殺原虫作用ペプチドの新規開発と宿主作用機作に関する研究

（課題番号　０７４０６０１４）

平成７年度～平成10年度科学研究費補助金（基盤研究Ａ）研究成果報告書

平成11年３月

研究代表者　齊藤篤志

（帯広畜産大学畜産学部教授）

はしがき

本報告書は、平成７年度から平成10年度にわたり交付を受けた
文部省科学研究費補助金「基盤研究(A)(2)」
（課題番号　０７４０６０１４）
の研究成果を取りまとめたものである。

研　究　組　織

研究代表者：齊藤　篤志（帯広畜産大学畜産学部教授）
研究分担者：鈴木　直義（帯広畜産大学名誉教授）
研究分担者：西村　昌数（帯広畜産大学畜産学部教授）
研究分担者：小俣　吉孝（帯広畜産大学畜産学部助教授）
研究分担者：見上　彪（帯広畜産大学原虫病分子免疫研究センター教授）
研究分担者：五十嵐郁男（帯広畜産大学原虫病分子免疫研究センター助教授）
研究分担者：佐藤　基佳（帯広畜産大学畜産学部教授）
研究分担者：宮原　和郎（帯広畜産大学畜産学部助教授）
研究分担者：藤崎　幸蔵（帯広畜産大学原虫病分子免疫研究センター教授）
研究分担者：長澤　秀行（帯広畜産大学原虫病分子免疫研究センター教授）
研究分担者：玄　学南（帯広畜産大学原虫病分子免疫研究センター助手）
研究分担者：井上　昇（帯広畜産大学原虫病分子免疫研究センター助手）

研　究　経　費

平成７年度	１４、８００千円
平成８年度	９、４００千円
平成９年度	５、８００千円
平成10年度	６、０００千円
計	３６、０００千円

研究成果の概要

世界最大の感染症にも拘わらず、原虫病に対する治療・予防薬の開発は、原虫が真核生物であるため、困難を極めている。抗生物質は無効であり、ワクチン開発も成功していない現状である。最近は、BRL（免疫調整物質）あるいは薬剤などによる、宿主動物に毒性が無く、原虫を直接殺滅しうる殺原虫物質の発見が世界中で望まれている。本研究では、原虫病治療において、原虫を直接殺滅しうる毒性の極めて低いペプチドの合成に成功した。

（1）本ペプチドには細胞内寄生原虫の細胞内侵入阻止および増殖抑制効果が観察されることをトキソプラズマおよびネオスポーラ原虫を用いて明らかにした。また、in vitro 培養細胞を用いた実験から、本ペプチドが宿主細胞に対して極めて毒性の低いことを証明した。

（2）マウスを用いた感染実験から、合成ペプチド投与による感染防御効果が認められた。原虫の感染経路および合成ペプチドの投与経路の検討から、本ペプチドの殺原虫作用は、原虫に対する直接障害性というよりは、むしろ宿主の免疫反応を増強することにより、感染抵抗性を賦与する可能性が示唆されたため、BRL としての機能が有力視された。

（3）免疫賦活作用の機序を解明することは、結果として他の感染症に対する防御反応の解明および治療法の確立に応用することができる。従って、バベシア、ネオスポーラ、トリパノソーマ、クリプトスポリジウム原虫に対する感染防御効果が期待される。

（4）原虫感染防御に係わる原虫分子を同定する目的で、原虫遺伝子を導入したトランスジェニックマウスの作成を試み、トキソプラズマ原虫主要抗原であるP30トランスジェニックマウスの作成に成功した。

本研究における合成ペプチドの新規開発は、他に類例のない極めて画期的な免疫賦活物質として世界的に高い評価を受けた。また、原虫病に対する治療・予防薬については、非特異的な BRL が有力視されている。従って、本ペプチドは原虫感染症に対する広スペクトルなワクチン候補としての評価も高く、実用化へ向けての更なる研究推進が望まれる。今後、本ペプチドの実用化に際しては、生体内における作用機序についての詳細な分子免疫学的あるいは発生工学的検討が必要である。

Summary of Research Results

1. Studies on newly synthesized peptides

Newly synthesized peptide (Obioactin to Obiopeptides) of the activity units which were isolated from cytokines as an immunoregulator were examined in the effect of *Toxoplasma* chronically infected animals. Mice chronically infected with *Toxoplasma gondii* were treated with cyclophosphamide (Cyp), Obiopeptide-1 (Obio-1) and/or anti-CD4 monoclonal antibody to determine the effect of these immunosuppressive agents on the cysts in the brain. In the brain of non-treate, and infected Cyp-Obi-1 treated mice, with hematoxyline-eosine staining or anti-*Toxoplasma* ABC labelling staining, large typically rounded tissue cysts were mostly detected, and in some regions dividing microcysts were also observed in this experimental studies. In contrast, brain tissue from Cyp only or anti-CI treated infected mice had multiple degenerated cysts of varied sizes in some brain regions, as well as clusters of microcysts, however, such change was more striking in the anti-CD4 treated mice. Infected mice treated with a combination of Cyp and Obio-1 showed a significantly higher survival of 80% compared to 20% survival in mice treated with Cyp only. These results indicate that reactivation of rupture of tissue cysts in mice treated with Cyp, chronically infected with Toxoplasma, might be mainly mediated by CD4 positive cells rather than other CD8 and CI positive cells.

2. Production of transgenic mice carrying protozoan gene

SAG-1 (p30), the major surface protein of *Toxoplasma gondii*, is considered as an important ligand in the process of host cell invasion. If this protein is produced by host cells and interfere with that of parasites, then the host will become resistant to parasite invasion. Or, it may become more susceptible to infection due to the lack of immune response against p30 antigen. To generate transgenic mice carrying p30 gene, a 3.3kb DNA fragments, containing p3 cDNA fused with CAG promoter, that has been shown to direct a ubiquitous expression of a reporter gene in transgenic mice, were microinjected into one of the pronuclei of one-cell embryos of C57BL/6J, BALB/c or F1 (C57BL/6J x C3H/He) mice. The embryos were transferred to the oviducts of pseudopregnant ICR mice. In the first series of experiment using inbred strains, two p30-positive founders (male C57BL/6J and female BALB/c) were obtained among 159 mice that developed from the injected eggs. Furthermore, we have obtained several P30-founde mice expressing the gene product and transmitting the gene to the next generation, by using F1 (C57BL/6J x C3H/H embryos. As far as we are aware, this is the first transgenic mice bearing a protozoan gene derived from *T. gondii*.

研 究 発 表

（1）学会誌等

原著論文

平成７年

Aquino, F.P., Naito, K., Cruz, L.C., Sato, E. and Toyoda, Y. Effects of vanadate on meiotic maturation of porcine oocytes in vitro. J. Reprod. Dev., 41: 271-276, 1995.

Chai, J-G., Okamoto, M., Bando, T., Nagasawa, H., Hisaeda, H., Sakai, T., Himeno, K., Sato, M. and Ohkubo, S. Dissociation between the mitogenic effect and antitumor activity of seed extract from *Aeginetia indica* L. Immunopharmacology, 30: 209-215, 1995.

Daen, F.P., Sato, E., Nakayama, T. and Toyoda, Y. Serum factor(s) stimulating cumulus expansion in porcine oocyte-cumulus complexes matured and fertilized in vitro. Cell Struct. Funct., 20: 223-231, 1995.

Furukawa, A., Maeda, K-I., Miyasaka, M., Kagawa, S., Yasutomo, K., Hisaeda, H., Nagasawa, H. and Himeno, K. Establishment of xeno-chimera without GVHD in NK cell-depleted SCID mice by grafting rat fetal liver cells. Cellular Immunol., 164: 176-181, 1995.

Hisaeda, H., Nagasawa, H., Maeda, K., Maekawa, Y., Ishikawa, H., Good, R. A. and Himeno, K. γ T cells play an important roles in expression of HSP65 and in acquiring protective immune response against infection with *Toxoplasma gondii*. J. Immunol., 154: 244-251, 1995.

Imahie, H., Sato, E. and Toyoda, Y. Pathenogenetic activation induced by progesterone in cultured mouse oocytes. J. Reprod. Dev. 41: 7-14, 1995.

Inoue, M., Naito, K., Aoki, F., Toyoda, Y. and Sato, E. Activation of mitogen-activated protein kinase during meiotic maturation of porcine oocytes. Zygote, 3: 265-271, 1995.

Kawazu, S., Kamio, T., Sekizaki, T. and Fujisaki, K. *Theileria sergenti* and *T. buffeli*: polymeras chain reaction-based marker system for differentiating the parasite species from infected cattle blood and infected tick salivary gland. Exp. Parasitol., 81: 430-435, 1995.

Kawazu, S., Miyahara, T., Kamio, T. and Fujisaki, K. Immunization with synthetic peptide reduce the severe consequences of *Theileria sergernti* infection in cattle. Bull. Soc. Fr.-Jpn. Sci. Vet., 6: 135-140, 1995.

Kikuchi, K., Izaike, Y., Noguchi, J., Furukawa, T., Daen, F.P., Naito, K. and Toyoda, Y. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. J. Reprod. Fert., 105: 325-330, 1995.

Kikuchi, M.,Naito, K., Daen, F.P., Izaike, Y. and Toyoda, Y. Histone H1 kinase activity during in vitro fertilization of pig follicular oocytes matured in vitro. Theriogenology, 43: 523-532, 1995.

Nagasawa, H., Hisaeda, H., Igarashi, I., Horiuchi, M., Inoue, N., Himeno, K. and Suzuki, N. Role of Heat Shock Proteins in Protective Immunity against Infection with *Toxoplasma gondii*. Bull. Soc. Fr-Jpn. Sci. Vet., 6(2): 154-162, 1995.

Nagasawa, H., Hisaeda, H., Ishikawa, H., Sakai, T., Maekawa, Y. and Himeno, K. Role of 65 000 MW heat-shock protein in SCID mice infected with *Toxoplasma gondii* after transplantation of mouse fetal thymus. J. Protozol. Res., 5(3): 86-96, 1995.

Nagasawa, H., Hisaeda, H., Igarashi, I., Horiuchi, M., Inoue, N., Himeno, K. and Suzuki, N. Role of heat shock protein in protective immunity against infection with *Toxoplasma gondii*. Bull. Soc.

Franco-Japon. Sci. Vet., 6: 154-162, 1995.

Naito, K., Hawkins, C., Yamashita, M., Nagahama, Y., Aoki, F., Kohmoto, K., Toyoda, Y. and Moor, R. M. Association of p34 cdc2 and cyclin B1 during meiotic maturation in porcine oocytes. Develop. Biol., 168: 627-634, 1995.

Nakagata, N., Ueda, S., Yamanouchi, K., Okamoto, M., Matsuda, Y., Tsuchiya, K., Nishimura, M Oda, S., Koyasu, K., Azuma, S. and Toyoda, Y. Cryopreservation of wild mouse spermatozoa. Theriogenology, 43: 635-643, 1995.

Nishinakamura, R., Nakayama, N., Hirabayashi, Y., Inoue, T., Aud, D., McNeil, T., Azuma, S., Yoshida, S., Toyoda, Y., Arai, K., Miyajima, A. and Murrey, R. Mice deficient for the IL-3/GM-CSF/ IL-5 bc receptor exhibit lung pathology and impaired immune response, while bIL3 receptor deficient mice are normal. Immunity, 2: 211-222, 1995.

Ohta, M., kawazu, S., Tsuji, N., Terada, Y., Kamio, T. and Fujisaki, K. DNA probes for detection of *Babesia ovata* or the *Babesia* sp. 1, a newly isolated bovine *Babesia* parasite in Japan. J. Protozool. Res., 5: 58-65, 1995.

Ohta, M., Kawazu, S, Tsuji, N., Terada, Y., Kamio, T. and Fujisaki, K. Rapid and sensitive metho for detection of newly isolated *Babesia* parasite (*Babesia* sp.1) in the anticipated vector-tick using the polymerase chain reaction technique. J. Protozool. Res., 5: 108-117, 1995.

Ohta, M., Tsuji, M., Tsuji, N. and Fujisaki, K. Morpholoical, serological and antigenic characteristics, and protein profile of newly isolated Japanese bovine *Babesia* parasite with articular refernce to those of *B . ovata.* J. Vet. Med. Sci., 57: 671-675, 1995.

Okada, M., Sato, E., Miyamoto, H. and Toyoda, Y. 17a-hydroxyprogesterone supresses neovascularization induced by HPLC-purified ovarian hyaluronic acid-like glycosaminoglycan in mice. Endocrine J., 42: 675-681, 1995.

Omata, Y., Inoue, N., Yonematsu, K. K., Claveria, F.G., Igarashi, I., Saito, A. and Suzuki, N. Detection of collagen-cross reactive antigen component in *Toxoplasma gondii.* J. Protozool. Res., 33-39, 1995.

Sakai, T., Agui, T., Muramatsu, Y., Nagasawa, H., Himeno, K. and Matsumoto, K. Low level of immunoglobulin G2a subclass correlates with a deficiency in T helper cell function in LEC mutan rats. J. Vet. Med. Sci., 57(3): 527-529, 1995.

Sato, E., Ando, N., Takahashi, Y., Miyamoto, H. and Toyoda,Y. Structural changes in the oviduct wall during passage of unfertilized cumulus-oocyte complexes in mice. Anat. Rec., 241: 205-210, 1995.

Sato, E., Nakayama, T., Kamio, K., Tokahashi, Y. and Toyoda,Y. Immunohistochemical localization and possible physiological role of tumor necrosis factor-a in mouse cumulus-oocyte complexes. Develop. Growth Differ., 37: 413-420, 1995.

Sato, E., Xian, M-W., Valdivia, R.P.A. and Toyoda, Y. Sex-linked differences in developmental potential of single blastomeres from in vitro fertilized 2-cell stage mouse embryos. Hormone Res., 44 (suppl.2): 4-8, 1995.

Suzuki, H., Togashi, M., Adachi, J. and Toyoda, Y. Developmental ability of zona-free mouse embryos is influenced by cell association at the 4-cell stage. Biol. Reprod., 53: 78-83, 1995.

Takahashi, Y., Meno, C., Sato, E. and Toyoda, Y. Synchronous sperm penetration of zona-free mouse eggs in vitro. Biol. Reprod. 53: 424-430, 1995.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Ymamauchi, K., Takase, M., Kawase, K., Igarashi, I. and Suzuki, N. *Toxoplasma gondii:* Parasiticidal effects of bovine lactoferricin against parasites. Exp. Parasitol. 81: 614-617, 1995.

Tsukui, T., Miyake, S., Azuma, S., Ichise, H., Saito, I. and Toyoda, Y. Gene transfer and expressior in mouse preimplantation embryos by recombinant adenovirus vector. Mol. Reprod. Dev., 42: 291-297, 1995.

Yoshino, M., Sagai, T., Lindahl, K. F., Toyoda, Y., Moriwaki, K. and Shiroishi, T. Allele-dependent recombination frequency: homology requirement in mice recombination at the hotspot in the mouse major histocompatibility complex. Genomics, 27: 298-305, 1995.

Wakayama, T., Suto, J., Matubara, Y., Fukuta, K., Toyoda, Y., Kuromaru, M. and Hayashi, Y. In vitro fertilization and embryo development of Japanese field vole (*Microtus montebelli*). J. Reprod. Fert., 104: 63-68, 1995.

平成８年

Araki, K., Naito, K., Haraguchi, S., Suzuki, R., Yokoyama, M., Inoue, M., Aizawa, S., Toyoda, Y. and Sato, E. Meiotic abnormalities of c-mos knockout mouse oocytes:Activation after first meiosis or entrance into third meiotic metaphase. Biol. Reprod., 55: 1315-1324, 1996.

Araki, K., Suzuki, R., Yokoyama, M., Naito, K., Sato, E. and Toyoda, Y. Fertilization capacity of mouse oocytes matured in vitro:effect of PMSG, FSH and epidermal growth factor (EGF) in serum-free maturation medium. J. Reprod. Dev., 42: 29-33, 1996.

Chai, J-G., Sakai, T., Hisaeda, H., Nagasawa, H., Yasutomo, K., Furukawa, A., Ishikawa, H., Maekawa, Y., Uehara, H., Izumi, K., Matsumoto, K. and Himeno, K. Development of functional rat-derived T cells in SCID mice engrafted with the fetal thymus of LEC rats which are defective in CD4+ T cells. Microbiol. Immunol., 40(9): 659-664, 1996.

Chowdhury, M. G. M., Maeda, K., Yasutomo, K., Maekawa, Y., Furukawa, A., Azuma, M., Nagasawa, H.,and Himeno, K. Antigen-specific B cells are required for the secondary response of T cells but not for their priming. Eur. J. Immunol., 26: 1628-1633, 1996.

Haraguchi, S., Naito, K., Azuma, S., Sato, E., Nagahama, Y., Yamashita, M. and Toyoda, Y. Effects of phosphate on the in vitro 2-cell block of AKR/N mouse embryos based on changes in cdc2 kinase activity and phosphorylation state. Biol.Reprod., 55: 598-603, 1996.

Hisaeda, H., Sakai, T., Nagasawa, H.,Ishikawa, H., Yasutomo, K., Maekawa, Y. and Himeno, K. Contribution of extrathymic gd T cells to the expression of heat-shock protein and to protective immunity in mice infected with *Toxoplasma gondii*. Immunology, 88: 551-557, 1996.

Hisaeda, H., Sakai, T., Ishikawa, H., Maekawa, Y., Yasutomo, K., Nagasawa, H. and Himeno, K. Mechanisms of HSP65 expression induced by gd T cells in murine *Toxoplasma gondii* infection. Pathobiology, 64: 198-202, 1996.

Horiuchi, M., Yuri, K., Soma, T., Katae, H., Nagasawa, H. and Shinagawa, M. Differentiation of vaccine virus from field isolates of feline panleukopenia virus by polymerase chain reaction and restriction fragment length polymorphism analysis. Vet. Microbiol., 1996.

Inoue, N., Omata, Y., Yonematsu, K., Claveria, F.G., Igarashi, I., Saito, A. and Suzuki, N. Collagen cross-reactive antigen of *Sarcocystis cruzi*. Vet. Parasitol., 63: 17-23, 1996.

Inoue, N., Omata, Y., Yonematsu, K., Claveria, F.G., Igarashi, I., Saito, A. and Suzuki, N. Detection of collagen-cross reactive antigen component in *Toxoplasma gondii*. J. Protozool. Res., 5 33-39, 1996.

Ishikawa, M., Miyauchi, T., Tashiro, S., Nagasawa, H. and Himeno, K. Acetaminophen absorption Test as a useful indicator of small intestinal rejection in rats. Transplantation Proceedings, 28(3): 1845-1846, 1996.

Kyuwa, S., Machii, K., Okumura, A. and Toyoda, Y. Characterization of T cells expanded in vivo

during primary mouse hepatitis virus infection in mice. J. Vet. Med. Sci., 58: 431-437, 1996.

Maki, Y., Seng, S., Kato, M., Hoshi, Y., Igarashi, I., Nagasawa, H., Toyoda, Y. and Suzuki. Sequence analysisi of three major antigen (P30, P23, P22) of virulent and avirulent strains of *Toxoplasma gondii*. J. Protozool. Res., 6: 83-93, 1996.

Meno, C., Saijo, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y. and Hamada, H. Left-right asymmetric expression of the TGFβ-family member lefty in mouse embryos. Nature, 381: 151-155, 1996.

Nagasawa, H., Himeno, K. and Suzuki, N. Protective immunity in toxoplasmosis. Appl. Parasitol., 37: 284-292, 1996.

Nakai, S., Kawakita, T., Nagasawa, H., Himeno, K. and Nomoto, K. Thymus-dependent effect of a traditional Chinese medicine, Ren-shen-yang-rong-tang (Japanese name; Ninjin-youei-to), in autoimmune MRL/Mp-lpr/lpr mice. Int. J. Immunopharmacol., 18: 271-279, 1996.

Nakamae, H., Kishi, S., Fujisaki, K., Oshiro, S. and Furuta, Y. Effect of trimming feathers off abdominal and crural tracts of the hen on parasitism of mites. Jpn. Poult. Sci., 33: 377-382, 1996.

Ohta, M., Kawazu, S., Terada, Y., Kamio, T., Tsuji, M. and Fujisaki, K. Experimental transmission of *Babesia ovata oshimaensis* n.var. of cattle in Japan by *Haemaphysalis longicornis*. J. Vet. Med. Sci., 58: 1153-1155, 1996.

Okumura, A., Machii, K., Azuma, S., Toyoda, Y. and Kyuwa, S. Maintenance of pluripotency in mouse embryonic stem cells persistently infected with murine coronavirus. J. Virol., 70: 4146-4149, 1996.

Orita, J., Sato, E., Saburi, S., Nishida, T. and Toyoda, Y. Magnetic resonance imaging of the internal structure of the mouse fetus. Exp. Animals, 45(2): 171-174, 1996.

Otsuka,H. and Xuan, X. Construction of bovine herpesvirus-1 (BHV-1) recombinants which express pseudorabies virus (PRV) glycoproteins gB, gC, gD, and gE. Arch. Virol., 141: 57-71, 1996.

Otsuka,H. and Xuan,X. Protective immunity of bovine herpesvirus-1 (BHV-1) recombinants which express pseudorabies virus (PRV) glycoproteins gB, gC, gD and gE. J. Vet. Med. Sci., 58: 819-824, 1996.

Seng, S., Maki, Y., Yokoyama, M., Inoue, N., Igarashi, I., Nagasawa, H., Horiuchi, M., Omata, Y., Saito, A., Suzuki, N. and Toyoda, Y. 1996. Production of transgenic mice carrying p30 gene encoding major surface antigen of *Toxoplasma gondii*. J. Protozool. Res., 6: 121-128, 1996.

Shimazaki, K., Nam, M.S., Harakawa, S., Tanaka, T., Omata, Y., Saito, A., Kumura, H., Mikawa, K., Igarashi, I. and Suzuki, N. Monoclonal antibody against bovine lactoferrinand its epitope site. J. Vet. Med. Sci., 58: 1227-1229, 1996.

Suzuki, N., Igarashi, I., Abgaandorjiin, A., de Waal, D. T., Nagasawa, H., Omata, Y., Saito, A. and Oyamada, T. Preliminary survey on horse serum indirect fluorescent antibody titers in Japan against *Babesia equi* and *B. caballi* (Ondestepoort strain) antigen. J. Protozool. Res., 6:31-36, 1996.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Igarashi, I. and Suzuki, N. Growth inhibitory effects of bovine lactoferrin to *Toxoplasma gondii* parasites in murine somatic cells. J. Vet. Med. Sci., 58: 61-65, 1996.

Tsukui, T., Kanegae, Y., Saito, I. and Toyoda, Y. Transgenesis by adenovirus-mediated gene transfer into mouse zona-free eggs. Nature Biotech., 14: 982-985, 1996.

Valdivia, R.P.A., Kunieda, T., Hattori, M. and Toyoda, Y. Typing X-chromosomes bearing *Tabby* allele in mouse preimplantation embryos by detection of microsatellite marker. Exp. Animals,

45(4): 395-398, 1996.

Wakayama, T., Suto, J., Imamura, K., Toyoda, Y., Kuromaru, M. and Hayashi, Y. Effect of hypotaurine on in vitro fertilization and production of term offspring from in vitro-fertilized ova of the Japanese field vole, Microtus montebelli. Biol. Reprod., 54: 625-630, 1996.

Xuan, X., Maeda, K., Tohya, Y., Mikami, T., and Otsuka, H. Identification and nucleotide sequence of the thymidine kinase gene of canine herpesvirus. Virus Genes, 12(2): 185-188, 1996.

Xuan, X., Maeda, K., Mikami, T., and Otsuka, H. Characterization of canine herpesvirus glycoprotein C expressed by a recombinant baculoviurs in insect cells. Virus Res., 46(1): 57-64, 1996.

Yasutomo, K., Nagasawa, H., Hisaeda, H., Good, R.A., Kuroda, Y. and Himeno, K. Fas system-mediated apoptosis suppresses lymphopoiesis. J. Immunol., 157(5): 1981-1986, 1996.

平成９年

Avarzed, A., Igarashi, I., Kanemaru, T., Hirumi, K., Omata, Y., Saito, A., Oyamada, T., Nagasawa, H., Toyoda, Y. and Suzuki, N. Improved *in vitro* cultivation of *Babesia caballi*. J. Vet. Med. Sci., 59(6): 479-481, 1997.

Avarzed, A., deWaal, D.T., Igarashi, I., Saito, A., Oyamada, T., Toyoda, Y. and Suzuki, N. Prevalence of equine piroplasmosis in Central Mongoria. Onderstepoort J. Vet. Med., 64: 141-145, 1997.

Hirumi, H., Martin, S. K., Hirumi, K., Inoue, N., Kanbara, H., Saito, A. and Suzuki, N. Cultivation of bloodstream forms of *Trypanosoma brucei* and *T. evansi* in a serum-free medium. Trop. Med. Int. Health, 2 (3): 240-244, 1997.

Horiuchi, M., Ishiguro, N., Nagasawa, H.,Toyoda, Y. and Shinagawa, M. Alternative usage of exon 1 of bovine PrP mRNA. Biochem. Biophys.Res.Comm., 233(3): 650-654, 1997.

Horiuchi, M., Mochizuki, M., Ishiguro, N., Nagasawa, H. and Shinagawa, M. Epitope mapping of a monoclonal antibody specific to feline panleukopenia virus and milk enteritis virus. J. Vet. Med. Sci., 59(2): 133-136, 1997.

Inagaki, Y., Hayashi-Ishimaru, Y., Ehara, M., Igarashi, I. and Ohama, T. Algae of protozoa: Phylogenic position of euglenophytes and dinoflagellates as inferred from mitochondrial sequences. J. Mol. Evolution, 45 (3):295-300, 1997.

Ishikawa, H., Hisaeda, H., Maekawa, Y., Nagasawa, H., Sakai, T., Ota, F. and Himeno, K. Expression of heat shock protein in host macrophages correlates with a protective potential against infection with *Laishmania major* in mice. Parasitol. Int. 46(4): 263-270, 1997.

Kamada, T., Igarashi, I., Inoue, N., Nagasawa, H., Suzuki, H., Kodama, T., Suzuki, N. and Yoyoda, Y. The role of scavenger receptor type A (SR-A) during infection with *Babesia microti*. J. Protozool. Res., 7 (3): 81-89, 1997.

Kawazu, S., Okumura, T., Hirogari, Y., Miyahara, T., Terasaka, Y., Hida, M., Terada, Y., Kamio, T. and Fujisaki, K. A polymorphism observed in the experimentally successful peptide vaccine sequence derived from Theileria sergenti piroplasm major surface antigen (p33). J. Vet. Med. Sci., 59(9): 829-831, 1997.

Kyuwa, S., Xiao, Y., Toyoda, Y. and Sato, E. Characterization of embryonic stem-like cell lines derived from embryoid bodies. Exp. Animals, 46(1): 11-16, 1997.

Maeda, K., Xuan, X., Kawaguchi, Y., Ono, M., Yokoyama, N., Fujita, K., Tohya, Y. and Mikami, T. Characterization of canine herpesvirus glycoprotein D (hemagglutinin). J. Vet. Med. Sci., 59(11): 1003-1009, 1997.

Maeda, K., Yokoyama, N., Fujita, K., Xuan, X. and Mikami, T. Role of one N-linked oligosaccharide chain on canine herpesvirus gD on its biological activity. J. Vet. Med. Sci., 59(12): 1123-1128, 1997.

Miyauchi, T., Ishikawa, M., Tashiro, S., Hisaeda, H., Nagasawa, H. and Himeno, K. Acetaminophen absorption test as a marker of small bowel transplant rejection. Transplantation, 63 (8): 1179-1190, 1997.

Nakamae, H., Fujisaki, K., Kishi, S., Yashiro, M., Oshiro, S. and Furuta. The new parasitic ecology of chicken mites *Dermanyssus gallinae*, parasitizing and propagating on chickens even in the daytime. Jpn.Poult.Sci., 34: 110-116, 1997.

Nakamae, H., Kishi, S., Fujisaki, K., Oshiro, S. and Furuta. Incidence of the parasitism of chicken mite *Dermanyssus gallinae* parasitizing qand propagating on chicken even in the daytime and their life cycle. Jpn.Poult.Sci., 34: 240-247, 1997.

Remer, K., Igarashi, I., Inoue, N., Nagasawa, H., Toyoda, Y. and Suzuki, N. Different activation of lymphocytes in reaction to *Babesia microti* infection in iNOS-/- and wildtype mice. J. Protozool. Res., 7 (2): 63-69, 1997.

Shimazaki, K., Nam, M. O., Harakawa, S., Tanaka, T., Omata, Y., Saito, A., Kumura, H., Mikawa, K., Igarashi, I. and Suzuki, N. Monoclonal antibody against bovine Lactoferricin and its epitopic site. J. Vet. Med. Sci., 58: 1227-1229, 1997.

Suzuki, H., Kamada, N., Ueda, O., Jishage, K., Kurihara, Y., Kurihara, H., Terauchi, Y., Azuma, S., Kadowaki, T., Kodama, T., Yazaki, Y. and Toyoda, Y. Germ-line contribution of embryonic stem cells in chimeric mice:Influence of karyotype and in vitro differentiation ability. Exp. Animals, 46(1): 17-24, 1997.

Suzuki,H.,Kurihara,Y.,Takeya,M.,Kamada,N.,Kataoka,M.,Jishage,K.,Ueda,O.,Sakaguchi,H.,Higashi,T.,Suzuki,S.,Takashima,Y.,Kawabe,Y.,Cynshi,O.,Wada,Y.,Honda,M.,Kurihara,H.,Aburatani,H.,Doi,T.,Matsumoto,A.,Azuma,S.,Noda,T.,Toyoda,Y.,Itakura,H.,Yazaki,Y.,Horiuchi,S.,Takahashi,K.,Kruijt,J.K.,vanBerkel,T.J.C.,Steinbrecher,U.P.,Ishibashi,S.,Maeda,N.,Gordon,S. and Kodama,T. A role for macrophage scavenger receptors in artherosclerosis and susceptibility to infection. Nature, 386(6622): 292-296, 1997.

Tanaka, T., Omata, Y., Narisawa, M., Saito, A., Shimazaki, K., Igarashi, I., Hirumi, H. and Suzuki, N. Growth inhibitory effects of bovine lactoferrin on *Toxoplasma gondii* tachyzoites in murine macrophages: Role of radical oxygen and inorganic itrogen oxide in *Toxopaslma* growth-inhibitory activity. Vet. Parasitol., 68:27-33, 1997.

Tsuji, N., Kawazu, S., Ohta, M., Kamio, T., Isobe, T., Shimura, K. and Fujisaki, K. Discrimination of eight chicken Eimeria species using the two-step polymerase chain reaction. J. Parasitol., 83(5): 966-970, 1997.

Tsuji, N., Ohta, M. and Fujisaki, K. Expression of a 70-kDa heat-shock-related protein during transformation from free-living infective larvae to the parasitic stage in *Strongyloides venezuelensis*. Parasitol. Res., 83: 99-102, 1997.

Xuan, X., Kojima, A., Murata, T., Mikami, T. and Otsuka, H. Characterization of canine herpesvirus glycoproteins B, C and D expressed by a recombinant vaccinia virus. Arch. Virol., 142(5): 1003-1010, 1997.

Yoshitake,N., Xuan,X. and Otsuka,H. Identification and characterization of bovine herpesvirus-1 glycoproteins E and I. J. Gen. Virol., 78: 1399-1403, 1997.

平成10年

Avarzed, A., Igarashi, I., de Waal , T., Kawai, S., Oomori, Y., Omata, Y., Inoue, N., Saito, A.,

Nagasawa, H., Toyoda Y. and Suzuki, N. Monoclonal antibody against *Babesia equi:* its characterization and poteitial use of antigen recognized by monoclonal antibodiy for diagnosis. J. Clin. Micorbiol., 36: 1835-1839, 1998.

Choi, Y-H., Seng, S. and Toyoda, Y. Effect of Taurine on in vitro fertilization and embryo development of BALB/c mouse strain. J. Reprod. Dev., 44: 29-34, 1998.

Damiani, A.M., Matsumura, T., Yokoyama, N., Maeda, K., Miyazawa, T., Kai, C. and Mikami, T. Nucleotide sequence of glycoprotein I and E genes of equine herpesvirus type 4. J. Vet. Med. Sci., 60(2): 219-225, 1998.

Damiani, A.M., Jang, H.-K., Matsumura, T., Yokoyama, N., Miyazawa, T. and Mikami, T. Transcriptional analyses of the region of the equine herpesvirus type 4 genome encoding glycoproteins I and E. Arch. Virol., (In press).

Fujita, K., Maeda, K., Yokoyama, N., Miyazawa, T., Kai, C. and Mikami, T. In vitro recombinatioı of feline herpesvirus type 1. Arch. Virol, 143: 25-34, 1998.

Horiuchi, M., Yamaguchi, Y., Gojobori, T., Mochizuki, M., Nagasawa, H., Toyoda, Y., Ishiguro, N. and Shinagawa, M. Differences in the evolutionary pattern of feline panleukopenia virus and canine parvovirus. Virology, 249: 440-452, 1998.

Igarashi, I., Njonge, F.K., Kaneko, Y. and Nakamura, Y. *Babesia bigemina :* In vitro and in vivo effects of curdlan sulfate on growth of parasites. Exp. Parasitol., 90: 290-293, 1998.

Ikeda, Y., Inoshima, Y., Kawaguchi, Y., Maeda, K., Kohmoto, M., Kai, C., Miyazawa, T. and Mikami, T. Protein-binding properties of the putative AP-1 and ATF sequences in the feline immunodeficiency virus long terminal repeat. J. Gen.Virol., 79(1): 95-99, 1998.

Ikeda, Y., Shinozuka, J., Miyazawa, T., Kurosawa, K., Izumiya, Y., Nishimura, Y., Nakamura, K., Cai, J.-S, Fujita, K., Doi, K. and Mikami, T. Apoptosis in feline panleukopenia virus-infected lymphocytes. J. Virol., 72(8): 6932-6936, 1998.

Ikeda, Y., Miyazawa, T., Kurosawa, K., Naito, R., Hatama, S., Kai, C. and Mikami, T. New quantitative methods for detection of feline parvovirus (FPV) and virus neutralizing antibody against FPV using a feline T lymphoblastoid cell line. J. Vet. Med. Sci., 60(8): 973-974, 1998.

Inoshima, Y., Miyazawa, T. and Mikami, T. The roles of *vif* and ORF-A genes and AP-1 binding site in *in vitro* replication of feline immunodeficiency virus. Arch. Virol., 143: 789-795, 1998.

Inoshima, Y., Miyazawa, T., Kohmoto, M., Ikeda, Y., Sato, E., Hohdatsu, T., Mathiason-Dubard, C., Hoover, E.A. and Mikami, T. Cross virus neutralizing antibodies against feline immunodeficiency virus genotypes A, B, C, D and E. Arch. Virol., 143: 157-162, 1998.

Inoshima, Y., Miyazawa, T. and Mikami, T. In vivo functions of the auxiliary genes and regulatory elements of feline immunodeficiency virus. Review article. Vet. Microbiol., 60: 141-153, 1998.

Inoue, N., Narumi, D., Mbati, P. A., Hirumi, K., Situakibanza N-T. H. and Hirumi, H. Susceptibility of severe combined immuno-deficient (SCID) mice to *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. Trop. Med. Int. Health, 3(5): 408-412, 1998.

Isamida, T., Tanaka, T., Omata, Y., Yamauchi, K., Shimazaki, K. and Saito, A. Protective effects o lactoferricin against *Toxoplasma gondii* infection in mice. J. Vet. Med. Sci., 60(2): 241-244, 1998.

Iwatsuki, K., Miyashita, N., Yoshida, M., Shin, Y.-S., Ohashi, K., Kai, C. and Mikami, T. The nucleotide and predicted amino acid sequence of the fusion protein of recent isolates of canine distemper virus in Japan. J. Vet. Med. Sci., 60(3): 381-385, 1998.

Izumiya, Y., Jang, H.-K., Kashiwase, H., Cai, J.-S., Nishimura, Y., Tsushima, Y., Kato, K., Miyazawa, T., Kai, C. and Mikami, T. Identification and transcriptional analysis of the homologue:

of the herpes simplex virus type 1 UL41 to UL51 genes in the genome of nononcogenic Marek's disease virus serotype 2. J. Gen. Virol., 79, 1997-2001, 1998.

Jang, H.-K., Ono, M., Kim, T.-J.,Yokoyama, N., Niikura, M., Kai, C. and Mikami, T. The genetic organization and transcriptional analysis of the short unique region in the genome of Marek's disease virus serotype 2. Virus Res., (In press).

Kawai, S., Igarashi,I., Abganndorrjiin, A., Ikadai, H., Omata, Y., Saito, A., Nagasawa, H., Toyoda Y. and Suzuki, N. Tublar structure associated with *B. caballi* -infected erythocytes in vitro. Parasitol. Res., (In press).

Kohmoto, M., Uetsuka, K., Ikeda, Y., Inoshima, Y., Shimojima,. M., Sato, E., Inada, G., Toyosaki, T., Miyazawa, T., Doi, K. and Mikami, T. Eight -year observation and comparative study of specific pathogen-free cats experimentally infected with feline immunodeficiency virus (FIV) subtypes A and B : terminal acquired immunodeficiency syndrome in a cat infected with FIV Petaluma strain. J. Vet. Med. Sci., 60(3): 315-321, 1998.

Kohmoto, M., Sato, E., Nishimura, Y., Ikeda, Y., Inoshima, Y., Shimojima, M., Inada, G., Miyazawa, T. and Mikami, T. Establishment of vaginal infection with a molecular clone of feline immunodeficiency virus TM2 strain and its deletion mutant of AP-1 binding site. Vet. Microbiol., (In press).

Kohmoto, M., Miyazawa, T., Sato, E., Uetsuka, K., Nishimura, Y., Ikeda, Y., Inada, G., Doi, K. and Mikami, T. Cats are protected against feline immunodeficiency virus infection following vaccination with a homologous AP-1 binding site-deleted mutant. Arch. Virol., 143: 1839-1845, 1998.

Maeda, K., Horimoto, T. and Mikami, T. Properties and functions of feline herpesvirus type 1 glycoproteins. Review article. J. Vet. Med. Sci., 60(8): 881-888, 1998.

Miyauchi, T., Ishikawa, M., Tashiro, S., Hisaeda, H., Nagasawa, H. and Himeno, K. Effect of donor-specific splenocytes via portal vein and FK506 in rat small bowel transplantation. Transplantation, 65 (1): 27-32, 1998.

Miyazawa, T., Ikeda, Y., Maeda, K., Horimoto, T., Tohya, Y., Mochizuki, M., Vu, G.D., Vu, D., Cu, D.X., Ono, K., Takahashi, E. and Mikami, T. Seroepidemiological survey of feline retrovirus infection in domestic and leopard cats in northern Vietnam in 1997. J. Vet. Med. Sci., 60(11): (In press), 1998.

Niikura, M., Witter, D. R., Jang, H.-K., Ono, M., Mikami, T. and Silva, R. F. Marek's disease virus glycoprotein D expressed in feather follicle epithelium of infected chickens. Acta. Virol., (In press).

Nishikawa, Y., Xuan, X. and Otsuka, H. Identification and characterization of the glycoprotein E and I genes of canine herpesvirus. Virus Res., 56: 77-92, 1998.

Nishimura, Y., Miyazawa, T., Ikeda, Y., Izumiya, Y., Nakamura, K., Cai, J.-S., Sato, E., Kohmoto, M. and Mikami, T. Molecular cloning and expression of feline CD3e. Vet. Immunol. Immunopathol., (In press).

Nishimura, Y., Miyazawa, T., Ikeda, Y., Izumiya, Y., Nakamura, K., Cai, J.-S., Sato, E. Kohmoto, M. and Mikami, T. Molecular cloning and sequencing of feline stromal cell-derived factor-1a and b. Eur. J. Immunogenetics, 25: 303-305, 1998.

Sakai, T., Agui, T., Kaichun, W., Oka, M., Hisaeda, H., Nagasawa, H., Himeno, K. and Matsumoto, K. Unresponsiveness of CD4-8+/- thymocytes to lectin stimulation in LEC mutant rats. Immunology, 95: 219-225, 1998.

Sato, E., Yokoyama, N., Maeda, K., Inoshima, Y., Kohmoto, M., Ikeda, Y., Miyazawa, T. and Mikami, T. Construction of a recombinant feline herpesvirus type 1 expressing gag precursor protein of feline immunodeficiency virus. Arch.Virol., 143: 453-466, 1998.

Shimazaki, K., Kamio, M., Nam, M.S., Harakawa, S., Tanaka, T., Omata, Y., Saito, A., Kumura, H., Mikawa, K., Igarashi, I. and Suzuki, N. Structural and immunochemical studies on bovine lactoferrin fragments. Adv. Exp. Med. Biol., 443: 41-48, 1998.

Shimojima, M., Miyazawa, T., Kohmoto, M., Ikeda, Y., Nishimura, Y., Maeda, K., Tohya, Y. and Mikami, T. Expansion of CD8a+b- cells in cats infected with feline immunodeficiency virus. J. Gen. Virol., 79(1): 91-94, 1998.

Shimojima, M., Pecoraro, M.R., Maeda, K., Tohya, Y., Miyazawa, T. and Mikami, T. Characterization of anti-feline CD8 monoclonal antibodies. Vet. Immunol. Immunopathol., 61: 17-23, 1998.

Song, C.-S., Kim, J.-H., Lee, Y.-J., Kim, S.-J., Izumiya, Y., Tohya, Y., Jang, H.-K. and Mikami, T. Detection and classification of infectious bronchitis viruses isolated in Korea by dot-immunoblotting assay using monoclonal antibodies. Avian Dis., 42(1): 92-100, 1998.

Song, C.-S., Lee, Y.-J., Kim,. J.-H., Sung, H.-W., Lee, C.-W., Izumiya, Y., Miyazawa, T., Jang, H.-K. and Mikami, T. Epidemiological classification of infectious bronchitis virus isolated in Korea between 1986 and 1997. Avian Pathol., 27(4): 409-416, 1998.

Song, C.-S., Lee, Y-J., Lee, C.-W., Sung, H.-W., Kim, J.-H., Mo, I.-P., Izumiya, Y., Jang, H.-K. and Mikami, T. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. J. Gen. Virol., 79(4): 719-723, 1998.

Tanaka, T., Omata, Y., Isamida, T., Saito, A., Shimazaki, K., Yamauchi, K. and Suzuki, N. Growth inhibitory effects of bovine lactoferrin to *Toxoplasma gondii* tachyzoites in murine macrophages: Phosphorylation in murine macrophages induced by bovine lactoferrin. J. Vet. Med. Sci., 60(3): 369-371, 1998.

Tomonaga, K., Itagaki, S., Kashiwase, H., Kawaguchi, Y., Inoshima, Y., Ikeda, Y. and Mikami, T. Characterization of an integrase mutant of feline immunodificiency virus. Arch.Virol., 143: 1-14, 1998.

Xuan, X., Nishikawa, Y., Takashima, Y., Tuchiya, K., Ueda, S., Yokoyama, N., Maeda, K., Mikami, T. and Otsuka, H. Constraction of canine herpesvirus vector expressing foreign genes using a LacZ-TK gene cassette as a double selectional marker. Virus Genes, 17(1): 25-32, 1998.

Xuan, X., Tuchiya, K., Sato, I., Nishikawa, Y., Onodera, Y., Takashima, Y., Yamamoto, A., Katsumata, A., Iwata, A., Ueda, S., Mikami, T. and Otsuka, H. Biological and immunogenic properties of rabies virus glycoprotein expressed by canine herpesvirus vector. Vaccine, 16(9/10): 969-976, 1998.

Yokoyama, N., Fujita, K., Damiani, A., Sato, E., Kurosawa, K., Miyazawa, T., Ishiguro, S., Mochizuki, M., Maeda, K. and Mikami, T. Further construction of a recombinant feline herpesvirus type 1 expressing feline calcivirus immunogenic antigen. J. Vet. Med. Sci., 60(6): 717-723, 1998.

Yoshida, E., Iwatsuki, K., Miyashita, N., Gemma, T., Kai, C. and Mikami, T. Molecular analysis of the nucleocapsid protein of recent isolates of canine distemper virus in Japan. Vet. Microbiol., 59: 273-244, 1998.

（2）口頭発表

平成７年

Igarashi, I., Suzuki, R., Omata, Y., Saito, A., Toyoda, Y., and Suzuki, N. Protective immunity

ahainst challenge infection with babesia microti in mice. 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Inoue, N., Hirumi, K., Narumi, D., Saito, A., Suzuki, N. and Hirumi, H. *Trypanosoma brucei gambiense*: In vitro cultivation of bloodstream trypomastigotes of a low virulent strain (IL3253).15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Kawazu, S., Kamio, T., Sekizaki, T., and Fujisaki, K. Polymerase chain reaction-based marker system for differentiation *Theileria sergenti* and *T. buffeli*. 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Kawazu, S., Miyahara, T., Kamio, T. and Fujisaki, K. (1995) Immunization with synthetic peptide reduced the severe consequences of *Theileria sergenti* infection in cattle. Japan-OECD joint international workshop. Novel Immunological Approaches to the Control of Diseases in Animals. Sep. 10-11, 1995, Tokyo, Japan.

Nagasawa, H. and Suzuki, N. Parasites and immunodepression: Immunopathophysiology of the host with *Toxoplasma gondii*. XII Congreso Latino Americano de Parasitologia. Santiago, Chile, Oct. 21-27, 1995.

Nagasawa, H. and Suzuki, N. Aspects of *Toxoplasma* Immunity: Role of heat shock proteins in protective immunity against infection with *Toxoplasma gondii*. XII Congreso Latino Americano de Parasitologia. Santiago, Chile, Oct. 21-27, 1995.

Nagasawa, H., Hisaeda, Himeno, K., Horiuchi, M., Inoue, N. and Igarashi, I. Role of heat shock protein in protective immunity against infection with *Toxoplasma gondii*. Japan-OECD joint international workshop. Novel Immunological Approaches to the Control of Diseases in Animals. Tokyo, Japan, Sep. 10-11, 1995.

Nagasawa, H., Hisaeda, H., Sakai, T., Ishikawa, H., Maekawa, Y., and Himeno, K. γδ T cells play an important role in HSP65 expression and in acquiring protective immunity in mice infected with *Toxoplasma gondii*. 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Narumi, D., Inoue, N., Saito, A. Suzuki, N. and Hirumi, H. Susceptibility of immnodeficiency (SCID and Nude) mice to high and low virulent strains of *Trypanosoma brucei gambiense*. 15th International Conference of the World Association for the Advancement of Veterinary Parasitology. Yokohama, Aug. 30-Sept. 2, 1995.

Ohta, M., Tsuji, N., Kawazu, S., Terada, Y., Kamio, T., and Fujisaki, K. Morphological, serological and antigenic characteristics, and protein profile of newly isolated Japanese bovine *Babesia* parasite with particular reference to those of *B, ovata*. 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Tanaka, T., Shimazaki, K., Igarashi, I., Omata, Y., Saito, A. and Suzuki, N. Growth inhibitory effect of bovine lactoferrin to *Toxoplasma gondii* parasites in murine somatic cells. Lactoferrin structure and functions second international symposium, Honolulu, Hawai, February, 1995.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Yamauchi, K., Takase, M., Igarashi, I. and Suzuki, N. Parasiticidal effect of bovine lactoferricin to *Toxoplasma gondii*, 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Tsuji, N., Ohta, M., Kawazu, S., Sekizaki, T. and Fujisaki, K. Expression of heat shock proteins and heat shock cognate 70 gene during transformation from free-living infective larvae to the parasitic stage in *Strongyloides venezuelensis*. 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

Xuan,X., Ihara,T., Ueda,S. et al. Analysis of functional domains of pseudorabies virus glycoprotein gIII. 15th Intern. Congress of WAAVP, Yokohama, Japan, Aug. 30-Sept. 2, 1995.

平成８年

Igarashi, I., Tagawa, Y., Seyha, S., Nagasawa, H., Omata, Y., Saito, A., Suzuki, N., Iwakura, Y., and Toyoda, Y. *Babesia microti* infection in IFN-γ knock-out mice. 10th Japanese-German Cooperative Symposium on Protozoan Diseases. Hannover, Germany, Sep. 11-15, 1996.

Igarashi, I., Y. Tagawa, H. Nagasawa, Y. Omata, A. Saito, N. Suzuki, Y. Iwakura, Y. Toyoda. Role of IFN- in the protection against Babesia microti infection in mice. I4 th International Congress for Tropical and Malaria, Nagasaki, Japan, November 17-22, 1996

Nagasawa, H., Igarashi, I., Satoma, H., Carvalho, L. M., Gonchigoogiin, B., Inoue, N., Horiuchi, M., Oyamada, T., Saito, A., Toyoda, Y. and Suzuki, N. Immune responses in mice infected with *Neospora caninum*. 10th Japanese-German Cooperative Symposium on Protozoan Diseases. Sep. 11-15, 1996, Hannover, Germany.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Igarashi, I. and Suzuki, N. Growth inhibitory effect of bovine lactoferrin to *Toxoplasma gondii* parasites in murine somatic cells. The 9th International Congress of Immunology, San Francisco, California, USA, July, 1996.

Xuan, X., Kojima, A., Murata, T., Mikami, T. and Otsuka, H. Characterization of canine herpesvirus gB, gC and gD. 21st Herpesvirus Workshop, DeKalb Illinois, July 27 - Aug.2 , 1996.

Xuan,X., Shibata,I. and Otsuka,H. Immunogenecity of bovine herpesvirus-1 (BHV-1) recombinants which express pseudorabies virus (PRV) glycoprotein gB, gC, gD and gE. 第２回日中ウイルス学会, 成都市, June 3-6, 1996.

平成９年

Avarzed, A., Igarashi, I., de Waal, D.T., Saito, A., Nagasawa, H., Toyoda, Y. and Suzuki, N. Monoclonal antibody against Babesia equi. 15th Intl. Congress of WAAVP, Sun City, South Africa, August 10-15, 1997.

Avarzed, A., I. Igarashi, T. D. de Waal, A. Saito, H. Nagasawa, Y. Toyoda, and N. Suzuki.: Monoclonal antibody against *B. equi*. 16th International Conference of the World Association for the Advancement of Veterinary Parasitology, Sun City, South Africa , August 10-15, 1997.

Fujisaki, K. Babesiosis: Emerting and re-emerging diseases transmitted by arthropod vectors and rodents. 2nd International Symposium on Lime Diseases, Shizuoka, Oct. 27-28, 1997.

Fujisaki, K. Babesiosis, 第２回　国際ライム病シンポジウム, Shizuoka, Japan, Oct. 27-28, 1997.

Inoue, N., Narumi, D., Inoue, M., Hirumi, K., Igarashi, I., Nagasawa, H., Saito, A., Suzuki, N. and Hirumi, H. *Trypanosoma brucei gambiense*: Importance of CD4+ T cell. 16th International Conference of the World Association for the Advancement of Veterinary Parasitology. Sun City, South Africa, Aug. 10-15, 1997.

Nagasawa, H. and Suzuki, N. Sympodium: Coccidia, Protective immune responses in toxoplasmosis. 10th International Congress of Protozoology (ICOP). July. 21-25, 1997, Sydney, Australia.

平成10年

Igarashi. I., Marbella, C.O., Kamata, T., Omata, Y., Saito, A., Nagasawa, H., Fujisaki, K., Suzuki, N.,Mak, T. and Toyoda, Y. Mice deficient for TNF Receptor p55 are resistant to lethal infection with Babesia rodhaini. 9th International Congress of Parasitology, Chiba, Japan. August 24-28, 1998.

Igarashi, I., Abgaandrojiin, A. and Suzuki, N. The in vitro culture of equine *Babesia* parasites and it's application for serodiagnosis. 9th International Congress of Parasitology, Chiba, Japan. August

24-28, 1998.

Igarashi,I., X. Xuan, U. Asaba, H. Nagasawa, K. Fujisaki, A. Saito, N. Suzuki and T. Mikami. Expression of Babesia rodhaini antigen and induction of protection by recombinant antigen. 11th Japanese-German Symposium on Protozoan Diseases, Isehara, Japan, August 29-31, 1998.

Ikadai, H., X. Xuan, I. Igarashi, S. Kawai, H. Nagasawa, K. Fujisaki, T. Mikami, Y. Toyoda and N. Suzuki. Characterization of the 48 kDa Babesia caballi merozoite antigen. Isehara, Japan, August 29-31, 1998.

Inoue, N., Yamaguchi, H., Kuriki, K., Nagasawa, H., Suzuki, N. and Hirumi, H. Interleukin 4 is a crucial cytokine in control of parasitemia during *Trypanosoma brucei gambiense* infection in mice. 9th International Congress of Parasitology. Makuhari, Chiba, Japan, 24-28 Aug. 1998.

Inoue, N., Honzako, Y., Hirumi, K., Xuan, X., Agatsuma, T., Nagasawa, H., Mikami, T. and Hirumi, H. Kinetoplast DNA and procyclic acidic repetitive protein A-a (PARP A-a) gene of *Trypanosoma evansi*. RCPMI-Obihiro / OIE-Paris International Symposium on Strategies for Research and Control of SURRA, *Trypanosoma evansi* Infection. Obihiro, Hokkaido, Japan, Aug. 19-22, 1998.

Xuan,X., Igarashi,I., Avarzed,A., Nagasawa, H., Fujisaki, K., Mikami, T. and Suzuki, N. Diagnosis of babesia caballi infection in horses by polymerase chain reaction. 9th International Congress of dParasitology. Makuhari, Chiba, Japan, Aug. 24-28, 1998.

（3）出版物

平成７年

五十嵐郁男、鈴木直義、豚のトキソプラズマ病、獣医伝染病学（第４版、見上彪ら編）近代出版、1995.

長澤秀行、姫野國祐、免疫実験法、第８章　感染実験、３項　寄生虫、編集：高津聖志，滝　伸介、実験医学別冊　バイオマニュアルUPシリーズ、羊土社、1995.

長澤秀行、久枝　一、姫野國祐、ストレス蛋白質と原虫感染防御、生体の科学、46(4): 356-359, 1995.

Nagasawa, H., Hisaeda, H., Igarashi, I., Horiuchi, M., Inoue, N., Himeno, K., and Suzuki, N. Role of Heat Shock Proteins in Protective Immunity against Infection with *Toxoplasma gondii*. Proceedings of the international workshop on Novel Immunological approaches to the control of diseases in animals, 163-172, NIAH, 1995.

平成９年

藤崎幸蔵、外部寄生虫症、鶏病研究会編、カラーマニュアル　鳥の病気　第２版　鶏病研究会、東京、106-109, 1997.

藤崎幸蔵、南米諸国、とくにブラジルとアルゼンチンにおける畜産：現状と今後に関する考察、國分牧衛・藤崎幸蔵編、南米の農業と大豆：その現状と将来、JIRCAS、つくば、7-14, 1997.

豊田　裕、生殖工学の歴史と展望、科学、67(5): 348-352, 1997.

平成10年
五十嵐郁男、トキソプラズマ病、猫の病気、講談社、1998.

五十嵐郁男、鈴木直義、免疫学的検査、　獣医臨床病理学（小野憲一郎・太田亨二・鈴木直義.編）、近代出版、1998.

Inoue, N., Honzako, Y., Hirumi, K., Xuan, X., Agatuma, T., Nagasawa, H., Mikami, T. and Hirumi, H. Kinetolast DNA and procyclic acidic repetitive A-a (PARP A-a) gene of Trypanosoma evansi. J. Protozool. Res. (Proceedings of the Obihiro International Symposium on "Starategies for Research and Control of SURRA, Tryapnosoma evansi Infection", August 19-22, 1998, Obihiro). (In press).

見上　彪、動物用生物学的製剤の現状と未来の展望―２１世紀を見据えた動生剤の方向―，「(社)動物用生物学的製剤協会創立三十周年記念誌」、7-12, 1998.

見上　彪、わが国の口蹄疫発生予防に対する水際防止戦略、特集：世界における口蹄疫の現状と日本の対応、獣医畜産新報、51(8): 641-642, 1998.

見上　彪、２１世紀を見据えた我が国の動物用生物学的製剤の発展、動生協会会報、31(4): 19-27, 1998.

Roles of CD4 + T cells and IFN-γ in protective immunity against *Babesia microti* infection in mice

Running title: PROTECTIVE ROLES OF CD4+ T CELLS AND IFN-γ IN *B. MICROTI* INFECTION

IKUO IGARASHI,[1*] REIKO SUZUKI,[2] SEIJI WAKI,[3] YOH-ICHI TAGAWA,[4] SEYHA SENG,[1] SOTHYRA TUM,[1] YOSHITAKA OMATA,[2] ATSUSHI SAITO,[2] HIDEYUKI NAGASAWA,[1] YOHICHIRO IWAKURA,[4] NAOYOSHI SUZUKI,[1] AND YUTAKA TOYODA[1]

The Research Center for Protozoan Molecular Immunology[1] and Department of Veterinary Physiology,[2] Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan, Gunma Prefectural College of Health Science, Maebashi,[3] Japan and Institute of Medical Sciences, The University of Tokyo, Tokyo,[4] Japan.

* Corresponding author:

Ikuo Igarashi, The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan, Tel. +81-155-49-564, Fax: +81-155-49-5643, E-mail:igarcpmi@obihiro.ac.jp

ABSTRACT

Babesia microti produces a self-limiting infection in mice, and recovered mice are resistant to reinfection. In the present study, the role of T cells in protective immunity against challenge infection was examined. BALB/c mice which recovered from primary infection showed strong protective immunity against challenge infection. In contrast, nude mice which were failed to control the primary infection and were cured with anti-babesial drug did not show protection against challenge infection. Treatment of immune mice with anti-CD4 monoclonal antibody (MAb) diminished the protective immunity against challenge infection, but treatment with anti-CD8 MAb had no effect on the protection. Transfer of CD4+ T cell -depleted spleen cells resulted in higher parasitemia than transfer of CD8+ T cell -depleted spleen cells. A high level of IFN-γ was observed in the culture supernatant of spleen cells from immune mice, and treatment of immune mice with anti-IFN-γ MAb partially reduced the protection. Moreover, no protection against challenge infection was found in IFN-γ-deficient mice. On the other hand, treatment of immune mice with MAbs against IL-2, IL-4 or TNF-α did not affect protective immunity. These results suggest essential requirements for CD4+ T cells and IFN-γ in the protective immunity against challenge infection with *B. microti*.

INTRODUCTION

Babesia microti is an erythrocytic parasite of rodents and has also been shown infect humans (10, 25, 27). Mice infected with *B. microti* produce transient high parasitemias, but they subsequently recover from the acute infection (22, 12). The role of T cells in the resolution of primary infection in mice has been suggested. Congenitally athymic nude mice (4), lethally irradiated, thymectomized mice reconstituted with anti-theta serum-treated bone marrow cells (23) or hamsters administered with anti-lymphocyte serum (37) failed to suppress parasitemia of *B. microti*. Recently it has been demonstrated that CD4+ T cells play an essential role in the resolution of primary infection with *B. microti* (26) and that IFN-γ produced by CD4+ T cells is partially responsible for resolution of primary infection with *B.microti* (12).

After recovery from the primary infection, mice are protected against reinfection with *B. microti*. The spleen appears to play an important role in immunity to *Babesia* (13). Immunity to reinfection with *B. microti* was successfully transferred by immune spleen cells (5, 18, 23). The importance of T cells for the protection against reinfection was demonstrated using anti-theta-treated immune spleen cells (27), Sephadex G-10-adherent spleen cells (19) or T cell clones (9). These results suggest that T cell-mediated immunity plays a significant role in protective immunity against the reinfection with *B. microti* in mice. However, the specific subset of T lymphocytes and mechanism responsible for protective immunity against *B. microti* are not yet known. In the present study, the role of T cells in *B. microti* reinfection was examined in BALB/c mice and BALB/c nude mice. To identify T cell subsets, immune mice were treated with anti-CD4 or anti-CD8 monoclonal antibodies during the course of challenge infection, and

thereafter the subpopulation of T cells responsible for adoptive transfer of immunity was determined. The role of cytokines in protective immunity was also studied by administration of monoclonal antibodies against cytokines or by using IFN-γ-deficient mice.

MATERIALS AND METHODS

Mice: Female BALB/ c mice and BALB/c nu/nu mice were purchased from Clea Japan (Tokyo, Japan). IFN-γ-deficient mice were generated as previously described (31). Male and female IFN-γ-deficient mice were backcrossed to BALB/c for seven generations and maintained by interbreeding heterozygous animals. Homozygous(-/-), heterozygous(+/-), and wild-type littermates (+/+) were identified by isolation of genomic tail DNA by proteinase K digestion and one extraction with TE-saturated phenol. After precipitation with ethanol, the DNA was dissolved in distilled water. An aliquot of the genomic DNA was amplified in a PCR reaction using one sequence within the neomycin cassette (antisense, 5'-ACG TGC ATG GAT CTG CAA CAT GTC-3') and two adjacent sequences of the IFN-g gene (sense, 5'-AAC AGA GGA TGG TTT GCA TCT GGG-3' ; antisense, 5'-AAA GCC AAG ATG CAG TGT GTA GCG-3'). PCR conditions were as follows: one incubation at 94℃ for 4 min, and 40 cycles of 94℃ for 1 min, 66℃ for 2 min, and 72℃ for 3 min. The final incubation was at 72℃ for 7 min, followed by agarose gel separation and ethidium bromide staining of the products. All mice were between 5 and 7 weeks old at the time of the experiment. They were housed in filter-topped autoclaved cages and given autoclaved food and water.

Parasite passage and infection of mice. The Munich strain of *Babesia microti* was maintained by blood passage in mice as previously described (12).

Immune animals were prepared by infecting BALB/c mice with $1x10^7$ parasitized erythrocytes. Mice which resolved infections by 30 days after the primary infection were used for challenge infection, or as immune cell donors. Infected nude and IFN-γ -/- mice, were treated intramuscularly with diazoaminodi-benzamidine diaceturate (Ganaseg, Ciba-Geigy, 1mg/mouse) for 6 consecutive days starting at 30 days postinfection. These drug-cured mice were used for challenge experiments. Challenge infections in mice were initiated by the injection of $1x10^7$ infected erythrocytes. Parasitemia was monitored by examining between 200 and $1x10^4$ erythrocytes in Giemsa-stained thin blood films.

Treatment of mice with monoclonal antibodies. Immune mice were treated intraperitoneally (i.p.) with anti-CD4 (GK1.5) or anti-CD8 (53-6.72) monoclonal antibodies (MAb) which were prepared as previously described by Igarashi et al (12). Half a milligram of either anti-CD4 or anti-CD8 MAb was injected into each mouse for 3 successive days before challenge infection and twice weekly thereafter for the duration of the experiment. Greater than 96% depletion of CD4+ or CD8+ T cells in immune mice was indicated by flow cytometric analysis of spleen cells using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) as previously described (Igarashi et al 1994). Anti-IFN-γ (XMG1.2), anti-IL-2 (S4B6), anti- IL-4 (11B11) and anti-TNF-α (MP6-XT22) MAbs were prepared for neutralization of lymphokines as previously described (12, 36). After parasite inoculation, mice were injected intraperitoneally with 1 mg of anti-IFN-γ, anti-IL-2, or anti-IL-4 MAb or 2 mg of anti-TNF-α MAb for 3 consecutive days and thereafter every other day until day 10. Control mice were treated with 0.5, 1 or 2 mg of normal rat IgG (Caltag Laboratories, South San

Francisco, CA).

Passive transfer of immune spleen cells and immune serum. Immune spleen cells were taken from immune donors 2 to 3 weeks after resolution of primary infection. CD4+ or CD8+-depleted spleen cells were prepared by treating immune mice with anti-CD4 or anti-CD8 MAb. Depletion of CD4+ or CD8+ cells was confirmed by flow cytometry as described above. BALB/c naive mice were immunized by adoptive transfer of spleen cells ($5x10^7$ cells/mouse) and challenged with $1x10^7$ infected erythrocytes.

Immune sera were collected from immune mice prepared as described above. The antibody titer in the pooled sera was 1:4096, which was determined by the indirect immunofluorescent antibody test with slight modification as previously described (35). Naive BALB/c mice were injected intravenously (i.v.) with 0.5 ml immune serum or normal serum on days 8, 5, and 2 before infection and challenged with $1x10^7$ infected erythrocytes. The control group of mice received an identical regimen of saline.

Detection of IFN-γ and TNF-α in immune mice. IFN-γ activity was determined as described by Igarashi et al (12). Briefly, spleen cells from infected mice were cultured with *B. microti* antigen for 72 hours in RPMI 1640 (ICN Biomedicals, OH) containing 5% fetal bovine serum (FBS), 100 units penicillin/ml and 100 mg of streptomycin/ml in 6-well plates containing $25x10^6$ cells /well. Culture supernatants were harvested at 72 hr and stored at-80℃ until use. Concentrations of IFN-γ and TNF -α activities in plasma were measured with ELISA kits (Endogen, Cambridge, MA) according to the manufacturer's instructions.

Statistics. All statistical analyses were performed by using unpaired Student's *t* test.

RESULTS

The role of T cells in protection against challenge infection with *B. microti*. The effect of T cells on resistance to reinfection with *B. microti* was examined by comparing parasitemias in BALB/c and nude mice. The infection with *B. microti* in BALB/c was self-limiting and was controlled by 30 days postinfection. In contrast, nude mice failed to resolve acute infection, and persistent parasitemias of between 40-70% were observed until 30 days postinfection. After treatment with Ganaseg for 6 days from day 30 after infection, all parasites had disappeared in infected nude mice. Fifty days after primary infection, both BALB/c and nude mice were challenged with $1x10^7$ *B. microti*-infected erythrocytes and the percent parasitemia was determined (Fig. 1). BALB/c mice had strong protection against challenge infection. They showed only latent parasitemias, which reached a very low level with a maximum individual parasitemia of 0.15 %. However, nude mice showed significantly higher parasitemia on day 8 after challenge infection and thereafter developed high parasitemias between 30-60% by the 30th day. These results demonstrate that T cells are essential for protective immunity against reinfection with *B. microti* in mice.

Effects of anti-CD4 treatment in immune mice. Immune BALB/c mice were treated with anti-CD4 or -CD8 MAb to determine the T cell subset required for the protective immunity against *B. microti* challenge infection. On day 0 (fifty days after primary infection), mice were challenged with i.p injection of $1x10^7$ *B. microti*-infected erythrocytes. $CD4^+$ T cell-depleted mice had an average peak parasitemia of 27.2% on day 16 after challenge infection, and demonstrated a significantly higher parasitemia than control mice from day 6 to 24 postinfection (Fig. 2A). In contrast, $CD8^+$ T cell-

depleted immune mice showed a similar level of parasitemia to control mice, with a maximum parasitemia of 0.15%. The levels of *Babesia*-specific IgG antibodies in serum were compared in CD4+ and CD8+ T cell-depleted mice. All mice showed high antibody titers (1:4096) before challenge infection. High IgG titers (1:4096) were maintained in the control and CD8+-depleted mice after challenge infection. CD4+-depleted mice tended to show lower IgG titers than control and CD8+-depleted mice from 12 to 24 days after challenge infection, but differences were not significant (Fig. 2B) .

Effect of transfer of spleen cells and immune serum on the challenge infection with *B. microti*. The role of CD4+ T cells was also examined by passive transfer of spleen cells from immune donors. BALB/c naive mice received $5x10^7$ immune or normal spleen cells and were challenged with $1x10^7$ infected erythrocytes on the same day of cell transfer. BACB/c mice which received immune spleen cells developed parasitemias slowly and showed significantly lower peak parasitemia than the mice which received normal spleen cells from day 8 to 30 after challenge infection, and parasites were rapidly cleared (FIG. 3A). However, BACB/c mice which received normal spleen cells showed significantly higher peak parasitemia, and the clearance of parasites from the circulation was delayed. Mice which received CD8+-depleted spleen cells resolved their infections with similar kinetics to those that received immune spleen cells. However, mice which received CD4+-depleted spleen cells showed significantly higher parasitemia from day 6 to 16 after challenge infection, and the clearance of parasites from the circulation was also delayed (Fig. 3B). These results strongly suggested that CD4+ T cells are required for protective immunity against reinfection with *B. microti* in mice.

To determine the protective effect of antibody on challenge infection with *B. microti*, mice were injected i.v. with 0.5 ml of serum from *B. microti* immune mice before challenge infection. All of the four injected mice showed high antibody titers (1:4096) after three injections of immune serum. Higher parasitemias were observed in normal serum recipients than in immune serum recipients after peak parasitemia, but the differences were not significant except on day 22 after challenge infection (Fig. 3C).

Production of IFN-γ and TNF-α. The production of IFN-γ by spleen cells, and of TNF-α in serum was examined in mice challenged with *B. Microti*. Spleen cells had to be stimulated with parasite antigen to release IFN-γ in culture. The cells obtained from mice 2 days after parasite inoculation produced IFN-γ most efficiently (Fig. 4A), and the cells became refractory to IFN-γ induction by 10 days after challenge infection. On the other hand, a first minor peak of TNF-α was observed in serum 8 days after reinfection and a second major peak of TNF-α was observed in serum between days 16 and 28. (Fig. 4B).

Effect of anti-cytokine MAbs on the course of challenge infection with *B. microti*. Immune mice were challenged with *B. microti* and given neutralizing MAbs to analyze the participation of cytokines in the protective immunity. Mice treated with anti-IFN-γ MAb showed higher parasitemias than control mice treated with anti-TNF-α MAb or rat IgG (Fig. 5). Treatment of mice with Mabs against IL-2 or IL-4 did not cause an increase in parasitemia, as compared with treatment with normal rat IgG (data not shown).

The course of infection of *B. microti* in IFN-γ -deficient mice. Since the treatment of immune mice with anti-IFN-γ suggested the possible role of IFN-γ

in protective immunity, IFN-γ-deficient mice were used to further examine the role of INF -γ in protective immunity against *B. microti* infection. IFN-γ -/- mice failed to resolve the primary infection (data not shown). IFN-γ -/- mice were then treated with babesiacidal drug and challenged with *B. microti*. Wild-type mice showed strong protection against challenge infection. They showed only latent parasitemias which reached a very low level with a maximum individual parasitemia of 0.28 %. However, IFN-γ -/- mice showed significantly higher peak parasitemia on day 10 after challenge infection. Although parasitemia dropped once to a low of 15% on day 24, it increased again thereafter until the end of the experiment (Fig. 6). These results strongly suggested that IFN-γ is an essential mediator of protective immunity against challenge infection with *B. microti*.

DISCUSSION

In the present study, the importance of T cells against reinfection with *B. microti* was clearly demonstrated by using nude mice. BALB/c mice recovered from primary infection showed strong immunity to challenge infection, with maximum parasitemia of <0.15%, while nude mice which were infected and treated with anti-babesial drug did not develop such immunity, and showed very high parasitemia. The importance of T cells for the protective immunity against reinfection with *B. microti* has been suggested in earlier reports. For example, Ruebush et al (24) reported that delayed type hypersensitivity (DTH) response occurs in parallel with resistance against *B. microti* (24). Transfer of immune spleen cells to recipient mice resulted in lower peak parasitemia after challenge infection than transfer of normal spleen cells, and treatment with anti-theta serum abrogated the protective immunity of immune spleen cells (23).

Meeusen et al. (19) demonstrated adoptive transfer of immunity against reinfection with T-enriched spleen cells. Our findings agree with those previous findings and give clearer evidence of the absolute requirement for T cells in the protective immunity against *B. microti* infection.

In rodent malaria infection, the requirement for CD4+ T cells in protective immunity has been demonstrated for infection with *P. berghei* (36), *P. chabaudi* (1, 2, 15, 30) and *P. yoelii* (14, 34). In *B. microti* infection, protection was achieved by the passive transfer of in vitro-generated CD4+ T cell clones. However, the protection was partial and short-lived (9). In the present study, immune BALB/c mice were treated with either anti-CD4 or anti-CD8 MAb in order to identify the T cell subpopulation responsible for protective immunity against reinfection with *B. microti.* Depletion of CD4+ T cells decreased the protective immunity in immune mice, while depletion of CD8+ T cells did not affect the protective immunity. The requirement for CD4+ T cells was also demonstrated by the adaptive transfer of CD4-enriched spleen cells to naive mice. These results suggested that CD4+ T cell-mediated immunity plays a major role in the protection against reinfection with *B. microti.*

To examine the mechanism by which CD4+ T cells provide protective immunity, the effect of antibody was studied because the Th2 cells among the CD4+ T cells act as helper cells for antibody production (20). Antibody-mediated immunity was suggested to be important in the protection against *B. microti* in hamsters (11, 37) and mice (16). In the present study, high antibody titers were observed both in mice treated with anti-CD4 and in those treated with anti-CD8 MAb during challenge infection, although a decrease of protective immunity was observed only in mice treated with anti-CD4 MAb.

Protective immunity could not be conferred to naive mice with passive transfer of immune serum. In addition, treatment of immune mice with anti-IL-4 MAb did not affect the protective immunity. Cavacini et al (3) demonstrated that B cell-deficient mice could control primary infection with *B. microti*. Taken together, the results of our experiments suggest that antibody does not play a major role in the protective immunity against reinfection in mice.

The role of IFN-γ in the protective immunity against reinfection was examined in the present study. The importance of IFN-γ has been shown in *P. chabaudi* infection with neutralizing MAb treatment (17, 28) and a more profound effect of IFN-γ was observed with IFN-γ knockout mice (33). In *B. microti* infection, the administration of natural human interferon alpha to mice inhibited development of parasitemia (21). Our previous study showed that IFN-γ was detected in culture 4 to 5 days prior to the peak parasitemia, and that IFN-γ produced by CD4+T cells is responsible for the resolution of the primary infection (12). In the present study, IFN-γ was detected in cultures of immune spleen cells as early as day 2, and the amount of IFN-γ detected during challenge infection was much higher than that detected during primary infection. The higher IFN-γ production after challenge may contribute to the strong protective immunity. Mice treated with anti-IFN-γ MAb showed higher peak parasitemia compared to untreated mice or to the anti-TNF-α MAb-treated group. However, the decrease of protective immunity caused by anti-IFN-γ MAb treatment was not as great as that caused by anti-CD4 MAb. We suspected that the effect might have been partial because treatment with MAb was not sufficient to completely deplete the IFN-γ activity in mice. Therefore, we infected IFN-γ-deficient mice with *B. microti* after chemotherapy and observed a more profound effect of

IFN-γ on the protective immunity. IFN-γ-deficient mice had higher parasitemia than wild-type controls and could develop the protective immunity. Our present results indicate that IFN-γ plays an important role in the protective immunity against reinfection with *B. microti*, and suggest that Th1 cells are apparently involved in the protective immunity against *B. microti*.

Our results are not in agreement with those of Hanafusa's report (9) in which there was no direct correlation between IFN-γ production in vitro and protective activity in vivo. The difference in the role of IFN-γ in protective immunity may simply be due to the different strains of parasites used in the experiments (16). Alternatively, IFN-γ alone may not mediate all the effects of CD4+ cells in protective immunity. As IFN-γ can activate macrophages or the production of other cytokines, additional factors such as tumor necrosis factor, reactive oxygen intermediates or IL-12 may be involved in the control and elimination of parasites, as has been suggested in malaria (6,7, 29, 32, 38). TNF-α levels were low during the early phase of reinfection, and were gradually increased during later stages of reinfection, at which time a decrease of parasitemia was observed in IFN-γ-deficient mice. Therefore, TNF-α may be responsible to some extent for the decrease of parasitemia. Further studies will be necessary to clarify the role of CD4+ T cells in protective immunity against *B. microti*.

ACKNOWLEDGEMENTS

We thank Toshikazu Shirahata for supplying the MAb against TNF-α.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan.

REFERENCES

1. Brake, D. A., C. A. Long, and W. P. Weidanz. 1988. Adoptive protection against *Plasmodium chabaudi adami* malaria in athymic nude mice by a cloned cell line. J. Immunol. **140**:1989-1993.

2. Cavacini, L. A., C. A. Long, and W. P. Weidanz. 1986. T-cell immunity in murine malaria: adoptive transfer of resistance to *Plasmodium chabaudi adami* in nude mice with splenic T cells. Infect. Immun. **52**:637-643.

3. Cavacini, L. A., L. A. Parke, and W. P. Weidanz. 1990. Resolution of Acute malarial infections by T cell-dependent non-antibody-mediated mechanisms of immunity. Infect. Immun. **58**:2946-2950.

4. Clark, I. A., and A. C. Allison. 1974. *Babesia microti* and *Plasmodium berghei yoelii* infection in nude mice. Nature **252**:328-329.

5. Clark, I.A., A. C. Allison, and F. E. Cox. 1976. Protection of mice against *Babesia* and *Plasmodium* with BCG. Nature **259**:309-311.

6. Clark, I. A., N. H. Hunt, G .A. Butcher and W. B. Cowden. 1987. Inhibition of murine malaria (*Plasmodium chabaudi*) *in vivo* by recombinant interferon-γ or tumor necrosis factor, and its enhancement by butylated hydroxyaniole. J. Immunol. **139**: 3493-3496.

7. Dockrell, H.M., and J. H. L. Playfair. 1983. Killing of blood-stage murine malaria parasites by hydrogen peroxide. Infect Immun. **39**:456-459.

9. Hanafusa, Y., M. Onuma, and T. Kamiyama. 1998. Partial protection of severe combined immunodeficient mice against infection with Babesia microti by in vitro-generated CD4+ T cell clones. J. Vet. Med. Sci. **60**:401-404.

10. Healy, G., and M. Ristic. 1985. Human babesiosis, p209-225. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Boca Raton, Fl.

11. Hu, R., M-T. Yeh, K. E. Hyland, and T. N. Mather. 1996. Experimental *Babesia microti* infection in golden hamsters: Immunoglobulin G response and recovery from severe hemolytic anemia. J. Parasitol. **82**: 728-732.

12. Igarashi, I., S. Waki, M. Ito, Y. Omata, A. Saito, and N. Suzuki. 1994. Role of CD4+ T cells in the control of primary infection with *Babesia microti* in mice. J. Protozool. Res. **4**:164-171.

13. Irvin, A. D., E. R. Young, G. D. Osborne, and L. M. A. Francis. 1981. A comparison of *Babesia* infection in intact, surgically splenectomized, and congenitally asplenic (Dh/+) mice. Int. J. Parasitol. **11**:251-255.

14. Jayawardena, A. N., D. B. Murphy, C. A. Janeway, and R. K. Gershon. 1982. T cell-Mediated mediated immunity in malaria. I. The Ly phenotype of T cells mediating resistance to *Plasmodium yoelii.* J. Immunol. **129**:377-381.

15. Langhorne, J., B. Simon-Haarhaus, and S. J. Meding. 1990. The role of CD4+ T cells in the protective immune response to *Plasmodium chabaudi* in vivo. Immunol. Lett. **25**: 101-107.

16. Matsubara, J., M. Koura, and T. Kamiyama. 1993. Infection of immunodeficient mice with a mouse-adapted substrain of the gray strain of *Babesia microti.* J. Parasitol. **79**:783-786.

17. Meding, S.J., S. C. Cheng, B. Simon-Haarhaus, and J. Langhorne. 1990. Role of gamma interferon during infection with *Plasmodium chabaudi chabaudi.* Infect Immun. **58**:3671-3678.

18. Meeusen, E., S. Lloyd, and E. J. Soulsby. 1984. *Babesia microti* in mice. Adoptive transfer of immunity with serum and cells. Aust. J. Exp. Biol. Sci., **6 2**:551-566.

19. Meeusen, E., S. Lloyd, and E. J.Soulsby.1984. *Babesia microti* in mice. Subpopulations of cells involved in the adoptive transfer of immunity with immune spleen cells. Aust. J. Exp. Biol. Med. Sci. **6 2**:567-75.

20. Mosmann, T. R. and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7**:145-73.

21. Orinda, G. O., D. J. Waltisbuhl, A. S. Young, and I. G. Wright. 1994. Low doses of natural human interferon alpha inhibit the development of *Babesia microti* infection in BALB/c mice. Vet. Parasitol. **5 3**:53-58.

22. Ruebush, M. J., and W. L. Hanson. 1979. Susceptibility of five strains of mice to *Babesia microti* of human origin. J. Parasitol. **6 5**:430-433.

23. Ruebush, M. J., and W. L. Hanson. 1980. Transfer of immunity to *Babesia microti* of human origin using T lymphocytes in mice. Cell. Immunol. **5 2**:255-265.

24. Ruebush, M. J., E. H. Troutman, and D. A. Kennedy. 1986. Delayed-type hypersensitivity to *Babesia microti*-infected erythrocytes in mice. Cell. Immunol. **9 8**: 289-299.

25. Shih, C.M., L.P. Liu, W. C. Chung, S. J. Ong, and C.C. Wang. 1997. Human babesiosis in Taiwan: asymptomatic infection with a *Babesia microti*-like organism in a Taiwanese woman. J. Clin. Microbiol. **3 5**:450-454.

26. Shimada, T., S. Shikano, R. Hashiguchi, N. Matsuki, and K. Ono. 1996. Effects of depletion of T cell subpopulations on the course of infection and anti-parasite delayed type hypersensitivity response in mice infected with *Babesia microti* and *Babesia rodhaini*. J. Vet. Med. Sci. **58**:343-347.

27. Spielman, A., P. Etkind, J. Piesman, T. K. H. Ruebush, D. D. Juraneck, and M. S. Jacobs. 1981. Resevoir host of human babesiosis on Nantucket Island. Am. J. Trop. Med. Hyg. **30**:560-566.

28. Stevenson, M. M., M. F. Tam, M. Belosevic, P. H. van der Meide, and J. E. Podoba. 1990. Role of endogenous gamma interferon in host response to infection with blood-stage *Plasmodium chabaudi* AS. Infect. Immun. **58**:3225-3232.

29. Stevenson, M. M., M. F. Tam, S. F. Wolf, and A. Sher. 1995. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via a nitric oxide-dependent mechanism. J. Immunol. **155**:2545-2556.

30. Süss, G., K. Eichmann, E. Kury, A. Linke, and J. Langhorne. 1988. Roles of CD4- and CD-8 bearing T lymphocytes in immune response to the erythrocytic stages of *Plasmodium chabaudi*. Infect. Immun. **56**:3081-3088.

31. Tagawa, Y., K. Sekikawa, and Y. Iwakura. 1997. Suppression of concanavalin A-induced hepatitis in IFN-$\gamma^{-/-}$ mice, but not in TNF-$\alpha^{-/-}$ mice: role for IFN-γ in activating apoptosis of hepatocytes. J. Immunol. **59**:1418-1428.

32. Traverne, J., J. Tavernier, W. Fiers, and J. H. L. Playfair. 1987. Recombinant tumor necrosis factor inhibits malaria parasites *in vivo* but not *in vitro*. Cli. Exp. Immunol. **67**: 1-4.

33. van der Heyde, H.C., .B. Pepper, J. Batchelder, F. Cigel, and W.P. Weidanz.1997. The time course of selected malarial infections in cytokine-deficient mice. Exp Parasitol. **85**:206-213.

34. Vinetz, J. M., S. Kumar, M. F. Good, B. J. Fowlkes, J. A. Berzofsky, and L. H. Miller.1990. Adoptive transfer of CD8 + T cells from immune animals does not transfer immunity to blood stage *Plasmodium yoelii malaria.* J. Immunol. **44**:1069-1074.

35. Waki, S., and M. Suzuki. 1974. Development and decline of antiplasmodial indirect fluorescent antibodies in mice infected with *Plasmodium berghei* (NK65) And treated with drugs. Bull. World Health Organ. **50**:521-526.

36. Waki, S., K. Uehara, K. Kanbe, M. Suzuki, and H. Nariuchi. 1992. The role of T cells in pathogenesis and protective immunity to murine malaria. Immunology **75**:646-651.

37. Wolf, R. E. 1974. Effect of anti-lymphocyte serum and splenectomy on the resistance to *Babesia microti* infection in hamsters. Clin. Immunol. Immunopathol. **2**:381-394.

38. Yoshimoto, T., T. Yoneto, S. Waki, and H. Nariuchi. 1998. Interleukin-12-dependent mechanisms in the clearance of blood-stage murine malaria parasite *Plasmodium berghei* XAT, an attenuated variant of *P. berghei* NK65. J Infect Dis. **177**:1674-1681.

Figure legends

FIG. 1. Comparison of B. *microti* infection in BALB/c (○) and nude mice (□). BALB/c mice recovered from primary infection or nude mice cured with chemotherapy were challenged with 1×10^7 parasites. Each value represents the mean parasitemia ± SME for 6 mice. An asterisk indicates a significant difference ($p<0.05$) between two groups.

FIG. 2. Effect of MAb treatment on the course of infection (A) and antibody titers (B) in mice challenged with *B. microti*. Mice recovered from primary infection were challenged with 1×10^7 parasites. Untreated control (□), treated with anti-CD4 MAb (○), treated with anti-CD8 MAb (△). Each value represents mean parasitemia ± SME for 4 mice. An asterisk indicates a significant difference ($p<0.05$) between anti-CD4 MAb- treated and control groups.

FIG. 3. Effect of spleen cell transfer and immune serum on the protective immunity against *B. microti* infection in mice. A) Mice received spleen cells of mice recovered from primary infection (○) or normal mice (□). B) Mice received spleen cells from mice treated with anti-CD4 MAb (○), mice treated with anti-CD8 MAb (△), or non-treated mice (□). C) Mice received anti-*B.microti* immune serum (△) or normal mouse serum (□). Each value represents mean parasitemia ± SME. An asterisk indicates a significant difference ($p<0.05$).

FIG. 4. IFN-γ production in spleen cell cultures (A) and TNF-α production in serum of mice during the course of challenge infection with *B.microti*. Spleen cells from immune mice were cultured with *B. microti* antigen and IFN-γ concentrations were measured in supernatants at 72-h.

FIG. 5. Effect of MAb against IFN-γ (△), TNF-α (○) or normal rat IgG (□). Mice recovered from primary infection were challenged with 1×10^7 parasites on day 0 and in vivo treatment was started on the same day. Each value represents the mean parasitemia ± SME for 5 mice. An asterisk indicates a significant difference ($p<0.05$) between anti-IFN-γ MAb- treated and control groups.

FIG. 6. Time course of B. Microti infection in IFN-γ-deficient mice (□) and wild-type control mice (○). Wild-type mice recovered from primary infection or IFN-γ-deficient mice cured with chemotherapy were challenged with 1×10^7 parasites. Each value represents the mean parasitemia ± SME for 5 mice. An asterisk indicates a significant difference ($p<0.05$) between two groups.

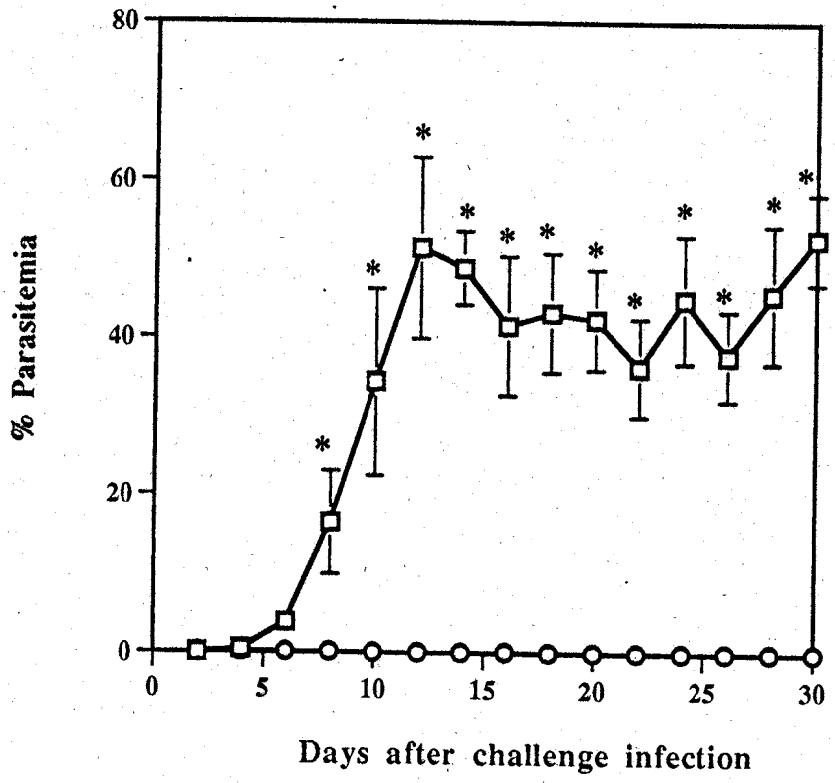

80
60
40
20
0
% Parasitemia
0
5
10
15
20
25
30
Days after challenge infection

Fig 1

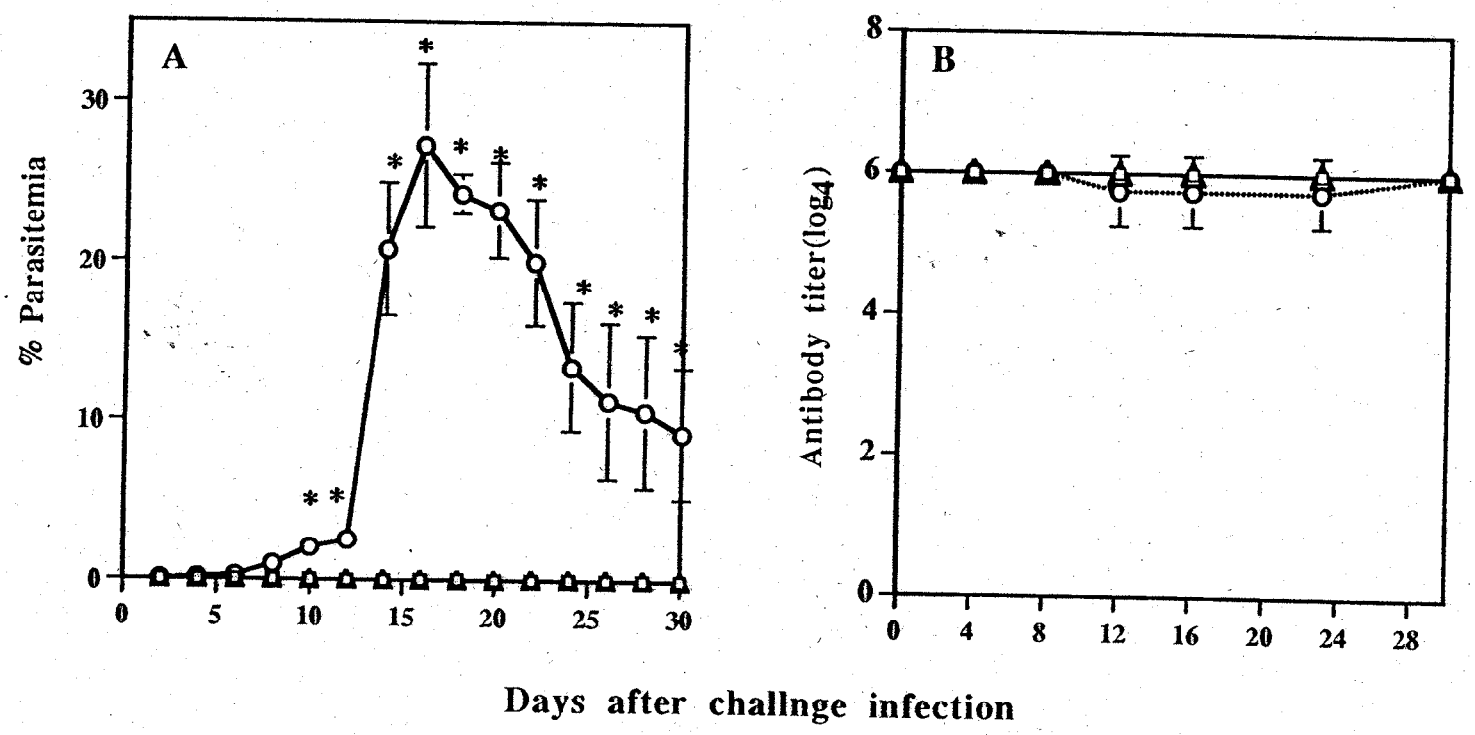

A
% Parasitemia
30
20
10
0
0
5
10
15
20
25
30
B
Antibody titer(log4)
8
6
4
2
0
0
4
8
12
16
20
24
28
Days after challnge infection

Fig. 2

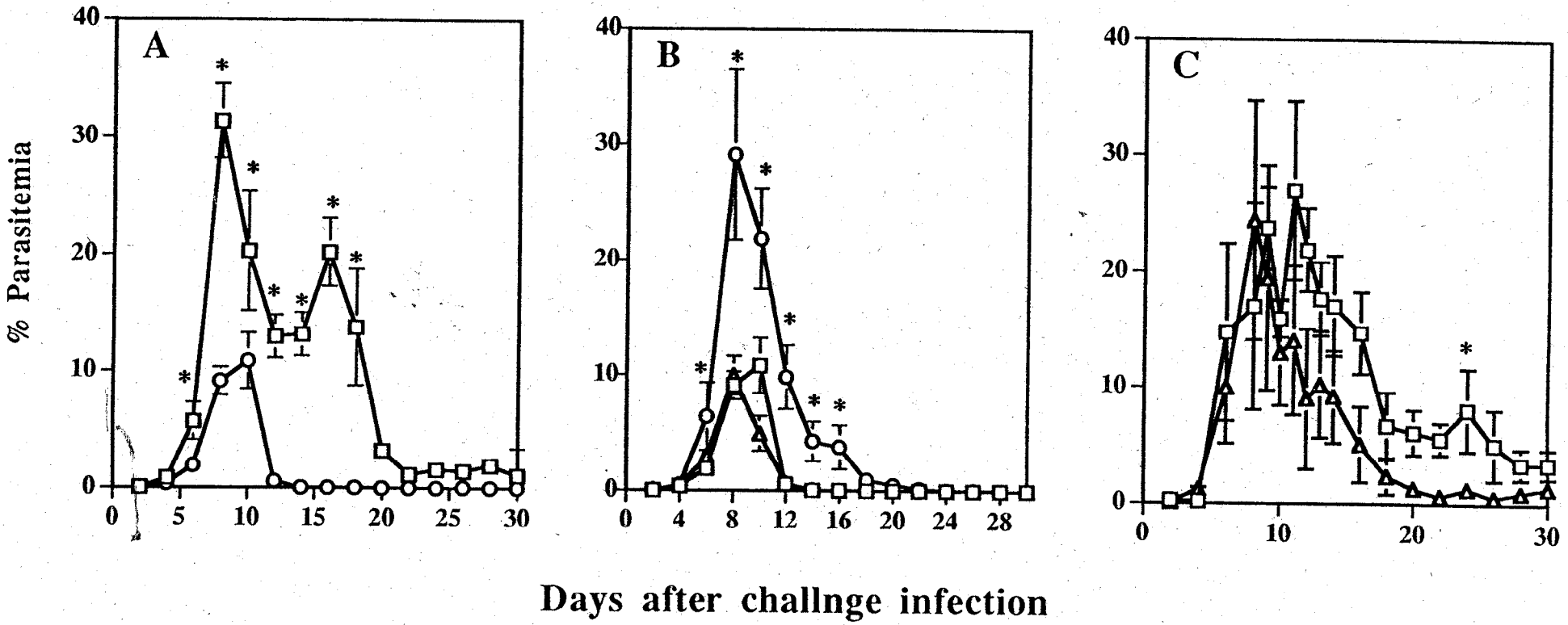
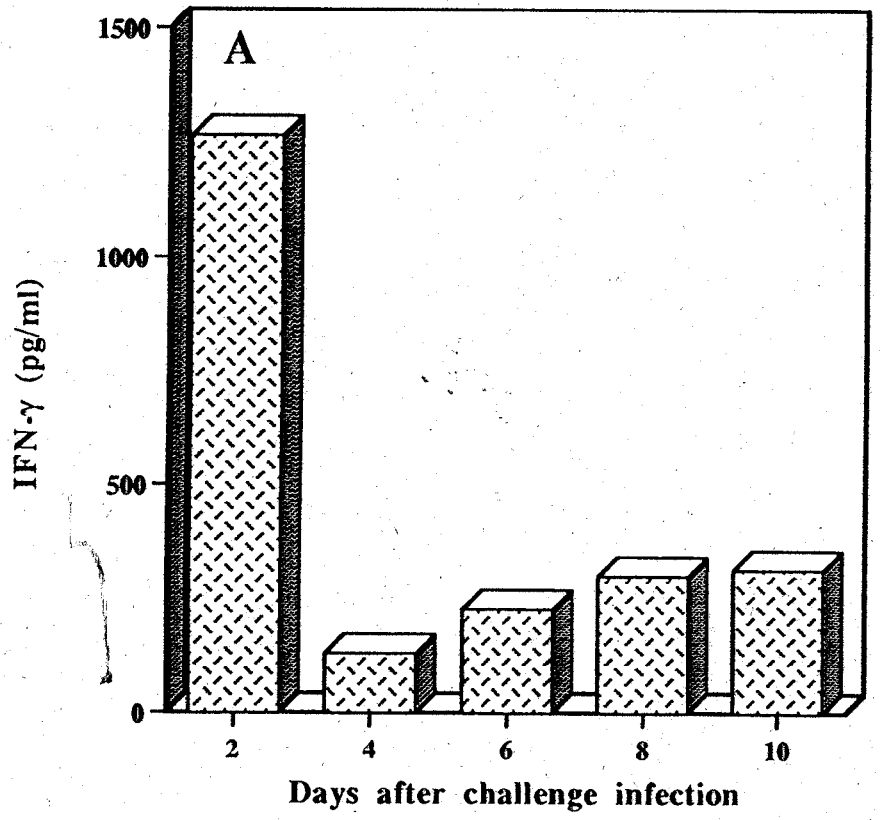


Fig. 3


A
1500
1000
500
0
IFN-γ (pg/ml)
2
4
6
8
10
Days after challenge infection

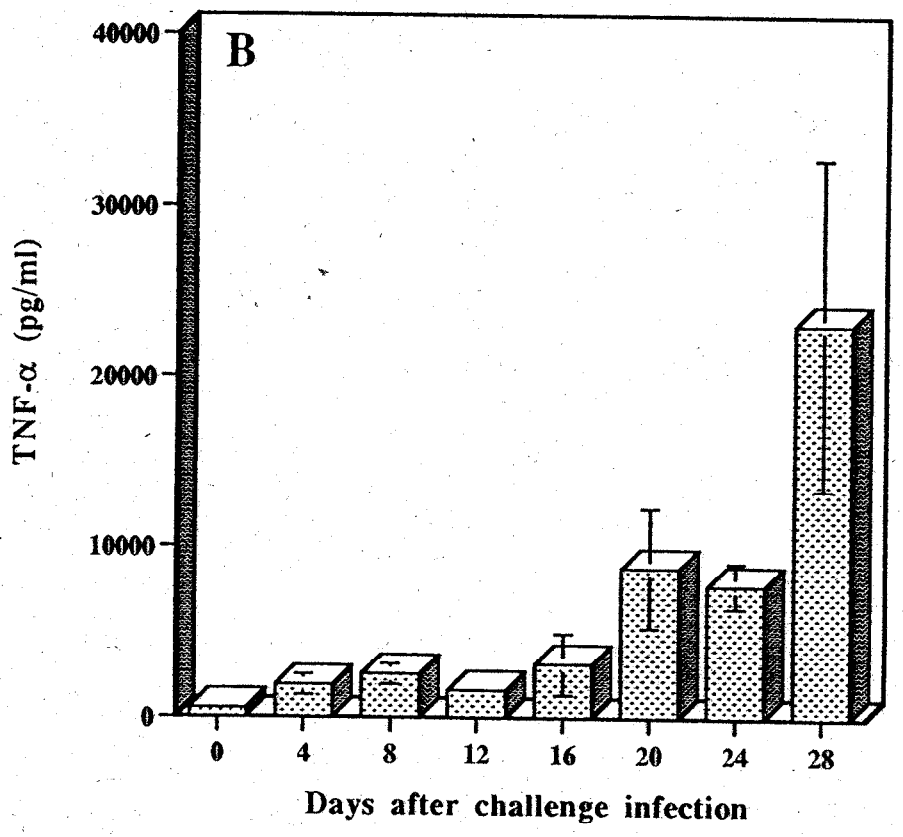

B
40000
30000
20000
10000
0
TNF-α (pg/ml)
0
4
8
12
16
20
24
28
Days after challenge infection

Fig. 4

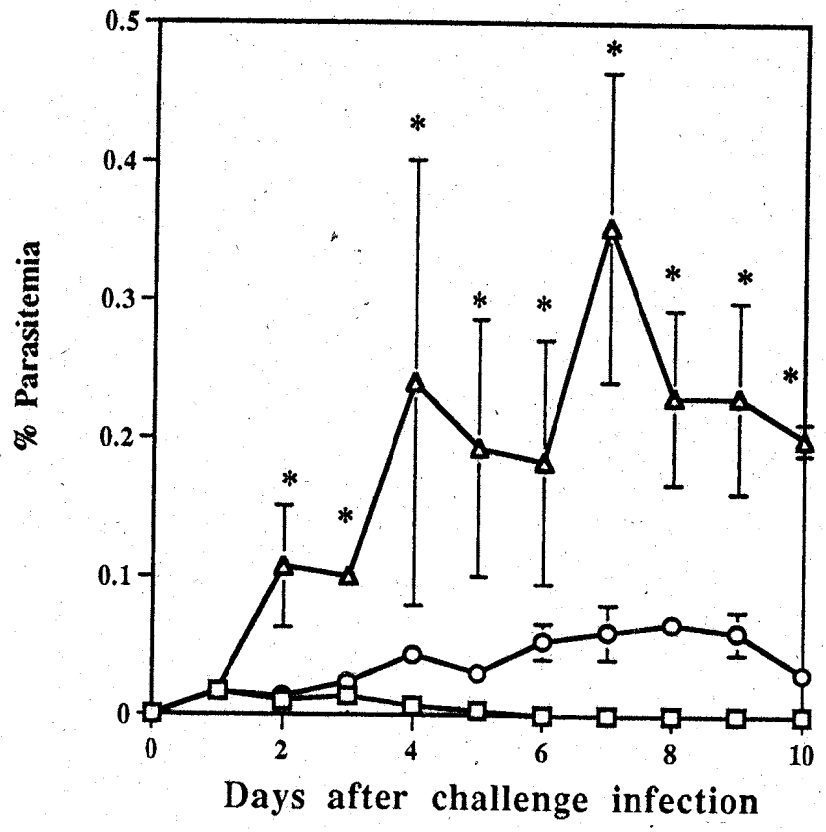

0.5
0.4
0.3
0.2
0.1
0
% Parasitemia
0
2
4
6
8
10
Days after challenge infection

Fig. 5

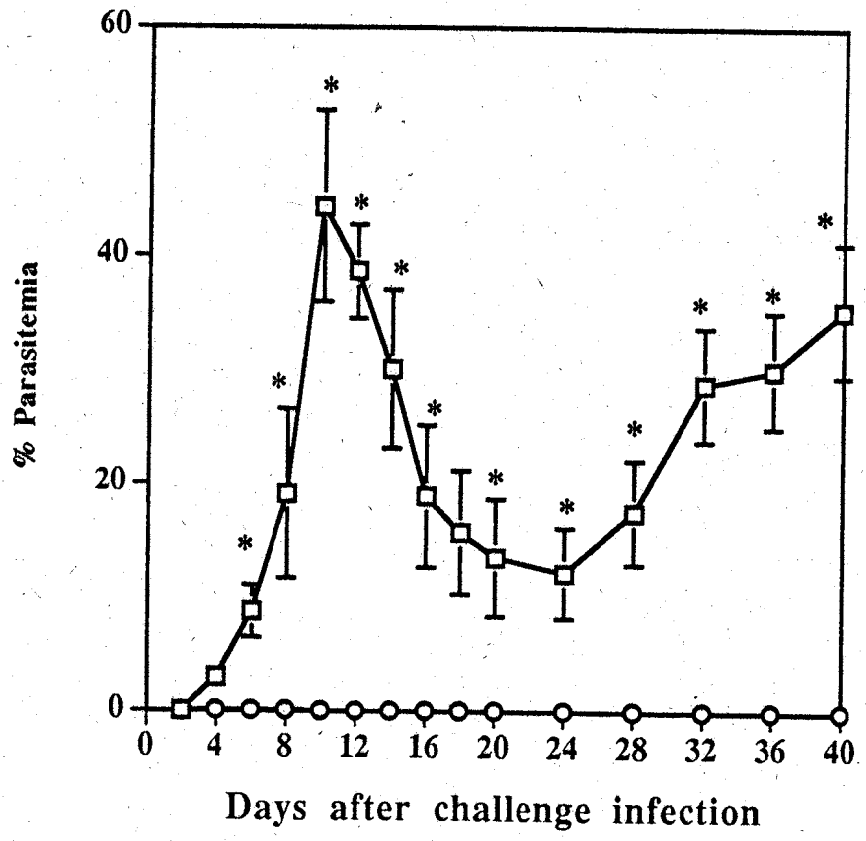

60
40
20
0
% Parasitemia
0
4
8
12
16
20
24
28
32
36
40
Days after challenge infection

Fig. 6

Parasitol Res (1999) 85: 171–175

ORIGINAL PAPER

Satoru Kawai · Ikuo Igarashi · Avarzed Abgaandorjiin
Hiromi Ikadai · Yoshitaka Omata · Atsushi Saito
Hideyuki Nagasawa · Yutaka Toyoda · Naoyoshi Suzuki
Hajime Matsuda

Tubular structures associated with *Babesia caballi* in equine erythrocytes in vitro

Received: 10 August 1998 / Accepted: 17 August 1998

Abstract In-vitro-propagated *Babesia caballi* parasites were examined by scanning and transmission electron microscopy. Many small pores were observed over the entire surface of infected erythrocytes on scanning electron microscopy, and on transmission electron microscopy these small pores were found to be openings of tubular structures. By the examination of a number of infected cells the tubular structures were found to be connected with the parasite, and this observation might indicate that the tubular structures arose from the edge of the parasite and terminated at an invagination on the surface of the erythrocyte. These findings suggest that intraerythrocytic stages of *B. caballi* come into direct contact with culture medium.

Introduction

Babesia caballi and *B. equi*, members of the phylum Apicomplexa, are intraerythrocytic protozoa that cause piroplasmosis in the horse, donkey, mule, and zebra (Purnell 1981). Piroplasmosis is characterized by fever, anemia, anorexia, and, during the later stages of the disease, icterus and hemoglobinuria. Equine piroplasmosis is endemic in tropical and subtropical regions of Central America, South America, Africa, Asia, and southern Europe (Knowles 1988). Due to the international horse trade there is a constant risk for the introduction of *Babesia* carrier animals into nonendemic areas such as North America, Australia, and Japan (Böse and Hentrich 1994; Knowles 1996).

As stated by Levine (1971), the genus *Babesia* can be divided into two groups: large types (more than 3 μm long) and small types (less than 3 μm long). According to Riek (1968), merozoites of *B. caballi* occur in erythrocytes as pyriform bodies measuring 2.15–4.0 μm in length and about 2.0 μm in width and as spherical or ameboid bodies measuring 1.5–3.0 μm in diameter. The pyriform parasites frequently occur in pairs at acute angles to each other. Therefore, *B. caballi* can be classified as a large-type *Babesia*, like *B. bigemina* (Purnell 1981).

In contrast to *B. caballi*, *B. equi*, whose merozoites measure about 2.0 μm in length and about 1.0 μm in width, belongs to the small-type *Babesia* group (Mahoney et al. 1977). Holbrook et al. (1968) have described the intraerythrocytic reproduction of *B. equi*, in which a "Maltese-cross arrangement" of merozoites occurs. This arrangement does not occur in the case of *B. caballi*. Thus, *B. caballi* and *B. equi* differ in size, shape, and development and can therefore easily be distinguished from one another in cases of double infection (Holbrook et al. 1968).

Recently, an in vitro *B. caballi* culture system for antigen production was developed (Bhushan et al. 1991; Avarzed et al. 1997). Relatively higher levels of parasitemia can be achieved in this in vitro system as compared with animal infection. Furthermore, the immune system affects the observed stages of parasites, whereas in the in vitro system, effects of the immune system are avoided. The rapid proliferation of in-vitro-cultured *Babesia* parasites enables the study not only of the fine structure of the parasite but also of its modes of replication (Droleskey et al. 1993).

S. Kawai (✉) · H. Matsuda
Department of Medical Zoology, Dokkyo University School of Medicine, Mibu, Tochigi 321-0239, Japan
e-mail: skawai@dokkyomed.ac.jp, Tel.: +81-282-87-2134, Fax: +81-282-86-6431

I. Igarashi · A. Abgaandorjiin · H. Ikadai · H. Nagasawa
Y. Toyoda · N. Suzuki
Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

Y. Omata · A. Saito
Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

Development of the intraerythrocytic stage of *B. caballi* has been documented by light microscopy, and primary reports on the fine structure of the intraerythrocytic stages of *B. caballi* have been published (Simpson et al. 1963, 1967; Holbrook et al. 1968). These reports include illustrations of the morphological features of merozoites, trophozoites, and dividing parasites. However, detailed descriptions of the ultrastructural changes in *B. caballi*-infected erythrocytes remain incomplete. Using scanning and transmission electron microscopy, the present study elucidated some morphological characteristics of equine erythrocytes infected with *B. caballi* and revealed the development of tubular structures in vitro.

Materials and methods

In vitro cultivation of *Babesia caballi*

The USDA strain of *B. caballi* was grown in vitro by the method of Avarzed et al. (1997). The parasite was cultured in equine erythrocytes [10% (v/v) hematocrit] in buffered RPMI 1640 medium (Dainippon Pharmaceutical Co., Ltd., Osaka) supplemented with 40% equine serum, L-glutamine, and antibiotics. Cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in humidified air. Development of the parasite in vitro was monitored by microscope observation of Giemsa-stained thin smears until the level of parasitemia reached about 12%.

Electron microscopy procedures

For scanning electron microscopy, blood specimens were fixed for 2 h in 2.5% (v/v) glutaraldehyde buffered with 0.1 *M* phosphate buffer (pH 7.4) at 4 °C. They were postfixed in 1% (w/v) osmium tetroxide for 1 h and then spread onto poly-L-lysine-coated coverslips (Matsunami Glass, Japan). They were then dehydrated in a graded ethanol series and dried in a JEOL JFD-300 freeze-drier (JEOL, Tokyo, Japan). Specimens were mounted on aluminum stubs, sputter-coated with gold, and observed and micrographed in a Jeol SC-630 scanning electron microscope.

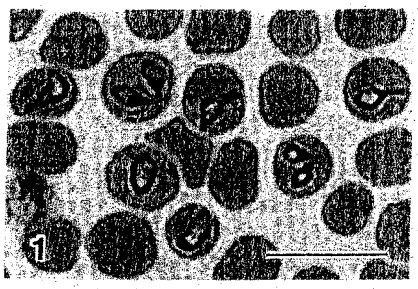
Specimens for transmission electron microscopy were fixed for 2 h in 2.5% (v/v) glutaraldehyde buffered with 0.1 *M* phosphate buffer (pH 7.4) at 4 °C They were postfixed in 1% (w/v) osmium tetroxide for 1 h, then dehydrated in a graded series of alcohols, treated with propylene oxide for 15 min and were then embedded in Epon 812. The blocks obtained were cut with an ultramicrotome (Porter-Blum MT-2; Ivan Sorvall, Inc.. Norwal, Conn., USA) equipped with a diamond knife (Diatome, USA). The sections were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM-200X transmission electron microscope.

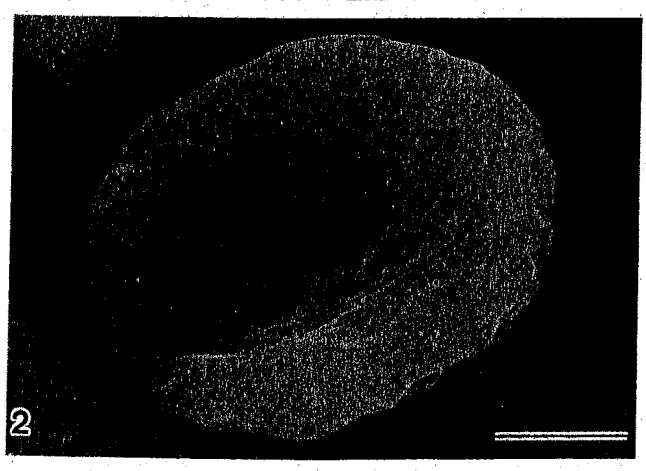
Results

Giemsa-stained thin smears of cultured parasites examined light microscopically revealed various intraerythrocytic stages such as single or paired pyriforms and single spherical or ameboid forms (Fig. 1). No change was seen on infected erythrocytes such as the dots and clefts observed on erythrocytes infected with *Plasmodium*.

Scanning electron microscopy revealed slight indentations and many small pores evenly distributed over the entire infected erythrocyte surface (Fig. 2). Each pore was round or oval-shaped, measuring approximately 40 nm in diameter.

On transmission electron microscopy, paired pyriform parasites, ameboid forms, and dividing forms of *Babesia caballi* were subcentrally located in host erythrocytes. In several infected erythrocytes, some vesicles were seen in the host cytoplasm (Fig. 3). The vesicles





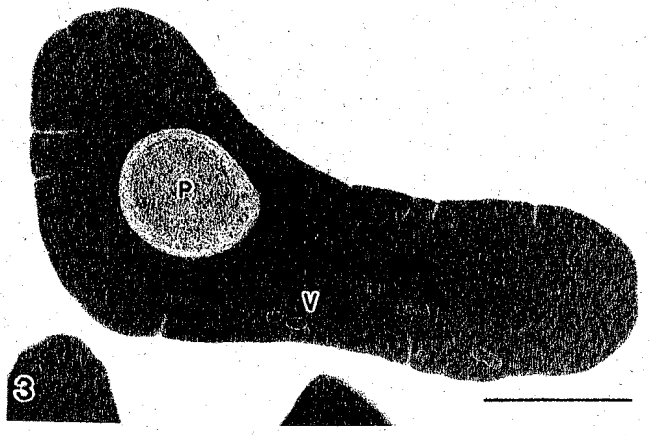


Fig. 1 Light micrograph of a Giemsa-stained thin smear of cultured parasites, showing various intraerythrocytic stages. Magnification X 2 400. *Bar* 10 μm

Fig. 2 Scanning electron micrograph of a *Babesia caballi*-infected equine erythrocyte. Magnification X 25 000. *Bar* 1 μm

Fig. 3 Transmission electron micrograph of a *B. caballi*-infected equine erythrocyte with many tubular structures (*P* Piroplasm, *V* vesicles). Magnification X 28 000. *Bar* 1 μm

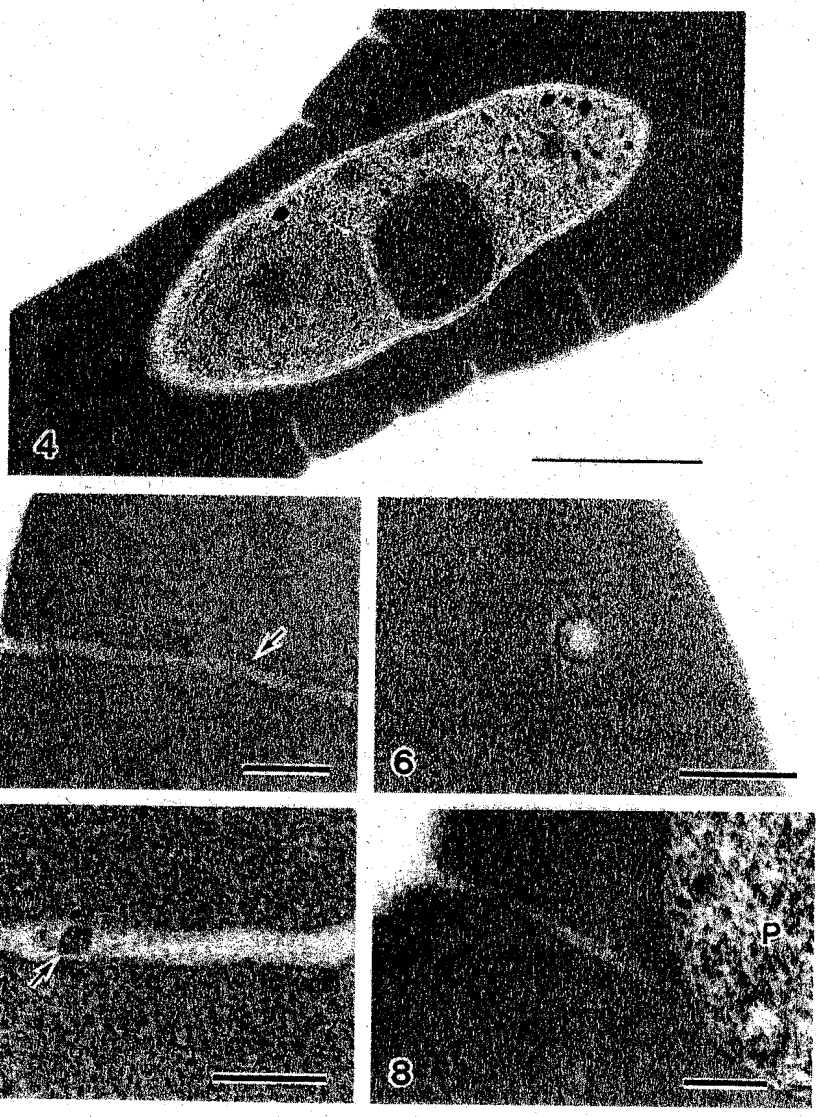


Fig. 4 Transmission electron micrograph of a *B. caballi*-infected equine erythrocyte, showing many tubular structures along the extreme edge of the host cell cytoplasm. Magnification X 30 000. *Bar* 0.5 μm

Fig. 5 High-magnification electron micrograph of a longitudinal section of a tubular structure. A unit membrane is clearly recognizable around the tubular structure (*arrow*). Magnification X 60 000. *Bar* 250 nm

Fig. 6 High-magnification electron micrograph of a transverse section of a tubular structure. Magnification X 84 000. *Bar* 250 nm

Fig. 7 High-magnification electron micrograph of a longitudinal section of a tubular structure. A particle is visible in the lumen of the tubular structure (*arrow*). Magnification X 85 000. *Bar* 250 nm

Fig. 8 Transmission electron micrograph of a *B. caballi*-infected equine erythrocyte (*P* Piroplasm). The tubular structures arise from the edge of the parasite and terminate at an invagination on the surface of the erythrocyte. Magnification X 55 000. *Bar* 250 nm

were surrounded by an area of low electron density measuring approximately 0.1 nm in width and were round or elongated. They were scattered throughout the host cytoplasm. The density of these vesicles was similar to that of the host cell cytoplasm (Fig. 3).

Many tubular structures were often seen in the infected cells (Fig. 3). Such structures were comparable with the small pores seen by scanning microscopy. When the structures were sectioned longitudinally, they were straight or slightly curved and were usually located along the extreme edge of the host cell cytoplasm (Fig. 4). These tubular structures had a common membrane with that of the host cell, and a unit membrane was clearly recognizable around the tubular structures (Fig. 5, arrow). On transverse sections the tubular structures were round and had a diameter of about 50 nm (Fig. 6). A unit membrane was observed around tubular structures, and the density of the central lumen was lower than that of the host cell cytoplasm (Fig. 6). Occasionally, some particles were found in the lumen of the tubular structure (Fig. 7, arrow). By the examination of a number of infected cells, tubular structures were found to be connected with the parasite (Fig. 8), and this observation might indicate that tubular structures arose from the edge of the parasite and terminated at an invagination on the surface of the erythrocyte.

Examination of nonparasitized equine erythrocytes by scanning and transmission electron microscopy revealed no surface indentation, tubular structure, or vesicle comparable with those observed in the parasitized cells.

Discussion

In this study we made ultrastructure observations of in-vitro-cultured *Babesia caballi* by scanning and transmission electron microscopy. Many small pores were observed for the first time over the entire surface of infected erythrocytes by scanning electron microscopy, and on transmission electron microscopy these small pores were found to be the openings of tubular

structures. Although Simpson et al. (1963, 1967) observed elongated microtubules in the cytoplasm of *B. caballi* parasites in specimens prepared from splenectomized ponies, they did not describe tubular structures in infected erythrocytes. The lack of tubular structures in Simpson et al.'s studies might be explained by the use of old methods of fixation specimen processing and the poor performance of electron microscopy about 30 years ago. Moreover, their observations were made using blood samples with low levels of parasitemia obtained from infected animals. In the present study, ultrastructure observations were made on cultured samples, including a great number of parasites in all stages of intraerythrocytic development. Previous studies have shown that the *B. divergens* and *B. bovis* fine structure in vitro is indistinguishable from that in vivo (Gorenflot et al. 1991; Holman et al. 1993). It is thus possible that the tubular structures associated with *B. caballi* infection were found in a higher percentage of parasitized cells prepared in vivo by the same fixation (and subsequent) methods used in the present study.

Changes on the surface of infected erythrocytes have been reported in *Babesia* and *Plasmodium* infections. Sun et al. (1983) observed perforations on the surface of erythrocytes infected with *B. microti*. Bodammer and Bahr (1973) observed deep invaginations or focal depressions on the surfaces of erythrocytes infected with *P. berghei* NYU-2; these structures were postulated to be "metabolic windows" permitting parasites access to metabolites outside the cell. However, these perforations and "metabolic windows" differ in size and number from the small pores observed in the present study on membranes of erythrocytes infected with *B. caballi*.

In the cytoplasm of *Babesia*-infected erythrocytes, some characteristic structures in addition to the parasites have been reported, including translucent single membrane-bound vesicles, aggregated tiny vesicles, tightly coiled membranes, and Maurer's clefts (Rudzinska 1981). The presence of Maurer's clefts has been reported in *B. bovis* trophozoites, especially in multiple infections of a single bovine erythrocyte (Todorovic et al. 1981). This structure has also been recognized in trophozoites of *B. bigemina*, *B. divergens*, *B. microti*, and some *Plasmodium* species (Saal 1964; Rudzinska 1981; Atkinson and Aikawa 1990).

According to Aikawa et al. (1986) and Atkinson and Aikawa (1990), cytoplasmic clefts associated with *P. falciparum* extended from the parasite and the surrounding parasitophorous vacuole membrane into the erythrocyte cytoplasm, and these authors suggested that they were a route of transport for parasite antigens through the erythrocyte cytoplasm to the erythrocyte membrane. However, in the present study, erythrocytes infected with *B. caballi* contained neither cytoplasmic clefts nor a parasitophorous vacuole membrane in the erythrocyte cytoplasm. These findings suggest that the tubular structures associated with *B. caballi* differ morphologically and functionally from the cytoplasmic clefts seen in erythrocytes infected with *P. falciparu* and other *Babesia* species.

The ultrastructure of *B. equi* has been described, unique morphological feature of which is the presence tubular food vacuoles that extend from intraerythrocyt parasites (Frerichs and Holbrook 1974). When the t bular food vacuoles of *B. equi* extended to the periphe of the erythrocyte, its central lumen was often in dire contact with plasma. Although the function of the t bular structures remains unknown, it has been spec lated that they play a role in the uptake of nutrients fro host plasma (Frerichs and Holbrook 1974).

In the present study we found that the tubul structures of *B. caballi*-infected erythrocytes extende from the surface of the cell membrane directly to th parasite, suggesting that intraerythrocytic stages c *B. caballi* come into direct contact with culture mediur or host plasma. As described for *B. equi*, if the tubula structures associated with *B. caballi* are related to in gestion by parasites, our findings would suggest that th erythrocytic stages of *B. caballi* obtain nutrients directl from the area outside of erythrocytes. Further studie are needed to clarify the process of formation and bio logical function of the tubular structures.

Acknowledgements The authors wish to thank Prof. H. Hidari fo supplying horse erythrocytes. This study was supported by Grant-in-Aid for Scientific Research on Priority Areas (06660397 from the Ministry of Education, Science, Culture and Sports Japan.

References

Aikawa M, Uni Y, Andrutis AT, Howard RJ (1986) Membrane associated electron-dense material of the asexual stages o *Plasmodium falciparum*: evidence for movement from the in tracellular parasite to the erythrocyte membrane. Am J Tro Med Hyg 35: 30–36

Atkinson CT, Aikawa M (1990) Ultrastructure of malaria-infecte erythrocytes. Blood Cells 16: 351–368

Avarzed A, Igarashi I, Kanemaru T, Hirumi K, Omata Y, Saito A Oyamada T, Nagasawa H, Totoda Y, Suzuki N (1997) Im proved in vitro cultivation of *Babesia caballi*. J Vet Med Sci 59 479–481

Bhushan C, Müller I, Friedhoff KT (1991) Enrichment of *Babesi caballi*-infected erythrocytes from microaerophilous stationary phase cultures using Percoll gradients. Parasitol Res 77: 177–179

Bodammer JE, Bahr GF (1973) Initiation of a "metabolic window" in the surface of host erythrocytes by *Plasmodium berghe* NYU-2. Lab Invest 28: 708–718

Böse R, Hentrich B (1994) Identification of antigens diagnostic fo European isolates of *Babesia equi* by two-dimensional electrophoresis and Western blotting. Parasitol Res 80: 182–185

Droleskey RE, Holman PJ, Waldrup KA, Corrier DE, Wagner GG (1993) Ultrastructural characteristics of *Babesia odocoilei* in vitro. J Parasitol 79: 424–434

Frerichs WM, Holbrook AA (1974) Feeding mechanisms of *Babesia equi*. J Protozool 21: 707–709

Gorenflot A, Brasseur P, Precigout E, L'Hostis M, Marchand A, Schrevel J (1991) Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man. Parasitol Res 77: 2–12

Holbrook AA, Johnson AJ, Madden PA (1968) Equine piroplasmosis: intraerythrocytic development of *Babesia caballi* (Nuttall) and *Babesia equi* (Laveran). Am J Vet Res 29: 297–303

Holman PJ, Waldrup KA, Droleskey RE, Corrier DE, Wanger GG (1993) In vitro growth of *Babesia bovis* in white-tailed deer (*Odocoileus virginianus*) erythrocytes. J Parasitol 80: 232–236

Knowles DP (1996) Control of *Babesia equi* parasitemia. Parasitol Today 12: 195–198

Knowles RC (1988) Equine babesiasis: epidemiology, control and chemotherapy. Equine Vet Sci 8: 61–64

Levine ND (1971) Taxonomy of the piroplasms. Trans Am Microsc Soc 90: 2–33

Mahoney DF, Wright IG, Frerichas WM, Groenendyk S, O'Sullivan BM, Roberts MC, Waddell AH (1977) The identification of *Babesia equi* in Australia. Aust Vet J 53: 461–464

Purnell RE (1981) Babesiosis of horses. In: Ristic M, Kreier JP (eds) Babesiosis. Academic Press, New York, pp 42–44

Riek RF (1968) Babesiosis. In: Weiman D, Ristic M (eds) Infectious blood diseases of man and animals. Academic Press, New York, pp 219–268

Rudzinska MA (1981) Changes in host cells. In: Ristic M, Kreier JP (eds) Babesiosis. Academic Press, New York, pp 127–132

Saal JR (1964) Giemsa stain for the diagnosis of bovine babesiosis. II. Change in erythrocytes infected with *Babesia bigemina* and *B. argentina*. J Protozool 11: 582–585

Simpson CF, Bild CE, Stoliker HE (1963) Electron microscopy of canine and equine *Babesia*. Am J Vet Res 24: 408–414

Simpson CF, Kirkham WW, Kling JM (1967) Comparative morphologic features of *Babesia caballi* and *Babesia equi*. Am J Vet Res 28: 1693–1697

Sun T, Tenenbaum MJ, Greenspan J, Teichberg S, Wang RT, Degnan T, Kaplan MH (1983) Morphologic and clinical observations in human infection with *Babesia microti*. J Infect Dis 148: 239–248

Todorovic RA, Wagner GG, Kopf M (1981) Ultrastructure of *Babesia bovis* (Babes, 1988). Vet Parasitol 8: 277–290

Growth Inhibitory Effect of Bovine Lactoferrin to *Toxoplasma gondii* Tachyzoites in Murine Macrophages: Tyrosine Phosphorylation in Murine Macrophages Induced by Bovine Lactoferrin

Tetsuya TANAKA*, Yoshitaka OMATA***, Tsuya ISAMIDA, Atsushi SAITO, Keiichi SHIMAZAKI[2], Koji YAMAUCHI[3] and Naoyoshi SUZUKI[1]**

Department of Veterinary Physiology and [1]The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro 080 [2]Department of Dairy Science, Faculty of Agriculture, Hokkaido University, Sapporo 060, and [3]Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd., Zama 228, Japan

(Received 12 June 1997/Accepted 23 October 1997)

ABSTRACT. Previous studies have shown that lactoferrin induces growth inhibitory effects in mouse macrophages against intracellular *Toxoplasma gondii*, and these effects were not mediated by the oxygen-dependent and inorganic nitrogen-dependent pathway. To clarify the mechanism of anti-*Toxoplasma gondii* activity induced by lactoferrin, we examined whether lactoferrin promoted the phosphorylation of tyrosine residues in macrophage proteins. In immunoblotting assays using anti-[phosphorylated tyrosine] monoclonal antibody, phosphorylation of tyrosine residues was detected in protein(s) of approximately 30 kDa in macrophages incubated with lactoferrin. Inhibition of the lactoferrin-induced tyrosine-phosphorylation by genistein led to loss of the lactoferrin-induced growth inhibitory effect against the parasites. These findings suggest that lactoferrin induces tyrosine-phosphorylation in macrophages, and the tyrosine-phosphorylation seems to be associated with the induction of the growth inhibitory activity exerted against intracellular *Toxoplasma gondii*. — KEY WORDS: lactoferrin, macrophage, tyrosine phosphorylation.

J. Vet. Med. Sci. 60(3): 369–371, 1998

Lactoferrin (LF), a cationic iron-binding protein produced and secreted by neutrophils, is known to have broad spectrum antimicrobial properties [10]. Other properties of LF also investigated include its activation of natural killer cytotoxicity for tumor cells [9] and induction of phagocytic activity and killing of amastigotes of *Trypanosoma cruzi (T. cruzi)*, an intracellular parasitic protozoan in macrophages [5, 6]. A recent study showed that murine macrophages incubated in media containing LF displayed growth inhibitory effects against the intracellular parasite *Toxoplasma gondii (T. gondii)* [11].

Macrophages possess oxygen-dependent and oxygen-independent anti-protozoan mechanisms and activation by Interferon-γ (IFN-γ) enhances the oxygen-dependent killing effect against intracellular *T. gondii* due to the induction of inorganic nitrogen oxide synthesis from L-arginine [1]. The growth inhibitory effects induced by LF in macrophages are not mediated by this oxygen-dependent pathway [12]. Thus, the mechanisms of the growth inhibitory effects induced by LF remain unknown.

Recently, some of the intracellular events triggered by lipopolysaccharide (LPS) that ultimately lead to the intracellular antimicrobial responses of macrophages have been identified. Murine macrophages stimulated with LPS and soluble antigens of *T. gondii* showed rapid induction of tyrosine-phosphorylation of several proteins [7].

In the present study, in an effort to further elucidate the intracellular events occurring in macrophages treated with LF, we examined whether LF promotes the phosphorylation of tyrosine residues in proteins of these cells.

Bovine lactoferrin was prepared from cow's milk by the method of Law and Reiter [4]. The amount of Fe^{2+} ion in the LF was 14.5 mg/100 g protein. Genistein (Wako Co., Osaka, Japan), which is an inhibitor of protein tyrosine kinase (PTKase), was dissolved at a concentration of 1 mg/ml in Dulbecco's modified essential medium (D-MEM) containing 10% fetal bovine serum (D-MEM-10FBS), and stored at −80°C until use. Seven week-old male and female ICR mice were used throughout the experiments.

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal cavity of mice inoculated with phosphate buffered saline (PBS) containing 0.2% glycogen on the 5th day post-inoculation (p.i.). They were centrifuged at 800 × g for 10 min and were suspended in D-MEM-10FBS. The macrophage suspension was applied to either round coverslips (15 × 15 mm diameter; Matsunami, Osaka, Japan), or 24-well tissue culture microplates (Corning IWAKI Inc., Osaka, Japan), at 1 × 10^6 cells/well. The macrophage suspensions were incubated at 37°C for 2 hr, then washed thoroughly to remove non-adherent cells, and further incubated at 37°C overnight in D-MEM-10FBS. To avoid the influence of LF present in FBS, each MPM culture medium was substituted with D-MEM containing 1% bovine serum albumin (D-MEM-BSA) for 24 hr before the experiments.

* PRESENT ADDRESS: TANAKA, T., Division of Veterinary Microbiology, Kyoto Biken Laboratories, 24–16 Makishima-cho, Uji, Kyoto 611, Japan.

** PRESENT ADDRESS: SUZUKI, N., The Kitasato Institute, Shirogane, 5–9–1, Minato-ku, Tokyo 108, Japan.

***CORRESPONDENCE TO: OMATA, Y., Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro 080, Japan.

Tachyzoites of the RH strain of *T. gondii* were harvested from the peritoneal cavity of mice under anesthesia on the 3rd day p.i. The parasites were washed by centrifugation at 1,200 × g for 10 min in PBS three times, and suspended in D-MEM-BSA at a concentration of 1×10^6 parasites/m*l*. Monolayers of MPM (1×10^6 cells/m*l*) were incubated in D-MEM-BSA supplemented with LF at concentrations of 1,10, 100 or 1000 μg/m*l* for 30 min, or at 1,000 μg/m*l* for 5, 15, 30 and 60 min. Under these conditions, the incubated cells showed no pathogenic effects by microscopic observation. As a negative control, MPM monolayers were incubated in D-MEM-BSA without added LF.

Growth of the parasites in the host cells was examined by the bioassay method described by Tanaka *et al.* [11]. Briefly, the MPM monolayers were preincubated for 24 hr with either D-MEM-BSA alone, or D-MEM-BSA supplemented with LF at 1,000 μg/m*l*, then were challenged with 10^5 parasites for 2 hr. After removing free parasites by washing, the monolayers were further incubated with the same medium for 24 hr. The development of intracellular parasites were examined by microscopic observation. To examine the effect of the tyrosine-phosphorylase inhibitor, genistein, MPM cultures were supplemented with this inhibitor at either 0.1 or 1.0 μg/m*l*.

To determine the molecular weight of tyrosine-phosphorylated protein(s), immunoblotting was performed.

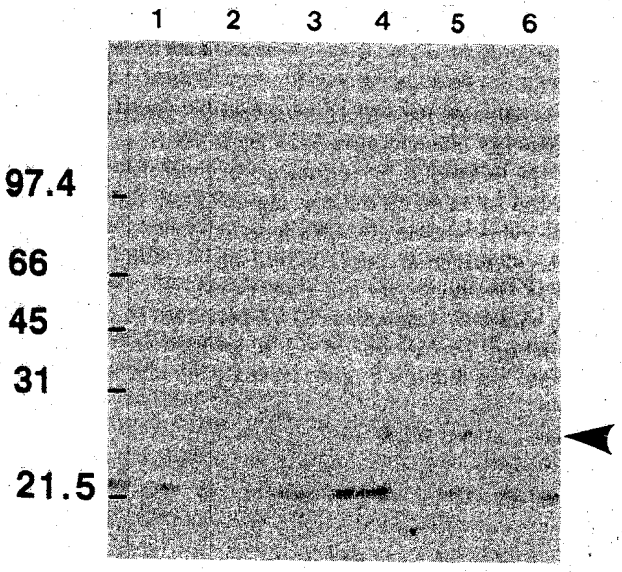


Fig. 1. Effect of genistein on tyrosine phosphorylation in macrophages. The macrophages were incubated in medium either with or without LF in the absence or presence of genistein for 30 min. After incubation, the monolayers were examined by immunoblotting. Lane 1: Medium alone. Lane 2: Medium supplemented with 0.1 μg/m*l* genistein. Lane 3: Medium supplemented with 1.0 μg/m*l* genistein. Lane 4: Medium supplemented with 1,000 μg/m*l* LF. Lane 5: Medium supplemented with 1,000 μg/m*l* LF and 0.1 μg/m*l* genistein. Lane 6: Medium supplemented with 1,000 μg/m*l* LF and 1.0 μg/m*l* genistein. Molecular weight standards are as indicated. Arrow indicates the 30 kDa protein band.

Macrophage-monolayered microplate wells were washed with chilled PBS, then the cells were solubilized in 50 μ*l* SDS-PAGE sample buffer. After shaking overnight, the samples were heated to 95°C for 5 min, ultrasonicated, and centrifuged at 12,000 × g for 5 min. The supernatant of each sample was applied to a 10% polyacrylamide gel for SDS-PAGE. The proteins separated by SDS-PAGE were transferred at 12 V for 50 min from the gels onto PVDF membranes (Immobilon-P, Millipore Corp., Tokyo, Japan). Then, the membranes were blocked with 3% BSA-PBS at 4°C overnight. The membranes were washed with PBS containing 0.25% Tween 20 (PBS-Tween 20) and incubated with anti-[phosphorylated tyrosine] monoclonal antibody (1/500 dilution) at 4°C overnight. The membranes were subsequently washed, and incubated with peroxidase-conjugated anti-mouse IgG at 4°C overnight. After washing with PBS-Tween 20, binding of secondary antibody was detected by the enhanced chemiluminescence detection method (DuPont NEM Res., Boston, MA, U.S.A.).

All experiments were done in triplicate and repeated at least twice. Data from each experiment were evaluated using the Student's *t*-test. The 95% level of significance was used in all analysis.

A tyrosine-phosphorylated protein(s) was detected by immunoblotting analysis. A specific band of protein with a molecular weight of approximately 30 kDa was detected in the lane containing the extract of MPM incubated with LF for 30 min. The density of the band increased dose dependently and the highest density appeared in the case of MPM incubated with 1,000 μg/m*l* LF. Levels of phosphorylated protein(s) of similar size, as evident in the lanes containing extracts of MPM incubated with 1,000 μg/m*l* LF, reached a maximum after either 30 or 60 min of treatment. To confirm whether the 30 kDa protein(s) is associated with tyrosine-phosphorylation, we examined the effect of genistein, a tyrosine-phosphorylation inhibitor, when added to the MPM cell culture. As shown in Fig. 1, the 30 kDa band of protein found in extracts of MPM incubated with LF for 30 min was not evident in extracts of cultures supplemented with 1.0 μg/m*l* genistein.

The number of tachyzoites in parasitophorous vacuoles (PV) of MPM in cultures supplemented with LF-genistein was shown to be higher than that in the case of cultures supplemented with LF alone (Fig. 2). This finding suggests that lactoferrin induces tyrosine-phosphorylation in macrophages, and the tyrosine-phosphorylation seems to be associated with the induction of the growth inhibitory activity exerted against intracellular *T. gondii*, and the tyrosine-phosphorylated protein(s) of 30 kDa observed may reflect the induction of this growth inhibitory activity.

Dong *et al.* [2] reported that LPS, a potent stimulant for macrophages, induces the expression of mRNA encoding IL-1b, TNF-a, and IL-12, leading to the phosphorylation-dephosphorylation of tyrosine residues in a group proteins with molecular weights of 39, 41, and 45 kDa in macrophages, and they suggested that the 39 and 45 kDa proteins are similar to, or identical to, mitogen-activated

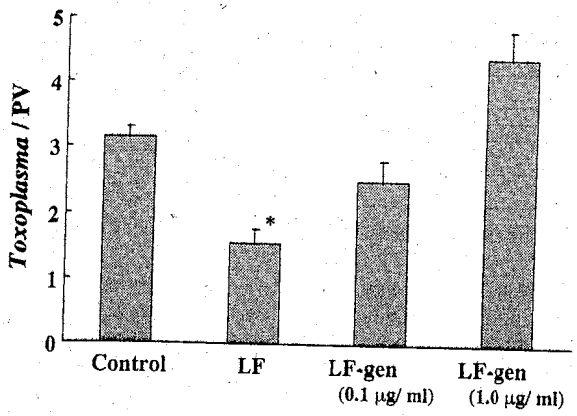


Fig. 2. Effect of genistein on the growth of *T. gondii* in macrophages. The macrophages cultured on cover slips were incubated for 24 hr post *T. gondii* challenge, then, were stained with Giemsa. Toxoplasma growth-inhibitory effect is expressed as the number of tachyzoites/parasitophorous vacuole among 100 vacuoles. Each value is the mean ± S.D. of triplicate measurements in a typical experiment. Control: Cultured in D-MEM-BSA alone. LF: incubated in medium supplemented with 1,000 μg/ml LF. LF+gen: incubated with medium supplemented with 1,000 μg/ml LF and genistein. The concentration of genistein is as indicated. *p<0.05, Control vs. Each sample

protein kinase. A recent study has documented that LF taken up by leukemia cells becomes bound to DNA and this binding event leads to transcriptional activation [3]. Thus, we speculate that LF might be taken up by MPM and act on tyrosine-phosphorylation of a 30 kDa protein which inhibits multiplication of the intracellular parasites. Another possibility to be considered is that the stimulatory effect of LF might be mediated by LF-receptors. Misra *et al.* [8] reported that binding of LF to low-density lipoprotein-related protein/alpha 2M receptor, a signalling receptor on macrophages failed to induce phosphorylation of phospholipase C gamma 1. Further experiments are necesarry to examine whether macrophages have specific receptors for LF to induce tyrosine-phosphorylation.

In this study, the characteristics and biological activity of the tyrosine-phosphorylated protein induced by LF were not determined. To clarify the mechanism by which LF enhances the growth inhibitory activity against intracellular parasites in macrophages further study is needed. We next aim to determine the kinetics of appearance of the tyrosine-phosphorylated protein.

REFERENCES

1. Adams, L. B., Hibbs, B. Jr., Janitor, R. R. and Krahenbuhl, J. L. 1990. *J. Immunol.* 144: 2725–2729.
2. Dong, Z., Qi, X. and Fidler, I. J. 1993. *J. Exp. Med.* 177: 1071–1077.
3. He, J. and Furmanski, P. 1995. *Nature (Lond.)* 373: 721–724.
4. Law, B. A. and Reiter, B. 1977. *J. Dairy Res.* 44: 595–599.
5. Lima, M. F. and Kierszenbaum, F. 1985. *J. Immunol.* 134: 4176–4183.
6. Lima, M. F. and Kierszenbaum, F. 1987. *J. Immunol.* 139: 1647–1651.
7. Li, Z-Y., Manthey, C. L., Yu, P., Perera, A. S. and Vogel, S. N. 1994. *Infect. Immun.* 62: 3434–3440.
8. Misra, U. K., Gaedi, G. and Pizzo, S. D. 1995. *Biochem. J.* 309: 151–158
9. Nishiya, K. and Horwitz, D. A. 1982. *J. Immunol.* 129: 2519–2523.
10. Sanchez, L., Calvo, M. and Brock, H. J. 1992. *Arch. Dis. Child.* 67: 657–661.
11. Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Igarashi, I. and Suzuki, N. 1996. *J. Vet. Med. Sci.* 58: 61–65.
12. Tanaka, T., Omata, Y., Narisawa, M., Saito, A., Shimazaki, K., Igarashi, I., Hirumi, H. and Suzuki, N. 1997. *Vet. Parasitol.* 68: 27–33.

VIROLOGY 249, 440–452 (1998)
ARTICLE NO. VY989335

Differences in the Evolutionary Pattern of Feline Panleukopenia Virus and Canine Parvovirus

Motohiro Horiuchi,*,†,[1] Yumi Yamaguchi,‡ Takashi Gojobori,‡ Masami Mochizuki,[§] Hideyuki Nagasawa,† Yutaka Toyoda,† Naotaka Ishiguro,* and Morikazu Shinagawa*

*Department of Veterinary Public Health, and †Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080 Japan; ‡Center for Information Biology, National Institute of Genetics, Mishima, Shizuoka 411 Japan; and §Laboratory of Clinical Microbiology, Kyoritsu Shoji Co., 1-12-4 Kudankita, Chiyoda-ku, Tokyo 102 Japan

Received May 6, 1998; returned to author for revision June 9, 1998; accepted July 14, 1998

Canine parvovirus (CPV) suddenly appeared in the late 1970s after which it showed continuous antigenic changes. Virological and molecular genetic analyses mainly focused on feline panleukopenia virus (FPLV) were conducted in this study because FPLV is the suspected ancestor of CPV; the way in which FPLV evolves may help to explain the emergence of CPV. Analysis of escape mutants against FPLV-specific monoclonal antibody showed that viruses possessing CPV-like properties were not easily detected in FPLV virus stocks. Phylogenetic analysis revealed that the nonstructural protein 1 (NS1) and capsid protein 2 (VP2) genes of FPLV changed with time. A similar tendency, however, was not observed in the FPLV VP2 proteins. In contrast, the topology of the phylogenetic tree of VP2 proteins of CPV basically concurred with that of the VP2 genes. Analysis of the ratio of nonsynonymous and synonymous substitutions revealed that synonymous substitutions exceeded nonsynonymous substitutions in both the NS1 and VP2 genes of FPLV, even when the analysis focused on specific regions in the VP2 gene that are known to be located on the capsid surface. Comparison of the CPV VP2 genes revealed that nonsynonymous substitution was found to dominate over synonymous substitution in one specific region in the VP2 gene. These results suggested that FPLV has changed mainly by random genetic drift. In contrast, after the appearance of CPV, changes in the CPV VP2 gene appear to be partly selected by certain positive selection forces. CPV and FPLV are known to be closely related viruses genetically and biologically, but the evolutionary mechanisms of the two viruses appeared to be different.

INTRODUCTION

The emergence of canine parvovirus (CPV), a pathogen for dogs, was first observed in 1978 (Parrish, 1990). The original CPV strain, CPV-type 2 (CPV-2), underwent extensive antigenic changes and has been fully replaced by the newer antigenic type strains, CPV-2a and CPV-2b (Parrish *et al.*, 1991). Retrospective serological studies showed that no anti-CPV antibodies were detected in the sera of domestic dogs or wild canine populations until the mid-1970s, indicating that CPV is a comparatively new pathogen for dogs (Schwers *et al.*, 1979; Koptopoulos *et al.*, 1986). Although the conclusive origin of CPV is unknown, the most widely accepted hypothesis for its emergence is that CPV is derived from feline panleukopenia virus (FPLV) or other FPLV-like viruses by natural genetic mutations.

CPV, FPLV, and mink enteritis virus (MEV) are classified as host range variants of feline parvoviruses belonging to the genus *Parvovirus* in the family *Parvoviridae* (Siegl *et al.*, 1985). FPLV, MEV, and a parvovirus isolated from blue fox (BFPV) are indistinguishable from each other by biological properties *in vitro* (Tratschin *et al.*, 1982; Veijalainen, 1988), but are distinguished by the host from which they were isolated. In this study, therefore, we call the viruses isolated from cats as FPLV, and the parvoviruses isolated from mink and blue fox with the same biological properties as FPLV are referred to as FPLV-like viruses. CPV, FPLV, and FPLV-like viruses are more than 98% similar in nucleotide and amino-acid sequences (Kariatsumari *et al.*, 1991), though CPV can be distinguished from the others by biological properties. In addition to differences in their host ranges, these viruses have different pH dependencies for hemagglutination (HA) (Carmichael *et al.*, 1980; Parrish *et al.*, 1988a), and their antigenicities are distinguishable by monoclonal antibodies (MAbs) (Parrish *et al.*, 1982; Parrish and Carmichael, 1983; Veijalainen, 1988; Horiuchi *et al.*, 1997). CPV can replicate in cultured canine cells, while FPLV and FPLV-like viruses either cannot replicate or replicate inefficiently in the cultured canine cells (Tratshir *et al.*, 1982; Parrish and Carmichael, 1983; Mochizuki and Hashimoto, 1986; Horiuchi *et al.*, 1992; Truyen and Parrish, 1992). Host range *in vivo* seems to be more complex; FPLV can replicate in feline tissue and in canine thymus but not in other canine tissues. CPV-2 cannot replicate in feline tissues, although antigenic type variants CPV-2a and 2b are reported to replicate

[1] Present address and to whom correspondence and reprint requests should be addressed at Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, 903 S. 4th St., Hamilton, MT 59840. Fax: (406) 363-9371. E-mail: mhoriuchi@atlas.niaid.nih.gov.

0042-6822/98 $25.00

in ileum and lymphoid tissues of cats (Truyen and Parrish, 1992; Truyen *et al.*, 1994, 1996).

The introduction of a virus into a new host species may happen in any number of ways: by genetic alteration of the virus through point mutation, recombination, or gene reassortment or by changes in the ecologies around the hosts and the viruses (Kilbourne, 1991). CPV is thought to have emerged suddenly in dogs (Parrish, 1990). CPV isolates appear to have evolved from a single common ancestor (Truyen *et al.*, 1995) indicating that the introduction of FPLV or FPLV-like viruses (i.e., an ancestral virus of CPV) into the dog population has probably been a single event. For instance, however, an interspecies transmission of influenza A virus is reported to be a frequent event (Kawaoka *et al.*, 1989; Gorman *et al.*, 1991). Perhaps FPLV and FPLV-like virus populations may give rise to another precursor of CPV, further change of which may result in a second CPV epidemic. The anticipation of probable outbreak of a new CPV is important to prevent the suffering of animals from a new pathogen. Elucidation of how FPLV and FPLV-like viruses evolve may aid in assessing the likelihood of new CPV epidemics. After the appearance of CPV, its antigenicity continuously changes by an antigenic drift type of mechanism (Parrish *et al.*, 1991). This type of continuous antigenic change has not been reported for FPLV and FPLV-like viruses, although some antigenic variation is reported among FPLV (Parrish *et al.*, 1983; Mochizuki *et al.*, 1989). MEV isolates are reported to be divided into three antigenic types when they are analyzed with MAbs, although they coexist in mink population (Parrish *et al.*, 1984). Recently we reported that restriction fragment length polymorphism analysis of PCR products reveals genetic changes in FPLV isolates over time (Horiuchi *et al.*, 1996). This finding led us to conduct more precise genetic and phylogenetic analyses of FPLV and FPLV-like viruses in an attempt to uncover an evolutionary pattern in FPLV variation.

Parvoviruses contain a linear ssDNA genome of about 5 kb. The genome has two open reading frames (ORF): a left-hand ORF encodes at least one nonstructural protein (NS1), and a right-hand ORF encodes the two capsid proteins, VP1 and VP2, which are translated from alternatively spliced mRNA. Defining the evolution of different genes among the same group of viruses helps to clarify their phylogenetic relationships more precisely. For this reason, we analyzed both the NS1 and VP2 genes.

Our aim in this study is to make clear the following questions: (1) Do FPLV virus populations have the potential to generate precursor viruses that have infectivity in dogs? (2) Is there any relationship between the evolutionary pathway of FPLV and the appearance of CPV? (3) What is the difference between the evolutionary patterns of CPV and FPLV? To address these questions, we analyzed mutants selected by escape from FPLV-specific monoclonal antibodies and estimated an evolutionary pattern for FPLV and FPLV-like viruses.

RESULTS

Characterization of MAb-selected escape mutants

Recently we produced MAb P2–215, which recognizes a specific epitope of FPLV and FPLV-like viruses that includes aa93-Lys in VP2 (Horiuchi *et al.*, 1997). One of the critical amino acids that influences the host range and antigenicity of CPV is aa93 (Chang *et al.*, 1992). Thus we analyzed the escape mutants selected by MAb P2–215. Direct infectious center assays revealed variant viruses that were not neutralized by MAb P2–215 but that were present in the original FPLV virus stocks, and the proportion of the presence of variant viruses varied from 2.2×10^{-4} to 2.3×10^{-5} (Table 1).

Properties of the escape mutants are summarized in Table 1. In enzyme-linked immunosorbent assay (ELISA), all the escape mutants reacted with MAb X1–251, the antibody that recognizes both CPV and FPLV, but they did not react with FPLV-specific MAb P2–215, indicating that the escape mutants lacked the FPLV-specific epitope. However, the *in vitro* host ranges of the escape mutants were the same as the parent viruses, i.e., none of the escape mutants could be propagated in A72 or Cf2Th canine cells. Because parvoviruses are difficult to clone by plaque purification, replicative form (RF) DNA was recovered from virus-infected cells, cloned into plasmid, and considered representative of plaque purified virus. The VP2-gene nucleotide sequences from two independent plasmids of each escape mutant were viewed as characteristic nucleotide sequences for each escape mutant. With the exception of escape mutant TU4 (TU4-es), the mutants had one nucleotide substitution at nt671; this was a change of a G to an A caused by an amino-acid change at aa224 from Gly to Glu. In addition to this substitution, TU4-es had another nucleotide substitution at nt694 which resulted in an amino acid change at aa232 from Val to Ile (Table 1). This means that variant viruses with aa224 as Glu predominated in the FPLV virus stocks and that substitution at aa224 from Gly to Glu affected the epitope recognized by MAb P2–215. A specific amino-acid substitution at aa93 from Lys, which is characteristic of FPLV and FPLV-like viruses, to CPV-specific Asn affected the epitope's recognition by MAb P2–215 (Horiuchi *et al.*, 1997); although the analysis in the present study was based on only 12 clones, no escape mutants with aa93-Asn were found in any FPLV virus stocks. In CPV, VP2 aa93 and a224 are included in one CPV-specific epitope (Strassheim *et al.*, 1994). The same position in FPLV VP2 seems to make up the FPLV-specific con-

TABLE 1

Incidence, Antigenic, Biological, and Genetic Properties of P2-215 Escape Mutants

Mutant	Incidence[a]	Reactivity against MAbs in ELISA (A405nm)[b]		Replication in canine cells[c]	Nucleotide and amino acid changes in VP2 (Parent virus → Mutant virus)	
		P2-215	X1-251		Nucleotide	Amino acid
TU2-es	7.9×10^{-5}	0.022	1.086	—	nt671:G→A	aa224:Gly→Glu
TU4-es	1.0×10^{-4}	0.009	0.971	—	nt671:G→A	aa224:Gly→Glu
					nt694:G→A	aa232:Val→ Ile
TU8-es	2.3×10^{-5}	0.011	1.102	—	nt671:G→A	aa224:Gly→Glu
TU10-es	1.9×10^{-4}	0.005	1.087	—	nt671:G→A	aa224:Gly→Glu
TU12-es	2.2×10^{-4}	0.009	1.026	—	nt671:G→A	aa224:Gly→Glu
FPV-483-es	9.1×10^{-5}	0.012	1.074	—	nt671:G→A	aa224:Gly→Glu

[a] Calculated by dividing the number of infectious center in the presence of MAb P2-215 by that of in the presence of negative control MAb (BSPX-54).
[b] Cut off value was 0.063.
[c] Determined by Southern blot analysis.

formational epitope, which is involved in virus neutralization.

Nucleotide sequence analysis

For estimation of viral phylogenetic relationships, analysis of several genes from the same isolates usually gives us more confident results. For this purpose, we determined the nucleotide sequences of the NS1 and VP2 genes from one FPLV vaccine strain originally isolated in France and from 12 FPLV isolates that had been collected in Japan between 1974 and 1995. Nucleotide sequences of both the NS1 and VP2 genes were already available for seven viruses in the feline parvovirus subgroup. Those combined with newer isolates yielded the 20 nucleotide sequences of the NS1 and VP2 genes that were the basis for our analysis.

The alignments of 20 NS1-gene sequences and 23 VP2-gene sequences are shown in Fig. 1, and the characterization of the nucleotide substitutions in those sequences are summarized in Table 2. When all the changes were assigned for comparison, synonymous substitutions predominated over nonsynonymous substitutions for both genes in each virus. Although the proportion of nonsynonymous substitutions increased when only the phylogenetically informative changes were compared, synonymous substitutions were still more than the nonsynonymous substitutions. Comparison of the NS1 and VP2 genes in FPLV and FPLV-like viruses did not show any significant difference in the proportion of nonsynonymous substitutions.

The VP1 and VP2 genes of FPLV and FPLV-like viruses are well characterized (Parrish *et al.*, 1988a; Truyen *et al.*, 1995), in contrast, little data are available for the NS1 gene. Comparison of the NS1-gene sequences of 16 FPLV and FPLV-like viruses with those from four CPV isolates revealed five nucleotide changes that were specific for CPV (Fig. 1A). Three of these resulted in amino-acid substitutions [nt741-NS1 (aa247), nt743-NS1 (aa248), and nt1785-NS1 (aa595)], and the others were silent (nt1224 and 1479-NS1).

Phylogenetic relationship

To analyze the phylogenetic relationships of the viral isolates, we constructed phylogenetic trees by using a branch-and-bound algorithm. To reduce the time needed for computer analysis, instead of full-length viral sequences, we used the nucleotide sequences of the VP2 and NS1 genes, as aligned in Fig. 1. Representative minimal trees for each gene and each protein are shown in Figs. 2 and 3. For NS1 genes, VP2 genes and VP2 proteins, more than 50 minimal trees were obtained by branch-and-bound algorithm; however, the differences among those trees in each gene and protein seemed not to be significant. Here, we selected the minimal trees showing the same topology as those obtained by neighbor-joining method as representative trees. For NS1 proteins, 19 minimal trees were obtained by branch-and-bound algorithm, and those were classified into two group on the basis of their topology (data not shown). So, we considered the minimal tree showing the same topology as that obtained by neighbor-joining method as representative minimal tree for NS1 proteins.

The phylogenetic tree of the FPLV-NS1 genes showed two branches with high confidence values (>70%) (Fig. 2A). One of the two groups consisted of four recent FPLV isolates that were isolated in the 1990s in Japan: 483, 94–1, Som1, and Som4. In the phylogenetic tree of the FPLV-VP2 genes, the same four isolates also constituted one group with a high confidence level (Fig 3A), and this group was located at the furthest position from the fiducial node in the phylogenetic trees for each gene. These results indi-

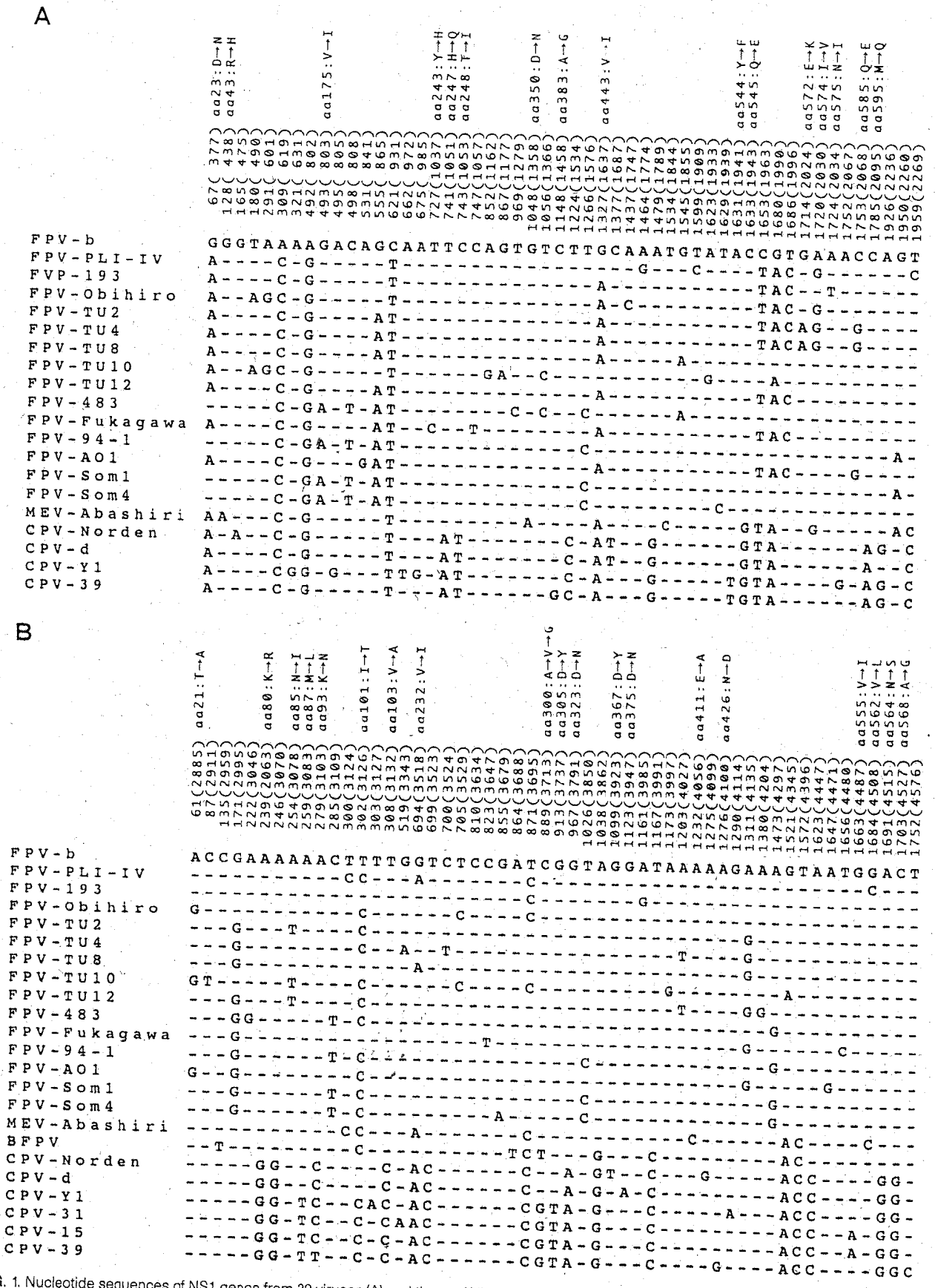


FIG. 1. Nucleotide sequences of NS1 genes from 20 viruses (A) and those of VP2 genes from 23 viruses (B). Differing nucleotides are indicated by a letter. The nucleotides that are identical to FPLV-b are indicated as dashes, while the nucleotides that differed from FPLV-b are indicated. Nucleotide positions in the NS1 and VP2 genes are numbered above the sequence from adenine of the initiation codon of each gene with the corresponding genomic positions in parentheses (nucleotide numbering followed the nucleotide sequence of MEV-Abashiri). Nucleotide changes that result in amino-acid substitutions are indicated with amino-acid positions of each protein and deduced amino acid changes [amino acid in FPLV-b to that in other virus(es)].

TABLE 2

Number of Sites Where Nucleotide Substitutions Were Observed

Viruses compared	Assigned change	Substitutions in NS1 gene		Substitutions in VP2 gene	
		Synonymous	Nonsynonymous	Synonymous	Nonsynonymous
All 20 viruses[a]	All	35 (0.67)[d]	17 (0.33)	32 (0.63)	19 (0.37)
	Phylo. infor.[c]	15 (0.58)	11 (0.42)	15 (0.54)	13 (0.46)
FPLV and FPLV-like[b]	All	26 (0.70)	11 (0.30)	23 (0.79)	6 (0.21)
	Phylo. infor.	11 (0.65)	6 (0.35)	9 (0.69)	4 (0.31)

[a] Twenty viruses with both NS1 and VP2 gene sequences available were used for comparison.
[b] A total of 16 FPLV and MEV viruses with both NS1 and VP2 gene sequences available were used for comparison.
[c] Phylogenetically informative nucleotide substitution.
[d] Number of sites where nucleotide substitutions were observed. Numbers in parentheses mean the ratio of the corresponding mutations to the total substitutions in each gene.

cated that both the NS1 and VP2 genes of FPLV varied with time. However, phylogenetic trees of the NS1 and VP2 genes also showed that isolates, AO1 and Fukagawa, which were isolated recently in 1994 and 1993, respectively, were closer to the older isolates than the four above-mentioned isolates (Figs. 2A and 3A). These data suggest that different genetic types of FPLV coexist in cat populations, at least in Japan.

Unlike the phylogenetic tree of their VP2 genes, the internal branches of the FPLV and FPLV-like virus lineages were not present in the phylogenetic tree of the VP2 proteins (Fig. 3), indicating that the branches of the phylogenetic tree of the VP2 genes are mainly composed of silent changes. In contrast, the topology of the phylogenetic tree of CPV VP2 proteins is similar to that of the VP2 genes. This suggested that the continuous antigenic type change that is well known in CPV is not present in FPLV, although several amino acid variations are found.

Relationship between the nucleotide difference and time of isolation

Porcine parvovirus (PPV) is reported to be the closest virus to the feline parvovirus subgroup (Chapman and Rossmann, 1993). We did not use PPV as a reference virus because of numerous sequence gaps that made aligning its nucleotide sequence with that of FPLV difficult. Instead, to estimate the evolutionary rate, we tentatively assigned the fiducial node for CPV and FPLV separately (as indicated in Figs. 2A and 3A), on the basis of the topology of the trees; the node under which all the FPLV or CPV isolates are located was used as a fiducial node for calculation of the number of substitutions in each virus lineage.

From the slope of regression curve (Fig. 4), the evolutionary rates of the NS1 and VP2 genes of FPLV were estimated to be 9.2×10^{-5} and 1.1×10^{-4} nt/site/year, respectively. The evolutionary rate of the FPLV VP2 gene looked a bit lower than that of CPV (1.69×10^{-4} nt/site/year) (Parrish *et al.*, 1991). When the graph was plotted with the isolation year against the number of amino-acid substitutions, the regression coefficient was −0.007 for the VP2 proteins.

Ratio of synonymous and nonsynonymous substitutions

To investigate the mechanism for the evolutionary change of FPLV and CPV, ratio of synonymous substitutions (*dS*) and nonsynonymous substitutions (*dN*) were estimated. In the case of no selection, *dS* and *dN* would be expected to be more or less the same. If certain amino acids were conserved by purified selection (conservation for functions), *dN* would be expected to be lower than *dS*. If positive selection was responsible for the variation of the gene, then *dN* would be higher than *dS* because such selection will accelerate amino acid changes. In the phylogenetic tree of the VP2 genes, CPV-d and FPLV-Obihiro were found to be the viruses that corresponded most closely to the fiducial node, so that *dS* and *dN* were calculated by comparison of CPV-d with other CPV isolates or by comparison of FPLV-Obihiro with other FPLV-isolates.

When whole nucleotide sequences of the NS1 gene were used for analysis, *dS* always predominated over the corresponding *dN*, and the ratios of *dN* to *dS* (*dN*/*dS*) were nearly 0.1 both among CPV or FPLV (Table 3). As with the VP2 gene, *dS* were still higher than the corresponding *dN*, however, the ratio of *dN*:*dS* among CPV was higher than that among FPLV. So we focused two regions within VP2 on the basis of the three-dimensional structure of the virion and the location of known amino-acid variations. One is Region 1, ranging from aa80 to 106 in VP2 (nt238 to 318 in VP2 gene); there is three CPV *vs* FPLV-specific amino acids within Region 1 (aa80, 93, and 103). The other is Region 2 ranging from aa295 to aa444 in VP2 (nt883 to 1332 in VP2 gene); there is one CPV *vs* FPLV-specific amino acid (aa323), two intra-CPV specific changes (aa300 and 305), and some linear B-cell epitopes within Region 2 (Tsao *et al.*, 1991; Agbandje *et al.*, 1993; Langeveld *et al.*, 1993). Both regions are known

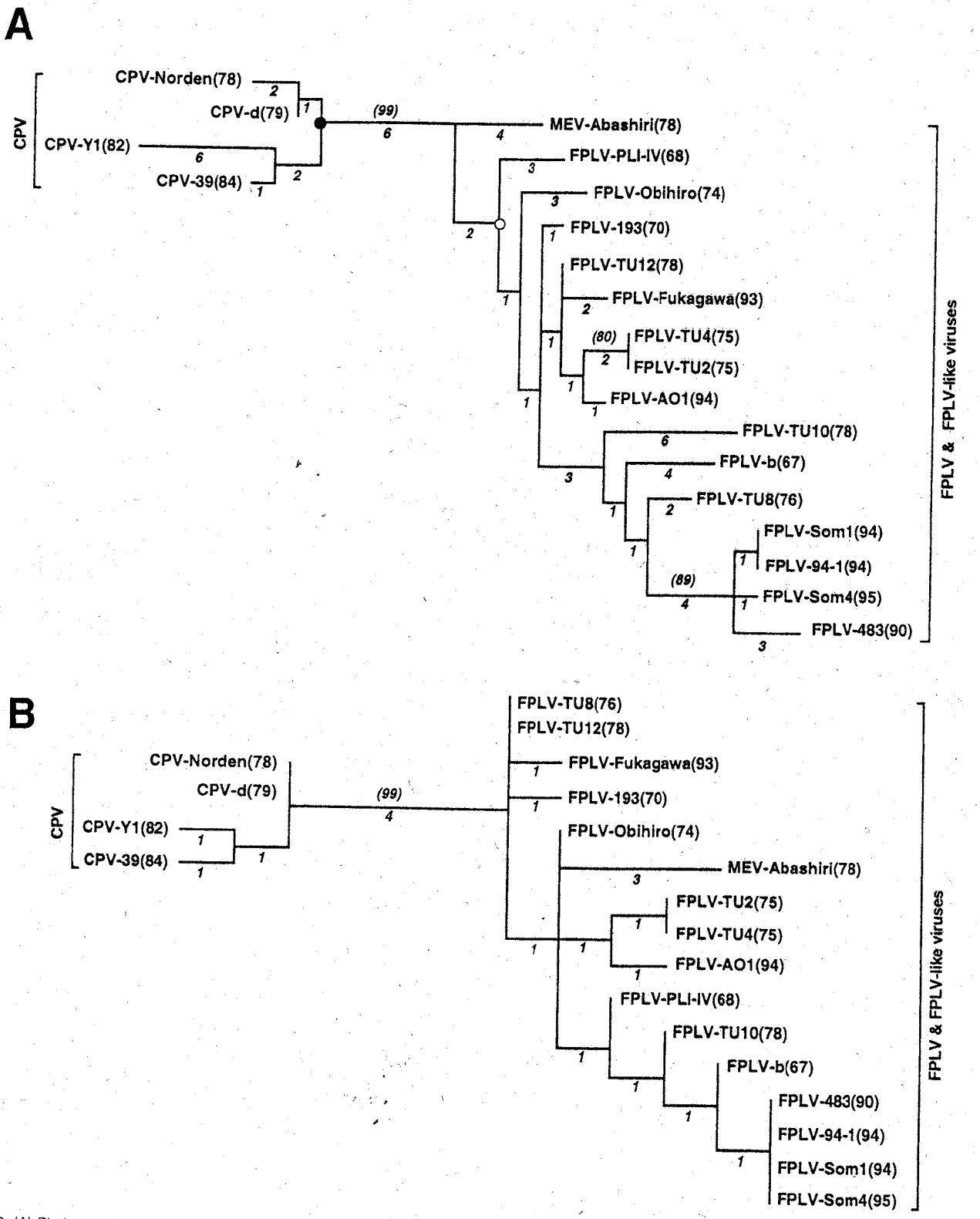


FIG. 2. (A) Phylogenetic tree constructed from the NS1-gene nucleotide sequences of 20 isolates. A representative of the shortest trees derive by the branch-and-bound analysis is shown. Italics indicate number of nucleotide differences in each branch. The significance of the branching orde was assessed by bootstrap resampling of 100 replicates. Resulting values of more than 70% are indicated on each branch (in parentheses). Ope and closed circles on the FPLV and CPV lineages, respectively, indicate the fiducial node from which the nucleotide substitutions were counted fc each isolate to calculate the evolutionary rates. (B) Phylogenetic tree constructed from the NS1-amino acid sequences of 20 isolates. A representativ of the shortest trees derived by the branch-and-bound analysis is shown. Italics indicate number of amino acid differences in each branch. Th significance of the branching order was assessed by bootstrap resampling of 100 replicates.

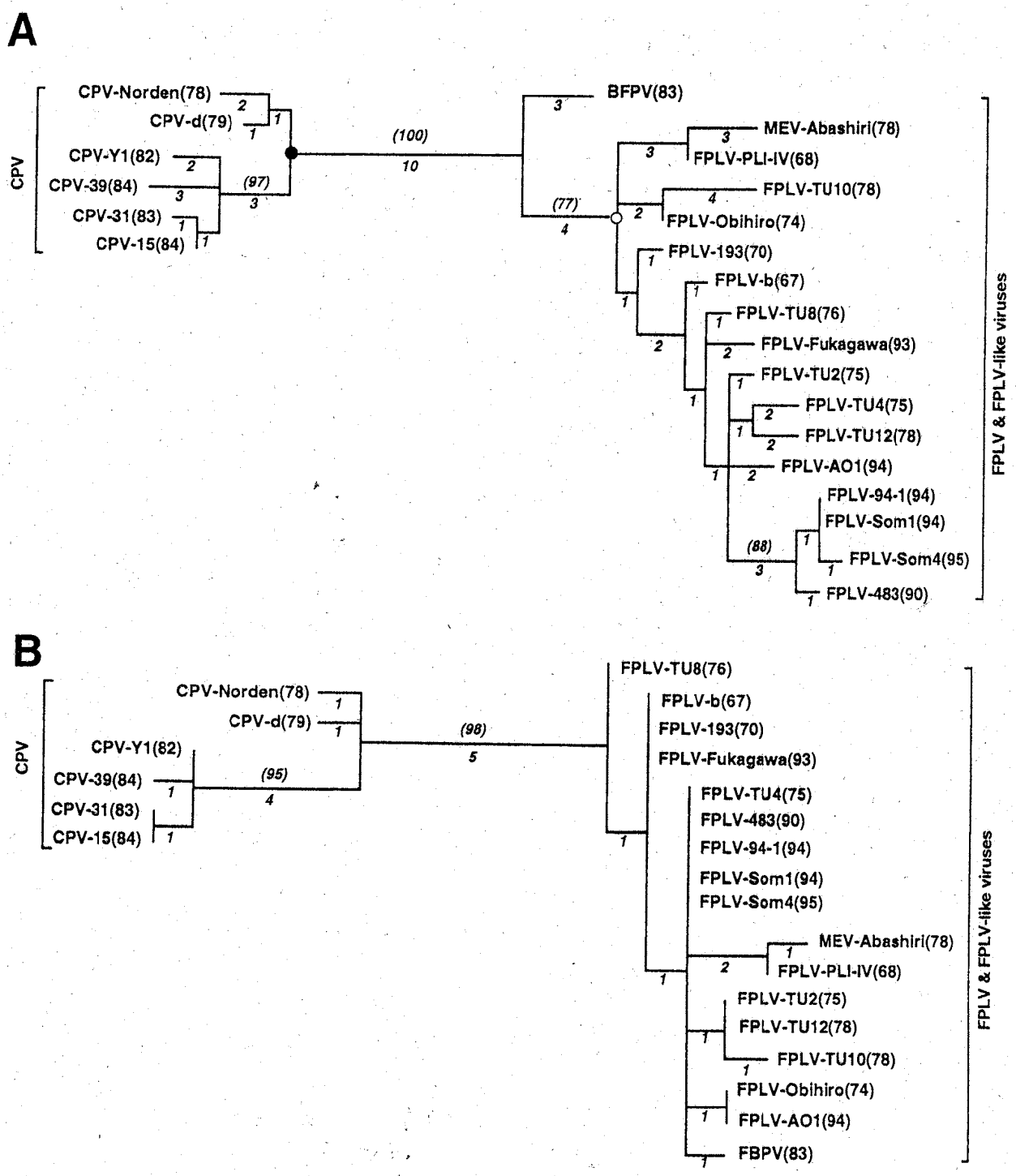


FIG. 3. (A) Phylogenetic tree constructed from the VP2-gene nucleotide sequences of 23 isolates. (B) Phylogenetic tree constructed from the VP amino-acid sequences of 23 isolates. The details are the same as in Fig. 2.

to form the 22 Å long spike of the capsid surface on the threefold axes (Tsao *et al.*, 1991; Agbandje *et al.*, 1993). The difference between CPV and FPLV became apparent when the analysis was focused to these two regions (Table 3). In Region 1, the *dN* was almost equal to the *dS* among CPV, while the *dN* was obviously lower than the *dS* among FPLV. For Region 2, the *dN* was higher than the *dS* (*dN*/*dS* = 2.42) among CPV, whereas the *dN* was lower than the *dS* among FPLV.

DISCUSSION

Evolutionary patterns of CPV and FPLV

In a way similar to antigenic drift of influenza A viruses in humans and horses (Webster *et al.*, 1992), CPV-2, the original CPV type, emerged in 1978 and has been replaced by newer antigenic types, CPV-2a and CPV-2b (Parrish *et al.*, 1985, 1988b, 1991; Senda *et al.*, 1988). Here we showed that, although they are closely related vi

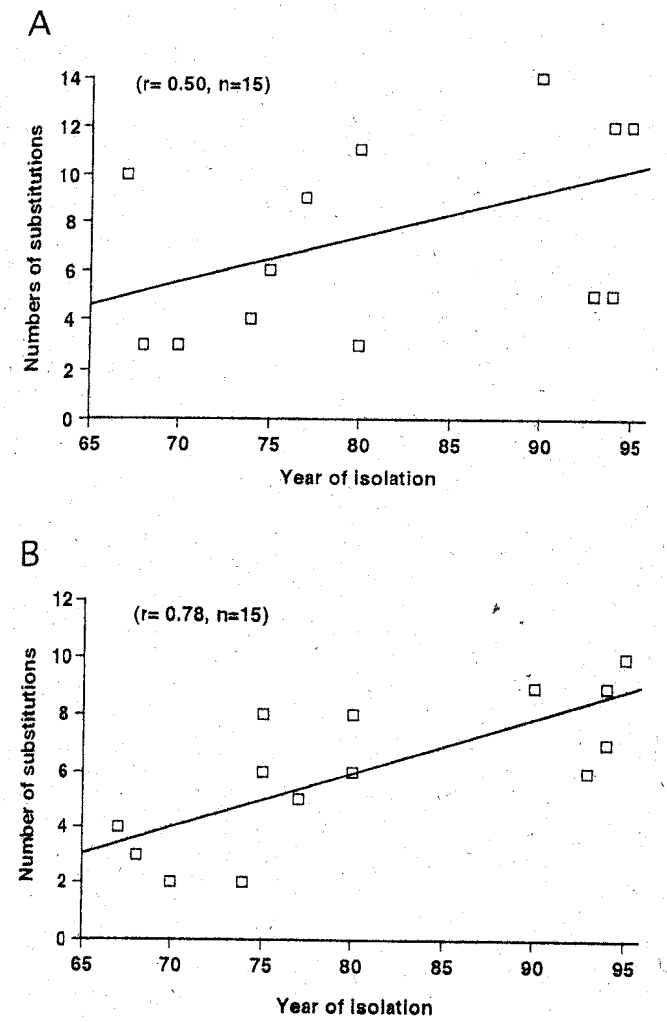


FIG. 4. Estimation of the temporal rate of nucleotide variation in FPLV NS1 (A) and VP2 (B) genes. Graphs were obtained by plotting the number of nucleotide differences from the fiducial node against the year of its isolation. The evolutionary rates are estimated by regression of the year of isolation against the number of nucleotide differences from the fiducial nodes (Figs. 2A and 2B). *r*, correlation coefficient, and *n*, number of viruses plotted on the graph.

ruses, the evolutionary pattern of FPLV differed from that of CPV. The results presented here would support the opinion that FPLV evolves with random genetic drift because of the following reasons.

Differences in phylogenetic trees

The phylogenetic analyses suggested that although genetic variations in FPLV and FPLV-like viruses occurred with time, VP2 antigenic type remained unchanged. In contrast for CPV, the topology of the phylogenetic tree of the VP2 genes was basically the same as that of its proteins. Among FPLV strains, few variations in VP2 amino-acid sequences were consistent with the results of the antigenic analyses with MAbs (Parrish *et al.*, 1983; Mochizuki *et al.*, 1989). The relatively high correlation coefficiency between the numbers of nucleotide substitutions and the year of isolation (Fig. 4) also supported the idea that the FPLV VP2 genes have been undergoing continuous genetic change for more than 20 years. For the VP2 proteins of FPLV, however, the slope of the regression curve was −0.007 when the year of isolation was plotted against the number of amino-acid changes, indicating no continuous antigenic change. Most of the FPLV isolates used here were isolated in Japan, so we can say that FPLV changes by random genetic drift at least in Japan. The evolution of the FPLV genes are similar to those of the nucleoprotein (NP), membrane proteins (M), and H3 hemagglutinin (HA) genes of avian influenza A viruses (Gorman *et al.*, 1990, 1991; Ito *et al.*, 1991; Bean *et al.*, 1992).

Evolutionary rates

Evolutionary rates of some viral surface antigens were found to be faster than those of their internal antigens (Li *et al.*, 1988; Fitch *et al.*, 1991; Webster *et al.*, 1992). One plausible explanation for this is that the surface antigen changes faster to escape immune surveillance by the host. The VP2 molecule of parvoviruses is a major component of the capsid (Cotmore and Tattersall, 1987) and thus VP2 may be subjected to selection pressure by neutralizing antibodies of host immune systems. The NS1 molecule, on the other hand, is not part of the capsid and thus is not related to virus neutralization (Cotmore and Tattersall, 1989). If the FPLV VP2 changes to escape host immune surveillance, then VP2 might be thought to change faster than NS1; however, the evolutionary rate of FPLV VP2 proteins calculated from its regression sta-

TABLE 3

Ratio of Nonsynonymous and Synonymous Substitutions

Viruses compared	NS1 (whole)			VP2 (whole)			VP2 Region 1			VP2 Region 2		
	dS	*dN*	*dN/dS*	*dS*	*dN*	*dN/dS*	*dS*	*dN*	*dN/dS*	*dS*	*dN*	*dN/dS*
CPV-d vs. other CPV[a]	8.1×10^{-3}	8.4×10^{-4}	0.10	4.1×10^{-3}	3.2×10^{-3}	0.78	2.3×10^{-2}	2.5×10^{-2}	1.10	3.6×10^{-3}	8.8×10^{-3}	2.42
FPLV-Ob[b] vs. other FPLV[c]	1.7×10^{-2}	1.6×10^{-3}	0.09	1.2×10^{-2}	1.3×10^{-3}	0.11	2.2×10^{-2}	6.8×10^{-3}	0.31	8.6×10^{-3}	5.5×10^{-4}	0.06

[a] $n = 4$ for NS1, $n = 6$ for VP2.
[b] FPLV-Obihiro.
[c] $n = 15$.

tistics (−0.007) was considered to be zero. This indicated that the positive selections for capsid functions including escape from immune pressure may not be the driving force of the FPLV evolutionary pathway.

dN and *dS* analysis

One of the striking differences between the evolutionary patterns of CPV and FPLV became obvious from the results of *dN* and *dS* analysis (Table 3). The *dS* was higher than *dN* when the entire NS1 and VP2 genes were compared among CPV or FPLV. Among CPV, however, *dN* was found to be higher than the *dS* when the comparison was focused on the Region 2, which is exposed on the surface of virion. When the entire gene regions are compared, the effect of conservation of function may predominate over that of positive selection, thereby leading to the interpretation that the gene is changing in a way that is consistent with the neutral theory of molecular evolution (Kimura, 1968), even though positive selection may be operating at least on specific regions of the gene (Gojobori *et al.*, 1990, 1994). Overdominant selection is found to operate on the antigen-recognition sites of major histocompatibility complex class I and II molecules on the basis of *dN* and *dS* analysis (Hughes and Nei, 1988, 1989). Furthermore, *dN* is also found to predominate over *dS* in the antigenic sites of some pathogens which may be subjected to immune response (Ina and Gojobori, 1994; Seibert *et al.*, 1995; Endo *et al.*, 1996), indicating that positive selection operates on such specific sites of the genes. Therefore, the results of *dN* and *dS* analysis suggested that positive selection may operate on at least one part of the VP2 gene of CPV in the canine population, as suggested by Parrish *et al.* (1991), although the real selection force remains to be determined. In contrast, no evidence for positive selection on the VP2 proteins of FPLV was obtained; rather, those seem to be in evolutionary stasis.

Comparison of evolution of FPLV with those of other viruses

Influenza A viruses and primate lentiviruses also display different types of evolution based on the host (Webster *et al.*, 1992; Seibert *et al.*, 1995). In the human immunodeficiency virus type 1 (HIV-1) that causes AIDS, the V3 loop of the envelope glycoprotein (gp120) is associated with various virus properties such as virus neutralization, cell tropism, and receptor recognition (McKeating, 1996). Rates of amino acid changes in the V3 loop of HIV-1 are found to be significantly higher than those of simian immunodeficiency virus from African green monkeys (SIM_{AGM}) (Shpaer and Mullins, 1993) that is considered to be the oldest primate lentiviruses (Johnson *et al.*, 1990) and avirulent for African green monkeys. Influenza A virus of aquatic birds appears to be fully adapted to waterfowls and is nonpathogenic to them. Some of the virus proteins of avian influenza A virus have remained unchanged for more than 50 years, while those of human influenza A virus change rapidly (Webster *et al.*, 1992). In both cases, fewer amino-acid variations appear in the viruses that are avirulent for their hosts. The evolutionary pattern of FPLV genes appears to be similar to those of influenza A virus of aquatic bird and SIV_{AGM}; however, FPLV is still pathogenic to felids. It is conceivable that the relatively low level of amino-acid substitutions in FPLV makes it difficult to elucidate the continuous change of the VP2 proteins.

Parvoviruses are remarkably stable when exposed to the environment, and their infectivity persists for a long period in feces. Thus changes in the VP2 protein that might help the virus escape immune surveillance may not be necessary for the continued survival of the virus; because the virus can survive in excreta without changing until its infects a naive host. Therefore, neutralizing antibodies may not be a major selection force for the VP2 gene of CPV, as suggested for the evolution of some other viruses by Wain-Hobson (1994). CPV has only a short history in the dog in contrast to FPLV in cats. We can hypothesize that CPV is undergoing changes that will result in its ability to replicate more efficiently in the dog. Support for this hypothesis lies in the finding that the NP gene newly introduced into swine populations evolved more rapidly than the classical NP gene of swine influenza A virus (Gorman *et al.*, 1991).

Implication of NS1 gene in host range

The VP2 gene affects the host range of parvoviruses (Parrish *et al.*, 1988a; Parrish, 1991; Chang *et al.*, 1992; Horiuchi *et al.*, 1994); characterization of CPV/FPLV chimeric viruses revealed that aa93, 103, and 323 in VP2 determine the host range of CPV (Chang *et al.*, 1992). In addition, our study of CPV/MEV chimeras showed that part of the NS1 gene contributes to the efficiency of virus replication in canine cells (Horiuchi *et al.*, 1994). Alignment of the NS1 genes from 20 isolates disclosed only three amino-acid residues that were CPV specific. Of those, aa595 is encoded within the region mentioned above. Perhaps aa595 influences viral growth in certain host cells.

How did CPV arise and how will new CPV arise?

Phylogenetic analyses of the CPV VP2 gene indicate origination of CPV from a common ancestor (Truyen *et al.*, 1995). Our phylogenetic tree of the CPV NS1 gene was consistent with that; all of the NS genes appeared to originate from a common ancestor. FPLV appeared to be in evolutionary stasis, which agrees with the idea that a CPV ancestor arose from FPLV or an FPLV-like virus as a result of random genetic mutations. However, this remains to be confirmed as do any similarities between the evolutionary patterns of FPLV-like viruses in each of their host to that established for FPLV in the cat. BFPV is suggested to be the closest virus to CPV on the basis of

TABLE 4

Viruses Used Here

Virus	Year of isolation	Place of isolation	Passage No.	Accession No. NS	Accession No. VP2	Reference of sequence
FPLV						
b	1967	U.S.	UK[a]	M38246	M38246	Parrish (1991)
PLI-IV	1968	France	UK	AB000057	D88287	This study
193	1970	Australia	UK	X55115	X55115	Martyn *et al.* (1990)
Obihiro	1974	Hokkaido*[b]	>20	AB000055	AB000056	This study
TU2	1975	Tokyo*	16	AB000065	AB000066	This study
TU4	1975	Tokyo*	15	AB000067	AB000068	This study
TU8	1976–1977	Tokyo*	14	AB000069	AB000070	This study
TU10	1978–1980	Tokyo*	18	AB000062	D78584	This study
TU12	1978–1980	Tokyo*	15	AB000063	AB000064	This study
483	1990	Hokkaido*	2	AB000048	D88286	This study
Fukagawa	1993	Fukushima*	2	AB000053	AB000054	This study
94-1	1994	Tottori*	1	AB000049	AB000050	This study
AO1	1994	Hokkaido*	1	AB000051	AB000052	This study
Som1	1994	Nara*	1	AB000058	AB000059	This study
Som4	1995	Saitama*	1	AB000060	AB000061	This study
FPLV-like						
MEV-Abashiri	1978	Hokkadio*	>20	D00765	D00765	Kariatsumari *et al.* (199
BFPV	1983	Finland	UK	NA[c]	U22185	Truyen *et al.* (1995)
CPV						
Norden	1978	U.S.	UK	M19296	M19296	Reed *et al.* (1988)
d	1979	U.S.	UK	M38245	M38245	Parrish (1991)
Y1	1982	Tokyo*	7	D26079	D26079	Horiuchi *et al.* (1994)
31	1983	U.S.	UK	NA	M24000	Truyen *et al.* (1995)
39	1984	U.S.	UK	NA	M74849	Truyen *et al.* (1995)
15	1984	U.S.	UK	NA	M24003	Truyen *et al.* (1995)

[a] Unknown.
[b] Asterisk means Japanese isolates (prefecture is indicated).
[c] Not available.

the phylogenetic relationship of VP2 genes (Truyen *et al.*, 1995). Based on the NS1 gene, MEV appeared to be the closest virus to CPV (Fig. 2A). Some animals in the family *Canidae* that are susceptible to FPLV-like viruses, such as mink and blue fox, may play a role as reservoirs for the ancestors of CPV.

Several interspecies transmissions of some genes of influenza A virus from aquatic bird to humans, horses, and swine are reported (Kawaoka *et al.*, 1989; Gorman *et al.*, 1991). The considerable genetic diversity of the influenza A virus of aquatic bird increases the chance for production of a source of new genes that would have a selective advantage in a particular host or altered circumstance (Gorman *et al.*, 1992; Webster *et al.*, 1992). We attempted, without success, to find a virus in FPLV populations that would have characteristics of CPV after selection by escape from FPLV-specific MAbs. The evolutionary rates of the FPLV genes is 10–100 times lower than those of RNA viruses (Domingo and Holland, 1994). Taken together with the proposal that accumulated mutations may be required for the virus to acquire infectivity in the dog (Chang *et al.*, 1992), interspecies transmission of the virus from cat to dog does not seem to take place readily.

CPV-2a and 2b were recently isolated from cats manifesting clinical signs of feline panleukopenia (Mochizuki *et al.*, 1996; Truyen *et al.*, 1996). This may mean that CPV and FPLV can infect host cells simultaneously, a situation that might provide the opportunity for intermolecular recombination between the two viruses. Such an occurrence might confer expanded host range or altered pathogenicity to the viruses. In some RNA viruses such as alphavirus and coronavirus, intermolecular recombination drives the emergence of new pathogenic virus (Hahn *et al.*, 1988; Olsen, 1993). Thus monitoring of recent isolates may be needed to anticipate and assess the risk caused by such newly emerging viruses.

MATERIALS AND METHODS

Viruses, cells, and antibodies

The virus isolates used here are listed in Table 4. Crandell feline kidney (CRFK) cells (Crandell *et al.*, 1973) and A72 canine fibroma cells (Binn *et al.*, 1980) were maintained as described previously (Horiuchi *et al.*, 1992). Anti-MEV rabbit serum and MAbs P2–215 and X1–251 were used in this study. The MAb P2–215 recognizes the FPLV and FPLV-like viruses-specific epitope

that includes aa93-Lys and the MAb X1-251 reacts equally with CPV and FPLV and FPLV-like viruses (Horiuchi *et al.*, 1997).

Selection of escape mutants

Virus stocks of field isolates of FPLV [approximately 10^6 plaque forming units (PFU)/100 μl] were incubated with an equal volume of ascitic fluid (diluted 1:100) containing MAb P2-215 for 1 h at room temperature (r.t.), and used to inoculate CRFK cells. After absorption, the cells were washed with medium, and growth medium containing 0.5% MAb P2-215-ascitic fluid was replaced. Blind passage was repeated in the presence of MAb P2-215 until viral CPE was observed.

Infectious center assay

To estimate the proportion of variant viruses in the original virus stocks, 100 μl of virus stock were incubated with an equal volume of a 1:100 dilution of MAb P2-215 ascites or negative control MAb BSPX-54 (Horiuchi *et al.*, 1995) for 1 h at r.t., and then viruses were adsorbed to CRFK cells for 1 h at 37°C. After absorption, the cells were overlaid with growth medium containing 1.2% methyl cellulose and 0.5% MAb P2-215-ascites, and cultured for 5 days at 37°C. Staining of infectious centers was carried out as described elsewhere (Horiuchi *et al.*, 1994).

Characterization of antigenicity

The antigenicity of the viruses was determined by antibody-sandwich ELISA as described elsewhere (Horiuchi *et al.*, 1997).

In vitro host range analysis

The host range of the viruses was determined by the ability of the viruses to replicate in canine cell lines, A72 canine fibroma cells and Cf2Th canine thymus cells. Low-molecular-weight DNA was extracted from virus-infected cells, and the presence of RF DNA was analyzed by Southern blot. The details were described previously (Horiuchi *et al.*, 1992).

Preparation of virion DNA and cloning of RF DNA

Virion DNA used as a template for PCR was prepared as described previously (Horiuchi *et al.*, 1996). RF DNA was recovered from virus-infected cells in two 100-mm dishes. Extraction of RF DNA was carried out by a modified Hirt's method as described previously (Horiuchi *et al.*, 1992). The RF DNA was digested with the restriction enzyme *Bst*I107 I and the resulting 4.3-kb fragment, which corresponds to map units (m.u.) 6.4-93.3, was cloned into the *Eco*RV site of the Bluescript SK+ vector (Stratagene).

PCR and nucleotide sequence analysis

The sequences of primers used for PCR are describec below. The region from nt245 to 2371, which contains the entire NS1 ORF (nt311 to 2314), was amplified by PCR in three sections with three sets of primers: primers N1 and N2, primers N3 and N4, and primers N5 and N6. The region from nt2726 to 4759, which contains the entire VP2 ORF (nt2825 to 4576), was amplified in five sections with five sets of primers: primers V51 and V55, primers V1 and V52, primers V5 and V9, primers V8 and V56, and primers V22 and V41. Amplifications were done with Expand™ High-Fidelity System (Boehringer Mannheim). The amplified fragments were purified through a S-300 HR spin column (Pharmacia) to remove the excess primers and then used for the direct sequencing of the PCR product. To determine the nucleotide sequence of the VP2 gene of the escape mutants, RF DNA cloned into the plasmid vector was used as a template for the sequencing reaction. Nucleotide sequences were determined with an automated DNA sequencer (ABI-373A, Applied Biosystems) and ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems). The sequences were analyzed with GENETYX-MAC/CD software (Software Development).

The sequences of primers are as follows: N1, 5′-CATAGACCGTTACTGACATTC-3′ (nt 245-265); N2, 5′-TATGTCTGTCTTGATACTTC-3′ [nt 1033-1052 [complementary (comp)]]; N3 5′-CATTTTGGAAATATGATAGCA-3′ (nt 926-946); N4, 5′-ATTTGCTTAGTTCCTTTACCT-3′ [nt 1720-1820 (compl)]; N5, N5′-AGCTGGTAACTTTGGTCAAC-3′ (nt 1645-1664); N6, 5′-TACTAACACACCCTTACCTC-3′ [nt 2352-2371 (comp)]; V1, 5′-GTACATTTAAATATGCCAGA-3′ (nt 3029-3048); V5, 5′-AGCTATGAGATCTGAGACA-3′ (nt 3388-3406); V8, 5′-AATACAAACTATATTACTGAAG-3′ (nt 3785-3806); V9, 5′-TCCTGCTGGATATCTTCCT-3′ [nt 4042-4060 (comp)]; V22, 5′-TGTCAAAATAATTGTCCTG-3′ (nt 4292-4310); V41, 5′-ATTGTATACCATATAACAAACC-3′ [nt 4738-4759 comp)]; V51, 5′-CCAACTAAAAGAAGTAAACC-3′ (nt 2726-2745); V52, 5′-ATTAATGTTCTATCCCATTG-3′ [nt 3461-3480 (comp)]; V55, 5′-ACTAACTAAATGCAACTCAC-3′ [nt 3224-3243 (comp)]; V56, 5′-AATTGGATTCCAAGTATGAG-3′ [nt 4449-4468 (comp)].

Molecular evolutional analysis

The phylogenetic relationship was analyzed by using the branch-and-bound algorithm of the PAUP (phylogenetic analysis using parsimony) package version 3.1.1 (Swofford, 1993). Evolutionary rates for NS1 and VP2 genes were estimated from the regression of the year of isolation against the number of differences between each isolate and the internal node that were tentatively assigned as a fiducial point (indicated in Figs. 2 and 3). Programs from the PHYLIP (Phylogeny inference package) version 3.5c (Felsenstein, 1993) were also used to infer the phylogenetic tree; pairwise genetic distances were calculated by using the DNADIST program under Kimura's two-parameter model, and phylogenetic trees

were constructed by the neighbor-joining method by using the NEIGHBOR program. A bootstrap analysis with 100 replicates was done to assess the confidence level of the branch pattern. A value of >70% is considered significant (Hills and Bull, 1993).

The ratio of synonymous (*dS*) and nonsynonymous (*dN*) substitutions were estimated by Nei and Gojobori's method (Nei and Gojobori, 1986).

ACKNOWLEDGMENT

We thank Dr. Byron Caughey (Rocky Mountain Laboratories, NIAID, National Institute of Health) for critical reading of this manuscript.

REFERENCES

Agbandje, M., McKenna, R., Rossmann, M. G., Strassheim, M. L., and Parrish, C. R. (1993). Structure determination of feline panleukopenia virus empty particles. *Proteins* **16**, 155–171.

Bean, W. J., Schell, M., Katz, J., Kawaoka, Y., Naeve, C., Gorman, O., and Webster, R. G. (1992). Evolution of the H3 influenza virus hemagglutinin from human and nonhuman hosts. *J. Virol.* **66**, 1129–1138.

Binn, L. N., Marchwicki, R. H., and Stephenson, E. H. (1980). Establishment of a canine cell line: Derivatization, characterization, and viral spectrum. *Am. J. Vet. Res.* **41**, 855–860.

Carmichael, L. E., Joubert, J. C., and Pollock, R. V. H. (1980). Hemagglutination by canine parvovirus: Serologic studies and diagnostic applications. *Am. J. Vet. Res.* **41**, 784–791.

Chang, S.-F., Sgro, J.-Y., and Parrish, C. R. (1992). Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. *J. Virol.* **66**, 6858–6867.

Chapman, M. S., and Rossmann, M. G. (1993). Structure, sequence, and function correlations among parvoviruses. *Virology* **194**, 491–508.

Cotmore, S. F., and Tattersall, P. (1987). The autonomously replicating parvoviruses of vertebrates. *Adv. Virus Res.* **33**, 91–174.

Cotmore, S. F., and Tattersall, P. (1989). A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. *J. Virol.* **63**, 3902–3911.

Crandell, R. A., Fabricant, C. G., and Nelson-Rees, W. A. (1973). Development, characterization, and viral susceptibility of a feline (*Felis catus*) renal cell line (CRFK). *In Vitro* **9**, 176–185.

Domingo, E., and Holland, J. J. (1994). Mutation rates and rapid evolution of RNA viruses. *In* "The Evolutionary Biology of Viruses," (S. S. Morse, Ed.), pp. 161–184. Raven Press, New York.

Endo, T., Ikeo, K., and Gojobori, T. (1996). Large-scale search for genes on which positive selection may operate. *Mol. Biol. Evol.* **13**, 685–690.

Felsenstein, J. (1993). PHYLIP (Phylogeny inference package) version 3.5c. University of Washington, Seattle.

Fitch, W. M., Leiter, J. M. E., Li, X., and Palese, P. (1991). Positive darwinian evolution in human influenza A viruses. *Proc. Natl. Acad. Sci. USA* **88**, 4270–4274.

Gojobori, T., Moriyama, E. N., and Kimura, M. (1990). Molecular clock of viral evolution, and the neutral theory. *Proc. Natl. Acad. Sci. USA* **87**, 10015–10018.

Gojobori, T., Yamaguchi, Y., Ikeo, K., and Mizokami, M. (1994). Evolution of pathogenic viruses with special reference to the rates of synonymous and nonsynonymous substitutions. *Jpn. J. Genet.* **69**, 481–488.

Gorman, O. T., Bean, W. J., Kawaoka, Y., Donatelli, I., Guo, Y., and Webster, R. G. (1991). Evolution of influenza A virus nucleoprotein genes: Implications for the origins of H1N1 human and classical swine viruses. *J. Virol.* **65**, 3704–3714.

Gorman, O. T., Bean, W. J., Kawaoka, Y., and Webster, R. G. (1990). Evolution of the nucleoprotein gene of influenza A virus. *J. Virol.* **64**, 1487–1497.

Gorman, O. T., Bean, W. J., and Webster, R. G. (1992). Evolutionary processes in influenza viruses: Divergence, rapid evolutionm, and stasis. *Curr. Top. Microbiol. Immunol.* **176**, 75–97.

Hahn, C. S., Lustig, S., Strauss, E. G., and Strauss, J. H. (1988). Western equine encephalitis virus is a recombinant virus. *Proc. Natl. Acad. Sci. USA* **85**, 5997–6001.

Hillis, D. M., and Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst Biol.* **42**, 182–192.

Horiuchi, M., Goto, M., Ishiguro, N., and Shinagawa, M. (1994). Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses. *J. Gen. Virol.* **75**, 1319–1328.

Horiuchi, M., Ishiguro, N., Goto, H., and Shinagawa, M. (1992). Characterization of the stage(s) in the virus replication cycle at which the host-cell specificity of the feline parvovirus subgroup is regurated in canine cells. *Virology* **189**, 600–608.

Horiuchi, M., Mochizuki, M., Ishiguro, N., Nagasawa, H., and Shinagawa, M. (1997). Epitope mapping of a monoclonal antibody specific to feline panleukopenia virus and mink enteritis virus. *J. Vet. Med. Sci.* **59**, 133–136.

Horiuchi, M., Yamazaki, N., Ikeda, T., Ishiguro, N., and Shinagawa, M. (1995). A cellular form of prion protein (PrP^C) exists in many non-neuronal tissues of sheep. *J. Gen. Virol.* **76**, 2583–2587.

Horiuchi, M., Yuri, K., Soma, T., Katae, H., Nagawsawa, H., and Shinagawa, M. (1996). Differentiation of vaccine virus from field isolates of feline panleukopenia virus by polymerase chain reaction and restriction fragment length polymorphism analysis. *Vet. Microbiol.* **53**, 283–293.

Hughes, A. L., and Nei, M. (1988). Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature* **335**, 167–170.

Hughes, A. L., and Nei, M. (1989). Nucleotide substitution at major histocompatibility complex class II loci: Evidence for overdominant selection. *Proc. Natl. Acad. Sci. USA* **86**, 958–962.

Ina, Y., and Gojobori, T. (1994). Statistical analysis of nucleotide sequences of the hemagglutinin gene of human influenza A viruses *Proc. Natl. Acad. Sci. USA* **91**, 8388–8392.

Ito, T., Gorman, O. T., Kawaoka, Y., Bean, W. J., and Webster, R. G. (1991) Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. *J. Virol.* **65**, 5491–5498.

Johnson, P. R., Fomsgaard, A., Allan, J., Gravell, M., London, W. T. Olmsted, R. A., and Hirsch, V. M. (1990). Simian immunodeficiency viruses from African green monkeys display unusual genetic diversity. *J. Virol.* **64**, 1086–1092.

Kariatsumari, T., Horiuchi, M., Hama, E., Yaguchi, K., Ishiguro, N., Goto H., and Shinagawa, M. (1991). Construction and nucleotide sequence analysis of an infectious DNA clone of the autonomous parvovirus mink enteritis virus. *J. Gen. Virol.* **72**, 867–875.

Kawaoka, Y., Krauss, S., and Webster, R. G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. *J. Virol.* **63**, 4603–4608.

Kilbourne, E. D. (1991). New viruses and new disease: mutation, evolution, and ecology. *Curr. Opin. Immunol.* **3**, 518–524.

Kimura, M. (1968). Evolutionary rate at the molecular level. *Nature* **217** 624–626.

Koptopoulos, G., Papadopoulos, O., Papanastasopoulou, M., and Cornwell, H. J. C. (1986). Presence of antibody cross-reacting with canine parvovirus in the sera of dogs from Greece. *Vet. Rec.* **118**, 332–333.

Langeveld, J. P. M., Casal, J. I., Vela, C., Dalsgaard, K., Smale, S. H. Puijk, W. C., and Meloen, R. H. (1993). B-cell epitopes of canine parvovirus: distribution on the primary structure and exposure on the viral surface. *J. Virol.* **67**, 765–772.

Li, W.-H., Tanimura, M., and Sharp, P. M. (1988). Rates and Dates of divergence between AIDS virus nucleotide sequences. *Mol. Biol. Evol.* **5**, 313–330.

Martyn, J., Davidson, B. E., and Studdert, M. J. (1990). Nucleotide sequence of feline panleukopenia virus: Comparison with canine

parvovirus identifies host-specific differences. *J. Gen. Virol.* **71,** 2747–2753.

McKeating, J. A. (1996). Biological consequences of human immunodeficiency virus type 1 envelope polymorphism: Does variation matter? *J. Gen. Virol.* **77,** 2905–2919.

Mochizuki, M., and Hashimoto, T. (1986). Growth of feline panleukopenia virus and canine parvovirus *in vitro*. *Jpn. J. Vet. Sci.* **48,** 841–844.

Mochizuki, M., Horiuchi, M., Hiragi, H., Gabriel, M. C. S., Yasuda, N., and Uno, T. (1996). Isolation of canine parvovirus from a cat manifesting clinical signs of feline panleukopenia. *J. Clin. Microbiol.* **34,** 2101–2105.

Mochizuki, M., Konishi, S., Ajiki, M., and Akaboshi, T. (1989). Comparison of feline parvovirus subspecific strains using monoclonal antibodies against a feline panleukopenia virus. *Jpn. J. Vet. Sci.* **51,** 264–272.

Nei, M., and Gojobori, T. (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* **3,** 418–426.

Olsen, C. W. (1993). A review of feline infectious peritonitis: molecular biology, immunopathogenesis, clinical aspects, and vaccination. *Vet. Microbiol.* **36,** 1–37.

Parrish, C. R. (1990). Emergence, natural history, and variation of canine, mink, and feline parvoviruses. *Adv. Virus Res.* **38,** 403–450.

Parrish, C. R. (1991). Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. *Virology* **183,** 195–205.

Parrish, C. R., Aquadro, C. F., and Carmichael, L. E. (1988a). Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink, and raccoon parvoviruses. *Virology* **166,** 293–307.

Parrish, C. R., Aquadro, C. F., Strassheim, M. L., Evermann, J. F., Sgro, J.-Y., and Mohammed, H. O. (1991). Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. *J. Virol.* **65,** 6544–6552.

Parrish, C. R., and Carmichael, L. E. (1983). Antigenic structure and variation of canine parvovirus type-2, feline panleukopenia virus, and mink enteritis virus. *Virology* **129,** 401–414.

Parrish, C. R., Carmichael, L. E., and Antczak, D. F. (1982). Antigenic relationships between canine parvovirus type 2, feline panleukopenia virus and mink enteritis virus using conventional antisera and monoclonal antibodies. *Arch. Virol.* **72,** 267–278.

Parrish, C. R., Gorham, J. R., Schwartz, T. M., and Carmichael, L. E. (1984). Characterization of antigenic variation among mink enteritis virus isolates. *Am. J. Vet. Res.* **45,** 2591–2599.

Parrish, C. R., Have, P., Foreyt, W. J., Evermann, J. F., Senda, M., and Carmichael, L. E. (1988b). The global spread and replacement of canine parvovirus strains. *J. Gen. Virol.* **69,** 1111–1116.

Parrish, C. R., O'Connell, P. H., Evermann, J. F., and Carmichael, L. E. (198). Natural variation of canine parvovirus. *Science* **230,** 1046–1048.

Reed, A. P., Jones, E. V., and Miller, T. J. (1988). Nucleotide sequence and genome organization of canine parvovirus. *J. Virol.* **62,** 266–276.

Schwers, A., Pastoret, P.-P., Burtonboy, G., and Thiry, E. (1979). Fréquence en Belgique de l'infection à parvovirus chez le chien, avant et après l'observation des premiers cas cliniques. *Ann. Med. Vet.* **123,** 561–566.

Seibert, S. A., Howell, C. Y., Hughes, M. K., and Hughes, A. L. (1995). Natural selection on the gag, pol, and env genes of human immunodeficiency virus 1 (HIV-1). *Mol. Biol. Evol.* **12,** 803–813.

Senda, M., Hirayama, N., Itoh, O., and Yamamoto, H. (1988). Canine parvovirus: Strain difference in haemagglutination activity and antigenicity. *J. Gen. Virol.* **69,** 349–354.

Shpaer, E. G., and Mullins, J. I. (1993). Rates of amino acid change in the envelope protein correlate with pathogenicity of primates lentiviruses. *J. Mol. Evol.* **37,** 57–65.

Siegl, G., Bate, R. C., Berns, K. I., Carter, B. J., Kelly, D. C., Kurstak, E., and Tattersall, P. (1985). Characteristics and taxonomy of Parvoviridae. *Intervirology* **23,** 61–73.

Strassheim, M. L., Gruenberg, A., Veijalainen, P., Sgro, J.-Y., and Parrish, C. R. (1994). Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. *Virology* **198,** 175–184.

Swofford, D. L. (1993). PAUP (phylogenetic analysis using parsimony) version 3.1. Laboratory of Molecular Systematics, Smithsonian Institution, Washington, DC.

Tratschin, J.-D., McMaster, G. K., Kronauer, G., and Siegl, G. (1982). Canine parvovirus: Relationship to wild-type and vaccine strains of feline panleukopenia virus and mink enteritis virus. *J. Gen. Virol.* **61,** 33–41.

Truyen, U., Agbandje, M., and Parrish, C. R. (1994). Characterization of the feline host range and a specific epitope of feline panleukopenia virus. *Virology* **200,** 494–503.

Truyen, U., Evermann, J. F., Vieler, E., and Parrish, C. R. (1996). Evolution of canine parvovirus involved loss and gain of feline host range. *Virology* **215,** 186–189.

Truyen, U., Grruenberg, A., Chang, S.-F., Obermaier, B., Veijalainen, P., and Parrish, C. R. (1995). Evolution of the feline-subgroup parvoviruses and the control of canine host range in vivo. *J. Virol.* **69,** 4702–4710.

Truyen, U., and Parrish, C. R. (1992). Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: Distinct host cell tropisms of each virus in vitro and in vivo. *J. Virol.* **66,** 5399–5408.

Tsao, J., Chapman, M. S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T. J., Rossmann, M. G., Compans, R. W., and Parrish, C. R. (1991). The three-dimensional structure of canine parvovirus and its functional implications. *Science* **251,** 1456–1464.

Veijalainen, P. (1988). Characterization of biological and antigenic properties of raccoon dog and blue fox parvoviruses: A monoclonal antibody study. *Vet. Microbiol.* **16,** 219–230.

Wain-Hobson, S. (1994). Is antigenic variation of HIV important for AIDS and what might be expected in the future? *In* "The Evolutionary Biology of Viruses," (S. S. Morse, Ed.), pp. 185–209. Raven Press, New York.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56,** 152–179.

Protective Effect of Lactoferricin against *Toxoplasma gondii* Infection in Mice

Tsuya ISAMIDA, Tetsuya TANAKA*, Yoshitaka OMATA, Koji YAMAUCHI[1)], Kei-ichi SHIMAZAKI[2)] and Atsushi SAITO

Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080, [1)]Nutritional Science Laboratory, Morinaga Milk Industry, Co., Ltd., Zama 228, and [2)]Dairy Science Laboratory, Animal Science Department, Faculty of Agriculture, Hokkido University, Sapporo 060, Japan

(Received 6 August 1997/Accepted 27 September 1997)

ABSTRACT. The protective effect of lactoferricin against *Toxoplasma gondii* infection was examined in experimental murine toxoplasmosis. All mice orally administered 5.0 mg of lactoferricin, and challenged with cysts of *T. gondii* at a dose of LD_{90} survived until the end of experiment (35 days post challenge). Intraperitoneal administration of 0.1 mg of lactoferricin also prevented death in 100 % of treated mice challenged with *T. gondii* cysts. In contrast, 80 % of untreated mice died of acute toxoplasmosis within 14 days post challenge. In the mice treated perorally with lactoferricin, the number of cysts in the brain was significantly lower than that in untreated mice. Levels of interferon-r in the serum of infected mice treated perorally with lactoferricin showed a tendency to lower than those in the infected mice without treatment. These results demonstrate that oral administration of lactoferricin induces resistance to *T. gondii* infection in mice.
— KEY WORDS: bovine lactoferricin, protection, *Toxoplasma gondii*.

J. Vet. Med. Sci. 60(2): 241–244, 1998

The obligate intracellular parasite *Toxoplasma gondii (T. gondii)*, a protozoan of the apicomplexa penetrates tissues of Vertebrates as tachyzoites and forms cysts containing a number of bradyzoites to escape host defense systems. Felidae, a definitive host of *T. gondii* shed environmentally resistant oocysts in their feces after primary infection. Ingestion of drinking water or uncooked meat contaminated with cysts or sporulated oocysts is a common source of *T. gondii* infection in humans. To prevent such transmission of toxoplasmosis, food additives which have anti-toxoplasma activity should be developed. Lactoferrin (Lf), an iron-binding protein found in exocrine secretions such as milk, saliva, and tears and in neutrophils, is thought to play an important role in the host defense against infectious disease. It has broad-spectrum antimicrobial properties and immunomodulatory properties [6, 7, 9]. Furthermore, it has been demonstrated that a potent antimicrobial peptide, named lactoferricin (Lfcin), is produced by gastric pepsin cleavage of Lf [1, 2]. It has recently been shown that Lfcin displays parasiticidal activity *in vitro* against *T. gondii* [12]. The present study aimed to examine the effect of administered Lfcin in mice acutely infected with this parasite. It remains obscure whether Lfcin can pass through the intestinal mucosa into tissues and serum, and exert biological activity in the organs of the animals to which it is administered.

Since IFN-r produced by both CD4+, CD8+ and NKcells plays an essential role in the protective response against *T. gondii* [5, 10], we also investigated whether the resistance is dependent on immune effector cells, especially T-cells, and whether administered Lfcin influences serum levels of IFN-r. Female ICR mice at 8 weeks of age, weighing 23–28 g were used throughout the experiments.

Lactoferricin was prepared from lactoferrin as described by Bellamy *et al.* [1], and dissolved in saline at a concentration of 10.0 mg/m*l* or 1.0 mg/m*l* just before use.

Cysts of the Beverley strain of *T. gondii* were obtained by homogenization of cerebral tissue from infected mice 2–3 months post infection. The number of cysts in the homogenates of cerebral tissue was determined by counting microscopically. Briefly, in each instance, 10 μl of homogenate made from one brain was placed on a slide glass and observed under an 18 × 18-mm coverslip. All samples were prepared in triplicate.

In vivo experiments examining the effects of administered Lfcin were performed by experimental infection of mice with *T. gondii*. Thirty mice were orally challenged with cysts at a dose of either LD_{50} or LD_{90} and divided into three groups. Thirty minutes before challenge, and once again on each of the first 7 days post infection, two groups of five mice were orally administered either 0.5 mg or 5.0 mg of Lfcin in 0.5 m*l* of saline. Another two groups of five mice were administered intraperitoneally either 0.1 or 1.0 mg of Lfcin in 0.5 m*l* of saline on each of the first 7 days post infection. As a negative control, five mice were orally administered saline. To examine a different route of the infection, five mice orally administered 5.0 mg of Lfcin were challenged intraperitoneally with cysts at a dose of LD_{50}. After challenge, the mortality and survival period of the mice were monitored up to 35 days post challenge. On day 35 post-infection the survivors were sacrificed and the brain of each mouse was removed and homogenized gently in 3 m*l* of phosphate buffered saline (PBS) with a glass homogenizer in order to count the number of cysts. To examine the serum levels of Interferon-r, each mouse in the experiment described above was bled and approximately 100 μl/mouse was obtained from the supraorbital vein at various times post inoculation. The sera were stored at −80°C until use. Assay of Interferon-r in the serum was performed by ELISA using an assay kit (mouse ELISA

* PRESENT ADDRESS: TANAKA, T., Division of Veterinary Microbiology, Kyoto Biken Laboratories, 24–16 Makishima-cho, Uji, Kyoto 611, Japan.

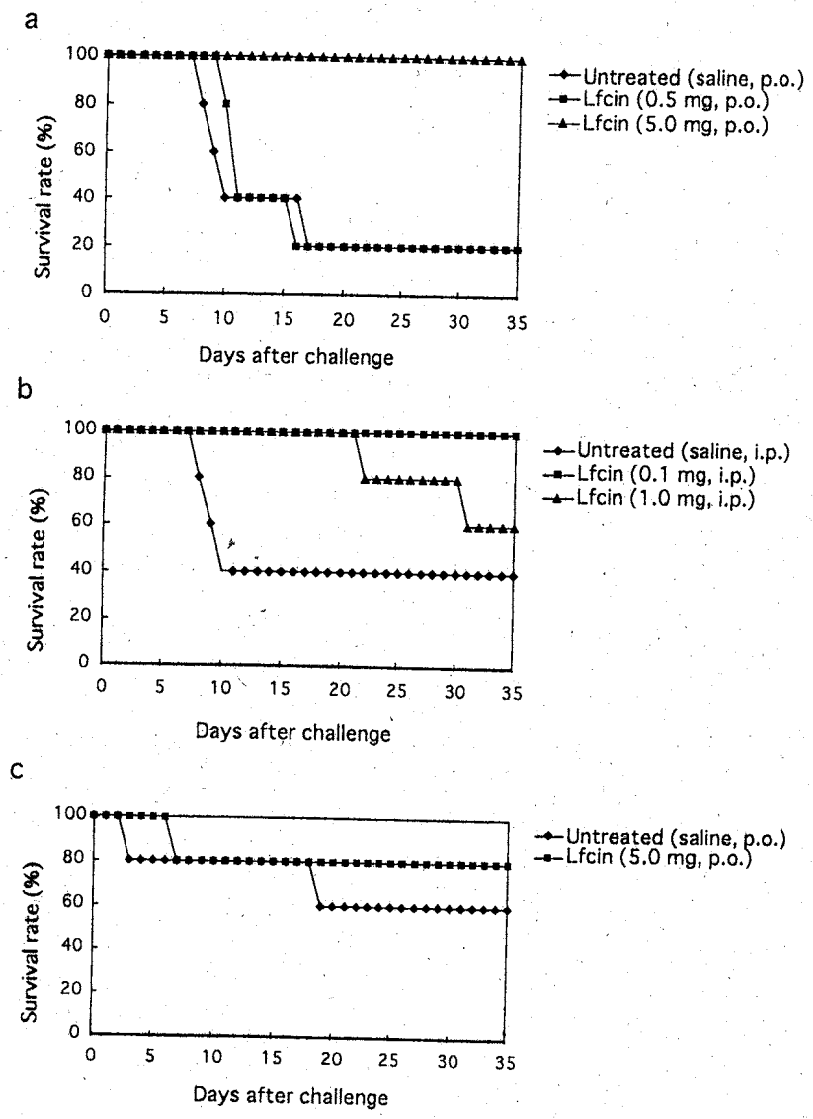


Fig. 1. Effect of administered Lfcin on resistance of mice to *T. gondii* infection. a: The mice were orally administered 0.5 mg or 5.0 mg of Lfcin in 0.5 m*l* of saline after oral challenge with *T. gondii*. b: The mice were administered intraperitoneally 0.1 mg or 1.0 mg of Lfcin in 0.5 m*l* of saline after oral challenge with *T. gondii*. c: The mice were orally administered 5.0 mg of Lfcin in 0.5 m*l* of saline after intraperitoneal challenge with *T. gondii*.

IFN-r; ENDOGEN,Inc., Cambridge, MA.). All experiments were done at least twice.

As shown in Fig. 1, in the case of mice orally inoculated with *T. gondii* cysts, all of the five mice orally administered 5.0 mg of Lfcin survived for at least 35 days post challenge, whereas, 60 % of the mice in the group orally administered 0.5 mg of Lfcin died of acute toxoplasmosis within 14 days post challenge (Fig. 1a). Substantial numbers of tachyzoites (approximately $\times 10^7$/m*l*) were found in the peritoneal cavity of mice administered 1.0 mg of Lfcin intraperitoneally, and 40% of the mice in this group died of toxoplasmosis within 22 to 31 days post challenge. In contrast, all of the mice administered 0.1 mg of Lfcin intraperitoneally survived until the end of the experiment (Fig. 1b). In the control group, 60% of the mice administered saline died of acute toxoplasmosis within 14 days post challenge. In the case of mice orally administered 5.0 mg of Lfcin and challenged intraperitoneally with one LD_{50} dose of cysts, 20% of the mice died of acute toxoplasmosis within 7 days post challenge. In the control group, 40% of the mice also died within 7 to 18 days post challenge (Fig. 1c).

The number of cysts found in the brain of mice in each group tested is shown in Table 1. The number of cysts in the case of mice orally administered 5.0 mg of Lfcin was significantly lower (147 ± 56 cysts/mouse brain), compared with that of survivors in the control group (3,900 cysts/

Table 1. The survival rate of mice and the numbers *T. gondii* cysts in cerebral tissue of mice in Lfcin-treated and untreated groups.

Group	Administration route of Lfcin	Average of total number of cysts[a] in the brain	Survival rate[b] (%)
Untreated (saline, n=1)	p.o.	3900	20
Lfcin (0.5 mg, n=1)	p.o.	600	20
Lfcin (5.0 mg, n=1)	p.o.	147 ± 56[c]	100
Untreated (saline, n=2)	i.p.	3300, 3400, (AVG=3350)	40
Lfcin (0.1 mg, n=5)	i.p.	2767 ± 2498	100
Lfcin (1.0 mg, n=3)	i.p.	2933 ± 1514	60
Untreated (saline, n=3)	p.o.	2100 ± 1552	
Lfcin (5.0 mg, n=4)	p.o.	1825 ± 618	80

a) Cerebral tissue per mouse. b) Survival up to day 35 post inoculation/total mice tested. c) Mean ± SD. p.o.: Peroral. i.p.: Intraperitoneal. n: Number of mouse brains examined.

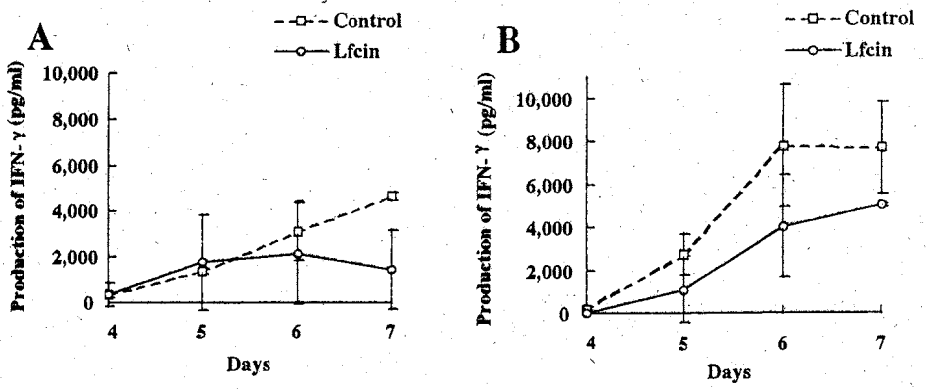


Fig. 2. Production of IFN-r in serum of mice (n=3). A: The mice were orally administered 5.0 mg of Lfcin for 7 days post p.o.infection. B: The mice were intraperitoneally administered 1.0 mg Lfcin for 7 days post p.o. infection.

mouse brain). The number of cysts in the case of survivors in the group orally administered 0.5 mg of Lfcin (600 cysts/mouse brain) was also lower than that of the control. In the case of mice administered 1.0 mg of Lfcin intraperitoneally, individual differences in the number of cysts (4,000, 3,600 and 1,200 cysts/mouse brain) were significant and the average number of cysts was similar to that of the control mice. The number of cysts in the case of mice administered 0.1 mg of Lfcin intraperitoneally also varied substantially (1,000–6,300 cysts /mouse brain). In the case of mice orally administered 5.0 mg of Lfcin following intraperitoneal challenge, the number of cysts found in the brain was similar to that of the control mice.

IFN-r was detected in the serum of all of the p.o. infected mice, whether untreated or treated with Lfcin. The levels of IFN-r in the untreated mice were 1,000 *pg/ml* on day 5 and 784 *pg/ml* on day 6 post challenge. In contrast, the serum levels of IFN-r in the infected mice treated orally with Lfcin showed a tendency to lower than those of untreated (Fig. 2). No IFN-r was detected in the serum of non-infected mice treated orally with Lfcin.

As described in our previous report [12], Lfcin has parasiticidal activity against *T. gondii* tachyzoites and bradyzoites in cysts *in vitro*. In the present study, orally administered Lfcin was shown to exert a protective effect against *T. gondii* infection *in vivo*, and its protective effect was dose dependent. Based on these results, two possibilities should be considered. One possibility is that Lfcin may interact directly with *T. gondii* cysts and bradyzoites in the intestinal tract, resulting in a decrease in infectivity of the parasites. Another possibility is that Lfcin itself and/or structural components released from the parasites as a result of its interaction with Lfcin may activate the host's defense systems. Regarding the former possibility, It has been reported that Lfcin is a cationic peptide having a strong capacity to bind to anionic surface components on the surface of biological membranes [2, 8]. The cell surface of *T. gondii* tachyzoites is known to have a strong negative charge and binds cationic substances [3, 4]. It has been reported that liposomes consisting of stearylamine, a positively charged lipid have parasiticidal activity against *T. gondii* [11]. Thus, it is plausible that Lfcin has the capacity to bind to the surface of the parasite in the intestinal tract, and this interaction may lead to a disruption of biological function of the parasites' membrane, resulting in loss of infectivity.

In addition to the oral route of challenge, we examined the resistance of mice to intraperitoneal infection with *T.*

gondii cysts at a dose of LD_{50}. The results showed that the resistance of mice orally administered 5.0 mg of Lfcin was incomplete, compared with the case of oral challenge. This suggests that orally administered Lfcin has little effect on parasites present in the peritoneal cavity and tissue organs.

When Lfcin was administered intraperitoneally, the group of mice given the lower dose (i.e. 0.1 mg of Lfcin) showed greater resistance against *T. gondii* infection than those given the higher dose (1.0 mg of Lfcin). A lower survival ratio was observed at the higher dose and the number of cysts in the brain was similar to that of control mice. We have insufficient data to explain these results in this report. It seems that the optimal dose of Lfcin against the parasite in the peritoneal cavity and abdominal organs is below 1.0 mg/mouse (perhaps near 0.1 mg/mouse) and an excess of Lfcin appears to result in suppressive effects or damage to the defense systems of the mouse.

With regard to the latter possibility, we investigated the levels of IFN-r in the serum of mice untreated or treated with Lfcin. Lower levels of IFN-r were observed in the serum of mice orally treated with Lfcin as compared with the levels in untreated mice. It is conceivable that the parasiticidal activity of Lfcin in the intestinal tract might be highly effective and, consequently, the number of parasites which penetrate the intestinal organs of the mice may be significant fewer than that in the case of control mice, and those few parasites would stimulate only a slight protective immune response resulting in lower serum levels of IFN-r.

Oral administration of Lfcin had no effect on serum levels of IFN-r in non-infected mice. We have no data in this report to suggest that Lfcin has the capacity to suppress IFN-r production. Further study is required to elucidate the biological activity of Lfcin, especially its regulatory role in the immune system.

ACKNOWLEDGMENT. We thank Dr. Wayne R. Bellamy for helpful editing.

REFERENCES

1. Bellamy, W. R., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S. and Tomita, M. 1993. *J. Appl. Bacteriol* 75: 478–484.
2. Bellamy ,W. R., Takase, M,. Wakabayashi, H., Kawase, K. and Tomita, M. 1992. *J. Appl. Bacteriol* 73: 472–479.
3. Cintra, W. M. and De Souza,W. 1985. *Eur J. of Cell Biol* 37: 63–69.
4. Cintra,F., Silva-Filho, C. and De Souza, W. 1986. *J. Submicroscopic Cytol* 18: 773–781.
5. Kasper, L. H., Currie, K. M. and Bradley, M. S. 1985. *J. Immunol* 134: 3426-3431
6. Lima, M. F. and Kierszenbaum, F. 1987. *J. Immunol.* 139: 1647–1651
7. Lima, M. F. and Kierszenbaum, F. 1985. *J. Immunol.* 134: 4176–4183.
8. Longhi, C., Conte, M. P., Bellamy, W., Seganti, I. and Valenti, P. 1994. *Med. Microbiol. Immunol* 183: 77–85.
9. Sanchez, L., Calvo, M. and Brock, H. J. 1992. *Arch. Dis . Child.* 67: 657–661.
10. Suzuki ,Y., Orellana, M. A., Schreiber, R. and Remington, J. S. 1988. *Science* 240: 516–519.
11. Tachibana, H., Yoshihara, E., Kaneda, Y. and Nakae, T. 1990. *J. Parasitol* 76: 352–355.
12. Tanaka, T., Omata, Y., Yamauchi, K., Takase, M., Shimazaki, K., Igarashi, I., Saito, A. and Suzuki, N. 1995. *Exp. Parasitol* 81: 614–617.

Immunology 1998 **95** 219–225

Unresponsiveness of $CD4^-8^{+/-}$ thymocytes to lectin stimulation in LEC mutant rats

T. SAKAI,* T. AGUI,† W. KAICHUN,‡ M. OKA,§ H. HISAEDA,* H. NAGASAWA,¶ K. HIMENO* & K. MATSUMOTO‡

**Department of Parasitology and Immunology, University of Tokushima School of Medicine, Tokushima, †Institute for Experimental Animal Science, Nagoya City University Medical School, Nagoya, and ‡Institute for Animal Experimentation, University of Tokushima School of Medicine, Tokushima, §Tokushima Research Institute, Otsuka Pharmaceutical Co. Ltd, Tokushima, and ¶The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan*

SUMMARY

A mutant strain of rat, LEC, shows a novel arrest of T-cell maturation from $CD4^+8^+$ to $CD4^+8^-$, but not to $CD4^-8^+$ cells in the thymus. The responsible mutant locus is designated the *thid*, which was acted upon in a recessive manner of inheritance. We found that LEC rat thymocytes failed to respond to interleukin (IL)-1, IL-6 and IL-7 in the presence of the mitogenic lectins, Allo A or concanavalin A (Con A). The unresponsiveness appeared to be due to unresponsiveness to the lectin stimulation rather than because of cytokine stimulation. Normal rat $CD4^-8^{+/-}$ (consisting of $CD4^-8^+$ and $CD4^-8^-$ thymocytes), $CD4^{+/-}8^-$ (consisting of $CD4^+8^-$ and $CD4^-8^-$ thymocytes), and $CD4^-8^-$ thymocyte subsets normally responded to mitogenic stimulation, while $CD4^+8^+$ thymocytes did not. In contrast, all LEC rat $CD4^-8^{+/-}$, $CD4^{+/-}8^-$, $CD4^-8^-$ and $CD4^-8^+$ thymocytes did not respond to the mitogenic stimulation, suggesting that the unresponsiveness of the $CD4^-8^{+/-}$ thymocytes seems to be responsible for the unresponsiveness of whole thymocytes in LEC rats. LEC rat $CD4^-8^{+/-}$ thymocytes normally expressed Con A receptor (R), lymphocyte function-associated antigen-1 (LFA-1), and CD45, which are thought to be important molecules for lectin stimulation. When backcross rats from $(F344 \times LEC)F_1 \times LEC$ were examined, the phenotype for the *thid* mutation correlated with the [^{3}H]thymidine deoxyribose (TdR) incorporation level in response to Con A stimulation; thymocytes from backcross rats showing +/thid phenotype responded to Con A stimulation normally, whereas thymocytes from backcross rats showing thid/thid phenotype showed significantly lower responsiveness compared with +/thid rats. However, in WKAH.C-*thid* congenic rat thymocytes that carry the *thid* mutation, the responsiveness to mitogenic stimulation was comparable to that of normal rat thymocytes. These results suggest that a defect in responsiveness to mitogenic stimulation in LEC rat thymocytes is controlled by multiple genetic loci and the *thid* locus is one of the important loci for the development of this abnormal phenotype.

INTRODUCTION

We have previously reported a novel mutant strain of rat, LEC, which shows a congenital maturational arrest from $CD4^+8^+$ to $CD4^+8^-$, but not to $CD4^-8^+$ thymocytes.[1,2] This mutation was caused by a single recessive mutant gene designated as *thid*[3] and it has been shown that bone marrow (BM)-derived cells, but not thymic stromal cells, carry this mutation both by means of rat fetal thymus transplantation and BM chimera rat experiments.[4,5]

Although LEC rats show specific maturational arrest of $CD4^+8^-$ thymocytes, $CD4^+8^+$, $CD4^-8^+$ and $CD4^-8^-$ thymocytes from these rats express T-cell receptor-αβ (TCR-α/β) and CD3 normally.[1] Furthermore, LEC rat peripheral $CD8^+$ T cells display the normal activity for cytotoxicity[1] and responsiveness to the lectin stimulation.[6] These facts suggest that differentiation from $CD4^-8^-$ to $CD4^+8^+$ thymocytes and further development of $CD4^-8^+$ thymocytes are normal in LEC rats. Thus, we have considered so far that thymocyte subsets, except for $CD4^+8^-$ thymocytes, must be normal in LEC rats. However, during the course of investigation for a cause of the *thid* mutation, we found that LEC rat thymocytes are unresponsive to cytokines, such as interleukin (IL)-1, IL-6 and IL-7 with costimulation of the mitogenic lectins. As these

Received 3 March 1998; revised 29 May 1998; accepted 29 May 1998.

Abbreviations: BM, bone marrow; Con A, concanavalin A; DMEM, Dulbecco's modified Eagle medium; FCM, flow cytometry; FITC, fluorescein isothiocyanate; IL, interleukin; LFA-1, lymphocyte function-associated antigen-1; M, modified; mAb, monoclonal antibody; MHC, major histocompatibility complex; PE, phycoerythrin; r, recombinant; R, receptor; TCR, T-cell receptor; TdR, thymidine deoxyribose.

Correspondence: Dr K. Matsumoto, Institute for Animal Experimentation, University of Tokushima School of Medicine, Kuramoto 3, Tokushima 770–8503, Japan.

cytokines are shown to play an important role in promoting growth and differentiation of thymocytes,[7–16] we further investigated this deficiency. We show in this paper that when LEC rat thymocytes are stimulated by lectin, unresponsiveness is observed and this defective response is attributed to a defect in the responsiveness of $CD4^-8^{+/-}$ thymocyte subsets. Furthermore, this deficiency is controlled by multiple genetic loci and the *thid* mutation, which causes the blockade of differentiation from $CD4^+8^+$ to $CD4^+8^{+/-}$ thymocytes, is one of the major factors for the development of this deficiency.

MATERIALS AND METHODS

Rats

LEC/Tj, WKAH/Tj, and F344/Tj inbred rats were bred in our laboratory under specific pathogen-free conditions. The generations of LEC/Tj, WKAH/Tj, and F344/Tj rats used in this experiment were 51, 275, and 150, respectively. Backcross rats were produced by mating $(F344 \times LEC)F_1$ rats with LEC rats. In this study we used *thid* congenic rats, WKAH.C-*thid*/Tj (N8F4), which were made by backcrossing eight times $(WKAH \times LEC)F_1$ rats to WKAH inbred rats.

Antibodies and reagents

Hybridoma cell lines producing anti-rat CD4 monoclonal antibody (mAb), W3/25 (ref. 17) and anti-rat CD8 mAb, OX8 (ref. 18) were gifts from Dr M. Miyasaka (Osaka University, Osaka, Japan). Anti-rat TCR-α/β mAb (R73) (ref. 19) was a gift from Dr T. Hünig (Universität Würzburg, Würzburg, Germany). Anti-rat CD45 (ref. 20) and anti-rat lymphocyte function-associated antigen-1 (LFA-1) (ref. 21) mAb were purchased from Serotec (Blackthorn, Bicester, GB) and Seikagaku Co. (Tokyo, Japan), respectively. Anti-CD4 mAb was purified using a protein G Sepharose column kit, MAB Trap® G (Pharmacia LKB, Uppsala, Sweden) and labelled with fluorescein isothiocyanate (FITC) by the standard procedure. Phycoerythrin (PE)-conjugated CD8 mAb was purchased from Serotec. Human recombinant (r)IL-1α, IL-6 and IL-7 were gifts from Dr S. Sogo (Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd, Tokushima, Japan), Dr T. Hirano (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan), and Dr G. LeGros (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, Bethesda, MD), respectively.

Cell preparation

Thymi were cut into small pieces and then pressed with the bottom of a syringe in Dulbecco's minimum essential medium (DMEM) supplemented with 5% fetal calf serum, 10 mM HEPES, 5·5 mM glutamine, 0·16 mM L-asparagine, 0·55 mM L-arginine-HCl, 1 × MEM non-essential amino acid, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0·5 mg/ml gentamycin [modified (M)-DMEM]. Purification of $CD4^-8^{+/-}$, $CD4^{+/-}8^-$, and $CD4^-8^-$ thymocytes was performed using Dynabeads M450 (Dynal AS, Oslo, Norway).[6] Briefly, thymocytes were incubated with the supernatant of either W3/25 hybridoma (anti-CD4), OX8 hybridoma (anti-CD8), or a mixture of W3/25 and OX8 for 30 min on ice, washed twice with culture medium, and then antibody-labelled cells were separated using Dynabeads precoated with sheep anti-mouse IgG_1. Purity of each thymocyte subset was >98%. Purification of $CD4^+8^+$, $CD4^+8^-$ or $CD4^-8^+$ thymocytes was performed with either a FACStar II Plus (Becton Dickinson & Co., Mountain View, CA) or with a EPICS ELITE (Coulter Co., Hialeah, FL), after staining with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD8 mAb. The $CD4^+8^+$, $CD4^+8^-$ or $CD4^-8^+$ subset was gated and then these subsets were sorted out. Purity of each thymocyte subset was >95%.

Proliferation assay

In this study, we used two kind of lectins, Allo A and concanavalin A (Con A), for proliferation assay. Allo A is β-D-glactoside-specific lectin from the beetle, *Allo Myrina dichotoma* and was reported to be a sensitive agent for IL-1 determination in thymocyte costimulator assays.[22] Thymocytes (5×10^5 cells/well) were stimulated with 5 μg/ml Allo A, or 2·5 μg/ml Con A in the absence or presence of either 1000 U/ml IL-1, 5 U/ml IL-6, or 500 U/ml IL-7. In the proliferation assay for $CD4^+8^+$, $CD4^+8^-$ and $CD4^-8^+$ thymocytes, syngeneic rat thymocytes that had been treated with mitomycin C were added as accessory cells. Cells were cultured in M-DMEM for 72 hr and then pulsed with 1 μCi [^{3}H]thymidine deoxyribose (TdR) for the last 16 hr. The incorporation of [^{3}H]TdR was counted by liquid scintillation counter.

Flow cytometry (FCM) analysis

For three-colour staining, thymocytes were first incubated with either anti-TCR-α/β, -LFA-1, or -CD45 mAb, and then with biotinylated anti-mouse IgG. After blocking free arms of biotinylated anti-mouse immunoglobulin with irrelevant mouse immunoglobulin, streptavidin RED670, FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD8 mAb were added. In the analysis for Con A receptor (R), cells were incubated with biotinylated Con A and then streptavidin RED670, FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs were added. Analyses were performed with a FACScan and either a Consort 30 or a FACScan software program (Becton Dickinson, Mountain View, CA).

RESULTS

Unresponsiveness of LEC rat thymocytes to mitogenic lectins and cytokines

Normal rat (WKAH) thymocytes showed DNA synthesis in response to IL-1, IL-6 and IL-7 with costimulation of mitogenic lectin, Allo A. LEC rat thymocytes, however, responded to neither Allo A only nor Allo A with cytokines (Fig. 1a). Con A, another mitogenic lectin, induced a full proliferative response without cytokine costimulation in normal rat thymocytes, unlike the results reported in murine thymocytes. LEC rat thymocytes did not respond to Con A, and furthermore, addition of cytokines could not restore the unresponsiveness (Fig. 1b).

Mitogenic effects of the lectins on thymocyte subsets

We purified various thymocyte subsets using magnetic beads and analysed their responsiveness to mitogenic stimulation. We used magnetic beads because cell purification using magnetic beads has the advantage of cell recovery over cell sorting and is free of antibodies on the purified cells. In normal rats,

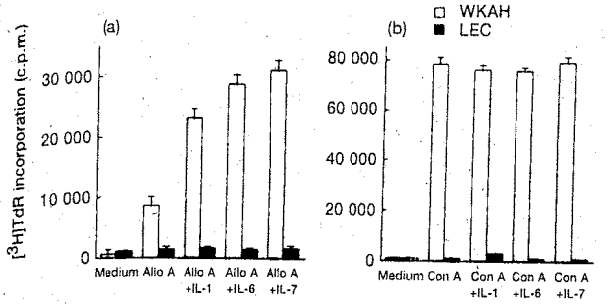


Figure 1. Unresponsiveness of LEC rat thymocytes to the mitogenic cytokines and lectins. WKAH and LEC rat thymocytes were stimulated by 5 μg/ml Allo A (a) or 2·5 μg/ml Con A (b) with costimulation of either 1000 U/ml IL-1α, 5 U/ml IL-6, or 500 U/ml IL-7. Thymocytes were cultured with stimulants for 72 hr and then pulsed with 1 μCi [^{3}H]TdR for the last 16 hr. Open and filled columns represent the data from WKAH and LEC rats, respectively. Columns and bars represent the means ± SD.

purified $CD4^-8^{+/-}$, $CD4^{+/-}8^-$ and $CD4^-8^-$ thymocytes showed DNA synthesis in response to Allo A with IL-1 or Con A and these responses were higher than those of whole thymocytes. In contrast to normal rats, all LEC rat $CD4^-8^{+/-}$, $CD4^{+/-}8^-$, and $CD4^-8^-$ thymocyte subsets did not respond to both Allo A with IL-1 and Con A stimulation (Table 1, Exp.1). Next, we investigated responsiveness of $CD4^+8^-$, $CD4^+8^+$, and $CD4^-8^+$ thymocyte subsets, as both $CD4^-8^{+/-}$ and $CD4^{+/-}8^-$ cells contain two subsets and it is difficult to determine which subset contributes to the response to the lectin stimulation. As in the case of CD3-crosslinking stimulation for $CD4^+8^+$ thymocytes, normal and LEC rat $CD4^+8^+$ thymocytes did not respond to the mitogenic stimulation (Table 1, Experiment 2). $CD4^+8^-$ and $CD4^-8^+$ thymocytes from normal rats were responsive to lectin stimulation, whereas LEC rat $CD4^-8^+$ thymocytes were not (Table 1, Experiments 2 and 3). These data show that $CD4^+8^-$, $CD4^-8^+$ and $CD4^-8^-$ thymocytes, but not $CD4^+8^+$ thymocytes, have the ability to respond to lectin stimulation in normal rats, while all thymocyte subsets of LEC rats did not. Thus, the unresponsiveness reaction to lectin stimulation in LEC rat thymocytes is not attributed to the absence of the $CD4^+8^-$ subset, but rather to other $CD4^-8^{+/-}$ thymocyte subsets in LEC rats.

Expression of Con A R, TCR-α/β, LFA-1, and CD45 on $CD4^-8^{+/-}$ thymocytes

It is possible that the LEC rat $CD4^-8^{+/-}$ thymocyte defect in the expression of lectin R is the cause of this unresponsiveness. Thus, we analysed the expression of Con A R of LEC rat thymocytes using FCM. As shown in Fig. 2, all LEC rat $CD4^-8^{+/-}$ thymocytes expressed Con A R and the mean fluorescence intensity was comparable to that of normal rat

Table 1. Proliferative response of thymocyte subsets to mitogenic stimulation in normal (WKAH) and LEC rats

	[^{3}H]TdR incorporation (c.p.m.)		
	Medium	Allo A* + IL-1†	Con A‡
Experiment 1			
WKAH			
Thymocytes	241 ± 43§	18 353 ± 2344	24 899 ± 2866
$CD4^-8^{+/-}$ thymocytes	1364 ± 841	39 180 ± 3252	78 048 ± 5172
$CD4^{+/-}8^-$ thymocytes	2146 ± 1240	87 153 ± 3252	78 048 ± 5172
$CD4^-8^-$ thymocytes	1641 ± 841	46 506 ± 3020	73 614 ± 2869
LEC			
Thymocytes	1112 ± 588	1486 ± 1026	1818 ± 457
$CD4^-8^{+/-}$ thymocytes	549 ± 193	1424 ± 714	1711 ± 1216
$CD4^{+/-}8^-$ thymocytes	428 ± 253	1601 ± 25	3539 ± 418
$CD4^-8^-$ thymocytes	381 ± 253	2113 ± 952	1705 ± 837
Experiment 2			
WKAH			
$CD4^+8^+$ thymocytes	169 ± 19§	650 ± 179	ND
$CD4^+8^-$ thymocytes	189 ± 31	50 816 ± 8706	ND
LEC			
$CD4^+8^+$ thymocytes	121 ± 12	221 ± 52	ND
Experiment 3			
WKAH			
$CD4^-8^{+/-}$ thymocytes	510 ± 31	19 741 ± 478	23 945 ± 541
$CD4^-8^+$ thymocytes	517 ± 102	14 906 ± 1158	16 351 ± 670
LEC			
$CD4^-8^{+/-}$ thymocytes	510 ± 187	3120 ± 629	2952 ± 690
$CD4^-8^+$ thymocytes	561 ± 121	1928 ± 1596	1794 ± 353

*5 μg/ml.
†1000 U/ml.
‡2·5 μg/ml.
§Triplicate observations (mean ± SD). ND, not done.

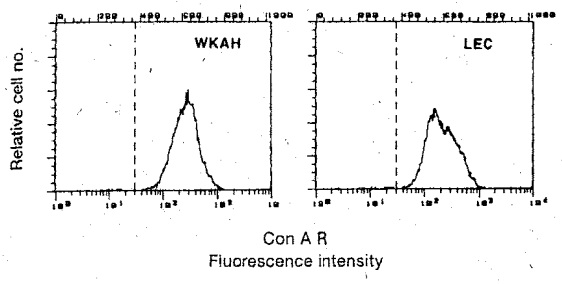


Figure 2. Three-colour FCM analyses of Con AR on $CD4^-8^{+/-}$ thymocytes. WKAH (left panel) and LEC (right panel) rat thymocytes were stained with biotinylated Con A and then with streptavidin RED670, FITC-conjugated anti-CD4, and PE-conjugated anti-CD8 mAbs. The data for the $CD4^-8^{+/-}$ subset were collected using a live gate and analysed with FL3. Dotted lines indicate the profile of the negative control.

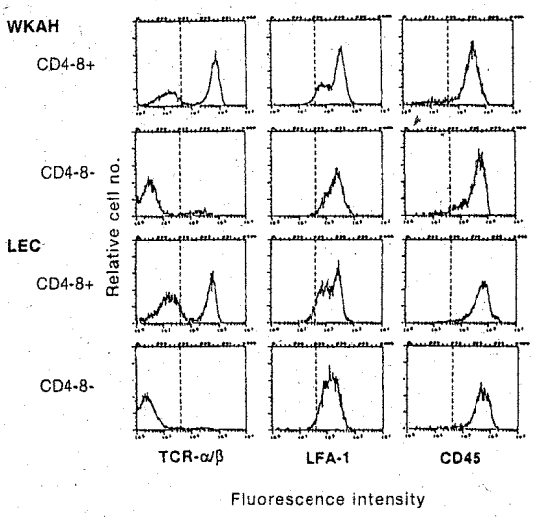


Figure 3. Expression of TCR-α/β, LFA-1 and CD45 on thymocyte subsets. Thymocytes stained with three colours were the first to be analysed with a FACScan using two parameters, FL1 (CD4) and FL2 (CD8), and then a $CD4^-8^+$ or a $CD4^-8^-$ subset was collected using a live gate. Collected cells were analysed using a parameter of FL3 (either anti-TCRα/β, anti-LFA-1 or anti-CD45). The dotted line represents the profile of the negative control.

$CD4^-8^{+/-}$ thymocytes. Addition of methyl-α-D-mannoside mannose that is a Con A-binding oligosaccaride, abrogated the detection of Con A R in FCM analysis (data not shown), suggesting that a detection of Con A R by FCM is specific. Thymocytes consist of mature ($TCR\text{-}\alpha/\beta^+$) and immature ($TCR\text{-}\alpha/\beta^-$) populations. Three-colour analysis of TCR-α/β expression on $CD4^-8^+$ and $CD4^-8^-$ thymocytes showed that the percentage of $TCR\text{-}\alpha/\beta^+$ cells on LEC rat $CD4^-8^+$ and $CD4^-8^-$ thymocytes is the same as that of normal rats (Fig. 3). There are reports that lectin R on T cells associates with CD45 molecules when activated by lectin,[23] and lectin-induced T-cell aggregation is inhibited by anti-LFA-1 mAb.[21] These reports suggest that LFA-1 and CD45 molecules play an important role in lectin-induced lymphocyte activation. The FCM analysis showed that the expression pattern for LFA-1 and CD45 was the same in both normal and LEC rat $CD4^-8^{+/-}$ thymocytes (Fig. 3). We further examined expression of other molecules (CD2, CD5, CD44, ICAM-1 and LECAM-1), but no difference was observed (data not shown). Our results show that LEC rat $CD4^-8^{+/-}$ thymocytes ($CD4^-8^+$ and $CD4^-8^-$ thymocytes) have a normal phenotype with respect to the expression of these cell surface molecules.

Linkage study between the *thid* mutation to a defect in the responsiveness to lectin stimulation

Maturational blockade from $CD4^+8^+$ to $CD4^+8^-$ cells in the thymus is the most characteristic immunodeficiency observed in LEC rats and this deficiency is caused by a single recessive gene referred as to *thid*.[3] To investigate whether a defect in the responsiveness to lectin stimulation correlates to the *thid* mutation, we produced backcross [$(F344 \times LEC)F_1 \times LEC$] rats and examined both phenotypes in these rats. Phenotype of +/thid or thid/thid was determined on the basis of the percentage of $CD4^+8^-$ cells and then these two phenotypes were clearly segregated in backcross rats as reported previously.[3] Rats with a percentage of $CD4^+8^-$ cells less than 1% were classified as thid/thid homozygotes, while those greater than 5% were classified as +/thid heterozygotes. As shown in Fig. 4, the responsiveness of thymocytes to Con A stimulation from +/thid rats was normal, while that from thid/thid rats was significantly lower compared with that of +/thid rats (means ± SD of +/thid and thid/thid phenotype rats were $54{\cdot}9 \pm 17{\cdot}9 \times 10^3$ c.p.m. and $8{\cdot}9 \pm 6{\cdot}9 \times 10^3$ c.p.m., respectively). A significant difference was observed between +/thid and thid/thid rats ($P < 0{\cdot}01$).

Responsiveness of WKAH.C-*thid* congenic rat thymocytes to lectin stimulation

In analysis of backcross rats, we showed that the *thid* mutation linked to unresponsiveness to lectin stimulation (Fig. 4). However, some rat thymocytes showing thid/thid phenotype showed moderate responsiveness, unlike LEC rats. Thus, to

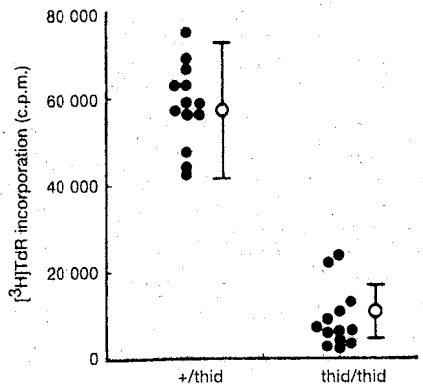


Figure 4. Linkage study between the *thid* mutation and a defect in the responsiveness of thymocytes to lectin stimulation. Thymocytes from [$(F344 \times LEC)F_1 \times LEC$] rats were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAbs. Backcross rats with a percentage of $CD4^+8^-$ thymocytes less than 1% were classified as thid/thid homozygotes, while those greater than 5% were classified as +/thid heterozygotes. Thymocytes from backcross rats were cultured with Con A (2·5 μg/ml) for 72 hr and then pulsed with 1 μCi [^{3}H]TdR for the last 16 hr. Open symbols and bars represent the means ± SD of values for +/thid and thid/thid rats.

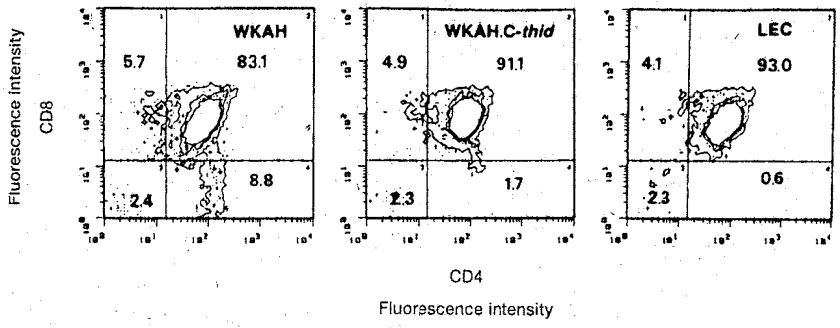


Figure 5. Two-colour FCM analyses of CD4/CD8 antigens on thymocytes of normal (WKAH) (left panel), WKAH.C-*thid* congenic (middle panel) and LEC rats (right panel). Thymocytes were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-CD8 mAb, and then analysed using FCM.

address whether the *thid* mutation only defines the unresponsiveness, we produced WKAH.C-*thid* congenic rats and analysed the responsiveness of thymocytes to the mitogenic stimulation. The immunodeficient phenotype of WKAH.C-*thid* congenic rats was similar to that observed in LEC rats; both maturational blockade of $CD4^+8^-$ thymocytes (Fig. 5) and a defect in the function of peripheral $CD4^+$ T cells[24] was observed in WKAH.C-*thid* congenic rats as well as LEC rats. As shown in Table 2, responsiveness of WKAH.C-*thid* rat thymocytes and $CD4^-8^{+/-}$ thymocytes to the lectin stimulation was comparable to that of WKAH rat thymocytes and was much higher than that of LEC rat thymocytes. Although responsiveness of WKAH.C-*thid* rat thymocytes to the mitogenic stimulation was slightly lower than that of WKAH rat thymocytes, this might be due to a lack of $CD4^+8^-$ cells as part of the responder cells. These results suggest that the *thid* mutation on its own cannot cause unresponsiveness of thymocytes to mitogenic stimulation and other gene(s) is (are) necessary to cause this deficient phenotype.

DISCUSSION

In LEC rat thymocytes, both Con A and Allo A could not act as a mitogenic stimulator. IL-1, IL-6 and IL-7 play an important role in the process of T-cell proliferation and maturation.[7–16] Addition of these cytokines, however, could not restore the responsiveness to mitogenic lectins in LEC rat thymocytes (Fig. 1). In this study, Con A stimulation was shown to be different from Allo A stimulation in rat thymocytes, unlike that observed in murine thymocytes. Thus, Con A could induce thymocyte proliferation by itself, whereas Allo A needed the assistance of cytokines (Fig. 1). It has been reported that Allo A is mitogenic to mature thymocytes but not to immature thymocytes.[25] However, in this study we showed that Allo A is also mitogenic to immature $CD4^-8^-$ but not $CD4^+8^+$ thymocytes (Table 1).

Responsiveness to the mitogenic stimulation was different in thymocyte subsets. $CD4^+8^-$, $CD4^-8^+$ and $CD4^-8^-$ thymocytes showed responsiveness to the stimulation of Allo A with IL-1 or Con A, whereas $CD4^+8^+$ thymocytes did not (Table 1, Experiment 2). Although FCM analysis showed that $CD4^+8^+$ thymocyte subsets express Con A R (data not shown), $CD4^+8^+$ cells did not respond to the mitogenic stimulation. The unresponsiveness of $CD4^+8^+$ thymocytes is also observed when they are stimulated with other mitogenic agents. When $CD4^+8^+$ thymocytes are stimulated with mitogenic agents, including anti-CD3 mAb or superantigens, activation of some tyrosine kinases and an elevation of intracellular Ca^{2+}

Table 2. Responsiveness of WKAH.C-*thid* congenic rat thymocytes to lectin stimulation*

	[^{3}H]TdR incorporation (c.p.m.)		
	Medium	Allo A† + IL-1‡	Con A§
WKAH			
Thymocytes	3521 ± 1547¶	41 937 ± 5854	37 700 ± 9805
$CD4^-8^{+/-}$ thymocytes	1409 ± 856	68 420 ± 8763	69 822 ± 4652
WKAH.C-*thid*			
Thymocytes	1830 ± 652	24 490 ± 4085	30 140 ± 3711
$CD4^-8^{+/-}$ thymocytes	1090 ± 671	63 200 ± 6721	64 098 ± 3607
LEC			
Thymocytes	1420 ± 363	2320 ± 1011	3480 ± 751
$CD4^-8^{+/-}$ thymocytes	1029 ± 161	1130 ± 177	1830 ± 177

*This is representative of three independent experiments.
†5 μg/ml.
‡1000 U/ml.
§2·5 μg/ml.
¶Triplicate observations (mean ± SD).

concentration occurred, but results in apoptosis, in contrast to the mature T cells.[26,27] Thus, $CD4^+8^+$ thymocytes are potentially sensitive to apoptosis following mitogenic stimulation.

Defective responsiveness of $CD4^-8^{+/-}$ thymocytes to mitogenic stimulation is responsible for unresponsiveness of LEC rat thymocytes. Although LEC rat $CD4^-8^+$ and $CD4^-8^-$ thymocytes expressed Con A R normally (Fig. 2), they did not respond to mitogenic stimulation (Table 1). Furthermore, they expressed CD45 and LFA-1, which are thought to play an important role for lectin-induced activation.[21,23] Although we examined expression of surface molecules (CD2, CD5, CD44, ICAM-1 and LECAM-1) on $CD4^-8^{+/-}$ thymocytes, no difference was observed between normal and LEC rats. This suggests that LEC rat $CD4^-8^{+/-}$ thymocytes have a normal phenotype with respect to these cell surface antigens. There is a possibility that LEC rat thymocytes have a defect in the function of accessory cells or in the production of lectin-induced cytokines. However, it seems unlikely because co-culture of LEC rat thymocytes with mitomycin C-treated syngenic rat thymocytes could not restore the responsiveness (Table 1). Furthermore, addition of supernatants from lectin-stimulated normal rat thymocytes also could not restore the responsiveness. Although LEC rat thymocytes exhibited less proliferative response to lectin stimulation, these cells showed normal DNA synthesis in response to IL-2 (data not shown), suggesting that these cells do not have an intrinsic defect in thymidine transport. Therefore, a defect in lectin-induced signals may be responsible for the unresponsiveness of thymocytes to mitogenic stimulation in LEC rats.

In this study, it was shown that the responsiveness of thymic $CD4^-8^+$ cells to the mitogenic stimulation is aberrant (Table 1). However, LEC rat peripheral $CD8^+$ T cells normally respond to Con A stimulation[6] and possess normal cytolytic activity,[1] suggesting that peripheral $CD8^+$ T cells from LEC rats are functionally normal. The reason for the variation in responsiveness to lectin stimulation between thymic $CD4^-8^+$ and peripheral $CD8^+$ T cells is not easy to explain. In the case of IL-2 production by $CD4^+8^-$ T cells, thymic $CD4^+8^-$ cells produce IL-2 10-fold lower than that of peripheral $CD4^+$ T cells. Induction of high-level IL-2 production in $CD4^+8^-$ cells was shown to require post-thymic development.[28] Therefore, one explanation for the discrepancy of responsiveness between thymic and peripheral $CD4^-8^+$ cells is that LEC rat $CD8^+$ T cells might acquire responsiveness to lectin stimulation by post-thymic maturation.

The *thid* mutation causes maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes and BM-derived cells carry this mutation.[4,5] In the analysis using backcross rats, the *thid* mutation correlated with the responsiveness to lectin stimulation (Fig. 4). However, in WKAH.C-*thid* congenic rats, responsiveness of thymocytes to lectin stimulation was normal. Although we do not have direct evidence, this discrepancy can be explained by an idea that a defect in responsiveness is caused by multiple genes. One indispensable gene for development of unresponsiveness is the *thid* mutation and other unidentified genes also may contribute. That the *thid* mutation alone could not cause defective responsiveness to lectin is shown by the analysis using WKAH.C-*thid* congenic rats (Table 2). No backcross rats that showed normal responsiveness were observed in thid/thid phenotype rats, unlike WKAH.C-*thid* rats (Fig. 4). These facts lead to the hypothesis that other candidate genes indispensable for the triggering unresponsiveness may closely link to the *thid* locus, and tl results in the correlation of the *thid* mutation in backcross r and no correlation in congenic rats to unresponsiveness.

It is possible that lineage-specific molecules for CD T-cell maturation is responsible for the *thid* mutation. Un now many gene-knockout mice were generated and it has be shown that CD4 or major histocompatibility complex (MH class II molecules play an important role in the differentiati of helper T cells.[29,30] Thus, a defect in the function of CD4 MHC class II molecules is a candidate for the *thid* mutatic However, the fact that the *thid* locus does not link to t MHC class II locus,[3] and that CD4 molecules on LEC r thymocytes associate with $p56^{lck}$ and can deliver signals nc mally,[31] excludes these possibilities. Recently, we mapped t *thid* mutation to the rat chromosome 1.[32] Rat CD4 and MH class II genes are mapped to rat chromosomes 4 and 2 respectively.[33,34] These genetic experiments also indicate th a defect in the function of CD4 and MHC class II is n responsible for the *thid* mutation. Although the nature of t *thid* mutation is not understood yet, the *thid* mutation affec the development from $CD4^+8^+$ to $CD4^+8^-$ thymocytes a is also correlated to a lectin-induced cell activation. Thu elucidation of the *thid* mutation provides a method for inves gating the mechanism of T-cell development and activation

ACKNOWLEDGMENTS

We thank Drs M. Miyasaka and T. Hünig for providing t hybridomas, Drs S. Sogo, T. Hirano and G. LeGros for the cytokin and Mr M. Oka (Exploratory Research Laboratories, Fujisa Pharmaceutical Co., Ltd, Osaka, Japan) for cell sorting.

REFERENCES

1. Agui T., Oka M., Yamada T. *et al.* (1990) Maturational arre from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in a mutant strain (LE of rat. *J Exp Med* **172**, 1615.
2. Agui T., Sakai T. & Matsumoto K. (1991) Ontogeny of T c maturation in LEC rats which bear a congenital arrest of mat ration from $CD4^+8^+$ to $CD4^+8^-$ thymocytes. *Eur J Immun* **21**, 2537.
3. Yamada T., Natori T., Izumi K., Sakai T., Agui T. Matsumoto K. (1991) Inheritance of T helper immunodeficien (*thid*) in LEC mutant rats. *Immunogenetics* **33**, 216.
4. Agui T., Sakai T., Himeno K. & Matsumoto K. (1991) Bo marrow-derived progenitor T cells convey the origin of mat rational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in LE mutant rats. *Eur J Immunol* **21**, 2277.
5. Sakai T., Agui T. & Matsumoto K. (1993) Maturational arre from $CD4^+8^+$ to $CD4^+8^-$ thymocytes is caused by the bo marrow-derived cells in LEC mutant rats. *Immunol Lett* **38**, 14
6. Sakai T., Agui T., Muramatsu Y., Yamada T. & Matsumo K. (1993) Dissociation of interleukin-2 production from the c activation in response to the mitogenic lectin in peripheral CD4 T cells of LEC mutant rats. *Immunology* **79**, 491.
7. Deluca D. & Mizel S.B. (1986) I-A positive nonlymphoid cel and T cell development in murine fetal thymus organ culture interleukin 1 circumvents the block in T cell differentiation induc by monoclonal anti-I-A antibodies. *J Immunol* **137**, 1435.
8. Takashi T., Gause W.C., Wilkinson M., MacLeod C.L. Steinberg A.D. (1991) Interleukin 1-induced maturation of pr genitor thymocytes. *Eur J Immunol* **21**, 1385.
9. Cotlieb W.H., Takacs L., Young H.A., Gusella L. & Duru

S.K. (1990) Growth of immature (double negative) rat thymocytes in the presence of IL-1. *J Immunol* **144,** 2072.
9. Damme J.V., Beeumen J.V., Decock B., Snick J.V., Ley M.D. & Billiau A. (1988) Separation and comparison of two monokines with lymphocyte-activating factor activity: IL-1β and hybridoma growth factor (HGF): identification of leukocyte-derived HGF as IL-6. *J Immunol* **140,** 1534.
11. Lotz M., Jirik F., Kabouridis P. *et al.* (1988) B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *J Exp Med* **167,** 1253.
12. Helle M., Brakenhoff J.P.J., Groot E.R.D. & Aarden L.A. (1988) Interleukin 6 is involved in interleukin 1-induced activities. *Eur J Immunol* **18,** 957.
13. Takeda S., Gillis S. & Palacios R. (1989) *In vitro* effects of recombinant interleukin 7 on growth and differentiation of bone marrow pro-B- and pro-T-lymphocyte clones and fetal thymocyte clones. *Proc Natl Acad Sci USA* **86,** 1634.
14. Watson J.D., Morrissey P.J., Namen A.E., Conlon P.J. & Widmer M.B. (1989) Effect of IL-7 on the growth of fetal thymocytes in culture. *J Immunol* **143,** 1215.
15. Uckun F.M., Tuel-Ahlgren L., Obuz V. *et al.* (1991) Interleukin 7 receptor engagement stimulates tyrosine phosphorylation, inositol phospholipid turnover, proliferation, and selective differentiation to the CD4 lineage by human fetal thymocytes. *Proc Natl Acad Sci USA* **88,** 6323.
16. Herbelin A., Machavoine F., Schneider E., Papiernik M. & Dy M. (1992) IL-7 is requisite for IL-1-induced thymocyte proliferation: involvement of IL-7 in the synergistic effects of granulocyte- macrophage colony-stimulating factor or tumor necrosis factor with IL-1. *J Immunol* **148,** 99.
17. Williams A.F., Galfrè. G. & Milstein C. (1977) Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell* **12,** 663.
18. Brideau R.J., Carter P.B., McMaster W.R., Mason D.W. & Williams A.F. (1980) Two sub-sets of rat T lymphocytes defined with monoclonal antibodies. *Eur J Immunol* **10,** 609.
19. Hünig T., Wallny H.J., Hartley J.K., Lawetzky A. & Tiefenthaler G. (1989) A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation: differential reactivity with subsets of immature and mature T lymphocytes. *J Exp Med* **169,** 73.
20. Sunderland C.A., McMaster W.R. & Williams A.F. (1979) Purification with monoclonal antibody of a predominant leukocyte common antigen and glycoprotein from rat thymocytes. *Eur J Immunol* **9,** 155.
21. Tamatani T., Kotani M. & Miyasaka M. (1991) Characterization of the rat leukocyte integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. *Eur J Immunol* **21,** 627.
22. Sakai Y., Kimura S., Yamashita T., Umetsu K., Suzuki T. & Sendo F. (1989) A sensitive IL-1 thymocyte co-stimulator assay using a novel β-D-galactoside specific lectin from the beetle, *Allomyrina dichotoma Microbiol Immunol* **33,** 569.
23. Bernabeu C., Carrera A.C., Landàzuri M.O.P. & Sànches-Madrid F. (1987) Interaction between the CD45 antigen and phytohemagglutinin: inhibitory effect on the lectin-induced T cell proliferation by anti-CD45 monoclonal antibody. *Eur J Immunol* **17,** 1461.
24. Sakai T., Agui T., Hisaeda H., Izumi K., Himeno K. & Matsumoto K. (1997) Expression of adhesion molecules on LEC rat peripheral $CD4^+$ T cells: unique expression of LFA-1 and LECAM-1. *J Trace Elem Exp Med* **10,** 81.
25. Kimura S., Umetsu K., Yamashita T., Suzuki T., Arai S. & Sendo F. (1987) T cell mitogenicity of a novel β-D-galactoside-specific lectin from the beetle, *Allomyrina dichotoma* (allo A). *Immunopharmacology* **13,** 181.
26. Cohen J.J. (1991) Programmed cell death in the immune system. *Adv Immunol* **50,** 55.
27. Kroemer G. (1995) The pharmacology of T cell apoptosis. *Adv Immunol* **58,** 211.
28. Ghang J.F., Thomas C.A. & Kung J. (1991) Induction of high level IL-2 production in $CD4^+8^-$ T helper lymphocytes requires post-thymic development. *J Immunol* **147,** 851.
29. Rahemtulla A., Fung-Leung W.P., Schilham M.W. *et al.* (1991) Normal development and function of $CD8^+$ cells but markedly decreased helper cell activity in mice lacking CD4. *Nature* **353,** 180.
30. Grusby M.J., Johnson R.S., Papaioannou V.E. & Glimcher L.H. (1991) Depletion of $CD4^+$ T cells in major histocompatibility complex class II-deficient mice. *Science* **253,** 1417.
31. Sakai T., Agui T., Muramatsu Y. *et al.* (1996) Function of CD4 molecules in LEC rat thymocytes. *J Vet Med Sci* **58,** 1125.
32. Wei K., Muramatsu Y., Sakai T., Yamada T. & Matsumoto K. (1997) Chromosomal mapping of T helper immunodeficiency (thid) locus in LEC rats. *Immunogenetics* **47,** 99.
33. Dissen E. & Fossum S. (1996) Chromosomal localization of the genes encording rat CD4, CD8α, and CD8β. *Immunogenetics* **44,** 312.
34. Locker J., Gill T.J., Kraus J.P. *et al.* (1990) The rat MHC and cystathionine beta-synthase gene are syntenic on chromosome 20. *Immunogenetics* **31,** 271.

STRUCTURAL AND IMMUNOCHEMICAL STUDIES ON BOVINE LACTOFERRIN FRAGMENTS

Kei-ichi Shimazaki,[1] Makoto Kamio,[1] Myong Soo Nam ,[2] Shinji Harakawa,[3] Tetsuya Tanaka,[3] Yoshitaka Omata,[3] Atsushi Saito,[3] Haruto Kumura,[1] Katsuhiko Mikawa,[1] Ikuo Igarashi,[4] and Naoyoshi Suzuki[4]

[1]Dairy Science Laboratory
Faculty of Agriculture
Hokkaido University
Sapporo, 060 Japan
[2]Korea Research Institute of Bioscience and Biotechnology
KIST, Taejon 305-600, Korea
[3]Department of Veterinary Physiology
Obihiro University of Agriculture and Veterinary Medicine
Obihiro, 080 Japan
[4]The Research Center for Protozoan Molecular Immunology
Obihiro University of Agriculture and Veterinary Medicine
Obihiro, 080 Japan

1. INTRODUCTION

Lactoferrin (Lf) is a metal-binding protein found in milk and other secretory fluids and also in blood. It shows multifunctional properties but the mechanism of developing its function in living systems has not been resolved yet. It is known to exert bacteriostatic effects due to its ability to bind environmental iron. Moreover, apo-lactoferrin has been shown to bind to microbial membranes and causes the direct destruction of microorganisms. Other biological functions attributed to lactoferrin include roles in modulation of the inflammatory response, activation of the immune system, and control of myelopoiesis or cell growth. This molecule is constructed with N- and C-lobes, each of which is composed of 3 domains[1]. The function of each lobe has been studying and there are certain differences. The biologically significant function has been found mainly in N-lobe. For the aids of resolving their functional analysis, the authors prepared the monoclonal antibodies (mAb) against N-lobe and C-lobe of bovine lactoferrin. To prepare the mAb specific to N-lobe, we used lactoferricin®

Advances in Lactoferrin Research, edited by Spik *et al.*
Plenum Press, New York, 1998.

B (bLfcin , an anti-microbial peptide isolated from N-lobe[2]) as an antigen[3]. In this paper, the characterization of the mAb against lactoferrin fragments has been examined and the structure of the mAb-recognition site on lactoferrin molecule was identified.

2. EXPERIMENTAL

2.1. Materials

Bovine lactoferrin (bLf) and bLfcin were kindly supplied by the Nutritional Science Laboratory, Morinaga Milk Industries Inc. C-lobe was prepared by mild tryptic digestion and isolated by ion-exchange chromatography as reported previously[4] or by reverse phase (RP) chromatography.

2.2. Preparation of Monoclonal Antibodies

Monoclonal antibodies against bLfcin or C-lobe were prepared according to the method of Oi et al.[5] bLfcin or C-lobe was injected intravenously, the spleen was aseptically removed and processed for screening positive hybridomas. bLfcin -keyhole limpet hemocyanin complex was used for immunization.

2.3. Reverse Phase HPLC

RP-HPLC was carried out using ODS column. For elution, a mixture of the eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) was employed, using a linear or a convex gradient of A and B.

2.4. Protein Concentration Determination

Concentrations of bovine lactoferrin, C-lobe and bLfcin were determined by UV absorption at 280 nm using the extinction coefficient (1 mg/ml) of 1.27[6], 1.23 and 3.02, respectively.

2.5. Chemical Modification and Cleavage Reactions

Disulfide bonds of peptides were reduced and then pyridylethylated (Pe) with 4-vinylpyridine or acetylated with monoiodoacetamide. Lys, Arg and Trp residues were modified using succinic anhydride, 1,2-cyclohexanedione and N-bromosuccinimide, respectively. CNBr cleavage of the peptide was carried out in 70% formic acid solution. The peptide bond cleavage between aspartic acid and proline was done with the acetic acid treatment in 10% acetic acid containing 7 M guanidium hydrochloride. The chemically treated peptide were separated from unreacted peptide by RP-HPLC.

2.6. Enzymatic Digestion

Completely denatured Pe-C-lobe by 8 M urea was digested by trypsin at 37°C for 9 hour in the presence of 2 M urea. Carbohydrate moiety of peptide was removed by endo-glycosidase H. The removal of sugar chain was detected by the staining method using periodic acid-schiff reagent after SDS-PAGE.

2.7. Amino Acid Sequence Analysis and Peptide Synthesis by SPOTs™

N-terminal amino acid sequences were determined using an Applied Biosystems Model 492A protein sequencer or Tosoh sequencing system. For the epitope determination, peptides were synthesized from F-moc amino acid active esters on a pre-activated cellulose membrane using SPOTs™ (Genosys Biotechnologies, Inc.).

2.8. Mass Spectrometry

Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry (MALDI-TOF MS Voyager™ RP, PerSeptive Biosystems/Vectec Products) was used to measure the molecular mass (m/z) of peptides. Sinapinic acid was used as the matrix and angiotensin I was used as the molecular mass standard.

2.9. Antibacterial Activity Measurements

E. coli O111 was used to measure the antibacterial activities of lactoferrin fragment with the modified method of Tomita et al.[7] After incubation, turbidity was measured.

3. RESULTS

3.1. Monoclonal Antibody Binding Site of bLfcin

After screening and cloning, 4 colonies were chosen and the mAb (IgG1) produced by 5F12.1.2 cells was used mainly for further experiments. All 4 mAb's showed reactivity against both native and chemically synthesized bLfcin, of which the sulfhydryl groups are acetamidomethylated, by ELISA. None of the 4 antibodies showed reactivity against human lactoferrin or hLfcin.

The reactivity of the mAb against bLfcin derivatives of chemically modified Lys, Trp or Arg residues, as compared with intact bLfcin, was 96.5, 30.1 and 27.9%, respectively. The ratio of Trp residue modified was estimated to be 70% from the decrease in absorbance at 280 nm. This observation suggests that the Trp and Arg residues of bLfcin are mainly involved in the epitopic region recognized by the mAb. By ELISA, the mAb did not show any reactivity against CNBr-cleaved fragments. Fifty kinds of peptides corresponding to the region around the Trp and Met residues of bLfcin were synthesized and the reactivity of anti-bLfcin mAb against each of these peptides was estimated. The common sequence found in each of the peptides recognized by the mAb is "QWR" as shown in Figure 1.

3.2. Monoclonal Antibody Binding Site of C-lobe

The colony displayed higher absorbance in the ELISA against C-lobe was chosen and this clone showed high specificity to anti-mouse IgG1 subclass antibody. The mAb showed reactivity against both C-lobe and intact lactoferrin by ELISA or Western-blotting. Human lactoferrin and transferrin, bovine transferrin and ovotransferrin did not react with this mAb. The partial N-terminal amino acid sequence of C-lobe used in this experiment (Figure 2A) was YTRVVWXAVX and this fragment begins from 342Tyr of bovine lactoferrin[8]. To determine the mAb-binding site of C-lobe, denatured C-lobe was digested into the smaller fragment by trypsin. Each fragment was fractionated by RP-HPLC and as-

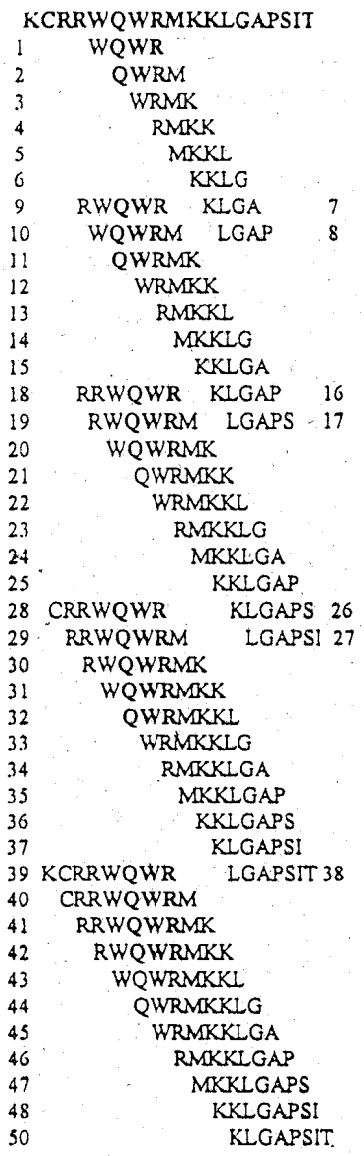


Figure 1. SPOTs™ analysis of bLfcin peptides. Peptides reacted with mAb are expressed in bold.

sayed with the reactivity against anti-C-lobe mAb by ELISA. The fraction eluted at 24 min. in Figure 2B showed reactivity against anti-C-lobe mAb and named CLT. The partial N-terminal amino acid sequences of both CLT and acetic acid-treated CLT were determined to be TAGWNIPMGLI. This fragment remained the reactivity against mAb after deglycosylation. The molecular mass of deglycosylated CLT was 4069.4 as determined by MALDI-TOF mass spectral analysis (Figure 3). No mAb-binding ability was changed on the chemically modified CLT with its Lys or Arg residues, although, the further digestion of CLT by pepsin, α-chymotrypsin or endoproteinase Glu-C made CLT very weak or not reactive against mAb. None of the peptides separated by RP-HPLC of CNBr-treated CLT showed any reactivities against anti-C-lobe mAb. Acetic acid-treated CLT lost its reactivity. Then, 38 kinds of peptides corresponding to the region from 464Thr to 508Asp of bovine lactoferrin were synthesized on the membrane and the reactivity of anti-C-lobe mAb was assayed (Figure 4). The common sequence found in the peptides recognized by the mAb is "WNIPMGL".

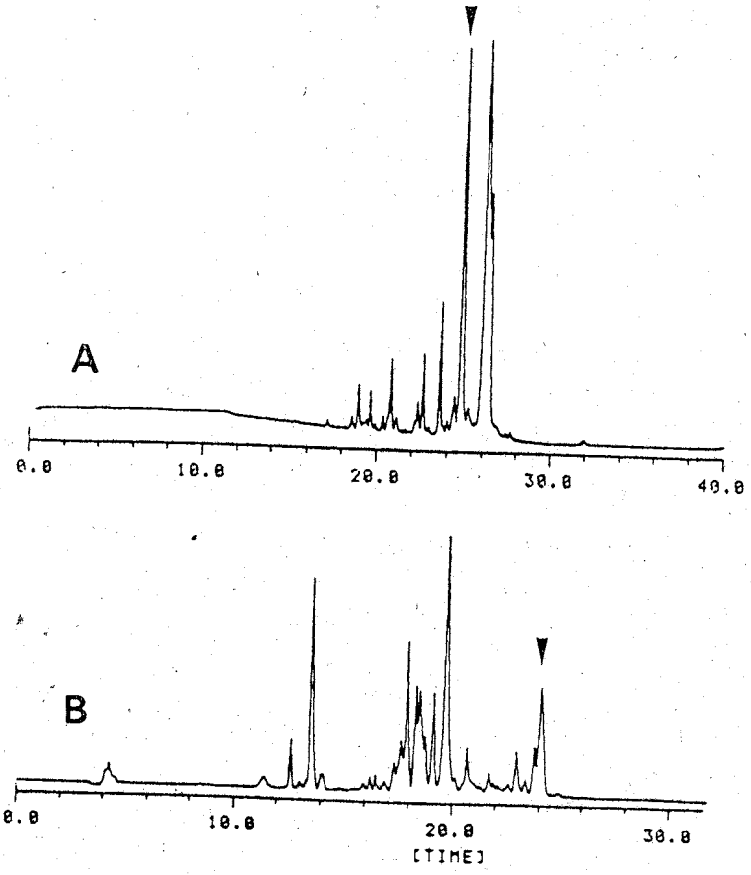


Figure 2. Separation of C-lobe (A) and CLT (B) by RP-HPLC using Capcell Pak C18 SG300 column (4.6 mm ID × 25 cm) at 40°C with a flow rate of 1 ml/min. C-Lobe and CLT are shown by arrows.

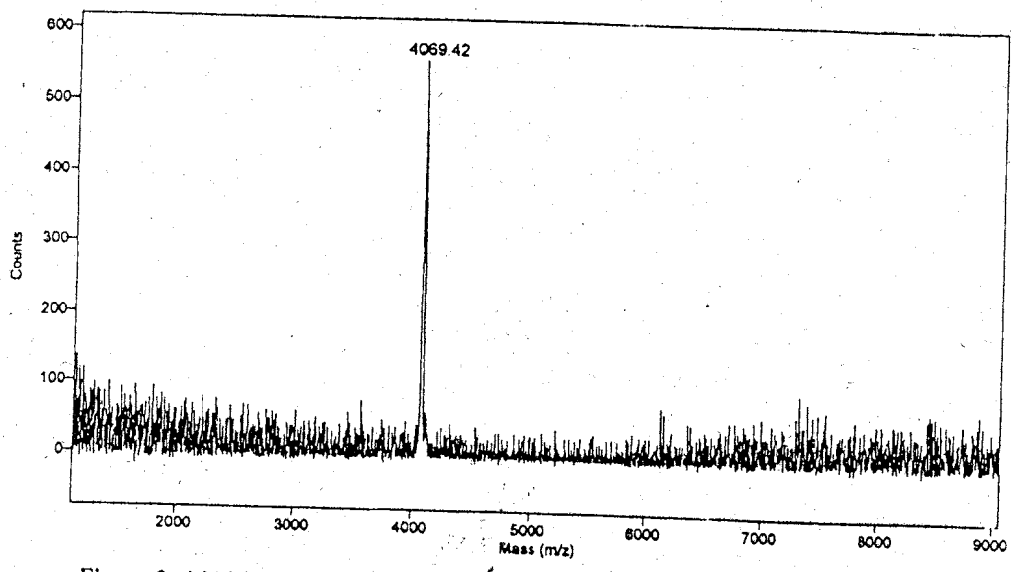


Figure 3. MALDI-TOF mass spectrum of pyridylethylated and deglycosylated CLT.

1 TAGWNIPM	9 GLIVNQTG	17 SCAFDEFF	25 SQSCAPGR	33 DPKSRLCA
2 AGWNIPMG	10 LIVNQTGS	18 CAFDEFFS	26 QSCAPGRD	34 PKSRLCAL
3 **GWNIPMGL**	11 IVNQTGSC	19 AFDEFFSQ	27 SCAPGRDP	35 KSRLCALC
4 **WNIPMGLI**	12 VNQTGSCA	20 FDEFFSQS	28 CAPGRDPK	36 SRLCALCA
5 NIPMGLIV	13 NQTGSCAF	21 DEFFSQSC	29 APGRDPKS	37 RLCALCAG
6 IPMGLIVN	14 QTGSCAFD	22 EEFSQSCA	30 PGRDPKSR	38 LCALCAGD
7 PMGLIVNQ	15 TGSCAFDE	23 FFSQSCAP	31 GRDPKSRL	
8 MGLIVNQT	16 GSCAFDEF	24 FSQSCAPG	32 RDPKSRLC	

Figure 4. Synthesized peptides for epitope determination by SPOTs™ analysis. The bold type peptides are reactive peptides.

3.3. Antimicrobial Activity of C-lobe

Antimicrobial activity of C-lobe was compared with apo- and holo-lactoferrin. C-lobe showed about 80 % turbidity of control and intact lactoferrin of apo- and holo-types showed 20–30% of control at the concentration of 5 mg/ml.

4. DISCUSSION

To investigate the relation between the biological function and structure of lactoferrin, mAb would be a useful tool. In this study, we prepared anti- bLfcin mAb and anti-C-lobe mAb. From the experiments described above, we concluded that the sequence "QWR" is the binding site with anti- bLfcin mAb. CNBr-cleaved fragments showed no reactivity against this mAb and this should be explained as follows: As the mAb binding site locates neighboring to the N-terminal side of Met, the peptidyl homoserine lactone formed as a result of CNBr treatment[9] hindered the antibody binding. This "QWR" sequence could not been found in human lactoferrin, human transferrin, melanotransferrin or ovotransferrin, all of which are a members of the transferrin family of proteins.

bLfcin has been shown to have an affinity for certain substances concerning living cells, membranes and others. It binds directly to lipopolysaccharide to disrupt the outer membrane of Gram-negative bacteria[10]. It is reported that the sequence "RRWQWR" is the subregion essential for antimicrobial activity of bLfcin[11]. The mAb binding site determined in this experiment is included in this subregion. Moreover, we have determined the heparin-binding site of bovine lactoferrin (in preparation). A fraction from pepsin hydrolysate of lactoferrin that binds to immobilized heparin column was separated. By sequence analysis and MALDI-TOF mass spectrometry, this heparin-binding peptide has the sequence of FKCRRWQWRMKKLGAPSITCVRRAFA, and this corresponds to the peptide of 17Phe to 42Ala, the same as bLfcin. We suggest that the heparin-binding site of bovine lactoferrin is at KCRR(18–21), RMKK(25–28) and RR(38–39) as shown in Figure 5. Heparin-binding site of human lactoferrin is reported to be at 1Gly-2–5Arg[12] or 5Arg, 25Arg-XX-28Arg-29Lys and 31Arg (BXXBBXB[13]). Here, B means basic residues. The consensus sequences of XBBXBX or XBBBXXBX has been reported for heparin-binding sites[14]. In bovine lactoferrin, the sequence of BXBB is found only at 2 locations of N-lobe and one in C-lobe.

bLfcin displayed no α-helix but rich in β-structure (ca. 50%) in the aqueous solution and circular dichroic spectra of bLfcin changed reversibly when heparin was mixed with bLfcin. In the presence of 50 % trifluoroethanol, a helix forming solvent, the peptide displayed only 6.5% of α-helical conformation and disulfide bond did show little restriction to prevent the formation of α-helix.

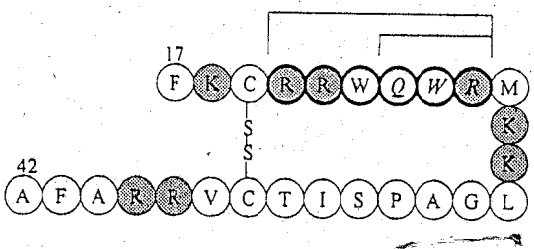


Figure 5. Binding sites of anti-bLfcin mAb (thick circle and *italic*) and heparin (thick circle), and antimicrobial activity subregion (gray dotted circle) of bLfcin.

As CLT fragment isolated by Figure 2B reminded the reactivity against mAb after removal of sugar moiety, it is excluded the possibility that carbohydrate chain is recognized by mAb. Partial N-terminal amino acid sequence of CLT was determined to be TAGWNIP-MGLI and the molecular mass of deglycosylated CLT was 4069.4 as determined by MALDI-TOF mass spectrometry. Due to the enzymatic specificity of the endoglycosidase H, one residual N-acetylglucosamine (GluNAc, MW 221.2) should be remained at 476Asn[15]. Therefore, it is concluded CLT is composed of the residues from 464Thr to 498Lys of lactoferrin, i.e., TAGWNIPMGLIVNQTGSDAFDEFFSQSCAPGRDPK which attach one residual GluNAc and 2 sulfhydryl groups of Cys pyridylethylated (Figure 6). This 464Thr should be adjacent to the 463Arg which is interacting with the carbonate ion for iron ion binding[16].

By SPOTs analysis, the common sequence found in these peptides recognized by the mAb is "WNIPMGL" (467 to 473 of bovine lactoferrin) as in Figure 4. This sequence contains no Lys nor Arg. Therefore, we concluded that this sequence is the antigenic determinant or epitopic site of the C-lobe. This "WNIPMGL" sequence is found in transferrin of human, horse, rabbit, rat and pig and in lactoferrin of pig, mouse, goat (Saanen) and Korean native goat[17]. Melanotransferrin or ovotransferrin have sequences that one amino acid residue is replaced. All of the proteins described above are members of the transferrin family proteins. By ELISA, the mAb against bovine C-lobe did not bind to some of these proteins, that are commercially available. This may mean that the location of the mAb binding site of these proteins are buried from the molecular surface or not accessible by antibody molecule. There observed some disagreement among the results of peptide bond cleavage or enzymatic treatment. These may be explained by the steric hindrance occurring among the bulky side chain groups apart to disturb the binding of mAb and epitopic site. The structural confirmation and biological significance of these epitopic site has not been resolved yet.

Additionally, antimicrobial activity of C-lobe was measured to see one of the biological functions of C-lobe, and it showed lower activity than those of intact apo- and holo-lactoferrin. As C-lobe purified by RP-HPLC was used for the assay, it remains the possibility that C-lobe was not in the native conformation even after the removal of acetonitrile followed by dialysis against phosphate buffer for 2 days at refrigerator. This is confirmed by the measurement of CD spectra at the range of 250 to 350 nm.

5. CONCLUSION

Monoclonal antibodies (mAb) against bovine lactoferrin C-lobe and N-lobe were prepared. To prepare the mAb specific to N-lobe, lactoferricin® (bLfcin) coupled with KLH was used as an antigen. The anti- bLfcin mAb showed reactivity against both natural

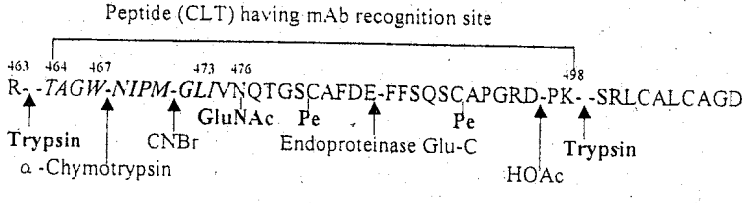


Figure 6. Amino acid sequence of CLT and mAb recognizing site (bold). The italic residues were determined by sequence analysis.

and chemically synthesized bLfcin, but not reacted with human lactoferrin nor hLfcin. By analyses with the synthetic peptides by SPOTs™ and the reactivity of chemically modified bLfcin, the mAb recognition site was identified to be the sequence of "QWR" of this peptide. C-lobe was cleaved into the smaller fragment by trypsin digestion and a small peptide that showed the binding activity against anti-C-lobe mAb was obtained. It is composed of the residues from 464 to 498 of lactoferrin judged from N-terminal amino acid sequence and molecular mass determined by MALDI-TOF mass spectrometry. By SPOTs™ analysis, the binding site of C-lobe was determined to be in the sequence of "WNIPMGL".

ACKNOWLEDGMENTS

We are very grateful to Dr. Y.C. Yoo, Mr.Y. Maki, Mr.Y. Hoshi, Miss T. Tazume and Mr. Y. Okouchi for their useful discussions and technical support.

REFERENCES

1. Baker, E.N., et al. Inter. J. Biol. Macromol. 13, 122–129 (1991).
2. Bellamy, W., et al. Biochim. Biophys. Acta 1121, 130–136 (1992).
3. Shimazaki, K., et al. J. Vet. Med. Sci. 58, 1227–1229 (1996).
4. Shimazaki, K., et al. J. Dairy Sci. 76, 946–955 (1993).
5. Oi, V.T. & Herzenberg, L.A. in Selected Methods in Cellular Immunology (eds. Mishell, B.B. & Shiigi, S.M.) 351–372 (W.H.Freeman and Co., New York, 1980).
6. Aisen, P. & Leibman, A. Biochim. Biophys. Acta 257, 314–323 (1972).
7. Tomita, M., et al. J. Dairy Sci. 74, 4137–4142 (1991).
8. Pierce, A., et al. Eur. J. Biochem. 196, 177–184 (1991).
9. Gross, E. Methods in Enzymology 11, 238–255 (1967).
10. Yamauchi, K., et al. Infect. Immun. 61, 719–728 (1993).
11. Tomita, M., et al. Acta Paediat. Japon. 36, 585–591 (1994).
12. Mann, D.M., et al. J. Biol. Chem. 269, 23661–23667 (1994).
13. Wu, H.F., et al. Arch. Biochem. Biophys. 317, 85–92 (1995).
14. Cardin, A.D. & Weinstraub, H.J.R. Arteriosclerosis 9, 21–32 (1989).
15. Spik, G., et al. Primary and three-dimensional structure of lactotransferrin (lactoferrin) glycans, 21–32 (Plenum Press, Hawaii, 1994).
16. Baker, E.N., et al. ibid, 1–12.
17. Yu, D.Y., et al. Animal Genetics, in press (1997).

Monoclonal Antibody against Bovine Lactoferricin® and Its Epitopic Site

Kei-ichi SHIMAZAKI, Myoung Soo NAM[1], Shinji HARAKAWA[2], Tetsuya TANAKA[2], Yoshitaka OMATA[2], Atsushi SAITO[2], Haruto KUMURA, Katsuhiko MIKAWA, Ikuo IGARASHI[3], and Naoyoshi SUZUKI[3]

Dairy Science Laboratory, Animal Science Department, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan, [1]Korea Research Institute of Bioscience and Biotechnology, Korea Institute of Science and Technology, P. O. Box 115, Yusong, Taejon, 305-600, Korea, [2]Department of Veterinary Physiology, and [3]The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan

(Received 11 July 1996/Accepted 21 August 1996)

ABSTRACT. Bovine lactoferricin® (LFcin B) is a strong antimicrobial peptide derived from N-lobe of lactoferrin. To study the immunochemical and structural properties of LFcin B, monoclonal antibody (mAb) was prepared and the amino acid sequence concerning with the binding to mAb has been identified. Mice injected with LFcin B showed no production of antibody specific to this peptide, whereas those with LFcin B-KLH conjugate produced anti-LFcin B antibodies. None of the mAb reacted with bovine lactoferrin C-lobe, human lactoferrin or LFcin H. By the reactivity of the mAb against the peptides synthesized on cellulose membranes using SPOTs™ and against chemically modified derivatives of LFcin B, the antigenic determinant of LFcin B was identified to be the sequence of "QWR".—
KEY WORDS: lactoferricin, lactoferrin, monoclonal antibody.

J. Vet. Med. Sci. 58(12): 1227–1229, 1996

Lactoferrin is a multifunctional protein. It is known to exert bacteriostatic effects due to its ability to bind environmental iron [1]. Also, apo-lactoferrin has been shown to bind to microbial membranes and causes the direct destruction of microorganisms [7]. Other biological functions attributed to lactoferrin include roles in modulation of the inflammatory response, activation of the immune system, and control of myelopoiesis [5]. Recently, hydrolysates produced by gastric pepsin cleavage of human or bovine lactoferrin were found to contain a potent bactericidal peptide, named lactoferricin (LFcin) H and B, respectively [2]. The microbial killing effect of these peptides was 10 to 100 times stronger than that of undigested lactoferrin and LFcin B is about 9-fold more effective than LFcin H [2]. These active peptides were found to display broad-spectrum antibacterial properties, having effectiveness against Gram-positive and -negative bacteria, yeast and filamentous fungi [15]. LFcin B consists of a single peptide chain of 25 amino acid residues having the sequence FKCRRWQWRMKKLGAPSITCVRRAF [2], derived from the N-terminal region (17–41) of bovine lactoferrin. On the other hand, LFcin H is composed of two peptides linked by a disulfide bond and is comprised of 47 amino acid residues. LFcin B has been shown to have an affinity for cell membranes and may exert its lethal effect by disruption of essential membrane functions. It binds directly to lipopolysaccharide and disrupts the permeability barrier of the outer membrane of Gram-negative bacteria [16]. Also, LFcin B acts to disrupt the permeability properties of the cytoplasmic membrane, as suggested by the observation that it inhibits bacterial uptake of proline [3].

To clarify the structural relationship of the biological functions of LFcin, it seems that antibodies specific to LFcin B would be a useful tool. Therefore, the antigenicity of LFcin B was examined, and monoclonal antibody (mAb) specific to this peptide was prepared. Then the antigenic determinant recognized by the mAbs prepared in this study was identified by several methods.

Bovine lactoferrin, LFcin B, and chemically synthesized LFcin B were kindly supplied by the Nutritional Science Laboratory, Morinaga Milk Industry Co., Ltd. (Zama, Japan). Bovine lactoferrin C-lobe was prepared as reported previously [13].

The carrier protein coupling method [10] was used to enhance the antigenicity of the peptides. For this purpose, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/HCl (Sigma Chem. Co., Ltd., St. Louis, MO) and keyhole limpet hemocyanin (KLH, Pierce Chem. Co., Rockford, IL) was used. To immunize mice with LFcin B or LFcin B-KLH conjugate, 100 μg/m*l* of the material was mixed with the same volume of Freund's complete adjuvant to form an emulsion. Eight week-old female BALB/c mice were injected intraperitoneally. On the days post primary inoculation, they were reinjected with the emulsified samples except in this instance Freund's incomplete adjuvant was used. Spleen cells isolated from the mice were fused with sp2/0-Ag-bearing mouse myeloma cells using polyethylene glycol (PEG 1500) [11]. Hybridoma cells were monoclonally selected twice by the limiting dilution culture method. Monoclonal antibody was obtained from the ascites fluid of BALB/c mice inoculated with the hybridoma cells.

Peptides were synthesized from Fmoc amino acid active esters on a pre-activated cellulose membrane [4] using SPOTs™ (Genosys Biotech. Inc., The Woodlands, TX). Detection of the mAb binding was carried out using horseradish peroxidase labelled secondary antibody. Functional groups of lysine, arginine and tryptophan residues of LFcin B were modified using succinic anhydride [9], 1,2-cyclohexanedione [12] and N-bromosuccinimide [14], respectively. The disulfide bond within LFcin B was reduced by treatment with dithiothreitol and then pyridylethylated (Pe-) with 4-vinylpyridine [6]. The Pe-LFcin B was separated from intact LFcin B by reverse phase (RP-) HPLC. For identification of Pe-LFcin B, partial N-

terminal amino acid sequences were determined using an Applied Biosystems Model 477A protein sequencer. Then, cyanogen bromide (CNBr) cleavage [8] of Pe-LFcin B was performed, and the fragments produced were separated by RP-HPLC. RP-HPLC was carried out using columns of TSKgel ODS-80T_S or ODS-120T_M (Tosoh Co., Ltd., Tokyo) at 40 °C. For elution, a mixture of eluents A (0.1% trifluoro acetic acid (TFA) in water) and B (0.1% TFA in 80% acetonitrile) was employed, using a linear gradient of A:B from 100:0 to 40:60 for 30 min at a flow rate of 1 ml/min.

None of the mice injected with LFcin B alone displayed detectable anti-LFcin B antibody production, even after the booster injection. Therefore, to produce mAbs specific to LFcin B, spleen cells were obtained from the mice inoculated LFcin B-KLH conjugate. After screening and cloning, four colonies were chosen for further study. One clone (5F12.1.2) showed reactivity to LFcin B at a concentration of 1 *ng/ml*. The other three clones showed reactivity to LFcin B at 100 *ng/ml*, but the reactivity was diminished at concentrations of 10 *ng/ml* or lower. Three clones showed high specificity to anti-mouse IgG1 antibody but the other clone (2F7.4.3) reacted with both anti-mouse IgG2b and IgM antibodies (Table 1). For further experiments, mAb produced by 5F12.1.2 cells was mainly used. These mAb showed reactivity against both chemically synthesized LFcin B, of which the sulfhydryl groups are acetamidomethylated, and natural LFcin B by ELISA. These antibodies showed a little reactivity against native state of bovine lactoferrin (Table 1), and they reacted with the denatured bovine lactoferrin (data not shown). None of them showed the reactivity with the C-lobe of bovine lactoferrin, human lactoferrin or LFcin H.

Fifty kinds of peptides corresponding to the LFcin B sequence were synthesized and their reactivity was studied (Fig. 1). Positive reactions were obtained with peptides 1, 2, 9, 10, 11, 18, 19, 20, 28, 29, 30, 31, 32, 39, 40, 41 and 42, and weak positive reactions were obtained with 21, 43 and 44. The common sequence found in each of the peptides recognized by the mAb is "QWR". The reactivity of the mAb against LFcin B derivatives having chemically modified lysine, tryptophan or arginine residues, as compared with intact LFcin B, was 96.5, 30.1 and 27.9%, respectively. The ratio of tryptophan residues modified was 70% estimated from the decrease in absorbance of the reaction mixture at 280 nm. The modified ratio of arginine and lysine residues has not been estimated. This observation supports the above result that the tryptophan and arginine residues of LFcin B are mainly involved in the epitope region recognized by the mAb. Therefore, we conclude that the sequence "QWR" is the antigenic determinant of LFcin B.

After the CNBr cleavage of the Pe-LFcin B fraction, the reaction mixture was separated into three fractions by RP-HPLC (Fig. 2). The last fraction (peak 3) eluted at the same position of the Pe-LFcin B having its disulfide bond cleaved and blocked. These three fractions were gathered, concentrated, and assayed for the reactivity against anti-LFcin B mAb. In the ELISA, the mAb did not show any reactivity against peaks 1 and 2, and showed only with the peak 3. This means that the epitope sequence is located very close to methionine residue, and the peptidyl

Table 1. Reactivity of mAbs to lactoferricin® B and lactoferrin and their subclass determination

Clones	lactoferricin B (*ng/ml*)			lactoferrin (*ng/ml*)	Subclass
	100	10	1	100	
5F12.1.2	0.55	0.42	0.25	<0.05	IgG1
2F7.4.2	0.64	0.08	0.01	<0.05	IgG1
2F7.4.3	0.48	0.04	0.00	<0.05	IgG2b, M
2F7.5.4	0.65	0.18	0.06	<0.05	IgG1

Reactivity of mAb is represented as the absorbance at 415 nm.

1 **WQWR**	14 MKKLG	26 KLGAPS	39 **KCRRWQWR**
2 **QWRM**	15 KKLGA	27 LGAPSI	40 **CRRWQWRM**
3 WRMK	16 KLGAP	28 **CRRWQWR**	41 **RRWQWRMK**
4 RMKK	17 LGAPS	29 **RRWQWRM**	42 **RWQWRMKK**
5 MKKL	18 **RRWQWR**	30 **RWQWRMK**	43 WQWRMKKL
6 KKLG	19 **RWQWRM**	31 **WQWRMKK**	44 QWRMKKLG
7 KLGA	20 **WQWRMK**	32 **QWRMKKL**	45 WRMKKLGA
8 LGAP	21 QWRMKK	33 WRMKKLG	46 RMKKLGAP
9 **RWQWR**	22 WRMKKL	34 RMKKLGA	47 MKKLGAPS
10 **WQWRM**	23 RMKKLG	35 MKKLGAP	48 KKLGAPSI
11 **QWRMK**	24 MKKLGA	36 KKLGAPS	49 KLGAPSIT
12 WRMKK	25 KKLGAP	37 KLGAPSI	50 LGAPSITC
13 RMKKL		38 LGAPSIT	

Fig. 1. Reactivity of anti-lactoferricin B mAb against synthetic peptides prepared by SPOTs™. Bold letters indicate that positive reactivity was observed (21, 43 and 44 were weakly positive). A common sequence in peptides reactive with the mAb is underlined. Antibodies obtained from clones 5F12.1.2 and 2F7.5.4 gave the same results.

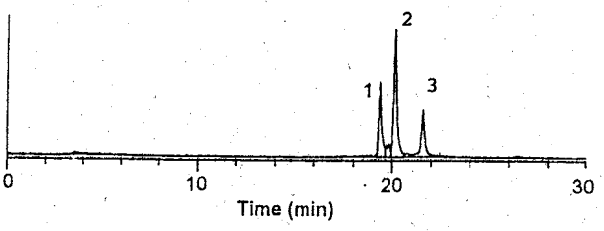


Fig. 2. Separation of the Pe-lactoferricin B (peak 3) and its fragments (peaks 1 and 2) after cyanogen bromide treatment. The column used was TSKgel ODS-120T (4.6 mm ID × 25 cm) and detection was at 210 nm.

homoserine lactone formed as a result of CNBr treatment [8] may hinder the binding of antibody. The antibody binding site of LFcin B is sequential or linear epitope, but the interaction of the bulky amino acid groups near this sequence would affect the antibody-peptide binding.

This "QWR" sequence could not been found in lactoferrin originated from human, pig, mouse and goat, transferrin originated from human, rabbit, pig, horse, rat, cockroach and African clawed frog, human melanotransferrin or ovotransferrin, all of which are members of the transferrin family proteins. These sequences were obtained from DNA Data Bank of Japan (Mishima). It has been reported that the sequence "RRWQWR" is the subregion essential for antimicrobial activity of LFcin B [15]. It is very interesting that the epitopic sequence "QWR" found in this study is located at the C-terminal half of this subregion sequence. The functional significance of this observation is now being explored in further experiments *in vivo* and *in vitro*.

ACKNOWLEDGMENTS. We are very grateful to Drs. Yung Choon Yoo, Shunsuke Iwanami, Keiji Kondo and Fumio Nakamura for their useful discussions and technical support.

REFERENCES

1. Arnold, R. R., Cole, M. F., and McGhee, J. R. 1977. *Science* 197: 263–265.
2. Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K., and Tomita, M. 1992. *Biochim. Biophys. Acta* 1121: 130–136.
3. Bellamy, W. R., Wakabayashi, H., Takase, M., Kawase, K., Shimamura, S., and Tomita, M. 1993. *J. Appl. Bacteriol.* 75: 478–484.
4. Blankemeyer-Menge, B., Nimtz, M., and Frank, R. 1990. *Tetrahedron Lett.* 31: 1701–1704.
5. Brock, J. 1995. *Immunol. Today* 16: 417–419.
6. Cavins, J. F. and Friedman, M. 1970. *Anal. Biochem.* 35: 489–493.
7. Ellison, R. T. 1994. pp. 71-90. *In*: Lactoferrin: Structure and Function (Hutchens, T. W., Rumball, S. V., and Lönnerdal, B. eds.), Plenum Press, New York.
8. Gross, E. 1967. *Methods Enzymol.* 11. 238–255.
9. Klotz, I. M. 1967. *Methods Enzymol.* 11. 576–580.
10. Maloy, W. L., Coligan, J. E., and Paterson, Y. 1991. pp. 9.4.1–11. *In*: Current Protocols in Immunology (Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W. eds.), John Wiley and Sons, New York.
11. Oi, V. T. and Herzenberg, L. A. 1980. pp. 351–372. *In*: Selected Methods in Cellular Immunology (Mishell, B. B. and Shiigi, S. M. eds.), W. H. Freeman and Co., New York.
12. Patthy, L. and Smith, E. L. 1975. *J. Biol. Chem.* 250: 565–569.
13. Shimazaki, K., Tanaka, T., Kon, H., Oota, K., Kawaguchi, A., Maki, Y., Sato, T., Ueda, Y., Tomimura, T., and Shimamura, S. 1993. *J. Dairy Sci.* 76: 946–955.
14. Spande, T. F. and Witkop, B. 1967. *Methods Enzymol.* 11: 498–506.
15. Tomita, M., Takase, M., Bellamy, W., and Shimamura, S. 1994. *Acta Paediatrica Japonica* 36: 585–591.
16. Yamauchi, K., Tomita, M., Giehl, T. J., and Ellison, R. T. 1993. *Infect. Immun.* 61: 719–728.

J. Protozool. Res., 7, 81-89 (1997)

The Role of Scavenger Receptor Type A (SR-A) during Infection with *Babesia microti*

TAKENORI KAMADA[1], IKUO IGARASHI[1], NOBORU INOUE[1], HIDEYUKI NAGASAWA[1], HIROSHI SUZUKI[2], TATSUHIKO KODAMA[3], NAOYOSHI SUZUKI[1] AND YUTAKA TOYODA[1]

[1] *The Research Center for Protozoan Molecular Immunology, Obihiro University Hokkaido*
[2] *FUJI GOTENBA Research Labs, Chugai Pharmaceuticed Co.Ltd, Gotenba-shi, Shizuoka*
[3] *Department of Molecular Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Komaba, Tokyo.*

Received 20 March 1998 / Accepted 26 April 1998

Key words : babesiosis; protective immune response; knock-out mouse; SRKO.

ABSTRACT

The class A macrophage scavenger receptor (SR) binds an extraordinary wide range of ligands including bacterial pathogens and mediates macrophage adhesion. In the present study, SR knock-out mice were infected with *Babesia microti* to clarify its role in host defense mechanisms against protozoan infection. Several aspects of the immunological responses of mice during infection were studied. In *B. microti* infection, the packed cell volumes of SR-/- mice at 16-30 days after infection were significantly higher than those of SR+/+ mice, although the course of parasitemia was not different between these mice. Increase in spleen weights after infection were less obvious in SR-/- mice than in SR+/+ mice at 20 days after infection. Flow cytometric studies revealed that the decreases in the percentages of Thy1.2$^+$ as well as CD4$^+$ T cells in spleen after *B. microti* infection were higher in SR+/+ mice than that in SR-/- mice, whereas there were no difference in the rates of B220$^+$ cells between SR-/-and SR+/+ mice. The peritoneal macrophages from SR+/+ mice exhibited higher phagocytic activity than those from SR-/- mice. These results indicate that scavenger receptor enhances immune responses and phagocytosis of parasitized erythrocytes, although it does not directly play an important role of protection against *B. microti* infection.

INTRODUCTION

Babesia, a tick-transmitted heamoprotozoa parasite, causes enormous economic losses in domestic animals throughout the world (McCosker 1981). *Babesia microti,* a species that parasitizes rodents, produces transient high parasitemia in mice and they naturally recover from the acute infection (Ruebush and Hanson 1979). The importance of cell-mediated immunity and IFN-γ in *B. microti* infection has been reported (Irvin et al. 1981; Eugui and Allison 1980). However, the mechanism of mediating control of the primary infection with *B. microti* has not been fully understood. We previously reported that $CD4^+$ T cell plays a very important role in immune response of host to protozoan infection (Igarashi et al. 1994). At the mean time, IFN-γ appears to take part in the response (Orinda et al 1994). However, not only IFN-γ, but other cytokines may involve in the immune response. Therefore, it is important to examine the other factors including TNF-α. In the present study, we focused on the role of macrophages in the control of primary infection with *B. microti* using knock-out mice. The role of macrophage for the resolution of acute primary infection with *B. microti* was investigated in this study.

The class A macrophage scavenger receptor (SR-A) is a trimeric integral membrane glycoprotein which exists in two forms, type I and II generated by alternative splicing of a single gene product (Naito et al. 1992). This family of receptors has recently been extended through the discovery of several additional scavenger receptors and now includes at least three independent SR classes (Kodama et al. 1988; Kodama et al. 1990). Scavenger receptor is defined according to their ability to bind and mediate uptake of modified low density lipoproteins (LDL), such as acetylated LDL (AcLAL) (Terpstra 1997). However, the range of ligands recognized by SR-A is wide, including lipopolysaccharide (LPS) and lipoeichonic acid. This broad ligand specificity has suggested that SR-A may play a role in wide range of macrophage-associated physiological and pathophysiological processes. In the present study, we focused on the possible role of macrophages in the control of primary infection with *B. microti* using SR-knock out mice (Suzuki et al. 1997).

MATERIALS AND METHODS

Mice

Mice deficient in type I and II SR-A (SRKO mice) were produced by disruption of exon 4 of the SR-A gene, which encodes the α-helical coiled coil domain, essential for the formation of functional trimeric receptors, by homologous recombination, these mice were bred onto a genetic background of 129 × ICR, and mice were cross bred on to an identical 129/ICR background.

Female SRKO mice were obtained from CSK Research Park (Gotenba, Japan) and were used at 8 to 15 weeks of age.

Parasites Seldom

Babesia microti was kindly provided by Prof. A. O. Heydorn of the Institute of Parasitology and Tropical Veterinary Medicine, Free University of Berlin,Germany. SRKO mouse were inoculated intraperitoneally with 1×10^7 *B. microti* parasites. Parasitemia was daily monitored by counting parasitized erythrocytes of Giemsa-stained mouse blood smears, everyday. Packed cell volume was monitored every 4 days after infection.

Preparation of spleen cells

Infected and uninfected control mice were killed under anesthesia, and spleens were removed aseptically, minced with scissor, and squeezed between two frosted glass slides. The cell suspension was filtered through the mesh (#200) to removed tissue fragments. Contaminated erythrocytes were lyzed with 0.83% ammonium chloride, washed with phosphate buffered saline (PBS, pH7.4) and resuspended to concentration of 5×10^6 cells/ml in PBS.

Flow cytometric analysis of T cell subset

Spleen cells which suspended in PBS containing 2.0% fetal bovine serum (FBS: BIO WHITTAKER Walkersville, Maryland) were incubated with FITC-conjugated anti-Thy1.2, FITC-conjugated anti-CD4, PE-conjugated anti-CD8, PE-conjugated anti-B220 or PE-conjugated NK1.1 mAb for 30min on ice. After washing with PBS, cell samples were analyzed by flow cytometry (EPICS XL, COULTER CORPORATION, Miami, Florida).

Peritoneal macrophage

Mouse peritoneal macrophages were taken by washing the peritoneal cavity with 5 ml of sterile PBS, pH 7.4. The cells were centrifuged once and counted total cell number. At some experiment cells were resuspended to adjust cell concentration in DMEM (GIBCO BRL, Grand Island, New York) containing 10% heat-inactivated FBS. Some animals were infected with *B. miroti* for10 days before harvesting the macrophages. The cells were seeded on 15-mm round glass coverslips on 24-well plate at a density of 1×10^6 cells/well.

Phagocytosis

Phagocytosis was assayed by overlaying the peritoneal macrophage with 0.1ml of parasitized erythrocytes, suspended to 2×10^8 cells/ml. Coverslips were

incubated for 45 min at 37°C, 5% CO_2 in air. They were rinsed in PBS, fixed in methanol for staining and quantification. After being stained with May-Grunwald solution for 10 min, and with Giemsa solution for 25 min, coverslips were washed with distilled water. The percentage of ingested red blood cells was determined by counting 200 macrophage/coverslip.

Parasitized erythrocytes

Parasitized erythrocytes were obtained from SR+/+ mice 10 days after infection. They were washed three times with DMEM and resuspended to 2×10^8 cells/ml.

Statistics

Data were analyzed statistically by Student's t-test.

RESULTS

Fluctuations of parasitemias and packed cell volumes in SR+/+ and SR-/- mice that had been inoculated intraperitoneally with 1×10^7 *B. microti* are shown in Figures 1 and 2. SR-/- mice developed acute infection registering 47.0% peak parasitemia on 10 days after infection, while SR+/+ mice developed registering 52.9% peak parasitemia on 10days. Recover of parasitemia was similar for both strains of mice during 30 days after infection. However, the packed cell volumes of SR+/+ mice at 16 -30 days after infection were significantly lower than those of

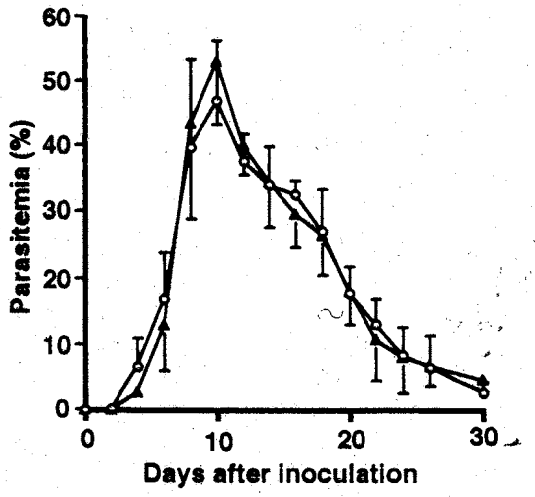


Fig. 1 Infection course of *Babesia microti* in SR+/+ mice (○) and SR-/- mice (▲). Mean parasitemia were obtained from groups of four mice. Data represents the mean ± SD.

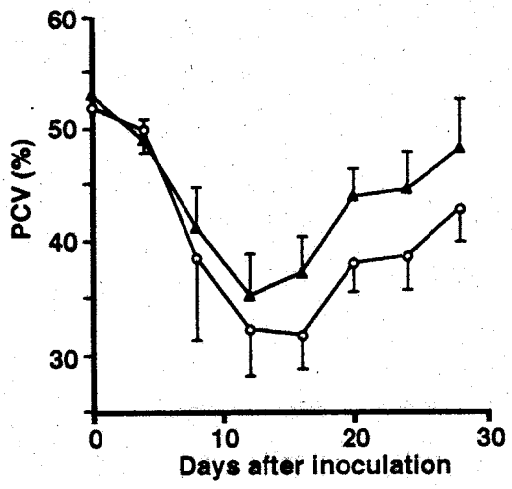


Fig. 2 Infection course of *Babesia microti* in SR+/+ mice (○) and SR-/- mice (▲). Mean PCV were obtained from groups of eight mice. Data represents the mean ± SD. *Student t-test $P<0.05$.

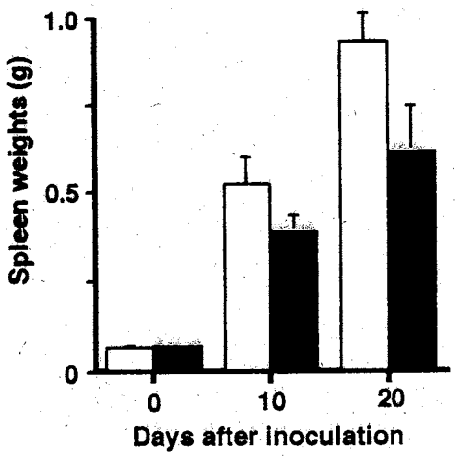


Fig. 3 Spleen weights in SR+/+ mice (□) and SR-/- mice (■) during infection course of *Babesia microti*. Data represents the mean ± SE.

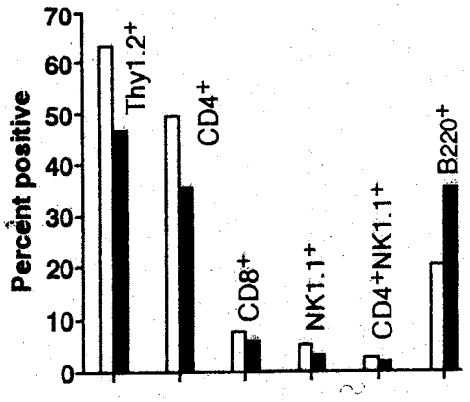


Fig. 4 Phenotypic distribution of spleen cells in SR+/+ mice (□) and SR-/- mice (■). Cell surface expression of the indicated markers was determined by flow cytometer.

SR-/- mice. Spleen weights of SR+/+ mice were higher than those of SR-/- mice at 20 days after infection, as show Figure 3. Phenotypic compositions of spleen cells in non-infected SR+/+ and -/- mice are shown in Figure 4. Percentage of CD4+ T cells from SR+/+ mice was higher than that from SR-/-, while percentage of B220 positive B cells from SR+/+ mice was lower than that from SR-/-. Total cell number and spleen weight were not different between two strains. Figures 5 and 6 show the rates of splenic lymphocyte subsets after infection, as examined by flowcytometer. Percentages of B220+ positive B cells at 10 days after infection were 35.9% and 38.8% from SR+/+ and SR-/- mice, respectively. They became

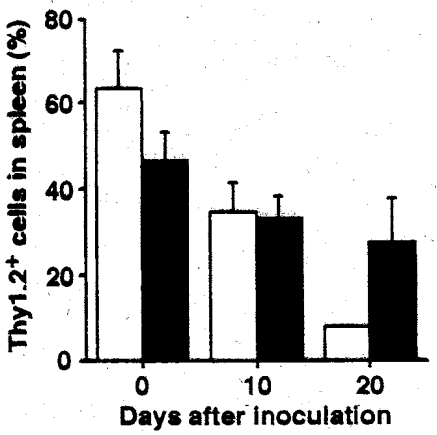


Fig. 5 Infection course of Thy1.2+ cell population in SR+/+ mice (□) and SR-/- mice (■) during infection course of *Babesia microti*. Data represents the mean ± SD.

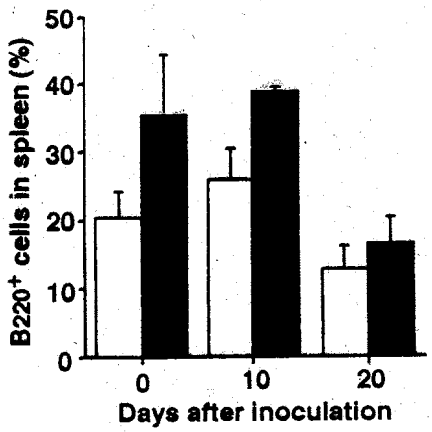


Fig. 6 Infection course of B220+ cell population in SR+/+ mice (□) and SR-/- mice (■) during infection course of *Babesia microti*. Data represents the mean ± SD.

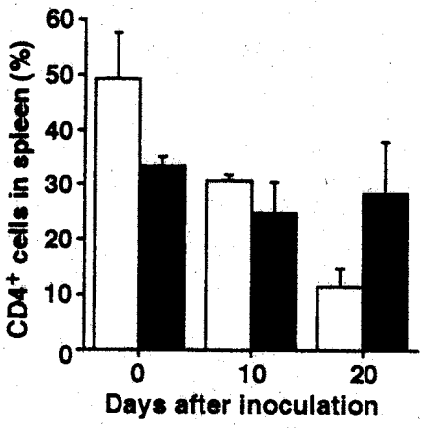


Fig. 7 Infection course of $CD4^+$ cell population in SR+/+ mice (□) and SR-/- mice (■) during infection course of *Babesia microti*. Data represents the mean ± SD.

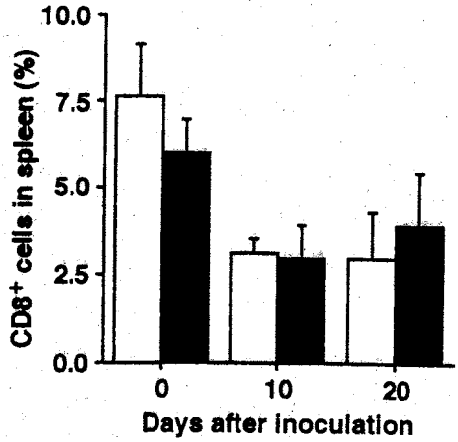


Fig. 8 Infection course of $CD8^+$ cell population in SR+/+ mice(□) and SR-/- mice (■) during infection course of *Babesia microti*. Data represents the mean ± SD.

lower than half of 10 days in both SR+/+ and SR-/-mice at 20 days after infection. There were no significant differences in the rate of B220 positive B cells between SR+/+ and SR-/- mice. Percentage of $Thy1.2^+$ positive T cells reduced percent change from 63.2% to 7.9% at 20 days after infection in SR+/+ mice. The reduction was less drastic from 46.6% in SR-/- mice to 16.5%. This period corresponded to the time when splenomegary occurred in SR+/+ mice. As show in Figures 7 and 8, fluctuations of $CD8^+$ positive T cell were less evident in both strains; changed from 7.6% and 6.0% to 2.9 and 3.9% at 20 days after infection in SR+/+ and SR-/- mice, respectively. There were no significant differences in the rate of $CD8^+$ positive T cells between them. On the other hands, the rates of $CD4^+$ positive T cells changed from 49.4% to 11.5% at 20 days after infection in SR+/+ mice and 33.5% to 28.3% in SR-/-mice. The percentages of $NK1.1^+$ cell and $NK1.1^+$ $CD4^+$ cells did not differ both strains (data not shown). As shown in Table 1, peritoneal macrophages obtained from infected mice at 10 days after infection or from non-infected SR+/+ mice ingested 26.0% or 20.3% erythrocytes infected with *B. microti*. In SR-/- mice, percents of peritoneal macrophages that ingested the infected erythrocytes were 16.8% and 15.0% when obtained from 10 days after infection and from non-infected mice. The rates of peritoneal macrophages that ingested infected erythrocytes were significantly higher in SR+/+ mice than those from SR-/- mice.

DISCUSSION

The importance of cell-mediated immunity and IFN-γ in intercellular protozoa infection has been reported (Shear et al. 1989; Sally et al. 1990; Sedegah

et al. 1994). It has been shown that $CD4^+$ T-cell plays a very important role in host immune response to protozoan infection (Süss et al. 1988; Podoba et al. 1991; Waki et al 1992). Mice treated with anti-CD4 mAb fail to control *B. microti* infection. However, the mechanism mediating the primary infection with *B. microti* has not been fully understood. The role of macrophage for the resolution of acute primary infection with *B. microti* was investigated in this study. The development of mice deficient in type I and type II Scavenger receptors, through disruption of exon 4 of the SR-A gene, has provided a method for investigation. Fluctuations of parasitemia were not different between SR-/- and SR+/+ mice after *B. microti* infection. Based on this result, scavenger receptor seems not to play an important role for exclusion of parasitized erythrocytes with *B. microti.* However, the packed cell volume of SR+/+ mice at 16-30 days after infection were significantly lower than those of SR-/- mice, and spleen weights of SR+/+ mice were heavier than those of SR-/- mice at 20 days after infection. Interestingly, the peritoneal macrophages from SR+/+ mice were more phagocytic than those from SR-/- mice in vitro. This suggests that scavenger receptor mediates uptake of both of infected and non-infected erythrocytes, although the phagocytic activity of macrophages against *B. microti*-infected erythrocytes in the present study is lower than that reported for the uptake of modified-LDL. In the same time, flow cytometric studies revealed that the percentage of lymphocytes in spleen from SR+/+ mice was lower than that from SR-/- mice after *B. microti* infection.

Based on our results, we conclude that scavenger receptor does not directly play an role in protection against *B. microti* infection. However, scavenger receptor, as a binding factor of macrophage, presumably enhance immune responses and induce macrophage for elimination of parasitized and damaged erythrocyte.

ACKNOWLEDGEMENTS

This work was supported by a scholarship of the Ministry of Education, Science and Culture, Japan (07406014, 08306014).

REFERENCES

Eugui, E. M. & Allison, A. C. 1980. Differences in susceptibility of various mouse strains to haemoprotozoan infections: possible correlation with natural killer activity. *Parasite Immunol. 2:* 277-292.

Hannah, L., Shear, H., Srinivasan, R., Nolan, T. & Chui, N. G. 1989. Role of IFN-γ in lethal and nonlethal malaria in susceptible and resistant murine hosts. *J. Immunol. 143:* 2038-2044.

Hoffeman, S. L., Crutcher, J. M., Puri, S. K., Ansari, A. A.,Villinger, F., Franke, E. D. et al. 1997. Sterile protection of monkey against malaria after administration of interleukin-12. *Nature Medicine 3:* 80-83.

Igarashi, I., Waki, S., Ito, M., Omata, Y., Saito, A. & Suzuki, N. 1994. Role of $CD4^+$ T cells in the control of primary infection with Babesia microti in mice. *J. Protozool. Res. 4:* 164-171.

Irvin, A. D., Young, E. R., Osborne, G. D. & Francis, L. M. A. 1981. A comparison of *Babesia* infection in intact, surgically splectomized and congenitally asplenic (Dh/+) mice. *Int. J. Parasitol. 11:* 251-255.

Kodama, T., Freeman, M., & Rohrer, L. 1990. Type 1, macrophage scavenger receptor contains α-helical and collagen like coiled coils. *Nature 343:* 531-535.

Kodama, T., Reddy, P., Kishimoto, C. & Purification, M. 1988. Purification and characterization of a bovine acetyl low density lipoprotein receptor. *Proc. Natl. Acad. Sci. USA. 85:* 9238-9242.

McCosker, P. J. 1981. The global importance of babesiosis. pp. 1-24. *In:* Babesiosis. M. Rustic and J. Freie, eds. Academic Press, New York.

Ruebush, M. J. & Hanson, W. L. 1979. Susceptibility of five strains of mice to *Babesia microti* of human origin. *J. Parasitol. 65:* 430-433.

Mzembe, S. A. T., Lioyd, S., & Soulsby, E. J. L. 1984. Macrophage mediated resistance to *Babesia microti* in Nematospiroides dubis -infected mice. *Z. Parasitenkd. 70:* 753-761.

Naito, M., Naito, H., Suzuki, H., Mori, T., Matsumoto, A., Kodama, T. & Takahashi, K. 1992. Coexpression of Type 1 and Type 2 human macrophage scavenger receptors in macrophage of various organs and foam cells in atherosclerotic lesions. *Am. J. Pathl. 141:* 591-599.

Orinda, G. O., Waltisbuhl, D. J., Young, A. S. & Wright, I. G. 1994. Low doses of natural human interferon alpha inhibit the development of *Babesia microti* infection in BALB/c mice. *Vet. Parasitol. 53:* 53-58.

Podoba, J. E. & Stevenson, M. M. 1991. $CD4^+$ and $CD8^+$ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. *Infect. Immun. 59:* 51-58.

Sally, J. M., Cheng, S. C., Simon-Haarhajs, B. & Langhorne, J. 1990. Role of Interferon during Infection with *Plasmodium chabaudi chabaudi. Infect. Immun. 58:* 3671-3678.

Sedegah, M., Finkelman, F., & Hoffman, S. 1994. Interleukin 12 induction of interferon γ-dependent protection against malaria. *Proc. Natl. Acad. Sci. USA. 91:* 10700-10702.

Shear, H. L., Srinivasan, R., Nolan, T. & Chui, N. G. 1989. Roles of IFN-γ in

lethal and nonlethal malaria in susceptible and resistant murine hosts. *J. Immunol. 143:* 2038-2044.

Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Ueda, O. et al. 1997. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature 386:* 292-296.

Süss, G., Eichamann, K., Kinke, A. & Langhorne, J. 1988. Role of CD4-and CD8-bearing T lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi. Infect. Immun. 56:* 3081-3088.

Terpstra, V., Kondratenko, N. & Steinberg, D. 1997. Macrophage lacking scavenger receptors A show decrease in binding and uptake of acetylated low-density lipoprotein and of thymocytes, but not of oxidatively damaged red blood cells. *Proc. Natl. Acad. Sci. USA. 94:* 8127-8131.

Waki, S.,Uehara, S., Kanbe, K., Ono, K., Suzuki, M. & Nariuchi, H. 1992. The role of T cells in pathogenesis and protective immunity to murine malaria. *Immunology 75:* 646-651.

ELSEVIER

Veterinary Parasitology 68 (1997) 27–33

veterinary parasitology

Growth inhibitory effect of bovine lactoferrin on *Toxoplasma gondii* tachyzoites in murine macrophages: Role of radical oxygen and inorganic nitrogen oxide in *Toxoplasma* growth-inhibitory activity

Tetsuya Tanaka [a,*], Yoshitaka Omata [a], Mutsumi Narisawa [a], Atushi Saito [a], Keiichi Shimazaki [c], Ikuo Igarashi [b], Hiroyuki Hirumi [b], Naoyoshi Suzuki [b]

[a] *Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan*

[b] *The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan*

[c] *Department of Dairy Sciences, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan*

Received 15 March 1996; accepted 11 June 1996

Abstract

To study the effector pathway of *Toxoplasma* growth-inhibitory activity induced by lactoferrin in murine macrophage, the role of reactive oxygen intermediates (O_2^-) and inorganic nitric oxide (NO) was examined. Production of O_2^- was diminished in cultures of macrophages supplemented with lactoferrin and the effect of lactoferrin was dose and time dependent. Production of NO was enhanced in cultures of macrophages supplemented with interferon-γ, but not with lactoferrin. These findings suggest that this *Toxoplasma* growth-inhibitory activity induced by lactoferrin in macrophages is not mediated by O_2^- or NO molecules. A competitive inhibitor of the L-arginine dependent effector pathway, N^G-monomethyl-L-arginine (N^GMMA), virtually abolished the inhibitory effects induced by interferon-γ. Similarly, the inhibitory activity induced by lactoferrin was also diminished in cultures supplemented with N^GMMA. From these findings, it appears that

* Corresponding author. Tel: (+81-155) 49-5115 ext. 5356; fax: (+81-155) 49-5359.

PII S0304-4017(96)01069-2

the *Toxoplasma* growth-inhibitory activity induced by lactoferrin in macrophages may be mediated by an L-arginine-dependent effector pathway that does not involve NO production.

Keywords: *Toxoplasma gondii*; Lactoferrin; Macrophages; Radical oxygen; Nitrogen oxide; Immune response–Protozoa

1. Introduction

Lactoferrin (LF) is a cationic, iron-binding protein produced and secreted by neutrophils and mammary glands. It is known to have broad spectrum antimicrobial properties. Also, its ability to activate the immune response and enhance natural killer cytotoxicity for tumor cells has been investigated (Nishiya and Horwitz, 1982; Sanchez et al., 1992). Lima and Kierszenbaum (1985) reported that LF stimulates the phagocytic activity of mouse peritoneal macrophages (MPM) and increases the intracellular killing of amastigotes of *Trypanosoma cruzi*, an intracellular parasitic protozoan in macrophages. Using scavengers of intermediates of oxygen reduction, they demonstrated that H_2O_2, and O_2^- were involved in the killing capacity of LF-treated MPM.

A recent study showed that murine somatic cells incubated with media containing LF display inhibitory activity against intracellular parasites of *Toxoplasma gondii* (Tanaka et al., 1996). The mechanism of this inhibitory activity induced by LF is, however, still unclear. Macrophages possess oxygen-dependent and oxygen-independent anti-protozoan mechanisms (Murray et al., 1984, 1985; Liew et al., 1990). Murine macrophages activated with IFN-γ and/or lipopolysaccharide display killing activity against *T. gondii*, and this activity is associated with increased production of inorganic nitrogen oxides derived from L-arginine. A competitive inhibitor of the L-arginine-dependent effector pathway, N^G-monomethyl-L-arginine acetate (N^GMMA) abolishes such killing activity of activated MPM (Adams et al., 1990).

To clarify the effector pathway of *Toxoplasma* growth-inhibitory activity induced by LF in macrophages, we examined the production of free radical oxygen products, O_2^- and nitrogen oxide (NO) derived from L-arginine in murine macrophages stimulated with LF. To evaluate the role of NO derived from L-arginine in the mechanism of this activity, the culture medium was supplemented with N^GMMA. In these bioassays, the viability of the intracellular parasites in mouse macrophages was monitored.

2. Materials and methods

2.1. Preparation of LF and reagents

Bovine lactoferrin was prepared from cow's milk by the method of Law and Reiter (1977). The amount of Fe^{2+} ion in the LF was 14.5 mg/100 g protein. Apo- and holo-lactoferrin were prepared by the method of Shimazaki and Hosokawa (1991). Recombinant murine interferon-γ (Genzyme, Cambridge, MA, USA) was diluted in culture medium to obtain a final concentration of 500 unit/ml. N^G-monomethyl

L-arginine acetate (N^GMMA; RBI Res. Biochemicals, Natick, MA, USA) was dissolved in phosphate buffered saline (PBS) at a concentration of 100 mM and stored at −80°C until use. Nitrate and nitrite concentrations were measured using a Nitrite/Nitrate assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations.

2.2. Animals

Seven weeks old male and female ICR mice were used throughout the experiments.

2.3. Monolayer cultures of peritoneal macrophages

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal cavity of mice inoculated with phosphate buffered saline (PBS) containing 0.2% glycogen on the fifth day post-inoculation (p.i.). They were centrifuged at $800 \times g$ for 10 min and were suspended in Dulbecco's modified essential medium (D-MEM) containing 10% fetal bovine serum (D-MEM–10FBS). The macrophage suspension was applied to round coverslips (15 × 15 mm diameter; Matsunami, Osaka, Japan) and 24-well tissue culture microplates (Corning IWAKI, Osaka, Japan), at 5×10^5 cells/well. The macrophage suspensions were incubated at 37°C for 2 h, then washed thoroughly to remove non-adherent cells, and further incubated at 37°C overnight in D-MEM–10FBS. To avoid the influence of LF present in FBS, each MPM culture medium was substituted with D-MEM containing 1% bovine serum albumin (D-MEM–BSA) for 24 h before the experiments.

2.4. Toxoplasma parasites

The RH strain of *T. gondii* tachyzoites were harvested from the peritoneal cavity of mice under anesthesia on the third day p.i. The parasites were washed by centrifugation at $1200 \times g$ for 10 min in PBS three times, and suspended in D-MEM–BSA at a concentration of 5×10^5 parasites/ml.

2.5. Measurement of O_2^- and NO

To assess the effects of O_2^-, MPM on monolayered coverslips were incubated with LF at 10, 100 and 1000 μg/ml or without LF for 24 h. After incubation, the coverslips were washed in Krebs-Ringer phosphate buffer solution free of glucose (KRS) as described by Murray and Cartelli (1983). Each coverslip was incubated in 1 ml of KRS containing 100 μM cytochrome *c* and 0.1 μg/ml phorbol myristate acetate (PMA; Sigma, Louis, MO, USA) at 37°C for 1 h. Before triggering of O_2^- production with PMA, and after incubation, 400 μl of the culture supernatant was taken out and mixed with 1600 μl of chilled H_2O. Measurement of O_2^- production in the cell culture was carried out by the method of McCord and Fridovich (1969) immediately after collecting the samples.

Measurement of nitrite and nitrate production in the culture media was performed

using a Nitrite/Nitrate assay kit as indicated above. The concentrations of nitrite and nitrate were calculated using a standard absorbance curve. The values are expressed as the ratio of the amount per 100 μg of total protein of the MPM tested.

To measure the total amounts of protein contained in the MPM tested, the MPM on coverslips were washed in PBS thoroughly, and dissolved in 6 M urea at room temperature for 3 h. The total amount of protein in the solvent solution was measured using Coomassie^{R} protein assay reagent (Pierce, Rockford, IL, USA).

Assays of the growth activity of the parasites in the host cells were performed by microscopic observation as described by Tanaka et al. (1996). Prior to observation in this assay, MPM monolayers were incubated for 24 h with either D-MEM–BSA alone, D-MEM–BSA supplemented with LF at 10–1000 μg/ml, or D-MEM–BSA supplemented with 100 μM N^{G}-MMA.

2.6. Statistical analysis

All experiments were done in triplicate and repeated at least twice. Data from each experiment were evaluated using Student's *t*-test. The 95% level of significance was used in all analysis.

3. Results

3.1. Effect of O_2^- in macrophages treated with LF

Based on our previous study of the *T. gondii* growth inhibitory effect of MPM treated with LF (Tanaka et al., 1996), we anticipated that O_2^- and/or NO would play a role in this activity. To examine the O_2^- production capacity of macrophages incubated with LF, the concentration of O_2^- in the culture of MPM incubated with 1000 μg LF was compared with that of MPM incubated without LF. As shown in Fig. 1, the amount of

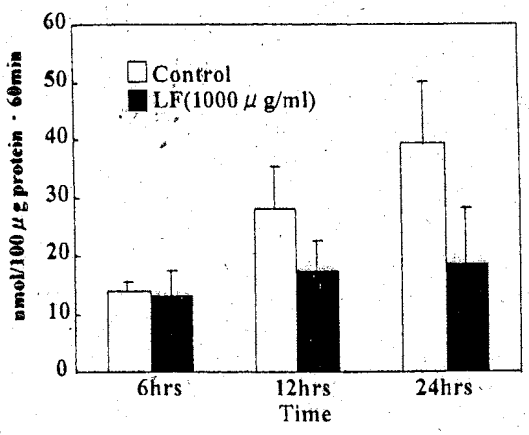


Fig. 1. Production of reactive oxygen intermediates in macrophages incubated with LF for various time periods. Each value is the mean ± S.D. of triplicate samples.

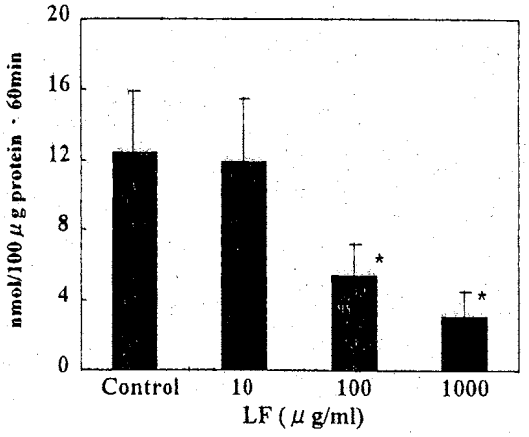


Fig. 2. Production of reactive oxygen intermediates in macrophages incubated with various concentrations of LF for 24 h. Each value is the mean ± S.D. of triplicate samples. * $P < 0.05$, Student's t-test vs control group.

O_2^- produced in the culture of MPM without LF increased gradually, and at 24 h post incubation the rate of production was 39.2 ± 11.0 nmol/100 μg protein/60 min. In contrast, the MPM incubated with LF showed a lower O_2^- production capacity, and at 24 h post incubation the rate of production was 18.2 ± 9.72 nmol/100 μg protein/60 min ($P < 0.05$). We next examined the effects of different doses of LF at 24 h post incubation. As shown in Fig. 2, MPM incubated with LF showed a tendency to have a reduced rate of O_2^- production and the effect of LF was dose dependent. Since LF has iron binding capacity, one possibility to be considered was that the O_2^- may have reacted with Fe^{2+} ions, resulting in a reduction in the concentration of O_2^- in the culture medium. The suppressive effect of LF was similar, however, in the case of MPM incubated with the same dose of apo- or holo-LF (data not shown). Thus, the reduction of O_2^- production in MPM incubated with LF was not mediated by Fe^{2+} ion contained in the LF. These results indicate that *Toxoplasma* growth-inhibitory activity of MPM incubated with LF is not associated with O_2^- production. The data obtained in this study appear to contradict the results reported by Lima and Kierszenbaum (1985). Probably, however, the susceptibility to oxygen intermediates differs between *T. gondii* tachyzoites and *T. cruzi* amastigotes (Hughes et al., 1989).

3.2. *Effect of NO macrophages treated with LF*

The effect of LF on NO production in MPM was also examined. As compared with the level of NO production in MPM incubated with medium alone, MPM incubated with 500 units of IFN-γ for 48 h showed a higher level (432 ± 51.6 nmol/100 μg/protein). A slight increase in the level of NO production in MPM incubated with LF at 1000 μg/ml for 24 h was detected (32.9 ± 11.2 nmol/100 μg/protein), but the amount of NO produced was significantly lower than that in the presence of IFN-γ ($P < 0.05$). Supplementation of the culture medium with N^GMMA strongly inhibited the production of NO in MPM incubated with IFN-γ. A decrease in NO production following treatment of MPM with N^GMMA was observed both in the presence and absence of LF (Fig. 3a).

To further evaluate the effect of N^GMMA on the *Toxoplasma* growth-inhibitory

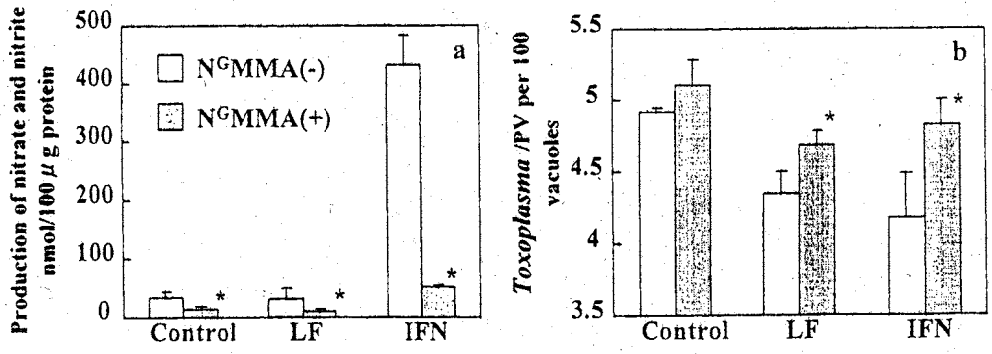


Fig. 3. Effects of N^GMMA on production of nitrate and nitrite in activated macrophages (a) and the microbiostatic capacity of activated macrophages against *T. gondii* (b). Control: Macrophages challenged with tachyzoites were incubated with medium alone for 24 h in the absence (−) or presence (+) of N^GMMA (100 μM). LF: Macrophages challenged with tachyzoites were incubated with LF (1000 μg/ml) for 24 h in the absence or presence of N^GMMA. IFN: Macrophages incubated with IFN (500 units/ml) for 24 h were challenged with tachyzoites. Then, these macrophages were incubated with IFN for 24 h in the absence or presence of N^GMMA. Toxoplasma growth-inhibitory capacity is expressed as the number of tachyzoites/parasitophorous vacuole (PV) among 100 vacuoles. Each value is the mean ± S.D. of triplicate samples. * $P < 0.05$, Student's *t*-test vs N^GMMA (−) group.

activity of MPM, the viability of parasites in MPM incubated with IFN-γ was compared to that in LF-treated MPM. The inhibitory activity of MPM incubated with IFN-γ was reduced in cultures supplemented with N^GMMA. Likewise, the *Toxoplasma* growth-inhibitory activity of MPM incubated with LF decreased in cultures supplemented with N^GMMA. The possibility is negligible that the concentration of NO produced by MPM treated with LF is sufficient to induce this *Toxoplasma* growth-inhibitory activity, because the NO concentration of MPM treated with IFN-γ in medium supplemented with N^GMMA was higher than that in the case of MPM treated with LF (Fig. 3b).

4. Discussion

From these results, we speculate that the *Toxoplasma* growth-inhibitory activity induced by lactoferrin in macrophages might be mediated by another L-arginine-dependent effector pathway that does not lead to NO production. In our previous study (Tanaka et al., 1996), MPM showed *Toxoplasma* growth-inhibition when the MPM were treated with LF after challenge with *T. gondii*. In contrast, the MPM pretreated with LF showed no such activity against *T. gondii* after challenge. This means that LF acts against the intracellular parasites, but not directly on extracellular parasites. LF has no parasiticidal effect, as shown in our previous report. Regarding the host–parasite relationship, intracellular *T. gondii* tachyzoites are enveloped by a parasitophorous vacuole membrane (PVM) which accounts for their resistance to phagolysosomal fusion and this membrane may play an important role in the exchange of nutrients and metabolic products. A recent study has demonstrated that host cell vimentin binds to the parasitophorous vacuole and may serve to dock the parasite compartment to the host cell nuclear surface (Halonen and Weidner, 1994). It has been observed that LF interacts

avidly with nucleic acids. A recent study has documented that LF taken up by leukemia cells becomes bound to DNA and this binding event leads to transcriptional activation (He and Furmanski, 1995). One possibility that should be considered is that LF might be taken up by MPM and then act on PVM to block its biological functions. Further studies of the effects of LF should address the relevance of host cell conditions.

References

Adams, L.B., Hibbs, Jr., B., Janitor, R.R. and Krahenbuhl, J.L., 1990. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-Arginine. J. Immunol., 144: 2725–2729.

Halonen, S.K. and Weidner, E., 1994. Overcoating Toxoplasma parasitophorous vacuoles with host cell vimentin type intermediate filaments. J. Euk. Microbiol., 41: 65–71.

He, J. and Furmanski, P., 1995. Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. Nature, 373: 721–724.

Hughes, H.P.A., Boik, R.J., Gerhardt, S.A. and Speer, C.A., 1989. Susceptibility of *Eimeria bovis* and *Toxoplasma gondii* to oxygen intermediates and a new mathematical model for parasite killing. J. Parasitol., 75: 489–497.

Law, B.A. and Reiter, B., 1977. The isolation and bacteriostatic properties of lactoferrin from bovine milk whey. J. Dairy Res., 44: 595–599.

Liew, F.Y., Millott, S., Parkison, C., Palmer, R.M. and Moncada, S., 1990. Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. J. Immunol., 144: 4794–4797.

Lima, M.F. and Kierszenbaum, F., 1985. Lactoferrin effects on phagocytic cell function. I. Increased uptake and killing of an intracellular parasite by murine macrophages and human monocytes. J. Immunol., 134: 4176–4183.

McCord, J.M. and Fridovich, I., 1969. Superoxide dismutase, an enzymatic function for erythrocuporein (hemocuprein). J. Biol. Chem., 244: 6049–6055.

Murray, H.W. and Cartelli, D.M., 1983. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes: evidence for oxygen-dependent and -independent leishmanicidal activity. J. Clin. Invest., 72: 32–44.

Murray, H.W., Rubin, B.Y., Carriero S. and Acosta, A.M., 1984. Reversible defect in antigen-induced lymphokine and γ-interferon generation in cutaneous leishmaniasis. J. Immunol., 133: 2250–2254.

Murray, H.W., Rubin, B.Y., Carriero, S.M., Harris, A.M. and Jaffee E.A., 1985. Human mononuclear phagocyte antiprotozoal mechanisms: oxygen-dependent vs oxygen-independent activity against intracellular *Toxoplasma gondii*. J. Immunol., 134: 1982–1988.

Nishiya, K. and Horwitz, D.A., 1982. Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody -dependent cell-mediated cytotoxicity. J. Immunol., 129: 2519–2523.

Sanchez, L., Calvo, M. and Brock, H.J., 1992. Biological role of lactoferrin. Arch. Dis. Child., 67: 657–661.

Shimazaki, K. and Hosokawa, T., 1991. A tentative method for rapid preparation of iron-saturated lactoferrin by affinity chromatography. Anim. Sci. Technol., 62: 354–356.

Tanaka, T., Omata, Y., Saito, A., Shimazaki, K., Igarashi, I. and Suzuki, N., 1996. Growth inhibitory effects of bovine lactoferrin to *Toxoplasma gondii* parasites in murine somatic cells. J. Vet. Med. Sci., 58: 61–65.

J. Protozool. Res., 6, 83-93 (1996)

Sequence Analysis of Three Major Antigens (P30, P23 and P22) of Virulent and Avirulent Strains of *Toxoplasma gondii*

YOSHIYUKI MAKI[1,2], SEYHA SENG[1], MIHOKO KATO[1,2], YUKIHIRO HOSHI[2], IKUO IGARASHI[1], HIDEYUKI NAGASAWA[1], YUTAKA TOYODA[1], AND NAOYOSHI SUZUKI[1]

[1] *Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido and* [2] *Nihon Genosys Biotechnologies, Inc., Ishikari, Hokkaido, Japan.*

Received 16 October 1996 / Accepted 30 December 1996

Keywords: *Toxoplasma gondii*; SAG-1 (p30); cDNA.

ABSTRACT

We have sequenced the cDNA encoding the major surface antigens P30 and P22, and the excreted-secreted major antigen P23 of the virulent RH strain and avirulent Beverley and S-273 strains of *Toxoplasma gondii* by direct PCR. The amplified sequences of antigen cDNA were compared. Complete homology was found in the cDNAs encoding the P22 antigen in the RH and S-273 strains of *T.gondii*, and high homology but not complete was found between the cDNA encoding P23 and P30 in the Beverley and S-273. An interesting finding was the amino acid sequence differences relating to surface charge of P30 of virulent and avirulent strains of *T.gondii*. These phenomena might be associated with infectious difference in cells and/or the differences in pathogenicity.

INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite is an important ubiquitous pathogen in veterinary and human medicine. It is known to cause transplacental infections that can lead to abortion or severe neonatal malformation (Remington and Desmonts 1983). Congenital toxoplasmosis remains a major health problem in developed countries and also causes great economic loss in animal breeding, particularly sheep and pig farming. Acute toxoplasmosis has been also observed in patients immunocompromised as a result of drug therapy

(Cohen 1970), neoplastic disease (Frenkel et al. 1975), or infection (Luft et al. 1984) and is a cause of severe complications and death in AIDS patients (Navia et al. 1986). *Toxoplasma gondii* is a successful parasite of immunocompetent hosts because it readily forms cysts within brain and muscle tissue. These tissues are impervious to the immune system, which otherwise effectively controls acute infection and disease symptoms. This organism is considered an opportunistic pathogen because the cysts, which may exist for the life of the host, occasionally rupture and release thousands of highly invasive parasites which may cause a disseminated and potentially fatal disease if the host is in a state of immune deficiency. Therefore, the development of a vaccine against toxoplasmosis and specific knowledge on the host defense response would be of great value both in human and in veterinary medicine. Two major surface antigens, P22 and P30 (SAG1) that anchor the plasma membrane via a glycophospatidylinositol anchor, are expressed on the surface membrane of *T.gondii.* (Kasper et al. 1984; 1989, Tomavo et al. 1988, 1992; Nagel et al. 1989). Several investigators have produced monoclonal antibodies (mAb) which react specifically with the P30 antigen (Ag) (Kasper et al. 1992; Kim and Boothroyd 1993; Mineo and Kasper 1994; Bulow and Boothroyd 1991; Johnson et al. 1983; Rodriguez et al. 1985; Burg et al. 1988) and P23-24 Ag (Cesbron-Delauw 1989). A major excreted-secreted major antigen, termed P23, that has Ca binding domains, is suggested to be a posttranslationally processed product of the 24kDa polypeptide (P24) (Cesborn-Delauw et.al. 1989).

Here we report the comparison of the cDNA encoding P30, P23 and P22 of three different *T. gondii* strains.

MATERIALS AND METHODS

Toxoplasma gondii growth

Tachyzoite of the RH, Beverley and S-273 strains of *T. gondii* were grown in tissue culture using a HeLa cell monolayer in vitro culture system with modified Dulbecco's MEM + 10% heat inactive fetal calf serum (FBS) (Valkoun 1983). Purified parasites were obtained as previously described (Kato et al. 1994) and washed with ice cold phosphate buffer saline solution (PBS).

cDNA Synthesis

Approximately $5x10^7$ *T. gondii* and HeLa cells (negative control) were used for cDNA synthesis. Total RNA of each *T. gondii* strain was prepared using RNA Isolator (Genosys, Houston, USA). Messenger RNA (mRNA) was purified using BioMag Oligo (dT)20 (Nihon Perseptive, Tokyo, Japan). As a template for the amplification reaction, cDNA were prepared from 1mg of mRNA using Oligo

dT12 primer and 200U of Superscript II reverse transcriptase (Life Technologies, Tokyo, Japan) in 20ml of reaction mixture according to the manufactures's instructions. The cDNA reaction mixture was heated at 70℃, 15min and diluted to 100ml with TE buffer (10mM Tris-HCl, 1mM EDTA , pH 7.4) and stored -20℃ until use.

PCR Amplification of cDNA encoding antigens

All PCR primers were synthesized based on previously reported sequences (Prince et al. 1990; Cesbron-Delauw et al. 1989; Burg et al. 1988) using a Cyclone DNA synthesizer (Millipore Co., Tokyo, Japan) and purified on TSKgel Oligo DNA-RP HPLC column, 4.6x150mm (TOSOH, Tokyo, Japan). A nested PCR was performed for P30 cDNA amplification to improve specificity. For first step PCR, 100ml PCR reaction mixture containing 5ml of diluted cDNA reaction mixture as a template, 50pmol each primer (P30F1:AATGTGCACCTG TAGGAAGCTG,P30R2: GCACATGCTGCACGAAGTGTG), 0.2mM of each dNTPs, 10mM Tris-HCl (pH 8.85), 25mM KCl, 5mM $(NH_4)_2SO_4$, 2mM $MgSO_4$ and 2.5U of Pwo DNA polymerase (Boehringer Mannheim) was prepared. For the nested PCR step, 100ml PCR reaction mixture containing 5ml of first PCR amplified mixture as a template, 50pmol each primer (P30 S2nd:GTACAGTTTT TGTGGGCAGAGC, P30AS2nd: TTGTCGATTTGA-GAAGTGAGCA), 0.2mM of each dNTPs, 10mM Tris-HCl(pH 8.3), 50mM KCl, 1.5mM $MgCl_2$ and 2.5U of Taq DNA polymerase (Perkin-Elmer, Tokyo) was prepared. PCR reaction for P22 and P23 cDNA amplification was performed with 5ml of diluted cDNA reaction mixture and specific primers (P22Fd:ACAATTGCGGTGTGACACCTTC, P22Rv: GGTGCATATCTTGGTGTGACCT for P22 cDNA amplification, P23Fd: TGT-TTGGTGGCTGGCCAAATCA, P23Rv: GCAGGTGAAGTAACATGGGGTA for P23 cDNA amplification) in the same manner as P30 nested PCR reaction. All amplification reactions were performed as follows: denaturation, at 93℃ for 3min in the first cycle and for 1min in all 25 subsequent cycles: annealing, at 54℃ for 1min : extension, at 72 ℃ for 2min and for 10 min in the only last cycle on Quick Thermo II (Nippon Genetics, Tokyo, Japan). 10ml of PCR mixture was electrophoresed in 2% agarose gel and detected by ethidium bromide staining.

Purification of PCR Product

The PCR Reaction mixture was purified directly on TSKgel DNA NPR anion exchange column, 4.6x7.5cm (Elena D Katz et.al. 1990) using CCPM HPLC system and UV-8020 micro flow cell UV detector operated at 260nm (TOSOH) at 0.9ml/min flow rate. The 90μl of PCR production mixture were injected onto the column equilibrated with 55% of Buffer A (10mM Tris-HCl, 1mM EDTA pH9.0),

and 45% of Buffer B (Buffer A containing 1M NaCl). The eluant conditions were followed by 0.5min linear gradient from 45% to 55% and 12min gradient from 55% to 65%B. The 2.5 volumes of ethanol, 0.1 volumes of 3M Sodium acetate pH 5.2 and 10mg of glycogen were added into the purified fraction by HPLC. The purified products were allowed to precipitate at -20℃ for several hours and centrifuged at 15,000rpm 15min at 4℃. The pellet was washed with 1ml of 70% ethanol and dissolved in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 7.4).

Sequence Analysis

Sequences of all PCR amplified antigen cDNA were analyzed by the 373A automated DNA sequencer (Applied Biosystem, Tokyo, Japan) using the dye terminator cycle sequencing kit and several internal primers. The sequence data were analyzed and compared by Genetyx Mac and Geneworks program (IntelliGenetics, U.S.A).

RESULTS

The common laboratory strain, RH, is well known for its exceptional virulence in mice and its unusually rapid growth in vivo and in vitro. Therefore, it may not be an appropriate model for experimental infection and protection studies. The S-273 strain is a wild-type isolated from a infected pig in Japan. As it is capable of infecting pigs, shows the typical properties of slow growth in tissue culture, and, at low doses results in a nonlethal, chronic infection in mice with the appearance of brain cysts several weeks post infection, it is a typical *T. gondii* strain found in nature. To determine the homology between several cDNAs encoding major antigens of two avirulent and a typically virulent *T. gondii* strain we sequenced the P22, P23 and P30 cDNA from Beverley, S-273 strains and the RH strain. The sequences of the cDNA encoding P22, P23 and P30 of these strains are shown in Fig.1. Amino acid sequences translated from the cDNA sequences are shown in Fig.2.

The cDNA and amino acid sequence of the P22, P23 and P30 cDNA from the RH strain in our laboratory were identical with those of previous reports (Burg et al. 1988; Cesbron-Delauw et al. 1989; Prince et al. 1990).

There were a total of 14 nt differences among 1135 nt which resulted in 12 AA changes among 336 AA that differed between the RH and S273 strain P30 cDNA in the region sequenced. Nine AA of those , position #78 Phe-Ser, #195 Asp-Asn, #213 Lue-Phe, #251T-Ser, #263 Lys-Asn, #270 Lys-Asn,#298 Lys-Gln, #311 Ala-Ser, 325 Ile-Thr, were in the portion encoding mature peptide. The Sequence of the Beverley strain P30 cDNA revealed differences in 18 nt which resulted in 12 AA changes from that of the RH strain : position #67 Ala-Gly, #195

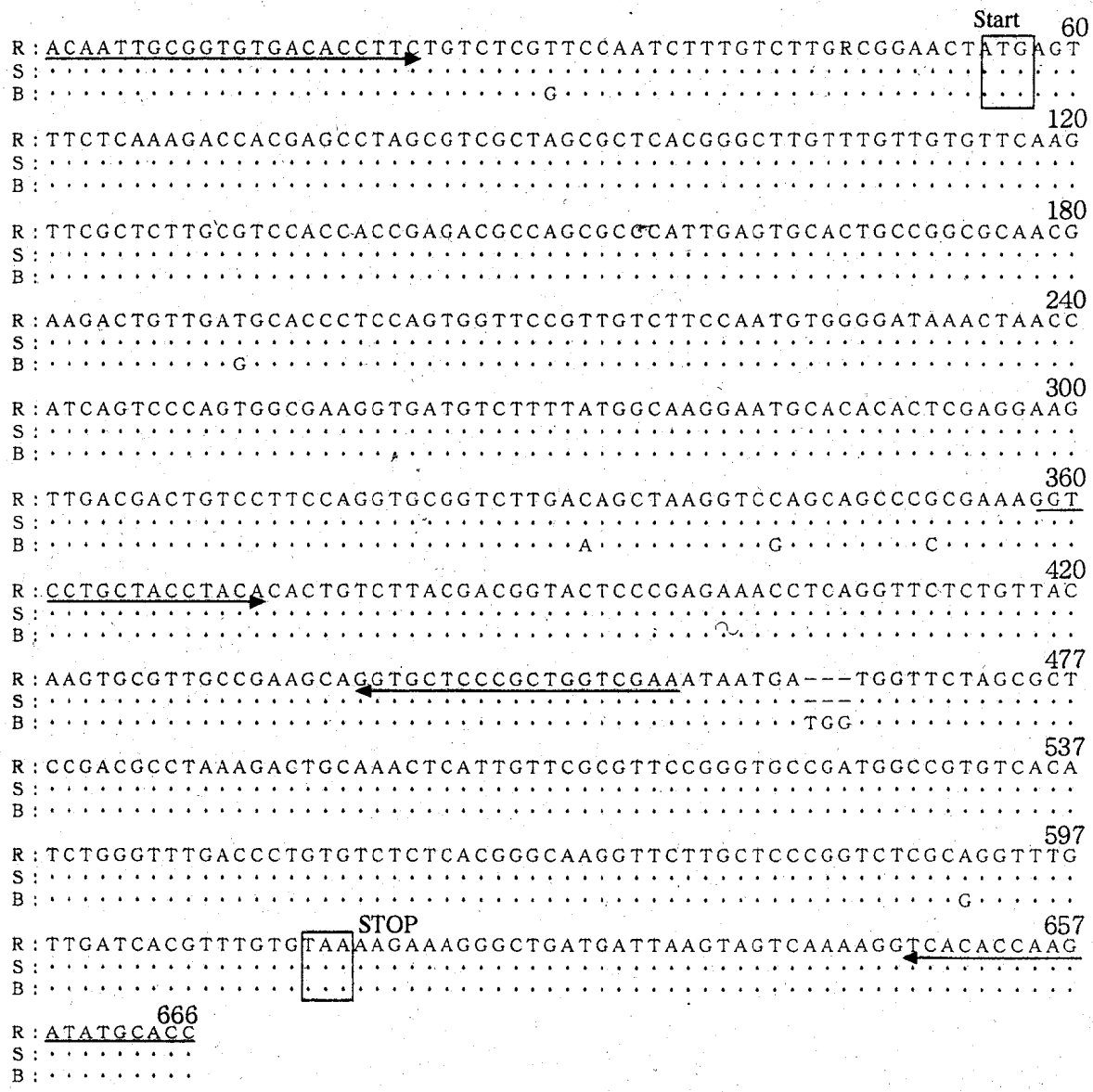


Fig. 1a cDNA Sequence comparison of P22 antigen. Arrows are showing primer site for PCR amplification and sequencing. R: RH strain, S: S-273 strain, B: Beverley strain.

Asp-Asn, #251Thr-Ser, #263 Lys-Asn, #270 Lys-Asn,#280 Ser-Gly, #298 Lys-Gln, #311 Ala-Ser, 325 Ile-Thr of the portion encoding mature peptides.

For the P22 cDNA comparison, no difference was observed between the RH strain and the S-273 strain, however, P22 cDNA between the RH strain and the Beverley strain differed in 6 nt and a 3nt insertion among 666 nt. As a result, 5 AA, position #46 Asp - Glu, #93 Thr-Lys, #97 Gln-Glu, #100 Ala-Pro in the common sequence and #138 Gly in the insertion occurred in the peptide of the Beverley strain.

The sequence of the P23 cDNA of the S-273 strain differed in 9 nt among 691 nt (which resulted in 6 AA differences among 190 AA) from that of the RH strain : position #42 Phe-Lue, #60 Asp-Glu, #86 Ala-T, #99 Val-Met, #114 Asp-

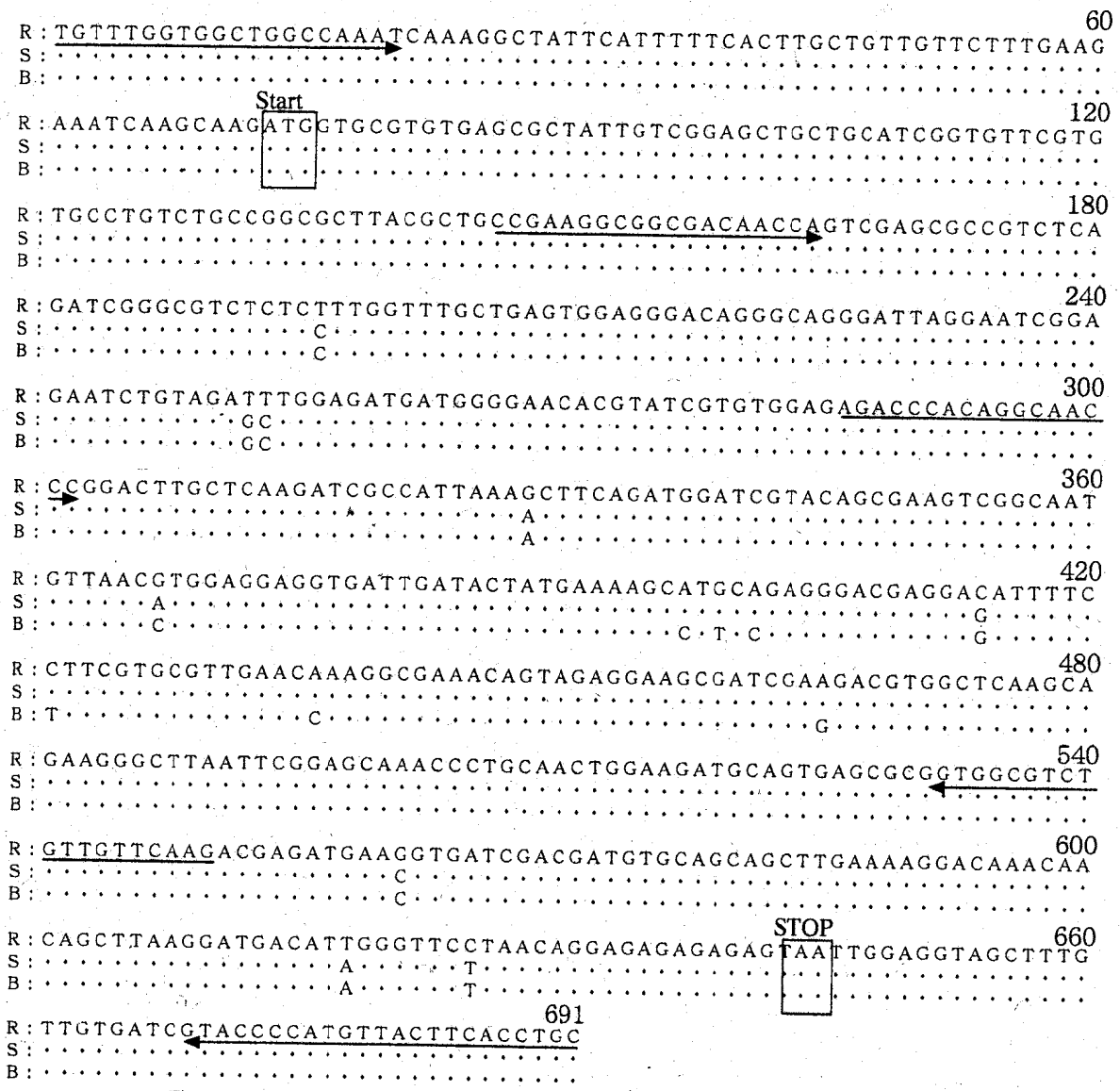


Fig. 1b cDNA Sequence comparison of P23 antigen. Arrows are showing primer site for PCR amplification and sequencing.

Glu, #163 Lys-Asn. There were a total of 15 nt among 691 nt (which result in 10 AA among 190 AA) that differed between the RH strain and the Beverley strain P23 cDNA (#42Phe-Lue, #60 Asp-Glu, #86 Ala-Thr, #99 Val-Lue, #109 Met-Lue, #110 Gin-Pro, #114 Asp-Glu, #117 Lue-Phe, #122 Lys-Gln, #163 Lys-Asn).

DISCUSSION

We describe here the sequence analysis of the cDNA encoding the major surface Ags (P30 and P22) and ES Ag (P23) of the RH, Beverley and S-273 strain of *T. gondii.*

The P30 and P23 cDNA sequences from the S-273 strain and the Beverley strain which are both avirulent *T. gondii* were observed in high similarity and homology. An interesting finding in two of the three strains studied was the

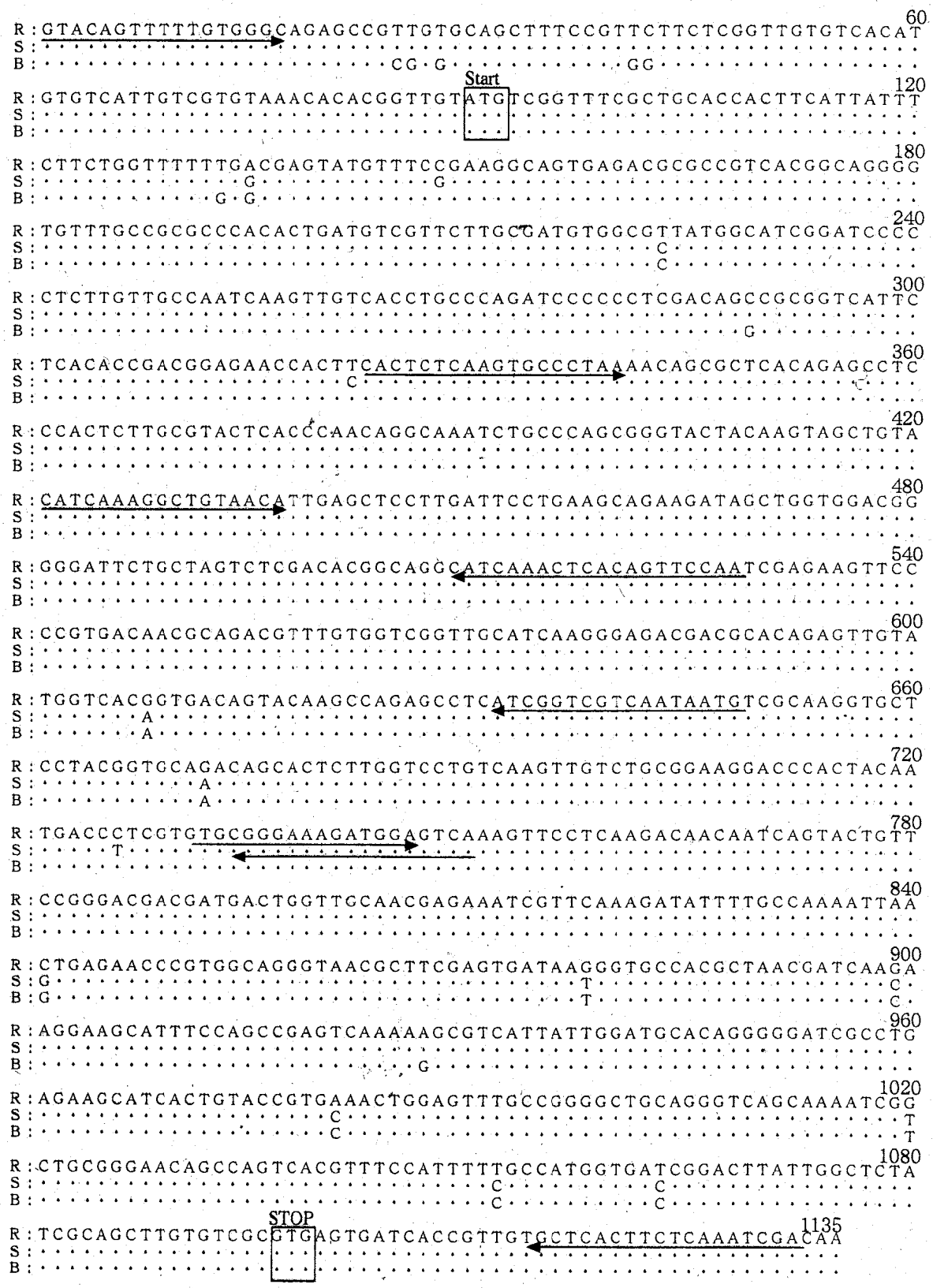


Fig. 1c cDNA Sequence comparison of P30 antigen. Arrows are showing primer site for PCR amplification and sequencing.

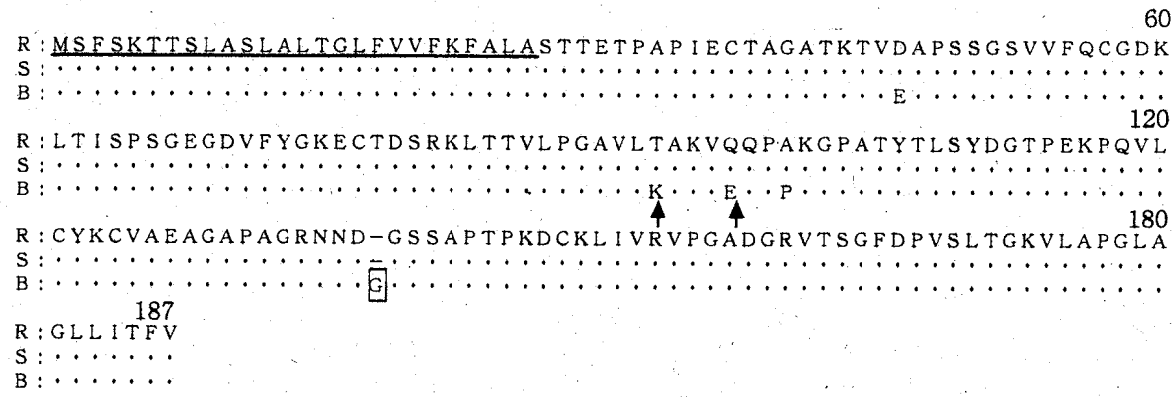


Fig. 2a Amino Acid Sequence comparison of P22 antigen. Signal peptide is underlined. Arrows Show the residue that changes the ionic charge. Inserted Gly are boxed.
R: RH strain, S: S-273 strain, B: Beverley strain.

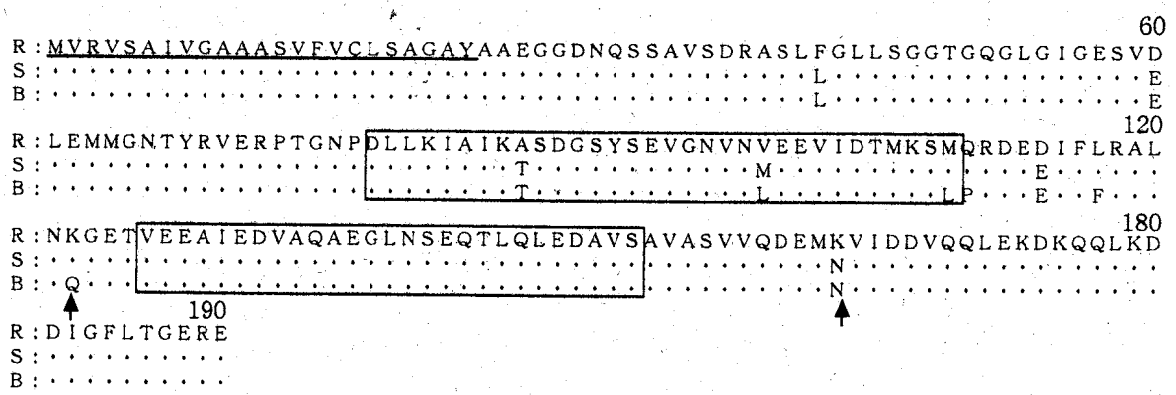


Fig. 2b Amino Acid Sequence comparison of P23 antigen. Signal peptide is underlined. Ca^{2+} binding domains are boxed. Arrows show the residue that lost anionic charge.

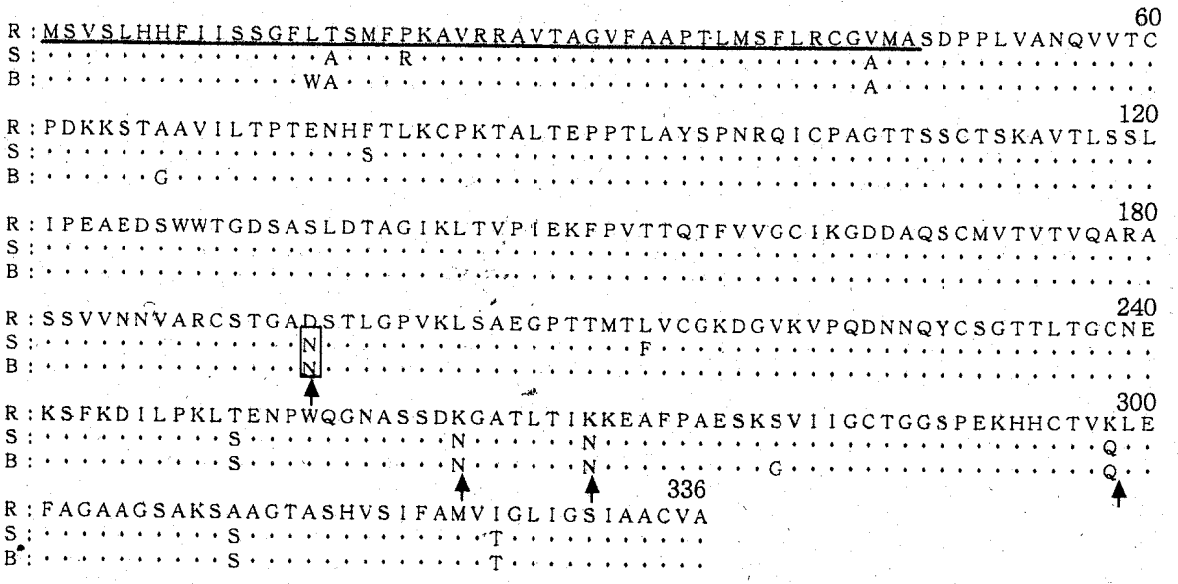


Fig. 2c Amino Acid Sequence comparison of P30 antigen. Signal peptide is underlined. Avirulence strain specific glycosylation site is boxed. Arrows Show the residue that lost anionic charge.

change in the same AA in #263 and #270 Asn and #298 Gln, while the RH strain was Lys, which may result in a reduction in the molecular surface positive charge in this region. On the other hand, the change of #195 Asp-Asn found in the S-273 and Beverley strains resulted in an Asn-X-Thr composition, which may result in glycolization of the #195 Asn. P30 has been reported as an important parasite ligand involved in the process of attachment to host cells (Mineo et al. 1994), and this difference in amino acid sequence may be associated with the infection and pathogenicity of *T. gondii*. Several observations suggested that P30 is a complex protein with considerable higher order structure and multiple forms (Handman and Remington 1980; Kasper 1987; Rodrigues et al. 1985; Santoro et al. 1985; Lawrence-Burg et al. 1988).

Here, we have confirmed that the major surface Ags P30 and P22, and ES Ag P23 of T.gondii are appropriate for further studies producing transgenic animals, which will be extremely valuable to examine the host immune mechanisms against Toxoplasma infection.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. A. M. Johnson of University Technology Sydney, Australia, and Prof. N. Matsushima of School of Health Science, Sapporo Medical University for critical review of the manuscript. This work was supported in part from the Grants-in-aid for Scientific Research (07406014, 08306014), Ministry of Education, Science, Sports and Culture in Japan.

REFERENCES

Bulow, R & Boothyroyd, J. C. 1991. Protection of mice from fatal *Toxoplasma gondii* infection by immunization with P30 antigen in liposomes. *J. Immunol. 147:* 3496-3500.

Burg, J. L., Perelman, D., Kasper,L. H., Ware, P. L. & Boothroyd, J. C. 1988. Molecular analysis of the gene encoding the major surface antigen of *Toxoplasma gondii. J. Immunol. 141:* 3584-3591.

Cohen, S. N. 1970. Toxoplasmosis in patients receiving immunosuppressive therapy. *J. Am. Med. Assoc. 211:* 657.

Cesbron-Delauw, M. F, Guy, B., Torpier, G., Pierce, R. J., Lenzen, G., Cesbron, G. Y., Charif, H., Lepage, P., Darcy, F., Lecocq, J. P. & Capron, A. 1989. Molecular characterization of a 23-kilodalton major surface antigen secreted by *Toxoplasma gondii. Proc. Natl. Acad. Sci. USA 86:* 7537-7541.

Frankel, J. K., Nelson, B. M. & Ariss-Stella, J. 1975. Immunosuppression and toxoplasmic encephalitis clinical and experimental aspects. *Human Pathol. 6:* 97-101.

Johnson, A.M., McDonald, P. J. & Neoh, S. H. 1983. Monoclonal antibodies to *Toxoplasma* cell membrane surface antigens protect mice from toxoplasmosis. *J. Protozool. 30:* 351-356.

Kasper, L. H., Bradly, M. S. & Pfefferkorn, E. R. 1984. Identification of stage specific sporozoite antigens of *Toxoplasma gondii* by monoclonal antibodies. *J. Immunol. 132:* 443-449.

Kasper, L. H. 1989. Identification of stage specific sporozoite antigens of *Toxoplasma gondii. Infect. Immunol. 57:* 668-672.

Kasper, L. H., Khan, I. A., Ely, K. H., Buelow, R. & Boothroyd, J. C. 1992. Antigenic-specific (P30) mouse $CD8^+$ T cells are cytotoxic against *Toxoplasma gondii*-Infected peritoneal macrophages. *J. Immunol. 148:* 1493-1498

Katz. E. D. & Dong, M. W. 1990. Rapid analysis and purification of Polymerase Chain Reaction products by High-Performance Liquid Chromatography. *BioTechniques 5:* 546-554.

Kato, M., Maki, Y., Inoue, N., Omata, Y., Claveria, F. G., Igarashi, I., Saito, A. & Suzuki, N. 1994. *Toxoplasma* lysate antigen (TLA144)-Ig8 antigenic component: Binding and induction of cytotoxic cell activity in mouse spleen cells. *J. Protozool. Res. 4:* 149-157.

Kim, K. & Boothroyd, J. C. 1995. *Toxoplasma gondii:* Stable complementation of sag1 (P30) mutants using sag1 transfection and fluorescence-activated cell sorting. *Exp. Parasitol. 80:* 46-53.

Luft, B. J., Brookds, R. O., Conley, F. K., McCabe, R. E. & Remington, J. S. 1984. *Toxoplasma* encephalitis in patients with acquired immune deficiency syndrome. *J. Am. Med. Assoc. 252:* 913-920.

Mineo, J. R. & Kasper, J. K. 1994. Attachment to *Toxoplasma gondii* to host cells involves major surface protein, SAG-1(P30). *Exp. Parasitol. 79:* 11-20.

Navia, B. A., Petito, C. K., Gold, J. W. M., Cho, E. S., Jordan, H. D. & Price, R. W. 1986. Cerebral toxoplasmosis complicating the acquired immune deficiency syndrome clinical and neuropathological findings. *Ann. Neurol. 19:* 224-232.

Nagel, S. D. & Boothroyd, J. C. 1989. The major surface antigen, P30, of *Toxoplasma gondii* is anchored by a Glycolipid. *J. Biol. Chem. 264:* 5569-5574.

Prince, J. B., Auer, K. L., Huskinson, J., Parmley, S. F., Araujo, F. G. & Remington, J. S. 1990. Cloning, expression, and cDNA sequence of surface antigen P22 from *Toxoplasma gondii. Mol. Biochem. Parasitol. 43:* 97-106.

Remington, J. S. & Desmonts, G. 1983. *In:* Infectious diseases of the Fetus and Newborn infant, eds., Remington, J. S., Saunders, Philadelphia, pp.143-161.

Rodrigues, C. D, Afehain, D., Capron, A., Disous, C. & Santoro, F. 1985. Major surface protein of *Toxoplasma gondii* (P30) contains an immunodominant region with repetitive epitopes. *Eur. J. Immunol. 15:* 747.

Tomavo, S., Schwarz, R. T. & Doubremetz, J. F. 1989. Evidence for glycosylphosphatidy-linositol anchoring of *Toxoplasma gondii* major surface antigen. *Mol. Cell Biol. 9:* 4576-4580.

Tomavo, S., Dubremetz, J., & Schwarz, R. T. 1992. A Family of glycolipids from *Toxoplasma gondii. J. Biol. Chem. 267:* 11721-11728.

Valkoun 1983. Continual cultivation of *Toxoplasma gondii* on Hela cells. *Folia. Parasitol. (Praha). 30:* 289-294.

J. Protozool. Res., 6, 121-128 (1996)

Production of Transgenic Mice Carrying p30 Gene Encoding Major Surface Antigen of *Toxoplasma gondii*

SEYHA SENG[1], YOSHIYUKI MAKI[1,2] MINESUKE YOKOYAMA[3], NOBORU INOUE[1], IKUO IGARASHI[1], HIDEYUKI NAGASAWA[1], MOTOHIRO HORIUCHI[1], YOSHITAKA OMATA[4], ATSUSHI SAITO[4], NAOYOSHI SUZUKI[1], AND YUTAKA TOYODA[1]

[1] *The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido,* [2] *Nihon Genosys Biotechnologies, Inc., Ishikari, Hokkaido,* [3] *Reproductive Engineering Section and Experimental Animal Center, Mitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo, and* [4] *Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.*

Received 16 May 1996 / Accepted 30 December 1996

Keywords: *Toxoplasma gondii*; SAG-1 (p30); transgenic mice.

ABSTRACT

To generate transgenic mice carrying a protozoan gene, 3.1 kb DNA fragments carrying the CAG promoter ligated with a protozoan gene encoding a major surface antigen SAG-1 (p30) of *Toxoplasma gondii*, were microinjected into one of pronuclei of embryos of C57BL/6J and BALB/c mice. The embryos were transferred to the oviducts of ICR pseudopregnant recipients. The transgene was detected by polymerase chain reaction in DNA sequence purified from tissue biopsies. Out of 159 mice that developed from injected eggs two p30-founders (BALB/c and C57BL/6J) were obtained. The transgene was not detected in 52 pups of F1 progeny of C57BL/6J founder. However, 3 (17.6%) out of 17 F1 progenies from BALB/c founder were found to have p30 gene and they inherited the transgene to 39 (55.7%) of 70 F2 progenies. These results may afford the opportunity to study the role of SAG-1(p30) gene in *Toxoplasma gondii* infection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite

responsible for toxoplasmosis that infects most species of warm-blooded animal including domestic animals, bird and human, in most parts of the world. Th disease has been known to result in blindness, mental retardation and death o congenitally infected infants (Dubey 1977; McCabe 1970) and AIDS patient (Mills 1986).

Mammalian cells can be infected by the parasite in vitro, althougl intracellular growth occurs principally within nucleated cells (Joiner anc Dubremetz 1993; McLeod et al. 1991; Buckley 1973). Attachment to the host cel is the first step required in the process of invasion. SAG-1(p30), the major surface protein of *T. gondii* (Kasper and Boothroyd 1993), is involved in the process of host cell infection (Mineo et al. 1993; Grimwood and Smith 1992; Robert et al. 1993) and is considered as an important ligand for binding to the host cell in the process of *T. gondii* invasion (Mineo and Kasper 1994).

It has been shown that the susceptibility to *T. gondii* infection varied among inbred mouse strains; BALB/c, with Ld gene has lower cyst burdens and less encephalitis than those, C57BL/10J, without the Ld gene (Brown et al. 1995). Mice that were resistant to *Toxoplasma* infection had little detectable cytokine mRNA expression in brain, while mice that were susceptible had elevated levels of mRNA for a wide range of cytokines, consistent with their greater amounts of inflammation (Brown et al. 1995). So far, there have been no report on the generation of transgenic animals carrying protozoan DNA, although Moleon et al. (1994) described the cloning of p30 gene by PCR for the purpose of transgenic mice production.

The purpose of this study was to generate transgenic mice carrying p30 gene of *T. gondii* protozoan parasite using BALB/c and C57BL/6J mice in order to analyze the role of p30 in *T. gondii* infection on different genetic background.

MATERIALS AND METHODS

In Vitro Fertilization

BALB/c and C57BL/6J mice, 8 weeks of age, purchased from a commercial supplier, CLEA, Japan,Inc., were superovulated by i.p. injection of 5 I.U of pregnant mare's serum gonadotropin (PMSG; Sankyo Zoki Co., Japan) and human chorionic gonadotropin (hCG; Sankyo Zoki Co.) with an interval of 48 hrs. In vitro fertilization and embryo culture were performed at 37 °C in a 5% CO_2 in air atmosphere. Unfertilized eggs were collected from oviducts of treated mice approximately 16 hrs post hCG injection into a drop of 400 μl of the fertilization medium (TYH) (Toyoda et al. 1971). A small amount of 2 hrs-preincubated sperm suspension was introduced to TYH medium containing cumulus-enclosed oocytes.

Approximately 5 hrs after insemination, fertilized eggs were harvested and washed several times and transferred to a microdrop of 100 μl of Whitten's medium (Whitten 1971) supplemented with 100 μM ethylenediaminetetraacetic acid (EDTA) (Hoshi and Toyoda 1985).

DNA Preparation for Microinjection

p30 gene was cloned from the cDNA obtained by RT-PCR amplification of the *T.gondii* mRNA according to the published sequence of the gene (Burg et al. 1988) as described elsewhere (Maki et al. 1996). A plasmid pexCANLacZ (Kanegae et al. 1995), that has been used for successful generation of LacZ-expressing transgenic mice (Tsukui et al. 1996), was digested with ClaI and the p30 gene was inserted to the ClaI site instead of LacZ. The plasmid pCAGP30, thus constructed, was digested with SalI and HindIII and the expression unit fragment containing CAG (Cytomegarovirus enhancer-chicken β-actin hybrid) promoter (Niwa et al. 1991) and p30 gene was isolated on TSKgel DNA-NPR anion exchange column, 4.6×7.5cm, using a CCP&8020 HPLC system (TOSOH, Japan) and precipitated in ethanol. Purified fragment was dissolved in 10mM Tris-HCl, 0.1mM EDTA solution (pH 7.4) at a concentration of 10 mg/ml.

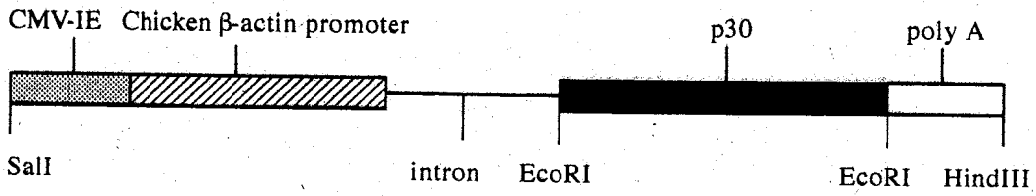


Fig.1 p30 Transgene Construct. The p30 transgene contains the CMV-IE enhancer, the chicken β actin promoter, p30 and rabbit β-globin poly A.

Pronucleous Microinjection

Pronuclear microinjection (Gordon et al. 1980) was performed under Hoffman modulation contrast optics on an inverted microscope (Nikon, Japan) armed with a micromanipulator (Narishige), and in Hepes-buffered mWM at approximately 10 hrs post insemination. One of visible pronuclei of the fertilized eggs was microinjected with the prepared DNA solution. Volume of the DNA solution injected into each pronucleus was estimated to be 2 pl (Allen et al. 1987). The injected embryos were washed with fresh medium and then cultured to the

two-cell stage in 100 μM EDTA supplemented Whitten's medium. The embryos were transferred to the infundibulum of oviducts of 0.5 day pseudopregnant ICR (CLEA, Inc., Japan) recipients.

Preparation of DNA

Distal 1 cm of tail from 4 weeks old mice under anesthetization with diethyl ether was excised with a sterile scissors. Tail biopsies were placed in 1.5 ml microcentrifuge tubes and digested for 4 hrs to overnight at 55 °C with 700 μl of digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, and 1 % sodium dodecyl sulfate) and 35 μl of proteinase K (10 mg/ml). The digests were extracted with 700 μl of TE-satured phenol, 700 μl of phenol/ chloroform, and 700 μl of chloroform. DNA was precipitated from the aqueous phase with 2.5 volumes of absolute ethanol and 1/10 volumes of 3M sodium acetate. Precipitates were washed with 70 % ethanol and air-dried. The pellets were then redissolved in 100 μl of sterile water.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in 50 μl total volume containing 250 ng of isolated DNA. The p30 gene was amplified using oligonucleotide primers consisting of 20 and 21 base pairs and corresponded to nucleotides 544-564 (cactctcaagtgccctaaaa) and 960-939 (gaactttgactccatctttcc). The PCR product was a 416 base pair fragment. The 50 μl reaction mixture contained a final concentration of 25mM $MgCl_2$, 2 mM of each dNTP, 1.6 units of Taq polymerase (Perkin Elmer) and 50 pmol of each oligonucleotide primer. Amplification was performed in an automated thermocycler (GeneAmp PCR system 2,400, Perkin Elmer). The condition for temperature cycling were: 94 °C for 5 min followed by 40 cycles of 94 °C for 20 sec, 53 °C for 30 sec, and 72 °C for 1 min. Cycling was followed by a final extension step at 72 °C for 10 min, and the reactions were held at 4 °C. Amplification products were then electrophoresed on a 1.5 % agarose gel, stained with ethidium bromide, and photographed under UV illumination.

RESULTS

Among 159 pups at 4 weeks of age, 32 BALB/c and 127 C57BL/6J, that had been weaned and were analyzed by polymerase chain reaction (Fig. 2A), two p30-founders, 1 BALB/c female and 1 C57BL/6J male, were identified. The success rate of the founder generated was 1.2 % (Table 1).

The p30-founders were bred with littermates and also with wild type mice of the same strain. The resultant F1 progeny was screened for the exogenous p30

Table 1 Success rate of generating p30-founder mice.

Mice	Number of weaned pups resulted from microinjection	p30-founder number	p30-founder rate (%)
BALB/c	32	1	3.1
C57BL/6J	127	1	0.7
Total	159	2	1.2

transmission by PCR (Fig. 2B).The exogenous gene was not detectable in 52 pups from C57BL/6J founder line (Table 2). For the BALB/c founder line, however, the p30 gene was detected in 3 of 17 pups, corresponding to 17.6% of transmission rate (Table 2). The mice were mated to obtain homozygous F2 progeny. PCR results (Fig. 2C) showed that 39 (55.7%) out of 70 pups were inherited the transgene from parents (Table 3).

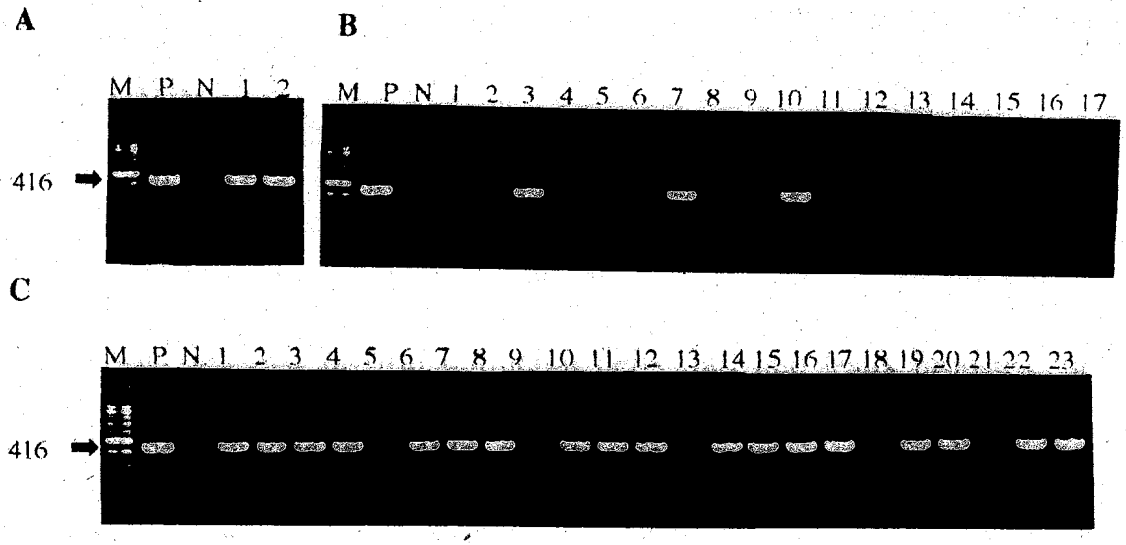


Fig. 2 Identification of p30 transgene by PCR. Mouse genomic DNA purified from tail biopsy amplified with oligonucleotide primers S-544 and AS-960. PCR products: 416 bp. The product (10 μl) were loaded onto a 1.5% agarose gel and visualized by ethidium bromide staining. A: DNA of mice resulted from microinjection, Lane 1: BALB/c, Lane 2: C57BL/6J. B: DNA derived from BALB/c F1 progeny, Lane 1-17. C: DNA derived from BALB/c F2 progeny of trangenic F1 male (No. 3), Lane 1-23. M: marker, P: positive control (microinjected DNA), N: negative control (DNA isolated from normal mouse).

Table 2 p30 transmission to F1 progeny.

p30-founder	Number of weaned progeny	Gene transmission	
		number	rate (%)
Female BALB/c	17	3	17.6
Male C57BL/6J	52	0	0.0

Table 3 p30 transmission to F2 BALB/c mice.

Transgenic F1 mice	Number of F2 pups	Gene transmission	
		number	rate (%)
Male No. 3	28	18	64.2
Male No. 7	33	16	48.4
Female No.10	9	5	55.5
Total	70	39	55.7

DISCUSSION

Introduction of the exogenous p30 transgene into fertilized eggs of BALB/c and C57BL/6J mice at pronucleus stage resulted in a p30-founder mouse of each strain. However, none out of 52 pups derived from C57BL/6J male founder was shown to have the transgene. Investigation on the C57BL/6J founder incapable of transferring genetically the p30 gene to his offspring was carried out. The transgene was detected in oral epithelial cells, tail and testis biopsies, but not in sperms recovered from uterine horns after being mated (data not shown). It stood reason for the founder incapable of transferring the gene to the progeny; and spermatogenesis might be involved. On the other hand, approximately 18% (Table 2) and 56% (Table 3) of progeny F1 and F2, respectively, derived from the BALB/c female founder were identified to inherit the p30 transgene. It indicated that the transgene was stably transmitted in BALB/c mice line. As far as, the authors are aware this is the first report of generation of transgenic mice carrying protozoan gene. Studies on the p30 gene product expression and its function in *Toxoplasma gondii* infection are being conducted.

ACKNOWLEDGMENTS

We thank Dr. J. Miyazaki for the CAG promoter, and Dr. I. Saito for

pexCANLacZ plasmid. This work was supported in part from the Grants-in-aid for Scientific Research (07406014, 08306014), Ministry of Education, Science, Sports and Culture in Japan.

REFERENCES

Allen, N. D., Barton, S., Surani, M. A. H. & Reik, W. 1987. Production of transgenic mouse, pp 217-233, *In:* Mammalian Development. Monk, M (ed). Oxford, IRL Press.

Buckley, S. M. 1973. Survival of *Toxoplasma gondii* in mosquito cell lines and establishment of continuous infection in Vero cell cultures. *Exp. Parasitol. 64:* 474-484.

Burg, J. L., Perelman, D., Kasper, L. H., Ware, P. L. & Boothroyd, J. C. 1988. Molecular analysis of the gene encoding the major surface antigen of *Toxoplasma gondii. J. Immunol. 141:* 3584-3591.

Brown, C. R., Hunter, C. A., Estes, R. G., Beckmann, E., Forman, J., David, C., Remington, J. S. & Mcleod, R. 1995. Definitive identification of a gene that confers resistance against *Toxoplasma* cyst burden and encephalitis. *Immunology 85:* 419-428.

Dubey, J. P. 1977. *Toxoplasma, Hammondia, Besnoitia, Sarcosystis,* and other tissue cyst-forming coccidia of man and animals. p.166, *In:* Parasitic Protozoa. Vol. 3. J. B. Kreier, (ed). Academic Press, New York.

Furtado, G. C., Slowik, M., Kleinman, H. K., & Joiner, K. A. 1992. Laminin enhances binding of *Toxoplasma gondii* tachyzoites to J774 murine macrophage cells. *Inf. Immun. 60:* 2337-2342.

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Basrobosa, J. A. & Ruddle, F. H. 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci. USA 77:* 7380-7384.

Grimwood, J., & Smith, J. E. 1992. *Toxoplasma gondii:* The role of 30 KDa surface protein in host cell invasion. *Exp. Parasitol. 74:* 106-111.

Joiner, K. A. 1991. Cell attachment and entry by *Toxoplasma gondii. Behring Institute Mitteilungen 88:* 20-26.

Joiner, K. A. & Dubremetz, J. F. 1993. *Toxoplasma gondii:* A protozoan for the ninties. *Inf. Immun. 61:* 1169-1172.

Mineo, J. R. & Kasper, L. H. 1994. Attachment of *Toxoplasma gondii* to host cells involves major surface protein, SAG-1(P30). *Exp. Parasitol. 79:* 11-20.

Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. & Saito, I. 1995. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic.*

Acids. Res. 23: 3816-3821.

Kasper, L. H. & Boothroyd, J. C. 1993. *Toxoplasma gondii:* Immunology and molecular biology. pp. 269-301, *In:* Immunology and Molecular Biology of Parasitic Infections. Warren, K. S. (Ed), Cambridge. MA.

McCabe, R. E. & Remington, J. S. 1983. The diagnosis and treatment of toxoplasmosis. *Eur. J. Clin. Microbiol. 2:* 95.

McLeod, R., Mack, D. & Brown, C. 1991. *Toxoplasma gondii:* New advance in cellular and molecular biology. *Exp. Parasitol. 72:* 109-121.

Mills, J. 1986. *Pneumocystis carinii* and *Toxoplasma gondii* infection in patients with AIDS. *Rev. Infect. Dis. 8:* 1001.

Mineo, J. R., McLeod, R., Mack, D., Smith, J., Khan, I. A., Ely, K. H. & Kasper, L. H. 1993. Antibodies to *Toxoplasma gondii* major surface protein (SAG-1, P30) inhibit infection of host cells and are produced in murine intestine after per-oral infection. *J. Immunol. 150:* 3951-3964.

Moleon, I., Beuchamp, P., Cesbron, F. M. & Tourvieille, B. 1994. Cloning of the gene encoding the p30 antigen of *Toxoplasma gondii* by PCR to insert it in transgenic mice. *Arch. Med. Res. 25:* 463-466.

Niwa, H., Yamamura, K. & Miyazaki, J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene 108:* 193-199.

Takeda, S. & Toyoda, Y. 1991. Expression of SV40-lacZ gene in mouse preimplatation embryos after pronuclear microinjection. *Mol. Rep. Del. 30:* 90-94.

Toyoda, Y., Yokoyama, M. & Hoshi, T. 1971. Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. *Jpn. J. Anim. Reprod. 16:* 147-151.

Toyoda, Y. 1995. In vitro culture and transfer of mammalian early embryos. Protein, nucleic acid and enzyme, Journal 40: 14 (in Japanese).

Tsukui, T., Kanegae, Y., Saito, I. & Toyoda, Y. 1996. Transgenesis by adenovirus-mediated gene transfer into mouse zone-free eggs. *Nature Biotechnol. 14:* 982-985.

Whitten, W. K. 1971. Nutrient requirements for the culture of preimplantantion embryo in vitro. *Adv. Biosci. 6:* 129-139.

J. Protozool. Res., 7, 63-69 (1997)

Different Activation of Lymphocytes in Reaction to *Babesia microti* Infection in iNOS-/- and Wildtype Mice

KATHARINA REMER, IKUO IGARASHI, NOBORU INOUE, HIDEYUKI NAGASAWA, YUTAKA TOYODA AND NAOYOSHI SUZUKI

The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan,

Received 12 December 1997 / Accepted 3 April 1998

Key words : lymphocytes, *Babesia microti*, spleen, iNOS-/- mice, $CD4^+$ cells, $CD8^+$ cells, IFN-γ, antibody-titer.

ABSTRACT

The reaction of lymphocyte populations, in iNOS-/- mice and wildtype control mice, to *Babesia microti* infection was investigated in this study. During the course of infection, in both groups of mice there was gross enlargement of the spleens but the spleen cell population decreased by 50%. At peak parasitemia iNOS-/- mice had a low lymphocyte count and on overall, a slight increase in leukocyte population in peripheral blood. On the other hand control mice showed a significant increase in leukocyte population but lymphocyte numbers remained unchanged. During infection the iNOS-/- mice had a higher percentage of B lymphocytes and $CD4^+$ T cells in the spleen, compared to the control mice. In response to *B. microti* infection iNOS-/- mice produce less IL-4, but more IFN-γ compared to control mice. Parallel to the higher percentage of B cells, iNOS-/- mice also produced higher amounts of *B. microti*-specific antibodies. These results suggest that in the early stages of infection NO protects the lymphocytes against *B. microti* invasion and that the immune defense in iNOS-/- mice involves to a large extent humoral immune response.

INTRODUCTION

Babesia microti is a rodent pathogen, species of the hemoprotozoan parasite *Babesia. Babesiae* invade erythrocytes where they multiply and cause hemolysis and anemia in the host (Callow and Dalgliesh 1982). *Babesia microti* and *B. equi* are the only *Babesia* species which invade the host's lymphocytes and multiply

there initially before they invade the erythrocytes. This characteristic places them close to the genus *Theileria* (Mehlhorn and Schein 1984). Cell-mediated immunity by $CD4^+$ T cells and IFN-γ plays an important role in the protection against primary infection with *B. microti* (Igarashi et al. 1994; Shimada et al. 1996). Our earlier experiments with iNOS-/- mice unable to produce nitric oxide in response to *B. microti* infection demonstrated that NO is involved in early immune defense against the parasite. Differences in the expression patterns of IFN-γ and TNF-α during the course of infection proved that iNOS-/- mice use different immune defense mechanisms against the invading parasites as compared to those employed by the wildtype control mice with the ability to produce NO (Remer et al. 1998, in print). The objective of the present study is to identify the lymphocyte subsets involved in immune defense against *B. microti* in iNOS-/- and wildtype mice.

MATERIALS AND METHODS

Mice and parasites

Breeding pairs of mice with disrupted iNOS gene (iNOS-/-) were generously provided by Dr. J. S. Mudgett (Merck Research Laboratories). The C57BL/6 mice used as controls for this study were purchased from CLEA (Tokyo, Japan). All mice were between 6-9 weeks of age at the beginning of the experiments and all experiment groups were sex and age matched. *B. microti* (Munich strain) was kindly provided by Prof. A.O. Heydorn from the Institute of Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin. The mice were inoculated intraperitoneally (i.p.) with 1×10^7 parasitized erythrocytes (PRBC) and the course of parasitemia was monitored every 2 days by counting the percentage of PRBC on Giemsa-stained blood smears.

Spleen size and cell numbers in the spleen

Groups of uninfected mice and infected mice at the peak and at the end of the parasitemia were sacrificed. The spleens were removed and their weight measured with an electronic balance. The spleens were homogenized between two frosted glass slides with Turk's solution (KANTO Chemical Co. Inc., Tokyo, Japan) and the cells in the suspension quantified using a modified Neubauer chamber. The cell-numbers were calculated as cells/mg spleen weight.

Leukocyte-numbers in the blood

Blood from infected and uninfected mice was collected by cardiac puncture. For leukocyte-numbers the blood was diluted with Turk's solution in a haemocytometer and the cells counted with a modified Neubauer chamber. For the proportion of lymphocytes in the total leukocyte population Giemsa-stained blood

smears were examined under light microscope.

Flow cytometrical analysis of lymphocyte subsets

Infected and uninfected mice were sacrificed and the spleen removed. The spleens were then homogenized between two frosted glass slides with phosphate buffered saline (PBS) + heparin (100 U/ml) and the suspension filtered through a nylon mesh to remove tissue fragments. Erythrocytes in the suspension were lysed with 0.83 % ammonium chloride, the cells were washed 2 times with PBS and resuspended with PBS to a concentration of 2×10^7 cells/ml. Cell suspensions were incubated with FITC-conjugated anti-T cell and anti-$CD8^+$ mAb or PE-conjugated anti-B cell and anti-$CD4^+$ mAb for 30 min on ice, washed 3 times and analyzed by flow-cytometer (COULTER® Epics®XL, Coulter TM, Miami, USA).

Measurement of IFN-γ and IL-4

Blood samples of infected and uninfected mice were collected by cardiac puncture. The serum was separated and stored in -80°C until use. The concentrations of IFN-γ and IL-4 in the serum were measured by ELISA test kits (ENDOGEN Inc., Cambridge, USA).

Antibody titer

Babesia microti-specific antibody-titers were determined by indirect immuno-fluorescence antibody test (IFAT). *Babesia microti*-antigen covered slides were kindly provided by Dr. Avarzed. The antigen-spots were covered with the PBS-diluted serum from infected and uninfected mice for 30 min, incubated with FITC-conjugated anti-mouse IgG for 30 min, washed in PBS and examined with a fluorescence microscope (Microphot-FX, NIKON, Tokyo, Japan).

RESULTS AND DISCUSSION

The spleens of uninfected iNOS-/- mice were slightly smaller in size and weight but contained relatively more cells than the spleens of normal C57BL mice. During the course of infection with *B. microti* the spleens increased in size in both iNOS-/- and wildtype mice. The increase in iNOS-/- mice at the end of the parasitemia was only slightly more than in normal mice (Fig. 1). In the same period the relative numbers of cells in the spleen decreased in both groups, however the decrease was greater in iNOS-/- mice as compared to wildtype mice (Fig. 2). These findings correspond with histology results of earlier experiment's which showed that during *B. microti* infection there is little follicle activation in the spleen of iNOS-/- mice as compared to that observed in C57BL controls (Remer et al. 1998, in print). Therefore, the increase in iNOS-/- mice spleen

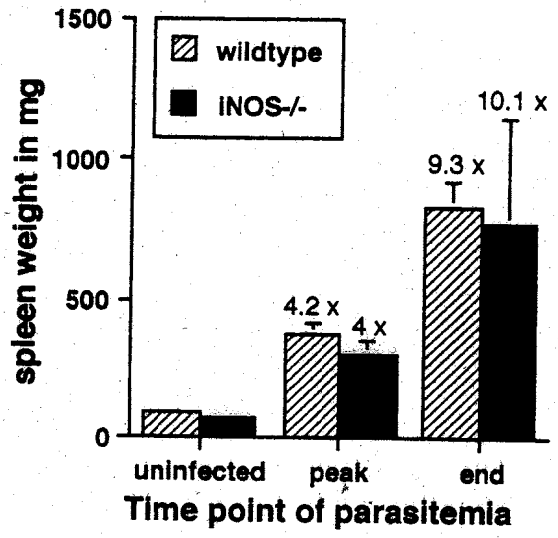


Fig. 1 Increase in spleen weight in iNOS-/- mice and wildtype C57BL mice during *B. microti* infection. The numbers over the columns show the relative increase in weight compared to the spleen of uninfected animals.

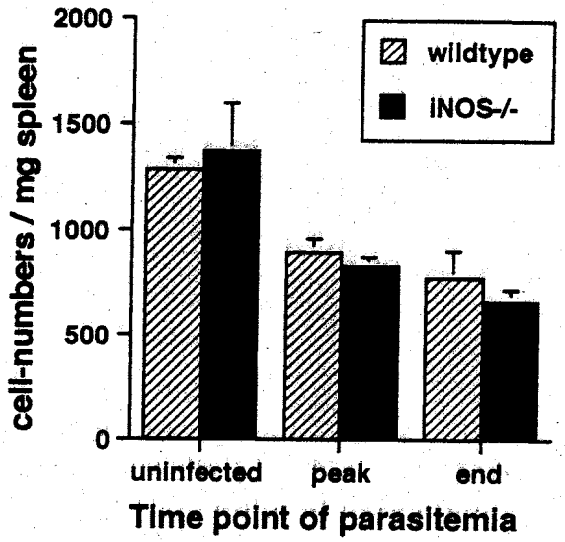


Fig. 2 Relative cell-numbers in the spleen of iNOS-/- and wildtype C57BL mice during *B. microti* infection. Cell-numbers calculated in cells/mg spleen weight.

weights, whilst relative cell numbers remain relatively lower, is caused by an increase in red pulp size rather than proliferation of the white pulp in reaction to the parasites (Hildebrandt 1981).

Uninfected iNOS-/- mice had lower leukocyte-numbers in the blood than their wildtype controls. During the course of infection leukocyte-numbers in the controls increased markedly but returned to normal before the end of parasitemia. In contrast, there was only a modest rise in leukocyte-numbers of iNOS-/- mice, which was maintained until the end of parasitemia (Fig. 3). The percentage of lymphocytes in iNOS-/- mice decreased during the course of parasitemia, while in C57BL control mice it remained unchanged (Fig. 4).

Taking into consideration that *B. microti* is one of the *Babesiae* that has an early phase of multiplication in the host's lymphocytes (Mehlhorn and Schein 1984), the higher percentage of lymphocytes in wildtype mice suggests that NO has the ability to protect the lymphocytes against destruction by the parasite. How these findings are connected with the differences in spleen activation (Remer et al. 1998, in print) and blood leukocyte-numbers is yet not well known and deserves closer examination.

To differentiate the subsets of the lymphocytes flowcytometry was performed. Here it turned out that uninfected iNOS-/- and wildtype control mice had almost the same proportions of T to B lymphocytes (1 : 1.2 and 1 : 0.81,

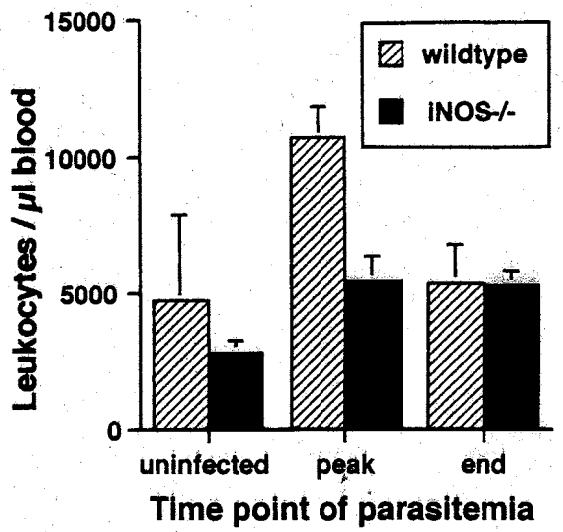


Fig. 3 Leukocyte numbers in the blood of iNOS-/- mice and wildtype C57Bl mice during *B. microti* infection.

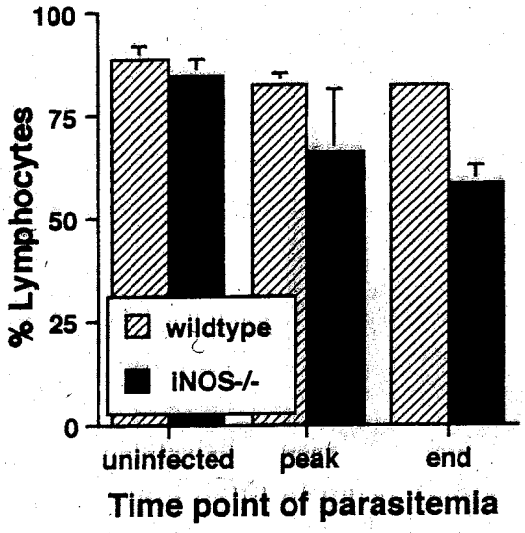


Fig. 4 Percentage of lymphocytes in the differential-blood smear of iNOS-/- mice and wildtype mice during B. *microti*-infection.

respectively), but there were significant differences in ratios between the two groups during infection (Table 1). There was also a distinct difference in the proportions of $CD4^+$ to $CD8^+$ cells: iNOS-/- mice had initially a lower percentage of $CD4^+$ cells, but developed a higher $CD4^+$: $CD8^+$ ratio during infection.

Table 1. Ratio of lymphocyte subsets in iNOS-/- mice and their C57BL controls during the course of infection with *B. microti*.

	T / B ratio		$CD4^+$ / $CD8^+$ ratio	
	wild type	iNOS-/-	wild type	iNOS-/-
uninfected	1 : 0.81	1 : 1.20	3.27 : 1	2.57 : 1
peak	1 : 2.47	1 : 3.90	4.83 : 1	6.30 : 1
end	1 : 1.53	1 : 2.27	3.63 : 1	4.57 : 1

To find out the relative dominance of Th1 or Th2 subsets among the $CD4^+$ cells, the concentrations of IFN-γ, IL-4 and *B. microti*-specific antibody-titer in the serum were determined. iNOS-/- mice had higher amounts of IFN-γ, lower amounts of IL-4 and a higher titer of specific antibodies, compared to wildtype C57BL mice (Figs. 5 and 6, and Table 2).

The importance of the $CD4^+$ T cells in resolving *B. microti* primary infection in normal mice has already been reported (Igarashi et al. 1994; Shimada

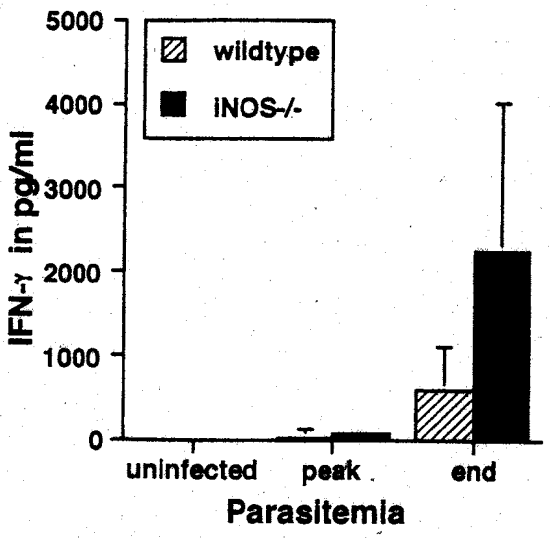


Fig. 5 IFN-γ in the serum of iNOS-/- and wildtype C57BL mice during *B. microti* - infection.

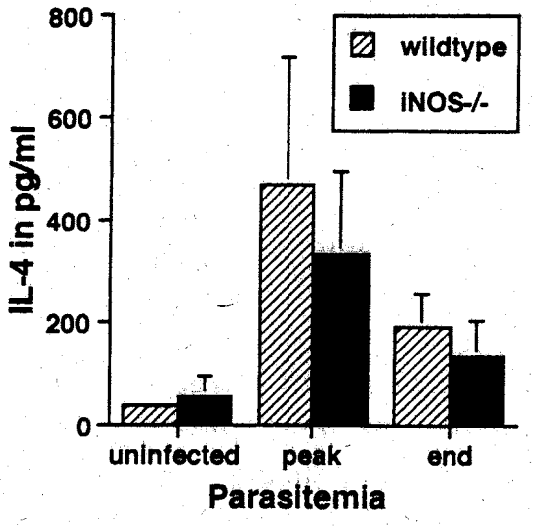


Fig. 6 IL-4 in the serum of iNOS-/- and wildtype C57BL mice during *B. microti*- infection.

Table 2. *Babesia microti*-specific antibody-titer in iNOS-/- and wildtype C57BL mice during the course of infection.

mouse	uninfected	peak	end
wildtype	-	1 : 1,024	1 : 4,096
iNOS-/-	-	1 : 1,024	1 : 16,384

et al. 1996). The results of our experiments demonstrate that $CD4^+$ T cells also play an important role in the immune defense in iNOS-/- mice. Wei et al. (1995) reported that a high concentration of NO prevents the overexpansion of the Th1 subset following a strong antigenic challenge. This may explain the high concentrations of IFN-γ found in iNOS-/- mice with *B. microti*-infection (Remer et al. 1998, in print; Wei et al. 1995). The higher proportions of B lymphocytes and the high *B. microti*-specific antibody-titer despite the general lower numbers of total lymphocytes in iNOS-/- mice indicate a greater importance of humoral immune response to primary infection in iNOS-/- mice.

ACKNOWLEDGEMENTS

This work was supported by a scholarship of the Ministry of Education,

Science and Culture, Japan (No. 07406014, 08306014). We especially acknowledge the generous gift of 5 breeding pairs of iNOS-/- mice from Dr. J. S. Mudgett from the Merck Research Laboratories, New Jersey. We further thank Dr. A. Avarzed who helped to perform the IFAT and Dr. M. L. He for his help with the statistics.

REFERENCES

Callow, L. L. & Dalgliesh, R. J. 1982. Immunity and Immunopathology in Babesiosis. pp. 475-526. *In:* Immunology of parasitic infections. S. Cohen and K. S. Warren, Blackwells Scientific Publications, Oxford.

Hildebrandt, P. K. 1981. The organ and vascular pathology of Babesiosis. pp. 459-473. *In:* Babesiosis. M. Ristic and J.P. Kreier, Academic Press, New York.

Igarashi, I., Hosomi, T., Kaidoh, T., Omata, Y., Saito, A., Suzuki, N. & Aikawa, M. 1993. Comparison of damage to kidneys and liver caused by lethal *Babesia rodhaini* and non-lethal *Babesia microti* infections in mice. *J. Protozool. Res. 3:* 144-155.

Igarashi, I., Waki, S., Ito, M., Omata, Y., Saito, A. & Suzuki, N. 1994. Role of $CD4^+$ T cells in the control of primary infection with *Babesia microti* in mice. *J. Protozool. Res. 4:* 164-171.

McMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q.-W., Sokol, K., Hutchinson, N., Chen, H. & Mudgett, J. S. 1995. Altered responses to bacterial infection and endotoxin shock in mice lacking inducible nitric oxide synthase. *Cell 81:* 641-650.

Mehlhorn, H. & Schein, E. 1984. The piroplasms: live cycle and sexual stages. *Adv. Parasitol. 23:* 37-103.

Remer, K., Igarashi, I., Toyoda, Y. & Suzuki, N. 1998. Nitric oxide is involved in early host defense against a primary infection with *Babesia microti* in mice. *J. Protozool. Res*: in press.

Shimada, T., Shikano, S., Hashiguchi, R., Ohashi, F., Matsuki, N. & Ono, K. 1996. Expression of Gamma-Interferon and Interleukin-4 messenger RNA in splenic $L3T4^+$ T Cells from *Babesia microti*- and *B. rodhaini*-infected mice. *J. Protozool. Res. 6:* 25-30.

Wei, X.-Q., Charles, I. G., Smith, A., Ure, J., Feng, G.-J., Huang, F.-P., Xu, D., Müller, W., Moncada, S. & Liew, F. Y. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature 375:* 408-411.

Mohammed Golam Mowla Chowdhury[1, 2], Ken-ichi Maeda[1], Koji Yasutomo[1, 3], Yoichi Maekawa[1], Atsuko Furukawa[1, 2], Miyuki Azuma[4], Hideyuki Nagasawa[1] and Kunisuke Himeno[1]

[1] Department of Parasitology and Immunology, School of Medicine, The University of Tokushima, Tokushima, Japan
[2] Department of Urology, School of Medicine, The University of Tokushima, Tokushima, Japan
[3] Department of Pediatrics, School of Medicine, The University of Tokushima, Tokushima, Japan
[4] Department of Immunology, School of Medicine, Juntendo University, Juntendo, Japan

Antigen-specific B cells are required for the secondary response of T cells but not for their priming

We studied the potential role of B cells in T cell responses using severe-combined immunodeficient (SCID) mice grafted with the thymus from fetal C.B-17 mice (TG mice). These mice developed both $CD4^+$ and $CD8^+$ T cells, but not B cells within 2 months after transplantation. TG mice showed normal delayed-type hypersensitivity responses against the immunizing antigen ovalbumin (OVA). Lymph node (LN) cells of TG mice proliferated well in response to concanavalin A (Con A). Further, Con A stimulation induced the production of interleukin (IL)-2, IL-6 and interferon (IFN)-γ and the expression of IL-4 mRNA. Thus, TG mice were reconstituted without remarkable immunodeficiency. However, these T cells failed to proliferate to OVA stimulation. Response to OVA was also inhibited in SCID mice grafted with fetal C.B-17 liver cells when B cells were depleted in the proliferation assay. Unresponsiveness against immunizing antigen was restored by the addition of antigen-primed B cells, but not by naive B cells, lipopolysaccharide-activated B cells or B cells primed with sheep red blood cells. Next, we examined whether antigen-primed B cells could induce T cell responses without professional antigen-presenting cells (APC). T and B cells were purified from OVA-immunized mice by cell sorter. These T cells proliferated in response to OVA and produced IFN-γ in the absence of non-B APC. When anti-CD80 or anti-CD86 was added in the assay, proliferation and IFN-γ production was inhibited. These results indicate that B cells activated specifically with antigen are required for the secondary response of T cells, but not for their priming.

1 Introduction

APC function is mediated by various cell types including peritoneal and splenic macrophages [1], dendritic cells [2], epidermal Langerhans cells [3] and B cells. B cells are efficient APC due to the stable expression of antigen-specific Ig receptors and class II molecules that present antigens [4–6]. The importance of B cells as APC has been investigated in B cell-depleted mice established by the chronic administration of anti-IgM (anti-μ mice) [7–11]. T cells of these mice fail to proliferate in response to immunizing antigens when restimulated *in vitro* with antigens [7–9]. This unresponsiveness is restored *in vivo* by transferring B cells with antigens [7, 10, 11]. In particulary, antigen-specific B cells have an important role in the restoration of T cell proliferation [11]. Recently, Constant et al. [12] reported that B cells were required for the priming of T cells to protein antigen using B cell knockout mice. These findings indicate that B cells are essential for both priming and activation of T cells. In contrast, other investigators have argued that B cells are necessary neither for priming nor for activation [13, 14]. Due to these contradictory findings, the role of B cells as APC is still not fully elucidated.

We previously reported that T lymphopoiesis was reconstituted in SCID mice by grafting a fetal thymus under the kidney capsule [15]. These mice (TG mice) lack B cells without continuous treatment with anti-IgM. We reassessed the APC function of B cells using the TG mice. T cells of these mice did not proliferate in response to immunizing antigen. The unresponsiveness of TG mice was restored *in vitro* by adding antigen-primed B cells, but not by B cells nonspecifically activated with LPS or B cells primed with an unrelated antigen. The activation could also be induced in the absence of professional APC. The role of B cells in the priming and the activation of T cells is discussed.

Received February 14, 1995; in final revised form April 22, 1996; accepted May 6, 1996.

Correspondence: Kunisuke Himeno, Department of Parasitology and Immunology, School of Medicine, The University of Tokushima, Kuramoto-cho 3, Tokushima 770, Japan
Fax: +81-886-33-7114

Abbreviations: LN: Lymph node **RT-PCR:** Reverse transcriptase-polymerase chain reaction **SCID:** Severe combined immunodeficiency **TG:** Thymus graft recipient **FLT:** Fetal liver cell transplant recipient

Key words: Antigen presentation / B cell function / SCID mouse / T cell activation / Chimera mouse

2 Materials and methods

2.1 Animals

C.B-17 *scid/scid* (SCID) and C.B-17 +/+ (C.B-17) mice were obtained from the Central Institute for Experimental Animals (Hamamatsu, Japan) through the permission of Dr. M. J. Bosma (Fox Chase Cancer Center, Philadelphia, PA). These mice were bred under specific pathogen-free (SPF) conditions.

2.2 Antibodies

PE-labeled anti-CD4 and FITC-labeled anti-CD8 mAb were purchased from Becton Dickinson (Mountain View, CA). PE-labeled anti-Thy-1.2 mAb was obtained from

0014-2980/96/0707-1628$10.00 + .25/0

Caltag Laboratories (San Francisco, CA). Anti-Thy-1.2 mAb (30-H12), anti-B220 mAb (RA3-6B2), anti-CD80 (RM80) and anti-CD86 (PO3.1) were purified from ascites of mice injected with each hybridoma. Anti-B220 mAb was labeled with FITC according to the standard procedure.

2.3 Transplantation of fetal thymus and fetal liver cells to SCID mice

Fetal thymuses and livers were taken from 15-day-old embryos of C.B-17 mice. One lobe of the fetal thymus was inserted under the left kidney capsule of 6–8-week-old SCID mice using a 21-gauge needle. Fetal livers were minced into a finely dispersed single-cell suspension and injected via the tail vein (1×10^7 cells). All mice were maintained under SPF conditions.

2.4 FACS analysis

LN cells were stained with a combination of PE-labeled anti-CD4 and FITC-labeled anti-CD8 mAb or a combination of PE-labeled anti-Thy-1.2 and FITC-labeled anti-B220 mAb, and were analyzed on a FACScan (Becton Dickinson). Nonviable and nonlymphoid cells were excluded from the analysis by light scatter gating. The profiles are shown by the integration of 1×10^4 cells using two-color analysis.

2.5 Purification of T and B cells

To collect T cells, LN cells were depleted of B cells by panning. Briefly, LN cells were incubated in plates coated with anti-B220 for 1 h and nonadherent cells were collected. These procedures were repeated twice. B cells were prepared by depleting T cells using anti-Thy-1.2 and guinea pig complement (ICN Biomedicals). In a separate experiment, LN cells were stained with a combination of PE-labeled anti-Thy-1.2 and FITC-labeled anti-B220 mAb and were sorted using an EPICS instrument (Coulter, Hialeah, FL). Purity of T and B cells was estimated at > 99 %.

2.6 Measurement of serum Ig by ELISA

Goat anti-mouse Ig was coated onto 96-well tissue culture plates. After blocking with 0.1 % BSA in PBS, serum samples serially diluted with PBS were incubated in the plates for 2 h at room temperature. The results were visualized by using alkaline phosphatase-conjugated anti-mouse IgG (Sigma, St. Louis, MO) followed by *p*-nitrophenyl phosphate (Sigma). After terminating the reaction with 3N NaOH, the absorbance at 415 nm was read on a microplate reader.

2.7 Measurement of DTH reaction

OVA (1 mg/ml; 200 µl) emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco, Detroit, MI) was injected subcutaneously in the dorsal flank of mice. Seven days later, the mice were challenged in the right footpad with 50 µl OVA (0.1 mg/ml). An identical volum of PBS was injected into the left hind footpad as a contro Footpad thickness was measured with a dial gauge calip 24 h after the challenge.

2.8 Proliferation *in vitro* assay

Mice were injected with 100 µl SRBC (50 %) or OV (1 mg/ml) emulsified in CFA at the base of the tail and both hind footpads. Seven days later, the cells collecte from popliteal and inguinal LN (5×10^5/well) were cu tured with SRBC (5×10^5/well), OVA (500 µg/ml) c Con A (2.5 µg/ml) in 0.2 ml of RPMI 1640 (Flow, Irvin Scotland) containing 10 % fetal bovine serum (IC Biomedicals, Costa Mesa, CA), 100 U/ml penicilli 100 mg/ml streptomycin and 5×10^{-5} M 2-me captoethanol in 96-well flat-bottom microplates for 72 Eight hours before harvesting, [^{3}H] dThd (0.5 µCi) wa added to each well. The incorporation of [^{3}H] dThd wa measured in a liquid scintillation counter (Aloka, Tokyo In some experiments, the proliferation assay was pe formed using purified cells.

2.9 Assay of cytokines

LN cells were cultured with or without 2.5 µg/ml Con for 24 h or 48 h. The activities of IL-2, IL-6 and IFN in th supernatant were measured by means of bioassays. IL- was measured as the proliferation of CTLL-2 cells in th presence of anti-IL-4 mAb. IFN activity was measured b the inhibitory effect from the infection of vesicular stom titis virus using L929 cells. The major activity of IFN i these samples was IFN-γ, since anti-IFN-γ mAb almo completely inhibited the activity. IL-6 activity was me sured as the proliferation of MH60BSF2 cells. The prolife ation of these cells was measured by the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid (MTT) assay. The amounts of cytokines in the superna ants were calculated according to the standard curve plo ted using recombinant cytokines. In a separate exper ment, IFN-γ production in the supernatant was measure using ELISA kit EM-IFNG (Endogen, Cambridge, MA)

2.10 RT-PCR

Total RNA (100 ng) extracted from LN cells was converte to cDNA in a reaction mixture containing reverse tra scriptase (Takara, Kyoto, Japan). Oligonucleotide primer were synthesized on a 392 DNA/RNA synthesize (Applied Biosystems, Warrington, GB) as described [16 The cDNA mixed with dNTP (Takara), Taq polymeras (Takara) and primer pairs were amplified by a programm able thermal cycle (MJ Research, Inc., Watertown, MA for a total of 35 cycles. PCR products were analyzed b 1.5 % agarose gel electrophoresis. Hae III-digested φX17 DNA (BRL, Gaithersburg, MD) was the molecular weigh marker.

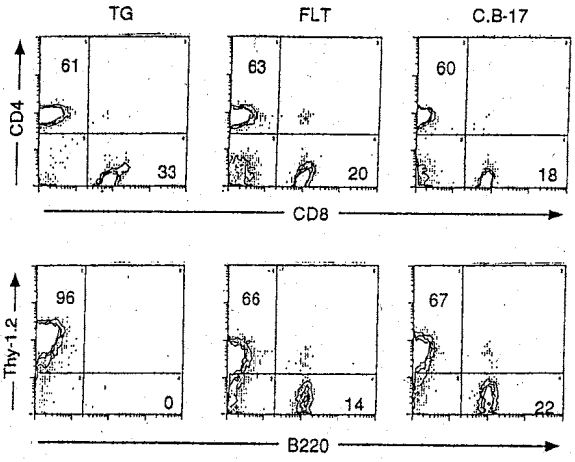


Figure 1. FACS analysis of lymphoid cells in TG and FLT mice. LN cells from TG, FLT and C.B-17 mice were stained with a combination of PE-labeled anti-CD4 and FITC-labeled anti-CD8 or a combination of PE-labeled anti-Thy-1.2 and FITC-labeled anti-B220. Numbers indicate the percentage of each fraction.

3 Results

3.1 Reconstitution of lymphopoiesis in SCID mice grafted with fetal thymus or fetal liver cells of C.B-17 mice

SCID mice were transplanted with fetal thymus (TG mice) or fetal liver cells (FLT mice) obtained from 15-day-old embryos of C.B-17 mice. After 2 months, the reconstitution was examined by staining LN cells with a combination of anti-CD4 and anti-CD8 mAb, or with a combination of anti-Thy-1.2 and anti-B220 mAb (Fig. 1). In TG mice, T (Thy-1.2$^+$) cells with the CD4$^+$ and CD8$^+$ phenotype were both reconstituted, whereas B (B220$^+$) cells were undetectable. There was no remarkable abnormality in the phenotype of the T cell subpopulation. In FLT mice, both T and B cells were reconstituted, and the phenotype was indistinguishable from that of C.B-17 mice. We also measured serum Ig by ELISA in TG and FLT mice (Fig. 2). Ig was produced in FLT mice at the level comparable with C.B-17 mice, but barely detectable in that of TG mice. Therefore, the TG mouse is useful model to examine the role of B cells in T cell response.

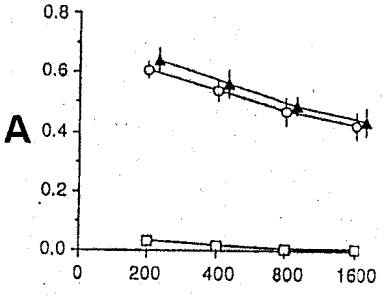


Figure 2. Serum Ig level in TG and FLT mice. Sera isolated from TG (□), FLT (○) and C.B-17 (▲) mice were serially diluted with PBS. The amounts of Ig in these samples were measured by ELISA. Data are expressed as mean absorbance at 415 nm ± SD of more than three mice in each experimental group. x-axis: fold of dilution.

3.2 DTH reaction in TG and FLT mice

TG, FLT and C.B-17 mice were immunized subcutaneously with OVA. After 7 days, footpad swelling was measured 24 h after the challenge with the immunizing antigen (Table 1). The magnitude of the DTH reaction was comparable in TG, FLT and C.B-17 mice. In contrast, naive SCID mice did not show any detectable DTH reaction (data not shown). Thus, the DTH reaction detected in TG mice was mediated by the reconstituted T cells. This result indicated that the reconstituted T cells were immunologically competent and that the reconstitution was complete.

Table 1. DTH reaction to OVA in TG and FLT mice[a)]

Responders	Footpad swelling (mm)	
	PBS	OVA
TG	0.02 ± 0.01	0.55 ± 0.11
FLT	0.05 ± 0.02	0.58 ± 0.11
C.B-17	0.03 ± 0.01	0.55 ± 0.07
SCID	0.01 ± 0.01	0.02 ± 0.01

a) Data are expressed as the mean swelling of three mice ± SD.

3.3 T cell proliferation to immunizing antigen in TG and FLT mice

Seven days after the immunization with OVA, LN cells of TG and FLT mice were restimulated *in vitro* with the immunizing antigen or Con A (Table 2). LN cells of TG mice did not show a significant proliferation response to the immunizing antigen. However, these T cells were functionally mature and competent, since the Con A response of these cells was normal. On the other hand, LN cells of FLT mice proliferated well not only to Con A but also to OVA. Thus, the failure of the T cell response to OVA in TG mice cannot be attributed to the defective environment of SCID mice in which T cells differentiate.

Table 2 Proliferation response of T cells to OVA and Con A in TG and FLT mice[a)]

Responders		Stimulated with		
		(−)	OVA	Con A
TG	(1)[b)]	0.3 ± 0.1	0.2 ± 0.1	152.3 ± 8.9
TG	(2)	0.4 ± 0.1	0.4 ± 0.3	162.5 ± 25.7
FLT	(1)	0.6 ± 0.2	9.8 ± 0.2	65.7 ± 2.4
FLT	(2)	0.5 ± 0.2	14.6 ± 0.7	64.0 ± 3.0
C.B-17	(1)	4.4 ± 0.4	29.1 ± 5.7	32.0 ± 2.2
C.B-17	(2)	2.8 ± 0.3	36.9 ± 0.5	38.1 ± 4.1

a) Data are expressed as the means of triplicates (1×10^{-3} cpm) ± SD.
b) Experiment was performed in two independent mice of each group.

3.4 Restoration of T cell proliferation by antigen-primed B cells

We examined whether the unresponsiveness of TG mice to OVA was restored *in vitro* by the addition of B cells. B cells from naive C.B-17 mice could not restore T cell prolif-

Table 3. Proliferation response of T cells to OVA in the presence or absence of B cells in TG and FLT mice[a)]

Responders	B cells	Stimulated with (−)	OVA	Con A
TG	−	0.2 ± 0.1	0.2 ± 0.0	79.9 ± 32.3
	+	0.2 ± 0.0	3.1 ± 0.5	ND[b)]
FLT	−	0.3 ± 0.3	0.3 ± 0.1	87.1 ± 10.3
	+	0.4 ± 0.0	3.2 ± 0.2	ND[b)]

a) Data are expressed as the means of triplicates (1×10^{-3} cpm) ± SD.
b) Not determined.

Table 4. Restoration of T cell proliferation by B cells specifically primed with antigen[a)]

T cells prepared from[b)]	B cells added[c)] (−)	Nonimmunized	LPS-stimulated	SRBC-primed	OVA-primed
Nonimmunized	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
OVA-primed	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	2.0 ± 0.1
SRBC-primed	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.0

a) Experiment was performed in the presence of OVA. Data are expressed as mean of triplicate (1×10^{-3} cpm) ± SD.
b) T cells were prepared from naive (nonimmunized), OVA-primed and SRBC-primed C.B-17 mice.
c) B cells were prepared from naive (nonimmunized), SRBC-primed and OVA-primed C.B-17 mice. LPS-stimulated B cells were prepared from naive C.B-17 mice by culturing LN cells with 10 μg/ml LPS (*S. typhimurium* 14028) for 48 h.

eration (data not shown). Next, we used B cells prepared from C.B-17 mice immunized with OVA by depleting T cells with anti-Thy-1.2 plus complement. T cells of TG mice recovered their ability to proliferate to OVA when these B cells were present in the assay (Table 3). The proliferation was induced only when priming antigen was added, indicating that the proliferation was antigen-specific. This restoration was not attributable to contamination by T cells not efficiently eliminated from the B cell pool, since these cells did not respond to Con A or OVA (data not shown). These results show that antigen-activated B cells are necessary for the activation of T cells. This notion is further supported by the finding that T cells of FLT mice became unresponsive to immunizing antigen when B cells were removed from the LN cell population. This lack of response was again restored by the addition of antigen-primed B cells.

3.5 Importance of antigen-specific B cells for the restimulation of T cells

We examined which type of B cells can restore T cell proliferation. B cells were prepared from naive C.B-17 mice and mice primed with OVA or SRBC. B cells stimulated with LPS were also used. T cells were prepared by depleting B cells by panning. Naive B cells or B cells activated with LPS or primed with SRBC did not restore the proliferation of OVA-primed T cells. In contrast, B cells primed with OVA specifically restored the response of these T cells (Table 4). This result indicates that antigen-specific B cells play an important role in the restoration of T cell proliferation.

3.6 Antigen-specific B cells can induce the T cell response in the absence of professional APC

We showed above that B cells primed specifically with antigen were required for the secondary response of T cells.

Table 5. Restoration of T cell proliferation by B cells in the absence of professional APC[a)]

OVA	APC added[b)] (−)	B cells	Non-B APC
(−)	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
(+)	0.5 ± 0.0	5.1 ± 0.3	0.7 ± 0.2

a) Data are expressed as the means of triplicates (1×10^{-3}) ± SD.
b) T and B cells were prepared from LN cells of OVA-immunized mice by cell sorter. Non-B APC were prepared by adhesion to plastic plates.

However, it is not certain whether B cells themselves can induce the secondary response. To examine this, T and B cells were purified from OVA-primed C.B-17 mice by cell sorter using anti-Thy1.2 and anti-B220. The purity of the preparation was above 99 %. Non-B APC were collected by adhesion to plastic. Contamination of B cells in the preparation was less than 4 %. Purified B cells restored the response of T cells, but non-B APC could not (Table 5). However, the response induced by purified B cells was less than that of unpurified spleen cells. Thus, B cells are sufficient for the induction of the T cell response, although non-B cell APC are required for the maximum response.

3.7 Contribution of the CD28/B7 co-receptor system for the APC function of B cells

LN cells of TG and FLT mice were cultured with or without Con A for 24 or 48 h. IL-2 activity was measured in the supernatant cultured for 24 h. The activities of IL-6 and IFN-γ were assayed using the supernatant from a 48 h culture. Without Con A stimulation, these cytokines were not

Table 6. Production of cytokines in TG and FLT mice[a)]

Responders		Cytokines (U/ml)[b)] IL-2	IFN-γ	IL-6
TG	(1)[c)]	1.35	144	3.98
TG	(2)	1.38	158	3.23
FLT	(1)	1.47	170	5.23
FLT	(2)	1.44	151	4.24
C.B-17	(1)	1.51	162	4.17
C.B-17	(2)	1.44	166	4.04

a) Cytokine activities were measured in the supernatants of LN cells cultured with Con A as described.
b) The units of cytokines were calculated according to the standard curve.
c) Experiment was performed in two independent mice of each group.

detected in any of the samples (data not shown). LN cells of both TG and FLT mice produced IL-2, IL-6 and IFN-γ in response to Con A at comparable levels (Table 6). The expression of IL-4 and IFN-γ mRNA was also detected by RT-PCR in both TG and FLT mice (Fig. 3), showing that TG mice are not defective in their ability to produce these cytokines. Then, we examined the contribution of CD28/B7 co-receptor system in the activation of T cells by measuring proliferation and IFN-γ production. T and B cells were purified by cell sorting from OVA-immunized mice and co-cultured in the absence or presence of anti-CD80 or anti-CD86. IFN-γ production as well as proliferation was induced by OVA stimulation in the absence of professional APC. When anti-CD80 or anti-CD86 was present in the assay, both proliferation and IFN-γ production were clearly inhibited. Combined addition of anti-CD80 and anti-CD86 showed the cooperative effect (Fig. 4). Thus, CD28/B7 are involved in the APC function of B cells.

4 Discussion

Findings using mice treated with anti-mouse IgM (anti-μ mice) have indicated that B cells are crucial APC for the antigen-priming of T cells [7–11]. In this study, we used SCID mice grafted with the fetal thymus of C.B-17 mice (TG mice) to assess the APC function of B cells. TG mice were reconstituted by T cells without the development of B cells. These mice showed normal function of T cells as described [15]. TG mouse is a better model than anti-μ mouse to examine the APC function of B cells, since it

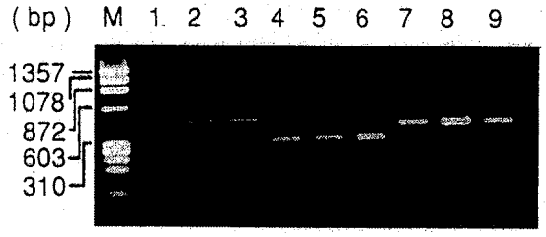


Figure 3. Detection of cytokine gene expression in TG and FLT mice. RNA was isolated from Con A-stimulated LN cells of TG (lane 1, 4, 7), FLT (lane 2, 5, 8) and C.B-17 (lane 3, 6, 9) mice. The expression of IL-2 (lane 1–3), IFN-γ (lane 4–6) and IL-4 (lane 7–9) was examined by RT-PCR. φX174 DNA digested with Hae III is shown as molecular weight marker.

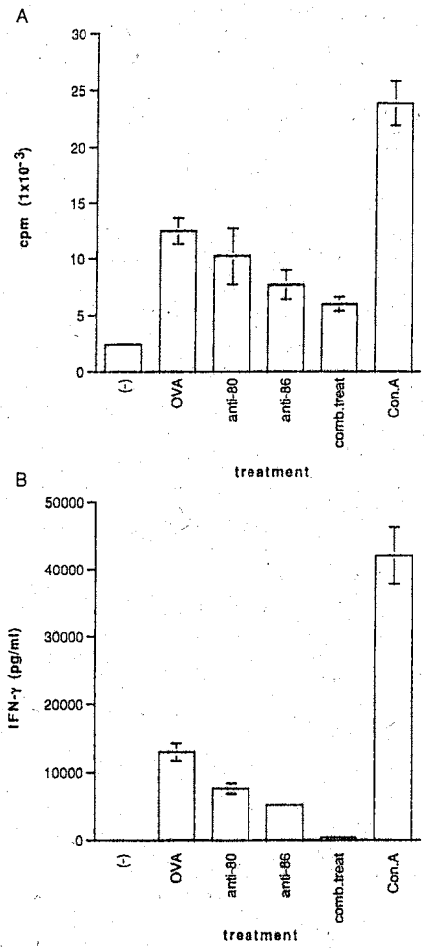


Figure 4. Inhibition of the APC function of B cells by anti-CD80 and anti-CD86. T and B cells were purified by cell sorter. T and B cells were co-cultured with anti-CD80, anti-CD86 (5 μg/ml) or the combination (comb. treat) in the presence of OVA. After culture for 48 h, the proliferation of T cells (A) and the production of IFN-γ in the supernatant (B) was measured. As controls, the results without stimulation (−) or stimulation with Con A are shown. Data are expressed as the means of triplicates ± SD.

does not require continuous treatment with anti-IgM, which may induce immunosuppression.

In TG mice, T cells of draining LN failed to proliferate in response to immunizing antigen, although normal Con A responses were readily inducible. As the Con A response is an APC-dependent phenomenon, unresponsiveness against immunizing antigen was not due to the absence of or lower numbers of APC in LN populations. DTH, which is mediated mainly by Th1 cells [17], was in the normal level and IL-4 mRNA, which is produced by Th2 cells, was detected. Thus, the unresponsiveness against immunizing antigen was not due to the aberrant development of Th1 or Th2 cells in TG mice. These findings are almost consistent with those obtained from anti-μ mice.

The response of TG mice to immunizing antigen was restored when T cells were co-cultured with antigen-primed B cells. This finding indicates that T cells of TG mice have already been primed *in vivo*. Recently, Constant et al. [12] reported that B cells were important for priming against protein antigen using B cell knockout mice, similar

to findings obtained with anti-μ mice. In contrast, other investigators have reported that B cells are required neither for the antigen priming nor for the induction of secondary responses against peptide antigen [13, 14]. A plausible explanation for this discrepancy is that B cells have an important role for the response to protein antigens that require processing, but not for peptide antigen as discussed by Constant et al. [12]. Otherwise, B cells would have a separate role for the type of T cell activation such as DTH reaction, IL-2 production, and proliferation. Furhter, the leakiness of B cells in SCID mice [18, 19] may support the priming of T cells, although they are not sufficient for the induction of seconary response. Due to the leakiness of SCID mice, we cannot state that B cells are not required for the priming. However, the leaky B cells in SCID mice are nonfunctional so antigen-activated B cells should not be induced in TG mice. Thus, we can at least say that antigen-specific B cells are not required for the priming of protein antigen.

The proliferation restored by B cells was less than that found in unpurified LN cells of normal C.B-17 mice or FLT mice. When B cells of FLT mice were depleted in the proliferation assay, T cell proliferation to the immunizing antigen was also disturbed. The proliferation of these T cells was not fully restored by re-addition of B cells. Since professional APC as well as B cells are eliminated during T cell preparation, we assume that cooperation of B cells and non-B APC is required for the maximum proliferation of T cells.

Malynn et al. [20] have reported that naive B cells cannot function as APC in the secondary response [20]. We assessed in our system which type of B cells could support for the recovery of T cells proliferation. Naive B cells or B cells activated with LPS or SRBC cells could not restore the proliferation of OVA-primed T cells, while OVA-primed B cells specifically did so. B cells reportedly present Ig-specific antigen 1000–10 000-fold more efficiently than other nonspecific antigens [21, 22]. We assume that the antigen-specific Ig receptor binds and concentrates the antigen in the secondary response. Further, we examined the importance of antigen-specific B cells using cell-sorter-purified T cells. The experiment indicated that professional APC such as dendritic cells or macrophages were not crucial for the restimulation of T cells. The lack of a requirement for non-B APC for T cell activation was confirmed by the production of IFN-γ. In contrast, antigen-specific B cells are critical for the secondary response. Further, when purified T cells were stimulated in the presence of anti-CD80 or anti-CD86, both the proliferation and IFN-γ production of T cells was suppressed. A CD28-mediated costimulatory signal has an important role for the activation of T cells. The counter receptor of both CD80 and CD86 is expressed selectively on activated B cells [23]. Thus, th finding supports the contribution of antigen-activated cells to the activation of T cells.

In conclusion, we show that antigen-specific B cells wer important as APC for the restimulation of T cells, but wer not essential for priming the T cells. Kupfer et al. [24] hav shown that the antigen-specific interaction of T and B cel is required for the proliferation of B cells [24]. This type c interaction would be important not only for B cells, bu also for T cell proliferation.

5 References

1 Unanue, E. R., *Annu. Rev. Immunol.* 1984. *2:* 395.
2 Steinman, R. M., Kaplan, G., Witmer, M. D. and Cohn, Z A., *J. Exp. Med.* 1979. *149:* 1.
3 Stingl, G., Katz, S. I., Clement, L., Green, I. and Shevach E. M., *J. Immunol.* 1978. *121:* 2005.
4 Rock, K. L., Benacerraf, B. and Abbas, A. K., *J. Exp. Mec* 1984. *160:* 1102.
5 Tony, H.-P. and Parker, D. C., *J. Exp. Med.* 1985. *161:* 223.
6 Ashwell, J. D., *J. Immunol.* 1988. *140:* 3697.
7 Hayglass, K. T., Naides, S. J., Scott, C. F., Jr., Benacerraf, E and Sy, M.-S., *J. Immunol.* 1986. *136:* 823.
8 Ron, Y., De Baetselier, P., Gordon, J., Feldman, M. an Segal, S., *Eur. J. Immunol.* 1981. *11:* 964.
9 Ron, Y., DeBastselier, P., Tzehoval, E., Gordon, J., Feldmar M. and Segel, S., *Eur. J. Immunol.* 1983. *13:* 167.
10 Ron, Y. and Sprent, J., *J. Immunol.* 1987. *138:* 2848.
11 Kurt-Jones, E. A., Liano, D., HayGlass, K. A., Beracerra B., Sy, M.-S. and Abbas, A. K., *J. Immunol.* 1988. *140:* 3773
12 Constant, S., Schweitzer, N., West, J., Ranney, P. and Bo tomly, K., *J. Immunol.* 1995. *155:* 3734.
13 Ronchese, F. and Hausmann, B., *J. Exp. Med.* 1993: *177:* 679
14 Epstein, M. M., Di Rosa, F., Jankovic, D., Sher, A. and Ma zinger, P., *J. Exp. Med.* 1995. *182:* 915.
15 Maeda, K., Nagasawa, H., Furukawa, A., Hisaeda, H., Mac kawa, Y., Manabe, T., Kudo, E., Good, R. A. and Himenc K., *Eur. J. Immunol.* 1993. *23:* 3151.
16 Montogomery, R. A. and Dallman, M. J., *J. Immunol.* 199 *147:* 554.
17 Cher, D. J. and Mosmann, T. R., *J. Immunol.* 1987. *137* 3688.
18 Bosma, G. C., Fried, M., Custer, R. P., Carroll, A., Gibsor D. M. and Bosma, M. J., *J. Exp. Med.* 1988. *167:* 1016.
19 Carroll, A. M., Hardy, R. R. and Bosma, M. J., *J. Immuno* 1989. *143:* 1087.
20 Malynn, B. A. and Wortis, H. H., *J. Immunol.* 1984. *132* 2253.
21 Rock, K. L., Benacerraf, B. and Abbas, A. K., *J. Exp. Mec* 1984. *160:* 1102.
22 Abbas, A. K., Haber, S. and Rock, K. L., *J. Immunol.* 1985 *135:* 1661.
23 Linsley, P. S., *J. Exp. Med.* 1995. *182:* 289.
24 Kupfer, H., Monks, C. R. F. and Kupfer, A., *J. Exp. Mec* 1994. *179:* 1509.

Growth Inhibitory Effects of Bovine Lactoferrin to *Toxoplasma gondii* Parasites in Murine Somatic Cells

Tetsuya TANAKA, Yoshitaka OMATA, Atsushi SAITO, Keiichi SHIMAZAKI[2], Ikuo IGARASHI[1], and Naoyoshi SUZUKI[1]

Department of Veterinary Physiology and [1]The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080 and [2]Department of Dairy Science, Faculty of Agriculture, Hokkaido University, Sapporo, 060, Japan

(Received 31 May 1995/Accepted 13 September 1995)

ABSTRACT. Lactoferrin (LF) is known to have broad spectrum antimicrobial properties. In regards to its defense mechanism against parasitic infection, it has shown phagocytic activity in the destruction of amastigotes, an intracellular parasitic form of *Trypanosoma cruzi* in macrophages. The effect of bovine lactoferrin on the intracellular growth *Toxoplasma gondii* parasites was examined in murine macrophage and embryonal cells. Co-cultures of host cells with the parasites were supplemented with either lactoferrin, apo-lactoferrin, holo-lactoferrin or transferrin in the culture media for varying periods. The growth activity of intracellular parasites in the host cells was determined by the measurement of selective incorporation of ^{3}H-uracil. Supplement of lactoferrin had no effect on the penetration activity of the parasites, while development of intracellular parasites was inhibited linearly in concentration of lactoferrin. Supplement of apo-lactoferrin and holo-lactoferrin, but not transferrin showed similar effects. These suggest that lactoferrin induces the inhibitory effects on the development of intracellular parasites. Pretreatment of lactoferrin to the macrophages, however, did not show any inhibitory effects. Whereas, mouse embryonal cells preincubated with lactoferrin suppressed the intracellular growth. Thus, the action of lactoferrin to macrophages would be different from that of mouse embryonal cells. — KEY WORDS: bovine-lactoferrin, host defense, *Toxoplasma gondii*.

J. Vet. Med. Sci. 58(1): 61–65, 1996

Lactoferrin (LF), a cationic, iron-binding protein which is produced and secreted by mammary glands and neutrophils, is known to have broad spectrum antimicrobial properties [2, 3, 6]. It has been shown to have activation properties of natural killer cytotoxicity in tumor cells [8, 12, 16] and induction of phagocytic activity and killing of amastigotes, an intracellular parasitic form of *Trypanosoma cruzi (T. cruzi)* in macrophages [13, 14, 21].

Toxoplasma gondii (T. gondii), an intracellular parasitic protozoa, can also penetrate in mammalian and avian somatic cells. In the host cells, the parasites of *T. gondii* are enclosed in parasitophorous vacuoles allowing them to evade the fusion of lysosomes, resulting in its growth even in macrophages [10]. Thus, the intracellular parasitism of *T. gondii* is different from that of *T. cruzi*. Macrophages possess oxygen-dependent and oxygen-indepentent anti-protozoan mechanisms and activation by interferon-γ (IFN-γ) enhance oxygen-dependent killing effect to the intracellular *T. gondii* due to synthesis of inorganic nitrogen oxide from L-arginine. While, human fibroblast activated by IFN-γ suppress the growth of the intracellular parasites due to starvation for tryptophan [18].

In the present study, we focused our interests to determine the possibility of LF inducing activation properties of macrophages and examined the effects of LF on the development of *T. gondii* parasites in mouse peritoneal macrophage and mouse embryonal cell.

MATERIALS AND METHODS

Bovine lactoferrin was prepared from bovine milk whey by the method of Law and Reiter [11].

Seven weeks old male and female ICR mice were used throughout the experiments.

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal cavity of mice inoculated with phosphate bufferd saline (PBS) containing 0.2% glycogen on the 5th day post inoculation (p.i.). They were centrifuged at 800 g for 10 min and were suspended in Dulbecco's modified essential medium (D-MEM) containing 10% fetal bovine serum (FBS). In the 24 wells tissue culture microplate (Cornig IWAKI Inc. Osaka, Japan), 10^5 cells/well of macrophages suspension was incubated at 37°C for 2 hrs, then were washed thoroughly to remove non-adherent cells, and were further incubated overnight at 37°C in D-MEM containing 10% FBS.

Mouse embryo cells (MEC) were prepared as described elsewhere [17].

The RH strain parasites of *T. gondii* were harvested under anestesia from the peritoneal cavity of mice on the 3rd day p.i. The parasites were washed by centrifugation at 1,200 g for 10 min in PBS 3 times, then were suspended in D-MEM containing 1% bovine serum albumin (D-MEM-BSA).

The growth activity of the parasites in the host cells were performed by the measurement of ^{3}H-uracil incorporation assay [7, 20]. Briefly, MPM and MEC monolayers were incubated with 1.0 m*l* of 10^5 parasites suspension at 37°C for 2 and 6 hrs, respectively. After removing free parasites by several washing in D-MEM, the monolayers were further incubated with either D-MEM-BSA alone, or supplemented with 100, 500 and 1,000 μg/m*l* of LF for 24 hrs. One m*l* of

D-MEM-BSA containing 0.146 MBp of ^{3}H-uracil was added to the culture and incubated at 37°C for last 6 hrs. After washing thoroughly in PBS to remove free ^{3}H-uracil, the monolayers were treated with 0.5 m*l* of 1% SDS for 10 min at room temperature and precipitated with 1 ml of 25% TCA at 4°C overnight. The precipitates were collected on glass filters, dried at room temperature, and counted for radioactivity in a liquid scintillation spectrometer. To determine the role of Fe^{2+} ion on the inhibitor effect of LF to the parasites, some of monolayers were supplemented with 1,000 μg/m*l* of apo-LF, holo-LF or Transferrin (TF).

To confirm the effect of LF, the development of intracellular parasites was examined by microscopic observation. Co-culture of MPM or MEC with the parasites on cover slips were supplemented with the same dose of LF in the culture media at 37°C for varying period of time. After incubation, the cover slips were washed several times with PBS, stained with Giemsa and monitored microscopically to determine the percentage of infected cells per 500 total cells in each group. All experiments were done in triplicate and repeated at least twice.

RESULTS

Lactoferrin did not produce any damage of the host cells, because there are no morphologic change of the incubated cells (data not shown). Parasites pretreated with LF sustained penetration activity of host cells and grew in them, indicating that LF had no parasiticidial effects to *T. gondii* directory (data not shown).

To determine the putative anti-toxoplasma effect of LF for MPM, ^{3}H-uracil incorporation was examined in infected MPM during 3 hrs intervals (Fig. 1). Higher incorporation of ^{3}H-uracil was shown between 0–3, 9–12, 15–18 and 21–24 hrs post incubation, guessing that the nucleic acids synthesis of the parasites would be approximately 6 hrs interval. Whereas, supplement with 1,000 μg/m*l* LF suppressed ^{3}H-uracil incorporation, especially between 21–24 hrs post incubation. The growth inhibitory effect was also evaluated at different concentrations of LF in the culture up to 30 hrs post incubation. As shown in Fig. 2, the growth inhibitory effect was shown in a dose dependent manner. At concentration of 1,000 μg/m*l*, ^{3}H-uracil incorporation of the parasites was 27.8% in comparison to untreated control. From this, we examined the effect of LF at concentration of 1,000 μg/m*l* in the following experiments. Mouse peritoneal macrophages incubated with LF for 24 hrs before inoculation with *T. gondii* did not show any inhibitory effects to intracellular parasites, compare *T. gondii* with untreated ones on the incorporation of ^{3}H-uracil (Fig. 3a). However, the number of infected cells in the LF treated group had shown lower percentage than that of the control (Table 1A). Probably, some infected MPM in the control might be removed to lose in the process of washing for ^{3}H-uracil incorporation assay. While, MPM incubated with LF for 24 hrs after inoculation with *T. gondii* LF treated group inhibited the growth of intracellular parasites as well that of the simultaneous incubation group (Fig. 3b and 3c). The number of infected cells in the LF treated group had tendency to show lower percentage than that of the control (Table 1B and 1C). Thus, MPM produced an inhibitory effect to the intracellular parasites in the presence of LF in the culture, and required the existence of LF in the culture to maintain the activity. This suggests one possibility that MPM incubated with LF may have suppressed the growth activity of the parasites, but not killed the parasites. Such inhibitory effect was also observed in the group of MPM treated with apo-LF or holo-LF. Oppositely, MEC preincubated with LF for 24 hrs showed

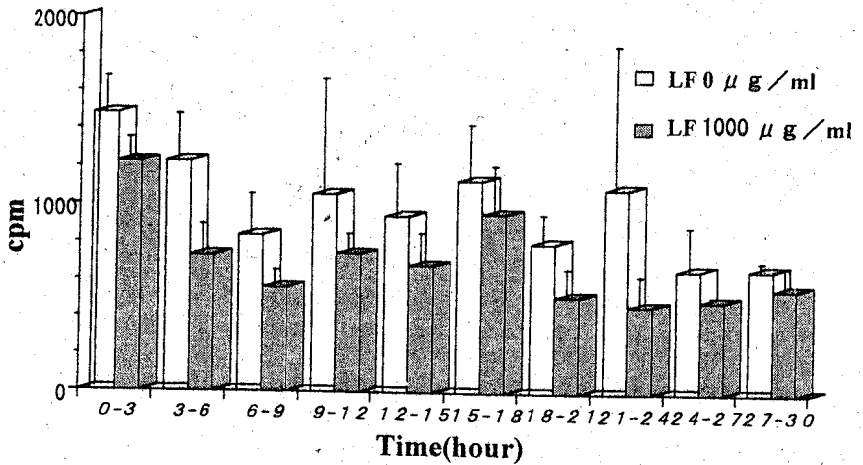


Fig. 1. Effect of lactoferrin to the growth of *T. gondii* parasites. Incorporation of ^{3}H-uracil in macrophages inoculated with *T. gondii* was examined at 3 hrs intervals for 30 hrs of incubation. Each value is the mean ± S. D. of triplicate measurement in a typical experiment (replicated twice). Control: cultured D-MEM-BSA alone.

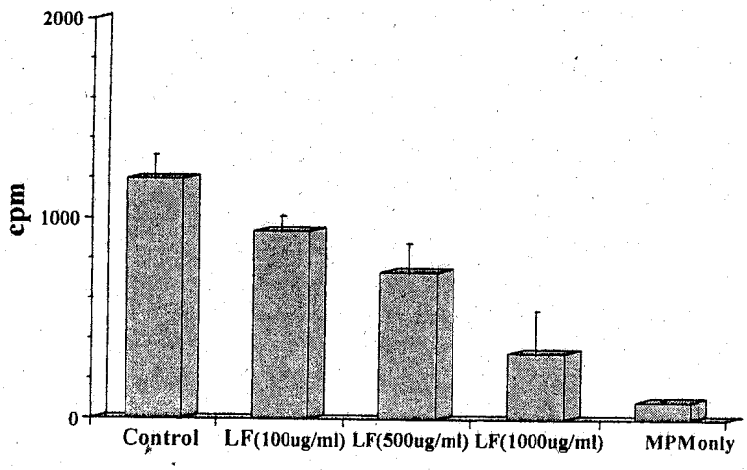


Fig. 2. Effect of various concentration of lactoferrin to the growth of intracellular parasites in macrophages. Incorporation of ^{3}H-uracil in macrophages inoculated with *T. gondii* were examined for last 6 hrs before harvesting. Each value is the mean ± S. D. of triplicate measurement in a typical experiment (replicated twice). Control: cultured D-MEM-BSA alone.

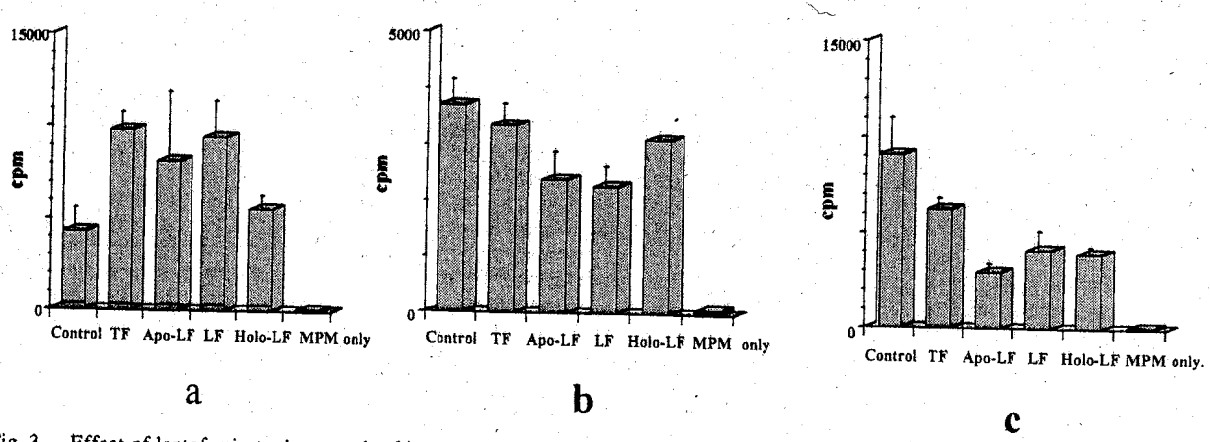


Fig. 3. Effect of lactoferrin to the growth of intracellular parasites in macrophages. a: The macrophages were incubated with 1,000 μg/m*l* lactoferrin for 24 hrs before *T. gondii* inoculation. b: The macrophages were incubated with 1,000 μg/m*l* lactoferrin simultaneously with inoculation of *T. gondii*. c: The macrophages were incubated with 1,000 μg/m*l* lactoferrin for 24 hrs after *T. gondii* inoculation. Each value is the mean ± S. D. of triplicate measurement in a typical experiment (replicated twice). Control: cultured D-MEM-BSA alone.

Table 1. Percent of infected macrophages post incubation for 24 hrs

	A				B				C		
Samples	0 Tp	1–5 Tp[a]	≧6 Tp[b]	Samples	0 Tp	1–5 Tp[a]	≧6 Tp[b]	Samples	0 Tp	1–5 Tp[a]	≧6 Tp[b]
Control	53.2[c]± 1.1[d]	32.7 ±1.3	14.1 ±2.4	Control	71.9[c]± 9.3[d]	3.9 ±2.5	24.3 ±11.8	Control	70.4[c]±8.8[d]	26.9 ±6.9	2.7±1.8
TF	48.4 ± 0.3	33.9 ±2.4	17.7 ±2.1	TF	70.7 ±15.3	0.9 ±0.1	28.5 ±15.3	TF	61.6 ±0.5	33.3 ±0.3	5.2±0.8
Apo-LF	59.0 ±11.0	32.9 ±6.6	8.1 ±4.4	Apo-LF	90.6 ± 0.8	2.5 ±1.9	7.0 ± 1.1	Apo-LF	84.2 ±2.5	15.0 ±2.8	0.8±0.3
LF	63.2 ±13.9	25.6 ±5.4	11.2 ±8.5	LF	95.3 ± 1.1	1.1 ±0.1	3.7 ± 1.2	LF	87.2 ±2.0	12.5 ±1.8	0.3±0.1
Holo-LF	62.5 ± 5.8	23.4 ±9.6	14.1 ±3.8	Holo-LF	93.4 ± 2.2	0.5 ±0.1	6.2 ± 2.1	Holo-LF	84.4 ±0.3	14.8 ±1.1	0.8±0.8

A: The macrophages were incubated with 1,000 μg/m*l* lactoferrin for 24 hrs before *T. gondii* inoculation. B: The macrophages were incubated with 1,000 μg/m*l* lactoferrin simultaneously with inoculation of *T. gondii*. C: The macrophages were incubated with 1,000 μg/m*l* lactoferrin after *T. gondii* inoculation. a) Cells found 1–5 parasites in the cytoplasm. b) Cells found more than 6 parasites in the cytoplasm. c) Percentage of infected cells were calculated in 500 total cells. d) Each value is the mean ± S. D. of triplicate measurement in a typical experiment that was replicated two times.

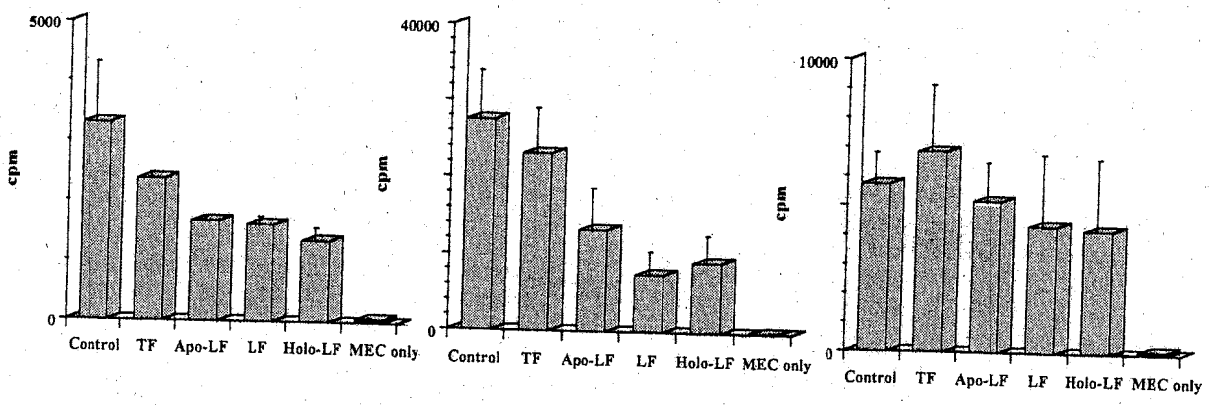


Fig. 4. Effect of lactoferrin to the growth of intracellular parasites in mouse embryonal cells. a: The mouse embryonal cells were incubated with 1,000 μg/m*l* lactoferrin for 24 hrs before *T. gondii* inoculation. b: The mouse embryonal cells were incubated with 1,000 μg/m*l* lactoferrin simultaneously with inoculation of *T. gondii*. c: The mouse embryonal cells were incubated with 1,000 μg/m*l* lactoferrin for 24 hrs after *T. gondii* inoculation. Each value is the mean ± S. D. of triplicate measurement in a typical experiment (replicated twice). Control: cultured D-MEM-BSA alone.

Table 2. Percentage of infected mouse embryonal cells post incubation for 24 hrs

	A				B				C		
Samples	0 Tp	1–5 Tp[a)]	≧6 Tp[b)]	Samples	0 Tp	1–5 Tp[a)]	≧6 Tp[b)]	Samples	0 Tp	1–5 Tp[a)]	≧6 Tp[b)]
Control	74.6[c)]± 8.8[d)]	2.3 ±0	23.1 ±0	Control	73.6[c)]± 4.2[d)]	9.3 ±1.6	17.6 ± 3.4	Control	32.4[c)]±2.4[d)]	23.6 ±7.8	44.2 ±5.4
TF	81.4 ± 3.6	1.5 ±0	17.1 ±3.5	TF	74.0 ± 0.7	5.3 ±3.3	20.7 ± 2.5	TF	53.2 ±4.2	22.1 ±5.5	24.8 ±1.3
Apo-LF	87.8 ± 4.2	2.2 ±0.7	10.1 ±3.5	Apo-LF	92.5 ± 1.6	2.2 ±0.1	5.3 ± 1.7	Apo-LF	54.0 ±11.1	18.1 ±6.6	28.0 ±4.5
LF	83.0 ± 5.4	2.6 ±1.3	14.5 ±4.2	LF	90.7 ± 1.8	4.9 ±1.2	4.4 ± 0.6	LF	50.1 ±8.4	13.0 ±1.5	37.0 ±6.9
Holo-LF	83.8 ± 5.9	2.2 ±1.1	14.1 ±4.7	Holo-LF	87.4 ± 1.8	3.3 ±0.2	9.4 ± 1.6	Holo-LF	55.6 ±13.2	20.3 ±1.3	20.4 ±11.8

A: The mouse embryonal cells were incubated with 1,000 μg/m*l* lactoferrin for 24 hrs before *T. gondii* inoculatio. B: The mouse embryonal cells were incubated with 1,000 μg/m*l* lactoferrin simultaneously with inoculation of *T. gondii*. C: The mouse embryonal cells were incubated with 1,000 μg/m*l* lactoferrin after *T. gondii* inoculation. a) Cells found 1–5 parasites in the cytoplasm. b) Cells found more than 6 parasites in the cytoplasm. c) Percentage of infected cells were calculated in 500 total cells. d) Each value is the mean ± S. D. of triplicate measurement in a typical experiment that was replicated two times.

the inhibitory effect as well that of simultaneous inoculation group (Fig. 4a and 4b, Table 2A and 2B). While, MEC incubated with LF after *T. gondii* inoculation did not induce any inhibitory effects (Fig. 4c, Table. 2C). Thus, one of non-phagocytic cells, MEC also showed the growth inhibitory effect to the intracellular parasites. This action seemed to be, however, different from that of MPM, because pretreatment of LF in MEC induced the growth inhibitory effect to the intracellular parasites.

DISCUSSION

In the present study, we have no data or other observations to explain such different actions of the inhibitory effect of activated host cells. In regard with killing mechanisms against *T. gondii*, as host defense, human mononuclear macrophages possess oxygen-dependent and oxygen-independent anti-protozoan mechanism [4, 9, 12, 19]. Primary response to intracellular *T. gondii* is mainly oxygen-dependent [15] and activation by lymphokines (interferron-γ) enhances the effect due to synthesis inorganic nitrogen oxide derived from l-arginine [1]. While, suppression of the growth of *T. gondii* in human fibroblast activated by IFN-γ act through starvation for tryptophan [18]. Thus, the inhibition of the growth of intracellular parasite is due to host cell defense system, and it may be also influenced by host cell condition. LF has binding capacity of divalent cations (Ca^{2+} and Mg^{2+}) to neutralized stabilize the negative charge of lipopolysaccharide [5]. It is plausible to explain that the LF acts on parasites and/or host cells' metabolic function, resulting in the suppression of intracellular parasite growth. Hence induction of such anti-toxoplasma activity is Fe^{2+}-ion independent, interaction of the Fe^{2+}-ion would be negligible.

The region of LF molecule responsible for activating properties to MPM and MEC infected with *T. gondii* is not so obvious. To clarify these function and metabolisms, further experiments are under way to examine the interaction of LF and cell metabolic functions.

REFERENCES

1. Adams, L. B., Hibbs, B. Jr., Tanitar, R. R., and Krahenbuhl, J.L. 1990. Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxides from L-Arginine. *J. Immunol.* 144: 2725–2729.
2. Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase,K., and Tomita,M. 1992. Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta* 1121: 130–136
3. Bullen, J. J., Rogers, H. J., and Leigh, L. 1972. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br. Med. J.* 1: 69–75
4. Carvalho, L. and Souza W.1989. Cytochemical localization of plasma membrane enzyme markers during interiorization of tachyzoites of *Toxoplasma gondii* by macrophages. *J. Parasitol.* 36: 164–170.
5. Ellison, R. T., LaForce F. M., Giehl, T. J., Boose, D. S., and Dunn, B. E. 1990. Lactoferrin and transferrin damage of the Gram-negative outer membrane is modulated by Ca^{2+} and Mg^{2+}. *J. Gen. Microbial.* 136: 1437–1446.
6. Ellison, R. T., Giehl T. J., and LaForce, F. M. 1988. Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infect. Immun.* 56: 2774–2781.
7. Harris, C., Salgo, M. P., Tanowitz, H. B., and Wittner, M. 1988. *In vitro* assessment of antimicrobial agents against *Toxoplasma gondii*. *J. Infectious Dis.* 157: 14–22.
8. Horwitz, D. A., Bakke, C. A., Abo, W., and Nishiya, K. 1984. Monocyte and NK cell cytotoxic activity in human adherent cell preparations: Discriminating effects of interferon and lactoferrin. *J. Immunol.* 132: 2370–2374.
9. Hughes, H. P. A., Boik, R. J., Gerhardt, S. A., and Speer, C. A.1989. Susceptibility of Eimeria bovis and *Toxoplasma gondii* to oxygen intermediates and a new mathematical model for parasite killing. *J. Parasitol.* 75: 489–497.
10. Jones, T. C. and Hirsch, J. G. 1972. The interaction between *Toxoplasma gondii* and mammalian cells. 2. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J. Exp. Med.* 136: 1173–1194.
11. Law, B. A. and Reiter, B. 1977. The isolation and bacteriostatic properties of lactoferrin from bovine milk whey. *J. Dairy Res.* 44: 595–599.
12. Liew, F. Y., Millott, S., Parkison, C., Palmer, R. M., and Moncada, S. 1990. Macrophage killing of Leishmania parasite *in vivo* is mediated by nitric oxide from L-arginine. *J. Immunol.* 144: 4794–4797.
13. Lima, M. F. and Kierzenbaum, F.1987. Lactoferrin effects on phagocytic cell function. II. The presence of iron is required for the lactoferrin moleccule to stimulate intracellular killing by macrophages but not to enhance the uptake of particles and microorganisms. *J. Immunol.* 139: 1647–1651.
14. Lima, M. F. and Kierszenbaum, F. 1985. Lactoferrin effects on phagocytic cell function. I. Increased uptake and killing of an intracellular parasite by murine macrophages and human monocytes. *J. Immunol.* 134: 4176–4183.
15. Murray, H. W., Rubin, B. Y., Carriero, S. M, Harris, A. M., and Jaffee, E. A. 1985. Human mononuclear phagocyte antiprotozoal mechanisms: Oxygen-dependent vs oxygen-independent activity against intracellular *Toxoplasma gondii*. *J. Immunol.* 134: 1982–1988.
16. Nishiya, K. and Horwitz, D. A. 1982. Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. *J. Immunol.* 129: 2519–2523.
17. Omata, Y., Igarashi, M., Ramos, M. I., and Nakabayashi, T. 1989. *Toxoplasma gondii*: Antigenic differneces between endozoites and cystozoites defined by monoclonal antibodies. *Parasitol. Res.* 75: 189–193.
18. Pfefferkon, E. R. 1984. Interferon γ blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc. Natl. Acad. Sci. U.S.A.* 81: 908–912.
19. Saito, A., Igarashi, I., Miyahara, K., Venturini, C., Claveria, F. G., Hirose, T., Suzuki, N., and Ono, K. 1992. Effects of reactive oxygen intermediate scavengers on the antitoxoplasmic activity of activated macrophages. *Parasitol. Res.* 78: 28–31.
20. Schwartman, J. D. and Pfefferkorn, E. R. 1981. Pyrimidine synthesis by intracellular *Toxoplasma gondii*. *J. Parasitol.* 67: 150–158.
21. Villalta F. and Kierszenbaum, F. 1984. Role of inflammatory cells in Chagas' diease. II. Interactions of mouse macro phages and human monocytes with intracellular forms of *Trypanosoma cruzi*; Uptake and mechanism in destruction. *J. Immunol.* 133: 3338–3343.

Microbiol. Immunol., 40(9), 659-664, 1996

Development of Functional Rat-Derived T Cells in SCID Mice Engrafted with the Fetal Thymus of LEC Rats Which Are Defective in CD4$^+$ T Cells

Jian-Guo Chai[1], Tohru Sakai[1], Hajime Hisaeda[1], Hideyuki Nagasawa[1], Koji Yasutomo[1], Atuko Furukawa[1], Hiroyuki Ishikawa[1], Yoichi Maekawa[1], Hisanori Uehara[2], Keisuke Izumi[2], Kozo Matsumoto[3], and Kunisuke Himeno*[,1]

[1]*Department of Parasitology and Immunology,* [2]*Second Department of Pathology, and* [3]*Institute for Animal Experimentation, University of Tokushima School of Medicine, Tokushima, Tokushima 770, Japan*

Received April 18, 1996. Accepted June 17, 1996

Abstract: **We reported that LEC rats are genetically deficient in the development of thymic CD4$^+$8$^-$ cells and that this defect is caused by bone marrow (BM)-derived stem cells. To determine which BM-derived cells are responsible for the arrest of T-cell development in LEC rats, fetal thymuses of LEC rats, or LEA rats which bear the same major histocompatibility complex (MHC) as LEC rats but are immunologically normal, were engrafted under the kidney capsule of severe combined immunodeficiency (SCID) mice (LEC-TG and LEA-TG mice, respectively). We then examined the differentiation of T cells and their immunological functions in the SCID mice. A large number of rat-derived CD4$^+$ T cells appeared in the peripheral blood, lymph nodes (LN) and spleens in LEC-TG mice. Furthermore, the peripheral LN cells in LEC-TG mice appeared to be functional. These cells produced IL-2 upon Con A stimulation, whereas LN cells from LEC rats produced no IL-2 in the same conditions. Thymopoiesis was observed at 3 weeks in LEC-TG as well as LEA-TG mice. The distribution of thymocyte subsets with respect to CD4 and CD8 expression in LEC-TG mice closely resembled that of LEA rat thymus and that in LEA-TG mice, suggesting that normal T-cell differentiation occurred in LEC-TG mice. The results indicated that BM-derived progenitor T cells of LEC rats could differentiate to functional CD4$^+$ T cells.**

Key words: **LEC rats, CD4$^+$8$^-$ T cells, Thymus graft, Rat-mouse chimeras**

After homing to the thymus, pre-T cells undergo complicated selection processes including positive (4, 17, 18) and negative selection (16, 19, 22), then they differentiate into functional T cells (20). This selection is supposed to occur at the stage of transition from CD4$^+$8$^+$ to CD4$^+$8$^-$ or CD4$^-$8$^+$ cells. Interactions between thymocytes and thymic stromal cells are critical for the differentiation process because mice with disrupted CD4, CD8, major histocompatibility complex (MHC) class I or class II molecules show maturational arrest from immature to mature T cells. Consequently, some T-cell functions of those mice become defective (7, 11, 12, 21, 24, 25, 31).

LEC rats exhibit maturational arrest from CD4$^+$8$^+$ to CD4$^+$8$^-$ cells in the thymus (1, 2) and this defect is caused by a single recessive gene designated as *thid* (T helper immunodeficiency) (30). Despite the blockade of CD4$^+$8$^-$ thymocyte differentiation, CD4$^+$ T cells appear in peripheral lymphoid organs. However, they are dysfunctional since they are defective in IL-2 production and helper T function in terms of specific antibody production against T cell-dependent antigens (1, 26). This dysfunction appears to be caused by an abnormal maturational pathway. We showed by means of fetal thymus or bone marrow transplantation that bone marrow (BM)-derived cells are responsible for the maturational arrest of CD4$^+$8$^-$ cells (3, 27). However, even with those systems, we could not identify the cell population that carries the *thid* mutation because BM-derived cells contain progenitors of T cells, macrophages, and thymic dendritic cells, all of which contribute to T-cell differentiation. Thus, it is essential to define which BM-derived cells are abnormal and cause maturational arrest

**Address correspondence to* Dr. Kunisuke Himeno, Department of Parasitology and Immunology, University of Tokushima School of Medicine, Kuramoto 3, Tokushima, Tokushima 770, Japan.

Abbreviations: BM, bone marrow; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LN, lymph node; MHC, major histocompatibility complex; PE, phycoerythrin; SCID, severe combined immunodeficiency; TdR, thymidine deoxyribose; TG, thymus graft.

from $CD4^+8^+$ to $CD4^+8^-$ T cells in the LEC rat thymus. To address the above question, we transplanted the thymus from fetal LEC rats into the severe combined immunodeficiency (SCID) mouse and analyzed the differentiation of $CD4^+8^-$ T cells in the xenogenic chimeras.

Materials and Methods

Animals. C.B-17 SCID mice at 6 weeks of age were obtained from Japan CLEA, Inc. (Tokyo). LEC/Tj ($RT1^u$) and LEA/Tj ($RT1^u$) rats were maintained in the Institute for Animal Experimentation, University of Tokushima (Tokushima, Japan). All animals were bred under specific pathogen-free conditions.

Transplantation of rat fetal thymus. Thymuses were transplanted according to the method of Maeda et al (23). Briefly, fetal thymuses were dissected from 16-day-old LEA and LEC rat embryos and inserted under the kidney capsules of SCID mice using a 21-gauge needle (one lobe/recipient). SCID mice transplanted with LEC or LEA rat thymus were designated LEC-TG or LEA-TG mice, respectively.

Antibodies and flow cytometry analysis. Anti-rat CD4 mAb (W3/25) (6) and anti-rat CD8 mAb (OX8) (29) hybridoma cell lines were gifts from Dr. M. Miyasaka (Osaka University, Osaka, Japan). Antibodies were labeled with fluorescein isothiocyanate (FITC) or biotin by standard procedures. Thymocytes, lymph node (LN) cells and peripheral blood mononuclear cells from thymus graft (TG) mice or rats were stained with a combination of FITC-conjugated and biotin-conjugated mAbs plus phycoerythrin (PE)-conjugated streptavidin for two-color flow cytometry. Stained cells were analyzed using a FACScan and the Consort 30 software program (Becton Dickinson, Mountain View, Calif.,U.S.A.) after gating out dead cells using forward and side light scattering.

IL-2 bioassay. IL-2 activity was assayed by using the IL-2-dependent cell line, CTLL-2. LN cells were cultured with 2.5 μg/ml Con A for 24 hr. The culture medium was RPMI 1640 containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 U/ml streptomycin. The supernatants were collected, diluted serially with fresh culture medium and mixed with growing CTLL-2 cells (5×10^3 cells/well). The proliferative response of CTLL-2 cells was determined by measuring the [^{3}H]thymidine deoxyribose (TdR) incorporation by direct β counting (Packard Instrument B. V. Chemical Operations, The Netherlands).

Results

Appearance of Rat-Derived T Cells in TG Mice

As reported (1-3, 27), the LEC rat has defective development of $CD4^+8^-$ thymocytes and a consequently reduced proportion of $CD4^+$ T cells in the peripheral blood (Table 1). The appearance of rat-derived $CD4^+$ and $CD8^+$ T cells in peripheral blood was examined by staining peripheral blood mononuclear cells with a combination of anti-rat CD4 and anti-rat CD8 mAb at various periods after grafting. In LEC-TG mice, rat-derived $CD4^+$ and $CD8^+$ T cells were detectable at 4 weeks after grafting. After 6 to 8 weeks, the percentages of $CD4^+$ and $CD8^+$ T cells increased and the CD4/CD8 ratio was within the normal range. In LEA-TG mice, the appearance of rat-derived $CD4^+$ and $CD8^+$ T cells in the blood was earlier than that in the LEC-TG mice. $CD4^+$ and $CD8^+$ T cells were detected after 3 weeks in LEA-TG mice; the ratio increased at 4 weeks and reached a maximum at 6 weeks after grafting the thymus (Table 1). Furthermore, we found that these rat-derived T cells expressed a normal density of TCRαβ and CD3 molecules, suggesting that they are mature type T cells (data not shown).

The LN in SCID mice contains only a few cells (1-2×10^5 cells/mouse). The LN of LEC-TG mice showed only minimal enlargement and the total cell number was quite low ($<5\times10^5$ cells/mouse) at 4 to 6 weeks after grafting. By 10 weeks, however, LN of LEC-TG mice enlarged moderately and the cell number also increased ($>2\times10^6$ cells/mouse). In LEC-TG mice, the number of $CD4^+$ T cells was significant in LN and the proportion of $CD4^+$T cells was markedly different

Table 1. Expression of rat CD4 and CD8 molecules on peripheral blood lymphocytes in TG mice

		$CD4^+$ T cells (%)	$CD8^+$ T cells (%)
LEC-TG mice	4 weeks[a]	9.3[b]	4.2
	6 weeks[a]	25.0	7.4
	8 weeks[a]	27.5	14.1
LEA-TG mice	4 weeks[a]	36.5	8.7
	6 weeks[a]	39.0	20.8
LEC rats	6 weeks[c]	3.4	3.3
	8 weeks[c]	4.4	3.4
LEA rats	6 weeks[c]	10.2	4.8
	8 weeks[c]	20.4	10.2

[a] Weeks after grafting with rat fetal thymus.
[b] Values represent the means of 5 to 10 mice or rats and the SD are below 15% of the mean values.
[c] Weeks after birth.

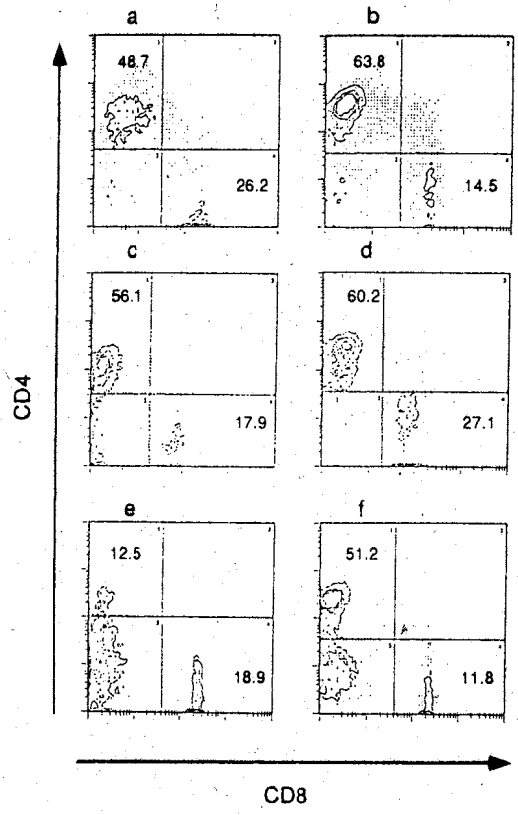


Fig. 1. Expression of CD4 and CD8 on LN cells from LEC-TG and LEA-TG mice. LN cells from a and c , LEC-TG mice (8 and 12 weeks after thymus grafting, respectively); b and d, LEA-TG mice (4 and 8 weeks after grafting, respectively); e and f, LEC and LEA rats (10 weeks after birth) were stained with anti-CD4 and anti-CD8 mAb as described in "Materials and Methods."

Table 2. Expression of rat CD4 and CD8 molecules on LN cells in TG mice

		$CD4^+$ T cells (%)	$CD8^+$ T cells (%)
LEC-TG	10 weeks[a]	27.2[b]	6.1
	12 weeks[a]	52.0	12.5
LEA-TG	5 weeks[a]	71.1	10.0
	8 weeks[a]	49.7	21.9
LEC	12 weeks[c]	12.5	18.8
LEA	12 weeks[c]	44.0	12.5

[a] Weeks after grafting with rat fetal thymus.
[b] Values represent the means of 3 mice or rats and the SD are below 15% of the mean values.
[c] Weeks after birth.

from that of LEC rats, which only contained a few $CD4^+$ T cells (Fig. 1 and Table 2). Similar results were obtained in the spleen of LEC-TG mice (data not shown). The proportion of peripheral $CD4^+$ and $CD8^+$ T cells in LEA- or LEC-TG mice was higher than that in LEA or LEC rats, respectively, but this seems to indicate that a compensatory increase in the proportion of T cells because only T cells, and not B cells, can be reconstituted in TG mice (23).

Analysis of Thymopoiesis in TG Mice

We showed that LEC rats exhibit a congenital maturational arrest from $CD4^+CD8^+$ to $CD4^+CD8^-$ cells in the thymus. To investigate whether LEC rat fetal thymuses undergo normal thymopoiesis in the xenogeneic environment of SCID mice, we assessed the kinetics of thymopoiesis in the grafted thymus by means of two-color flow cytometry. As shown in Fig. 2, in LEC-TG mice, the grafted thymus enlarged at 2 weeks after grafting, and most cells in the graft were $CD4^+8^+$, whereas $CD4^+8^-$ and $CD4^-8^+$ cells were rare. By 3 weeks, the grafted thymus enlarged obviously and reached a peak of cellularity. The proportion of $CD4^+8^+$ cells decreased but that of $CD4^+8^-$ and $CD4^-8^+$ cells increased. The distribution of these subsets was comparable to that in the LEA rat. It is unlikely that the $CD4^+$ cells detected in the grafted thymus at this stage migrated from the peripheral blood, since rat-derived $CD4^+$ T cells in the blood of the LEC-TG mice were very rare ($<2\%$) at an early period of reconstitution. Thus, we supposed that these $CD4^+$ T cells differentiated from $CD4^+8^+$ cells and emigrated from the grafted thymus. In contrast to the thymopoiesis in LEC-TG mice, the differentiation from $CD4^+8^+$ cells to $CD4^+8^-$ cells was blocked completely in the LEC rat thymus, and the percentage of $CD4^+8^-$ cells remained extremely low. Thymopoiesis in the grafted thymus of LEC-TG mice decreased abruptly at 4 weeks after grafting. At this time, the grafted thymus became very small and contained only a few cells ($<1\times10^5$ cells/mouse). The proportion of $CD4^+8^+$ thymocytes reduced rapidly and these cells became undetectable 4 to 5 weeks after grafting. A similar thymopoiesis profile was also seen in LEA-TG mice, which were grafted with LEA rat fetal thymus (data not shown).

Function of Rat-Derived T Cells in TG Mice

The above data show that LEC-TG mice exhibited normal thymic maturation and distribution of CD4/CD8 T cells in the periphery, although the maturation of $CD4^+8^-$ cells in the thymus was blocked in LEC rats. Next, we examined whether the T cells in LEC-TG mice can produce IL-2, since LEC rat T cells cannot produce IL-2 in response to Con A stimulation. As reported (26), LN cells from LEC rats proliferated well (data not shown) but failed to secrete IL-2 upon Con A stimulation (Fig. 3). In contrast to the LN cells of LEC rats, those of LEC-TG mice produced IL-2, the level of which was higher than that of LEA rats, suggesting that LN cells in LEC-TG mice are functionally normal. The

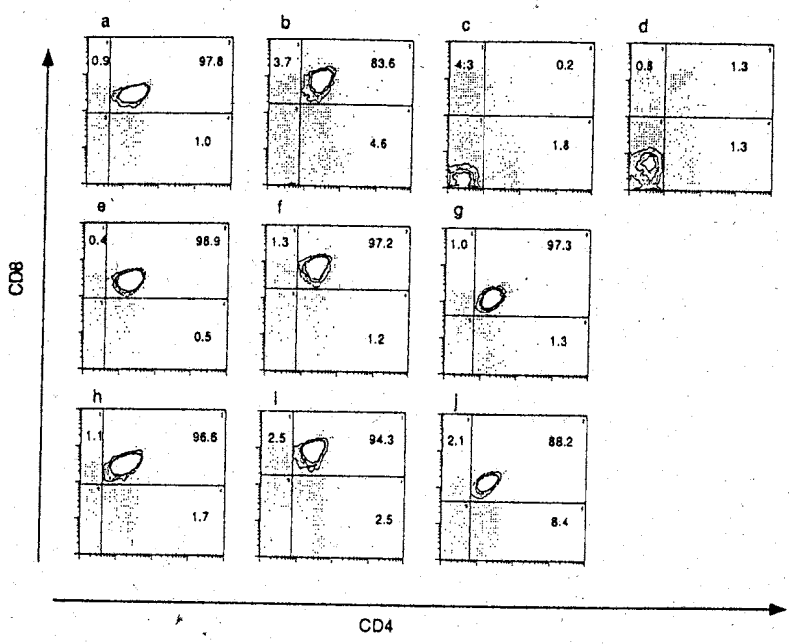


Fig. 2. Expression of CD4 and CD8 on grafted thymus from LEC-TG and LEA-TG mice. Thymocytes from a, b and c, LEC-TG mice (2, 3 and 4 weeks after thymus grafting, respectively); d, LEA-TG mice (4 weeks after grafting); e, f and g, LEC rats (2, 3 and 4 weeks after birth, respectively); h, i and j, LEA rats (2, 3 and 4 weeks after birth, respectively) were stained with anti-CD4 and anti-CD8 mAb as described in "Materials and Methods."

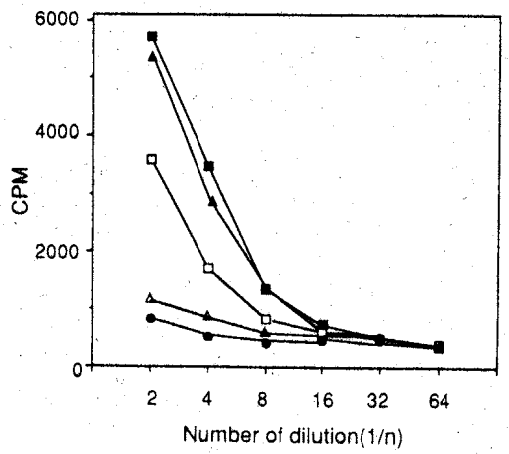


Fig. 3. IL-2 production in LN cells form LEC-TG and LEA-TG mice. LN cells at 8 and 10 weeks after grafting with thymus. LN cells from LEA and LEC-TG mice were cultured with Con A (2.5 μg/ml) for 24 hr and IL-2 activity in the culture supernatant was estimated using the IL-2-dependent cell line CTLL-2. LEC-TG mice (closed triangles); LEA-TG mice (closed squares); LEC rats (open triangles); LEA rats (open squares), SCID mice (closed circles).

IL-2 activity observed in supernatants from LEC-TG mice was of rat origin, since it was completely blocked by anti-rat IL-2 mAb and since the CTLL-2 cell line used in this experiment showed weak responsiveness to IL-4 (data not shown). Furthermore, age-matched SCID mice failed to produce any detectable IL-2 under the same conditions. LN cells from LEA-TG mice also produced IL-2 normally upon Con A stimulation (Fig. 3).

Discussion

LEC rats show a novel maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ cells in the thymus, which is caused by the *thid* mutation (1, 30). We reported that thymic stromal cells of LEC rats can nurse nude rat BM-derived progenitor T cells. Furthermore, when the normal rat fetal thymus was transplanted under the LEC rat kidney subcapsule, the maturation from $CD4^+8^+$ to $CD4^+8^-$ cells was arrested in the grafted thymus (3). These results indicated that BM-derived, but not thymic epithelial cells carry the *thid* mutation in LEC rats. However, the cell population responsible for the *thid* mutation was not identified since BM-derived cells in the thymus include progenitor T cells, macrophages, and dendritic cells. One of the most important findings in the present study is that rat-derived $CD4^+$ T cells developed normally in LEC-TG mice. In LEC-TG mice, the grafted thymus became visibly enlarged after 3 weeks and showed clear signs of thymopoiesis (Fig. 2). The distribution of thymocyte subsets in the LEC-TG mice closely resembled that in the LEA rat thymus with respect to the expression of CD4 and CD8. Following thymopoiesis, a large number of rat-derived $CD4^+$ and $CD8^+$ T cells appeared in the peripheral blood and LN (Tables 1, 2, and Fig. 1). This fact indicated that BM-derived progenitor T cells of LEC rats could differenti-

ate into mature $CD4^+8^-$ cells and were not influenced by the *thid* mutation.

In LEC-TG mice, rat T cells from grafted rat thymuses might have matured in host SCID mouse thymuses and exported to peripheral lymphoid organs. However, this is not likely because rat-derived $CD4^+8^+$ cells were not found and only mature cells ($CD4^+8^-$ and $CD4^-8^+$ thymocytes) were identified in the host thymus. $CD4^+8^-$ and $CD4^-8^+$ cells in the SCID mouse thymus should re-enter from the peripheral circulation (13). These results suggest that the host SCID mouse thymus does not participate in the differentiation process of rat T cells. This notion was also supported by an experiment using athymic SCID mice, since rat-derived T cells also developed in athymic SCID mice grafted with the rat fetal thymus (data not shown).

We did not determine whether there were stromal cells derived from mouse BM in the grafted rat thymus. When athymic nude mice are used as recipients instead of SCID mice, the generated T cells are exclusively of mouse origin (15), suggesting that mouse BM-derived cells migrated to the rat thymus continually in that system. Some groups have reported that rat BM and fetal liver cells can differentiate into mature T cells in a xenogenic microenvironment (14, 28). From these findings, it appears that thymic stromal cells derived from SCID mice can interact with xenogenic rat pre-T cells efficiently and support rat T-cell differentiation in LEC-TG mice. Thus, mouse BM-derived stromal cells compensate for the function of rat stromal cells. The absence of T and B cells in SCID mice has been established in cellular and functional properties (5, 8) while other types of bone marrow-derived cell populations are supposed to be normal (9, 10), supporting again that BM-derived stroma compensate for the *thid* mutation. However, we cannot exclude the possibility that some other factors allow the differentiation of the arrested $CD4^+$ T cells under the rat-mouse xenogenic environment.

Expansion and differentiation of T cells in grafted fetal thymus decreased or terminated around one month after transplantation. Progenitor cells in the thymus have limited capacity for self renewal. The reconstitution in secondary lymphoid organs reached a maximum within three months after transplantation. Reconstitution subsequently declined in TG mice. The limited period of reconstitution in TG mice may be due to autoreactivity, otherwise the mouse environment may not be sufficient to make continuing expansion of the rat T-cell populations possible.

As shown in Fig. 1, Table 1 and Table 2, both LEA and LEC rat-derived T cells appeared in the periphery in SCID mice. However, the timing was different between LEA- and LEC-TG mice. The total number of thymocytes in LEC rats was lower than that in LEA rats aft day 19 of gestation and this tendency continued until tl adult stage (2). A potent low supply of T cells from tl LEC rat thymus would result in the delayed reconstit tion of T cells in LEC-TG mice. Alternatively, it possible that the *thid* mutation affects the differentiatic of $CD4^+8^-$ thymocytes even in LEC-TG mice.

The second important finding is that rat-derived CD4 T cells in LEC-TG mice are functional. LEC rats cann produce specific antibody against T cell-dependent ant gens (1) and, furthermore, LN cells from these rats do n secrete IL-2 or IL-4 upon lectin stimulation (1, 26 These facts show that LEC rats are defective in helper cell function. Although LN cells from LEC rats coul not produce any detectable IL-2, those from LEC-T mice secreted a significant amount of IL-2 when stimu lated with Con A and the levels were comparable t those of LEA-TG mice (Fig. 3). The levels of IL- production from LN cells in both LEA- and LEC-T mice were higher than that of LEA rat LN cells. We cai not explain this finding completely. However, it is po: sible that the high IL-2 production observed in TG mic resulted from the appearance of autoreactive T cells i the xenogenic environment since some T cells expresse an activation maker, IL-2 receptor, in both TG mic (data not shown). These results indicated that the prc genitor cells of LEC rat $CD4^+$ T cells are not geneticall defective in the signal transduction component necessar for IL-2 production and that the IL-2 deficiency in LE rats is caused by a defect in thymic education for CD4 T cells.

In conclusion, we found that the complete reconst tution of LEC rat-derived $CD4^+$ and $CD8^+$ T cells wa achieved in SCID mice grafted with a thymus from fet LEC rats. T cells in LEC-TG mice produced IL-2 upo Con A stimulation, suggesting that these T cells ar functionally immunocompetent. These results indicate that BM-derived progenitor T cells of LEC rats could di ferentiate to $CD4^+8^-$ thymocytes irrespective of th *thid* mutation.

This work was supported by research grants from the Ministr of Education, Science and Culture, Japan.

References

1) Agui, T., Oka, M., Yamada, T., Sakai, T., Izumi, K., Ishid Y., Himeno, K., and Matsumoto, K. 1990. Maturation arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in a mutai strain (LEC) of rat. J. Exp. Med. **172**: 1615–1624.
2) Agui, T., Sakai, T., and Matsumoto, K. 1991. Ontogeny of cell maturation in LEC mutant rats which bear a congenit arrest of maturation from $CD4^+CD8^+$ to $CD4^+CD8^-$ thy mocytes. Eur. J. Immunol. **21**: 2537–2541.

3) Agui, T., Sakai, T., Himeno, K., and Matsumoto, K. 1991. Bone marrow-derived progenitor T cells convey the origin of maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in LEC mutant rats. Eur. J. Immunol. **21**: 2277–2280.
4) Bill, J., and Palmer, E. 1989. Positive selection of $CD4^+$ T cells mediated by MHC class II bearing stromal cell in the thymic cortex. Nature **341**: 649–651.
5) Bosma, G.C., Custer, R.P., and Bosma, M.J. 1983. A severe combined immunodeficiency mutation in the mouse. Nature **301**: 527–530.
6) Brideau, R.J., Carter, P.B., McMaster, W.R., Mason, D.W., and Williams, A.F. 1980. Two-subsets of rat T lymphocytes defined with monoclonal antibodies. Eur. J. Immunol. **10**: 609–615.
7) Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C., and Mathis, D. 1991. Mice lacking MHC class II molecules. Cell **66**: 1051–1066.
8) Custer, R.P., Bosma, G.C., and Bosma, M.J. 1985. Severe combined immunodeficiency (SCID) in the mouse: pathology, reconstitution, neoplasms. Am. J. Pathol. **120**: 467–477.
9) Czitrom, A.A., Edwards, S., Phillips, R.A., Bosma, M.J., Marrack, P., and Kappler, J.W. 1985. The function of antigen-presenting cells in mice with severe combined immunodeficiency. J. Immunol. **134**: 2276–2280.
10) Dorshkind, K., Keller, G.M., Phillips, R.A., Miller, R.G., Bosma, G.C., O'Toole, M., and Bosma, M.J. 1984. Functional status of cells from lymphoid and myeloid tissues in mice with severe combined immunodeficiency disease. J. Immunol. **132**: 1804–1808.
11) Fung-Leung, W.P., Schilham, M.W., Rahemtulla, A., Kundig, T.M., Vollenweidere, M., Potter, J., Van Ewijk, W., and Mak, T.M. 1991. CD8 is needed for development of cytotoxic T cells but not helper T cells. Cell **65**: 443–449.
12) Grusby, M.J., Auchincloss, R., Lee, R., Johnson, R., Spencer, J., Zijlstra, M., Jaenisch, R., Papioannou, V., and Glimcher, L. 1993. Intrathymic maturation of murine T lymphocytes from $CD8^+$ precursors. Proc. Natl. Acad. Sci. U.S.A. **86**: 7542–7546.
13) Hilbert, D.M., Holmes, K.L., Anderson, A.O., and Rudikoff, S. 1993. Long-term thymic reconstitution by peripheral CD4 and CD8 single-positive lymphocytes. Eur. J. Immunol. **23**: 2412–2418.
14) Ildstad, S.T., Wren, S.M., Boggs, S.S., Hronakes, M.L., Vecchini, F., and Van den Brink, M.R.M. 1991. Cross-species bone marrow transplantation: evidence for tolerance induction, stem cell engraftment, and maturation of T lymphocytes in a xenogenic stromal environment (rat→mouse). J. Exp. Med. **174**: 469–478.
15) Iwasaki, A., Yoshikai, Y., Sakumoto, M., Himeno, K., Yuuki, H., Kumamoto, M., Sueishi, K., and Nomoto, K. 1990. Sequential appearance of host-derived T cell subsets during differentiation in nude mice grafted with rat fetal thymus. J. Immunol. **145**: 28–35.
16) Kappler, J.W., Staerz, U., White, J., and Marrack, P.C. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature **332**: 35–40.
17) Kaye, J., Hsu, M.-L., Sauron, M.-E., Jameson, S.C., Gascoigne, N.R.J., and Hedrick, S.M. 1989. Selective development of $CD4^+$ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. Nature **341**: 746–749.
18) Kisielow, P., Teh, H.S., Blüthmann, H., and Von Boehmer, H. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature **335**: 730–733.
19) Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M., and Von Boehmer, H. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. Nature **333**: 742–746.
20) Kisielow, P., and Boehmer, H. 1994. Development and selection of T cells: facts and puzzles. Adv. Immunol. **58**: 87–209.
21) Koller, B.H., Marrack, P., Kappler, J.W., and Smithies, O. 1990. Normal development of mice deficient in β2M, MHC class I proteins, and $CD8^+$ T cells. Science **248**: 1227–1230.
22) MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M., and Hengartner, H. 1988. T-cell receptor Vβ use predicts reactivity and tolerance to Mls^a-encoded antigens. Nature **332**: 40–45.
23) Maeda, K., Nagasawa, H., Furukawa, A., Hisaeda, H., Maekawa, Y., Manabe, T., Kudo, E., Good, R.A., and Himeno, K. 1993. Development of T cells in SCID mice grafted with fetal thymus from AKR mice or F344 rats. Eur. J. Immunol. **23**: 3151–3157.
24) Nakayama, K., Nakayama, K., Negishi, I., Kuida, K., Louie, M.C., Kanagawa, O., Nakauchi, H., and Loh, D.Y. 1994. Requirement for CD8β chain in positive selection of CD8-lineage T cells. Science **263**: 1131–1133.
25) Rahemtulla, A., Fung-Leung, W.P., Schilham, M.W., Kundig, T.M., Sambhara, S.R., Narendran, A., Arabin, A., Wakeham, A., Paige, C.J., Zinkernagel, R.M., and Mak, T.M. 1991. Normal development and function of $CD8^+$ cells but markedly decreased helper cell activity in mice lacking CD4. Nature **353**: 180–184.
26) Sakai, T., Agui, T., Muramatsu, Y., Yamada, T., and Matsumoto, K. 1993. Dissociation of interleukin-2 production from the cell activation in response to the mitogenic lectin in peripheral $CD4^+$ T cells of LEC mutant rats. Immunology **79**: 491–497.
27) Sakai, T., Agui, T., and Matsumoto, K. 1993. Maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes is caused by the bone marrow-derived cells in LEC mutant rats. Immunol. Lett. **38**: 145–152.
28) Surh, C.D., and Sprent, J. 1991. Long-term xenogenic chimeras: full differentiation of rat T and B cells in SCID mice. J. Immunol. **147**: 2148–2154.
29) Williams, A.F., Galfrè, G., and Milstein, C. 1977. Analysis of cell surfaces by xenogenic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. Cell **12**: 663–673.
30) Yamada, T., Natori, T., Izumi, K., Sakai, T., Agui, T., and Matsumoto, K. 1991. Inheritance of T helper immunodeficiency (*thid*) in LEC mutant rats. Immunogenetics **33**: 216–219.
31) Zijlstra, M., Bix, M., Simister, N.E., Loring, J.M., Raulet, D.H., and Jaenisch, R. 1990. β2-Microglobulin deficient mice lack $CD4^-8^+$ cytolytic T cells. Nature **344**: 742–746.

Immunology 1996 **88** 551–557

Contribution of extrathymic γδ T cells to the expression of heat-shock protein and to protective immunity in mice infected with *Toxoplasma gondii*

H. HISAEDA, T. SAKAI, H. NAGASAWA, H. ISHIKAWA, K. YASUTOMO, Y. MAEKAWA & K. HIMENO *Department of Parasitology and Immunology, School of Medicine, The University of Tokushima, Japan*

SUMMARY

We demonstrated that γδ T cells contribute to protective immunity against *Toxoplasma gondii* by inducing the expression of a 65 000 MW heat-shock protein (hsp 65) in host macrophages. Here we examined the role of extrathymic and intrathymic γδ T cells in protective immunity and hsp 65 expression in mice infected with *T. gondii*. Intrathymic γδ T cells were obtained from severe combined immunodeficiency (SCID) mice grafted with syngeneic fetal thymus (TG-SCID), in which only T cells derived from the donor thymus developed, whereas extrathymic γδ T cells were obtained from nude mice that lack thymus. Extrathymic γδ T cells from *T. gondii*-infected nude mice differed from intrathymic γδ T cells of infected TG-SCID mice, in terms of Thy1.2 expression and V-region gene usage of T-cell receptor (TCR) γδ. Extrathymic γδ T cells expressed extremely high levels of Thy1.2, and had Vγ7 repertoire but lacked Vγ5,6 and Vδ1,5. On the other hand, intrathymic γδ T cells express intermediate and low levels of Thy1.2. These cells possessed Vγ5,6 and Vδ1,5 but failed to rearrange the Vγ7 gene. Peritoneal macrophages from infected nude mice contained hsp 65, whereas this protein was scarcely expressed in those of infected TG-SCID mice. Transfer of extrathymic, but not of intrathymic γδ T cells to SCID mice enabled their macrophages to express hsp 65. Athymic nude mice were significantly resistant to the infection compared with SCID mice which lack γδ T as well as αβ T cells. The resistance was dependent upon extrathymic γδ T cells, since nude mice depleted of γδ T cells using a corresponding monoclonal antibody became extremely susceptible. These results indicated that extrathymic rather than intrathymic γδ T cells play some crucial roles in protection against *T. gondii* and in hsp 65 expression.

INTRODUCTION

Toxoplasma gondii is one of the most common pathogens of opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS).[1,2] Cellular immunity mediated by T cells is indispensable for eradicative immune responses to this protozoan,[3–5] although some T-cell-independent protective mechanisms reportedly exist.[6–8] T cells are phenotypically divided into two distinct subsets. The majority of T cells in the periphery bears T-cell receptor (TCR)-αβ which recognizes conventional antigens in the context of major histocompatibility complex (MHC) gene products.[9] A minor T-cell population expresses TCR-γδ, which is supposed to recognize MHC-like molecules[10] and mycobacterial antigens, including the 65 000 MW heat-shock protein, hsp 65.[11–13] In contrast to αβ T cells, the antigen recognition mechanism of γδ T cells and their role in protective immunity is not fully understood. This type of T cell reportedly participates in host defence against some pathogens, especially intracellular pathogens.[14–16] In the peripheral blood of patients with acute toxoplasmosis, γδ T as well as αβ T cells increase.[17,18]

Recently, we found that γδ T cells play a crucial role in the expression itself of hsp 65 in host macrophages and its expression is essential for the protective immunity to *T. gondii* at the early phase of infection.[19]

Nude mice lack a thymus which is the central organ for T-cell development. However, a significant level of T cells develops through a thymus-independent (extrathymic) pathway in these mice.[20,21] In contrast, only thymus-derived (intrathymic) T cells develop in severe combined immunodeficiency (SCID) mice by grafting them with a murine fetal thymus.[22] Here, we compare the roles played by extrathymic and intrathymic γδ T cells in protective immunity and hsp 65 expression in mice infected with *T. gondii*. The phenotype, V gene usage of TCR and the ability to induce host hsp 65 were

Received 7 February 1996; revised 17 April 1996; accepted 19 April 1996.

Abbreviations: AIDS, acquired immunodeficiency syndrome; hsp 65, 65 000 MW heat-shock protein; mAb, monoclonal antibody; MHC, major histocompatibility complex; RT-PCR, reverse transcription–polymerase chain reaction; SCID, severe combined immunodeficiency; TCR, T-cell receptor; TG-SCID, fetal thymus grafted-SCID; V, variable.

Correspondence: Dr H. Hisaeda, Department of Parasitology and Immunology, The University of Tokushima, School of Medicine, 3 Kuramoto-cho, Tokushima, 770 Japan.

examined in $\gamma\delta$ T cells from those mice. Some mice were depleted of $\gamma\delta$ T cells by giving them a corresponding monoclonal antibody (mAb) prior to infection with *T. gondii*.

MATERIALS AND METHODS

Animals

Euthymic and athymic BALB/c mice were obtained from Japan SLC Inc. (Hamamatsu, Japan), and CB-17 +/+ and *scid/scid* (SCID) mice were from CLEA Japan Inc. (Tokyo, Japan). The CB-17 and BALB/c mice have the same genetic background, except for the immunoglobulin heavy chain gene. Then CB-17 mice were used as normal control in some experiments. These mice were studied at 7–10 weeks old.

Establishment of fetal thymus-grafted SCID (TG-SCID) mice

Fetal thymuses were isolated under aseptic conditions from 15-day-old CB-17 +/+ mouse embryos. One lobe of the fetal thymus was grafted under the subcapsular space of the left kidney of SCID mice using a 21-gauge needle as described.[22,23] To prove the reconstitution with T cells, peripheral blood mononuclear cells from TG-SCID mice were analysed with flow cytometry 3 weeks after the operation.

Micro-organisms

Bradyzoites of the Beverley strain of *T. gondii* were used as described.[24] In brief, the brains of chronically infected mice were homogenized with RPMI-1640 (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS; Flow, MacLean, VA), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES buffer. The homogenate was centrifuged at 800 *g* for 20 min at 15° through a 1·057/1·070 gum arabic (resolved with 0·02% ethylenediaminetetraacetic acid (EDTA) saline) (Sigma Chemical Co., St Louis, MO) discontinuous gradient. To obtain the bradyzoites, precipitated cysts were exposed to 0·25% trypsin (Difco Laboratories, Detroit, MI) in phosphate-buffered saline (PBS) for 5 min at 37°, followed by centrifugation at 590 *g* for 10 min at 4° and resuspension in RPMI-1640. Mice were infected with 70–100 bradyzoites of the Beverley strain of *T. gondii* by intraperitoneal injection.

Monoclonal antibodies

Anti-TCR-$\gamma\delta$ [UC7–13D5 (hamster IgG)] and anti-TCR-$\alpha\beta$ [H57–597 (hamster IgG)] were provided by Dr G. Matsuzaki, Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan. Anti-Thy1.2 [30-H12 (rat IgG)] was purchased from the American Type Culture Collection (Rockville, MA). Phycoerythrin (PE)-anti-CD3 was purchased from Pharmingen (San Diego, CA). Anti-TCR-$\gamma\delta$ and anti-TCR-$\alpha\beta$ were conjugated with fluorescein isothiocyanate (FITC). Anti-CD4 [GK1.5 (rat IgG)], anti-CD8 [53–6.7 (rat IgG)], and anti-Thy1.2 were biotinylated according to the standard procedure. The murine IgG mAb, termed IA10, specific for amino acids 172–224 of hsp 65 derived from *Mycobacterium bovis* was provided by Dr J. DeBruyn, Institute Pasteur de Brabant, Belgium.

Flow cytometry analysis and cell sorting

Non-adherent peritoneal exudate cells (PEC) were stained with various combinations of fluorescence-conjugated mAb and analyzed by two or three-colour flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). Three-colour an was achieved using Streptavidin-Cy-Chrome (Pharmi Before analysis, the lymphocyte population was gated b scatter signals to exclude dead and non-lymphoid cells. subsets were purified with a cell sorter (EPICS ELITE, C Electronics, Hialeah, FL). For use *in vivo*, $\gamma\delta$ T cells negatively sorted.

Reverse transcriptase–polymerase chain reaction (RT–PC

Non-adherent PEC from mice infected with *T. gondii* collected for RNA preparation. Non-adherent PEC were in 0·5 ml of lysis solution D containing 4 M guan thiocyanate, 25 mM sodium citrate (pH 7·0), 0·1 M 2-me toethanol, and 0·5% (wt/vol) sarcosyl. To shear high mole weight DNA, the solution was drawn through a 22-g needle fitted onto a 1-ml syringe. Thereafter, 50 μl o sodium acetate (pH 4·0), 500 μl of phenol and 100 μ chloroform/isoamylalcohol (49 : 1) were added, and n well. The mixture was chilled on ice and centrifuge 12 000 *g* for 20 min at 4°, and RNA in the supernatant extracted with 1 ml of ice-cold ethanol at −20° for 60 min. aliquot was centrifuged as described above, then the precip was incubated with 150 ml of solution D and 1 ml of ice- ethanol at −20° for 60 min. After centrifugation, precipitated RNA was washed with 75% ice-cold ethanol was dissolved with sterile diethyl pyrocarbonate-treated w

RNA (100 ng) was reverse-transcribed using hexanucle random primers (Boehringer Mannheim, Mannheim, Germ in a reaction mixture (Takara Shuzo, Tokyo, Japan), ther cDNA was amplified with Taq DNA polymerase (Ta Shuzo). The PCR consisted of 34 cycles of relaxation denaturation of cDNA for 30 seconds at 94°, primer annea for 30 seconds at 60° and a primer extension for 1 min at followed by a prolonged extension cycle for 10 min at 72° DNA thermal controller (Funakoshi, Tokyo, Japan). The pri were as follows: 3′ primers for Vγ and Vδ usage are CTTATG GATTTGTTTCAGA, and CGAATTCCACAATCTTCT respectively. The 5′ primers were as follows: Vγ 1/2, AC AGCTATACATTGGTAC; Vγ 2, CGGCAAAAAACAA CAACAG; Vγ 4, TGTCCTTGCAACCCCTACCC; V TGTGCACTGGTACCAACTGA; Vγ 6, GGAATTCAA GAAAACATTGTCT; Vγ 7, AAGCTAGAGGGGTCCT GC; Vδ 1, ATTCAGAAGGCAACAATGAAAG; Vδ AGTTCCCTGCAGATCCAAGC; Vδ 3, TTCCTGGCT TGCCTCTGAC; Vδ 4, CCGCTTCTCTGTGAACTTCC; 5, CAGATCCTTCCAGTTCATCC; Vδ 6, TCAAGTCC CAGAATTGTC; and Vδ 7 CGCAGAGCTGCAGTGT CT. TCR nomenclature is according to Reilley *et al.*[25] microliters of PCR products were electrophoresed on a 1· agarose gel containing ethidium bromide and visualized un ultraviolet fluorescence. *Hin*cII digested ϕX174 was used molecular weight marker.

Western blotting

hsp 65 was detected by Western blotting as describe Briefly, protein extracts of a plastic-adherent fraction PEC from infected mice were separated by sodium dode sulphate–polyacrylamide gel (SDS–PAGE), then gels w electroblotted onto a polyvinylidene difluoride membr (Millipore Co., Bedford, MA). The mAb IA10 cult supernatant diluted 1 : 200 was used as the first antibody, a

goat peroxidase-conjugated anti-mouse IgG (Pierce, Rockford, IL) as the second. Binding antibodies were detected using a Konica immunostaining horseradish peroxidase kit (Konica, Tokyo, Japan).

T-cell depletion

Mice were depleted of γδ T cells using a mAb (UC7–13D5) specific for TCR-γδ. Five hundred micrograms of mAb was inoculated intraperitoneally 1 and 3 days prior to infection and 200 mg of mAb was further administered every 3 days from day 5 after infection. The administration of a control antibody (immunoglobulin fraction of hamster serum) did not alter the course of infection with *T. gondii*.[19]

RESULTS

γδ T-cell expansion in the peritoneal cavity of nude mice infected with *T. gondii*

Nude mice reportedly possess T cells developed via the extrathymic pathway.[20,21] To investigate whether nude mice have γδ T cells responding to infection with *T. gondii*, T-cell subsets in the peritoneal cavity of nude mice were analysed by flow cytometry 10 days after infection (Fig. 1a). The number of γδ T cells ($CD3^+$, TCR-$\gamma\delta^+$ fraction) increased in the peritoneal cavity of nude as well as in normal mice. Although the number of αβ T cells ($CD3^+$, TCR-$\gamma\delta^-$ fraction) also increased, this increase was not comparable to that of γδ T cells. This finding indicated that extrathymic γδ T cells expanded in response to *T. gondii* infection.

Characteristics of extrathymic γδ T cells responding to *T. gondii* infection

Extrathymic γδ T cells were found in the peritoneal cavity of nude mice infected with *T. gondii* as described above. Therefore, we characterized these T cells in comparison with thymus-derived γδ T cells (intrathymic γδ T cells) obtained from SCID mice grafted with a syngeneic fetal thymus (TG-SCID). TG-SCID mice possess only T cells derived from grafted thymus.[22] Fluorescence-activated cell sorter (FACS) analysis revealed an increase in the number of intrathymic γδ T cells in these mice after challenge with *T. gondii* (Fig. 1b). There was no difference in TCR expression between extra- and intrathymic γδ T cells, since the levels of TCR and CD3 in γδ T cells from both nude and TG-SCID mice were comparable to those in γδ T cells from control mice. Further phenotypic analysis revealed that neither extra- nor intrathymic γδ T cells expressed CD4 or CD8 (data not shown). The expression of Thy1.2 antigen on γδ T cells was markedly different between euthymic, nude and TG-SCID mice (Fig. 2). The γδ T cells from euthymic normal mice were composed of three subsets expressing Thy1.2 antigen with high, intermediate or low intensity, and those from TG-SCID mice expressed this antigen with intermediate or low intensity, whereas most of the γδ T cells from nude mice were composed of a single population expressing high levels of Thy1.2. These data indicated that intrathymically developed γδ T cells are composed of subsets with low and intermediate levels of Thy1.2, while most γδ T cells developed extrathymically express Thy1.2 antigen at high level.

The cDNA constructed from non-adherent PEC of mice infected with *T. gondii* was analysed for usage of the V region of TCR-γδ, by means of PCR (Fig. 3). The γδ T cells from euthymic BALB/c mice infected with *T. gondii* used all of the Vγ gene segment and Vδ1,4,5 and 6. Intrathymic γδ T cells from infected TG-SCID mice showed rearrangement similar to those of BALB/c mice but lacked Vγ7, whereas extrathymic γδ T cells from nude mice lacked Vγ5,6 and Vδ5 repertoires but possessed Vγ7. Uninfected mice also possessed the similar repertoires of the γδ T cells in terms of Vγ gene usage in each group of mice, but Vδ1,4,5 and Vδ4,5,6 were not transcribed in BALB/c and TG-SCID mice, respectively. These findings indicated that extrathymic and intrathymic γδ T cells differ in the V gene repertoire as well as Thy1.2 expression, and that intrathymic T

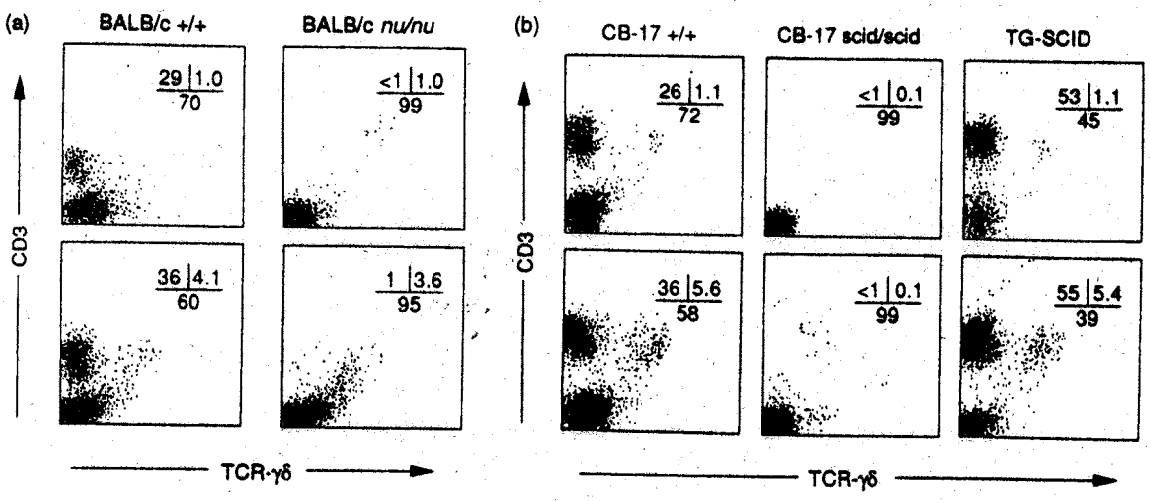


Figure 1. γδ T cells in the peritoneal cavity of nude and TG-SCID mice infected with *T. gondii*. Flow cytometry analysis of non-adherent PEC from uninfected (upper panels) and *T. gondii*-infected (lower panels) nude mice (a) and TG-SCID mice (b). Cells were collected 10 (nude) and 12 days (TG-SCID) after infection with 100 bradyzoites and stained with a combination of FITC–anti-TCR-γδ and PE–anti-CD3. These panels show typical two-colour profiles. The numbers represent the percentages of each subpopulation. Similar results were obtained when cells were stained with FITC–anti-TCR-αβ and PE–anti-CD3 (data not shown).

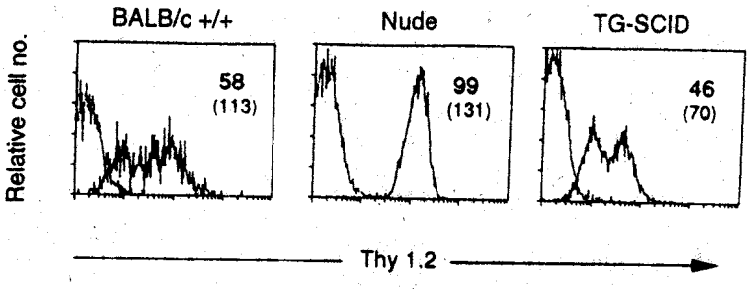


Figure 2. Thy1.2 expression by intrathymic and extrathymic γδ T cells. FACS three-colour analysis using the above combination plus biotin–anti-Thy1.2 (bold line) or control antibody (line). Cells from the indicated mice 12 days after infection with 100 bradyzoites were stained. $CD3^+$, TCR-$\gamma\delta^+$ cells were gated and analysed for Thy1.2 expression. Numbers represents the percentage of $Thy1.2^+$ cells and those in parentheses indicate the mean fluorescence intensity of Thy1.2 high and intermediate γδ T cells. Thy1.2 expression by γδ T cells from uninfected mice was comparable to that from infected mice, although the absolute number was much less than that of infected mice (data not shown).

cells bearing Vδ4,5 expanded or were recruited in the peritoneal cavity.

hsp 65 expression in/on macrophages of nude and TG-SCID mice

We examined the biological functions of intrathymic and extrathymic γδ T cells. We showed that host protective immunity to *T. gondii* closely correlates with the intensity of hsp 65 expression in/on host macrophages, and that γδ T cells play a central role in its expression.[19,26,27] We thus analysed whether hsp 65 is expressed in/on macrophages in nude and TG-SCID mice. This protein was expressed in macrophages of normal mice 9 days after infection with 70 bradyzoites of the Beverley strain of *T. gondii*, but not in SCID mice that completely lack γδ T cells. Peritoneal macrophages of nude mice expressed hsp 65, while those of TG-SCID mice showed only marginal expression (Fig. 4a). These results indicated that extrathymic γδ T cells have more potential than intrathymic γδ T cells to induce hsp 65. To confirm this notion, macrophages from SCID mice given 10^6 sorted extrathymic γδ T cells from nude mice or intrathymic γδ T cells from fetal thymus were examined for hsp 65 expression after infection with *T. gondii*. As shown in Fig. 4(b), the extrathymic γδ T cells transferred to SCID mice enabled their peritoneal macrophages to express hsp 65, whereas those given intrathymic γδ T cells did not. This finding indicated that extrathymic γδ T cells are responsibl hsp 65 expression in macrophages, and supports our pre finding[19] that depletion of $Thy1.2^+$ T cells including e thymic γδ T cells markedly reduced hsp 65 expres regardless of the presence of $Thy1.2^{low}$ γδ T cells devel via the intrathymic pathway.

Contribution of extrathymic γδ T cells to the protection ag *T. gondii* infection

Nude mice are susceptible to *T. gondii* infection because T-cell defect.[28,29] Here, all of the infected nude mice die acute infection after being given a dose sublethal for euth BALB/c mice (Fig. 5). Thus, nude mice were susceptible t *gondii* infection compared with their euthymic counterp but were significantly more resistant than SCID mice w lack γδ T as well as αβ T cells, with respect to duratio survival (21·0 ± 1·83 versus 18·2 ± 2·11 days, $P <$ significant according to Student's *t*-test). This resistanc nude mice should be dependent on extrathymically derive cells, especially γδ T cells. To examine this possibility, r mice were depleted of γδ T cells using a mAb specific for T γδ (UC7–13D5). The efficacy of this *in vivo* treatmen depleting the corresponding T-cell subset has t described.[19,27] Nude mice treated with anti-TCR-γδ n

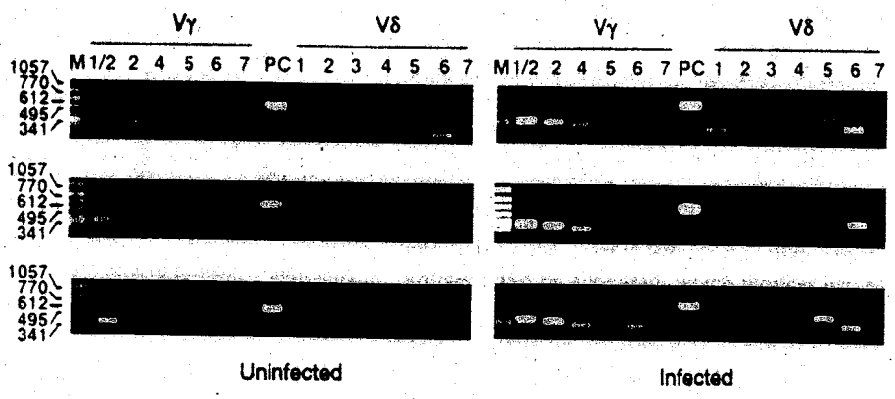


Figure 3. V gene usage by intrathymic and extrathymic γδ T cells. The cDNA was constructed from non-adherent PEC of BALB/c (upper panels), nude (middle panels) and TG-SCID mice (bottom panels) before and 12 days after infection with 70 bradyzoites of *T. gondii*. Primers for β-actin were used as the internal control indicated as PC. M indicates molecular markers shown in the left in base pairs.

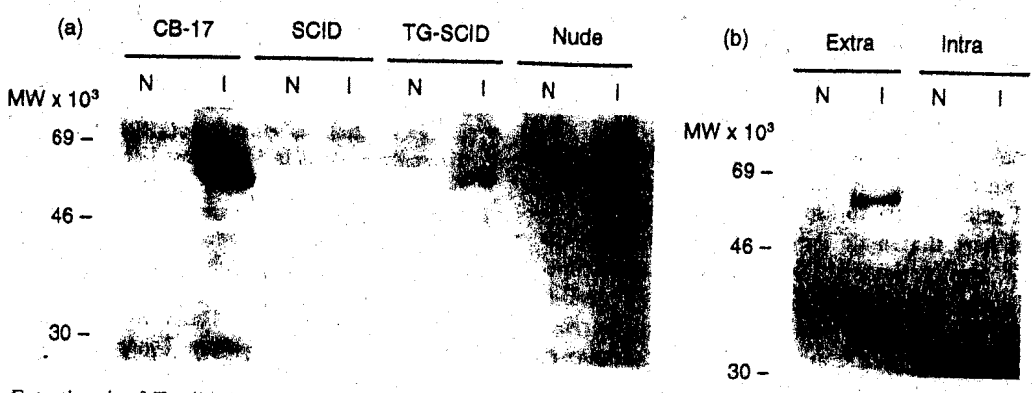


Figure 4. Extrathymic γδ T cells play a crucial role in the induction of hsp 65. Cell lysates prepared from peritoneal macrophages of mice 10 days after infection with 100 bradyzoites of the Beverley strain of *T. gondii* were Western blotted. (a) Nude and TG-SCID mice, (b) SCID mice transferred with sorted extrathymic (extra) or intrathymic (intra) γδ T cells were infected. N and I refer to naive and infected mice, respectively. Protein extracts of the parasites and macrophages from uninfected mice did not contain demonstrable levels of hsp 65 as previously described.[19] Eight micrograms of protein was loaded on lanes. Normal mouse IgG did not react with this protein. Standard molecular weight markers are shown on the left in kilodaltons.

became susceptible to *T. gondii* infection compared with naive nude mice (18·3 ± 1·86 versus 21·3 ± 2·05 days, $P < 0.05$ significant with Students's *t*-test). On the other hand, this treatment never altered the course of infection in TG-SCID mice that possessed intrathymic, but not extrathymic γδ T cells. In addition, less hsp 65 was expressed in γδ T cell-depleted, than in untreated nude mice (data not shown). These results indicate that extrathymic, rather than intrathymic γδ T cells play an essential role in the protection and induction of host hsp 65 in/on macrophages.

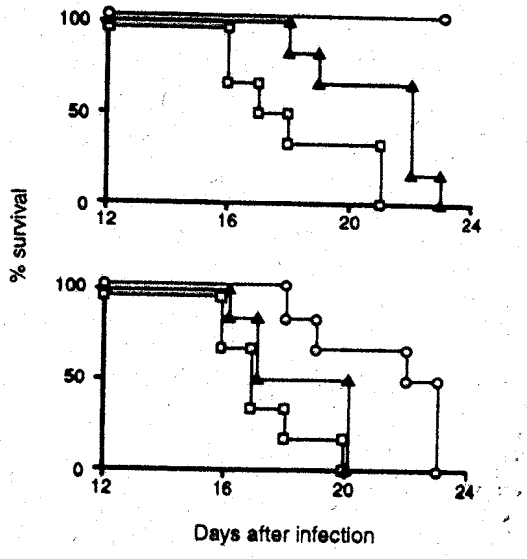


Figure 5. Contribution of extrathymic γδ T cells to protective immunity against *T. gondii*. Upper panel, nude mice (closed triangles) exhibited significant resistance to *T. gondii* compared with SCID mice (open squares), but were much more susceptible to a sublethal dose in euthymic mice (open circle). Six mice from each group were infected with 70 bradyzoites of the Beverley strain of *T. gondii*. Lower panel, nude mice treated with mAb to TCR- γδ (closed triangles) or without (open circles) and SCID mice (open squares) were infected with 100 bradyzoites. Each group consisted of six animals. Two repeated experiments yielded similar results.

DISCUSSION

We investigated the involvement of extra- and intrathymic γδ T cells in protective immunity and hsp 65 expression in *Toxoplasma* infection using nude and TG-SCID mice. Phenotypically, the levels of Thy1.2 antigen expression is extremely different from each other, although that of TCR is similar. This discrepancy between extra- and intrathymic γδ T cells may reflect the state of cell activation,[30] suggesting that extrathymic γδ T cells are highly activated from an early phase of infection. RT-PCR analyses revealed that these two subsets also differ in the V gene usage. The relationship between Vγ repertoire usage, tissue distribution and the origin of γδ T cells is well defined.[31] That is, the Vγ5 is used preferentially by γδ T cells located within epidermis, while γδ T cells with Vγ6 locate under the epithelium of the tongue and in the female reproductive tract, and those with Vγ7 are found under that of the intestine. In contrast to Vγ5 and Vγ6, both of which are supposed to be derived from fetal thymus, Vγ7 develops via the extrathymic pathway. This concept is consistent with our observations that nude mice lack Vγ5, 6 and TG-SCID mice lack Vγ7. It is not known whether and how epidermic or intestinal γδ T cells migrate into the peritoneal cavity. However, the relationship between the homing ability of γδ T cells to epithelia and their Vγ repertoire usage is not stringent.[32,33] We suppose that these cells originally locate in the peritoneal cavity.

Recently, a part of the γδ T cells participating in immune surveillance recognizes ubiquitous compounds in pathogens and mammalian cells in an MHC-independent fashion.[34,35] Thus, our data suggest that extrathymic γδ T cells, which have not undergone thymic education, may recognize protozoan antigens and be activated, which induces hsp 65 expression in/on macrophages. Intrathymic γδ T cells, especially $V\delta4^+$ and $V\delta5^+$ T cells in BALB/c and TG-SCID mice, also expanded after infection, although they seemed not to function in host-defence and hsp 65 expression. As is generally known, hsp 65 is one of the ligands for γδ T cells.[11,12] Thus, these γδ T cells may recognize host-derived hsp 65 on macrophages induced by extrathymic γδ T cells. In fact, our speculation is supported by some reports as follows. That is, $V\delta5^+$ γδ T cells recognize

murine HSP60 in the context of MHC in self mixed lymphocyte reaction,[36] and the early and late expansion of γδ T cells consisting of Vδ6$^+$ and Vδ4$^+$ cells, respectively, are observed in schistosomiasis.[37] Taken together, extrathymic γδ T cells bearing Vδ6 may be activated and induce hsp 65 in macrophages at an early stage of infection, then intrathymic Vδ4 and 5$^+$ γδ T cells reactive to hsp 65 may expand.

We described here that nude mice are more resistant to infection with *T. gondii* than SCID mice, suggesting that extrathymic γδ T cells are responsible for resistance to *T. gondii* infection. One of the causes of this difference may be the existence of antibodies, but specific antibodies are not produced in nude mice because of a defect in helper T-cell function exerted by CD4$^+$ T cells.[29] Thus, this possibility is negligible and extrathymic γδ T cells should be important. However, this resistance preferentially operated in the early stages after infection and could not completely control the infection after all. Alternatively, extrathymic γδ T cells contribute to protection but sequential defence mechanisms from extrathymic γδ T cells to intrathymic αβ T cells are required for the complete resolution of *T. gondii* infection. We found that γδ T cell-depleted euthymic BALB/c mice become extremely susceptible to infection with this organism, despite the fact that αβ T cells increase even in absolute number,[19] indicating again that γδ T cells are indispensable to the early stages of protection and to the accomplishment of sequential defence mechanisms. It is reported that αβ and γδ T cells play different roles in protective immunity against intracellular pathogens.[38] Thus, it is of importance to elucidate the relationship between αβ and γδ T cells in protective immunity. Several studies have demonstrated the existence of host hsp 65-reactive αβ T cells,[39–41] indicating that hsp 65 derived from host macrophages and/or parasites provides a link between these two types of T cell. The biological role and expression mechanism of hsp 65 in host macrophages is still under investigation. This protein is expressed by cytokines secreted from γδ T cells, and preserves macrophage functions in terms of evading apoptosis induced by intracellular noxious agents such as nitric oxide (manuscript in preparation).

ACKNOWLEDGMENTS

This study was supported by grants from the Ministry of Education, Science and Culture (05268229, 05670227, 05770175 and 04044129). We would like to thank Dr Y. Okamura for technical instruction for cell sorting methods.

REFERENCES

1. Luft B.J. & Remington J.S. (1988) AIDS commentary: toxoplasmic encephalitis. *J Infect Dis* **157**, 1.
2. Kasper L.H. & Boothroyd J.C. (1993) *Toxoplasma gondii* and toxoplasmosis. In: *Immunology and Molecular Biology of Parasitic Infection* (ed. K. S. Warren), 3rd edn, p. 269. Blackwell Scientific Publications, Boston.
3. Nagasawa H., Manabe T., Maekawa Y., Oka M. & Himeno K. (1991) Role of L3T4$^+$ and Lyt-2$^+$ T cells subsets in protective immune responses of mice against infection with a low or high virulent strain of *Toxoplasma gondii*. *Microbial Immunol* **35**, 215.
4. Gazzinelli R., Xu Y., Hieny S., Cheever A. & Sher A. (1992) Simultaneous depletion of CD4 and CD8 T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J Immunol* **149**, 175.
5. Kasper L.H., Khan I.A., Ely K.H., Buelow R. & Boothroyd J.C. (1992) Antigen-specific (p30) mouse CD8$^+$ T cells are cy against *Toxoplasma gondii*-infected peritoneal macroph *Immunol* **148**, 1493.
6. Denkers E.Y., Gazzinelli R.T. & Martin M. (1993) Eme of NK1.1 cells as effectors of IFN-γ dependent immu *Toxoplasma gondii* in MHC class I-deficient mice. *J Exp M* 1465.
7. Johnson L.L., Van der Vegt F.P. & Havell E.A. (1993) C interferon-dependent temporary resistance to acute *Toxo gondii* infection independent of CD4$^+$ or CD8$^+$ lymph *Infect Immun* **61**, 5474.
8. Sher A., Oswald I.P., Hieny S. & Gazzinelli R.T. *Toxoplasma gondii* induces a T-independent IFN-γ respo natural killer cells that requires both adherent accessory cel tumor necrosis factor-α. *J Immunol* **150**, 3982.
9. Janeway C.A. (1993) How the immune system recognizes inv *Sci Am* **269**, 231.
10. Strominger J.L. (1989) The γδ T cell receptor and class Ib l restricted proteins: enigmatic molecules of immune recogn *Cell* **57**, 895.
11. O'Brien R.L., Happ M.P., Dallas A., Palmer E., Kubo R. & W.K. (1989) Stimulation of a major subset of lymph expression T cell receptor γδ by an antigen derived *Mycobacterium tuberculosis*. *Cell* **57**, 667.
12. O'Brien R.L., Fu X-Y., Cranfill R. *et al.* (1992) Heat protein Hsp60-reactive γδ T cells: a large, diversified T-lymph subset with highly focused specificity. *Proc Natl Acad Sci US* 4348.
13. Beagley K.W., Fujihashi K., Black C.A. *et al.* (1993) *Mycobacteria tuberculosis* 71-kDa heat-shock protein in proliferation and cytokine secretion by murine gut epit lymphocytes. *Eur J Immunol* **23**, 2049.
14. Rosat J.P., MacDonald H.R. & Louis J.A. (1993) A role for cells during experimental infection of mice with *Leishmania n J Immunol* **150**, 550.
15. Emoto M., Danbara H. & Yoshikai Y. (1992) Induction of cells in murine salmonellosis by an avirulent but not by a vir strain of *Salmonella choleraesuis*. *J Exp Med* **176**, 363.
16. Hiromatsu K., Yoshikai Y., Matsuzaki G. *et al.* (199 protective role of γ/δ T cells in primary infection with *Lis monocytogenes* in mice. *J Exp Med* **175**, 49.
17. De Paoli P., Basaglia G., Gennari D., Crovatto M., Mo M.L. & Santini G. (1992) Phenotypic profile and functi characteriztics of human gamma and delta T cells during a toxoplasmosis. *J Clin Microbiol* **30**, 729.
18. Scalise F., Gerli R., Castellucci G. *et al.* (1992) Lympho bearing the γδ T-cell receptor in acute toxoplasmosis. *Immunc* **76**, 668.
19. Hisaeda H., Nagasawa H., Maeda K. *et al.* (1995) γδ T cells an important role in expression of hsp 65 and in acqui protective immune responses against infection with *Toxopla gondii*. *J Immunol* **155**, 244.
20. Speiser D.E., Stubi U. & Zinkernagel R.M. (1992) Extrathy positive selection of αβ T-cell precursors in nude mice. *Nature* 170.
21. Rocha B., Vassalli P. & Guy-Grand D. (1992) The extrathy T-cell development pathway. *Immunol Today* **13**, 449.
22. Maeda K., Nagasawa H., Furukawa A. *et al.* (1993) Developn of T cells in SCID mice grafted with fetal thymus from AKR r of F344 rats. *Eur J Immunol* **23**, 3151.
23. Yasutomo K., Maeda K., Nagata S. *et al.* (1994) Defective T c from *gld* mice play a pivotal role in development of Thy1.2$^+$B22 cells and autoimunity. *J Immunol* **153**, 5855.
24. Hisaeda H., Nagasawa H., Maeda K. *et al.* (1993) Correlat between heat shock protein expression and the acquisition protective immunity to *Toxoplasma gondii* in rats. *Jpn J Paras* **42**, 461.

25. Reilley E.B., Kranz D.M., Tonegawa S. & Eisen H.N. (1986) A functional γ gene formed from known γ-gene segments is not necessary for antigen-specific responses of murine cytotoxic T lymphocytes. *Nature* **321**, 878.
26. Nagasawa H., Oka M., Maeda K. *et al.* (1992) Induction of heat shock protein closely correlates with protection against *Toxoplasma gondii* infection. *Proc Natl Acd Sci USA* **89**, 3155.
27. Nagasawa H., Hisaeda H., Maekawa Y. *et al.* (1994) γδ T cells play a crucial role in the expression of 65 000 heat-shock protein in mice immunized with *Toxoplasma* antigen. *Immunology* **83**, 347.
28. Murray H.W., Teitelbaum R. & Haripràshad J. (1993) Response to treatment for an intracellular infection in a T cell-deficient host: toxoplasmosis in nude mice. *J Infect Dis* **167**, 1173.
29. Lindberg R.E. & Frenkel J.K. (1977) Toxoplasmosis in nude mice. *J Parasitol* **63**, 219.
30. LeFrancois L. & Goodman T. (1989) *In vivo* modulation of cytolytic activity and Thy-1 expression in TCR-$\gamma\delta^+$ intraepithelial lymphocytes. *Science* **243**, 1716.
31. Haas W., Pereira P. & Tonegawa S. (1993) Gamma/delta cells. *Annu Rev Immunol* **11**, 637.
32. Goodman T. & LeFrancois L. (1989) Intraepithelial lymphocyte, anatomical site, not T cell receptor form, dictates phenotype and function. *J Exp Med* **170**, 1569.
33. Bonneville M., Itohara S., Krecko E.G. *et al.* (1990) Transgenic mice demonstrate that epithelial homing of γ/δ T cells is determined by cell lineages independent of T cell receptor specificity. *J Exp Med* **171**, 1015.
34. Penninger J.M., Wen T., Timms E. *et al.* (1995) Spontaneous resistance to acute T-cell leukaemias in TCRVγ1.1Jγ4Cγ4 transgenic mice. *Nature* **375**, 241.
35. Tanaka Y., Morita C.T., Tanaka Y., Nieves E., Brenner M.B. & Bloom B.R. (1995) Natural and synthetic non-peptide antigens receognized by human γδ T cells. *Nature* **375**, 155.
36. Kobayashi N., Matsuzaki G., Yoshikai Y., Seki R., Ivanyi J. & Nomoto K. (1994) $V\delta5^+$ T cells of BALB/c mice recognize the murine heat shock protein 60 target cell specificity. *Immunology* **81**, 240.
37. Sandor M., Sperling A.I., Cook G.A., Weinstock J.V., Lynch R.G. & Bluestone J.A. (1995) Two waves of γδ T cells expressing different Vδ genes are recruited into Schistosome-induced liver granulomas. *J Immunol* **155**, 275.
38. Mombaerts P., Arnoldi J., Russ F., Tonegawa S. & Kaufmann S.H.E. (1993) Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. *Nature* **365**, 53.
39. Koga T., Wand-Wuttenberger A., DeBruyn J., Munk M.E., Schoel B. & Kaufmann S.H.E. (1989) T cells against a bacterial heat shock protein recognize stressed macrophages. *Science* **245**, 1112.
40. Anderton S.M., van der Zee R. & Goodacre J.A. (1993) Inflammation activates self hsp60-specific T cells. *Eur J Immunol* **23**, 33.
41. Anderton S.M., van der Zee R., Prakken B., Noordzij A. & van Eden W. (1995) Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med* **181**, 943.

Reprinted from

veterinary parasitology

An International Scientific Journal

Veterinary Parasitology 64 (1996) 3–11

Research and Education of Veterinary Parasitology in Japan

Naoyoshi Suzuki

The Research Center for Protozoan Molecular Immunology, Obihiro University, Obihiro, Hokkaido, Japan

ELSEVIER

Veterinary Parasitology

An International Scientific Journal

Aims and scope. This journal is concerned with those aspects of helminthology, protozoology and entomology which are of interest to animal health investigators, veterinary practitioners and others with a special interest in parasitology. Papers of the highest quality dealing with all aspects of disease prevention, pathology, treatment, epidemiology and control of parasites in all animals which can be regarded as being useful to man, fall within the scope of the journal. It is intended to give special attention to new aspects of veterinary parasitology, such as problems arising from the intensification of industrialized production systems and mass production, problems of game animals which may be of epizootiological significance in domestic animals, etc. Parasitological studies on laboratory animals fall within the scope of the journal only if they provide a reasonably close model of a disease of domestic animals. Although the journal primarily publishes experimental and clinical research papers, occasionally unique case histories may also be published. Papers on the taxonomy of parasites do not fall within the scope of the journal.

Circumstances relating to animal experimentation must meet the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for International Organizations of Medical Sciences. (Obtainable from: Executive Secretary C.I.O.M.S., c/o W.H.O., Via Appia, CH-1211 Geneva 27, Switzerland.)

Publication information: *Veterinary Parasitology* (ISSN 0304-4017). For 1996 volumes 60–65 are scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where Air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available on request. Claims for missing issues should be made within six months of our publication (mailing) date. Please address all your requests regarding orders and subscription queries to: **Elsevier Science B.V., Order Fulfilment Department, P.O. Box 211, 1000 AE Amsterdam, The Netherlands, tel. (+31-20)4853642, fax (+31-20)4853598.**
In the USA and Canada: For further information on this and other Elsevier journals please contact: **Elsevier Science Inc., Journal Information Center, 655 Avenue of the Americas, New York, NY 10010, USA. Tel. (212)6333750; fax (212)633 3764; telex 420-643 AEP UI.**
Back volumes: Please contact the Publisher.
US mailing info—*Veterinary Parasitology* (0304-4017) is published monthly by Elsevier Science B.V. (Molenwerf 1, Postbus 211, 1000 AE Amsterdam). Annual subscription price in the USA is US$ 1390.00 (valid in North, Central and South America), including air speed delivery. Second class postage paid at Jamaica, NY 11431.
USA POSTMASTER: Send address changes to *Veterinary Parasitology,* Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003.
AIRFREIGHT AND MAILING in the USA by Publications Expediting Inc., 200 Meacham Avenue, Elmont, NY 11003.

ELSEVIER

Veterinary Parasitology 64 (1996) 3–11

veterinary parasitology

Research and Education of Veterinary Parasitology in Japan

Naoyoshi Suzuki

The Research Center for Protozoan Molecular Immunology, Obihiro University, Obihiro, Hokkaido, Japan

1. Introduction

Welcome to Yokohama, one of the oldest port cities in Japan. This is the 15th Conference of the W.A.A.V.P., and it is the first time to take place in Asia, as is the World Veterinary Congress, which will be held following this Conference at Yokohama. I would like to take this opportunity to express appreciation to you all for coming all the way to attend this conference. It was the 1967 or 2nd W.A.A.V.P. Conference at Lyon, that I first participated in this conference, and since then I have attended several conferences. I still cannot forget the marvellous conference organized by Lord Soulsby at Cambridge, UK, in 1993.

Since having agreed to host this conference, all the members of the Japanese Association of Veterinary Parasitology have worked to prepare for the conference with the valuable suggestions and advice from Dr. Slocombe, President of the W.A.A.V.P. On behalf of the Association, I would like to express my deep and special thanks to him, and all the staff members for their continuous and devoted efforts.

The main purpose of this conference is to have high-level scientific discussions, as well as to promote understanding and friendship among the attendants, but in addition to that, I do hope you will have opportunities to come in contact with the oriental culture, especially our traditional Japanese culture. I, and all the staff members of the Local Organizing Committee, would be so happy, if you would thoroughly enjoy your stay here as much as your time allows.

2. History of veterinary parasitology in Japan

The history of research and education of Veterinary Parasitology in Japan is a very short period, in fact, it has been only about 100 years since the education system for Veterinary Science was established. The outline of its progress and development is given by focusing on the leading Japanese researchers.

PII S0304-4017(96)00989-2

Fig. 1. A Japanese traditional poem with the picture, expressing our welcome to all participants.

The oldest paper reported on research of parasitic diseases in veterinary medicine is the one on bovine schistosomiasis written in Japanese (chinese characters) by Medical Dr. Katayama in 1847. In it, he reported that some cattle had shown similar symptoms tc those of human schistosomiasis in an area where infection of *Schistosoma japonicum* was prevalent among humans.

After the intermediate host of *Schistosoma japonicum* was found to be *Oncomelanic nosophora*, measures were taken against this disease by establishing diagnostic, treatment and protective methods. As a result, in present-day Japan, new cases of this disease have not been found either in humans or in animals. In the old days, the Japanese use tc write sentences from the upper right to the lower right proceeding from the right to the left, using a brush and black ink.

As shown in Fig. 1 I want to explain a little bit more about Japanese. Since the end o the 2nd World War in 1945, the Japanese way of writing has been changed. We started to write from the upper left to the upper right, proceeding from top to bottom. Despite this change in the Japanese way of writing in general, some of the writings, such as formal official documents, are still written in the traditional way. On the paperboarc with Haiga, which means the picture to go along with the Japanese poem, that has developed together with the Japanese traditional green tea ceremony, I have written a piece of Haiku on which my wife made the picture. Haiku is a Japanese traditiona poem, in which one expresses what he or she feels, using only seventeen syllables in principle. The poem in Fig. 1 reads: ''To-o-ku-ka-ra, Hi-no-de-ru-ku-ni-e, A-ri-ga-to-o,'' as a total of only 17 syllables and it means, ''Thank you so much for coming all the way to the eastern country of the rising-sun-Japan.''

Fig. 2. Professor Shiro Itagaki — Pioneer and leader of research and education vetinary parasitology in Japan.

believe it is of great significance that, 60 years ago, in a pig's stomach as paratenic host, he found *Anisakis* larval parasitism, a disease which is similar to human anisakiasis larvae infection, now widely known as one of the parasitic zoonoses.

Since 1930, Dr. S. Itagaki had long conducted research on canine heartworm disease with Dr. S. Kume who was a Professor of the Department of Veterinary Internal Medicine, Tokyo University of Agriculture and Technology (Tokyo No-ko University). Later, together with Dr. I. Ohishi, his successor, and Dr. M. Hayasaki, they worked on patho-physiological studies and established diagnostic methods and treatment for this disease. This research is still continued as one of the major themes at their university.

During the 2nd World War, from 1940–1945, there were outbreaks of lumbar paralysis in sheep in the Peninsula of Korea, and it became a big problem. Dr. Jinkichi Fujita (shown in Fig. 4), one of the young followers of Dr. S. Itagaki, was sent from the University of Tokyo to the Veterinary Epidemiology Research Center in Korea. This was for the purpose of researching lumbar paralysis and other parasitic infections. Eventually Dr. Fujita found out that sheep lumbar paralysis is caused by the cerebro-spinal migration of *Setaria digitata* larvae. After returning to Japan, he trained or taught many scientists, first as Director-General of the National Institute for Animal Health, and later as a Professor of Parasitology at Nippon Veterinary and Zootechnical University. Besides that, he also made significant contributions to the development of scientific

A NEW LARVAL NEMATODE (ANISAKINAE)
FOUND IN THE STOMACH OF SWINE.

SHIRO ITAGAKI

(*From the Veterinary Department of the Faculty of Agriculture, Tokyo Imperial University, Tokyo, Japan.*)

(Received for publication May 2, 1928.)

The parasites described in this paper were offered to me by Mr. Otani, an inspector of the Tokyo slaughterhouse, who, during the winter of 1926, obtained them from the stomach of three slaughtered swines. The worms, I considered, may be a species of the subgenus Anisakinae *Raillet et Henry* belonging to Ascaroidea, owing to that they have the posterior part of the oesophagus. It is impossible, however, to determine whether they belong to Anisakis or Paranisakis unless the adult worms are discovered.

In examining these shree stomachs of the infested swines I found that the parasites attached firmly to the mucosa. From the gross appearance of the worms it seemed to be the adults of Physocephalus or Arduenna, but a microscopical examination of the worms revealed they were larval staged nematode of a hitherto nndescribed species.

So far as the writer is aware, no cases of this infestation with such larval nematode in swines have been reported. It may be therfore, of great interest to report these cases in detail.

The parasites were found attached chiefly to the anterior part and rarely other parts of the stomach. The affected stomach showed a feebly inflamed zone surrounding the infested areas and deep lineal ulcers in the mucous membrane from few millimeters to three centimeters long, which were dark red in colour as a result of chronic haemorrhage. Along these long ulcers several openings were seen, through which the worms projected only the caudal ends above the mucous membrane. The infected areas presented small circumscribed elevations on the mucosa and were found to be thickened by cell-infiltrations of the submucosa, but no lesions were found in the muscular layers.

Description of the parasites. The worms were all in en-

Fig. 3. The very first and important report on Anisakiasis presented in English by Professor S. Itagaki.

research as a president of the Veterinary Parasitology Association. Today, as a scientific advisor, he still gives invaluable advice and consultation to the association. During the period from 1941 to 1945, almost the same period that Dr. Fujita worked in Korea, there were outbreaks of horse-lumbar paralysis in Japan. Through various infection experiments, Professor S. Itagaki and Dr. C. Sho-ho found out that the cause of horse-lumbar paralysis was also caused by the cerebro-spinal migration of *Setaria digitata* larvae. Even today, there are occasions when Dr. Sho-ho presents the results of his research on *Setaria*. I believe his epidemiological research on *Setaria* is highly acclaimed world-wide.

Professor S. Itagaki not only made brilliant achievements as a scientist of parasitology, but he also, as president of Azabu University, made great contributions to the establishment and development of the educational system of veterinary science in Japan. His strong will regarding research in veterinary parasitology was continued by his son, Professor Hiroshi Itagaki. Dr. H. Itagaki is a Professor of Parasitology at the Veterinary School of Azabu University, and is conducting developmental and comparative morphological research on various helminths, using morphological and molecular biological methods. He is also a successor to Dr. J. Fujita and works very hard in developing young scientists in his role as president of Veterinary Parasitology Association.

Fig. 4. Professor Jinkicki Fujita — An elder scientific advisor in the Japanese Association of Veterinary Parasitologists.

At the present time, the scientific level of veterinary parasitology in Japan has reached highest levels through the efforts of many senior scientists and professors. They also have made contributions to developing young researchers and enhancing the stature of the Veterinary Parasitology Association. These senior researchers are the ones who took initiative in deciding to host the 15th World Conference here in Japan. If Professor H. Itagaki had been in good health, he is one individual who should have been the Chairperson of the LOC of the 15th Conference. On behalf of the Veterinary Parasitology Association in Japan, I would like to express my deep thanks to Professor H. Itagaki and all the other senior parasitologists who determined to have this conference in our country.

As I have explained so far, the students of Dr. S. Itagaki, who scattered to various universities and research institutes, continued and expanded his research, and established research on animal helminths as a main theme in Japan. Among them, at the Nippon Veterinary and Zootechnical University, Professors. J. Fujita and Toshio Ishii primarily investigated chicken coccidiosis and toxoplasmosis, Professors S. Imai and Saeki studied rumen protozoa and *Babesia* infections. These scientists are one of a few groups that

still place major emphasis on research of protozoan diseases of domestic animals. Of course, in the western part of Japan, at Osaka Prefectural University, Prof. A. Arakawa and his group have a most active research program on chicken coccidiosis.

Other students of Dr. S. Itagaki, Professors J. Yamashita and M. Ohbayashi, at the Veterinary School of Hokkaido University, energetically conducted research on echinococcosis and contributed to establishing diagnostic methods, applicable not only to domestic animals, but also humans. This research was taken over first by Professor Ohbayashi and later by Professors M. Kamiya and Y. Oku. It is one of the outstanding scientific achievements made in Japan.

In 1990 at Obihiro University, the Research Center for Protozoan Immunology was founded by the Government, as the only one of its kind at 16 universities of veterinary medicine and science in Japan. The purpose of this research center was the basic research of zoonotic protozoan diseases, using molecular immunological methods, as well as offering scientific cooperation to other countries. The Research Center is equipped with levels 1, 2 and 3 safety rooms for zoonotic protozoan infections. The Center has its own quarterly journal publication, *The Journal of Protozoology Research.* Its publication covers a wide range of research activities and outputs of international scientific cooperation in the fields of protozoology, not only in Japan, but also in developing countries in Asia, South America and Africa, and in Europe and North America. For the period 1995 to 2005, the Research Center initiated on Advanced Study Course of Protozoan Diseases, which is offered to 10 university scientists from each of 10 foreign countries for one year. The research and organisnal emphasis in this course is pathophysiological and molecular research on toxoplasmosis, trypanosomiasis, babesiosis and neosporosis. Professors A. Saito, H. Nagasawa, Y. Omata, I. Igarashi and M. Horiuchi constitute the professional staff of the Research Center studies on the theme mentioned above. Professor H. Hirumi (many of you probably know his name well) was the first in the world to succeed in reproducing a life cycle of *Trypanosoma* species by using an in vitro cultivation system. He is now conducting experimental research on african trypanosomiasis as a visiting professor invited by the Japanese government. Professor Y. Toyoda and his group are now conducting developmental embryo-engineering research on transgenic and/or targetting knockout animals, and consequently, I am personally expecting very significant results of the comprehensive research conducted by both research groups.

There are two primary veterinary parasitology research groups in Japan one of which is largely a university faculty that mainly conducts basic research. The other is a group of researchers at the National Institute for Animal Health who conduct a broad range of basic and applied developmental research. The number of researchers in the latter group is almost the same as that of parasitology researchers at universities and its research facilities are far better than those of the universities. From the time of its foundation up to the present the National Institute has made numerous remarkable achievements in research on the diagnosis, treatment and protective methods of parasitic diseases of domestic animals.

In particular, they have succeeded in solving problems of such protozoan diseases as coccidiosis, toxoplasmosis and piroplasmosis, as well as various helminth diseases. Starting with research on toxoplasmosis of animals, Dr. Shingo Ito and his group

conducted comparative biological research on the life cycles and therapeutic drugs for *Toxoplasma*, *Sarcocystis*, *Hammondia* and *Besnoitia* of various domestic animals. Needless to say, these achievements made great contributions to stimulating and activating the research on protozoan diseases in our country. Presently, Dr. Shimura and his group have taken over this research work. The research on bovine piroplasmosis, especially in theileriosis, was also mainly conducted at the National Institute. Since 1950, this research has been continued, first by Dr. T. Ishihara, later by Dr. T. Minami and most recently by Dr. K. Fujisaki as the reseach leader. Their research currently ranges widely from isolation and identification, infection response and protective mechanisms, and treatment through new research using advanced biotechnology techniques. It is not too much to say that the research group of the National Institute is a core research group of veterinary protozoan diseases in our country.

Since 1987, there have been outbreaks reported of sudden death of unknown cause in group-breeding calves in the southern part of Japan. This brought great financial loss to the stock-raising industry and occurred mainly in the summer. Groups of calves suddenly died without any symptoms in calf-houses for group breeding with saw-dust litter laid on pen floors. In 1991, Dr. N. Taira and his group found out, through extensive investigation, that calves died from severe infection with the nematode parasite *Strongyloides papillosus*. The exact cause of death because of natural or experimental infections seems not to have been found, but Dr. Taira and his group recognized outbreaks of this sudden death type of Strongyloidisis over most of Japan and therefore emphasized the need for and importance of hygienic control for calves in this management system. Thus, the Ministry of Agriculture and its related research institutes has established a research system combining basic research and applied developmental research, and acts as a center of excellence for present veterinary parasitology research in Japan.

3. Conclusion

I have given an outline of the history of veterinary parasitology research and education in Japan, tracing back the main Japanese researchers. Finally, on behalf of the Local Organizing Committee, I would like to summarize some aspects of the 15th Conference in Japan to all the participants.

The final numbers of people registered were a total of 291, including accompanying persons. Total number of scientists registered was 261. Among the 261 scientists, 128 registrants were from abroad and the number of participating countries was 38. One hundred and thirty-three persons attended from Japan. The final number of oral presenters was 93, and that of poster presenters was 51 persons. Speakers in nine plenary sessions and 11 workshops are included. I can indicate that plenary papers and workshop summaries will be published in a special issue of Veterinary Parasitology, and will be sent to all scientists registered. A copy will be also sent to all W.A.A.V.P. members, who did not attend the Yokohama Conference. I do hope that participants might expect to receive their copies in the first half of 1996. Special thanks to Pfizer, Inc. for making this possible.

In the course of this conference, various pending issues have been presented regarding solution of problems of animal parasitic disease. It is hoped that vigorous investigation of these and others will be continued and discussed in future W.A.A.V.P. conference.

I'd like to take this opportunity to express my deep and special thanks to all the members of Executive Committee and LOC, and President Dr. Slocombe, for their continuous efforts and valuable advice, and the financial support from many companies.

Veterinary Parasitology

Submission of manuscripts
Manuscripts should be submitted in triplicate to the Editorial Office of *Veterinary Parasitology*, P.O. Box 181, 1000 AD Amsterdam, The Netherlands. Authors in the Americas and Japan should submit their manuscripts directly to Dr. J.C. Williams, Department of Veterinary Science, Louisiana State University, 111 Dalrymple Building, Baton Rouge, LA 70803-6002, USA.

Authors in Japan please note: Upon request, Elsevier Science Japan will provide authors with a list of people who can check and improve the English of their paper (*before submission*). Please contact our Tokyo office: Elsevier Science Japan, 1-9-15 Hagashi-Azabu, Minato-ku, Tokyo 106; Tel. (03)-5561-5032; fax (03)-5561-5045.

Electronic manuscripts: Electronic manuscripts have the advantage that there is no need for the rekeying of text, thereby avoiding the possibility of introducing errors and resulting in reliable and fast delivery of proofs.
For the initial submission of manuscripts for consideration, hardcopies are sufficient. For the processing of *accepted papers*, electronic versions are preferred. After *final acceptance*, your disk plus two, final and exactly matching printed versions should be submitted together. Double density (DD) or high density (HD) diskettes (3.5 or 5.25 inch) are acceptable. It is important that the file saved is in the native format of the wordprocessor program used. Label the disk with the name of the computer and wordprocessing package used, your name, and the name of the file on the disk. Further information may be obtained from the Publisher.

All questions arising after acceptance of the manuscript, especially those relating to proofs, should be directed to Elsevier Editorial Services, Mayfield House, 256 Banbury Road, Oxford OX2 7DH, UK, tel. (01865) 314900, fax (01865) 314990.

***Veterinary Parasitology* has no page charges.**

For a full and complete Guide for Authors, please refer to *Veterinary Parasitology*, Vol. 60, Nos. 1–2, pp. 177–182.

0304-4017/96/$15.00

♾ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper).

Printed in The Netherlands

Appl. Parasitol. 37 (1996), 284–292
Gustav Fischer Verlag Jena

Mini review: Protective immunity in toxoplasmosis*

HIDEYUKI NAGASAWA[1], KUNISUKE HIMENO[2] and NAOYOSHI SUZUKI[1]

[1] The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Japan,
[2] Department of Parasitology and Immunology, School of Medicine, The University of Tokushima, Japan.

Accepted: 15 May 1996

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the subclass Coccidia. Toxoplasmosis is widespread in human beings and many other warm-blooded animals. The cat, *Felis catus*, and many other felines (e. g. lynx and bobcat), are the only hosts known to harbor the sexual stage of the parasite. Although *T. gondii* is an uncommon cause of disease in individuals with a normal functional immune system, immunocompromised hosts such as AIDS patients are at high risk of developing severe toxoplasmosis, especially toxoplasmic encephalitis as opportunistic infection (SIBLEY & BOOTHROYD 1992).

We showed earlier that T cells play an important role in protective immunity against infection with *Toxoplasma* parasites (SETHI et al. 1975; NAGASAWA 1984). However, we also found that the protective mechanisms involved in resisting infection with a strain of low virulence (Beverley strain) of *T. gondii* differ greatly from those involved in resisting infection with a highly virulent strain (RH strain) by the following results. When mice were immunized with homogenate of *Toxoplasma* tachyzoites before infection with a lethal dose of Beverley strain bradyzoites (1×10^4), the mice acquired resistance and survived. By contrast, vaccination with a sublethal dose of live Beverley strain bradyzoites (1×10^4) was required for acquisition of resistance to infection with the highly virulent RH strain (NAGASAWA et al. 1991). These findings seem consistent with our observations that immunization with *Toxoplasma* homogenate along with complete Freund's adjuvant failed to prevent infection of mice by tachyzoites of the RH strain. Therefore, it remains to be determined whether protective mechanisms against *T. gondii* are different between resistant and susceptible host species.

Recently, heat shock proteins (HSPs) have attracted the attention of immunologists as target of T cells for specific recognition by antibodies and T cells of the immune system. Exposure of cells to a veriety of stressful conditions such as elevated temperature, stressful chemical intoxication, or infection lead to the transcription of a highly conserved set of genes and, subsequently, to the synthesis of a family of polypeptides calles HSPs (LINDQUIST 1986; SCHLESINGER 1986; PELHAM 1988).

* This publication is dedicated to Prof. Dr. M. ROMMEL (Hannover) on the occasion of his 60th birthday.

HSPs have been identified as major immunogens in certain infectious diseases, for example leprosy and tuberculosis (YOUNG et al. 1988), filariasis (ROTHENSTEIN et al. 1989), schistosomiasis (HEDESTROM et al. 1987), malaria (BIANCO et al. 1986) and Chagas' disease (ENGMAN et al. 1990). Immunodominant antigens from a wide variety of bacteria and parasites have been identified by sequence homology as belonging to the family of HSPs (YOUNG et al. 1988). Among the various HSPs, a 65 kD mycobacterial HSP has been identified as a target of T cells, including γδ T cells (VAN EDEN et al. 1988; RES et al. 1988; KOGA et al. 1989; O'BRIEN et al. 1989; HOLOSHITZ et al. 1989; HAREGEWIN et al. 1989). This HSP contains a significant sequence similarity and cross-reactivity with antigens from a variety of other microbes. T cells reactive to HSP65 derived from pathogen exhibit cytotoxicity to macrophages expressing host-derived HSP65 (KOGA et al. 1989), suggesting that HSP contributes to the elimination on intracellular parasites as target antigen on the infected cells. Moreover, HSP65 is known as one of the ligands of γδ T cells (O'BRIEN et al. 1991), which participate in protection against early phase of infections with intracellular pathogens (HIROMATSU et al. 1992; RAZIUDDIN et al. 1992).
In this paper, we present that HSP65 is expressed in host peritoneal macrophages of mice after infection with *T. gondii*. The degree of expression of this apparent HSP correlates with protection that occurred in exposed hosts, regardless of differences in virulence or strain specificity of this protozoan or in species of host. We have also shown here that γδ T cells rather than αβ T cells participate in the induction of HSP65 on macrophages of hosts which have acquired resistance against toxoplasmosis.

Expression of HSP65 in mice infected with a low virulent strain of *T. gondii*

The role of HSPs in infection and immunity is receiving much attention, and it has been postulated that HSP response to stress during inflammation actually plays a role in the host defense against certain infections (POLLA & KANTENGWA 1991). HSPs in parasite infection appear, on the one hand, to play important roles in adaptation of microorganisms. For example, they may play a role in differentiation of parasites and in development of infectivity (SMEJKAL et al. 1988; BUCHMEIER & HEFFRON 1990). On the other hand, HSPs function as prominent antigenic proteins that can activate the host immune system (HAREGEWOIN et al. 1989).
We have reported that HSP65, measured by electroblot assay with specific mAb (IA 10), is expressed in peritoneal exudate cells (PEC) of mice infected with a low-virulent strain of *Toxoplasma* (Beverley strain) but is not expressed if infected with a high-virulent RH strain (NAGASAWA et al. 1992). Also, the expression of HSP65 decreases in the PEC lysate from mice infected with serially passaged tachyzoites of the Beverley strain, which, in turn, increases in pathogenicity to approximate the RH strain in terms of infectivity. These findings suggest that expression of virulence in these parasites correlates with interaction between the host and parasites. This correlation occurs despite major differences between the strains of *Toxoplasma*. It is, therefore, postulated that the low-virulent strain of *T. gondii* with the capacity to persist in host macrophages for prolonged periods may generate production of abundant quantities of host-derived HSP that can then be expressed on the surface of macrophages and, thus, presented effectively to T cells to induce immunity. By contrast, mice infected with a highly virulent strain of *T. gondii* (RH strain) seem unable to produce HSP on the macrophages they infect (Table 1).

Table 1. Relationship between the expression of 65 kD-heat shock protein (HSP65) and protection against *Toxoplasma* infection.

	Host	Infection with	Expression of HSP65	Resistance to infection
mouse	normal	–	–	
	normal	RH straintachyzoites	–	–
	normal	Beverley strain bradyzoites	+	+
	normal	Beverley strain 1st tachyzoites	±	±
	normal	Beverley strain 6th tachyzoites	±	–
	homogenate-immune	–	+	
	homogenate-immune	Beverley strain bradyzoites	+	+
	homogenate-immune	RH strain tachyzoites	±	–
	bradyzoite-immune	–	+	
	bradyzoite-immune	RH strain tachyzoites	+	+
rat	normal	RH strain tachyzoites	+	+
	nu/nu	RH strain tachyzoites	–	–
	LEC	RH strain tachyzoites	–	±

Mammalian cells may synthesize HSPs in response to infections and/or physiological stimulation. With regard to phagocytes, these cells protect themselves from noxious molecules that they produce, such as highly reacitve oxygen metabolites. Phagocytosis and physiological activators of the oxidative burst induce HSP synthesis in macrophages (POLLA & KANTENGWA 1991). However, *Toxoplasma* parasites may survive and replicate within macrophages after phagocytosis. The survival of *Toxoplasma* within certain phagocytic cells has been studied by WILSON et al. (1980). These investigators showed that survival of tachyzoites within human monocyte-derived macrophages and normal mouse peritoneal macrophages can be attributed fo failure of this parasite to stimulate an oxidative burst that normally occurs with phagocytosis of *Candida, Staphylococcus* spp., or latex particles. During early phase post-infection, infectivity or virulence of intracellular parasites should be defined by two factors. One factor will be determined by the parasites themselves as cited above and another factor should be regulated by natural resistance genes expressed in host macrophages (BLACKWELL et al. 1991). At late stages, T cells will mainly control infections, activating or directly destroying infected macrophages.

Relationship between the expression of HSP65 on host macrophages and protective immunity in mice infected with *T. gondii*

When mice were immunized with *Toxoplasma* cell homogenate, HSP65 was detectable in PEC from these mice after immunization but was not detectable in either unimmunized controls or *Toxoplasma* cell homogenate itself. To determine which cell fraction of PEC expressed HSP65, PEC from mice immunized with *Toxoplasma* homogenate were separated into adherent and non-adherent cell fractions. As a result, HSP65 was expressed by the adherent cell fraction. Similar results were obtained by an immuno-histochemical electron microscopy using gold parti-

cles. The specific labeling was seen on the cell surface of peritoneal macrophages from mice after immunization with *Toxoplasma* homogenate, while very few gold particles were also found in the cytoplasm (NAGASAWA et al. 1994). Furthermore, mice that acquired resistance against a high-virulent RH strain after the resolution of infection with Beverley strain bradyzoites strongly expressed HSP65 in their peritoneal macrophages (Table 1). We also reported that T cells play a major role in mediating protective immunity against low- and high-virulent strains of *T. gondii* from studies in which T cells were depleted *in vivo* with a mAb against Thy1.2 (NAGASAWA et al. 1991). Further evidence comes from the finding that interferon γ released from sensitized T cells acts as a major mediator of the host defenses against *T. gondii* infection (GAZZINELLI et al. 1991; SUBAUSTE & REMINGTON 1991). Taking these results together, one might suggest that the expression of HSPs in mice immunized by *Toxoplasma* homogenate may be attributable to cytokines like interferon γ. Such cytokines may induce T cells to react to and attack macrophages infected with *Toxoplasma* parasites. Indeed, macrophages subjected to interferon γ activation are recognized by class-I-restricted $CD8^+$ T cells raised against HSP65 (KOGA et al. 1989). Perhaps, bradyzoite-induced immunity in mice provides a constant source of HSP antigens for T cell stimulation and thus cannot cause clinical disease. Once activated, macrophages would be expected to rapidly eliminate the highly virulent parasites before clinical disease develops.

HSPs expressed within host cells may also participate in the elimination of pathogens, either by anonimmunological self-nonself discrimination mechanism, as hypothesized by FORSDYKE (1985), or as a consequence of processing and presentation of foreign antigens for effective immunity (VAN BUSKIRK et al. 1989). In the course of characterizing T-cell stimulatory antigens of tubercle or leprosy bacilli, T cells with reactivity to HSP65 have frequently been identified. In mice immunized with killed *Mycobacterium tuberculosis*, > 10% of T cells that exhibit reactivity to whole *M. Tuberculosis* particles recognize HSP65 (KAUFMANN 1989). Moreover, a significant number of healthy individuals possess T cells specific for the mycobacterial HSP65 (MUNK et al. 1988). Thus, the cellular immune response to HSP65 cannot be taken as an identification of immunity to tuberculosis or leprosy, and use of this antigen is inappropriate for diagnosis of these diseases. Still, HSP may contribute to acquired resistance against a variety of intracellular pathogens (KAUFMANN 1989). Because of their high degree of conservation in microbes, HSP65, as well as other HSPs is likely to be seen by the immune system quite frequently.

Correlation between the expression of HSP65 and acquisition of protective immunity to *T. gondii* exists across the barrier of species of host

We intended to clarify the correlation between the resistance to *T. gondii* and the expression of HSP65 on host macrophages by comparing these two parameters in various strains of rats including athymic nude rats and $CD4^+$ T cell-deficient rats (LEC). In contrast to mice, normal rats (F344 and LEA) were strongly resistant to infection even with a high-virulent strain (RH) of *T. gondii* and HSP65 was strongly expressed on their macrophages. However, only half of LEC rats which were genetically defective in $CD4^+$ T cells (AGUI et al. 1990) could control the infection and HSP65 was only faintly expressed. Furthermore, all of athymic nude rats died of acute infection and this protein was scarcely expressed on their

macrophages (HISAEDA et al. 1993). Thus, in rats, T cells especially $CD4^+$ T cells, appear to contribute to pretection against infection with the RH strain of *T. gondii*, although they are extremely resistant as compared with mice. HSP65 was not expressed on either *T. gondii* themselves or peritoneal macrophages of uninfected rats. These results indicate that expression of HSP65 closely correlates with protection against *Toxoplasma* infection not only in mice but in rats as well. Moreover, T cells, especially $CD4^+$ T cells, appear to be important in acquiring resistance against *T. gondii* infection, and play a crucial role in the expression of HSP65.

Role of γδ T cells in protection against *Toxoplasma* infection

Recently, a subset of γδ T cells has been shown to recognize HSP65 (O'BRIEN et al. 1989; HOLOSHITZ et al. 1989; HAREGEWOIN et al. 1989) and was found to increase rapidly in peripheral blood of patients with acute *T. gondii* infection (DE PAOLI et al. 1992; SCALISE et al. 1992). Thus, it is not surprising that γδ T cells recognizing HSP65 are involved in protective immunity in some kinds of infection including toxoplasmosis. This T cell subset is thought to possibly represent a first line of defense against infection and is probably demonstrable in normal individuals. Furthermore, these HSP65-reactive γδ T cells should have been primed previously by contact with many different microbes or by exposure to HSP generated in host cells under various stressful conditions. The role of HSPs as a selective target for γδ T cells remains more speculative than the role of HSPs in other forms of immunologic reactions, particularly nonspecific resistance to parasites.

We showed earlier that treatment of BALB/c mice with anti-Thy1.2 mAb one day before immunization with *Toxoplasma* homogenate led to an almost complete loss of the expression of HSP65. To determine the subsets of T cells responsible for induction of this protein, mice were depleted of γδ T cells, αβ T cells, $CD4^+$ T cells or $CD8^+$ T cells by treatment with corresponding mAbs before immunization. From these experiments, γδ T cells were shown to be essential for the expression of HSP65, although $CD4^+$ αβ T cells also contributed to some extent (NAGASAWA et al. 1994).

Several investigators have found that the number of γδ T cells increases in mice infected with intracellular pathogens, e.g., *Plasmodium, Leishmania, Salmonella*, and *Listeria*, and they have suggested that the γδ T cells play a crucial role in protection against these pathogens (VAN DER HEYDE et al. 1993; ROSAT et al. 1993; EMOTO et al. 1992; HIROMATSU et al. 1992). We have also reported that the nzumber of γδ T cells expanded in the peritoneal cavity and spleen in the early stage of infection with *T. gondii*, and HSP65 was expressed in their peritoneal macrophages (HISAEDA et al. 1995). In addition, when mice were depleted of γδ T cells by the administration of a mAb before infection with *Toxoplasma* bradyzoites, HSP65 expression was markedly decreased. In contrast, the expression of this protein was rather enhanced and γδ T cells were prominently expanded in mice depleted of αβ T cells. The protection in mice treated with the mAb parallel the magnitude of HSP65 expression. Mice depleted of γδ T cells died most frequently in the early stages of infection, whereas most of those depleted of αβ T cells survived the early stages of lethal infection with *T. gondii*. However, the latter group of mice did not definitely control the *T. gondii* infection in its late stages. From these findings, γδ T cells appear to play an important role in protective immunity against infection with *T. gondii* through mediating the expression of HSP65 in host macrophages.

Conclusion

Characterization of effector and regulatory functions of HSPs in other hosts and microbial systems should provide insight into mechanisms of virulence and protective adaptations that control virulence. Thus, it seems likely that HSPs could be of a critical importance in numerous host-parasite relationships, including resistance of

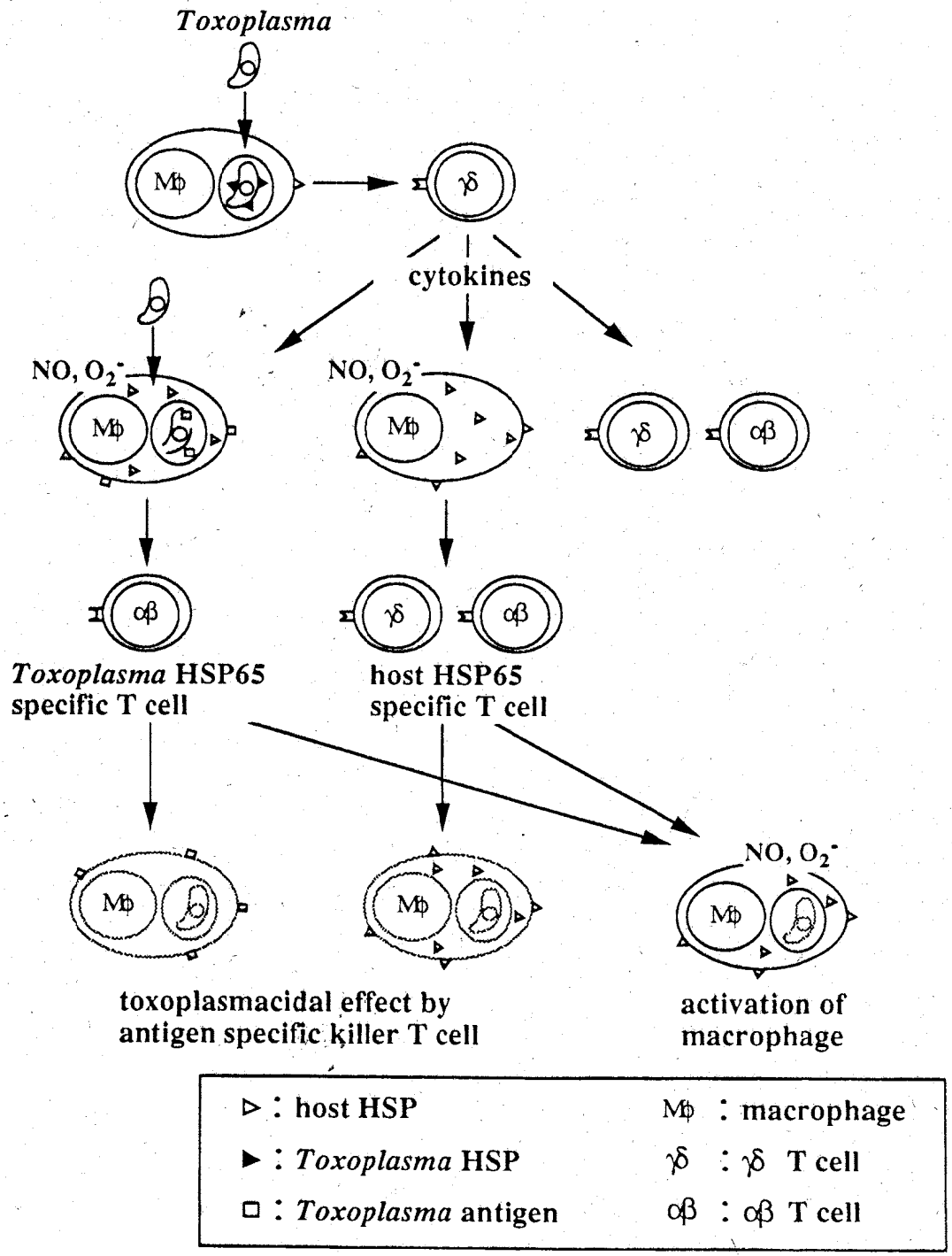


Figure 1. Role of HSP65 in protective immunity against infection with *Toxoplasma gondii*.

host to otherwise destructively virulent parasites. At any rate, it seems clear from a series of our experiments that expression of HSP65 on peritoneal macrophages correlates dramatically with the capacity to inhibit destructive consequences of infection with both low-virulent and high-virulent strains of *T. gondii*.

From these bases, the expression mechanism and the biological role of HSP65 may be as follows. As the first step after infection with a low-virulent strain of *Toxoplasma*, circulating γδ T cells recognize either *Toxoplasma*-derived HSP65 or host-derived HSP65, and then they accumulate and are activated. As the second step, macrophages activated by γδ T cells probably via certain cytokine pathways exhibit an enhanced respiratory burst releasing noxious molecules, e.g. oxygen metabolites, a major protective mechanism against intracellular pathogens like *T. gondii*. As the third step, activated macrophages synthesize endogenous HSP65 for protection against these toxic molecules, for repairment of damaged functions of themselves or for effective antigen-presentation. Finally, either γδ T cells and αβ T cells reactive to HSP65 or αβ T cells specific for *Toxoplasma* antigen further accumulate and are activated. Such T cells directly destroy the host macrophages or activate macrophages to kill the intracellular *Toxoplasma* parasites (Fig. 1).

We showed here that γδ T cells rather than αβ T cells participate in the induction of HSP65 on macrophages of hosts which have acquired resistance against toxoplasmosis. However, the still remain undetermined mechanisms of expression of HSP65 and the role of this protein in the protection against this infection.

Furthermore, it is very important to elucidate whether γδ T cells which induce the expression of HSP65 and those which accumulate recognizing HSP65 involve in the same functional subset, and whether the former and the latter subset(s) of γδ T cells recognize different antigens or the same epitope of HSP65. Moreover, it is of interest to investigate the relationship between γδ T cells and αβ T cells in protective immunity, both of which have an ability to induce this protein.

References

AGUI, T.; OKA, M.; YAMADA, T.; SAKAI, T.; IZUMI, K.; ISHIDA, S.; HIMENO, K.; MATSUMOTO, K. (1990): Maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in mutant strain (LEC) of rat. – J. Exp. Med. **172**: 1615–1624.

BIANCO, A. E.; FAVALORO, J. M.; BURKOT, T. R.; CULVENOR, J. G.; CREWTHER, P. E.; BROWN, G. B.; ANDRES, R. F.; COPPEL, R. L.; KEMP, D. J. (1986): A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein of *Drosophila melanogaster*. – Proc. Natl. Acad. Sci. USA **83**: 8713–8717.

BLACKWELL, J. M.; ROACH, T. I. A.; ATKINSON, S. E.; AJIOKA, J. W.; BARTON, C. H.; SHAW, M.-A. (1991): Genetic regulation of macrophage priming/activation: the Lsh gene story. – Immunol. Letters **30**: 241–248.

BUCHMEIER, N. A.; HEFFRON, F. (1990): Induction of *Salmonella* stress proteins upon infection of macrophages. – Science **248**: 730–732.

DE PAOLI, P.; BASAGLIA, G.; GENNARI, D.; CROVATTO, M.; MODOLO, M. L.; SANTINI, G. (1992): Phenotypic profile and functional characteristics of human gamma and delta T cells during acute toxoplasmosis. – J. Clin. Microbiol. **30**: 729–731.

EMOTO, M.; DANBARA, H.; YOSHIKAI, Y. (1992): Induction of γδ T cells in murine salmonellosis by an avirulent but not by a virulent strain of *Salmonella choleraesuis*. – J. Exp. Med. **176**: 363–372.

ENGMAN, D. M.; DRAGON, D. A.; DONELSON, J. E. (1990): Human humoral immunity to hsp70 during *Trypanosoma cruzi* infection. – J. Immunol. **144**: 3987–3991.

FORSDYKE, D. R. (1985): Heat shock proteins defend against intracellular pathogens; a non-immunological basis for self/nonself discrimination. – J. Theor. Biol. **115**: 471–473.

GAZZINELLI, R. T.; HAKIM, F. T.; HIENY, A. S.; SHEARER, G. M.; SHER, A. (1991): Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-γ production and protective immunity induced by an attenuated *Toxoplasma gondii vaccine*. – J. Immunol. **146**: 286–292.

HAREGEWOIN, A.; SOMAN, G.; HOM, R. C.; FINBERG, F. W. (1989): Human γδ+ T cells respond to mycobacterial heat-shock protein. – Nature (London) **340**: 309–312.

HEDESTROM, R.; CULPEPPER, J.; HARRISON, R. A.; AGABIAN, M.; NEWPORT, G. (1987): A major immunogen in *Schistosoma mansoni* infection is homologous to the heat-shock protein Hsp70. – J. Exp. Med. **165**: 1430–1435.

HIROMATSU, K.; YOSHIKAI, Y.; MATSUZAKI, G.; OHGA, S.; MURAMORI, K.; MATSUMOTO, K.; BLUESTONES, J. A.; NOMOTO, K. (1992): A protective role of γ/δ T cells in primary infection with *Listeria monocytogenes* in mice. – J. Exp. Med. **175**: 49–56.

HISAEDA, H.; NAGASAWA, H.; MAEDA, K.; ISHIKAWA, H.; CHAI, J.-G.; MAEKAWA, Y.; ITO, Y.; AGUI, T.; MATSUMOTO, K.; HIMENO, K. (1993): Correlation between heat shock protein expression and the acquisition of protective immunity to *Toxoplasma gondii* in rats and mice. – Jpn. J. Parasitol. **42**(6): 461–467.

HOLOSHIZ, J.; KONING, F.; COLOGANM, J. E.; BRUYN, J. D.; STROBER, S. (1989): Isolation of CD4- CD8- mycobacteria-reactive T lymphocytes clones from rheumatoid arthritis synovial fluid. – Natrue (London) **339**: 226–229.

KAUFMANN, S. H. E. (1989): Immunity to bacteria and fungi. – Curr. Opin. Immunol. **1**: 431–440.

KOGA, T.; WAND-WURTTENBERGER, A.; DEBRUYN, J.; MUNK, M. E.; SCHOEL, B.; KAUFMANN, S. H. E. (1989): T cells against bacterial heat shock protein recognized stressed macrophages. – Science **245**: 1112–1115.

LINDQUIST, S. (1986): The heat-shock responses. – Ann. Rev. Biochem. **55**: 1151–1191.

MUNK, M. E.; SCHOEL, B.; KAUFMANN, S. H. E. (1988): T cell responses of normal individuals towards recombinant protein antigens of *Mycobacterium tuberculosis*. – Eur. J. Immunol. **18**: 1835–1838.

NAGASAWA, H. (1984): Immune responses in experimental toxoplasmosis in mice treated with carrageenan. – Jpn. J. Parasitol. **33**: 265–270.

– MANABE, T.; MAEKWA, Y.; OKA, M.; HIMENO, K. (1991): Role of L3T4+ and Lyt-2+ T cell subsets in protective immune responses of mice against infection with a low and high virulent strain of *Toxoplasma gondii*. – Microbiol. Immunol. **35**: 215–222.

– OKA, M.; MAEDA, K.; CHAI, J.-G.; HISAEDA, H.; ITO, Y.; GOOD, R. A.; HIMENO, K. (1992): Induction of heat shock protein closely correlates with protection against *Toxoplasma gondii* infection. – Proc. Natl. Acad. Sci. USA. **89**: 3155–3158.

– HISAEDA, H.; FUJIOKA, H.; AIKAWA, M.; HIMENO, K. (1994): γδ T cells play a crucial role in the expression of 65 000 heat-shock protein in mice immunized with *Toxoplasma* antigen. – Immunology **83**: 347–352.

O'BRIEN, R. L.; HAPP, M. P.; DALLA, A.; PALMER, E.; KUBO, R.; BORN, W. K. (1989): Stimulation of a major subset of lymphocytes expressing T cell receptor γδ by an antigen derived from *Mycobacterium tuberculosis*. – Cell **57**: 667–674.

– – – CRANFILL, R.; HALL, L.; LANG, J.; FU, Y. X.; KUBO, R.; BORN, W. (1991): Recognition of a single hsp-60 epitope by an entire subset of γδ T lymphocytes. – Immunol. Reviews **121**: 155–170.

PELHAM, H. (1988): Heat shock proteins: Coming in from the cold. – Nature (London) **332**: 776–777.

POLLA, B. S.; KANTENGWA, S. (1991): Heat shock proteins and inflammation. – Curr. Trop. Microbiol. **167**: 93–105.

RAZIUDDIN, S.; TELMASANI, A. W.; EL-AWAD, M. E. H.; AL-AMARI, O.; AL-JANADI, M. (1992): γδ T cells and the immune response in visceral leishmaniasis. – Eur. J. Immunol. **22**: 1143–1148.

RES, P. C. M.; SCHARR, C. G.; BREEDVELD, F. C.; VAN EDEN, W.; VAN EMDEN, J. D.; COHEN, I. R.; DE VRIES, R. R. P. (1988): Synovial fluid T cell reactivity against 65 kd heat shock protein of mycobacteria in early chronic arthirits. – Lancet **11**: 478–480.

ROSAT, J. P.; MACDONALD, H. R.; LOUIS, J. A. (1993): A role for γδ+ T cells during experimental infection of mice with *Leishmania major*. – J. Immunol. **150**: 550–555.

ROSTEIN, N. M.; HIGASHI, G.; YATES, J.; RAJAN, T. V. (1989): *Onchocerca volvulus* heat shock protein 70 is a major immunogen in amicrofilaremic endemic area. – Mol. Biochem. Parasitol. **33**: 229–236.

SCALISE, F.; GERLI, R.; CASTELLUCCI, G.; SPINOZZI, F.; FABIETTI, G. M.; CRUPI, S.; SENSI, L.; BRITTA, R.; VACCARO, R.; BERTOTTO, A. (1992): Lymphocytes bearing the γδ T-cell receptor in acute toxoplasmosis. – Immunology **76**: 668–670.

SCHLESINGER, M. J. (1986): Heat shock proteins: The research for functions. – J. Cell. Biol. **103**: 321–325.

SETHI, K. K.; PELSTER, B.; SUZUKI, N.; PIEKARSKI, G.; BRANDIS, H. (1975): Immunity to *Toxoplasma gondii* induced in vitro in non-immune mouse macrophages with specifically immune lymphocytes. – J. Immunol. **115**(4): 1151–1158.

SIBLEY, L. D.; BOOTHROYD, J. C. (1992): Virulent strain of *Toxoplasma gondii* comprise a single clonal lineage. – Nature (London) **359**: 82–85.

SMEJKAL, R. M.; WOLFF, R.; OLENICK, J. G. (1988): *Leishmania braziliensis panamensis*: Increased infectivity resulting from heat shock. – Exp. Parasitol. **65**: 1–9.

SUBAUSTE, C. S.; REMINGTON, J. S. (1991): Role of gamma interferon in *Toxoplasma gondii* infection. – Eur. J. Clin. Microbiol. Infect. Dis. **10**: 58–67.

VAN BUSKIRK, A.; CRUMP, B. L.; MARGOLIASH, E.; PIERCE, S. K. (1989): A peptide binding protein having a role in antigen presentation is a member of the hsp70 heat shock protein family. – J. Exp. Med. **170**: 1799–1808.

VAN DER HEYDE, H. C.; ELLOLO, M. M.; ROOPENIAN, D. C.; MANNING, D. D.; WEIDANZ, W. P. (1993): Expansion of the CD4, CD8-γδ T cell subset in the spleens of mice during non-lethal blood-stage malaria. – Eur. J. Immunol. **23**: 1846–1850.

VAN EDEN, W.; THOLE, J. E. R.; VAN DER ZEE, R.; NOORDZIJ, A.; VAN EMDEN, J. D.; HENSEN, E. J.; COHEN, I. R. (1988): Cloning of the mycobacterial epitope recognized by T-lymphocytes in adjuvant arthritis. – Nature (London) **331**: 171–173.

WILSON, C. B.; TSAI, V.; REMINGTON, J. S. (1980): Failure to trigger the oxidative metabolic burst by normal macrophages: possible mechanism for survival of intracellular pathogens. – J. Exp. Med. **151**: 328–346.

YOUNG, D. B.; LATHIGRA, R.; HENDRIX, R.; SWEETSER, D.; YOUNG, R. A. (1988): Stress proteins are immune target in leprosy and tuberculosis. – Proc. Natl. Acad. Sci. USA. **85**: 4267–4270.

Address of the authors: c/o Dr. HIDEYUKI NAGASAWA, The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan.

EXPERIMENTAL PARASITOLOGY 81, 614–617 (1995)

RESEARCH BRIEF

Toxoplasma gondii: Parasiticidal Effects of Bovine Lactoferricin against Parasites

TETSUYA TANAKA,* YOSHITAKA OMATA,* ATUSHI SAITO,* KEIICHI SHIMAZAKI,† KOJI YAMAUCHI,‡ MITSUNORI TAKASE,‡ KOUZOU KAWASE,‡ IKUO IGARASHI,§ AND NAOYOSHI SUZUKI§

**Department of Veterinary Physiology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080 Japan; §The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, 080 Japan; †Department of Dairy Science, Faculty of Agriculture, Hokkaido University, Sapporo, 060 Japan; and ‡Nutritional Science Laboratory, Morinaga Milk Industry Co. Ltd., Zama, 228 Japan*

TANAKA, T., OMATA, Y., SAITO, A., SHIMAZAKI, K., YAMAUCHI, K., TAKASE, M., KAWASE, K., IGARASHI, I., AND SUZUKI, N. 1995. *Toxoplasma gondii:* Parasiticidal effects of bovine lactoferricin against parasites. *Experimental Parasitology* 81, 614–617.

INDEX DESCRIPTORS: Lactoferricin; *Toxoplasma gondii;* Parasiticidal effect.

Lactoferrin is a cationic iron-binding protein with broad spectrum antimicrobial properties, produced by the mammary glands and neutrophils (Bullen *et al.* 1972).

Recently, an antimicrobial peptide generated by pepsin digestion of bovine lactoferrin was isolated and sequenced. The active peptide, named lactoferricin B (LFcin-B), is derived from the N-terminal region of the molecule and is composed of 25 amino acid residues. It inhibits the growth of gram-negative and gram-positive bacteria due to its ability to cause direct damage to microbial membranes (Bellamy *et al.* 1993; Jones *et al.* 1994). Considerable potential seems to exist for use of this peptide to inactivate foodborne pathogens.

Toxoplasma gondii (*T. gondii*) is an obligatory intracellular parasitic protozoan transmitted by ingestion of uncooked infected meat. This organism causes congenital infections and opportunistic infections in immunodeficient individuals. It is unknown whether LFcin-B is effective against such parasitic protozoa.

In the present study, we have examined the effect of LFcin-B on the viability and infectivity of *T. gondii* parasites both *in vitro* and *in vivo*. LFcin-B was prepared from bovine lactoferrin as described by Bellamy *et al.* (1993). The purity of the LFcin-B used was confirmed by high performance liquid chromatography and by amino acid sequencing.

The C-terminal region of bovine lactoferrin was prepared by procedures described by Shimazaki *et al.* (1993). *T. gondii* parasites of the RH strain were obtained from the peritoneal cavity of infected mice on the third day postinoculation. The parasite-enriched peritoneal exudate was washed by centrifugation at 1200*g* for 10 min in PBS three times. The parasites were isolated by Percoll–sucrose solution density gradient centrifugation (gravity; 1.070) at 40,000*g* for 20 min and then suspended in Dulbecco's modified minimum essential medium (D-MEM) containing 1% bovine serum albumin (D-MEM–1%BSA). Cysts of the S-273 strain of *T. gondii* were obtained from the brains of chronically infected mice by Percoll–PBS density gradient centrifugation as described by Cornelissen *et al.* (1981) and suspended in D-MEM containing 10% fetal bovine serum (D-MEM–10FBS)

Mouse embryonal cells (MEC) were prepared as described elsewhere (Omata *et al.* 1990) and cultured in D-MEM–10FBS. For experiments, MEC were harvested from culture flasks by incubation with PBS containing 0.025% trypsin, centrifuged at 800*g* for 10 min, and suspended in D-MEM–10FBS at 5×10^4 cells/ml. Aliquots of 0.2 ml of this cell suspension were mounted on an oval coverslip (15 mm in diameter, Matsunami glass Inc. Osaka, Japan) and incubated at 37°C overnight.

To examine the parasiticidal effect of LFcin-B, bovine lactoferrin, and a peptide from the C-terminal region of bovine lactoferrin, suspensions of the parasites in D-MEM–1%BSA in a series of tubes were treated with LFcin-B at 100 or 1000 μg/ml, bovine lactoferrin at 1000 μg/ml, the C-terminal peptide at 1000 μg/ml, or were untreated (control). These suspensions were incubated at 37°C for 15 min to 4 hr. The parasites in each series of tubes were then washed thoroughly by centrifugation at 1200*g* for 10 min, three times in PBS, and finally resuspended in D-MEM–1%BSA at 1×10^6/ml parasites.

To examine the exclusion of trypan blue dye as a criterion of parasite viability, a 5-μl aliquot of the parasite suspension was mixed with the same volume of PBS containing 0.5% trypan blue, and staining was immediately assessed under a light microscope. The number of stained parasites was counted among a total of 200 parasites in four samples

0014-4894/95 $12.00

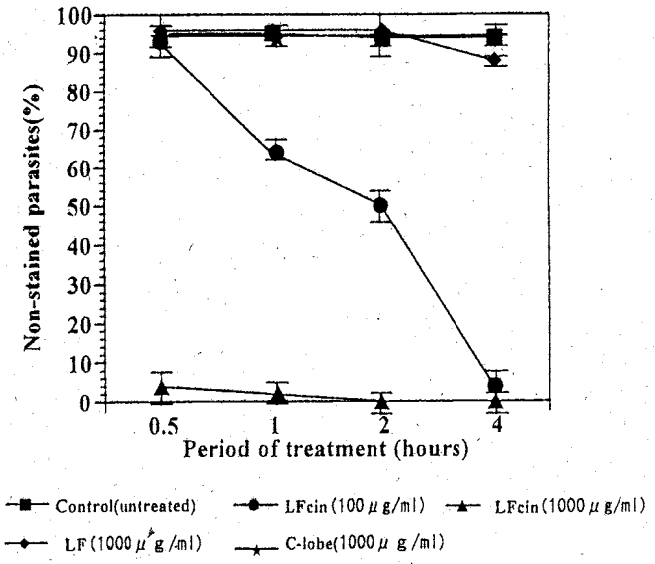


FIG. 1. Effect of LFcin-B on the viability of *T. gondii*. Parasites were incubated with LFcin-B at defined concentrations for the periods indicated, and then viability was examined by the trypan blue dye exclusion assay. The mean percentage of nonstained parasites is shown. Values shown are mean ± SD of triplicate samples.

of each series. The ratio of stained parasites was represented as the mean of percentages.

For assay of infectivity of the parasite to host cells, 0.1 ml of either treated or untreated parasites was mounted onto the MEC monolayered coverslips and incubated at 37°C. At 18 hr post inoculation (p.i.), the coverslips were washed in PBS thoroughly, fixed with methanol, and stained with Giemsa. The number of infected cells was counted among a total of 500 cells and the percentage of infected cells was calculated.

The infectivity of treated parasites and/or cystozoites in cysts after incubation with LFcin-B for 4 hr was also examined by an inoculation test in mice. Five mice in each group were inoculated intraperitoneally with 10^2 parasites, either untreated or pretreated with various concentrations of LFcin-B, and the survival time and mortality of the mice were monitored for up to 30 days p.i. Samples from the mice that died during the monitoring period were examined microscopically for the presence of parasites in the peritoneal cavity. To examine the infectivity of cysts, five mice per group were inoculated with 10^2 cysts, either untreated or pretreated with LFcin-B as described above, and the survival time of the mice was monitored for up to 30 days p.i. The number of cysts in the brains of individual mice was counted as described by Mcleod *et al.* (1989). To confirm the infection, the level of anti-*T. gondii* IgG in the serum of the mice was measured by an indirect immunofluorescence assay as described elsewhere (Omata *et al.* 1989).

All experiments described above were done in triplicate.

A preliminary experiment was carried out to determine the putative anti-toxoplasma activity of LFcin-B. Viability of *T. gondii* was examined after incubation of the parasite suspension with LFcin-B for various periods. As shown in Fig. 1, after treatment of *T. gondii* with LFcin-B at 100 μg/ml for 1 hr, 64% of the parasites became oval in shape and had lost the ability to exclude trypan blue dye. Their nuclei showed little staining by Giemsa. At 4 hr p.i., more than 96% of the parasites showed no dye exclusion ability. Similarly, approximately 96% of the parasites treated with LFcin-B at 1000 μg/ml for 0.5 hr lost the dye exclusion ability. After 2 hr of incubation with LFcin-B at 1000 μg/

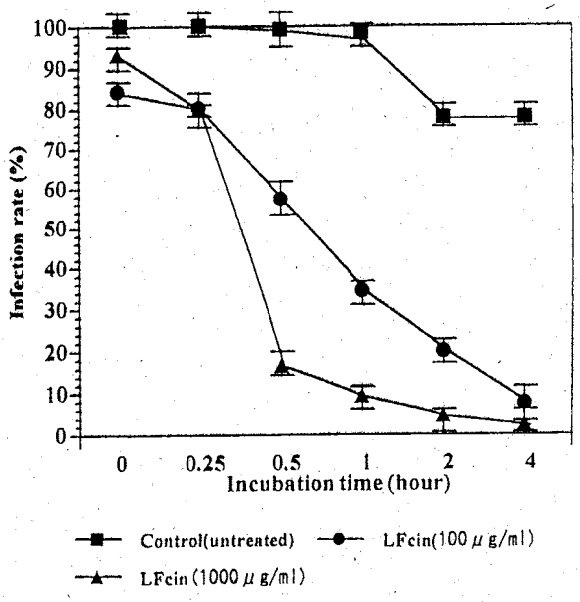


FIG. 2. Effect of LFcin-B on the infectivity of *T. gondii* in MEC. Parasites were incubated with LFcin-B at 37°C for the time indicated and then were applied onto MEC monolayered coverslips for 18 hr. The infection rate shown is the percentage of infected cells among a total of 500 cells. Mean ± SD of triplicate samples.

TABLE I
Infectivity of *T. gondii* in Mice after Treatment with LFcin-B

Treatment of parasites	No. of mice inoculated	Mortality (%)	Anti-*T. gondii* IgG in serum[a]
Untreated	5	100	ND
LFcin (100 μg/ml)	5	80	<4
LFcin (1000 μg/ml)	5	20	<4
LF (1000 μg/ml)	5	100	ND

Note. ND, not done.
[a] Anti-*T. gondii* IgG titers in the serum of surviving mice.

ml, no unstained parasites were detected. In contrast, more than 80% of the parasites incubated with bovine lactoferrin or C-terminal peptide at 1000 μg/ml for 4 hr retained the dye exclusion ability. Likewise, the parasites incubated with D-MEM alone for 4 hr maintained this ability.

The ability of the parasites to penetrate MEC was represented by the percentage of infected cells. The parasites had significantly less penetration activity after preincubation with LFcin-B at 1000 μg/ml for 0.5 hr (Fig. 2). The parasites treated with LFcin-B at 100 μg/ml showed gradually decreasing penetration activity and the percentage of infected cells became less than 10% after 4 hr of treatment. However, the untreated parasites retained this ability after similar incubation. It was considered that some of the parasites treated with LFcin-B at 100 μg/ml had lost the penetration ability within 30 min after the treatment, although they seemed to sustain trypan blue dye exclusion activity.

The loss of infectivity of the parasites and/or cystozoites in cysts was confirmed by inoculation of mice (Table 1). All five mice inoculated with 10^2 untreated parasites died within 9 days postchallenge. Similarly, parasites pretreated with bovine lactoferrin at 1000 μg/ml caused 100% mortality of inoculated ice within 9 days postchallenge. In contrast, four of five mice inoculated with the same dose of parasites pretreated with LFcin-B at 1000 μg/ml survived for more than 30 days postchallenge. In the case of parasites pretreated with LFcin-B at 100 μg/ml, one of five mice survived up to 30 days postchallenge. All mice inoculated with cysts, either pretreated with LFcin-B or untreated, survived for more than 30 days. The mean number of cysts in the brains of the mice inoculated with untreated cysts was approximately 1500 per mouse while that of the group inoculated with LFcin-treated cysts was only 300 per mouse.

In the present study, treatment of *T. gondii* with LFcin-B caused an irreversible loss of trypan blue dye exclusion activity and inactivation of the host cell penetration capacity of the parasites. These observations are positive evidence of the parasiticidal effects of LFcin-B. Concentrations of LFcin-B of 100 μg/ml or higher achieved marked efficacy against *T. gondii*, and this is consistent with the effective dose against other microorganisms (Bellamy *et al.* 1993). LFcin-B contains asymmetric clusters of basic amino acid residues such as lysine and arginine. Similar cationic peptides, such as defensins, magainins, and the antibiotic polymyxin, are known to show affinity for biological membranes and act to disrupt the cytoplasmic membrane of various microorganisms. The biochemical similarity and/or dissimilarity of the membranes of *T. gondii* and microbial membranesis is obscure. Nonetheless, it is conceivable that LFcin-B may have affinity for membranes of *T. gondii* and act to disrupt their normal functional properties. Further studies aimed at evaluating the susceptibility of other parasitic protozoa to LFcin-B are under way.

Lactoferrin and a peptide derived from the C-terminal region of lactoferrin had no inhibitory effect on the parasites, whereas LFcin-B displayed this activity. These results suggest that an anti-*T. gondii* sequence within lactoferrin, located near the N-terminus, is released from the folded protein molecule by certain proteolytic enzymes. It is plausible that proteolytic enzymes in the phagosomes of host cells cleave the lactoferrin molecules and generate potent parasiticidal peptides (Tanaka *et al.*, unpublished). In order to clarify the mechanisms of the parasiticidal effect of LFcin-B, further studies are necessary to investigate the kinetics of interaction between LFcin-B and the membrane of the parasite.

References

APPELMELK, B. J., YUN-QING, A., GEERTS, M., THIJS, B. G., DEBOER, H. A., MACLAREN, D. M., GRAAFF, J., AND NUIJENS, J. H. 1994. Lactoferrin is a lipid A-binding protein. *Infection and Immunity* **62,** 2628–2632.

BELLAMY, W. R., WAKABAYASHI, H., TAKASE, M., KAWASE, K., SHIMAMURA, S., AND TOMITA, M. 1993. Role of cell-binding in the antibacterial mechanism of lactoferrin B. *Journal of Applied Bacteriology* **75,** 478–484.

BULLEN, J. J., ROGERS, H. J., AND LEIGH, L. 1972. Iron-binding proteins in milk and resistance to *Escherichia coil* infection in infants. *British Medical Journal* **1,** 69–75.

CORNELISSEN, A. W. C. A., OVERDULVE, J. P., AND HOENDERLOOM, J. M. 1981. Separation of *Isospora* (*Toxoplasma*) *gondii* cysts and cystozoites from mouse brain

tissue by continuous density gradient centrifugation. *Parasitology* **83,** 103–108.

COUZINET, S., DUBREMETZ, J. F., DAVID, L,. AND PRENSIER, G. 1994. *Toxoplasma gondii:* Activity of the polyether ionophorus antibiotic nigericin on tachyzoites in cell culture. *Experimental Parasitology* **78,** 341–351.

HARRIS, C., SALGO, M. P., TANOWITZ, H. B., AND WITTNER, M. 1988. In vitro assessment of antimicrobial agents against *Toxoplasma gondii.* 3. *Infectious Diseases* **157,** 14–22.

JONES, E. M., SMART, A., BLOOMBERG, G., BURGESS, L., AND MILLAR, M. R. 1994. Lactoferricin, a new antimicrobial peptide. *Journal of Applied Bacteriology* **77,** 208–214.

LONGHI, C., CONTE, M. P., BELLAMY, W., SEGANTI, L., AND VALENTI, P. 1994. Effect of lactoferricin B, a pepsin-generated peptide of bovine lactoferrin, on *Escherichia coil* HB101 (pR1203) entry into HeLa cells. *Medical Microbiology and Immunology* **183,** 77–85.

SHIMAZAKI, K., TANAKA, T., KON, H., OOTA, K., KAWAGUCHI, A., MAKI, Y., SATO, T., UEDA, U., TOMIMURA, T., AND SHIMAMURA, S. 1993. Separation and characterization of the C-terminal half molecule of bovine Lactoferrin. *Journal of Dairy Science* **76,** 946–955.