原虫感染症の発症予防と早期診断法の分子免疫学的開発研究

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1。課題番号：　０３５５６０４１

2。研究課題：　原虫感染症の発症予防と早期診断法の分子免疫学的開発研究

3。研究組織：

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5。研究経過

A）はじめに

原虫感染牛のＴ－リンパ球産生物質、リンホカインから分子量５０００以下のマクロファージ活性化作用を有し殆ど毒性のない天然作用物質をオビオアクチンとして研究代表者らが世界に公表（１９８４）し広領域の動物に対する非特異的免疫調整物質として追試確認されている。ここに来て、日鉱共石生物研の研究グループによって大略、数種の本物質活性単位が解明され化学合成されようとしている（特願昭６２－３３０１４２）。衆知の如く、天然および合成免疫調整・賦活物質は、その作用効果と併行して強い副作用を有することで人においても使用法が極めて限定されているのが実情である。したがって、研究代表者らが発見した毒性のないペプチドの開発研究は極めて魅力的かつ有効な新規の免疫調整作用物質として重要な意義を有する。　同様にトキソプラズマ原虫抗原成分の一部（TLA）にも感染症のみならず癌増殖などを抑制する非特異的免疫賦活作用物質が存在することを研究代表者らは明らかにしている（１９７８）。　これらの作用物質を用いて基礎と応用の両領域から、社会的対策要望の強い感染死を必須とする原虫感染症の発病予防あるいは発病軽減に対する作用機構ならびに生物学的意義を早急かつ総合的に検討すべき時期に至っていると考察した。一方、諸種原虫感染の早期診断法の確立と予防対策課題も早急に解決しなければならなく現在の日本における原虫免疫学者の参加による研究分担プロジェクトを組み込んだ。

B)研究発表

1。 原著論文

1-1) Omata,Y., Oikawa,H., Kanda,M., Mikazuki,K., Claveria,F.G., Takehara,T., Saito,A. & Suzuki,N.: Humoral immune response to Isospora felis and Toxoplasma gondii in cats experimentally infected with Isospora felis, J. Vet. Med. Sci., 53, 163-165, 1991.

1-2) Matsuki,N., Tamura,S., Ono,K., Watari,T., Goitsuka,R., Yamanobe, A., Hiraga,A., Kubo,K., Takagi,S., Hasegawa,A. & Suzuki,N.: Excercise-induced phospholipid degradation in the equine skeletal muscle and erythrocytes, J. Vet. Med. Sci., 53: 1001-1007, 1991.

1-3) Omata,Y., Oikawa,H., Kanda,M., Mikazuki,K., Takehara,T., Venturini,C., Saito,A. & Suzuki,N.: Enhancement of humoral immune response of Isospora felis-infected cats after inoculation with Toxoplasma ghondii, J. Vet. Med. Sci., 53: 1071-1073, 1991.

1-4) Shimada,T., Igarashi,I., Maki,Y., Claveria,F.G., Saito,A. & Suzuki,N.: Cellular subsets involved in protective immunity to Babesia rodhaini infectioin in BALB/c mice, J. Protozool. Res., 1, 35-44, 1991.

1-5) Miyahara,K., Honda,U., Tose,S., Sakukrai,H., Igarashi,I., Saito,A., Hirose,T. & Suzuki,N.: Therapeutic effects of TLA on 20-methylcholanthrene induced BALB/c mouse tumors, J.Vet. Med. Sci., 54: 7-12, 1992.

1-6) Miyahara,K., Matsumoto,T., Tose, S.,Sakurai,H., Iarashi,I., Saito,A., Hirose,T. & Suzuki,N.: Characteristics of cytotoxic cells induced by Toxoplasma lysate antigen (TLA) in mouse spleen, J.Vet. Med. Sci., 54: 87-93, 1992.

1-7) Miyahara,K., Yokoo,N., Sakurai,H., Igarashi,I., Sakata,Y., Yoshida,Y., Saito,A., Hirose,T. & Suzuki,N.: Antitumor activity of TLA in methycholanthrene induced tumor-bearing rats, J. Vet. Med. Sci.,54: 221-228, 1992.

1-8) Fujii,Y., Maki,Y., Claveria,F.G., Igarashi,I., Saito,A., Ono,K. & Suzuki,N.: Effects of Obiopeptide and Obioactin on the toxoplasmacidal activity, glucose consumption and rufle formation in mouse macrophages, J. Vet. Med. Sci., 54: 351-353, 1992.

1-9) Kono,M., Fujii,Y., Shikano,S., Ono,K. & Suzuki,N.: Influence of a newly synthesized peptide, Obiopeptide-1, as a biological response modifier, upon the lysosomal enzyme activities and chemotactic responses of mouse macrophages, J. Vet. Med. Sci., 54: 793-795, 1992.

1-10) Shimada,T., Shikano,S., Ono,K., Saito,A. & Suzuki,N.: Changes of splenic lymphocyte subpopulation in mice inoculated with Babesia microti and Babesia rodhaini, J. Vet. Med. Sci., 54: 1071-1075, 1992.

1-11) Saito,A., Igarashi,I., Miyahara,K., Ventrurini,C., Claveria,F.G., Hirose,T., Suzuki,N. & Ono,K.: Effects of reactive oxygen intermediate scavengers on antitoxoplasmic activity of activated macrophages, Parasitol. Res., 78: 28-31, 1992.

1-12) Fujii,Y., Maki,Y., Ito,M., Saito,A., Igarashi,I.,Ono,K., Itoh,K., & Suzuki,N., Restorative effects of a newly synthesized peptide, Obiopeptide-1, in cyclophosphamide or carragenan-pretreated mice infected with opportunistic bacteria, J. Protozool. Res., 2, 74-83.

1-13) Claveria,F.G., Omata,Y., Takehara,T., Saito,A., Maki,Y., Igarashi,I. , & Suzuki,N., Anti-Babesia rodhaini monoclonal antibodies: effect against Babesia rodhaini and cross-reactivity with human strain of Babesia microti, J. Protozool.Res., 2, 112-122, 1992.

1-14) Omata,Y., Yonemasu,K., Sasaki,T., Saito,A., Nakabayashi,T. & Suzuki,N.: Toxoplasma gondii; antibody-independent binding of human complement subcomponent C1q to the paasite, J. Protozool. Res., 2: 141-148, 1992.

1-15) Omata,Y., Heydorn,O.A., Heidrich,H.G., Igarashi,I., Saito,A., Toba,H. & Suzuki,N.: Survey of Sarcocystis spp. infection in slaughtered pigs in east Hokkaido, Japan, J. Protozool. Res., 3: 29-30, 1993.

1-16) Ikenaga,H., Mizuta,Y., Ono,K., Sawazaki,T., Suzuki,N. & Tomoda,I.: Placental and plasma cystine aminopeptidase in pregnant animals, J. Vet. Med. Sci., 55: 479-480, 1993.

1-17) Ito,M., Igarashi,I., Fujita,Y., Omata,Y., Saito,M., Nakagawa,M., Claveria,F.C. & Suzuki,N.: Studies related to immunosuppression in mice with chronic toxoplasmosis, J. Protozool. Res., 3: 99-109, 1993.

1-18) Takahashi,M., Omata,Y., Oikawa,H., Claveria,F.G., Igarashi,I.,

Saito,A. & Suzuki,N.: Protective immune response of Isospora felis-infected mice against Babesia microti infection, J. Vet. Med. Sci., 55: 587-590, 1993.

1-19) Omata,Y., DiLorenzo,C., Venturini,C., Venturini,L., Igarashi,I., Saito, A. & Suzuki,N.: Correlation between antibody levels in Toxoplasma gondii infected pigs and pathogenicity of the isolated parasite, Veterinary Parasit., (in press), 1993.

1-20) Omata,Y., Xu Shen-Zhi, Igarashi,I., Saito,A., Toba,H. & Suzuki,N.: Survey of Sarcocystis infection in cattle in east Hokkaido,Japan, J. Vet. Med. Sci., (in press), 1993.

2。学会発表　（国際学会）

2-1) Fujii,Y., Maki,Y., Igarashi,I., Omata,Y., Ono,K. & Suzuki,N.: Restorative effects of an immunoregulator, Obiopeptide-1, in cyclophosphamide pretreated mice inoculated with P. aeruginosa or K. pneumonia, 8th Japanese-German Symposium on Protozoan Diseases, Berlin, Germany , Sep. 9-14, 1991.

2-2) Omata, Y.: Toxoplasmosis, A la 6 Jornada Provinical de Zoonosis, La Plata, Argentina, Sep. 16, 1991.

2-3) Omata,Y., Dilorenzo,C., Boren,J.L. & Venturini,L.: Toxoplasma gondii; diagnostic serologico y aislamiento en cerdos de consumo, 7th Argentina Veterinary Diagnosis Symposium, Buenos Ailes, Argentina, Dec. 5-6, 1991.

2-4) Saito,A., Ito,M., Igarashi,I., Takahashi,M. & Suzuki,N.: Effects of reactive oxygen intermediate scavengers on the anti-toxoplasma activity of activated mouse macrophages, 8th Intern. Cong. of Immunology, Budapest, Hungary, Aug. 23-28, 1992.

2-5) Shimazaki, K., Maki,Y., Kato, M., Igarashi,I., Miura,H. & Suzuki,N.: Isolation and characterization of anti-tumor component from Toxoplasma lysate antigen (TLA144), 8th Intern. Cong. of Immunol., Budapest, Hungary, Aug. 23-28, 1992.

2-6) Igarashi,I., Waki,S.,Ito,M., Saito,A. & Suzuki,N.: Major role of CD4 positive T-cells in primary infection with Babesia microti in mice, 13th Intern. Cong. for Tropical Medicine and Malaria, Pattaya, Thailand, Nov. 29-Dec. 4, 1992.

2-7) Igarashi,I., Waki,S., Omata,Y., Saito,A., Shinagawa,M. & Suzuki,N.: Contributions of CD4 positive T-cells in control of Babesia microti infection in mice, 9th Intern. Cong. of Protozoology, Berlin, Germany, July 25-31, 1993.

2-8) Shikano,S., Shimada,T., Saito,A., Suzuki,N. & Ono,K.: Enzyme activities related to glucose metabolism in Babesia microti and B. rodhaini, 9th Intern. Cong. of Protozoology, Berlin, Germany, July 25-31, 1993.

2-9) Shimada,T., Shikano,S., Igarashi,I., Suzuki,N. & Ono,K.: The role of Lyt-2 and L3T4 positive cells in Babesia microtic and B. rodhaini infected

mice, 9th Intern. Cong. of Protozoology, Berlin, Germany, July 25-31, 1993.

2-10) Fujii,Y., Igarashi,I., Omata,Y., Ono,K., Saito,A. & Suzuki,N.: Restorative effects of a newly synthesized peptide in cyclophosphamide pretreated mice infected with opportunistic bacteria, 14th Intern. Cong. of Veterinary Parasitology (WAAVP), Cambridge, UK, Aug. 8-13, 1993.

2-11) Igarashi,I., Fujita,Y., Omata,Y., Saito,A. & Suzuki,N.: The effect of immunosuppression caused by cyclophosphamide to mice chronically infected with Toxoplasma gondii, 14th Intern. Cong. of Veterinay Patasitology (WAAVP), Cambridge, UK, Aug. 8-13, 1993.

2-12) Igarashi,I. & Suzuki,N.: Immunity to Babesia infection, 2nd Meeting on the Cooperative Rsearch Project in the Area of Veterinary Sciences between National Univ. of La Plata & Japan International Cooperative Agency, La Plata, Argentina, Aug. 12-14, 1993.

3：総説

3－1）五十嵐郁男、鈴木直義：トキソプラズマ症の感染と免疫、Modern Media　（モダンメヂイア）、１２（３９）、５８１－５９０、１９９３

4：国内特別講演

4－1）鈴木直義：免疫の仕組み　－　人・動物共通原虫病から考える、第９回北海道地区大学保健管理業務職員研修会、帯広、北海道、６月２３日、１９９３

4－2）鈴木直義：人畜共通原虫トキソプラズマ症の病態生理、第４０回北日本寄生虫学会、旭川、北海道、９月２４日、１９９３

4－3）鈴木直義：　人畜共通原虫病の現状と課題、農林水産省研究特別推進会議、家畜衛生試験場、筑波、茨城県、２月８日、１９９４

C）研究成果

研究成果については、研究発表原著論文に詳しく述べられているが、本研究期間の成績を要約すると以下の如くである。

１９８９年頃から世界は微生物感染症の発病予防及び軽減作用を有する免疫調整・賦活物質の開発研究が最重要課題の一つに取り上げられ、世界の諸種関連学会での中心研究領域になっている。一連の基礎研究から研究代表者らがリンフォカイン中から研究開発した天然物質を母体として日鉱グループが作出した新規の合成ペプチドはアミノ酸が２－７個の単純ペプチドである。本ペプチドの宿主生体内作用機構の全体像は未だ明らかではないが、現在までに得られた実験成績から健康動物の血液中白血球総数、とくに好中球、単球、リンパ球の軽度増数と細胞機能活性化が観察されている。なかでも微生物感染症時には、その傾向が強まり健康値維持作用を持つ血液細胞刺激物質であろうと現在考えられている。

世界で人および動物の臓器移植時に常用される免疫抑制剤の大量投与による常在細菌および薬剤耐性菌（MRSA）の顕性化により人および動物が死亡することが良く知られている。このことが無毒かつ安定な免疫調整剤の開発研究に集中している一理由であるが、世界が未だ開発研究途上にあると言うのが真実である。本合成ペプチドの投与により一部の常在細菌の顕性化を抑制し、結果として免疫抑制剤投与による死亡を最小限にくいとめる事が判明してきた。この効果の主役は生体内マクロファージの活性化機構による事も明らかになって来た。一方、生体内マクロファージを薬物処理してマクロファージ機能障害を与えて免疫抑制剤投与するとMRSA感染ではほぼ１００％宿主は死亡する。しかし、これに新規の合成免疫調整ペプチドを投与すると明らかに生残例が増加する。このことは、本合成ペプチド投与による微生物感染に対する生体防御機構にはマクロファージのみならずキラー細胞を含むリンパ球や好中球の活性化が関与していることが十分に推測されている。したがって、研究代表者らが発見した新規の合

成ペプチドはマクロファージのみならず諸種血液細胞活性化作用物質と考察している所以でもある。

世界の人類および動物の３０％以上はトキソプラズマ原虫に既に感染していると一般的に考えられている。人エイズ患者の３０－４０％はエイズ免疫能低下による脳内トキソプラズマ　シスト破壊に伴うトキソプラズマ増殖性劇症脳炎により例外なく１００％急性死する。この脳内トキソプラズマ　シスト破壊の生物学的シグナル伝達作用機構は現在全く不明であり、その解決法は皆無の状態にある。トキソプラズマ　シストならびにエイズ感染症に対する完全な予防薬ならびに治療薬の全くない現在、先進国のみならず世界的規模で最も緊急対策研究課題であることは衆目の一致したところで、全世界の関連研究者がそれぞれの立場から研究遂行中である。従って、本研究グループは「本合成免疫調整作用物質によるトキソプラズマ感染宿主体内の免疫能調整有無と脳内トキソプラズマ　シスト破壊防御能」について総合的に検討を加えた。トキソプラズマ　シスト保有動物に免疫抑制剤の大量投与を一定期間継続すると、動物の８０－９０％がほぼ同時日に死亡する。一方、これに合成ペプチドを小量継続的に投与すると、興味あることに８０－９０％が生残する。免疫抑制剤（サイクロフォスファミド）投与マウス脳内トキソプラズマ　シスト破壊像は、抗L3T4血清投与では死亡しないマウス脳内シスト破壊像に比較して顕著ではないが、限局的にシスト破壊と増殖型トキソプラズマの散在像が認められた。しかし、合成ペプチドの併用投与マウスの脳内シスト像は全般的に静止型でありトキソプラズマ虫体増殖像は検出されなかった。現在までの実験成績から、トキソプラズマ　シスト破壊のシグナル発進の一つにはリンパ球比率（CD4:CD8リンパ球）の最低維持値が存在し、それ以下になると脳内トキソプラズマ　シスト破壊とトキソプラズマ原虫増殖が進行し劇症トキソプラズマ性脳障害に伴う生体防御免疫能不全による斃死と推察している。しかし乍ら、これらの感染発病機構は、未だ完全には実験的に証明されていない部分が少なくないので、今後継続して分子免疫学的領域から考究する必要がある。

一方、トキソプラズマ原虫由来成分（TLA）は正常マウスへの投与により、そのマウスはマラリア、トキソプラズマ、ピロプラズマなどの原虫感染に対して強いアジュバント効果による感染抵抗性を示し一部のマウスは生残することを研究代表者らは現象的に認め既に世界に公表している（

SUZUKI et.al., 1986）。しかし、その防御機構に関しては殆ど未解明であるために、非特異的免疫賦活作用効果の基礎的解明としてマウス同種可移植腫瘍およびMethylcholantren　誘発自家腫瘍、猫白血病、猫骨肉腫、そして実験感染原虫としてPlasmodium berghei, Babesia microti, Babesia rodhainiなどを用いて多種の異物ならびに微生物抗原に対する生体応答を検討した。その結果、とくにTLA投与によるマウス腫瘍および骨肉腫に対しては腫瘍部位への大型Thy-1陽性キラー細胞の集簇が顕著に観察された。これら大型キラー細胞の生物学的細胞回転を検討すると、いわゆるＴ－リンパ球由来Lymphkine Activated Killer (LAK) 細胞の一種であることが明らかになった。同系列の実験検索から生体へのTLA　感作は Natural Killer (NK)　細胞の産生増加と活性の誘導にも強く関与し、大型NK細胞の誘導に作用することが判明した。これらのキラー細胞誘導には脾臓内マクロファージの存在が必須であり、マクロファージ産生物質の介在よりもマクロファージが直接にNK細胞あるいはリンパ球との接触が第一義的に重要条件であることも実験的に明らかにした。現在、この事実は世界から追試確認されている。基礎的実験事実の応用開発の一部として、TLA　を放牧牛に被害をもたらす牛ピロプラズマ発病予防実験あるいは臨床患畜の乳腺腫瘍や猫骨肉腫などに投与した。少なくとも、免疫賦活作用物質としてのTLAは疾病予防、治療効果において予期以上に顕著に認められると判定されている。猫白血病および猫骨肉腫と診断された難治療病に対して、TLA投与による骨肉腫の増殖停止ならびに縮小、あるいは猫白血病の症状回復現象をLAK 細胞およびNK大型細胞の誘導効果のみでの解釈は難しい。上述の生体反応を主にした全身性抵抗性賦活効果と推察しているが、現在までの全ての実験評価系では論拠を提示できる成績は皆無であり、今後継続して研究すべき重要課題と結論した。本試験研究の最終目標の一つとして提示した早期診断法の確立の為の研究は現在も進行中であり、１－２の基礎研究報告に留まり総合的にはさらなる継続研究期間を必要とした。

(別紙様式2)　　平成5年度科学研究費補助金研究成果報告書概要

1。研究機関番号　10105　2。研究機関名　帯広畜産大学

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6。研究課題　原虫感染症の発症予防と早期診断法の分子免疫学的開発研究

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9．研究成果の概要　（最終年度のまとめ）　（600字～800字）

1989年頃から世界は微生物感染症の発病予防及び軽減作用を有する免疫調整・賦活物質の開発研究が最重要課題の一つに取り上げられ、世界の諸種関連学会での中心研究領域になっている。一連の基礎研究から研究代表者らがリンフォカイン中から研究開発した天然物質を母体として日鉱グループが作出した新規の合成ペプチドはアミノ酸が2－7個の単純ペプチドである。本ペプチドの宿主生体内作用機構の全体像は未だ明らかではないが、現在までに得られた実験成績から健康動物の血液中白血球総数、とくに好中球、単球、リンパ球の軽度増数と細胞機能活性化が観察されている。なかでも微生物感染症時には、その傾向が強まり健康値維持作用を持つ血液細胞刺激物質であろうと現在考えられている。

世界の人類および動物の30%以上はトキソプラズマ原虫に既に感染していると一般的に考えられている。人エイズ患者の30－40%はエイズ免疫能低下による脳内トキソプラズマ　シスト破壊に伴うトキソプラズマ増殖性劇症脳炎により例外なく100%急性死する。この脳内トキソプラズマ　シスト破壊の生物学的シグナル伝達作用機構は現在全く不明であり、その解決法は皆無の状態にある。トキソプラズマ　シストならびにエイズ感染症に対する完全な予防薬ならびに治療薬の全くない現在、先進国のみならず世界的規模で最も緊急対策研究課題であることは衆目の一致したところで、全世界の関連研究者がそれぞれの立場から研究遂行中である。従って、本研究グループは「本合成免疫調整作用物質によるトキソプラズマ感染宿主体内の免疫能調整有無と脳内トキソプラズマ　シスト破壊防御能」について総合的に検討を加えた。トキソプラズマ　シスト保有動物に免疫抑制剤の大量投与を一定期間継続すると、動物の80－90%がほぼ同時日に死亡する。一方、これに合成ペプチドを小量継続的に投与すると、興味あることに80－90%が生残する。免疫抑制剤（サイクロフォスファミド）投与マウス脳内トキソプラズマ　シスト破壊像は、抗L3T4血清投与では死亡しないマウス脳内シスト破壊像に比較して顕著ではないが、限局的にシスト破壊と増殖型トキソプラズマの散在像が認められた。しかし、合成ペプチドの併用投与マウスの脳内シスト像は全般的に静止型でありトキソプラズマ虫体増殖像は検出されなかった。現在までの実験成績から、トキソプラズマ　シスト破壊のシグナル発進の一つにはリンパ球比率（CD4:CD8リンパ球）の最低維持値が存在し、それ以下になると脳内トキソプラズマ　シスト破壊とトキソプラズマ原虫増殖が進行し劇症トキソプラズマ性脳障害に伴う生体防御免疫能不全による斃死と推察している。しかし乍ら、これらの感染発病機構は、未だ完全には実験的に証明されていない部分が少なくないので、今後継続して分子免疫学的領域から考究する必要がある。

10．キーワード

(1)	免疫調整物質	(2)	リンフォカイン	(3)	オピオペプチド
(4)	トキソプラズマ	(5)	免疫不全	(6)	TLA
(7)	血液細胞刺激因子	(8)	生理活性物質		

(裏面に続く)

(未発表 発表予定も含む)

雑誌論文：

著者名	Saito, A.,et. al.	論文標題	Effects of reactive oxygen intermediate scavengers on antitoxoplasmic activity of activated macrophages		
雑誌名	Parasitology Research	巻	発行年	ページ	
		78	1992	28	31

著者名	Fujii, Y., et.al.	論文標題	Restorative effects of a newly synthesized peptide in cyclophosphamide or carragenan pretreated mice infected with opportunistic bacteria		
雑誌名	Journal of Protozoology Research	巻	発行年	ページ	
		2	1992	74	83

著者名	Omata, Y., et.al.	論文標題	Toxoplasma gondii:antibody independent binding of human complement C1q to the parasite		
雑誌名	Journal of Protozoology Research	巻	発行年	ページ	
		2	1992	141	148

著者名	Itou,M., et.al.	論文標題	Studies related to immunosuppression in mice with chronic toxoplasmosis		
雑誌名	Journal of Protozoology Research	巻	発行年	ページ	
		3	1993	99	109

著者名	Tkahashi,M.,et.al,	論文標題	Protective immune response of Isospora felis-infected mice against Babesiamicroti infection		
雑誌名	Journal of Vet. Medical Science	巻	発行年	ページ	
		55	1993	587	590

著者名	Omata, Y. et.al.	論文標題	Correlation between antibody levels in T.gondii infected pigs and pathogenicity of the isolated parasites		
雑誌名	Veterinary Parasitology	巻	発行年	ページ	
			1993	(In press)	

図　書：

著者名	五十嵐郁男、鈴木直義	出版者	Modern Media　(モダンメヂイア)
書名	：トキソプラズマ症の感染と免疫	発行年	総ページ数
		1993	10

著者名	鈴木直義	出版者	大学保健管理業務職員研修会
書名	免疫の仕組み　－　人・動物共通原虫病から	発行年	総ページ数
		1993	7

(別紙様式３)

ABSTRACTS OF RESEARCH PROJECT. GRANT-IN-AID FOR SCIENTIFIC RESEARCH (1993)

1. RESEARCH INSTITUTION NUMBER: 10105
2. RESEARCH INSTITUTION:
   OBIHIRO UNIVERSITY,RES. CENTER FOR PROTOZOAN MOLECULAR IMMUNOLOGY
3. CATEGORY: Grant-in-Aid for Seientific Research (B)
4. TERM OF PROJECT (1991-1993)
5. PROJECT NO. 03556041
6. TITLE OF PROJECT: Molecular Immunological Studies on The Prevention and Diagnoses of Protozoan Infection
7. HEAD INVESTIGATOR

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9. SUMMARY OF RESEARCH RESULTS

Using newly synthesized peptides of the activity units which were isolated from lymphokines as an immunoregulator, Obiopeptide, the effect of immunosuppression caused by cyclophosphamide to animals cronically infected with Toxoplasma gondii was examined. Mice chronically infected with Toxoplasma, were treated with cyclophosphamide or anti-CD4 monoclonal antibody to identify the effect of these immunosuppressive reagents on the cysts in the brain of infected mice. The effect of obiopeptide as an immunomodulator treatment of the immunosuppressed mice was also analyzed. In the brain of non-treated and cyclophosphamide-treated, chronically Toxoplasma infected mice mainly typicallarge tissue cysts, and sometimes divided cysts, were detected after staining with anti-Toxoplasma ABC technique. In contrast, the brain from anti-CD4 treated, chronically infected mice, contained multiple deeneated Toxoplasma tissue cysts of different size in some partialregions in the brain. Mice chronicaly infected with Toxoplasma and treated with a combination of cyclophosphamide and obiopeptide showed a significantly higher survival rate than those treated with cyclophosphamide alone. The percentage of neutrophils and lymphocytes in mice treated with a combination of Obiopeptide and anti-CD4, or obiopeptide and cyclophosphamide, was higher than that of mice treated withanti-CD4 or cyclophosphamide alone. These results indicate that reactivaion or rupture of tissue cysts in mice treated with cyclophosphamide, chronically infected with Toxoplasma, might be mainly mediated by CD4 positive cells rather than other immunocomponent cells. The increase of neutrophilic leukocytes might contribute tothe induction of the resistance to Toxoplasma gondii in mice, after treatment with obiopeptide and cyclophosphamide in combination.

On the other hand, an immunopotentiator such as TLA (Toxoplasma Lysate Antigens) was tested in our experimetal studies. Growth of the tumor autoinduced by 20-methylcholanthrene (MC) in rats was inhibited after administration of TLA. The antitumor activity of TLA was most obvious in the early stage of tumoral growth. When TLA was administered to rats before the appearance of tumor, tumor formation was delayed slightly. According to the immunohistological examination of tumor tissue with anti-Thy-a antibody,the rats treated with TLA showed large Thy-1 positive or Lymphokine-Activated Killer (LAK) cells, whereas the untreated rats indicated only a few small Thy-a positive or NK cells. These observation indicate that TLA is a useful modifier of biological responses to MC-induced tumors. In a series of these studies, TLA induces NK cells, cytotoxic T-lymphocytes and LAK cells in animals after injection, however, the induction of these killer cells needs to combine with monocyte-macrophages in in vivo.

10. KEY WORDS

(1) Immunoregulator (2) Lymphokines (3) Obiopeptides (4) Toxoplasma
(5) TLA (6) Immunodeficiency (7) Blood Cell Stimulating Factor (8) Physio-Active substance

11. REFERENCES

AUTHORS , TITLE OF ARTICLE | JOURNAL, VOLUME-NUMBER, PAGES CONCERNED, YEAR

1-1) Omata,Y., Oikawa,H., Kanda,M., Mikazuki,K., Claveria,F.G., Takehara,T., Saito,A. & Suzuki,N.: Humoral immune response to Isospora felis and Toxoplasma gondii in cats experimentally infected with Isospora felis, J. Vet. Med. Sci., 53, 163-165, 1991.

1-2) Matsuki,N., Tamura,S., Ono,K., Watari,T., Goitsuka,R., Yamanobe, A., Hiraga,A., Kubo,K., Takagi,S., Hasegawa,A. & Suzuki,N.: Excercise-induced phospholipid degradation in the equine skeletal muscle and erythrocytes, J. Vet. Med. Sci., 53: 1001-1007, 1991.

1-3) Omata,Y., Oikawa,H., Kanda,M., Mikazuki,K., Takehara,T., Venturini,C., Saito,A. & Suzuki,N.: Enhancement of humoral immune response of Isospora felis-infected cats after inoculation with Toxoplasma ghondii, J. Vet. Med. Sci., 53: 1071-1073, 1991.

1-4) Shimada,T., Igarashi,I., Maki,Y., Claveria,F.G., Saito,A. & Suzuki,N.: Cellular subsets involved in protective immunity to Babesia rodhaini infectioin in BALB/c mice, J. Protozool. Res., 1, 35-44, 1991.

1-5) Miyahara,K., Honda,U., Tose,S., Sakukrai,H., Igarashi,I., Saito,A., Hirose,T. & Suzuki,N.: Therapeutic effects of TLA on 20-methylcholanthrene induced BALB/c mouse tumors, J.Vet. Med. Sci., 54: 7-12, 1992.

1-6) Miyahara,K., Matsumoto,T., Tose, S.,Sakurai,H., Iarashi,I., Saito,A., Hirose,T. & Suzuki,N.: Characteristics of cytotoxic cells induced by Toxoplasma lysate antigen (TLA) in mouse spleen, J.Vet. Med. Sci., 54: 87-93, 1992.

1-7) Miyahara,K., Yokoo,N., Sakurai,H., Igarashi,I., Sakata,Y., Yoshida,Y., Saito,A., Hirose,T. & Suzuki,N.: Antitumor activity of TLA in methycholanthrene induced tumor-bearing rats, J. Vet. Med. Sci.,54: 221-228, 1992.

1-8) Fujii,Y., Maki,Y., Claveria,F.G., Igarashi,I., Saito,A., Ono,K. & Suzuki,N.: Effects of Obiopeptide and Obioactin on the toxoplasmacidal activity, glucose consumption and ruffle formation in mouse macrophages, J. Vet. Med. Sci., 54: 351-353, 1992.

1-9) Kono,M., Fujii,Y., Shikano,S., Ono,K. & Suzuki,N.: Influence of a newly synthesized peptide, Obiopeptide-1, as a biological response modifier, upon the lysosomal enzyme activities and chemotactic responses of mouse macrophages, J. Vet. Med. Sci., 54: 793-795, 1992.

1-10) Shimada,T., Shikano,S., Ono,K., Saito,A. & Suzuki,N.: Changes of splenic lymphocyte subpopulation in mice inoculated with Babesia microti and Babesia rodhaini, J. Vet. Med. Sci., 54: 1071-1075, 1992.

1-11) Saito,A., Igarashi,I., Miyahara,K., Ventrurini,C., Claveria,F.G., Hirose,T., Suzuki,N. & Ono,K.: Effects of reactive oxygen intermediate scavengers on antitoxoplasmic activity of activated macrophages, Parasitol. Res., 78: 28-31, 1992.

1-12) Fujii,Y., Maki,Y., Ito,M., Saito,A., Igarashi,I.,Ono,K., Itoh,K., & Suzuki,N., Restorative effects of a newly synthesized peptide, Obiopeptide-1, in cyclophosphamide or carragenan-pretreated mice infected with opportunistic bacteria, J. Protozool. Res., 2, 74-83.

1-13) Claveria,F.G., Omata,Y., Takehara,T., Saito,A., Maki,Y., Igarashi,I. , & Suzuki,N., Anti-Babesia rodhaini monoclonal antibodies: effect against Babesia rodhaini and cross-reactivity with human strain of Babesia microti, J. Protozool.Res., 2, 112-122, 1992.

Humoral Immune Response to *Isospora felis* and *Toxoplasma gondii* in Cats Experimentally Inoculated with *Isospora felis*

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KEY WORDS: cat, *Isospora felis*, *Toxoplasma gondii*.

Feline apicomplexan parasitic protozoans, *Toxoplasma gondii* (*T. gondii*) and *Isospora felis* (*I. felis*), are prevalent all over the world. The biological and epidemiological significance of the latter species is well-documented, since *I. felis* infection has been shown to trigger oocyst re-shedding in cats chronically infected with *T. gondii* [2, 3].

We reported in a previous study that cats naturally infected with *I. felis* produced lower anti-*I. felis* antibody and, following *T. gondii* inoculation, showed an increase in anti-*I. felis* antibody titer [10]. This finding suggests either immunoproliferating phenomenon due to *T. gondii* infection or the presence of similar antigen(s) between these two species.

In order to understand more the immune reactions in cats inoculated with *I. felis* in this paper, we examined anti-*I. felis* and anti-*T. gondii* antibody reactivities in cats experimentally inoculated with *I. felis*.

Cysts of S-273 *T. gondii* strain were prepared from brains of mice from the 5th to 7th week post-exposure (p.e.) and density was adjusted to 10^2 cysts in one m*l* of phosphate buffered saline (PBS). Trophozoites of RH and S-273 strains of *T. gondii* were obtained from infected mouse embryonal cells (MEC) as described previously [9]. *I. felis* oocysts were collected from feces of infected cats by the flotation method with $ZnSO_4$ solution, followed by sucrose density gradient centrifugation [1] and stored at 4°C in PBS supplemented with 100 μg/m*l* of Kanamycin. Oocysts were examined within two months after collection. Excystation was done by incubating the oocysts in PBS containing 0.5% taurocholic acid and 0.1% trypsin at 37°C for 30 min. Sporozoites of *I. felis* and *T. gondii* were purified by the percoll-sucrose density gradient centrifugation [1].

Two female six-week-old cats born to a mother that shed no *I. felis* oocyst were used as *I. felis*-free animals, and were kept in individual cages strictly isolated in the animal care facilities at Shionogi-Aburahi Laboratories, Shiga. The cats were orally inoculated with 5×10^4 oocysts of *I. felis*. Cat No. 1 was orally inoculated with 5×10^4 oocysts of *T. gondii* orally on day 28 p.e. and cat No. 2 was also inoculated at the same dose as of *T. gondii* on day 56 p.e.

Feces of each cat were daily collected with the floating method as described previously [4] and were examined microscopically for the presence of coccidian oocysts.

Sera obtained weekly from cats at the jugular vein were used to measure anti-*T. gondii* and anti-*I. felis* antibody IgG titer, and to determine the common antigen(s) to the two species. Anti-*T. gondii* and anti-*I. felis* antibody IgG was titrated by the indirect immunofluorescence assay (IFA) described in a previous report [9].

To define the antigenicity between *T. gondii* and *I. felis*, the extracts of both parasites were examined by the immunoblotting assay. Oocysts of *I. felis* or S-273 or RH strain of *T. gondii* were suspended in a fixed amount of solubilizing buffer [2% 2-mercaptoethanol, 12.5 mM Tris-HCl buffer pH 6.8, 4.6% sodium dodecyl sulfate (SDS), 2 mM phenyl methyl sulfonyl fluoride] for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the suspension was boiled for 5 min, ultrasonicated for 10 sec and centrifuged at 12,000 g for 5 min. The supernatants were applied on a 10% gel of SDS-PAGE. After electrophoresis, the separated proteins were electrophoretically transferred into nitrocellulose membrane (GVHP, Nihon Millipore, Yonezawa, Japan) as described by Towbin *et al.* [12]. The membrane sheets were immersed in PBS containing 5% skim milk (Milk-PBS) for blocking at room temperature for 1 hr and then washed in PBS containing 0.05% Tween 20. Cat sera tested were incubated with either MEC fixed with 1% paraformaldehyde in PBS or parasites of *T. gondii* S-273 strain at 4°C overnight to remove non-specific or cross-reactive antibodies, and then were diluted 50-fold in Milk-PBS. Membrane sheets were incubated with the sample sera at 4°C overnight, followed by reaction with peroxidase-conjugated anti-cat IgG (American Qualex, La Mirada, CA) diluted with Milk-PBS. The antigens were visualized by reaction with 0.02% diaminobenzidine-4HCl and 0.1% H_2O_2 in 0.1 M Tris-HCl buffer at pH 7.4.

As shown in Fig. 1a, cat No. 1 showed two peaks of *I. felis* oocyst shedding between days 7 and 10 and between days 14 and 16 p.e. The number of oocysts in the first peak was approximately 1.3×10^7, which was significantly greater than that in the second peak. Cat No. 2 showed also transient oocyst shedding on days 8 to 21 p.e. (Fig. 1b). The number of oocysts in the first peak on day 10 p.e. was 1.4×10^7 and that in the second peak on day 18 p.e. was 0.4×10^7. After challenged with *T. gondii* oocysts, cat No. 1 showed transient shedding of *T. gondii* oocysts on days

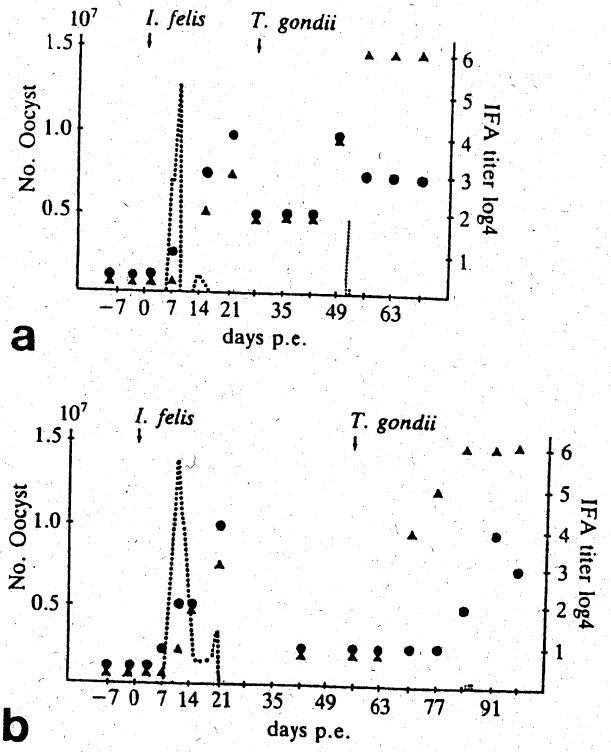

Fig. 1. Oocyst excretion and antibody production in cat No. 1, a, and No. 2, b, exposed perorally to *I. felis* oocysts. Both cats never infected with *I. felis* were inoculated with 5×10^4 *I. felis* oocysts and subsequently with 5×10^4 *T. gondii* oocysts. *I. felis* oocysts/day (• • •), *T. gondii* oocysts/day (····), anti-*I. felis* IgG antibody (●), anti-*T. gondii* IgG antibody (▲).

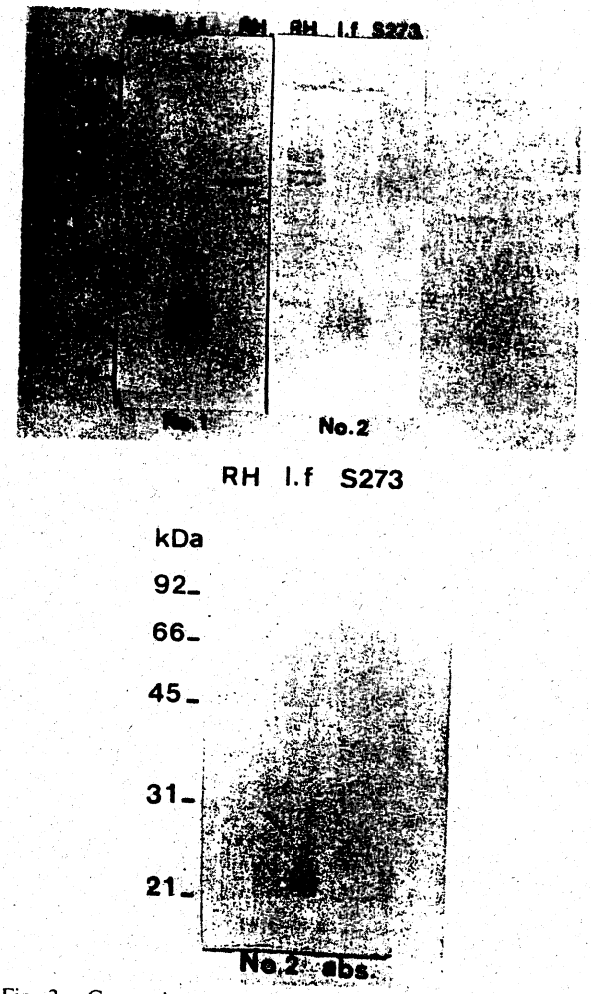

Fig. 2. Comparison of antigenicity between of *T. gondii* and *I. felis* by immunoblotting analysis with serum of cats exposed to *I. felis*. The membrane transblotted with each parasite extract (RH and S-273 strains of *T. gondii* and *I. felis* oocysts) was incubated with one of the following sera: cat No. 1 (No. 1), cat No. 2 (No. 2) and cat No. 2 absorbed with *T. gondii* (No. 2 abs). Molecular markers are given in kilodaltons (kDa).

24 and 25 p.e. to *T. gondii*. The total number of oocysts was 5.5×10^6. Cat No. 2 also showed transient shedding of *T. gondii* oocysts from days 30 to 33 p.e. to *T. gondii*. The number of oocysts per day was below 10^4 and most of the oocysts were not completely sporulated.

By indirect immunofluorescence assay, both *I. felis* sporozoites and *T. gondii* parasites incubated with non-infected cat serum showed fluorescence at the on terminal part of parasites, which was recognized as non-specific reaction and observed up to a serum dilution of 4^2. Specific fluorescence on the whole body of parasites was found in cat No. 1 up to a titer of 4^4 on days 7 and 21 p.e., and then decreased to a titer of 4^2 on day 28 p.e. Likewise, anti-*T. gondii* IgG increased to a titer of 4^3 on day 21 p.e. Anti-*I. felis* IgG antibody in cat No. 2 also appeared on day 7 p.e., and it increased transiently to a titer of 4^4 on day 21 p.e. and subsequently, decreased to 4^1 on day 42 p.e. Anti-*T. gondii* IgG appeared on day 10 p.e. and on day 21 p.e., it increased to 4^3 in titer, and then decreased to 4^1 on day 42 p.e. After cat No. 1 was challenged with *T. gondii*, anti-*I. felis* IgG increased to 4^4 in titer on day 21 p.e. and decreased to 4^3 on day 28 p.e. to *T. gondii*. Anti-*T. gondii* IgG also increased to 4^4 on day 21 p.e. to *T. gondii* and was maintained at 4^6 from day 28 p.e. to the end of experiment. Anti-*I. felis* IgG in cat No. 2 also increased to 4^2 on day 28 and further to 4^4 on day 35 p.e. to *T. gondii*. Anti-*T. gondii* IgG also increased to 4^4 on day 14 p.e. and was maintained at 4^6 up to the end of experiment.

To examine the reactivity of cat sera to *T. gondii* and *I. felis* by immunoblotting method, the extracts were prepared by boiling parasites with solubilizing buffer. Whole parasites of *T. gondii* and *I. felis* oocysts containing sporozites were dissolved with solubilizing buffer, and oocyst walls were removed by centrifugation. Transblotted membrane made to react with cat No. 2 serum collected on day 28 p.e., showed a broad band with a m.w. of 22 kDa in the *I. felis* extract lane. In the lanes of *T. gondii* strains (RH and S-273) extracts, specific bands with a m.w. of 22, 45, 58 and 62 kDa appeared. Membranes made to react with cat No. 1 serum showed similar band in

the lane of *I. felis* extract, while a faint band with a m.w. of 45 kDa was detected in the lane of RH strain *T. gondii*. The same serum sample (cat No. 2) preincubated with *T. gondii* parasites showed only one band of a m.w. of 22 kDa in the lane of *I. felis* extract, but not in that of *T. gondii*. The membrane made to react with the serum obtained from the cats pre-inoculated with *I. felis* showed no specific bands (the data not shown).

Results of the present study demonstrate that *I. felis* can survive considerably for a long time in the host, but it does not reproduce in enormous numbers just like *T. gondii*; consequently, the antigenic stimulation by the parasite is likely to be quantitatively weaker than that by other pathogenic microorganisms. In recent years, stage-specific antigenicity *T. gondii* has been documented [6–8]. It is plausible that *I. felis* also is antigenically transformed from sporozoites to trophozoites or hypnozoites. This antigenic transformation, if it really occurs, may be associated with the variation of anti-*I. felis* antibody titer in cats infected with *I. felis*, as noted in this study. In a previous report, cats naturally infected with *I. felis* showed low antibody production [10]. One reason for the difference in anti-*I. felis* antibody titers between experimentally and naturally infected cats noted by us, may be explained by the difference in the inoculum dose of parasites to which the cats were exposed. We surmise that the relatively lower anti-*I. felis* antibody titer in naturally infected cats may be due to the past exposure to lower inoculum dose.

Furthermore, cats inoculated orally with *I. felis* oocysts showed a transient appearance and increase in anti-*T. gondii* antibody titer on day 7 p.e. The prepatent period and the pattern of anti-*T. gondii* antibody titer in cats in the present study are somewhat different from those of naturally infected cats [9, 10]. Contamination of the materials used in this experiment with *T. gondii* is probably negligible, because it has been shown that cats infected with *T. gondii* can sustain a high anti-*T. gondii* antibody production for a long period of time. Our finding suggests that cats infected with *I. felis* would very likely show the positive range of anti-*T. gondii* antibody titer useful to serodiagnosis within the first 21 days after exposure. The titer, however, is relatively lower than that of *T. gondii* infected cats [9, 10]. It seems reasonable to do serodiagnostic test at least twice at more than three week interval in order to determine whether cats showing relatively lower titer of anti-*T. gondii* antibody IgG is infected with *T. gondii* or not.

By immunoblotting assay with *I. felis*-infected cat sera, we have noted the presence of cross reactive antigens with m.w. of 45, 58 and 62 kDa between *T. gondii* and *I. felis*. Absorption test indicated that the band of a m.w. of 22 kDa is specific to *I. felis*. The increase in anti-*T. gondii* IgG antibody titer observed in cats infected with *I. felis* may have resulted from an accelerated production of antibody to the cross-reactive antigen. Recently, cross reactive antigen located in the apical region of sporozoan parasites has been documented [11]. Cat serum is recognized as "positive" by the fluorescence pattern against the whole parasite. This suggests that some of the cross-reactive antigens observed by immunoblotting analysis in this study may be associated with apically-located antigens [11] or antigens with similar antigenicity. The reason why common antigen(s) distributed on the whole body was not detectable by immunoblotting analysis may be explained that the sensitivity of immunoblotting analysis for detection of antigen is lower than that of immunofluorescence method. Further studies are necessary to examine whether antibody production to these cross reactive antigens has protective roles and some biological significance.

REFERENCES

1. Arrowood, M. J. and Sterling, C. R. 1987. *J. Parasitol.* 73: 314–319.
2. Chessum, B. S. 1972. *Br. Vet. J.* 128: 33–36.
3. Dubey, J. P. 1976. *Nature (Lond.)* 262: 213–214.
4. Dubey, J. P. and Frenkel, J. K. 1972. *J. Protozool.* 19: 89–92.
5. Freyre, A., Dubey, J. P., Smith, D. D., and Frenkel, J. K. 1989. *J. Parasitol.* 75: 750–755.
6. Kasper, L. H. 1989. *Infect. Immun.* 57: 668–672.
7. Lunde, M. N. and Jacobs, L. 1983. *J. Parasitol.* 69: 806–811.
8. Omata, Y., Igarashi, M., Ramos, M. I., and Nakabayashi, T. 1989. *Parasitol. Res.* 75: 189–193.
9. Omata, Y., Oikawa, H., Kanda, M., Mikazuki, K., Nakayashi, T., and Suzuki, N. 1990. *Jpn. J. Vet. Sci.* 52: 865–867.
10. Omata, Y., Oikawa, H., Kanda, M., Mikazuki, K., Takehara, T., Venturini, C., Saito, A., and Suzuki, N. 1991. *J. Vet. Med. Sci.* 53: 163–165.
11. Taylor, W. P., Evans, C. B., Aley, S. B., Barta, J. R., and Danforth, H. D. 1990. *J. Protozool.* 37: 540–546.
12. Towbin, H., Staehlin, T., and Gordon, J. 1979. *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350–4354.

Exercise-Induced Phospholipid Degradation in the Equine Skeletal Muscle and Erythrocytes

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ABSTRACT. To understand the pathogenesis of equine exercise-induced myopathies and hemolysis, changes o phospholipid peroxidation products in the equine middle gluteal muscle and erythrocytes following the high-spee treadmill exercise were studied. In the skeletal muscle, the peroxidized phosphatidylethanolamine (PE) were increased a 24 hours after the exercise. The malondialdehydes (MDAs) were also increased as the protein-bound MDAs followin exercise. In the erythrocytes, the peroxidized PE were significantly decreased at 24 hours after the exercise. Th protein-bound MDAs were significantly increased at 5 min after the exercise and returned to the base values at 24 hour after the exercise. These findings indicate that the PE is more susceptible to *in vivo* oxidative effects than the othe phospholipid classes, and the accumulation of the protein-bound MDAs is considered to play some cytotoxic roles in th equine skeletal muscle and erythrocytes following exercise.—KEY WORDS: exercise, horse, malondialdehyde, phospholipi peroxidation.

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Strenuous exercise is widely accepted to cause the acute myopathies and/or the hemolysis in horses [9], however, the pathogenesis of these exercise-induced disorders is still unclear. The exercise-induced acute myopathies have been suggested to be resulted from the enhanced free radical generation and subsequent phospholipid peroxidation in the skeletal muscle [7, 15]. In addition, such muscular oxidative changes have been suspected to induce the phospholipid peroxidation in the erythrocytes [4].

On the other hand, some products derived from the phospholipid peroxidation are known to play cytotoxic roles. Phospholipid hydroperoxides, the primary products of peroxidation, have been considered to perturb the cellular homeostasis *via* the activation of phospholipase As [1, 10, 18] and the subsequent accumulation of lysophospholipids in membranes [14]. The end products of the peroxidation, mainly malondialdehydes (MDAs), were demonstrated to be associated with the polymerization and aggregation of membrane compounds [6, 8], and the reduction of the cellular function [2]. Therefore, the selective quantitation of the each peroxidation products as well as the detection of the free radical generation are required to understand the pathogenesis of exercise-induced disorders in horses.

The present study deals with the phospholipid peroxidation products, the peroxidized phosphlipids and MDAs, in the equine skeletal muscle and erythrocytes following exercise.

MATERIALS AND METHODS

Animals: Four clinically healthy adult thoroughbred horses (aged 5 to 7 years; 1 male, 2 neutered male and 1 female; 483 to 500 kg body weight) were used in this study. These horses were kept in Institute for Japan Racing Association under common feeding, and none of them had been in training for at least 2 weeks prior to the investigation. The 2 of 4 horses were studied again at 3 weeks after the first trials.

Exercise: The horses received the exercise on a high-speed treadmill (Deltalong, Australia) at a slope of 10%. The exercise-program was set at 4 m/s for the first 5 min and then 2 m/s for the next 5 min for the warming-up. After 10 min, the speed was increased to 10 m/s and held constant for 5 min. Finally, the speed was set at 4 m/s for 3 min and 2 m/s for 17 min for the cooling down.

Sample collection: Tissue specimens were obtained from the middle gluteal muscle by the technique of Snow and Guy [20] with a percutaneous needle immediately prior to, and at 10 min and 24 hours after the exercise. The tissue speci-

mens were collected with the constant depth (5 cm) from the skin, since Kai [11] reported that the distribution of fiber types in the equine middle gluteal muscle are nonhomogenous with their depth from the skin. Blood samples were collected from the jugular vein immediately prior to, and at 5 min and 24 hours after the exercise. The packed cell volume (PCV: micro hematocrit method), blood lactate concentration (electrode method) and plasma creatine phosphokinase (CPK) activities (kinetic method) were measured.

Sample preparation for the peroxidized phospholipids and MDAs: A piece (100 to 150 mg) of the tissue specimen was homogenized with 2.5 m*l* of ice-cold 0.1 M phosphate buffer (pH 7.4, containing 10 mM ethylendiamine-tetraacetic acid) in a glass homogenizer on ice for 1 min. The 1.2 m*l* of the homogenate was used for the extraction of whole lipids and free MDAs. The 1.0 m*l* of the rest homogenate was subjected for the extraction of protein-bound MDAs. The erythrocytes were prepared from the whole blood as described in our previous report [16]. The 300 μl of the packed erythrocytes suspended in 900 μl of the 0.1 M ice-cold phosphate buffer (as described above) were used for the extraction of whole lipids and free MDAs. The 300 μl of the rest packed erythrocytes were suspended in 700 μl of the same phosphate buffer and subjected for the extraction of protein-bound MDAs.

The whole lipids and free MDAs were extracted with the Bligh-Dyer lipid extraction method [3] according to the report of Schmedes and Hølmer [17]. In brief, the 1.2 m*l* of the tissue homogenate or erythrocyte suspensions was placed in a screw-capped glass test tube. The 4.5 m*l* of ice-cold chloroform-methanol mixture (1:2, v/v) containing 0.02% tert-buthylhydroxytoluene (Sigma, St. Louis, MO, U.S.A.) was added and mixed vigorously for 1 min. Then 1.5 m*l* of ice-cold chloroform and 1.5 m*l* of ice-cold 1.15% KCl were added. The mixture was vigorously mixed again for 1 min. Finally, the mixture was centrifuged at 2,300 G for 10 min. The aqueous phase (upper layer) including free MDAs was collected and immediately used for assay. The chloroform phase (lower layer) including whole lipids was collected, filtrated with a membrane filter (Dismic 13Jp, Advantec, Tokyo, Japan) and stored at −20°C until the HPLC analysis of peroxidized phospholipids.

For the preparation of protein-bound MDAs, an alkaline pretreatment of the sample [12] was performed prior to the Bligh-Dyer extraction. In brief, 1.0 m*l* of the tissue homogenate or erythrocyte suspension was placed in a screw-capped glass test tube. The 0.1 m*l* of 0.5 N NaOH was added, and the tightly capped test tube was incubated at 60°C for 30 min for the release of protein-bound MDAs. After cooling in tap water, the mixture was neutralized to pH 7.8 with 0.1 m*l* of 0.5 N HCl. Then, the 1.2 m*l* of the mixture was subjected to the Bligh-Dyer extraction as described above. The aqueous phase (upper layer) including protein-bound MDAs with free MDAs was collected and immediately used for assay.

HPLC analysis for peroxidized phospholipids: The HPLC method for the analysis of peroxidized phospholipids was described in our previous report [13]. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were detected in the equine skeletal muscle and PE, PC, phosphatidylinositol (PI) and phosphatidylserine (PS) were detected in the equine erythrocytes by this HPLC method.

MDA quantitation: The concentration of free MDAs and protein-bound MDAs was determined with a thiobarbituric acid (TBA) method as described by Schmedes and Hølmer [17]. The TBA reagent was prepared by dissolving 1% (w/v) TBA (Merck, Darmstadt, B. R. D.) in 5% trichloroacetic acid (Wako Pure Chemical, Osaka, Japan) solution. The 1.0 m*l* of the extracts (the Bligh-Dyer aqueous phase) was mixed with 1.0 m*l* of the TBA reagent in a screw capped test tube. The tightly capped test tube was heated in a water bath at 95°C for 30 min, cooled in tap water and centrifuged at 1,500 G to assure a clear solution. The fluorescent intensity was measured by a Model 650–10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at Ex 515 nm and Em 553 nm. The MDA value was obtained with the standard curve prepared in the each assay. The concentration of protein-bound MDAs was calculated from the MDA concentration in a sample subjected to the alkaline hydrolysis, and the corresponding free MDA concentration.

Statistical analysis: The statistical differences were analyzed by paired *t*-test.

RESULTS

After the exercise, neither the clinical acute myopathy nor hemolysis was observed in any case. The PCV was increased at 5 min after the exercise

Table 1. Changes of the packed cell volume, blood lactate concentration and plasma creatine phosphokinase (CPK) activities following the treadmill exercise

Measurement		Horse No. No. 1	No. 2	No. 2[a)]	No. 3	No. 4	No. 4[a)]	Mean±SD
Packed cell volume (%)	Pre	45	44	43	40	49	51	45± 4
	5 min	58	57	57	53	54	55	56± 2**
	24 hr	46	43	41	41	49	52	45± 5
Blood lactate (mg/dl)	Pre	2	3	6	3	4	5	4± 1
	(JBC[b)])	(120)	(120)	(174)	(79)	(73)	(106)	(112±36***)
	5 min	90	92	148	20	18	70	73±49*
Plasma CPK (IU/l)	Pre	113	62	70	45	64	74	71±23
	5 min	209	130	158	132	82	122	138±42**
	24 hr	233	172	212	168	157	173	185±30***

a) The second trials.
b) Just before cooling down.

* $p<0.05$.
** $p<0.01$.
*** $p<0.001$.

Table 2. Changes of the peroxidized phospholipids in the equine skeletal muscle following the treadmill exercise

Class ($\times 10^{-2}$ A/A[a)])		Horse No. No. 1	No. 2	No. 2[b)]	No. 3	No. 4	No. 4[b)]	Mean±SD
Phosphatidyl-ethanolamine	Pre	8.19	6.76	6.23	6.49	10.0	5.37	7.17±1.52
	10 min	8.64	7.03	8.39	8.03	7.44	7.11	7.77±0.62
	24 hr	13.6	9.71	7.40	7.79	7.69	9.01	9.20±2.13
Phosphatidyl-choline	Pre	2.76	3.22	2.04	3.00	2.89	1.64	2.59±0.56
	10 min	5.39	2.63	0.86	2.91	2.62	1.43	2.64±1.43
	24 hr	3.51	3.43	2.13	3.52	2.33	1.85	2.80±0.71

a) The ratio of peak areas simultaneously detected at 233 nm and 206 nm (A233 nm/A206 nm).
b) The second trials.

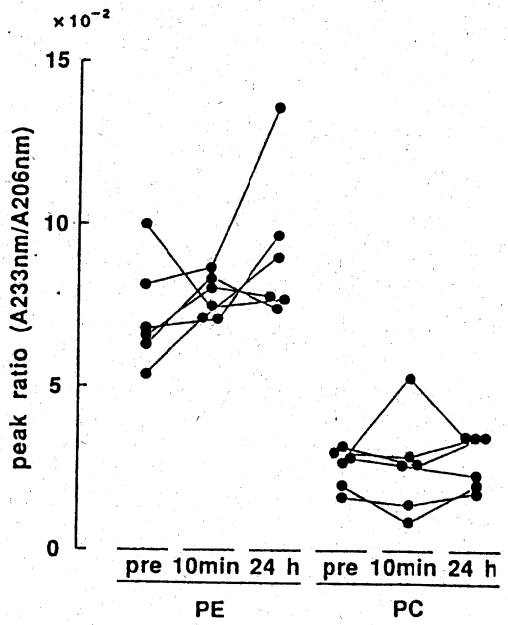

Fig. 1. Changes of the peroxidized phospholipids in the equine skeletal muscle following the treadmill exercise in 6 cases. PE; phosphatidylethanolamine, PC; phosphatidylcholine.

and decreased to the base values at 24 hours after the exercise (Table 1). The blood lactate concentration was increased just before cooling down (Table 1), and reduced to the base values at several hours after the exercise (data not shown). The plasma CPK activities were also increased after exercise (Table 1).

The changes of peroxidized PE and PC in the middle gluteal muscle are shown in Table 2 and Fig. 1. The peroxidation levels in PE were higher than those in PC before exercise. The peroxidized PE showed an increasing tendency at 24 hours after exercise, whereas the peroxidized PC concentration seemed not to be changed. The changes of the MDA concentration in the skeletal muscle are shown in Table 3 and Fig. 2. The total MDAs (protein-bound and free MDAs) were increased in all of 4 horses at 10 min or 24 hours after exercise. While the MDA concentration was increased in different manners in each horse, the increase in the protein-bound MDAs appeared to mainly contribute to the increase in the total MDA concentration.

Table 3. Changes of the total, free and protein-bound malondialdehydes in the equine skeletal muscle following the treadmill exercise

Malondialdehydes (nmol/g wet tissue)		Horse No. 1	No. 2	No. 3	No. 4	Mean±SD
Total	Pre	3.26	2.12	2.63	2.12	2.53±0.54
	10 min	3.39	4.45	6.15	6.78	5.19±1.55*
	24 hr	6.30	2.67	2.32	3.81	3.78±1.80
Free	Pre	3.98	1.42	1.16	0.85	1.85±1.43
	10 min	2.61	3.05	0.74	1.23	1.90±1.10
	24 hr	3.80	1.15	0.47	2.23	1.91±1.45
Protein-bound[a)]	Pre	—	0.70	1.47	1.27	0.86±0.66
	10 min	0.78	1.40	5.41	5.55	3.29±2.54*
	24 hr	2.50	1.52	1.85	1.58	1.86±0.45

a) Calculated from (Total MDA)-(Free MDA).
* $P<0.05$

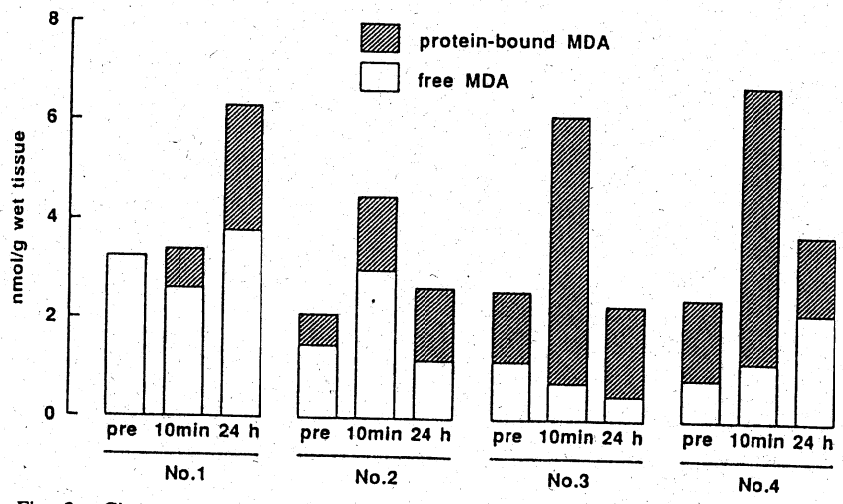

Fig. 2. Changes of the free malondialdehydes (MDA) and protein-bound MDA in the equine skeletal muscle following the treadmill exercise in 4 horses.

The changes of the peroxidized phospholipids in erythrocytes are shown in Table 4 and Fig. 3. The peroxidized PE were continuously and significantly decreased at 24 hours after exercise, whereas the peroxidation levels in the other 3 phospholipid classes were not changed. However, the peroxidation levels in PI showed the extremely low values. The changes of the MDA concentration in the erythrocytes are shown in Table 5 and Fig. 4. No change was observed in the free MDA concentration following exercise. The protein-bound MDA concentration was significantly increased at 5 min after the exercise and returned to the base values at 24 hours after the exercise.

DISCUSSION

The increase of the PCV, blood lactate concentration and plasma CPK activities following exercise warranted the enough load of treadmill exercise, while neither the clinical acute myopathy nor hemolysis was observed. The blood lactate, derived from the anaerobic metabolism in the skeletal muscle, were increased after the exercise. Davies *et al.* [7] reported that the exercise-induced anaerobic condition in the rat skeletal muscle caused the enhancement of free radical generation *via* the reduced mitochondrial respiratory control.

Since the peroxidation levels of PE were specifically elevated following exercise, it was considered that the muscular PE was particularly susceptible to the *in vivo* oxidative stress caused by the exercise.

Table 4. Changes of the peroxidized phospholipids in the equine erythrocytes following the treadmill exercise

Class ($\times 10^{-1}$ A/A[a])		Horse No. 1	No. 2	No. 2[b]	No. 3	No. 4	No. 4[b]	Mean±SD
Phosphatidyl-inositol	Pre	0.98	0.62	0.97	0.31	0.25	1.10	0.71±0.37
	5 min	0.73	0.47	0.67	ND	0.38	0.90	0.63±0.21
	24 hr	0.66	0.56	0.45	ND	ND	0.66	0.58±0.10
Phosphatidyl-serine	Pre	10.0	7.54	8.76	10.4	8.00	12.4	9.52±0.73
	5 min	7.72	8.49	8.32	7.45	11.0	12.5	9.24±0.83
	24 hr	7.97	10.2	8.25	8.02	10.0	9.67	9.02±0.43
Phosphatidyl-ethanolamine	Pre	7.63	7.53	8.85	7.55	7.22	12.0	8.46±0.74
	5 min	7.17	7.68	7.35	7.39	6.74	8.72	7.51±0.27
	24 hr	5.10	7.12	6.48	6.75	3.73	8.42	6.27±0.67*
Phosphatidyl-choline	Pre	6.72	7.08	8.24	4.23	6.47	10.4	7.19±0.84
	5 min	5.37	6.38	7.20	6.85	4.74	8.14	6.45±0.51
	24 hr	7.29	6.22	6.20	6.39	6.56	8.63	6.88±0.39

a) The ratio of the peak areas simultaneously detected at 233 nm and 206 nm (A233 nm/A206 nm).
b) The second trials.
ND not detected at 233 nm.
* $P<0.01$.

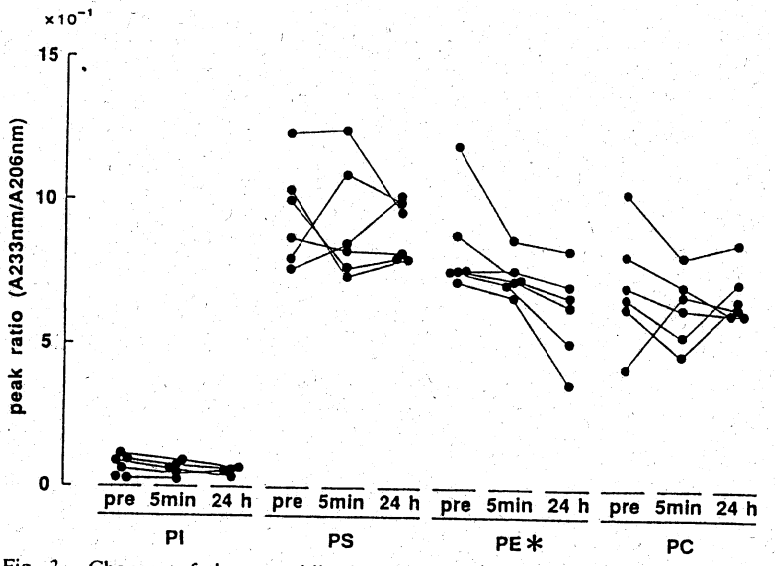

Fig. 3. Changes of the peroxidized phospholipids in the equine erythrocytes following the treadmill exercise in 6 cases. PI; phosphatidylinositol, PS; phosphatidylserine, PE; phosphatidylethanolamine, PC; phosphatidylcholine. *$p<0.01$ (pre vs 24 hr).

The peroxidized phospholipids were shown to activate phospholipase as in rats and were considered to perturb the membrane *via* the enzyme-mediated phospholipid catalysis [1, 10, 18]. Thus, the accumulation of the peroxidized PE in the equine skeletal muscle following exercise would be able to cause the myopathies *via* the degradation of muscular membranes.

The rate of the PE peroxidation might be higher than that of the degradation of the peroxidized PE into MDAs or other end products. The MDA produced in the skeletal muscle seemed to cause mainly the increase in the protein-bound MDAs. This finding supported previous reports that the MDA was highly reactive to proteins [5, 6]. The transient increase in protein-bound MDAs, which could mean the polymerization and aggregation of cellular compounds [6, 8], would also induce the

Table 5. Changes of total, free and protein-bound malondialdehydes in the equine erythrocytes following the treadmill exercise

Malondialdehydes (nmol/ml packed erythrocytes)		Horse No. No. 1	No. 2	No. 2[b]	No. 3	No. 4	No. 4[b]	Mean±SD
Total	Pre	2.36	2.16	2.12	2.00	2.14	2.17	2.16±0.05
	5 min	3.15	2.84	2.72	2.90	2.64	2.72	2.83±0.75**
	24 hr	2.77	2.59	2.33	1.78	1.68	2.10	2.21±0.18
Free	Pre	0.94	0.89	1.06	1.21	1.20	1.18	1.08±0.14
	5 min	0.90	1.20	0.96	1.36	1.14	1.22	1.13±0.17
	24 hr	0.89	1.22	0.97	1.16	1.15	1.18	1.10±0.13
Protein-bound[a]	Pre	1.42	1.27	1.06	0.79	0.96	0.99	1.08±0.23
	5 min	2.25	1.64	1.76	1.54	1.50	1.50	1.69±0.29*
	24 hr	1.88	1.37	1.36	0.62	0.53	0.92	1.11±0.51

a) Calculated from (Total MDA)-(Free MDA).
b) The second trials.
* $p<0.01$.
** $p<0.001$.

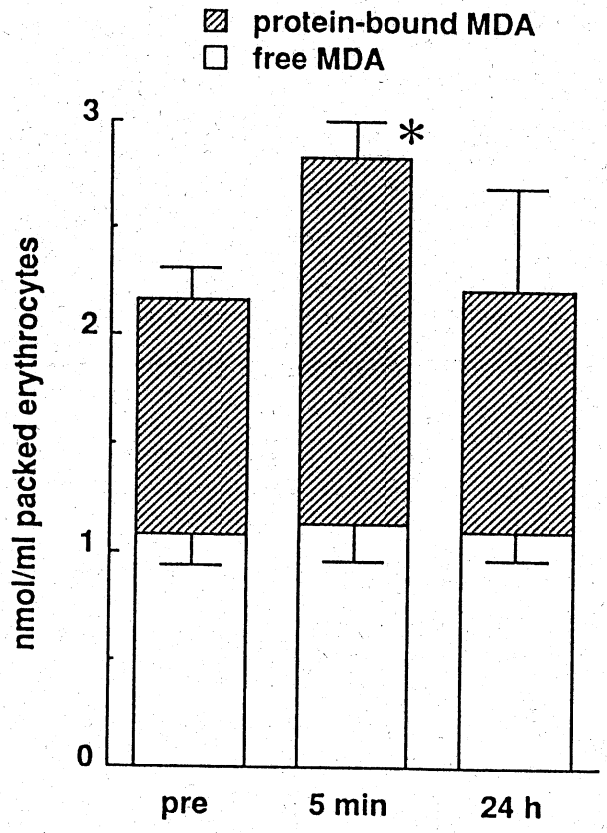

Fig. 4. Changes of the free malondialdehydes (MDA) and protein-bound MDA in the equine erythrocytes following the treadmill exercise in 6 cases.
*$p<0.01$ (pre vs 5 min).

reduced cellular homeostasis followed by tissue damages. In this experiment, the protein-bound MDAs were returned to the base values in 2 of 4 horses at 24 hours after exercise. However, the *in vivo* catabolic pathway of the protein-bound MDA is unclear, while the free MDA was demonstrated to be broken down rapidly by the mitochondrial enzymes *in vitro* [2, 19].

On the other hand, the peroxidized PE in the erythrocytes was significantly decreased at 24 hours after the exercise, whereas the peroxidation levels in the other phospholipid classes were unchanged. The protein-bound MDAs were specifically increased at 5 min after the exercise like as in the course of the skeletal muscle, and diminished to the base values at 24 hours. These findings would indicate the enhancement of specific nonenzymic degradation of the peroxidized PE into MDAs, or the clearance of the peroxidized PE by the activated phospholipases [1, 18] followed by the nonenzymic degradation of fatty acid hydroperoxides into MDA. The catabolic pathway of MDAs in the erythrocytes has not been elucidated. However, it could be considered that the erythrocytes with accumulated protein-bound MDAs were susceptible to the entrapment in the spleen, because of the changes in the rheological properties of erythrocytes *via* the polymerization of band 1 and 2 proteins, and/or the accumulation of fluorescent pigments [8].

These results suggested that the exercise-induced myopathies and hemolysis in horses were due to, at least in part, the accumulation of the protein-bound MDAs related with the membrane PE degradation.

REFERENCES

1. Beckman J. K., Borowith, S. M., and Burr, I. M. 1987. The role of phospholipase A activity in rat liver microsomal lipid peroxidation. *J. Biol. Chem.* 262: 1479–1481.
2. Bird, R. P. and Draper, H. H. 1982. Uptake and oxidation of malonaldehyde by cultured mammalian cells. *Lipids* 17: 519–523.
3. Bligh, E. G. and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911–917.
4. Brady, P. S., Ku, P. K., and Ullrey, D. E. 1978. Lack of effect of selenium supplementation on the response of the equine erythrocyte glutathione system and plasma enzymes to exercise. *J. Anim. Sci.* 47: 492–496.
5. Buttkus, H. and Bose, R. J. 1972. Amine-malonaldehyde condensation products and their reactive color contribution in the thiobarbituric acid test. *J. Am. Oil Chem. Soc.* 49: 440–443.
6. Crawford, D. L., Yu, T. C., and Sinnhuber, R. O. 1967. Reaction of malonaldehyde with protein. *J. Food Sci.* 32: 332–335.
7. Davies, K. J. A., Quintanilha, A. T., Brooks, G. A., and Packer, L. 1982. Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* 107: 1198–1205.
8. Hochstein, P. and Jain, S. K. 1981. Association of lipid peroxidation and polymerization of membrane proteins with erythrocyte aging. *Fed. Proc.* 40: 183–188.
9. Hodgson, D. R. 1987. Exertional rhabdomyolysis. pp. 487–490. *In*: Current Therapy in Equine Medicine, 2nd ed. (Robinson, N. E. ed.), W. B. Saunders Company, Philadelphia.
10. Itabe, H., Kudo, I., and Inoue, K. 1988. Preferential hydrolysis of oxidized phospholipids by peritoneal fluid of rats treated with casein. *Biochim. Biophys. Acta* 963: 192–200.
11. Kai, M. 1984. Distribution of fiber types in equine middle gluteal muscle. *Bull. Equine Res. Inst.* 21: 46–50.
12. Lee, H. and Csallany, A. S. 1987. Measurement of free and bound malondialdehyde in vitamin E-deficient and -supplemented rat liver tissues. *Lipids* 22: 104–107.
13. Matsuki, N., Tamura, S., Ono, K., Watari, T., Goitsuka, R., Takagi, S., and Hasegawa, A. 1991. The high-performance liquid chromatographic analysis for the peroxidized phospholipids in equine erythrocytes and skeletal muscle. *J. Vet. Med. Sci.* 53: 717–719.
14. Mock, T. and Man, R. Y. K. 1990. Mechanism of lysophosphatidylcholine accumulation in the ischemic canine heart. *Lipids* 25: 357–362.
15. Novelli, G. P., Bracciotti, G., and Falsini, S. 1990. Spin-trappers and vitamin E prolong endurance to muscle fatigue in mice. *Free Radical Biol. Med.* 8: 9–13.
16. Ono, K., Inui, K., Hasegawa, T., Watanabe, H., Takagi, S., Hasegawa, A., and Tomoda, I. 1989. Superoxide dismutase, glutathione peroxidase, and catalase activities in equine erythrocytes. *Jpn. J. Vet. Sci.* 51: 656–658.
17. Schmedes, A. and Hølmer, G. 1989. A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. *J. Am. Oil Chem. Soc.* 66: 813–817.
18. Sevanian, A., Muakkassah-Kelly, S. F., and Montestruque, S. 1983. The influence of phospholipase A_2 and glutathione peroxidase on the elimination of membrane lipid peroxidation. *Arch. Biochem. Biophys.* 223: 441–452.
19. Siu, G. M. and Draper, H. H. 1982. Metabolism of malonaldehyde *in vivo* and *in vitro*. *Lipids* 17: 349–355.
20. Snow, D. H. and Guy, P. S. 1976. Percutaneous needle biopsy in the horse. *Equine Vet. J.* 8: 150–155.

Enhancement of Humoral Immune Response of *Isospora felis*-Infected Cats after Inoculation with *Toxoplasma gondii*

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Toxoplasma gondii (T. gondii), an obligate intracellular protozoan parasite, is prevalent in man and animals throughout the world. Several workers have reported that cats shed transiently a number of oocysts during the acute phase of infection, while chronically infected cats seldom pass out oocysts, even with reinfection [5]. One of the feline coccidia, *Isospora felis (I. felis)* commonly found in wild and domestic cats is recognized as non-pathogenic. Both parasitic species can penetrate extra-intestinal tissues, and survive for long periods. The relationship between *T. gondii* and *I. felis* is biologically and epidemiologically significant, since superinfection with *I. felis* has been shown to induce reshedding of *T. gondii* oocysts in cats chronically infected with *T. gondii* [2, 6]. However, it is still unclear whether such mixed coccidial infections have some influence on the immune response of cats to either *I. felis* or *T. gondii* infection.

In the present study, we attempted to examine anti-I. felis antibody in sera of cats infected with *I. felis* using indirect immunofluorescence assay (IFA). The effect of *T. gondii* infection on the humoral immune response against *I. felis* of the cats was likewise studied.

Cysts of S-273 strain of *T. gondii* were obtained from brains of mice on the 5th to 7th week post-infection and suspended to adjust cyst density to 10^2 cysts in one m*l* of saline. Trophozoites of RH strain of *T. gondii* were prepared from infected mouse embryonal cell culture as described previously [11]. Oocysts of *I. felis* and *T. gondii* were isolated from the feces of infected cats by floating in $ZnSO_4$ solusion and suspended to a concentration of 10^5 oocysts in one m*l* of saline. Sporulated oocysts of *I. felis* were isolated by sucrose density gradient centrifugation [1] and excystation was done by incubating the oocysts in phosphate buffered saline (PBS), containing 0.5% taurocholic acid and 0.1% trypsin at 37°C for 30 min. Sporozoites were then isolated by percoll-sucrose density gradient centrifugation [1]. Some of the isolated parasites were fixed in 1.0% paraformaldehyde in PBS at 4°C for 15 min and washed in PBS three times.

Male and female six-week-old cats were kept in individual cages under strict isolation in the Animal Care Facilities at Shionogi-Aburahi Laboratories, Shiga. Three groups of cats used are as follows; Group 1) one cat that shed no oocysts of either *T. gondii* or *I. felis* was inoculated orally with 10^5 oocysts of *T. gondii* and on the 46th day post inoculation (p. i.), the cat challenged with 10^5 oocysts of *I. felis* (negative control); Group 2) two cats that shed intermittently *I. felis* oocysts as natural infection were inoculated with 10^5 of oocysts of *T. gondii*; and Group 3) consisted of three cats that shed *I. felis* oocysts as natural infection, two of which were inoculated intramuscularly with 100 mg of freeze-thawed killed *T. gondii* together with Freund's complete adjuvant three times at three-weeks interval, and one cat was injected with the same volume of saline instead of killed parasites. On the 1st week post-final injection, they were inoculated with 10^2 cysts of S-273 strain of *T. gondii* orally.

For the detection of excretion of oocysts, cats' feces were collected daily, and examined microscopically [4].

For measuring anti-*I. felis* and anti-*T. gondii* antibodies, sera were obtained weekly from each cat from the jugular vein, and stored at −20°C until use. For testing, serum samples were diluted four-fold serially in PBS. Anti-*T. gondii* IgG antibodies were examined by IFA [11] and their titers are expressed in the text and figures as reciprocal of serum dilution.

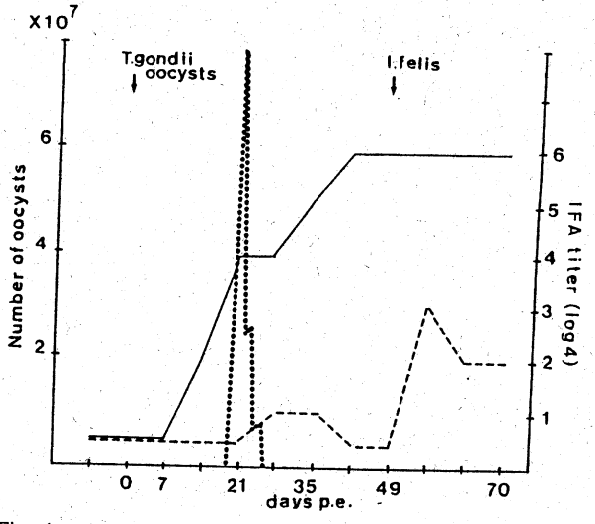


Fig. 1. Aspects of oocyst excretion and antibody production in a cat inoculated with oocysts of *T. gondii* followed by inoculation with oocysts of *I. felis*.

A cat which had never been infected with both of *T. gondii* and *I. felis* was inoculated with 10^5 oocysts of *T. gondii* (S-273 strain), subsequently with 10^5 oocysts of *I. felis* on the 46th day p. e. with *T. gondii*.

Anti-*T. gondii* IgG antibody (solid line); anti-*I. felis* IgG antibody (broken line); *T. gondii* oocysts per cat per day (dotted line); post-exposure (p.e.)

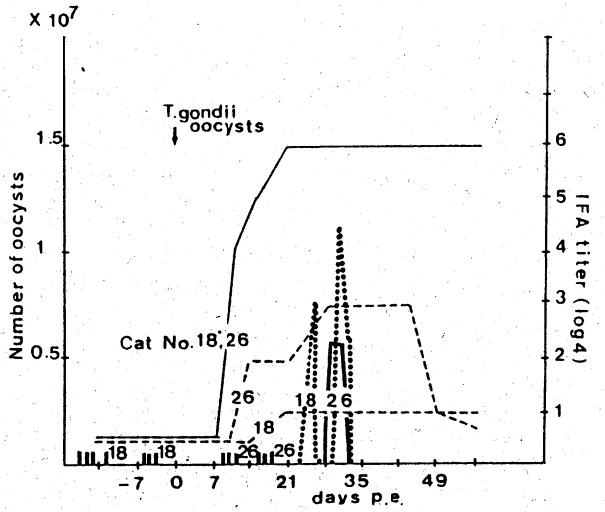


Fig. 2. Profile of anti-*I. felis* or anti-*T. gondii* antibody production in cats naturally infected with *I. felis* but experimentally with *T. gondii*.

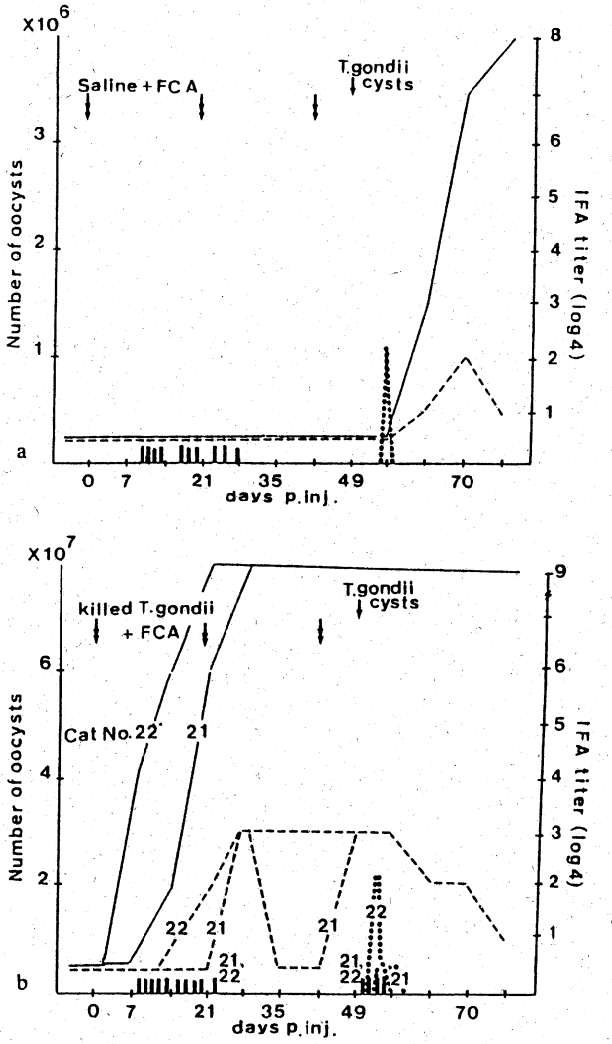
An asterisk refers to the identification of cat used. Anti *T. gondii* IgG antibody (solid line); anti-*I. felis* IgG antibody (broken lines); *I. felis* oocysts (thick solid lines) and *T. gondii* oocysts (dotted lines) per cat per day.

Anti-*I. felis* IgG antibody titre was determined by IFA following the same procedure used for anti-*T. gondii* IgG determination, using sporozoites instead of *T. gondii* RH strain trophozoites.

Cat of Group 1 shed *T. gondii* oocysts between 20 and 26 days post-exposure (p. e.) with *T. gondii* (Fig. 1). Peak of shedding was on the 21st day p. e. and the total number of oocysts was approximately 18.6×10^7. Shedding of *I. felis* ooycsts was not observed until the end of the experiment, despite inoculation with *I. felis* oocysts on the 46th day p. e. with *T. gondii*.

As shown in Fig. 2, one of the two cats (cat No. 26) in group 2, shed *I. felis* oocysts irregularly from the 9th to the 18th and from the 30th to the 32nd day p. e., and oocyst counts per day ranged from 10^5 to 10^7. The other cat (cat No. 18) also shed *I. felis* oocysts transiently from the 4th to the 8th day p. e. prior to inoculation with *T. gondii* oocysts. After challenge with oocysts of *T. gondii*, cats shed *T. gondii* oocysts from the 23rd to 27th day p. e. (cat No. 18), or on the 30th day until the 33rd day p. e. (cat No. 26) respectively. Peak of shedding was on the 24th day (cat No. 18) and on the 31st day (cat No. 26) p. e. The total number of oocysts varied from 0.8×10^7 (cat No. 18) to 2×10^7 (cat No. 26).

As shown in Fig. 3a, a cat exposed to cysts showed transient shedding of *I. felis* oocysts on the 21st to the 42nd day before inoculation with *T. gondii* cysts. Thereafter, the cat began to shed *T. gondii* oocysts on the 5th to the 8th day p. e. The total number of oocysts was about 1.2×10^6. Reshedding of *I. felis* oocysts was not observed. In Group 3b, both cats shed *I. felis* oocysts transiently from the 27th to the 41st day before *T. gondii* infection.



Figs. 3a and 3b. Effect of inoculation of *T. gondii* parasites: oocysts excreted and antibody production of *I. felis*-infected cats immunized with either saline (3a) or killed *T. gondii* (3b), and post-challenged with live cysts of S-273 strain of *T. gondii*.

Anti-*T. gondii* IgG antibody (solid lines); anti-*I. felis* IgG antibody (broken lines); oocysts of *I. felis* (thick solid lines) and oocysts of *T. gondii* (dotted lines) per cat per day; post injection of killed *T. gondii* (p. inj.).

Shedding of oocysts of *T. gondii* was observed on the 3rd (Cat No. 21) or the 4th (Cat No. 22) day p. e., and continuously shed for four days. Shedding of *I. felis* oocysts was also detected on the 2nd day before shedding of *T. gondii* oocysts, and continued for four days (Fig. 3b).

Anti-*T. gondii* IgG antibody in Group 1 (Fig. 1) appeared on the 14th day p. e., then increased its IFA titre to 4^6, and remained at the same level until the end of the experiment. Anti-*I. felis* IgG antibody was detected on the 28th day to the 35th day p.e., even prior to inoculation of the cat with *I. felis* oocysts. On the 46th day p.e. to *T. gondii*, the cat was inoculated with *I. felis* oocysts. An

increase of anti-*I. felis* IgG antibody titre was observed as 4^3 on the 9th day p. e.

In Group 2 (Fig. 2), cats exposed to *T. gondii* oocysts also showed similar pattern of anti-*T. gondii* IgG antibody production to that of Group 1. Anti-*I. felis* IgG antibody also appeared on the 14th day p. e. (cat No. 26) or on the 21th day p.e. (cat No. 18).

Likewise, a cat inoculated with *T. gondii* cysts showed an increase of anti-*T. gondii* IgG antibody on the 14th day p. e. and the titre reached to 4^8 on the 28th day p. e. (Fig. 3a). Anti-*I. felis* IgG antibody peaked to 4^3 on the 21st day p. e., and thereafter gradually decreased to 4^1 on the 77th day p. e.

Cats immunized with killed parasites of *T. gondii* registered a high level of anti-*T. gondii* IgG titre from the second injection of killed parasites (Fig. 3b). Anti-*I. felis* IgG antibody also appeared transiently after the second and third injection of killed *T. gondii*. After the cats were inoculated with *T. gondii* cysts, they showed a transient increase of anti-*I. felis* IgG titre, while anti-*T. gondii* IgG antibody sustained the high titre level until the end of the experiment.

Our findings demonstrate that in cats infected with *I. felis*, anti-*I. felis* IgG antibody titre is seemingly low or non detectable during the period of oocyst shedding as well as during chronic phase of infection. Several authors have reported that antigenic differences were found between *T. gondii* sporozoites and trophozoites and the rapid loss of sporozoite specific antigens was observed after sporozoite invasion into the host tissues [8, 9, 10]. Also, *I. felis* has been shown to possess the ability to survive in host cells as hypnozoites and induce no host immune reactions [3]. In view of this, our data suggest that antibody-production response to the antigenic stimulation of *I. felis* sporozoite in cats is apparently low to detect the presence of antibody by IFA. We have no data, however, to show the antigenic differences between *I. felis* sporozoites and hypnozoites, as well as between *I. felis* and *T. gondii*. Thus, further studies are necessary to clarify these questions. Moreover, other assay systems should be developed to detect host immune reactions against *I. felis*.

Anti-*I. felis* antibody production was enhanced by exposing the *I. felis*-infected cats to *T. gondii*. Interestingly, cats immunized with killed *T. gondii* also showed the increase of anti-*I. felis* antibody titre even before inoculated with live *T. gondii*. This phenomenon may have resulted from the immunopotentiating activity of *T. gondii* antigens. Our findings seem to agree with studies that documented enhanced anti-tumour and anti-parasite effect(s) of *T. gondii* and its extracts in animals [7, 12, 13]. Furthermore, cross-immune reaction between *T. gondii* and *I. felis* may be another plausible explanation for such a phenomenon. However, studies on the comparative analysis of antigenicity between *T. gondii* and *I. felis* are needed.

It is also interesting to note that cats immunized with killed *T. gondii* did not appreciably suppressed shedding of *T. gondii* and *I. felis* oocysts, despite the cats' production of high anti-*T. gondii* antibody titre. This finding suggests the apparent absence of the effect of humoral antibodies in suppressing oocyst excretion after inoculation with *T. gondii* cysts. A better understanding of this phenomenon warrants further studies related to mechanism of local immune reaction(s) and cell-mediated immunity involving intestinal parasitism.

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REFERENCES

1. Arrowood, M. J. and Sterling, C. R. 1987. *J. Parasitol.* 73: 314–319.
2. Chessum, B. S. 1972. *Br. Vet. J.* 128: 33–36.
3. Dubey, J. P. and Frenkel, J. K. 1972. *J. Protozool.* 19: 89–92.
4. Dubey, J. P., Swan, G. V., and Frenkel, J. K. 1972. *J. Parasitol.* 58: 1005–1006.
5. Dubey, J. P. and Frenkel, J. K. 1974. *Vet. Pathol.* 11: 350–379.
6. Dubey, J. P. 1976. *Nature (Lond.)* 262: 213–214.
7. Dubey, J. P. and Beattie, C. P. 1988. p. 27. *In*: Toxoplasmosis of Animals and Man. CRC Press, Boca Raton, Florida.
8. Kasper, L. H., Bradley, M. S., and Pfefferkorn, E. R. 1984. *J. Immunol.* 132: 443–449.
9. Kasper, L. H. and Ware, P. L. 1985. *J. Clin. Invest.* 75: 1570–1577.
10. Kasper, L. H. 1989. *Infect. Immun.* 57: 668–672.
11. Omata, Y., Oikawa, H., Kanda, M., Mikazuki, K., Nakabayashi, T., and Suzuki, N. 1990. *Jpn. J. Vet. Sci.* (in press)
12. Saito, A., Sakurai, H., Terada, S., Igarashi, I., Hirose, H., Suzuki, N., Osaki, H., Kudo, H., Sakata, Y., Okamoto, K., and Yoshida, Y. 1989. *Zentralbl. Bakteriol. Hyg., A* 271: 114–126.
13. Tadros, W. and Laarman, J. J. 1981. pp. 321–327. *In*: Advance of Parasitology. Academic Press, London.

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Cellualr Subsets Involved in Protective Immunity to *Babesia rodhaini* Infection in BALB/c Mice

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ABSTRACT: Protective immune responses and the functional role of spleen cells in mice infected with *Babesia rodhaini* were examined with an in vitro proliferation assay systems and by in vivo passive transfer of spleen cells to uninfected mice. Mice that resolved primary babesial infection after chemotherapy (*Babesia* immune mice) had transient and low parasitemia after challenge infection and high rates (75%) of survival. *Babesia* hyperimmune mice, by contrast, had no detectable parasitemia after challenge and 100% survival. Proliferative response of spleen cells to *Babesia* lysate antigen (BLA) were determined for mice from both groups. This proliferative response was inhibited by treatment of spleen cells with anti-T cell serum and monoclonal antibody (MAb) to Lyt1 antigen. Spleen cells of hyperimmune mice produced larger amount of IL-2 production than those of immune mice. Transfer of spleen cells from immune mice to nonimmune mice provided protection against babesial infection and recipient mice had high titers of anti-babesial antibody. When these spleen cells were treated with anti-T cell serum or anti-mouse Ig serum, protection against challenge was abolished. By contrast, transfer of hyperimmune spleen cells was capable of protecting recipient mice. Treatment of hyperimmune spleen cells with antiserum to mouse Ig or MAb against Lyt 1 and Lyt 2 antigens did not interfere with their ability to protect recipient mice against infection, even though recipient mice had low levels of antibody production. These results indicate that humoral immune response is important in establishing protection after primary infections while the participation of Lyt 1^+ cells and Lyt 2^+ cells and other aspects of the cell-mediated immune response is important in controlling secondary infections.

INTRODUCTION

Babesia rodhaini and *B. microti* have been widely used as experimental models for investigating babesiosis in domestic animals. These two rodent parasites differ widely in pathogenicity. Infections with *B. microti* are non-lethal and self-limiting while *B. rodhaini* are virulent and usually fatal. Mechanisms mediating the clearance of these parasites are not fully understood, but involve both humoral and cell-mediated aspects of immune system.

Adoptive transfer of spleen cells from mice with chronic infections of *B.microti* was effective in reducing parasitemia in recipient mice(Meeusen et al,1984; Ruebush and

Hanson,1980). In this experiment, B cells were necessary to establish protection during primary infections while T cells played an important role in secondary infections. The role of NK cells in establishing protective immunity to babesial infections is controversial (Eugui and Allison,1980; Wood and Clark, 1982). Mice are not able to completely clear primary infections with *B. rodhaini*. However, sensitization of mice with *Toxoplasma* lysate antigen increases survival to as 40%. Numbers of T cells, B cells, and NK cells increase in surviving mice while numbers of thymic Thy-1 cells decrease (Igarashi et al., 1990). After drug treatment, mice could control primary infections (Cox and Young,1969) and mice were resistant to subsequent challenge infections. Spleen cells isolated from these animals were capable of transferring protection (Roberts, 1968; Zivkovic et al.,1983). The mice that resolved reinfection with *B. rodhaini* were strongly protected against challenge infections. Spleen cells from these mice, however, could not transfer protection to recipient mice (Honda, 1989). These results suggest that subpopulations of spleen cells function at different times during development of infections and play important roles in development of protective immunity to *Babesia*. Protective immune responses and functional role of s pleen cells were examined in the present study through use of in vitro proliferation assays and in vivo passive transfer of spleen cells to uninfected mice.

MATERIALS AND METHODS

Animals and Parasites: Female BALB/c mice were bred in our facilities from breeding pairs obtained from CLEA Laboratory (Tokyo) and were 4-5 wk old at the start of experiment. *Babesia rodhaini* of Australian strain, kindly provided by Kyushu Branch, National Institute of Animal Health, Ministry of Agriculture, Forestry and Fishery, was maintained by blood passage in BALB/c mice. Parasitemias and the course of infections were monitored by examination of Giemsa-stained thin smears of tail blood.

Immunization procedure: Mice were inoculated intraperitoneally (i.p.) with $1x10^5$ parasitized erythrocytes (PE) followed by repeated intramuscular (i.m.) injection of 0.125ml of diminazene diaceturate (Ganaseg, 5 mg/ml in distilled water) over the course of 1 week. Four weeks after inoculation (a.i.), mice did not have detectable PE in the peripheral blood and were considered to be immune. Immune mice were inoculated again with $1x10^4$ PE. Individuals that survived more than 4 wk were classified as hyperimmune (Fig. 1). Uninfected mice of the same age were used as controls.

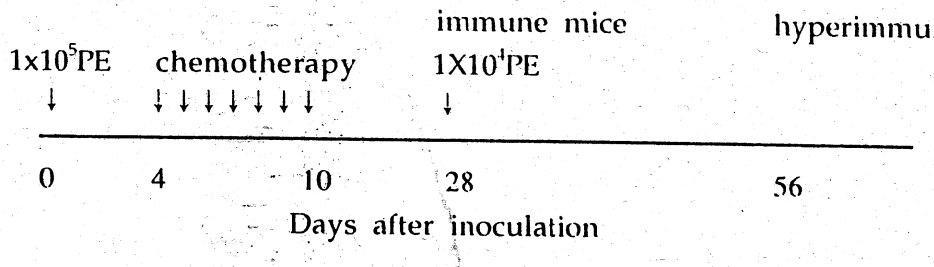

Fig.1. Immunization schedule used in the preparation of *Babesia* immune and hyperimmune mice.

Preparation of Babesia lysate antigen(BLA): Blood with parasitemias exceeding 80% was collected by cardiac puncture in heparinized syringes and was washed three times with saline by centrifugation (400g, 8 min, 4℃). Pelleted PE were subjected to 3 repeated freeze-thaws with liquid nitrogen. The thawed material was centrifuged at 144,000g for 30 min at 4 ℃. The supernatant was used as BLA.

Preparation of spleen cells: Spleen cells of immune, the hyperimmune and control mice were removed aseptically, minced with scissors, and squeezed between two frosted slides. The cell suspension was filtered through a sterile stainless mesh to remove tissue fragments. Erythrocytes were lysed with warm 0.83% NH_4Cl. After two washes in Hanks balanced salt solution, the cells were suspended in RPMI 1640 (Flow Laboratories, Inc., Irvine, Scotland) supplemented with 12 mM HEPES, 150 µl of 2-mercaptoethanol, penicillin G (100 units/ml), and streptomycin sulfate (100 µg/ml).

Antibodies: Antibodies used for in vitro cell depletion included rabbit anti-T cell and rabbit anti-mouse immunoglobulins(Ig) serum and anti-Lyt1.2 and anti-Lyt 2.2 monoclonal antibodies (MAb). All antibodies as well as low-toxic-M rabbit complement were purchased from Cedarlane Laboratories Limited, Horby, Ontario, Canada. For the depletion in vitro, splenn cells were incubated with antibodies for 60 min at 4℃, washed once, and then incubated for 60 min at 37℃ with low-toxic-M rabbit complement.

Proliferative response of spleen cells: Spleen cells were prepared as described above and cell density was adjusted to $1x10^6$ cells/ml with RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS). Aliquots of 0.1 ml of cell suspension were incubated in 96-well flat-bottom tissue culture plate (Falcon 3079, Becton Dickinson, U.S.A.) in a 5% CO_2 incubator at 37℃ for 5 days. Wells containing 0.1 ml of medium alone or medium containing BLA at 1:150 dilution were run in triplicate. During last 6-7 h incubation, 0.2 µ Ci of [^{3}H]-thymidine in 50 µl of complete medium was added to each well. Cells were harvested on filter paper with an automatic cell harvester. Incorporated radioactivity was measured in a liquid scintillation spectrometer.

Interleukin 2 activity. Interleukin 2 (IL-2) activity of the immune, the hyperimmune, and control mice was examined by incubating spleen cells in RPMI 1640 containing 10% FCS at a cell density of $2x10^6$ cells/ml. After 24 h incubation, spleen cells were cultured with or without BLA (1:300 dilution) for another 48 h, and supernatant was collected for measurement of IL-2 activity.

IL-2 activity was measured according to the method of Theander et al. (1986). IL-2 dependent CTLL were kindly provided by Prof. Hideo Nariuchi, Institute for Medical Science, University of Tokyo, and cultured in a Celgrosser-H medium (Sumitomo Pharmaceutical Co., Osaka) supplemented with 10% FCS and 5% rat IL-2. Rat IL-2 was prepared by incubating rat spleen cells ($5x10^6$ cells/ml) with Concanavalin A (2 µg /ml) for 24 h. After incubation, the supernatant was collected and mixed with methyl alpha-D-mannopyranoside (Sigma Chemical Co., St. Louis, U.S.A.) at a concentration of 10-20 mg/ml. This mixture was used for culture of CTLL. CTLL were collected 3 days after subculture and washed 3 times in RPMI 1640. One hundred µ l of aliquots of Celgrosser-H medium containing a suspension of CTLL cells at a density of $1x10^5$ cells/ml were incubated for 24 h in 96-well culture plate with 100 µl serial dilution of standard mouse IL-2 (Inter-cell Technologies Inc., Somerville, New Jersey) or with culture supernatants described earlier. Blastogenic response was measured by the incorporation of [^{3}H]-thymidine as described above. IL-2 activity was expressed with Probit analysis by

Gillis et al .(1981).

Adoptive Cell transfer: Spleen cells prepared from the immune mice, the hyperimmune, and the control mice were adjusted to a density of 2.5×10^7 cells/ml. Two hundred µl of each cell suspension was transferred intravenously to untreated mice. Following transfer, 1×10^4 PE were inoculated i.p. The parasitemia, delayed type hypersensitivity and humoral antibody titers were measured.

To determine whether parasites were present in spleen cell suspension, spleen cells from the immune or hyperimmune mice were injected into untreated mice. Blood smears were prepared and examined regularly for more than 20 days to monitor parasitemia, but no evidence of transmission was detected.

Indirect immunofluorescence test: Antibody levels were detected by an indirect fluorescent antibody method (Waki et al.,1974). PE were washed three times in 0.85% saline, applied to 12 spots in 2 rows on individual glass slides, air dried, and stored at -70 ℃ until use. Drops of sera at dilutions ranging from 1:4 to 1:4096 were placed over antigen spots and incubated for 30 min at 37℃ in a moist chamber . The slide were washed 3 times in phosphate buffered saline (PBS) and drops of fluorescein isothiocyanate-labeled anti-mouse IgM or IgG were incubated on the spots for additional 30 min at 37℃. The slides were then washed as above and coverslipped with glycerin-PBS before examination with a fluorescence microscope.

Measurement of delayed-type hypersensitivity: Delayed-type hypersensitivity (DTH) response was measured as described by Katsura (1975). Six days after cell transfer and challenge infection, all mice were injected subcutaneously with 50 µl of BLA in the right footpad and 50 µl of 0.85% saline in the left footpad. Twenty four hours after administration of challenge inoculation, thickness of both footpads was measured. Swelling was quantified with the following formula: Rate of food swelling = 100 x [thickness of footpad 24 h after inoculation (mm) -thickness of footpad before inoculation (mm)] / [thickness of footpad before inoculation (mm)].

RESULTS

Course of challenge infection: Immune and control mice were divided into groups of four and inoculated i.p. with 1×10^4 PE. Percent parasitemia was monitored in all animals every 2 days(Fig. 2) . The control and immune groups had average parasitemia less than 1% by days 8 a.i. By 12 days a.i., mice in the control group had parasitemias that averaged 90.6 ± 5.6% while those in the immune group had significantly lower parasitemias of only 10±7.1% ($P<0.001$). All animals in the control group were dead by 14 days a.i. One of 4 immune mice was dead by 14 days a.i., but 3 remaining mice survived without subsequent mortality or detectable parasitemias to the end of the experiment. There were no fatalities in the group of hyperimmune mice. Two of these mice had transient parasitemias on the 8th (0.011±0%) and 10th day (0.015±0%) a.i. Differences in parasitemia between immune and hyperimmune mice were significant ($P<0.001$).

Proliferative response to BLA of the spleen cells: Data of proliferative response of spleen cells to the specific antigen BLA are shown in Table 1. Proliferative responses of spleen cells from immune and hyperimmune mice were significantly higher than those of control mice ($P< 0.001$). After treatment with anti-T cell serum and anti-Lyt 1.2 MAb, proliferative response of spleen cells from the immune mice was significantly reduced. MAb to Lyt 2.2 antigen, however, had no effect on proliferative response. Incubation of

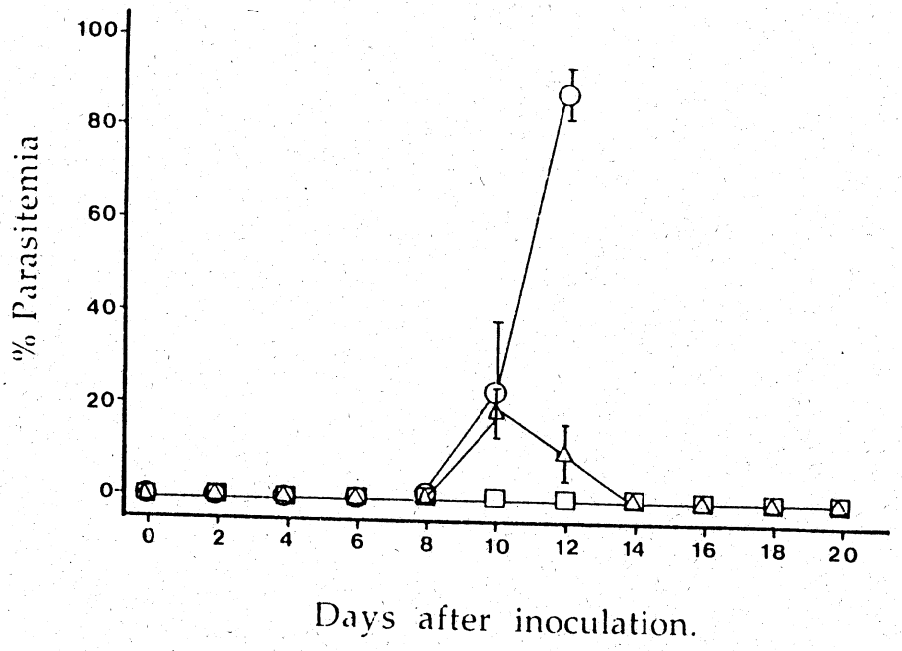

Fig. 2. Progression of parasitemia of *B.rodhaini* infection in control(○), immune (△),and hyperimmune (□) mice (Mean; ⊢⊣ SD, n=4).

Table 1. The effect of antibody treatment on proliferative response of spleen cells to *Babesia* lysate antigen

Spleen cells	Antibody treatment	^{3}H-Thymidine incorporation (Mean cpm ± SD, n=3)	
		medium	BLA
Normal mice		4324±615	3477±545
Immune mice	medium	6798±1044	44570±5425
	anti-mouse Ig	1469±474	21477±4511
	anti-T cell	1461±65	254±115
	anti-Lyt 1.2	1044±501	306±55
	anti-Lyt 2.2	11203±525	53015±2647
Hyperimmune mice	medium	198±22.8	33156±5937
	anti-mouse Ig	238±44.3	34371±3126
	anti-T cell	202±18.5	339±14.3
	anti-Lyt 1.2	185±40	267±20
	anti-Lyt 2.2	524±419	37707±283

Table 2. Interleukin 2 activities in the supernatant of *Babesia* lysate antigen activated spleen cells obtained from normal, immune and hyperimune mice.

	normal	immune	hyperimmune
medium	0.085[a)]	0.11	0.05
BLA	0.098	0.22	0.55

a) Units of IL-2 per ml of cell supernatant

Table 3. Transfer of immunity against *B.rodhaini* infection in recipient mice by injection of subpopulations of spleen cells from immune and hyperimmune mice.

Antibody treatment	Survival rate	Peak * parasitemia	Antibody ** titer IgM	IgG	DTH *** response (%)
Normal mice	0/3	90%≦	1:256	1:4	14.1±4.0
Immune mice					
medium	2/3	42%	1:256	1:4096	39.6±7.9
anti-mouse Ig	0/3	90%≦	1:4	1:64	33.6±3.3
anti-T cell	0/3	90%≦	1:16	1:64	10.2±0.7
Hyperimmune mice					
medium	0/3	90%≦	1:16	1:64	15.4±0.4
anti-mouse Ig	2/3	10%	1:4	1:64	28.0±3.0
anti-T cell	1/3	24%	1:256	1:4096	16.0±4.2
anti-Lyt 1.2	2/3	0%	1:4	1:16	29.8±8.5
anti-Lyt 2.2	2/2	13%	1:16	1:256	43.0±1.4

* Examined on 10 days after inoculation (a.i.).

** Examined by indirect immunofluorescence test at 12 days a.i.

*** Examined on 7 days a.i..

babesial infections is still not clear. Since IL-2 is produced by Lyt 1^+cells in mice (Miller and Stutman, 1982), its increased production in immune and hyperimmune mice indicates activation of this cell type in infected mice. These in vitro results suggest that T cells, especially Lyt 1^+cells, are activated by babesial antigen in immune or hyperimmune mice and may play an important role in development of immunity to babesial infections.

To examine this hypothesis, adoptive spleen cell transfer was done. Studies of *B. microti* have shown that transfer of cellular fractions rich in B cells was effective in producing strong resistance to primary infections, while transfer of fractions rich in T cells was effective in producing resistance to reinfection (Meeusen et al, 1984). In the present study, spleen cells from the immune mice could transfer the protective immunity to recipient mice. The recipient mice produced high titers of IgG antibody. Treatment of spleen cells with antibody to T cell or mouse Ig abolished protection, and mice that received these cells had low antibody titers. These data indicate that antibodies play a major role in the development of protective immunity after primary infection and that cooperation of T cells is essential for the production of antibody by B cells.

Transfer of spleen cells from hyperimmune mice failed to protect uninfected mice from infection even though the cells were more effective than those from immune mice in providing resistance to challenge infections. Hyperimmune spleen cells treated with antibody against T cells or mouse Ig, however, were still able to confer protection to recipient mice after challenge. Mice that received spleen cells treated with antiserum to T cells had higher antibody titers, but lower rates of survival than those that received cells treated with antiserum to mouse Ig. This suggests that T cells are more important than B cells development of defensive mechanisms after secondary infection.

We were unable to identify the subpopulation of T cells from hyperimmune mice that plays the most important role in development of cell-mediated immunity. Spleen cells treated with anti-Lyt 1.2 or anti-Lyt 2.2 MAb could still transfer protection to recipient mice, although there was some difference in degree of protection. Transfer of Lyt $1^+,2^-$ (mostly $CD4^+,CD8^-$) or Lyt $1^-,2^+$ (mostly $CD4^-,CD8^+$) splenic T cells from immune to nonimmune animals provides protection in malarial infection 1-2 wk after spontaneous cure (Mogil et al., 1987). Jayawardena et al. (1982) found that Lyt $1^+,2^-$ but not Lyt $1^-,2^+$ T cells could transfer protection 8-10 wk after resolution of infection. Although kinetics of $CD4^+$ or $CD8^+$ cells was not examined in the present study, similar changes in splenic T cell populations may occur at different times in mice that are hyperimmune to babesial infections.

DTH response is a well-characterized cell-mediated phenomenon that involves complex interactions between T lymphocytes and macrophages. It is often used as a measure for expression of cell-mediated immunity. Ruebush et al. (1986) found that anti-parasite DTH reactions are correlated with resistance to infection with *B. microti*. Its importance as a protective mechanism in infections with *B. rodhaini* is not clear, however, because the correlation between DTH response and protection was only found when spleen cells from hyperimmune mice were used for transfer. Cher et al. (1987) found recently that the DTH response was mediated by Th1 clones, indicating that DTH responses in babesial infections should be correlated with functional ability of Th1 cells in future studies. Nonspecific protective effects of tumor necrotic factor (TNF), one of lymphotoxins, have been reported (Clark et al.,1987). In malarial infections, lymphokines such as gamma-interferon (Shofield et al.,1987), TNF (Taverne et al.,1987), and IL-2 (Theander et

immune spleen cells with antiserum to mouse Ig reduced to mitogenic response by 45%. Proliferative response of spleen cells from hyperimmune mice were also reduced significantly when they were treated with antiserum to T cell or MAb to Lyt 1.2 antigen ($P<0.001$). Treatment with antiserum to mouse Ig or MAb to Lyt 2 .2 antigen had no effect on proliferative response of hyperimmune mice.

Production of IL-2: Incubation of spleen cells from the immune mice and hyperimmune mice with specific antigen BLA resulted in production of IL-2. The hyperimmune mice produced larger amounts of IL-2 than immune mice (Table 2).

Active transfer of spleen cells from immune mice : Twenty four hours after intravenous transfer of spleen cells from control and immune mice to recipient normal mice, $1x10^4$ PE were inoculated i.p. into the recipients. All recipient mice that received normal mouse spleen cells died by 14 days a.i. (Table 3). By contrast, 2 of 3 recipient mice that received transfers of immune spleen cells survived the challenge infection. These mice had higher titers of IgG antibody and a larger DTH response than control mice. Treatment of immune spleen cells with antiserum to T cells or mouse Ig eliminated their protective ability. A DTH response was present in mice that received spleen cells treated with antiserum to mouse Ig, but did not occur in mice that received T cell depleted spleen cells.

Active transfer of spleen cells from hyperimmune mice: Recipient mice that transferred with hyperimmun spleen cells were unable to survive challenge infections with *Babesia* (Table 3). When hyperimmune spleen cells were treated with antiserum to T cell or mouse Ig, however, one of 3 and 2 of 3 recipient mice survived, respectively. Antibody titers in mice that received spleen cells depleted with anti-mouse Ig had lower titers (1:4 for IgM and 1:64 for IgG) than mice that received T cell-depleted spleen cells (1:256 for IgM and 1:4096 for IgG). Furthermore, spleen cells treated with anti-Lyt 1.2 MAb or anti-Lyt 2.2 MAb could transfer protection to recipient mice (Table 3). However, no increase of antibody titers was observed in these mice. Mice that received spleen cells treated with anti-mouse Ig serum , anti-Lyt 1.2 or anti-Lyt 2.2 MAb had significantly higher DTH responses than that received T cell -depleted spleen cells.

DISCUSSION

The present study was undertaken to examine the differences between the protective mechanisms of immune (able to control primary infections) and hyperimmune (able to survive reinfection) mice. Both immune and hyperimmune mice were resistant to challenge infection, although hyperimmune mice were more strongly protected than immune mice. Two different in vitro assays were used to measure the functional ability of spleen cells from immune and hyperimmune mice. In malaria infections, the high proliferative responses of immune mice are believed to result from activation of cell-mediated immunity (Weinbaum et al.,1976). In the present study, proliferative responses of spleen cells to BLA were observed in both the immune and hyperimmune mice. The blastogenic response was reduced significantly by the treatment of spleen cells with antibody to T cell or Lyt 1 antigen , but not with antibody to mouse Ig or Lyt 2 antigen. These results indicate that proliferative response to babesial antigen is a T cell dependent reaction.

High IL-2 levels was also detected in immune and hyperimmune mice. It has been shown that IL-2 is important in malarial infections (Theander et al, 1986), but its role in

al.,1986) have also been reported to be important in development of protective immunity. All of these factors are produced by Th1 cells(Cherwinski et al.,1987).

The present study suggests that different protective mechanisms occur in immune and hyperimmune mice. T cells provide protective immunity to babesial infections in both types of mice, but have different functions at various stages of infection. The role of specific subsets of T cells should be examined in future studies of protective mechanisms.

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REFERENCES

Cher,D.J. and Mosmann,T.R. 1987. Two types of murine helper T cell clone.II. Delayed-type hypersensitivity is mediated by Th1 clones. *J. Immunol.138*:3688-3694.

Cherwinski, H.M., Schumacher, J.H., Brown,K.D. 1987. Two types of murine helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J.Exp.Med.166*:1229-1244.

Clark,A.T., Hunt,H.N., Buchter, A.G., and Cowden, B.E.1987. Inhibition of murine malaria (*P. chabaudi*) in vivo by recombinant interferon-gamma or tumour necrosis factor, and its enhancement by butylated hydroxyanisole. *J. Immunol. 139*:3493-4396.

Cox, F.E.G. and Young, A.S. 1969. Acquired immunity to *Babesia microti* and *Babesia rodhaini* in mice. *Parasitol. 59*:257-268.

Eugui,E.M. and 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.

Gillis, S, Kozak, R.,Durante, M., and Weksler, M.E. 1981. Immunological studies of aging. Decreased production of and response to T cell growth factor by lymphocytes from aged humans. J.*Clin. Invest. 67*:937-942.

Honda, R. 1989. Studies on the protective mechanism of *Babesia* chronically infected mice against *Babesia rodhaini* infection. Master thesis, Obihiro University, Obihiro, Japan ,(in Japanese).

Igarashi,I., Honda, R., Shimada, T., Miyahara, K., Sakurai, H., Saito, A., and Suzuki, N. 1990. Changed of lymphocyte subpopulations and natural killer cells in mice sensitized with *Toxoplasma* lysate antigen before and after *Babesia* infection. *Jpn.J. Vet. Sci. 52*:969-977.

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

Katsura, Y. 1975. Footpad test for delayed type hypersensitivity in mice caused by protein antigen. pp.1201-1205. *In* :Technics for immunological Practice.Maede, I., (Ed), Print Co., Kanazawa (in Japanese).

Meeusen,E., Lloyde,S., and Soulsby, L.J.E. 1984. *Babesia microti* in mice. Adoptive

transfer of immunity with serum and cells. *Aust. J. Exp. Biol.Med. Sci.62*:551-556.

Miller,R.A., and Stutman, O. 1982. Use of positively selected Lyt-2^+ mouse splenocytes to examine interleukin 2-secretion in response to alloantigen and to TNP-modified syngeneic cells. *Cell. Immunol. 68*:114-127.

Molgi, R.J., Patton, C.L. and Green, D.R. 1987. Cellular subsets involved in cell- mediated immunity to murine *Plasmodium yoelii* 17X malaria. *J.Immunol. 138*:1933-1393.

Reubush,J.M., and Hanson, L.M. 1980. Transfer of immunity to *Babesia microti* of human origin using of T lymphocytes in mice. *Cell. Immunol. 52*:255-265.

Reubush,J.M., Troutman, E.H. and Kenned,D.A. 1986. I.Delayed-typre hypersensitivity to *Babbesia microti*-infected erythrocytes in mice. *Cell. Immunol. 98*:289-299.

Roberts, A.J. 1968. Adoptive transfer of immunity to *B. rodhaini* by spleen cells from immune rats. *Aust. J. Exp. Biol.Med. Sci.46*:807-808.

Schofield, L., Ferreira, A., Altszuler, R., Nussensweig, V., and Nussensweig,S.R. 1987. Interferon-gamma inhibits the intrahepatocytic development of malaria parasites in vitro. *J. Immunol.139*:2020-2025.

Taverne, J., Tavernier,J., Fierst W., and Playfair,L.H.J. 1987. Recombinant tumor necrosis factor. *Clinic.Exp. Immunol. 67*:1-4.

Theander,G.T., Bygbjerg,C.B., Jepsen, S., Sevenson,M.,Khaazmi, A., Larsen, B.P. and Bendtzen,K. 1986. Proliferative response induced by *P. falciparum* antigen and Interleukin-2 production by lymphocytes isolated from malaria individuals. *Infect .Immunity. 53*:221-226.

Weinbaum,F.I., Evans, C.B. and Tigelaar, R.E. 1976. An in vitro assay for T cell immunity to malaria in mice. *J.Immunol. 116*:1280-1283.

Wood,P.R. and Clark, I.A. 1982. Apparent irrelevance of NK cells to resolution of infections with *Babesia microti* and *Plasmodium vinckei petteri* in mice. *Parasite Immunol.4*:319-327.

Zivkovic, D., Seinen,W., Kuil, H., Albers-van Bemmel,C.M.G. and Speksnijder,J.E. 1983/1984. Immunity to *Babesia* in mice. I. A doptive transfer of immunity to *Babesia rodhaini* with immune spleen cells and the effect o f irradiated on the protection of immune mice. *Veterianry Immunol. Immunopathol. 5*:343-357.

Therapeutic Effects of *Toxoplasma* Lysate Antigen on 20-Methylcholanthrene-Induced BALB/c Mouse Tumors

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ABSTRACT. Therapeutic effects of *Toxoplasma* lysate antigen (TLA) were studied in mice bearing the tumor in the second passage of 20-methylcholanthrene (MC)-induced tumor cells. Intramuscular administration of TLA 7 days after the tumor-cell inoculation caused apparent inhibition of the tumor growth on day 14. The second treatment facilitated the therapeutic effects. Intravenous transfer of spleen cells prepared from TLA-sensitized mice into tumor-bearing mice also represented the growth inhibitory effects. Prominent effects were seen when the transferred cells were prepared 5 days after sensitization of donor animals. The inhibitory effects were absent in the groups transferred only the adherent cells or the non-adherent cells prepared from sensitized mice. The strongest inhibitory effect was observed in the group to which both adherent and non-adherent spleen cells were transferred simultaneously from sensitized mice. In *in vitro* experiments, spleen cells obtained from sensitized mice showed cytolytic effect on P-815 or YAC-1 cells after the secondary stimulation *in vitro* with TLA. Large non-adherent cells containing densely packed granules were induced when cultured with the adherent cells obtained from sensitized mice. These results revealed that TLA can inhibit the growth of the chemically-induced transplantable tumors by activation of adherent and non-adherent spleen cells.—KEY WORDS: methylcholanthrene-induced tumor, mouse, spleen cell, therapeutic effect, *Toxoplasma* lysate antigen.

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It has recently been reported that antigen extracted from *Toxoplasma Gondii* (Tp) is able to activate human natural killer (NK) cells *in vitro* [12]. Mice treated with *Toxoplasma* lysate antigen (TLA) and infected with *Plasmodium berghei* or *Babesia rodhaini* are capable of surviving infections that would otherwise be fatal [4–6, 11, 13]. Interferon-gamma (IFN-gamma) and other lymphokines (LKs) are present in the serum of TLA-sensitized animals [3, 7, 14]. Incubation of TLA-sensitized mouse spleen cells with TLA *in vitro* causes induction of nonspecific cytotoxic cells that are capable of lysing target cells [2, 9]. The growth of allograftable Sarcoma-180 (S-180) tumors and isograftable Meth A tumors in BALB/c mice is also strongly inhibited by the administration of TLA [8]. These observations indicate that TLA causes a nonspecific stimulation of immunoprophylactic responses in mice. In this study, the therapeutic effect of TLA on mice with 20-methylcholanthrene (MC)-induced tumors was studied as a model for examining of the effects of TLA on spontaneous tumors.

MATERIALS AND METHODS

Experimental animals: Inbred male BALB/c mice were reared and maintained in this laboratory, and 4–6-week-old mice (10–20 g) were used in this experiment.

Preparation of Toxoplasma lysate antigen (TLA): TLA was prepared as described by Sakurai *et al.* [10] and Saito *et al.* [8]. For *in vivo* experiments, TLA was dissolved in 0.85% sodium chloride solution to make a final concentrations of 300, 1,000, or 2,000 μg/m*l* protein. The solutions were filter-sterilized with disposable syringe filter units (0.45 μm, Dismic-25, Toyo Roshi Co., Ltd., Japan) and stored at −20°C until use. For *in vitro* incubations, TLA was dissolved in RPMI-1640 (Flow Laboratories, U.K.) containing 12 mM HEPES, 100 units/m*l* potassium penicillin G, 100 μg/m*l* streptomycin sulfate, 0.1 μM sodium pyruvate, and 50 μM 2-mercaptoethanol to make a final concentration of 1,000 μg/m*l* protein. The solution was filter-sterilized as described above and stored at −20°C until use.

Preparation of methylcholanthrene-induced tumors: A paraffin pellet containing 0.2 mg of MC (Wako Pure Chemi. Ind., Tokyo, Japan) was inserted subcutaneously into the back of mouse. Tumors induced at the site of the implants approximately 3 months later were removed and divided into pieces of 10 mm^2 each. These were grafted

subcutaneously on the backs of additional mice 8–12 weeks of age. Tumors from these animals were excised, chopped with scissors on ice, triturated between a pair of glass slides, and suspended in Dispase (Dispase grade II, Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan). After stirring for 30 min at 37°C to digest connective tissue, the cell suspension was filtered twice through #40 stainless steel mesh and passed through a glass fiber column to eliminate large pieces. The cells were washed 3 times with HBSS (250×g, 6 min, 4°C) and suspended in RPMI-1640 to prepare a final concentration of 4×10^6 or 5×10^6 viable cells/m*l*. The cell viability was determined by the dye exclusion test using trypan blue. Additional mice were inoculated with 0.025 m*l* (1×10^5 cells/mouse) or 0.1 m*l* (5×10^5 cells/mouse) of these cell suspension.

Measurement of tumor growth: Nodular tumors formed by subcutaneous inoculation of MC tumor cells were measured with a pair of calipers. The area of each tumor was calculated as the product of the major and minor axes. Mean values from each group were compared with a Student's *t*-test.

Preparation of spleen cells: Spleens were excised from mice immediately after sacrifice, chopped with scissors, triturated between two glass slides, and suspended in HBSS. The spleen cells were passed through a #40 stainless steel mesh to eliminate large pieces of tissue. Red blood cells were hemolyzed with 0.83% ammonium chloride solution at 37°C. The cell suspension was filtered through a glass fiber column to obtain the lymphocyte fraction according to the method of Conray-Ficoll [15]. Lymphoid cells were washed twice with HBSS and once with RPMI-1640 and suspended in RPMI-1640 before transfer to other animals.

For separation of spleen cell fraction, cell suspensions prepared as described above were resuspended in RPMI-1640 containing 10% heat inactivated fetal calf serum (FCS; Flow Laboratories, Australia) (10% FCS-RPMI) to make a final cell density of 3.0×10^6 cells/m*l*. The suspension was incubated in a culture flask for 12 hr. The culture flask was washed gently with HBSS to collect non-adherent (NA) cells. Adherent (A) cells were removed from the flask with a rubber policeman. Both fractions were washed once with HBSS before transfer to mice.

Preparation of effector cells: Spleen cells prepared from TLA-sensitized or non-sensitized mice were suspended to make a density of 3×10^6 cells/m*l* in 10% FCS-RPMI containing with or without 30 μg/m*l* TLA and incubated for 6 days. The cells were then washed twice with RPMI-1640, resuspended to the same density, and used as effector cells.

Cytotoxicity test: The mouse mast cell sarcoma P-815 and the mouse T cell lymphoma YAC-1 were kindly supplied by Dr. Tsuneo Kamiyama, Department of Animal Epidemiology, National Institute of Health, Japan, and used as target cell which is resistant or sensitive to natural killer cells.

Cell suspensions prepared at densities of 1×10^7 viable cells/m*l* were incubated with 5.55 MBq of ^{51}Cr (Na_2CrO_4, specific activity of 1.5 GBq/m*l*, Japan Atomic Energy Research Institute, Tokyo, Japan) with occasional agitation for 60 min. The ^{51}Cr-labeled cells were washed with phosphate buffered physiological saline (PBS) and then with RPMI-1640 [1]. The pellet was resuspended in 10% FCS-RPMI to make a final density of 1×10^5 cells/m*l*.

Culture tubes were filled with 150 μl of the ^{51}Cr-labeled target cell suspension (1.5×10^4 cells) and 150 μl of the effector cell suspension (3.0×10^2 cells) to adjust the ratio of effector: target cells (E:T ratio) to 50:1. One m*l* of 10% FCS-RPMI was added to each tube and incubated for 20 hr. Each tube was centrifuged (1,000×g, 5 min, room temperature) after incubation and 1.2 m*l* of the supernatant and 0.1 m*l* of the pellet were sampled for counting in a gamma-scintillation counter (Packard auto-gamma 5650; United Technologies Packard, U.S.A.). The release of ^{51}Cr from each sample was measured in triplicate. Non-specific release of ^{51}Cr was measured in samples to which 150 μl of 10% FCS-RPMI was added in place of the effector cells.

Cytotoxicity of effector cells was calculated according to the following formula:

$$\text{Cytotoxicity (\%)} = 100 \times \{\text{Release of sample } ^{51}Cr \text{ (cpm)} - \text{release of nonspecific } ^{51}Cr \text{ (cpm)}\} / \{\text{Release of total } ^{51}Cr \text{ (cpm)} - \text{Release of nonspecific } ^{51}Cr \text{ (cpm)}\}$$

Analysis of mouse spleen cells by automatic cell sorting: Cultured mouse spleen cells were washed once with PBS and suspended in 1 m*l* of 10 mM HEPES buffer containing 0.1% BSA. The suspension was filtered through a 40 μm nylon filter and analyzed with an automatic cell sorter CS-20, Argon ion laser (400 mw, 488 nm, Showa Denko Co., Tokyo, Japan). Approximately 2,000 cells were processed in each group.

RESULTS

Therapeutic effects of TLA: As shown in Fig. 1, each group of 5 mice was administered intramuscularly with either 0.1 m*l* of physiological saline (control) or 0.1 m*l* of physiological saline containing 30 (T30), 100 (T100), and 200 (T200) μg TLA weekly starting on day 7.

Mean tumor size 14 days after the inoculation was 48 mm^2 in control group and 25 mm^2, 22 mm^2 and 28 mm^2 in T30, T100, and T200, respectively. Groups receiving TLA had significantly smaller tumors than control group. On day 21, the differences among the control and TLA treated groups were highly significant ($p<0.01$). Mean tumor size of T100 was smallest whereas no statistical differences were observed among the TLA treated groups.

Effect of TLA-sensitized spleen cells on tumor growth: As shown in Fig. 2, 21 mice were divided into 5 groups on day 7 after inoculation of 5×10^5 tumor cells per mouse. Each animal received 2×10^6 spleen cells (containing both adherent and non-adherent cells) from TLA-sensitized or non-sensitized mice via the caudal vein. Cells prepared from the mice on day 1, 3, 5 and 7 after intramuscular administration of TLA were transferred into the tumor-bearing mice of 4 groups, 1DT, 3DT, 5DT, and 7DT, respectively. Transfer of spleen cells was carried out on day 7 and day 14 after tumor transplantation. Changes in tumor size were measured every 2 days starting on day 8. Mean tumor size on day 21, was 512 mm^2 in control group, 396 mm^2 in 1DT, 373 mm^2 in 3DT, 302 mm^2 in 5DT, and 405 mm^2 in 7DT. Tumor growth was inhibited significantly in 1DT and 3DT ($p<0.05$) and in 5DT ($p<0.01$), but not in 7DT.

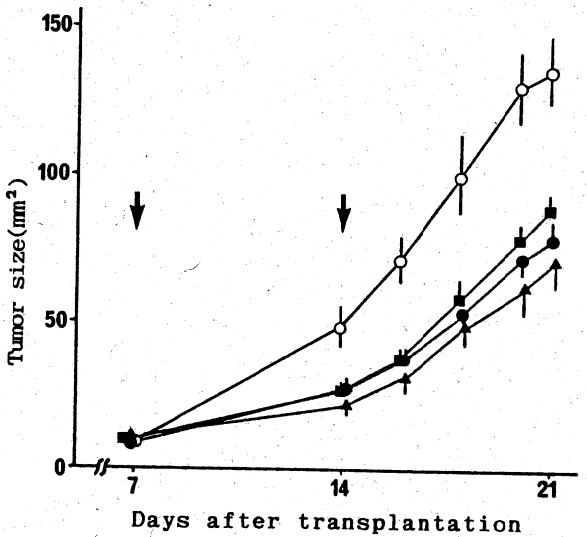

Fig. 1. Growth inhibitory effects of TLA on the Methylcholanthrene-induced tumors (2nd passage). (○), Control; (●), TLA 30 μg/mouse; (▲), TLA 100 μg/mouse; (■), TLA 200 μg/mouse. Each point and vertical bar represent the mean ±SE. Arrows indicate the day of TLA administration.

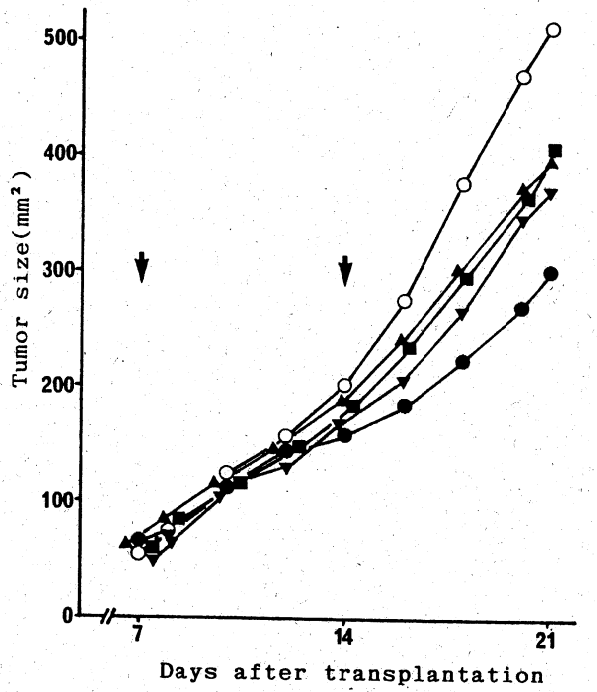

Fig. 2. Tumor growth inhibition by transferring the spleen cells prepared from TLA-sensitized mice. Spleen cells were prepared on day 1 (▲), day 3 (▼), day 5 (●), or day 7 (■) after intramuscular administration of TLA, and the control group (○) received unsensitized cells. Arrows indicate the day when the spleen cells were transferred.

In order to clarify the population of spleen cells that might play a role in tumor inhibition, tumor-bearing mice received the adherent (A) cells and/or non-adherent (NA) cells prepared from the mice 14 days after TLA-sensitization or from the non-sensitized mice (Fig. 3). Proportions of Thy-1, Ig, or asialo GM1 positive cells in NA cells prepared from non-sensitized mice (NAC) and from TLA-sensitized mice (NAT) were 50.4, 23.3 or 4.5%, and 50.2, 25.4 or 4.7%, respectively. Proportions of macrophage in adherent cells from the former (AC) and the latter (AT) animals were 68.0 and 66.0%, respectively. The animals of each group received 2×10^6 NA cells and/or 2×10^5 A cells at weekly intervals for five consecutive weeks.

Mean tumor size 33 days after tumor inoculation was 180.6 mm^2 in control group, 156.6 mm^2 in Group NAC, 133.4 mm^2 in Group AC, 189.6 mm^2

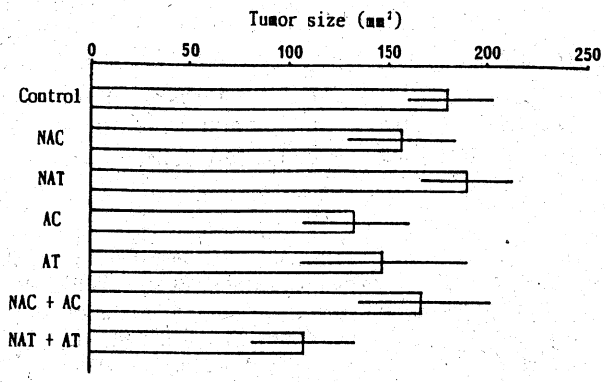

Fig. 3. Tumor growth in mice receiving adherent (A) and/or non-adherent (NA) spleen cells from either TLA-sensitized mice (T), or non-sensitized mice (C), respectively. The data shown are the mean tumor size±SE of five mice 33 days after inoculation.

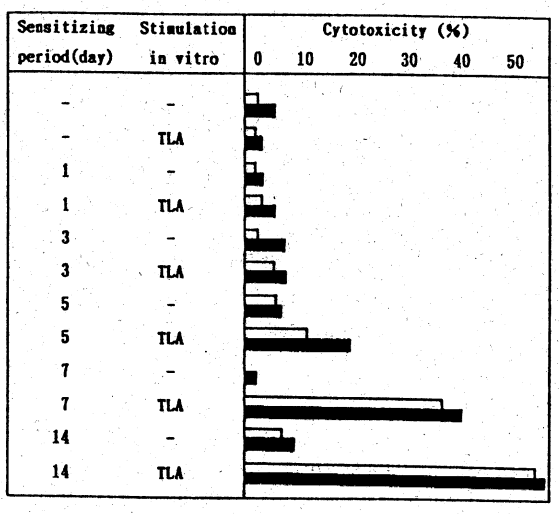

Fig. 4. Effect of cytotoxicity in terms of TLA-sensitizing procedure. Spleen cells prepared from the mice sensitized with TLA 1, 3, 5, 7, and 14 days before sacrifice were restimulated *in vitro* with TLA. Cytotoxic activities against P-815 (□) and YAC-1 (■) were measured 6 days after secondary stimulation.

in Group NAT, 148.5 mm^2 in Group AT, 169.0 mm^2 in Group NAC + AC, and 108.3 mm^2 in Group NAT + AT. Inhibition of tumor growth in Group NAT + AT was the most prominent among other Groups.

Relationship between cytotoxicity and sensitizing period: Spleen cells were prepared from TLA-sensitized mice on 1, 3, 5, 7, and 14 day and restimulated with TLA *in vitro*. The cytotoxicity test was carried out 6 days after the incubation with target cells. Spleen cells prepared within 3 days after sensitization showed little cytotoxicity to P-815 or YAC-1 target cells (Fig. 4). However, secondary stimulation with TLA *in vitro* induced marked cytotoxicity 5 days after sensitization *in vivo*, especially in the groups of 7 and 14 days-sensitizing periods. These values were significantly different from those in the same groups without secondary stimulation ($p<0.001$).

Morphological analysis of nucleated cells: The cell size and the quantity of intracellular granules of NA cells were measured in the four cases where NA and A cells prepared from TLA-sensitized or non-sensitized mice were cultured concurrently, i.e. NAC + AC, NAC + AT, NAT + AC, and NAT + AT (Fig. 5). After a 6-day incubation, NA cells in each group were collected and analysed with a cell sorter. The proportion of cells larger than the 40th channel on the abscissa (ca. 21.6 μm) was calculated for each group. Proportions were 61.0% for Group NAC + AC, 68.4% for Group NAC + AT, 65.2% for Group NAT + AC, and 77.8% for Group NAT + AT. Large mononuclear cells containing densely-packed intracellular granules were present in Group NAT + AT.

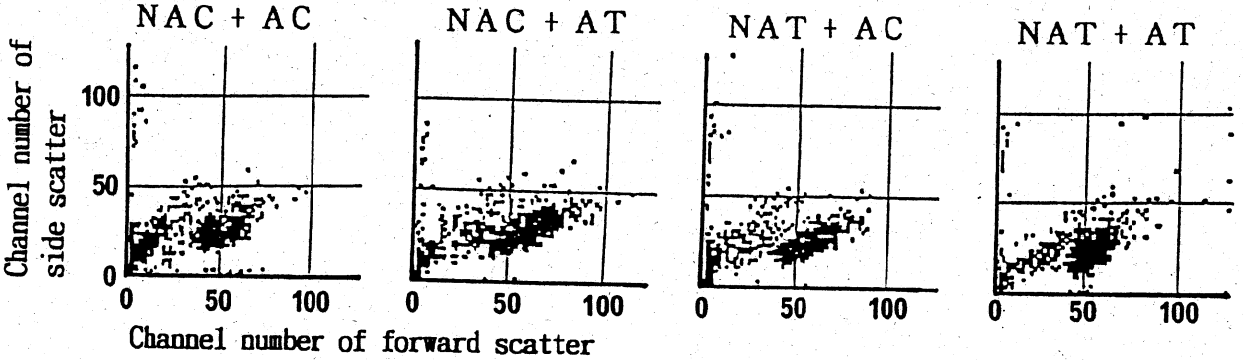

Fig. 5. Cell-sorter profiles of non-adherent spleen cells cultured with adherent cells in various combinations indicated in the figures (Abbreviations mean the same groups as in Fig. 3). The axis of abscissas and ordinate show the cell size and the quantity of intracellular granules, respectively.

DISCUSSION

The therapeutic effects of TLA on MC-induced tumor cells were studied as a model for evaluating the clinical availability of this compound for spontaneous tumors. Weekly intramuscular doses of TLA to mice with MC-induced tumors inhibited tumor growth in the cases treated 7 days or later after tumor transplantation. Previous studies have reported that TLA inhibits the *in vivo* growth of established allograftable Sarcoma 180 tumor cells and established isograftable Meth A tumor cells [8]. Results in this study using second passaged tumor suggest that TLA can be applied in veterinary medicine for the control of spontaneous tumors.

Significant inhibition of tumor growth occurred in our study when TLA was administered at doses of 30, 100, and 200 μg/mouse. While inhibition of tumor growth was most prominent at doses of 100 μg/mouse, there were no significant differences among any of the three regimens. Since cytotoxicity of lymphoid cells collected from the spleens of TLA-sensitized mice was larger in mice that received 100 μg than 30 μg of TLA at biweekly intervals [9], we consider a single 100 μg dose of TLA to be sufficient for stimulating biological effects.

The growth of tumors was inhibited within 7 days by administration of a single dose of TLA at 100 μg/mouse and by transfer of spleen lymphoid cells from mice that had been sensitized with 100 μg of TLA 5 days earlier. Spleen cells from unsensitized mice and mice that had been sensitized with 2 doses of TLA at biweekly intervals were collected and separated into A and NA cell populations in order to determine the underlying mechanisms of TLA activity. Adherent and NA cells were transferred to mice with MC-induced tumors in a number of different combinations. Tumor growth was inhibited evidently in mice that received a mixture of A and NA cells from TLA-sensitized mice. Co-incubation of these two cell populations and subsequent analysis by cell sorter revealed the induction of large NA cells that contained large numbers of intracellular granules. An increase in the cytotoxic activity of spleen cells harvested between 5 and 14 days after sensitization with TLA and restimulation with TLA *in vitro* indicated that cytotoxic cells, such as NK sensitive and/or resistant killer cells, also participate in the anti-tumor activity of TLA. Anti-tumor immunity is said to involve the response of immunologically competent cells after induction by antigen-presenting cells. These results, in consequence, revealed that TLA can inhibit the growth of the chemically induced transplantable tumor on the basis of activation of A and NA cells, especially in the spleen.

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REFERENCES

1. Grabstein, K. 1982. Cell mediated cytolytic response. pp. 124–133. *In*: Selected Methods in Cellular Immunology (Mishell, B. B. and Shiigi, M. S. eds.), W. H. Freeman Co., San Francisco.
2. Igarashi, I., Honda, R., Shimada, T., Miyahara, K., Sakurai, H., Saito, A., and Suzuki, N. 1990. Changes of lymphocyte subpopulations and natural killer cells in mice sensitized with *Toxoplasma* lysate antigen before and after *Babesia* infection. *Jpn. J. Vet. Sci.* 52: 969–977.
3. Jacob, L. and Lunde, M. N. 1975. Assessment *in vitro* of immunity against *Toxoplasma gondii*. *J. Exp. Med.* 141: 446–482.
4. Ogawa, R., Sato, M., Hirose, T., and Suzuki, N. 1985. Preventive effect on *Toxoplasma* lysate antigen (TLA) on fatal infection with mouse piroplasma. *Jpn. J. Vet. Sci.* 47: 225–236.
5. Omata, Y., Nakabayashi, T., and Suzuki, N. 1979. Different appearance of parasitized erythrocytes in blood between normal and *Toxoplasma*-infected rats after infection of *Plasmodium berghei*. *Zentralbl. Bakteriol. Hyg., A* 244: 362–373.
6. Omata, Y., Yagami, K., Takei, N., Suzuki, N., and Nakabayashi T. 1981. Protective reaction against malaria infection in mice sensitized with frozen-thawed *Toxoplasma* tachyzoites. *Zentralbl. Bakteriol. Hyg., A* 250: 223–235.
7. Omata, Y., Yagami, K., Sakurai, H., Saito, A., and Suzuki, N. 1983. Interferon induction in *Toxoplasma gondii* infected mice. *Zentralbl. Bakteriol. Hyg., A* 225: 392–398.
8. Saito, A., Sakurai, H., Terada, S., Igarashi, I., Hirose, T., Suzuki, N., Osaki, H., Kudo, H., Sakata, Y., Okamoto, K., and Yoshida, Y. 1989. Inhibitory effect of *Toxoplasma* lysate antigen on the multiplication of transplanted tumor in mice. *Zentralbl. Bakteriol. Hyg., A* 271: 114–126.
9. Saito, A., Sakurai, H., Igarashi, I., Suzuki, N., and Osaki, H. 1989. Fundamental studies on characteristics of *Toxoplasma* lysate antigen (TLA)-induced killer cells. *Biotherapy* 3: 320–324 (in Japanese with English abstract).
10. Sakurai, H., Igarashi, I., Omata, Y., Saito, A., and Suzuki, N. 1983. Effects of neonatal spleen cell products on the multiplication of *Toxoplasma* in rat peritoneal macrophages. *J. Immunol.* 131: 1527–1530.
11. Sato, M., Sekiguchi, S., Miyagami, T., Sakurai, H., Saito, A., Hirose, T., and Suzuki, N. 1987. With time changes of T-lymphocytes after *Babesia* infection in mice pretreated

with *Toxoplasma* lysate antigen. *Zentralbl. Bakteriol. Hyg., A* 264: 435–445.
12. Sharma, S. D., Verhoef, J., and Remington, J. S. 1984. Enhancement of human natural killer cell activity by subcellular components of *Toxoplasma gondii*. *Cell. Immunol.* 86: 317–326.
13. Suzuki, N., Kikushima, K., Miyagami, T., Igarashi, I., Sakurai, H., Saito, A., and Osaki, H. 1987. Modulator effect of *Toxoplasma* lysate antigen in mice experimentally infected with *Plasmodium berghei*. *Zentralbl. Bakteriol. Hyg., A* 264: 422–434.
14. Suzuki, N., Sakurai, H., Saito, A., Omata, Y., and Osaki, H. 1988. Interferon induction in mice treated with *Toxoplasma* lysate antigen. *Biotherapy* 2: 96–98 (in Japanese with English abstract).
15. Tsuji, K. 1971. Separation method for lymphocytes by gravity centrifugation. pp. 443–446. *In*: Technics for Immunological Procedure I (The Japanese Soc. Immunol. ed.), Maeda Print Co., Kanazawa (in Japanese).

1-7

Antitumor Activity of *Toxoplasma* Lysate Antigen against Methylcholanthrene-Induced Tumor-Bearing Rats

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ABSTRACT. Growth of the tumor autoinduced by 20-methylcholanthrene (MC) in rats was inhibited after administration of *Toxoplasma* lysate antigen (TLA). The antitumor activity of TLA was most obvious in the early stage of tumoral growth. When TLA was administered to rats before the appearance of tumor, tumor formation was delayed slightly. Histopathological studies revealed dense growths of spindle tumor cells in untreated control rat, while enlarged central necrosis with the infiltration of lymphocytes and neutrophils was apparent in TLA-treated rats. According to the immunohistological examination of tumor tissue with anti-Thy-1 antibody, the rats treated with TLA showed large Thy-1 positive granular cells, whereas the untreated rats indicated only a few small Thy-1 positive cells. These observations indicate that TLA is a useful modifier of biological responses to MC-induced tumors.—KEY WORDS: antitumor activity, methylcholanthrene-autoinduced tumor, rat, Thy-1 positive granular cell, *Toxoplasma* lysate antigen.

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Mice treated with *Toxoplasma* lysate antigen (TLA) was protected preferentially from the infection with *Plasmodium berghei* or *Babesia rodhaini*, and survived the challenge fatal to non-treated animals [7, 8, 14, 16]. Interferon-gamma (IFN-gamma) and other lymphokines (LKs) were present in the serum of these animals [5, 7, 9, 17]. Furthermore, cytotoxic cells were induced when spleen cells harvested from TLA-sensitized mice were incubated with TLA *in vitro* [4, 6, 12]. Sharma *et al.* [15] have also reported that TLA activated human natural killer (NK) cells *in vitro*. In the light of these observations, it can be presumed that TLA might cause nonspecific activation of immunoprophylactic mechanisms.

Further studies on the antitumor activity of TLA revealed that the intramuscular administration of TLA strongly inhibited the growth of allogenic or isogenic tumors, i.e. Sarcoma-180 (S-180) or Meth A [11]. Moreover, an apparent antitumor effect of TLA was observed even in the mice bearing 20-methylcholanthrene (MC)-induced tumor [6].

In this report, rats with the MC-autoinduced tumors were examined as a therapeutic model for the TLA treatment.

MATERIALS AND METHODS

Rats: A total of 39 male rats of the Wistar-Imamichi strain of 6 to 8 weeks of age were used for the experiments except for the hematological study, in which 3 mature female rats of the Rowett strain were used. All animals were raised and maintained in our laboratory.

Preparation of Toxoplasma lysate antigen (TLA): TLA was prepared according to the method described previously [3, 13].

In brief, after the centrifugation of crude antigen solutions at 144,000 × g for 120 min, the supernatant was used as TLA preparation throughout the experiment.

Induction of tumors with 20-methylcholanthrene (MC): A solution of MC (Wako Pure Chemi. Ind., Tokyo) in paraffin at a final concentration of 5 mg/ml was divided into 0.1 ml aliquots, and allowed to cool. A pellet was implanted subcutaneously on the back of rat, which was kept under observation until the tumoral growth (for about 4 or 5 months).

Hematology: Blood sample was collected by heart puncture from the rat with or without TLA treatment. Red blood cell counts, white blood cell counts, hematocrit (Ht; capillary method), total serum protein (STP; refractometry), glutamic-oxaloacetic transaminase activity (SGOT; karmen's method), glutamic-pyruvic transaminase (SGPT; Karmen's method), lactate dehydrogenase (LDH; Wroblewski-Radu method), alkalinephosphatase (AlP; p-nitrophenol test), total bilirubin (Malloy-

Evelyn test), creatinine (alkaline picric acid test), and uric acid (urate oxidase test), were measured routinely.

Measurement of tumor growth: The major and minor axes of each nodule were measured using a pair of calipers and the tumor area calculated as the product of both axes. Mean values in each group were compared with a Student's *t*-test.

Calculation of mononuclear cell distribution: Spleen or liver was ground and the mononuclear cells were collected by the method of Conray 400-Ficoll [18]. After the number of nucleated cells in each organ was counted, the smears were prepared for the determination of lymphocyte subcalsses.

Calculation of lymphocyte subclasses: Rabbit anti-rat Thy-1 serum (ATS) was prepared according to the method of Golub [2]. Confirmation of the specificity of this serum for T cells was carried out with the cytotoxicity tests according to the method described by Barker *et al.* [1]. Smears of mononuclear cells prepared from spleen or liver were incubated with ATS or fluorescein-isothiocyanate conjugated goat anti-rabbit IgG (Cappel Inc., U.S.A.). The ratio of ATS positive cells to the total mononuclear cell count was calculated. The ratio of IgG positive lymphocytes was determined in a similar manner by incubating the preparations with fluorescein-isothiocyanate conjugated anti-mouse IgG (Nippon Kotai Kenkyusho, Japan).

Histopathology and immunohistochemistry: Subcutaneous tumors were excised from rats and divided in half with a sharp scalpel. One piece of tissue was routinely processed for HE-staining and the other half was processed for immunohistochemical studies as described by Saint-Marie [10]. Fluorescein-isothiocyanate (FITC) conjugated mouse IgG antibodies (Sera-Lab Ltd., U. K.) against rat Thy-1, rat T helper cells and rat T cells (non-helper subset) were used.

RESULTS

Effects of TLA on the growth of MC-induced tumor: Twenty rats with MC-induced tumors were divided into four groups of the same number according to the criteria listed in Fig. 1. Treatment with TLA was started when the tumor size reached to the diameter specified for the each group, i.e. about 3 mm, 10 mm or 18 mm. TLA dissolved in physiological saline was injected at a dose of 500 μg/rat into the femoral muscle once a week for 5 weeks.

Effects of TLA depended on the tumor size at the beginning of treatment (Fig. 1). In the group "b" with tumors less than 10 mm in diameter, prominent inhibiton of growth was observed in 2 of 5 animals, and growth delay occurred in the remaining 3 cases. In the group "c" with tumors about 10 mm in diameter, tumor growth was inhibited in 1 animal and only partially suppressed in 2 others. TLA had no effect on tumor growth in the group "d" with tumors larger than 10 mm in diameter.

In the second experiment, 5 rats bearing tumors about 10 mm in diameter were treated with TLA at a dose of 100 μg/rat once a week for 8 weeks. Other 4 rats bearing the tumor of the same size were given intramuscularly physiological saline as a control group.

As shown in Fig. 2, the mean tumor area in the control group was 110 mm^2 at the beginning of the experiment and increased markedly to 421 mm^2 after 6 weeks and to 948 mm^2 after 9 weeks. Tumor growth was inhibited significantly ($p<0.05$) in the TLA-treated group, i.e. the initial tumor size (115 mm^2) was followed by slight increase to 191 mm^2 after 6 weeks and to 311 mm^2 after 9 weeks.

Effect of TLA on the tumor induction by MC: Ten male Wistar rats subcutaneously implanted of MC-containing paraffin pellets were divided into two equal groups. Animals in one group were not treated while those in the other group were administered 500 μg of TLA once a month immediately after the implantation of the pellet. Tumor nodule was observed first in the untreated control group 124 days after the implantation (Fig. 3). Within the following 21 days, tumors were induced in all other untreated rats. TLA treatment slightly delayed the tumoral growth and one animal remained tumor-free throughout the experimental period.

In vivo reactions to administration of TLA: For the hematological, biochemical, immunological, and histopathological studies, MC-containing pellets were implanted into the femoral muscles of three Rowett rats. One animal was used as an untreated control (No. I), and other two rats (No. II, III) were treated with TLA according to the schedule indicated in Fig. 4. Hematological characteristics are listed in Table 1 where differences of the value in WBC, SGOT, LDH and uric acid were distinctive among the three rats. SGOT of the control rat was about three times as high as those of TLA treated

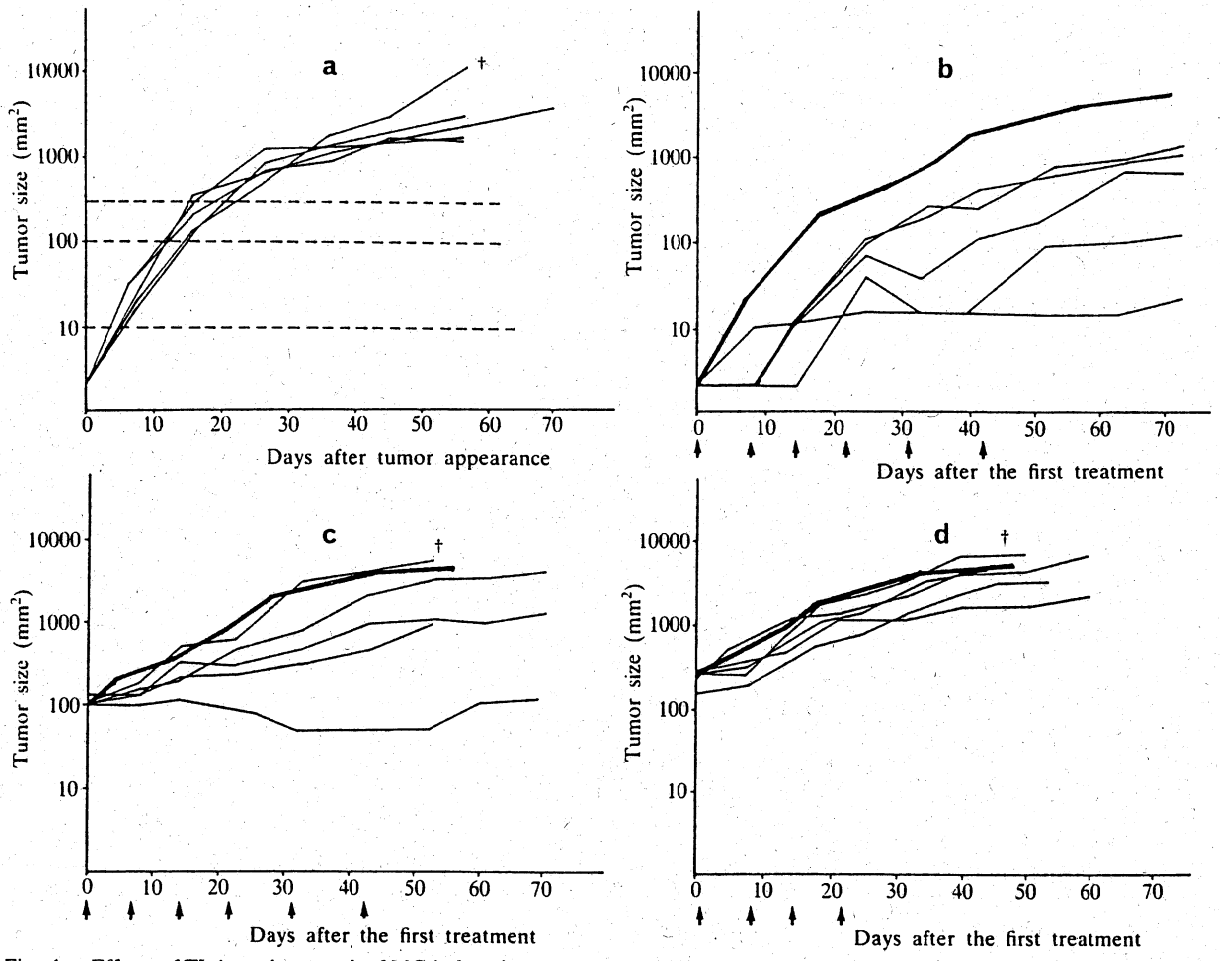

Fig. 1. Effects of TLA on the growth of MC-induced tumors at the various stages of growth. Group "a" was untreated control and groups "b", "c", or "d" were treated with TLA when the tumor diameter reached about 3, 10, or 18 mm, respectively. Each thick line in "b", "c" and "d" represents the average growth of control. Each dotted line in "a" represents tumor size when the treatment was started in the group "b", "c", or "d". Arrows indicate the day of TLA (500 μg/rat) administration.

rats, No. II and No. III. LDH of the rat treated with TLA after tumor induction (No. II) was about one third that of control one, whereas LDH of the rat treated immediately after MC-pellet implantation (No. III) was about two times as high as that of control one.

Total lymphocytes counts of the spleen and liver were highest in No. II and lowest in No. III (Fig. 5). There were no marked differences among these rats concerning in the proportion of Ig positive cells, ATS positive cells and others.

Histopathological section of tumor tissue from No. I represented the characteristics of fibrosarcoma in which small necrotic foci were observed (Fig. 6). Tumor tissue of No. II, by contrast, showed honeycomb appearance with large necrotic foci, and intensive infiltration of lymphocytes and neutrophils was observed in the boundary of necrotic area (Fig. 7). Tumor of No. III was smaller and the central necrosis was more prominent than that of No. II (Fig. 8). Severe cell infiltration was also observed around the residual tumor tissue in the margin of the mass. Degenerative signs of tumor cells such as pyknosis and vacuolization were apparent in No. II and No. III.

Immunohistochemical examination revealed the existence of a few small Thy-1 positive cells in tumor tissue of No. I (Fig. 9a). In case of No. II, large granular Thy-1 positive cells were detected sporadically (Fig. 9b). Although no reactor was observed to the antibody against rat T helper cells, occasional labeling was evident with the antibody for non-helper subset. Immunohistochemical findings in tumor tissue from No. III were similar to those of No. II, where large granular Thy-1 positive cells were present in sporadic numbers (Fig. 9c).

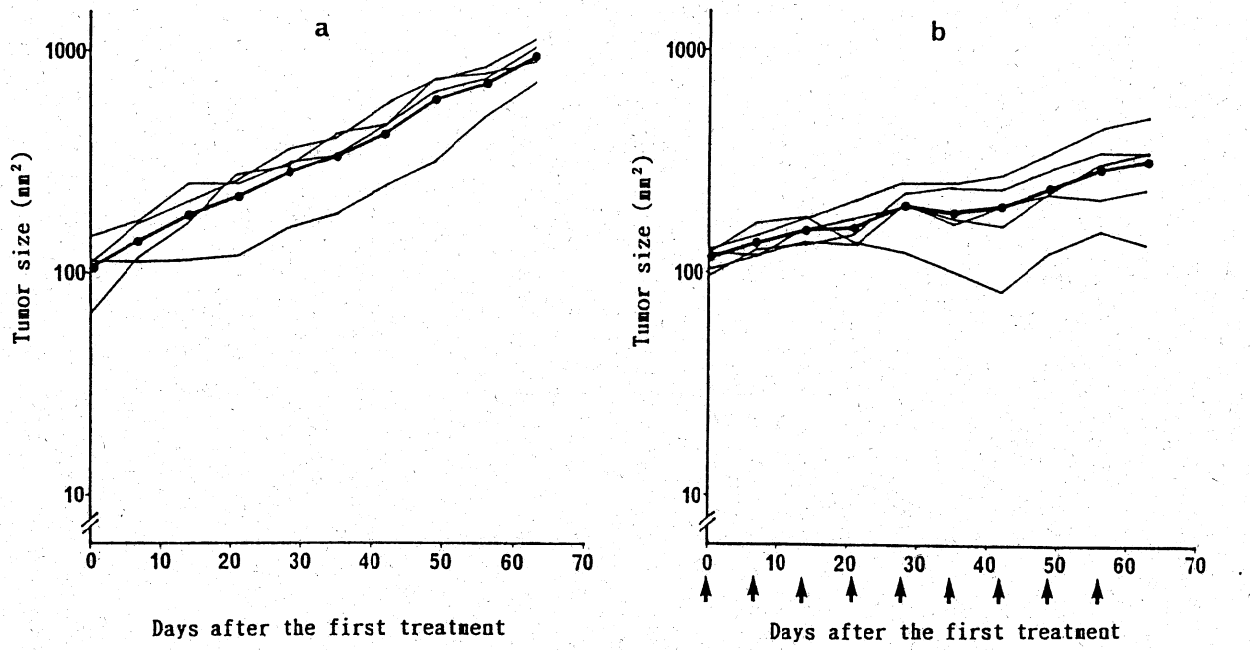

Fig. 2. Effects of the lower dose of TLA on the growth of MC-induced tumor. The experimental group "b" was intramuscularly administered 100 μg of TLA once a week and the control group "a" was treated with saline as a same manner. Each thick line (●—●) represents the mean size and the arrows indicate the day of TLA administration.

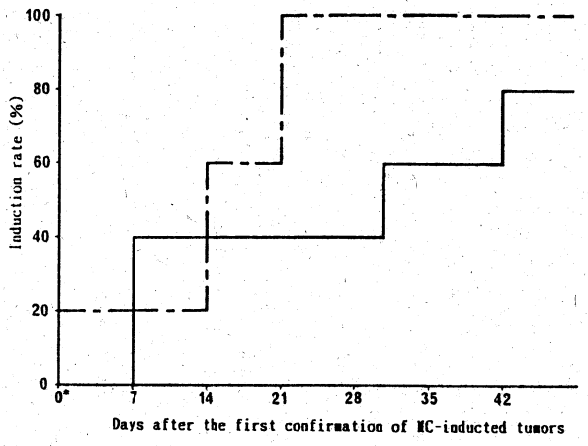

Fig. 3. Effects of TLA on the induction of tumor by MC. Two groups of 5 rats were implanted with a MC-treated paraffin pellet. One group was untreated (— · —). The other was given TLA i. m. at a dose of 500 μg/rat at monthly intervals for 4 months (—).
0*: 124 days after insertion of paraffin pellets.

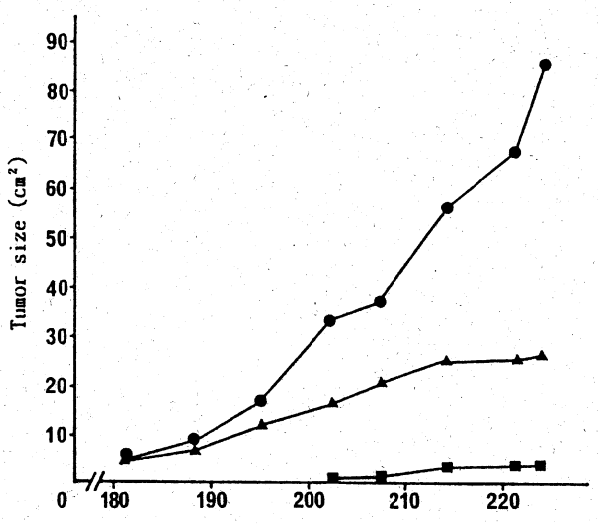

Fig. 4. Tumor growth of the rat implanted with MC-pellet for the further oncological studies.
(●—●): Untreated rat (No. I).
(▲—▲): The rat treated with TLA (500 μg/rat) at 2-week intervals for 6 weeks. Treatment began after the tumoral growth was observed (No. II).
(■—■): The rat treated with TLA (500 μg/rat) weekly for 5 weeks. Treatment began immediately after the MC-containing paraffin pellet was implanted (No. III).

Histological section of No. I liver showed focal necrosis where severe degenerative changes such as cloudy swelling and pyknosis were prominent (Fig. 10). The livers of TLA-treated rats (No. II and No. III) seemed more vivid than those of control animals while moderate degenerative changes of liver cells were still observed (Figs. 11 and 12). Intensive interstitial infiltrations composed of many neut-

Table 1. Hematological characteristics of the tumor-bearing rat with or without TLA treatment

Case number[a)]	No. I	No. II	No. III
RBC ($\times 10^4$/mm^3)	312	328	387
WBC (mm^3)	6,700	11,500	4,100
Ht (%)	27	17	20
STP (g/dl)	5.2	3.6	5.3
SGOT (Karmen Unit)	304.2	88.6	98.5
SGPT (Karmen Unit)	24.0	27.4	23.7
LDH (Wrobleski Unit)	1,058	294	1,813
AlP (King-Armstrong Unit)	10.7	17.7	10.7
T-bilirubin (mg/dl)	0.33	0.19	0.14
Creatinine (mg/dl)	1.46	0.99	1.13
Uric acid (mg/dl)	3.99	3.73	11.56

a) Case numbers were identical to those in Fig. 4.

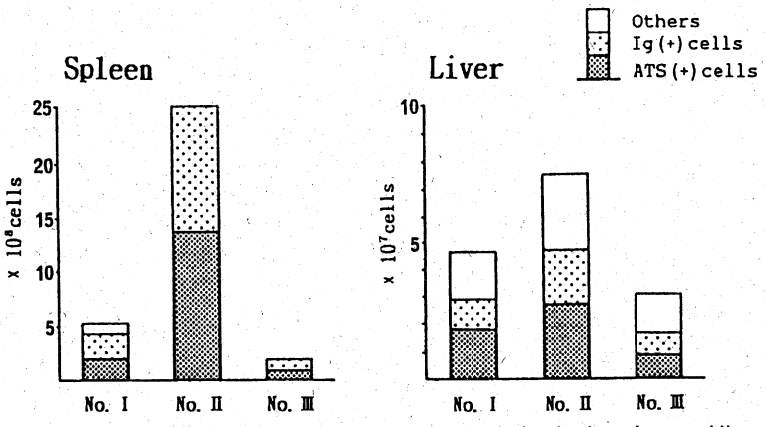

Fig. 5. Distribution ratio of mononuclear cell population in the spleen and liver of the MC-induced tumor bearing rat with or without TLA treatment. Case numbers (I-III) were identical to those in Fig. 4.

rophils, a few lymphocytes and large mononuclear cells were observed in cases of No. II and No. III. Furthermore, swelling of arteriolar endothelial cells was observed in No. III (Fig. 12).

DISCUSSION

Results of this study demonstrate that TLA inhibits the growth of MC-induced murine tumors. The inhibitory effects were most prominent in the case with the tumor smaller than 100 mm^2. Weekly intramuscular administration of TLA at dosages of 100 μg/rat was sufficient to cause these effects. Furthermore, slight inhibitory effects on the induction of tumor were evident when TLA was administered at the time of tumor induction.

Lymphocyte composition of the spleens and livers of these animals was examined to determine the mechanisms for the antitumor activity of TLA. When TLA was administered after the formation of tumors, the number of splenic and hepatic lymphocytes was larger in TLA-treated animals than in untreated controls or rats dosed with TLA before formation of tumors. We reported significant increases in the numbers of B and T lymphocytes in the spleens and livers of TLA-treated mice within 10 days after infection with *Babesia* [4]. Similar responses occurred in the present study, although the number of animals in each experimental group was very limited.

Serum concentrations of SGOT and LDH were high in untreated rats, which corresponded to the histological observations of the liver. On the other hand, normal level of SGOT and moderate injury of liver cells were observed in the TLA-treated rats. These findings suggest the therapeutic effects of TLA concomitant of tumor inhibitory effect. Elevation of LDH is considered to result from the muscular disorders, and the rat treated with TLA just after MC-pellet implantation showed unexplain-

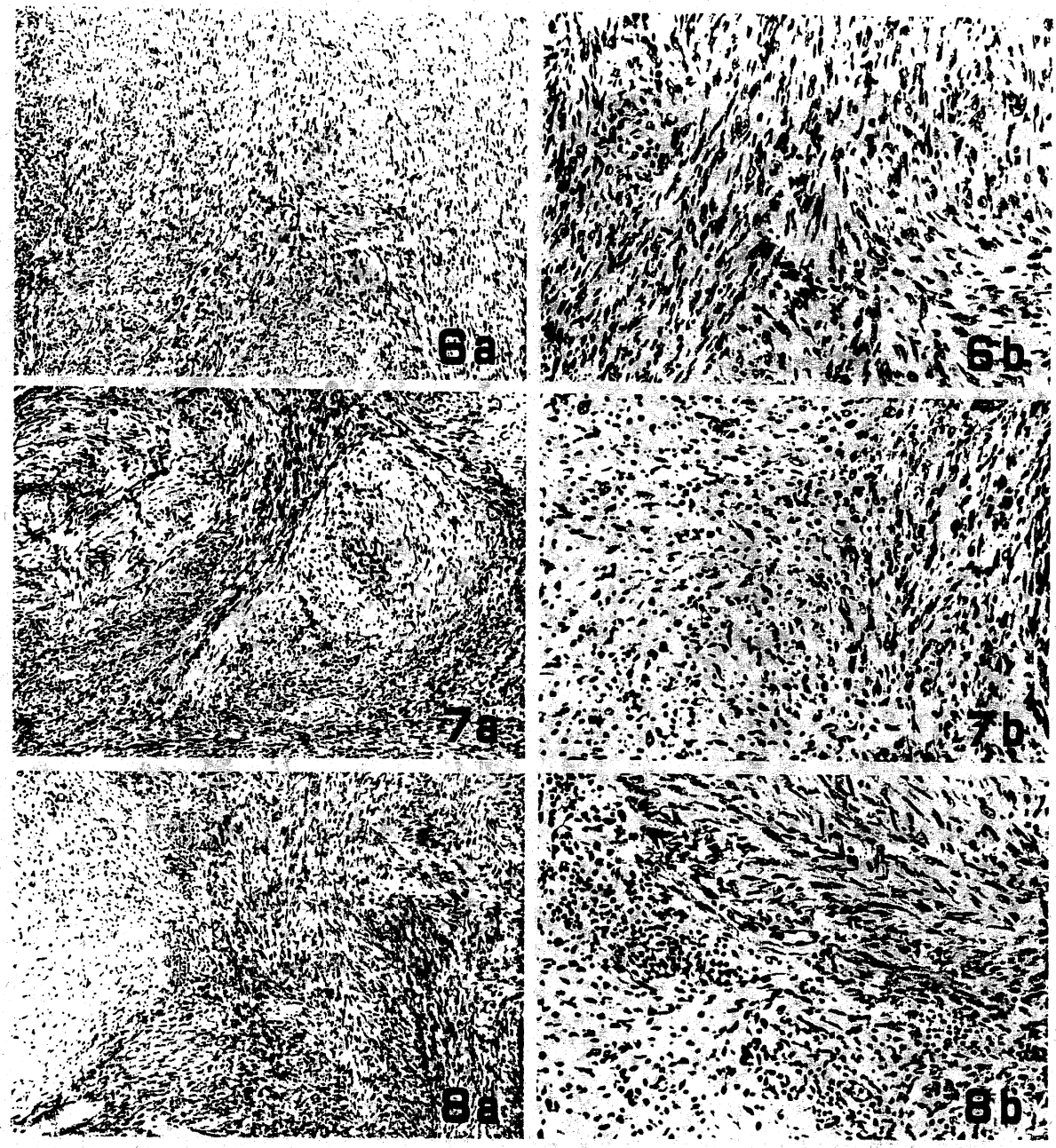

Fig. 6. Histological appearance of MC-induced tumor of control animal (No. I). Tumor cells of spindle type are arranged in bundles or a knot-like pattern (6a, × 80). Nuclei are rich chromatin and contain one or two prominent nucleoli (6b, × 200).

Fig. 7. MC-induced tumor of the rat treated with TLA (No. II) fallen into focal necrosis (7a, ×80). Infiltration of lymphocytes and neutrophils were observed at the inner side of residual tumor cells (7b, × 200).

Fig. 8. Central necrosis was enlarged in case of No. III whereas viable tumor cells were still resident (8a, × 80). Prominent cell infiltration and degenerative change of tumor cells were also observed (8b, × 200).

ably high level of LDH.

Histopathological studies revealed dense growths of spindle tumor cells in untreated control rats, while enlarged central necrosis with the infiltration of lymphocytes and neutrophils was apparent in TLA-treated rats. Of particular interest was the presence of large granular Thy-1 positive cells in TLA-treated rats that were not observed in the untreated control group. Immunohistochemical examination demonstrated that these cells were occa-

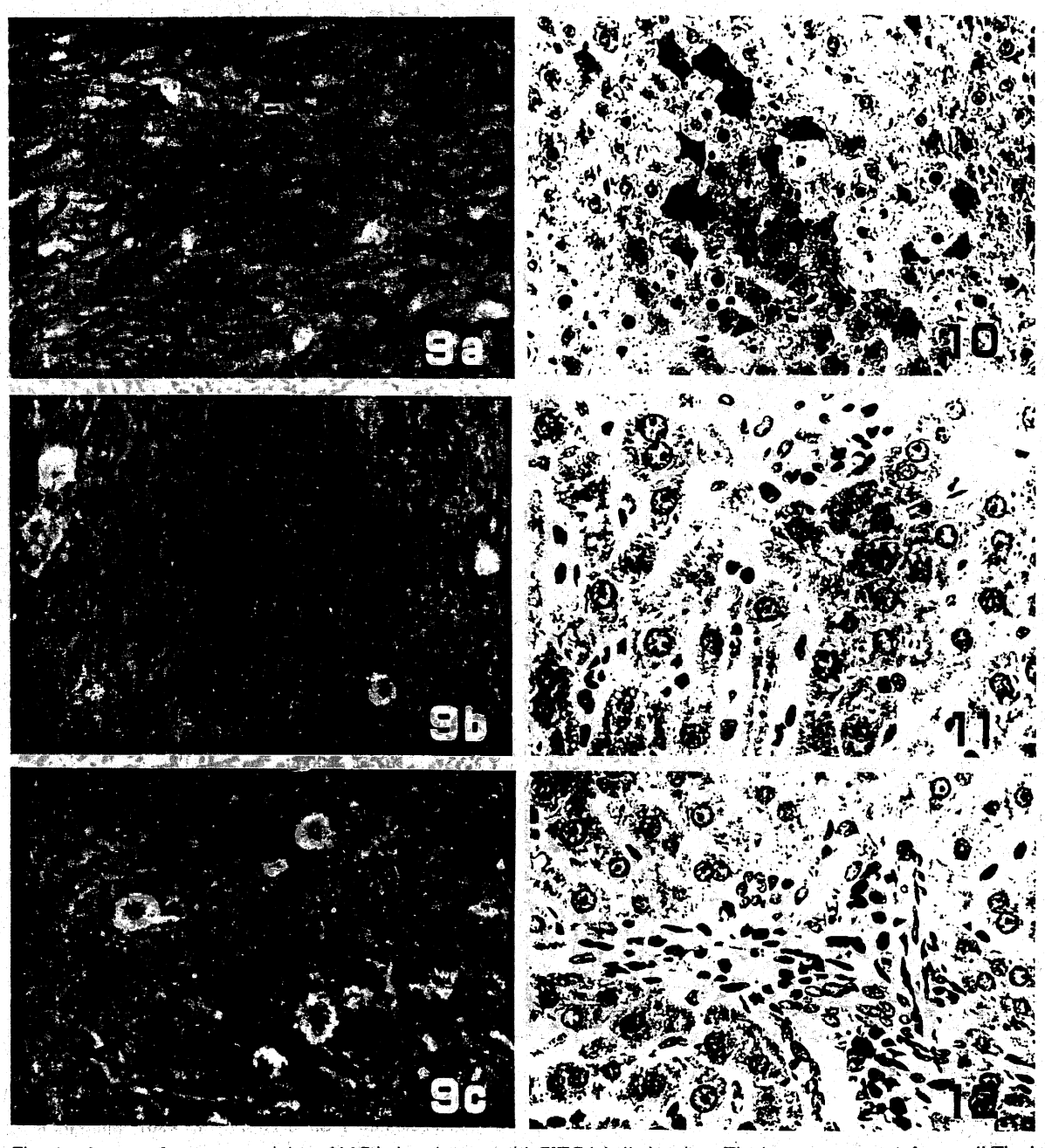

Fig. 9. Immunofluorescent staining of MC-induced tumor with FITC-labelled anti-rat Thy-1 mouse serum. A few small Thy-1 positive cells are present in the tumor tissue of the control animal, No. I (9a, × 350). In the tumor tissues of TLA-treated animals, No. II (9b) or No. III (9c), large granular labelled cells were detected sporadically.

Fig. 10. Histological appearance of the liver of control animal, No. I (× 300). Note the focal necrosis with cloudy swelling and pyknosis of liver cells.

Fig. 11. Interstitial infiltration of neutrophils, lymphocytes and large mononuclear cells were prominent in case of No. II (× 500).

Fig. 12. Degenerative changes of liver cells were moderate and cell infiltrations were observed in case of No. III as well as No. II (× 500). Swelling of arteriolar endothelial cells was also observed.

sionally positive for markers for non-helper T-cells. Authors have reported that incubation of spleen cells from TLA-sensitized mice with TLA caused an increase in the numbers of large Thy-1.2 positive cells and asialo GM_1 positive cells. These cells are highly cytotoxic to P-815 target cells (nonsensitive to NK cells) and YAC-1 target cells (sensitive to NK cells) [4, 12]. Results of this study suggest that large granular Thy-1 positive cells observed in tumor tissue might be identical to the cells reported earlier [6] and might participate in the inhibition of tumor growth.

Histological section of the liver of untreated tumor-bearing rat showed focal necrosis where severe degenerative changes such as cloudy swelling and pyknosis were prominent. The livers of TLA-treated rats seemed more vivid than control animals while moderate degenerative changes of liver cells were still observed. Intensive interstitial infiltrations composed of many neutrophils, a few lymphocytes and large mononuclear cells were observed in cases of TLA-treated rats. Previous studies have indicated that counts of sIg(+), Thy-1.2(+), Lyt-1.2(+), and asialo GM_1 (+) cells in the liver and peripheral blood of TLA-sensitized mice are likely to be higher than those of non-sensitized animals [4]. The cellular infiltrates observed in this study may correspond to the increases in specific cell populations that were observed in TLA-sensitized mice above mentioned. The *in vivo* effects of TLA on MC-induced tumors indicate that this substance may be a useful modifier of biological responses to tumor growth.

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REFERENCES

1. Barker, A. D., Rhains, M. S., and St. Pierre, R. L. 1973. The effect of rabbit anti-mouse brain-associated θ serum on the immunologic responsiveness of AKR mice. *Cell. Immunol.* 7: 85–91.
2. Golub, E. S. 1971. Brain-associated θ antigen: Reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* 2: 353–361.
3. Igarashi, I., Taguchi, M., and Suzuki, N. 1979. Fundamental studies on macrophage migration inhibitory factors in the supernatant from spleen cells in mice infected with *Toxoplasma gondii*. *Zentralbl. Bakteriol. Hyg., A* 244: 374–382.
4. Igarashi, I., Honda, R., Shimada, T., Miyahara, K., Sakurai, H., Saito, A., and Suzuki, N. 1990. Changes of lymphocyte subpopulations and natural killer cells in mice sensitized with *Toxoplasma* lysate antigen before and after *Babesia* infection. *Jpn. J. Vet. Sci.* 52: 969–977.
5. Jacob, L. and Lunde, M. N. 1975. Assessment *in vitro* of immunity against *Toxoplasma gondii*. *J. Exp. Med.* 141: 446–482.
6. Miyahara, K., Honda, U., Tose, S., Sakurai, H., Igarashi, I., Saito, A., Hirose, T., and Suzuki, N. 1992. Therapeutic effects of *Toxoplasma* lysate antigen on 20-methylcholanthrene-induced BALB/c mouse tumors. *J. Vet. Med. Sci.* 54: 7–12.
7. Ogawa, R., Sato, M., Hirose, T., and Suzuki, N. 1985. Preventive effect on *Toxoplasma* lysate antigen (TLA) on fatal infection with mouse piroplasma. *Jpn. J. Vet. Sci.* 47: 225–236.
8. Omata, Y., Yagami, K., Takei, N., Suzuki, N., and Nakabayashi, T. 1981. Protective reaction against malaria infection in mice sensitized with frozen-thawed *Toxoplasma* tachyzoites. *Zentralbl. Bakteriol. Hyg., A* 250: 223–235.
9. Omata, Y., Yagami, K., Sakurai, H., Saito, A., and Suzuki, N. 1983. Interferon induction in *Toxoplasma gondii* infected mice. *Zentralbl. Bakteriol. Hyg., A* 225: 392–398.
10. Saint-Marie, G. 1962. A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* 10: 250–256.
11. Saito, A., Sakurai, H., Terada, S., Igarashi, I., Hirose, T., Suzuki, N., Osaki, H., Kudo, H., Sakata, Y., Okamoto, K., and Yoshida, Y. 1989. Inhibitory effect of *Toxoplasma* lysate antigen on the multiplication of transplanted tumor in mice. *Zentralbl. Bakteriol. Hyg., A* 271: 114–126.
12. Saito, A., Sakurai, H., Igarashi, I., Suzuki, N., and Osaki, H. 1989. Fundamental studies on characteristics of *Toxoplasma* lysate antigen (TLA)-induced killer cells. *Biotherapy* 3: 320–324 (in Japanese with English abstract).
13. Sakurai, H., Igarashi, I., Omata, Y., Saito, A., and Suzuki, N. 1983. Effects of neonatal spleen cell products on the multiplication of *Toxoplasma* in rat peritoneal macrophages. *J. Immunol.* 131: 1527–1530.
14. Sato, M., Sekiguchi, S., Miyagami, T., Sakurai, H., Saito, A., Hirose, T., and Suzuki, N. 1987. With time changes of T-lymphocytes after *Babesia* infection in mice pretreated with *Toxoplasma* lysate antigen. *Zentralbl. Bakteriol. Hyg., A* 264: 435–445.
15. Sharma, S. D., Verhoef, J., and Remington, J. S. 1984. Enhancement of human natural killer cell activity by subcellular components of *Toxoplasma gondii*. *Cell. Immunol.* 86: 317–326.
16. Suzuki, N., Kikushima, K., Miyagami, T., Igarashi, I., Sakurai, H., Saito, A., and Osaki, H. 1987. Modulator effect of *Toxoplasma* lysate antigen in mice experimentally infected with *Plasmodium berghei*. *Zentralbl. Bakteriol. Hyg., A* 264: 422–434.
17. Suzuki, N., Sakurai, H., Saito, A., Omata, Y., and Osaki, H. 1988. Interferon induction in mice treated with *Toxoplasma* lysate antigen. *Biotherapy* 2: 96–98 (in Japanese with English abstract).
18. Tsuji, K. 1971. Separation method for lymphocytes by gravity centrifugation. pp. 443–446. *In*: Technics for Immunological Procedure I (The Japanese Soc. Immunol. ed.), Maeda Print Co., Kanazawa, Japan (in Japanese).

48

Effect of Obiopeptide and Obioactin on the Toxoplasmacidal Activity, Glucose Consumption and Ruffle Formation in Mouse Macrophages

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Obioactin derived from *Toxoplasma* superinfected cattle serum hydrolyzed with both pronase and alkali shows a higher inhibitory effect on the multiplication of microorganisms inside homologous and heterologous macrophages and somatic cells than that of non-treated with obioactin [1, 4, 6, 7]. Recently, some active components from obioactin called obiopeptides were analyzed and chemically synthesized [10, 11]. In this paper, we report some findings on the effect of obioactin and one of its active components, obiopeptide-1 to inhibit *Toxoplasma* multiplication in mouse macrophages, and on the amount of oxygen intermediates released, glucose consumption and ruffle formation in mouse macrophages during the activation period. A six-month old holstein calf was inoculated with 1×10^6 tachyzoites of the *Toxoplasma* RH strain in the cervical muscles, and 4 weeks later, the calf was infected with 2×10^6 tachyzoites of the S-273 strain. Two weeks after infection, 10 μg of *Toxoplasma* lysate antigen (TLA)/kg was injected intravenously, and serum was obtained from blood collected 24 hr later. Serum from normal calf was also obtained. Antibody titer to *Toxoplasma* was measured using the latex agglutination test and indirect fluorescence method [2, 3, 6]. Obioactin was prepared by hydrolysis of the serum as previously reported by Suzuki *et al.* [8]. Obiopeptide-1 was synthesized using the Biosearch automatic peptide synthesizer [10–12]. Glucose concentrations were measured using glucose test Wako Kit (Wako Pure Chemical Co., Tokyo). Samples for electron microscopy were prefixed in 2.5% glutaraldehyde, treated with 1.0% OsO_4, and dehydrated with a series of upgraded ethyl-alcohol. The solvent was replaced with penthyl acetate for further dehydration, and then dried using the critical point dryer (Hitachi HPC-2). The dried samples were fixed, coated with Au-Pd metal using an IB-3 ion coater (Eiko Engineering, Tokyo), and then were observed by the Hitachi S-430 scanning electronmicroscope. Preparation of macrophage monolayers and the measurement of toxoplasmacidal activity and active oxygen intermediates of cells were carried out following the methods as previously reported [4, 6, 7]. Significantly differences were examined by the Student's-t-test.

Table 1 shows glucose consumption of macrophages in the presence of either obiopeptide-1 or obioactin. When

Table 1. Glucose consumption of mouse macrophages incubated with either obiopeptide-1 or obioactin for 48 hr

Groups	Glucose concentration (mg/dl)		Glucose consumption (mg/dl)
	Pre-incubation	48 hr post-incubation	
Control[a)]	100	75.2±3.9	24.8±1.6
Obioactin[b)]	100	62.8±3.6	37.2±1.5*
Obiopeptide-1[c)]	100	62.0±8.2	38.0±3.4**

Note: Mean±SE results of 6 independent experiments. Significant differences between a) and b), and a) and c) were $p<0.001$ and $p<0.01$, respectively.
a) Cell medium (Tc−199+10% calf serum) alone.
b) Cell medium+5.0 mg/ml Obioactin.
c) Cell medium+0.5 mg/ml Obiopeptide-1.

Table 2. Superoxide releases from mouse peritoneal macrophages incubated with either obiopeptide-1 or obioactin for 48 hr

Groups	Dose	nmol/1×10^8 cells/60 min	nmol/100 μg protein/60 min
Control	—	1.34±0.12[a)]	3.72±0.34[a)]
Obioactin	5.0 mg/ml	1.58±0.12	6.27±0.19
Obiopeptide-1	0.5 mg/ml (0.7 mM)	2.01±0.18	5.96±0.89
	0.05 mg/ml (0.07 mM)	1.58±0.12	4.36±0.52

Note: a) Mean±SD of 5 independent experiments.

Table 3. Toxoplasmacidal activity of *Toxoplasma*-exposed normal mouse macrophages incubated with obiopeptide-1 or obioactin

Groups	Dose	Mean % mouse macrophages with *Toxoplasma* (Tp) 48 hr post-incubation			*Toxoplasma*-growth inhibitory effect (%)
		0 Tp	1–5 Tp	≥ 6 Tp/cell	
Control	—	54.4±12.7[a)]	19.8±2.6	25.8±11.3	0
Normal hydrolyzed cattle serum	5.0 mg/ml	68.2± 7.1	22.6±6.9	9.2±3.4	30.3
Obioactin	5.0 mg/ml	86.4± 7.3[b)]	5.8±3.4	7.8±4.2	65.8
Obiopeptide-1	0.5 mg/ml	80.4± 8.9[c)]	14.8±5.4	5.0±3.5	57.5
	0.05 mg/ml	78.0± 6.6	14.6±5.8	7.4±3.7	51.8

Notes: a) Mean±SD of 5 independent experiments.
Significant differences between a) and b), and a) and c) were $p<0.001$, respectively.

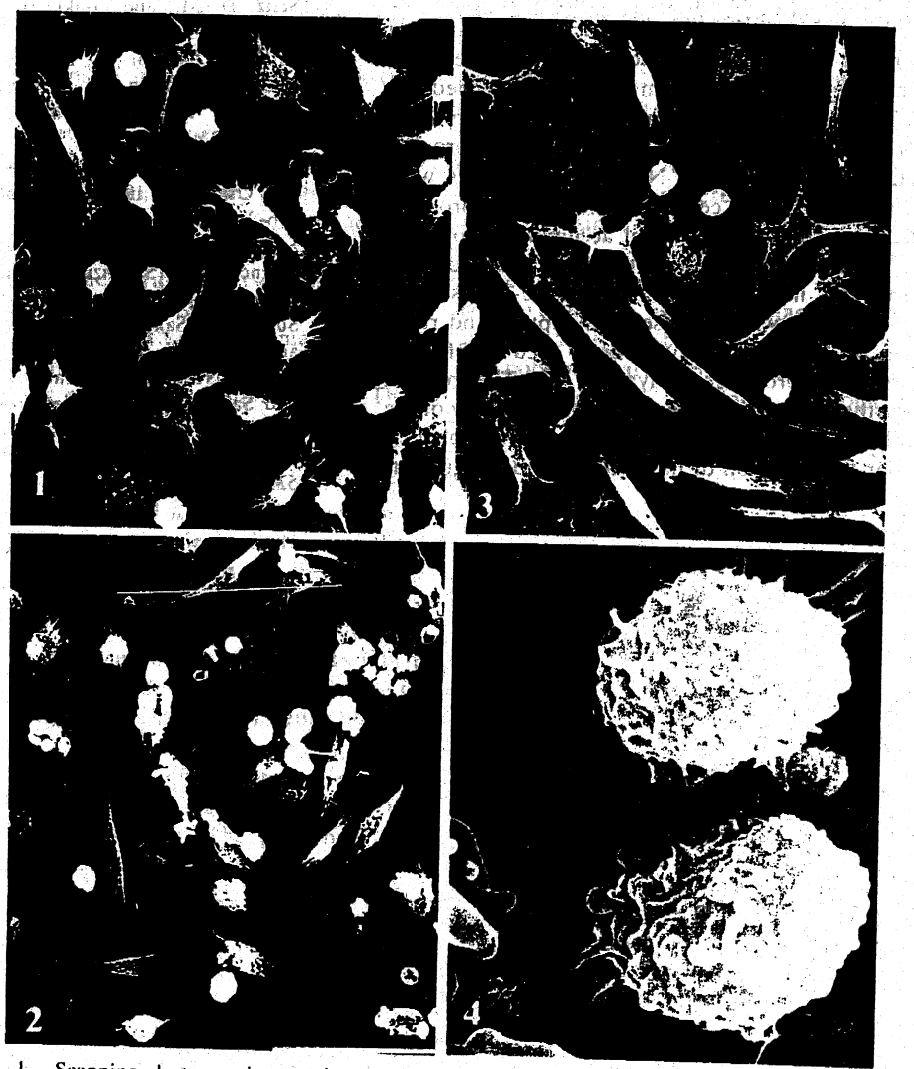

Fig. 1. Scanning electron micrographs of macrophages incubated with obioactin (1), obiopeptide-1 (2, 4) and hydrolyzed normal cattle serum (3) for 48 hr. Fig. 1–1, 2 & 3 (× 500); Fig. 1–4 (× 5,000).

50

compared with the glucose consumption of the control group at 24.8 mg/48 hr of cultivation, cell cultures containing either obiopeptide or obioactin registered greater glucose consumption of 38.0 mg and 37.2 mg/48 hr of cultivation ($p<0.01$ and $p<0.001$), respectively. Likewise, superoxide anion (O_2^-) releases from macrophages incubated with either obiopeptide-1 (0.5 mg/m*l*) or obioactin (5.0 mg/m*l*) for 48 hr were 2.01 ± 0.18 nmoles and 1.58 ± 0.12 nmoles, respectively, while that of the control was 1.34 ± 0.12 nmoles (Table 2).

Toxoplasma growth inhibitory effect of obioactin and obiopeptide-1 incubated with normal mouse macrophages is presented in Table 3. Cell cultures incubated with obioactin and obiopeptide-1 (0.5 mg/m*l*) showed a significantly higher number of normal uninfected macrophages compared to that of the control group ($p<0.001$). Clearly, the *Toxoplasma* growth inhibitory factor (Toxo-GIF) was more than 50% in cells incubated with either obioactin or obiopeptide-1.

Numerous round mononuclear cells were noted in cultures incubated with either obioactin (Fig. 1–1) or obiopeptide-1 (Fig. 1–2), and those cells incubated with hydrolyzed normal cattle serum appeared streched out (Fig. 1–3). Macrophages incubated with either obiopeptide-1 or obioactin showed numerous ruffles and prominent pseudopodia (Fig. 1–4). No marked changes were noted on the surface of cells incubated with hydrolyzed normal cattle serum. A significant accumulation of large rounded macrophages or monocytes in the spleen of mice intramuscularly injected with obiopeptide-1 has been reported [12]. Likewise, glucose consumption and ruffle formation of macrophages have been used as efficient indicators of macrophage activation [5]. The present finding further strengthens the activation capability of the chemically synthesized obiopeptide-1 on macrophages, was similar to the action of the native immunoregulator, obioactin.

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REFERENCES

1. Frenkel, J. K. 1982. *Lyon Medical* 248: 67–73.
2. Matsumoto, Y., Nagasawa, H., Sakurai, H., Sasaki, S., and Suzuki, N. 1981. *Zentralbl. Bakteriol. Hyg., A* 250: 383–391.
3. Nagasawa, H., Igarashi, I., Matsumoto, Y., Sakurai, H., Marbella, C., and Suzuki, N. 1980. *J. Immunobiol.* 157: 307–319.
4. Nagasawa, H., Takei, Y., Miyagami, T., Omata, Y., and Suzuki, N. 1982. *Jpn. J. Parasitol.* 31: 155–164.
5. Onozaki, K. 1985. *Jpn. J. Bacteriol.* 40: 811–817 (in Japanese with English summary).
6. Saito, A., Sakurai, H., Saito, T., Miyagami, T., Suzuki, N., Seitz, H. M., and Osaki, H. 1987. *Zentralbl. Bakteriol. Hyg., A* 264: 445–454.
7. Saito, A., Sakurai, H., Terada, S., Igarashi, I., Suzuki, N., Osaki, H., Kudo, H., Sakata, Y., Okamoto, K., and Yoshida, Y. 1989. *Zentralbl. Bakteriol. Hyg., A* 271: 114–126.
8. Suzuki, N., Izumo, A., Sakurai, H., Saito, A., Miura, H., and Osaki, H. 1984. *Zentralbl. Bakteriol. Hyg., A* 256: 356–366.
9. Suzuki, N. 1987. pp. 76–90. *In*: Progress in Veterinary Science 1987 (Shimizu, Y. and Izawa, H. eds.), Kindai Press, Tokyo (in Japanese).
10. Suzuki, N., Sakurai, H., Saito, A., Igarashi, I., Omata, Y., and Osaki, H. 1990. *Jpn. J. Vet. Sci.* 52: 907–914.
11. Suzuki, N., Fujii, Y., Maki, Y., Sakurai, H., Igarashi, I., and Saito, A. 1990. *Allergy & Immunol.* 9: 149–158.
12. Sakurai, H., Fujii, Y., Maki, Y., Igarashi, I., Omata, Y., Saito, A., Ono, K., and Suzuki, N. 1991. *J. Vet. Med. Sci.* 53: 823–831.

51

Influence of a Newly Synthesized Peptide, Obiopeptide-1, as a Biological Response Modifier (BRM), upon the Lysosomal Enzyme Activities and Chemotactic Responses of Mouse Macrophages

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There have been many recent developments in studies of biological response modifiers (BRM) which activate the defense systems of immunodeficient individuals or regulate immunological responses [1, 2, 8, 9]. Especially in scientific and medical fields, they have been found to be effective immunomodulators against infection with parasites and bacteria, and tumorgenesis.

Suzuki *et al.* [8] purified the polypeptide from serum hydrolysates of *Toxoplasma*-immune cattle and named it obioactin. Obioactin nonspecifically activates somatic cells without any toxicity or antigenecity. Recently, some low molecular weight peptides, obiopeptides, were newly synthesized on the bases of the active sites which were included in obioactin [10]. The functions of obiopeptides have not been clarified. In this study, we examined the influence of obiopeptide-1 (Obi-1) on the activation of mouse macrophages by measuring the lysosomal enzyme activities and chemotactic response.

Mice used for all experiments were ddY, male, eight to ten weeks of age. Immunosuppressed mouse was made by injection of 250 mg/kg of cyclophosphamide (Cyp). They did not survive more than ten days after being infected with two species of bacteria (*K. pneumoniae* 1×10^6 CFU per mouse, *P. aeruginosa* 1×10^8 CFU per mouse). When the mice were treated with 100 μg of Obi-1 per mouse together with Cyp there was significant decrease in their mortalities (Fig. 1). Fujii *et al.* reported that the numbers of neutrophils, lymphocytes, and monocyte-macrophages in peritoneal fluid of the mice treated with Obi-1 were increased [1]. This resistance in mice treated with Obi-1 against bacterial infection was thought to be partly due to the activation of phagocytic cells such as neutrophils or macrophages.

In this study we examined the lysosomal enzyme activities in peritoneal and splenic macrophages after the administration of Obi-1 and Cyp. The peritoneal and splenic macrophages were obtained by the methods of Suzuki *et al.* [9], and Matsuyama *et al.* [5], respectively. To prepare these two macrophages for an enzyme assay were obtained by adhesion for 6 hours and ultrasonicated. In order to elucidate the mechanism of the resistance of Obi-1 treated mice against bacterial infection, the activities of three lysosomal enzymes, acid phosphatase [15], β-*N*-acetyl-glucosaminidase [14], and β-glucuronidase [3] were measured. These enzyme activities increased in peritoneal but not in splenic macrophages of mice treated with Obi-1 (Fig. 2). In mice treated with Cyp, the numbers of mononuclear cells and macrophages in the spleen decreased to about one half (Table 1), therefore the splenic macrophage enzyme activities per mouse decreased. Although the decrease of the number of macrophages in the peritoneal fluid of mice treated with Cyp was severe than that in the splenic fluid, the enzyme activities of mice treated with Obi-1 were elevated to the same levels as those of intact mice. These results suggest that the mechanism of the activation with Obi-1 is different between peritoneal and splenic macrophages. Previously, Suzuki *et al.* [6] reported that the numbers of monocyte-macrophages in the spleen of tumor bearing mice increased when Obi-1 was injected. It is possible that the lysosomal enzyme activities of the peritoneal macrophages were higher than those of the splenic macrophages because the former were more mature than the latter which contained many blast cells.

Chemotactic responses of peritoneal and splenic macrophages were measured by the modified methods of Tokoro *et al.* [13], using chemotactic chamber in which the upper (0.5 m*l*) and bottom (0.7 m*l*) wells were separated by a polycarbonate membrane (pore size 5.0 μm, Nucleopore, U.S.A.) [4]. Maximum chemotactic responses of these two macrophages were obtained at 10^{-7} M concentration of Obi-1 (Fig. 3). It should be noted that splenic

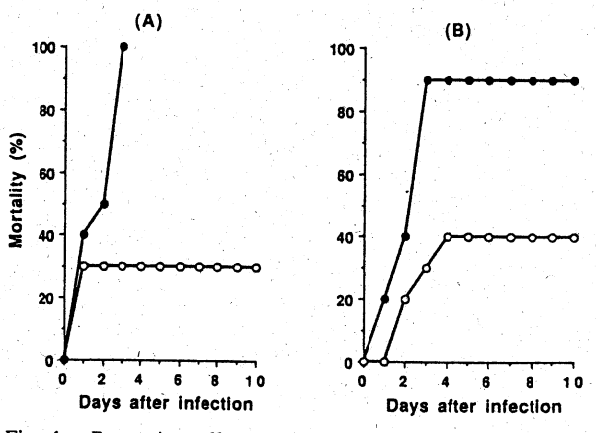

Fig. 1. Protective effect of Obi-1 in Cyp pretreated mice against bacterial infection. (A): *P. aeruginosa* (1×10^8 CFU/mouse, intraperitoneal) infection, (B): *K. pneumoniae* (1×10^6 CFU/mouse, intraperitoneal) infection, ●: Mice were treated with Cyp alone 4 days before, ○: Mice were treated with Cyp 4 days, and Obi-1 7 and 1 days before. Cyp (250 mg/kg) was injected intraperitoneally and Obi-1 (100 μg/mouse) was injected intramuscularly.

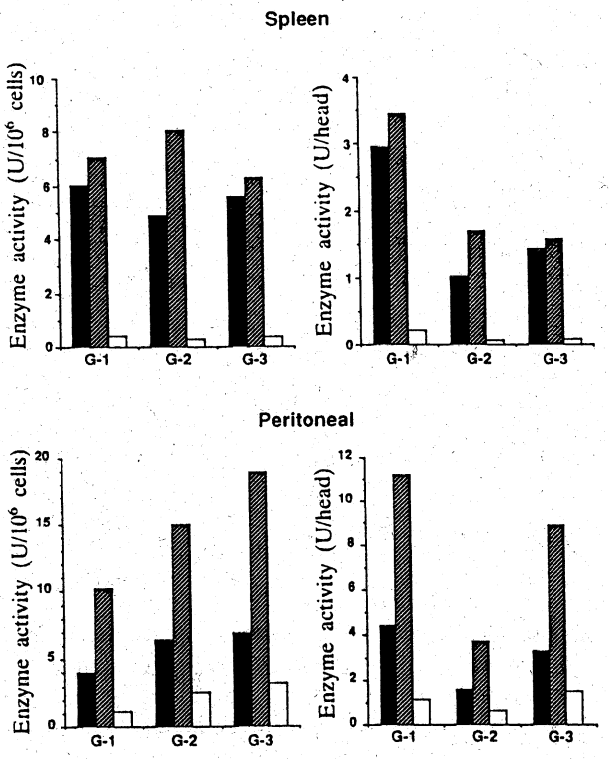

Fig. 2. Lysosomal enzyme activities of spleen and peritoneal monocyte-macrophages in mice treated with Obi-1 and Cyp. Cyp (250 mg/kg) was injected intraperitoneally and Obi-1 (100 μg/mouse) was injected intramuscularly. ■: Acid phosphatase, ■: β-*N*-Acetylglucosaminidase, □: β-Glucuronidase. G-1, G-2, G-3, see notes of Table 1.

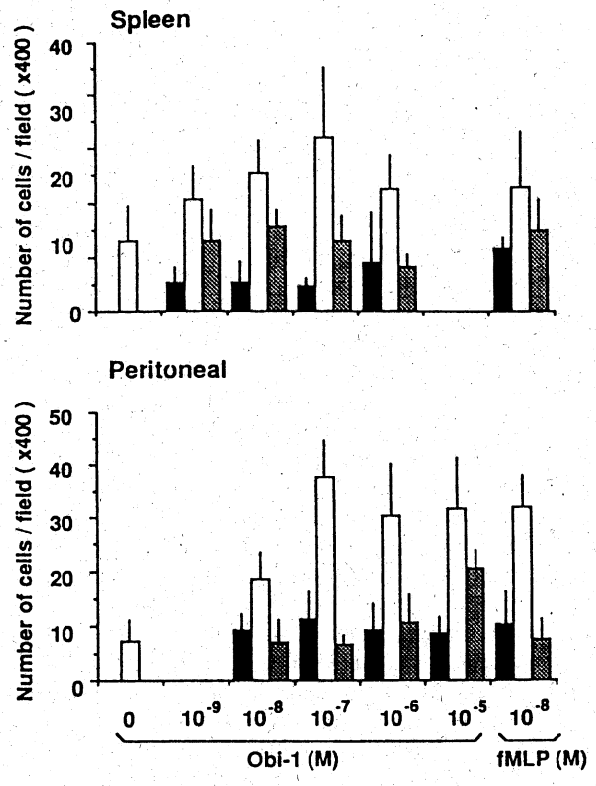

Fig. 3. Migration of mouse monocyte-macrophages in response to various concentration of Obi-1. Spleen monocyte-macrophages (7.0×10^5/0.5 m*l*) and peritoneal monocyte-macrophages (7.5×10^5/0.5 m*l*) were added to the upper wells, and incubated 2 h at 37°C under 5% CO_2. ■: Negative gradient, □: Positive gradient, ▨: No gradient.

Table 1. Spleen and peritoneal monocuclear cells and macrophages in mice treated with Obi-1 and Cyp

Group	Administration		Body weight	Spleen weight	Mononuclear cells (× 10^5)		Macrophages (× 10^5)	
	Obi-1	Cyp	(g)	(g)	Spleen	Peritoneal	Spleen	Peritoneal
G-1	−	−	36.3	0.116	1890	46	4.9	11.0
G-2	−	+	33.6	0.066	413	20	2.1	2.6
G-3	+	+	33.0	0.064	313	16	2.5	4.7

G-1: Mice were not treated with Obi-1 and Cyp, G-2: Mice were treated with Cyp alone 4 days, G-3: Mice were treated with Cyp 4 days, and Obi-1 7 and 1 days before preparation of mononuclear cells and macrophages, respectively.

macrophages migrate at lower concentrations of Obi-1 than peritoneal macrophages. Formyl-methionyl-leucyl-phenylalanine (fMLP) used as a control is one of the formyl peptide that has been identified as the major chemotactic peptide originating from bacteria. The receptors for fMLP were recognized on the neutrophils and monocytes of many species [7, 11, 12]. It has been postulated that the receptor affinities for Obi-1 are different between peritoneal and splenic macrophages. We are now studying the peritoneal and splenic macrophage receptors for Obi-1 by using tritiated Obi-1.

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REFERENCES

1. Fujii, Y., Maki, Y., Ito, M., Saito, A., Igarashi, I., Ono, K., Itoh, K., and Suzuki, N. 1992. *J. Protozool. Res.* 2: 74–83.
2. Fujii, Y., Maki, Y., Claveria, F. G., Igarashi, I., Saito, A., Ono, K., and Suzuki, N. 1992. *J. Vet. Med. Sci.* 54: 351–353.
3. Fishman, W. H. 1974. pp. 929–948. *In*: Methods of Enzymatic Analysis, 2nd Eng. ed. (Bergmeyer, H. U. ed.), Academic Press, New York and London.
4. Horwitz, D. A. and Garrett, M. A. 1971. *J. Immunol.* 106: 649–655.
5. Matsuyama, S., Ohta, M., and Takahashi, M. 1987. *Endocrinol Japon.* 34: 849–855.
6. Sakurai, H., Fujii, Y., Maki, Y., Igarashi, I., Omata, Y., Saito, A., Ono, K., and Suzuki, N. 1991. *J. Vet. Med. Sci.* 53: 823–831.
7. Schiffmann, E., Corcoran, B. A., and Wahl, S. M. 1975. *Proc. Natl. Acad. Sci. U.S.A.* 72: 1059–1062.
8. Suzuki, N., Izumo, A., Sakurai, H., Saito, A., Miura, H., and Osaki, H. 1984. *Zentralbl. Bakteriol. Hyg., A* 256: 356–366.
9. Suzuki, N., Fujii, Y., Maki, I., Sakurai, H., Igarashi, I., and Saito, A. 1990. *Allergy & Immunol.* 9: 149–158.
10. Suzuki, N., Sakurai, H., Saito, A., Igarashi, I., Omata, Y., and Osaki, H. 1990. *J. Vet. Med. Sci.* 52: 907–914.
11. Snyderman, R. and Pike, M. C. 1984. *Ann. Rev. Immunol.* 2: 257–281.
12. Snyderman, R. and Fudman, E. J. 1980. *J. Immunol.* 124: 2754–2757.
13. Tokoro, A., Suzuki, K., Matsumoto, T., Mikami, T., Suzuki, S., and Suzuki, M. 1988. *Microbiol. Immunol.* 32: 387–395.
14. Verporte, J. A. 1972. *J. Biol. Chem.* 247: 4787–4793.
15. Walter, K. and Schutt, C. 1974. pp. 857–870. *In*: Methods of Enzymatic Analysis, 2nd Eng. ed. (Bergmeyer, H. U. ed.), Academic Press, New York and London.

54

1-10

Changes of Splenic Lymphocyte Subpopulation in Mice inoculated with *Babesia microti* and *Babesia rodhaini*

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ABSTRACT. Changes of splenic lymphocyte subpopulation after *Babesia microti* and *Babesia rodhaini* inoculation in mice were examined by flow cytometric analysis. The *B. microti* inoculated mice showed a longer period of time from inoculation to the onset of increase or decrease parasitaemia (%), packed cell volume, total spleen cell numbers and surface immunoglobulin positive splenic cell numbers than respective periods in *B. rodhaini* inoculated mice. The Thy-1 positive cell numbers in *B. microti* inoculated mice and *B. rodhaini* inoculated mice pre-immunized with homologous parasites were significantly higher than that of *B. rodhaini* inoculated mice. The ratio of L3T4 positive cell/Lyt-2 positive cell after inoculation with *B. microti* was quite similar to that in *B. rodhaini* mice pre-immunized. However, the ratio in *B. rodhaini* inoculated mice revealed a lack of an increasing phase. These results suggested that the T-cell dependent early immune response, especially suppressor activity, was closely related to the difference in the course of infection between the non-lethal *B. microti* and the lethal *B. rodhaini* infection in mice.—KEY WORDS: *Babesia microti, Babesia rodhaini,* mouse, splenic lymphocyte, subpopulation.

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It has been widely accepted that the enlargement of the spleen is always observed in mice infected with the intraerythrocytic protozoan parasite, *Babesia* spp. [1, 4–7]. Since splenectomized mice were found to be more susceptible to *Babesia* infection than mice with intact spleens [8], the spleen cells were considered to play important roles on the protective mechanism. The transfer of spleen cells obtained from immunized mice showed a protective activity [5, 9–13], and induced significantly lower parasitemia compared with the transfer of immunized lymph node cells [12].

There are 2 major species of *Babesia* in rodents and it is well documented that these 2 species show a different course of infection [1, 7]. Briefly, the *B. microti* infection is non-lethal and chronic, whereas *B. rodhaini* is lethal in mice. These differences in infection were considered to be mainly dependent on the immune response of host against infectious organisms [13, 14]. However, there are few reports of the dominant cell population in splenic cells providing protection from *B. microti* or *B. rodhaini* infection.

This paper consequently deals with the changes of splenic lymphocyte subpopulation after *B. microti* and *B. rodhaini* inoculations in mice by flow cytometric analysis.

MATERIALS AND METHODS

Inoculation and immunization: Male BALB/c mice aged 8 weeks were supplied from Nippon SLC Inc. (Shizuoka, Japan). *Babesia microti* (Munich strain: BM) and *B. rodhaini* (Australia strain: BR) were maintained in our laboratory by serial passages of parasitized blood to the BALB/c mice. Inoculations were performed by a peritoneal injection with 1×10^4 parasitized erythrocytes (PRBC) in 0.2 m*l* of physiological saline. The experimental mice were divided into 3 groups: mice inoculated with *B. microti* (BM mice), mice inoculated with *B. rodhaini* (BR mice), and mice inoculated with *B. rodhaini* and preimmunized with homologous parasite (BR immunized mice) as another non-lethal control. The immunization was carried out by the method described previously [5]; that is, approximately 1×10^5 *B. rodhaini* PRBC were injected intraperitoneally to normal mice. If the percentage of parasitaemia was more than 1% in peripheral blood, 4–4' diazoaminodibezamidine diaceturate (Ganasec, E, R, Squibb & Sons Inc., Manila, Philippines) was administered intramuscularly at a dose of 0.75 mg/head for 5 days. The mice were examined randomly for parasitaemia. Mice with no parasitaemia at 6 weeks after inoculation qualified as immunized mice.

Analysis: Parasitaemia (%, calculated by Giemsa's staining blood smear), packed cell volume

(PCV), total spleen cell numbers, and splenic lymphocyte subpopulation were examined in 3 mice from each group at an interval of 3 days after inoculation (ai). The antibody titer against homologous parasites inoculated was also measured by the indirect fluorescent antibody technique [14]. The spleen cell preparation for total spleen cell counts and flow cytometric analysis was carried out according to the method described by Inchley [6]. Briefly, the spleen was removed from each mouse, cut into small piecies, and forced through a 200 stainless screen mesh with phosphate buffered saline (PBS, 0.01 M, pH 7.2). The cell suspension was mixed with 0.83% NH_4Cl solution for lysing erythrocytes and centrifuged at 800 G for 7 min. Spleen cells were collected and washed 3 times with PBS and counted with a hemocytometer.

A suspension of splenic cells was prepared at a concentration of 2×10^6 cells/m*l* and stained with various optimally diluted fluorescein-isothiocyanate- or phycoerythrin-conjugated antibodies, such as anti-Thy-1.2 monoclonal antibody (mAb), anti-L3T4 mAb, anti-Lyt-2 mAb (Becton Dickinson Immunocytometry System, California, U.S.A.), anti-IgM Ab, and anti-IgG Ab (Cappel, Westchester, Penna., U.S.A.). After incubation for 30 min in ice, splenic cells stained were washed 3 times with cold PBS. Then, each sample was resuspended with 1 m*l* of PBS and the surface phenotype was examined with the use of a Flow Cytometer (Nihon-Bunko Inc., Tokyo, Japan).

RESULTS

Parasitaemia, PCV and antibody response: The parasitaemia in BM mice increased from day 12, showing a maximum value of 60% at day 18, and then decreased. The parasitaemia in BR mice increased from days 9 to 12, inducing the death of hosts (Fig. 1). The appearance of parasitaemia in BR mice was earlier than that in BM mice. In BR immunized mice, no parasitaemia was observed after the challenge with homologous parasite. The PCV in BM mice decreased, showing a minimum value of 30% at day 18 and then increased. In BR mice, the PCV decreased from days 9 to 12 ai (Fig. 2). Decrease in PCV was seen earlier in BR mice than that in BM mice. Antibody responses against homologous parasites of 3 groups are shown in Table 1. The antibody titer in BM mice increased from days 6 to 12 ai, while in BR mice increased from days 3 to 12 ai.

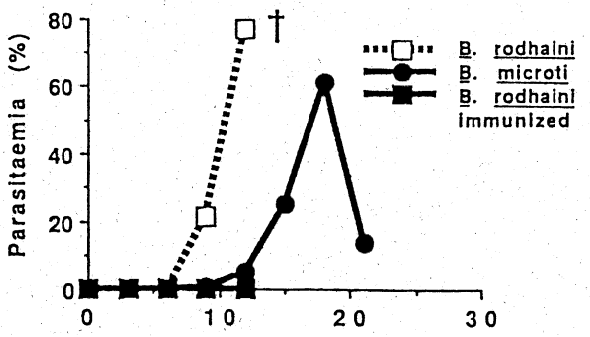

Fig. 1. Changes of parasitaemia in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group.

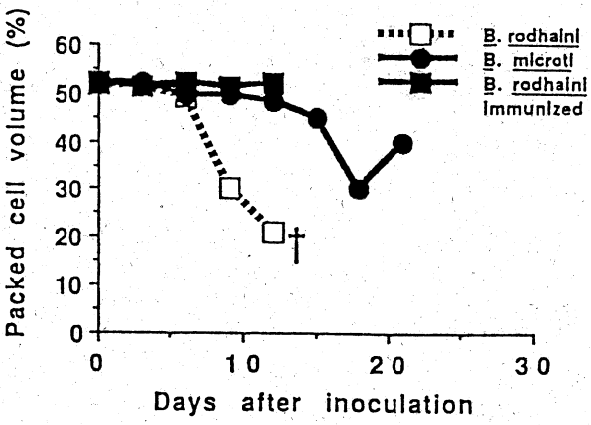

Fig. 2. Changes of packed cell volume in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group.

Total spleen cell numbers: Total spleen cell numbers in both BM mice and BR mice increased gradually in a peak value of approximately 2.3×10^8 cells at days 12 and 15 ai, respectively (Fig. 3). The BR immunized mice showed no changes of total spleen cell numbers during the challenge inoculation.

Splenic lymphocyte subpopulation : Numbers of the IgM positive cells in both BM mice and BR mice increased to the maximum value of approximately 1.1×10^8 at days 10 and 12 ai, respectively. Numbers of the IgG positive cells also increased to the

Table 1. Antibody response against homologous parasites

Group	Subclass	Days after inoculation 3	6	9	12
B. microti inoculated mice	IgM	1:16	1:64	1:256	1:256
	IgG	1:4	1:16	1:256	1:256
B. rodhaini inoculated mice	IgM	1:64	1:256	1:1024	1:256
	IgG	1:16	1:256	1:1024	1:1024
B. rodhaini immunized mice	IgM	1:16	1:16	1:16	1:16
	IgG	1:4096	1:4096	1:4096	1:4096

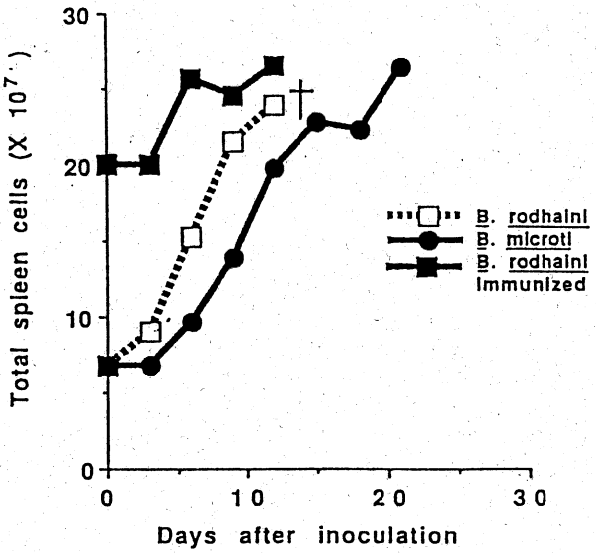

Fig. 3. Changes of total splenic cell numbers in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized. Each point represents the mean value of 3 mice in each group.

maximum value of approximately 1.4×10^8 cells at days 12 and 15, respectively (Fig. 4). The peak number of IgM and IgG positive cells in both inoculated mice were similar to each other, the only exception of the difference of the day of onset for increasing cell numbers (at days 6 and 9 ai, respectively). The cell numbers in BR immunized mice were same at the initial onset of the experiment as they did throughout the rest of the experiment. The Thy-1 positive cell numbers in BM mice increased from days 9 to 12 ai, and continued at a value of 6.5×10^7 cells (Fig. 5). The Thy-1 positive cell numbers in BR mice increased from days 6 to 9, keeping at a value of 5.5×10^7 cells. The peak number of Thy-1 positive cells in BM mice was significantly higher than that in BR mice. No change of Thy-1 positive cell numbers was observed in BR immunized mice during the experiment, showing a value of 6.5×10^7 cells. Note that this is the similar cell numbers to that found in BM mice. The ratio of L3T4 positive cells to Lyt-2 positive cells in BM mice

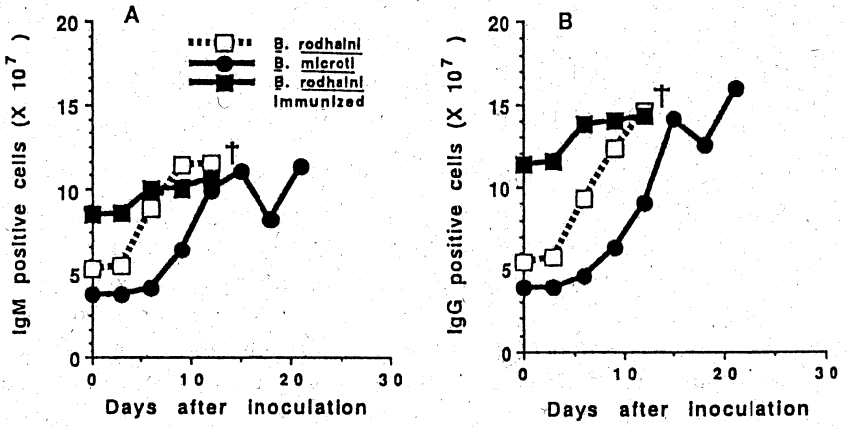

Fig. 4. Changes of splenic surface IgM positive (A) and surface IgG positive (B) cell numbers in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized. Each point represents the mean value of 3 mice in each group.

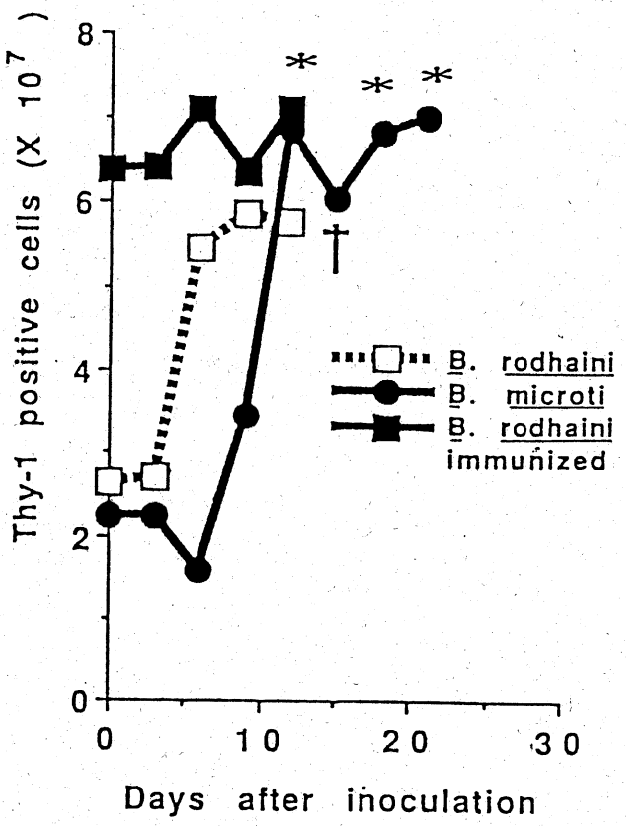

Fig. 5. Changes of splenic Thy-1 positive cell numbers in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group. *: $p<0.001$

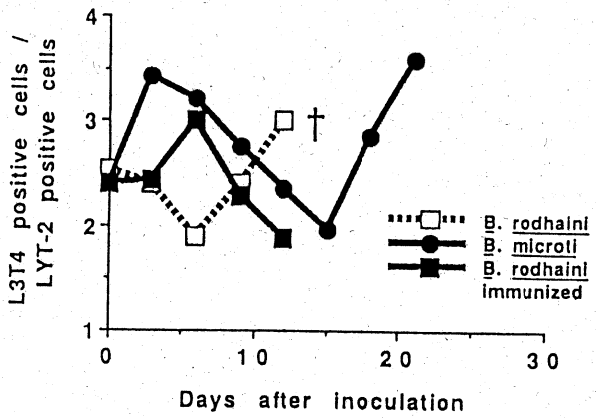

Fig. 6. Changes of L3T4 positive cell/Lyt-2 positive cell ratio in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group.

initially increased at day 3, decreased from days 6 to 15, and then increased gradually until day 21 ai (Fig. 6). The ratio in BR mice decreased from days 3 to 6, showing a minimum value, and then increased from days 9 to 12 ai. The patterns in both BM mice and BR mice were similar to each other, with exceptions of their initial increasing phase observed in BM mice and the day at which they showed a minimum value. The ratio in BR immunized mice increased to day 6 and then decreased gradually until day 12 ai. The pattern in BR immunized mice was quite similar to that in BM mice.

DISCUSSION

The data presented in this paper showed the differences of splenic lymphocyte subpopulation between lethal and non-lethal *Babesia* infection. The increasing or decreasing patterns of parasitaemia, packed cell volume, and antibody response observed in this study were similar to the results previously reported [1, 7]. It was also shown that the onset day of increasing or decreasing cell volume in BM mice was later than that in BR mice [7]. Many investigators have suggested that the spleen cells play important roles in the protective mechanisms against *Babesia* infection [1, 4, 8, 9, 11]. It has also been suggested that the spleen cells have an effect on the course of *Babesia* infection, being non-lethal in the case of BM mice and lethal in BR mice. Inchley *et al.* [7] reported that the enlargement of the spleen observed in BM mice was later than that in BR mice. The spleen enlargement after BM inoculation resulted from B-cell proliferation [6]. However, Igarashi *et al.* [5] reported that no differences in both total spleen cell numbers and B-cell numbers was observed between BR mice and BR immunized mice. In this experiment, no difference was observed in the peak numbers of total spleen cells and of IgM and IgG positive cells among BM and BR mice, and also BR immunized mice with the only exception being the initial day of the onset for increase in cell numbers. The onset day in BR mice was earlier than those in others, like as shown in the antibody response. These results suggested that the B-cell proliferation might be a minor reason for the different course of the resistance and/or parasitaemia observed in these 2 species.

Ruebush and Hanson [12] reported that the splenic T-cells had a major effect on the protective

mechanisms against *Babesia* infection. They showed that the protective effects of the tranferred spleen cells was abrogated by pretreatment with anti-T-cell serum. It was also noted that athymic nude mice were more susceptible to *Babesia* infection [5, 12]. These observations led to the conclusion that the splenic T-cells might be reason for the difference in the course of infection between BM and BR infected mice. In this study, the Thy-1 positive cell numbers in both BM mice and BR immunized mice were significantly higher than that in BR mice. In addition, L3T4 positive cell ratios to Lyt-2 positive cells increased at the initial phase in BM mice and BR immunized mice, while in BR mice they decreased. These results indicate that the immuno suppressive response did not occur in the early phase of inoculation in BM mice and BR immunized mice. Inchley [6] suggested that early death observed in BR infected mice was closely related to the lack of early phase reduction in T-cell proliferation. Zivkovic *et al.* [13] also reported that 8 mice out of 14 pretreated with cyclophosphamide as a complete suppressive dose for immunoglobulin production survived against BR infection and suggested that the protective effect of cyclophosphamide was caused by elimination of suppressor T cells. Therefore, the difference in the course of infection between BM mice non-lethal and BR mice lethal was considered to be mainly dependent upon the onset of suppressor T- cell proliferation after infections.

On the other hand, Habicht *et al.* [3] reported that the incidence of *Babesia* spp. infection was remarkably enhanced in an immunocompromised host. The variation of immunosuppressive activities between BM mice and BR mice might be related to the difference in the respective course of infection, since Gray and Phillips [2] reported that the BM infection developed immunosuppressive effects on host immune system. Further studies are necessary for the understanding of *Babesia* spp. infection on the immunosuppressive phenomena, as well as on the initiation and memory of immunity, especially macrophage responses suggested by Inchley [7].

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REFERENCES

1. Cox, F. E. G. and Young, A. S. 1969. Acquired immunity to *Babesia microti* and *Babesia rodhaini* in mice. *Parasitology* 59: 257–269.
2. Gray, G. D. and Phillips, R. S. 1983. Suppression of primary and secondary antibody responses and inhibition of antigen priming during *Babesia microti* infection in mice. *Parasite Immunol.* 5: 123–134.
3. Habicht, G., Benanch, J., Leichling, K. D., Gocinski, B. L., and Coleman, J. L. 1983. The effect of age on the infection and immunoresponseness of mice to *Babesia microti*. *Mech. Ageing Dev.* 23: 357–369.
4. Hussen, H. S. 1979. *Babesia microti* and *Babesia hylomysei*: Spleen and phagocytosis in infected mice. *Exp. Parasitol.* 47: 1–12.
5. Igarashi, I., Shimada, T., Omata, Y., Claveria, F. G., Saito, A., Ono, K., and Suzuki, N. 1990. Defference in resistance between hetero and nude Rowell rats and BALB/c mice to *Babesia rodhaini* infection. *Allergy and Immunol.* 9: 95–103.
6. Inchley, C. J. 1987. The contribution of B cell proliferation to spleen enlargement in *Babesia microti* infected mice. *Immunology* 60: 57–60.
7. Inchley, C. J., Greieve, E. M., and Preston, P. M. 1987. The proliferative response of mouse lymphoid tissues during infections with *Babesia microti* or *Babesia rodhaini*. *Int. J. Parasitol.* 17: 945–950.
8. Ivin, A. D., Young, E. R., Osborn, G. D., and Francis, L. M. A. 1981. A comparison of Babesia infections in intact, surgically splectomized, and congenitally asplenic (Dh/f) mice. *Int. J. Parasitol.* 11: 251–255.
9. Meeusen, E., Lloyd, S., and Soulsby, E. J. L. 1984. *Babesia microti* in mice. Subpopulations of cells involved in the adaptive transfer of immunity with immune spleen cells. *Aust. J. Exp. Biol. Med. Sci.* 62: 567–575.
10. Meeusen, E., Lioyd, S., and Sousby, E. J. L. 1984. *Babesia microti* in mice. Adaptive transfer of immunity with serum and cells. *Aust. J. Exp. Biol. Med. Sci.* 62: 551–566.
11. Roberts, J. A. 1968. Adoptive transfer of immunity to *Babesia rodhaini* by spleen cells from immune rats. *Aust. J. Exp. Biol. Med. Sci.* 46: 807–808.
12. Ruebush, M. J. and Hanson, W. L. 1980. Transfer of immunity to *Babesia microti* of Human Origin using T lymphocytes in mice. *Cell. Immunol.* 52: 255–265.
13. Zivkovic, D., Seinen, W., Kuil, H., Albers-van Bemmel, C. M. G., and Spekshijder, J. E. 1983. Immunity to Babesia in mice. Adaptive transfer of immunity to *Babesia rodhaini* with immune spleen cell. *Vet. Immunol. Immunopathol.* 5: 343–358.
14. Zivkovic, D., Speksnijder, J. E., Kuil, H., and Seinen, W. 1983. Immunity to *Babesia* in mice. Cross protection between various *Babesia* and *Plasmodium* species and its relevance to the nature of *Babesia* immunity. *Vet. Immunol. Immunopathol.* 5: 359–368.

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Parasitology Research

Effects of reactive oxygen intermediate scavengers on the antitoxoplasmic activity of activated macrophages

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Abstract. Obioactin, Lonomycin A, muramyl dipeptide, and scavengers of hydrogen radicals and of singlet oxygen were used to study the participation of ·OH and $^1O_2$ in the killing of *Toxoplasma* in cultures of glycogen-induced peritoneal macrophages. Both the scavengers of OH (diazabicyclooctane and histidine) and those of $^1O_2$ (mannitol and sodium benzoate) failed to inhibit the multiplication of *Toxoplasma* in macrophages that were incubated with either Obioactin, Lonomycin A, or MDP. The results of these experiments demonstrate the apparent lack of an inhibitory effect of ·OH and $^1O_2$ on the multiplication of *Toxoplasma*, whereas the scavengers alone inhibited the growth of the parasites.

Reactive oxygen intermediates have been reported to produce strong cytotoxic effects and to play important roles in the antimicrobial and antitumor activity of murine macrophages (Schaffner et al. 1982). Previous studies have shown that Obioactin acts as an immunoregulator, inhibiting the growth of *Toxoplasma* in mouse peritoneal macrophages and accelerating the production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2; Suzuki et al. 1984a). Although the production of O_2^- and H_2O_2 increases in monolayers of macrophages incubated with muramyl dipeptide (MDP), the growth of *Toxoplasma* is not inhibited (Saito et al. 1987). By contrast, the ionophore antibiotic Lonomycin A markedly inhibits the growth of *Toxoplasma* in macrophages without increasing the production of reactive oxygen intermediates. In view of these findings, Saito et al. (1987) reported that the multiplication of *Toxoplasma* in macrophages may be inhibited by two distinct mechanisms that are either related or unrelated to the production of reactive oxygen intermediates. In the present study, we attempted to use scavengers of reactive oxygen to examine the intracellular killing of *Toxoplasma* in macrophages that were incubated with either Obioactin, Lonomycin A, or MDP.

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Materials and methods

Test animals and Toxoplasma

Female BALB/c mice aged 7–9 weeks were raised at the Department of Veterinary Physiology. *Toxoplasma* tachyzoites (RH strain) were obtained by periodic subculture from the peritoneal cavities of mice.

Obioactin, Lonomycin A, and MDP

Obioactin was dissolved in TC-199 culture medium containing 10% calf serum (TC-CS) to yield a final concentration of 0.5%. Lonomycin A (Taisho Pharmaceutical Co., Tokyo) was added to TC-CS to obtain a final concentration of 1 ng/ml. MDP (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine; Choay Chimiem Rectifs, France) was dissolved in TC-CS to yield a final concentration of 1 μg/ml. All solutions were sterilized by filtration before use.

Scavengers

The hydroxyl radical (·OH) scavengers benzoic acid (sodium benzoate) and D-mannitol (mannitol) were obtained from Sigma Chemical Co. (St. Louis, Mo.). The singlet oxygen ($^1O_2$) scavengers L-histidine hydrochloride (histidine) and diazabicyclooctane (DABCO) were obtained from Sigma Chemical Co. and Eastman Kodak Co. (Rochester, N.Y.), respectively. All compounds were dissolved in TC-CS at the following concentrations: sodium benzoate, 10 mM; mannitol, 50 mM; histidine, 10 mM; and DABCO, 1 mM (Murray and Cohn 1979, 1980; Murray et al. 1979). The solutions were sterilized by filtration before use.

Monolayer cultures of peritoneal macrophages

Monolayer cultures of glycogen-induced mouse peritoneal macrophages were prepared as previously described by Suzuki et al. (1984a, b).

Measurement of H_2O_2 and O_2^-

H_2O_2 was measured according to the method of Nakano et al. (1968). The cytochrome C method of McCord and Fridovich (1969) was used to measure O_2^-. Volumes produced were expressed in nanomoles per 1×10^6 macrophages per 60 min.

Estimation of growth

Monolayers of macrophages were incubated with *Toxoplasma* tachyzoites (1×10^5/well) for 1 h at 37° C in an incubator containing 5% CO_2 in air. Extracellular tachyzoites were removed by washing, and cultures were subsequently incubated for 24 and 48 h with TC-CS containing different test substances. Cultures were then fixed and stained. A total of 500 macrophages were examined in each culture using light microscopy as described by Suzuki et al. (1984a, b). The percentages of uninfected macrophages, macrophages exhibiting 1–5 tachyzoites each, and those displaying ≥6 tachyzoites each were calculated.

Phagocytic activity of macrophages

Latex particles (Polybead-dyed polystyrene microspheres measuring 3 μm in diameter; Polysciences Inc., Warrington, Pa.) were added to monolayers of macrophage that had been cultured for various periods in media containing scavengers. Cultures exhibiting 1×10^5 particles/well were incubated for 1 h in an incubator containing 5% CO_2 in air and were then washed, fixed, and stained. The percentage of macrophages displaying intracellular particles was determined using light microscopy.

Viability of tachyzoites

Tachyzoites were incubated for various periods in TC-CS medium containing the scavenger of interest. The viability of tachyzoites was determined by the trypan blue dye test and was expressed as the percentage of viable tachyzoites per 300–500 organisms.

Statistical analysis

Measurements of multiplication, phagocytosis, and viability were evaluated by chi-square test. Data from experiments on reactive oxygen intermediates were evaluated using Student's *t*-test. The 95% level of significance was used in all analyses.

Results

Effect on parasite growth and production of reactive oxygen intermediates

Monolayers of *Toxoplasma*-infected macrophages were incubated for 24 and 48 h in media containing either histidine, sodium benzoate, or mannitol to study the effect of scavengers on parasite growth and survival. At 24 h postinoculation, both treated and control cultures exhibited similar percentages of infected and uninfected macrophages (Table 1). However, after 48 h the percentages of macrophages displaying intracellular tachyzoites decreased significantly in cultures that were incubated with scavengers ($P<0.001$). Fewer than 10% of the macrophages exhibited six or more parasites.

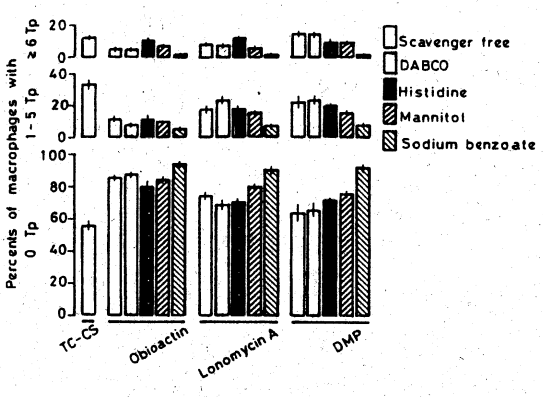

Fig. 1. Growth of *Toxoplasma* tachyzoites in macrophage cultures incubated with different combinations of various oxygen scavengers and Obioactin, Lonomycin A, or MDP in culture medium. Data represent the mean values ±SEM for 9 experimental trials

Cultures treated with Obioactin showed higher percentages of uninfected macrophages. However, the use of scavengers in combination with this immunoregulator had little effect on the rates of infection (Fig. 1). Incubation of scavengers with Lonomycin A also failed to influence the inhibitory effect of the latter on parasite growth. Cultures exposed to combinations of MDP or Lonomycin A and different scavengers exhibited higher percentages of uninfected macrophages than did those exposed to MDP or Lonomycin A alone (Fig. 1). In particular, significantly higher percentages of uninfected macrophages were found in cultures that were exposed to combinations of Lonomycin A ($P<0.005$) or MDP ($P<0.001$) and sodium benzoate.

Production of H_2O_2 decreased significantly in macrophage cultures containing either mannitol and Obioactin ($P<0.05$), sodium benzoate and Obioactin ($P<0.001$), DABCO and MDP ($P<0.005$), or mannitol and MDP

Table 1. Comparison of the percentage of infected macrophages found in monolayer cultures incubated with different scavengers

Treatment	Mean percentage of infected macrophages					
	24 h incubation[a]			48 h incubation[a]		
	0 Tp	1–5 Tp	≥6 Tp	0 Tp	1–5 Tp	≥6 Tp
TC-CS (control)	96.8±0.8	0.8±0.2	2.4±0.7	39.8±6.3	27.2±3.7	33 ±4.1
Histidine (10 mM)	99 ±0.2	0.8±0.5	0.2±0.2	79.2±4.1	15.6±3.1	5.2±1.5
Sodium benzoate (10 mM)	99.8±0.2	0.2±0.2	0	98.8±1.6	1.2±0.6	0
Mannitol (50 mM)	97.2±1	0.8±0.4	2 ±0.9	76.6±5	16.4±3.6	7 ±1.7

Data represent mean values ±SEM for 5 experimental trials

[a] Number of *Toxoplasma* (Tp) per macrophage

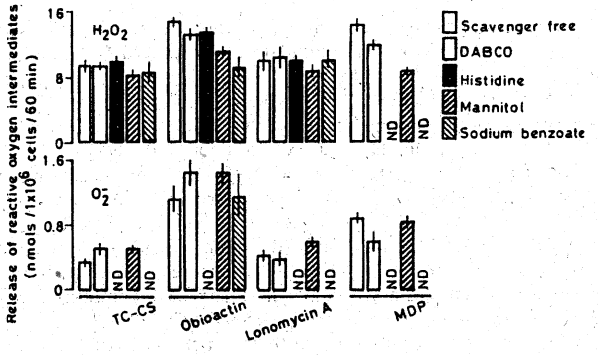

Fig. 2. Production of reactive oxygen intermediates from macrophage cultures incubated with different combinations of various oxygen scavengers and Obioactin, Lonomycin A, or MDP in culture medium. Data represent the mean value ±SEM for 9 experimental trials. ND, Not determined

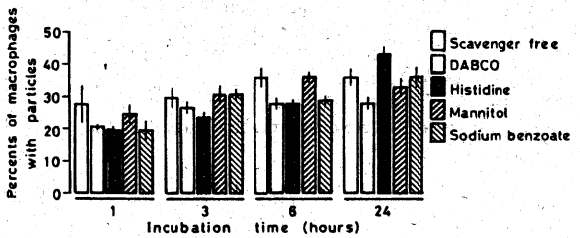

Fig. 3. Phagocytic activity of macrophage cultures incubated with various oxygen scavengers in culture medium. Data represent the mean values ±SEM for 5 experimental trials

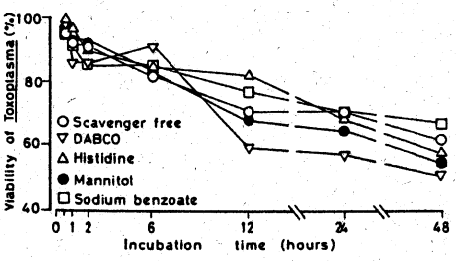

Fig. 4. Viability of *Toxoplasma* tachyzoites in culture medium containing various oxygen scavengers. Data represent the mean values for 3 experimental trials

($P < 0.001$) as compared with control cultures incubated with either Obioactin or MDP alone. However, cultivation of macrophages either with Lonomycin A and scavengers or with scavengers alone had no effect on the production of H_2O_2. Cultivation of macrophages with scavengers and either Obioactin, Lonomycin A, or MDP did not significantly influence the production of O_2^- (Fig. 2).

Effect of scavengers on phagocytic activity

The phagocytic activity of macrophages incubated with either DABCO, histidine, sodium benzoate, or mannitol for 1, 3, 6, and 24 h was related to incubation time. As the incubation period was increased, the number of cells exhibiting internal latex particles increased as well. However, there was no significant difference between the control and the treated cultures when comparisons were made at the end of each incubation period (Fig. 3).

Effect of scavengers on parasite viability

The viability of tachyzoites was studied after 0.5, 1, 2, 6, 12, 24, and 48 h incubation in the presence of either DABCO, histidine, sodium benzoate, or mannitol. Control cultures that were not exposed to scavengers showed a decrease in parasite viability over time that dropped to about 65% after 48 h. Similar decreases in viability (52%–68%) occurred in cultures that were incubated with scavengers for 48 h (Fig. 4). Comparisons of control and treated cultures at the end of each incubation period revealed no appreciable difference ($P > 0.05$).

Discussion

The intracellular growth of *Toxoplasma* was inhibited by Obioactin and Lonomycin A; however, the latter did not increase the production of reactive oxygen intermediates by macrophages. Saito et al. (1987) reported that MDP increased the production of reactive oxygen intermediates to a level similar to that obtained using Obioactin but failed to inhibit the growth of *Toxoplasma*. These findings suggest that the increased production of reactive oxygen intermediates is not always correlated with the intracellular killing of *Toxoplasma*. Thus, it is possible that other oxygen-independent mechanisms exist that may be capable of inhibiting the growth of this parasite.

The killing of *Toxoplasma* in the presence of both xanthine and xanthine oxidase was inhibited by scavengers of ·OH and $^1O_2$, indicating that these scavengers are capable of quenching ·OH and $^1O_2$ generated in a cell-free system (Murray and Cohn 1979). Likewise, the growth of *Toxoplasma* in lymphokine-activated macrophages is also inhibited by scavengers (Murray and Cohn 1980). However, in the present study, the growth of tachyzoites was not inhibited in cultures treated with either Obioactin, Lonomycin A, or MDP, even when cultures were also exposed to scavengers. Differences between our findings and those of both Murray et al. (1979) and Murray and Cohn (1980) may be related to differences in the preculture period, in the substances that were used to elicit peritoneal macrophages, and/or in the activating substances that were used in vitro.

In the present study, initial data showed that the multiplication of tachyzoites was suppressed by scavengers. This finding suggests that scavengers themselves may directly affect either the viability of parasites or the phagocytic activity of macrophages, thus reducing the ability of tachyzoites to invade and establish themselves in new macrophages. However, our data revealed that scavengers had no effect on the viability of *Toxoplasma* (Fig. 4) or the phagocytic activity of macrophages (Fig. 3). These

observations are consistent with those of Murray et al. (1979).

The present results also show that in macrophages, antimicrobial mechanisms exist that are independent of reactive oxygen. Further elucidation of these mechanisms is essential to an understanding of the functions of macrophages. The mechanism by which scavengers alone inhibit the growth of *Toxoplasma* remains unclear; however, it is possible that they influence the cellular metabolism associated with oxygen-independent microbicidal mechanisms.

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References

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

Murray HW, Cohn ZA (1979) Macrophage oxygen-dependent antimicrobial activity: I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. J Exp Med 150:938–949

Murray HW, Cohn ZA (1980) Macrophage oxygen-dependent antimicrobial activity: III. Enhanced oxidative metabolism as an expression of macrophage activation. J Exp Med 152:1596–1609

Murray HW, Juangbhanich CW, Nathan CF, Cohn ZA (1979) Macrophage oxygen-dependent antimicrobial activity: II. The role of oxygen intermediates. J Exp Med 150:950–964

Nakano M, Tsutsumi Y, Danowski TS (1968) Enzymatic determination of small quantities of hydrogen peroxide. Proc Soc Exp Biol Med 129:960–963

Saito A, Sakurai H, Saito T, Taji S, Miyagami T, Suzuki N, Seitz HM, Osaki H (1987) Correlation between release of reactive oxygen intermediates and inhibition of *Toxoplasma* multiplication in mouse peritoneal and alveolar macrophages and kidney cells after in vitro incubation with Obioactin, Lonomycin A, muramyl dipeptide, lipopolysaccharide or *Toxoplasma* lysate antigen. Zentralbl Bakteriol Mikrobiol Hyg [A] 264:446–454

Schaffner T, Kekker HU, Hess MW, Cottier H (1982) Macrophage functions in antimicrobial defense. Klin Wochenschr 60:720–726

Suzuki N, Izumo A, Sakurai H, Saito A, Miura H, Osaki H (1984a) Toxoplasmacidal activity of Obioactin derived from hydrolyzed *Toxoplasma* immune bovine serum in heterologous cell cultures. Zentralbl Bakteriol Mikrobiol Hyg [A] 256:356–366

Suzuki N, Espinas FM, Sakurai H, Saito A, Sasaki H, Kato G, Manba K, Taniguchi K, Mochizuki K, Osaki H (1984b) Studies on the activity of Obioactin with acetylspiramycin on mice infected with *Toxoplasma gondii*. Zentralbl Bakteriol Mikrobiol Hyg [A] 256:367–380

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Restorative Effects of A Newly Synthesized Peptide, Obiopeptide-1, in Cyclophosphamide- or Carrageenan-pretreated Mice Infected with Opportunistic Bacteria

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ABSTRACT

Large numbers of cyclophosphamide- or carrageenan-pretreated immunosuppressed adult ddY mice died within 10 days after inoculation with several species of opportunistic bacteria, including *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *S.aureus* and methicillin resistant *S. aureus*. When immunosuppressed mice were administered bacteria in combination with two 100 μg/mouse intramuscular doses of a newly synthesized peptide, Obiopeptide-1 (OP-1), survival rates increased significantly. At 24 and 48 hours after intraperitoneal inoculation with *P. aeruginosa*, counts of viable organisms from the livers, spleens, lungs, hearts, and kidneys of mice that were administered cyclophosphamide in combination with OP-1 were significantly lower than counts from mice that were administered cyclophosphamide alone. Bacteriocidal activity of peritoneal cells, neutrophils and monocyte-macrophages was higher in OP-1 pretreated mice than in non-OP-1 treated mice. This newly synthesized peptide, OP-1, is a potential immunomodulator which increases host resistance against bacterial infection. The peptide may also function as a nonspecific blood stimulating factor.

INTRODUCTION

During organ transplants and tumor surgery, experimental animals and clinical patients are commonly given immunosuppressive agents such as cyclophosphamide (Cyp) and carrageenan(Carra) to suppress the normal immune function. This treatment often provokes *Mycobacterium bovis* (Takahashi, & Collins, 1987), *Listeria monocytogenes* (Hugin et al, 1986), *Pseudomonas aeruginosa* (Saegusa et al., 1990; Urano, 1978; Yokota, 1984), Methicillin resistant *Staphylococcus aureus* (MRSA, Kouda et al., 1987), *Trypanosoma cruzi* (McCabe et al., 1985) and other microorganisms.

Obioactin is a native immunoregulatory substance found in lymphokines produced by the lymphocytes of *Toxoplasma*-immune cattle which has been shown to inhibit the multiplication of *Toxoplasma gondii* in macrophages and somatic cells in a variety of

animal species (Suzuki et al., 1984; Fujii et al., 1992). This immunomodulator has a molecular weight of less than 5,000 daltons.

Newly synthesized Obipeptides 1,2,3, and 4 have shown significantly higher biological activity than native Obioactin on a per weight or per mole basis (Fujii et al., 1992; Sakurai et al,1991; Suzuki et al.,1984, 1990a,b.). Some of our synthesized Obiopeptides are similar to partial peptides of prothymosin alpha, a polypeptide with 110 amino acide residues (Fangaou et al., 1988; Watts et al., 1989). Recently, prothymosin alpha has been shown to protect mice against an opportunistic infection caused by *Candida albicans* that is particularly threatening to AIDS patients (Eschenfeld et al.,1988; Gomez et al., 1989). For these reasons, experiments in the present study were conducted to study the immunoregulatory effects of this peptide on cyclophosphamide- or carrageenan-treated immunosuppressed mice with opportunistic bacterial infections.

MATERIALS AND METHODS

Animals used: Male mice of the ddY strain that were 8-10 weeks of age were used for all experiments (Carely Co., Tokyo). Animals were maintained under standard conditions at room temperature (22±1℃ with 60±5% humidity), fed ad lib with pellets (Clea Japan Co., Tokyo) and water. Separate groups of mice were used for the mortality test and for the tests to measure persistence of viable bacteria within internal organs.

Obiopeptide-1 (OP-1): Obiopeptide-1, Gly-Glu-Glu-Glu-Glu-Glu, was synthesized chemically with a synthesizer (Biosearch 9600 type, Biosearch Co., USA) (Suzuki et al., 1990a, b).

Cyclophosphamide(Cyp): Cyclophosphamide monohydrate (Janssen Chemica, 2340, Beerse, Belgium) was dissolved in a physiological saline solution at a concentration of 25 mg/ml and sterilized by filtration before use.

Carrageenan (Carra): Carrageenan (Wako Pure Chemical Co., Tokyo) was mixed with a physiological saline solution in a glass mortar. A final concentration of 6 mg/ml was prepared and kept at 4℃ until used.

Preparation of bacterial suspensions: Standard agar cultures of *Pseudomonas aeruginosa* (IFO 12689), *Klebsiella pneumoniae* (PCI 602,ATCC 10031) *Escherichia coli* (K-12), *Staphylococcus aureus* (209P, IFO 12732, ATCC 6538P), and Methicillin resistant *Staphylococcus aureus* (MRSA, strain Nos. 910308-508, 910309-502, 910309-504, 910311-520, 910312-521, 910313-591) were cultivated more than 3 times (BBL, Becton Dickinson Microbiology Systems, USA). The 6 strains of MRSA were kindly shared by Dr. M.Kouda, Department of Central Clinical Lab., Tokyo Metropolitan Police Hospital, Tokyo. Bacteria were cultured on a standard agar plate 2 days prior to preparation of a suspension. A loop of bacteria was collected with a Stickmate 10 (Ono Pharmaceutical Co., Tokyo), and suspended in 10 ml of a physiological saline solution. The suspension was standardized with a Hitachi double beam spectrophotometer at 600 nm to give a constant turbidity and used as a bacterial stock suspension. The concentration of bacteria was determined precisely by preparing serial 10-fold dilutions of the suspension according to the plate dilution method.

Tests of infectivity: Mice were inoculated intraperitoneally (ip) with 0.1 mg of Cyp solution per 10 g body weight 4 days prior to receiving injections of bacteria. Final dosage of Cyp was 250 mg/kg. Other experimental mice were inoculated ip with 0.1 ml of Carra solution per 10 g of body weight 2 days prior to receiving infection of

MRSA. Final dosage of Carra was 60 mg/kg. Mice were inoculated ip with 1 ml of bacterial suspension. This experimental scheme followed the same method as Yokota (1984).

Treatment of Inoculated mice: Obiopeptide-1 was injected intramuscularly (im) in the femoral area of mice that has been treated with Cyp or Carra. Dosage of 100 µg/mouse were administered 7 days and 1 day prior to intraperitoneal inoculation with bacteria.

Counts of viable bacteria: Mice were killed at specified times after inoculation(ai) with bacteria and injected ip with 3 ml of Hanks balanced salt solution (HBSS). The abdomens were massaged and 1.5 ml of peritoneal exudate was collected with a heparinized syringe from each mouse. Animals were then laparotomized and blood was collected from the inferior vena cava. Livers, spleens, kidneys, stomachs, lungs and hearts were excised, weighed and cut in half. One half of each organ was fixed in 10 % phosphate buffered formalin (pH7.0) for histological examination. Remaining halves were weighed and used to estimate numbers of viable bacteria. Serial 10-fold dilutions were prepared of peritoneal exudates and blood with sterile physiological saline. Other organs were homogenized in 10 ml of sterilized saline with a glass homogenizer. Serial 10-fold dilutions were prepared from homogenates with sterile physiological saline. One ml aliquots of the dilutions were placed in Petri dishes and mixed with warm 40℃ agar. After the agar solidified, the dishes were incubated at 37℃ for 2 days. The number of bacterial colonies were counted in each dish and used to estimate numbers of viable bacteria in each organ.

Preparation of spleen lymphoide cells: Spleens were removed under sterile conditions from mice that had been killed by cervical dislocation. The organs were chopped into fine pieces at ice-cold temperatures, ground between glass slides, and suspended in HBSS. The tissue suspension was filtered through a # 40 stainless steel mesh to remove large pieces of tissue and centrifuged at 800×g for 10 min at 4℃. Red blood cells were hemolyzed with 0.83% ammonium chloride at 37 ℃. Non-hemolyzed cells were filtered through a glass fiber column to eliminate filamenteous tissue and adherent cells and centrifuged. The pellet was resuspended in HBSS and applied to a Conray-ficoll density gradient for collection of the lymphocyte rich fraction (Suzuki et al., 1984). The lymphocyte fraction was washed twice by centrifugation at 800×g for 7 min at 4℃, once with HBSS and once with RPMI 1640. These cell suspensions were analyzed by flow cytometry (Cell Sorter FACS, Showadenko Co., Tokyo).

RESULTS

Protective effects of OP-1 on Cyp-treated immunosuppressed mice that were administered bacteria:

When untreated control mice (P-G-1) were inoculated ip with 8×10^6 *P. aeruginosa*, all individuals survived 10 days ai and had an overall increase in body weight of 12.3% (Table 1). None of the 10 Cyp-treated mice (P-G-2) survived to 10 days ai. By 8 days ai, their body weights had an overall decrease of 23.3%. Seven of the 10 mice that were treated with Cyp and OP-1 survived 10 days ai, but had overall decline in body weight of 12.8%.Similar results were obtained when mice were inoculated ip with 6×10^6 *K.pneumoniae*. All untreated control mice survived 10 days ai with an overall weight gain of 12.3%. Only one of the 10 Cyp-treated mice survived to 10 days ai. Mice in this group had an overall weight loss of 20.8%. An overall decrease in body weight of 22.3% occurred in mice that were injected with both Cyp

EFFECTS OF OP-1 IN IMMUNOSUPPRESSED MICE

Table 1. Changes in Body Weights and Survival Rates of Mice Inoculated with Bacteria after Administration of Cyclophosphamide Alone or in Combination with Obiopetide-1

Number of Bacxteria		Administration (Days) of OP-1 and Cyp				Days after inoculation							Percent survival
		-8	-7	-4	-1	0	1	2	3	4	6	10	(%)
P.aeruginosa(8×10^6)													
P-G-1	OP-1		-	-	-								
	Cyp		-	-	-								
	B.W. (g)	34.2							37.0			38.4	
	(change %)	(0)							(+8.2)			(+12.3)	
	Survival of mice					10	10	10	10	10	10	10	100
P-G-2	OP-1		-	-	-								
	Cyp		-	+	-								
	B.W. (g)	34.8							33.4		26.7		
	(change %)	(0)							(-4.0)		(-23.3)		
	Survival of mice					10	6	5	1	1	1	1	0
P-G-3	OP-1		+	-	+								
	Cyp		-	+	-								
	B.W. (g)	34.5							33.7			28.0	
	(change %)	(0)							(-4.0)			(-12.8)	
	Survival of mice					10	7	7	7	7	7	7	70
K.pneumoniae(6×10^6)													
K-G-1	OP-1		-	-	-								
	Cyp		-	-	-								
	B.W. (g)	34.2							37.0			38.4	
	(change %)	(0)							(+8.2)			(+12.3)	
	Survival of mice					10	10	10	10	10	10	10	100
K-G-2	OP-1		-	-	-								
	Cyp		-	+	-								
	B.W. (g)	34.6							32.6			27.1	
	(change %)	(0)							(-5.8)			(-20.8)	
	Survival of mice					10	8	6	1	1	1	1	10
K-G-3	OP-1		+	-	+								
	Cyp		-	+	-								
	B.W. (g)	34.5							33.0			26.8	
	(change %)	(0)							(-4.4)			(-22.3)	
	Survival of mice					10	10	8	7	6	6	6	60

Notes: Approximately 6-8x10^6 *P.aeruginosa* or *K.pneumoniae* were inoculated intraperitoneally (ip) into a mouse : Cyclophosphamide(Cyp, 250mg/kg) was injected ip, and Obiopeptide-1(OP-1,100 μg/mouse) was injected intramuscularly. G-1, 10 mice were not treated with OP-1 and Cyp; G-2, 10 mice were treated with Cyp alone 4 days before inoculation of bacteria; and G-3, 10 mice were treated with Cyp 4 days before inoculation of bacteria and with OP-1 7 and 1 days before inoculation of bacteria.

and OP-1. Only 6 of the 10 mice in this last group survived until 10 days ai.

Estimates were made of viable *P.aeruginosa* in organs of mice that were administered Cyp alone (G-Cyp) or Cyp in combination with OP-1 (G-Cyp+OP-1) (Table 2). Large numbers of bacteria were initially present in peritoneal exudates and peripheral blood of mice in group G-Cyp between 3 and 24 hrs ai and then tended to decrease. Bacteria showed a clear tendency to shift from the liver to the kidney between 3 and 48 hrs ai. Numbers of bacteria in group G-Cyp+OP-1 were substantially lower at 3 and 24 hrs ai than those in group G-Cyp. By 48 hrs ai, the most notable differences between the 2 groups in numbers of viable bacteria were in

Table 2. Average Number of Viable *P.aeruginosa* in the Organs of Mice that were Administered Cyclophosphamide Alone or in Combination with Obiopeptide-1

Hours after inoculation	Average numbers of viable organisms after inoculation							
	Peritoneal fluids	Peripheral blood	Liver	Spleen	Lungs	Heart	Kidney	Stomach
3 hrs								
G-Cyp*	$464x10^3$	8200	850	200	550	85	25	0
G-Cyp+OP-1**	$173x10^3$	4550	725	220	650	25	0	0
24 hrs								
G-Cyp	$680x10^3$	1365	0	305	105	945	1350	2
G-Cyp+OP-1	$143x10^3$	1210	0	10	25	800	1080	3
48 hrs								
G-Cyp	$146x10^3$	290	10	15	$160x10^3$	2875	$107x10^3$	3
G-Cyp+OP-1	10	0	2	0	1	0	1	0
216 hrs								
G-Cyp	0	0	65	40	0	0	20	215
G-Cyp+OP-1	0	0	35	8	0	0	7	0

Notes: All averages were calculated from 3 mice independently in each group.
*:G-Cyp, mice were treated with Cyclophosphamide(Cyp) alone 4 days before intraperitoneal inoculation with $2x10^6$ *P.aeruginosa*.
**:G-Cyp+OP-1, mice were treated with Cyp 4 days before inoculation of bacteria and with Obiopeptide-1(OP-1) 7 and 1 days before inoculation with *P.aeruginosa*.

peritoneal exudates, lungs, hearts and kidneys with OP-1 treated animals having significantly fewer bacteria.

When Cyp-treated and Cyp+OP-1 treated mice were inoculated ip with 5.5×10^6 *E.coli* or 5.4×10^9 *S.aureus*, survival rates were 50% and 60%, or 60% and 70%, respectively (Table 3). Mice receiving Cyp+OP-1 combinations had slightly higher rates of survival. However, rates of survival of groups treated with Cyp and Cyp+OP-1 were similar after treatment with different strains of MRSA. Survival was 20% and 20%, respectively, after administration of strains 2 and 3, 20% and 40% after administration of strains 4 and 5, and 20% and 0% after administration of strains 6 and 7.

***Protective effects of OP-1 on carrageenan pretreated mice against MRSA infection*:**

When untreated mice (G-1-1-1) were inoculated ip with 1.6×10^9 of the No.2 strain, 4 of 5 mice survived until 5 days ai (Table 4). Three of the 5 Carra-treated mice (G-1-1-2) survived until 5 days ai. All of the 5 mice that were treated with Carra+OP-1 (G-1-1-3) survived until 5 days ai.

After inoculation with 1.2×10^9 of the No.3 strain, one of 5 mice in G-2-2-2 survived, and 4 of 5 Carra+OP-1 treated mice (G-2-2-3) survived, respectively. When 5 Carra-treated mice were inoculated with $8x10^8$ of the No. 3 strain (G-2-3-2), 3 of 5 mice survived, and all of 5 Carra+OP-1 treated mice (G-2-3-3) survived until 5 days ai.

After inoculation of 1.1×10^{10} organisms of MRSA, one of 5 Carra-treated mice (G-3-2-2) survived, and 3 of 5 Carra+OP-1 treated mice (G-3-2-3) survived. When 5 Carra-treated mice were inoculated with 2.2×10^9 of the No. 4 strain (G-3-3-2), 3 of 5.

Table 3. Survival Rates of Mice Inoculated with *E.coli*, *S.aureus* and MRSA after Administration of Cyclophosphamide Alone or in Combination with Obiopeptide-1

Number of Bacteria	Number of Mice examined	Survival of mice after bacterial inoculation (Days)						Percent Survival
		0	1	2	3	4	5	(%)
E.coli(5.5×10^6CFU/mouse)								
Cyp*	10	10	8	5	5	5	5	50
Cyp+OP-1**	10	10	10	7	6	6	6	60
S.aureus(5.4×10^6CFU/mouse)								
Cyp	10	10	8	6	6	6	6	60
Cyp+OP-1	10	10	9	7	7	7	7	70
MRSA***								
No.2(521)(3.2×10^6CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	5	1	1	1	1	1	20
No.3(502)(7.5×10^6CFU/mouse)								
Cyp	5	5	3	1	1	1	1	20
Cyp+OP-1	5	5	3	1	1	1	1	20
No.4(520)(5.1×10^6CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	5	2	2	2	2	2	40
No.5(508)(3.2×10^6CFU/mouse)								
Cyp	5	5	3	1	1	1	1	20
Cyp+OP-1	5	5	4	2	2	2	2	40
No.6(504)(8.8×10^6CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	0	0	0	0	0	0	0
No.7(591)(6.5×10^6CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	0	0	0	0	0	0	0

*: Treated with Cyclophosphamide(Cyp) 4 days before bacterial inoculation.

**: Treated with Cyp in Combination with Obiopeptide-1(OP-1) 7 and 1 days before bacterial inoculation.

***: Methicillin Resistant *Staphylococcus aureus*.

mice survived, and all of the 5 Carra+OP-1 treated mice survived

When Carra-treated mice were inoculated with either 8×10^8 of the No.5 strain(G-4-2-2) or 1.4×10^9 of the No.6 strain (G-5-2-2), 3 of the 5 mice in the former group and none of the 5 mice in the latter group survived to 5 days ai. Among Carra+OP-1 treated mice in G-4-2-3 and G-5-2-3, all mice in the former and 4 of the 5 mice in the latter group survived.

When mice treated with either Carra alone (G-6-2-2 or G-6-3-2) or Carra+OP-1(G-6-2-3 or G-6-3-3) and inoculated with either 1.3×10^9 or 2.8×10^8 of the No.7 strain, one of the 5 in G-6-2-2 and G-6-3-2 survived until 5 days ai, while 2 of the 5 in G-6-2-3 and 3 of the 5 in G-6-3-3 survived until 5 days ai, respectively.

***Bacteriocidal activities of peritoneal cells*:**

Numbers of *P.aeruginosa* from peritoneal exudates of groups 1,2,3, and 4 were compared at 0.5, 1 and 3 hrs after bacterial inoculation (Table 5). The numbers of organisms in non-treated control mice from group 1 and OP-1 treated mice from group 2 were 269×10^4 and 148×10^4 at 0.5 hr, 121×10^4 and 18×10^4 at 1 hr ai. Numbers of mouse peritoneal cells at 5 hrs after ip injection of 0.5% glycogen solution were 20.3×10^2 in group 1 and 34.0×10^2 in group 2. A higher percentage of neutrophils was found in group 2.

EFFECTS OF OP-1 IN IMMUNOSUPPRESSED MICE

Table 4. Changes in Survival Rates of Mice Inoculated with *Staphylococcus aureus* (MRSA strains) after Administration of Carrageenan Alone or in Combination with Obiopeptide-1

No. of strains	Inoculated number of the strain	Administration of OP-1 and Carrageenan			Survival of mice after bacteria inoculation						No. of survival /no. examined (%)	Percent survival
		-7	-2	-1(days)	0	1	2	3	4	5		
No.2(521)												
G-1-1-1	1.6×10^{9}											
	OP-1	-	-	-	5	4	4	4	4	4	4/5	80
	Carra	-	-	-								
G-1-1-2	1.6×10^{9}											
	OP-1	-	-	-	5	3	3	3	3	3	3/5	60
	Carra	-	+	-								
G-1-1-3	1.6×10^{9}											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.3 (502)												
G-2-2-2	1.2×10^{9}											
	OP-1	-	-	-	5	5	3	3	3	1	1/5	20
	Carra	-	+	-								
G-2-2-3	1.2×10^{9}											
	OP-1	+	-	+	5	5	4	4	4	4	4/5	80
	Carra	-	+	-								
G-2-3-2	8.0×10^{8}											
	OP-1	-	-	-	5	4	4	3	3	3	3/5	60
	Carra	-	+	-								
G-2-3-3	8.0×10^{8}											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.4(520)												
G-3-2-2	1.1×10^{10}											
	OP-1	-	-	-	5	4	1	1	1	1	1/5	20
	Carra	-	+	-								
G-3-2-3	1.1×10^{10}											
	OP-1	+	-	+	5	3	3	3	3	3	3/5	60
	Carra	-	+	-								
G-3-3-2	2.2×10^{9}											
	OP-1	-	-	-	5	3	3	3	3	3	3/5	60
	Carra	-	+	-								
G-3-3-3	2.2×10^{9}											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.5(508)												
G-4-2-2	8.0×10^{8}											
	OP-1	-	-	-	5	5	5	5	4	3	3/5	60
	Carra	-	+	-								
G-4-2-3	8.0×10^{8}											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.6(504)												
G-5-2-2	1.4×10^{9}											
	OP-1	-	-	-	5	4	3	1	0	0	0/5	0
	Carra	-	+	-								
G-5-2-3	1.4×10^{9}											
	OP-1	+	-	+	5	4	4	4	4	4	4/5	80
	Carra	-	+	-								
No.7(591)												
G-6-2-2	1.4×10^{9}											
	OP-1	-	-	-	5	1	1	1	1	1	1/5	20
	Carra	-	+	-								
G-6-2-3	1.4×10^{9}											
	OP-1	+	-	+	5	2	2	2	2	2	2/5	40
	Carra	-	+	-								
G-6-3-2	2.8×10^{8}											
	OP-1	-	-	-	5	2	2	1	1	1	1/5	20
	Carra	-	+	-								
G-6-3-3	2.8×10^{8}											
	OP-1	+	-	+	5	5	4	4	3	3	3/5	60
	Carrag	-	+	-								

Six strains (No.2-No.7) of *Staphylococcus aureus* were isolated clinically as the MRSA from the human patients at the Police hospital, Tokyo. Approximately 2.8×10^{8}-1×10^{10} MRSA were inoculated ip into each mouse. Carrageenan (Carra.,60mg/kg as 6 mg/ml of saline solution) was injected ip 2 days, and Obiopeptide-1(OP-1,100μg/mouse) was injected im 7 days and 1 day before bacterial inoculation, respectively.

Table 5. Bacteriocidal Activities of Peritoneal Cells Collected from Mice Injected with 0.5% Glycogen Solution 5 hours before Bacterial Inoculation.

Group	Administration (Days) of OP-1 and Cyp			Average number($1x10^4$) of viable organisms in peritoneal exudates after inoculation			Number of peritoneal cells	Differentiation of peritoneal exudate cells just before inoculation				
	-7	-4	-1	0.5	1	3 (hrs)	($x10^3$)	Eos.	Neut.	Baso.	Lymph.	Mono.(%)
Group 1												
OP-1	-	-	-	269±100	121±40		20.3±9.7	1.7±1.2	24.7±15.1	0	72.0±14.0	2.3±0.9
Cyp	-	-	-									
Group 2												
OP-1	+	-	+	148±48	18±3		34.0±5.0	1.7±1.7	54.3±23.5	0	43.3±24.4	0.7±0.9
Cyp	-	-	-									
Group 3												
OP-1	-	-	-			46.6±12	8.0±2.0	0	2.5±0.5	0	97.5±0.5	0
Cyp	-	+	-									
Group 4												
OP-1	+	-	+			17.3±5	7.6±2.0	0	11.4±7.5	0	88.5±7.5	0
Cyp	-	+	-									
Significant difference between Group 1 and 2				P<0.001	P<0.001							
Significant difference between Group 3 and 4						P<0.05						

Note: Mean± SD values were calculated from 10 mice in Group 1 and 2. Each mouse was injected intraperitoneally with 2.0 ml of 0.5% glycogen solution 5 hours before inoculation with *P.aeruginosa*. Collection of peritpneal exudates was done by injection of 5 ml of Hank's balanced salt solution.

Table 6. Bacteriocidal Activities of Peritoneal Cells Collected from Mice Injected with 0.2% Glycogen Solution 5 days before Bacterial Inoculation.

Group	Administration (Days) of OP-1 and Cyp			Average number($1x10^4$) of viable organisms in peritoneal exudates after inoculation			Number of peritoneal cells	Differentiation of peritoneal exudate cells just before inoculation				
	-7	-4	-1	1	4	24(hrs)	($x10^3$)	Eos.	Neut.	Baso.	Lymph.	Mono.(%)
Group 1												
OP-1	-	-	-	1072±115	468±176		20.0±11.0	0.3±0.5	0	0	95.0±2.9	4.7±2.6
Cyp	-	-	-									
Group 2												
OP-1	+	-	+	538±213	323±153		37.0±23.7	1.0±1.4	15.3±17.4	0	80.0±19.1	3.7±1.2
Cyp	-	-	-									
Group 3												
OP-1	-	-	-			68±56	9.5±5.0	0.3±0.8	1.8±4.3	0	95.8±2.4	2.5±1.1
Cyp	-	+	-									
Group 4												
OP-1	+	-	+			14±12	10.5±3.9	0.5±0.5	4.0±1.4	0	93.5±2.9	2.2±2.3
Cyp	-	+	-									
Significant difference between Group 1 and 2				P<0.002	P<0.5							
Significant difference between Group 3 and 4						P<0.5						

Note:Mean± SD values were calculated from 10 mice in Group 1 and 2. Each mouse was injected intraperitoneally with 2.0 ml of 0.2% glycogen solution 5 days before inoculation with *P.aeruginosa*. Collection of peritoneal exudates was done by injection of 5 ml of Hank's balanced salt solution.

Numbers of *P.aeruginosa* from peritoneal exudates of Cyp-treated mice from group 3, and Cyp+OP-1 treated mice from group 4 were compared at 3 hrs ai. Numbers of organisms were 46.4×10^4 in group 3 and 17.3×10^4 in group 4 at 3 hrs ai. Numbers of neutrophils from the exudate cells in group 4 were larger than those in group 3. Numbers of viable bacteria in peritoneal exudates from OP-1 treated mice in groups 2 and 4 were significantly lower than those in groups 1 and 3 at 0.5, 1 and 3 hrs ai.

Numbers of bacteria in peritoneal exudates in mice injected with 0.2 % glycogen

71

solution 5 days before inoculation is presented in Table 6. Numbers of organisms in non-treated mice (group 1) and OP-1 treated mice (group 2) were 1072×10^4 and 538×10^4 at 1 hr, and 468×10^4 and 323×10^4 at 4 hrs ai, respectively. Numbers of organisms in Cyp-treated mice (group 3) and Cyp+OP-1 treated mice (group 4) were 68×10^4 and 14×10^4 at 24 hrs ai. Numbers of peritoneal exudate cells and neutrophils in OP-1 treated mice in groups 2 and 4 were larger than those from non-OP-1 treated groups 1 and 3.

DISCUSSION

Yokota (1984) and Ogata (1983) reported a correlation between increased susceptibility to opportunistic pathogens and reduced leukocytes counts in mice that were administered Cyp or Carra. In these experiments, marked reduction of body weight and total numbers of spleen mononuclear cells were not noted until 4 to 6 days after administration of a single 250 mg/kg dose of Cyp. These values recovered in time to normal values or values that were higher than normal. Most mice that were administered Cyp died within 5 days after they were inoculated with bacteria. Accordingly, the authors suggested that reduced resistance to infection may be related mainly to a reduction of leukocytes and macrophages.

Obiopeptide (Suzuki et al., 1990) has an activity as an immunomodulator and is capable of increasing resistance to the growth of tumor cells (Sakurai et al., 1991). In the present study, doses of 100 µg /mouse were capable of increasing resistance to bacterial infection, especially against *P.aeruginosa* and *K.pneumoniae*, and reducing mortality. This was demonstrated by the almost complete elimination of live bacteria from organs of mice that were administered Cyp and OP-1 within 2 days ai. It is still not known whether the antibacterial mechanism of OP-1 stems from increases in the activity of macrophages and neutrophils. Nevertheless, increases in the numbers and the functional activation of the cells in OP-1 treated mice is important evidence suggesting that the hosts showed a strong resistance against bacterial infection.

In recent years, immunosuppressive agents such as Cyp have been used routinely worldwide after organ transplantation or for leukemia therapy and treatment of some tumors. Agents that can prevent opportunistic infections in immunosuppressed individuals are urgently needed. Results of our study demonstrate that OP-1 is capable of moderating opportunistic bacterial infections in immunosuppressed animals and has potential as a nontoxic immunomodulator. Further studies on the mode of action of Obiopeptide and application of this immunomodulator in conjunction with antibiotics and other chemotherapeutic agents may make it possible to develop treatments for infectious diseases that are currently intractable.

REFERENCES

Eschenfeld W.H. Marrow, R.E. Krug, M.S. and Berger L.S. 1989. Isolation and partial sequencing of human Prothymosin alpha gene family. *J.Biol. Chemist.* 264:7546-7555.

Fanganou-Lazarides,M.,Clinton, M., Goodall, G.J. and Horecker, B.L. 1988. Prothymosin alpha and Parathymosin: amino acide sequences deduced from cloned rat spleen cDNAs. *Arch. Biochem. Biophy.* 263:305-310.

Fujii, Y., Maki, Y., Sakurai, H., Igarashi,I., Omata, Y., Saito, A. and Suzuki, N. 1992.

Growth inhibitory effect of a newly synthesized peptide, Obiopeptide-1, on mice bearing methylcholanthrene induced murine tumors. *Jpn.J.Vet. Sci.* 54: 351-353.

Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J.G., Bustelo, X.R., and Freire, M. 1989. The expression of Prothymosin alpha gene in T-lymphocytes and leukemic lymphoide cells is tied to lymphocyte proliferation. *J.Biol.Chem.* 264:8451-8454

Hugin, A.W., Cerny, A., Wran, M., Hengarter, H. and Zinkernagel, R.M. 1986. Effect of cyclosporin A on immunity to *Listeria monocytogenes. Infect. Immunit.* 52: 12-17.

Kouda,M., Kobayashi, J., Kumagai, I., Uemura, C., and Mathuzaki, H. 1987. Detection rate of *Staphylococcus aureus* isolated from blood cultures and evaluation of in vitro activity of various antibiotics against methicillin-resistant *S. aureus* isolated from blood cultures. *J.Jpn.Assoc. Infect. Dis.* 61:1230-1238 (in Japanese).

MaCabe, A.E., Remington, J.S., and Araujo, F.G. 1985. In vivo and in vitro effects of cyclosporin A on *Trypanosoma cruzi. Am. J. Trop. Med. Hyg.* 34: 861-865.

Nozawa, T. 1986. Phagocyte cells and defense system against microbiology. *Jpn J. Bact.* 41: 783-795 (in Japanese).

Ogata, N., 1983. Analysis of protective mechanisms against infection by *Pseudomonas aeruginosa. Med. J. Fukuoka* 74: 335-350.

Saegusa,J., Ueda, K. and Fujiwara, K, 1979. Fatal infection with *Pseudomonas aeruginosa* in mice treated with cyclophosphamide and *Propionibacterium acnes. Exp. Anim.* 28: 61-64.

Sakurai, H., Fujii, Y., Maki, Y., Igarashi,I., Omata, Y., Saito, A., Ono,K., and Suzuki, N. 1991. In Vitro and In Vivo studies of growth inhibitory effect of a newly synthesized peptide, Obiopeptide-1, on mice-bearing methycholanthrene-induced murine tumors. *J.Vet. Med. Sci.* 53: 823-831.

Suzuki, N., Izumo,A., Sakurai, H., Saito, A., Miura, H., and Osaki, H. 1984. Toxoplasmacidal activity of Obioactin derived from hydrolyzed Toxoplasma immune bovine serum in heterologous cell culture. *Zbl. Bakt. Hyg.A.* 256: 356-366.

Suzuki, N. 1987. Obioactin as a native immunoregulatory factor in the biological response modifiers.pp 76-90. *In*: Progress in Veterinary Science 1987. Izawa,H. & Shimizu, Y. (Eds), Kindai Shuppan, Tokyo (in Japanese).

Suzuki, N., Sakurai, H., Saito, A., Igarashi,I., Omata, Y., and Osaki,H. 1990. Biological activity of Obiopeptide-1, a synthetic peptide derived from the native immune-regulator Obioactin. *Jpn.J.Vet.Sci.* 52: 907-914.

Suzuki, N., Fujii, Y., Maki, Y., Sakurai, H., Igarashi, I. and Saito, A. 1990. In vitro cytocidal effect of novel synthetic Obiopeptides 1,2, and 3 on *Toxoplasma gondii. Allergy & Immunol.* 9: 159-158.

Takashima, T. and Collins, F.M.1987. Immunosuppressive effect of cyclosporin A on *Mycobacterium bovis* infections in mice. *Infect. Immunt.* 55: 1701-1706.

Urano, T. and Maejima,K. 1978. Provocation of pseudomoniasis with cyclophosphamide in mice. *Lab. Animals* 12:159-161

Watts, J.D., Cary, P.D. and Crane-Robinson, C. 1989. Prothymosin alpha is a nuclear protein, *FEBS letter* 245:17-20.

Yokota, Y. 1984. Host defense mechanism in experimentally immunocompromised animals and restorative effect of immunopotentiators. *Jpn. J. Bact.* 39: 29-46 (in Japanese).

J. Protozool. Res., 2, 112-122 (1992)

1-13

Anti-*Babesia rodhaini* Monoclonal Antibodies: Effect against *Babesia rodhaini* and Cross-reactivity with Human Starin of *Babesia microti*

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ABSTRACT

Anti-*Babesia rodhaini* monoclonal antibodies(mAb), namely: 1-E7, 2-H2 and 3-B8, significantly suppressed the development of high parasitemia in BALB/c mice infected with *B.rodhaini* and all mAb-treated mice survived the infection. While, only monoclonal antibody 3-B8 showed some inhibitory effect against *Babesia microti*, with mice showing high parasitemia of 18.04 ± 2.69 %, at 9 days post-exposure. Westernblot analysis of *B. rodhaini* and *B. microti* parasite extract reacted with anti-*B. rodhaini* monoclonal antibodies showed cross-reactive bands of molecular weights 62 and 55 kilodaltons. Comparison of the antigenic components of *B. rodhaini* and *B. microti* using polyspecific sera revealed several shared or common parasite antigens of molecular weights 62, 55, 45-47, 41, 30-31 and 26-28 kilodaltons.

INTRODUCTION

Several studies have documented protection against *Babesia* spp. either by passive transfer of immune serum (Kurtzuhals et al., 1988; Ishimine et al., 1979; Bautista and Kreier, 1979; Abdahlla et al., 1978; Rogers 1974; Philips 1969; Mahoney 1967), or by adaptive transfer of splenocytes and lymph node cells from immunized mice (Meeusen et al., 1984a, 1984b; Reubush and Hanson 1980), or by passive transfer of immune spleen cells and injection of non-viable antigenic materials to recipient mice (Meeusen et al., 1985; Smith and Ristic 1981). Although, some workers are convinced of the role of non-specific factors particularly in cases related to cross-protection induced by heterologous agents (Zivkovic et al., 1983/1984; Clark et al., 1976, 1977; Cox and Turner 1970), voluminous data from previous studies have provided strong evidence to suggest the principal involvement of antibodies (Ab) in host protection against babesiosis.

Until recently, in most vaccine-related research such as that of Tetzlaff et

74

al. (1990) on *Babesia microti* using polyspecific sera, and those of Figueroa et al. (1989), Wright et al. (1985, 1983), Smith et al. (1979) on bovine babesiosis, greater emphasis has been directed towards the immunochemical analysis of *Babesia* antigens (Ag) and their capacity to induce protective immunity. In this paper, we report our findings using monoclonal antibodies against two protective *B. rodhaini* Ag and their cross-reactivity with the human isolate of *B. microti.*

MATERIALS AND METHODS

Experimental animals and parasites: Six to eight wks old BALB/c mice were used in all experiments and were reared and kept in our animal facility. *Babesia rodhaini* and *Babesia microti* were maintained in BALB/c mice and both parasite species were syringe-passaged through intraperitoneal (ip) inoculation of infected blood in heparin and phosphate-buffered saline (PBS). *B. microti* was generously supplied by Dr. Heydorn of the Institute for Parasitology and Tropical Veterinary Medicine, The Free University of Berlin. This *B.microti* was originally isolated from a human case of babesiosis and was subsequently adapted to mice.

Hybridoma and monoclonal antibody screening: BALB/c mice were primed by ip inoculation of $1x10^4$ *B.rodhaini* parasitized red blood cells (PRBC). One wk after exposure, infected mice were given three intramuscular (IM) injections of 100 µl Ganaseg (5mg/ml, Squibb & Sons Inc., Argentina) per injection, spaced out in one wk. Mice that survived the treatment regimen were inoculated with a booster dose of $1x10^4$ PRBC. Cell hybridization protocol follows essentially that described by Oi and Herzenberg (1979). Three days after the boost, immune spleen cells were fused with P3Ns1-1, a BALB/c myeloma cell line, using 50 % polyethylene glycol 1500 in 75 mM Hepes (Boehringer Mannheim GmbH, Germany). Cell fusion suspension in 2 % hypoxanthine aminopterin thymidine (HAT) + 10 % fetal calf serum (FCS) + Cellgrosser culture medium (Meguro Laboratories, Osaka, Japan) was plated into five 96-wells plates, and was kept in a CO_2-supplied incubator at 37 ℃. Wells that showed healthy growth of cell colonies were checked for antibody (Ab) production using indirect fluorescence antibody test (IFAT). Positive colonies were subcultured and were screened for clones that produced Ab specific against the parasite and/or against PRBC membrane. Hybridomas that produced relevant Ab were expanded in 24-wells trays, and were maintained in Freund's incomplete adjuvant (FIA) and/or pristane primed BALB/c mice. Ascitic fluid obtained from mice showing ascitic tumors was examined for mAb with IFAT. Of the 13 relevant clones inoculated into mice, only three clones got established, namely; mAb 1-E7, 2-H2 and 3-B8. Monoclonal Ab were precipitated from ascitic fluid diluted with PBS (v/v 50:50) using 50 % ammonium sulfate, and were dialized in three changes of PBS at 4 ℃. Protein concentration was determined with the double wavelength beam spectrophotometer (Hitachi, Tokyo, Japan). Precipitated mAb were stored at -70 ℃ until use. Isotyping of mAb was done using the mouse mAb isotyping kit (Amersham, International pLc, UK).

Indirect fluorescence antibody test (IFAT):

A. Fixed parasites and PRBC: Infected blood diluted in PBS was applied on wells and fixed in methanol, and then was incubated with titrated (4^1-4^{12}) hybridoma culture-supernatant, ascitic fluid, precipitated mAb or polyspecific Ab (normal and *Babesia* immune mouse serum) at 37 ℃ for 30 min. After three washings of PBS and once with distilled water, the wells were air-dried and then incubated with fluorescein isothiocyanate(FITC) conjugated anti-mouse IgG (Tago Inc., Berlingame, CA) for 30 min. The slides were washed three times with PBS and mounted in 90 % glycerol-PBS.

B.Live parasites and PRBC: Infected blood obtained through cardiac puncture using heparinized syringe was transferred into microcentrifuge tubes, washed once with citrate buffer (pH 7.0), and three times with PBS by centrifugation at 5,000 rpm, 10 min/wash. To the tubes, mAb (1:10) or polyspecific Ab (1:100) in PBS were added and incubated for 30 min in a 37 C waterbath with constant gentle shaking. After three washings with PBS, the cell pellets were incubated with FITC conjugated anti-mouse IgG, as described above. To remove excess FITC conjugated anti-mouse IgG, the cells were washed three times with PBS, resuspended in 90 % glycerol-PBS, and then mounted on glass slides. Both fixed and unfixed preparations were examined with a Nikon fluorescence microscope.

Sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE): Parasite Ag mixed with an equal amount of sample buffer (2.5 M Tris-HCl, pH 6.8, 4.6 % SDS, 10 % glycerol, 10 % 2 mercaptoethanol, 0.05 % bromophenol-blue) was heated over boiling water for 5 min and kept in a 37 ℃ water bath with shaking for another 5 min. The sample was ultrasonicated at 70 W, 3 x 10 s each time and centrifuged for 5 min at 15,000 rpm. Per well, 10-15 µl of the soluble parasite extract obtained was loaded on a standard 10 % acrylamide gel. Fractionated proteins were immunoblotted and/or stained with Coomassie blue. Low molecular weight markers used were purchased from BIO-RAD Laboratories, Richmond, CA.

Immunoblotting: Electrophoretic transblotting of fractionated proteins or Ag into nitrocellulose paper (0.22 µm pore size, GVHP, Nippon Millipore Kogyo, Yonezawa, Japan) followed essentially that of Towbin et al. (1979). Transblotted nitrocellulose sheets were stained with 5 % amidoblack black, destained with 10 % acetate and washed three times with PBS-0.05 % Tween 20 (PBS-T20), 10 min/wash. To remove nonspecific reactants, the membrane sheets were washed with 10 % methanol for 10 min, followed by three washings in PBS-T20, and reacted with the different test Ab (normal and immune mouse serum) and mAb. Polyspecific Ab and mAb were diluted 1:100 in 5 % skim milk (Difco Co., Michigan, USA), respectively. Reaction time with mAb lasted 2 - 6 h, about half the time at room temperature (RT) with constant gentle shaking, and the remaining time at 4 ℃. The membranes were washed three times with PBS-T20, and incubated with 1.5 % peroxidase-conjugated anti-mouse IgG (BIO-RAD Laboratories, Richmond, CA), in 5 % skim milk, for 1 h at RT, with constant gentle shaking. After 3 washings in PBS-T20, the membranes were reacted with freshly prepared substrate

consisting of 0.02 % 3,3-diaminobenzidine-4HCl and 15 µl H_2O_2 in 0.1 M Tris-HCl (pH 7.4).

Babesia rodhaini and Babesia microti parasite extract preparation: Blood was drawn out from infected mice through cardiac puncture using heparinized syringe with PBS and Alsever solution. Parasite Ag were processed following the procedure described by Sugimoto et al. (1991) with some modifications, Blood was overlaid on Ficoll-Conray solution (1:1 ratio) and centrifuged at 1,440 rpm for 20 min. The cell pellets were washed three times in 10 mM Tris-HCl (pH 7.5) - 150 mM NaCl buffer at 2,200 rpm, 10 min/wash. Cells were hemolyzed using 27 units hemolysin (Asao et al., 1984; Kozaki et al., 1987) per ml of Tris- NaCl buffer, generously provided by Dr. Sugimoto of the National Institute of Animal Health, Tsukuba, Japan. Hemolysin-treated PRBC were incubated for 2 h in a 37 ℃ waterbath with occasional shaking. The level of hemolysis was checked after 1 h of incubation using a hemocytometer. To obtain maximum hemolysis, incubation was allowed to continue longer depending on the level of parasitemia obtained from blood, with the addition of 13 - 17 hemolysin units/ml. The lysate was kept in ice-cold water for about 30 min, and ethylenediamine tetraacetic acid (EDTA) solution (pH 7.5) was added to make 5 mM final lysate concentration. Five ml of lysate/tube was overlaid on a 10 ml 60 % and 40 % Percoll discontinuous density gradient (Pharmacia LKB Biotechnology, Uppsala, Sweden) prepared with Tris-(HCl)-NaCl-EDTA buffer (pH 7.4), and centrifuged at 20,000 rpm for 20 min. Parasites obtained from the visible interphase band between 60 % and 40 % Percoll solution were washed with Tris-NaCl-EDTA buffer 3 times at 10,000 rpm, first wash for 10 min and the last two washings for 5 min/wash. To the parasite suspension, an equal amount of lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5 % Triton X-100, Noninet P-40 and 1 mM PMSF was added and kept at -70 ℃ until use.

Purification of mAb: Monoclonal Ab were purified from ascitic fluid by ion-exchange chromatography using DEAE Toyo Pearl 650M (Toso, Tokyo, Japan) in 50 mM Tris-HCl buffer (pH 7.5) with gradient concentration of 0 - 400 mM NaCl. The eluted mAb in the fractions were detected with IFAT and the protein concentration was determined using a double wavelength beam spectrophotometer.

Immunoaffinity purification of Ag :Immunoabsorbent column was prepared using 30 mg of purified mAb covalently bound to 300 mg TSK gel Tresyl-5PW (Toso, Tokyo, Japan) in 1 M phosphate buffer (PB), pH 7.5, at 4 C for 16 h with gentle shaking. The remaining binding sites were blocked by incubation with 0.02 M Tris-HCl buffer (pH 8.0) for 1 h at RT, and then washed with 0.1 M PB (pH 7.4). Ultrasonicated parasite extract that was passed through the affinity column was extensively washed with 0.1 M PB (pH 7.4), and the bound Ag were eluted with 0.1 M citrate-HCl buffer (pH 1.6). Eluted fractions were neutralized immediately with 1 M Tris solution and were concentrated by ultrafiltration, and then stored at -70 ℃.

Passive transfer of immunity :Tubes containing 300 µl each of the precipitated mAb 1-E7 (1.0 mg),2-H2 (2.7 mg) or 3-B8 (3.84 mg) were

717

incubated with $3x10^4$· *B. rodhaini* PRBC suspended in 1.2 ml of PBS and 10.8 ml of 20 % FCS-Eagle's minimum essential medium (MEM) for 1 h in a CO_2-supplied incubator at 37°C. The cell suspensions were centrifuged for 20 min at 2,000 rpm, and the supernatant was aspirated leaving 1 ml of the content to which 2 ml of PBS was added. In the experimental groups (3 mice per group), each mouse was ip-inoculated with 1 ml of mAb-treated PRBC. Control groups, likewise, consisted of 3 mice per group, each mouse was ip-injected with incubated PRBC without mAb (control group A). For control B, each mouse was injected with the same dose of PRBC which were neither incubated nor treated with mAb. Each mouse in the experimental groups was ip-injected 0.2 ml mAb suspension containing mAb titers of 0.67 mg, 1.8 mg and 2.56 mg for 1-E7,2-H2 and 3-B8, respectively. Preparation of *B. microti* inoculum was prepared similar to that described for *B. rodhaini.* Each mouse was inoculated with $18x10^6$ PRBC. Experimental groups mAb 1-E7, 2-H2 and 3-B8 were injected daily mAb titers of 1.26 mg, 2.48 mg and 3.32 mg, respectively. Thin blood smears were prepared at 2 - 3 day interval. Percent parasitemia was calculated by counting the number of PRBC out of 500 - 1,000 (depending on the level of parasitemia) total red blood cells covering various microscopic fields.

RESULTS AND DISCUSSION

Monoclonal antibodies 1-E7, 2-H2 and 3-B8 were of the isotype IgG2b.With fixed parasite material, we observed strong fluorescence on PRBC membrane and against both extracellulay- and intracellulary-located parasites. With live material fluorescence was likewise, noted on PRBC membrane, and against extracellular parasites only.

Mice exposed to *B.rodhaini* parasite incubated with precipitated mAb preparation and administered daily injections of mAb exhibited significantly lower percent parasitemia ($p< 0.05$) starting the 5th day post-exposure (PE), compared to the control groups (Fig. 1A). At 19 day PE, percent parasitemia of mAb-treated groups were 15.0 ± 0.86% (1-E7), 12.95 ± 4.9% (2-H2) and 13.32 ± 7.15% (3-B8) respectively. After the last injection, however, parasitemia increased in all mAb-treated groups, and at 38 day PE, registered peak percent parasitemia of 65.0 ± 17.2% (1-E7), 69.6 ± 16.5% (2-H2) and 46.0 ± 8.1% (3-B8). Afterwards parasitemia decreased in all experimental groups and ranged from 6-21 % at 43 - 48 day PE, and all mAb-treated mice survived the infection. While all control mice died between 27 and 30 day PE with peak percent parasitemia ranging from 87.2 - 93.7 %.

Fig. 1B shows the effect of anti-*B. rodhaini* mAb on *B.microti* infection. The first 5 day after exposure showed no significant difference between mAb-treated and control groups. At 7 day PE, however, percent parasitemia for mAb 1-E7- and 3-B8-treated mice were 3.92 ± 3.23 % and 3.69 ± 2.0%, respectively. Those of mAb 2-H2-treated mice and the control groups A and B were slightly higher at 13.7 ± 3.45%, 19.34 ± 10.65% and 12.96 ± 9.47%, respectively. Days following the 7th day PE, parasitemia in all groups

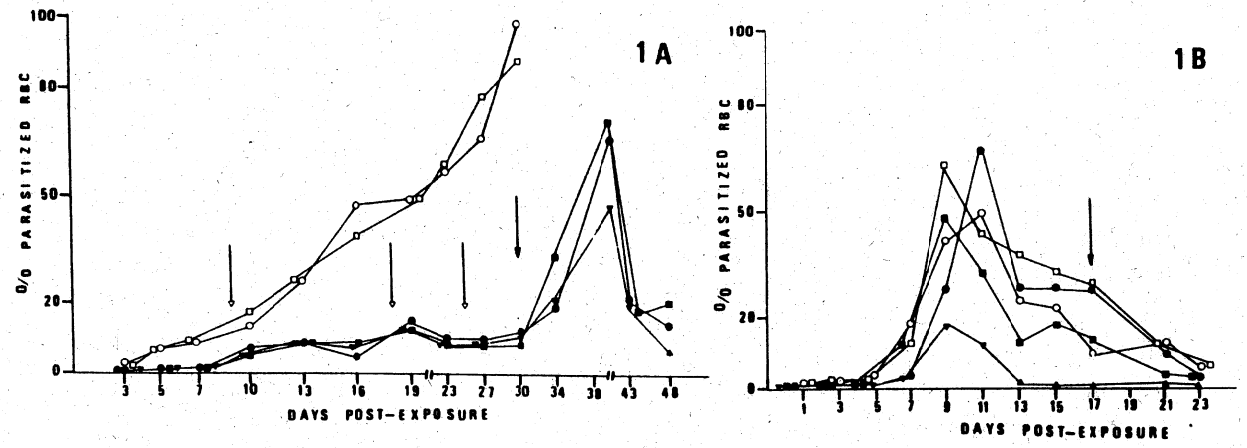

Figure 1. Passive transfer of immunity using anti-*Babesia rodhaini* mAb against *B. rodhaini* parasite (1A) and against *Babesia microti* parasite (1B). Per experimental group consisted of three mice, each mouse was exposed to mAb-incubated parasitized red blood cells (PRBC) and was injected daily with mAb 1-E7 (●), mAb 2-H2 (■), or mAb 3-B8 (▲). Inoculation dose: *B.rodhaini* PRBC 1 x 10^4 ; *B. microti* PRBC 18 x 10^6. Control group A (○) exposed to the same inoculum dose of incubated, non-mAb-treated PRBC. Control group B (□) exposed to the same inoculum dose of non-incubated, non-mAb-treated PRBC. Last day of mAb injection (↓); no mAb administration (⇣). Each points represents the mean value per three mice.

increased significantly, except for mAb 3-B8-treated mice which showed peak parasitemia of 18.01 ± 2.69 % at 9 day PE, and was kept at levels less than 0.5 % from the 13th to the 23rd day PE. In contrast, peak parasitemia of mAb 1-E7- and 2-H2-treated groups were 66.47 ± 15.42%, 48.78 ± 8.95%, and those of controls A and B were 48.97 ± 27.5% and 61.69 ± 28.24%, respectively. All control and experimental mice survived the infection.

Results of westernblot analysis showed cross reactive Ag between *B. rodhaini* and *B. microti.* As shown in Fig. 2 (lanes B), parasites extracts of *B. microti* and *B. rodhaini* reacted with *B. rodhaini* hyperimmune serum revealed common bands of M.W 66, 62, 55, 45 - 47 and 30 - 31 kilodaltons (kDa). Reaction with *B. microti* polyspecific Ab showed similar bands as indicated above, in addition to two other bands of M.W 26 - 28 kDa. Another parasite Ag of M.W 72 kDa was apparent in the *B. rodhaini* lanes which was detected with both *B. rodhaini* and *B. microti* immune sera.

Likewise, westernblot analysis (Fig. 2) showed mAb 1-E7 and 2-H2 reactive with the 62 and 55 kDa *B. microti* parasite components. With mAb 3-B8, we noted a weak band of M.W 62 kDa. With *B. microti* parasite extract, similar bands were detected by all three mAb. A fine band M.W 72 kDa was noted. This band seemed to be a non-specific reactant detected by certain Ab presumably present in crude mAb preparation obtained from ascitic fluid, since no such band was detected when immunoaffinity purified Ag was reacted with purified mAb.

Repeated westernblot analysis consistently yielded weak 62 and 55 kDa bands. We tried to verify our findings by reacting purified mAb with parasite Ag obtained through immunoaffinity chromatography. As shown in Fig. 3, parasite Ag collected during the first run and second run of immunopurified

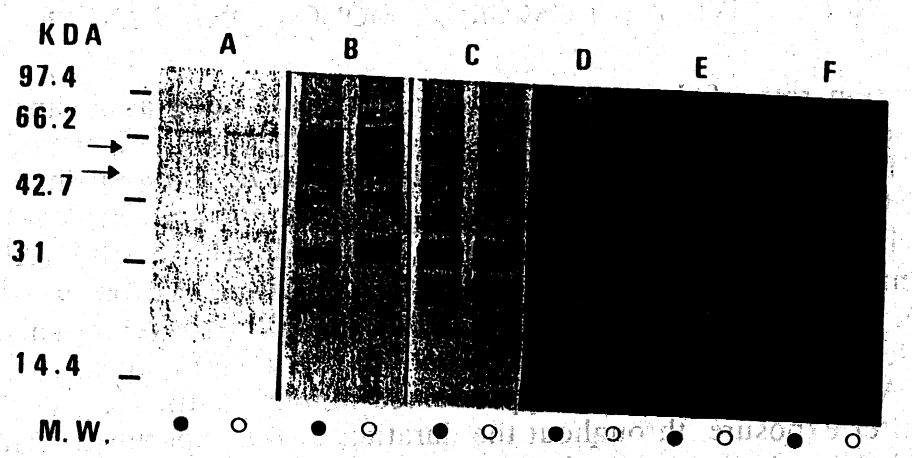

Figure 2. Westernblot analysis of *B. rodhaini* (○) and *B.microti* (●)parasite extract reacted with normal serum (A), *B. rodhaini* hyperimmune serum (B), *B. microti* immune serum (C), mAb 1-E7 (D), mAb 2-H2 (E), mAb 3-B8 (F). Kilodaltons (kDa) : Molecular weight (M.W). Arrows indicate the 62 and 55 kDa parasite antigens reactive with mAb.

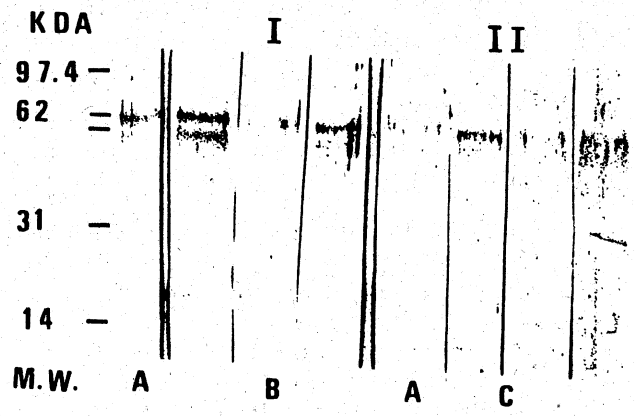

Figure 3. Westernblot analysis using immunoaffinity-purified *B. rodhaini* antigens. Crude parasite extract (lane A). Purified antigens of the first cycle (Group I, lane B) and second cycle (Group II, lane C) reacted with mAb 1-E7 and mAb 2-H2, respectively.

B. rodhaini extract using a mixture of mAb 1-E7 and 2-H2, both yielded two distinct bands of M.W 62 and 55 kDa. when reacted with mAb 1-E7 and 2-H2.

Although, the establishment of *B. rodhaini* infection in mice injected with mAb was not totally suppressed, the significantly lower percent parasitemia suggests some protective or inhibitory effect of mAb against the parasite. The daily injection of mAb seemed to have augmented and helped sustained the level of these specific protective Ab against the parasite, in addition to all other protective Ab in circulation. This may effect a slowing down in the

80

penetration rate of the parasite inside red blood cells as gleaned from an increased parasitemia, a few days after the last injection, and on certain day of mAb (Fig. 1a). Abdalla et al. (1978), noted that *B. rodhaini* immune serum delayed the onset of parasitemia, but it neither prevented the development of injection nor protected the mice from death, even with further supplementation of immune serum during infection. We observed a similar pattern of the progress of infection, however, all mAb-treated mice outlived the infection (Fig. 1a). While, we administered a daily injection of mAb to mice, Abdalla et al. (1978) supplemented immune serum two to three times only after exposure, throughout the duration of the experiment. The survival of mAb-treated mice infected with the usually fetal *B. rodhaini* noted by us is interesting. Thoongsuwan and Cox (1973) had recovered three antigenic strains of *B. rodhaini* from the virulent parent strain by means of in vitro treatment with immune globulin. They alluded the loss of virulence of the recovered strains to loss of antigenicity (i.e. either loss or decreased immunogenicity), rather than to selection of a variant with antigen(s) that differed from that of the parent strain. Timms et al. (1990), likewise, attributed the appearance and loss of *Babesia bovis* virulence to the mixture of subpopulations of varied virulence consisting a parent strain. They, too, maintained that the mechanisms of differential gene expression and phenotypic selection of subpopulations have to operate for virulence in *B. bovis* to be altered. In the present study, whether the loss of virulence in mAb-treated mice resulted from the loss or diminished immunogenicity as a consequence of mAb-treatment (Thoongsuwan and Cox 1973) or from the combined mechanisms of differential gene expression and phenotypic selection (Timms et al., 1990), and/or from continuous passage of the parasite in mice for a long time (Howard et al., 1980) is an interesting question that warrants further investigation.

Against *B. microti*, only mAb-3-B8 showed some protective activity to mice, although , less significantly compared to its effect against *B. rodhaini.* The absence of inhibitory effect of anti-B. rodhaini mAb-1-E7 and 2-H2 against *B. microti*, seemingly suggests a difference in the antigenic determinants or epitopes of both the 62 and 55 kDa between these species. Thus, these mAb may be alluded to, as non-protective against *B. microti.* Likewise, our data suggest common epitope for the M.W 62 kDa Ag recognized by mAb-3-B8 in both species.

Westernblot analysis of both *B.rodhaini* and *B.microti* parasite extracts revealed shared Ag. The 66, 62, 55 and 45 - 47 kDa Ag may share similarities with the immunochemically determined surface Ag of *Babesia bigemina* (Figueroa et al. 1989), and to the 60 kDa protein obtained from B. rodhaini-infected blood (Howard et al., 1980).Tetzlaff et al. (1990) had isolated and characterized a gene of M.W 55 kDa which he hypothesized to be likely associated with a virulent strain of *B.microti.* Interestingly, we noted, a 55 kDa parasite component in *B. rodhaini* and *B. microti*, which reacted with both mAb 1-E7 and 2-H2, and with immune sera.

At present, we have started purifying the 62 and 55 kDa *B.rodhaini* Ag.

81

Antigens will be used for further experimentations related to the findings reported in this paper.

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REFERENCES

Abdalla, H., Hussein, H. & Kreier, J. 1978. *Babesia rodhaini* : Passive protection of mice with immune serum. *J. Tropenmed. Parasitol. 29*: 295-306.

Asao, T., Kinoshita, Y., Uemura, T. & Sakaguchi, G. 1984. Purification and some properties of *Aeromonas hydrophilia* hemolysin. *Infect. Immun.46*: 122-127.

Bautista, C. & Kreier, J. 1979. Effect of immune serum on the growth of *Babesia microti* in hamster erythrocytes in short term culture. *Infect. Immun. 25*: 470-472.

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

Clark, I., Richmond, J., Wills, E. & Allison, A. 1977.Intraerythrocytic death of the parasite in mice recovering from infection with *Babesia microti. Parasitol. 75*:189-196.

Cox, F. & Turner, S.,1970. Antigenic relationships between the malarial parasites and piroplasma of mice as determined by the fluorescence antibody technique. *Bull. Wld. Hlth. Org. 43*: 337-340.

Figueroa, J., Buenig, G., Kinden, D. & Green, T. 1989. Identification of common surface antigens among *Babesia bigemina* isolated by using monoclonal antibodies. *Parasitol. 100*: 161-175.

Howard, E., Clark, I. & Allison, A. 1980. Characterization of surface proteins and glycoproteins on red blood cells from mice infected with haemosporidia: *Babesia rodhaini* infections of BALB/c mice. *Parasitol. 81*: 251-271.

Ishimine, T., Nagasawa, H. & Suzuki, N. 1979,.An in vitro study of monocyte phagocytosis in the peripheral blood of healthy and *Babesia*-infected beagles. *Jpn. J. Trop. Med. Hyg. 4*: 487-493.

Kozaki, S., Kato, K., Asao, T., Kamata, Y. & Sakaguchi, G. 1987. Activities of *Aeromonas hydrophilia* hemolysin and their interaction with erythrocyte membranes. *Infect. Immun. 55*: 1594-1599.

82

Kurtzhals, J., Anderson, J. & Christensen, N. 1988. Effect on the in vitro growth of *Babesia microti* by cells and serum from *Babesia microti* and *Schistosoma mansoni* infection in mice. *Acta Veterinaria Scandinavia 29*: 357-362.

Mahoney, D. 1967. Bovine babesiosis: the passive immunization of calves against *Babesia argentina* with special reference to complement-fixing antibodies. *Exp. Parasitol. 20*: 119-124.

Meeusen,D., Lloyd, S. & Soulsby, E. 1985. Antibody levels in adoptively immunized mice after infection with *Babesia microti* or injection with antigen fractions. *Aust. J. Exp. Biol. Med. Sci. 63*: 261-272.

Meeusen, D., Lloyd, S. & Soulsby, E. 1984a. *Babesia microti* in mice: Adoptive transfer of immunity with serum and cells. *Aust. J. Exp. Biol. Med. Sci. 62*: 551-566.

Meeusen, D., Lloyd, S. & Soulsby, E. 1984b. *Babesia microti* in mice: Subpopulations of cells involved in the adoptive transfer of immunity with immune spleen cells. *Aust. J. Exp. Biol. Med. Sci. 62*: 567-575.

Oi, V. & Herzenberg, L. 1979. Immunoglobulin-producing cell lines., pp. 351-372. *In*: Selected Methods in Cellular Immunology, Mishell, B. & Shiigi, S. (Eds), W.H. Freeman and Co., New York.

Phillips, R. 1969. The protective activity of serum from immune rats against *Babesia rodhaini. Parasitol. 56*: 357-364.

Rogers, R. 1974. Function of the spleen in controlling infections of *Babesia rodhaini* in rats. *Internat. J. Parasitol. 4*: 197-201.

Ruebush, M. & Hanson, W. 1980. Transfer of immunity to *Babesia microti* of human origin using T-lymphocytes in mice. *Cell Immunol. 52*: 255-265.

Sugimoto, C., Sato, M., Kawazu, S., Kamio, T. & Fujisaki, K. 1991. Purification of merozoites *Theileria sergenti* from infected bovine erythrocytes. *Parasitol Res.* 77:129-1312.

Smith, R. & Ristic, M. 1981. Immunization against bovine babesiosis with culture derived antigens. pp. 495-507. *In* : Babesiosis, Ristic, M. & Kreier, J.P. (Eds), Academic Press, New York.

Smith, R., Carpenter, J., Cabrera, A., Gravely, S., Erp, E., Osorno, M. & Ristic, M. 1979. Bovine babesiosis: Vaccination against tickborne challenge exposure with culture-derived *Babesia bovis* immunogens. *Amer. J. Vet. Res. 40*: 1678-1682.

Tetzlaf, C., McMurray, D. & Rice-Ficht, A. 1990. Isolation and characterization of a gene associated with a virulent strain of *Babesia microti. Mol. Biochem.Parasitol. 40*: 183-192.

Timms, P.,Stewart, N. & De Vos, J. 1990. Study of virulence and vector transmission of *Babesia bovis* by use of cloned parasite lines. *Infect. Immun. 58*: 2171-2176.

Thoongsuwan, S. & Cox, H. 1973. Antigenic variants of the haemosporidian parasite, *Babesia rodhaini*, selected in vitro treatment with immune globulin. *Ann. Trop. Med. Parasitol. 67*: 373-385.

83.

Towbin, H., Staehelin, T. & Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci., USA. 76*: 4350-4354.

Wright, I., Mirre, G., Rode-Bramanis, K., Chamberlain, M., Goodger, B. & Waltisbuhl, D. 1985. Protective vaccination against virulent *Babesia bovis* with a low-molecular weight antigen. *Infect. Immun. 48*: 109-113.

Wright, I., White, M., Tracet-Patte, P., Donaldson,R., Goodger, B., Waltisbuhl, D. & Mahoney, D. 1983. *Babesia bovis*: Isolation of a protective antigen by using monoclonal antibodies. *Infect. Immun. 41*: 244-250.

Zivkovic, D., Sperksnijder, J.E., Kuil, H. & Seinen, W. 1983/84. Immunity to *Babesia* in mice. II. Cross protection between various *Babesia* and *Plasmodium* species and its relevance to the nature of *Babesia* immunity. *Vet. Immunol. Immunopathol. 5*: 359-368.

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Toxoplasma gondii: Antibody-independent Binding of Human Complement Subcomponent C1q to the Parasite

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ABSTRACT

The binding capacity of C1q onto *Toxoplasma gondii (T.gondii)* parasites at each infectious stage was examined by using the immunofluorescence assay (IFA) with purified human C1q, rabbit anti-human C1q antibodies and fluorescein -isothiocynate conjugated goat anti-rabbit IgG antibodies in parallel with the IgM-binding to the parasite.

Any possibly contaminated natural antibodies to the parasite were completely removed from the anti-human C1q-preparations by repeated absorption with the parasite in advance. Parasites incubated with C1q before adding anti-C1q antibodies have shown the specific fluorescence on their outer surface membrane, whereas those without any addition of C1q have no specific fluorescence. The intensity of fluorescence differed in stages, and cystozoites and trophozoites seemed to have significantly higher C1q-binding activities than sporozoites. Furthermore, C1q-binding sites have been differently located from IgM-binding sites, or anterior poles, on the parasite.

These observations indicate that *T.gondii* parasite has the antibody- independent binding capacity to human C1q on its outer surface membrane.

INTRODUCTION

Intracellular parasitic organisms have evolved a wide variety of mechanisms that allow them to grow and multiply within cells and escape defense mechanisms of their hosts. *Toxoplasma gondii* (*T. gondii*) an obligate intracellular protozoa is well adapted to an intracellular growth and development during the extraintestinal phase of its life cycle. This apicomplexan parasite can penetrate phagocytic and non-phagocytic mammalian cells, and survive within parasitophorous vacuoles. Several organelles are believed to participate in its active penetration of the parasite into host cells. Recent studies have shown that substances released from rhoptries enhance the penetration into host cells during invasion process (Nichols et al. 1983; Schwartzman 1986; Kimata

and Tanabe 1987; Sadak et al 1988). Moreover, the existence of Fc receptor (Budzko et al. 1989) and C3 receptor (Fuhrman and Joiner 1989a) on *T. gondii* has been documented. Biological functions of these receptors may play an important role in internalization and infectivity of the parasite (Fuhman and Joiner 1989b). To date, however, the interaction between the parasite and host defense system after cell invasion, is still unclear.

C1q, a subcomponent of the first component of complement(C1) is an unusual plasma protein with the N-terminal half of the molecule composed of a triple helix with collagen-like amino acid sequence. The C-terminal end of the molecule lacks the collagen-like sequence, and is believed to contain the site that interacts with Fc regions of aggregated IgG and/or immune complexes. The collagen-like regions of C1q appear to be involved in the interaction with and activation of C1r and C1s proenzymes of C1 (Reid and Porter 1981). Substances such as polyinosinic acid, bacterial lipid A and antivenom polysaccharide (Loss 1982) and some constituents of the extracellular matrix such as fibronectin (Isliker et al. 1981) and laminin (Bohnsack et al 1985) have been shown to interact directly with C1q. Moreover, some of these substances activate subsequent components in the classical complement pathway. Thus, it has become apparent that C1q plays an important role in the triggering and activation of the classical complement pathway by immune complexes or other antibody-independent activators. Also C1q may carry out the essential function by clearing immune complexes and other antibody-independent activators via phagocytic mechanism.

To have a better understanding of the mechanism of intracellular parasitism, in this study, we examined the binding of C1q to each infectious stage of *T. gondii*.

MATERIALS AND METHODS

Adult BALB/c mice bred in the Department of Veterinary Physiology, Obihiro University, Hokkaido, Japan, were used. Mouse embryonal cells (MEC) were obtained from embryos on the 12th-14th day of gestation, and cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and antibiotics.

To examine the C1q binding capacity of high virulent and low virulent strains of *T. gondii*, RH and S-273 strains were used. Trophozoites of RH and S-273 strain were obtained from infected MEC cultures. S-273 strain cystozoites were collected from physically ruptured cysts from infected mouse brain. Trophozoites were isolated by Percoll (Pharmacia, Tokyo, Japan) sucrose density gradient centrifugation (Cornelissen et al. 1981). To remove host immunoglobulins from cysts surface, isolated cysts were incubated for 30 min in 50 mM acetate buffered saline (pH 2.9) at 4℃ and washed three times with phosphate buffered saline (PBS).

To examine the quantitative difference of C1q binding capacity at each infectious stage of *T.gondii*, only S-273 strain sporozoites were used, inasmuch as cats inoculated with RH strain did not shed any oocysts. Oocysts of the S-273 strain were isolated from cats' feces using sucrose density gradient centrifugation and excysted at 37℃ through incubation in PBS containing 0.5% taurocholic acid and 0.1% trypsin for 30 min. Sporozoites were isolated from oocyst suspension

using Percoll-sucrose density gradient centrifugation and washed extensively with cold PBS (Arrowood and Sterling 1987). Isolated parasites were fixed in PBS with 1% paraformaldehyde at 4℃ for 15 min.

Purification of C1q from healthy human serum was done following the method of Yonemasu and Stroud (1971), then stored at -105℃, until use. Absence of immunoglobulins in the material was confirmed by immunoblotting analysis using peroxidase-conjugated anti-human Immunoglobulins.

Rabbit anti-human C1q antiserum was prepared as described by Yonemasu and Stroud (1971) and stored at -105℃, until use. All antiserum preparations were adsorbed repeatedly with fixed parasites to remove any possibly contaminating natural antibodies to *T. gondii*. F(ab')2 fragments were prepared and purified from the IgG fraction of antiserum by pepsin digestion and subsequently by Sephadex G-150 chromatography (Nisonoff 1964).

The binding of C1q to fixed smears of the RH and the S-273 strains and a live suspension of the RH strain were examined by indirect IFA (IIFA). Fixed or live parasites were incubated with different concentrations of C1q or without C1q at 37 ℃ for 30 min in PBS containing 5% skim milk. The parasites were subsequently incubated at 37℃ for 30 min with an appropriate dilution of F(ab')2 fragments prepared from anti-human C1q antiserum. The parasites were extensively washed to remove nonspecifically bound F(ab')2 and incubated at 37℃ for 30 min with an appropriate dilution of FITC-conjugated goat anti-rabbit IgG.

The capacity of *T. gondii* to bind IgM was examined with a direct IFA (DIFA) technique with various dilutions of normal human serum and with goat FITC-conjugated anti-human IgM antibodies. All dilutions were prepared in PBS. The healthy adult human serum was inactivated at 56℃ for 30 min to destroy all C1q antigenicity (Yonemasu and Stroud 1971). Observation was carried out using a fluorescence microscope.

Trophozoites of the RH strain and human erythrocytes obtained from healthy individuals were incubated at 37℃ for 30 min in PBS containing C1q(50 ug/ml). Control preparations were incubated without C1q. After extensive washing, the parasites were solubilized at 100℃ for 3 min with 125 mM Tris buffer, pH 6.8, containing 10% 2-mercaptoethanol and 4.6% sodium dodecyl sulfate(SDS). The preparations were centrifuged to remove insoluble aggregates and the supernatant was applied to a 10% polyacrylamide gel containing SDS(SDS-PAGE) (Fairbanks et al. 1971). The peptide chains of C1q (A: 27,500; B: 25,200; C: 23,800) were used as protein standards for estimation of molecular weights. Proteins were transferred from SDS-PAGE gels to nitrocellulose filters with a Trans-Blot apparatus (Bio-Rad, Richmond, Calf., U.S.A.) as described by Towbin et al. (1979). The membrane was blocked with 1% bovine serum albumin (BSA) in PBS and incubated for 60 min at room temperature with appropriate dilutions of rabbit anti-human C1q and/or rabbit anti-human IgG (H and L chains) antiserum. After washing with PBS containing 0.05% Tween-20, the membrane was incubated for another 60 min with horseradish peroxidase-conjugated F(ab')2 fragments prepared from the IgG fraction of goat anti-rabbit IgG antibodies. After extensive washing, the nitrocellulose membrane was stained with

4-chloro-1-naphthol containing H_2O_2.

RESULTS AND DISUCUSSION

Incubation with C1q revealed a rim-like specific fluorescence on the membrane of both RH and S-273 strains of *T. gondii* (Figure 1). Also, membrane associated fluorescence was observed with live parasites of RH and/or S-273 strains incubated with C1q; while the parasites incubated without C1q did not show any specific fluorescence. Trophozoites of RH (Figure 1a) and S-273 (Figure 1b) strains had diffuse and slightly patchy fluorescence. Sporozoites from fractured oocysts (Figure 1c) also exhibited fluorescence on their membrane, but were stained less intensely than trophozoites.

Fig. 1a

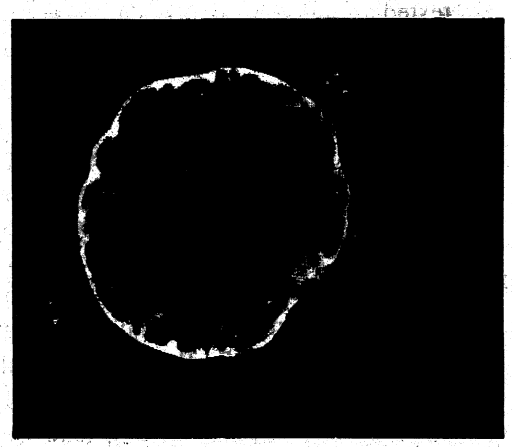

Fig. 1b

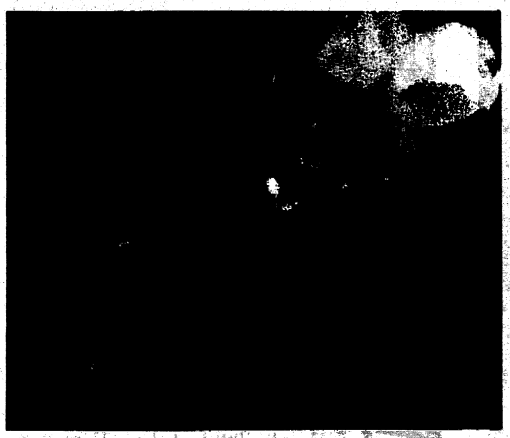

Fig. 1c

Fig. 1d

Fig.1a-d. Immunofluorescence localization of human C1q binding sites on fixed parasites. Parasites were incubated for 30 min at 37℃ with purified human C1q and then incubated with rabbit anti-human C1q F(ab')2 fragments, and reacted with FITC-conjugated anti rabbit IgG.
Extracellular trophozoites of RH strain (1a). trophozoites were harvested from cultured MEC cells. Magnification:× 1,000. Trophozoites of S-273 strain (1b). Parasites were collected from infected mice brains. Magnification:×1,000. Sporozoites of S-273 strain (1c). Magnification: ×400.
Human IgM binding sites on fixed trophozoites of RH strain. Parasites were incubated with heat-inactivated human serum from healthy adults and reacted with FITC-conjugated anti-human IgM (1d). Magnification: × 1,000.

Table 1.

Localization of human C1q on *Toxoplasma gondii* by immunofluorescence assay

parasite or host cell		Localization and intensity of staining host cell		
		living	fixed	concentration of C1q*
MEC		NT	-	
Trophoozoite	(RH)	rim +	rim +	60 ng/ml
	(S-273)	rim +	rim +	60 ng/ml
Sporozoite	(S-273)	NT	rim ±	240 ng/ml

Intensity of fluorescent staining: negative, - ; slightly positive, ± ; positive, +; NT, not tested.

*Values are expressed as minimum concentrations of C1q giving a distinct positive staining.

Specific and distinct immuno-staining was detected on the membrane of trophozoites incubated with C1q at concentrations higher than 60 ng/ml. Weak, but significant immuno-staining was detected on the membrane of sporozoites incubated with C1q at concentrations higher than 240 ng/ml (Table 1). Treatment of sporozoites with trypsin and taurocholic acid during excystation had no effect on the binding of C1q to the parasites (data not shown).

Trophozoites incubated with heat-inactivated human serum and stained by the DIFA procedure exhibited dot-like specific fluoresence on their anterior end (Figure 1d).

Immunoblotting analysis of bound C1q is shown in Figure 2. Bands corresponding to the A-, B-, and C-chains of purified C1q (shown with arrows) were only detected in lane of trophozoites of RH strain incubated with C1q (lane 2); while, no other bands associated with A-, B-, C-chains of C1q were detected in the other lanes of trophozoites without C1q (lane 4). Human erythrocytes

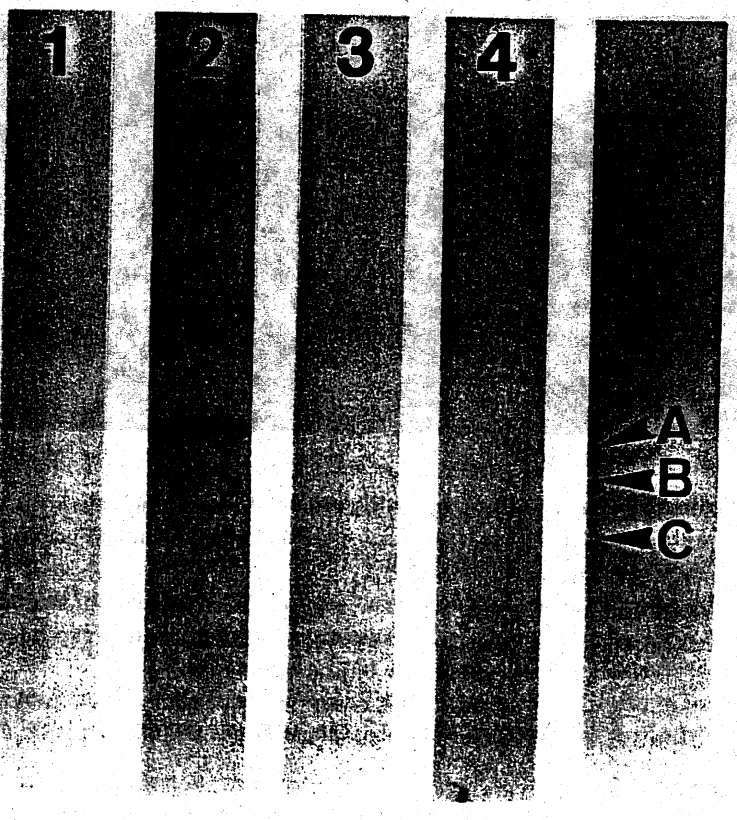

Figure 2. Immunoblotting of trophozoites of RH strain or human erythrocytes incubated either with or without C1q. Parasites incubated with C1q (lane2) or without C1q (lane4),human erythrocytes incubated with C1q (lane 3) or without C1q (lane 1), and purified C1q were electrophoresed in 10% SDS-PAGE gel. Transblotted membrane sheet was reacted with rabbit anti-C1q antiserum, followed with peroxidase-conjugated F(ab')2 anti-rabbit IgG.

89

incubated with (lane 3) or without C1q (lane 1) also showed no bands similar with those of C1q components.

Results of the immunofluorescence and immunoblotting analysis demonstrate that the binding sites of human C1q on the parasite are morphologically and immunochemically different from those of the IgM molecules. The use of purified C1q and anti-human C1q F(ab)'2 fragments adsorbed with parasites, effected the elimination of immunoglobulins that exhibited non-specific binding with *T. gondii*. Budzko et al. (1989) described the presence of receptors for the Fc portion of human immunoglobulins on the surface of *T. gondii* by IIFA technique. These receptors, however, exhibited a polar pattern of immunofluorescence which is different from the C1q binding sites noted in the present study. Our findings suggest the binding of C1q to *T. gondii* seems not to be mediated by Fc receptors.

It is of interest that the intracellular proliferative forms of the parasites, such as trophozoites of both low virulence (S-273) and high virulence (RH) strains showed higher C1q-binding activity than sporozoites (Figure 1 and Table 1). This observation seems to suggest that constituents or receptor(s) on the membrane of *T. gondii* responsible for C1q-binding has function(s) associated with intracellular survival, rather than with pathogenicity or virulence. Our finding is consistent with a related study on C1q receptors on fibroblastoid cells by Bordin et al. (1983). For now, we may hypothesize that binding of native C1q to the membrane of trophozoites must be important in bringing parasites in contact with host cells during the initial phase of their penetration. Moreover, the binding of C1q may also assist the parasite in terms of its adaptation to an intracellular survival by way of physico-chemical and metabolic modification on its cell membrane. To clarify these speculations, topographical and quantitative studies concerned with C1q-binding site on the parasite are inevitably necessary.

The present study was not able to determine whether C1q-binding substances are derived from the parasites themselves, from host cell products, or from extracellular matrix components, such as fibronectin or laminin. Furtado et al (1992) demonstrated that tachyzoites of *T.gndii* recognized multiple laminin receptors in attaching to different target cells. Further experiments to clarify these points, and the determination of the biological significance of C1q binding are in progress.

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REFERENCES

Arrowood, M.J., and Sterling, C.R. 1987. Isolation of cryptosporidium oocyst and sporozoites using discontinuous sucrose and isopycnic percoll gradients. *J. Parasitol., 73;* 314-319.

Bohnsack, J.F., Tenner, A.J., Laurie, G.W., Kleinman, H.K., Martin, G.R., and Brown, E.J. 1985. The C1q subunit of the first component of complement binds to laminin: a mechanism of

the disposition and retention of immune complexes in basement membrane. *Proc. Natl. Acad. Sci. USA., 82;* 3824-3828.

Bordin, S., Kolb, W.P., and Page, R.C. 1983. C1q receptors on cultured human gingival fibroblasts: analysis of binding properties. *J. Immunol., 130;* 1871-1875.

Budzko, D.B., Tyler,L., and Armstrong, D. 1989. Fc receptors on the surface of *Toxoplasma gondii* trophozoites: a confounding factor in testing for anti-toxoplasma antibodies by indirect immunofluorescence. *J. Clin. Microbiol., 27;* 959-961.

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.

Fairbanks, G., Stech, T.L., and Wallsch, D.F.E. 1971. Electrophoretic analysis of the polypeptides of the human erythrocyte membrane. *Biochemistry 10;* 2606-2617.

Fuhrman, S.A., and Joiner, K.A. 1989a. *Toxoplasma gondii*: Mechanism of resistance to complement-mediated killing. *J. Immunol., 142;* 940-947.

Fuhrman, S.A., and Joiner, K.A. 1989b. Complement evasion by protozoa. *Exp. Parasitol.,68;* 474-481.

Furtado, G.C., Slowik, M., Kleinman, H.K., and Joiner, K.A. 1992. Laminin enhances binding of *Toxoplasma gondii* tachyzoites to J774 murine macrophage cells. *Infect. Immun., 60;* 2337-2342.

Isliker, H.,Bing, D.H.,Laham, J., and Hynes, R.O. 1982. Fibronectin interacts with C1q, a subcomponent of the first component of complement. *Immunol. Lett., 4;* 39-43.

Kimata, I.; and Tanabe, K. 1987. Secretion by *Toxoplasma gondii* of an antigen that appears to become associated with the parasitophorous vacuole membrane upon invasion of the host cell. *J. Cell Biol., 88;* 231-239.

Loos, M. 1982. Antibody independent activation of C1, the first component of complement. *Ann. Immunol. (Paris) (Annales De L' Institut Pasteur) i33C;* 165-179.

Nicholas, B.A., Chiappino, M.L., and O'Connor, G.R. 1983. Secretion from the rhoptries of *Toxoplasma gondii* during host cell invasion. *J. Ultrastruct. Res., 83;* 85-89.

Nisonoff, A. 1964. Enzymatic digestion of rabbit gamma globulin and antibody and chromatography of digestion products. *Methods Med. Res., 10;* 134-149.

Reid, K.B.M., and Porter, R.R. 1981. The proteolytic activation systems of complement. *Ann. Res. Biochem., 50;* 433-464.

Sadak, A., Taghy, Z., Fortier, B., and Dueremeitz, J.F. 1988. Characterization of a family of rhoptry proteins of *Toxoplasma gondii. Mol. Biochem. Parasitol., 29;* 203-212.

91

Schwartzman, J.D. 1986. Inhibition of penetration enhancing factor of *Toxoplasma gondii* by monoclonal antibody specific for rhoptries. *Infect. Immun., 51;* 760-764.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA., 76;* 4350-4354.

Yonemasu, K., and Stroud, R.M. 1971. C1q: Rapid purification method for preparation of monospecific antisera and biochemical studies. *J. Immunol., 106;* 304-313.

1-16

Placental and Plasma Cystine Aminopeptidase in Pregnant Animals

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ABSTRACT. The placental and plasma cystine aminopeptidase (CAP) in pregnant animals was examined on stability after the treatment with L-methionine, ethylene diamine tetra-acetic acid (EDTA) and heat. Inhibitory effects of these treatments on enzyme activities were different among CAPs from the animal species, however, significant correlation in those effects between placental and plasma CAPs was observed. These results suggested that plasma CAP might reflect placental CAP and seemed to be available for estimating maternal gestational conditions.—KEY WORDS: cystine aminopeptidase, placenta, plasma.

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Cystine aminopeptidase (CAP) [EC 3.4.11.3] synthesized in placental syncytiotrophoblast has been widely accepted to play an important role on the maintenance of gestation by inactivation of oxytocin in pregnant women [1, 4]. Since the enzymatic characteristic of plasma CAP is the same as those of placental CAP, plasma CAP levels are considered to be closely related with placental weight and functions, especially amino acids uptake, and also with fetal growth [11]. Plasma CAP activities in pregnant women increased remarkably at the late gestational period and these values were also found to be available for estimating maternal gestational conditions [4].

However, there are few informations on CAP in pregnant animals in spite of few markers for evaluation of gestational conditions [3, 12]. This note deals with enzymatic characteristics of placental and plasma CAP in pregnant animals with regard to their placental structures.

Samples: The full term placentas were obtained from the pregnant animals at delivery. Maternal blood were collected into heparinized tubes from clinically healthy pregnant women, cynomolgus monkeys, marmosets, dogs, pigs, goats, cows and horses just before the full gestational period. The plasma was separated by centrifugation at 600 g for 10 min and stored at −80°C until assay.

Sample preparation: The placental CAP was extracted by Oya's method [11]. All procedures were carried out at 4°C. Briefly, the placentas were washed with cold physiological saline, followed by removal of the fetal membrane and umbilical cord. Then, they were minced in small piecies and added with 5 volumes of Tris-HCl buffer (5 mM, pH 7.4) containing 0.25 M sucrose. The homogenates prepared with Potter-Elvehjem homogenizer were filtrated through gauze and centrifuged at 600 g for 10 min. The supernatants were stored at −80°C until use.

Enzyme activities: The CAP activities were measured by CAP color test Sankyo (Sankyo Co., Ltd.) using S-benzyl-L-cystein-p-dimethylaminoanilide as a substrate. The protein concentration was determined by Lowry's method [8].

Inhibition test: Inhibitory effects of ethylene diamine tetra-acetic acid (EDTA, 0.1 and/or 10 mM) and L-methionine (10 and/or 20 mM) on placental and plasma CAP activities, and their heat stability (60°C, 30 min) were examined.

As shown in Fig. 1-a, EDTA revealed a significant inhibitory effect on both placental and plasma CAP in women, cynomolgus monkeys, marmosets, whereas it showed no inhibitory effect in the other animals. Many investigators reported that placental and plasma CAP from pregnant women was completely inhibited by EDTA [6, 7]. Furthermore, Hayashi and Oshima [2] reported a significant inhibitory effect of EDTA on placental CAP from monkeys (*Macaca fuscata fuscata*). Since such an inhibitory effect on CAP activity was considered to be associated with metal ions on its biologically active site [6], our results suggested that characteristics of CAP as a metalloenzyme were different among animal species. On the other hand, the L-methionine was reported to have no competitive inhibitory effect on neither placental nor plasma CAP in women, whereas it showed a complete inhibitory effect on leucine aminopeptidase [2, 5–7, 9–11]. In our study, L-methionine also exerted a remarkable inhibitory effect on both placental and plasma CAP from all of the pregnant animals except for only human (Fig. 1-b). These results also suggested that there was a difference in the competitive status of the biologically active site of CAP among animal species. The remaining activities of placental CAP were very low in human, cynomolgus monkeys, marmosets, pigs, and horses after incubation at 60°C for 30 min, whereas those of plasma CAP varied among animal species (Fig. 1-c). Placental and plasma CAP in pregnant women had been shown to lose completely their activities by preincubation at 60°C for 30 min [5, 9–10]. In this study, an inhibitory effect of heat treatment on placental and plasma CAP was observed in human, cynomolgus monkeys, marmosets, and pigs.

The correlation of inhibitory effects by EDTA, L-methionine, and heat treatment on placental and plasma CAP in pregnant animals is shown in Fig. 2. The significant correlation ($p<0.001$, $n=24$, $r=0.843$) was observed between them. Oya *et al.* [9–11] suggested plasma CAP in pregnant women may be originated from the placental lysosomal CAP according to the fact that they were not inhibited by L-methionine, but by heat treatment, and that they had the same electrophoretical mobility. Kleiner *et al.* [5] also reported that plasma CAP reflected placental CAP which might be released from

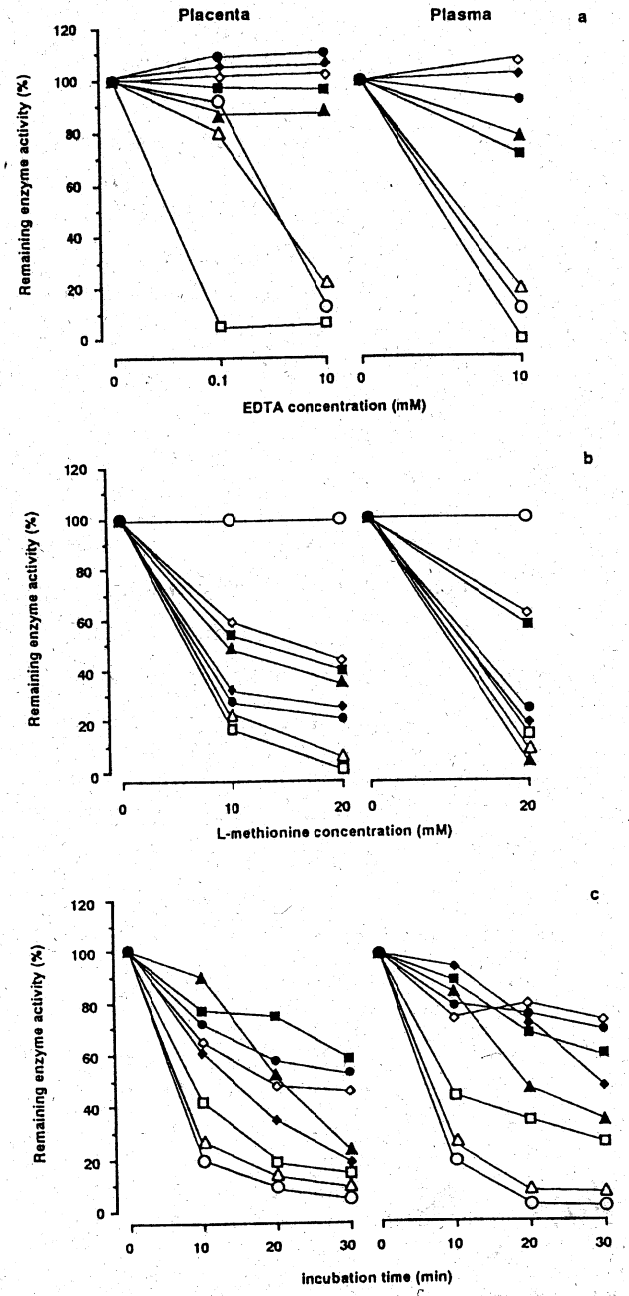

Fig. 1. Inhibitory effects of EDTA, L-methionine, and heat treatment on placental and plasma CAP activities in pregnant animals (human: -○-, cynomolgus monkey: -□-, marmoset: -△-, dog: -◊-, goat: -●-, cow: -■-, pig -▲-, horse: -◆-).

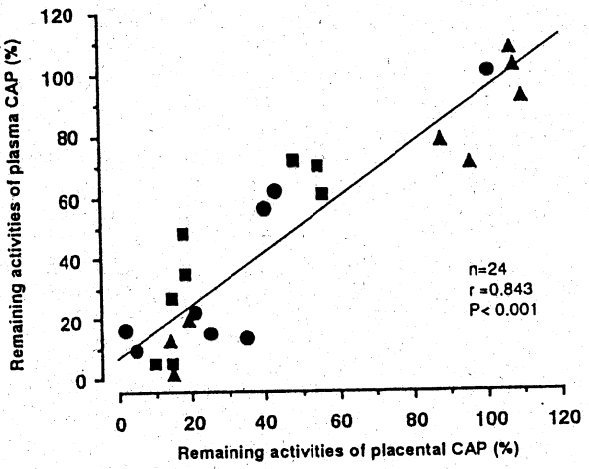

Fig. 2. The correlation of inhibitory effects by EDTA (▲), L-methionine (●), and heat treatment (■) on placental and plasma CAP in pregnant animals.

placenta.

In conclusion, plasma CAP activities in pregnant animals might reflect placental CAP activities and seemed to be available for estimating maternal gestational conditions.

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REFERENCES

1. Hashimoto, T. 1961. *J. Jpn. Obstet. Gynec. Soc.* 8: 87–104.
2. Hayashi, M. and Oshima, K. 1976. *J. Biochem.* 80: 389–396.
3. Hiwada, K., Saeki-Yamaguchi, C., Inaoka, Y., and Kokubu, T. 1978. *Biochem. Med.* 20: 305–314.
4. James, N. T. 1966. *Nature (Lond.)* 210: 1276–1277.
5. Kleiner, H., Dictus-Vermeulen, C., May-Cocriamont, C., Brouet-Yager, M., Popowski, A., Mosselmans, R., and Graff, G. 1980. *Clinica. Chimica. Acta* 101: 113–123.
6. Lampelo, S. and Vanha-Perttula, T. 1979. *J. Reprod. Fertil.* 56: 285–296.
7. Lampelo, S. and Vanha-Perttula, T. 1980. *J. Reprod. Fertil.* 58: 225–235.
8. Lowry, O. H., Rosbrough, N. J., Farr, A. L., and Randall, R. J. 1951. *J. Biol. Chem.* 193: 265–275.
9. Oya, M., Yoshino, M., and Asano, M. 1974. *Experientia* 15: 985–986.
10. Oya, M., Yoshino, M., and Mizutani, S. 1975. *Acta Obst. et Gynaec. Jpn.* 22: 49–50.
11. Oya, M., Yoshino, M., Mizutani, S., and Wakabayashi, T. 1974. *Gynecol. Invest.* 5: 276–283.
12. Reynolds, S. R. M. and Mackie, J. D. 1961. *Proc. Soc. Exp. Biol. Med.* 108: 649–651.

94

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Survey of *Sarcocystis* spp. Infection in Slaughtered Pigs in East Hokkaido, Japan

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Key words: pig, *Sarcocystis*, survey

Sarcocystis spp. being obligate heteroxenous coccidians, require definitive hosts in the course of their transmission. Prevalence of *Sarcocystis* infection in domestic animals have been reported in many countries (Barrows et al. 1981; Bottner et al. 1986; Munday 1975; Pereia and Bermejo 1988; Prestwood et al. 1980; Saleque and Bhatia 1991). Saito et al. (1986) documented the isolation of *Sarcocystis miescheriana* in 17 culled older pigs (17 / 200 = 8.5%) and none among fattened pigs in Saitama Prefecture, Honsyu, Japan. In the northen part of Japan, specifically in Hokkaido, to date, there are no published reports of *Sarcocystis* infection. Thus, the present study was sought to survey *Sarcocystis* infection among slaughtered pigs in East Hokkaido, Japan.

Samples of diaphragm muscle tissues were obtained from 104 sows, and 40 fattened pigs at a slaughter house in Obihiro, between October and November, 1992.

Figure 1. Cyst of *Sarcocystis suicanis* recovered from diaphragm of sow. Note perpendicular protrusions on the outer surface of cyst wall. x 400.

Nine to fifteen pieces of muscle tissues, weighing approximately 0.5 g each, were excised from each host muscles and teased in phosphate buffered saline and examined microscopically. Identification of *Sarcocystis* spp. was based on morphological features, specifically on the structural dissimilarity of the cyst wall.

Cysts of *Sarcocystis suicanis* were detected in 17 out of 104 sows. Cysts were fusiform in shape and the cyst wall has radial, striated and perpendicular protrusions on the outer surface (Figure 1) containing numerous banana-shaped cystozoites. Among 40 fattened pigs, cysts of the more pathogenic and zoonotic *Sarcocystis suihominis* which have cyst walls characterized as having hair-like villar and slanted protrusions on their outer surface were not found.

Results of the present study show a prevalence rate of 16.5 % of *S.suicanis* among sows and 0.0% occurence of the pathogenic and zoonotic *S.suihominis* in East Hokkaido. Non-utilization of human stool as manure for the past 10 years in Japan could be attributed as one major factor influencing the absence of contamination of soil with the zoonotic *S. suihominis.* Furthermore, while this study does not provide information related to infection route of *S.suicanis* in sows in Hokkaido, the absence of wild pigs in the Hokkaido area, may lend support to the hypothesis that Honsyu areas would most likely be a source of *S. suicanis* in Hokkaido.

REFERENCES

Barrows, P. L., Smith, H. M. Prestwood, A. K. & Brown, J. 1981. Prevalence and distribution of *Sarcocystis* sp among wild swine of southeastern United States. *JAVMA.*, 179:1117-1118.

Bottner, A., Charleston, W. A. G., Pomroy, W. E. & Rommel, M. 1987. The prevalence and identity of *Sarcocystis* in beef cattle in New Zealand. *Vet. Parasitol.* 24: 157-168.

Munday, B. L. 1975. The prevalence of sarcosporidiosis in Australian meat animals. *Austral. Vet. J.* 51: 478-480.

Pereira, A. & Bermejo M. 1988. Prevalence of *Sarcocystis* cysts in pigs and sheep in Spain. *Vet. Parasitol.* 27: 353-355.

Prestwood, A. K., Cahoon, R. W. & McDaniel, T. H. 1980. *Sarcocystis* infections in Georgia swine. *Am. J. Vet. Res.* 41: 1879- 1881.

Saito, M., Nakajima, T., Watanabe, A. & Itagaki, H. 1986. *Sarcocystis miescheriana* infection and its frequency in pigs in Japan. *Jpn. J. Vet. Sci.* 48: 1083-1090.

Saleque A. & Bhatia B. 1991. Prevalence of *Sarcocystis* in domestic pigs in India. *Vet. Parasitol.* 40: 151-153.

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Studies Related to Immunosuppression in Mice with Chronic Toxoplamosis

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Key words: *Toxoplasma*, cysts, mice, immunosuppression, immunoregulator, obiopeptide-1

ABSTRACT

Mice chronically infected with *Toxoplasma gondii* were treated with cyclophosphamide, obiopeptide-1 (Obi-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-treated, and infected cyclophosphamide-Obi-1 treated mice, with hematoxylin-eosin, and anti-*Toxoplasma* avidin-biotin-conjugate labelling techniques, large typically rounded tissue cysts were mostly detected, and sometimes with dividing microcysts. In contrast, brain tissue from cyclophosphamide only or anti-CD4 treated infected mice had multiple degenerate cysts of varied size in some brain regions, as well as clusters of microcysts, however, such change was more striking in the anti-CD4 treated group. Infected mice treated with a combination of cyclophosphamide and Obi-1 showed a significantly higher survival of 80% compared to 20% survival in mice treated with cyclophosphamide only. Percent neutrophilic leucocytes, monocytes and lymphocytes in mice treated with a combination of Obi-1 and anti-CD4, or Obi-1 and cyclophosphamide were higher compared to those groups treated with anti-CD4 antibody, or cyclophosphamide only. The increase in neutrophilic leucocyte and lymphocyte counts after a combined cyclophosphamide and Obi-1 treatment may, likewise, contribute to the induction of resistance in mice against *T. gondii*. Furthermore, these results seem to suggest that the reactivation or rupture of tissue cysts in mice chronically infected with *T. gondii* is not principally correlated with the death of cyclophosphamide treated mice.

INTRODUCTION

Within three wk post-infection *Toxoplasma* tachyzoites invade other organs like the brain and muscles of animals forming cysts, with the infection course exhibiting no apparent clinical manifestations. Generally, hosts that are less resistant as a result of acquired immunodeficiency syndrome (AIDS) in humans, distemper in dogs, leukaemia virus infection or administration of immunosuppressive drugs in cancer patients and others, may activate *T. gondii* proliferation within cysts showing clinical signs which can kill the hosts. As to what mechanism(s) activates *T. gondii* to multiply rapidly and form cystozoites, is still not well elucidated. Vollmer et al. (1987) had reported the appearance of *T. gondii* cysts of varied size in the mouse brain, and tachyzoite migration following experimental administration of anti-CD4 antibodies (Ab's). The importance of both CD4 and CD8 positive cells in chronic toxoplasmosis has been likewise, reported by Ricardo et al. (1991), Araujo (1991), and Suzuki et al. (1988).

The present study sought to carry out two objectives. First, was to determine the effect of cyclophosphamide, a widely used immunosuppressant (Makim et al. 1991; Stockman et al. 1973), a drug for cancer treatment with OK-432 (Ogawara et al. 1992), and a drug for the treatment of opportunistic infections in mouse model (Fujii et al. 1992b) on cyst movement in the brain when administered together with anti-CD4 antibody. Second, was to determine the life prolongation effect of synthetic obiopeptide-1 (Obi-1), as an immunoregulator on immunosuppressed mice with chronic toxoplasmosis.

MATERIALS AND METHODS

Experimental host and parasite: Five wk old male BALB/c mice and cysts of *T. gondii* (Beverley strain) maintained at the Department of Veterinary Physiology were used.

Preparation of T. gondii chronic carriers: An emulsion of infected mouse brain was inoculated into the mouse peritoneal cavity. Mice were intramuscularly injected 1 mg/ml/day of prednisolone for 5 days. Seven days later, the peritoneal cavity was washed with 0.9% physiological saline solution (PSS) to obtain tachyzoites. Mice were intraperitoneally (IP) inoculated 1 x 10^2 tachyzoites, and on the 5th, 7th and 9th day post-inoculation (PI), they were orally administered 0.5 mg/ml of Daimeton (sulfamonomethoxine). Mice that survived the initial infection 28 days PI were challenged with 1 x 10^4 tachyzoites. Those that survived for 56 days were used as *T. gondii* chronic carriers. Tachyzoites that were used for the challenge dose were obtained from subcultures in ICR-JCL mice which were treated with prednisolone.

Administration of cyclophosphamide and anti-CD4 antibodies: Cyclophosphamide

(Janssen Chimica, Beerse, Belgium) dissolved in 0.9% PSS was IP- inoculated to chronic carrier mice (250 mg/kg) five times at 7 days interval or four times at 10 days interval. In both experiments, the control groups consisted of non-infected mice but treated similarly as the experimental animals. Experimental and control mice were administered 300 µg/head of anti-CD4 monoclonal AB's (mAb's) four times at 10 days interval. Anti-CD4 mAb's were obtained by inoculating GKL.5 cell hybridoma to nude mice, and Ab's were purified from ascitic fluid using ammonium sulfate precipitation.

Administration of Obi-1: Synthetic Obi-1 (Suzuki et al. 1990) was dissolved in 100 mM $NaHCO_3$ (10 mg/ml) pH 6.4, diluted in PSS (1 mg/ml) and sterilized using a micropore filter. Each mouse was injected 100 µg (0.1 ml) of Obi-1 into the femoral muscle on the 3rd and 7th day post-administration of cyclosphosphamide and anti-CD4 Ab's.

Preparation of mouse spleen cells: Spleen cells from infected and non-infected mice were prepared following the method of Igarashi et al. (1990). Mice were bled to death and immediately the spleen were excised, finely cut and suspended in Hanks balanced salt solution (HBSS) containing 5 units/ml heparin, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate, and filtered using a steel mesh. To the sediment obtained by centrifugation at 800xg for 7 min at 4°C, warmed (37°C) 0.83% ammonium chloride solution was added to lyse the erythrocytes. The cells were washed twice with HBSS and once with RPMI-1640 (containing 5 units/ml heparin, 100 units/ml penicillin G, 100 µg/ml streptomycin sulfate, 12 mM Hepes, 0.1 µM 2-mercaptoethanol) by centrifugation at 800xg for 7 min at 4 °C.

Calculation of spleen cell subpopulations: Twenty mice were IP-inoculated about 100 tachyzoites/head. At 14, 28, and 162 days PI, five mice per group were used as *T. gondii* - infected group. Unexposed normal mice comprised the control group. Cell density ratio (I/N) was obtained by dividing the cell number in infected by the cell number in the non-infected mice. To the spleen cell suspension Ab's diluted in RPMI 1640 containing 0.3% bovine serum albumin (BSA) was added to approximate 1 x 10^6 cells/ml, then allowed to stand for 60 min at 4°C with stirring at 15 min interval. Monoclonal Ab's used were anti-Thy-1,2 (x500), anti-CD8 (x60), and anti-CD4 (x50) (Cedarlen Laboratories Limited, Ontario, Canada) , and Asialo GM1 (x60) (Wako Pure Chemical Industries, Tokyo). The cell suspension was centrifuged and washed once, and rabbit complement (x15 diluted in RPMI 1640 & 0.3% BSA) was added with stirring to approximate a cell density of 1 x 10^6 cells/ml, then allowed to stand for 60 min at 37 °C with stirring at 15 min interval. For the control 1 x 10^6 cells/ml were used. To the cells, trypan blue was added to approximate a 10% concentration, then placed on ice for 2-3 min, and the ratio of positive cells was obtained following the formula: % dead cells upon addition of Ab's - % dead cells in control divided by 100% - % dead cells in the control.

Calculation of leucocyte count: Heparinized blood obtained from the mouse caudal vein was diluted in Türk's solution using a melangeur. Leucocyte count per mm^3 was noted using a hemocytometer. With Giemsa-stained blood smear, the ratio of neutrophils, eosinophils, basophils, monocytes and lymphocytes to the total leucocyte count was obtained.

Calculation of cyst density in the brain: Using a sharp knife, the intact mouse brain was divided into the right lobe for histological examination, and the left lobe for the calculation of cyst density. To the left lobe, PSS was added and then homogenized using glass slides. Cyst density was calculated by placing 1/20 of the brain tissue emulsion on a plankton counter. A mean value was obtained from three counts and multiplied by 40 to get the cyst number per mouse. The right lobe was fixed in 15% formalin solution, processed and embedded in paraffin. Tissue sections were stained with hematoxylin & eosin (H&E). Parasites in tissues were, likewise, pathohistologically examined with light microscopy. Tissue sections were stained with avidin-biotin-peroxidase, and thereafter, were soaked in 0.3% methanol hydrogen peroxide for 30 min, washed with buffer solution and reacted with goat normal serum for 30 min. Sections were reacted with *T. gondii* - immunized rabbit serum for 30 min, washed and reacted with diluted biotinylated second antibody for 30 min, and with avidin-biotin conjugate (ABC) for another 30 min (Funakoshi Co., Tokyo). After another wash, the sections were reacted with peroxidase substrate solution (0.01% H_2O_2 and 0.05% DAB) (Wako Pure Chemical Industries, Tokyo) and then counterstained with H&E.

RESULTS

Table 1 shows the cell phenotype population ratios (I/N) in both experimental and control (pre-inoculation time) groups. Fourteen days PI, the I/N ratio of $\geqq 2.0$ was noted in total monocytes, Lyt-1,2, Lyt-2,2 and aGM1-positive cells in the spleen. A similar ratio was also recorded with Lyt-1,2, Lyt-2,2 and aGM1-positive cells in the peripheral blood, and in aGM1-positive cells in the liver. At 28 days PI, a ratio of 2.2 for Lyt-2,2 in the spleen and > 2.0 for aGM1-positive cells in the spleen and liver were, likewise, noted. At 162 days PI, the number of Lyt-1,2 and Lyt-2, 2, and aGM1-positive cells in the liver more than doubled compared to the non-infected group.

The effect of Obi-1 in prolonging the survival of chronically-infected mice after the administration of cyclophosphamide is shown in Figure 1. In experiment I (Fig. 1A), all mice in the non-infected control, non-infected-cyclophosphamide treated, and infected control groups survived until the last day of experimentation. With the infected cyclophosphamide-Obi-1 treated mice, one out of 5 died on the 28th day post-treatment (PT), while four out of five died between the 27th and 32nd day PT among the infected cyclophosphamide treated mice. In experiment 2 (Fig. 1B) two

100

IMMUNOSUPPRESSION IN MICE WITH CHRONIC TOXOPLASMOSIS

Table 1. Cell phenotype population ratios (I/N) in *T. gondii* (Beverley strain) inoculated mice

	pre-inoculation cell count	Days post inoculation 14 I/N*	28 I/N	162 I/N
Total cells (x 10^6)				
spleen	13.9	2.1	1.2	1.7
Liver	12.0	1.5	1.2	1.7
Blood	2.5	1.8	1.2	1.1
Thymus	27.3	0.3	0.3	0.7
Thy-1.2(+)cells (x 10^6)				
Spleen	3.9	2.2	1.3	1.9
Liver	5.0	1.5	1.3	1.7
Blood	1.3	1.9	1.2	1.1
Thymus	24.2	0.3	0.3	0.7
sIg(+) cells (x 10^6)				
Spleen	4.0	1.8	1.0	1.9
Liver	0.6	1.6	1.3	1.7
Blood	0.5	1.8	1.0	1.0
Lyt-1.2(+) cells (x 10^6)				
Spleen	2.6	2.5	1.5	1.8
Liver	5.0	1.8	1.5	2.0
Blood	0.2	2.0	1.3	1.0
Lyt-2,2(+) cells (x 10^6)				
Spleen	1.4	2.0	2.2	1.9
Liver	1.8	1.9	1.2	2.6
Blood	0.1	2.0	1.0	1.0
αGM1(+) cells (x 10^6)				
Spleen	1.0	3.0	2.3	1.7
Liver	0.4	2.6	2.2	2.0
Blood	0.1	2.0	1.0	1.0

* Number of cells in infected mice / Number of cells in non-infected mice

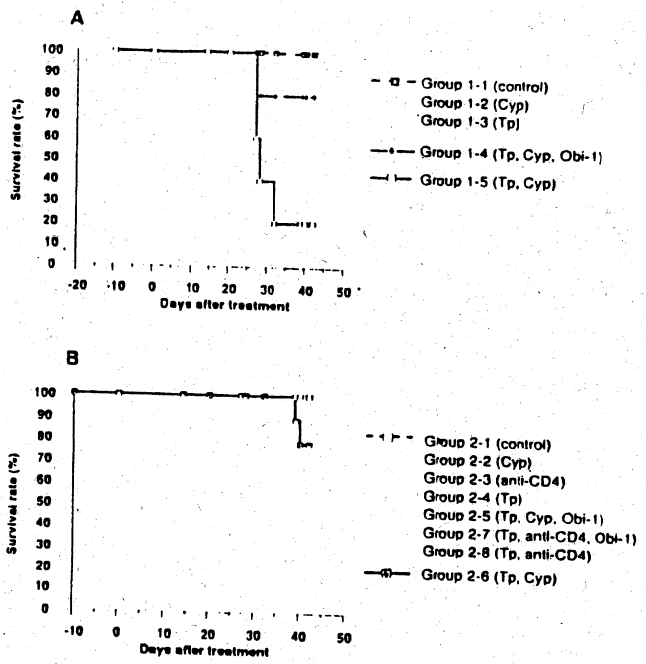


Figure 1. Survival of treated *T. gondii*-chronically infected mice. Mice exposed to $1x10^7$ parasites/head and at 28 days post-exposure were challenged with a similar dose. Cyclophosphamide (Cyp) treated at 7 days (Fig.1A) and at 10 days (Fig. 1B) interval. Obi-1 treatment (100ug/head) every 3, 7 days post-Cyp treatment.

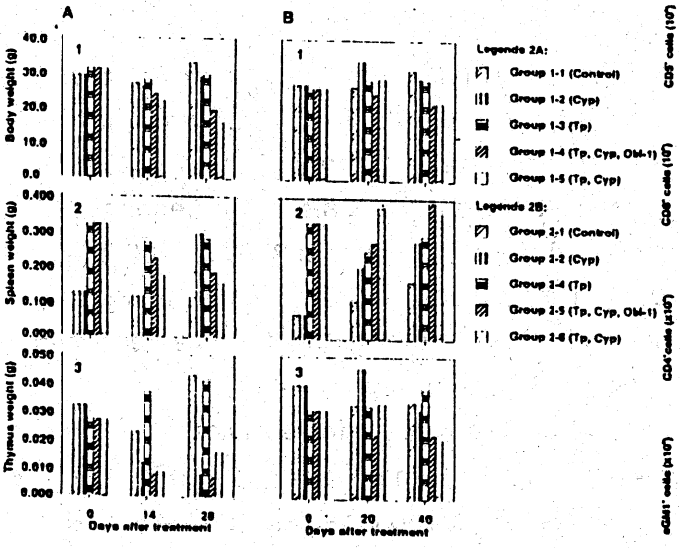


Figure 2. Changes in body (1), spleen (2) and thymus (3) weight of mice chronically infected with *T. gondii* and treated with cyclophosphamide (Cyp), or with Cyp and Obi-1. Treated with Cyp (250mg/kg) at 7 days interval, or with Obi-1 (100ug/head) every 3, 7 days after treatment with Cyp.

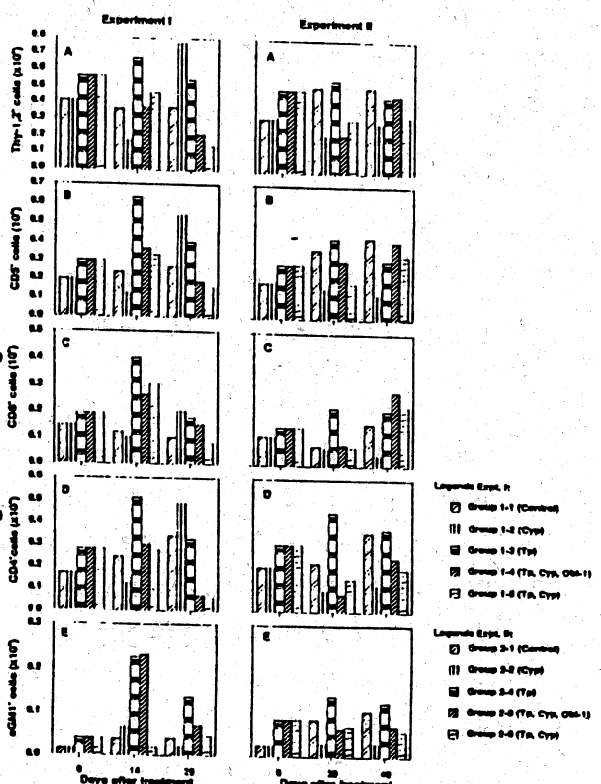


Figure 3. Number of spleen cells in *T. gondii* chronically infected mice treated with cyclophosphamide(Cyp), or with Cyp and Obi-1. Treated with Cyp (250mg/kg) at 10 days interval and with Obi-1 (100ug) every 3, 7 days after treatment with Cyp.

101

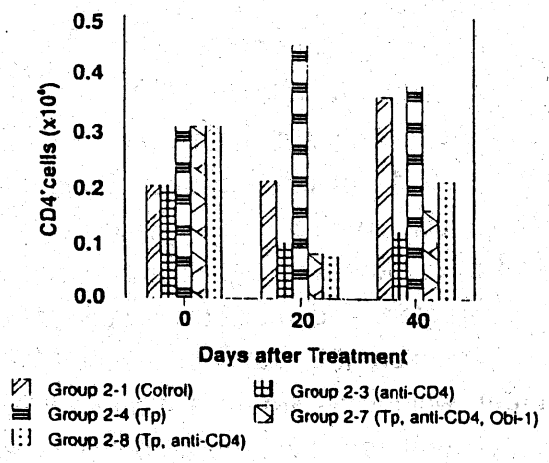


Figure 4. Number of CD4'cells in the spleen of mice chronically infected with *T. gondii* and treated with anti-CD4, or with anti-CD4 and Obi-1:
Treated with anti-CD4 (300ug/head) at 10 days interval and with Obi-1 (100ug/head) every 3, 7 days after treatment with anti-CD4.

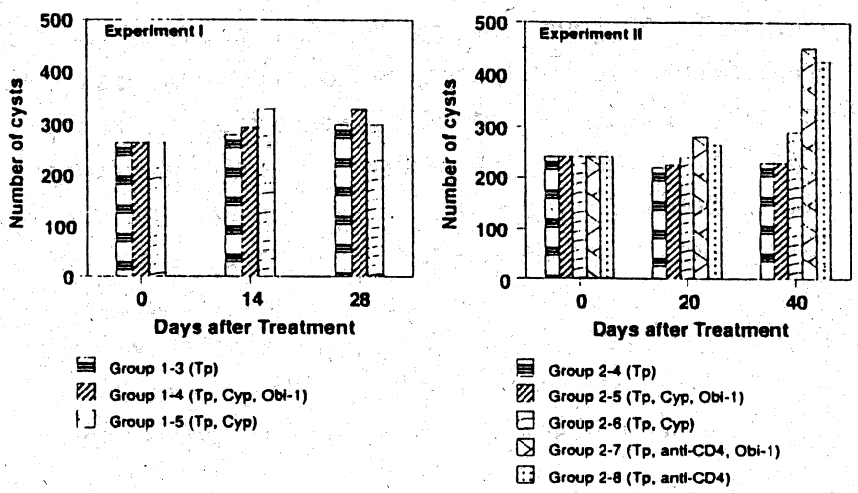
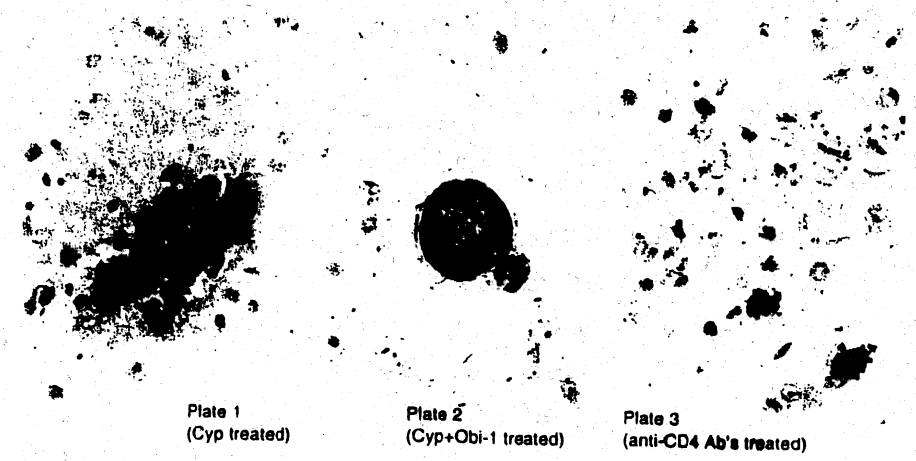


Figure 5. Number of cysts in mouse brain chronically infected with *T. gondii* and treated with cyclophosphamide(Cyp), with Cyp and Obi-1, with anti-CD4 or with anti-CD4 and Obi-1.
Experiment 1: Treated with Cyp(250mg/kg) at 7 days interval, and with Obi-1(100ug/head) every 3, 7 days after treatment with Cyp.
Experiment 2: Treated with anti-CD4(300ug/head), or Cyp(250mg/kg) at 10 days interval; Treated with Obi-1(100ug/head) every 3, 7 days after treatment with anti-CD4 or Cyp.



Parasites in brain tissues of mice stained with avidin-biotin-peroxidase.

out of five infected mice treated with cyclophosphamide only, succumbed to death on the 39th and 40th day PT ; while Groups 2-1, 2-5, 2-7 and 2-8 had a 100% survival.

Changes in the weight of body and immune organs of mice with chronic toxoplasmosis administered treatments are summarized in Figures 2A and 2B. Infected cyclophosphamide treated, and infected cyclophosphamide-Obi-1 treated mice exhibited weight loss (Figs. A1 & B1). Spleen weight of non-infected cyclophosphamide treated mice increased significantly on the 28th PT; while those of infected cyclophosphamide treated, and infected cyclophosphamide and Obi-1 treated exhibited spleen weight reduction (Fig. A2). In another experiment, however, we noted an increase in spleen weight in Groups 1-4 and 1-5 at 40 days PT. Mice that received cyclophosphamide treatment exhibited reduction in thymus weight compared to the non-treated groups (Fig. A3). A similar pattern is evident in Figure B3.

Figure 3 shows the changes in lymphocyte subpopulation counts in the spleen of *T. gondii*-chronically infected mice given cyclophosphamide and Obi-1 treatment. Results of experiment 1 (Fig. 3) gave consistently higher counts of Thy-1,2, CD5, CD8 and CD4- positive cells in Group 1-3, compared to all other groups at 14 days PT (Figs. A-D). At 28 days PT, Groups 1-4 and 1-5 showed a reduction in Thy-1,2, CD5, CD4, CD8 and aGM1-positive cells, demonstrating a state of immunosuppression. A similar pattern of cell subpopulation reduction was noted in experiment 2, at 20 days PT . At 40 days PT, however, cell counts increased in all groups except in Group 1-2.

The number of CD4- positive cells in mice administered anti-CD4 Ab's is shown in Figure 4. At 20 days PT, infected non-CD4 Ab treated mice showed a significant increase in CD4- positive cells; while those of the non-infected, and infected and Ab treatment exhibited a decrease in CD4- positive cells. A similar pattern was noted at 40 days PT.

Intracerebral cyst density is shown in Figure 5. In experiment 1 at 14 days PT, infected mice treated with cyclophosphamide manifested a marked increase in cysts count compared with the infected (control), and infected cyclophosphamide and Obi-1 treated mice. At 28 days PT, however, infected mice treated with cyclophosphamide and Obi-1 showed an increase in cyst density. In experiment 2, while no significant difference in cyst number was noted between groups at 20 days PT, a significant change in cyts density was observed 20 and 40 days among infected mice administered anti-CD4 Ab's, and among infected mice treated with anti-CD4 Ab's and Obi-1. In cyclophosphamide treated mouse, clusters of small cysts were observed in some brain regions and tachyzoites could hardly be found (Plate 1). However, in cyclophosphamide and Obi-1 treated mice, pathohistological detection of cysts revealed them to mainly possess clear cysts walls, and sometimes with dividing microcysts (Plate 2). Mice administered anti-CD4 Ab's were morphologically similar to those observed in mice treated with cyclophosphamide only. However, some

microcyst exhibited faint cyst wall with H&E. Degenerate cysts of varied size and small cysts in colonies , and some tachyzoite-like organisms were, likewise, observed with ABC staining (Plate 3).

Total leucocyte counts and the ratio of neutrophils demonstrate no marked difference between groups at 20 days PT. At 40 days PT, the non-infected cyclophosphamide treated mice had the lowest white blood cell (WBC) count and percent neutrophil, while those of infected mice treated with cyclophosphamide only , or cyclophosphamide and Obi-1, exhibited higher WBC count, and percent neutrophil, lymphocyte and monocyte ratios.

DISCUSSION

The suppression of all immunologically competent cells including T and B lymphocytes at the stage of DNA synthesis has been reported (Igarashi et al. 1990; Miyauchi et al. 1990; Schwartz et al. 1978; Turk et al. 1972). Ogawara et al. (1992) contend that the administration of cyclophosphamide at a dose of 250 mg/kg can cause rapid reduction of granulocytic and splenic cells and their disappearance in 3-5 days, returning to the normal level within 7 to 14 days, therafter. In the present study, the administration of cyclophosphamide to mice with chronic toxoplasmosis effected a state of depression, loss of body weight and reduction in Thy-1,2, CD5, CD8, CD4 and aGM1 positive cells, as well as atrophy of the thymus and spleen as a result of immunosuppression. In contrast, cyclophosphamide treated non-infected mice exhibited splenoma and a marked increase in Thy-1,2, CD5, CD4 positive cells and intrasplenic nucleated cells.

Intracerebral cysts from non-infected untreated, and non-infected treated groups exhibited numerous intracerebral cysts of similar large size, and with H&E and ABC staining, the cysts showed clear cyst walls. In the treated group, at 28 days PT, cyst density declined. Vollmer et al. (1987) and Mark et al. (1991) had documented the appearance of cysts of varied sizes and sometimes dividing cysts mainly in the brain, as well as the migration of tachyzoites in chronic mice treated with anti-CD4 Ab's. In our study, microscopic examination of brain tissue sections of chronic mice treated with anti-CD4 Ab's revealed faint cyst walls containing degenerate irregular cysts undergoing fission and clusters of small cysts. In infected cyclophosphamide treated mouse brain most of the cysts were of large size, nonetheless, in some regions there were degenerate irregular small cysts in clusters. However, tachyzoite migration was hardly observed. Furthermore, the non-infected control, and infected cyclophosphamide and Obi-1 treated groups showed typical intracerebral cysts with clear or sharp cyst walls, and sometimes mutiple dividing cysts of various sizes.

Monitoring the movement of intrasplenic T- lymphocyte subpopulations with the

use of their specific Ab's revealed a reduction in Thy-1,2, CD5, CD8, CD4 and aGM1-positive cells in non-infected mice as immunosuppression progressed with cyclophosphamide administration. In contrast, among chronically treated infected mice, there was no marked change in the values of lymphocyte subpopulations, however, the number of CD4 cells was significantly low, about half the density noted among clyclophosphamide untreated mice. Mice with chronic toxoplasmosis and administered cyclophosphamide exhibited loss of weight and depression. The authors tend to suppose that the rupture of intracerebral cysts in likely caused by a reduction in CD4 positive cells more than the effect of a reduction of all immunocompetent cells, including T and B- lymphocytes.

Obi-1 has been shown to inhibit T. *gondii* growth in cultured cells of bovine peripheral blood monocytes, mouse intraperitoneal macrophages and renal cells, human myocardial and cerebral cells, and exhibits a non-specific immunoregulating effect on tumours (Sakurai et al. 1982; Suzuki et al. 1990). In the present study, we noted a marked difference in survival rate between cyclophosphamide and Obi-1 treated, and Obi-1 untreated mice. The regular administration of cyclophosphamide to mice with chronic toxoplasmosis may not strongly activate the cysts to proliferate, and percent neutrophilic leucocytes, monocytes and lymphocytes in mice treated with Obi-1 were of higher values compared to those obtained from mice treated with cyclophosphamide only. These observations demonstrate the possible role of Obi-1 in the presence of cyclophosphamide, in prolonging the lifespan of chronically infected mice. From these results, we tend to think that the difference in host resistance and/or a difference in host life threshold limit against the fatal effect of acute toxoplasmosis could have been influenced by a leucocyte count below the normal lower threshold value, which is otherwise needed for survival. Fujii et al. (1992b) reported enhanced susceptibility of mice administered cyclophosphamide to bacteria and such susceptibility correlated clearly with a reduction in leucocytes. In the present study, the ratio of neutrophils and monocytes to total WBC count was highest among mice treated with cyclophosphamide and Obi-1. Fujii et al. (1992b) and Kono et al. (1992) have shown that the addition of Obi-1 does not only reverse the effect of leucocyte reduction due to cyclophosphamide, but Obi-1, also, accelerates phagocytic bactericidal activity by functioning as a hematopoeitic cell stimulating-like factor in a host under a state of immunosuppression. It can be speculated, moreover, that there could be a factor influencing the difference in effect between cyclophosphamide and Obi-1, thus influencing the increase in number and activation of neutrophils, lymphocytes and monocytes.

The administration of anti-CD4 Ab's to mice with chronic toxoplasmosis resulted to a reduction in CD4 positive cells in the spleen, irregular sized cysts undergoing fission, and small cysts in colonies scattered in the brain. Furthermore, the administration of

cyclophosphamide alone to chronic mice also caused a decrease in CD4- positive cells, however, immediately after fission, irregular sized cysts showed less obvious changes compared with those noted among anti-CD4 treated mice. As to how anti-CD4 Ab's cause the disruption and multiplication of intracerebral cysts, and as to what mechanism(s) is involved in reducing CD4 positive cells in the presence of cyclophosphamide or of any other immunosuppressive agents are questions that warrant further investigations.

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REFERENCES

Araujo, F. G. 1991. Depletion of CD4+ T lymphocytes prevents development of resistance to *Toxoplasma gondii* in mice. *Infect. Immun. 59*:1614-1619.

Fujii, Y., Maki, Y., Claveria, F. G., Igarashi, I., Saito, A., Ono, K. & Suzuki, N. 1992a. Effect of obioactin and obiopeptide on the toxoplasmacidal activities, glucose consumption and ruffle formation in mouse macrophages. *J. Vet. Med. Sci. 54*: 351-353.

Fujii, Y., Maki, Y., Ito, M., Saito, A., Igarashi, I., Ono, K., Itoh, K. & Suzuki, N. 1992b. Restorative effects of a newly synthesized peptide, obiopeptide-1 in cyclophosphamide or carrageenan-pretreated mice infected with opportunistic bacteria. *J. Protozool. Res. 2*: 74-83.

Igarashi, I., Shimada, T., Omata, Y., Claveria, F. G., Saito, A., Ono, K. & Suzuki, N. 1990. Difference in resistance between hetero and nude Rowett rats and BALB/c mice to *Babesia rodhaini* infection. *Allergy & Immunol. 9*: 95-103.

Kono, M., Fujii, Y., Shikano, S., Ono, K. & Suzuki, N. 1992. Influence of a newly synthesized peptide, Obiopeptide-1, as a biological response modifier (BMR) upon the lysosomal enzyme activities and chemotactic responses of mouse macrophages. *J. Vet. Med. Sci. 54*: 793-795.

Miyauchi, A., Hiramine, C. & Houjoh, K. 1990. Differential effects of a single dose of cyclophosphamide on T cell subset of the thymus and spleen in mice: Flow cytofluorometry analysis. *Tohoku J. Expt. Med. 162*: 147-167 (In Japanese with English summary).

Makim, T. F., Ricardo, G. T., Denkers, E., Hieny, S., Shearer, M. G. & Sher, A. 1991. CD8+ T cells from mice vaccinated against *Toxoplasma gondii* are cytotoxic for parasite-infected or antigen-pulsed host cells. *J. Immunol. 147*: 2310-2316.

Mark, H. J., Araujo, J. F. & Remington, S. J. 1991. Evaluation of the effect of drug on the cysts of *Toxoplasma gondii*. *J. Infect. Dis. 164*: 170-177.

Ogawara, T., Sakai, K., Sekikawa, T. & Matsumoto, Y. 1992. Lymphocyte subsets of regional lymph nodes and spleen after immunochemotherapy with OK-432 and cyclophosphamide. *Biotherapy. 6*: 697-699. (In Japanese with English summary).

Ricardo, G., Xu, Y., Hieny, S., Cheever, A. & Sher, A. 1991. Simulation depletion of CD4 and CD8 T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J. Immunol. 142*: 175-181.

Sakurai, H., Satoh. M., Hirose, T., Saito, A. & Suzuki, N. 1982. Microbicidal activity of hydrolyzed toxoplasma immune bovine serum in heterologous cell culture. *Jpn. J. Trop. Med. Hyg. 10*: 183-195.

Schwartz, A., Askenase, P. W. & Richard, K. G. 1978. Regulation of delayed type hypersensitivity reaction by cyclophosphamide-sensitized T cells. *J. Immunol. 118*: 1573-1577.

Stockman, D. G., Hein, R. L., South, A. & Trentin, J. J. 1973. Differential effects of cyclophosphamide on the B and T cell compartment of adult mice. *J. Immunol. 110*: 272-282.

Suzuki, N., Fujii, Y., Maki, Y., Sakurai, H., Igarashi, I. & Saito, A. 1990. In vitro cytocidal effect of novel synthetic obiopeptides 1, 2 and 3 on *Toxoplasma gondii*. *Allergy & Immunol. 9*: 149-158.

Suzuki, Y. & Remington, F. J. 1988. Dual regulation of resistance against *Toxoplasma gondii* infection by Lyt-2+ and Lyt-1+, CD4+ T cells in mice. *J. Immunol. 140*: 3943-3946.

Turk, J. L. & Poulter, L. W. 1972. Selective depletion of lymphoid tissue by cyclophosphamide. *Clin. Expt. Immunol. 10*: 285-289.

Vollmer, L. T., Walder, K. M., Steinman, L. & Conley, K. F. 1987. Depletion of lymphocytes with monoclonal antibody reactivates *Toxoplasma gondii* in the central nervous system: A model of superinfection in AIDS. *J. Immunol. 138*: 3737-3741.

Protective Immune Response of *Isospora felis*-Infected Mice against *Babesia microti* Infection

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ABSTRACT. Protective response against *Babesia microti* was studied in *Isospora felis*-infected mice. *Isospora felis*-infected mice which were exposed to *B. microti* on the 28th day post-infection showed absolute resistance against *Babesia microti*. Interestingly, these mice showed no anti-*B. microti* antibodies. Mice that received spleen cells from *I. felis*-infected donors that were subsequently exposed to *B. microti* showed lower peak parasitemia (10.3% ± 2.6) compared to those mice that received normal spleen cells (60.9% ± 15.0), and no spleen cells at all (47.3% ± 8.5). Treatment of *I. felis*-infected mice with monoclonal antibodies against L3T4$^+$ cells resulted to a depression of their resistance to *B. microti*, as clearly manifested by high levels of parasitemia. Findings of the present study demonstrate the role of cell mediated immunity, specifically by L3T4$^+$ T-cells induced by *I. felis* infection, in providing mice protection against *B. microti*.—KEY WORDS: *Babesia microti*, immune response, *Isospora felis*, mouse, natural resistance.

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Isospora felis, a feline coccidian parasite, is commonly found in feces of domestic and stray cats. In mammals, this parasite can penetrate and survive in extra-intestinal organs [5]. One of the possible modes of transmission of *I. felis* in cats is the ingestion of infected rodents, as intermediate hosts. Experimental studies of *I. felis* infection in rodents are wanting, probably because of the absence of pathogenicity in the intermediate hosts, and due to the work's low economic value. Nevertheless, the investigation of immune responses to *I. felis* infection in intermediate hosts is necessary to gain additional knowledge of its transmission mechanism, and to attract epidemiologic interest.

Coxiella burnetii, Nematospiroides dubius (N. dubius) and other species have been shown to induce non-specific immunity to *Babesia microti* infection in mice [2, 6]. Although, the precise defence mechanisms are still unknown, natural killer cells and/or macrophages are believed to interfere with the parasite development within host erythrocytes [6–7].

In the present study, we examined the immune response of *B. microti* infection in mice experimentally inoculated with *I. felis* oocysts. Specifically, we sought to determine whether *I. felis* infection in mice can stimulate the host immune system to induce protective immune reactions against *B. microti*.

MATERIALS AND METHODS

Oocysts of *I. felis* were obtained from cats' feces using $ZnSO_4$ flotation method, followed by sucrose density gradient centrifugation [1], and stored at 4°C in phosphate buffered saline (PBS). Absence of other coccidian oocysts was microscopically confirmed. Some oocysts were hatched by incubation in PBS supplemented with 0.5% taulochoilic acid and 0.25% trypsin at 37°C for 30 min.

Babesia microti parasitized red blood cells (*B. microti* PRBC) obtained from Dr. O. A. Heydon, Berlin Free University, were prepared from infected mice (0.6–0.8 m*l* blood with a hematocrit of 20–35% and a parasitemia of 50–70%) on the 7th–10th day post infection (p.i.) by heart puncture in heparinized PBS at 4°C, then were washed with PBS three times by centrifugation at 1,200 g for 10 min at 4°C.

Male and female BALB/c and ICR mice, 5–7 weeks of age were used in all experiments. Each group consisted of four mice which were infected perorally with 5×10^5 oocysts of *I. felis*. On the 7th and 28th day p.i., they were challenged intraperitoneally with 1×10^6 *B. microti* PRBC. Thereafter, survival of exposed mice was monitored. Percent parasitemia per mouse was examined at one day interval beginning on the 3rd and ending on the 21st day p.i. Level of parasitemia was determined by counting the number of PRBC per 400 cells in tail-blood thin smears stained with Giemsa.

Anti-*I. felis* and anti-*B. microti* IgG titers in sera were measured by indirect immunofluorescence antibody test (IFAT). Test sera were obtained weekly from each mouse and stored at −20°C until use. Serum samples were diluted four-fold in PBS and mounted on glass slides coated with *B. microti* PRBC, and incubated at 37°C for 30 min, washed in PBS for 10 min, then reacted with FITC-conjugated anti-mouse IgG diluted in PBS, at 37°C for 30 min. Anti-*I. felis* IgG antibody titers in the same sera samples were performed following similar procedure.

Spleen cells from either *I. felis* non-inoculated or inoculated BALB/c mice were suspended in RPMI 1640 medium at a concentration of 5×10^6/m*l*. Four BALB/c

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mice were intravenously injected 10^6 spleen cells of *I. felis* infected mice, and another four recipient mice received a similar inoculum of spleen cell suspension from *I. felis* non-infected donors. For the control group, four mice were not injected spleen cells. These three groups of mice were inoculated with 10^6 *B. microti* PRBC on the same day of spleen cell transfer. Thereafter, the level of parasitemia in each mouse was checked. To establish the absence of *I. felis* in the spleen cell suspension, two C57BL/6 mice were also inoculated with the same inoculum dose of spleen cells. Negative anti-*I. felis* antibody titers were noted in these mice on the 30th day post-spleen cells transfer.

Anti-mouse L3T4 monoclonal antibody (mAb) (rat hybridoma GK-1, 5) and anti-mouse Lyt 2, 2 mAb (rat hybridoma 53–6,72) were purified using 33% ammonium sulfate precipitation method. Each of the *I. felis*-infected mice on the 28th day p.i. were depleted of T-cell subset through inoculation with 0.5 mg/0.25 m*l* of anti-L3T4 mAb or anti-Lyt2,2 mAb intraperitoneally for three successive days prior to challenge with 10^6 *B. microti* PRBC. Treatment with mAb was continued for the next 27 days post-challenge with *B. microti* at two days interval. Depletion of L3T4 or Lyt2,2 positive ($L3T4^+$, $Lyt2,2^+$) cells in mouse spleen was monitored by fluorocytometric analysis using FITC-conjugated mAbs.

RESULTS

As shown in Fig. 1a, *B. microti*-infected mice which were inoculated with *I. felis* on the 7th day p.i. showed a peak parasitemia of 29.8% ± 6.48 on the 11th day *B. microti* p.i. The control group, likewise, registered a peak parasitemia of 42.5% ± 0.7 on the same day. BALB/c mice inoculated with *I. felis* and then exposed to *B. microti* on the 7th day p.i. also showed lower parasitemia compared to that of the control (Fig. 1b). Interestingly, *I. felis*-inoculated mice which were exposed to *B. microti* PRBC on the 28th day *I. felis* p.i. registered absolute resistance to the establishment of *B. microti* infection (Fig. 1c). With ICR mice, we noted less than 1.0% and 60.0% parasitemia in the experimental and control groups, respectively (Fig. 1d). Also, ICR mice showed anti-*I. felis* antibody IgG titer of 4^3. Anti-*B. microti* antibody IgG was detected on the 7th day post-inoculation with *B. microti*.

Figure 2 shows mice that received spleen cells from *I. felis*-infected mice had lower peak parasitemia (10.3% ±

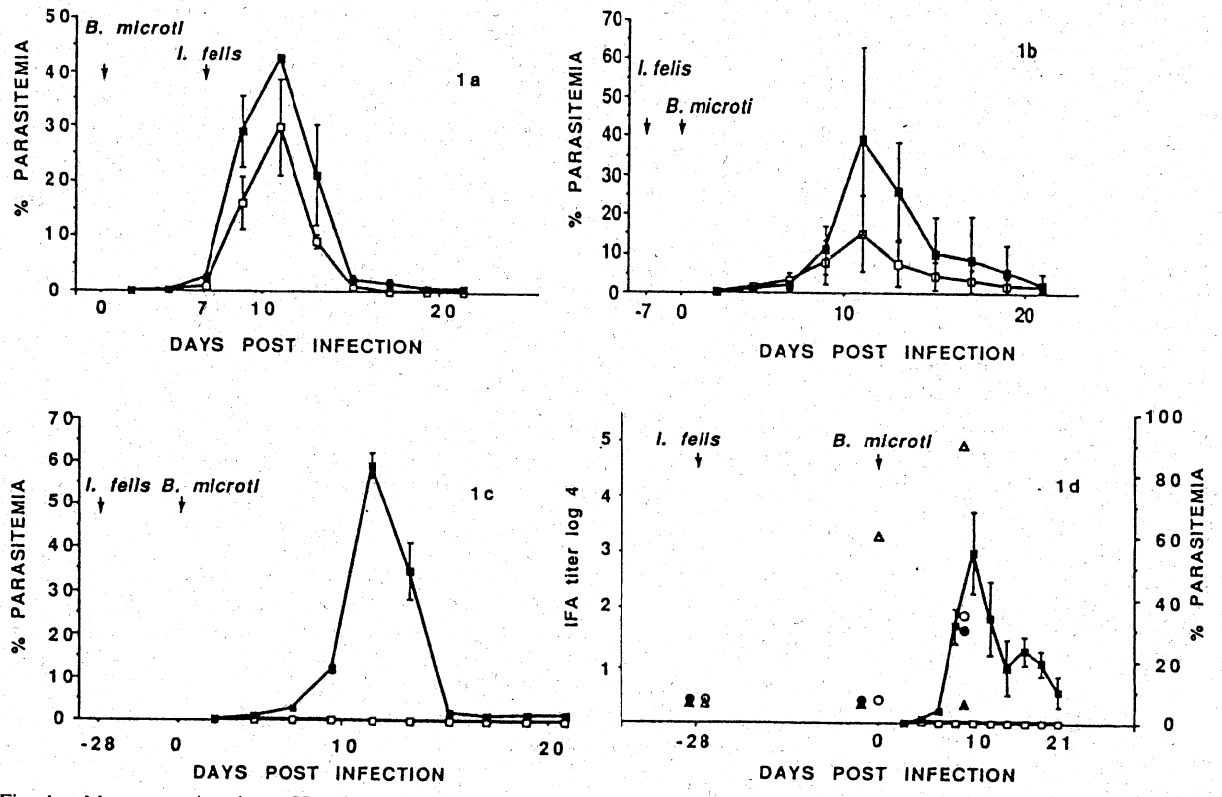

Fig. 1. Mean parasitemia ± SD of *B. microti* infected mice. ■: *B. microti* exposed only. Bar: standard deviation per group samples. 1a. □: BALB/c mice infected with *B. microti* and inoculated with *I. felis* 7 days post-infection. 1b. □: BALB/c mice inoculated with *I. felis* and then exposed to *B. microti* 7 days post-inoculation. 1c. □: BALB/c mice inoculated with *I. felis* and exposed with *B. microti* 28 days post-inoculation. 1d. □: ICR mice inoculated with *I. felis* and exposed with *B. microti* at 28 days post inoculation. IFA titer of anti-*B. microti* IgG (○) and anti-*I. felis* IgG (△) in *I. felis*-infected mice; anti-*B. microti* IgG (●) and anti-*I. felis* IgG (▲) in control.

2.6) on the 11th day *B. microti* p.i., compared to those that received normal spleen cells (60.9% ± 15.0) and to those mice that received no spleen cells at all (47.3% ± 8.5).

The effects of *in vivo* treatment with anti-L3T4 mAb or anti-Lyt 2,2 mAb on the resistance pattern of *I. felis*-infected mice against *B. microti* are shown in Figure 3. The treatment of *I. felis*-infected mice with anti-L3T4 mAb (Fig. 3a) resulted in a depressed resistance against *B. microti*, as evidenced by severe parasitemia. Two of the four mice in this group had about 85% parasitemia on the 13th day post challenged and was sustained at 50% parasitemia until the end of the experiment. One mouse also showed high parasitemia at about 70%, on the 23rd day post-challenged. In comparison, *I. felis*-infected mice inoculated with anti-Lyt 2,2 mAb (Fig. 3b), with normal rat IgG (Fig. 3c), or without-inoculation (Fig. 3d) either had significantly low parasitemia or no parasitemia until the end of the observation period. Using fluorocytometric analysis, the treatment of mice with either anti-L3T4 mAb or anti-Lyt 2,2 mAb resulted in more than 90% of the restricted depletion of the corresponding population (data not shown).

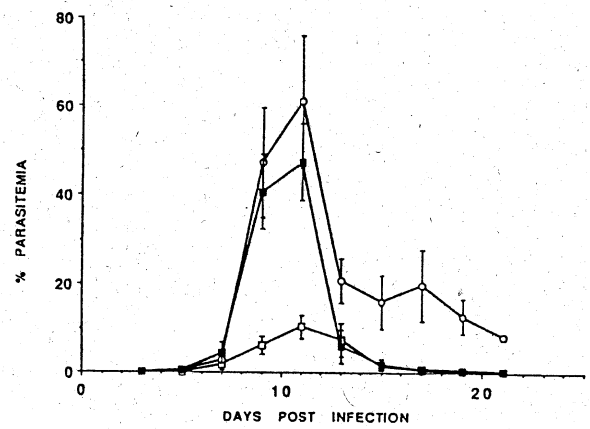

Fig. 2. Effect of adoptive transfer of spleen cells from *I. felis*-inoculated BALB/c mice against *B. microti* challenge. ○: non-inoculated. ■: inoculated with spleen cells of normal mice. □: inoculated with spleen cells from *I. felis*-infected BALB/c mice.

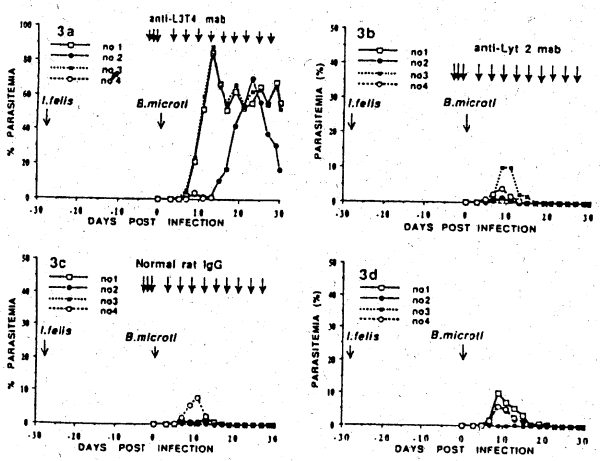

Fig. 3. Course of *B. microti* infection in *I. felis* inoculated mice treated with either anti-L3T4mAb (Fig. 3a), anti-Lyt2,2 mAb (Fig. 3b), normal rat IgG (Fig. 3c) or non-treated (Fig. 3d). Nos. 1, 2, 3 and 4 are mice designations.

DISCUSSION

Despite the numerous findings of the existence of natural resistance of hosts to babesiosis microti, the precise defence mechanism underlying such phenomenon remains controversial, to date. Mzembe *et al.* [6] reported the role of macrophages in suppressing the growth of *B. microti* in *N. dubius*-infected mice by releasing soluble factors which show parasiticidal effect when tested *in vitro*. On the contrary, macrophages obtained from *Schistosoma mansoni*-infected mice failed to suppress the *in vitro* growth of *B. microti* [4]. Studies by Ruebush and Burgess [7] on the determination of interferon-r (INF-r)levels and parasitemia in experimental *B. microti* infection in beige mice provided valuable information that led them to suggest the probable contribution of NK cells in the expression of host natural resistance.

In the present study, infection of mice with *I. felis* induced significant host protection against *B. microti* infection. Interestingly, we found no cross reactive antibodies with *B. microti*, thus indicating no apparent participation of humoral antibodies. Likewise, results of spleen cells transfer and the control of T-cell subsets with mAb's revealed the importance of spleen cells, specifically the L3T4$^+$ cells in providing protection against *B. microti*. Our findings strongly demonstrate that non-pathogenic parasites such as *I. felis* has the capability to stimulate host's immune response.

While L3T4$^+$ cells are generally associated with production of INF-r, tumor necrotic factor (TNF), IL-2 and of other lymphokines in the presence of antigens and induction of activation of macrophages, NK cells or L3T4$^+$ own cells through lymphokines, we have no data to explain the role of L3T4$^+$ cells in the present study. Thus, studies related to the interaction between L3T4$^+$ cells and effector cells are necessary to understand the mechanism(s) underlying natural resistance of mice against *B. microti*.

REFERENCES

1. Arrowood, M. J. and Sterling, C. R. 1987. Isolation of *Cryptosporidium* oocyst and sporozoites using discontinuous sucrose and isopycnic percoll gradients. *J. Parasitol.* 73: 314–319.
2. Clark, I. A. 1979. Resistance to *Babesia* spp. and *Plasmodium* sp. in mice pretreated with an extract of *Coxiella burnetii*. *Infect. Immun.* 24: 319–324.
3. Eugui, E. M. and Allison, A. C. 1982. Natural cell-mediated immunity and interferon in malaria and babesia infections. pp. 1491–1502. *In*: NK Cells and Other Natural Effector Cells (Herberman, R. B. ed.), Academic Press, New York.

4. Kurtzhals, J., Andersen, B. T., and Christensen, N. 1988. Effects of *in vitro* growth of *Babesia microti* by cells and serum from *B. microti* and *Schistosoma mansoni* infected mice. *Acta Vet. Scand.* 29: 357–362.
5. Levine, N. D. 1985. Veterinary Protozoology. p. 208. IOWA State University Press.
6. Mzembe, S. A. T., Lioyd, S., and Soulsby, E. J. L. 1984. Macrophage mediated resistance to *Babesia microti* in *Nematospiroides dubius*-infected mice. *Z. Parasitenkd.* 70: 753–761.
7. Ruebush, M. J. and Burgess, D. E. 1982. Induction of natural killer cells and interferon production during infection of mice with *Babesia microti* of human origin. pp. 1483–1489. *In*: NK Cells and Other Natural Effector Cells (Herberman, R. B. ed.), Academic Press, New York.

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Correlation between antibody levels in *Toxoplasma gondii* infected pigs and pathogenicity of the isolated parasite

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Abstract

Sera and diaphragm muscle tissues were obtained from 109 commercial pigs between September 1991 and May 1992 from the slaughterhouse at La Plata, Provincia Buenos Aires, Argentina. Anti-*Toxoplasma gondii* IgG antibody reactivity to *T. gondii* antigens were assayed using sera by indirect immunofluorescence assay and immunoblotting technique. Anti-*T. gondii* IgG titers at serum dilutions of 1:1024 and higher were noted in 11.0% of the tested sera, and at dilutions of 1:16 and lower in 36.7% of the serum samples. Using mouse inoculation test, *T. gondii* was isolated from 14 pig diaphragm samples. Of five samples derived from pigs with antibodies at dilutions of 1:1024 and higher, four contained trophozoites which, when inoculated into mice intraperitoneally, killed all recipient hosts within 15 days post inoculation. Parasites detected in seven out of eight samples from pigs with antibodies at serum dilutions of 1:64 and lower formed cysts in the brain, and mice survived longer than 13 days post inoculation. Immunoblotting demonstrated antibody reactivity in pig sera samples with relatively high titers for parasite antigens. Results of the present study suggest that antibody production in infected pigs is apparently dependent on the pathogenicity of the parasite strain.

Introduction

The intracellular protozoan parasite *Toxoplasma gondii* causes not only congenital infection, but also intensive encephalitis in immunocompromised individuals, especially in AIDS patients. In Argentina, approximately 30–40% of adult women are serologically *Toxoplasma* positive (Lazarte et al., 1988; Sortino et al., 1988). The survey of *T. gondii* infection in domestic animals is of epidemiologic importance considering that one source of *T. gondii* infec-

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1/12

tion is eating raw or uncooked infected meat. Epidemiological investigations using serological tests in domestic animals in Mendoza, Santa Fe and Cordova, Argentina, have been reported (Wynne de Martini and Martin, 1977; Benvissuto et al., 1988; Pizzi et al., 1988).♦1 In the present study, we examined pigs from a slaughterhouse in Provincia Buenos Aires, for serological and parasitological evidence of *T. gondii* infection. We determined the IgG antibody titers and the pathogenicity of the isolated parasites to mice.

Materials and methods

Male and female ICR mice, 5–7 weeks of age, were used in all experiments.

Toxoplasma gondii parasites of RH strain were obtained from mouse embryonal cell culture, and were isolated using Percoll–sucrose density gradient centrifugation at 22 000×*g* for 20 min. Parasites which floated at the 1.112 gravity interface were collected, centrifuged at 2000×*g* for 10 min and washed three times in phosphate buffered saline (PBS).

From September 1991 to May 1992, sera and diaphragm muscle tissues, chosen randomly at the La Plata slaughterhouse, where pigs were kept from unlimited livestock producing centers in Argentina, were obtained from 109 adult pigs (body weights ranged from 90 to 110 kg). Serum samples were stored at −20°C until use. Diaphragm muscle tissue samples were stored at 4°C and examined within 48 h post sampling. Titers of anti-*T. gondii* antibodies were determined by indirect immunofluorescence assay test (IFAT) as described elsewhere (Omata et al., 1990). Briefly, the test serum was serially diluted in PBS, placed on tachyzoites-coated glass slides and incubated at 37°C for 30 min. The coated glass slides were washed with PBS, and then reacted with goat anti-pig IgG (Kirkegaard & Perry Laboratories, MD, USA), and incubated with FITC-conjugated rabbit anti-goat IgG antibody (Sigma, St. Louis, MO, USA). The recognition of a fluorescence pattern on the parasite surface was scored as a specific reaction.

Immunopredominant parasite antigens detected by anti-*T. gondii* IgG antibody of pigs were examined by immunoblotting following the method of Towbin et al. (1979). Ten million parasites of RH strain was extracted in 500 μl sample buffer (4.6% sodium dodecyl sulfate 12.5 mM Tris–HCl (pH 6.8), 10% 2-mercaptoethanol) by incubation at 100°C for 5 min, followed by ultrasonication and centrifugation at 12 000×*g* for 5 min. The supernatant was electrophoresed in 12.5% gel SDS–PAGE and the separated proteins were electrophoretically transblotted on membrane sheets (GVHP, Nihon Millipore, Yonezawa, Japan). Thereafter, the membrane sheets were blocked in PBS containing 5% non-fat skim milk (skim milk–PBS) at room temperature for 1 h, then washed in PBS containing 0.05% Tween-20. Each test serum was diluted in 50-fold volume of skim milk–PBS. Membranes were incubated with pig sera at room temperature for 2 h and then followed by incubation in 200-

fold diluted peroxidase-conjugated goat anti-pig IgG antibody (Sigma, St. Louis, MO, USA) at room temperature for 1 h. Peroxidase reaction was visualized using 0.02% diaminobenzidine–4HCl and 0.01% H_2O_2 in 0.1 M Tris–HCl buffer, pH 7.4. Molecular weight estimates were based on SDS–PAGE molecular weight standard low range (Bio-Rad Laboratories, Richmond, VA, USA).

Parasites in the meat samples were detected by the mouse inoculation method. Thirty grams of each meat sample were minced and homogenized in a stainless steel blender (Nihon Seiki, Tokyo, Japan) and suspended in pepsin–HCl solution as described by Dubey (1981). After incubation at 37°C for 1 h, the homogenates were washed three times with PBS containing 10^4 units of potassium-G penicillin and 100 mg of streptomycin in 100 ml) to remove pepsin–HCl by centrifugation at 2000×*g* for 10 min. The homogenate was resuspended in PBS and 1.0 ml of the suspension was inoculated intraperitoneally into two mice (0.5 ml per mouse). Exposed mice were monitored for 28 days post-inoculation (p.i.). Mice that died during the monitoring period were examined microscopically for the presence of *T. gondii* in the peritoneal exudates and in the brain. Those mice that survived were examined for anti-*T. gondii* antibody levels by IFAT using FITC-conjugated rabbit anti-mouse IgG antibody (Organon Teknika Corp.-Cappel Products, Westchester, PA, USA) and for the presence of *T. gondii* in the brain as previously described.

Results

Anti-*T. gondii* IgG antibody titers and incidence of persistent *T. gondii* infection in 109 serum samples were assayed. Twelve of the 109 serum samples tested had significantly high anti-*T. gondii* antibody titers at dilutions of 1:1024 to 1:16 386, and 57 of the serum samples were positive for IgG antibodies against *T. gondii* at dilutions of 1:64 to 1:256. Antibodies at dilutions of 1:16 and lower were found in 36.7% of all tested sera. *Toxoplasma gondii* parasites were isolated in only 14 out of 109 pig diaphragm tissue samples (12.8%). Parasites were detected in five out of 12 pigs that showed anti-*T. gondii* antibodies at dilutions of 1:1024 and higher in seven out of 57 pigs positive for antibodies at dilutions of 1:64 to 1:256, and in two out of 10 pigs at dilutions of 1:16 and lower.

As shown in Table 1 mice inoculated with pig tissue sample Nos. 35, 31, 36, 39 and 67 derived from pigs with high antibody titers except No. 67, died on Days 9–14 p.i. due to unrestricted multiplication of trophozoites in the peritoneal cavity. Likewise mice inoculated with sample Nos. 65, 66, 60, 84 and 5 derived from pigs with low antibody titers (1:16 to 1:64, except for No. 65) died between Days 13 and 20 p.i. showing numerous cysts in the brain. Mice inoculated with sample Nos. 38, 40, 85 and 63 derived from pigs

Table 1
Pathogenicity of *T. gondii* isolated from pigs through mouse inoculation method

Pig sample no.	Pig antibody titer	Mouse survival period (days p.i.)[a]	Mouse *T. gondii* stage detected (antibody IgG titer)[b]
35	16000	9	Troph
65	16000	15	Cyst
31	4096	10	Troph
36	1024	9	Troph
39	1024	14	Troph
38	256	28	Cyst (4096)
67	64	12	Troph
66	64	14	Cyst
60	64	15	Cyst
40	64	28	Cyst (4096)
85	64	28	Cyst (16000)
63	16	28	– (4096)
84	16	13	Cyst
5	16	20	Cyst

[a]Post-inoculation.
[b]Antibody titer in inoculated mice was examined on the 28th day p.i. Cyst, detected in the brains of the inoculated mice; Troph, detected in the peritoneal cavity of the inoculated mice; –, non-detected.

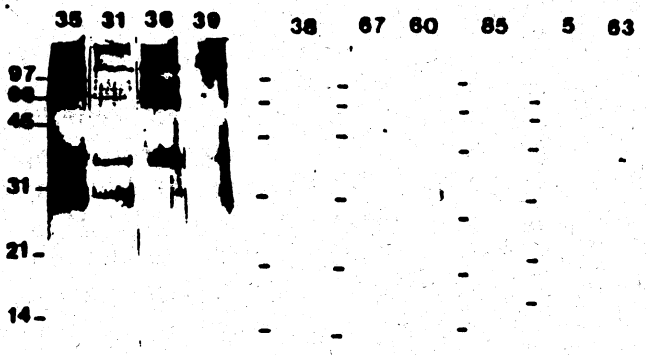

Fig. 1. Immunoblotting: *T. gondii* antigens reacted with pig serum samples. Numbers on top of lanes indicate pig serum sample number. Numbers on the left side represent molecular masses in kilodaltons (kDa).

with low antibody titers (1:16 to 1:64, except for No. 38) survived the entire monitoring period without any clinical manifestations of infection. These mice had significantly high anti-*T. gondii* antibody titers and numerous cysts in the

brain except for No. 63.

The immunoblotting reactivity of anti-*T. gondii* IgG antibodies with the parasite antigens is shown in Fig. 1. Serum samples Nos. 35, 31, 36 and 39 exhibited strong reactivity to several antigens of molecular masses ranging from approximately 120 to 29 kDa. Likewise serum samples Nos. 38, 67, 60 and 85 showed reactivity to some antigens of molecular masses of approximately 120 to 14 kDa. No specific bands were noted in serum samples Nos. 5 and 63, or in sera obtained from pigs that showed no development of infection in mice (data nut shown).

Discussion

Wynne de Martini and Martin (1977) reported 22% of porcines in Buenos Aires as *Toxoplasma* positive. Benvissuto et al. (1988) and Pizzi et al. (1988) also noted 36–38% of the pigs in Provincia Santa Fe and Cordoba as positive for toxoplasmosis. Our data likewise reveal approximately 40% of pigs infected with *T. gondii* during upkeep, and about 15.7% of the examined pigs had latent and/or persistent infection as evidenced by the results of the mouse inoculation test. The present study, likewise, indicates the risk of *T. gondii* transmission during the period of rearing and upkeep of pigs in Argentina. Most of the farms are open ranges, with negligible improvements for the control and prevention of infectious diseases during the past 20 years. Under the present conditions, contamination of farms with oocysts from infected cats and/or the presence of infected rodents or other animals in swine houses must be considered as a possible major sources of infection. In order to protect animals from contacting toxoplasmosis, preventive measures similar to those employed in Japan, such as keeping the animals in barns equipped with barriers, are thus necessary.

It is of interest to note that some of the pigs from which *T. gondii* was isolated had lower titers of anti-*T. gondii* antibodies and showed no clinical symptoms. *Toxoplasma gondii* is generally isolated from serologically positive rather than from sero-negative pigs; nonetheless, findings are consistent with those reported by Beverley et al. (1978). In our study, the possibility of acute infection in animals with low antibody titers cannot be excluded. Diaphragm samples were treated with pepsin–HCl solution, and according to Dubey (1981), trophozoites which multiply in the acute phase loose their viability when treated with this method. Verhofstede et al. (1988) have reported reactivity of anti-*T. gondii* IgG to the parasite antigens as being related to the infection stage. Immunoblotting patterns of samples Nos. 5 and 63 showed, however, less reactivity to *T. gondii* antigens. In our study, the isolated parasites from sero-negative pigs showed a tendency to have relatively lower pathogenicity in mice compared with isolated from serologically positive pigs. It must be realized, however, that the pig's antibody response is very

weak, and thus, animals with low or even negative *T. gondii* antibody titers may be infected with parasites (Oliver et al., 1983; Prickett et al., 1985; Takahashi and Konishi, 1986).

Acknowledgments

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References

Benvissuto, G., Pizzi, H. and Carballada, H., 1988. Estudio serologico de toxoplasmosis en porcinos provincia de Santa Fe. ano 4, 2/5, 20 pp.♦2

Beverley, J.K., Henry, L. and Hunter, D., 1978. Experimental toxoplasmosis in young piglets. Res. Vet. Sci., 24: 136–146.

Dubey, J.P., 1981. Pepsin digestion of meat for detection of *Toxoplasma gondii* cysts. Am. J. Vet. Res., 42: 128–130.

Lazarte, E., Lazarte, J. and Benvisutto, G., 1988. Investigacion serologica para toxoplasmosis en dadores de sangre hospital nuestra senora del valle de la misericordia Cordoba-Argentina.♦3

Oliver, D.G., Dreesen, D.W., Prickett, M.D., Waltman, W.D., Poy, D.S. and Blue, J.L., 1983. Comparative study of enzyme linked immunoassay with standard serological tests for the diagnosis of toxoplasmosis in swine. Proc. Intern. Symp. Wld. Assoc. Vet. Lab. Diagnosticians, pp. 163–170.♦4

Omata, Y., Oikawa, H., Kanda, M., Mikazuki, K., Nakabayashi, T. and Suzuki, N., 1990. Experimental feline toxoplasmosis: Humoral responses of cats inoculated orally with *Toxoplasma gondii* cysts and oocysts. Jpn. J. Vet. Sci., 52: 865–867.

Pizzi, H., Bianchini, N., Barrionuevo, L., Ongini, R. and Robledo, S., 1988. Estudio serologico de toxoplasmosis en porcinos de la provincia de Cordoba. Revista de la Fundacion Argentina para el estudio de la toxoplasmosis. ano 4, 2/5, 8 pp.♦5

Prickett, M.D., Dresen, D.W., Waltman, W.D., Blue, J.L. and Brown, J., 1985. Correlation of tissue infection and serologic findings in pigs fed *Toxoplasma gondii* oocysts. Am. J. Vet. Res., 46: 1130–1132.

Sortino, H., Buchacra, O., Elias, P. and Durigutti, M., 1988. Estudio serologico de toxoplasmosis en embarazadas de la ciudad de San Martin, Provincia de Mendoza Argentina.♦3

Takahashi, J. and Konishi, E., 1986. Quantitation of antibodies to *Toxoplasma gondii* in swine sera by enzyme-linked immunosorbent assay. J. Immunoassay., 7: 257–272.

Towbin, H., Staehelin, T. and Gordon, J., 1979. Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheet: procedure and some applications. Proc. Natl. Acad. Sci. USA, 76: 4350–4354.

Verhofstede, V., Gelder, V.P. and Rabaey, M., 1988. The infection-stage-related IgG responses to *Toxoplasma gondii* studied by immunoblotting. Parasitol. Res., 74: 516–520.

Wynne de Martini, G.J. and Martin, A.M., 1977. Prueba de hemoaglutinacion para toxoplasmosis en distintos sueros animales. Rev. Med. Vet., 58: 437–439.

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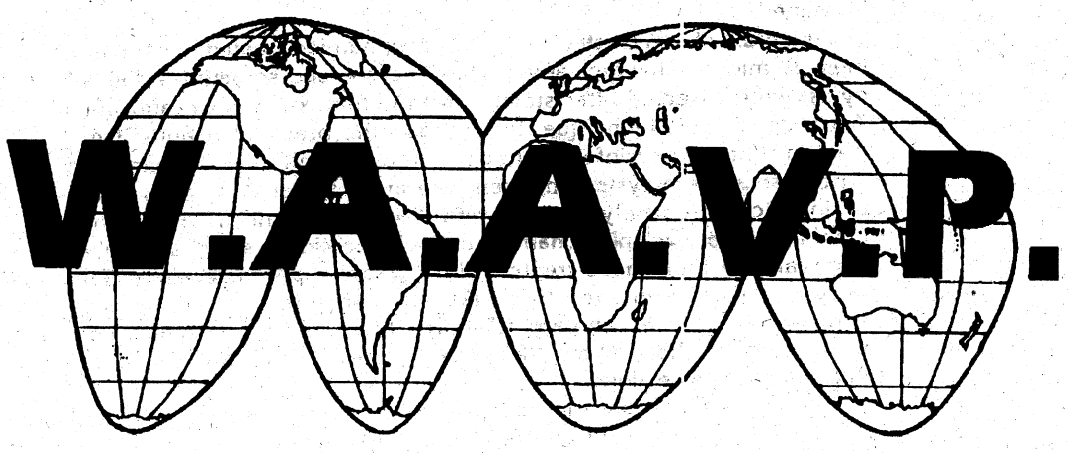

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Understanding and control of parasitic diseases of animals

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THE EFFECT OF IMMUNOSUPPRESSION CAUSED BY CYCLOPHOSPHAMIDE TO MICE CHRONICALLY INFECTED WITH *TOXOPLASMA GONDII*

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Mice chronically infected with *Toxoplasma gondii*, were treated with cyclophosphamide or anti-CD4 monoclonal antibody to identify the effect of these immunosuppressive reagents on the cysts in the brain of infected mice. The effect of Obiopeptide-1 as an immunomodulator treatment of the immunosuppressed mice was also examined.

In the brain of non-treated and cyclophosphamide-treated, chronically infected mice mainly typical large tissue cysts, and sometimes divided cysts, were detected after staining with hematoxylin-eosin and anti-*Toxoplasma* ABC technique. In contrast, the brain from anti-CD4 - treated, chronically infected mice, contained multiple degenerated *Toxoplasma* tissue cysts of different size in some partial regions in the brain. Mice chronically infected with *Toxoplasma* and treated with a combination of cyclophosphamide and Obiopeptide-1 showed a higher survival rate than those treated with cyclophosphamide alone. The percentage of neutrophilic leukocytes in mice treated with a combination of Obi-1 and anti-CD4, or Obi-1 and cyclophosphamide, was higher than that of mice treated with anti-CD4 or cyclophosphamide alone.

These results indicate that reactivation or rupture of tissue cysts in mice treated with cyclophosphamide, chronically infected with *Toxoplasma*, might be mainly mediated by CD4 positive cells rather than other immunocomponent cells. The increase of neutrophilic leukocytes might contribute to the induction of the resistance to *Toxoplasma gondii* in mice, after treatment with Obi-1 and cyclophosphamide in combination.

モダンメディア　39巻12号 '93〔人畜共通感染症〕581

トキソプラズマ症の感染と免疫

Infection and Immunity in toxoplasmosis

五十嵐郁男：鈴木直義
Ikuo IGARASHI　Naoyoshi SUZUKI

はじめに

トキソプラズマ原虫 *Toxoplasma gondii* は1908年，チェニジアの首都チェニスにあるパスツール研究所で Nicolle & Manceaux によりリーシュマニア症などの実験に使用されていたヤマアラシの1種（*Ctenodactylus gundi*）より発見された[1]。その学名は，弓型（toxon）をした生きもの（plasma）の意味から命名された。同じ年にブラジルのサンパウロにあるポルトガル総合病院の Splendore も実験に用いた家兎に本原虫を認めていた[2]。それ以来，ヒトを含めたほとんどの哺乳類および鳥類に感染することが判明し，本原虫は人獣共通感染症の重要な病原体のひとつである。トキソプラズマ原虫の分布は世界的であるが，ヒトおよび動物の分布は必ずしも一様でなく，気候風土（高温多湿の地域は乾燥低温の地域よりも感染率が高い），食生活，動物種により異なる。ヒトでは成人の約3分の1は抗体陽性であり，加齢とともに感染率が増加する。動物のうち，イヌ，ネコ，ブタ，ヒツジがヒトと同様の高い抗体保有率を有しており，ウマ，ウシなどは比較的低い抗体保有率である[3]。

トキソプラズマに感染しても，多くは臨床的にほとんど症状を示さず不顕性感染の経過をとる。しかし，妊娠期間中に初めてトキソプラズマに感染すると，胎児が先天性トキソプラズマ症にかかり，流・死産，脳水腫，網膜脈絡炎や精神・運動障害などの症状を表す[4]。また，トキソプラズマ症は，最近の後天的免疫不全症候群（AIDS）感染を初めとして宿主の免疫力が低下した場合，トキソプラズマ性脳炎などの急性症状をあらわす症例が増加し，改めてその重要性を認識する必要のある原虫

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性疾患である[5]。

Ⅰ．形態・生活史

トキソプラズマは発見以来，栄養型と嚢子しか認められていなかったため，その生活史および分類学上の位置は長い間不明であった。1960年代後半にHutchisonらは，トキソプラズマに感染させたネコの糞便をマウスに食わせた結果，感染が成立したことを報告した[6,7]。この研究が契機になり，ネコおよびネコ科の動物がトキソプラズマの終宿主であり，小腸粘膜上皮細胞内で有性生殖を行って糞便中にオーシストを排泄し，他の動物が中間宿主となるコクシジウムの1種であることが判明した[8]。ネコを除いた哺乳動物や鳥類では急性感染期に見られるタヒゾイトと慢性感染期に見られるシスト内のブラディゾイトが認められる。終宿主であるネコでは，タヒゾイトとブラディゾイトのほかに，腸管上皮細胞内で有性生殖が行われ糞便中にオーシストが認められる（**図1**）。

タヒゾイト（tachyzoite）はトキソプラズマの栄養型虫体で急性期に認められ，その名称は細胞内で急速に分裂増殖することに由来する（tachyはspeedyの意味）[9]。タヒゾイトは長径5～7 μm，短径2～3 μmで中央よりやや後端に核が存在する三ヶ月状の虫体である（**写真1**）。電子顕微鏡でみるとタヒゾイトの先端部には，ボーラーリング，カップ状のコノイドやロプトリーなどの胞子虫特有の微細構造が認められる（**写真2**）。タヒゾイトは

写真1　タヒゾイト（ギムザ染色）

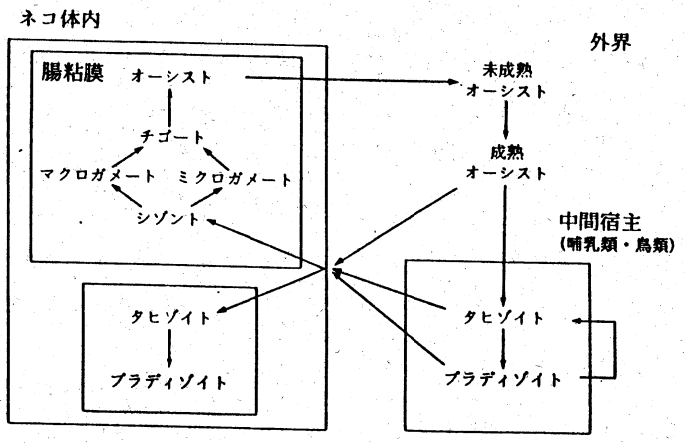

図1　トキソプラズマの生活史

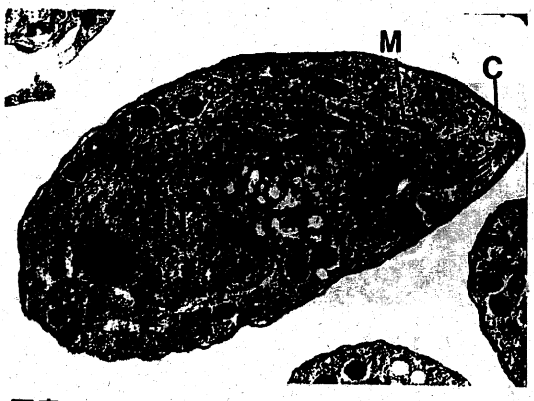

写真 2　タヒゾイト電顕像

N：核　　M：マイクロネーム
R：ロプトリー　C：コノイド

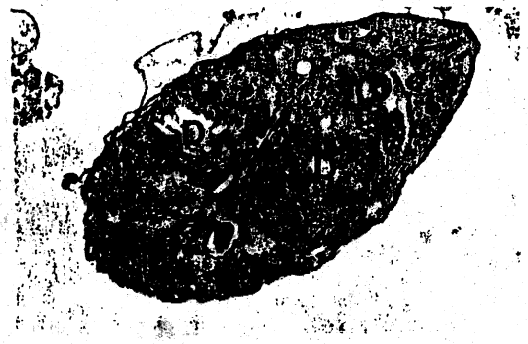

写真 3　内部出芽による虫体の分裂像。母虫体(M)に 2 個の娘虫体(D)が認められる。

生細胞内でしか分裂・増殖できなく，母虫体の中に 2 個の娘虫体が生じて，これが母虫体を破壊する内部出芽法（**写真 3**）で分裂する。虫体はこれを反復して細胞内に 16～32 個のタヒゾイトが充満し，いわゆるコロニーを形成する。増殖した虫体により宿主細胞は最終的に破壊され，タヒゾイトは細胞外に遊離し，再び他の細胞に侵入し分裂増殖を繰り返して組織に障害を与え，急性症状を呈する。

ブラディゾイト（bradyzoite）は慢性期に認められる虫体でその名称は緩慢な増殖を行うことに由来する（brady は slow の意味）[9]。急性期を過ぎ，宿主のトキソプラズマに対する免疫が成立すると，タヒゾイトは脳内や筋肉中で球形の強靭な膜を有するシスト形成に移行し，このシスト内にブラディゾイトが充満している（**写真 4**）。ブラディゾイトの増殖も内部出芽であるが，分裂に要する時間が長く緩慢に増殖するので種々の大きさのシストが生体内に認められる。成熟したシストは直径

写真 4　マウス脳内のシスト像(スタンプ・ギムザ染色)

20～50 μm のものが多いが，なかには 100 μm に達するものもある。シスト内には 100～1,000 個以上のブラディゾイトが含まれる。組織内のシストの周囲には炎症性の反応も認められず，またペプシンやトリプシンに対しても比較的抵抗性が強い。不顕性感染や慢性感染の宿主内でのトキソプラズマ虫体は一般に組織内シストの状態で存在し，他の動物への感染源となることが多い。

オーシストは終宿主であるネコの糞便内に

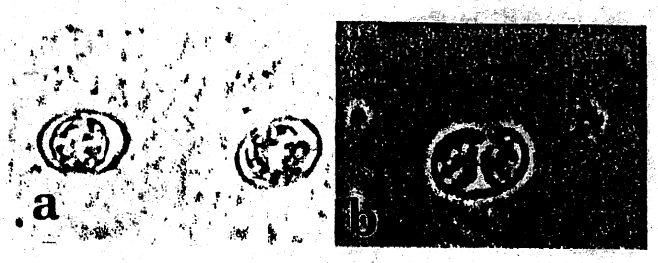

写真5　糞便中に排泄されたオーシスト

a：未成熟オーシスト
b：成熟オーシスト

認められる虫体である。ネコ体内にトキソプラズマが経口的に入ると，腸粘膜上皮内において有性生殖が行われ，その結果オーシストが形成される。しかし，糞便に排泄された直後のオーシストは10×12 μm 程度の大きさで感染力のない未熟オーシストである（**写真5 a**）。外界で発育するにつれて，2個のスポロシストが形成され，その中に各々4個のスポロゾイトができて，感染力を持った成熟オーシストとなる（**写真5 b**）。

栄養型，嚢子，オーシストのいずれかが経口的にネコの体内に入ると，虫体は小腸特に空腸から回腸粘膜上皮細胞に穿入して分裂増殖する。この後，トキソプラズマは2経路の発育過程に分かれる（**図1**）。ひとつは無性生殖で，腸間膜リンパ節を経て全身に広がりタヒゾイトとして増殖し，ついで全身の臓器にシストを形成する。その2はトキソプラズマが腸粘膜内において有性生殖を行う。すなわち，小腸上皮粘膜に穿入したトキソプラズマは初代分裂体（シゾント）を形成し，そこから遊離したメロゾイトのうち，分裂せずに雄性と雌性配偶子母細胞に分化し，雄性配偶子母細胞から多数の鞭毛を持った雄性配偶子が生ずる。一方，雌性配偶子母細胞からは1個の雌性配偶子ができ，やがて両者が融合し接合子（チゴート）となり，さらにその周囲に無色の2層の壁を形成し胞嚢体（オーシスト）となる。小腸上皮細胞内で形成された未熟オーシストは上皮細胞を破り，小腸内腔に出て糞とともに体外に排泄されるが，排泄直後のオーシストは感染力がない。しかし，十分な温度と湿度のある条件下（20 ℃で3～4日）で感染力を有する8個のスポロゾイトを含む成熟オーシストに発育し，次の感染の機会を待つ。

中間宿主である動物には，ネコから外界に排泄されて成熟したオーシストや他の感染動物中のシストが経口的に体内に侵入することにより感染する。これらの虫体からスポロゾイトやブラディゾイトが遊離し，消化管壁から宿主細胞内に侵入する。細胞内に侵入した虫体は急速に分裂・増殖する。これらの虫体はやがて宿主細胞を破壊して細胞外に遊離し再び新しい細胞に侵入して分裂増殖を繰り返し，急性感染の症状を表す。やがて，脳や筋

細胞内にはゆっくりと分裂するブラディゾイトを包含するシストが形成され，慢性感染に移行する。これらのシストも他の動物への感染源となる。

II．感染

トキソプラズマの感染方法としては，先天性（経胎盤）と後天性に起こることがよく知られている。先天性感染は妊娠中のヒト，ブタ，イヌ，ネコ，マウス等の母体が初感染を受けた場合，トキソプラズマ虫体（タヒゾイト）は胎盤を通過した子宮内の胎児に感染し，医学，獣医学上重要な問題となる[10]。ヒトの感染の場合，妊娠初期では流・死産などの重篤な症状を呈し，中期・後期では脳内石灰化像，脳水腫（**写真6**）や網膜脈絡炎などの症状を表す。また，出生時に無症状であったとしても，数年後に精神的あるいは運動的な障害を残すことが多いとされている。したがって結婚適齢期の婦人は感染の検査をする必要がある。その結果すでに抗体価が陽性である慢性感染症の場合，宿主が妊娠してもトキソプラズマ虫体が胎盤を通過して胎児に感染する確率は極めて低いと考えられ，妊娠や分娩率の低下もないとされている。しかし，抗体価が陰性であれば，生肉・未調理の肉およびネコの糞便に十分注意して感染しないようにすることが必要である。

写真6　脳水腫の患者
（アルゼンチン Cecchini 博士より提供）

出生後のトキソプラズマ感染は第1にネコ糞便中のオーシストに汚染された食物の摂取が考えられる。オーシストは通常の自然環境や薬剤に対しても極めて抵抗力が強く，湿潤状態では長期間感染力を有する。たとえば，4～22 °C の水中では550～580日，－21 °C で28日生存する。また，10％ホルマリンで24時間，95％エタノールで1時間，5.5％アンモニア液で1時間生存する[10]。このためネコの糞便中のオーシストの汚染された飼料や土壌から経口的にブタやヒツジが感染すると考えられる。また，ハエ，ゴキブリ，ミミズなどによってもオーシストは機械的に伝播される。最近，公園などの砂場が高率にネコ糞便に汚染されていることが明らかになってきており，砂場のトキソプラズマのオーシストの汚染についても今後は注意を払っていく必要があると思われる。

第2に未調理または生鮮トキソプラズマ（主としてシスト）感染肉の摂取が感染源としてあげられる。シストはオーシストに比較すると抵抗力が弱い。しかし，肉を70 °C で加熱すると死滅するが，4～6 °C では2か月生存し，－20°C の冷凍保存では必ずしも死滅せず，16日間シストは生存していたという報告もあ

る[11]。それゆえ，肉類を調理不完全なままの状態で食べると感染の確率が高くなるといえる。このことは，肉の生食を好むフランス人は20歳前で抗体陽性率が80％を超えており，他の国民に比べて抗体陽性率が非常に高いことからも説明される[10]。

タヒゾイトは先述のごとく，細胞内でのみ分裂・増殖が可能で，細胞外に出ると極めて抵抗力が弱く，単に水中に放置しても浸透圧の差により虫体は破壊される。また，経口的に体内に摂取されても，消化液に弱いのでそのために感染することはほとんど考えられない。しかし，実験中に，眼，鼻，皮膚の傷口などから感染した例が知られている。また，胎盤感染の場合は遊離しているタヒゾイトが胎盤を通過し胎児に移行して感染し重篤な症状を引き起こす。

III．免疫

トキソプラズマ原虫感染に対してマウスのほかに新世界ザルやカンガルーも感受性が高いが，ほとんどは抵抗性である。感染後タヒゾイトは細胞内で急速に分裂・増殖し，次々と細胞を破壊し新しい細胞に侵入していく。しかし，免疫応答が始動し体液性抗体が出現する3週以後では，タヒゾイトは体内諸組織から消失して症状は軽快し，心筋，横隔膜，などの筋肉組織中で緩慢に増殖するシストが認められる。脳脊髄液および脳内には他の諸臓器よりも免疫応答の遅延と減弱からタヒゾイトの存在はやや長くまで認められる。シストが形成されると，臨床的には症状は認められなくなり，再感染に対して強い抵抗性を示す。

トキソプラズマ感染に伴う体液性免疫応答はいずれの動物においても感染初期にIgM抗体のみが出現する。ついでIgMとIgG抗体が認められ，感染後5～6週以後ではIgG抗体のみが検出される。細胞外の虫体にはこれら体液性抗体が直接関与するが，マウスを用いての実験で大量の新鮮免疫血清を健康マウスに注射しても，トキソプラズマ強毒株原虫を感染させると，マウスは全例死亡する[12]。したがって，体液性抗体のみではトキソプラズマ感染に対する感染死防御能を賦与することはできない。通常細胞内で分裂増殖し，宿主の免疫機構から逃れて生存しているトキソプラズマ原虫に対する感染抵抗性に関しては体液性抗体よりも細胞性免疫が重要である。

細胞性免疫をになう機構として，Tリンパ球およびその産生物であるリンフォカイン，そしてマクロファージの活性化等が総合的に強く関わり合っている。トキソプラズマ症における免疫リンパ球およびリンフォカインとマクロファージの生理機能を中心にした細胞性免疫の機作は*in vitro*試験細胞の研究で明らかにされてきている。健康マウスの腹腔マクロファージ単層培養にタヒゾイトを添加すると，貪食機能と虫体の穿孔性によりトキソプラズマはマクロファージ内に取り込まれるが，虫体とライソゾームとの融合は阻止されて消化酵素の作用を受けない。トキソプラズマは細胞内において6～7時間で2分裂し，

原虫の約70％は12時間で2回分裂を繰り返す（**写真1a**）。虫体を免疫新鮮血清あるいは非働化免疫血清で37℃で30分感作してから正常マクロファージに添加すると，虫体の一部は増殖できずに死滅したり，その他の虫体は増殖が遅延する。しかし，正常マクロファージ内にすでに侵入した虫体には新鮮免疫血清を添加培養しても，細胞内のトキソプラズマ虫体の分裂増殖は何ら抑制されない。

一方トキソプラズマ虫体に対して免疫が成立しているマウスからのマクロファージ内で虫体増殖は軽度に抑制され，2分裂に要する時間は16～18時間に延長する。この増殖抑制作用は正常のマクロファージに免疫マウスからのTリンパ球あるいはその産生物であるリンフォカインを添加して培養するとより顕著になり，マクロファージの機能が活性化される[13]。その結果，細胞内トキソプラズマ虫体はライソゾームと融合し増殖は明らかに抑制され，多くは殺滅・消化される（**写真1b**）。さらに細胞外虫体を新鮮免疫血清にさらさせた後に活性化マクロファージに貪食させると虫体の増殖はほぼ完全に阻止される。このような活性化マクロファージでは殺機能に主として関与するとされている活性酸素中間体の産生が増加している[14]。またマクロファージ由来の窒素中間体の発生など，活性酸素中間体の介在しない機構も存在することが明らかになってきている[15,16]。

また，Tリンパ球により産生されるガンマーインターフェロン（IFN-γ）もトキソプラズマに対する免疫に重要な役割を演じていることが，IFN-γの投与やIFN-γに対するモノクローナル抗体を用いた実験により明らかになってきている[17,18]。また，ナチュラルキラー（NK）細胞やリンフォカイン活性化キラー（LAK）細胞も防御免疫に関与するらしい[19,20]。しかし，これらのサイトカインや免疫

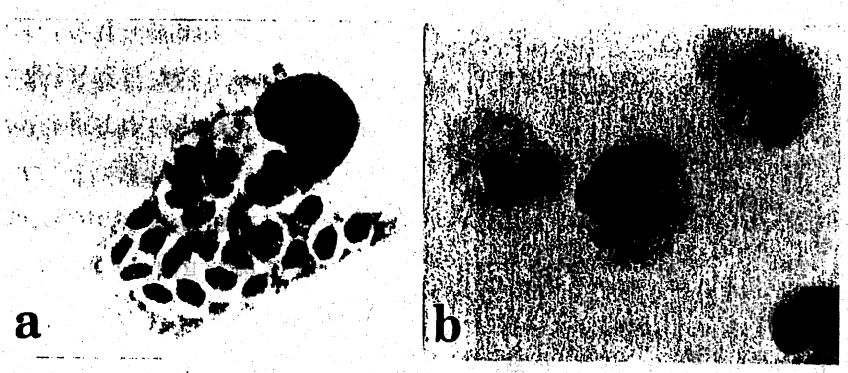

写真1a　正常マクロファージ内のトキソプラズマ増殖像

写真1b　活性化マクロファージ内の消化されつつあるトキソプラズマ虫体

担当細胞がどのように相互に関わり合いながらトキソプラズマに対する免疫が成立するのか，その全体像は明らかにされてない。

トキソプラズマ感染が長期間続くと抑制性マクロファージやTリンパ球が出現し，免疫抵抗性の低下が起きることがある。その結果，シスト壁が破壊され，ブラディゾイトが遊出し，再びシストが形成されるため，新旧のシストの存在が認められる。さらに悪性腫瘍の治療や臓器移植における拒絶反応を押えるための免疫抑制剤の投与およびAIDS感染などにより免疫状態が強度に低下した場合，シスト壁を破壊して遊出したブラディゾイトは隣接の細胞に侵入する。その結果，再び活発にタヒゾイトが増殖して急性の症状を呈し，最悪の場合患者は死亡する（**写真8**）。たとえばAIDS患者の場合，3～40％がトキソプラズマ性脳炎をおこし，特にトキソプラズマ抗体陽性率が高いフランス（80％以上の人が感染）では25％以上のAIDS患者がトキソプラズマ性脳炎を発症している[5]。HIVはCD4陽性細胞に感染し，そのため宿主の免疫力が低下してトキソプラズマ性脳炎が引き起こされると推察される。実験的にトキソプラズマ感染させたシスト保有マウスにモノクローナル抗体を投与し，CD4陽性細胞の機能を抑制すると，脳内に種々の大きさのシストが現れ，タヒゾイトが遊出することが報告されている[21]。しかし，CD4陽性細胞がいかなるメカニズムでブラディゾイトの活性化に関与しているか具体的には不明である。

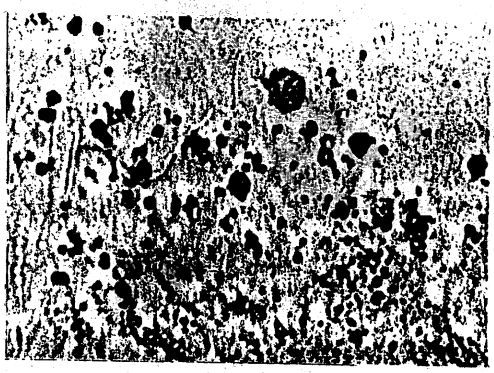

写真8　ヒトAIDS患者の脳内シストおよびタヒゾイト像
(Avidin-Biotin-Peroxidase染色法)

IV. 治療・予防

感染初期の急性期に活発に増殖するタヒゾイトに対してサルファー剤やピリメサミンは古くから治療薬として使用されている。サルファー剤は宿主細胞膜を通過しやすく，細胞内増殖の阻止に有効である。しかし時として長期連用のため，血小板や白血球の減少など骨髄機能の低下が認められる。ブラディゾイトは強靱なシスト膜に保護されているために治療は困難であるとされてきたが，近年いくつかの薬剤がシスト治療剤として検討されており[22,23]，これらの薬剤の中から組織内シストに有効な薬剤の実用化が期待されている。

ヒツジ，ヤギ，ブタなどの家畜において，先天性トキソプラズマ性による流産や幼獣の消耗による経済的な損失が大きいため，ワクチンによる予防が重要な手段として獣医学領域では検討されてきている。最近，ニュージーランドやイギリスにおいてヒツジに対する

トキソプラズマワクチンが実用化されてきている[24,25]。これは流産したヒツジの胎児から分離した原虫をマウスで3,000回以上も継代した結果，シストを形成する能力を失い，タヒゾイトがネコに摂取されてもそのネコはオーシスト排泄がみられない“不完全”な株を生ワクチンとして使用したものである。ワクチン後少なくとも18か月は良好な効果がある。もちろんこの生ワクチンをヒトに直ちに応用できないので，現在防御に有効なタヒゾイトのエピトープの検討が行われている。そのような抗原を特定できればヒトにも使用できる安全なワクチン開発が可能となるかも知れない。

おわりに

従来高い抗体陽性率にも拘わらず，健康な固体では症状を表さないため，トキソプラズマ症に対する関心は低かった。しかし，最近免疫抑制状態やエイズ感染における脳炎の発症により今後注意を払うべき疾患として見直す必要があり，脳内シストの活性化の機構，シストに対する治療薬やワクチンの開発が検討事項として重要である。

文　献

1) Nicolle, C. and Manceaux, L.: Sur une infection a corps de Leishman (ou organisms voisins) du gondii, C. R. Acad. Sci., **147**: 763, 1908.
2) Splendore, A.: Un nuovo protoaoz parassita de'conigli incontrato nelle lesioni anatomiche d'une malatti che ricorda in molti punti il Kala-azar dell'umo. Nota preliminaire pel. Rev. Soc. Sci. Sao Paulo., **3**: 109, 1908.
3) 鈴木直義．：トキソプラズマ，新編獣医微生物学，梁川良ら編：p 993，1989.
4) Krahenbuhl, J. L. and J. S. Remington.: The immunology to *Toxoplasma* and toxoplasmosis, In The Immunology of Parasitic Infections. 2nd ed. S. Cohen and K. S. Waren, eds.: p356, Blackwell Scientific, Oxford, 1982.
5) Luft, B. L. and J. S. Remington.: Toxoplasmic encephalitis. J. Infect. Dis., **157**: 1, 1988.
6) Hutchison, W. M.: Experimental transmission of *Toxoplasma gondii*. Nature, **206**: 961, 1965.
7) Hutchison, W. M.: The nematode transmission of *Toxoplasma gondii*. Trans. R. S. Trop. Hyg., **61**: 80, 1967.
8) Frenkel, J. K. et al.: *Toxoplasma gondii*: The oocyst, sporozoite, and infection of cultured cells. Science, **167**: 892, 1970.
9) Frenkel, J. L.: *Toxoplasma* in and around us. Bioscience, **23**: 343, 1973.
10) Dubey, J. P. and C. P. Beattie.: Toxoplasmosis of animals and man. CRC Press, Boca Raton Florida. p 41, 1988.
11) 板垣　博，大石　勇．：新版家畜寄生虫病学，p 289，朝倉書店，1984.
12) 鈴木直義．：原虫感染と免疫，日獣会誌，**35**：687，1982.
13) Sethi, K. K. et al.: Immunity to *Toxoplasma* induced *in vitro* in non-immune mouse macrophages with specifically

immune lymphocytes. J. Immunol. **115**: 1151, 1975.

14) Nathan, C. F. et al.: Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. **158**: 670, 1983.

15) Adams, L. B. et al.: Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii*: Role for synthesis of inorganic nitrogen oxides from L-arginine. J. Immunol., **144**: 2725, 1990.

16) Saito, A. et al.: Effects of reactive oxygen intermediate scavengers on antitoxoplasmic activity of activated macrophages. Parasitol. Res., **78**: 28, 1992.

17) McCabe, R. E. et al.: Effect of murine interferon gamma on murine toxoplasmosis. J. Infect. Dis., **150**: 961, 1984.

18) Suzuki, Y. et al.: Importance of endogeneous IFN-γ for prevention of toxoplasmic encephalitis in mice. J. Immunol., **143**: 2045, 1989.

19) Subaute, C. S. et al.: Murine $CD8^+$ cytotoxic T lymphocytes lyse *Toxoplasma gondii* infected cells. J. Immunol. **147**: 3955, 1991.

20) Subaute, C. S. et al.: Human lymphokine-activated killer cells are cytotoxic against cells infected with *Toxoplasma gondii*. J. Exp. Med., **176**: 1511, 1992.

21) Vollmer, L. T. et al.: Depletion of lymphocytes with monoclonal antibody reactivates *Toxoplasma gondii* in central nervous system: A model of superinfection in AIDS, J. Immunol. **138**: 3737, 1987.

22) Harris, C. et al.: *In vitro* assessment of antimicrobial agent against *Toxoplasma gondii*, J. Infect. Dis., **157**: 14, 1988.

23) Huskinson-Mark, J. et al.: Evaluation of the effect of drugs on the cyst form of *Toxoplasma gondii*. J. Infect. Dis., **164**: 170, 1991.

24) Wilkinson, M. F. et al.: Toxoplasmosis in sheep III. Further evaluation of the ability of a live *Toxoplasma gondii* vaccine to prevent lamb losses and reduce congenital infection following experimental oral challenge. N. Z. Vet. J., **36**: 86, 1988.

25) Buxton, D. et al.: Vaccination of sheep with a live incomplete strain (S48) of *Toxoplasma gondii* and their immunity to challenge when pregnant. Vet. Rec., **129**: 89, 1991.

4-1

特別講演　第9回北海道地区大学保健管理業務職員研修会、帯広、北海道、6月23日、1993

「免疫の仕組み ― ヒト・動物共通原虫病から考える」

帯広畜産大学原虫病分子免疫研究センター長　鈴木直義　教授

ご紹介頂きました，鈴木直義でございます。私の体験，私を例にとりながらお話しをさせて頂きます。

原生動物という言葉は一般的に原虫とも呼ばれます。この原生動物は人間を高等動物の一番右端に置くとしたら，最も下等な動物といえます。昨日の講演は微生物の感染症 ― エイズの話しでしたが，原生動物は微生物の中にも含まれる，ちょうど微生物と動物の境界領域の生物なのであります。

動物でもありますので，1個の個体で栄養補給を行い，代謝・排泄そして生殖を行って生存する形態をとっています。

世界には約6万5千種程いるのですけれど，その中で，原生動物（原虫）が高等動物に寄生して栄養補給をしたり，あるいは，病原を発現したりする寄生性のものが，約1万種います。その中で，寄生しながら他動物に対し病原性を発揮するもので有名なものには，まずマラリアがあります。

マラリアは世界規模で言うと何億という人間が感染し，年間300万人～400万人が死んでいます。またニウモシスカリニ，現在エイズの約40%位はカリニ肺炎で死ぬと言われている，そのカリニ，あるいはトリパノゾーマー，そして動物から人間に容易に感染するトキソプラズマなどがあります。

マラリアは先生方もご存じのとおり，赤血球の中で増殖し赤血球を破壊するものです。そしてトリパノゾーマーは，プラズマ中に生存し急性死するものです。

トキソプラズマ

今日はトキソプラズマに的を絞ったお話をすることにします。1969年まではまだトキソプラズマが動物の肉から人間へ行くということでは，その種・族は不明であったのですが，1967～69年にハチソンのグループの研究で，終宿主すなわち体内で増殖を行って出す動物というのがネコ及びネコ科動物であるというのがハッキリいたしました。

1970年以降，そのネコ科動物から出てきた成熟オーシストは，外界での抵抗性が強く，−20°でも，土中に少し入っているだけでも，感染力を持つことが分かり，オーシストで汚染した乾燥

した草とか野菜とか，そのようなものを介して動物あるいは人間に感染すると言うのが感染ルートとされたのです。

人間をもう少しつめてみますと，この動物の肉の中に感染があると，細胞の中で増殖して，それが筋肉とか脳に嚢胞を形成して，その中で虫が沢山，半永久的に生存する訳です。その肉を生肉，あるいは未調理の肉で食べますと人間に感染する。と言うことで以上の2つが人への感染ルートでございます。

しかし，肉食を主体とする欧米も含めまして，人為的な感染（事故）も少なくありません。実験的にトキソプラズマ虫体をマウスに実験感染させたりする時に，自分で自分の手に打ったりすることがあります。これは私の話ですが1966年にドイツ医学寄生虫病研究所にいた時に，ドクターがちょうどネズミに注射を打とうとしている時に，「おはようございます。」と挨拶したら，振り向きざまに注射薬が私の目に入ってしまったという訳です。日本でも独協医大の眼科がトキソプラズマの研究をやっておりますが，失明寸前になりました私は，現在でも脳とか特に心筋にシストとして生存しています。

世界の人間のトキソプラズマ感染，過去に感染していたかどうかを抗体で調べる調査を行いますと，フランスのパリの80％以上の人間が陽性だというので，1972年の国際学会で非常に問題になったのですが，パリの特に上流社会のご婦人達が生肉を好むのが原因です。生肉を食べないグループですと，これが60％以下に下がるということで問題になりました。

フランスに限って言えば，第2の都市のリオンでも追跡調査で同じことをやるとやはり，今から4～5年前の国際学会で約65％位陽性との報告です。

日本，アメリカまたはイギリスでは，だいたい成人以上の約30％の人が陽性で年令と共に増える，主として2つの感染ルートから感染していると言われ，そして，世界の成人の平均は，約30％と言われています。

ネコは，先程終宿主がネコであることを申し上げましたが，世界のネコあるいは日本の一部で調べた結果では約30％が抗体を持っています。

ブタ・ヒツジまたはウシとかを調べると，ブタ・ヒツジの肉は30％近くが陽性で，その中でウシは何度調査しても10％以下です。安全度から言えば，同じ生肉を食べるにしてもウシの方が安全です。少し値が高いと言えますが，いずれにしましても，トキソプラズマはあらゆる動物に感染していると言うこと，世界中の規模で，凡そ3分の1の動物が感染していると行っても過言でないと言うのが専門家の常識です。

昨日もそうでしたが，帯広では何かあると肉を食べる，学生諸君には「肉は良く焼いて裏表黒

くなるようにして食べなさい」と指導しています。学生には言うのですが，家庭で言うと「そんな肉は食べれない」といわれます。東京のあるホテルに家族で行った時に「ミデアムウエル，よく焼いてくれ」と言ったところ，家族から非難されました。

肉は余り焼かない方が旨いと言いますが，この講義を本日聞いた人は，良く焼いた方がよろしいと言うことを覚えていてほしいと思います。

先程も申し上げましたが，ネコが終宿主であるという事が分かる以前から，人及び動物に流産死がありました。その流産死の中からトキソプラズマ虫体が検出されることで，流産・死産そのものにトキソプラズマが関係あるだろうということが古くから言われていた訳です。

これは先生方が詳しいのですが，脳に水が溜る水頭症がありますが，南米等の肉を食べる地方では決して珍しい例ではありません。

ドイツの例ですが，子供が入院しておりまして，約半年後に風邪をこじらして死んだのですが，調べますと脳内にトキソプラズマのシストが破裂しているのが確認されました。

妊娠とトキソプラズマの感染がどんな因果関係があるのか，1970年頃までに臨床例では認められているのですが，実験的データーがない。ネズミを使って実験したところ，一度トキソプラズマに感染した約3分の1のネズミについてはトキソプラズマが感染しても，期間に係わらず，その胎児への移行は認められません。しかし，一度も感染経験のないネズミに，交配前に感染させると，2例（8分の2）が感染し，その感染した胎児からは虫体が検出されました。

これらの者は，妊娠交配させてから一週間目の，妊娠前半に感染させると，新生児は生まれるのですが，その発育は健康な者に比べ非常に劣り，これからは虫体分離あるいは血清抗体が陽性となり，確実に100%が感染いたします。

もし分娩直前に感染させると，マウス・ラッテの実験によると，出生後約2週間位で全例が死にました。

以上をまとめると，マウス・ラッテでは少なくとも，一度感染経験のある母親が再感染しても，胎児への移行は100%近くない，ところがトキソプラズマに感染しないように注意して育てた母親の妊娠中の感染では，ほとんど，どの妊娠ステージでも感染の可能性があること，そして新生児の発育が感染と妊娠の時期との関係によって違ってくることが分かってまいりました。

抗体――昨日の話と同じですが，このトキソプラズマも微生物の一つとして，感染すると感染宿主の応答としては身体の中にＩＧＭ抗体ができます。

トキソプラズマに限って言いますと，約2週間細胞内で2分裂増殖しながら最終的には，シスト形成に入ります。このような状態の時に，他の一般的な感染症と同じように防御機構は，細胞

性免疫，とくにマクロファージーまたはTリンパ球系が関与するということが分かっています。これは，他の原虫でもほぼ同じ状態を示します。

細胞の中で増殖している栄養型虫体は，2分裂増殖しますが，感染経験の無い細胞の中では，8～10時間で2分裂して増殖し，その細胞を破壊して，次の細胞に移行する，というのがトキソプラズマの特徴です。

細胞性免疫とかリンフォカイン・サイトカインと言う言葉を知らない人は現在ではいないと思います。実は，1960年代に，恩師から「本実験前の基礎実験や計画・立案には，できるだけ使い古しのマウスを，使用しなさい。」と言われ，ある実験で一度トキソプラズマを使用したマウスの細胞を取って，同じように行ったところ，先ほどの説明とちがい，虫が正しく分裂しないで虫が死にかかる現象が見られました。少しオーバーに言うと，これが1966～67年にかけてマクロファージーまたは他の細胞が，他のTリンパ球・酸性のリンフォカインのような物質によってアクチベイトとされて虫を殺すという実験を始めるきっかけになったもので，センター長の「あまり良いマウスばかり使用しないで，使用済マウスを使いなさい。」の恩師の言葉のおかげでした。

要約すると，良好なマウスから取ったマクロファージーを例にとると，そのマクロファージーの中では，先程言ったように，一個の虫が10時間で2分裂し細胞を破壊して次の細胞に移行する，ところが，一度トキソプラズマに感染したマウスのマクロファージーに一個の虫を入れると，この分裂が非常に遅くなる。ある細胞は増殖しなかったり，2分裂するのに20時間位かかるようになります。また，免疫マウスにこの退化したマウスのリンパ球，あるいはリンパ球からの酸性リンフォカインを入れると，この正常マウスのマクロファージーでも，虫が増殖しないで殺されてしまう。このような物質があるということが1969～73年に分かってきたのです。

一般的に，マウスのマクロファージーは感染症で異物を鈍食する細胞ですが，そのマクロファージーの活性化，異物を鈍食して殺していくという作用を活性化する物質が，リンパ球の中の酸性Tリンパ球から産生されてくることを知ったのです。私達は，そのような物質とインターフエロンガンマーが同じように出てくるトキソプラズマの増殖抑制因子と，マクロファージー（活性化因子）インターフエロンガンマーと非常に似ている部分がオーバーラップしていると敢えて考えたのです。こう分けたのはこの物質はマクロファージーだけでなく，体細胞の中の虫も押さえるという物質だからです。

これらは，1982年に認められたのですが，これらがトキソプラズマに感染して，その生体が反応をおこして，そしてトキソプラズマ自体が脳とか筋肉にシストを作り，その状態のマクロファージーでもアクチベイトされて，異物を殺す能力が亢進している。なおかつ，Tリンパ球から産生

された物質を入れると，より亢進することが分かってまいりました。

その物を何とか精製分離したく，1980年代にこれ以上分けられないひとつの活性単位を生物物質から取り出した訳です。結論からいうと，シングルピークを分析していくと，また2つ3つとなり，我々の手に負えなくなります。

ハンブルクのある学会で専門の先生と相談したときに，そこから類似の合成物質を作り活性化に持っていくほうが早いと思い実行したところ，先程の活性物質は分子量が5,000以下であり，非特異的にマクロファージーや体細胞を活性化させ異物を殺す，そのうち一番活性の強い部分は非常に小さな単純なペプチドで，非常に強く作用するということが分かってきました。

これは人の脳細胞のインビトロカルチャーの実験です。インビトロでは非常に早く，虫がありとあらゆる所に増殖します。しかし，先程の活性物質を入れますと虫が殺されます。インビトロでも押さえるということです。同じようなことはインビトロの心筋でも空胞形成を押さえることで証明されます。

ところで次に，マラリア原虫のマウスに対する病態と，それに対する前述の活性物質・合成物質の作用についてですが，これは同時平行的に分かってきたことなのですが，実験過程を少し飛ばして説明しますと，マラリアに感染させたマウスは100%死ぬ，しかし，このトキソプラズマの原虫成分の中の非常に特異的に我々の動物体を賦活する作用物質を与えるか，その合成した類似ペプチドを作用させるかしますとどうなるか？　その物質の約500マイクログラムを感染前に2回打つと，100%死んでいくマウスの約30%生き残りますし，合成ペプチドを投与すると約60%位が生き残ることが分かってきた訳です。

少なくとも我々の体に致命的な症状を現すマラリア系統でも，原虫に属しているこのトキソプラズマの適応性の成分の中に，非常に範囲の広い別のマラリアまでも，ある程度死ぬのを押さえる物質があることが分かってきました。

それに今のをやるとなお賦活される，その賦活形態のメカニズムがどうなのかは80年代の仕事です。少なくともトキソプラズマが我々にとって，ただ悪いだけでなくその賦活する物質があるということを，どこか意に留めておいてほしいと思います。

抗腫瘍作用―トキソプラズマに関係のない異物に対しての反応をみると，マウスの同種細胞（主要細胞）を移植したものに打つとどうなるかという実験では，マウスに打ってそのコントロールでは，移植腫瘍が増殖していく，そして最終的に約80～100日位で死んでいくのに，今の原虫から取り出したある成分を，一週間に一回打つとその腫瘍の増殖面積が顕著に押さえられる。そして押さえられるために死ぬ割合も非常に少ない，ということが色々な固形腫瘍の実験でわかり

ました。ただし，私達はあくまでも原虫生態の防御免疫・防御機構の追跡を主にしておりますので，あまり腫瘍の問題には入っておりません。

OK 432が，実は10年程前アメリカのＮＩＨで，バイオロジカルレスポンスモデファイア（ＢＲＭ）の評価判定で，OK 432が良く評価されていたのでそれを使用したところ，OK 432よりもこの腫瘍に限っていえば，その増殖は異なっておりました。このように，トキソプラズマのある成分が，非特異的に我々動物体の免疫応答を賦活するというデーターです。

同じようにＴＬＡだけでなく，合成のペプチドを行いますと面白いことに，ＴＬＡを打つとその腫瘍の増殖が遅延します。この組織の切片を見ると，この中に非常に大型の細胞が出現することが，1983～84年に分かってきました。サイワン陽性染色で調べますとやはり大きな細胞が見られます。

普通のコントロールで上がっていく腫瘍細胞では，ＮＫ細胞が散在的にありますが，先程のような大型細胞は全く見られません，したがって大型の細胞はトキソプラズマ原虫成分の一部の免疫賦活作用のあるものが誘導するのだろうということが分かってきております。

そのような誘導細胞，このＰ815という腫瘍細胞は，ＮＫナチュラルキラー細胞非感受性の細胞ですし，ヤークワンはナチュラルキラー細胞の感受性のターゲット細胞ですが，これらを使って行いますと，いまのＴＬＡというのは濃度依存的に殺すのが分かると思います。

先程の大型細胞が誘導されるのですが，これはＮＫ由来だけではなくて，非由来の大型キラー細胞であるのが分かると思います。

普通のリンパ球，そして大型の細胞を見ますと，この大型の細胞は直系が24～44ミクロンです。このような細胞はどんなものかというと，先程言いましたようにナチュラルキラー細胞のマーカーであるアシアルＧM1，或はT細胞由来のマーカーとしてサイワン，これらで処理しますと，いまの何もしないものの活性に比べ非常に下がってしまいます。このような事でＴＬＡで誘導される非特異的なキラー細胞は，大型のアシアルＧM1でポジテブなもの，あるいはサイワンでポジテブなものと，色々なものが入り込んでいます。

そのうちの一つの成分を精製分離し，配列が分かったのですが，分かってみると，哺乳動物体のユビキチンの構造式と２カ所異なるだけで，作用はいくら行ってもキラー細胞は誘導されませんが，全てではありませんが，精製したひとつとして若干異なるだけで，作用が非常に異なることが分かってきました。

このことから要約したいのは，私はいわば実験エラーでトキソプラズマに感染したわけですが，身体にシストとして脳の中と筋肉の中にいます。

これらの原虫がホストの私に対し微弱な抗原性を持続しており，T細胞からは常にサイトカインを出せる状態，あるいはモノサイト等が異物に対し闘える状態でおりますので，私が健康である限りトキソプラズマに感染していても，仲良く共存している状態です。したがって学生にも，私は異物としての腫瘍・ガンにはならないだろう，あるいは他の病気にも簡単には伝染しないだろうと説明しています。しかし，最近は風邪等で医師の世話になる回数が多いので，少し身体の状態が下がっていると感じています。

トキソプラズマが我々動物体に感染しても，悲しむのではなく共存できるのですが，ところが免疫能が下がると，免疫能の下がり具合によっては，共存していたトキソプラズマシストが破裂して，もう一度細胞の中で，増殖するのが分かってきています。このことは，実験でも明らかです。

エイズ・臓器移植・ＭＲＳＡとの関連―昨日のエイズの話にあったように，我が国でのエイズ患者死亡例はかなりの%がカリニ肺炎の併発ですが，トキソプラズマでのエイズ死は，欧米では，約30～35%がシストの脳内破裂です。

たまたま1984年に，アメリカのＮＹＨのギャロー教授が，1982年にこのエイズウイルスを分離したことで，1984年にドイツで微生物の関係者が特別講演を聞いたのですが，そのときドイツのボン大学で，エイズ患者の２人の青年が同じ時期に，トキソプラズマによる劇症脳障害を発し３週間位で死亡した人の組織像を示しました。ハッキリしたシストが確認でき，そのシストが破裂し虫が出現しています。ただこれだけでは分かりませんので，トキソプラズマを確認するのに，特殊染色を行うと，シストが破壊され脳内に増殖型の虫体が全て２分裂増殖しています。つまり免疫能が落ちていたのです。

もう一つ，臓器移植が今日普通に行われるようになりましたが，この臓器移植に対し免疫抑制剤の大量投与があります。この免疫抑制剤の大量投与によって臓器移植は落ち着くのに，脳内のトキソプラズマは活動し，敵対行為を働かすことで，結果として死亡することも少なくありません。

動物でも同じ問題があり，一度トキソプラズマに感染したマウスは，脳内にシストを持っているのですが，そのマウスに免疫抑制剤のサイクロホストマイドを一週間に一度づつ投与していくと，ある時期にバタバタと全滅します。

ところが，合成ペプチドを同時投与すると，１例が死に４例が生き残りました。それが組織標本の顕微鏡写真で見られ，脳の中のシストがエイズ患者のようになっているからだと考えられます。

色々な実験で，エイズ患者のヘルパーT細胞がウイルス感染により殺され破壊される。マクロファージーがウイルスを飲み込んでもマクロファージーが殺されます。特にCD8とCD4の問題が大事だということは分かっています。先程のネズミが5例中5例が，ある時期に死んでしまうのが，そしてペプチドを同時投与したものは5例中4例が生き残りますが，これは全体のリンパ球系の細胞は，どれもがサイクロホストマイドだけを投与したほうが少ない，一つは数の差，生体の防御を考えた時の数の閾値があるものと現在考えます。

ただサイクロホストマイドで免疫抑制だけをかけるのではなく，CD4だけを潰していく，またはCD8だけを潰していく実験を行うと，脳内のトキソプラズマの破裂だけを考えると，こちらの方がより著明に出てくるのが分かってきました。

昨日の話のメチシリン耐性菌（MRSA）等でも免疫機能を下げたりあるいはマクロファージーをブロックする70%位は死んでしまいます。このような実験証明もあります。

最後に話をまとめますと，このような病原性の原虫が動物体に感染すると，多くはその病気の程度の差はあれ，主だった原虫感染では最終的には死を招くことになります。ところが，この病気にトキソプラズマから取り出した成分，あるいはリンフォカインから精製した成分を投与すると，この病気は非常に軽くなる。ただ先程言ったように，我々の身体が，より，免疫反応を防御効果として発現できないように破壊されたり無くなっていったならば，いくら投与しても最終的には死に導かれることになり，その代表的なものが，いまエイズという感染症としてあることになります。

こうなりますと，我々の世界規模での動物・人間を含めて考えてみると，原虫性疾患には感染したとしても，元気な抵抗性のある遺伝子とそうでない遺伝子があることになる。その解析の問題がひとつの将来の夢としてあることになります。最後に夢のまた夢を申し上げますが，原虫は全て蚊・ダニ・蠅等の媒介昆虫系統がいて，血を吸ってそれを別の者に感染させるのがルートです。従いまして，もし将来，媒介昆虫の蚊・ダニ・蠅等が動物の血を吸わないで，水なんかでも飲んで生き延びられるようにもしなれば，環境・農薬等の環境破壊につながりませんし，環境保全の意味からも本当に共存できると思うのでありますが……この話しは学生は真面目には聞いてくれませんでした。

私達は，開発途上国への人材養成と学術協力で視野を拡げざるをえない。これからも他の国々と国際協力を行い，さらに研究を進めて行きたいと考えております。

〔この講演記録は録音テープから抄録された。したがって豊富なスライド資料の提示や，その指示説明を略記したものとなった。また読み易いよう，文中に適宜タイトルを入れさせていただいた。〕

4-2(3)

3－2）鈴木直義：人畜共通原虫トキソプラズマ症の病態生理、
第４０回北日本寄生虫学会、旭川、北海道、９月２４日、１９９３
3－3）鈴木直義：　人畜共通原虫病の現状と課題、農林水産省研究
特別推進会議、家畜衛生試験場、筑波、茨城県、２月８日、１９９４

人畜共通トキソプラズマ原虫症の病態生理学的研究

鈴　木　直　義

原生動物（原虫）は生活に必要なすべての機能（代謝・摂食・運動・排泄・生殖など）を一個の細胞で営む動物で、地球上には約65000種の原虫が存在し、そのうち約10000種が寄生性のものである。動物やヒトに共通に感染し、病原性を示す原虫に起因する疾患は人畜共通原虫症と呼ばれている。トキソプラズマ原虫は、ヒトや動物の細胞内で寄生・増殖する人畜共通原虫症の原因となる原虫で、肉食の摂取の多い欧米人を初めとして、世界の成人の約30％が脳や筋肉内にトキソプラズマ嚢胞（シスト）を保有（感染）しているといわれている。とくに妊娠期にトキソプラズマに初感染すると、殆どの場合、死産・流産あるいは脳水腫の原因になることが知られている。動物およびヒトへの感染はウシ、ブタ、ヒツジ等の感染生肉の摂食のみならず、感染ネコ糞便内のオーシストによっても成立することが明らかにされている。したがって本症は、欧米では古くから畜産食品に関する獣医公衆衛生研究の最重要課題の一つとして指定されてきた。これまで主として感染生肉排除に努力が傾けられてきたが、その一方で、本症の病態生理の解明は世界的にも極めて遅れていた。このような背景から我々は、1966年以降現在まで本原虫感染宿主の病態生理学的基礎研究を系統的に行うとともに、中でも未解明であった宿主の免疫応答を実験的に明らかにする努力を続けて来たので、以下にその経過と知見の概要を説明する。

1.　トキソプラズマ症一般感染応答に関する研究

トキソプラズマ原虫の分布は世界的に広がり、ヒトでは世界人口の約30％が原虫感染者と考えられており、動物でもブタ、ヒツジ、イヌ、ネコはヒトと同様のトキソプラズマ抗体保有率を有している。トキソプラズマ感染には妊娠中母体から胎児に感染するという、いわゆる先天性感染が存在し、ヒトの小児トキソプラズマ症を初め各種動物の流産胎仔からも虫体が分離されている。しかしながら、その感染メカニズムには未解明の部分も多かった。

そこで、トキソプラズマ原虫をウシ、イヌおよびマウスに実験的に感染させその経過を観察した。感染初期にはIgM抗体のみ、ついでIgMとIgG抗体、５－６週以降ではIgG抗体のみが検出され、いずれの動物でも、ほぼ３週以降では栄養型虫体が体内組織から消失し、心筋、横隔膜筋等の筋組織および脳内にシストを形成し、不顕性状態になった。そこで、妊娠ラットを用いて、トキソプラズマの胎仔移行について検討したところ、妊娠前に感染した慢性シスト保有ラットでは、胎仔への虫体移行は認められないが、妊娠前期および中期にトキソプラズマに初感染すると、容易に胎仔に移行し、

虫体保有の新生仔は発育不良の経過をたどること、また妊娠後期にトキソプラズマに初感染した場合は、全ての胎仔は虫体保有のまま死産あるいは流産されることが判明した。以上の結果から、先天性感染の成立要件、胎児（仔）に与える影響の違いを、宿主の免疫応答と関連して説明することが可能となった。

2. 細胞性免疫応答およびリンホカインに関する研究

トキソプラズマであらかじめ免疫したマウス（免疫マウス）および健康マウスを用い、それらの血液あるいは腹腔内に生原虫を接種して、虫体の細胞内侵入過程を観察したところ、健康マウスとは異なり、免疫マウス細胞中では虫体の増殖抑制あるいは殺滅像が認められた。その後の研究から、マクロファージ内での虫体の増殖抑制には、感作T-リンパ球が産生する可溶性媒介物質（リンホカイン）が必須の役割を持つことが明らかにされた。

トキソプラズマ感染を耐過した動物では、事後のトキソプラズマ抗原刺激により各種のリンホカインが産生されるが、この中にはマクロファージ活性化因子（MAF）以外に、トキソプラズマ増殖を抑制する未同定の物質（Toxo-GIF）の存在することが明らかにされた。Toxo-GIFは分子量3～4万の糖蛋白で、産生宿主と同じ動物種の細胞内原虫の増殖は阻止するが、異種動物の細胞内での増殖は阻止しないことが判かり、インターフェロンあるいはMIFなどとの異同について種々の面から検討することで、多くの知見が得られた。

Toxo-GIFおよび感染耐過動物血清を緩徐に加水分解して得られた低分子ポリペプチドの特定画分に、Toxo-GIF様活性が存在し、このものの効果発現には種属特異性が消失していることが判明した。さらに、本物質は細胞内のトキソプラズマ増殖を抑制するばかりでなく、一部の細菌・ウイルスに対しても抗微生物活性を発揮するとの知見が得られたので、本物質の作用は、いわゆる免疫調整物質のそれに類似のものであろうと考えられた。この物質の作用は、異なるバッチの標品でも良く再現されることから、以下オビオアクチンと称することとした。

3. オビオアクチンとトキソプラズマ虫体成分由来免疫賦活物質（TLA）の精製と合成ペプチドの開発研究

トキソプラズマ感染が長期間続くと、第1次および第2次免疫応答におけるマクロファージやT-リンパ球が抑制性に働き、時として免疫抵抗性の低下を引き起こす。一方、ヒトの後天性免疫不全症候群（AIDS）、イヌジステンパー、白血病ウイルス感染および免疫抑制剤投与などで、免疫不全がもたらされると、長期感染の末期と同様に、不顕性の慢性シストが破壊され、多数の虫体が出現増殖し、感染者（動物）が急速に斃死することが知られている。したがって、トキソプラズマ既感染者（動物）の生命維持には、感染者（動物）の免疫能の低下を抑制することが極めて大切である。このような観

点から、以下の免疫賦活能を持つ2種のペプチドを合成した。

オビオアクチン標品から出発して、活性を持つ最小の配列を求める実験を繰り返したところ、最終的にアミノ酸7個以下の4種のペプチドを同定することができ、オビオペプチド1—4と名付けた。

トキソプラズマ虫体成分の一部（TLA）を動物に投与するとインターフェロン-γ、MAF等のリンホカインが産生されるのみならず、Natural Killer, Lymphokine Activated Killer（LAK）細胞など多種のキラー細胞の増殖が生体内に誘導されることを見いだし、このTLA構成成分から、最も強く非特異免疫賦活効果を惹起する成分を目標に精製をかさね、アミノ酸73個のペプチド配列を決定し、合成した（TLA-H6E)。なお、TLAには牛馬の放牧飼育に多大な被害を与える、ピロプラズマ原虫症の発症を予防あるいは軽減する強い免疫賦活作用が存在することも示された。

4. オビオペプチドおよびTLA-H6Eペプチドの作用効果に関する研究

オビオペプチド（30—100 μg/head）を筋肉内投与すると、実験的自家発ガン肉腫の増殖遅延が認められ、マクロファージと好中球の増多・活性化、また脾臓内の幼若円形マクロファージの集簇が特徴的に観察された。一方、免疫抑制剤を大量投与すると、不顕性原虫および常在細菌、とくにメチシリン耐性菌が顕性化して斃死することが知られているが、通常飼育下のマウスに、免疫抑制剤とオビオペプチドを併用投与すると、種々の実験的細菌感染にも有意の差をもって、よりよく耐過した。この効果は、主としてマクロファージ、好中球活性化による異物貪食と殺滅機能の亢進によるものと推察された。また、トキソプラズマ慢性感染マウスに免疫抑制剤を投与するとトキソプラズマの増殖によって動物は死亡するが、オビオペプチド併用投与により動物の生残が有意に向上することも証明された。

免疫賦活物質としてのTLA-H6Eペプチドの家畜に対する投与実験によって、トキソプラズマ以外の原虫についても感染予防および発症軽減が認められ、本物質が単球—リンパ球—リンホカイン系の液性免疫を増強する作用に併せて、各種キラー細胞増殖を促進する作用のあることが推察された。

5. 産業家畜原虫病感染予防に関する研究

わが国におけるウシのピロプラズマ、ブタ・ヒツジのトキソプラズマ原虫の被害は甚大であるが、その感染予防および治療薬の開発は未だ世界的にも成功していない。われわれが開発した免疫賦活剤TLA-H6Eは非特異的かつ強い免疫増強効果を示し、牛のピロプラズマ発症を軽減することが明らかになっている。現在、原虫生ワクチンの作成とTLA併用の応用開発研究を遂行中であり、本研究の成果は我が国のみならず、発展途上国の畜産業への応用開発研究としても期待しうるものと考えている。

以上のように、本研究は長い間未解明であったトキソプラズマ原虫感染宿主の免疫応答を主体とした病態生理学的研究の成果に基づき、それらの知見を発展させるとともに、免疫調整物質（オビオペプチド）ならびに虫体抽出成分（TLA）由来免疫賦活物質（H6E）の合成に成功したものである。すなわち、このことは資源動物の生産性向上に大いに寄与すると考えられ、さらには原虫感染症の予防と治療に関する基礎を世界に先駆けて提示したものといえる。