後天性免疫不全症（ＡＩＤＳ）の治療における
トキソプラズマ抗原の役割に関する研究

（研究課題番号：６２４４００２０）

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研究代表者：鈴　木　直　義
（帯広畜産大学畜産学部　教授）
（東京大学農学部　教授）

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3．研究組織：

研究代表者：鈴木　直義（帯広畜産大学・東京大学教授）
研究分担者：三浦　弘之（帯広畜産大学教授）
広瀬　恒夫（帯広畜産大学教授）
齋藤　篤志（帯広畜産大学助教授）
佐藤　基佳（帯広畜産大学助教授）
三上　正幸（帯広畜産大学助教授）
五十嵐郁男（帯広畜産大学助手）
宮原　和郎（帯広畜産大学助手）
小俣　吉孝（帯広畜産大学助手）
桜井　治久（帯広畜産大学学振特研）

4．研究経費：

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5．研究経過

（A）はじめに

トキソプラズマ原虫（Toxoplasma gondii, Tp）は全世界的な哺乳動物に広がり、ヒトでは世界人口のうち約１０億人がＴｐ抗体を所有している。動物のうちイヌ、ネコ、ブタ、ヒツジなどの抗体保有率からはヒトと同様に世界各所から報告されているが、ヒツジ、ブタの本原虫感染による流産など以外に典型的症状を現すのは少なく、大部分はシスト保有の不顕性感染である。

先天性感染は妊娠中母体からのヒト胎児感染に伴う小児トキソプラズマ症が代表的であるが、マウス、ラット、モルモット、ハムスター、イヌ、ブタ、ヒツジ、家ウサギなどでも認められている。世界の動物のＴｐ感染率はヒツジで８～３５％、ブタで２４～４３％の肉から虫体分離、ウシでは約９％の感染率が世界の平均として報告されている。このような、ヒトおよび動物のＴｐ感染は第１にネコ糞便中オーシスト混入の食物摂取、第２に未調理または生鮮トキソプラズマ、シスト感染肉の摂食によると考えられている。感染成立後のＴｐは細胞内で増殖し、リンパおよび血流を介して諸臓器から全身へ分布し、最終的に脳内および筋肉内にシストを形成し長期間、不顕性の状態で生残する。

このような慢性Ｔｐ感染宿主が他疾病罹患、とくにＴ－細胞由来の免疫抑制状態になると、その作用機序は不明であるが脳内シストが破裂し、Ｔｐの脳内分散と増殖をおこない、著しい組織障害を伴った急性Ｔｐ症で多くの宿主は激症状態で斃死する。ヒトでの後天性免疫不全症候群（エイズ）、犬ジステムパー、猫白血病などでの本原虫感染は上述の発症過程で重篤症状を呈し宿主は急性死する。

Ｔｐ原虫感染による宿主体内のシストに対する完全治療方策は皆無である。しかし、ヒトの治療での多くは Sulfone 剤や Sulfamin 剤などを基剤として数種の抗生物質の併用療法が用いられている。生体

内Ｔｐシストの根絶を目的に、近年、抗Ｔｐ血清の生体への応用が注目されている。とくに、難治療病としてのＴｐシスト感染宿主の免疫能低下阻止をも含めてＩＦＮ－γなど免疫賦活調整剤と単一抗体（モノクローナル抗体）の応用開発が世界の研究の主体になりつつある。

本研究においても、研究代表者らが長年に亘り基礎的研究を遂行して来たＴｐ感染宿主由来感作Ｔ－リンパ球産生物質の１つ（オビオアクチン）、そしてＴｐ抗原由来免疫賦活成分（ＴＬＡ）による生体細胞賦活作用と免疫能正常値調節機構への影響を基礎的に検討を加えた。そして、それらの基礎研究から免疫不全症に対する治療応用の可能性について総合的な研究を企画した。

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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。

トキソプラズマ（Ｔｐ）原虫感染耐過動物体内のリンパ球、主としてＴ－リンパ球はＴｐ原虫由来の特異抗原成分（ＴＬＡ）に感作されるとサイトカイン（リンホカイン、ＬＫｓ）を産生放出することは現在良く知られていることである。このＬＫｓ中に正常マクロファージのみならず体細胞内Ｔｐ増殖を抑制する因子を Toxoplasma - Growth Inhibitory Factor (Toxo - GIF)として、世に公表されて久しい（Matsumoto et al., 1981）。この Toxo - GIF は極めて種属特異性が強く、同種細胞以外は活性化しない。その Toxo - GIF 含有牛血清の加水分解処理物質から、新規な天然免疫調整物質を摘出し、オビオアクチンと命名した（Suzuki, et al, 1984)。

一方、Ｔｐ由来の抗原成分（ＴＬＡ）の一部は、正常マウス投与によって、そのマウスはマラリアおよびピロプラズマ感染に対して抵抗性を示し、一部のマウスは生残することを研究代表者らは現像的に認め既に報告している（Suzuki, et al., 1986)。しかしながら、その防御機構に関しては殆ど不明であるために、猫白血病ウイルス、マウス接種スクレピーウイルス、マウス同種可移植腫瘍、Methylcholantren 誘発腫瘍、および感染原虫として Plasmodium berghei, Babesia rodhaini, おるいは Babesia microti を用いて、多種の異物抗原に対する宿主の感染抵抗性に及ぼす生体免疫賦活効果について検討した。

その結果、とくにＴＬＡ投与によるマウス腫瘍の増殖抑制には、腫瘍部位に大型Ｔｈｙ－１（＋）細胞の集族像が顕著に認められた。これら大型Ｔｈｙ－１陽性細胞の細胞回転など生物学的検討をおこなうと、いわゆるＴ－リンパ球由来 Lymphokine-Activated Killer 細胞の一種であることが明らかになった。同様の実験検索から、ＴＬＡ感作は Natural Killer (NK) 細胞の増生と活性にも強く関与し、大型

ＮＫ細胞の誘導にも関与することが判明した。そして又、これらのKiller 細胞の誘導には脾内マクロファージ（Ｍｐ）の存在が必須であり、それもＭｐはＮＫおよびリンパ球との接触が第一義的に重要であることも明らかになった。少なくとも、猫白血病および猫骨肉種など全く難治療病とされていた例のなかにＴＬＡ感作によって骨肉種の増殖停止、縮小あるいは白血病ウイルス感染猫の症状回復などの現像を単にＴＬＡ投与の効果として表現されるＬＡＫ細胞、大型ＮＫ細胞の誘導にみによると判定することは難しい。上述の生体反応を中軸とした全身性抵抗性賦与と考察しているが、今のところあらゆる実験成績を包含しても明白な論拠を提示出来る成績は皆無である。したがって、研究代表者らは天然免疫調整賦活物質（Obioactin, ＴＬＡ）の作用効果の有効性は現像として例示して来たが、最終年度に発見した本研究の類似化学合成物質（Obiopeptide-1, Suzuki; et al., 1990）の使用によってのみその生体内作用機構の根幹に近ずけ得る実験計画を企画出来ると結論した。

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Modulator Effect of Toxoplasma Lysate Antigen in Mice Experimentally Infected with Plasmodium berghei*

N. SUZUKI[1], K. KIKUSHIMA, T. MIYAGAMI, I. IGARASHI, H. SAKURAI, A. SAITO, and H. OSAKI[2]

Department of Veterinary Physiology, Obihiro University, Obihiro, Hokkaido, and Kochi Medical School[2], Kochi, Japan

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Summary

Normal mice were pretreated twice at an interval of 2 weeks with an emulsion of TLA (*Toxoplasma* lysate antigen), PLA (*Plasmodium* lysate antigen) or both in LMO (light mineral oil) or with a combination of the emulsion and Obioactin or Tp-LKs (*Toxoplasma* lymphokines) as an immunopotentiator. They were then given Obioactin or Tp-LKs 3 and 25 days after the first treatment and were further given parasitized erythrocytes with 1×10^2–10^4 *P. berghei* 2 weeks after the second treatment. Thirty (3/10, number of survival/number of examined) per cent of mice treated with TLA, 50 (5/10) % of those treated with a combination of TLA and Tp-LKs and 60 (6/10)% of those treated with a combination of TLA and Obioactin survived as long as 20 days postinfection while none of untreated controls survived more than 15 days postinfection. Only 18.2 (2/11)% of mice treated with PLA or TLA + PLA survived and 20 (2/10), 18.2 (2/11) and 60 (6/10)% of those treated with TLA + Obioactin, PLA + Obioactin or TLA + PLA + Obioactin survived throughout the experiment, respectively while none of controls survived more than 13 days postinfection. Five mice of each group were killed right before infection, and 5, 10 and 15 days postinfection. In mice treated with TLA + Obioactin, more macrophage phagocytosis and macrophage migration inhibition induced by sensitized T-cells were observed than in those treated otherwise. No appreciable differences were noted according to the method of treatment in blood examination values. Cross immunities between *Toxoplasma* and *Plasmodium* antigens were tested by counter-immunoelectrophoresis and indirect fluorescent antibody technique. By using counter-immunoelectrophoresis, a specific precipitin line was observed between TLA and anti-PLA which was absorbed by mouse erythrocytes, leucocytes and liver powder. By the indirect fluorescent antibody technique, anti-*Plasmodium* IgM and IgG titers were detected in sera from mice treated with TLA or TLA-Obioactin before infection.

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Introduction

When mice were sensitized with an emulsion of *Toxoplasma* lysate antigen (TLA) in Freund's incomplete adjuvant (FIA), they developed a strong ability to defend themselves against infection with *Plasmodium berghei* or *Babesia rodhaini* (*Omata* et al., 1979, 1980, 1981; *Ogawa* et al., 1985). In other words, to mice sensitized with FIA alone no such ability was conferred. In mice sensitized with FIA and TLA, however, it is known that the circulating blood contained such lymphokines produced by sensitized T-lymphocytes as interferon-gamma (IFN-r), macrophage activating factor (MAF), and macrophage migration inhibitory factor (MIF).

In the present experiment, mice were sensitized with TLA or *Plasmodium* lysate antigen (PLA) alone. Other mice were administered only with the immunopotentiator, lymphokines originated from splenic cells of *Toxoplasma*-immune mice (Tp-LKs), or simultaneously with Tp-LKs and Obioactin (*Suzuki* et al., 1984). The two groups of mice were examined mainly for responses to infection with *Plasmodium berghei*.

Materials and Methods

Mouse and protozoan strains. Three protozoan strains were used. They were the NK 65 strain of *Plasmodium berghei* (supplied by Professor *Mamoru Suzuki*, Department of Medical Parasitology, Gunma University) and the RH and the S-273 strains of *Toxoplasma gondii*, which had been inoculated into mice for passage. All the mice used were females 8–10 weeks old of the ICR/JCL strain which had been bred and raised at the laboratory of the Protozoan Cell Immunology of the Department of Veterinary Physiology, Obihiro University.

Preparation of Toxoplasma-immune mice, Toxoplasma-immune mouse lymphokines (Tp-LKs), Toxoplasma lysate antigen (TLA), and Plasmodium lysate antigen (PLA). *Toxoplasma*-immune mice were prepared by essentially the same method as described by *Makimura* and *Suzuki* (1982) and *Ogawa* et al. (1985). Tp-LKs were prepared and MIF contained in the lymphokines was measured by the methods of *Igarashi* et al. (1979) and *Sakurai* et al. (1983). TLA was prepared by the method of *Igarashi* et al. (1979) and PLA by that of *Itoh* et al. (1981). In preparing PLA, 40 ml of phosphate buffered saline (PBS; pH 7.2) was added to 20 ml of heparinized *Plasmodium*-infected mouse blood and washed twice by centrifugation (750 g, 10 min, 4 °C). The resulting precipitate was warmed at 37 °C to induce hemolysis. It was further washed twice with 0.83% ammonium chloride solution by centrifugation (45,000 g, 45 min, 25 °C). Then, it was washed three times with PBS by centrifugation (45,000 g, 45 min, 4 °C). To the final precipitate was added 3 ml of thrice-distilled water and subjected to the ultrasonic treatment (Kubota ultrasonic vibrator, Tokyo, 100W, 5 min). The resulting suspension was frozen and thawed five times at −80 °C and room temperature, respecitvely. It was again subjected to the ultrasonic treatment (100 W, 5 min). To it was added 3 ml of 1.7% NaCl to prepare an isotonic suspension. This suspension was centrifuged at 25,000 g for 45 min at 4 °C. The resulting supernatant was collected and used as PLA. The total protein content of PLA was estimated by *Lowry's* method (*Lowry* et al., 1951).

Preparation of anti-PLA. Two one-year-old male rabbits of the Japanese White breed were administered with immunogen, which was a dilution of PLA the total protein content of which was 35.7 mg/ml in 0.9% NaCl. In the first sensitization the rabbit was injected with 1 ml of 1 : 4 dilution of PLA into the auricular vein. On the same day of injection it was injected intramuscularly into the foot pad with a mixture of 0.5 ml of 1 : 6 dilution of PLA and 0.5 ml of Freund's complete adjuvant (PLA-FCA emulsion). One week after the first sensitization it was injected intravenously into the auricular vein and subcutaneously with

1.0 and 0.6 ml of 1 : 4 dilution of PLA, respectively. Furthermore, it was injected intramuscularly into the buttock with 2 ml of PLA-FCA emulsion twice, or 4 and 8 weeks after the first sensitization. Blood was collected from it 10 weeks after the first sensitization to separate serum.

At the time of use, anti-PLA serum was allowed to absorb (at 37 °C for 30 min) stepwise to the equivalents of normal mouse erythrocytes, leukocytes, and liver powder. As a result, it absorbed the corresponding antigen completely. In this manner, anti-PLA serum having absorbed erythrocytes (anti-PLA abs. RBC), anti-PLA serum having absorbed erythrocytes and leukocytes (anti-PLA abs. RBC and WBC), and anti-PLA serum having absorbed erythrocytes, leukocytes, and liver powder (anti-PLA abs. RBC, WBC, and L. P.) were obtained.

Estimation of Plasmodium and Toxoplasma antibody titers. Heparinized *Plasmodium*-containung erythrocytes were washed three times with 0.9% NaCl by centrifugation (630 g, 10 min, 4 °C). The resulting precipitate was smeared on a slide, fixed in methanol, and stored at −20 °C until use. *Toxoplasma* organisms were washed similarly three times with 0.9% NaCl by centrifugation. Then, they were fixed in 0.9% NaCl containing 10% formalin at 4 °C for an h. They were washed three times with 0.9% NaCl by centrifugation (190 g, 10 min, 4 °C). Finally, they were dropped on a slide, dried in air, fixed in methanol, and stored at −20 °C until use.

Serum to be tested was dropped on a *Plasmodium* or *Toxoplasma* smear preparation, which was allowed to stand in a humid box for an h. After that, the preparation was washed well with PBS and exposed to fluorescein isothiocyanate-labelled anti-IgG (Cappel Co., USA) or anti-IgM (Cappel Co., USA). It was again allowed to stand for an hour and washed well with PBS. It was sealed with carbonate buffer solution containing 10% glycerin and observed by a fluorescent microscope (Nikon Microphot-FX, Nikon Co., Tokyo). The serum was judged to be positive when organisms were stained to show fluorescence.

Physical examination of blood and serum. General blood examination was performed and enzymes contained in serum were estimated by the methods mentioned by *Ogawa* et al. (1985). Counter-immunoelectrophoresis was conducted by essentially the same method as reported by *Sakuarabayashi* (1979). Macrophages in the peritoneal cavity of the mouse were examined for phagocytosis in vitro of *Plasmodium*-containing erythrocytes by the methods mentioned by *Ishimine* et al. (1979) and *Ogawa* et al. (1985).

Effect of administration with TLA emulsion alone or simultaneous administration with TLA and Tp-Lks or with TLA and Obioactin on prevention of clinical Plasmodium infection of mice. Three groups, I to III, of 10 mature mice each were injected intraperitoneally with an emulsion containing 500 μg of TLA and 0.5 ml of LMO per head twice at an interval of 2 weeks. Moreover, the mice of group II were injected intraperitoneally with 1 ml of Tp-LKs twice, or 3 and 25 days after the first injection with the TLA emulsion, and those of group III intramuscularly with 10 mg of Obioactin twice on the same days as these. Each mouse of the three groups was inoculated intraperitoneally with 1×10^2 erythrocytes infected with *Plasmodium* (PE) 28 days after the first injection with the TLA emulsion. The number of surviving mice and the occurrence of parasitemia were examined in these groups every other day beginning with 5 days after inoculation. On the other hand, a control group was set up with 5 untreated healthy mice, which were inoculated intraperitoneally with 1×10^2–1×10^4 PE per head. In addition, to study the response of mice administered with Obioactin alone to mouse *Plasmodium*, 10 mice were injected intramuscularly with 10 mg of Obioactin 25 and 3 days before they were inoculated intraperitoneally with 1×10^2 PE. Then, they were examined for the number of surviving mice and the occurence of parasitemia.

Response of mice sensitized with TLA, PLA, or TLA + PLA antigen to infection with mouse Plasmodium. Four groups of 50 mature mice each were set up. Of them, one served as an untreated control. The other three, groups I, II and III, were injected intraperitoneally with 100 μg of TLA, 200 μg of PLA and a combination of 100 μg of TLA and 200 μg of PLA, respectively, twice at an interval of 2 weeks. Each mouse of all the groups was

inoculated intraperitoneally with 1×10^2 PE 28 days after the first injection with the emulsion. The general examination of blood and physicochemical examination of serum were carried out on 5 mice of each group 1 day before inoculation and 5, 10, and 15 days after inoculation with PE. In addition, the sensitized mice were administered with 10 mg of Obioactin to examine the effect of this substance given after sensitization.

Results

Ability of TLA emulsion, Obioactin, or Tp-LKs administered simultaneously to prevent mice from dying of infection with mouse Plasmodium. As shown in Table 1, 40 mature mice were divided equally into four groups, I to III and untreated control. Group I was administered with 500 μg of TLA emulsion per head alone. Group II was administered simultaneoulsy with 500 μg of TLA emulsion and 1 ml of Tp-LKs. Group

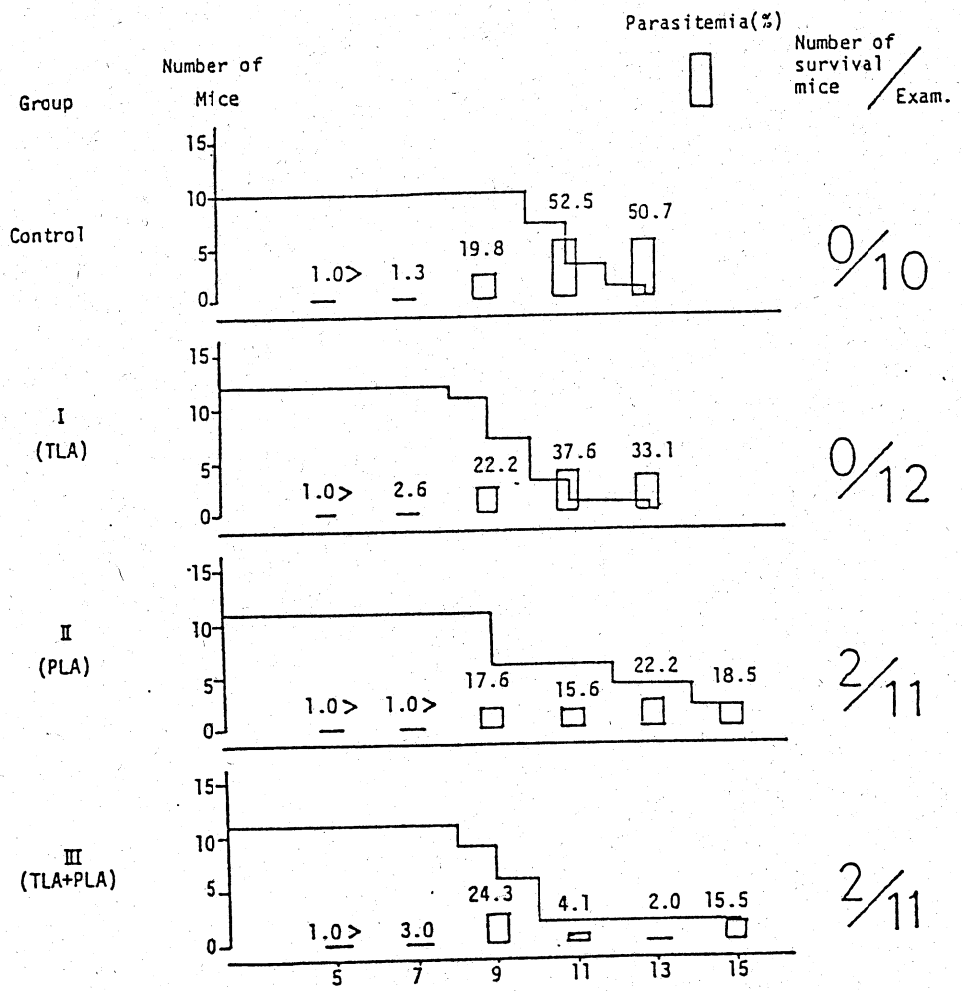

Fig. 1. Preventive effect against *P. berghei* infection in mice to pretreatment with TLA, PLA or TLA + PLA emulsion (Parasitemia and survival of infected mice). Remarks: Percentage of parasitemia was average of 5 mice.

Table 1. Adjuvant effect of TLA emulsion or a combination of Tp-LKs or Obioactin in mice infected with *P. berghei*

Group	Mice treated with	Days treated b. i. Infection[d]	Days after infection												Percent of mouse survival
			5		9		11		13		15		17		
			PE(%)[e]	S/E[f]	PE(%)	S/E	PE(%)	S/E	PE(%)	S/E	PE(%)	S/E	PE(%)	S/E	
	Untreated	−28 −25 −14 −3	0 1.0 (0.5)	10/10	2.6 39.6 (21.2)	6/10	24.3 44.5 (34.4)	5/10	(44.5)	2/10	(37.9)	1/10		0/10	0
I	TLA	TLA[a] TLA[a]	(0)	10/10	1.0 17.4 (8.2)	10/10	4.5 18.6 (11.3)	8/10	9.9 40.7 (24.9)	5/10	0 22.2 (11.1)	3/10	(0)	3/10	33.3
II	TLA+Tp−LKs	LKs[b] LKs[b]	(0)	10/10	1.0 6.8 (3.9)	10/10	5.3 12.4 (8.9)	8/10	10.5 16.0 (13.3)	7/10	10.5 12.9 (11.7)	7/10	0 14.3 (7.2)	5/10	50.0
III	TLA+Obioactin	Obio.[c] Obio.[c]	(0)	10/10	0 4.0 (2)	10/10	0 26.5 (13.2)	9/10	0 25.6 (12.3)	9/10	0 18.7 (9.4)	7/10	0 18.1 (9.0)	6/10	60.0

Remarks: [a] TLA, *Toxoplasma* lysate antigen (500 μg) emulsified in light mineral oil (0.2 ml) was injected into a mouse intramuscularly.
[b] Tp-LKs, *Toxoplasma* lymphokines (1 ml) or
[c] Obioactin (10 mg/0.1 ml physiological saline solution) was injected into a mouse intraperitoneally.
[d] Each mouse was inoculated with *Plasmodium berghei*-parasitized erythrocytes (PE), 1×10^2–10^4, intraperitoneally.
[e] PE, percentage of parasitized erythrocytes was shown in a range and (average).
[f] S/E, number of survivals/number of examined mice.

III was administered with 500 μg of TLA emulsion and 10 mg of Obioactin. The 10 mice of the untreated control group were inoculated intraperitoneally with 1×10^2–1×10^4 PE. After exhibiting a high rate of parasitemia, all of them died of infection within 16 days after inoculation. In group I, parasitemia began to appear 7 days after PE inoculation, but its incidence rate was lower than in the control group, and 3 mice were alive 17 days after inoculation and later. In groups II and III the mice presented a rate of survival of 50 and 60%, respectively, after inoculation.

Responses of mice sensitized with TLA, PLA, or TLA + PLA antigen to Plasmodium infection. As illustrated in Fig. 1, three groups, I, II and III, of mice were sensitized with 100 μg of TLA per head, 200 μg of PLA, and 100 μg of TLA + 200 μg of PLA, respectively. A control group of mice was also set up. All the four groups were inoculated intraperitoneally with 1×10^2 PE per head. As a result, all the mice died of infection in group I and the control group up to 13 days after inoculation. In groups II and III, two of the 11 mice were alive even 15 days after inoculation and later. When the incidence of parasitemia was examined, it was high, or 50.7%, in the control group and low, or 33.1%, 22.2% and 2.0%, in groups I, II and III, respectively, 13 days after inoculation. Especially, there was a statistically significant difference ($p < 0.05$) in it between group III and the control group.

Table 2 presents the results of estimation of the activity of macrophage migration inhibitory factor (MIF) contained in lymphokines produced by T-lymphocytes which were originated from splenic cells of these mice infected with mouse *Plasmodium.* When the mice of the untreated control group were infected with *Plasmodium,* they showed an MIF activity value of 4% 10 days after infection. In groups I, II and III the MIF activity value was 12%, 12%, and 8%, respectively, 1 day before infection and tended to increase with the lapse of time after infection, as indicated by the values estimated 5, 10, and 15 days after infection.

Table 2. Response of mice to treatment with TLA, PLA or TLA-PLA emulsion in macrophage migration inhibitory factor (MIF) activity after *Plasmodium* infection

Splenic cell culture supernatant derived from	Percent[a] activity of macrophage migration inhibitory factor: / Days after infection			
	−1	5	10	15
Control	0	0	4	
Group I (TLA)	12	32	44	
Group II (PLA)	12	20	40	32
Group III (TLA + PLA)	8	32	16	44

Remark: [a] $\text{MIF}\ (\%) = 100 \times \left(1.0 - \frac{\text{Average distance of migration in the test material}}{\text{Average distance of micration in the Tc-199 + 10\% calf serum medium}}\right)$

Table 3 exhibits the rate of phagocytosis of foreign body (canine red blood cells) by macrophages in the peritoneal cavity. In this experiment, splenic cells were collected from the mice of each group 1 day before and 5, 10, and 15 days after infection with ***Plasmodium.*** They were cultured with Con A for 48 h to collect a supernatant of the culture. The supernatant was used to culture macrophages harvested from the perito-

Table 3. Phagocytic activity of macrophages sensitized with splenic cell culture supernatant to normal beagle erythrocytes

Splenic cell culture supernatant derived from	Minutes incubated after addition of beagle RBC	Mean percentage of macrophages with normal beagle RBC: / Days after infection			
		−1	5	10	15
Control	30	9.2± 5.4	9.4± 6.8		
Group I (TLA)		18.8±11.9	16.2±13.3		
Group II (PLA)		14.6± 6.5	16.6± 4.4	17.8± 8.9	–
Group III (TLA+PLA)		13.4±11.1	9.4±14.5	–	–
Control	60	16.2± 8.6	33.4±11.7	12.8± 2.9	–
Group I (TLA)		20.2± 6.5	22.8± 9.6	15.4± 4.4	–
Group II (PLA)		11.8± 2.9	23.8± 8.4	15.2± 7.8	19.2±7.3
Group III (TLA+PLA)		22.4±10.6	26.4± 8.8	19.6±10.3	15.6±3.6

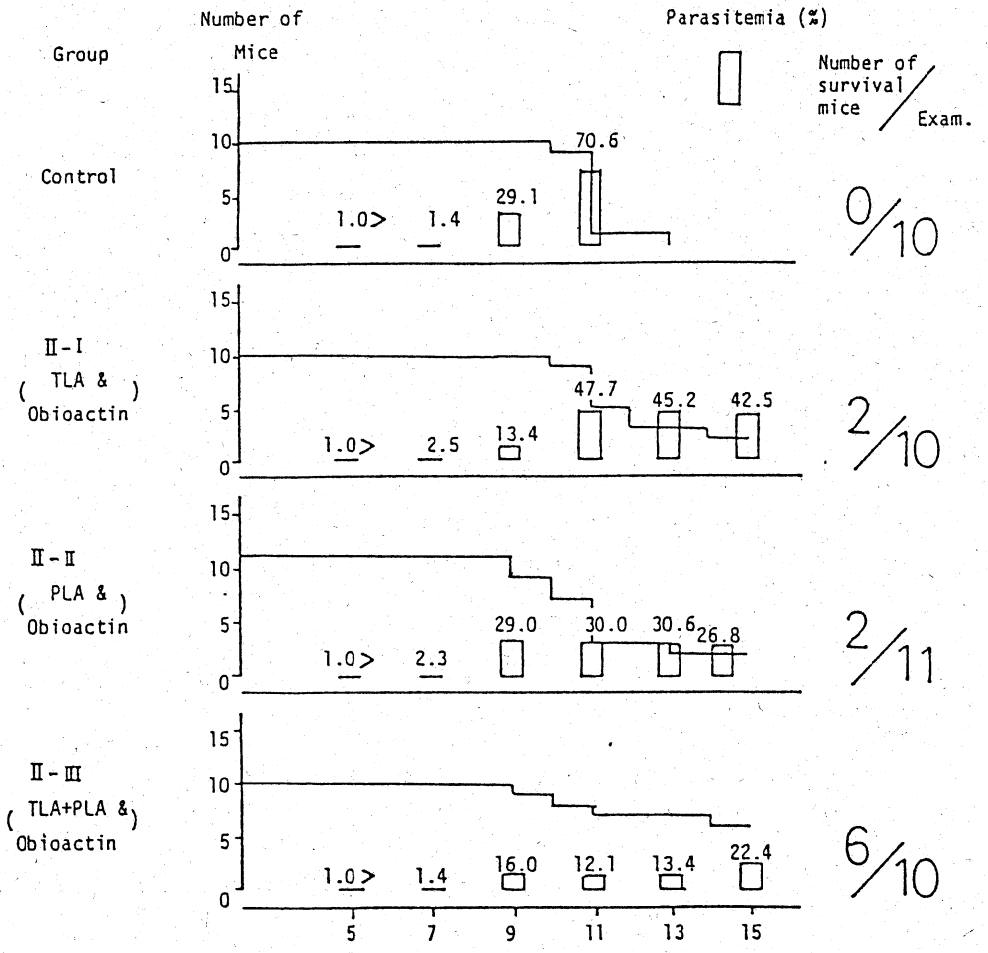

Fig. 2. Preventive effect against *P. berghei* infection in mice to pretreatment with TLA, PLA or TLA + PLA emulsion and these combination with Obioactin (Parasitemia and survival of infected mice). Remarks: Percentage of parasitemia was average of 5 mice.

neal cavity of a healthy mouse for 24 h. To the resulting macrophage monolayers were added canine red blood cells to determine the rates of phagocytosis of these cells by the macrophages for 30 and 60 min. In the control group these rates were 9.2% for 30 min and 16.2% for 60 min and increased to 9.4% and 33.4% respectively, 5 days after infection with *Plasmodium*. In groups I to III, sensitized with TLA and others, they were a little higher than in the control group 1 day before infection. There were, however, little differences in them between any sensitized group and the control 5 days after infection and later.

Responses of mice administered simultaneously with Obioactin and TLA, PLA, or TLA + PLA to Plasmodium infection. As shown in Fig. 2, 4 groups, II–I to II–III and an untreated control, of 25 mice each were set up. Group II-I was administered simultaneously with 100 μg of TLA and 10 mg of Obioactin, group II–II with 200 μg of PLA and 10 mg of Obioactin, and group II–III with 100 μg of TLA, 200 μg of PLA, and 10 mg of Obioactin. Then, every mouse was inoculated intraperitoneally with 1×10^2 PE. All the mice of the control group manifested a high rate of parasitemia and died of infection by 13 days after inoculation with PE. Even more than 15 days after inoculation were alive two of 10 mice of group II–I two of 11 mice of group II–II and six of 10 mice of group II–III.

Table 4. Body reaction against *Plasmodium* infection in mice by pretreatment with a combination of TLA, PLA or TLA-PLA emulsion and Obioactin

Splenic cell culture supernatant derived from	Percent activity of macrophage migration inhibitory factor: / Days after infection			
	−1	5	10	15
Control	0	15	92	
Group II-I (TLA + Obioactin)	15	58	85	96
Group II-II (PLA + Obioactin)	50	62	75	98
Group II-III (TLA-PLA + Obioactin)	31	92	73	98

Table 4 presents the results of chronological estimation of the activity of MIF contained in lymphokines originated from splenic cells of these mice infected with mouse *Plasmodium*. In the control group, MIF was not detectable 1 day before infection, but was proved to be rather high 10 days after infection. In groups II–I to II–III MIF activity value was 15–50% 1 day before infection and began to increase at the start of *Plasmodium* infection. Normal macrophages were activated when cultured in the presence of the lymphokines mentioned above. This activation was examined in beagles by estimating the rate of phagocytosis of red blood cells by macrophages. The results obtained are shown in Table 5. There was no difference in the degree of activation of macrophages between any two groups.

Titration of Plasmodium and Toxoplasma antibodies in serum of mice administered with TLA emulsion, PLA emulsion, or mixed emulsion of TLA and PLA or simultaneously with one of these emulsions and Obioactin. Table 6 shows *Plasmodium* antibody titers in the serum of these mice. IgM or IgG antibody against *Plasmodium* was not detected in 1 : 12 or 1 : 32 serum dilution of untreated control mice in these experi-

Table 5. Phagocytic activity of macrophages sensitized with splenic cell culture supernatant to normal beagle erythrocytes

Splenic cell culture supernatant derived from	Minutes incubated after addition of beagle RBC	Mean percentage of macrophages with normal beagle RBC: / Days after infection			
		−1	5	10	15
Control	30	3.0± 1.0	4.0± 2.0	4.8±3.9	
Group II-I (TLA + Obioactin)		5.4± 3.8	6.6± 4.7	6.8±2.4	–
Group II-II (PLA + Obioactin)		3.8± 2.7	4.4± 3.4	–	–
Group II-III (TLA + PLA + Obioactin)		7.2± 4.1	8.2± 2.9	–	7.4±7.0
Control	60	8.0± 7.3	10.8± 6.2	8.8±4.9	
Group II-I (TLA + Obioactin)		10.4±13.8	13.4±10.6	9.0±3.0	4.2±1.1
Group II-II (PLA + Obioactin)		15.2± 5.1	10.4± 7.4	13.2±4.4	3.0±2.0
Group II-III (TLA-PLA + Obioactin)		6.0± 3.7	21.4± 7.8	9.8±2.2	5.8±2.2

ments one day before infection, however, these IgM and IgG antibodies against *Plasmodium* presented a titer higher than 1 : 32 and 1 : 192 five and 10 days after infection. In groups I, II, and III which had been sensitized with TLA, PLA, and TLA + PLA with or without Obioactin, respectively, both of them presented titers ranging from 1 : 12 to 1 : 192 one day before infection, showing a tendency to increase in titer with the establishment of infection.

Table 6. *Plasmodium* antibody titers in the sera of mice to pretreatment with TLA, PLA or TLA + PLA emulsion and these combinations with Obioactin after *P. berghei* infection

Serum source	Antibody fraction	*Plasmodium* antibody titers Days after infection			
		−1	5	10	15
Control	IgM	< 1: 12	1: 32	1:192	
Group I (TLA)	IgM	1: 12	1: 32	1:192	
Group II (PLA)	IgM	1: 12	1: 32	1:192	1: 32
Group III (TLA + PLA)	IgM	1: 12	1:192	1:192	1:192
Control	IgM	< 1: 32	1: 12	1: 32	
Group II-I (TLA + Obioactin)	IgM	1: 32	1: 12	1: 12	1:192
Group II-II (PLA + Obioactin)	IgM	1: 32	1: 12	1: 12	1:192
Group II-III (TLA + PLA + Obioactin)	IgM	1: 32	1: 12	1: 12	1:192
Control	IgG	< 1: 12	1: 32	1:192	
Group I (TLA)	IgG	1:192	1:192	1:192	
Group II (PLA)	IgG	1: 32	1:192	1:192	1:192
Group III (TLA + PLA)	IgG	1: 32	1:192	1:192	1:192
Control	IgG	< 1: 32	1: 32	1:128	
Group II-I (TLA + Obioactin)	IgG	1:192	1:192	1:192	1:192
Group II-II (PLA + Obioactin)	IgG	1: 32	1:192	1:192	1:192
Group II-III (TLA + PLA + Obioactin)	IgG	1: 32	1: 32	1:192	1:192

Remarks: These levels are titers of pooled sera.

Table 7 gives the reults of estimation of *Toxoplasma* antibody titers. No *Toxoplasma* antibody was detected in 1 : 32 serum dilution of group II, sensitized with PLA, or the untreated control group before infection or 5, 10, or 15 days after infection with

Table 7. *Toxoplasma* antibody titers in the sera of mice to pretreatment with TLA, PLA or TLA + PLA emulsion and these combinations with Obioactin after *P. berghei* infection

Serum source	Antibody fraction	*Toxoplasma* antibody titers Days after infection			
		−1	5	10	15
Control	IgM	< 1:32	< 1:32	< 1:32	
Group I (TLA)	IgM	< 1:32	< 1:32	< 1:32	
Group II (PLA)	IgM	< 1:32	< 1:32	< 1:32	< 1:32
Group III (TLA + PLA)	IgM	< 1:32	< 1:32	< 1:32	< 1:32
Control	IgM	< 1:32	< 1:32	< 1:32	
Group II-I (TLA + Obioactin)	IgM	1:32	1:32	1:32	1:32
Group II-II (PLA + Obioactin)	IgM	< 1:32	< 1:32	< 1:32	< 1:32
Group II-III (TLA + PLA + Obioactin)	IgM	1:32	1:32	1:32	1:32
Control	IgG	< 1:32	< 1:32	< 1:32	
Group I (TLA)	IgG	1:32	1:32	1:32	< 1:32
Group II (PLA)	IgG	< 1:32	< 1:32	< 1:32	< 1:32
Group III (TLA + PLA)	IgG	1:32	1:32	1:32	1:32
Control	IgG	< 1:32	< 1:32	< 1:32	
Group II-I (TLA + Obioactin)	IgG	1:32	1:32	1:32	1:32
Group II-II (PLA + Obioactin)	IgG	< 1:32	< 1:32	< 1:32	< 1:32
Group II-III (TLA + PLA + Obioactin)	IgG	1:32	1:32	1:32	1:32

Remarks: These levels are titers of pooled sera.

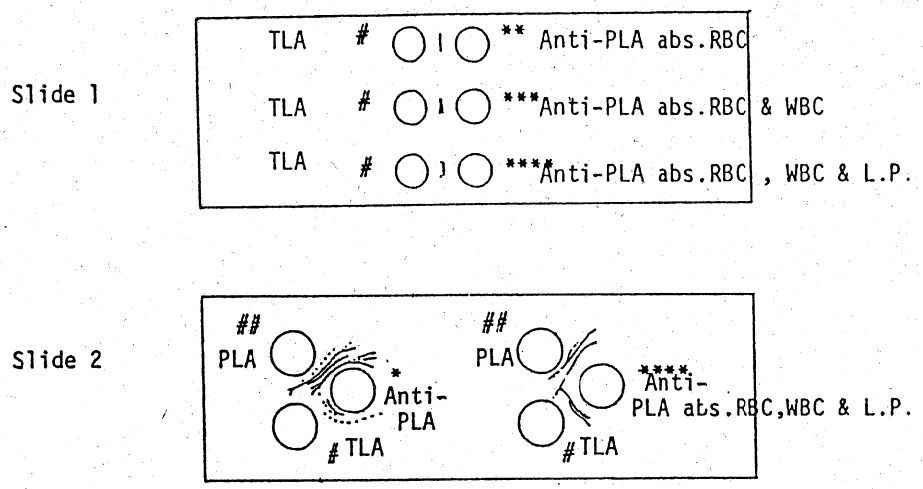

Fig. 3. Precipitin reaction by counter immunoelectrophoresis between TLA or PLA and anti-PLA or anti-PLA absorbed with mouse RBC, WBC and liver powder.

Plasmodium. When sensitized with TLA alone, groups I and III presented an IgG antibody titer, which was 1 : 32 or higher throughout the experimental period, but had no detectable IgM antibody. In groups II–I and II–III, where Obioactin was added to TLA, not only IgG but also IgM antibody were positive exhibiting a titer of 1 : 32.

To examine the cross immunogenicity between *Plasmodium* antigen and *Toxoplasma* antigen, counter-immunoelectrophoresis was carried out between TLA or PLA and anti-PLA serum. The results obtained are presented in Fig. 3. It was conducted on two slides. To the spots on one side of slide 1 was added TLA. To three spots on the other side of the slide were added anti-PLA abs. RBC, anti-PLA abs. RBC + WBC, and anti-PLA abs. RBC + WBC + L. P., respectively. On slide 2, TLA and PLA were added to spots on one side and anti-PLA and anti-PLA abs. RBC + WBC + L. P. to spots on the other side. The same experiment was repeated several times. The number of precipitin lines exhibited was not the same in all the experiments repeated. Nevertheless, distinct precipitin lines were detected among all the samples used in these experiments.

Discussion

It has already been reported that mice sensitized with TLA are resistant to infection with *Plasmodium* or *Babesia* (*Omata* et al., 1979, 1980, 1981; *Ogawa* et al., 1985). Besides, it has been reported that Obioactin activates macrophages in an experiment in vitro, and that it is an immunopotentiator with anti-microbial activities, including those against *Toxoplasma gondii* and *Trypanosoma cruzi* (*Suzuki* et al., 1984; *Osaki* et al., 1984). In the present experiments, it was clarified that simultaneous administration with TLA emulsion and Obioactin had a greater effect of preventing mice from infection with *Plasmodium berghei* than administration with TLA alone.

Two groups, A and B, of mice were set up and sensitized with TLA alone and simultaneously with TLA and Obioactin, respectively. Splenic cells were collected from them and cultured. The supernatant of the culture was examined for the presence of MIF activity. As a result, this activity was found in it from each of the two groups of mice. After these groups were infected with *Plasmodium*, MIF activity increased more remarkably in group B than in group A. Another experiment was performed to examine macrophages in the peritoneal cavity collected from mice sensitized with the supernatant of the splenic cell culture derived from the two groups of mice, for ability to phagocytize heterologous red blood cells. In it, the rate of these cells phagocytized by those macrophages was higher in the culture for 60 min than in that for 30 min. Essentially the same results as mentioned above have been reported by *Ockenhouse* and *Shear* (1982). The rate of red blood cells phagocytized by macrophages was a little higher in group B than in group A. In group B, it tended to increase distinctly at the same time when MIF activity rose remarably with the lapse of time after infection with *Plasmodium*. These results suggest that sensitization with T-lymphocytes, especially with Td-lymphocytes, and production of lymphokines may participate partially in the machanism of mice administered with TLA emulsion alone to prevent themselves from clinical infection with *Plasmodium* and from dying of this infection, and that Obioactin may have an immunizing action to enhance the sensitization with Td-lymphocytes and the active function of macrophages.

Then, cross immunogenicity between TLA and PLA was examined by counter-immunoelectrophoresis and the indirect fluorescent antibody technique. As a result, it was elucidated that a portion of TLA had cross immunogenicity with *Plasmodium*.

Accordingly, antibody against *Plasmodium* was detected in the serum of mice sensitized with TLA by the indirect fluorescent antibody technique. Therefore, it is highly probable that the participation of humoral antibody may be a cause for the resistance of mice sensitized with TLA to *Plasmodium* infection.

Recently, importance has been attached to deciphering genes with *Plasmodium* antigen as a code in studies on the prevention of malarial infection (*Coppel* et al., 1984; *Dame* et al., 1984; *Enea* et al., 1984; *Hall* et al., 1984). Efforts have been concentrated to prepare a *Plasmodium* vaccine. Under these circumstances it is necessary to isolate and purify TLA which has cross immunogenicity with a portion of PLA, and study the effect of simultaneous administration with TLA and Obioactin and the action mechanism of TLA on the living body.

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Dr. *N. Suzuki,* Department of Veterinary Physiology, Obihiro University, Obihiro, Hokkaido 080, Japan

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With Time Changes of T-Lymphocytes After Babesia Infection in Mice Pretreated with Toxoplasma Lysate Antigen*

MOTOYOSHI SATO[1], SHINICHI SEKIGUCHI[2], TADATOSHI MIYAGAMI[2], HARUHISA SAKURAI[2], ATSUSHI SAITO[2], TSUNEO HIROSE[1], and NAOYOSHI SUZUKI[2]

[1] Department of Veterinary Clinical Radiology and
[2] Department of Veterinary Physiology, Obihiro University, Obihiro, 080, Japan

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Summary

Adult female mice of the ICR/JCL strain were injected intraperitoneally with an emulsion of *Toxoplasma* lysate antigen (TLA; 100 μg per head) in light mineral oil (LMO) twice at 2 weeks' interval. Each mouse was inoculated intraperitoneally with 10^2 erythrocytes infected with *Babesia rodhaini* 2 weeks after the second injection of the emulsion. The rates of decrease in the erythrocyte count, hemoglobin concentration, and hematocrit value 10 days after inoculation, as compared with the counterparts determined 1 day before inoculation, were distinctly lower in these treated mice than in untreated control mice. In the course of *Babesia* inoculations, substantial reduction in size and weight of the thymus became higher in untreated control than in TLA-treated mice as the infection progressed. In untreated controls, the number of Thy-1 positive (Thy-1 (+)) cells was about 91.9% of the normal value being 3.7×10^7 cells in the whole thymus 1 day before inoculation while those in TLA-treated mice were 6.5×10^7 and 0.9×10^7 cells 1 day before inoculation and 10 days after inoculation, respectively. The size and weight of the spleen increased in both groups by day 10 after inoculation. The total number of Thy-1 (+) cells in the spleen was 3.9×10^7 and 11.7×10^7 1 day before and 10 days after inoculations, respectively in untreated and 12.9×10^7 and 15.6×10^7, respectively in TLA-treated mice. The number of Thy-1 (+) cells in the liver was 0.5×10^7 and 1.2×10^7 1 day before and 10 days after inoculations, respectively in untreated and 0.3×10^7 and 3.4×10^7, respectively, showing a rate increase of 1033.3% in TLA-treated mice. The rate of increase in count of Thy-1 (+) cells contained in the peripheral blood was 61.4% in the former and 64.1% in the latter. In untreated mice, degeneration and destruction of lymphocytes in the thymus and spleen follicles and aggregation of lymphocytes, which were not found in Thy-1 (+) staining, around pericapillary ducts in the liver were seen histopathologically 10 days after inoculation. In TLA-treated mice, however, perivascular infiltration by Thy-1 (+) lymphocytes and activation of Kupffer cells in the liver and activation of spleen follicles were observed locally 10 days after inoculation.

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Introduction

It has been reported by a group of investigators, including some of the authors, that when administered with *Toxoplasma* lysate antigen (TLA), mice acquire a strong resistance to experimental infection with mouse malaria *(Plasmodium berghei)* and mouse babesiosis *(Babesia rodhaini)* (*Omata* et al., 1979, 1981; *Ogawa* et al., 1985). When mice are administered with *Toxoplasma* lymphokines (Tp-LKs) or *Babesia* lymphokines (B-LKs) at the same time with TLA, they show a distinctly higher rate of surviving *Babesia* infection than those administered with TLA alone. Tp-LKs and B-LKs stand for lymphokines originated from the splenic cells of mice immunized to *Toxoplasma* (Tp) and *Babesia,* respectively. The increase in this rate is interpreted as follows. In the mice, macrophages and lymphocytes are sensitized by TLA and a lymphokine-like substance is produced in the body. Moreover, Tp-LKs or B-LKs administered simultaneously with TLA may activate the immune mechanism of the living body and enhance the effect of prevention of death from infection.

In the present studies, attention was paid to the behaviors of macrophages and lymphocytes among various responses of the living body to which had been conferred an ability to prevent itself from experimental infection with *Babesia,* as mentioned above. An attempt was made to clarify changes in lymphocytes, mainly T-cells, in mice administered with TLA and untreated control mice in *Babesia* infection.

Materials and Methods

Host animals and Babesia protozoa. The host animals used were adult female mice of the ICR/JCL strain 8–10 weeks old weighing 20–30 g each. The protozoa of *Babesia rodhaini* (Australian strain) used were supplied from the Kyushu Regional Laboratory, National Institute of Animal Health, Ministry of Agriculture, Forestry and Fisheries, and stored by passage into mice at the Department of Veterinary Physiology, Obihiro University.

Administration with TLA and inoculation with Babesia. TLA was prepared by the method of *Igarashi* et al. (1979) and emulsified with light mineral oil (LMO) for injection. Each mouse was injected intraperitoneally with 100 μg of TLA twice at 2 weeks' interval. It was inoculated intraperitoneally with *B. rodhaini* protozoa 2 weeks after the second injection. The inoculum per capita contained 1×10^2 erythrocytes infected with *B. rodhaini.* It was able to kill the mouse inoculated about 12 days after inoculation. Samples were collected from every mouse 1 day before and 10 days after inoculation with protozoa.

Preparation of visceral cell suspension. Thymic, splenic, and hepatic tissues were chopped finely and triturated between two slides. Then they were filtered through 40-mesh stainless steel filter to eliminate tissue debris. A cell suspension was prepared from the filtrate of each tissue. It was washed with Hanks' balanced salt solution (HBSS), pH 7.2, by centrifugation at 400 × g for 5 min. The precipitated cells were suspended in 0.83% ammonium chloride solution warmed at 37 °C to remove erythrocytes entering erroneously by hemolysis. This cell suspension was centrifuged at 400 × g for 5 min at room temperature. The precipitated cells were suspended again in HBSS and washed twice by centrifugation at 400 × g for 5 min each at 4 °C. Thymic, splenic, and hepatic cells were suspended in liquid culture medium 199 (TC-199). After their numbers were calculated, they were washed by centrifugation at 400 × g for 5 min at 4 °C. The precipitated cells were suspended in TC-199 to such extent that the resulting suspension might contain 5×10^7 cells per milliliter. This suspension was used for the cytotoxicity test. Moreover, some cells precipitated after washing with TC-199 by centrifugation were washed once with phosphate buffer solution (PBS), pH 7.2, by centrifugation at 400 × g for 5 min at 4 °C. They were suspended again in PBS and smeared on a slide for the measurement of fluorescence to carry out the indirect fluorescent antibody test (IFAT).

Preparation of anti-Thy-1 serum. *Golub's* methods (1971) was used to prepare anti-mouse Thy-1 serum. Briefly, the brain was collected from a Balb/c mouse and triturated after addition of physiological saline. The resulting brain suspension was filtered through a glass filter and emulsified with an equal amount of Freund's complete adjuvant (FCA). The resulting emulsion was injected into the foot pad and muscle of a rabbit three times at 2 weeks' intervals. The rabbit was injected subcutaneously with the brain suspension alone 2 weeks after the third injection. When immunized, it was bled totally 1 week after the fourth injection. Serum was separated from the blood harvested, and inactivated by heating at 56 °C for 30 min. Five volumes of serum were mixed with one volume of well-washed mouse erythrocyte pellet and allowed to stand at 0 °C for 60 min. Then, they were treated with mouse liver powder at 0 °C for 60 min. In this manner absorbed anti-mouse Thy-1 serum was prepared.

Test of cytotoxicity. Thy-1 positive (Thy-1 (+)) cells were calculated in the mice administered with TLA and untreated control mice by the methods of *Barker* et al. (1973) and *Igarashi* and *Wàki* (1983). In these methods, (1) 0.1 ml of cell suspension, (2) 0.1 ml of 1:32 dilution of anti-Thy-1 serum or normal rabbit serum (NRS) in physiological saline, and (3) 0.1 ml of complement which was 1:5 dilution in physiological saline of fresh guinea pig serum absorbed with thymic cells were mixed well in a test tube and incubated at 37 °C for 30 min. After incubation 0.03 ml of 0.5% trypan blue staining solution was added to the cell suspension. Under the light microscope a total of 200 cells in the suspension were observed to count the number of dead cells stained among them. In each sample the rate of dead cells were calculated from following formula:

Rate of dead cells (%) = [(Dead cells among cells treated with anti-Thy-1 serum) – (Dead cells among cells treated with NRS)] ÷ (Dead cells among cells treated with NRS)× 100.

The number of Thy-1 (+) cells was calculated as the number of cells found dead in the cytotoxicity test among all the cells contained in a given tissue sample.

Indirect fluorescent antibody test (IFAT). When this test was carried out on cells in a smear sample, the sample was first fixed in methanol for 5 min. As primary antibody, anti-mouse Thy-1 rabbit serum was added to the sample and incubated at 37 °C for 1 h. Then, the sample was washed with PBS. As secondary antibody, FITC-labelled anti-rabbit IgG goat serum (Goat Anti-Rabbit IgG, Fluorescein Conjugated IgG Fraction, Cappel Inc., U.S.A.) was added to the sample and incubated in the same manner at 37 °C for 1 h. After incubation the sample was washed with PBS and observed by the fluorescent microscope.

When the IFAT was carried out on histological sections, thymic, splenic and hepatic tissue pieces were fixed by three steps. First in an ice-cooled 1:1 mixture of acetone and ethanol for 90 min, secondly in the same but renewed mixture for 90 min, and thirdly in absolute ethanol at –20 °C for 12 h. Then the tissue pieces were dipped in a 1:1:2 mixture of ethanol, acetone and benzene for 3 h, the mixture being renewed at the begining of the second and the third hour. After that, they were dipped in benzene at 0 °C for 3 h, the chemical being renewed in the same manner after an hour passed. They were soaked in 54 °C paraffin (mp. 52–54 °C) for 30 min, the paraffin being renewed every 10 minutes. Finally, they were embedded in paraffin to prepare paraffin blocks. For fluorescent staining, each block was cut into sections 3 μm thick and mounted on slides. The sections were deparaffinized and dipped in PBS. Like the smear samples, they were labelled with anti-mouse Thy-1 rabbit serum, as primary antibody, and with FITC-labelled anti-rabbit IgG goat serum, as secondary antibody. Finally, they were examined under the fluorescence microscope. Concurrently, serial sections of the same specimen were double-stained with hematoxylin and eosin, and observed by the light microscope. By comparing the results between the two staining methods, it was possible to confirm the presence of Thy-1 (+) cells and anti-Ig positive cells in the tissue.

Histopathological examination. Thymic, splenic, and hepatic tissue pieces were fixed in 10% formalin solution and embedded in paraffin. Histological sections were prepared from them and double-stained with hematoxylin and eosin. They were examined histopathologically.

Results

1. *General properties of blood*

As shown in Table 1, the erythrocyte count (RBC), hemoglobin concentration (Hb), and hematocrit value (Ht) determined 10 days after inoculation with *Babesia* showed a decrease, as compared with the counterparts determined 1 day before the inoculation. The rate of decrease was 30.6% for RBC, 50.9% for Hb, and 33.1% for Ht, in the untreated control group. In the group treated with TLA, it was 10.1% for RBC, 32.0% for Hb and 19.8% for Ht. In other words, the treated group exhibited lower rates of decrease in RBC, Hb and Ht than the control group. In addition, it was affected with milder parasitemia than the control group.

Table 1. Changes of erythrocytes, hemoglobin concentration and hematocrit in mice after *Babesia* infection

Item of examination	TLA treated group			Untreated group		
	Days postinfection		Rate of change (%)[c]	Days postinfection		Rate of change (%)[c]
	− 1[a]	10[b]		− 1[a]	10[b]	
RBC × $10^4/\mu l$	845	760	− 10.1	886	615	− 30.6
Hb g/dl	16.9	11.5	− 32.0	17.5	8.6	− 50.9
Ht %	43.0	34.5	− 19.8	47.8	32.0	− 33.1
Parasitemia %	0	40.1		0	57.5	

Note: $c\,(\%) = \frac{b-a}{a} \times 100$

2. *Changes in lymphocytes in thymus, spleen and liver*

As shown in Table 2, the total number of Thy-1 (+) cells was 3.4×10^7 and 3.9×10^7 in the thymus and spleen, respectively, in the control group 1 day before inoculation with *Babesia*. It was 6.5×10^7 and 12.9×10^7 in the thymus and spleen, respectively, in the treated group. In brief, it was clearly larger in this group than in that. The number of Thy-1 (+) cells in the thymus was 0.9×10^7 and 0.4×10^7, presenting a rate of decrease 86.2 and 88.2%, in the treated and control group, respectively, 10 days after inoculation. There was no significant difference in the rate of decrease between the two groups. The number of Thy-1 (+) cells in the spleen was 15.6×10^7 and 11.7×10^7 in the treated and control group, respectively, 10 days after inoculation. It was larger in these groups 10 days after inoculation than 1 day before inoculation. The difference in it, however, was small between the two periods of time in the treated group, in which it was considerably large 1 day before inoculation.

The number of Thy-1 (+) cells in the liver was 0.3×10^7 and 0.5×10^7 in the treated and control group, respectively, 1 day before inoculation. In short, it was a little smaller in the treated group than in the control. It was 3.4×10^7 and 1.2×10^7 in the treated and control group, respectively, 10 days after inoculation, showing a distinct increase. Especially in the treated group, such increase was outstanding, showing a rate of increase of 1033.3%.

Table 2. Changes of number of Thy-1(+) cells (IFAT)

Organs		TLA treatment	Days postinoculation − 1[a]	10[b]	The rate of change (%)[c]
Thymus[d]					
	No. of mononuclear cells × 10^7	Untreated	3.7	0.5	−86.5
		Treated	7.8	1.5	−80.8
	No. of Thy-1(+) cells × 10^7	Untreated	3.4	0.4	−88.2
		Treated	6.5	0.9	−86.2
Spleen[d]					
	No. of mononuclear cells × 10^7	Untreated	8.0	33.0	312.5
		Treated	33.0	48.0	45.5
	No. of Thy-1(+) cells × 10^7	Untreated	3.9	11.7	200.0
		Treated	12.9	15.6	20.9
Liver[d]					
	No. of mononuclear cells × 10^7	Untreated	1.2	2.4	100.0
		Treated	0.7	4.7	571.4
	No. of Thy-1(+) cells × 10^7	Untreated	0.5	1.2	140.0
		Treated	0.3	3.4	1033.3
Peripheral blood[e]					
	No. of mononuclear cells × 10^5	Untreated	9.7	36.0	73.1
		Treated	23.0	50.0	54.0
	No. of Thy-1(+) cells × 10^5	Untreated	6.8	17.6	61.4
		Treated	9.9	27.6	64.1

Note: $c\,(\%) = \frac{b-a}{a} \times 100$

d; Each value was calculated as the mean from 4 mice.
e; Each value was calculated as per ml of pooled blood from 5 mice.

The number of Thy-1 (+) cells was 6.8×10^5 and 17.6×10^5 1 day before inoculation and 10 days after inoculation, respectively in untreated and 9.9×10^5 and 27.6×10^5, respectively in TLA treated group.

When mice which had not been administered with TLA were examined for Thy-1 (+) cells 1 day before inoculation, and 3,6 and 10 days after inoculation by the cytotoxicity test, they decreased remarkably in the thymus and increased obviously in the spleen. Fig. 1 exhibits the rise and fall in the number of these cells in both organs with the lapse of time. The cells began to decrease remarkably in the thymus immediately after inoculation with *Babesia*. In the spleen, however, they increased gradually with the lapse of time after the inoculation.

3. Changes in serum antibody titer

As shown in Table 3, the titer of antibody against *Toxoplasma* was 1:32 and 1:128 in the treated group 1 day before and 10 days after inoculation with *Babesia*, respectively, when estimated by the IgG-IFAT and the latex agglutination reaction test. It was lower than 1:32 and lower than 1:16 in the control group before and after inoculation, respectively.

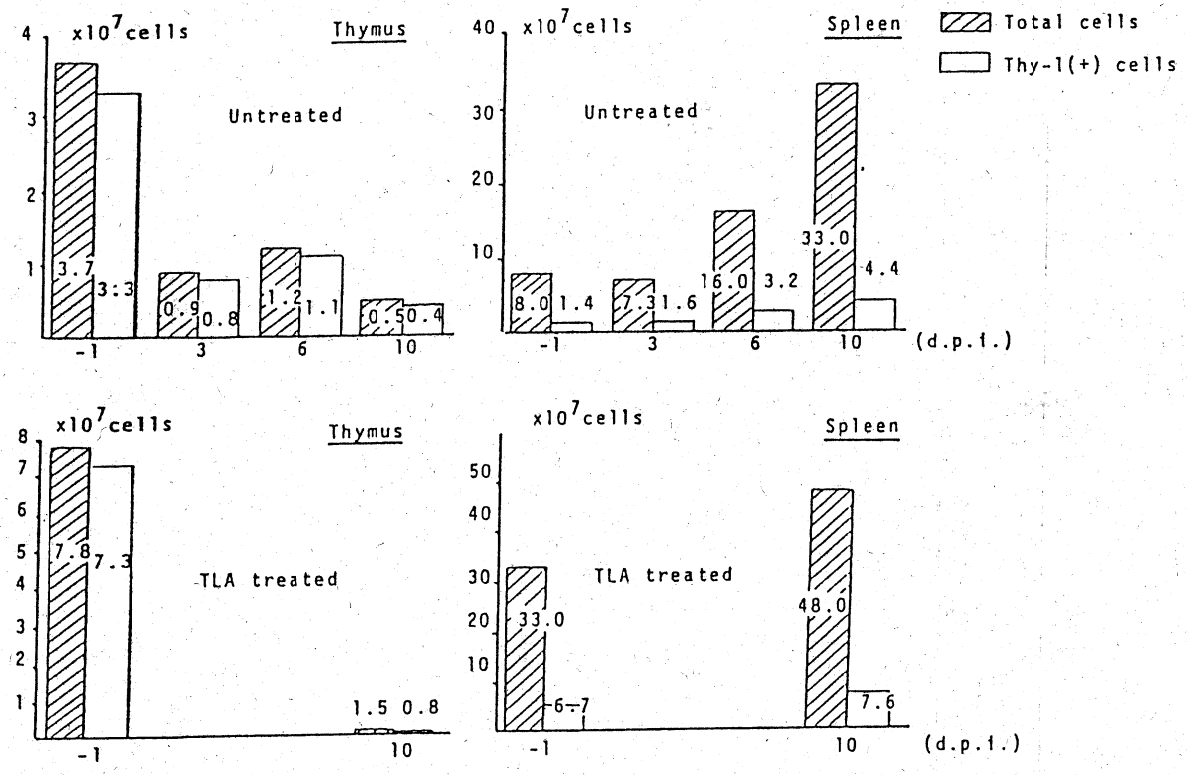


Fig. 1. Changes of Thy-1 (+) cells on the thymus and spleen (cytotoxicity test).

Fig. 2. Vacuolization in cytoplasm of hepatic cells, perivascular cell infiltration, and activation of RES in the liver of a mouse (No. 1) administered with TLA, 1 day before infection. Hematoxylin and eosin staining (HE), × 238.

Fig. 3. Same as above.

Fig. 4. Lucidification at the center of follicles in the spleen of mouse No. 1. HE, × 238.

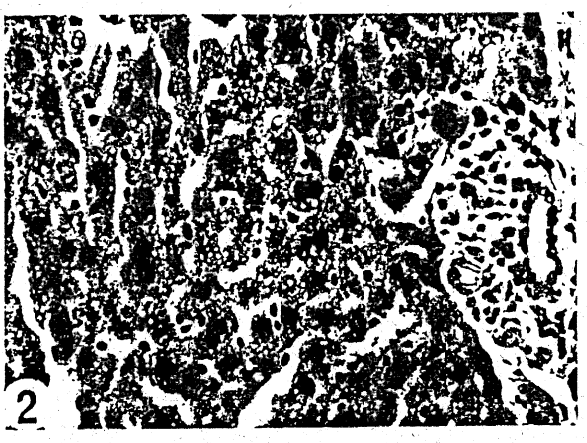
Fig. 5. Turbid swelling and necrosis of hepatic cells in a mouse (No. 2) not administered with TLA, 10 days after inoculation with *B. rodhaini*. HE, × 238.

Fig. 6. Loosening of lymph follicles and degeneration of mouse No. 2. HE, × 238.

Fig. 7. Same as above. HE, × 119.

Fig. 8. Degeneration of lymphocytes in the thymus of mouse No. 2. HE, × 238.

Fig. 9. Same as above. HE, × 476.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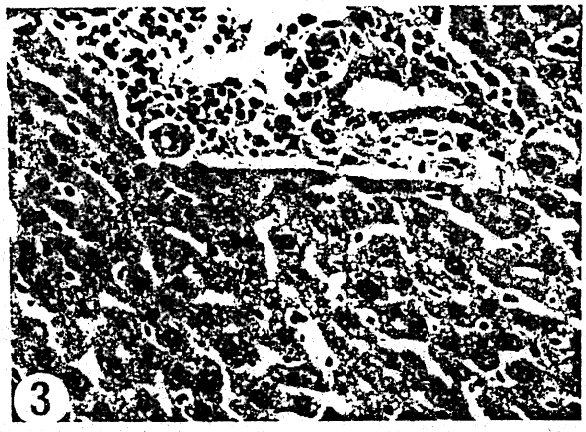
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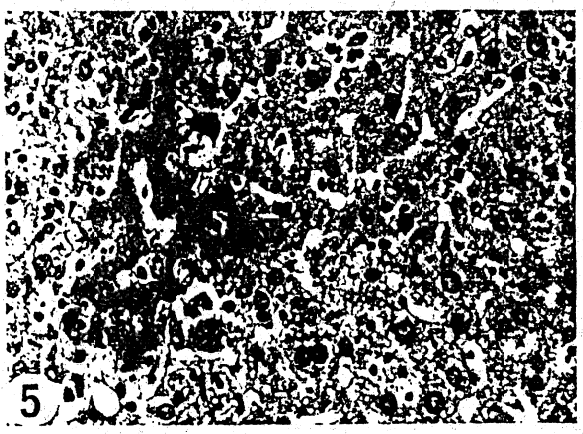
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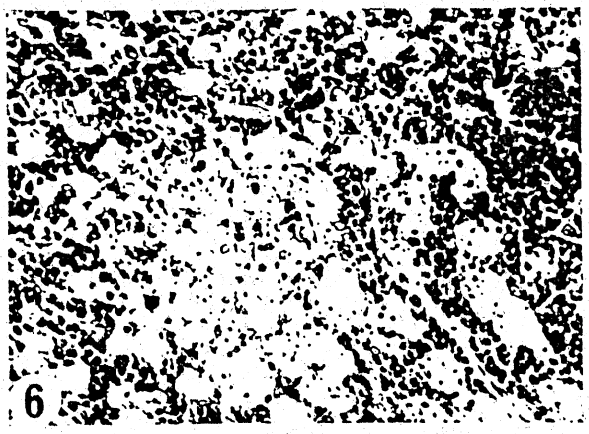
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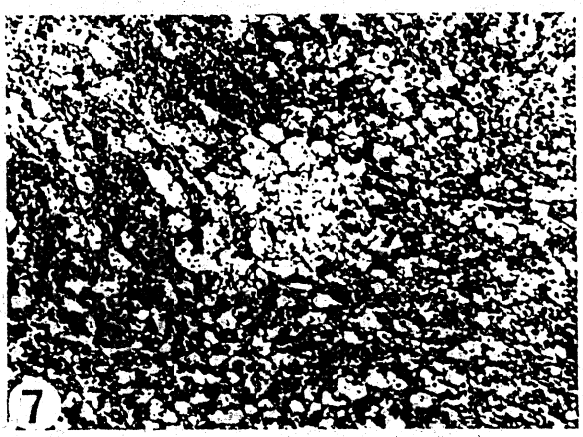
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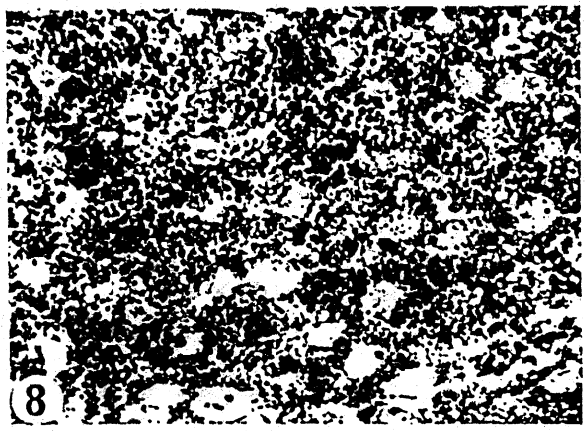
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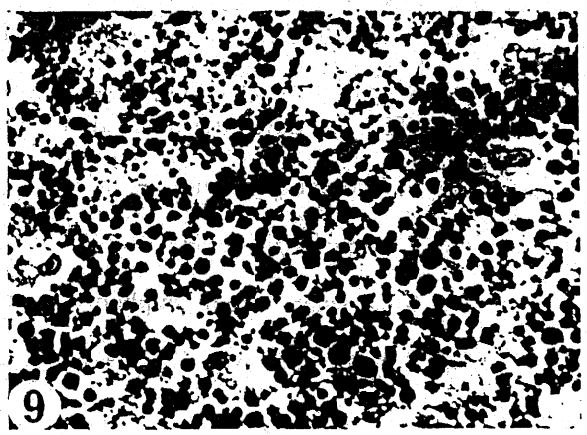
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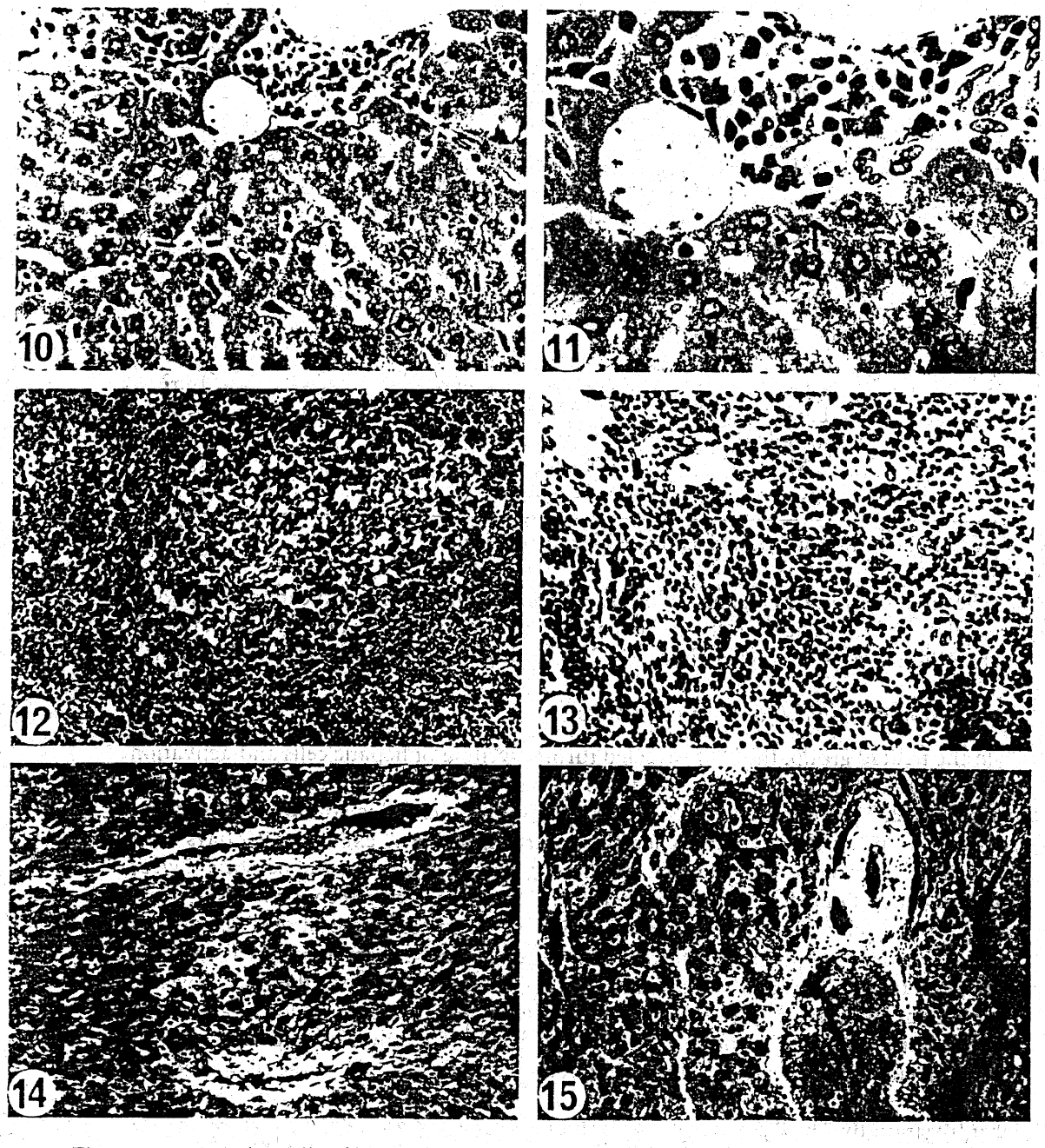

Fig. 10. Perivascular cell infiltration and activation of RES in a mouse (No. 3) administered with TLA, 10 days after inoculation with *B. rodhaini*. HE, × 238.
Fig. 11. Same as above. HE, × 476.
Fig. 12. Activity of follicles in the spleen of mouse No. 3. HE, × 119.
Fig. 13. Proliferation of reticular cells in the thymic cortex of mouse No. 3. HE, × 238.
Fig. 14. Perivascular infiltration by Thy-1 positive cells in mouse No. 1. Indirect fluorescent antibody test (IFAT), × 135.
Fig. 15. Perivascular infiltration by Thy-1 positive cells in mouse No. 3. IFAT, × 180.

Table 3. Changes of antibody titer in mice treated with TLA by *Babesia*-infection

TLA Treatment	Antibody titers	Days postinoculation			
		− 1	3	6	10
Untreated	Anti-*Toxoplasma*				
	IgM	< 1:32	< 1:32	< 1:32	< 1:32
	IgG	< 1:32	< 1:32	< 1:32	< 1:32
	Latex	< 1:16	< 1:16	< 1:16	< 1:16
	Anti-*Babesia*				
	IgM	< 1:12	1:12	1:12	1:192
	IgG	< 1:12	1:32	1:32	1:192
Treated	Anti-*Toxoplasma*				
	IgM				
	IgG	1:32			1:32
	Latex	1:128			1:128
	Anti-*Babesia*				
	IgM	1:12			1:192
	IgG	< 1:12			1:192

Note: Antibody titer was tested as pooled sera from 4 mice.

The titer of antibody against *Babesia* was lower than 1:12, indicating negativity, regardless of IgM or IgG, in the treated and control group 1 day before inoculation. It increased, however, to higher than 1:192 in both groups 10 days after inoculation.

4. Histopathological findings of various organs

In the treated group, the liver revealed turbid swelling of hepatic cells and activation of the reticulo-endothelial system and had perivascular foci of aggregation of lymphocytes (Figs. 2, 3). The spleen presented activation of follicles (Fig. 4). No marked changes were found in the thymus. When the control group was examined 10 days after inoculation, the liver revealed turbid swelling of hepatic cells and focal necrosis (Fig. 5) and had perivascular foci of aggregation of lymphocytes. The spleen exhibited loosening of lymph follicles, degeneration of lymphocytes, and proliferation of reticular cells (Figs. 6, 7). The thymus was affected with degeneration of lymphocytes (Figs. 8, 9). When the treated group was examined 10 days after inoculation, the liver revealed perivascular infiltration of lymphocytes, activation of the reticulo-endothelial system, and edematous swelling of hepatic cells (Figs. 11, 12). The other findings were activation of splenic follicles (Fig. 12) and proliferation of reticular cells in the thymic cortex (Fig. 13). In the staining of the Thy-1 (+) cells by the IFAT in the liver tissue, perivascular infiltration by Thy-1 (+) cells was found in the treated group 1 day before and 10 days after infection (Figs. 14, 15). In the control group, however, Thy-1 positive cells were not found in the perivascular spaces 10 days after infection.

Discussion

It has been reported up to this time that as responses of the living body to the administration with TLA, the production of such LKs as γ-interferon (γ-IFN) by the sensitization of T-lymphocytes, and the activation of macrophages (MΦ) take place

(*Omata* et al., 1979, 1981; *Ogawa* et al., 1985). The administration with TLA induces a resistance to experimental infections with mouse babesiosis and mouse malaria. This resistance is presumed to be caused by sensitization of T-lymphocytes. Moreover, it has been suggested that some of the components of antigen originated from *Toxoplasma* protozoa may cross-react with antigen of parasitic protozoa in erythrocytes. TLA is assumed to be an "immunomodulator" which has such effect that it activates cellular and humoral immune responses to parasitic protozoa in erythrocytes nonspecifically. In the present experiment, studies were made on changes in lymphocytes, mainly T-cells, which play a principal part in the cellular immune response.

The number of Thy-1 (+) cells in the thymus was approximately more than two times as large in the mice of the treated group as in those of the control group. After *Babesia* was inoculated, the thymus showed a remarkable atrophy and Thy-1 positive cells were reduced obviously in number in this organ. In the mice of the control group, lymphocytes were swollen and disintegrated very clearly in the thymus. On the contrary, the thymus was slightly atrophic and reticular cells proliferated in those of the treated group.

No papers have been published to report a decrease in the number of Thy-1 (+) cells in the thymus after infection with *Babesia*. *Igarashi* and *Waki* (1983) mentioned that in mice infected with mouse malaria *(P. berghei)* the thymus fell in atrophy after infection, that most of the lymphocytes contained in this organ were released in the circulating blood, and that finally they accumulated in the liver, showing a remarkable increase in number with the lapse of time. They assumed that lymphocytes in the thymus might be destroyed by cytotoxins and others, and that this organ might be atrophic accordingly.

On the other hand, the number of Thy-1 (+) cells in the spleen was approximately more than five times as large in the mice of the treated group as in those of the control group. After infection with *Babesia* it increased in both groups. Especially in the control group it exceeded 200% of the number of these cells determined before infection.

Histopathologically, loosening of lymph follicles and disintegration of lymphocytes were pronounced in the control group, and activation of lymph follicles was noticed in the treated group. It is of interest to note that these results were contrary to those obtained by *Igarashi* and *Waki* (1983) from mice infected with *P. berghei* and exhibiting a decrease in the number of Thy-1 (+) cells in the spleen. Therefore, it was clarified that there was a difference in the immune response of the host, especially changes in Thy-1 (+) cells, between *B. rodhaini* and *P. berghei,* both of which parasitized erythrocytes in the same manner. *Aikawa* et al. (1980) reported that there was a difference in changes in Thy-1 (+) cells in the thymus between *P. chabaudi* and *P. berghei* infections. Taking their report into consideration, it is of great interest to note that Thy-1 (+) cells accumulated in the spleen in mice infected with *B. rodhaini* in the present experiment.

There was a marked increase in the number of Thy-1 (+) cells in the liver of mice infected with *B. rodhaini.* This increase was particularly noticeable in the treated group. *Dockrell* et al. (1980) presumed that there might be a defence mechanism in mice infected with *P. yoelii,* and that in this mechanism the accumulation of T-lymphocytes in the liver might participate in the destruction of *P. yoelii* protozoa in the liver. *Igarashi* and *Waki* (1983) observed that T-lymphocytes increased remarkably in the liver of mice infected with *P. berghei.* They suggested that these lymphocytes might act as ergogenic cells for host cells harboring malaria protozoa, and that they might exert an action to disturb not only malaria protozoa parasitizing host cells but also host cells themselves harboring these protozoa. In the present experiment, histophatological

examination of the liver revealed distinct turbid swelling of hepatic cells, serious focal necrobiosis, and scattered perivascular aggregations of lymphocytes. These findings seem to lend support indirectly to the suggestion of *Igarashi* and *Waki* (1983). On the other hand, the mice of the treated group were affected with edematous swelling of hepatic cells, perivascular infiltration of lymphocytes, and activation of the reticuloendothelial system. These changes, however, were so mild that the severity of disturbances induced by infection was much lower in the treated group than in the control.

In conclusion, when mice were administered with TLA, Thy-1 positive cells were found to accumulate remarkably in the thymus and spleen and there was an increase in the immune response of the host to infection with *B. rodhaini*. Histological disturbances caused by infection were distinctly milder in these mice than in those not administered TLA.

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Dr. *Motoyoshi Sato,* Department of Veterinary Clinical Radiology, Obihiro University, Obihiro 080, Japan

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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*

ATSUSHI SAITO[1], HARUHISA SAKURAI[1], TOSHIO SAITO[1], SHUICHI TAJI[1], TADATOSHI MIYAGAMI[1], NAOYOSHI SUZUKI[1], HANNS M. SEITZ[1]**, and HUMIO OSAKI[2]

[1] Department of Veterinary Physiology and Laboratory of Protozoan Immunology, Obihiro University, Obihiro, Hokkaido and [2] Kochi Medical School, Kochi, Japan

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Abstract

The inhibition of *Toxoplasma* multiplication inside cells does not correlate with an enhanced release of oxygen intermediates except in the case of peritoneal macrophages treated with Obioactin. The inhibition observed in alveolar macrophages treated with Obioactin, in kidney cells treated with Obioactin or lonomycin A and in peritoneal macrophages treated with lonomycin A was not accompanied by an increment of release of oxygen intermediates. Lipopolysaccharide (LPS) and muramyl-dipeptide (MDP) enhanced the release of toxic oxygen intermediates in peritoneal macrophages, but did not have any toxoplasmacidal effect. Adenosine triphosphate (ATP) content increased during Obioactin, MDP or *Toxoplasma* lysate antigen (TLA) treatment. The actual oxygen consumption of the peritoneal macrophages treated with Obioactin increased dose dependently, but that of TLA-, lonomycin A- or MDP-treated cells did not change. These results suggest that the relationship between the intracellular killing of *Toxoplasma* protozoa and the release of oxygen intermediates differs according to the cells and/or the stimuli, and that the cellular mechanism of *Toxoplasma* killing in the peritoneal macrophages treated with Obioactin involves an energy-dependent mechanism.

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Indroduction

It is reported that *Toxoplasma* parasites which penetrate or were phagocytized into the macrophages were killed by toxic oxygen intermediates inside macrophages (12, 13, 21). *Toxoplasma* multiplication inside macrophages was inhibited by treatment with the antibiotic lonomycin A (11) and with Obioactin (21), one of the immunopotentiators which can be obtained from *Toxoplasma*-immune cattle serum by hydrolyzation. The release of oxygen intermediates from the peritoneal macrophages was increased by treatment with Obioactin (21). However, it is not clear to date whether the release of oxygen intermediates is enhanced by lonomycin A treatment. On the other hand, muramyl dipeptide (MDP) and lipopolysaccharide (LPS) enhanced the release of oxygen intermediates from the peritoneal macrophages (6, 20) and activated the glucose oxidation by the peritoneal macrophages (6). The superoxide releasing capacity of the peritoneal macrophages from mice pretreated with *Toxoplasma* lysate antigen (TLA) was higher than that from non-treated mice (18). However, it is also unclear whether the multiplication of *Toxoplasma* inside cells is inhibited by in vitro treatment with MDP, LPS or TLA.

It is the purpose of this paper to disclose the relationship between the release of oxygen intermediates (H_2O_2 and O_2^-) and the inhibition of *Toxoplasma* multiplication inside cells, and the effects of Obioactin and other substances on the cellular metabolism of glycogen-induced peritoneal macrophages.

Material and Methods

Mice and Toxoplasma strains used. ICR-JCL mice, 8–10 weeks old, were used. *Toxoplasma gondii* of the RH strain were used in this study.

Stimuli used. MDP was obtained from Peninsula Lab., U.S.A. LPS was obtained from Difco Lab., U.S.A. Lonomycin A was obtained from Taisho Pharm., Japan. TLA and Obioactin were prepared in the same manner as described previously (3, 21).

Cell monolayers

Peritoneal macrophages. Mice were injected i.p. with a 0.2% glycogen solution. Five days later, peritoneal exudate cells were harvested by washing the peritoneal cavity with heparinized *Hanks'* balanced salt solution (HBSS). The cells were suspended to a concentration of 1×10^6 nucleated cells/ml in TC-199 medium containing 10% heat-inactivated calf serum (TC-199-CS), penicillin (100 U/ml) and streptomycin (100 μg/ml). One ml of the suspension was placed in each well of a multidish tray containing a round coverslip and incubated at 37 °C for 24 h in air with 5% CO_2.

Alveolar macrophages. Lungs and kidneys (see below) were isolated from the same mice. The lungs were filled with heparinized HBSS using a syringe fitted with a 27 gauge needle, and minced with a pair of scissors. The fragments were suspended in HBSS. The suspension was sieved through a nylon mesh (No. 40) to remove large debris. The cells were adjusted to a concentration of 1×10^6 nucleated cells/ml in TC-199-CS and cultured as described above.

Kidney cells. Mouse kidney cell monolayers were prepared in the same manner as described previously (8). The cells were suspended in TC-199-CS to a concentration of 5×10^5 cells/ml and cultured as described above. The cultures were used as cell monolayers for the assay of microbicidal activity, the release of oxygen intermediates of ATP content.

Assessment of microbicidal activity of cells. The toxoplasmacidal activity was determined in the same manner as described by previous paper (16). Briefly, after the co-cultivation of cell monolayers on coverslips and 1×10^5 tachyzoites of the RH strain per each monolayer

for 1 h, the coverslips were washed to remove extracellular organisms. The infected monolayers were then cultivated for 48 h with plain medium or medium-containing Obioactin (5 mg/ml), Ionomycin A (1 ng/ml), MDP (1 μg/ml), LPS (10 ng/ml) or TLA (100 μg/ml). Then, the cultures were stained with *May-Grünwald-Giemsa* stain and examined by light microscopy. The infection rate was grouped into cells with 1 to 5 tachyzoites and cells with more than 6 tachyzoites.

Assay of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). H_2O_2 and O_2^- were assayed on the same manner as described by previous paper (21). Briefly, the *Nakano* method (17) was employed to determine the triggering activity of phorbol myristate acetate on H_2O_2 release from the monolayers which were incubated with medium containing Obioactin, Ionomycin A, MDP, LPS or TLA or with plain medium for 48 h at 37 °C in 5% CO_2 in air. The O_2^- generation was determined by the cytochrome c reduction assay (10).

Measurement of oxygen consumption. The glycogen-induced peritoneal macrophages were placed in a glass *Petri* dish and incubated at 37 °C for 3 h in air with 5% CO_2. The adherent cells were removed by scraping with a rubber policeman and suspended to a concentration of 1×10^6 cells/ml in HBSS. The suspension was filled into a 650 μm^3 chamber for measurement of oxygen tension. The oxygen tension was measured polarographically by using a *Clark* style oxygen electrode (Single channel oxygen uptake system, model 102A, Instech Lab., U.S.A.). After oxygen uptake reached a steady state, the stimuli were added to the chamber.

Measurement of ATP. The peritoneal macrophage monolayers incubated with several stimuli for 48 h were homogenized by a sonicator (Heat System-Ultrasonics Inc., model W-220 f, U.S.A.) in ice-cold HBSS at 70 W for 15 sec. The sonicated mixture was deproteinized by addition of equal volume of 1 N perchloric acid, and centrifuged at 1,700 *g* at 4 °C for 10 min. Then the supernatant was used as a sample for ATP assay. ATP content was assayed by use of an ATP-test kit (Boehringer Mannheim, FRG).

Results

Toxoplasma growth inhibition inside peritoneal macrophages and release of oxygen intermediates

The *Toxoplasma* multiplication was inhibited in peritoneal macrophages treated with Obioactin or Ionomycin A for 48 h (Fig. 1). The percentage of *Toxoplasma*-free

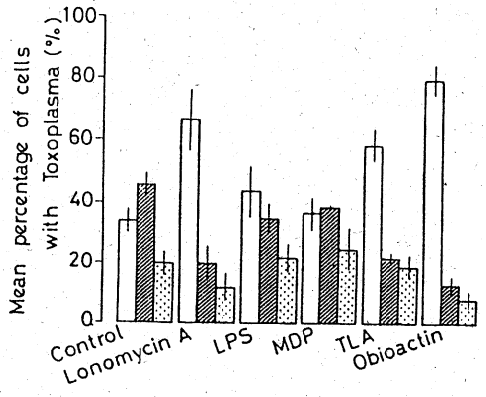

Fig. 1. Toxoplasmacidal activity of glycogen-induced peritoneal macrophages cultivated with Ionomycin A (1 ng/ml), LPS (10 ng/ml), MDP (1 μg/ml), TLA (100 μg/ml) and Obioactin (5 mg/ml) for 48 h in vitro. The white columns indicate the percentage of *Toxoplasma* free cells, the hatched columns indicate the cells containing 1 to 5 tachyzoites, and the dotted columns indicate the cells containing more than 6 tachyzoites. Each value represents the mean (± SE) of five experiments.

cells in the non-treated controls was 34.4 ± 4.0%. That in the Obioactin- or lonomycin A-treated cells was 79.6 ± 4.9% or 66.8 ± 10.2%, respectively, and was significantly higher than that in non-treated controls ($P < 0.001$ and $P < 0.02$, respectively). The amount of oxygen intermediates released from the Obioactin-treated cells was more than that from the non treated control. However, the release from the lonomycin A-treated cells was not enhanced (Fig. 2). On the other hand, the *Toxoplasma* multiplication in the cells treated with LPS or MDP was not inhibited (Fig. 1), but the release of oxygen intermediates increased as in the Obioactin-treated cells (Fig. 2). The percentage of *Toxoplasma*-free cells in the TLA-treated cells was higher than that in LPS- or MDP-treated cells. But, the rate of tachyzoites was almost the same in the LPS- or MDP-treated cells, or the non-treated cells.

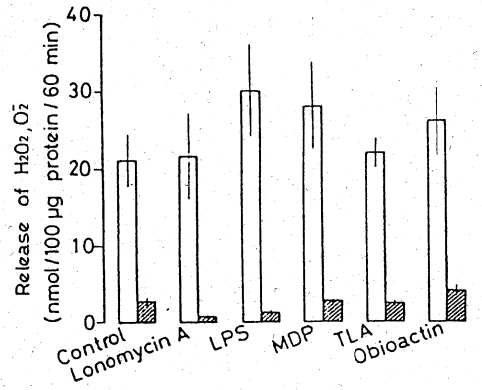

Fig. 2. Oxygen intermediates released from glycogen induced peritoneal macrophages cultivated with lonomycin A (1 ng/ml), LPS (10 ng/ml), MDP (1 µg/ml) TLA (100 µg/ml) and Obioactin (5 mg/ml) for 48 h in vitro. The white columns indicate the release of H_2O_2, and the hatched columns indicate the relase of O_2^-. Each value represents the mean (± SE) of five experiments.

Effects of Obioactin, MDP or TLA on the multiplication of Toxoplasma and the release of oxygen intermediates in alveolar macrophages.

Obioactin enhanced the toxoplasmacidal activity of the alveolar macrophages without increasing the release of oxygen intermediates (Fig. 3, 4). The inhibition of the *Toxoplasma* multiplication in the cells treated with Obioactin was stronger than that in the MDP- or TLA-treated cells. The release of oxygen intermediates from the MDP-, TLA- or Obioactin-treated cells did not differ from that of the non-treated controls (Fig. 4).

Effects of Obioactin, lonomycin A, LPS, MDP or TLA on the Toxoplasma multiplication and the release of oxygen intermediates in the mouse kidney cells

Treatment with Obioactin or lonomycin A inhibited the *Toxoplasma* multiplication inside kidney cells, but treatment with MDP, LPS or TLA (Fig. 5) had no significant effect. The release of oxygen intermediates from the kidney cells was not increased by the treatment with any of the substances (Fig. 6).

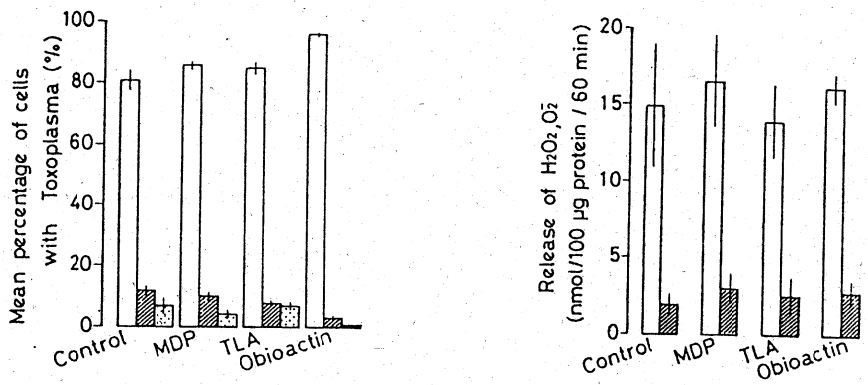

Fig. 3. Toxoplasmacidal activity of alveolar macrophages cultivated with MDP (1 µg/ml), TLA (100 µg/ml) and Obioactin (5 mg/ml) for 48 h in vitro. Symbols are as in Fig. 1. Each value represents the mean (± SE) of five experiments.

Fig. 4. Oxygen intermediates released from alveolar macrophages cultivated with MDP (1 µg/ml), TLA (100 µg/ml) and Obioactin (5 mg/ml) for 48 h in vitro. Symbols are as in Fig. 2. Each value represents the mean (± SE) of five experiments.

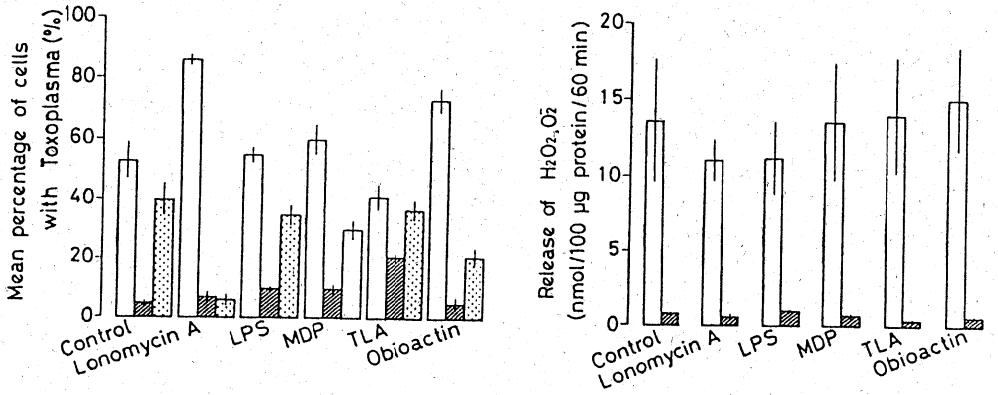

Fig. 5. Toxoplasmacidal activity of kidney cells cultivated with lonomycin A (1 ng/ml), LPS (10 ng/ml), MDP (1 µg/ml), TLA (100 µg/ml) and Obioactin (5 mg/ml) for 48 h in vitro. Symbols are as in Fig. 1 Each value represents the mean (± SE) of five experiments.

Fig. 6. Oxygen intermediates released from kidney cells cultivated with lonomycin A (1 ng/ml), LPS (10 ng/ml), MDP (1 µg/ml), TLA (100 µg/ml) and Obioactin (5 mg/ml) for 48 h in vitro. Symbols are as in Fig. 2. Each value represents the mean (± SE) of five experiments.

ATP content and oxygen consumption of the peritoneal macrophages

As shown in Table 1, the ATP content was increased during the Obioactin, MDP or TLA treatment. The ATP content of the Obioactin-treated cells was significantly higher than that of the non-treated controls ($P < 0.05$). The oxygen consumption of peritoneal macrophages at the steady state was 6.38 ± 0.60 nl/1×10^6 cells/min (mean ± SE of individual 30 measurement). The dose-dependency of the oxygen consumption was

Table 1. ATP content in the glycogen induced peritoneal macrophages cultivated with or without MDP, TLA or Obioactin for 48 h in vitro

	ATP content (nmol/mg protein)
Control (non treated)	4.6 ± 0.7
MDP (1 μg/ml)	5.3 ± 0.5
TLA (100 μg/ml)	6.8 ± 0.5
Obioactin (5 mg/ml)	7.9 ± 0.6

Mean ± SE, n = 3

expressed by the actual value after subtraction of the steady state value from the experimental value (Fig. 7). The actual oxygen consumption of cells treated with Obioactin increased dose-dependently, but that of TLA-, lonomycin A- or MDP-treated cells was almost zero for the dosage used.

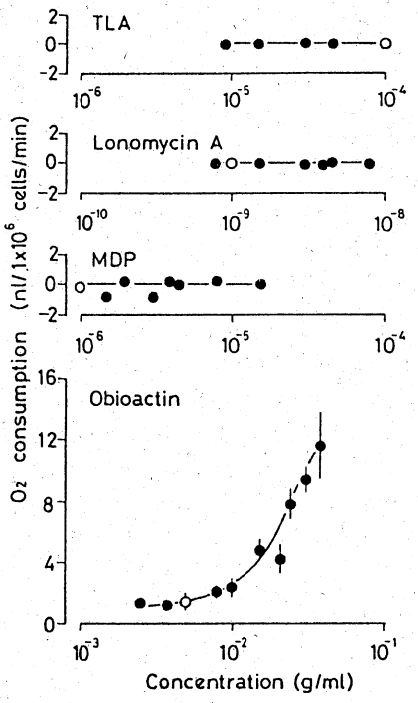

Fig. 7. Relationship between the actual oxygen consumption of peritoneal macrophages and dose of TLA, lonomycin A, MDP or Obioactin. The vertical axis indicates the oxygen consumption. The horizontal axis indicates the concentration of each substances. The open circles indicate the oxygen consumption at the doses which were used to test the inhibition of *Toxoplasma* multiplication inside cells. Each value represents the mean (± SE) of five experiments.

Discussion

At present, it is known that the toxic oxygen intermediates play an important role in the microbicidal system of cells (2, 4, 12, 14). However, the results in our experiments suggest that the toxoplasmacidal phenomena in the lonomycin A-treated peritoneal macrophages, the Obioactin-treated alveolar macrophages, and the Obioactin- or lonomycin A-treated kidney cells differ from oxygen-dependent mechanisms. But, an effect of oxygen intermediates cannot be excluded absolutely, because there is a possibility that our assay system for oxygen intermediates does not trap the oxygen intermediates completely. If toxoplasmacidal phenomena without an increment of the release of oxygen intermediates as shown in this experiment would not be abolished by treatment with scavengers for oxygen intermediates, these phenomena might suggest to be due to the oxygen-independent mechanism.

Recently, many reports suggested the oxygen-independent mechanism for protozoa killing inside cells. *Trypanosoma* killing trehalose 6, 6'-dimycolate-treated macrophages (5), the intracellular killing of *Leishmania* parasites by electron carrier stimulated macrophages (9), toxoplasmacidal mechanism by the fresh monocytes from patients with chronic granulomatous disease (15) and anti-*Toxoplasma* activity in MDP-treated human macrophages (22) were not accompanied by an enhancement of the oxygen-dependent system. In addition, recent investigations suggested the participation of intracellular Ca^{2+} (19) and receptor mechanisms (1, 23, 24) in oxygen-independent activation of macrophages. To obtain more conclusive results on the correlation between *Toxoplasma* multiplication inside cells and the release of oxygen intermediates, additional experiments have to be done.

An increment of ATP content in the peritoneal macrophages during Obioactin, MDP or TLA treatment may suggest that these substances stimulate the oxidative phosphorylation in mitochondria and/or glycolysis in the cytosol. Since the actual oxygen consumption during the treatment with MDP or TLA did not change when the dosage of MDP or TLA was progressively increased, MDP- or TLA-induced increment of ATP content in the peritoneal macrophages might not result from an acceleration of oxidative phosphorylation. However, the experimental result that Obioactin induced oxygen consumption with a dose-dependent increase, which was not abolished by potassium cyanide (Saito, unpublished data) suggests that the increment of ATP content during Obioactin treatment was due to the activation of ATP synthetic systems, oxidative phosphorylation and glycolysis.

The present experimental results on the cellular metabolism and the report by *Loike* et al. (7) that the peritoneal macrophages consumed oxygen during phagocytosis may indicate that the toxoplasmacidal phenomena in the peritoneal macrophages treated with Obioactin involve the energy-dependent mechanism.

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Dr. *Atsushi Saito,* Dept. of Veterinary Physiology, Obihiro University, Obihiro, Hokkaido 080, Japan

Zbl. Bakt. Hyg. A 264, 468–477 (1987)

Long Term in vitro Suspension Culture of Toxoplasma and HeLa Cells*

HARUHISA SAKURAI, HIROSHI SAKURAI, TATSUYA TEGOSHI, TAKAO TAKAGI, ATSUSHI SAITO*[1], NAOYOSHI SUZUKI*[2], and HANNS M. SEITZ*[3]

Department of Veterinary Physiology, Obihiro University, Obihiro, Hokkaido, Japan

With 6 Figures · Received May 22, 1986

Summary

To establish a method of mass and long-term propagation of *Toxoplasma* in suspension cultures and to find a technique for separating the organisms from host cells, basic investigations were carried out in a microcomputer controlled system. By using an arbitrary unit "the medium index" host HeLa cells were continuously supplied to *Toxoplasma* organisms for about 30 days, at cycles of 4 days. HeLa cells were well infected with *Toxoplasma* in the suspension cultures by leaving the medium unstirred for 15 min intervals. The separation of *Toxoplasma* organisms from cells was improved by treating toxoplasmas and HeLa cells mixtures with 0.4% trypsin. The total number of separated organisms obtained from 29 days cultivation period was 4.9×10^9. The mean generation time of *Toxoplasma* organisms on the 0, 10, 20 and 30 day's cultivation was calculated approximaetly 7.4, 9.4, 9.3 and 7.8 h, respectively.

Introduction

In order to clarify the defence mechanisms which can be induced by *Toxoplasma* antigen against various infections (1, 2, 11, 12, 14, 16), the mass cultivation of *Toxoplasma* is necessary. At present, *Toxoplasma* is generally propagated by passages in mice or embroyonated hen's eggs. This makes it impossible to collect *Toxoplasma* organisms free of contamination with heterologous antigens. It has been reported that non-contaminated *Toxoplasma* can be obtained from *Toxoplasma* grown in tissue culture (3, 4, 18, 19).

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To establish a method of mass and long-term propagation of *Toxoplasma* in suspension cultures and to find a technique for separation of the organisms from host cells, experiments were carried out in a microcomputer-controlled system.

Materials and Methods

Cell and Toxoplasma strain used

HeLa cells grown in suspension cultures were selected as host cells, since they are suitable for mass and long-term cultivation. These cells were given by Prof. Dr. *Akio Nomoto* (Dept. of Bacteriology, School of Medicine, Tokyo University, Tokyo, Japan). They had been cultivated in minimum essential medium including 5% heat-inactivated fetal calf serum (5% FCS-MEM).

RH strain of *Toxoplasma gondii* was used for inoculation.

Equipment for mass cultivation

The apparatus, designated for automatically controlled fermentation (Tokyo Rika Co. Ltd., Tokyo, Japan) has a medium tank, 3 culture vessels (one serves only as a reservoir for the completed medium), a water circulator, a few tube pumps, and a control unit which is connected to a personal computer. As shown in Fig. 1, the medium tank is connected with the 1st vessel and the 1st vessel is connected with the 2nd and 3rd vesssel by silicone tubes. The water jackets of the culture vessels are connected by tubes with a water circulator which circulates water of 37 °C. Each vessel has sensors for pH, dissolved oxygen and temperature. The tubes are controlled by the personal computer. A propeller is suspended from the

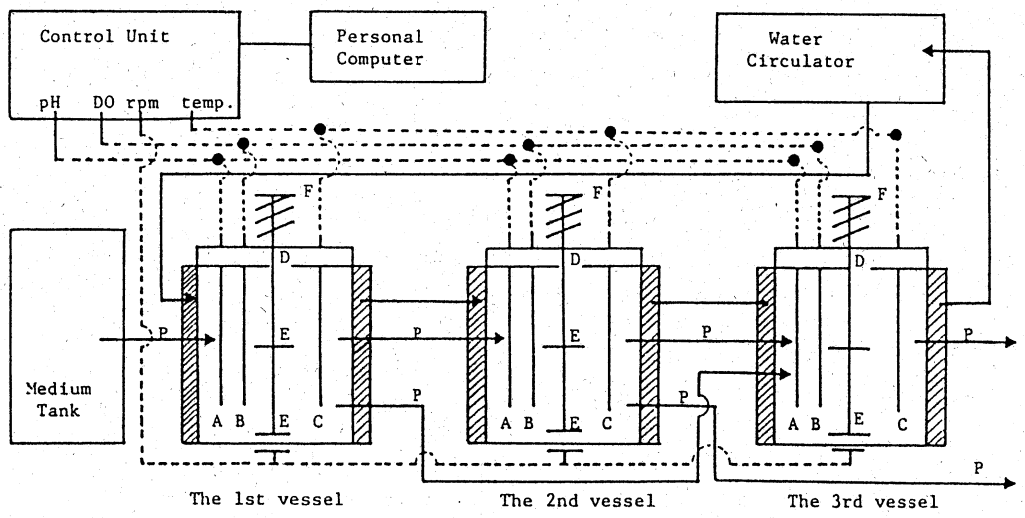

Fig. 1. Microcomputer fermentation controller system.
A: pH sensor
B: DO sensor
C: temperature sensor
D: stirrer shaft
E: propeller
F: mist separator
P: tube pump

cover of the vessel. The medium powder, minimum essential medium (MEM), is dissolved in water in the tank, and it is sterilized by autoclaving. The sterilized medium is transferred to the 1st vessel. In the 1st vessel, the medium is supplemented with L-glutamine and fetal calf serum, with a final concentration of 5%. The propeller mixes the components at a speed of 100 rev/min. After that, a 10% sodium hydrogen carbonate solution is added to the medium, to bring the pH up to 7.4 ± 0.2. The completed medium is transferred to the 2nd vessel. Cultivation of the host cells was carried out in the 2nd vessel. The cells were seeded into the 2nd vessel. Propagated HeLa cells were later transferred to the 3rd vessel to be infected with *Toxoplasma*. The propagation of toxoplasmas took place in the 3rd vessel. In the 3rd vessel, the stirring of the culture medium was interrupted at 15 min intervals to enable toxoplasmas to contact and to penetrate into host cells. The medium remained unstirred for 15 min intervals.

Medium index

A trial was made to estimate the growth-supporting capacity of the medium by using the medium index (MI) as an arbitrary unit. One MI was assumed to be the amount of the consumed factors in 1 ml of the medium, in which 1×10^5 cells were growing for one day. Fresh MEM was calculated as 10 MI per ml. Total consumption in the culture medium was then calculated as follows:

Actual MI = Previous day's MI – Consumption MI

Consumption MI (MI/ml) =

$$\frac{\text{Incubation time (days)} \times \text{Average number of HeLa cells } (\times 10^5 \text{ cells})}{\text{Volume of medium (ml)}}$$

Fresh MEM = 10 MI/ml

The separation of Toxoplasma organisms from cells

The separation of *Toxoplasma* organisms from cells was improved by treating toxoplasmas and HeLa cells mixtures with 0.4% trypsin in phosphate-buffered saline solution (7). The mixtures suspended in trypsin solution were kept at 37 °C for 40 min and washed by centrifugation (800 *g*, 10 min, 4 °C). This treatment was repeated 3 times.

The generation time for Toxoplasma multiplied in the cultivation

The generation time for *Toxoplasma* organisms multiplying in the long-term cultures was investigated. On day 0, 10, 20 and 30 of cultivation, extracellular organisms were collected from vessel 3, and transferred to macrophage monolayers (2×10^5 organisms to 1×10^6 macrophages). Macrophage monolayers were prepared as described previously (8, 17). One hour after infection with toxoplasmas, the monolayers were washed to remove extracellular organisms and then the monolayers were reincubated with fresh medium. At 0, 6, 12, 24 and 36 h after reincubation, the number of organisms that had multiplied in macrophages was counted in 500 infected cells in a preparation stained with *May-Grünwald-Giemsa* stain. The generation time of *Toxoplasma* organisms multiplying in macrophages was calculated according to the procedure used by *Kaufman* and *Maroney* (5) and *Kusunoki* (6).

Results

The relationship between MI and the concentration or the viability of the cells was investigated. HeLa cells were propagated in the 2nd vessel, and the medium was not transferred. The number and the viability were calculated for 6 days. As shown in Fig. 2, when MI value turned negative, the number and the viability of the cells decreased.

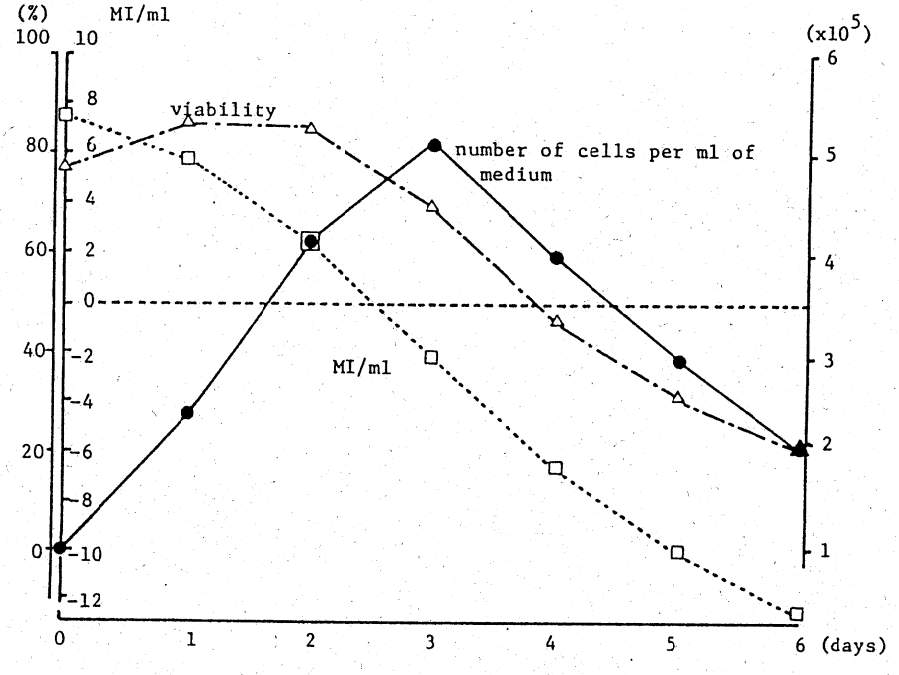

Fig. 2. Changes of viability or concentration of HeLa cells and MI without medium transfer.

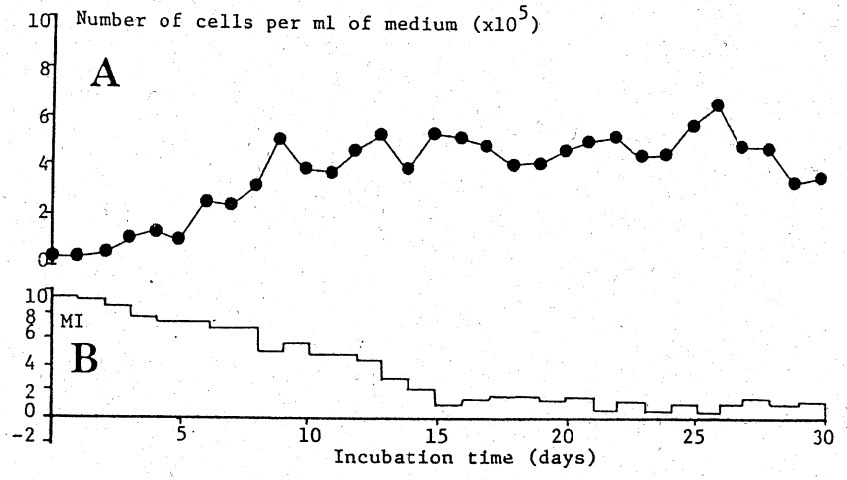

Fig. 3. Growth of HeLa cells in the 2nd vessel.
A: Number of HeLa cells per ml of medium.
B: Relationship between MI and incubation time.

Fig. 3 shows the growth of HeLa cells incubated in the 2nd vessel for 30 days. Medium was renewed to keep MI values positive. In Fig. 3 A, the number of cells per ml of medium is shown, in Fig. 3 B, the decline of the MI is indicated. After 9 days cultivation, HeLa cells reached a concentration of approximately 4–5 × 10^5 per ml and continued to keep that concentration for 30 days cultivation.

In the following experiment, HeLa cells in the 2nd vessel were propagated to provide host cells which were transferred to the 3rd vessel every 4 days, as shown in Fig. 4. Fresh medium from the 1st vessel was added to supply the remaining cell suspension in vessel 2 for further multiplication. Transferred HeLa cells were exposed to toxoplasmas in the 3rd vessel.

As shown in Fig. 5 B, toxoplasmas multiplied vigorously while at the same time, the number of HeLa cells was reduced as shown in Fig. 5 A. The propagation of the organisms was carried out for 33 days continuously. The maximum yield per cycle of 4 days was approximately 1.4 × 10^9 organisms. The highest rate of infected cells was 48% (Fig. 5C).

The separation of *Toxoplasma* organisms from cells was improved by treating toxoplasmas and HeLa cells mixtures with 0.4% trypsin in phosphate buffered saline solution. As shown in Table 1 the total number of separated organisms obtained from 29 days cultivation period was 4.9 × 10^9 and was approximately 3.2 times higher than the number of inoculum. The average yield of toxoplasmas after trypsin treatment was 130.4%. The viability of the organisms after treatment was 99.6%. After trypsin treatment 1.5% of the HeLa cells remained intact.

The generation time for toxoplasmas multiplying in vessel 3 was investigated. Organisms were collected from the 3rd vessel and inoculated to macrophage monolayers. The number of organisms inside macrophages was calculated for 500 infected macrophages at 0, 6, 12, 24 and 36 h after inoculation to macrophage monolayers (Table 2).

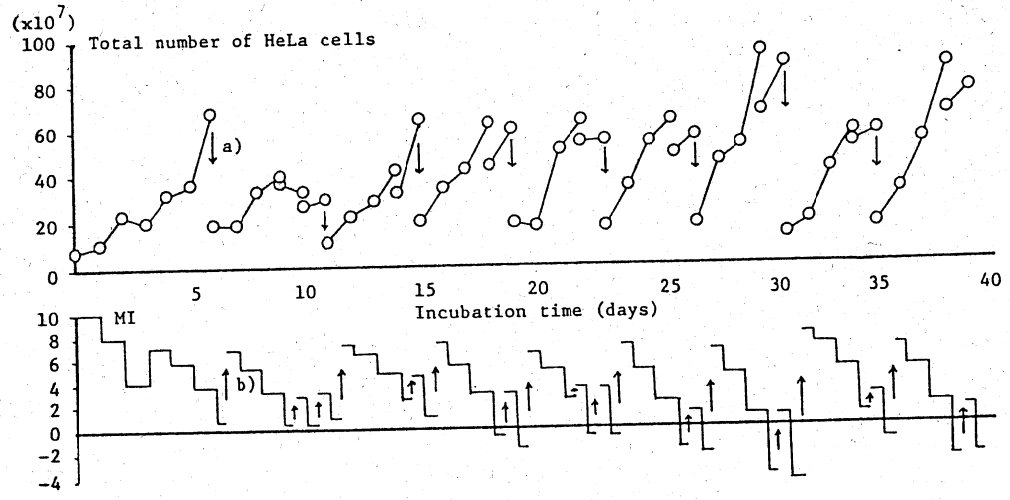

Fig. 4. Growth of HeLa cells in the 2nd vessel.
a) HeLa cells were transferred to the 3rd vessel.
b) Fresh medium was added.

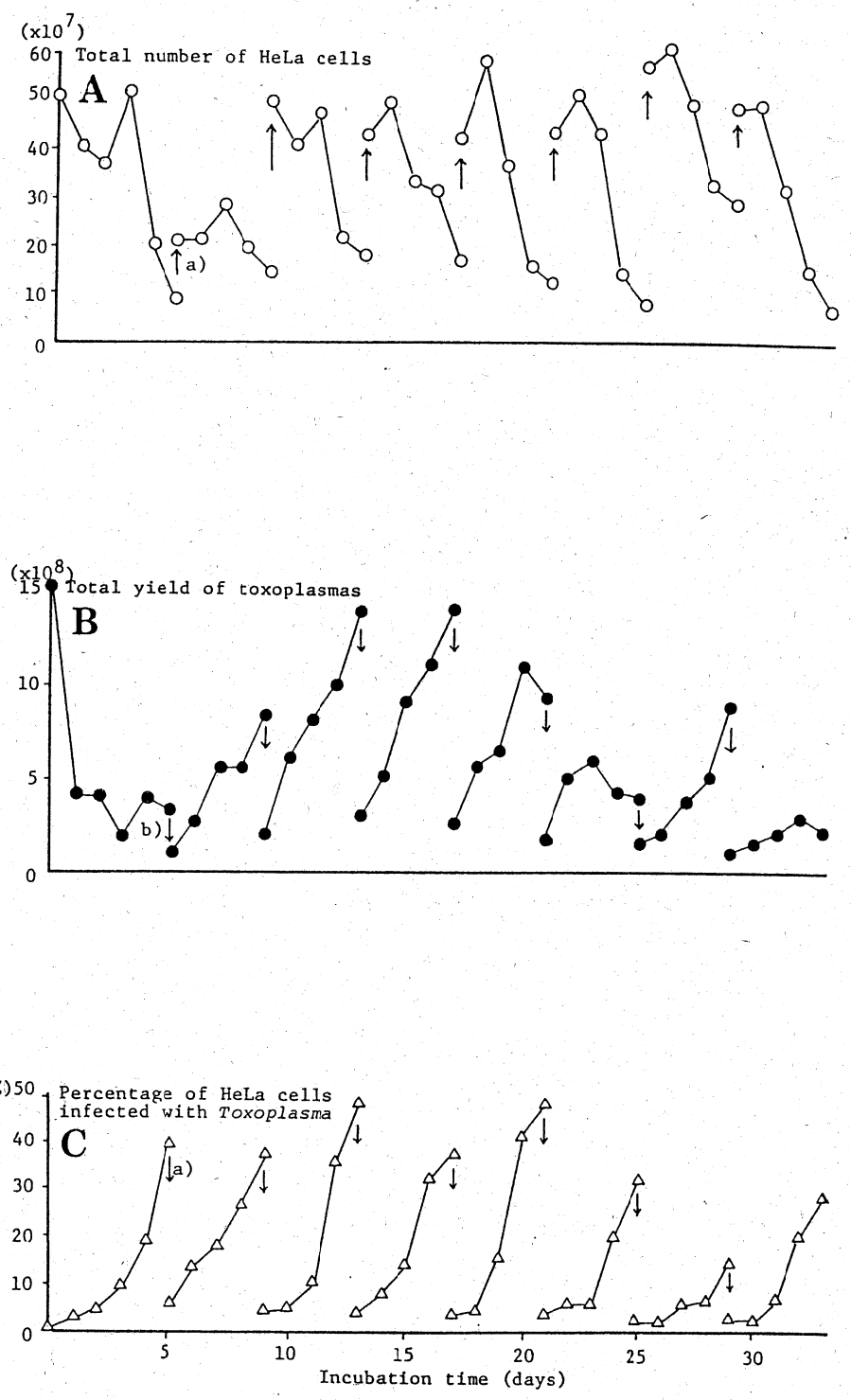

Fig. 5. Propagation of *Toxoplasma* on HeLa cells in the 3rd vessel.
a) HeLa cells were transferred from the 2nd vessel.
b) Toxoplasmas were harvested.

Table 1. Separation of toxoplasmas from HeLa cells by treatment with trypsin

Incubation time (days)	0	5	9	13	17	23	29	Average value	Total
Number of free *Toxoplasma*									
before treatment ($\times 10^7$)	–	23.5	54.9	95.9	92.5	64.1	72.8	67.3	403.7
after treatment ($\times 10^7$)	112.5	44.4	63.5	111.0	108.0	98.2	67.2	82.0	492.3
yield in (%)[a]	–	188.9	115.7	115.7	116.7	153.1	92.3	130.4	–
HeLa cells remaining after treatment (%)[b]	–	1.2	0.6	2.6	2.8	1.4	3.0	1.5	–

[a] $\frac{\text{Number of toxoplasmas yield after treatment}}{\text{Number of free toxoplasmas yield before treatment}} \times 100$

[b] $\frac{\text{Number of HeLa cells remaining after treatment}}{\text{Number of toxoplasmas and HeLa cells yield after treatment}} \times 100$

Table 2. The number of *Toxoplasma* tachyzoites in macrophage monolayers

Time after inoculation (h)	Number of tachyzoites/100 infected macrophages 0	10	20	day's 30 cultivation
0	143.2 ± 6.9	190.2 ± 21.5	136.2 ± 20.5	175.0 ± 17.5
6	109.4 ± 3.0	196.3*	355.8 ± 42.8	262.8 ± 37.1
12	196.6 ± 1.3	234.0 ± 14.5	602.8 ± 67.9	359.8 ± 80.6
24	599.4 ± 54.7	713.2 ± 53.9	1374.8 ± 107.7	1239.0 ± 49.2
36	1778.4 ± 160.8	1442.4 ± 246.0	1554.2 ± 274.4	1440.8 ± 192.5

* Failure of staining (Average ± Standard deviation)

Table 3. Number of generations and mean generation time for *Toxoplasma* organisms multiplied in macrophages

Time after inoculation (h)	0	10	20	day's 30 cultivation
0	0.51 ± 0.07	0.92 ± 0.16	0.43 ± 0.21	0.80 ± 0.15
6	0.13 ± 0.04	0.98*	1.82 ± 0.18	1.38 ± 0.21
12	0.98 ± 0.01	1.22 ± 0.09	2.58 ± 0.17	1.82 ± 0.34
24	2.58 ± 0.13	2.83 ± 0.11	3.78 ± 0.11	3.63 ± 0.06
36	4.15 ± 0.13	3.83 ± 0.24	3.94 ± 0.25	3.84 ± 0.20
Mean generation time (h)	7.35	9.35	9.26	7.75

* Failure of staining (Average ± Standard deviation)

The number of organisms in macrophages were plotted in logarithm (Table 3 and Fig. 6). The generation time of organisms was calculated for the logarithmic phase expressed as a nearly straight line in Fig. 6. The mean generation time of *Toxoplasma* organisms on day 0, 10, 20 and 30 cultivation was calculated to be approximately 7.4, 9.4, 9.3 and 7.8 h, respectively (Table 3).

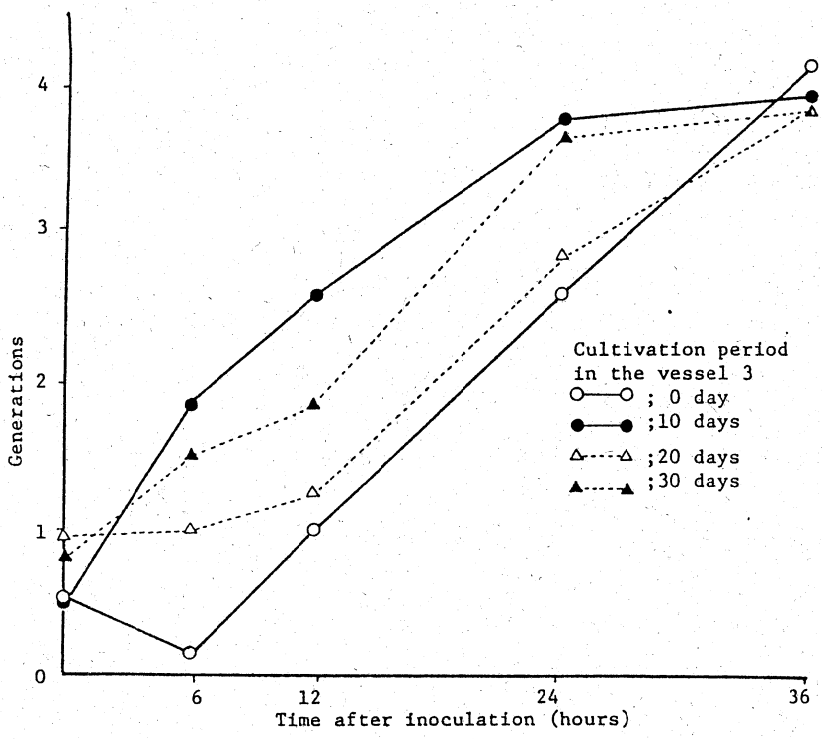

Fig. 6. Generations of *Toxoplasma* organisms in macrophage monolayers.

Discussion

The growth-supporting capacity of the medium was estimated by using the medium index (MI) as arbitrary unit which was assumed to reflect the mutual relation between incubation period and cell concentration or viability. When the MI showed negative values, the concentration or viability of HeLa cells tended to decrease. It was assumed that if a certain volume of the culture medium was renewed which was determined by using the MI to keep its value positive, HeLa cells are able to grow steadily. *Peraino* et al. (15) reported that they were able to culture HeLa cells automatically by using a system with a nephelometer. Our results suggest that our equipment is able to culture HeLa cells automatically by using the MI. HeLa cells were supplied to the multiplying *Toxoplasma* organisms at 4 days intervals. It is known that the penetration of *Toxoplasma* organisms is influenced by the opportunity to contact host cells (10, 18). In our suspension cultures the culture medium was repeatedly kept still at 15 min intervals to

make it possible for the toxoplasmas to contact and penetrate into host cells. Using this procedure the percentage of HeLa cells infected with *Toxoplasma* was more than 40% as compared to 10–20% in continuously stirred culture medium (not shown). This suggests that the repetition of stir-still cycles is useful to promote contact and penetration of parasites into host cells in suspension culture.

The generation time of toxoplasmas in its logarithmic phase did not change for the 30 days cultivation period.

These results suggest that the mass and long-term propagation of *Toxoplasma* is possible by using a computer-controlled in vitro suspension culture.

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Dr. *Haruhisa Sakurai,* Department of Veterinary Physiology, Obihiro University, Inada-Cho, Obihiro, Hokkaido 080, Japan

Immunity in Neonates of Mice Chronically Infected with *Babesia rodhaini*

Haruhisa SAKURAI, Hiroaki TAKAHASHI, Motoyoshi SATO[1)], Tsuneo HIROSE[1)], Atsushi SAITO, and Naoyoshi SUZUKI

Departments of Veterinary Physiology and [1)]Veterinary Clinical Radiology, Obihiro University, Obihiro, Hokkaido 080, Japan

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ABSTRACT. The resistance of newborn mice to infection with *Babesia rodhaini* was studied. No parasites were detected in the neonates or fetuses of both acutely infected and chronically infected mothers. However, the neonates born of chronically infected mice and nursed by normal uninfected mothers or by chronically infected mice were significantly more resistant to the infection than those from normal uninfected mice. In experimentally infected adult mice, the activities of macrophage migration inhibitory factor (MIF) in the supernate of concomitant cultivation of spleen cells with the lysate antigen of *Babesia* increased up to 6 weeks after infection. The degree of peritoneal macrophage phagocytosis in infants from chronically infected mothers was significantly greater than that of normal mice. The macrophage phagocytosis of parasitized erythrocytes was enhanced remarkably when the erythrocytes were exposed to immune serum just before addition to the macrophages.—KEY WORDS: babesiosis, lymphokines, mouse, neonatal immunity.

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In some protozoan infection, most of the neonates of chronically infected mother animals show a high degree of immunity to the homologous parasites as compared to those born of normal mothers [1, 10, 14, 16–19]. A foster experiment indicated that passive immunity was transferred primarily through the milk of the rodent [22]. Palmer [14] showed, however, that in rats infected with *Plasmodium berghei,* a degree of protection was transferred during pregnancy supporting the suggestion of Desowits [2] that neonates were sensitized by the soluble antigen of *P. berghei* which passed the placental barrier.

In our experimental studies on toxoplasmosis [18, 19], most of the neonates from immune mothers showed a significant resistance to *Toxoplasma* infection compared to these born of normal rats.

These phenomena demonstrated that T-lymphocytes had been sensitized transplacentally with a lymphokine to *Toxoplasma* before birth, and the lymphocytes returned to a non-sensitized state within several weeks after birth. Additionally, Omata *et al.* [11–13] suggested that the specific antigen-antibody complex could stimulate lymphocytes to induce proliferation and maturation of the peripheral immue system during the neonatal life. The goal of this study was to determine whether the protective immunity against *Babesia* infection was present in neonates of chronically infected mothers.

MATERIALS AND METHODS

Animals and infection: Laboratory bred conventional ICR/JCL, 6–8 weeks old female mice were used in this study. The Australian strain of *B. rodhaini* was obtained from the Kyushu Branch Laboratory of the National Institute of Animal Health. The parasite was maintained in the laboratory by passage at 4–5 day intervals. *B. rodhaini* parasitized erythrocytes (PE) were obtained from these mice and were

used to infect the experimental mice intraperitoneally (i.p.) at a dose of 1×10^2 PE per mouse. The severity of infection with *B. rodhaini* was assessed by the number of PE per 1,000 erythrocytes counted on a smear from tail blood stained with May-Crünwald and Giemsa.

Mice were treated with 4,4-diaminodibenzamidine diaceturate (Ganaseg, E. R. Squibb and Sons Inc., Manila, Phillipines) administered intramuscularly (i.m.) at a dosage of 1.0 mg per mouse per day on 7, 8, 9, 11, 12 and 13 days postinoculation (p.i.). They were challenged with 1×10^4 PE per mouse i.p. 4 weeks after the first infection and the animals which survived 4 weeks after the challenge were used throughout the experiment as *Babesia* immune mice.

Babesia lysate antigen: *Babesia* lysate antigen (BLA) was prepared by the method described previously by Ishimine *et al.* [6] and Ito *et al.* [7]. *Babesia*-PE were washed 3 times with heparinized Hanks' balanced salt solution (HBSS, with 10 units heparin/m*l*) and once with Tc-199 by centrifugation at 750×G for 10 min at 4°C. The sediment was hemolyzed 5 or 6 times with warm 0.83% ammonium chloride solution, centrifuged at 45,000×G for 45 min at 20°C, washed 3 times with PBS, and centrifuged at 45,000×G for 45 min at 4°C. The sediment was resuspended in physiological saline. The suspension was frozen at −20°C and thawed at room temperature for 7 to 8 cycles, and then sonicated for 5 min at 100 W (Kubota Insonator, Model 200, Tokyo). The lysate product was centrifuged at 45,000×G for 45 min at 4°C. The supernate was filtered through a millipore membrane filter (0.3 μ, type HA, Millipore Co., Bedford, Mass, USA) and stored at −70°C until use. Total protein content of the BLA was estimated by the Lowry method [8] using bovine serum albumin fraction V as the standard.

Spleen cells and lymphokines: Cells were collected from the spleens of normal or infected mice. The cell separation was done by the Conray-Ficoll method [21]. The cells were washed twice with HBSS. After centrifugation at 450×G for 10 min, the resulting cells were resuspended in a medium (Tc-199) containing 10% heat inactivated calf serum (M+10%CS), and the concentration was adjusted to approximately 1×10^7 cells/m*l*. Immune and normal spleen cells were cultured with M+10%CS containing BLA (100 μg/m*l*) at 37°C for 48 hr in a humidified 5% CO_2 incubator. The supernate of immune spleen cell cultures, lymphokines (LKs), or normal spleen cell cultures were filtered through a membrane filter and store at −70°C until use.

Assay for macrophage migration inhibitory factor: The assay was performed by the modified agarose droplet method [5, 9, 15]. Guinea pigs were injected i.p. with 10 m*l* of 3% thioglycolate agar to stimulate peritoneal exudation. Three days later, peritoneal exudate cells (PEC) were harvested by washing the peritoneal cavity with 50 to 60 m*l* of HBSS. The cells obtained were wahsed 3 times with HBSS and resuspended in Tc-199 medium. The cell suspension was then mixed with agarose saline solution and maintained in a water bath at 37°C. One microliter of the PEC suspension was dropped into each well of the chamber (Lab Tek Product, USA) and cooled for 5 min at 4°C. Lymphokines (0.3 m*l*) were gently poured into each of two wells containing PEC agarose droplets. Wells containing M+10%CS served as controls. The chambers were incubated at 37°C for 24 hr in a humidified CO_2 incubator. The migration distance of macrophages was measured from the periphery of the droplet to that the radially migrated macrophages. The activity of macrophage migration inhibitory factor (MIF) of each of the LKs was calculated by the formula described by Sakurai *et al.* [18].

Phagocytosis test: Macrophage mono-

layers were prepared as described by Nagasawa *et al.* [9]. Peritoneal exudates containing a large number of macrophages were harvested by peritoneal washing with HBSS. The cells were centrifuged at 320×G for 5 min at 4°C. The sediment was suspended in M+10%CS and adjusted to a concentration of 1×10^6 mononucrear cells per m*l*. One milliliter of this suspension was placed in each well of a multidish tray (FB-15-24. Linbro Flow Lab., Inc., Conn., USA) containing a round coverslip, and incubated for 6 hr in a humidified 5% CO_2 incubator at 37°C. The cultures were washed 3 times with the same medium to remove non-adherent cells at 2 hr intervals. The cultures were reincubated with the same medium for 18 hr in a 5% CO_2 incubator and used as macrophage monolayers.

Babesia-PE were washed 3 times with HBSS and once with Tc-199 by centrifugation at 750×G for 10 min at 4°C. *Babesia* immune mouse fresh serum (BIFS), normal mouse fresh serum (NFS) or Tc-199 was added to the sediment at a volume ratio of 2:3. These mixtures were incubated at 37°C for 30 min in a water bath, and washed 3 times with HBSS and once with Tc-199. These treated erythrocytes were suspended in M+10%CS and added to the macrophage monolayers at a final concentration of 1×10^7 cells per well. The coverslips were taken out of the culture trays and stained with May-Grünwald and Giemsa at 30, 60 and 120 min after inoculation [6]. Macrophages with phagocytized PE were identified as "positive" macrophages and the rate was calculated from a total of 1,000 macrophages. Student's t-test was used to evaluate the significance of the results.

Vertical transmission of Babesia parasites from the acutely infected or immune mice to their fetuses: Parasite isolation was attempted by subinoculation of homogenized organs of fetuses or neonates i.p. into normal adult mice. The whole body of fetuses used at 2 weeks of gestation were homogenized. Only in the case of 3-week-old fetuses and neonates, the internal organs excluding gastrointestinal tract were homogenized. Mice at the end of the 1st week of pregnancy were infected with *Babesia* parasites. The fetuses were used for subinoculation 1 week after infection of the mother. The subinoculated mice were designated as group I. In group II, other pregnant mice were infected at the end of the 2nd week of pregnency and divided into 2 groups. The fetuses of one group were used for subinoculation at the end of the 3rd week of pregnancy. These subinoculated mice were designatd as group II-A. The newborn mice from the other group, as well as the *Babesia* immune mice were used for subinoculation, and were designated as group II-B and group III, respectively. The mortality rate of these subinoculated mice of each group was observed for 4 weeks.

Babesia infection of neonates of normal or Babesia immune mice: The *Babesia* immune female mice were challenged with *Babesia*-PE 1×10^2 per mouse. They were mated for 4 weeks following the challenge. Neonates and mothers were treated according to the method described by Palmer [14], but a slight modification was made as follows: (1) neonates born of normal mothers were nursed by normal mothers (N/n), and those born of immune mothers were nursed by immune mothers (I/i), (2) neonates born of immune mothers were put to normal mothers (I/n), and those born of normal mothers were put to immune mothers (N/i). The mothers of neonates were exchanged within 8 hr post partum. Each litter of neonates was divided into 2 groups. One group was inoculated with 1×10^2 *Babesia*-PE neonate within 1 week after birth and challenged with the similar number of PE 4 weeks after primary infection. These neonates were designated as group-A. The other

Table 1. The activity of macrophage migration inhibitory factor (MIF) in mice after primary infection and challenge with *B. rodhaini*

	Weeks after primary infection[a]					
	0 (Normal)	1	2	3	4[b]	6
MIF activity (%)	28[c]	48	56	36	33	51

a) Infected with PE (1×10^2 per mouse).
b) Challenged with PE (1×10^4 per mouse) 4 weeks after primary infection.
c) $100\times\left(1-\frac{\text{Average distance of migration in the test material}}{\text{Average distance of migration in the control}}\right)$.

Table 2. MIF activity in infected mice[a] after BLA[b] injection

	Hours after BLA injection			
	3	6	12	24
MIF activity (%)	42[c]	55	54	69

a) Infected 4 weeks before BLA injection (1×10^2 PE/mouse).
b) *Babesia* lysate antigen (100 μg of protein/mouse) injected intraperitoneally.
c) See Table 1.

half (group-B) was infected with 1×10^2 *Babesia*-PE per mouse 5 weeks after birth, at the time of the challenge in group-A. They were examined for mortality and parasitemia. Chi-square test was used to evaluate the results of mortality.

RESULTS

Appearance and degree of MIF activity: As shown in Table 1, MIF activity increased

Table 3. Phagocytic abilities of normal or *Babesia* immune mouse macrophages against parasitized erythrocytes treated with antibodies

Macrophages collected from	Incubation time (min)	Phagocytic percentage[a] of macrophages against parasitized erythrocytes treated with		
		BIFS[b]	NFS[c]	Tc-199
Babesia immune mice	30	3.7±1.5	2.2±0.5	3.5±0.6
	60	14.6±4.4	6.4±1.7	10.1±1.4[d]
	120	47.5±3.2[d]	16.7±3.5	22.6±7.8
Normal mice	30	8.9±3.5	3.7±1.3	2.8±0.4
	60	8.8±3.0	5.0±2.8	4.2±1.8
	120	26.1±6.4	11.9±3.8	12.8±7.0

a) Treated erythrocytes were suspended in M+10%CS and added to the macrophage monolayers at a final concentration of 1×10^7 cells per well. The coverslips were stained at 30, 60 and 120 min after incubation. Macrophages with phagocytized erythrocytes were identified as "positive" macrophages for the phagocytosis and the rate of phagocytosis was calculated from 1,000 macrophages. Mean(percentage)±SE was calculated from the results of 3 independent experiments.
b) BIFS, *Babesia* immune mouse fresh serum.
c) NFS, normal mouse fresh serum.
d) Statistically significant difference compared to the case of normal mice macrophage at the same period ($p<0.05$).

Table 4. Examination for vertical transmission of *Babesia* parasites

Adult female mice	Number of fetuses or neonates	Parasitemia[a] (%)	Mortality of recipient mice (number dead/ number injected)
Acute infected[b]			
group I	4	0.20	0/ 2
	11	0.72	0/ 2
	16	0.14	0/ 2
group II-A	7	0.10	0/ 2
	13	0.51	0/ 2
	9	0.22	0/ 2
	6	2.40	0/ 2
group II-B	10	–[c]	0/10
	13	–[d]	0/13
	16	–[e]	0/16
Babesia immune			
group III	6	–	0/ 6
	7	–	0/ 7
	5	–	0/ 5

a) Number of parasitized erythrocytes per 100 erythrocytes in tail blood smears before sacrifice.

b) Group I. Normal mice in the 1st week of pregnancy infected with *Babesia* parasites: fetuses used for subincoulation 1 week after infection of the mother.
Group II-A. Normal mice infected at the end of the 2nd week of pregnancy; fetuses used for subinoculation at the end of the 3rd week of pregnancy.
Group II-B. Normal mice infected at the end of the 2nd week of pregnancy.

c~e) Died of babesiosis at the 7th, 2nd and 4th day after birth, respectively.

up to the 2nd week p.i. in *Babesia* infected mice. In the succeeding weeks, MIF activity tended to decrease during the 3rd and 4th weeks p.i. When the infected mice were challenged with *B. rodhaini* 4 weeks after primary infection (*Babesia* immune mice), MIF activity increased up to 51% 2 weeks after the challenge. Table 2 shows the MIF activity in the *Babesia* immune mice injected i.p. with BLA (100 μg/mouse). MIF activity was examined at 3, 6, 12 and 24 hr after injection. MIF activity increased up to the maximum of 69% at 24 hr.

Phagocytic ability of macrophages from normal and Babesia immune mice to the PE: The percentages of phagocytosis for the PE treated with Tc-199 medium (Tc-PE) in normal mouse macrophage monolayers at 30, 60 and 120 min incubations were 2.8, 4.2 and 12.8%, respectively (Table 3). However, in immune mouse macrophage monolayers, phagocytosis was 3.5, 10.1 and 22.6%. The phagocytosis of normal mouse macrophages incubated with normal-fresh-serum-treated PE (NFS-PE) at 30, 60 and 120 min after inoculation, was 3.7, 5.0 and 11.9%, respectively. In case of *Babesia*-immune-fresh-serum-treated PE (BIFS-PE), phagocytosis increased to 8.9, 8.8 and 26.1%. Phagocytosis of immune mouse macrophages incubated with NFS-PE at 30, 60 and 120 min after inoculation was 2.2, 6.4 and 16.7%, respectively. Phagocytic ability of immune macrophages was remark-

Table 5. Comparison of resistance against *Babesia* infection between infants from normal and *Babesia* immune mice

Group	Mortality of neonates after infection			
	Challenged (A)[a]		Not challenged (B)[b]	
	Number of dead/ number of infected	(%)	Number of dead/ number of infected	(%)
Infants born of immune mice,				
and nursed by immune mice (I/i)	6/35	17.1[c]	16/38	42.1[d]
and nursed by normal mice (I/n)	1/ 9	11.1[e]	8/16	50.0[f]
Infants born of normal mice,				
and nursed by immune mice (N/i)	20/33	60.6	30/35	85.7
and nursed by normal mice (N/n)	19/30	63.3	28/32	87.5

a) Neonates inoculated with *Babesia*-PE 1×10^2 per neonate within 1 week after birth and challenged in a similar fashion 4 weeks after primary infection.
b) Infants infected with *Babesia*-PE 1×10^2 per mouse 5 weeks after birth.
c~f) The statistically significant difference of mortality compared to N/i mice ($p<0.01$).

ably increased for BIFS-PE with respective percentages of 3.7, 14.6 and 47.5. Phagocytosis in immune mouse macrophages was greater than those in normal mouse macrophages. The percentage of phagocytized cells in immune mouse macrophages incubated with Tc-199 for 60 min was significantly greater than that in normal mouse macrophages (10.1 and 4.2%, $p<0.05$).

Vertical transmission and host resistance against Babesia infection: As shown in Table 4, 3 female mice and their 31 fetuses in group I, 4 female mice and 35 fetuses in group II-A, 3 female mice and 39 neonates in group II-B, and 3 female mice and 18 neonates in group III were examined in this experiment. None of the recipient mice, which were injected with homogenized material, died of babesiosis.

Immune resistance of the infants against *Babesia* infection was examined (Table 5). Mortality in group I/i-A and I/n-A was as low as 17.1 and 11.1%, respectively. On the other hand, 60.6 and 63.3% of the infants died in group N/i-A and N/n-A, respectively. Mortality in group N/i-B reached 85.7%. In contrast, mortality in group I/n-B and I/i-B reached 50.0% and 42.1%, respectively. It was noted that mortality in the four groups, I/i-B, N/i-A, I/n-B and N/n-A were close to each other (Table 5).

DISCUSSION

No vertical transmission of *Babesia rodhaini* was found in this experiment. However, the neonates from the immune mice both in group A (neonates infected with *Babesia*-PE 1 week after birth and challenged 4 weeks after primary infection) and group B (neonates infected with *Babesia*-PE 5 weeks after birth) showed a higher resistance against *Babesia* infection as compared with that from the normal mice. In our *in vitro* experiment, with the addition of BIFS-PE, the phagocytic ability of immune macrophages was higher as compared with phagocytosis of NFS-PE. This suggests that the serum antibody might play a part in the resistance against *Babesia* infection in neonates born of immune mice. It is accepted that a large proportion of protective antibodies is transferred from mother to infants mainly through the milk in the rodents [3, 16, 19, 22]. Furthermore, in rodents with *Plasmodium berghei*, the IgG antibodies in

the serum of neonates from immune mothers decreased gradually, with no serum IgG antibodies remaining at the 5th week after birth [10]. On the contrary, the infants born of normal and nursed by immune mothers showed no resistance against *Babesia* infection (N/i-A), while those born of immune and nursed by normal mothers showed remarkable protection (I/n-A). Furthermore, the immune infants fostered by normal mothers (I/n-B) also showed more resistance against single *Babesia* infection than mice in N/i-B. These findings suggest that the resistance of neonates born of immune mice is dependent on a factor, other than antibody, transferred during the fetal period. The antibody-parasite complex has possibly sensitized the immunocompetent cells of the neonates, and the challenge has triggered the secondary immune responses [11–13]. Desowits [2] suggested that a soluble antigen crosses the placental barrier and sensitizes the fetal immunocompetent cells in rats with *P. berghei*. In addition, Flynn *et al.* [4] and Teranishi [20] reported that the interleukin-1 (IL-1) was released from mononucrear phagocytes isolated from mouse and human placentae into culture medium and that its release was increased by stimulation of phagocytosis. These macrophages are stimulated by the soluble antigen opsonized by specific antibody.

The report that neonates of chronically infected mother rats showed a significant resistance to *Toxoplasma* infection compared to those born of normal rats [18] may support our hypothesis that macrophages and spleen cells may be possibly sensitized by soluble antigen or by lymphokines which have been transferred from the imuune mother. A possible explanation for this finding might be that fetal lymphocytes had been sensitized to *Babesia* before birth and that they returned to a non-sensitized state within several weeks after birth. Our experimental results, in which MIF activity increased in *Babesia* infected mice, may suggest that the cell-mediated immune response might play a large part in the resistance against *Babesia* infection. In consequence, the high resistance against *Babesia* immune mice might probably be caused by the same action of T-lymphocyte sensitization as in the neonates from *Toxoplasma* immune mice.

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要　　約

バベシア感染マウス新生仔における *Babesia rodhaini* 感染に対する抵抗性：桜井治久・高橋宏昌・佐藤基佳[1)]・広瀬恒夫[1)]・斎藤篤志・鈴木直義（帯広畜産大学家畜生理学教室，[1)]獣医臨床放射線学教室）——*Babesia rodhaini*（以下，バベシア）感染耐過マウスの脾臓細胞をバベシア抗原存在下で培養すると，培養上清（LKs）は，非感染マウス由来の脾臓細胞培養上清に比べて高い MIF 活性を示した。感染耐過マウスの腹腔マクロファージ（Mφ）のバベシア貪食能は，非感染マウス Mφに比較して高進していた。バベシア感染急性期および感染耐過母マウスからの胎仔あるいは新生仔からは，原虫は分離されず，垂直感染は証明できなかった。感染耐過母マウスからの新生仔は，1週齢でバベシア感染に対して強い感染死防御能を示した。感染耐過母マウスからの新生仔は，非感染マウスで授乳しても，1～5週齢の攻撃に対して有意の感染抵抗性を示した。

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Inhibitory Effect of Toxoplasma Lysate Antigen on the Multiplication of Transplanted Tumor in Mice

A. SAITO[1], H. SAKURAI[1], S. TERADA[1], I. IGARASHI[1], T. HIROSE[2], N. SUZUKI[1], H. OSAKI[3], H. KUDO[4], Y. SAKATA[5], K. OKAMOTO[5], and Y. YOSHIDA[5]

[1] Dept. of Veterinary Physiology and Protozoan Immunology and
[2] Dept. of Veterinary Clinical Radiology, Obihiro University
[3] Yagi Hospital, Hyogo
[4] Dept. of Pathology and
[5] Dept. of Medicine, School of Medicine, Hirosaki University, Japan

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Summary

Tachyzoites of the RH strain of *Toxoplasma gondii* were pretreated by a process of freezing and thawing followed by ultrasonication. After ultracentrifugation at 144,000 g for 120 minutes, the resulting supernatant contained a protein (TLA 144), which consisted mainly of a component of protozoan origin with a molecular weight of 10,000–20,000, and additional sugars, peptides, andd amino acids. TLA 144 was soluble in water and of very low toxicity. Mice that had been inoculated with allogeneic (S-180) and syngeneic (Meth A) transplantable tumor cells, were injected intramuscularly with 100 μg of TLA 144 once a week for some time beginning one week after transplantation. It was found that after TLA treatment the multiplication of tumor cells was more intensely inhibited than following administration of OK-432, one of the biological response modifiers (BRMs).

Zusammenfassung

Tachyzoiten der RH-Stammes von *Toxoplasma gondii* wurden durch Gefrieren und Auftauen sowie nachfolgende Ultraschallexposition vorbehandelt. Nach Ultrazentrifugierung bei 144 000 g über 120 min enthielt der sich ergebende Überstand ein Protein (TLA 144), das hauptsächlich aus einer von dem Protozoon stammenden Komponente mit einem Molekulargewicht von 10 000 bis 20 000 sowie aus Zuckern, Peptiden und Aminosäuren bestand. TLA 144 war wasserlöslich und von sehr geringer Toxizität. Mit allogeneischen (S-180) und syngeneischen (Meth A) transplantierbaren Tumorzellen geimpfte Mäuse erhielten i.m. Injektionen von 100 μg TLA 144 einmal wöchentlich über einige Zeit, beginnend eine Woche nach der Transplantation. Es wurde festgestellt, daß nach TLA-Behandlung die Vermehrung der Tumorzellen stärker gehemmt wurde als nach Gabe von OK-432, einem biologischen Reaktions-Modifikator (biological response modifier – BRM).

Introduction

Animals inoculated with *Toxoplasma* lysate antigen (TLA) were sensitized and survived infections with *Plasmodium berghei* or *Babesia rodhaini* (5, 6, 7, 14). In TLA-treated cattle, infections with *Theileria sergenti* led to less severe clinical symptoms than found in untreated cattle (10). As a response of the mice to the sensitization with TLA, the sensitized delayed-hypersensitivity T-lymphocytes (T-dh cells) produced lymphokines (Lks), including interferon-gamma (IFN-γ), macrophage activating factor (MAF), macrophage migration inhibitory factor (MIF), and Toxo-GIF in the circulating blood (5, 6, 7). In TLA-treated mice the thymus, spleen, and the peripheral blood contained considerably more T-lymphocytes than controls without TLA. It has been shown that in *Babesia* infections T-lymphocytes increase remarkably in spleen and liver. These T-lymphocytes aggregated perivascularly and consisted mainly of so-called helper T-cells (Th cells) and inducer T-cells, the phenotypes of which were Lyt1.2(+) and Lyt2.2(–), respectively (11). In addition, it is known from an *in vitro* experiment that murine and human natural killer (NK) cells are also activated by sensitization with TLA (1, 12). These results prove that some components of the cell body of *Toxoplasma gondii* tachyzoites have a strong modulator effect on animals infected by various protozoans (8). It has also been shown that some components of TLA have cross immunogenicity effects on infectious protozoa (14). As a consequence an experiment was started with allogeneic (S-180) and syngeneic (Meth A) transplantable tumor cells of mice, in which components of the *Toxoplasma* cell are certain to have no cross immunogenicity effects. We examined, whether or not TLA as a biological response modifier (BRM), with its strong immunity-activating effect had an influence on the multiplication of tumor cells.

Materials and Methods

1. *Mice and protozoans.* The RH strain of *Toxoplasma gondii* was inoculated intraperitoneally into mice which had not infected to *Toxoplasma gondii.* Tachyzoites were collected from the peritoneal cavity 2 days after inoculation and processed further. Mice of the ICR-JCL and the BALB/c strains were bred at the senior author's laboratory.

2. *Preparation of TLA.* An original TLA suspension was prepared using the methods of *Igarashi* et al. (2) and *Sakurai* et al. (9). The process of preparation is summarized in Table 1. Tachyzoites were washed three times with Hanks' balanced salt solution (HBSS) by centrifugation at 750 g for 10 min at 4 °C. The sediment was washed in sterilized water so that the resulting suspension contained 2×10^9 tachyzoites/ml. This suspension was frozen three times at –80 °C and thawed at 37 °C. Then the included tachyzoites were sonified five times (at 40W for 1 min; Sonicator, model W-220 F, Heat Systems-Ultrasonics Inc., Plainview, New York, U.S.A.). The resulting suspension was centrifuged at 16,000 g for 60 min at 4 °C. An equal volume of 1.7% NaCl solution was added to the supernatant obtained in order to prepare an isotonic suspension, which was used as a *Toxoplasma* lysate antigen (TLA 16). The concentration of protein within TLA 16 was determined by the method of *Lowry* et al. (4) with bovine serum albumin as a standard.

For purification, TLA 16 was centrifuged at 100,000 g for 60 minutes; the resulting supernatant was called TLA 100 and its sediment TLA 100-Sed. Subsequently, TLA 100 was centrifuged at 144,000 g for 120 min. The resulting supernatant and sediment were called TLA 144 and TLA 144-Sed., respectively. These supernatants and sediments were subjected to a process of freeze-drying in vacuum and stored at 4°–8 °C until use. To verify the purification of TLA 16, TLA 100, and TLA 144, a TLA solution in physiological saline

at a concentration of 500 mg/ml was subjected to liquid chromatographic separation (Hitachi high-speed liquid chromatograph, model 655; UV, 280 nm and 220 nm). For this technique Toyo-pearl HW 55 Fractgel (Toso Co., Tokyo) or Gelpack GL-W 550 (Hitachi Co., Tokyo) resin was used as the gel filtration method applied with 0.01 M PBS, pH 7.0, as an eluent. For the partial purification and the assay of purity, the same sample as used for the liquid chromatographic separation was subjected to elution by ion exchange chromatography. Gelpack GL-K 55D (Hitachi Co., Tokyo) was used for this technique, which was performed by the linear gradient method. In this method eluents A and B were used. The former was a 20 mM tris hydrochloric acid buffer solution, pH 8.0, the latter was a mixture of the eluent A and 500 mM NaCl. Transfer from a 100% solution of A to a 100% solution of B was completed by this method within 30 min.

Ion exchange resin prepared for DEAE-5 PW analysis (Toso Co., Tokyo) was used to purify the final product TLA 144 and to extract an active fraction. In the technique employed, elution was carried out with the initial basic eluent (A) and 0.02 M PBS, pH 7.0, at a flow velocity of 1 ml/min for 60 min. After that, TLA 144 was fractionated by the linear gradient method with a mixture of 1.0 M NaCl and 0.02 M PBS. The resulting fractions were examined for the presence or absence of antitumorigenic effects.

3. *Production of mice bearing Sarcoma 180 and Meth A tumor cells.* Adult female mice (8 weeks old) of the ICR-JCL or BALB/c strain were transplanted intraperitoneally with homotypic or allogeneic (S-180) or homologous or syngeneic (Meth A) transplantable tumor cells. To prepare S-180 tumor cells, ascites was collected from mice, into which these cells had been transplanted, for passage. Cells were separated by centrifugation at 150 g for 6 min at 4 °C representing 98% of S-180 cells. They were washed three times with HBSS by centrifugation at 150 g for 6 min at 4 °C. Then they were suspended in HBSS so that the resulting suspension contained 5×10^6 S-180 cells/ml. Mice were injected subcutaneously on the back or at the side of the abdominal region with 0.2 ml (containing 1×10^6 cells) of this suspension. They served as mice bearing S-180 tumor cells. A suspension of Meth A tumor cells was prepared in the same manner. Other mice were each inoculated subcutaneously on the side of the abdominal region with 1×10^6 Meth A tumor cells. All tumor-bearing mice were injected intramuscularly once a week with 100 μg of TLA suspended in 0.2 ml of light mineral oil.

4. *Indication of inhibitory effect on tumor multiplication.* The size of a subcutaneous transplant of S-180 or Meth A tumor cells in a mouse was examined at given intervals by measuring tumor length (a) and width(b) with a vernier caliper. The lenght was multiplied by the width to calculate the extent of the tumor. The volume (V) of the tumor was determined by the following formula: $V = \frac{1}{2}(ab^2)$.

5. *Preparation of OK-432 suspension.* One KE of OK-432 (Chugai Pharmaceutical Co., Tokyo) was suspended in 2.0 ml of physiological saline as BRM. Mice were administered with 0.2 ml (0.1 KE) of the resulting suspension. The OK-432 suspension was prepared just before use.

Results

1. *Gel filtration and elution pattern by ion exchange chromatography of TLA 144*

TLA 144 was subjected to a process of elution with Gelpack GL-W 550 resin. By comparing with the elution pattern of a known substance, it was confirmed that the molecular weight of the principal component of TLA 144 was within a range of 15,000 to 20,000 (Fig. 1-A). To confirm the degree of partial purification, the same sample was subjected to ion exchange chromatography with Gelpack GL-K 55D resin. When the elution pattern was examined, it became clear that TLA 144 appeared as a single peak with a purity of more than 95% (Fig. 1-B).

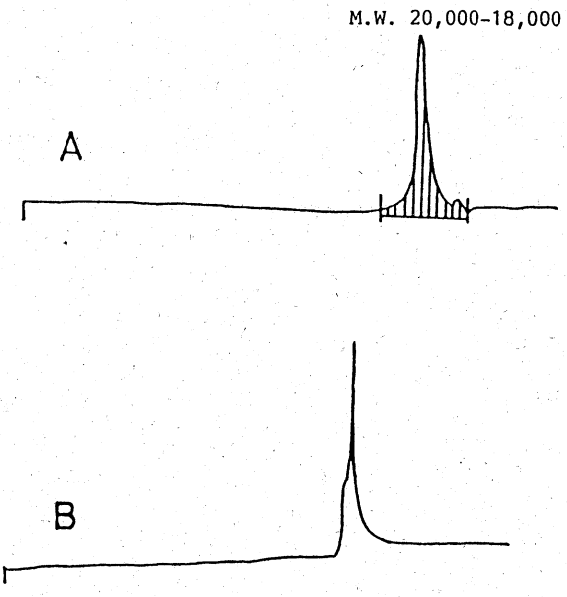


Fig. 1. Elution patterns of TLA-144 by gel filtration and ion exchange chromatography. A; Elution pattern of TLA-144 by gel filtration with Gelpack GL-W550 resin. B; Elution pattern of TLA-144 by ion exchange chromatography with Gelpack GL-K55D resin.

TLA 144 was proven to consist mainly of protein components with molecular weights of 18,000 to 20,000. It was subjected to elution with ion exchange resin for analytical use (DEAE-5 PW). When the results of elution were examined at 220 nm and 280 nm, they could be divided into almost 10 absorption fractions represented by the same number of peaks (Fig. 2). Therefore, TLA 144 is not a single protein but an aggregate of several proteins varying in molecular weight and ion strength.

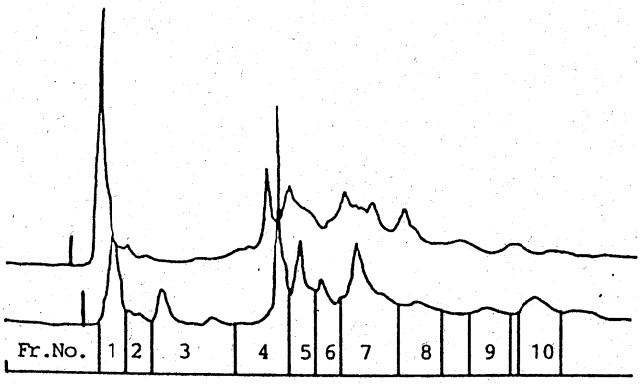
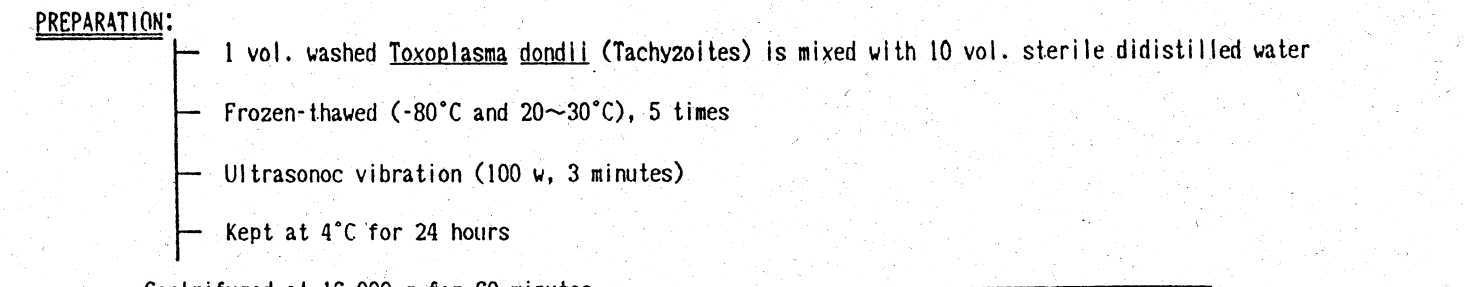


Fig. 2. Elution pattern of TLA-144 by ion exchange resin for analytical use (DEAE-5PW). Column chromatography was carried out by linear gradient with Buffer A to Buffer B and then Buffer B to Buffer C. Column: DEAE-5PW (7.5 × 750 mm). Elution buffer: Buffer A, 0.02 M phosphate buffer, pH 7.0; Buffer B, 0.1 M NaCl in Buffer A; Buffer C, 1.0 M NaCl in Buffer A. Flow Rate: 1.0 ml/min. Pressure: 9 Kg/cm^2.



PHYSICO-CHEMICAL PROPERTIES

Substances	TP-WS	TLA 16	TLA-I	TLA-II	TLA-III	TLA 100	TLA 144
Molecular weights			100,000	100,000~20,00	4,000		20,000~15,000
Solubility in water:	−		±	±	±		+
Solubility in organic solvents:	Non or practically insoluble in methanol, ethanol and chloroform						
6N HCl solubility:			+	+	+	+	+
pH:	6.3		6.4	6.4	6.5		6.5
UV absorption: (Maximum, nm)	278~280		278~280	270~274	260~270		260~270

Color reactions					
Lowry-Folin process	+	+	+	+	+
Ninhydrin reaction	+	+	+	+	+
Phenol-sulfuric acid r.	+	+	+	+	+
Elson-Morgan's reaction	+	+	+	+	+
Color and appearance:	Pale yellow	Yellow-white	Yellow-white	Yellow white	Pale white
Constituent amino acids:	Aspartic acis, Threonine, Serine, Glutamic acid, Glycine, Alaninee, Cystine, Valine, Methionine, Isoleucine, Leucine, Tyrosine, Phenylalanine, Tryptophan, Histidine, Arginine, Proline				
EFFECTIVENESS OF TLA					
Interferon induced:	γ type	γ	γ	α & β	
Antibody produced:	+	+	+	+	+
Lymphokines produced:	+	+	+		+
Macrophage activation:	+	+	+		+
Intracellular cAMP level:			++		++
Intracellular cGMP level:			±		±
Intracellular ATP level:			+		+
ATP:ADP ratio			±		±
O_2 consumption			±		±

Table 1. An outline of the preparative method of TLA from *Toxoplasma* parasites

2. Physiochemical properties of TLA 144

The physicochemical properties of TLA are summarized in Table 1. When a 0.1% (w/v) aqueous solution of TLA 144 was prepared and subjected to two-wavelength ultraviolet absorption spectrophotometry (type 200-20, Hitachi Co., Tokyo), the absorption spectrum curve showed maximum absorption in the vicinicity of 260 nm with a shoulder at 278 nm. When the nucleic acid content of TLA 144 was determined by liquid chromatography (type 655-A, Hitachi, Tokyo), such principal components as hypoxanthine, uridine monophosphate (UMP), adenosine monophosphate (AMP), and guanosine monophosphate (GMP) were less than 1%. TLA 144 was readily soluble in water. One weight percent aqueous solution of TLA 144 was positive for the phenol sulfuric acid reaction with 0.1 weight percent aqueous solution for the Lowry-Folin and the ninhydrin test. Thus, is was confirmed that TLA 144 consisted mainly of sugar, peptide bond, and protein including amino acids.

3. Acute toxicity test of TLA 144

An experiment was carried out with adult female mice of the ICR-JCL strain (weighing 20 g each) using the method of *Litchfield* and *Wilcoxon* (3). Six groups of 3 mice each were injected intraperitoneally with 1 ml of physiological saline containg 50, 100, 500, 1,000, 10,000, or 20,000 μg of TLA 144. No mortality occurred within 24 h after injection. Thus, it was shown that the LD 50 of TLA 144 was more than 1,000 mg/kg for mice after intraperitoneal injection, indicating that the toxicity of TLA 144 was extremely low.

4. Effect of administration of TLA 16 or TLA 100 on the multiplication of S-180 tumor cells

TLA 16 and TLA 100 were examined for their antitumorigenic effects on mice of the ICR-JCL strain bearing S-180 tumors (Fig. 3). For this purpose TLA 100 was prepared from TLA 16 in such a manner that 77.5% of the protein was recovered. Therefore, considering the inoculation dose of 100 μg of TLA 16 per animal, the amount of included TLA 100 was judged to be 77.5 μg of protein per animal. Fifteen mice bearing S-180 tumors were divided into three groups of five each. Group I served as a control without TLA. Groups II and III received TLA 16 or TLA 100, intramuscularly, five times at weekly intervals beginning one week after transplantation of S-180 tumor cells. On day 35 after transplantation the mean extension of tumor measured 871.4 mm^2 in group I, 715.8 mm^2 in group II, and 170.0 mm^2 in group III. The survival rate of mice on day 49 after transplantation was 20%, 80% or 100% in groups I, II or III. Thus, administration of TLA 100 led to a higher inhibition of tumor cell multiplication and an increase in the survival rate of mice compared with the effects of TLA 16.

5. Effect of administration of TLA 100 or TLA 144 on the multiplication of S-180 tumor cells

When 100 μg of protein in TLA 16 was taken as 100%, the recovery rate of protein was 67% for TLA 100, 33% for the sediment of TLA 100 (TLA 100-Sed.), 61% for TLA 144, and 6% for the sediment of TLA 144 (TLA 144-Sed.). The amount of TLA administered was 100 μg per animal in the case of TLA 100. The amount for any other product of TLA was calculated by using the rate of recovery of protein for conversion. Five groups of 5 mice each were used for the following experiments. Group I served as

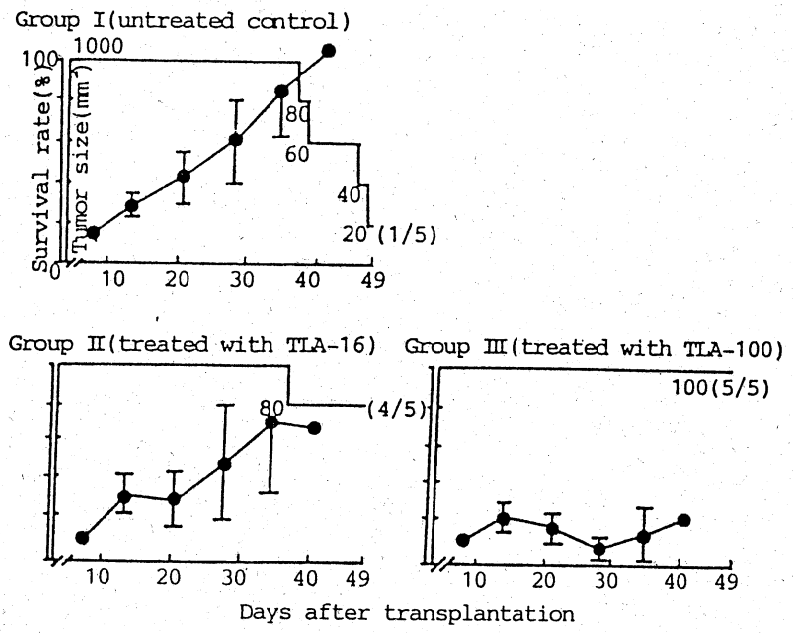


Fig. 3. Antitumorigenic effect of TLA-16 or TLA-100 administration on mice of the ICR-JCL strain bearing S-180 tumor. The vertical axis represents survival rate of mice bearing tumor and tumor size. The horizontal axis represents days after transplantation of S-180 tumor cells (1×10^6 cells/head). The dots and vertical bars represent tumor size (mean ± S.E.).

a control consisting of tumor bearing mice without any TLA product. Group II received 100 μg of TLA per animal, group III 50 μg of TLA 100-Sed., group IV 90 μg of TLA 144, and group V 10 μg of TLA 144-Sed. Each mouse was injected intramuscularly in the right thigh with the respective dose five times at weekly intervals beginning one week after transplantation of S-180 tumor cells. Fig. 4 shows the time-dependent changes in the multiplication of tumor cells in these groups. The mean extensions of tumor on day 35 after transplantation were 546.6 mm^2 in group I, 416.0 mm^2 in group II, 420.0 mm^2 in group III, 334.8 mm^2 in group IV, and 871.7 mm^2 in group V. On day 49 after transplantation the survival rate of tumor-bearing mice was 56% in group I, 40% in group II, 40% in group III, 80% in group IV and 20% in group V. In short, group IV, which had been given TLA 144, exhibited a better antitumorigenic effect of the agent and a higher survival rate of mice than any other group.

6. Effect of administration of TLA 144 or OK-432 on the transplantation of S-180 or Meth A tumor cells

After transplantation of S-180 or Meth A tumor cells, mice were injected intramuscularly in the thigh with 100 μg of TLA 144 or 0.1 KE of OK-432 per head five times at weekly intervals beginning on the day after transplantation. The results are illustrated in Figs. 5-A and 5-B. Administration of TLA 144 had a greater inhibitory effect on tumor multiplication, and led to a better life prolongation, than did administration of OK-432. No side effects, such as loss in body weight, were observed at all in any mouse during the period of 48 experimental days. As documented in Fig. 5-B, administration

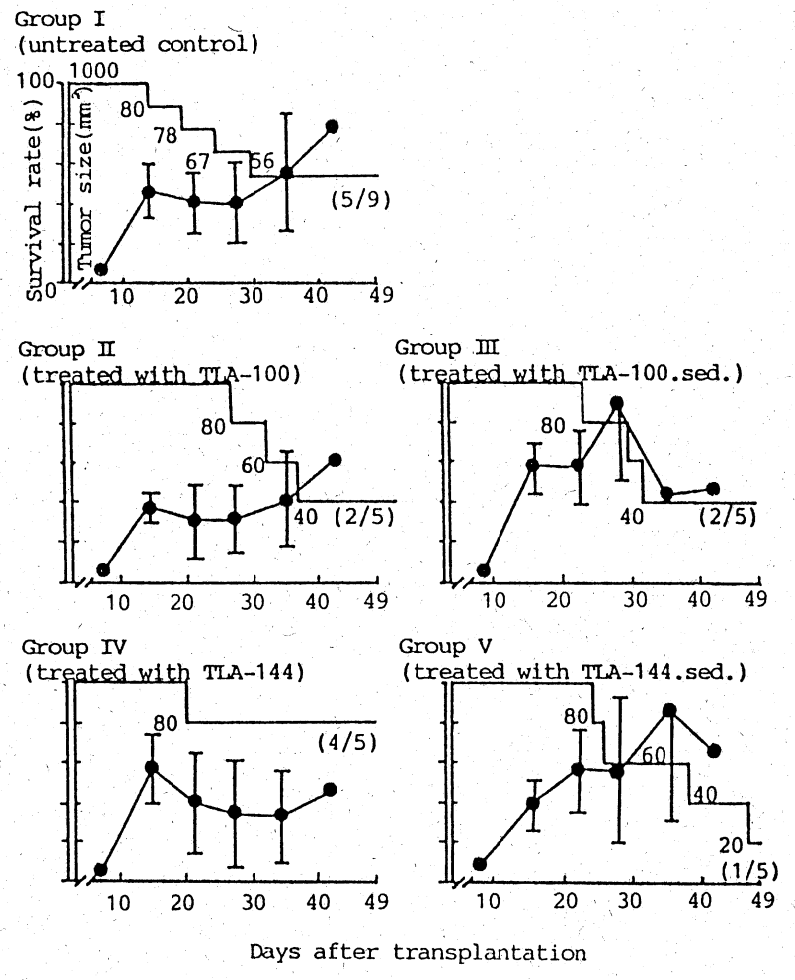


Fig. 4. Antitumorigenic effect of TLA-100 or TLA-144 administration on mice bearing S-180 tumor. Symbols are as in Fig. 3.

of OK-432 had a distinct inhibitory effect on tumor multiplication in those mice transplanted with the homotypic tumor to BALB/c mice, Meth A. Administration of TLA 144 led, however, to a greater inhibitory effect on the multiplication of Meth A tumor cells compared to that of OK-432. TLA 144 and OK-432 both had a significant effect on life prolongation of the treated mice bearing Meth A tumor compared to untreated mice bearing this tumor.

7. Effect on S-180 tumor cells of constituent fractions of TLA 144 obtained by ion exchange chromatography with DEAE-5 PW for analytical use

The elution pattern produced is shown in Fig. 2. TLA 144 was separated into ten fractions (called OT-1 to OT-10) by ion exchange chromatography for analytical use. Antitumorigenic activity was compared for a single administration of each fraction of TLA 144 and an administration of TLA 144 alone (33 μg/mouse). A curve of tumor

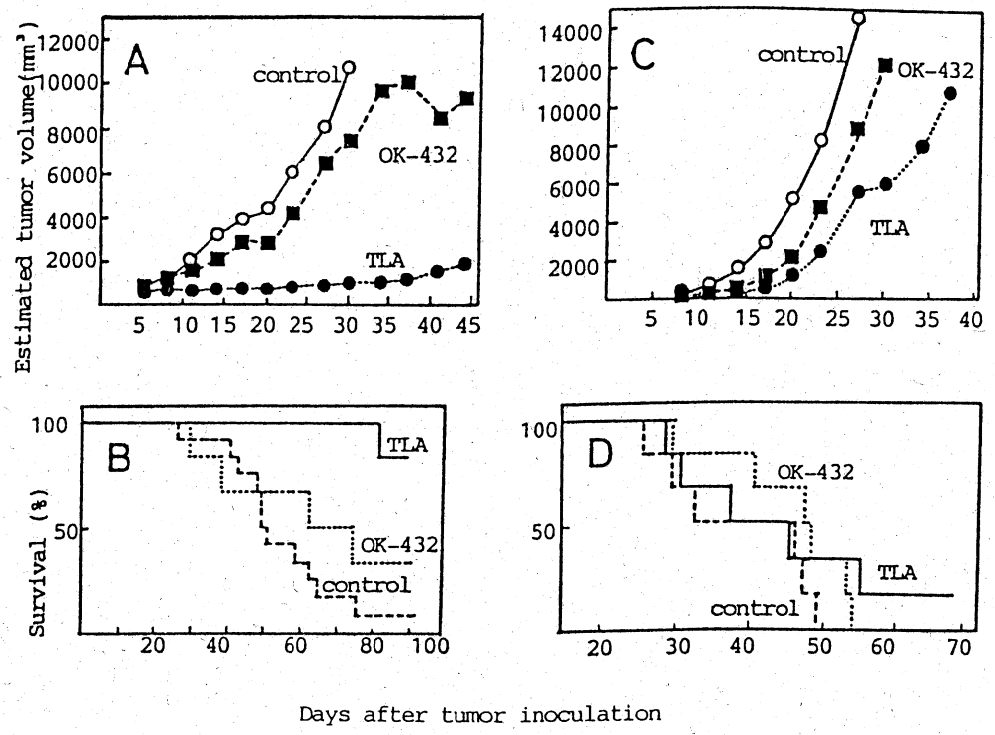


Fig. 5. Effect of TLA-144 or OK-432 administration on mice transplanted with S-180 or Meth A tumor cell. A and B; Effect of TLA-144 and OK-432 on tumor growth and survival time of ICR-JCL mice inoculated s.c. with S-180. C and D; Effect of TLA-144 and OK-432 on tumor growth and survival time of BALB/c mice inoculated s.c. with Meth A. TLA; 100 μg/mouse, i.m. weekly for 5 weeks. OK-432; 0.1 KE/mouse, i.m. weekly for 5 weeks.

multiplication was drawn for the single administration of each fraction, as shown in Fig. 6. Some fractions were found to have an antitumorigenic effect. Fractions OT-1, 2, 5, and 8 had an inhibitory effect on tumor multiplication 30 days after tumor transplantation. Fraction OT-10 exhibited the best antitumorigenic effect. Fractions OT-1, 5, 8 and 10 showed a clear life prolongation. TLA 144 is an aggregation of protein constituents, and its antitumorigenic activity was found to be distributed among its fractions.

Discussion

In the present study fractions obtained by centrifugation of the constituents of antigens of *Toxoplasma* origin were examined primarily for an inhibitory effect on the multiplication *in vivo* of allogeneic (S-180) or syngeneic (Met A) transplantation tumor cells. Previous studies have shown that when mice were injected with a TLA 16 emulsion, the number of T-lymphocytes increased (11) as did the production of lymphokines (6, 7), the activation of NK cells (1), and the activation of macrophages (5). However, there is no previously published discussion on the effects of TLA, as a nonspecific immunity-activating substance or BRM, on the multiplication of tumor cells.

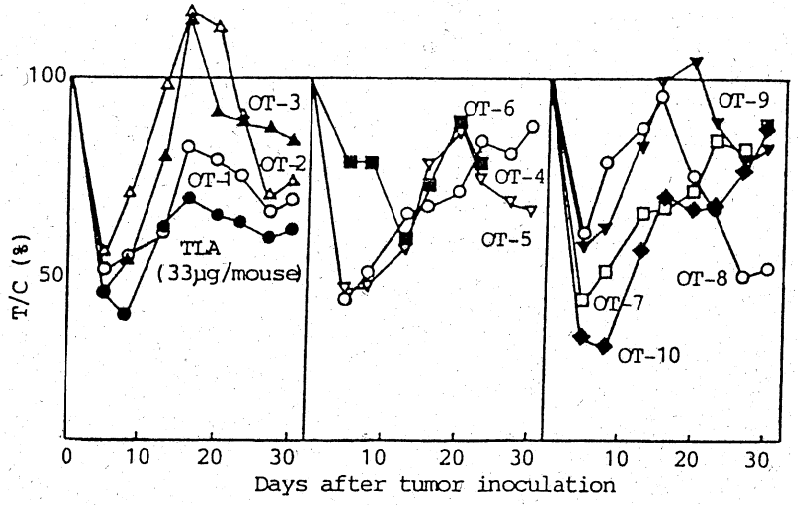


Fig. 6. Effects on S-180 tumor of 10 fractions of TLA-144 obtained by ion exchange chromatography with DEAE-5PW. Antitumorigenic activities were tested against S-180 implanted in ICR-JCL mice. OT-1, -2, -3, -4, -5, -6, -7, -8, -9 and -10 indicate name of fraction of TLA separated by ion exchange HPLC (see Fig. 2). Vertical axis represents the percentage of tumor size in mice treated with each fraction or TLA-144 to tumor size in non-treated control mice at same days after tumor inoculation.

When mice bearing S-180 tumor received a TLA 16 emulsion, the tumor was clearly prevented from increasing. When TLA 16 was subjected to ultracentrifugation at 100,000 g for 60 min, the resulting supernatant had a distinctly greater inhibitory effect on tumor multiplication than TLA 16 alone. It is considered that microsomes are generally a principal component of the supernatant obtained from TLA 16 by centrifugation at 100,000 g (called TLA 100). In the present study it was shown that an antitumorigenic effect was induced by at least one component differing from the crude antigenic substance, which had an antitumorigenic effect due to its protozoan constituents. TLA 100 was subjected to ultracentrifugation at 144,000 g for 120 min. When the resulting supernatant (called TLA 144) and the sediment were examined for antitumorigenic effects, the supernatant had a much greater efficacy. The partial purification of TLA 144 was attempted, but was not successful. TLA 144 still remained a multiphasic protein bond. Further studies are required for purification of TLA 144 and for determination of the mechanisms of the physiological activity of TLA 144 in the living body.

The antitumorigenic effects of TLA and OK-432 on the multiplication of allogeneic and syngeneic tumor cells transplanted to mice of the BALB/c strain were compared. As a result, mice administered with TLA 144 showed a significantly greater inhibitory effect on the multiplication of S-180 and Meth A tumor cells, and displayed significantly higher survival rates than did mice administered with OK-432. In its versatile evaluation studies on biological response modifiers (BRMs), the National Cancer Institute, U.S.A., stated that OK-432 is one of the most effective immunity-activating substances in the world (15). In general, there are three methods of immunochemical treatment of infections and tumors. The first is carried out with the immunity-adjusting or immunoregulatory substances (such as Obioactin, IFN-γ and IL-2) produced in the

body of the host. The second is performed using anticarcinogens and other chemotherapeutics. A third method utilizes immune-potentiatory substances (such as BCG, MDP, OK-432, and lentinan) as their main BRMs. The present study verifies that TLA belongs to these substances. It is presumed that BRMs may participate in the inhibition of tumor multiplication in the host by supporting either the production of an immunity-adjusting substance in the living body (5, 7, 13) or by exhibition of a chemotherapeutic effect. In this manner, BRMs are considered to enhance the non-specific resistance of the host. TLA 144 is of low toxicity, but is an active substance which possesses a very strong BRM effect. The most important problem remains that of clarifying the primary structural formula of this substance.

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Dr. *Atsushi Saito,* Department of Veterinary Physiology and Protozoan Immunology, Obihiro University, Obihiro, Hokkaido 080, Japan

Protection against *Babesia* Infection in Beagles Immunized with *Toxoplasma* Lysate Antigen

Motoyoshi SATO, Ikuo IGARASHI[1)], Atushi SAITO[1)], Tsuneo HIROSE, and Naoyoshi SUZUKI[1)]
Department of Veterinary Clinical Radiology and Department of Veterinary Physiology and Protozoan Immunology[1)], Obihiro University, Obihiro, Hokkaido 080, Japan
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Toxoplasma gondii has been reported to stimulate non-specific resistance to rodent malaria [6]. Mice that were treated systemically with *Toxoplasma* lysate antigen (TLA) were highly resistant to infection with *Plasmodium berghei* [7] and *Babesia rodhaini* [5]. The purpose of this study was to confirm the protective effects of TLA or *Babesia* lysate antigen (BLA) against *Babesia gibsoni* infection in beagles.

Four 150-day-old parasite-free beagles of the same litter were injected subcutaneously with canine distemper vaccine at 70 to 90 days of age. To maintain constant conditions, the dogs were housed in parasite-proof cages, and given a mesured volume of commercial dog diet every day. Tap water was supplied *ad libitum* during the experiment. Blood containing 1×10^9 *B. gibsoni*-infected erythrocytes (IE) per m*l* was collected from a splenectomized and chronically infected beagle. The blood was washed 3 times by centrifugation with phosphate buffered saline (PBS) and resuspended in PBS to a final concentration of 1×10^8 IE per 2 m*l*. TLA and BLA were prepared essentially as described by Itoh *et al.* [4] and Sakurai *et al.* [9], respectively.

Beagles 1, 2, and 3 were pre-treated with either BLA, TLA or TLA plus levamisol HCL (Japan Lederle Co., Tokyo), respectively (Table 1). Beagle No 4 was used as a non-treated control (Table 1). Beagle 1 was injected intramusucularly in the shoulder and gluteal regions with the first dose of 1,000 μg BLA/kg in 1 m*l* PBS mixed with the same volume of Freund's incomplete adjuvant (FIA). This was followed 2 weeks (wks) later with a second injection of the same quantity of BLA and FIA. Beagles 2 and 3 received two injections of the same quantity of 1,000 μg TLA/kg and FIA 3 wks and 1 wk before infection. Beagle 3 was orally administered 7.5 mg/kg of levamisol 2 wks before infection. One week after the second injection all four beagles were infected with *B. gibsoni* by injection of 1×10^8 IE into the antebrachial part of the cephalic vein. Each beagle was examined daily for clinical signs of infection, including body temperature, pulse, hematuria and jaundice. At each examination whole blood was collected with potassium EDTA as an anticoaglant for estimation of hematocrit (Ht), hemoglobin concentration (Hb), and differential cell counts. Parasitemia was estimated by counting the number of IE per 1,000 erythrocytes on May-Grünwald and Giemsa stained blood smears. Glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), bilirubin, and blood urea nitrogen (BUN) values in the sera were determined by the RaBA-super system (Chugai Pharma. Co., Tokyo). Total serum protein and serum protein fractions were determined with a refractometer and a micro-electrophoretic apparatus, respectively. Antibody titers were measured by the indirect fluorescent antibody (IFA) test, using anti-canine immunoglobulins conjugated with FITC [3]. Lymphokines (LKs) were prepared by the method of Ishimine *et al.* [3]. Macrophage migration inhibitory factor (MIF) was estimated as described by Igarashi *et al.* [2].

The results are summarized in Table 1. Beagles 1, 2, and 3 were protected against lethal infection with *B. gibsoni*, while beagle 4 (non-immunized control) was euthanized 23 days after infection (AI), just before death from severe babesiosis. Pyrexia, however, presented essentially the same pattern in all the beagles for 2 wks AI. Body temperature of the control dog fell from 39.2°C before infection to 36.2°C at 3 wks AI, with a 7.6% reduction. In the BLA immunized beagle, a maximum reduction in body temperature of 4.7% was observed during the 4th week AI, but returned in pre-immunization levels by 5 wks AI. At 8 wks AI, no differences in temperature reduction were observed between the 2 TLA immunized beagles. Jaundice was first noticeable in all beagles at 2 wks AI, however, beagle 3 exhibited the mildest signs. Infected erythrocytes were present in all beagles 1 week AI, and

Table 1. Summary of experimental design and results in 4 beagles infected with *Babesia gibsoni*

Beagle No	Immunogen used	Modulator used	Blood			Temperature	Serum					
			RBC maximum reduction <x10⁴/μl>	Ht maximum reduction <%>	Parasitemia maximum rate (‰)	maximum reduction <°C>	GOT max.rate of increment <KU>	GPT max. rate of increment <KU>	TP max. rate of variation <g/dl>	A/G ratio max. reduction	Alb. maximum reduction <g/dl>	γ-glob. max. increment <g/dl>
1	BLA[a)]+FIA[b)]	NU[c)]	81.4 d) (3W) <115>h)	76.1 (4W) <11>	29 (2W)	4.7 (4W) <36.8>	96.7 (3W) <53.77>	169.8 (4W) <50.37>	+45.5 (1W)<6.4> — e)	63.2 (3W) <0.05>	40.3 (3W) <1.94>	423.8 (3W) <1.27>
2	TLA[f)]+FIA	NU	88.3 (3W) <78>	76.1 (3W) <11>	7.3 (2W)	(-3W) <38.1>	35.5 (8W) <38.68>	141.4 (5W) <41.25>	+28.6 (-1W)<7.2> -3.6 (2W)<5.4>	71.4 (5,6) <0.50>	47.2 (6W) <2.25>	462.2 (6W) <1.92>
3	TLA+FIA	Levamisol	70.7 (3W) <173>	62.0 (2W) <16>	4.2 (2W)	1.0 (5W) <38.5>	39.9 (3W) <36.77>	111.1 (3W) <43.60>	+44.7 (0W)<6.8> —	67.6 (3W) <0.56>	43.3 (3W) <1.93>	534.6 (4W) <0.92>
4[g)]	Control	NU	86.9 (3W) <91>	74.5 (3W) <12>	34 (2W)	7.6 (3W) <36.2>	45.1 (1W) <43.76>	5.4 (0W) <29.65>	+6.9 (0,1W)<6.2> -13.8 (3W)<5.0>	67.3 (3W) <0.55>	43.3 (2W) <1.99>	309.3 (3W) <0.65>

a) *Babesia* lysate antigen.
b) Freund's incomplete adjuvant.
c) Modulator was not used.
d) Week at the maximum (max.) reduction or increment after infection.
e) Not recorded.
f) *Toxoplasma* lysate antigen.
g) Control beagle No. 4 was euthanized just before death on 23rd day after *Babesia*-infection.
h) Value of the maximum (max.) reduction or increment week.

Table 2. Gamma-globulin in concentration and immunofluorescent antibody titers in sera, and macrophage migration inhibitory factors in the spleen of beagles infected with *Babesia gibsoni*

Beagle No	Weeks postinfection	Gamma-globulins (g/dl)	Antibody[a] titers	MIF[b] (%)
1 (BLA+FIA)	-3	4.2	1: <4	60.3
	-2	3.5	—[c]	65.7
	-1	4.4	—	—
	0	5.2	1: 20	66.8
	1	5.9	1: 320	75.1
	2	14.0	1: 1280	—
	3	22.0	1: 1280	69.7
	4	16.1	1: 1280	80.8
	6	16.9	1: 1280	69.7
	8	10.6	1: 1280	63.0
2 (TLA+FIA)	-3	4.5	1: <4	46.8
	-2	6.4	—	71.1
	-1	6.3	—	—
	0	7.1	1: 10	70.3
	1	7.8	1: 160	81.5
	2	10.0	1: 320	—
	3	20.6	1: 640	84.6
	4	18.7	1: 1280	—
	6	25.3	1: 1280	88.1
	8	13.9	1: 1280	73.6
3 (TLA+FIA) + Levamisol	-3	2.6	1: <4	61.6
	-2	3.1	—	56.2
	-1	2.6	—	—
	0	6.9	1: 20	71.4
	1	7.6	1: 320	80.5
	2	11.9	1: 1280	—
	3	15.3	1: 2560	85.9
	4	16.5	1: 1280	88.9
	6	12.3	1: 2560	64.3
	8	10.0	1: 1180	49.5
4 Control	-3	3.2	—	—
	-2	3.5	—	—
	-1	3.1	—	—
	0	3.5	1: <4	46.8
	1	4.1	1: 20	69.7
	2	12.3	1: 160	—
	3	13.1	1: 640	88.9

a) Antibody titers were examined by the indirect fluorescent antibody (IFA) test using anti-canine immunoglobulins conjugated with FITC.
b) Activities of MIF, percentage of macrophage migration inhibitory factors in the lymphokines producted by the sensitized spleen cells incubated with Concanavalin A for 24 hours.
c) Not examined/recorded.

reached a peak in numbers 2 wks AI. Maximum parasitemia was markedly lower in beagles 2 and 3 (7.3‰ and 4.2‰, respectively) than in beagles 1 and 4 (29‰ and 34‰, respectively). A decrease in erythrocyte count and Ht in all the beagles at 2 wks AI during the period of high parasitemia,

was less marked than at 3 wks AI. The erythrocyte count and Ht in beagles 1, 2, and 3 returned to pre-immunization levels by 8 wks AI. Beagle 3 had the lowest parasitemia. Total protein and GPT were markedly higher in the immunized beagles than in the control dog.

Plasma gamma-globulins increased gradually in all the beagles after infection (Table 2). Just before infection with *B. gibsoni*, antibody titers in beagles 1, 2, and 3 rose to 1:20, 1:10 and 1:20, respectively. The antibody titers in beagles 1 and 3 rose sharply within 1 wk AI, and reached 1:1280 2 wks AI. Titers reached only 1:320 in beagle 2 and 1:160 in the control. The antibody titers in beagle 2 subsequently rose to 1:1280 by 4 wks AI. MIF activity in beagle 4 (control) was 46.8% before infection. It increased markedly to 69.7% and 88.7% by 1 and 3 wks AI. When MIF activities were measured in beagles 1, 2, and 3, 1 wk after the 2nd immunization, they were 66.8%, 70.3% and 71.4% respectively. After infection, the MIF activities increased markedly between the 3rd and 4th wk AI. In the succeeding wks, however, there was a noticeable decline in MIF activity in beagle 3, especially during the 8th wk AI.

The present study indicates that immunization of beagles with lysate antigen from *T. gondii* stimulates non-specific protection against *B. gibsoni* infection. Lysate antigens of *T. gondii* have been reported to alter immune functions such as processing of cross-reactive antigens, activation of macrophages and release of lymphokines such as MIF and macrophage activating factor [5, 9]. However, the specific components of *Toxoplasma* which determine biological activity and stimulate an increase in protective effects are not yet known. The consistently lower *B. gibsoni* parasitemias and higher erythrocyte counts and Hts in the TLA immunized beagles as compared with those in the non-treated control suggest that resistance against *Babesia* infection was enhanced. Parasitemia was lowest in beagle 3, suggesting that treatment of this dog with levamisol enhanced non-specific immunity by activation of various types of immunological effector cells [1, 8–12].

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要　約

トキソプラズマ溶解抗原によるビーグル犬のバベシア感染に対する免疫賦与(短報)：佐藤基佳・五十嵐郁男[1]・斎藤篤志[1]・広瀬恒夫・鈴木直義[1]（帯広畜産大学獣医臨床放射線学教室，[1]家畜生理学教室）——*Babesia gibsoni* 感染に対する *B. gibsoni* 溶解抗原（BLA）と *Toxoplasma gondii* 溶解抗原（TLA）の感染死防御効果について4頭の同腹ビーグル犬を用いて検討した．無処置，健康ビーグル（No. 4）は *B. gibsoni* 感染後23日目に重篤症状を示したので殺処分した．その他の3頭は感染3週前よりそれぞれBLA，TLAおよびTLA＋レバミゾールを2週間隔で2回投与した．これらのビーグル犬は *B. gibsoni* 感染に対して感染死防御効果を示し，生残した．とくに，TLAとレバミゾールの併用投与では低い原虫寄生率と高い抗体価の値を示し，種々のタイプの免疫功奏細胞の増強効果が示唆された．

Isospora felis: Possible Evidence for Transmission of Parasites from Chronically Infected Mother Cats to Kittens

Yoshitaka, OMATA, Hiroshi, OIKAWA[1)], Masanori KANDA[1)], Kouji, MIKAZUKI[1)], Toshio, NAKABAYASHI[2)], and Naoyoshi, SUZUKI

Department of Veterinary Physiology, Obihiro University, School of Veterinary Medicine, Obihiro 080, [1)]Aburahi Laboratories, Shionogi & Co., Ltd., Kohka-cho, Shiga 520–34, and [2)]Department of Parasitology, Fujita-Gakuen Health University, School of Medicine, Toyoake, Aichi 470–11, Japan

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KEY WORDS: cat, *Isospora felis*, transmission.

Although the feline coccidian parasite, *isospora felis (I. felis)*, is thought to be non-pathogenic, its ability to stimulate the reshedding of *Toxoplasma* oocysts in cats with chronic toxoplasmosis is of public health significance [1, 3]. Oocysts of *I. felis* are often found in the feces of domestic and wild cats, however, the parasite can invade extra-intestinal tissues and persist for long periods of time without the shedding of oocysts.

It is believed that *I. felis* is probably not transmitted congenitally in the chronic phase of the infection [4]. In order to approach eradication of *I. felis* in a breeding colony of laboratory cats, in this study, we examined whether kittens born from cats with chornic *I. felis* infections were infected with *I. felis* or not.

Three female cats were obtained at 6–10 weeks of age and kept under strict isolation in animal care facilities at Aburahi Laboratories of Shionogi & Co., Ltd., Kohka-cho, Shiga prefecture, Japan. The cats were housed in individual cages except for brief periods when they were mated. All cages were sterilized daily. Newly born kittens were housed with their mothers until they were weaned at the 4th week after birth.

Feces from mother cats and kittens were collected daily, concentrated by the method of Dubey *et al.* [2], and examined microscopically for the presence of coccidian oocysts. On the 12th week after birth, kittens were challenged orally with either 10^2 cysts or 10^5 oocysts of the S-273 strain of *Toxoplasma gondii (T. gondii)* to induce the shedding of *I. felis* oocysts. Feces were monitored daily for up to 4 weeks after the challenge.

All of three mother cats (Nos. 88–013, 88–016, 89–007) showed no shedding of oocyst during this experiment. Whereas, as shown in Table 1, two kittens that were littermates (Nos. 4 and 5) began to shed very low number of *I. felis* oocysts between 42 to 59 days after birth. When these kittens were challenged orally with 10^2 *T. gondii* cysts on the 79th day after birth, one of two passed a few *I. felis* oocysts on the 3rd day after challenge. This kitten subsequently shed thousands of *T. gondii* oocysts on the 7th day after challenge. The remaining two kittens (Nos.

Table 1. Detection of oocysts of *I. felis* and *T. gondii* in the feces of kittens

No. kitten (mother)	Days *I. felis* oocysts shed (after birth)	Days *T. gondii* oocysts shed (after birth)/ {after challenge}
4[b)] (88–013)	42–59[a)], —	85–88[a)]/{6–9[a)]}
5[b)] (88–013)	42–59, 79–83, —	83–87/{4–8}
7[b)] (88–016)	46–58, 80–85, —	83–87/{3–7}
8[c)] (89–007)	46–58, 90–97, 104–108, —	105–114/{18–27}

a) First and last day oocyst shed.
b, c) All kittens were inoculated with either cysts[b)] or oocysts[c)].

7 and 8) also shed *I. felis* oocysts spontaneously between 46 and 60 days after birth. Reshedding of *I. felis* oocysts was stimulated by oral challenge with both *T. gondii* cysts (No. 7) and oocysts (No. 8).

Transmission of *I. felis* between mother and offspring is believed to occur by either fecal contamination, transplacental infection, or through breast milk during nursing. Transmission by fecal contamination is unlikely in our study because oocysts were not detected in fecal examinations of mother cats and because cages were sterilized daily. Fecal contamination from other cats was not possible because the animals were housed in isolation facilities. Transplacental infection during chronic phases of toxoplasmosis is rare as are reports on the infection of *T. gondii* via the milk of lactating females [5]. Our observations suggest, however, that *I. felis* may have the ability to pass from mother to young via either of these latter two routes of transmission. Unfortunately we have no data to indicate that hypnozoite of *I. felis* can excyst and appear in blood, saliva or milk during the nursing period of host. Further studies on the presence and persistence of the parasite in saliva, blood and milk are necessary to answer this question. In addition, serological studies of mothers and offspring may help to determine the prevalence of congenital infections.

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要　約

Isospora felis 慢性感染母ネコから仔ネコへの虫体移行の可能性(短報)：小俣吉孝・及川　弘[1]・神田政典[1]・三日月幸治[1]・中林敏夫[2]・鈴木直義(帯広畜産大学家畜生理学教室，[1]塩野義製薬油日ラボラトリーズ，[2]藤田学園保健衛生大学医学部寄生虫学教室)——*Isospora felis* 慢性感染母ネコおよび新生仔におけるオーシスト排泄の有無について継時的検索を行った．仔ネコでは，生後6ないし7週令においてオーシスト排泄が観察され，*Toxoplasma gondii* 接種による誘発によっても一過性のオーシスト排泄が認められた．一方，母ネコでは実験期間中，オーシスト排泄は観察されなかった．以上の事から，母親から新生仔への虫体移行の可能性が示唆された．

Changes in immune serum of *Toxoplasma gondii* following administration of *T. gondii* lysate antigen

Tomoko YODA[1], Haruhisa SAKURAI[2], Ikuo IGARASHI[3]
Yoshitaka OMATA[3], Atsushi SAITO[3] and Naoyosi SUZUKI[3]

Abstract

The relationship between cell-mediated immunity and changes in the serum components in *Toxoplasma*-immune cattle after injection of *Toxoplasma* lysate antigen (TLA) was investigated. The serum albumin decreased from 0 hour (approx. 40%) to 12 hours (approx. 37%) and returned by 48 hours (40%). In three of 5 cases, particular protein fraction (M. W. of approx. 12,000) were detected intensively in sera tested 6, 12 and 24 hours but less apparent in sera tested 48 hours after TLA stimulation. Two substances were detected in the culture supernatant of peripheral lymphocytes from *Toxoplasma*-immune cattle stimulating *in vitro* with TLA, but not in the control. The electrophoretic mobility of the substances was faster than albumin. A peak activity of macrophage migration inhibitory factor (MIF) in sera was showed at 3 hours after stimulation, and *Toxoplasma* growth inhibitory factor (Toxo-GIF) 12 hours after stimulation. Toxo-GIF activity showed mainly in the β-and γ-globulin fractions. MIF activity was transferred from the β-globulin fraction 3 hours to the albumin fraction 48 hours after stimulation. These results led to the conclusion that MIF and Toxo-GIF activities changed in response to TLA stimulation increasing initially. There was no relationship between these activities and the proportions among the albumin and α-, β-and γ-globulin fractions.

Key words; *Toxoplasma*, Serum components, Bovine, *Toxoplasma* lysate antigen, Lymphokines

Introduction

It was reported that the survival rate increased in mice inoculated with *Listeria monocytogenes* or *Salmonella typhimurium* when mice were previously inoculated with *Toxoplasma gondii*[13]. These infections were known to relate to cell-mediated immunity of the hosts. Serum level of lymphokines (LKs) increased in animals which were immunized

[1] Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University. Suita, Osaka 565 Japan.
[2] Laboratory of Protozoan Diseases and Cellular Immunology, Obihiro University of Agriculture and Veterinary Medicine. Obihiro, Hokkaido 080 Japan.
[3] Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine. Obihiro, Hokkaido 080 Japan.

with *T. gondii* and later injected with *Toxoplasma* lysate antigen (TLA)[11,14,21,22,23].

Mechanisms of cell-mediated immune response have also been studied *in vitro*. It has been repoted that *Toxoplasma* multiplication in normal macrophages was inhibited by the culture supernatant of *Toxoplasma* immune lymphocytes incubated with *Toxoplasma* antigen[1,3,8]. This phenomenon has also been observed in macrophages of human[2,3], murine[9,15,16,17,18] and hamster[8]. The inhibition of *Toxoplasma* multiplication is apparently caused by substances from immune lymphocytes. These phenomenon were found in *Toxoplasma* infections of many animal species. Since large amounts of serum are needed to examine for quantitative and qualitative changes of the immune serum, cattle are therefor highly suitable experimental animals. This report deals with the appearance of increased immune mediators in cattle chronically infected with *T. gondii* and later inoculated with TLA. Quantitative and qualitative canges of these mediators in the serum were examined at given intervals after inoculation of TLA by using several electrotion.

Materials and methods

Preparation of Toxoplasma Lysate Antigen

Toxoplasma lysate antigen (TLA) was prepared using the method of Jacobs and Lunde[5], modified by Igarashi et al[4].

Preparation of serum

Five Holstein bulls were inoculated intramuscularly with 2.5×10^8 *T. gondii* tachyzoites twice with 4 weeks interval. Sera were collected 4 weeks after the second inoculation and they were used as the chronic-stage *Toxoplasma*-infected sera.

The same bulls were used to examine the effect of TLA on chronically infected cattle. They were given 1,000 μg of TLA intravenously each, and their blood was obtained at 0, 3, 6, 12 and 48 hours later. The sera were fractionated individually by cellulose acetate electrophoresis and sodium dodecyl sulfate plyacrylamide gel electrophoresis (SDS-PAGE). The sera for determination of the macrophage migration inhibitory factor (MIF), and the *Toxoplasma* growth inhibitory factor (Toxo-GIF) activities, and raw-starch gelelectrophoresis were pooled.

Preparation of bovine peripheral lymphocytes

Peripheral lymphocytes and supernatants of lymphocytes of chronically *T. gondii*-infected cattle were collected by the Conray 400-Ficoll method[24]. The cells (1×10^6 cells/ml) was incubated at 37°C in a humid incubator of 5% CO_2 for 12 hours. Nonadherent cells were collected and the cell mixtures were centrifuged at 750 g and 4°C for 10 min. The sedimented cell concentrations were adjusted to 1×10^7 cells/ml and divided equally into two portions, one of which was treated with 50 μg of TLA per ml and the other was control. Then the cells were incubated at 37°C in a humidified incubator for 48 hours. For concentrating the protein content, the resulting supernatants was dialysed in 30% polyethylene glycol 6,000 (Wako Pure Chemical Industries, Lid., Osaka) using seamless cellulose tubing (Visking Co., U. K.).

Assay for macrophage migration inhibitory factor (MIF)

The agarose droplet method of Postlethwaite and Kang[12] modified by Igarashi et al.[4] was used.

Assessment of Toxoplasma growth inhibitory factor (Toxo-GIF)

Activity of Toxo-GIF was assessed using the method of Takei et al.[23]. Monolyer of bovine peripheral monocytes were inoculated with *Toxoplasma* tachyzoites. After incubation for 1 hour, monolyers were rinsed with TC-CS to remove non-adherent tachyzoites, then they were cultured with 1 ml of fresh full bovine serum (6,000~8,000 μg/ml), or serum albumin or globulin fractions (2,000 μg/ml) within TC-CS, for 48 hours. The cultured monocytes were stained with the May-Grünwald/Giemsa stain for light microscopy.

Electrophoresis

1) Cellulose acetate membrane electrophoresis.

Cellulose acetate membrane electrophoresis was

carried out using the standard method of the Japanese Electrophoresis Association[10] with minor modifications in the buffer and the staining solution. The composition of veronal buffer was as follows, 0.83g of 5,5-diethylbarbituric acid and 6.38 g of 5,5-diethylbarbiturate (Daiichi Pure Chemicals Co., Ltd., Tokyo) in DDW of 500 ml, pH 8.6, $\mu = 0.07$. The staining solution contained 0.4% ponceau 3R (Kanto Chemicals Co., Inc., Tokyo), 6.0 g of trichloracetic acid (Wako Pure Chemical Industries, Ltd., Osaka) in DDW of 100 ml.

Oxoid membrane (Separax, Jookoo Co., Ltd., Tokyo) was used as an electrophoresis carrier.

2) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Slab-type SDS-PAGE was performed using Laemmli's method[7], modified as Suzuki[20]. Samples for electrophoresis (1-2 mg protein/ml) were treated in solution containing 1% SDS (Wako Pure Chemical Industries Ltd., Osaka), 1% β-mercaptoethanol (2-mercaptoethanol, Wako Pure Chemical Industries Ltd., Osaka) and 20% glycerin in 10 ml tris buffer (pH 6.8) at 80°C for 5 min. Three percent polyacrylamide gel in 0.125 M this-HCl buffer (pH 6.8) containing 0.1% SDS was used as concentration gel and 5~20% gradient polyacrylamlde gel in 0.375M tris-HCl buffer (ph8.8) containing 0.1% SDS as separation gel. The running buffer consisted of 0.05 M tris, 0.37 M glycine and 0.1% SDS. Electrophoresis through the concentration gel was performed at 5 mA (15 V) and at 10 mA (30~60 V) for approximately 20 hours.

3) Raw-starch gel electrophoresis.

Granular starch gels were washed with distilled water (DW) for 2 days, which was changed 3~4 times daily. Then, the gels were dried and kept overnight in veronal buffer (consisting of 1.85 g of 5, 5-diethylbarbituric acid and 10.3 g of sodium 5,5-diethylbarbiturate in DDW to a final volume of 1, 000 ml, pH 8.6, $\mu = 0.05$).

The starch gels in the distance proportion of 15 : 25 from the negative side (i. e. closer to the cathode) were replaced by dry gel (approx. 12×40×2 cm), and 10 ml of serum was placed on this place. Electrophoresis was performed at 24 mA (200~300 V) for 24~26 hours at room temparature. After that, the block of starch was divided into 20 sections. Each section was left overnight in 30 ml of physiological saline and then centrifuged at 250 g for 5 min to eliminate containing starch gel particles from supernatant of decanted solution. The supernatants were dialysed to concentrate. The total protain concentration was estimated spectrophotometrically (Hitachi, Perkin-Elmer UV-Vis) at 280 μm using bovine serum albumin fraction V as standard. The each fraction V as standard. The each fraction was analyzed by cellulose acetate membrane electrophoresis and immunoelectrophoresis.

4) Immunoelectrophoresis

Glass slides precoated with a thin film of 0.6% ager in DDW (Ager Noble, Difco Laboratories, Detroit, Mich., U. S. A.) were coated a second layer of gel (3ml of 1.2% agar in veronal buffer). Electrophoresis was carried out in veronal buffer at a constant voltage of 60 V for approximately 70 min. When a precipitate was produced within 48 hours after the addition of antiserum, the slides were rinsed with physiological saline for 3 days, washed with DW, and dried at 37°C (covered with wet filter paper to ensure that the grooves were filled with water). The slides were stained by the staining solution which consisted of 0.5 g of amido-black 10B, 50 ml of methanol, 10 ml of acetic acid (99%) and 40 ml of DDW, and destained overnight in a destaining solution which consisted of 50 ml of methanol, 10 ml of acetic acid and 40 ml of DDW.

Results

Changes in components of Toxoplasma-immune serum

When the sera of the bulls were examined before and 48 hours after injection with TLA using the Eiken latex hemagglutination test (Eiken Chemical, Co., Ltd., Tokyo), they showed no increase in antibody titer against *T. gondii* antigen. These titers

were stable at 1 : 256 in one bull, 1 : 512 in two and 1 : 1024 in the two animals.

1) Cellulose acetate membrane electrophoresis.

As shown in Fig. 1, the ratio of alubumin to globulin (A/G) decreased significantly to a minimum value of 0.62 ± 0.03 after 12 hours ($p < 0.05$). Then, it returned gradually to reach the pretreatment value (about 0.71) by 48 hours after TLA injection. The albumin fraction showed the lowest value ($37.2 \pm 1.3\%$) after 12 hours and returned to its original value (about 40%) 48 hours after TLA injection. The value of α-globulin fraction showed a rise (about 25%) on 12 hours and returned to reach the pretreatment value by 24 hours after TLA injection. The β-globulin fraction showed high value 3 to 6 hours and 48 hours after TLA injection. The percentage of γ-globulin showed slightly increase from 6 hours after TLA injection and it reached about 30% on 24 hours after TLA injection, then it returned the pretreatment value.

2) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In three of the 5 investigated cases particular protein bands were stained strongly, when the serum was tested after TLA injection (Fig. 2). This tendency was especially obvious for the sera tented 6, 12 and 24 hours after injection. In some cases the apparent band was seen at 24 and 48 hours after stimulation with TLA. The electrophoretic mobility of these protein was similar to that of cytochrome C used here as a molecular marker. Their molecular weights were entimated to be approximately 12,000.

Comparison of components between the supernatants of Toxoplasma-immune lymphocytes incubated with and without TLA

Peripheral lymphocytes of *Toxoplasma*-immune cattle were cultured with TLA. Components of the

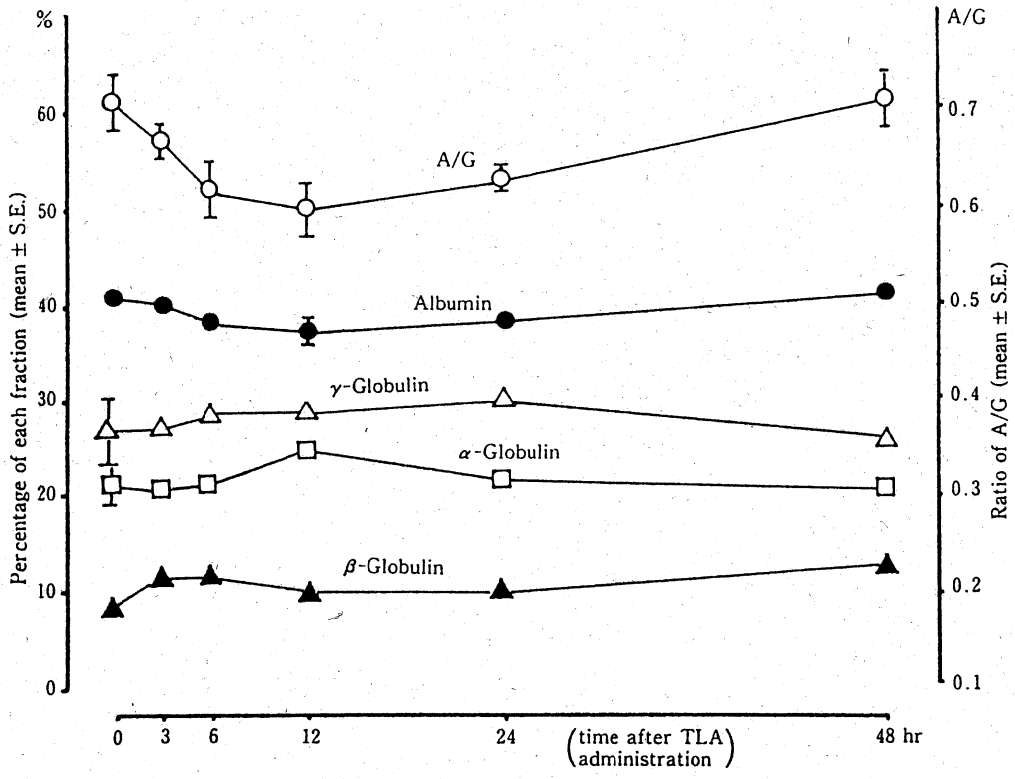

Figure 1. Quantitative changes of serum fractions in *Toxoplasma*-immune cattle at various intervals following TLA administration.

culture supernatant were separated by SDS-PAGE (Fig. 3). The culture supernatant of the TLA-treated lymphocytes contained 2 protein fractions which were not found in that of the untreated lymphocytes. Mobility of two protein bands was similar to that of cytochrome C (M. W. 12,000).

MIF and Toxo-GIF activity in Toxoplasma-immune cattle serum at different intervals after TLA administration

MIF activity (being 11% at 0 hour) in the sera increased to 58% within 3 hours following TLA stimulation *in vivo*. It dropped to a minimum of 23% in another 9 hours. Later on, it tended to increase in quantity. but its value remained below 30% (Fig. 4). Toxo-GIF activity reached to a peak 3 hours after TLA administration and decreased suddenly to −105% 6 hours after administration. It increased again to 66% by 12 hours and return to the unstimulated level of nearly 30% at 24 hours (Table 1).

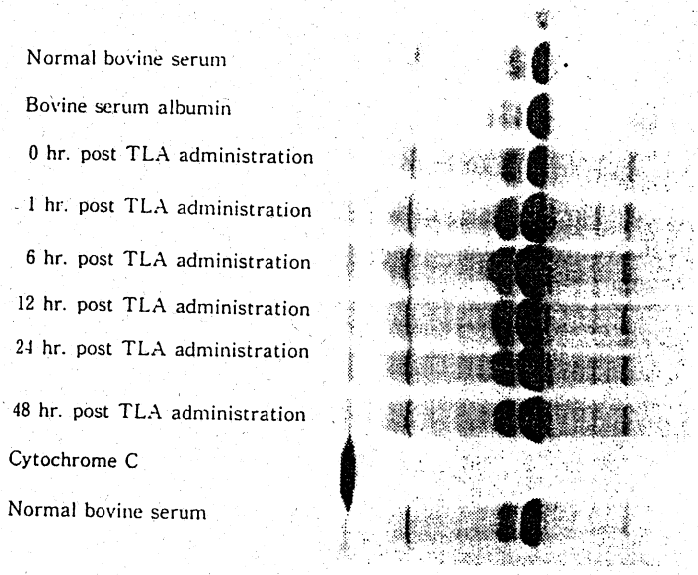

Figure 2. Quantitative changes in *Toxoplasma*-immune cattle serum at various intervals following TLA administration using SDS-PAGE.

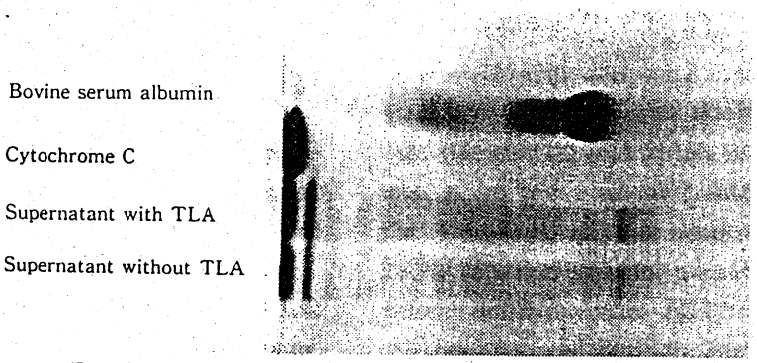

Figure 3. Comparison of components in the supernatant of *Toxoplasma*-immune lymphocytes incubated with or without TLA.

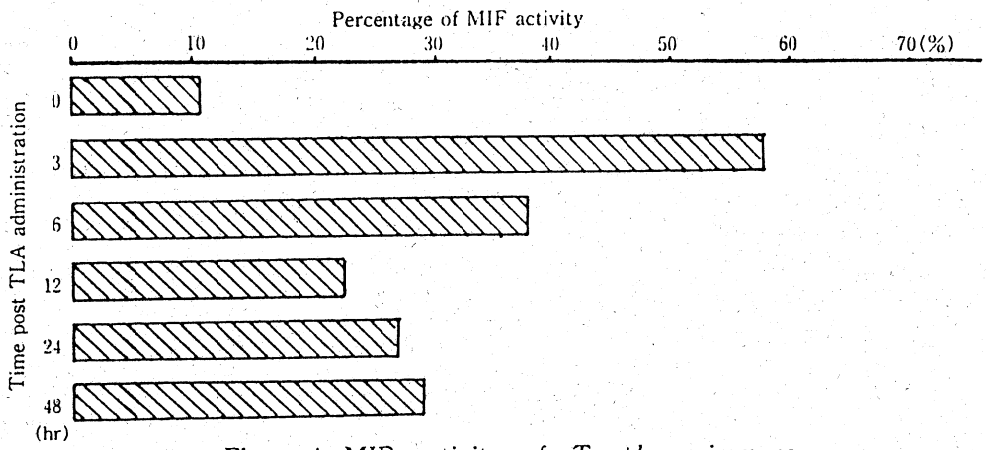

Figure 4. MIF activity of *Toxoplasma*-immune cattle serum post TLA administration at various intervals.

Table 1. Toxo-GIF activity at various intervals post TLA administration in *Toxoplasma*-immune bovine serum. Cells were grouped into three categories according to whether they contained ≧6, 1-5, or no tachyzoites. The values below are the percent of cells which belong each group.

Serum collected/ hours after TLA admin.	Tachyzoites/cell			GIF(%)*
	0	1-5	≧6	
Tc-199 20% CS (blank)	82.2±10.3	16.8±9.5	1.0±1.0	0
0 hr	87.2± 3.7	11.6±3.0	1.2±1.6	28
3 hr	93.6± 0.5	5.4±0.5	1.0±0.0	64
6 hr	63.6± 9.4	34.2±8.2	2.2±2.5	−105
12 hr	94.0± 2.7	5.6±2.6	0.4±0.5	67
24 hr	88.0± 2.3	11.0±2.7	1.0±1.2	33
48 hr	88.4± 2.3	10.8±2.3	0.8±1.3	35
Normal serum	Most of cells were destroyed.			

$$*\text{GIF}(\%) = 100 \times \left(1 - \frac{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in sample}}{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in blank}}\right)$$

Distribution of MIF and Toxo-GIF activity in fractions of Toxoplasma-immune cattle serum following TLA administration

The sera of *Toxoplasma*-immune cattle 3, 6, 24 and 48 hours after TLA administration were pooled, and their albumin and α-, β- and γ-globulin fractions were separated by raw-starch gel electrophoresis. Table 2 shows the MIF activity of the full serum and the globulin fractions examined 3, 6 and 48 hours after TLA treatment. MIF and Toxo-GIF activities were found in all fractions, but they varyed among the components. Thus, MIF activities of the whole serum 3 hours after treatment, was showed 58%, but the β-globulin fraction was 61%. The MIF activities of albumin and globulins fractions 6 and 48 hours after treatment, were 68 and 97%, but both of the full serum samples showed not remarkable MIF activ-

Table 2. MIF activity of each serum fraction at 3, 6 and 48 hour following TLA administration in *Toxoplasma*-immune cattle serum. All value is the percentage of MIF activity.

Time	Albumin	Fraction			Whole serum
		α-Globulin	β-Globulin	γ-Globulin	
3 hr	39	37(Alb)*	61	44	58
6 hr	93	97	79	93	38
48 hr	83	68($\beta-$)*	70	75($\beta-$)*	29

* These fractions are including ( ).

Table 3. Toxo-GIF activity at 3 and 24 hr after TLA administration are demonstrated. Cells were grouped into three categories according to whether they contained ≧6, 1-5 or no tachyzoites. The value is the percent of cells which belong to each group.

Serum fraction	Tachyzoites/cell			GIF(%)*
	0	1-5	≧6	
Tc-199 20% CS (blank)	74.6± 5.5	24.0± 4.3	1.4±1.5	0
3 hr Albumin	72.8± 8.2	26.0± 7.9	1.2±1.3	− 7
α-globulin(Alb)	····· Most of cells were destroyed.·····			
β-globulin	90.0± 3.7	9.4± 3.4	0.6±0.9	61
γ-globulin	78.6±12.3	19.6±10.9	1.8±1.9	16
24 hr Albumin	71.6±10.1	25.0± 7.3	3.6±3.6	−12
α-globulin(β-)	····· Most of cells were destroyed.·····			
β-globulin(γ-)	95.6± 1.5	3.8± 1.3	0.6±0.5	83
γ-globulin	····· Most of cells were destroyed.·····			

$$*\text{GIF}(\%) = 100 \times \left(1 - \frac{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in sample}}{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in blank}}\right)$$

ities. Table 3 demonstrates for Toxo-GIF activities 3 and 24 hours after TLA stimulation. The remarkable Toxo-GIF activities were found in both of the β-globulin fractions 3 and 24 hours after treatment.

Discussion

It has been reported that serum of infected human[2,3] and mice[15,16,17] with *Toxoplasma gondii* had a factor which suppresses *Toxoplasma* multiplication. Among the factors related to cell-mediated immunity, macrophage migration inhibitory factor (MIF) and *Toxoplasma* growth inhibitory factor (Toxo-GIF) may play some roles of host diffence mechanisms in *Toxoplasma* infection.

In this study, the decrease of albumin fraction and the increase of globulin fractions were seen in the *Toxoplasma*-immune cattle serum during 24 hours after stimulation with *Toxoplasma* lysate antigen (TLA).

To our knowledge, there are no reports on the distribution of MIF activity in bovine serum fractions. If there is a species variation, the results of the present studies suggest that MIF detected 3

hours after stimulation was different in physicochemical properties from that detected after 48 hours.

Klinkert and Sorg[24] proposed that MIF might be composed of indipendent subunits and they determined molecules with MIF activity to have an M. W. of 30,000, 45,000 or 60,000. However, taking into consideration the molecular weight determined by different workers, an alternative explanation is required. MIF activity may be related to a subunit, which binds to various heterologous proteins or other substances present in the serum or medium. These findings might support to the hypothesis that MIF activity may be a property of a subunit, which may be attached to various other serum/medium components causing a change in molecular weight and electrophoretic mobility.

Youngner and Salvin[25] showed that the properties of MIF were very similar to those of immune IFN (IFN-γ). According to Shirahata and Shimizu[19], Toxo-GIF from splenic cells of immune murine share physicochemical properties with IFN. Takei et al.[23] found the maximum activity of Toxo-GIF in the plasma of *Toxoplasma*-immune beagles 24 hours after intravenous injection with TLA. It has been speculated that Toxo-GIF released by *Toxoplasma*-immune T-lymphocytes[18] may not be part of the immunoglobulin or albumin fractions of the serum[19]; using ager zone electrophoresis they found Toxo-GIF activity in the post-albumin position.

In the present studies, however, there was an increase in the α-globulin fraction at the time of the peak of Toxo-GIF activity (12 hours after stimulation), and raw-starch gel electrophoresis revealed that the activity is mainly related to the β- and γ-globulin fractions. The results of these studies led to the conclusion that these factors may change in properties with the lapse of time after TLA administration. Further investigations are required to clarify the relationships of MIF, Toxo-GIF and IFN-γ.

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トキソプラズマ抗原投与による トキソプラズマ感染血清蛋白の推移

依 田 知 子
大阪大学微生物病学研究所免疫化学研究室
(大阪府吹田市山田丘)

櫻 井 治 久
帯広畜産大学原虫病細胞免疫学研究室
(北海道帯広市稲田町)

五十嵐郁男・小俣吉孝・齋藤篤志・鈴木直義
帯広畜産大学家畜生理学教室
(北海道帯広市稲田町)

摘 要

トキソプラズマ慢性感染牛にトキソプラズマ抗原(TLA)を投与した後の血清成分の質的及び量的変化の推移を経時的に検討した。セルロース・アセテート膜電気泳動法から,TLA 投与後48時間以内にグロブリン画分の増加とアルブミンの減少が認められた。SDS-ポリアクリルアミドゲル電気泳動(SDS-PAGE)により,TLA 投与6,12及び24時間後の血清中に,他の時間や健常牛の血清には認められない,分子量1万2千の物質が観察された。感染牛末梢血リンパ球を TLA 存在下で培養し,培養上清を SDS-PAGE にて検討したところ,TLA 添加培養上清中に,分子量1万2千の特異的物質が観察された。マクロファージ遊走阻止因子(MIF)とトキソプラズマ増殖阻止因子(Toxo-GIF)活性については,MIF は,3及び48時間後に活性の増加を示し,Toxo-GIF は,3及び12時間後に高い活性を示した。これらの血清を生澱粉電気泳動法で4分画(アルブミン,α-,β-,γ-グロブリン)に分離し,それぞれの活性の分布を検討したところ,MIF 活性は,TLA 投与後3時間の血清ではβ-グロブリン分画に,48時間ではアルブミン画分に高い活性を認めた。一方,Toxo-GIF 活性は,β-,γ-グロブリン画分に認められた。

Experimental Feline Toxoplasmosis: Humoral Immune Responses of Cats Inoculated Orally with *Toxoplasma gondii* Cysts and Oocysts

Yoshitaka OMATA, Hiroshi OIKAWA[1)], Masanori KANDA[1)], Kouji MIKAZUKI[1)], Toshio NAKABAYASHI[2)], and Naoyoshi SUZUKI

Department of Veterinary Physiology, Obihiro University, School of Veterinary Medicine, Obihiro 080, [1)]Aburahi Laboratories, Shionogi & Co., Ltd., Kohka-cho, Shiga 520–34, and [2)]Department of Parasitology, Fujita-Gakuen Health University School of Medicine, Toyoake, Aichi 470–11, Japan

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KEY WORDS: antibody production, feline toxoplasmosis, oocyst.

A number of rapid, sensitive and specific serological methods for diagnosis of *Toxoplasma gondii (T. gondii)* infection have been described [2, 7]. There are few reports, however, described about serological responses of cats to experimental infection induced by the inoculation of *T. gondii* sporozoites. Since prepatent period of oocyst shedding is influenced by the infective stage of the parasite that is ingested [5], it may be important to examine immune responses to the parasite in cats infected with either cysts or sporozoites. In the present study, we focused our interest to determine when the humoral antibody production was induced in experimental infection of cats fed either cysts or oocysts.

Six-week-old cats of both sexes, and weighing 400–450 g, were used in this experiment. The cats were born from toxoplasmosis negative mothers. They were kept in individual cages under strict isolation in animal care facilities at Aburahi Laboratories.

Three cats were inoculated orally with 10^2 cysts and two were inoculated orally with 10^5 oocysts of the S-273 strain of *T. gondii*. Feces were collected daily, and examined microscopically for the presence of coccidian oocysts as described by Dubey *et al.* [4]. Cats were bled weekly from the jugular vein up to 8 weeks post inoculation (p.i.) for measurement of toxoplasma antibodies. On the 4th week p.i., cats were reinoculated orally with 10^3 cysts of the same strain and the same examinations as mentioned above were done for up to 4 weeks post reinoculation.

Titration of anti-*T. gondii* IgG and IgA antibodies was performed by indirect immunofluorescence assay (IFAT) as described elsewhere [8]. Briefly, serial dilutions of the test serum were prepared in PBS, mounted onto glass slides coated with parasite antigen and incubated for 30 min at 37°C. Slides were washed in PBS and reincubated after covered with sufficient

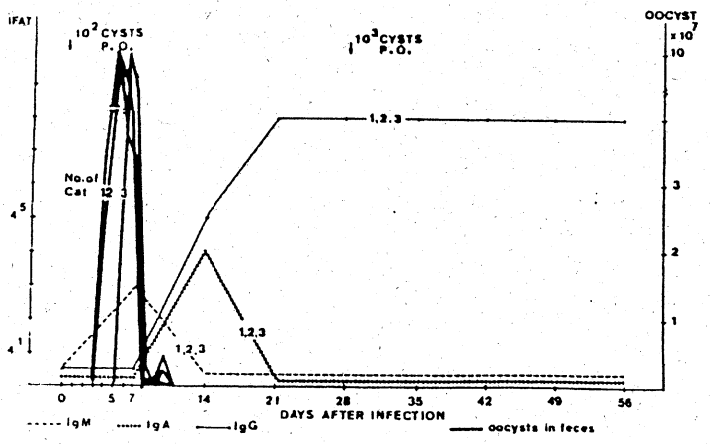

Fig. 1. Number of oocyst per day per cat and antibody titers of 3 cats inoculated orally with cysts. Parasite antigen was incubated with four-fold serial diluted test serum, subsequently reacted with either anti-cat IgM, anti-cat IgA, or anti-cat IgG.

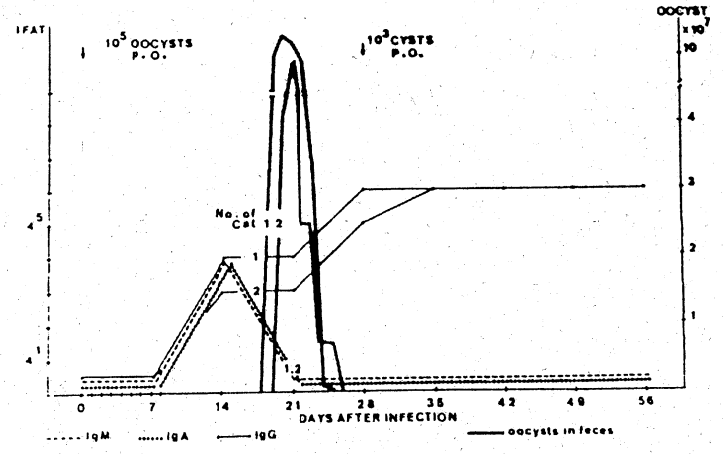

Fig. 2. Number of oocyst per day per cat and antibody titers of 3 cats inoculated orally with oocysts. Antibody titers were measured as described in Fig. 1.

amount volume of either FITC conjugated rabbit anti-cat IgG (Organon Teknika Corp.-Cappel Products, West Chester, U.S.A.) or rabbit anti-cat IgA (Bethyl Laboratories, Inc., Montgomery, U.S.A.). Slides were then washed in PBS. Slides reacted with anti-cat IgA were further incubated with FITC-conjugated goat anti-rabbit IgG (E.Y. Laboratory Inc., San Mateo, U.S.A.). Specific fluorescence was observed with a fluorescence microscope. Titration of IgM antibodies was done in a similar manner with an indirect immunoperoxidase assay using peroxidase-conjugated anti-cat IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, U.S.A.). Peroxidase reaction was visualized by incubation of the slide with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan) and 0.1% hydrogen peroxide in 0.1 M Tris-HCl buffer (pH 7.4).

All cats inoculated with *T. gondii* cysts shed oocysts between 4 and 11 days p.i. (Fig. 1). Peak oocyst shedding of the two was at 5 days p.i. The other one was at 6 days p.i. and the total number of oocysts shed by individuals exceeded 10^8. Reshedding of oocysts was not observed even after cats were reinoculated with tissue cysts at 4 weeks p.i.

Antibodies against *T. gondii* in all cats became detectable after the peak oocyst shedding. Both IgM and IgA antibody titers appeared transiently and their titers reached peaks during the first and second weeks p.i., respectively. Reinoculation of cats with tissue cysts at 4 weeks p.i. had no effect on both IgM and IgA titers and they remained at undetectable levels throughout the experiment. By contrast, IgG antibody titers began to increase on the 2nd week p.i. and remained at high levels until the end of the experiment (Fig. 1).

Whereas, cats inoculated with oocysts shed oocysts between 19 and 27 days p.i. with peak oocyst shedding at 21 days p.i. The total number of oocysts per cat during oocyst shedding was more than 10^8. Both cats did not show reshedding of oocysts after the reinoculation of cysts. IgM and IgA antibodies were only detectable on the 2nd week p.i.. IgG antibody titers also increased during the 2nd week p.i., and remained at high levels until the end of the experiment (Fig. 2).

From these results, it was indicated that production of IgM and IgA antibodies was restricted during an acute phase of the infection in both cats fed cysts and oocysts as a primary immune response. Antibody production was induced before oocyst shedding in the cat fed sporulated oocysts. Oppositely, the cat inoculated orally with cystozoites started antibody production when oocyst excretion had almost ceased.

Thus, it is confirmed that the measurement of IgM and IgA antibody titers in the serum is useful for diagnosis of acute toxoplasmosis in cats, though the antibodies production is not associated with oocyst shedding.

Dubey and Frenkel demonstrated that in the case of cats inoculated orally with tissue cysts, parasites penetrated extra-intestinal tissues within a few hours after ingestion and speculated that the extra-intestinal infection might be necessary for antibody production [3]. Our results indicate that it takes approximately 1 week for detectable levels of anti-*T. gondii* antibodies to appear in the blood after the parasites invade extra-intestinal sites. In the present data, anti-*T. gondii* antibody production needed at least 2 weeks p.i. in cats fed oocysts. These findings suggest that sporozoites require to perform extra-intestinal development for at least 1 week p.i. At the present time, the reason why the long term of 3 weeks is required for prepatent period is unclear. The size of the initial inoculum did not effect on the duration of prepatent period [1, 3]. Thus, it seems that sporozoites require longer period for their development of pregametocytic stages in intestinal tissue than cystozoites do it. According to the recent study where distribution and development of parasites were examined in tissues of cats inoculated orally with sporozoites, transformation of sporozoites to endozoites or cystozoites may be necessary to occur the gametogenesis following to oocyst production [6]. This finding may support the necessity of long developmental term for sporozoites as described above hypothesis.

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要　　約

ネコの実験的トキソプラズマ症：シストならびにオーシスト経口接種ネコにおける体液性免疫応答(短報)：小俣吉孝・及川　弘[1]・神田政典[1]・三日月幸治[1]・中林敏夫[2]・鈴木直義(帯広畜産大学家畜生理学教室，[1]塩野義製薬油日ラボラトリーズ，[2]藤田学園保健衛生大学医学部寄生虫学教室)——*Toxoplasma gondii* のシスト経口接種ネコならびにオーシスト経口接種ネコにおけるオーシスト排泄時期と，血清中抗体価を継時的に検索した．シスト接種ネコではオーシスト排泄後，血清中抗体価が出現，上昇するのに対し，オーシスト接種ネコでは，オーシスト排泄前から血清中抗体価が出現し，上昇する事が観察された．

Effect of *Toxoplasma* Lysate Antigen (TLA) on Feline Cytotoxicity against FeLV Positive Lymphoma Cells

Mhan-Pyo YANG, Ryo GOITSUKA, Kenichiro ONO[1)], Naoyoshi SUZUKI[1)], and Atsuhiko HASEGAWA

Department of Veterinary Internal Medicine and [1)]Department of Veterinary Clinical Pathobiology, Faculty of Agriculture, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT. The cytotoxic activities of feline spleen cells treated with *Toxoplasma* lysate antigen (TLA) were assayed against feline leukemia virus (FeLV)-producing lymphoma, FL74 cells, and xenogeneic target lymphoma, mouse YAC-1 cells. The TLA treatments were performed *in vivo* alone, *in vitro* alone, and *in vivo* plus *in vitro*, respectively. *In vivo* plus *in vitro* treatments with TLA induced a marked augmentation in cytotoxic activity of spleen cells to FL74 cells. The treatment with TLA *in vivo* alone showed an enhancement of cytotoxic activity but *in vitro* alone did not. The cytotoxic effects of TLA-treated spleen cells obtained from the cats which had been previously immunized with live FL74 cells were similar to those of spleen cells from non-immunized cats treated with TLA. However, no increase of cytotoxicity was shown in the response to mouse YAC-1 cells regardless of TLA treatments. These results indicated that the *in vivo* TLA treatment augmented the cytotoxicity of feline spleen cells against FeLV-producing lymphoma cell.—KEY WORDS: feline cytotoxicity, FeLV-producing lymphoma cell, *Toxoplasma* lysate antigen.

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The *Toxoplasma* lysate antigen (TLA), an antigen derived from *Toxoplasma* tachyzoites is also critical in the augmentation of NK activity by inducing interferon (IFN)-α and-γ [7, 17, 19, 21]. The components of TLA responsible for enhancing the NK activity were proved to be proteins associated with cytoplasm and membrane of *Toxoplasma* but not DNA and RNA [21]. The infection with live *Toxoplasma* also causes the remarkable enhancement of the cytotoxic activity [6, 11].

The immune cytotoxic response of the peripheral blood lymphocytes (PBL) and spleen cells to FeLV-producing lymphoma cells, i.e., FL74 cells, has been studied as an effector mechanism that mediates the tumor protection [10, 14, 15, 24, 25]. The results on the cytotoxicity of feline PBL to FL74 cells differ from one investigator to another [10, 14, 15, 25]. These investigators, however, agreed that cytotoxicity of spleen cells to FL74 cells is present in normal cats.

In this study, we determined whether TLA exerts any effect on the cytotoxic activity of feline PBL and spleen cells against FL74 cells.

MATERIALS AND METHODS

Animals: The healthy cats of 2–3 years old were housed at our laboratory. All cats utilized in this study were free from FeLV infection as examined by an enzyme-linked immunosorbent assay using a Leukassay F kit (Pitman Moore Inc., Washington Crossing, NJ).

Effector cells: The PBL and spleen cells were used as sources of effector cells in cytotoxicity assay. Briefly, the cells prepared by Ficoll-Hypaque density gradient were incubated for 1 hour at 37°C to remove adherent cells, and thereafter the nonadherent cells were cultured at a concentration of

1×10^6/m*l* in medium RPMI-1640 (Nissui Pharmaceutical Co., Tokyo) supplemented with 2 mM L-glutamine, 0.02 mg/m*l* of gentamycin and 10% heat-inactivated fetal bovine serum, referred hereafter to as culture medium.

Target cells: The target cells utilized were FL74 and YAC-1 which were maintained in continuous culture with the culture medium.

Preparation of TLA: The TLA was prepared as for the procedure described previously [19, 20]. Ten milligram of TLA in sterile physiological saline as a stock solution were adjusted to 1 mg/m*l* and 0.5 mg/m*l* with culture medium.

TLA treatments of effector cells: 1) *in vivo* treatment; The TLA was administered intravenously (iv) to cats at a concentration of 0.1 mg per kilogram of body weight twice (7 and 3 days) prior to effector cell preparations, and the equal volume of sterile physiological saline instead of TLA was administered iv to the control animals. Before administration of TLA in the above manner, some cats were immunized *in vivo* by iv inoculation with 1×10^8 viable FL74 cells on three occasions with 1 week interval between inoculations. 2) *in vitro* treatment; The PBL was treated with TLA at a concentration of 0.05 mg/m*l* for 20 hours and the spleen cells were incubated with TLA for 20 hours or 5 days at 37°C under 5% CO_2 humidified atmosphere.

Cytotoxicity assay: The cytotoxic activity of feline PBL and spleen cells was tested by standard ^{51}Cr releasing assay as described elsewhere [12, 22]. Briefly, the effector cells (0.1 m*l*) were distributed into each well of 96 well-microplates. The target cells were pelleted and labeled with 0.03 mCi of ^{51}Cr sodium chromate (New England Nuclear, Boston, MA) for 1 hour at 37°C. To minimize the spontaneous release of target cells, ^{51}Cr labeled cells were preincubated in 10 m*l* of culture medium for 2 hours at 37°C. Subsequently, these target cells were plated in each well at a final volume of 0.2 m*l* with effector to target (E:T) ratios ranging from 12.5:1 to 50:1. The plates were centrifuged at 55 × G for 3 minutes to increase E:T contact and then incubated for 6 hours or 18 hours at 37°C. At the end of incubation the plates were recentrifuged at 500 × G for 6 minutes and half-aliquots of supernatant from each well were removed to measure the radioactivity. The percentages of specific release were calculated as follows: % specific release = (cpm in test release - cpm in spontaneous release/cpm in maximal release - cpm in spontaneous release) × 100, where the maximal release indicates the release of target cells by adding 0.1 m*l* of 1% sodium dodecyl sulfate solution instead of effector cell suspensions, and the spontaneous release was the counts released by target cells in culture medium alone.

Statistics: The significance of differences between experimental groups was determined by Student's *t*-test and values of $p<0.05$ were considered significant.

RESULTS

Effect of TLA treatment on cytotoxicity of feline PBL and spleen cells: The cytotoxicity of PBL and spleen cells to FL74 cells is shown in Table 1. All the PBL tested in 6-hour assay failed to lyse FL74 cells regardless of the treatments with TLA. In contrast, the cytotoxic activity of spleen cells was augmented by *in vivo* plus *in vitro* treatments with TLA as compared with that of untreated control animals ($p<0.02$).

Cytotoxicity of spleen cells against FL74 cells: In order to determine the possibility that *in vivo* TLA-induced cytotoxicity of spleen cells is further augmented by prolonged culture periods in the presence of *in vitro* TLA (0.05 mg/m*l*), an experiment in which culture periods of splenic effector cells were prolonged to 5 days was performed (Fig. 1). No increase in the treat-

Table 1. Cytotoxic activity of PBL and spleen cells sensitized with TLA against FL74 cells

Effector cells	TLA treatments		% specific ^{51}Cr release[c]		
	in vivo[a]	*in vitro*[b]	12.5:1	25:1	50:1
PBL	−	−	−2.9±4.1[d]	−2.5±3.6	−5.8±5.6
	+	−	−0.6±1.9	−0.4±8.4	−6.6±0.3
	+	+	0.9±3.2	−4.2±2.7	−5.0±5.9
Spleen cells	−	−	1.8±2.7	4.6±3.6	4.8±1.3
	+	−	2.1±0.9	5.5±3.2	10.3±3.2
	+	+	3.4±3.0	5.5±2.3	18.2±3.0

a) Effector cells from cats administered with TLA (0.1 mg/kg, body weight, iv) twice (7 and 3 days) prior to effector cell preparations(+) or saline solution(−), respectively.
b) After *in vivo* TLA treatments, effector cells were cultured with TLA (0.05 mg/ml) (+) or without TLA(−) for 20 hours before cytotoxicity assay.
c) Cytotoxicity assay was performed for 6 hours at E: T ratios of 12.5:1, 25: 1 and 50: 1.
d) Values represent mean ± SE.

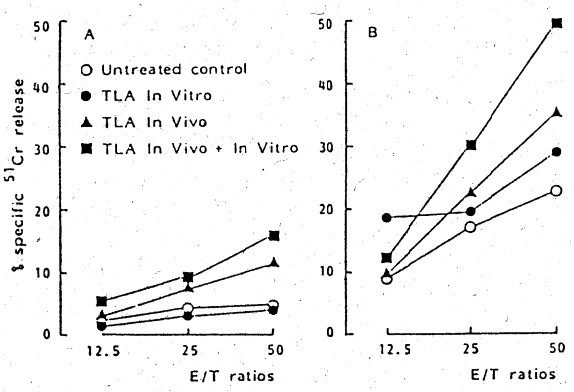

Fig. 1. Effect of TLA treatments on the cytotoxicity of spleen cells against FL74 cells. TLA (0.1 mg/kg, body weight, iv) was administered to cats twice (7 and 3 days) prior to spleen cell preparation, respectively (*in vivo* treatment), and spleen cells were cultured with TLA (0.05 mg/ml) for 5 days thereafter (*in vitro* treatment). The cytotoxicity assay for FL74 cells was performed for 6 (A) and 18 hours (B).

ment with TLA *in vitro* alone was observed in 6-hour response with FL74 cells when compared with untreated cells. Spleen cells taken from cats treated with TLA *in vivo* alone and *in vitro* plus *in vivo* showed significant cytotoxicity against FL74 cells ($p<0.05$ and $p<0.001$, respectively) when compared with that of *in vitro* alone at an E:T ratio of 50:1. There was, however, no significant difference between treatments with TLA *in vivo* alone and *in vitro* plus *in vivo*, although *in vitro* plus *in vivo* treatments showed higher cytotoxicity than *in vivo* alone. The cytotoxicity of 18-hour assay as compared with 6-hour assay in each group resulted consistently in the enhanced activity as E:T ratios increased. The spleen cells of untreated control revealed 22.5% cytotoxicity to FL74 cells at an E:T ratio of 50:1, but its cytotoxic activity tended to be enhanced when treated with TLA *in vivo* or *in vitro*, and furthermore *in vivo* plus *in vitro* treatments with TLA induced a significant augmentation of cytotoxicity ($p<0.05$).

Lytic effect on xenogeneic YAC-1 target cells: The YAC-1 cells in 6-hour assay were resistant to lysis by feline spleen cells regardless of treatments with TLA as shown in Table 2. The TLA-induced cytotoxic cells of spleen cells were also not capable of killing mouse YAC-1 cells in longer assay of 18-hour although there were some variations by *in vitro* alone and *in vivo* plus *in vitro* treatments of TLA in one experiment.

Effect of TLA treatment on cytotoxicity of PBL from FL74-immunized cats: To determine whether feline cytotoxic function

Table 2. Cytotoxic activity of feline spleen cells sensitized with TLA against xenogeneic mouse YAC-1 cells

Expt[a]	TLA treatments		% specific ^{51}Cr release[d]		
	in vivo[b]	*in vitro*[c]	12.5:1	25:1	50:1
1	−	−	0.8±2.5[e]	0.0±0.8	−0.5±0.5
	−	+	0.5±1.6	3.9±0.6	4.2±2.1
	+	−	−5.6±2.6	−7.6±2.2	−1.4±4.4
	+	+	−7.1±2.0	−5.3±2.5	−1.3±2.8
2	−	−	1.1±0.5	1.7±1.1	1.9±1.9
	−	+	8.3±6.1	8.2±6.0	9.9±2.4
	+	−	2.7±12.0	0.4±7.0	3.5±16.0
	+	+	0.4±7.0	0.8±19.0	9.7±13.0

a) Cytotoxicity assay was performed for 6 hours in experiment 1 and for 18 hours in experiment 2.
b) Spleen cells from cats administered with TLA (0.1 mg/kg, body weight, iv) twice (7 and 3 days) prior to spleen cell preparation (+) or saline solution(−), respectively.
c) After *in vivo* TLA treatments, spleen cells were cultured with TLA (0.05 mg/m*l*) (+) or without TLA(−) for 5 days before cytotoxicity assay.
d) Cytotoxicity assay was performed at E: T ratios of 12.5: 1, 25: 1 and 50: 1.
e) Values represent mean ± SE.

Table 3. Effect of TLA treatments on the cytotoxic activity of FL74-immunized PBL[a] against FL74 cells

Expt[b]	TLA treatments		% specific ^{51}Cr release[e]		
	in vivo[c]	*in vitro*[d]	12.5:1	25:1	50:1
1	−	−	3.0±4.9[f]	−0.9±4.8	1.7±0.5
	−	+	−2.2±1.8	−3.1±2.2	1.1±4.0
	+	−	4.1±4.1	4.1±5.4	−2.6±3.2
	+	+	1.5±6.1	2.0±6.4	−0.9±2.8
2	−	−	7.5±1.3	4.8±1.9	6.1±2.8
	−	+	6.9±2.5	4.6±1.8	5.6±2.9
	+	−	3.9±4.0	4.6±2.2	3.9±2.5
	+	+	6.8±1.9	7.1±1.3	8.0±2.4

a) Cats were immunized with 1×10^8 viable FL74 cells/head (iv) on 3 times with 7-day interval before *in vivo* TLA administration.
b) Cytotoxicity assay was performed for 6 hours in experiment 1 and for 18 hours in experiment 2.
c) PBL from FL74-immunized cats administered with TLA (0.1 mg/kg, body weight, iv) twice (7 and 3 days) prior to PBL preparation (+) or saline solution (−), respectively.
d) After *in vivo* TLA treatments, PBL was cultured with TLA (0.05 mg/m*l*) (+) or without TLA(−) for 20 hours before cytotoxicity assay.
e) Cytotoxicity assay was performed at E: T ratios of 12.5: 1, 25: 1 and 50: 1.
f) Values represent mean ± SE.

could be enhanced by immunization with live FL74 cells, a portion of PBL from cats immunized with FL74 cells was followed by TLA treatment as described in materials and methods. The PBL from cats immunized with FL74 cells did not cause the killing of FL74 cells in spite of TLA inoculation (Table 3).

Effect of TLA treatment on FL74-immunized spleen cells against FL74 cells: When spleen cells were treated with TLA *in vivo* and then incubated with TLA for 20 hours *in vitro*, the cytotoxicity was increased in proportion to the increase of ratios of E:T (Fig. 2). And the cytotoxic activity of immune splenic effector cells that were sensitized with TLA *in vivo* and thereafter incubated with TLA for 5 days *in vitro* was shown in Fig. 3. The treatment with TLA revealed a tendency to increase the cytotoxicity in 6-hour assay. A significant cytotoxic activity of immunized spleen cells showed in *in vivo* plus *in vitro* TLA treatments when compared with that of untreated cells at E:T ratios of 25:1 and 50:1 ($p<0.02$). All the immunized spleen cells in 18-hour assay showed higher cytotoxicity than those of 6-hour assay. However, the TLA-induced cytotoxic activity of FL74-immunized spleen cells was not enhanced beyond that of non-immunized cats treated with TLA.

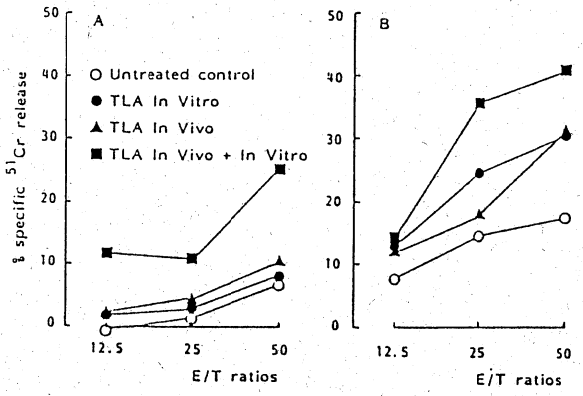

Fig. 2. Effect of TLA treatments on the cytotoxicity of spleen cells from FL74-immunized cats against FL74 cells. Cats were immunized with 1×10^8 live FL74 cells (iv) on 3 times with 1-week interval between injections. TLA (0.1 mg/kg, body weight, iv) was administered to FL74-immunized cats twice (7 and 3 days) prior to spleen cell preparation, respectively (*in vivo* treatment), and spleen cells were cultured with TLA (0.05 mg/ml) for 20 hours thereafter (*in vitro* treatment). The cytotoxicity assay for FL74 cells was performed for 6 (A) and 18 hours (B).

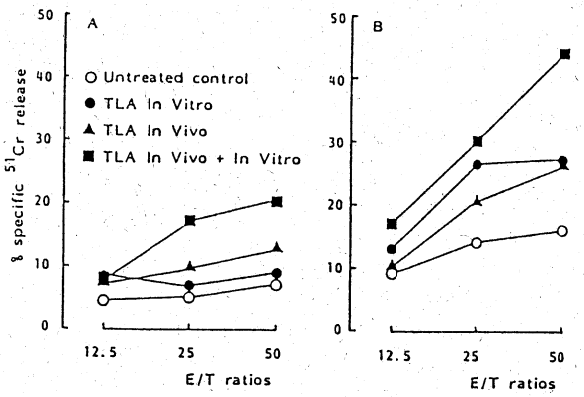

Fig. 3. Effect of TLA treatments on the cytotoxicity of FL74-immunized spleen cells cultured for prolonged periods (5 days *in vitro*). Cats were immunized with 1×10^8 live FL74 cells (iv) on 3 times with 1-week interval between injections. TLA (0.1 mg/kg, body weight, iv) was administered to FL74-immunized cats twice (7 and 3 days) prior to spleen cell preparation, respectively (*in vivo* treatment), and spleen cells were cultured with TLA (0.05 mg/ml) for 5 days thereafter (*in vitro* treatment). The cytotoxicity assay for FL74 cells was performed for 6 (A) and 18 (B).

DISCUSSION

The results presented in this study demonstrated that TLA prepared from tachyzoites of *Toxoplasma* is capable of enhancing the cytotoxic activity of feline spleen cells against FL74 cells *in vivo*. It is conceivable that (i) antigenic components of TLA are responsible for the direct augmentation effect on splenic cytotoxic activity in cats; (ii) spleen cells sensitized with TLA may be able to react with target cells, FL74 cells, by producing soluble factors such as IFN and interleukin (IL)-2 that can induce cytostasis and proliferation on themselves or other cells; and (iii) TLA may induce the expression of surface receptors on spleen cells as a portion of endogenous

cytotoxic cells. Thus, it is most likely that spleen cells sensitized with TLA *in vivo* alone may be cytotoxic rather than those *in vitro* alone, and that spleen cells treated with TLA *in vivo* plus *in vitro*, would be more efficient to lyse FL74 target cells than those treated with TLA either *in vivo* alone or *in vitro* alone. Treatment with TLA *in vitro* alone did not augment the cytotoxicity of spleen cells against FL74 cells. It is not surprising that spleen cells treated with TLA *in vitro* alone would not be sufficient for cytotoxic activity against target cells, since the adherent cells such as macrophages were previously removed before *in vitro* TLA treatment. The macrophages are also involved in the mechanisms of cytotoxicity [4, 16]. When macrophages were activated by any stimulation, they produce a series of immunoregulatory products, including IFN [1] and lymphocyte activating factors, which have the activating effects on T cells [3, 5]. It is also suggested that macrophages, when treated with TLA *in vivo*, also seemed to play an important role in the enhancement of splenic cytotoxicity. From these points of view, the spleen cells and macrophages *in vivo* may interact with each other in a complex variety of ways of cytotoxic activity.

In the TLA-induced feline PBL and FL74-immunized PBL, no lysis of FL74 cells was seen. In contrast, NK-like activity of PBL is shown to be present in humans [23], rats [8], dogs [9] and pigs [2, 13]. This resistance of FL74 cells to PBL and FL74-immunized PBL is not due to a lack of TLA stimulation and a low level of IFN production but may be due to the weakness or inability to lyse FL74 cells with feline PBL by itself or the absence of NK-like cells in feline PBL as reported in previous studies [10, 25]. The absence of cytotoxicity in feline PBL is not consistent with the reports of McCarty and Grant [14, 15] who were able to measure NK-like lysis of FL74 cells. The discrepancy between their results and our findings might be explained by ratios of PBL versus target cell and variation of cat breeds selected. The E:T ratios of 400:1 to 50:1 in their experiment were much higher as compared with 50:1 to 12.5:1 in the present study.

The ability of cytotoxic cells to kill the tumor target cells is substantially dependent on the intrinsic susceptibility of targets and whether the effector cells are stimulated by any agent capable of augmenting NK activity or not. Thus, we determined if TLA exerts any effect on cytotoxicity of feline spleen cells against mouse YAC-1 cell, a classical NK target cell [18]. This study showed that the cytotoxic activity of spleen cells by TLA treatments nearly exhibited no lysis of xenogeneic YAC-1 target cells. This finding, therefore, confirmed that the feline cells can not cross the species barrier and that lysis of xenogeneic target cells is dependent on the ability in which the effector cells recognize and bind with them.

McCarty and Grant [14] demonstrated that cytotoxic T lymphocyte (CTL) activity to FL74 cells was not detectable in spleen cells from cats immunized with the same cells *in vivo* when compared with those from normal cats. The cytotoxic activity found in TLA-treated spleen cells from immunized cats revealed a significant increase than that of cats without TLA treatment, but these levels of cytotoxic activity were, of interest, almost similar to the level of lysis detected in TLA-stimulated spleen cells of non-immunized cats, even when at an E:T ratio of 50:1 showing the highest levels of killing in each group. These results suggest that a significant portion of cytotoxicity in feline spleen cells is primarily mediated by NK-like cells rather than by CTL-like cells, which may be due to the lack of alloreactive CTL-like activity in cats. The possibility is existent that FL74 cells as a target tumor cell may be sensitive to NK-like cells but resis-

tant to CTL-like cells.

Further studies are warranted to determine whether TLA-induced NK cells contribute to the defence of FeLV-producing lymphoma cells or feline sarcoma virus (FeSV) related tumor cells.

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要　　約

ネコリンパ球のFeLV産生腫瘍細胞株に対する細胞障害活性へのトキソプラズマ溶解抗原(TLA)の及ぼす影響：梁　萬長・後飯塚　僚・小野憲一郎[1]・鈴木直義[1]・長谷川篤彦(東京大学農学部獣医内科学教室，[1]獣医臨床病理学教室)——TLAを*in vivo*あるいは*in vitro*で処置したネコの末梢血リンパ球および脾細胞のネコ白血病腫瘍細胞株(FL74)に対する細胞障害活性を検索した．末梢血リンパ球のFL 74細胞に対する細胞障害活性はTLA処置によっても変化は認められなかったが，脾細胞の細胞障害活性は*in vivo*のTLA投与により増強される傾向がみられ，特に*in vivo*および*in vitro*の両方で処理した場合は有意な増強効果が観察された．ネコにFL 74細胞を免疫した場合にも細胞障害活性は免疫しなかった時とほぼ同様であった．以上の結果からTLAはネコの脾細胞の細胞障害活性を増強させることが明らかになった．

Biological Activity of Obiopeptide-1, a Synthetic Peptide Derived from the Native Immune-Regulator Obioactin

Naoyoshi SUZUKI, Haruhisa SAKURAI, Atsushi SAITO, Ikuo IGARASHI, Yoshitaka OMATA, and Humio OSAKI

Department of Veterinary Physiology and Protozoan Immunology, Obihiro University, Obihiro, Hokkaido, Japan

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ABSTRACT. Tachyzoites of *Toxoplasma gondii* were killed in mouse macrophage and human somatic cell monolayers by a novel synthetic peptide (Obiopeptide-1) which is a Glycil-penta-Glutaminate (GpG) derivative of native Obioactin. In view of the worldwide prevalance of this protozoan disease and the lack of effective treatments, Obiopeptide-1 may be a new and unique antimicrobial active substance of non-antibiotic chemotherapeutic agents for intracellular parasites, *T. gondii* and associated nonspecific hypoimmune responses that occur in infected hosts.—KEY WORDS: immunoregulator, macrophage activation, nonantibiotic antimicrobial agent, synthetic obiopeptide, *Toxoplasma* killing.

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It has been shown that cultivation of spleen cells from a *Toxoplasma*- hyperimmune animal in the presence of *Toxoplasma* lysate antigen (TLA) causes the release of a lymphokine or cytokine which inhibits the multiplication of *Toxoplasma* in homologous cell lines [6, 10, 11]. This lymphokine, referred to as Toxo-GIF, is a glycoprotein with a molecular weight of approximately 30,000 to 40,000 daltons which is believed to be a product of T-lymphocytes [4, 12]. Toxo-GIF inhibits multiplication of *Toxoplasma* in somatic cells as well as macrophages, but is active only against intracellular protozoa in cells of the same animal species, i.e. it does not inhibit multiplication of *Toxoplasma* in cells of different animal species [5, 6]. Because of this host specificity, Toxo-GIF is not effective against human toxoplasmosis or infections that occur in other species of animals.

We have previously described a novel polypeptide with a molecular weight of less than 5,000 daltons which inhibits the multiplication of *Toxoplasma* in a variety of animal species [13]. This polypeptide is prepared by hydrolysis of serum of *Toxoplasma*-immune animals [7–9, 13]. Serum hydrolysates which originate from *Toxoplasma*-immune cattle are generally referred to as Obioactin [13]. Obioactin has antitumor activity as well as antimicrobial activity against a variety of microorganisms and is effective as a modifier of biological responses [8, 14, 15]. Since the only current source of Obioactin is *Toxoplasma*-immune serum, it is important to determine the primary structure and active sites of this polypeptide for eventual mass production by synthetic processes.

MATERIALS AND METHODS

Source of Obioactin: Native Obioactin with a molecular weight of less than 5,000 daltons was obtained from *Toxoplasma*-immune cattle serum and hydrolyzed with proteinase, HCl and NaOH [13].

Purification: Partially purified Obioactin [13] was fractionated in a DEAE-5PW column (21.5 mm ID X 15 cm, Tosoh Co., Tokyo) by NaCl gradient ion-exchange

chromatography with a High Pressure Liquid Chromatography (HPLC) apparatus (Hitachi Co., Tokyo). A NaCl gradient ranging from 0 M to 1 M in a basic solution of 0.02 M ammonium acetate was used for the fractionation. An active fraction obtained by ion-exchange chromatoraphy was subjected to reverse-phase HPLC with an ODS-120T column (4.6 mm ID X 250 mm, Tosoh Co., Tokyo) over an acetonitrile concentration gradient of 10–100% in 0.1% trifluoroacetic acid.

Characterization of refined Obioactin: The HPLC fraction of refined Obioactin was sealed in a tube containing a constant boiling point solution of hydrochloric acid and 0.1% thioglycol. The contents of the tube were heated to 110°C for 24 hr to hydrolyse the Obioactin fraction. The amino acid composition was analyzed by the OPA method [1, 2] with a Hitachi amino acid analyzer (Model 835, Hitachi Co., Tokyo).

Peptide synthesis: Obiopeptide analogs were synthesized by the t-Boc method (Biosearch 9600 type, Biosearch Co., U.S.A.). All reagents were obtained from Biosearch Distribution, Kubota-Shoji Co., Tokyo.

After extraction and Tosoh-column HPLC, the identification of the peptides was determined with a Type CCP & 8010 HPLC unit (Tosoh Co., Tokyo) that employed a TSKgel ODS-80TM column (21.5 mm X 300 mm) and the purity was checked by an analytical TSKgel ODS-80TM column (4.6 mm X 250 mm). HPLC profiles of these peptides indicated that they had a purity of more than 95%. To confirm that synthesis progressed to completion, an amino-terminus sequence analysis was performed on all peptides with an Applied Biosystems 470-A gas phase protein sequence apparatus (Applied Biosystems Co., USA). PTH-derivatized amino acids, generated from the sequencer, were analyzed with the Tosoh HPLC CCP & 8010 super system (Tosoh Co., Tokyo).

Preparation of Obiopeptide: A stock solution of Obiopeptide was prepared by dissolving 10 mg of synthetic polypeptide in 1 m*l* of 100 mM $NaHCO_3$ (pH 6.4). Aliquots from the stock solution were diluted with TC-199 medium containing 10% calf serum (Medium) just prior to use to give final concentrations of 500, 100, 10 and 1 μg/m*l*. A solution of Medium was used alone as a control.

Preparation of macrophage and somatic cell monolayers: Two milliliters of sterile 0.2% glycogen saline solution were injected intraperitoneally (i.p.) into adult mice (BALB/c male mice, 8–10 weeks age). Five days later, peritoneal exudate containing elicited macrophages were harbested by washing the peritoneal cavities of the mice with heparinized HBSS. The cell suspension was centrifuged at 200 X G for 5 min and the sediment was suspended in Medium at a concentration of 1×10^6 nucleated cells per m*l*. One milliliter of this suspension was placed in a multidish tray (FB-16–24-TC, Linbro Chemical Co., Inc.) containing round coverslips and incubated in a humidified 5% CO_2 incubator at 37°C for 4 hr. Cells that did not adhere to the coverslips were removed by two rinses with Medium. The coverslips were then reincubated overnight with the same medium in a CO_2 incubator. Thereafter, the cell cultures were rinsed with Medium and used 24 and 48 hr after preparation for assays of *Toxoplasma* growth inhibition.

Assessment of antiprotozoal activity: Tachyzoites of the RH strain of *T. gondii* were obtained from the peritoneal cavity of mice 2 days after inoculation (ai). Tachyzoites were cultured in 1 m*l* aliquots of various concentrations of Obiopeptide at 37°C in a 5% CO_2 incubator. Each aliquot contained approximately 5×10^6 tachyzoites. At intervals of 1, 2 and 3 hrs after exposure to

Obiopeptide, 0.2 m*l* aliquots were removed from each culture, stained with 1 drop of 0.2% trypan blue and examined. Two counts of the number of dead, stained tachyzoites per 100 tachyzoites were made and averaged to calculate average mortality (%).

Obiopeptide solution was added in 1.5 m*l* aliquots to individual wells of multidish trays that contained mouse macrophage or somatic cell monolayers. One hour prior to the addition of Obiopeptide, the monolayers were infected with approximately 5×10^4 tachyzoites per well, giving an approximate ratio of one tachyzoite per 20 mononuclear cells. One hr ai, the monolayers were washed thoroughly to remove excess parasites and then incubated at 37°C for 24 and 48 hrs in a CO_2 incubator. Obiopeptide solution (0.05 mg/m*l*) was also added in 1.5 m*l* aliquots to multidish trays containing cultures of guinea pig macrophages, canine monocyte-macrophages, or human heart cells that had been infected with 5×10^4 tachyzoites per dish one hr earlier. These cultures were also incubated at 37°C for 24 and 48 hrs in a 5% CO_2 atmosphere.

Coverslips were fixed and stained with May-Grüenwald Giemsa double stain at regular time intervals and intracellular parasites in all cultures were counted by phase contrast microscopy. *Toxoplasma* can be readily identified by its characteristic morphology within cytoplasmic vacuoles of infected cells. Infection rates were calculated by counting the number of parasites in phagocytic vacuoles of 1,000 individual cells on each coverslip. The number of tachyzoites per individual cell was recorded as O Tp for cells devoid of parasites, 1–5 Tp for cells containing 1–5 organisms, and 6 Tp for cells containing 6 or more organisms. Experiments were repeated at least 3 times.

RESULTS

Gel filtration and ion-exchange chromatography: Obioactin prepared as described by Suzuki *et al.* [13] was fractionated by NaCl gradient ion-exchange chromatogra-

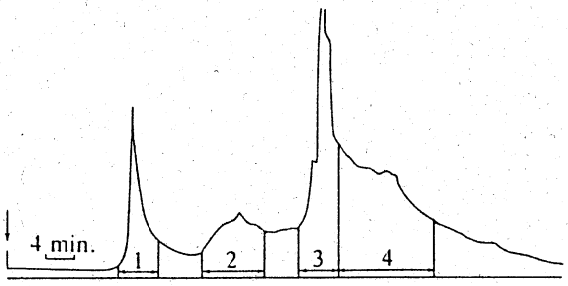

Fig. 1. HPLC elution pattern from a DEAE-5PW column after application of Obioactin.

Note: A typical trace for Obioactin (total 100 mg). Operating conditions are as described in Materials and Methods. (Flow rate, 3.0 m*l*/min; Eluate, 0.02 M CH_3COOH_4, O to 1 M NaCl, linear gradient to 2 hrs; wave-length, 280 nm)

Table 1. Inhibition of *Toxoplasma* by DEAE-5 PW fractions

Sample	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF	
	0 Tp	1 to 5 Tp	≧6 Tp/cell	Activity (%)	Cytotoxicity
Control (Tc-199)	62.8± 7.5	22.6±4.9	14.6±3.9	–	–
Fr. 1	48.6± 9.3	29.0±3.8	22.4±8.8	−38.2	–
Fr. 2	66.8±15.7	17.8±8.6	15.4±8.8	10.8	–
Fr. 3	81.4±11.4	11.8±4.8	6.8±7.4	50.0	–
Fr. 4	68.8±10.8	17.4±5.9	13.6±5.6	16.1	–

Note: Results are the means (± standard deviation) from 5 independent experiments. Operating conditions are described in Materials and Methods. See Fig. 1.

phy (Fig. 1). Each fraction was lyophilized, desalted by gel filtration through Sephadex G15 (Pharmacia Co., Sweden) and then re-lyophilized. Each powdered fraction was dissolved in Medium to a final concentration of 5 mg/m*l* and evaluated for Toxo-GIF activity with mouse peritoneal macrophages. Toxo-GIF activity was −38.2% for fraction 1, 10.8% for fraction 2, 50.0% for fraction 3 and 16.1% for fraction 4 (Table 1). Because of its higher Toxo-GIF activity, the 3rd fraction was collected and refined by reverse-phase liquid chromatography with an ODS-120 T column as described earlier (Fig. 2). Each fraction was concentrated under reduced pressure and lyophilized. These sub-fractions were then tested for Toxo-GIF activity (Table 2). Activities were 97.6% in fraction 3-I, 96.8% in fraction 3-II, 97.2% in fraction 3-III, and 99.2% in fraction 3-IV. Fraction 3-IV was selected for further characterization because of its high activity.

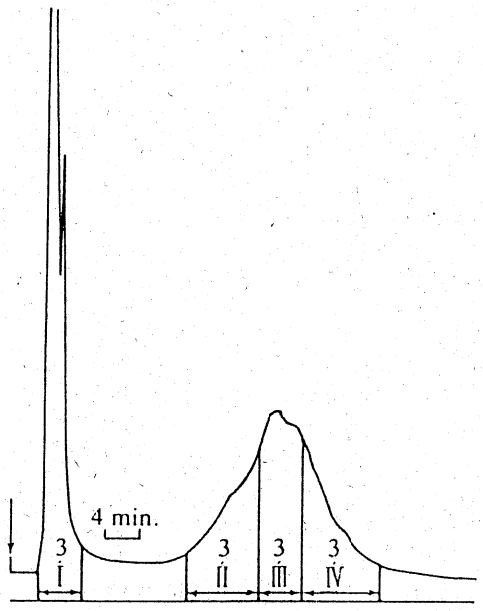

Fig. 2. HPLC-separation of fraction-3 (Fig. 1) with an ODS-120 T column by reverse phase chromatography.

Note: A typical trace of the eluation pattern after application of 10 mg of fraction 3 from Fig. 1. Operations are described in Materials and Methods. (Flow rate, 1.0 m*l*/min; Eluate, initial stage-acetonitrile (10%) +0.1% TFA (90%) to final stage-acetonitrile (100%), linear gradient to 1 hr; wave length, 230 nm)

Composition and amino acid sequence: Refined Obioactin in fraction 3-IV was hydrolysed in a constant boiling point tube as described earlier and analyzed with a Hitachi amino acid analyzer. The fraction contained 30% Asx (total of Asn + Asp), 43% Glx (total of Glu + Gln), 7% Gly, 6% Ala, 5% Val and 3% Pro. Predominant amino acids in fractions 3-I, 3-II and 3-III were also Asx, Glx, Gly, Val, Lys, and Ala. Pro and Leu were also detected as minor components.

The N-terminal amino acids of fraction 3-IV as determined by the Edman degradation method included Glu, Asp, Gly and

Table 2. Inhibition of *Toxoplasma* by ODS-120 T fractions

Sample	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF	
	0 Tp	1 to 5 Tp	≧6 Tp/cell	Activity (%)	Cytotoxicity
Control (Tc-199)	49.8±16.3	26.8±7.1	23.4±9.3	–	–
Fr. 3-I	98.8± 1.1	1.2±1.1	0	97.6	–
Fr. 3-II	98.4± 1.5	1.6±1.5	0	96.8	–
Fr. 3-III	98.6± 2.1	1.2±1.6	0	97.2	–
Fr. 3-IV	99.6± 0.5	0.4±0.5	0	99.2	–

Note: See Table 1 and Fig. 2. Each eluated fraction, Fr. 3-I to Fr. 3-IV, was concentrated under reduced pressure, and then lyophilized. Each powdered fraction was reconstituted to a concentration of 0.5 mg/m*l* and then evaluated for Toxo-GIF activity.

Table 3. Arrangement of amino acids in refined Obioactin, Frac. 3-IV by ODS 120 T fractionation

Amino Acid No.	(n mol) Glu	Asp	Gly	Ala	Val	Pro	Tyr	Leu	Ile	Les
1	2.6	1.2	1.0	0.8	0.4	0.3	0.2	0.1	0.1	0.1
2	2.1	0.7	0.8	0.3	0.4	0.2	–	0.2	–	–
3	1.2	0.5	–	0.2	0.3	0.3	–	0.2	–	–
4	0.5	0.3	–	–	0.1	0.1	–	0.1	–	–
5	0.3	0.2	–	–	–	–	–	–	–	–
6	0.2	0.1	–	–	–	–	–	–	–	–
7	0.1	0.1	–	–	–	–	–	–	–	–

Note: Operating conditions are described in Materials and Methods. (≧0.1 n mol)

Table 4. Inhibition of *Toxoplasma* by synthetic peptides

Sample (0.5 mg/ml)	Percentage of cells containing *Toxoplasma* (%) 0 Tp	1 to 5 Tp	≧6 Tp/cell	Toxo-GIF Activity (%)	Cytotoxicity
10%CS-Tc-199 (Control)	83.6±3.6	7.6±1.8	8.8±3.5	–	–
Glu-Glu-Glu-Glu-Glu	89.4±8.5	5.2±4.9	5.4±3.9	35.4	–
Gly-Glu-Glu-Glu-Glu-Glu	96.6±3.4	2.6±2.6	0.8±0.8	79.3	–
Ala-Glu-Glu-Glu-Glu-Glu	76.0±20.5	10.8±7.6	13.0±12.9	−46.3	–
Asp-Asp-Asp-Asp-Asp	90.0±8.5	3.8±2.4	6.2±6.4	39.0	–
Gly-Asp-Asp-Asp-Asp-Asp	83.2±7.5	9.8±4.6	7.0±3.7	−2.4	–
Asp-Asp-Asp-Asp-Asp-Asp	90.0±6.0	5.8±3.1	4.2±3.3	39.0	–

Note: Each of the synthetic peptides was dissolved in Medium + 10%CS to a concentration of 0.5 mg/ml. Toxo-GIF activity was measured using mouse peritoneal macrophages.

Table 5. Dose dependency of glycil-penta-glutaminate (GpG) in the inhibition of *Toxoplasma*

Concentration of GpG (mg/ml)	Percentage of cells containing *Toxoplasma* (%) 0 Tp	1 to 5 Tp	≧6 Tp/cell	Toxo-GIF Activity (%)	Cytotoxicity
0	84.0±1.6	26.8±7.1	5.0±2.3	–	–
1.000	99.8±0.4	0.2±0.4	0	98.8	±~–
0.500	99.4±0.5	0.6±0.5	0	96.3	–
0.250	93.4±6.3	4.2±4.1	2.4±2.4	58.8	–
0.100	90.8±6.4	5.2±3.4	4.0±3.4	42.5	–
0.050	88.4±7.6	7.0±4.5	4.6±4.2	27.5	–
0.025	82.6±10.8	9.8±6.3	7.6±4.8	− 8.8	–

Note: See Table 4. Operating conditions are described in Materials and Methods.

Ala as major constituents of the first cycle (first residue) and Val, Pro, and Tyr as minor constituents. Glu and Asp were major constituents of the second cycle. The second cycle also contained Ala, Val, and Pro. Glu and Asp were the major constituents of the third to seventh cycles (Table 3). Similar results were obtained from analysis of fractions 3-I, 3-II and 3-III. None of the four fractions obtained by reverse phase chromatography could be refined further by additional chromatography.

Synthesis and biological activity of peptides: Oligopeptides that were estimated to be the smallest constitutive units of Obioactin were synthesized as described earlier [1,

3]. Each of the peptides was dissolved in Medium to give a final concentration of 0.5 mg/m*l* and evaluated for Toxo-GIF activity with mouse peritoneal macrophages (Table 4). Toxo-GIF activities of the respective peptides were 35.4% for penta-Glutaminate (pG), 79.3% for Glycil-penta-Glutaminate (GpG), −46.3% for Alanyl-penta-Glutaminate (ApG), 39.9% for penta-Asparaginate (pA), −2.4% for Glycil-penta-Asparaginate (GpA) and 39.3% for Alanyl-penta-Asparaginate (ApA). None of the peptides were cytotoxic at concentrations of 0.5 mg/m*l*.

The dose dependency of GpG was examined because of its high Toxo-GIF activity (Table 5). This peptide showed Toxo-GIF activity at concentrations above 0.05 mg/m*l* with saturation at a concentration of 0.5 mg/m*l*. Shrinkage and exfoliation of cultured cells were observed at a concentration of 1.0 mg/m*l*. Significant Toxo-GIF activity was detected at GpG concentrations of 0.25 mg/m*l*. By contrast, the Toxo-GIF activity of native Obioactin is not appreciable at a concentration of below 5 mg/m*l*.

GpG exhibited Toxo-GIF activity in canine monocytes, human cardiac muscle cells and mouse macrophages (Table 6), indicating that this peptide did not exhibit the host restriction that is characteristic of native Obioactin. Combinations of GpG and other synthesized peptides were also examined for possible synergistic effects in stimulating Toxo-GIF activity (Table 7). Synergistic effects were not observed, even when 0.5 mg/m*l* concentrations of various peptides were added to 0.5 mg/m*l* concentrations of GpG.

Table 6. Inhibition of *Toxoplasma* by glycil-penta-glutaminate (GpG) in heterologous cells

Cell	Concentration of GpG (mg/m*l*)	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF Activity (%)	Cytotoxicity
		0 Tp	1 to 5 Tp	≧6 Tp/cell		
Mouse Macrophages	0	76.6±9.2	16.5±5.1	7.2±4.3	–	–
	0.05	88.4±7.6	7.0±4.5	4.6±4.2	50.4	–
Dog Monocytes	0	40.1±15.5	28.5±6.8	31.3±8.2	–	–
	0.05	80.5±6.4	12.8±5.4	6.7±3.3	67.4	–
Human Cardiac Muscle	0	54.4±18.3	23.5±9.3	22.1±9.7	–	–
	0.05	80.7±6.1	10.6±3.4	8.7±2.1	57.7	–

Note: Operating conditions are described in Materials and Methods.

Table 7. Synergistic effect of synthetic peptides

Sample A (0.5 mg/m*l*)	Sample B (0.5 mg/m*l*)	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF Activity (%)	Cytotoxicity
		0 Tp	1 to 5 Tp	≧6 Tp/cell		
Control	(Tc-199)	76.6±9.2	16.2±5.1	7.2±4.3	–	–
GpG	GpG	100	0	0	100	±
GpG	–	100	0	0	100	–
GpG	pA	95.6±2.3	4.0±1.9	0.4±0.5	81.2	+
GpG	pG	98.0±1.0	2.0±1.0	0	91.5	±
GpG	GpA	91.4±4.7	7.8±4.8	0.8±0.4	63.2	±
GpG	ApA	91.4±5.7	8.6±5.2	0.2±0.4	61.5	–
GpG	ApG	100	0	0	100	++

Note: See Materials and Methods.

DISCUSSION

Results of this study indicate that active fractions of Obioactin have an N-terminal containing Glu, Asp, Gly, or Ala and biologically active regions that contain mainly 4 to 5 molecules of Glu or Asp. The high biological activity of GpG indicates that it is the major active unit of the native Obioactin molecule. GpG has a molecular weight of about 720. Penta-Glutaminate, ApA and pA also exhibit Toxo-GIF activity and may have potential uses in inhibiting the intracellular multiplication of *Toxoplasma* and promoting host immunoregulation. The biological activity of GpG was 10 to 20 times higher than that of native Obioactin on a per weight basis, and 40 to 140 times higher on a per mol basis.

Refined Obioactin is probably a mixture of peptides that resemble each other and have heterologous N-terminals. It is not clear why low molecular weight oligopeptides can be separated from native Obioactin which has a major molecular weight of approximately 3,000. It is believed, though, that active regions of Toxo-GIF and other natural lymphokines are composed of 10 to 20 active units of low molecular weight that are weakly bound to each other to form a high molecular weight aggregate. During the refining operation, these dissociate into several units with different molecular weights. This is one reason why it is difficult or rather impossible to purify native Obioactin as a single primary structure [4, 6, 13].

Our objective has been to refine Obioactin and identify the sequence of amino acids in this immunoregulator that is capable of enhancing cell functions. We synthesized the oligopeptide GpG, termed Obiopeptide-1, which has significantly higher biological activity than native Obioactin on a per weight or per mol basis. This peptide does not exhibit species specificity and has important potential applications in the treatment of protozoan infections and modulation of hypoimmune responses in infected hosts.

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要　　約

天然免疫調整物質オビオアクチン由来合成ペプチド，Obiopeptide-1，の生物活性：鈴木直義・桜井治久・斎藤篤志・五十嵐郁男・小俣吉孝・尾崎文雄(帯広畜産大学獣医学科家畜生理学教室・原虫病細胞免疫研究室)——トキソプラズマ原虫栄養型虫体はマウスマクロファージ及びヒト体細胞単層培養細胞内において合成ペプチド(Obiopeptide-1)添加によって著しく増殖抑制され，多くは死滅する．本ペプチドは1 Glycil と5 Glutaminate よりなり，天然オビオアクチンの有する細胞内殺トキソプラズマ原虫活性単位の1つとして合成された．世界の動物および人に認められるトキソプラズマ感染症に対する有効な治療薬のない現在，Obiopeptide-1は新規の抗微生物活性を有する非抗生物質治療補助剤として細胞内寄生原虫および一般感染症に随伴する宿主免疫能の低下に対する有効な免疫調整作用物質になり得るかも知れない．

Changes of Lymphocyte Subpopulations and Natural Killer Cells in Mice Sensitized with *Toxoplasma* Lysate Antigen before and after *Babesia* Infection

Ikuo IGARASHI, Ryusuke HONDA, Teruo SHIMADA, Kazuro MIYAHARA, Haruhisa SAKURAI, Atsushi SAITO, and Naoyoshi SUZUKI

Department of Veterinary Physiology and Protozoan Immunology, Obihiro University, Obihiro, Hokkaido, Japan

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ABSTRACT. When 8-week-old BALB/c mice were sensitized with two intramuscular injections of *Toxoplasma* lysate antigen (TLA) at 2 week interval, the numbers of sIg(+), Thy-1,2(+), Lyt-1, 2 (+) Lyt-2, 2(+), and Asialo GM1(ASGM1)(+) cells in the spleen, liver and peripheral blood increased by 2 to 4 times over those found in unsensitized mice of the same age. When TLA-sensitized and unsensitized mice were infected with *Babesia*, 4 of 10 (40%) of the TLA-sensitized mice survived infection, while none of the unsensitized control mice lived longer than 14 days after *Babesia* infection. By contrast, sensitization of nude mice with TLA had no effect on survival, and mice did not live more than 12 days. The number of thymic Thy-1,2(+) cells decreased in TLA-sensitized and unsensitized BALB/c mice by almost 80% within 10 days after infection (AI). During the same time, the numbers of B cells, T cells, and NK cells increased in the spleen, liver and peripheral blood of both sensitized and unsensitized mice. Especially notable were increases in numbers of Lyt-2,2 (+) cells in the spleen and blood and increases in numbers of NK cells in the spleen, liver and blood in both TLA-sensitized and unsensitized mice. When spleen cells from TLA-sensitized and unsensitized mice were cultured in the presence or absence of TLA for 6 days, assays for cytotoxicity using NK-insensitive P-815 target cells and NK-sensitive YAC-1 target cells demonstrated higher rates of cytotoxicity in cultures of TLA-sensitized spleen cells.—KEY WORDS: *Babesia*, immunomodulator, killer cell, NK cell, *Toxoplasma* lysate antigen.

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It has been reported that mice acquire strong resistance to experimental infection with parasites of malaria (*Plasmodium berghei*) and babesia (*B. rodhaini*) when TLA is administered [7–9, 13, 14]. Satoh [13] found that Thy-1,2 positive cells accumulate remarkably in the thymus and spleen of TLA inoculated mice and observed a corresponding increase in the immune response of the hosts to infection with *B. rodhaini*. Consequently, studies of the behavior of lymphocytes, killer and natural killer (NK) cells from TLA sensitized animals may clarify the factors responsible for resistance to infection with *Babesia* and *Plasmodium*. In the present study, differences in lymphocyte phenotypes between TLA-sensitized and unsensitized control mice were measured with monoclonal antibodies and the sensitive analytical capabilities of a fluorescence-activated cell sorter after the animals were infected with *Babesia*.

MATERIALS AND METHODS

Host animals: Adult mice (BALB/c nu/+ hetero and nu/nu nude mice) which had been bred and raised at the Department facilities were used for all experiments.

Preparation of Toxoplasma lysate antigen (TLA): TLA was prepared as described by Igarashi [2] and Sakurai [12]. TLA supernatant (TLA144) was prepared by centrifuging TLA at 144,000 × g for 120 min at 4°C and then freeze-dried and stored until use. Freeze-dried TLA144 was reconstituted

with 0.85% sodium chloride to a final protein concentration of either 500 μg/m*l* or 5,000 μg/m*l* as determined by the method of Lowry *et al* [6]. Bovine serum albumin was used as a protein standard.

Sensitization of mice with TLA: Mice were sensitized by two intramuscular injections of 100 μg of TLA in physiological saline at 2 week interval. Four weeks after the first TLA injection, mice were inoculated intraperitoneally (IP) with 1×10^2 *Babesia*-infected erythrocytes. Each of 10 unsensitized control animals was also inoculated IP with 1×10^2 parasitized erythrocytes. This inoculum is capable of killing a mouse within about 2 weeks. Tissue samples were collected from every animal 1 day before and 10 days after inoculation with *Babesia*. Average parasitemia and number of surviving animals were also monitored for each group.

Preparation of visceral cell suspension: Thymic, splenic, and hepatic tissues were chopped finely, triturated between two slides and then pressed through a 40-mesh stainless steel filter to eliminate large tissue debris. Mononuclear cells (MNC) were separated from the crude suspension with Conray 400-Ficoll [15]. The remaining suspension was pelleted by centrifugation at 400 × g for 5 min and washed with Hank's balanced salt solution (HBSS), pH 7.2. After a second centrifugation, the pellet was resuspended in warm (37°C) 0.83% ammonium chloride solution to hemolyze any extraneous erythrocytes and then centrifuged at 400 × g for 5 min at room temperature. The pelleted cells were resuspended in HBSS and washed with HBSS at 4°C by two additional 5 min centrifugations at 400 × g. Thymic, splenic, and hepatic cells were suspended in liquid culture medium 199 supplement with 10% heat-inactivated fetal calf serum (TC-199). After the number of cells was calculated, the suspension was pelleted and resuspended in TC-199 to a final density of approximately 5×10^7 cells per m*l*. MNC were isolated by the Conray-Ficoll method from peripheral blood that had been collected in a heparinized syringe. These cell suspensions were used for the cytotoxicity test and the flow cytometry analysis (Showadenko Co., cell sorter FACS, Tokyo).

Complement-mediated cytolysis: Complement-mediated cytolysis was used to deplete Asialo GM1+(ASGM1), surface Ig+(sIg) and Thy-1,2(+) cells from the MNC suspension. Rabbit antiserum against ASGM1 antigen, rabbit anti-mouse Ig(polyvalent) antiserum, and monoclonal anti-mouse Thy-1,2 antibody (IgM) were purchased from Wako, Tokyo, Japan, DAKO, Denmark and Olac, UK, respectively. MNC were incubated with anti-ASGM1 (0.2 mg/m*l*), anti-mouse Ig (0.2 mg/m*l*) or anti-Thy-1,2 (0.25 μg/m*l*) antibody at 4°C for 45 min, washed twice with the medium, and then incubated with a 1/10 dilution of low toxicity rabbit complement (Cedarlane Lab., Hornby, Ontario, Canada) at 37°C for 45 min.

Radiolabeling and cytotoxicity assays: Cytotoxic function was examined with a specific 51Cr release assay [5]. NK sensitive YAC-1 cells derived from a Moloney virus-induced lymphoma in A/Sn mice were used as target cells for NK assay. P-815 lymphoma cells were used as nonsensitive controls. Both cell lines were kindly shared by Dr. T. Kamiyama, National Institute for Health, Tokyo. Various numbers of splenic MNC were incubated with TLA for 6 days and then incubated with either 1×10^4 51Cr labeled YAC-1 or 1×10^4 P-815 cells in 96-well round-bottomed microtiter plates. Each well held 0.2 m*l*. Plates were centrifuged at 300 × g for 5 min and incubated for 4 hrs at 37°C in a 5% CO2 incubator. After incubation, 0.1 m*l* of the supernatant from each well was removed and counted in a gamma counter. 51Cr-specific release was determined as described by Kumagai [5]

with minor modifications. Spontaneous 51Cr release from YAC-1 and P-815 target cells ranged from 2–5%. Addition of TLA to the wells had no influence on the spontaneous release of label from target cells. All samples were assayed in triplicate.

Immunofluorescence microscopy: Cell surface antigens(Ag) on MNC was identified by monoclonal antibody (mAb) or polyclonal antibodies by direct or indirect immunofluorescence microscopy [1]. B cell sIg was labeled with FITC-conjugated rabbit anti-mouse Ig antiserum (DAKO). T cell associated Thy-1,2 Ag, Lyt-1,2 Ag, and Lyt-2,2 Ag was labeled with FITC-conjugated monoclonal antibodies (Becton Dickinson Immunocytometry Syst., Mountain View, Ca., U.S.A.). NK cells were detected with anti-ASGM1 antibody and FITC-conjugated anti-rabbit IgG antisera (Cedarlane). Enumeration of fluorescence-positive cells was performed with a cell sorter FACS analyzer and/or fluorescence microscope with differential interference equipment (Nikon-microphoto Fx-RFL-NTF, Nikon, Tokyo). Biotin-conjugated anti-mouse Thy-1,2, Lyt-1,2, and Lyt-2,2 mAb and peroxidase conjugated streptavidin (Standard Vectastain ABC Kit, Vector Lab., Burlington, Ca., USA) were also used to identify T cells with a Nikon FAX-2-Luzex image analyzer.

Morphologic study of MNC: Isolated MNC were smeared on glass slides and stained with May-Grüenwald-Giemsa method.

Single-cell cytotoxicity test: Fresh guinea pig serum was absorbed with thymus cells and diluted 1.5 times. 0.1 m*l* of the diluted guinea pig serum was combined in a test tube with either 0.1 m*l* of a 1:32 dilution of anti-Thy-1,2 serum or normal rabbit serum and mixed with 4×10^5 cells (0.1 m*l*). After a 30 min incubation at 37°C, 0.03 m*l* of a 0.5% solution of trypan blue dye was added to the cell suspension. Dead and viable MNC were counted under a microscope. Student's *t*-test was used to evaluate the results.

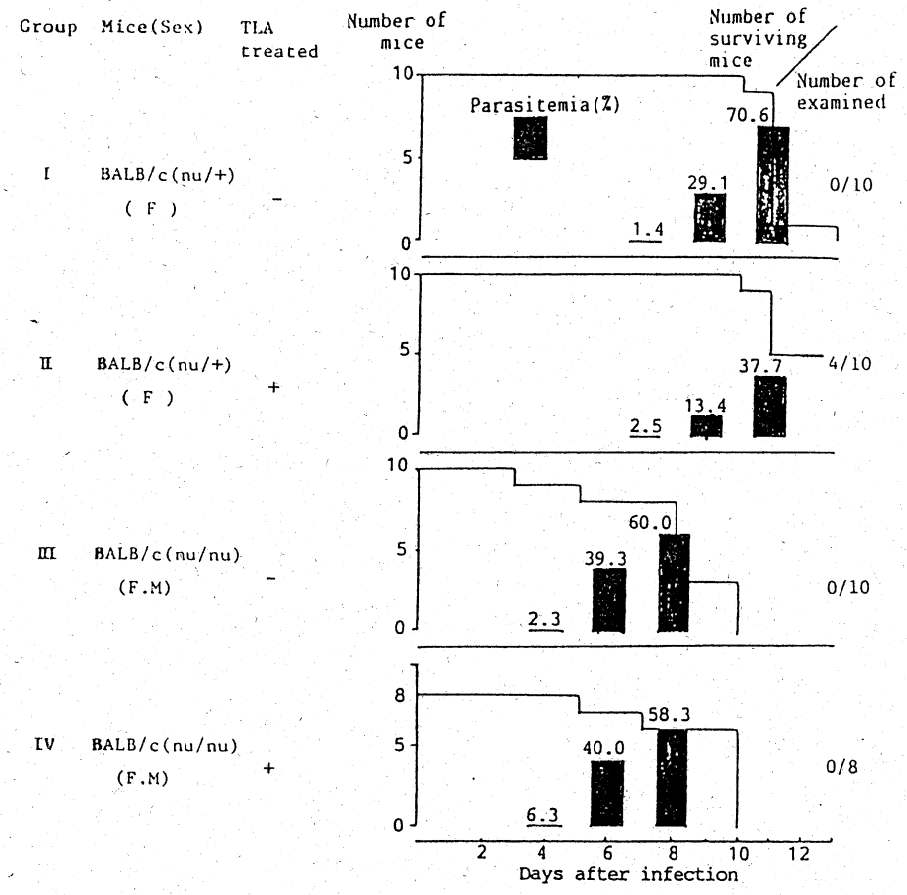
RESULTS

Effects of TLA administration on the mortality of B. rodhaini-infected mice: Mature BALB/c (nu/+) hetero mice (Groups I and II) and BALB/c (nu/nu) mice (Groups III and IV) were divided into four groups of 10. Mice in groups II and IV were sensitized with two 100 μg injection of TLA at 2 week interval. Groups I and III were used as unsensitized controls. Two weeks after the 2nd injection of TLA, mice in all four groups were inoculated IP with 1×10^2 parasitized erythrocytes (PE).

All 10 unsensitized control mice in Group I died from high parasitemias within 14 days after inoculation (AI). Parasites first appeared in Group II at 4 days AI, but parasitemia was clearly lower than that of Group I. Four mice (40%) in Group II were still alive at 14 days AI (Fig. 1).

TLA sensitization had no effect on survival of nude mice. All 8 of the TLA-sensitized nude mice in Group IV died within 14 days AI. The 10 unsensitized nude mice in Group III died of similar parasitemias within 14 days AI(Fig. 1).

Variations in spleen lymphocytes and NK cells in TLA sensitized BALB/c hetero mice: Comparisons of total numbers of MNC in BALB/c hetero mice 2 weeks after the 2nd injection of TLA revealed differences among control and sensitized groups (Table 1). The average number of MNC in unsensitized control mice was lower than those in the T30 and T100 groups (12.9×10^6 cells vs 22.5×10^6 and 15.1×10^6 cells, respectively). Larger numbers of Lyt-1,2 and Lyt-2,2 positive lymphocytes were found in the T30 group than those in the unsensitized control group. A distinct increase in the number of ASGM1 positive cells also occurred in both group of TLA sensitized mice, and the



Notes: Percent parasitemia was the average of 5 mice. F, Female and M, Male

Fig. 1. Resistance to *B. rodhaini* infection in BALB/c mice sensitized with TLA.

Table 1. Changes in cell phenotypes in the spleens of mice 4 weeks after the 1st injection of TLA

	Control	TLA 30 μg (T30)	TLA 100 μg (T100)
Spleen weight (mg)	104.2±14.9	130.8±11.2	131.8±6.3
Total cells ($\times 10^6$)	12.9± 5.9	22.5± 8.5	15.1±3.0
Analysis by Cell Sorter (CS-20)			
Lyt-1, 2 ($\times 10^6$)	2.6± 1.6	5.1± 3.4	2.6±0.5
(%)	17.6± 3.1	20.0± 7.2	17.9±3.5
Lyt-2, 2 ($\times 10^6$)	1.4± 0.8	2.2± 1.0	1.6±0.6
(%)	9.3± 1.9	9.3± 1.7	10.0±2.0
asialoGM$_1$ ($\times 10^6$)	1.2± 0.4	4.0± 2.1[a)]	1.8±0.5
(%)	9.2± 2.6	16.8± 4.0	11.7±2.8

Remarks: Results are the means (± standard deviation) from 5 independent experiments.
a) A significant difference ($P<0.02$) is shown as compared to control. Operating conditions as described in materials and methods.

Table 2. Cytotoxic activities[1] of spleen cells[2] on YAC-1 or P-815 cells

Mouse spleen cells from	Incubated with		Target cells YAC-1 (%)	Target cells P-815 (%)
Unsensitized group	None	(N-Med)	8.1±1.8	1.0±1.9
	TLA	(N-TLA)	11.0±2.4	9.2±2.0[c]
TLA-sensitized group	None	(T-Med)	10.4±1.4	3.8±2.7
	TLA	(T-TLA)	23.1±3.1[a,b]	8.1±1.5[d,e]

Remarks

1) Cytotoxicity (%) = 100×

$$\frac{\text{Experimental release of } {}^{51}\text{Cr (cpm)} - \text{Nonspecific release of } {}^{51}\text{Cr (cpm)}}{\text{Total release of } {}^{51}\text{Cr (cpm)} - \text{Nonspecific release of } {}^{51}\text{Cr (cpm)}}$$

2) Spleen cells were incubated for 6 days.

3) Significant differences are shown as follows, a, b) $P<0.001$, compared to N-Med and T-Med, c) $P<0.001$, compared to N-Med, d) $P<0.02$, compared to T-Med, and e) $P<0.001$, compared to N-Med.

increase in the T30 group was statistically significant ($p<0.02$).

Cytotoxicity of cultured spleen cells: When spleen cells were incubated with YAC-1 target cells as shown in Table 2, higher rates and significant differences ($P<0.001$) of cytotoxicity were observed in TLA-sensitized spleen cells incubated with TLA (T-TLA, 23.1%) than those in unsensitized spleen cells and TLA-sensitized ones incubated without TLA (N-Med, 8.1% and T-Med, 10.4%), respectively. When unsensitized spleen cells were incubated with TLA (N-TLA), the rate of cytotoxicity was 11.1%. This value was higher, but not statistically different from the rate calculated for unsensitized spleen cells incubated with N-Med.

The rate of cytotoxicity on P-815 cells was 8.1% in TLA sensitized spleen cells and 9.2% in unsensitized spleen cells incubated with TLA (T-TLA and N-TLA), respectively, and only 1.0% in unsensitized spleen cells incubated without TLA (N-Med). These differences were statistically significant($P<0.001$). When TLA sensitized spleen cells were incubated without TLA (T-Med), the rate of cytotoxicity was 3.8%. This value was approximately 4 times higher than that of unsensitized spleen cells incubated without TLA or with medium alone(N-Med), however, the difference was not statistically significant.

Variations in lymphocytes and NK cell populations in TLA-sensitized and unsensitized control mice after Babesia infection: Clear increase in the numbers of Thy-1,2(+) cells occurred in the thymus, spleen and liver of TLA-sensitized mice prior to inoculation with *Babesia* (Table 3). When comparisons were made before and after infection with *Babesia*, the number of thymic Thy-1,2(+) cells decreased from 45.8×10^6 to 10.1×10^6 in the TLA-sensitized group and from 24.2×10^6 to 5.0×10^6 in the unsensitized control mice, representing decreases of 77.9% and 79.3%, respectively.

The number of Thy-1,2(+) cells in the spleens of TLA-sensitized and unsensitized control mice were 178×10^6 and 83.8×10^6 respectively, at 10 days AI. These numbers represented increases of 1279.8% and 1535.9% over values at 1 day before inoculation (BI). Numbers of spleen Lyt-1,2(+) and Lyt-2,2(+) cells showed similar increases between 1 day BI and 10 days AI in TLA-sensitized and control mice. Numbers of Lyt-1,2(+) cells increased by 588.5% in unsensitized control mice and 931.4% in TLA-sensitized mice. Numbers

Table 3. Variations of mononuclear cells in the organs in BALB/c mice sensitized with TLA 10 days after *Babesia* inoculation

Organs	TLA(30μg) treated	Days after infection −1[a]	10[b]	The rate of change (%)[c]
Thymus				
Total No. of mononuclear cells (×10⁶)	Untreated	27.3	5.2	−81.1
	Treated	48.7	13.5	−72.3
No. of Thy-1, 2(+) cells (×10⁶)	Untreated	24.2	5.0	−79.3
	Treated	45.8	10.1	−77.9
Spleen[d]				
No. of mononuclear cells (×10⁶)	Untreated	13.9	120.0	763.3
	Treated	22.5	329.0	1382.2
No. of sIg (+) cells (×10⁶)	Untreated	4.0	25.4	535.0
	Treated	9.3	84.9	812.9
No. of Thy-1, 2(+) cells (×10⁶)	Untreated	3.9	83.8	1535.9
	Treated	12.9	178.0	1279.8
No. of Lyt-1, 2(+) cells (×10⁶)	Untreated	2.6	17.9	588.5
	Treated	5.1	52.6	931.4
No. of Lyt-2, 2(+) cells (×10⁶)	Untreated	1.4	81.2	4271.4
	Treated	4.2	181.0	4209.5
No. of asialo-GM1(+) cells (×10⁶)	Untreated	1.0	3.5	250.0
	Treated	2.1	14.7	600.0
Liver[d]				
No. of mononuclear cells (×10⁶)	Untreated	12.0	10.8	−10.0
	Treated	7.0	12.2	74.3
No. of sIg (+) cells (×10⁶)	Untreated	0.6	2.5	316.7
	Treated	1.2	3.2	166.7
No. of Thy-1, 2(+) cells (×10⁶)	Untreated	5.0	8.8	76.0
	Treated	6.8	15.8	132.4
No. of Lyt-1, 2(+) cells (×10⁶)	Untreated	3.1	4.7	51.6
	Treated	3.2	9.9	209.4
No. of Lyt-2, 2(+) cells (×10⁶)	Untreated	1.8	5.3	194.4
	Treated	2.2	6.1	177.3
No. of asialo-GM1(+) cells (×10⁶)	Untreated	0.4	3.9	875.0
	Treated	0.7	8.1	1057.1
Peripheral[e]				
No. of mononuclear cells (×10⁶)	Untreated	2.5	4.7	88.0
	Treated	2.8	4.8	71.4
No. of sIg (+) cells (×10⁶)	Untreated	0.5	0.7	40.0
	Treated	0.6	0.7	16.7
No. of Thy-1, 2(+) cells (×10⁶)	Untreated	1.3	2.8	115.4
	Treated	1.5	2.9	93.3
No. of Lyt-1, 2(+) cells (×10⁶)	Untreated	0.2	1.7	750.0
	Treated	0.4	1.9	375.0
No. of Lyt-2, 2(+) cells (×10⁶)	Untreated	0.1	1.7	1600.0
	Treated	0.1	1.9	1800.0
No. of asialo-GM1(+) cells (×10⁶)	Untreated	0.1	0.2	100.0
	Treated	0.3	1.1	266.7

Note: a) BALB/c mice, aged 8 weeks, 1 day before infection.
b) 10 days after infection.
c) %; $\frac{b-a}{a} \times 100$.
d) Each value was calculated as the mean from 5 samples of 5 independent mice.
e) Each value was calculated as the per m*l* of pooled blood from 5 mice.

of Lyt-2,2 (+) cells underwent the most remarkable changes and increased by 4271.4% in control mice and 4209.5% in TLA-sensitized mice. Numbers of ASGM1(+) cells increased by 250% in unsensitized control mice and 600% in TLA-sensitized mice by 10 days AI.

In the liver, Thy-1,2(+) cells increased

from 5.0×10^6 to 8.8×10^6 in unsensitized control mice and from 6.8×10^6 to 15.8×10^6 in TLA-sensitized mice between 1 day BI and 10 days AI. The largest increase was in the TLA sensitized group. Numbers of Lyt-1,2(+) lymphocytes and ASGM1 (+) cells also exhibited significant increases in TLA-sensitized mice before and after infection. Numbers of Lyt-1, 2 (+) lymphocytes increased by 51.6% and 209.4% in the control and TLA-sensitized groups, respectively. Numbers of ASGM1(+) cells increased by 875.0% and 1057.1% in the control and TLA-sensitized groups, respectively. Numbers of Lyt-2,2(+) lymphocytes increased by similar amounts in both groups before and after infection.

In peripheral blood, the number of Thy-1,2(+) cells increased from 1.3×10^6 to 2.8×10^6 cells in unsensitized control mice and from 1.5×10^6 to 2.9×10^6 cells in TLA-sensitized mice between 1 day BI and 10 days AI. In unsensitized control mice, numbers of Lyt-1,2(+) and Lyt-2,2 (+) lymphocytes increased by 750% and 1600%, respectively, before and after infection. Numbers of these two cell types increased by 375% and 1800% in the TLA-sensitized mice before and after infection. Numbers of ASGM1(+) cells increased by 100% in the unsensitized mice and by 266.7% in the TLA-sensitized mice before and after infection.

DISCUSSION

The present experiments confirm that the spleens of 8-week-old, unsensitized control mice contain similar proportions of sIg(+) B cells, Thy-1,2(+) T cells and ASGM1(+) NK cells. By contrast, relative populations of T,B, and NK cells in murine liver were distinguishable from those of the spleen and peripheral blood, and contained larger proportions of Thy-1,2(+) T cells. When unsensitized mice were infected with *Babesia*, the numbers of all of these lymphocyte cell types increased. Significant accumulations of Lyt-2,2 T cells, which contained mainly two subpopulations, Tdh and Tc or killer T cells, occurred in the spleen, the liver, and the blood by 10 days AI, even though all mice were only 1–2 days before death.

It is known that the number and activity of murine NK cells depend on the strain and age of the mouse they are isolated from [3, 4]. The BALB/c mice used in this experiment were 8 weeks old. The numbers of ASGM1(+) NK cells in unsensitized control mice were larger in the spleen than in the liver and peripheral blood before infection with *Babesia*. After infection with *Babesia*, control mice exhibited remarkable accumulations of NK cells in the spleen, the liver, and the blood by 10 days AI. Some large granular cells were ASGM1(+).

TLA is now known as a modifier of biological responses which is capable of stimulating prodution of cytokines such as IFN-γ, Toxo-GIF, and MIF, sensitizing T cells, and activating macrophages [7–9, 10, 11, 13]. In the present study, TLA sensitization decreased mortality in *Babesia*-infected BALB/c mice, but had no effect on mortality or survival of *Babesia*-infected nude mice. The observations indicate that resistance to *Babesia* is mediated mainly by T cell functions. Two injections of TLA at 2 week interval caused the number of thymic Thy-1,2(+) cells to more than double. Within 10 days after *Babesia* infection, however, the number of Thy-1,2(+) cells dropped to levels only1/5 as large as at 1 day BI. When TLA-sensitized and unsensitized control mice were infected with *Babesia*, numbers of sIg(+), Thy-1,2(+), Lyt-2,2(+), and ASGM1(+) cells increased in both groups, although numbers were much higher in the TLA-sensitized group. An increase in numbers of large granular cells were also evident in populations of Thy-1,2(+) or ASGM1(+)

cells from the spleens and livers of mice at 10 days AI.

It is known that some modifiers of biological responses as well as lymphokines (e.g. OK-432, IL-2, and IFN-γ) augment both the number of NK cells and the level of NK activity [8, 16, 17]. Larger numbers of Thy-1,2(+) or ASGM1(+) large cells were present in TLA-treated cultures of spleen lymphocytes from TLA-sensitized mice than those in culures of unsensitized spleen cells cultured with or without TLA [11]. The present results also clearly demonstrate that addition of TLA to cultures of TLA-sensitized spleen cells stimulates production of killer cells, such as lymphokine-activated killer(LAK)-like cells and NK-like LAK and/or NK cells. These cells showed high cytotoxic activities against both NK-insensitive P-815 tumor cells and NK-sensitive YAC-1 target cells. However, increased numbers of ASGM1 (+) cells in the spleens of TLA-sensitized and unsensitized control mice were not correlated with levels of NK activity against YAC-1 target cells. Consequently, resistance of TLA-sensitized mice to *Babesia* infection may be mediated not only by increases in the numbers of Thy-1,2(+) and ASGM1(+) cells, but also by quantitative and qualitative variations in these cell types.

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要　　約

トキソプラズマ溶解抗原(TLA)感作マウスの *Babesia* 感染に対するリンパ球およびNK細胞の動態：五十嵐郁男・本田龍介・嶋田照雄・宮原和郎・桜井治久・斎藤篤志・鈴本直義(帯広畜産大学家畜生理学教室，原虫病細胞免疫研究室)——8週令，雄，BALB/cマウスに2週間隔で2回，TLAを背部皮下に投与後，脾臓，肝臓および末梢血液中のsIg，Thy-1，2，Lyt-1，2，Lyt-2，2およびAsialo GM1陽性細胞数を測定した．TLA感作マウスの細胞数は，それぞれTLA非投与群マウスに比較して2－4倍に増加した．これらのTLA感作および非感作群マウスに *Babesia rodhaini* 感染赤血球(1×10^2)を接種すると，TLA感作マウス10例中4例が生存したが，非感作対照マウスは接種後14日までに全例死亡した．TLA感作ヌードマウスでは，TLA非感作ヌードマウスと同様に接種後12日までに全例死亡した．*Babesia* 接種後10日目の両群マウスの胸腺細胞数は両群共に接種前に比較して約80%減少した．一方，*Babesia* 接種10日目の脾臓，肝臓および血液中のBおよびTリンパ球数とNK細胞数は両群マウス共に増加したが，その増加の度合はTLA感作マウスにおいて著明であった．両群マウスの脾臓細胞をTLAと6日間 in vitro で培養した．それをEffector細胞として，標的細胞(P-815及びYAC-1腫瘍細胞)に対する細胞障害性試験を試みたところ，TLA感作脾臓細胞が強く両標的細胞を破壊した．

実験動物：バベシア病

実験動物，特にマウスやラットなどのげっ歯類では，同種動物が閉鎖環境下において，多数集団で，しかも衛生管理の充実した場所で飼育されていることが常態である。さらに同種動物でも，同一株，同一週齢などによって区分し，管理条件を同一にしたうえ，恒温・恒湿下で均一な感受性動物集団として一般には取り扱われている。したがって，実験動物における住血微生物の自然感染は例外的な場合を除いては存在しない。

このような実験動物は医学や獣医学領域における住血微生物病の解明や治療対策のためのモデルとして用いられている。

わが国においても，各種の住血微生物病のなかで牛および犬のバベシア病は重要疾病の一つであるため，近年，げっ歯類を用いて *Babesia rodhaini* 感染による種々の基礎的研究がなされ始めた[4~6, 8, 15]。

げっ歯類のバベシア病
Murine Babesiosis

バベシア Babesia の語は1888年，ルーマニアの Babès がアフリカの血色素尿牛の血液に認めた病原体を報告したことに端を発する[10]。

バベシア病はダニが媒介する *Babesia* 属原虫の感染による疾病で，家畜および野生動物において世界的に広くまん延する獣医学領域での重要疾患の一つである。また，*B. microti* などのヒトへの感染例が報告されて以来，人畜共通原虫病としても重要視されるに至った[16, 21]。

Levine (1971)[13]，Killick-Kendrick (1974)[11] および Momen ら (1979)[14] によると，げっ歯類目および食虫目寄生のバベシア種として約25～32種が記載されている。しかし，それらのなかには既報の同一種が新種として分類されているものもあり，現在のところ，げっ歯類に寄生するバベシア種のうちで世界的によく知られているものは，*B. microti* (＝*B. microtia*)，*B. colesi*，*B. muris*，*B. hylomysci*, *B. musculi* (＝*B. muratovi*), *B. rodhaini* などである（125頁表2）。

病　原　体

生活環および形態

Babesia 属は有性生殖をダニ体内で，無性生殖を宿主体内で行う。しかし，その全過程の詳細が明らかにされているわけではない。

1.　宿主体内における発育・形態

ダニの唾液腺細胞内で増殖したスポロゾイトは吸血の際に宿主の血液中に入って赤血球内に侵入する。原虫は宿主赤血球内での発育に伴って種々の形態をとる。

わが国でネズミ類から分離された *B. microti*（カラー写真1）類似のバベシア種について報告したShiotaら[20,21]によると，小型（1～2μm）および大型（4～6μm）の円形ないし楕円形のメロゾイトの中央部は空胞状で，ギムザ染色では周辺部の青染した細胞中に通常1個のクロマチンが認められる。次いで，円形の辺縁が不整形を呈したアメーバ状のトロホゾイトでは，細胞質の先端部にクロマチンが集塊として認められたり，あるいはまたメロゾイト形成過程と思われるそれぞれ独立したクロマチンがみられる。また，メロゾイトがほぼ完成し独立直前の状態と考えられる十字型のマルタクロス maltese cross がみられる。

メロゾイトの大きさは約0.5～1.2×1.2～2.0μm で紡錘形に近く，細胞質は青染し，クロマチンは中央より一方に位置する。

B. rodhaini（カラー写真2）感染赤血球によるマウスおよびラットにおける実験感染では脾臓，肝臓および末梢血液中赤血球内で，ほぼ上述のごとく円形，卵円形，細長形あるいはアメーバ状などさまざまな発育形態がみられ，虫体内には多数の不整形のクロマチン集塊を含む分裂・増殖像などが認められる。しかし，いわゆる洋梨子状の原虫や十字型のマルタクロス状原虫は赤血球中にみいだされない。

このように，赤血球内では種々の形態をした原虫が観察されるが，バベシアの発育については不明な点が多く，また赤血球以外の部位での発育は認められていない。

赤血球内で発育する *Plasmodium* 属原虫がマラリア色素を産生するのとは異なり，*Babesia* 属原虫はヘモグロビン消化による赤血球内ヘモゾイン hemozoin を産生しない。

2.　ダニ体内における発育・形態

バベシア感染動物から吸血によってマダニ腸管内に入った赤血球寄生原虫は，その大部分が死滅する。しかし，一部の原虫がガメトゴニーの後，ダニ中腸上皮細胞内でザイゴートを経て第一次キネートに成長する。キネートはヘモリンフ中に遊走し，他の細胞で分裂・増殖して次世代のキネートとなる。経卵的に幼ダニに移行したキネートはさらに分裂を繰り返し，最終的には唾液腺細胞内に侵入し，洋梨子状または円垂形のスポロゾイトに発育する。この過程および形態は基本的には牛に寄生するバベシアである *B. bigemina* に類似する。

感染能を有するダニの発育期はバベシアの種類および媒介ダニの種類によって異なる。たとえば，*B. microti* を媒介するマダニ属の *Ixodes scapularis* や *Ixodes pacificus* では媒介能を有する発育期は若ダニで，幼ダニと成ダニには媒介能がない[16]。

感　　染

本病の自然感染はマダニ科 Ixodidae のダニの媒介により成立する。

B. microti の媒介者として *Ixodes scapularis* が証明されている[5]。そのほか，実験的には *Ixodes pacificus* の若ダニが *B. microti* を媒介するが，カクマダニ属のダニは媒介しない[16]。

実験的には原虫感染血液の接種によって感受性動物は容易に感染する。

培養および保存

培養：*B. rodhaini* の短期培養は Jack らが開発成功している[9,10]。ヒトまたはラット血清添

加RPMI 1640培養液中に，ラットでの感染赤血球を非感染赤血球の約0.3～1.0%になるように浮遊し，その200μlずつをマイクロプレートの各穴に分注して5% CO_2 培養器で培養する。培養液は24時間ごとに交換する。3日間の培養で原虫は約3倍に増殖する。

B. microti においても4日間の連続培養が報告されている[2]。また，本法では牛胎仔血清とハムスター赤血球を用いて，5% CO_2 培養器で培養すると，原虫は約3倍量に増殖する。さらに連続培養を行うことによって，*B. microti* はハムスター赤血球に馴化し，その分裂・増殖期は原虫原株より短くなる。

保存：凍結防止剤としてグリセリン，ジメチルスルホキシド，糖などを用いることによって液体窒素内での長期間保存が可能である。

疫　　学

ネズミのバベシア種のなかでも *B. microti*，*B. rodhaini*，*B. hylomysci* などは北米，欧州，アフリカ，アジアなど世界的に認められている[7, 22, 23]。

その他，げっ歯類に感染するバベシアの分布は熱帯から温帯に及び，広く世界を覆っている。世界各地における野生のネズミ類のバベシアに関する疫学調査および日本からの発見例を含めて[20, 21]，多くは *B. microti* あるいはそれに近い種と同定されている。

ネズミにおけるバベシア感染の季節的変動では，冬が他の季節に比較して感染率が低い[12]。しかし，一方では四季に差がないとの報告[20, 21]もある。

わが国では野生小動物からのバベシアの報告はきわめて少ない。これまで滋賀県山中町で捕獲されたホンドアカネズミ *Apodemus speciosus speciosus* とホンドヒメネズミ *Apodemus argenteus argenteus* からの検出例[20, 21]，鹿児島県屋久島で捕獲され愛知県犬山市の日本モンキーセンターで5年から10年間放飼されていた4頭のヤクニホンザル *Macaca fuscuta yakui*[17, 19]，および北海道産エゾリス *Sciurus vulgaris orientis*[24]からの検出例が報告されているにすぎない。

病原性および宿主感受性の差

病　原　性

自然感染宿主の病状は明らかでない。*B. rodhaini* 実験感染での主な症状は貧血および血色素尿症であり，そのほか宿主の生体反応としては血小板減少症，続発性糸球体腎炎あるいは免疫複合体病などが一般に認められる[1]。

B. microti 寄生赤血球1×10^1～1×10^8個を成熟C57B1マウスに接種すると，1×10^1～1×10^3個接種群では末梢血液中における原虫の増殖は認められない。1×10^8個の接種では3.2日目に1×10^4個接種では15.4日目に原虫血症が観察される[3]（表1）。これら1×10^4～1×10^7個感染赤血球接種による原虫血症の出現期間は14.9～17.1日間である。1×10^8個感染赤血球接種マウスにおいても全例耐過生残する。

B. hylomysci 寄生赤血球を同様に成熟C57B1マウスに接種すると，1×10^8個の接種で3.0日目，1×10^4個接種では14.5日目に原虫血症が認められる。1×10^8個接種ではマウスは全例斃死するが，1×10^5個以下の接種では逆に全例生残する。

表1　*Babesia microti*, *Babesia hylomysci* および *Babesia rodhaini* 実験感染に対するマウスの応答

報告者	*B. microti* 寄生赤血球接種量				
	Gray & Phillips (1983)				
原虫寄生赤血球数	10^8	10^7	10^6	10^5	10^4
マウスの系統	C57 Bl	C57 Bl	C57/Bl	C57/Bl	C57/Bl
原虫最高寄生率(%)	67.7 + 8.0	54.9 + 4.6	42.0 + 4.1	33.0 + 8.2	30.5 + 3.4
原虫出現までの日数(日)	3.2 + 0.9	6.1 + 0.6	9.3 + 0.7	12.0 + 1.2	15.4 + 0.9
2%以上の原虫寄生率の期間(日)	21.3 + 1.2	17.1 + 1.0	14.9 + 1.2	16.1 + 2.4	15.9 + 2.1
感染後の生残日数	—	—	—	—	—
死亡数／感染数	0/6	0/6	0/6	0/6	0/6

報告者	*B. hylomysci* 寄生赤血球接種量				
	Gray & Phillips (1983)				
原虫寄生赤血球数	10^8	10^7	10^6	10^5	10^4
マウスの系統	C57/Bl	C57/Bl	C57/Bl	C57/Bl	C57/Bl
原虫最高寄生率(%)	54.3 + 10.2	49.8 + 13.4	32.0 + 3.9	32.5 + 5.5	20.8 + 3.7
原虫出現までの日数(日)	3.0 + 0.1	4.4 + 0.1	7.0 + 0.8	10.0 + 0.7	14.5 + 1.0
2%以上の原虫寄生率の期間(日)	—	12.2	15.3 + 1.8	16.4 + 3.6	15.8 + 1.7
感染後の生残日数	—	—	—	—	—
死亡数／感染数	6/6	5/6	2/6	0/6	0/6

報告者	*B. rodhaini* 寄生赤血球接種量		
	Han *et al.* (1982)		Ogawa *et al.* (1985)
原虫寄生赤血球数	10^8	10^4	10^2
マウスの系統	dd, ICR, BALAB/c		ICR-JCL
原虫最高寄生率(%)	60～70	60～70	41.5
原虫出現までの日数(日)	—	—	6.5
2%以上の原虫寄生率の期間(日)	—	—	—
感染後の生残日数	3～5	9～12	10～12
死亡数／感染数	15/15	15/15	14/14

—：報告なし

主要症状の貧血はバベシア寄生による赤血球膜破壊およびカリクレインの活性による赤血球減少に起因する。

循環血液中には可溶性抗原が原虫寄生赤血球とともに出現する。そのため，赤血球表面に免疫複合体が形成され，末梢赤血球の浸透圧抵抗が減弱し，赤血球の脆弱性が増加する。血漿中における上記複合体の形成はさらに免疫抗体による原虫破壊の回避にも関与する。

宿主感受性の差

Han らの報告（表2）によると[4]，6種のげっ歯類に *B. rodhaini* 寄生赤血球 1×10^8 個を接種すると，マウスでは高度な原虫血症と血色素尿症を呈して3～5日目に全例が死亡する。ハム

表2 *Babesia rodhaini* 感染に対するげっ歯類の生体応答 (Han *et al.*, 1982)

動物種	原虫寄生赤血球数	試験動物数	生体応答		死亡率(%)	生存期間(日数)
			原虫血症発現率(%)	血色素尿発現率(%)		
マウス						
dd	1×10^8	5	100	100	100	3, 4, 4, 4, 4
ICR	1×10^8	5	100	100	100	4, 4, 4, 4, 5
BALB/c/nu/nu	1×10^8	5	100	100	100	4, 4, 4, 4, 5
ゴールデンハムスター	1×10^8	5	100	20	20	11, S, S, S, S
スナネズミ	1×10^8	5	100	40	20	12, S, S, S, S
ラット	1×10^8	5	40	20	0	S, S, S, S, S
ハタネズミ	1×10^8	5	0	0	0	S, S, S, S, S
モルモット	1×10^8	5	0	0	0	S, S, S, S, S

S＝生残マウス

スター，スナネズミおよびラットでは原虫血症は全例に認められるが，血色素尿症および死亡率は減少し，ラットでは斃死しない。ハタネズミでは原虫血症は認められるが，血色素尿症はなく死亡例もない。モルモットでは全く感受性が認められない。

系統別(表1)には，*B. rodhaini* 寄生赤血球接種マウスでは，dd, ICR および BALB/c/nu/nu 系いずれのマウスにおいても1×10^4個接種で9～12日目に高度な原虫血症と重篤な貧血を伴って全例死亡する[4]。一方，ICR-JCL 系マウスに1×10^2個の *B. rodhaini* 寄生赤血球を接種すると，接種後約12日で重篤な貧血を呈して死亡する[15]。

このように，ネズミに寄生するバベシアは種属間で明らかに感受性の差が認められるが，同一原虫株に対しても宿主間で感染抵抗性の差がある。

病　　理

Satoh ら (1986)* の成績では，*B. rodhaini* 接種後10日目の胸腺は著しく萎縮し，結合織と細網細胞が大部分を占め，リンパ球の崩壊像などが認められる。脾臓の腫大は著明で，リンパ濾胞の疎鬆化と，リンパ球の変性および細網細胞の増殖が観察される。肝臓では肝細胞の混濁腫脹，巣状壊死，囲管性の単核球の集簇が随所に観察されるが，この単核球はTリンパ球ではない。

診断および治療

診　　断

原虫の確認：原虫の検出には一般に末梢血液を用いた血液薄層塗抹標本を作製し，ギムザ染色により赤血球内外の原虫を確認する。同時に，直径約1cm平方に広げた血液濃厚塗抹標本を

* Satoh,M. *et al.* (1986)： *Zentralbl, Bakteriol, Mikrobiol, Hyg. Ser.A.* (受理)

数枚作製し，十分に乾燥する。一方は，無固定のまま2～3%のギムザ染色液で4～5分染色し，溶血の起こったところでこれを捨て，新たに6～8%のギムザ染色液(pH6.5～7.0)で約30分～1時間染色する。他方は，アセトン固定後，蛍光抗体法によって原虫の検出を試みる。

ヒトなどへの感染が疑われる場合には，その血液（抗凝固剤添加）をマウスに接種し，経時的にマウスの血液を検査して原虫の有無を調べる。

血清診断：一般にはCF反応，間接赤血球凝集反応，毛細管内凝集反応，間接蛍光抗体法およびELISAなどによって特異抗体の有無を検索する。

治　療

バベシア原虫の駆虫には古くからアクリジン色素やキノリン誘導体など，種々の薬剤が用いられている。薬剤名と投与方法を下表に示す。

①アクリジン誘導体	Acriflavine, Trypaflavineなど	静脈内注射
②キノリン誘導体	Acaprin, Babesanなど	0.5～1.0mg/kg皮下注射
③ジアミンジン誘導体	ガナゼックなど	1.0mg/日/マウス連続3日間，筋肉内注射
④抗生物質	テトラサイクリン-A	0.05mg/日/マウス7日間連続，皮下注射で完治[8]

免疫および予防

免　疫

B. rodhaini 感染に伴うマウス体内での体液性抗体の消長は，感染後3～5日目にはIgM抗体が，5～7日目にはIgG抗体が出現する。感染後10日目以降では，IgG抗体価はIgM抗体価に比較してはるかに優勢であるが，IgM抗体も長期間検出される。このIgMの長期間の出現は，宿主体内におけるバベシア原虫の抗原変異が常時行われていることを示唆している[18]。

一方，Tリンパ球産生リンホカインおよびマクロファージが主役を演ずる細胞性免疫応答も *B. rodhaini* 感染10日目以降では明らかに発現している[15, 25]。*in vitro* の実験系では，バベシア免疫ハムスターのマクロファージあるいはマクロファージ培養上清の存在は，*B. microti* の *in vitro* 培養での分裂・増殖が有意に抑制される。

感染抵抗性に関与するリンパ球の動態では，特に胸腺内Tリンパ球が感染後10日目で感染前値の85%以上の減少率を示す。一方，脾臓および肝臓内Tリンパ球およびBリンパ球はともに感染後著増する。

このようなバベシア感染耐過ネズミでは相関免疫の状態にあり，長期間再感染に対して強い抵抗性を示す。

バベシア免疫マウスの抗体含有新鮮血清を実験的に健康マウスに移入すると，感染抵抗性はある程度賦与される。また，免疫マウスの脾臓内細胞を健康マウスに移入すると，そのマウスはバベシア感染に対して抵抗性を有する[26, 27]。

このようなことから，バベシア感染に対するマウスの感染防御能は体液性および細胞性免疫応答の相補的な共同作用によって形成されていると考えられる。

予　　防

媒介者であるマダニの予防と駆除（有機リン系，カーバメイト系などの殺虫剤散布）が第一義である。

ワクチン（生および不活化ワクチン）の開発が試みられているが，まだ完成されたものはない。他方，バベシア感染に対する宿主の感染死防御能の賦与効果の目的で非特異免疫原の開発が行われている[15]。

（本項の執筆に際し帯広畜産大学家畜生理学教室原虫病細胞免疫研究室・斉藤篤志氏および桜井治久氏の協力をいただいた。）

実験動物：マラリア

マラリア malaria とは mal(悪い)と aria(空気)の意味からなる。歴史的にみると，すでにヒポクラテスが紀元前400年にマラリアの熱型について記載している。原因が赤血球中に存在する *Plasmodium* 属原虫であることは1880年にフランスの軍医 Laveran により発見された。彼は北アフリカでマラリア患者の血液中に *Plasmodium falciparum*（熱帯熱型マラリア原虫）のガメトサイトや鞭毛放出をみつけた。1897年には，Ross がマラリア原虫はハマダラカ属 *Anopheles* の蚊によって媒介されると報告した。

本項では，主としてサルおよびマウスのマラリアについて概説するが，これらの動物に感受性を有する種類は多く，また共通する内容も多い。したがって，初めに総論として，これら実験動物のマラリアについての概要を述べ，次いで各種のマラリア原虫の特徴について述べる。

病　原　体

生活環および形態

Plasmodium 属原虫は有性世代を蚊の体内で，無性世代を脊椎動物（哺乳類，鳥類，爬虫類など）の組織と赤血球内で行う。ガメトサイトは必ず赤血球内に存在し，その赤血球内に血色素由来の褐色のマラリア色素 malaria pigment を産生する特徴を有している。

図にマラリア原虫の生活環を示した。

1．脊椎動物（中間宿主）体内における発育

蚊の吸血時に吻を通して注入されたスポロゾイトは直ちに血流を介して中間宿主の諸臓器，主として肝細胞に侵入する。ここで多数分裂しメロゾイトが生ずる。ここまでの過程は赤血球外発育 exo-erythrocytic multiplication あるいは pre-erythrocytic cycle と呼ばれ，感染後約1週間を要する。ただし，原虫の種類によっては肝細胞内にヒプノゾイトとして数カ月から数年にわたり存在するものがある。

肝細胞から放出されたメロゾイトは赤血球に侵入する。赤血球内原虫の初期発育期は環状の早期栄養体（環状体）となる。次いでトロホゾイトとなり，赤血球内に新しいメロゾイトが形成される。

メロゾイトの数は，種によってほぼ一定で，たとえば *P. inui* では通常約12個で最大18個，*P. knowlesi* では約16個，*P. malariae* では6～12個である。赤血球を破壊して流血中に遊出したメロゾイトはごく短時間内に未感染赤血球に侵入し，上記の分裂を繰り返す。この発育を赤血球内発育 intra-erythrocytic cycle と呼ぶ。

一方，きわめて少数ながら原虫の一部はミクロガメトサイトあるいはマクロガメトサイトとなり，蚊に吸血された場合にのみ発育を続ける。

2．蚊（終宿主）体内における発育

ガメトゴニー：ミクロガメトサイトは蚊の中腸内でミクロガメートとなるが，ミクロガメートはその形態が鞭毛に似ているため鞭毛放出と呼ばれる像を示す。

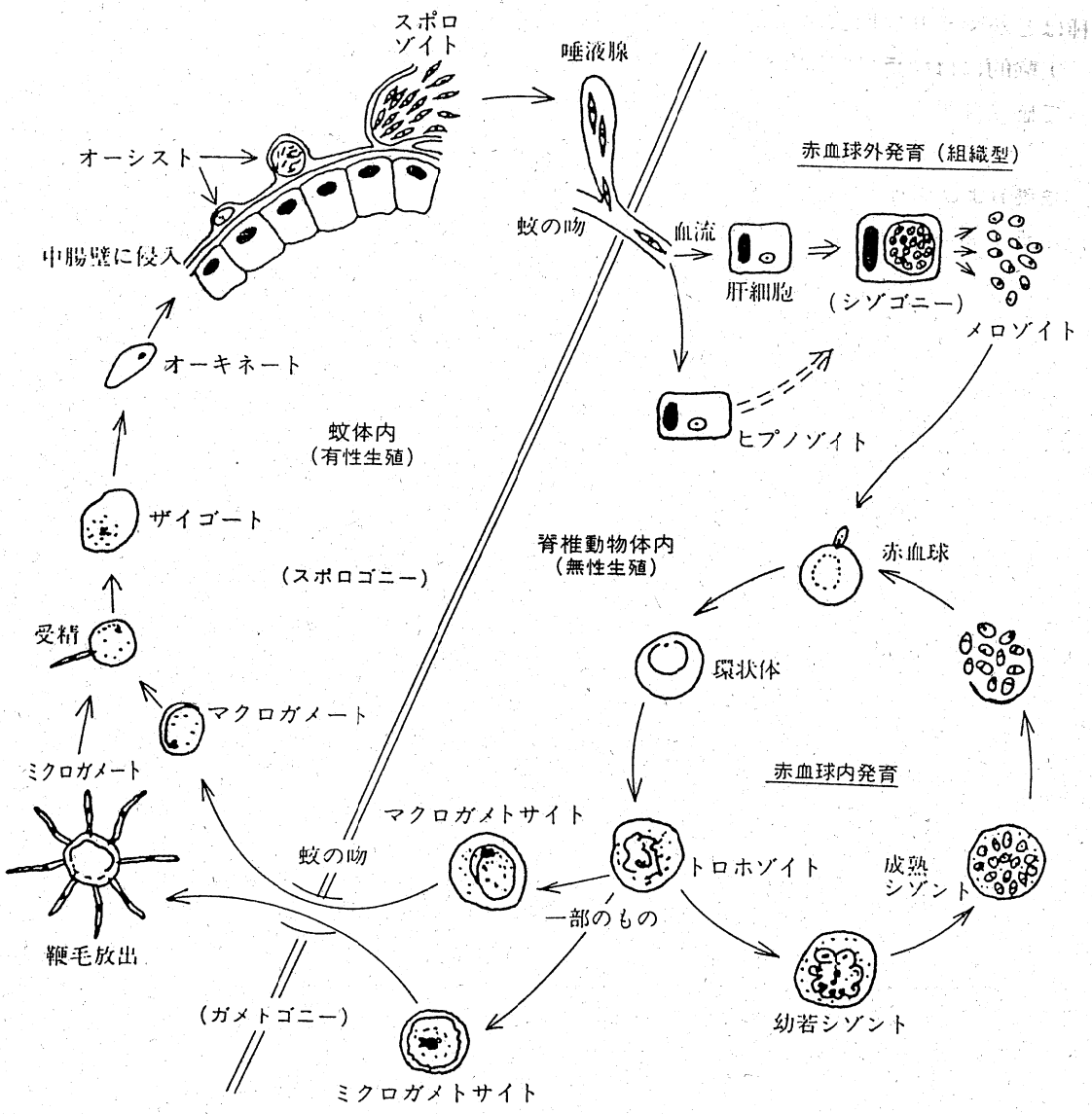


図　マラリア原虫の生活環

マクロガメトサイトがマクロガメートになる像はほとんど明らかでない。これまでの発育過程をガメトゴニーという。

スポロゴニー：ミクロガメートの受精後はザイゴートと呼ばれ，次いで運動性をもつオーキネートとなり中腸壁から最終的に腸管上皮に侵入し球状のオーシストを形成する。オーシスト内には徐々にスポロゾイトが形成される。この過程をスポロゴニーという。

オーシストが破れて放出されたスポロゾイトは蚊の唾液腺内に集まり，吸血時に吻を通じて脊椎動物体内に入る。

感　染

媒介昆虫であるハマダラカ属に属する蚊は世界に200種以上存在しているが，そのうちの60

種ほどがマラリア原虫の媒介に関与している。

実験的にはマラリア原虫感染動物の血液またはハマダラカ唾液腺内のスポロゾイト接種によって感受性動物は容易に感染する。

培養および保存

培養：赤血球内および赤血球以外のそれぞれの発育期での培養が数種のマラリア原虫で報告されている。

赤血球内発育段階　　1912年，Bass と Jhons により *P. falciparum* と *P. vivax* の短期間の培養が行われた。長期の連続培養は，現在までのところ *P. falciparum* のみが可能である。

サルマラリアでは *P. knowlesi* と *P. coatneyi* の短期間培養が可能である。

赤血球外発育段階　　1978年，Foley らがラットの胎仔肝細胞を培養し，*P. berghei* のスポロゾイトを感染させたところ，原虫がシゾントまで発育し，培養が可能となった。1983年には *P. vivax*，1985年には *P. falciparum* がヒト肝細胞を用いて可能となった。

保存：通常はグリセリンや糖を加えて−80℃や液体窒素中で長期間保存可能である。

① 感染血液に等量のグリセリンを加えて混合し，急速に凍結させる。

② グリセリン10%とした Alsever 液を用意し，この溶液3容量に対し血液1容量を加え混合し，急速に凍結させる。

培養中の *P. falciparum* 感染血球を凍結する場合には，感染血液を遠心後，凍害防止液(42%ソルビトール／生理食塩液180m*l* とグリセリン70m*l* を混合後，0.45mM フィルターで濾過し滅菌したもの)と赤血球沈渣とを等量に加えて5～10分室温においた後，急速に凍結する。

疫　　学

マラリア原虫は熱帯から温帯地方まで世界を広く覆っている。分布地域は一般にはハマダラカの存在と蚊体内での原虫発育に必要な温度，約16℃の夏期等温線が外周の限界と考えられている。すなわち，最高気温が月平均24℃以下で，ハマダラカの分布しない中央および南太平洋諸島などにはみられない。マラリアの発生状態には，一地域に急激に多発する流行性と常在的な浸淫性の2型があり，後者はしばしば前者へ移行する。

わが国のヒトでのマラリアは19世紀末まで全国的に流行し，北海道からも認められていた。三日熱マラリアが本土では土着していたが，沖縄県には熱帯熱，四日熱マラリアも流行した。1945年以降，ハマダラカによると推定される熱帯熱マラリアの散発，輸血マラリアあるいは熱帯・亜熱帯地方諸国との交流による輸入マラリアの症例が報告されている[12]。

症状および病理

症　　状

ヒトのマラリアでは3大徴候として，①発熱，②脾腫，③貧血があげられる。そのほかに肝腫，消化器障害，黄疸がみられ，熱帯熱マラリアでは脳障害，腎不全がみられる場合がある。

サル類の主要症状はヒトほど明瞭ではない。感染した原虫の種とサルの種類により発症の度合いが異なるが，一般に外見的には無症状の場合が多い。原虫血症が軽度から中等度，さらに

重度になるにつれて発熱，貧血，食欲減退がみられ，元気は沈衰し，やや憔悴を呈するようになる。摘脾した場合には一般的にマラリアは重度となる。

病　理

マラリア原虫感染による熱発作は定型的症状の一つであるが，これはマラリア・シゾント（繁殖体）が分裂を完了する時期に一致し，原虫分裂に際し放出されるメロゾイトとその代謝物，色素，崩壊赤血球残骸などによるとされる。したがって，貧血性無酸素血症，感染赤血球の毛細管栓塞，血管内皮細胞の障害などによる局所ないし全身的臓器組織の酸素欠乏が要因となる。特に，感染赤血球は互いに膠着しやすく，脳，肺，腎臓などの毛細管栓塞，小斑点状の出血斑を生じ，加えて内皮細胞の虫体貪食で閉塞像も認められる。

脾臓は充血腫大し，急性期にはうっ血状で軟らかい。慢性期の脾臓は硬く腫大し，ヘマチン沈着により暗黒褐色を呈する。肝臓は急性期に充血し色素沈着を認め，組織酸素欠乏による小葉中心性の壊死をきたす。腎臓は充血し，糸球体腎炎像を呈し各所に出血巣がみられる。肺毛細管は通常うっ血性で粘膜に点状出血，心筋の脂肪変性と冠状血管の閉塞性酸素欠乏がマラリアの主要死因になることが多い。

診断および治療

診　断

原虫の検出：血液中の原虫の存在を確認するのを第一とする。

血液中の原虫数が少ないときは濃厚塗抹標本を作り，固定せずにギムザ染色を30～45分間行う。原虫数がある程度多いときや形態を調べるときは薄層塗抹とし，メタノールで5分間固定後，ギムザ染色を行う。

採血時期により原虫の出現に大きな差のあるマラリア原虫があるので注意を要する。

血清学的診断：間接蛍光抗体法を用いて抗体の存在を検査する。

その他：発熱の周期性は臨床上有効な判定根拠となるので，体温の測定は重要である。

治　療

マラリアの治療には古くからキニーネ（Cinchona alkaloids）が使われているが，一般的には赤血球内原虫に対して4-アミノキノリン製剤であるクロロキン，レゾヒンが用いられている。またジアミノピリミジン（ピリメサミン，ダラプリム，トリメトプリム）やサルファ剤も赤血球内原虫に対して使用される。

8-アミノキノリン製剤（プリマキン，パマキン）は三日熱型，四日熱型および卵型マラリアの赤血球外（組織型）発育期原虫に対して用いられる。また熱帯熱マラリアの赤血球外発育期原虫に対してはbiguanideが有効である。

目安として体重あたりの投与量を示す。

クロロキン	5 mg/kg　筋肉内注射
ピリメサミン	15 mg/kg
プリマキン	0.64 mg/kg

付1　治療例

P. falciparum 感染ヨザルに対し

第1日目　クロロキン（5 mg/kg）筋肉内注射

ファンシダール*（0.1ml/kg）筋肉内注射

*Fansidar（Roche社）：2.5ml中にスルファドキシン500mgとピリメサミン25mgを含む

第5日目　同上の処置

なお，治療中の観察はもちろんのこと，治療後も回帰や再発に注意しなければならない[7]。*P. knowlesi* では回帰は起こらないが，*P. vivax, P. ovale, P. malariae, P. cynomolgi, P. fieldi, P. simiovale* では回帰が起こることが確認されている。

サルのマラリア
Simian Malaria

サルに寄生するマラリア原虫は世界中に約20数種と報告されている。ここではヒトのマラリア原虫の種を基本としたCoatneyら[1]の分類に基づき三日熱型，四日熱型，熱帯熱型，その他に分けて記述する。それぞれの型と原虫の種類を表1に示す。

三日熱型 vivax-type parasites

Plasmodium vivax（カラー写真7）：ヒトが自然宿主であるが，実験的にはチンパンジー，ヨザル，リスザルなどが感染可能である。

媒介蚊はハマダラカ属の *Anopheles stephensi*，*Anopheles balbacensis*，シナハマダラカ *Anopheles sinensis*，*Anopheles freeboni* などである。これらのハマダラカ属の分布は広く，

表1　サルに寄生する *Plasmodium* 属の種類

三日熱型	四日熱型	卵　型	熱帯熱型	その他
P. vivax	*P. malariae*	*P. ovale*	*P. falciparum*	*P. knowlesi*
P. cynomolgi	*P. brasilianum*	*P. fieldi*	*P. coatneyi*	*P. girardi*
P. eylesi	*P. inui*	*P. simiovale*	*P. fragile*	*P. lemuris*
P. gonderi	*P. rodhaini*		*P. reichenowi*	
P. hylobati				
P. jefferyi				
P. pitheci				
P. schwetzi				
P. simium				
P. youngi				

熱帯から亜熱帯，温帯に及び，中国東北部，北朝鮮，中近東北部など比較的寒冷な地方にも存在する。わが国にも以前から分布している。

感染赤血球は正常の赤血球に比べて大きくなり，1個の赤血球中に1個，ときとして2個の原虫が寄生する。ギムザ染色では感染赤血球内に赤色の小さなシュフナーの斑点 Schüffner's dots がみられる。

分裂体は12～18個のメロゾイトを有する。

赤血球内発育に要する時間は約48時間である。第1発熱日から数えて3日目に次の発熱があるので三日熱マラリア tertian malaria といわれる。

ミクロガメトサイトはマクロガメトサイトに比べてやや小さく，ギムザ染色で核は赤く染まり中央に存在するが，染色性は弱い。

マクロガメトサイトは直径12～14μm のほぼ円形で，核は辺縁に存在する。

肝臓内にあるすべての虫体がいちどきに血流中に出ず，ときとして数カ月から数年後に血流中に出現して回帰を起こす。

P. cynomolgi：ジャワからドイツに輸入されたカニクイザル *Macaca fasucularis* から検出された。赤血球内発育に48時間を要する。*P. cynomolgi* の感染は *Macaca* 属のサルに広く認められ，オマキザル，オナガザルなどにもみられる。ヒトの感染例も報告されている。

分布は東南アジア各地で，マレーシア，ビルマ，カンボジアなどである。

P. eylesi：マレーシアのテナガザルで発見された。アカゲザルに感染実験を行ったが成功していない。

P. gonderi：マンガベーやドリルが自然宿主で，赤血球内発育時間は48時間である。アフリカ西海岸とカメルーンに分布する。

P. hylobati：ジャワ産のテナガザルから検出され，ジャワ，ボルネオ地域に分布する。

実験的にヒトに対して感染蚊を吸血させても発症しない。

P. jefferyi：西マレーシアのテナガザルから検出された。

P. pitheci：1905年，パリで Laveran がオランウータンにサルマラリアとして初めてみつけた。次いで1907年，Harbertädter と von Prowazek がボルネオのオランウータンで発見し *P. pitheci* と名づけた。オランウータン以外のサルやチンパンジーでは発症しないと考えられている。

P. schwetzi：*P. vivax* に非常に似ている。感染はチンパンジー，ゴリラに限られるが，実験的にはヒトにも感染可能である。

アフリカのコンゴ，リベリア，シェラレオネなどに分布する。

P. simium：1939年，ブラジルで黄熱病の研究をしていた Fonseça がホエザルの血液中にみつけた原虫で，当初 *P. brasilianum* と考えていたが，その後1951年に別な種と分かり，彼は本原虫を *P. simium* と命名した。

ホエザル，クモザルにみられ，分布はブラジルの一部の地域に限られている。

P. youngi：テナガザルの類にみられ，タイ，マレーシアなどに分布する。

四日熱型 malariae-type parasites

P. malariae：自然宿主はヒトであるが，チンパンジーにも感染する。また実験的にはヨザル

も感染する。

分布は広く熱帯から亜熱帯に及び，わが国の八重山群島にも存在していた。

感染赤血球は大きくならないが，原虫の環状体は *P. vivax* に似ている。トロホゾイトは帯状体 band form と呼ばれる形態を呈し，赤血球を横断していることもある。通常，1赤血球中に1個の原虫が寄生するが，シュフナー斑点はみられない。

赤血球内発育には72時間を要する。

ヒトでは発熱が第1日目の次に第4日目に起こるため四日熱マラリア quartan malaria と呼ばれる。

分裂体内のメロゾイトは6～12個，ときとして14個みられ，菊花状に並ぶことが多い。ガメトサイトは *P. vivax* に似ているが少し小さい。

P. brasilianum：ブラジル（アマゾン）から西ドイツ，ハンブルグ市に輸入されたウアカリから発見された。

パナマ，ベネズエラ，ペルー，ブラジル，コロンビアなどの南米に分布し，ホエザル，クモザル，リスザル，オマキザル，ウーリーモンキーなどに感染する。

ヒトへの感染報告がある。

P. inui（カラー写真5）：ジャワ，ボルネオ，スマトラ，インドなどのアジア地域に広く分布する。カニクイザル，ブタオザル，クロサル，ハヌマンモンキーなどに感染がみられる。

ヒトへの感染例が報告されている。

同じ四日熱型である *P. brasilianum*（新世界ザルに感染）と *P. inui*（旧世界ザルに感染）のアイソザイム分画値は非常に異なっている。

P. rodhaini：*P. malariae* に非常に似ており，同一と考えられている。1920年，Reichenow がカメルーンのチンパンジーから発見した。ヒトに感染する。

卵型 ovale-type parasites

三日熱型および四日熱型マラリアが原虫の赤血球内発育時間の差異で区別されているのに対し，卵型は形態学的な相違から区別された。

P. ovale（カラー写真6）：赤血球内にトロホゾイトが寄生しているときに赤血球が卵型を呈することが多いことから卵型マラリア ovale malaria といわれる。感染赤血球はやや大きくなる。

シュフナー斑点が認められる。通常，赤血球中に1個の原虫が寄生する。

分裂体は6～12個のメロゾイトを有し，赤血球内発育時間は48時間である。ヒトが自然宿主であるがチンパンジーにも感染する。ヨザルにも感染は可能である。

卵型マラリアはアフリカの赤道を中心とした熱帯域が主な発生地であるが，その他，*P. ovale* はインド，フィリピン，タイ，ベトナム，パプアニューギニアでも検出されている。

P. fieldi：マレーシアのブタオザルから発見された。

赤血球内発育時間は48時間で，分裂体中のメロゾイトは4～16個，平均12個で大きい。

マレーシアの *Macaca* 属のサル（ブタオザル，アカゲザルなど）にみられる。

P. simiovale：スリランカの *Macaca sinica* から検出された。実験的には *Macaca mulatta* にも感染する。赤血球内発育時間は48時間である。

熱帯熱型 falciparum-type parasites

P. falciparum（カラー写真8）：本種によるマラリアは熱帯熱マラリア tropical malaria あるいは悪性マラリアといわれる。悪性のため適切な治療を要する。

ヒトが自然宿主であるが，実験的にはチンパンジー，ヨザル，テナガザル，マーモット，リスザル，オマキザルなどが感染する。

約66種のハマダラカにより媒介される。*P. falciparum* の分布は広く，熱帯から亜熱帯に及ぶ。わが国でも八重山群島に存在していたが，マラリア対策により1960年以後絶滅した。

本種の特徴は環状体が他のものより少し小さいことで，その環状体の原虫が赤血球に2～3個寄生しているのがよくみられる。感染赤血球にはマウレル斑点 Maurer's dots が観察される。分裂体は通常，末梢血液中にはみられず，脳や心臓などの毛細血管中でみられる[3]。

赤血球内発育時間は36～48時間であるが，三日熱型，四日熱型および卵型のマラリアと異なり，個々の原虫が同調化せず増殖するため，患者は不規則な熱型を示す。

ガメトサイトは鎌状，もしくは半月状を呈している。肝臓内で形成されたメロゾイトがすべていちどきに放出されるので，回帰はないが，薬剤治療後，血液中に生き残っていた原虫が増殖し再び発症することがある。これを再発という。

現在，クロロキンやピリメサミンなどの治療薬に対して耐性を示す原虫が出現している。

P. coatneyi：本種はカニクイザルなどに感染がみられ，フィリピン，マラヤなどに分布する。

形態学的には *P. knowlesi* に似るが，赤血球内発育時間は48時間で，周期性からみると三日熱型である。1赤血球中に数個（4個以内）の原虫が感染しているのがみられる。感染赤血球の膨化はみられない。寄生赤血球内にはマウレル斑点がみられる。ガメトサイトは三日熱型に似ているが，ミクロガメサイトの核は大きい。

P. fragile：*Macaca* 属のサルに感染する。1赤血球に2～3個の原虫が寄生しているのがよくみられる。赤血球内発育に48時間を要し，ガメトサイトは完全な半月形ではなく卵の形に似る。

P. reichenowi：形態学的に *P. falciparum* によく似ている。1917年に Reichenow がカメルーンのチンパンジーとゴリラに発見し，ヒトの *P. falciparum* と同一とみられた。その後，実験的にヒトに感染させても *P. falciparum* 感染のような発症はしないことが確認され，*P. reichenowi* と名づけられた。

その他の *Plasmodium* 属原虫

P. knowlesi：本種はアジアの広い地域に分布し，*Macaca* 属のサルやテナガザルなどに感染する。アカゲザルなどでは感染すると通常は死亡する。

環状体は *P. falciparum* に似るが，トロホゾイトの形態は四日熱型マラリアを思わせ，帯状形を呈することがある。分裂体中のメロゾイトはおよそ16個ぐらいである。赤血球内発育時間は24時間とみられており，quotidian species（一日熱型マラリア）といわれる。

P. girardi：本種はマダガスカルのキツネザルを摘脾した際，末梢血液中に原虫が増加し発見された。他のサルのマラリア原虫に比べ環状体は小さい。感染赤血球は膨化せず，分裂体は

10～12個のメロゾイトを有する。

P. lemuris：マダガスカルのキツネザルにみつかった。

早期のトロホゾイトは小さく，赤血球の1/3～1/4を占めるにすぎない。トロホゾイトは不整形を呈し，アメーバ状となる。ガメトサイトは非常に大きく不整形をとる。

注）キツネザルのマラリア原虫についての研究成果は少なく，今後の研究によっては分類学上変化することがあり得る。

付　わが国の輸入サルにおけるマラリア

わが国に輸入されたサルにマラリア原虫の感染がみられており，飼育には十分の注意が必要である。

サルのマラリアがヒトに自然感染した報告は少数ではあるが1960年代からあり，人畜共通原虫病の一つと考えられている。さらにサルのマラリア原虫の実験室内での感染例の報告もあり，感染血液の取り扱いに十分の注意を払うと同時に媒介蚊への配慮も必要である。

輸入カニクイザルのマラリア原虫の感染頭数

原産国	感染頭数/検査頭数	種類
1971年12月～1972年11月[16]		
マレーシア	49/115	*P. inui*
フィリピン	22/120	*P. inui*
インドネシア	50/354	*P. inui*
1973年～1975年[5]		
マレーシア	1/14	*Plasmodium* sp.
インドネシア	1/39	*Plasmodium* sp.

ネズミのマラリア
Murine Malaria

ネズミのマラリア原虫は通常，4つのグループに分けられ，それぞれ亜種をもつ[2]。すべてがアフリカで発見された。

ネズミに寄生するマラリア原虫を表2に示した。

表2 ネズミに寄生する *Plasmodium* 属の種類

	分布	自然宿主
P. berghei グループ		種々のマウス，ラットおよびハムスター
P. berghei Vincke and Lips, 1948	ザイール・カダンガ州高地，中央アフリカ	
*P. yoelii** *yoelii* Landau and Killick-Kendrick, 1966	中央アフリカ	
P. yoelii killicki Landau, Michel and Adam, 1968	コンゴ	
P. yoelii nigeriensis Killick-Kendrick, 1973	ナイジェリア	
P. vinckei グループ		種々のラットおよびマウス
P. vinckei vinckei Rodhain, 1952	ザイール・カダンガ州	
P. vinckei petteri Carter and Walliker, 1975	中央アフリカ	
P. vinckei lentum Landau, Michel, Adam and Boulard, 1970	コンゴ	
P. vinckei brucechwatti Killick-Kendrick, 1975	ナイジェリア，中央アフリカ	
P. chabaudi グループ		マウス，ラット
P. chabaudi chabaudi Landau, 1965 Partim, Carter and Walliker, 1975	中央アフリカ	
P. chabaudi adami Carter and Walliker, 1977	コンゴ	

* *P. yoelii* グループは主に *Thamnomys rutilans* に感染している

実験感染による症状および病理（病態）

ヒトのマラリアの基礎的研究における実験室内モデルとしてネズミに感染する *Plasmodium* 属，特に *P. berghei*（カラー写真3，4），*P. vinckei* および *P. chabaudi* が用いられる。特に，マラリア原虫感染に伴う宿主の病態生理学的研究にはマウスおよびラットが用いられているが，個体の経時的変化の検討にはラットがよい。

鈴木らの一連の実験において[8~11,13~15,18,20]，50頭のラットに 4×10^7 感染赤血球接種による *P. berghei* 感染ラットの血液・血清諸性状では，感染後14日目まで漸次貧血度を増し重篤な大球性高色素性貧血を示し，以後50日目までに感染前値に復する。

赤血球内マラリア原虫の出現は感染後14日目に，網赤血球は感染後21日目にそれぞれ最高値を示し，以後漸次減少し感染前値に戻る。

また，感染後1～2週では末梢血液中単球が著増し，それらの多くは空胞を有し退行性変化を示す。赤色骨髄内の骨髄球対赤芽球細胞比では感染後21日目を頂点とする赤芽球細胞の増加と，形質細胞の相対増加が観察されている。

血清諸成分の変動ではLDH，GOT，GPTおよびビリルビンが感染後著増し，以後減少する。ヘモシアニンは感染後減少するが，その後はあまり変動しない[8]。血清蛋白質分画像の推移では，感染後アルブミンの減少とグロブリンの増加，特に γ-グロブリンの増加が認められる。

病理組織学的所見では，感染初期に肝毛細血管内皮細胞の腫大と，壁着性に色素の貪食像を認める。脾髄における細網内皮細胞の増数と円形化，そして旺盛な造血像がしばしば観察される。感染後8週目に至っても肝臓および脾臓内の色素貪食はきわめて著しいが，造血像はほとんどなく，脾濾胞も正常に近く復する[18]。

P. berghei 感染ラットでは原虫接種後６日に IgM および IgG 抗体がともに検出され，８週以降も両抗体は比較的高値を維持する[9]。この８週経過したラットに再度 *P. berghei* を接種すると，両抗体価は上昇する。感染後60日および180日経過したラットを摘脾すると，前者のラットは軽度な原虫血症を示すが，後者は認めない。このようなラットに再び *P. berghei* を接種すると前者のラットは感染抵抗を示し生残するが，後者は貧血と原虫血症の進行とともに斃死する[9]。

一方，マラリア原虫慢性感染ラットから生まれた新生仔は出生時移行 IgG 抗体を保有しているが，生後５週まで漸減し，７週では検出されない[14]。ラットは妊娠中に *P. berghei* を感染しても胎仔への垂直感染は認められない[11]が，これらの新生仔は出産直後におけるマラリア原虫感染に対して強い抵抗性を示す[15]。しかし，出産直後に胸腺あるいは脾臓を摘出すると，これらの新生仔はマラリア原虫感染に対する抵抗性を失い高度の貧血および原虫血症を示し死亡する[14]。

このように，ラットのマラリア原虫感染に対する抵抗性には，液性抗体が重要であるが，それと同様に胸線，Ｔリンパ球およびマクロファージの相互作用が必要不可欠な条件である[8,10,13,15]。

マラリア原虫感染抵抗賦与物質やワクチンに関する研究は多い[9,15]。これらの基本は純粋かつ大量の原虫採集法の開発であり，*P. berghei* においても試験管内培養が成功している[19]。感染赤血球の分離法も感染赤血球膜蛋白構造の変化や表面荷電の差異による無担体電気泳動法や濃度勾配比重遠心法，セルロースカラム法など試みられているが，まだ完全な抗原精製法は実験的にも成功していないのが現状である[2,4,6,17]。

（本項の執筆に際し帯広畜産大学原虫病細胞免疫研究室宮土禎雄氏ならびに五十嵐郁男氏ら群馬大学医学部寄生虫学教室，長崎大学熱帯医学研究所原虫部門の協力をいただいた。）

実験動物：トリパノソーマ病

Trypanosoma 属は宿主の血液および組織内に寄生する鞭毛虫で，熱帯地方ではこの原虫によるヒトおよび家畜の被害は甚大である。*Trypanosoma* 属は吸血性無脊椎動物を媒介者とし，媒介者の体内で発育，増殖のうえ，それらの刺咬・吸血時にその吻から直接侵入するもの(Salivaria トリパノソーマ) と，媒介者の吸血時に糞便とともに排泄された原虫が皮膚の創傷面あるいは皮膚粘膜から宿主に侵入するもの(Stercoraria トリパノソーマ)がある(表1)。さらに形態学的にあるいは宿主特異性，媒介者の特異性，地理的分布などによって，多くの種に分類される。表2に Salivaria トリパノソーマの分類を示す。

トリパノソーマに関する研究には，*Trypanosoma brucei gambiense*, *T. b. brucei*, *T. evansi* などが多く用いられ，*T. b. brucei*，*T. evansi* はヒトへの病原性がなく，実験上安全性が高い。*T. evansi* は，ツェツェバエの刺咬・吸血によって媒介される *T. brucei* が，アブ，サシバエによって機械的に媒介されるように進化して世界中に広がった種であることが形態学的に示唆されている[4]。

病　原　体

形　態

トリパノソーマ科の原虫は，細胞膜の内側に位置する微細小管によって保持された紡錘形の単核細胞である。縦二分裂によって増殖するが，核外DNAを含むキネトプラストが核に先立って分裂し，発育段階によってその位置を変え，核との位置関係から，それぞれアマスティゴート型，プロマスティゴート型，エピマスティゴート型，トリポマスティゴート型と呼ばれる(図1)。

鞭毛は体の後端近くにある生毛体から出て虫体前端に伸び，虫体との間に波動膜を形成する。多くは体内に色素に濃染するボルチン顆粒を有する。

表1　*Trypanosoma* 属の性状

	Stercoraria トリパノソーマ	Salivaria トリパノソーマ
キネトプラストの形とその位置	大型．トリポマスティゴートの後端より離れたところ	小型．トリポマスティゴートの後端
虫体後端	鋭	鈍
自由鞭毛	常にある	ないものもある
哺乳動物での発育	アマスティゴート，エピマスティゴート型	トリポマスティゴート型
媒介者体内での発育	後腸．*T. rangeli* は唾液腺，吻でも発育する	唾液腺，吻
感染方法	接触感染．*T. rangeli* は接種感染も	接種感染．*T. equiperdum* は交接によるあり，強いものが多い
病原性	*T. cruzi* 以外はない	
種	*T. cruzi*, *T. rangeli*, *T. theileri*, *T. lewisi* など	*T.b. gambiense*, *T.b. rhodesiense*, *T.b. brucei*, *T. evansi*, *T. equiperdum* など

表2　Salivariaトリパノソーマの分類

種　類	感染する動物	感受性をもつ動物	媒介者	分　布	病原性
T. vivax	反すう動物，馬，犬	げっ歯類	ツェツェバエ	熱帯アフリカ，モーリシャス，南米	あり：スーマ
T. uniforme	反すう動物	なし	ツェツェバエ	中央・東アフリカ	あり
T. congolense	反すう動物，馬，豚	げっ歯類	ツェツェバエ	熱帯アフリカ	あり：ナガナ
T. simiae	豚	サル，ウサギ	ツェツェバエ	熱帯アフリカ	あり
T. suis	豚	なし	ツェツェバエ	ザイール，タンザニア	あり
T. b. brucei	あらゆる家畜	げっ歯類	ツェツェバエ	熱帯アフリカ	あり：ナガナ
T. b. rhodesiense	ヒト，カモシカ	げっ歯類	ツェツェバエ	東アフリカ	あり：ヒト睡眠病
T. b. gambiense	ヒト	げっ歯類	ツェツェバエ	熱帯アフリカ	あり：ヒト睡眠病
T. evansi	犬，馬，ラクダ	げっ歯類	アブ，サシバエ	世界的	あり：ズルラ
T. equiperdum	馬	ウサギ	性的接触	南欧，アジア，北アフリカ	あり：媾疫

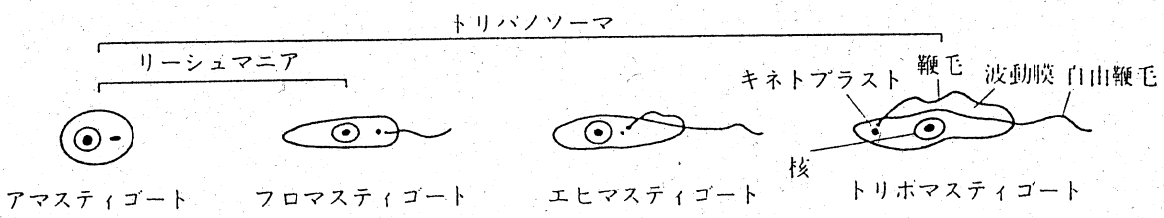


図1　トリパノソーマ科原虫の発育型

生活環

Salivariaトリパノソーマの多くはツェツェバエ（*T. evansi* ではアブ類）が，Stercorariaトリパノソーマ *T. cruzi*，*T. rangeli* ではサシガメ類が，*T. lewisi* ではネズミノミが媒介者となる。Salivariaトリパノソーマと Stercorariaトリパノソーマの生活環をそれぞれ図2と図3に示す。

T. congolense，*T. b. gambiense* などはツェツェバエによって媒介されるが，宿主血液中のトリポマスティゴートは細長型，中間型，ずんぐり型などの多形性を示し，分裂・増殖するのは細長型である。

宿主血液中のトリパノソーマ原虫では，TCA 回路が機能せずミトコンドリアは萎縮している。吸血によってツェツェバエの中腸に侵入した原虫では TCA 回路が作動し始め，それと同時に外被は消失し，この時期の原虫には感染力がない。その後，ハエの唾液腺に移行しエピマスティゴートとなり分裂・増殖した後，再び外被を備え感染能力を有するトリパノソーマ型となるが，これを特に発育終末トリパノソーマ型，発育終末トリポマスティゴート（metatrypomastigote）と称する。

T. evansi はアブ類により機械的に伝播されるので媒介者内における特有の発育形態は存在しない。シャガス病の病原体である *T. cruzi* は，宿主血液内ではトリポマスティゴート型を，

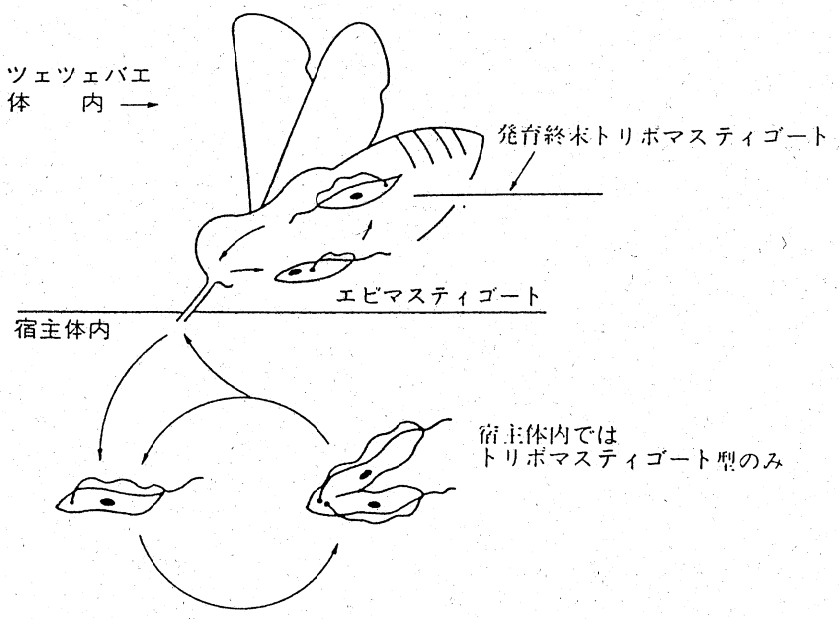


図2　*Trypanosoma b. gambiense* と *T.b. rhodesiense* の生活環

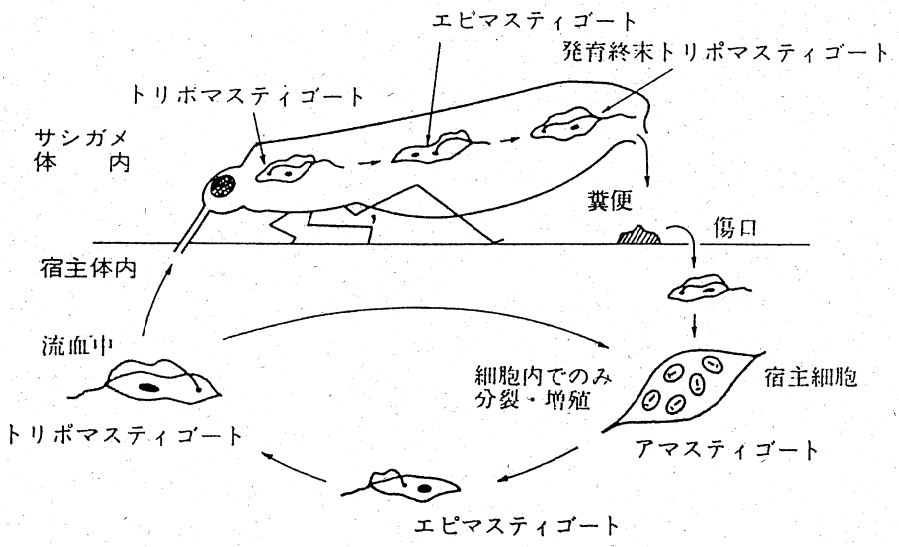


図3　*Trypanosoma cruzi* の生活環

筋肉その他の組織内ではアマスティゴート型をとり，媒介者のサシガメ腸管内ではエピマスティゴート型，アマスティゴート型を経て，発育終末トリポマスティゴート型となり糞便とともに外界に出る。

感染性

ヒトに寄生するものは*T. cruzi*（カラー写真9，10，163），*T. rangeli*, *T. lewisi*, *T.b. gambiense*, *T.b. rhodesiense*（カラー写真11）の5種である。*T. congolense*, *T. b. gambiense*などの実験動物への感染は感染血液の腹腔あるいは皮下接種により容易であるが，継代を続けると，多形性は失われ，ほとんどが細長型のいわゆる単形性となる。

ドブネズミやクマネズミなどのネズミ類の流血中にみられる*T. lewisi*は，実験的にモルモットには感染するが，マウスを含む他の動物には感染しないとされている。一般に非病原性であるが，研究室のラットに本種によると思われる四肢の関節炎がみられたという報告があり[5]，宿主に直接被害を与える可能性もあることから，実験成績への影響を考慮する必要がある。

*Trypanosoma*属の実験動物および媒介者からヒトへの感染の可能性は種により一定でないが，*T. cruzi*などでは重篤な実験室内感染が起こり得るので十分な注意が必要である。

培養および保存

培養：ほとんどの*Trypanosoma*属原虫は，培地あるいは培養細胞を用いて培養することができる。*T. b. gambiense*, *T. b. rhodesiense*などについてはTobieらの培地[12]，Weinman培地[13,14]などで患者からの分離培養は可能であるが，動物で継代を経た個体は培養できない。*T. cruzi*, *T. rangeli*, *T. theileri*などはN.N.N.培地などで容易に培養できる。また，ラット，マウスの胎仔心筋あるいは神経芽細胞などを用いて培養することも可能である。

培養については牛のトリパノソーマ病で詳述される。

保存：凍結保存は比較的容易で，感染血液に10%の割合でグリセリンを加え，液体窒素で急速凍結し保存できる。解凍にあたっては緩徐に行うことが必要である。

症状および病理

症　状

種により病原性は異なり，*T. rangeli*や*T. theileri*のように病原性がほとんどないものから，*T. b. rhodesiense*のように急性症状をとるものまでさまざまである。また，宿主となる動物によってもその症状・病理は異なる。一般に，媒介者による刺咬部位に痛み，あるいはかゆみを伴う腫瘤を形成するものもあり，1～3週間の潜伏期間をおいて，発熱，頭痛，リンパ節腫大，肝腫，貧血などの症状が進行する。末期には中枢神経障害を引き起こし，昏睡状態や知覚麻痺をきたし，放置すれば栄養失調，進行性衰弱，ついには悪液質で死亡することが多い。一般症状は原虫の消長が深く関与するとされている。

病理（病態）

トリパノソーマ病の病態を大別すると，1) *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense*などの体液，組織細胞内増殖による組織壊死および炎症性変化と続発性貧血，2) *T. congolense*, *T. vivax*などによる赤血球および血管障害による貧血，3)その他となる。

T. b. brucei 1 ×10²個をラット腹腔内に実験的に接種すると，約10日で骨髄，脾臓および肝臓内増殖像，脾腫と肝充血を認め，重度な貧血と末梢血液中単球増多を主徴に，重篤な原虫血症を呈し多くは20日以内に死亡する。Jenningsら（1974，1976）によると，ネズミへの *T. b. brucei* 感染に伴う貧血は骨髄および脾臓内での大球性正色素性溶血性貧血が主因で，赤血球寿命の短縮，特に脾臓内赤血球の貪食によるヘモジデリン沈着が認められ，血管外破壊が原因である[8,9]。しかし，マウスでの *T. b. brucei* 実験感染では，このような特徴的赤血球機能の変化はほとんど観察されていない[8]。そして，赤血球破壊の亢進は赤血球膜表面に付着したトリパノソーマ抗原と抗体および補体の免疫複合体による貪食細胞の貪食亢進と解釈されている。

トリパノソーマ自体の代謝産物あるいは毒素による宿主に対する直接影響について，Seed（1972）はモルモットを用いた実験で，肝臓内貯蔵グリコーゲンおよび血糖値の減少[10]，あるいは血清中全脂質，マクログロブリンおよび血清K^+の増加[3]，IgM濃度の増量[1]などをみている。同様に，*T.b. brucei* 感染に伴う毛細血管の病理変化は単核球の集積と血管破壊が主徴で[2]，原虫感染組織内単核球の集簇と壊死巣の発現には細胞性免疫の関与を示唆している[6]。

T. congolense 感染によるネズミでは，貧血とともにリンパ節腫脹や心筋の出血斑，脾腫，肝充血および骨髄うっ血障害などが認められる。

診断および治療

診　断

病原学的診断法：持続性頻脈，発熱，リンパ節腫脹などの臨床症状からの診断も可能であるが，確定診断は病原体の直接検出による。

発病初期には，血液または粘液中を活発に運動する原虫を容易にみつけることができるが，慢性期には，末梢血液の濃厚塗抹標本，骨髄およびリンパ節などの穿刺標本によっても検出は必ずしも容易ではない。

また，培養も併用される。

T. cruzi などでは実験室内で飼育した正常なサシガメに被検者を吸血させ，その体内で増殖した原虫を観察する外因（体外）診断法 xenodiagnosis が最も信頼度が高いとされている。

被検血液をマウス，ラットなどの実験動物に腹腔接種する方法も用いられるが，*T. b. gambiense* では，最初から小動物への感染が難しいので，まずサルへの接種が必要である。

種の鑑別は薄層塗抹標本をギムザ染色し，形態学的に観察する。

血清診断：CF反応，間接蛍光抗体法，ゲル内拡散沈降反応，間接赤血球凝集反応，，ELISAなどが用いられる。

抗原としては培養原虫が広く用いられているほか，濃厚感染血液からの原虫抽出抗原も用いられる。

T. b. gambiense では感染血液をDEAE-セルロースカラムに通すことによって原虫の純化が可能である。また，*T. b. gambiense* に感染した宿主のIgM量が著明に増加することを利用したNapierの血清膠化反応が診断に慣用されている。

治　療

T. b. gambiense および *T. b. rhodesiense* によるアフリカ睡眠病では，中枢神経が侵され

ない中期には，ヒ素を含まない suramin（商品名：Germanin，Antrypol），pentamidine（商品名：Lomidine），puromycine などが用いられる。後期にはヒ素剤の tryparsamide，melarsoprol（Mel B），melarsonyl（Mel W），ニトロフラゾン（商品名：Furacin）などが利用されるが，十分な効果は期待できない。

T. cruzi には nifurtimox（商品名：Lampit），benznidazole（商品名：Radanil）があるが，治療はなかなか困難であり，予防に力を注ぐべきである。

免疫および予防

免　疫

T. b. brucei，*T. b. gambiense* などは宿主体内で抗原変異を起こし免疫学的にも興味深い。血液中で増殖した原虫は，宿主の抗体作用によりほとんどが死滅するが，生き残った少数の原虫は抗原型を変えて増殖を始める。この抗原型に対する抗体が原虫に作用するとさらに別の抗原型をもつ原虫が出現するという具合に抗原変異を繰り返すので，治療によっても根治が難しい。この変異抗原は原虫表層を覆う外被に存在する糖蛋白質からなり，その解析もかなり進んできた。

予　防

予防は媒介する節足動物の防除およびその刺咬を避けるにあるが，一部予防的薬剤内服も短期間の効果をあげている。ツェツェバエは住間吸血性，サシバエは夜間吸血性であり，いずれも雌雄とも吸血する。シャガス病流行地では，輸血による感染，胎盤感染，母乳による感染にも十分な注意をする。

（本項の執筆に際し，徳島大学医学部寄生虫学教室長沢秀行氏の協力をいただいた）

実験動物：リーシュマニア病

リーシュマニア病はトリパノソーマ科 *Leishmania* 属原虫の感染によって起こるヒトおよび動物の感染病である。*Leishmania* 属原虫[7]は宿主の血液，肝臓，脾臓，骨髄，リンパ節，小腸などの細網内皮系細胞内に寄生する。

Leishmania 属原虫には，内蔵リーシュマニア症（カラ・アザール，ダムダム熱）の病原体である *L. donovani*（カラー写真12～14），皮膚リーシュマニア症（東洋腫瘤 Oriental sore）の病原体である *L. tropica*（カラー写真15），皮膚粘膜リーシュマニア症（アメリカリーシュマニア症；エスプンディア espundia）の病原体である *L. braziliensis*（カラー写真17）など多くの種がある（表）が，これらの形態はきわめて類似しており，形態学的に種を鑑別することは困難である。

病　原　体

生活環

Leishmania 属原虫の生活環の完結（図）には媒介者であるサシチョウバエを必要とし，

表　動物に寄生する主な *Leishmania* 属の種類

種	感染する動物	媒介者	分布	病名
L. donovani (Laveran and Mesnil, 1903) Ross, 1903	ヒト，犬	サシチョウバエ	中国，インド，中近東，中南米，地中海沿岸，熱帯アフリカ	カラ・アザール
L. tropica (Wright, 1903) Lühe, 1906	ヒト，犬，げっ歯類	サシチョウバエ	地中海沿岸，インド西北部，中近東，ソ連南部，赤道アフリカ	東洋腫瘤
L. mexicana mexicana (Biagi, 1953) Lainson and Shaw, 1972	ヒト，げっ歯類オポッサム	サシチョウバエ	メキシコ，グアテマラ，アマゾン川流域	ベイソール，チクレロ潰瘍
L. enriettii Muniz and Medina, 1948 **(カラー写真16)**	モルモット*	不　明	ブラジル	
L. peruviana Velez, 1913	ヒト，犬	サシチョウバエ	ペルー	
L. infantum Nicolle, 1908	犬，キツネ，ヤマアラシ，ヒト	サシチョウバエ	地中海沿岸，中近東，ソ連南部，中国，アフリカ	小児リーシュマニア症
L. chagasi Marques da Cunha and Chagas, 1937	キツネ，犬，猫，ヒト	サシチョウバエ	中央アフリカ，南米	アメリカカラ・アザール
L. braziliensis braziliensis (Vianna, 1911) Pessoa, 1961	ヒト，サル，げっ歯類	サシチョウバエ	中南米，特にブラジル，ペルー	フェリダブラボ，皮膚粘膜リーシュマニア症（ウタ uta）

* 実験用モルモットに発見された[11]

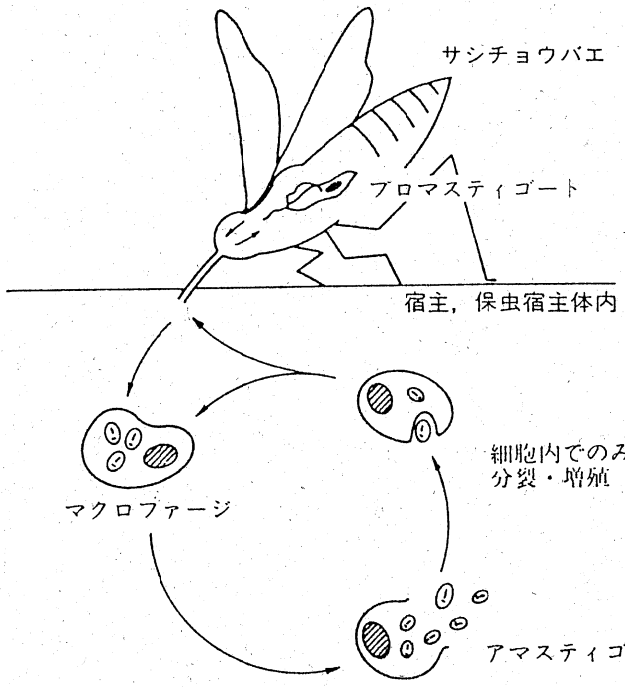


図　*Leishmania*属の生活環

吸血の際，宿主体内のアマスティゴート型原虫が取り込まれると，サシチョウバエの中腸内でプロマスティゴートとなり分裂・増殖とともに前方に移動し，4～5日後，食道に達して咽頭を経て口腔に充満し，吸血によって新しい宿主に感染する。

基本構造

*Leishmania*属原虫の発育型には宿主の細胞内にみられるアマスティゴート型と媒介者であるサシチョウバエの消化管内にみられるプロマスティゴート型の2型がある（図）。

アマスティゴート型は，Leishman-Donovan (L-D) 小体とも呼ばれ，円形ないし類円形（12～14μm）を呈し，細胞質内には1個の核と，それとほぼ直角に位置するキネトプラストを有する。自由鞭毛はもたないが，電子顕微鏡的には細胞質内に鞭毛構造が認められる。

プロマスティゴートは長紡錘形で，1個の核と1本の鞭毛を有する。

感　染

種により異なるが，媒介者を介してヒト，保虫宿主たる犬，ジャッカル，小型げっ歯類および有蹄類から感染する。幸い，わが国では媒介者がいないため自然感染はみられない。

培養および保存

培養：ほとんどの*Leishmania*属原虫はN.N.N.培地による培養が可能であり，*L. braziliensis*を除き継代培養も容易である。

保存：培養原虫あるいは組織にグリセリンを加えて凍結することによって可能である。

疫　学

リーシュマニアの分布は種によって異なるが，媒介昆虫はいずれもサシチョウバエ類で

ある。

L. donovani はアジア，アフリカ，欧州，中南米の各大陸に及んでいる。しかし，各地域によって病型に差がみられ，インド地域では成人の罹患率が高いが犬の自然感染は少ない。しかし，地中海沿岸，中国，中央アジア，中南米では小児と犬の自然感染が多い。一方，アフリカでは成人とネズミ類およびジャコウネコ科と猫科動物の感染率は高いが，犬は保虫宿主と考えられていない。

L. tropica は広くアジア地域，地中海沿岸，北アフリカおよび中南米諸国にみられ，犬，猫，アレチネズミ（gerbil）などが保虫宿主として知られている。

メキシコ，中南米のみに分布する *L. braziliensis* は犬および野生動物が保虫宿主である。

そのほか *L. infantum* は地中海沿岸，アジア，中近東，ソ連南部，アフリカの犬，キツネ，ジャッカル，ヤマアラシなどを宿主とし，ヒトは偶発宿主である。

L. chagasi は中南米に分布し，宿主はキツネ，犬，猫などでヒトは偶発宿主である。

リーシュマニア病は人畜共通原虫病の一つとして重要であり，ヒトの内臓リーシュマニア症（*L. donovani, L. infantum, L. chagasi* などを原因とする）のうち，*L. infantum* と *L. chagasi* は犬などの動物に自然感染がみられるため，動物およびヒト相互における感染源となり重要である。ヒトの皮膚リーシュマニア症は，*L. tropica* が病因原虫の一つであるが，犬およびネズミ類も保虫宿主でヒトへの感染源として重要視されている。

犬は地中海，中南米における *L. donovani* および広い地域における *L. tropica* の重要な保虫宿主であり，またキツネ，ジャッカルそのほか数多くの種類のげっ歯類がリーシュマニア病の発生に重要な役割を演じており，いまだ保虫宿主が十分に解明されていない地域もある。

症状および病理

種により寄生場所が異なるため，哺乳動物，主としてヒト，犬科動物およびげっ歯類に感染した原虫の種類により症状も異なる。

L. donovani による内臓リーシュマニア症（カラ・アザール）

L. donovani は肝臓，脾臓，骨髄などの細網内皮細胞に寄生するため，肝腫，脾腫，貧血と発熱などがみられ，末期には浮腫，下痢，黄疸が起こり悪液質に陥る。治療が不完全な場合，顔面その他に結節性皮疹が多発し，後カラ・アザール皮疹症 post-kala-azar dermal leishmanoid（PKDL）となる。

実験動物として最も多く用いられているハムスターでは，*L. donovani* 接種後約6週間で浮腫をきたし，脾臓，肝臓，腎臓が侵される。ラットでは感染後無症状の状態が長く続き，肝臓と骨髄はあまり侵されない。犬では当初リンパ節に充満していた原虫は徐々に少なくなり脾臓，肝臓，骨髄および皮膚に多くなる。サルでは継代とともに原虫の毒力が落ち，皮膚および粘膜の病変にとどまるようになる。

L. tropica による皮膚リーシュマニア症（東洋腫瘤）

L. tropica によるリーシュマニア症は病巣が皮膚に限局されており，丘疹から腫瘤を形成し，中心に潰瘍ができるが，細菌の二次感染がなければ瘢痕を形成して自然治癒し，免疫が成立する。

ハムスターに培養液を皮下接種すると4日目にはアマスティゴート型原虫がリンパ系細胞に現れる。犬では *L. donovani* の場合と鑑別の難しい皮膚の障害をきたす。マウスでは接種場所に60～90日後に肉芽のような病変を生じ，潰瘍化し，続いてカラ・アザール様に内臓も侵され，100～200日で死亡する。

***L. braziliensis* による粘膜および皮膚リーシュマニア症（アメリカリーシュマニア症，エスプンディア）**

サシチョウバエに刺されて2週間前後の潜伏期を経て，局所にできた腫瘤が潰瘍化し，さらに血流，リンパ流を経て皮膚と粘膜の境界面に移り，主として鼻咽頭，口腔粘膜が侵される。軟骨には達するが骨の侵されることはない。

ハムスターに病巣材料を皮内に接種すると局所障害しか生じないが，皮下および腹腔内接種ではカラ・アザール様の症状を高率に生じる。培養からの非経口接種では内臓の変化は起こりにくいか，きわめて軽度である。マウスでは，検体を腹腔内に接種すれば，ハムスター以上に内臓に変化を生じ，ラットでは変化がわずかである。マウスへの皮下接種では局所的に変化がみられる。サルの鼻に培養液を皮内接種した場合も局所反応にとどまるのが普通である。

***L. mexicana* による皮膚リーシュマニア症（チクレロ潰瘍）**

メキシコ，パナマ地帯，ブラジルなどにおける皮膚リーシュマニア症は，特に耳部に多く慢性経過をたどるが，潰瘍形成はまれであり，近年，*L. mexicana* complex によるとして区別されている。

診断および治療

診　断

末梢血液塗抹標本，骨髄，肝臓および脾臓の捺印標本などを用いて，病巣部からのアマスティゴート型原虫の検出を行う。または，原虫感染血液および臓器の穿刺材料をN.N.N.培地，田辺培地などで培養してプロマスティゴート型原虫を検出する。

また，幼若ハムスター，リスなどの実験動物の腹腔内に検体を接種して，約3カ月後に脾臓内のアマスティゴート型原虫を検出する。

血清学的診断法は，皮膚リーシュマニア症および粘膜・皮膚リーシュマニア症では抗体産生が弱いので診断価値は低い。内臓リーシュマニア症では間接蛍光抗体法，CF反応，間接ラテックス凝集反応，間接赤血球凝集反応，血清膠化反応，Montenegroの皮内反応などがある。しかし，いずれもリーシュマニア種相互間の，あるいは *Trypanosoma cruzi*（**カラー写真9，10，163**）などとの交差反応に留意する必要がある。

治療は5価アンチモン剤，ジアミジン製剤，抗生物質（アンホテリシンB）などが有効とされている。

サシチョウバエは小さくかやの目を通してなかに入るので危険であり，殺虫剤も欠かせない。また保虫宿主（犬，キツネ，ネズミなど）を調べ，感染動物の処置に努める。

患畜および患者は隔離治療する。

（本項の執筆に際し徳島大学医学部寄生虫学教室長沢秀行氏の協力をいただいた）

実験動物：その他の住血微生物病

ネズミに感染するエペリスロゾアおよびヘモバルトネラなどはリケッチア目のアナプラズマ科に属し，原虫とは区別されているが，ネズミの赤血球寄生微生物としては広く世界に分布しているので，その概略を記述する[5,6]。

げっ歯類のエペリスロゾーン病
Murine Eperythrozoonosis

病　原　体

本病の病原体は *Eperythrozoon* 属の *E. coccoides* である（カラー写真18）。

E. coccoides はマウス，ラット，ハムスターおよび家兎の赤血球表面および血漿中などに寄生し，その大きさは径0.5～3 μmの環状体で，ギムザおよびロマノウスキー染色では赤紫に染まる。

電子顕微鏡的観察では，両端に電子密度の高い物質の集簇を含む細胞質を単一膜で覆った環状あるいは桿状体として認められる。赤血球表面寄生では単独または2～8個の小体が集合したり，あるいは赤血球辺縁に連鎖状に配列している。血漿中に遊離しているものでは桿状体が多い。

自然界ではシラミなどの昆虫が媒介すると考えられているが，感染方法については経口および非経口の両方が考えられているがまだ不明な面が多い。感染は血液接種によって容易に成立する。以前は実験室内飼育マウスにおいても普通に認められ，広範囲に伝播していた。

症　　状

一般にネズミでは不顕性感染を呈し，明らかな症状を呈さずに耐過する。しかし，他の微生物との混合感染では臨床的にも微生物感染との併発によって重篤な貧血などを惹起し死の転機をとることが多い[6]。マウスの肝炎ウイルスと *E. coccoides* の混合感染によって斃死する例が少なくないが，その一因に *E. coccoides* はインターフェロンの産生を抑制するためと考えられている。しかし，*E. coccoides* とマラリア原虫の混合感染ではマラリアの単独感染による症状と有意な差は認められていない[1]。いずれにしても，エペリスロゾアは血液学的あるいは免疫学的研究において，その結果を複雑にすることは事実である。

診断および治療

診　断

血液塗抹による鏡検：エペリスロゾアとヘモバルトネラとの類症鑑別は容易ではない。一般にヘモバルトネラは固着性に赤血球に付着しているが，エペリスロゾアは容易に赤血球から離れ血漿中に遊離する。

血清診断法：CF 反応，間接血球凝集反応および蛍光抗体法などがある。ヘモバルトネラとアナプラズマおよびエペリスロゾーンとは血清による交差反応が陽性になることが多い[6]。

治　療

エペリスロゾアの増殖は含ヒ剤やテトラサイクリン投与で抑制されるが根治は難しい[4]。ペニシリン，ストレプトマイシン，サルファ剤は効果がない。

げっ歯類のヘモバルトネラ病
Murine　Haemobartonellosis

本病は *Haemobartonella* 属の *H. muris* の感染によって起こるげっ歯類の住血微生物病である。

H. muris は1924年，ラットの血液中から発見されたが，世界的に広く分布しており，野生や実験室飼育のラットの多くが感染しているものと考えられている。マウスやハムスターにも感染する。摘脾や強いストレスによって血液中に出現し，感染ラットに溶血性貧血を起こす。

形態学的には桿菌状，連鎖球菌状を呈し，赤血球表面および赤血球内に１個あるいは多数集団として観察されることが多い。血漿中に存在することはまれである。ロマノウスキー染色による血液塗抹上では染色性に乏しく，不鮮明な核構造物が単一あるいは二重膜によって覆われている[7]。

H. muris は全世界に広く認められ，ネズミのシラミ（*Polyplax spinulosa*）が媒介昆虫として知られている[5,6]。感染による症状は通常明らかではないが，摘脾したり免疫不全になると重篤な臨床症状を呈する。

診断および治療はエペリスロゾーン病と同じである。

獣医学

オビオアクチン

—生体応答修正・修飾物質（BRM）中の天然免疫調整剤として—

鈴木直義
帯広畜産大学畜産学部獣医学科家畜生理学教室
原虫病細胞免疫研究室
（〒080 北海道帯広市稲田町 ☎0155-48-5111）

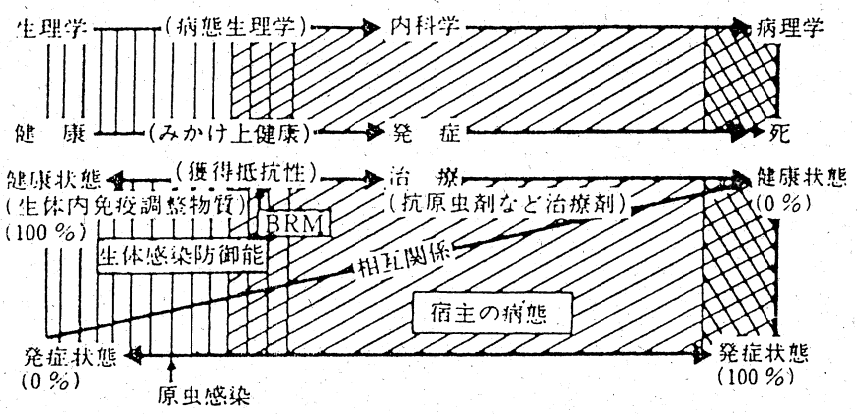
要旨：*Toxoplasma gondii* 感染耐過牛に再度トキソプラズマ特異抗原を投与し24時間を経た血清中には高いインターフェロン-ガンマ，マクロファージ活性化因子，トキソプラズマ増殖抑制因子などを含むリンホカインが出現する。その血清を加水分解して分子量3 000～5 000の部分精製蛋白成分を得た。本物質は毒性および抗原性をほとんど有さず，同種細胞のみならずヒトを含む異種の動物由来の食細胞ならびに体細胞内での一部の原虫，細菌およびウイルスの増殖をきわめて顕著に抑制する。加えて，ある種の腫瘍細胞に対しても増殖抑制傾向が認められる。さらに本物質は抗原虫剤や抗癌剤との併用投与によってこれら化学療法剤の効果を著しく増強する免疫調整物質としての効果を示す。本物質は，既知のリンホカインなどとは異なる新たな天然生理活性物質，オビオアクチンとして公表された。

76-90，*獣医学1987*

病態生理学とは，現代生理機能学の一領域として，生理学および内科学，両領域の派生学問を宿主側の生体の機能応答として研究する学問である（鈴木，1982；図1）。そして原虫感染に対する宿主の応答は先天的宿主抵抗性に加えて，宿主の恒常性に左右される。これらの関係は古くから，宿主・寄生体の関係として論じられてきた。宿主側は，先天的に健康宿主に備わっている自然抵抗性と後天的に獲得した獲得抵抗性をもって，寄生体の感染を防御する（表1）。これら，生体の抵抗性を修飾する因子としては遺伝因子，加齢因子，ホルモン，栄養，解剖的因子，生理的因子などがある。獲得抵抗性には周知のごとく，特異防御機構と非特異防御機構とがある。後者にはいわゆる自然抗体や，インターフェロン-γ，インターロイキン-2（IL-2）などのリンホカインも含まれる。自然抗体とは，生体が生来保有する抗体で，遺伝的支配を受けているもの，正常菌叢によるもの，共通抗原因子によるものなどがある。リンホカインはたとえば，トキソプラズマ感染宿主の感作Tリンパ球が産生する物質で，インターフェロン-γ，IL-2，マクロファージ活性化因子などの総称であり，免疫調整物質として包含される。非特異防御機構にあずかる物質としてはこのほかに，多糖，ポリI・C，トキソプラズマ溶解抗原などの免疫賦活物質がある。これら両物質に，免疫アジュバント物質などを加えたものは一括して，生体応答修正・修飾物質 biological response modifiers（BRM）と呼ばれている。

本題のオビオアクチンは，免疫調整物質に含まれるもので，著者らによって世界で初めて見出されたものである。本論文では著者らの研究を中心として，オビオアクチンの性状ならびにその作用について紹

Naoyoshi Suzuki：Obioactin—as a native immunoregulatory factor in the biological response modifiers—. 76-90, *Progress in Veterinary Science 1987.*



BRM：生体応答修正・修飾物質（表1を参照）

図1　宿主・寄生体（原虫）関係についての学問体系

表1　宿主の抵抗性と感染防御能

- 生体感染防御能
  - 自然抵抗性
    - 先天的抵抗性：非感受性
    - 固有の抵抗性
      - 解剖的因子（上皮組織など）
      - 細胞性因子（マクロファージ，ミクロファージなど）
      - 液性因子（リゾチームなど）
      - 機能的因子（毒物質の分離，排除など）
  - 獲得抵抗性
    - 特異免疫
      - 受身免疫
        - 自然（乳子の母体由来免疫グロブリンなど）
        - 誘導（異種抗体，同種γ-グロブリンなど）
      - 能動免疫
        - 自然
          - （液性免疫，抗体産生）
          - （細胞性免疫）
        - 誘導（トキソイド，死または弱毒原虫による免疫，免疫アジュバント）*
    - 非特異免疫
      - 免疫賦活物質*（多糖，ポリI・C，OK432，レンチナン，TLAなど）
      - 免疫調整物質*〔リンホカイン，インターフェロン，IL-2，Nor-MDP，オピオアクチン（CSP-II）など〕

* 生体応答修正・修飾物質
TLA：トキソプラズマ溶解抗原　　Nor-MDP：ムラミルジペプチド

介したい。

原虫病の病態生理学的総合研究プロジェクトの設立

1964年，ボン大学に西ドイツの医科系大学には唯一の寄生虫および原虫病に関する研究所が創設された。初代研究所所長（主任教授）には世界寄生虫学者連盟副会長（後に会長）Piekarski教授が任命された。研究所設立の主目的はヒトの寄生虫病および原虫病の基礎と臨床両面からの総合的研究教育による学術国際協力にあった。また，Piekarski教授の主研究の一つがヒトのトキソプラズマ症であったことから，その血清学的診断について，全ドイツのみならず欧州全域の中心的存在としての要素も強く含まれていた。このため，西ドイツ政府とDFG資金による原虫病の病態生理学的総合研究に関する特別研究プロジェクトが設立され，1965年，世界から生化学，免疫学，血液学領域の中堅研究者が募集された。そして，西ドイツ以外からは著者を含めて6名がプロジェクトに参画した。

設立された研究プロジェクトにおける原虫病の病態生理学的研究遂行に際しての出発理念および研究哲学は，周知の理論と必ずしも全面的に一致しているとは限らなかった。万人から受け入れられる理論および理念でなければその説得力は半減するが，1966年当時には，寄生虫病および原虫病学の分野で

感染に伴う宿主の生体応答を総合的に検討する実験研究は全世界的にきわめて少なかったのである。そこで，Piekarski博士を代表にわれわれは，マクロファージと感作Tリンパ球およびリンホカインの生理機能を主軸として細胞性免疫によるトキソプラズマ感染に対する宿主の防御能についての研究を主題に据え，プロジェクトを開始した。

リンホカインからオピオアクチン

感染防御能を有する健康な宿主にトキソプラズマが侵入しマクロファージに摂取されても，原虫の多くは一般微生物とは異なって，マクロファージの殺機能から逃避して，内部出芽による二分裂増殖を繰り返す。感染成立に伴う抗体の出現は感染初期にIgM抗体のみ，次いでIgMとIgG抗体，以後IgG抗体のみが認められる(Omata & Suzuki, 1975)。しかし，マウスを用いての実験では，大量のトキソプラズマ免疫血清を健康マウスに投与してもトキソプラズマ強毒株を接種すると全例斃死する。一方，トキソプラズマ弱毒株感染耐過マウスでは，強毒株攻撃に対してその多くが生残する。ヌードマウスでは，このような感染死防御能はみられないが，感染耐過マウスの脾細胞投与によって防御能が付与され，マウスは生き残る。このように，トキソプラズマ感染における宿主の抵抗性は液性免疫よりも細胞性免疫によるものが大きい。

1966年，著者らはトキソプラズマ免疫マウスおよび健康マウスの血液あるいは腹腔細胞中に生原虫を接種して，虫体の細胞内侵入過程を観察した。すると，免疫マウス由来細胞中では虫体の増殖抑制と殺滅像がみられたのに対して，健康マウス由来の細胞中では虫体の増殖像がみられ，またTリンパ球の接着像がしばしば観察された。しかし，以後の研究で虫体の増殖抑制にはマクロファージへのTリンパ球の接着は必須のものではなく，感作Tリンパ球から産生される可溶性の生物媒介物質の重要性が示唆されている(Sethi *et al.*, 1975)。

同時期にDumondeら(1969)は，感作リンパ球を感作に用いた抗原を加えて培養すると，リンパ球は幼若化し，生化学的変化をきたして種々の活性物質を培養液中に放出することを見出した。これらのうち，免疫グロブリンや遊出酵素などで生成された二次的作用物質を除く一連の可溶性作用物質を「リンホカイン」と名づけた。

トキソプラズマ感染により免疫された宿主はトキソプラズマ抗原の刺激により，各種のリンホカインを産生することが知られている(表2)。このなかには，マクロファージ活性化因子(MAF)のほかに，同種体細胞内でのトキソプラズマの増殖を抑制する物質が含まれており，防御に関与している。この物質はトキソプラズマ増殖抑制因子(Shirahata *et al.*, 1976)，トキソプラズマ抑制因子(Jones *et al.*, 1977)，抗トキソプラズマ武装因子(Sethi & Brandis, 1979)，あるいはトキソプラズマ免疫媒介因子(Chinchilla & Frenkel, 1978)と呼ばれている。トキソプラズマ増殖抑制因子は分子量30 000から40 000の糖蛋白で，その物質が由来した宿主と同じ動物種の細胞内における原虫の増殖は阻止できるが，異種の動物の細胞内での増殖は阻止できない(Nagasawa *et al.*, 1980; Matsumoto *et al.*, 1981)。本物質はマクロファージ遊走阻止因子と完全には分離されないために同一視される場合もある。しかし，マクロファージ遊走阻止因子そのものが単一物質ではなく多様性を有し，幾つかのサブユニットから構成されているために，その一部がトキソプラズマ増殖抑制因子と易動度を同じくする可能性が指摘されている(Igarashi *et al.*, 1979)。事実，マクロファージ遊走阻止因子は低分子のサブユニットが重合あるいは担体に結合しているものとの考えが，現在支持されている(須甲，1982)。一方，マクロファージはインターフェロンによってもマクロファージ活性化因子刺激と同様に活性化されることが知られており，両物質ともその一部は同一であろうと考えられている(Schultz *et al.*, 1977; 1978)。しかし，トキソプラズマ増殖抑制因子の活性はインターフェロン-I型(αおよびβ型)には認められない。また，リンホカイン中のインターフェロン-γとトキソプラズマ増殖抑制因子の発現の時間的推移が異なっており，インターフェロン-γとトキソプラズマ増殖抑制因子は同一ではない(Sakurai *et al.*, 1981; Omata *et*

表2　リンホカインに属する作用因子(メディエーター)

A.マクロファージに働く因子	
1.マクロファージ遊走阻止因子（MIF）	*in vitro*でマクロファージの遊走を阻止
2.マクロファージ凝集因子	マクロファージの凝集を惹起
3.マクロファージ走化因子（MCF）	*in vitro*で食細胞を遊走
4.マクロファージ活性化因子（MAF）	マクロファージの食殺菌作用を促進
5.特異マクロファージ武装因子（SMAF）	特異免疫細胞への傷害
マクロファージ遊走増強因子（MEF）	マクロファージの遊走を増強
B.リンパ球に働く因子	
1.幼若化またはマイトジェン因子（BF，MF）	小リンパ球の芽球化および分裂促進
2.賦活因子（PF）	リンパ球成熟（DNA合成）の促進
3.ヘルパー因子（ThF）	T細胞由来でB細胞活性化に関与
4.サプレッサー因子（TsF）	リンパ球の芽球化を抑制
5.T細胞因子（伝達因子TF）	細胞活性能を伝達
6.インターロイキン-2，-3（IL-2，-3）	T細胞を増殖し，*in vitro*で長期培養可能
C．顆粒球に働く因子	
1.白血球遊走阻止因子（LIF）	白血球の遊走を阻止
2.PMN走化因子（PMNCF）	好中球を局所に遊出，遊走させて集積させる
3.好酸球走化因子（ECF）	好酸球を局所に遊走させて集積させる
4.好酸球走化促進因子（ESP）	好酸球を刺激して増強させる
D.継代培養細胞に働く因子	
1.細胞傷害因子，リンホトキシン（CF，LT）	単層培養細胞を傷害剥離
2.細胞増殖阻止因子（PIF）	増殖を抑制する
3.クローニング阻止因子（CLIF）	細胞（HeLA）分裂増殖を抑制
DNA合成阻害因子（IDS)(？)	リンパ球DNA合成を阻害
4.インターフェロン（IFN）	ウイルスの病原性を干渉する
5.トキソプラズマ増殖抑制因子（Toxo-GIF）	体細胞内原虫の増殖を抑制
E.*in vivo*で働く因子	
1.皮膚反応性因子（SRF）	皮膚に炎症反応を起こす
2.マクロファージ消失因子（MDF）	腹腔内マクロファージの減少
3.リンパ節活性化因子（LNAF）	細胞性免疫の増強
4.血管透過因子（VPF)(？)	血管の透過性に影響

al., 1983a ; 1983 b)。トキソプラズマ免疫ハムスターのリンパ球と特異抗原を培養すると，トキソプラズマ増殖抑制因子と同様の作用因子トキソプラズマ免疫媒介因子が培養液中に含まれるようになる。本因子はハムスターのマクロファージのみならず，腎細胞ならびに線維芽細胞内での原虫の増殖を阻止する分子量4 000～5 000の体細胞活性因子で，伝達因子あるいはこれに最も類似した因子として報告されている(Chinchilla & Frenkel, 1978)。マウス，イヌおよびウシのトキソプラズマ免疫脾細胞由来リンホカイン中にはトキソプラズマ増殖抑制因子，マクロファージ遊走阻止因子，マクロファージ活性化因子，インターフェロン-γ，IL-2以外に，分子量3 000～5 000のマクロファージ遊走阻止因子-IIとリンホトキシンが認められている(Igarashi *et al*., 1979)。このようなトキソプラズマ増殖抑制因子などを含むリンホカインはトキソプラズマ免疫動物の循環血液中にも出現する（Takei *et al*., 1981)。リンホカインと正常脾細胞を*in vitro*で長時間培養すると，Tリンパ球は感作されリンホカインを産生する（Sakurai *et al*., 1983)。

リンホカインおよびトキソプラズマ免疫動物血清を加水分解して低分子量物質にすると，分子量3 000～5 000のポリペプチド成分にトキソプラズマ増殖抑制因子様活性が存在する(Nagasawa *et al*., 1981 ; 1982)。トキソプラズマ増殖抑制因子は前述のように種属特異性の強い同種体細胞活性物質であるが，加水分解して得られた物質は種属特異性を消失

表3　各種有機溶媒に対するオピオアクチンの溶解性と総窒素重量*

有機溶媒	上清画分(μg)	不溶画分(μg)	上清(%)
エタノール	2.10	56.06	3.6
メタノール	49.41	6.72	88.0
エーテル	0.01	59.75	0
ベンゼン	0.02	58.75	0
クロロホルム	1.72	63.79	2.6
アセトン	0.14	66.91	0.2

* オピオアクチン100 mgを有機溶媒5 m*l*とともに混合振盪後，遠心分画成分を乾燥し，純水1 m*l*に溶解した場合の総窒素重量

し，いわゆるspecies barrierを通過する。また，本物質は細胞内のトキソプラズマの増殖を抑制するばかりではなく，細菌，ウイルスに対する抗微生物活性をも有する(Osaki *et al.*, 1984; Suzuki *et al.*, 1984b)。著者らは，トキソプラズマ免疫牛血清由来加水分解物質をオピオアクチンと称した(Suzuki *et al.*, 1984a)。そして，オピオアクチンはインターフェロン-γなどとは異なる新規の天然生理活性物質として登録されている(US Patent No. 4482543)。

オピオアクチンの理化学的性状

pH，水および有機溶媒による溶解性：オピオアクチンは淡黄白色不定形粉末状の水易溶性の物質で，そのpHは7.0～7.2である。本物質100 mgを各種有機溶媒5 m*l*に加え，30分間振盪後3 000 rpmで10分間遠心分離して上清画分と不溶画分に分画し，両画分を乾燥後，純水1 m*l*を加えて総窒素量を求めると，表3に示すように，メタノールでのみ溶解される(Suzuki *et al.*, 1984a)。

糖および蛋白含量比：ヘキソース含量をフェノール硫酸法で，ヘキソサミン含量をエルソン・モルガン法で，蛋白質含量を総窒素法で測定すると，これらの含量比(重量%)はそれぞれ1～2%，9～13%および85～90%となる。このことは，オピオアクチンが中性およびアミノ糖含有ペプチド結合蛋白物質であることを意味する。

構成アミノ酸：アスパラギン酸，トレオニン，セリン，グルタミン酸，グリシン，アラニン，シスチン，バリン，メチオニン，イソロイシン，ロイシン，チロシン，フエニルアラニン，リジン，トリプトファン，ヒスチジン，アルギニンおよびプロリンを構成アミノ酸とする。このなかではアスパラギン酸，グルタミン酸およびイソロイシンの含量が特異的に多く，これら3種の全アミノ酸に対する含有率はアミノ酸アナライザーにより分析すると，45～65%となる。

温度安定性：1%水溶液(w/v)を60±0.1℃で30分間加熱しても異種細胞内原虫増殖抑制作用および免疫賦活作用は消失しない。

毒　　性：BALB/c雌成熟マウスに4 000 mg/kg腹腔内接種あるいは2 000 mg/kg静脈内接種で行った急性毒性試験ではいずれも死亡例を認めない。したがって，オピオアクチンのLD_{50}値は腹腔内接種で＞4 000 mg/kg，静脈内接種で＞2 000 mg/kgであるといえる。一方，100～500 mg/kgのオピオアクチンをラットに連続90日間経口投与したが，無投与群の体重増加曲線とほとんど差がなく，一般病理組織学的検索においても諸臓器に変化は認められない。

以上のことから，本物質は全く，あるいはほとんど毒性のない生理活性物質といえる(Suzuki *et al.*, 1984a)。

抗 原 性：0.8%オピオアクチンを同量のフロインド完全アジュバントと乳濁後，モルモット7匹に1匹あたり0.5m*l*，隔日3回，皮下接種した。接種後3週および4週に，処置モルモットにオピオアクチン10 mgを静脈内注入し，全身性アナフィラキシーの有無を観察したが，全例異常を認めない。また，これらのモルモットの回腸は，オピオアクチン0.2 mg/m*l*液を用いたSchultz-Dale反応でもアレルギー性収縮を示さない。対照としてウシ血清アルブ

表4　正常牛単球内原虫増殖に対するオビアクチン投与の影響
(Suzuki *et al.*, 1984b)

添加物質	添加物質濃度	トキソプラズマ保有細胞出現率*1(%)		
		0*2	1～5*2	≧6*2
無添加対照	0	51.0± 9.6	40.8±7.7	8.1±2.7
正常牛血清由来加水分解物質	0.25	65.0± 8.3	28.4±7.1	6.6±4.8
	0.5	66.2± 8.7	28.0±5.9	6.0±3.5
	0.75	67.2± 3.7	25.0±2.7	7.8±3.9
	2.0	—	—	—
オビオアクチン	0.25	65.8±10.5	25.8±5.4	8.6±5.5
	0.5	82.2±11.1	13.8±8.1	4.0±3.5
	0.75	84.6± 6.8	9.4±6.1	6.0±2.9
	1.0	90.4± 4.8	6.2±2.9	3.4±2.6
	2.0	—	—	—
	5.0	—	—	—

数字は5回の実験の平均値±標準偏差
*1 培養後48時間で判定
*2 1細胞あたりのトキソプラズマ数

ミン0.4％を同様に接種したモルモットはアナフィラキシーを起こし，全例死亡する。さらに，ウサギ免疫血清を用いて受身皮膚アナフィラキシーを観察した。0.8％オビオアクチンをフロインド完全アジュバントと乳濁後，ウサギ1匹あたり5 m*l*ずつを隔日3回皮下接種し，その1週後に0.8％オビオアクチンを2.5 m*l* 静脈内接種した。その後2週および3週目に血液を採取し，分離した血清を非働化して健康モルモットの皮内に0.2 m*l* 接種し，5時間後に2％オビオアクチン0.5 m*l* と0.5％エバンスブルー0.5 m*l* を静脈内接種した。その後30分でモルモットの皮膚を剥離して色素漏出斑の有無を観察したところ，オビオアクチン接種群は全例陰性であるのに対して，ウシ血清アルブミンで同様に実験を行った群では強陽性の色素漏出斑を認めた。すなわち，これらの方法ではオビオアクチンの抗原性は検出されていない(Suzuki *et al.*, 1984a)。

オビオアクチンの作用

同種細胞内トキソプラズマに対する増殖抑制効果： 正常牛末梢単球単層培養にトキソプラズマを接種後，各種濃度のオビオアクチンを添加して48時間培養し，単球内原虫増殖の有無を検討した(表4)。対照として，オビオアクチン分離の方法と同じ加水分解法を施した正常牛血清由来物質を用いた。その結果，オビオアクチン添加濃度1％までは濃度依存性に同種単球内における原虫の増殖は抑制されるが，2％以上では単球破壊像が認められる。一方，正常牛血清由来物質ではオビオアクチンのような作用効果は認められない(Suzuki *et al.*, 1984b)。

異種細胞内トキソプラズマに対する増殖抑制効果： 異種動物由来の細胞におけるトキソプラズマ増殖に対するオビオアクチンの作用を調べるため，マウスのマクロファージならびに腎細胞，イヌ単球，ヒト心筋ならびに脳細胞を用いて実験を行った(表5)。その結果，0.5％濃度にオビオアクチンを加えて48時間培養すると，いずれの細胞種でも全供試細胞の80％以上にトキソプラズマはみられず，無添加の場合に比べて原虫の増殖は明らかに抑制される(Suzuki *et al.*, 1984b)。

オビオアクチン経口投与に対するマウスの生体応答： オビオアクチン経口投与に対するマウスの生体応答を検討するために，オビオアクチンを20 mg/m*l* 濃度に加えた生理食塩液を0.5 m*l* ずつBALB/cマウス100匹に4週間にわたって，連日経口投与した。対照は生理食塩液0.5 m*l* を同様に投与したマウス

表5　オビオアクチン投与による異種細胞内原虫増殖抑制効果 (Suzuki *et al.*, 1984b)

供試細胞	オビオアクチン濃度(%)	トキソプラズマ保有細胞出現率*1(%)		
		0 *2	1～5 *2	≧6 *2
マウスマクロファージ	0	39.4±13.7	36.7±2.3	23.9±7.8
	0.5	87.3± 3.2	7.5±2.5	5.1±2.4
マウス腎細胞	0	11.1± 9.3	34.3±2.1	24.6±7.8
	0.5	83.1± 3.2	14.2±1.9	2.7±1.4
イヌ単球	0	40.1±15.5	28.5±6.8	31.3±8.2
	0.5	80.5± 6.4	12.8±5.4	6.7±3.3
ヒト心筋	0	54.4±18.3	23.5±9.3	22.1±9.7
	0.5	80.7± 6.1	10.6±3.4	8.7±2.1
ヒト脳細胞	0	55.4±11.1	21.3±2.9	29.3±4.2
	0.5	82.4±13.1	11.1±8.7	6.5±4.4

数字は3～6回の実験の平均値±標準偏差
*1 培養後18時間で判定
*2 1細胞あたりのトキソプラズマ数

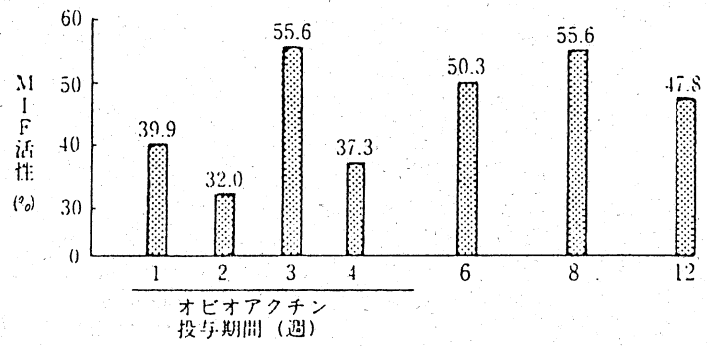
表6　オビオアクチン投与マウス脾細胞培養上清中のトキソプラズマ増殖抑制因子の消長*1(上田ら, 1984)

投与した物質	週*2	トキソプラズマ保有細胞出現率(%)		
		0 *3	1～5 *3	≧6 *3
TC-199(対照)	−	22.6±7.2	46.2±9.5	31.2±6.8
オビオアクチン	1	75.8±6.9	19.0±4.1	5.2±3.1
オビオアクチン	2	66.6±3.4	28.6±2.7	4.8±2.2
オビオアクチン	3	83.0±5.2	15.2±4.2	2.0±1.7
オビオアクチン	4	79.2±5.2	18.4±4.2	2.4±1.5
オビオアクチン	6	69.2±4.0	25.8±3.6	5.0±2.0
オビオアクチン	8	60.4±9.7	35.0±7.3	4.4±2.6
オビオアクチン	12	76.0±5.7	17.8±2.4	66.2±5.0

数字は平均値±標準偏差
*1 オビオアクチンはマウス1匹あたり10 mg/0.5 ml生理食塩液を連日4週間経口投与
*2 オビオアクチン投与初日からの週数
*3 1細胞あたりのトキソプラズマ数

とした。投与前，第1回投与後1，2，3，4，6，8および12週目に10匹ずつのマウスをと殺し，脾細胞を集めて培養して，培養上清由来リンホカイン中のマクロファージ遊走阻止因子およびトキソプラズマ増殖抑制因子活性ならびに一般病理組織学的検索を行った。オビオアクチン経口投与開始後1週で，脾細胞培養上清にトキソプラズマ増殖抑制因子活性の出現を認める(表6)。また，マクロファージ遊走阻止因子活性は投与開始後3週で高い値を示し，オビオアクチン投与終了後8週に至るも，比較的その活性が継続している（図2)。病理組織学的所見では，対照群と比較して特徴的変化は認められていない（上田ら，1984)。

トキソプラズマ急性感染マウスに対する治療効果： BALB/c雄マウス（体重18～20 g）10匹を1群とし，各マウスにトキソプラズマS-273株のタキゾイト5×10²個を腹腔内に接種した。その後，無処置対照群(I群）は1日1回生理食塩液0.25 mlを4週間連日腹腔内投与，II群はアセチルスピラマイシンを1日あたり0.2 g/kg，4週間連日経口投与，III群



$$*\mathrm{MIF}(\%) = 100 \times \left(1 - \frac{\text{検体におけるマクロファージ遊走距離}}{\text{対照におけるマクロファージ遊走距離}}\right)$$

図2　オビオアクチン投与マウス脾細胞培養上清中のマクロファージ遊走阻止因子(MIF)の消長（上田ら，1984）

表7　トキソプラズマ急性感染マウスに対するアセチルスピラマイシンとオビオアクチンの併用効果

実験群	脳あたりシスト数		抗体*2	ヌードマウス致死数
	数(個)	減少率(%)*1	陽性数/マウス数(%)	死亡例/マウス数
I群（無処置対照群）	4 302	0	10/10 (100.0)	16/16
II群（アセチルスピラマイシン投与群）	26	99.4	7/8 (87.5)	14/14
III群（アセチルスピラマイシンとオビオアクチン併用投与群）	0	100.0	0/8 (0)	0/13

*1 $\text{減少率}(\%) = \dfrac{\text{対照群脳内シスト数} - \text{実験群脳内シスト数}}{\text{対照群脳内シスト数}} \times 100$

*2 ラテックス凝集反応で検索

はアセチルスピラマイシン 0.2 g/kg の経口投与にあわせて，マウス1匹あたり 1 mg のオビオアクチンを4週間腹腔内投与した(表7)。S-273 株原虫接種後 80 日目に各マウスの血清抗体価をラテックス凝集法で，脳内シスト数をプランクトン計算盤法で測定し，シスト数の減少率を求めた。さらに，各群マウスの脳乳剤を BALB/c ヌードマウスに接種して，原虫の有無について検討した。その結果，感染マウス脳内シスト数の平均は対照群（I群）で4 302であるのに対して，II群のマウスでは 26 シストとI群に比べると激減している。しかし，これらの脳乳剤を接種したヌードマウス 14 匹全例から虫体が分離されている。すなわち，トキソプラズマ感染と同時にアセチルスピラマイシンを単独に投与しても，完全治療は困難である。一方，オビオアクチン併用投与群（III群）ではプランクトン計算法でシストが検出されず，脳乳剤接種ヌードマウス13 例においても，全例で虫体は分離されていない。また，これらのマウスの血中には抗体は認められず，アセチルスピラマイシンとオビオアクチンの併用で完全な治療を行うことができると考えられる(Espinas *et al.*, 1981; Suzuki *et al.*, 1984b)。

トキソプラズマシスト保有マウスに対する治療効果： トキソプラズマ S-273 株 1 $\times 10^2$個をマウスに接種し，4 週後に再度同株で攻撃した。その4週後から4週間，オビオアクチン（10 mg/マウス/日，腹腔内投与）およびアセチルスピラマイシン（8 mg/マウス/日，経口投与）を単独あるいは併用して連日投与した。オビオアクチン，アセチルスピラマイシンのいずれも投与しないI群を無処置対照群とした

表8　トキソプラズマ慢性感染マウスに対するアセチルスピラマイシンとオビオアクチンの併用効果（Suzuki *et al.*, 1984b）

実験群	検体数	脳あたりシスト数		抗体価*2
		数（個）	平均（減少率*1）	
I　群（無処置対照群）	4	3 943	1 609	128～512
		1 508		
		519		
		466		
II　群（アセチルスピラマイシン投与群）	4	1 257	1 052（34.61）	128～512
		1 186		
		938		
		828		
III　群（オビオアクチン投与群）	5	2 210	1 080（32.91）	256～512
		1 367		
		852		
		791		
		179		
IV　群（アセチルスピラマイシンとオビオアクチン併用投与群）	4	999	766（52.40）	512
		901		
		868		
		295		

*1 減少率（%）＝ $\frac{\text{対照群脳内シスト数} - \text{実験群脳内シスト数}}{\text{対照群脳内シスト数}} \times 100$

*2 ラテックス凝集反応で検索

（表8）。4週間の連日投与終了時に各実験群のマウスについて，脳内シスト数とその減少率および抗体価を検索したところ，対照群（I群）の脳内シスト数の平均が1 609個であるのに対して，アセチルスピラマイシン投与群（II群），オビオアクチン単独投与群（III群）およびアセチルスピラマイシンとオビオアクチン併用投与群（IV群）では，それぞれ1 052個，1 080個，766個に減少している。特に，併用投与群ではシストは約半数に減少し，病理組織学的に脳内シスト破壊像も観察されている（Espinas *et al.*, 1982；Suzuki *et al.*, 1984b）。

トキソプラズマ以外の原虫に対する増殖抑制効果：*Trypanosoma cruzi* をマウスに接種すると，通常9～12日で全例死亡する。*T. cruzi* 接種前にオビオアクチン100 mg/kgを投与しても予防効果はほとんどない。しかし，**表9**に示すように感染後，治療薬としてのランピットとオビオアクチンを併用投与すると生残率は上昇し，明らかに *T. cruzi* 感染に対する宿主の防御能が賦活される（Osaki *et al.*, 1984）。

一方，*Babesia rodhaini*（Ogawa *et al.*, 1985）や *Plasmodium berghei*（Suzuki *et al.*, 1987*）感染赤血球（10^2個）をマウスに接種すると，いずれの場合も2週以内に全例死亡する。しかし，生体応答修正・修飾物質（BRM）の一種であるトキソプラズマ溶解抗原100μgで2回前感作すると一部のマウスは耐過生残する。これにリンホカインやオビオアクチンを併用投与すると，その生残率は明らかに上昇する。

このように，オビオアクチンは抗原虫剤やBRMとの併用投与によって，致死的原虫感染に対して強く防御能を賦活する生理活性物質としての作用を有することが明らかになっている。

抗細菌効果：10%ウシ胎仔血清を加えたTC-199培地で24時間培養した健康マウス腹腔マクロファージに50倍量の *Escherichia coli*，*Proteus vulgaris*（グラム陰性菌）あるいは *Bacillus subtilis*（グラム陽性菌）を30分間作用させた後，オビオアクチンを0.75 mg/m*l* に加えた培養液あるいは無添加培養液で培養する。2時間あるいは4時間の培養後，マクロファージを蒸溜水中で破壊し，さらにプレートに培養してマクロファージ内の残存生菌数を計測する

*Suzuki, N. *et al.* (1987)：*Zbl. Bakt. Hyg. A.* 受理

表9　オビオアクチンによるマウスの抗トリパノソーマ効果（Osaki *et al.*, 1984）

投与時期	投与経路	投与物質	生存率	死亡マウスの平均生存日数*2
感染前	腹腔内	オビオアクチン（100 mg/kg）	0　（0　9）*1	13.1±0.6
		ランピット（3 mg kg）	0　（0　8）	12.8±0.6
		＋オビオアクチン（100 mg/kg）	12.5（1　8）	13.8±1.0
感染後	腹腔内	オビオアクチン（100 mg/kg）	16.0（4　25）	13.6±0.3
		オビオアクチン（200 mg/kg）	42.3（3　7）	15.8±1.0
		ランピット（10 mg/kg）	100　（11　11）	
		＋オビオアクチン（100 mg/kg）	100　（12　12）	
		ランピット（5 mg kg）	100　（7　7）	
		＋オビオアクチン（100 mg/kg）	100　（14　14）	
		ランピット（3 mg kg）	22.7（5　22）	16.0±0.4
		＋オビオアクチン（100 mg/kg）	54.5（12　22）	14.6±0.7
	経口	ランピット（10 mg/kg）	75.0（6　8）	16.0±0
		＋オビオアクチン（100 mg/kg）	100　（8　8）	
		ランピット（5 mg kg）	35.3（6　17）	18.7±1.7
		＋オビオアクチン（100 mg/kg）	23.5（4　17）	16.7±1.4
感染前および後	腹腔内	オビオアクチン（100 mg/kg）	14.3（1　7）	12.5±0.5
		ランピット（3 mg kg）	12.8（1　8）	14.1±0.9
		＋オビオアクチン（100 mg/kg）	50.0（4　8）	12.3±0.3

*1　生残頭数／供試頭数

*2　平均値±標準誤差

オビオアクチンは5％グルコース溶液中に溶解。ランピットは0.1％メチルセルロース溶液として使用

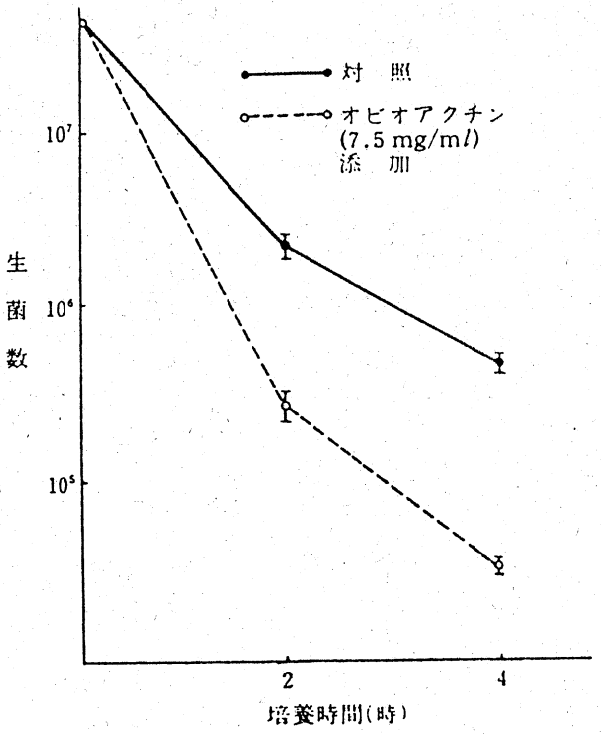


図3　オビオアクチン添加培養マクロファージ内 *E. coli* 生菌数（Osaki *et al.*, 1984）

と，オビオアクチンを加えて培養したマクロファージ中の *E. coli* 生菌数は対照に比べて著しく少なかった（図3）。

一方，*L. monocytogenes* 1×10^6をマウス腹腔内に接種すると，約1週間で全例死亡する。しかし，*L. monocytogenes* 接種の24時間前あるいは接種と同時にオビオアクチンをマウス1匹あたり5 mg投与すると一部のマウスは耐過生残する（図4）。しかも，その生存率は *L. monocytogenes* 接種前にオビオアクチンを投与した群で高い。

これらのことから，オビオアクチン投与によって各種グラム陰性菌および陽性菌の細胞内での増殖抑制ならびに生体内での増殖抑制効果のあることが認められている（Osaki *et al.*, 1984）。

ウイルス増殖抑制効果：FL細胞に単純ヘルペスウイルス1型（HSV-1）深山株，BHK-21細胞に

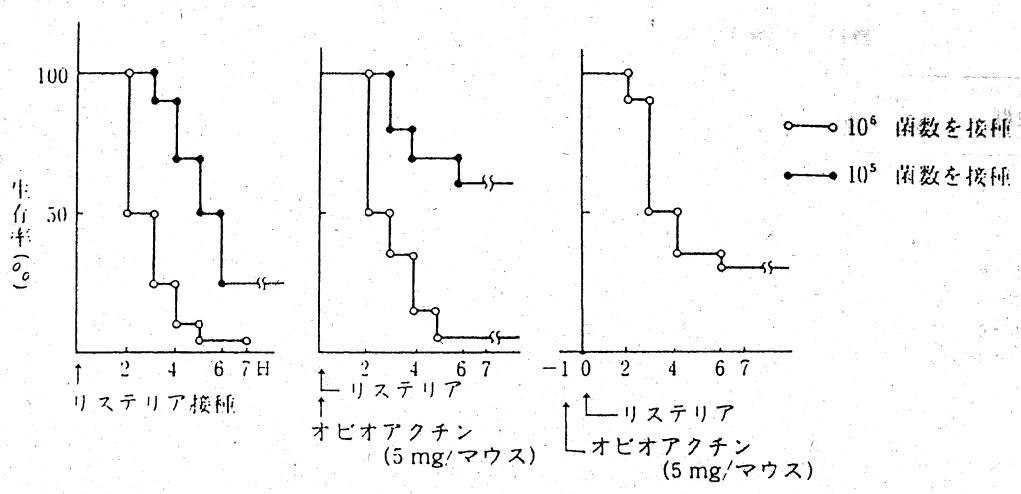


図4　*L. monocytogenes* 感染マウスに対するオビオアクチン投与の影響
(Osaki *et al.*, 1984)

表10　ウイルスの増殖に対するオビオアクチンの影響 (Osaki *et al.*, 1984)

オビオアクチン濃度 (mg ml)	ウイルス力価 (PFU)			
	BHK-21細胞		FL細胞	
	CHIKV*1	JEV*1	HSV-1*1(1)	HSV-1(2)*2
0	1.1×10^{8}	8.0×10^{5}	3.4×10^{8}	1.9×10^{8}
0.1	1.8×10^{8}	2.1×10^{5}	3.6×10^{8}	1.5×10^{8}
0.5	ND	ND	2.7×10^{8}	1.8×10^{8}
1	ND	ND	2.5×10^{8}	5.3×10^{7}
5	ND	ND	1.4×10^{8}	3.0×10^{7}
10	1.1×10^{8}	5.0×10^{5}	3.3×10^{7}	1.2×10^{7}

CHIKV: チクングニヤウイルス
JEV: 日本脳炎ウイルス
HSV-1: 単純ヘルペウイルス1型
*1 ウイルス接種後，オビオアクチンを加えた培養液で24時間培養
*2 ウイルス接種前に12時間，接種後24時間，オビオアクチンを加えた培養液で培養
ND: 試験せず

チクングニヤウイルス (CHIKV) アフリカ株あるいは日本脳炎ウイルス (JEV) JaGAr 01株を接種し，オビオアクチン添加あるいは無添加培養液で24時間培養後，ウイルス力価をプラック法で求めた(表10)。

BHK-21細胞に接種したCHIKVやJEVではオビオアクチンを10 mg/ml濃度で加えてもウイルス増殖は抑制されていない。しかし，FL細胞にHSV-1を接種した場合にはオビオアクチンを10 mg/mlに加えて培養した後のウイルス力価が対照の1/10に抑えられている。さらに，HSV-1は，接種前にもオビオアクチンで処理すると，1 mg/ml濃度でもウイルスの増殖が抑制される。

このように，オビオアクチンがある種のウイルスに対してもその増殖を抑制する効果をもつことが明らかになっている (Osaki *et al.*, 1984)。

抗腫瘍効果：10週齢の雄BDF1マウスの腹腔内に，マウス白血病由来P388細胞10^{6}個を移植し，移植後1日目より9日間連続して右側中腹部にフトラフール(FT207)(体重10 gあたり1.0 mg/0.1 ml生理食塩液)およびオビオアクチン(体重10 gあたり8.0 mg/0.1ml生理食塩液)をそれぞれ単独で，または併用して1日1回投与した(表11)。抗腫瘍効果は，無投与対照群の平均生存日数を基準として各群の延命率を算出して評価した。オビオアクチン単独では延命率20.29％とその効果は軽度であるが，公知の制癌剤(FT 207)との併用投与により，延命率は82.61％になるという高い効果をあげ，相乗的抗腫瘍効果を示す。

表11　P 388細胞移植マウスに対するオビオアクチンの抗腫瘍効果

実験群	マウス匹数	各生存日数(日)	平均生存日数(日)	延命率(%)	移植当日のマウス平均体重(g)
無投与	7	9, 9, 10, 10, 10, 11	9.86±0.69	0	25.9
オビオアクチン 200 mg/kg	7	11, 11, 12, 12, 12, 13	11.86±0.69	20.29	26.1
FT 207 100 mg/kg	7	12, 12, 13, 13, 14, 15, 16	13.57±1.51	37.68	26.1
オビオアクチン + FT207	7	16, 17, 18, 18, 18, 19, 20	18.0 ±1.29	82.61	25.9

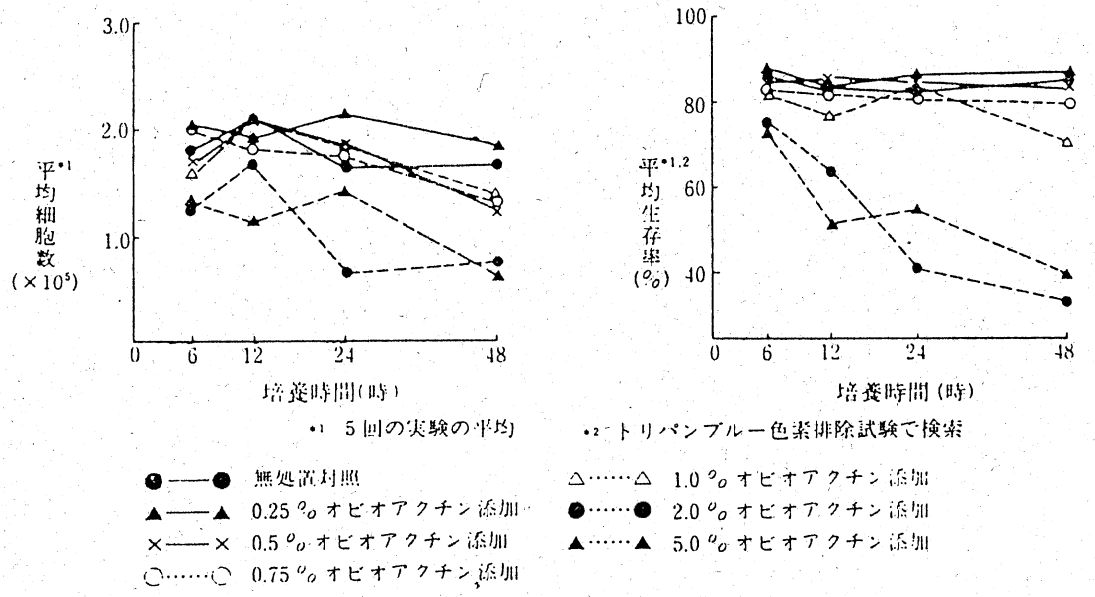


図5　*in vitro* 試験におけるS-180細胞に対するオビオアクチンの影響（上田ら，1984）

次に，マウス同種可移植性腫瘍S-180の増殖に対する効果を調べた。まず，*in vitro* でS-180細胞を2％以上のオビオアクチンとともに培養すると直接的細胞障害性が認められる（上田ら，1984）。一方，マウス腹腔マクロファージにS-180細胞を移入して培養するにあたり，移入前後に2％以上のオビオアクチンを添加した場合にS-180細胞の生残率が著しく低下した(図5)。また，オビオアクチンによって活性化されたマクロファージへのS-180細胞付着像がきわめて多く観察されている。次いで，S-180腫瘍細胞移植マウスに対するオビオアクチンの抗腫瘍効果を経口，腹腔内あるいは筋肉内投与によって検討したところ，筋肉内投与の場合に腫瘍の増殖が最も抑制されることが判明した。そこで，マウス1匹あたり1×10⁶個のS-180細胞を皮下移植した後，週1回，4 mg/kgから400 mg/kgまで指数関数関係になるように生理食塩液で調整したオビオアクチンを大腿部筋肉内に注射し，S-180細胞移植後40日目の平均腫瘍面積を調べた。無処置対照群が1 223.0 ± 51.6 mm²であるのに対し，オビオアクチン投与群は，オビオアクチン濃度4 mg/kgで857.7 ± 223.7 mm²，10 mg/kgで588.0 ± 286.5 mm²，40 mg/kgで195.3 ± 130.1mm²，100 mg/kgで594.0 ± 509.6 mm²，400 mg/kgで270.0 ± 243.5 mm²であり，オビオアクチン投与濃度が10 mg/kg以上の場合に腫瘍増殖抑制が認められている。

トキソプラズマ溶解抗原はトキソプラズマに作用するばかりではなく，マウスの同種あるいは同系肉腫やラットのメチルコラントレン誘発自家腫瘍に対して増殖抑制効果を示すBRMとして，注目を浴び

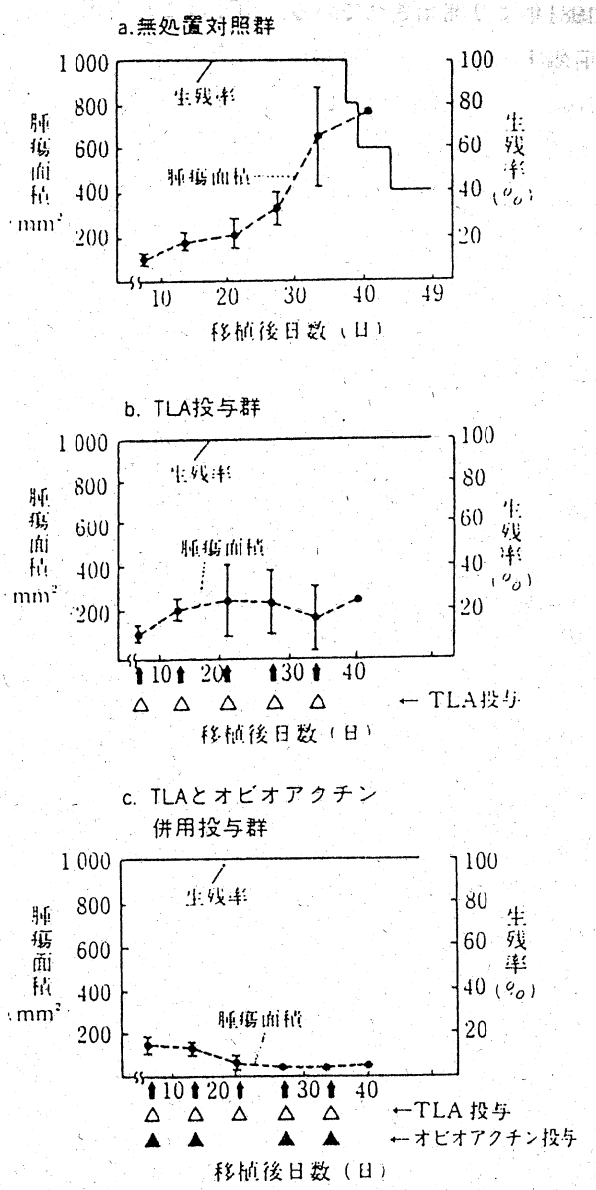


図6　S-180 細胞移植マウスに対するトキソプラズマ溶解抗原(TLA) とオビオアクチンの併用投与による増殖抑制効果

ている。そこで，トキソプラズマ溶解抗原とオビオアクチンの腫瘍増殖抑制に対する相乗効果について検討した。

8～10 週齢の ICR-JCL 系雄マウスの背部皮下に S-180 細胞を 1 匹あたり 1×10^6 個移植した。その後，週 1 回，トキソプラズマ溶解抗原を 1 匹あたり 100 μg 筋肉内注射した群(b)，トキソプラズマ溶解抗原100 μg とオビオアクチン40 mg を併用して筋肉内注射した群 (c)，無処置対照群 (a) の 3 群について S-180 腫瘍細胞の増殖程度およびマウスの生残率を調べた(図 6)。その結果，腫瘍移植後 35 日目における腫瘍面積はトキソプラズマ溶解抗原単独投与群が 198 mm^2 で，無処置対照群の 630 mm^2 に比較すると有意に腫瘍の増殖が抑制されている。さらに，トキソプラズマ溶解抗原とオビオアクチンの併用投与群では腫瘍移植後 20 日目以降，ほとんど腫瘍部位を触知できなく，腫瘍巣に強い壊死を認め，痂皮を形成した例もみられる。しかし，腫瘍が完全に消失した例はみられない。このように，オビオアクチンは，その単独投与でも軽度ながら腫瘍細胞増殖抑制傾向を示すが，制癌剤あるいは BRM などとの併用投与によってその抑制効果は著しく亢進されることが明らかとなっている。

オビオアクチンの細胞活性化機構

オビオアクチンはヒトおよび諸種動物由来マクロファージや体細胞内でのトキソプラズマ原虫の増殖を抑制する (Nagasawa, H. *et al.*, 1981；Sakurai, H. *et al.*, 1982；Suzuki, N. *et al.*, 1984a)。

まだ不明確な点が残るが，Saito ら (1987)* はオビオアクチン投与によるマクロファージの活性化と抗原虫効果は，オビオアクチン投与量とヒドロキシパーオキサイド (H_2O_2) やスーパーオキサイド基 (O_2^-) の産生放出量に濃度依存性があることを明らかにした。そしてごく最近，H_2O_2，O_2^- および一重項酸素 ($^1O_2$) とヒドロキシル基 (-OH) の 4 種の活性酸素中間体がマクロファージ内トキソプラズマ増殖抑制作用に関与する系について種々検討した結果，抗原虫作用には-OH が主役を演じ，H_2O_2 や O_2^- は前駆体であると現在，考えている。また，マクロファージの抗原虫作用における細胞内 cAMP や cGMP の役割について論じられている (Jones *et al.*, 1977)。オビオアクチン投与によるマクロファージ内 cAMP，cGMP は投与後 24 時間まで全く変化しない。そこで，オビオアクチンとともにジブチリル cAMP を 10^6 M 濃度で培養液中に添加してマクロ

*Saito, A. *et al.* (1987)：*Zbl. Bakt. Hyg. A.* 受理

ファージ内cAMP濃度や抗原虫作用を検討すると，cAMP濃度は増加するがトキソプラズマ増殖抑制率は明らかに減少する。したがって，オピオアクチン投与によるマクロファージの殺作用亢進には細胞内cAMPやcGMPは重要でないことがわかる。しかし，オピオアクチンは腎細胞など体細胞内原虫の増殖を明らかに抑制するにもかかわらず，H_2O_2やO_2^-など活性酸素中間体の放出はない（Suzuki *et al.*, 1984）。Saitoら（1987）は，Lonomycin-Aなどは細胞内抗原虫作用を有しながら活性酸素中間体の放出が認められないことから，細胞内抗原虫作用には活性酸素中間体によらない系があることを明らかにしている。このようなオピオアクチンを生体内に投与するとTリンパ球は感作されリンホカインを産生し，マクロファージの活性化と相まって非特異生体防御能の賦与に関与する。広義のBRMの作用を有するオピオアクチンは抗原虫治療剤との併用投与によって，抗原虫治療薬の毒性をも軽減することが明らかにされている（Apt *et al.*, 1986）。

おわりに

1986年6月に米国食品医薬局がインターフェロン-γを白血病治療薬として認可したが，この認可に先立ち米国癌研究所（NCI）はBRMに関する研究を4年間にわたって行い，その評価をCancer Treatment Reportに発表している（Talmadge & Herberman, 1986）。これまで欧米ではBRMの代表的物質であるBCGによる治療の効果がはっきりしなかったことと副作用の問題から，免疫療法に対して否定的な見方がなされていた。しかし，近年，生体の免疫機能が解明されるようになり，免疫療法に対する見直しが急速に高まり，非特異免疫治療剤の重要性が増してきている。1978年10月，NCIの癌治療部門は癌に対するBRMの評価研究のため，Mihich博士を委員長とする小委員会を発足させた。そして，1979年10月に小委員会はBRM評価のためのプログラムを発表した。それは，従来の化学療法剤のスクリーニング法とは異なり，動物細胞を用いて免疫学的評価を行うcommon trackと呼ばれるもので，1981年より開始されている。NCIはこれらBRMの前臨床試験評価を集中的かつ専門的に実施するためNCI Frederick癌研究施設にPreclinical Screening Laboratoryを新たに設立している。前臨床試験は免疫賦活物質および免疫調整物質について，これらの物質のそれぞれの効奏細胞に対する活性化作用と治療的効果との相関性を，脾細胞のNK活性，マクロファージの活性化，*in vitro*でのTリンパ球刺激性ならびにTリンパ球のアジュバント活性化，非特異免疫予防効果，治療効果などから多面的に検討し，その評価をしようとしている。一連の試験成績の評価から，これまで否定的であった免疫調整および賦活物質に対して新たな認識が得られており，BRMに対する研究開発が今後世界的に一段と活発化することになりそうである。

オピオアクチンは，異種動物由来細胞内原虫，細菌，ウイルスなどの増殖を抑制する作用を有する物質で，リンホカイン中のマクロファージ活性化因子，インターフェロン-γ，トキソプラズマ増殖抑制因子などとは異なる免疫調整物質と考えられている。このような異種細胞内微生物増殖抑制作用を有する免疫調整物質の存在はこれまでに知られておらず，この分子量約3000内外の糖ペプチドは著者らによって世界で初めて見出されたものである。さらに，オピオアクチンは各種微生物の増殖を抑制するばかりではなく，一部の腫瘍細胞の増殖抑制に関する免疫調整作用をもつことが確認されている。したがって，オピオアクチンは各種微生物による感染症の予防および治療補助剤として，また制癌剤やBRMとの併用投与によって生体防御能を強く賦活する物質として興味ある天然生理活性物質の一つといえよう。

一般に感染症や腫瘍に対する免疫化学療法には宿主の生体内で産生される蛋白質であるインターフェロン，IL-2，オピオアクチンなどの免疫調整物質を利用するものと，抗原虫剤，抗癌剤などの化学療法剤を利用するものとがある。この2つの間に位置するものが免疫賦活物質（OK432，レンチナン，トキソプラズマ溶解抗原など）を中心とした狭義のBRMであり，オピオアクチンはここにも含まれると考えてよい。これらBRMは生体内免疫調整物質の産生あるいは化学療法剤効果のいずれかを補助す

ることによって宿主の抵抗性を増強するものと推察される。このように，きわめて興味ある生体賦活作用を有するオピオアクチンであるが，現在のところ構造式は不明であり，生命工学技術による大量生産の方策も全く未解決であるために，詳細な生理作用に関する成績は得られていない。今後，新しい免疫調整物質としての生体作用を攻究するためには，本物質の構造式の解明と合成技術開発への展開が最重要課題であると考える。

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