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Specific and Nonspecific Mediation of Protective Immunity to *Toxoplasma gondii*

LILIANA REYES† AND J. K. FRENKEL*

Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kansas 66103

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We studied the specificity of protection conferred by *Toxoplasma gondii* immune lymphocytes and their supernatants on infected hamster kidney cells, using *Besnoitia jellisoni* immune lymphocytes as a nonspecific control. The intracellular growth of the organisms was measured by [^{3}H]uracil incorporation, and inhibition of multiplication was used as a measurement of immunity. Although the immune lymphocytes restricted principally the multiplication of homologous organisms, partial protection, expressed against the heterologous organism, was found. This was true for either parasite with intact lymphocytes or their supernatants. Exposure of immune lymphocytes to antigen for 18 to 24 h and treatment of kidney cells with supernatant fluids for 18 to 24 h were required for maximal protection. The specific protective mediator in supernatants of immune lymphocytes was characterized by dialysis as having a molecular weight between 3,000 and 12,000 and was found in the 3,000 to 5,000 peak after Sephadex G-50 chromatography. Nonspecific protective activity was greater than 12,000 by dialysis; it chromatographed in the excluded peak, measuring over 43,000, and was destroyed by exposure to pH 2. In vitro production of lymphokines from toxoplasma immune lymphocytes was first detected 7 to 10 days after vaccination of hamsters. At about the same time, hamsters began to resist challenge infection with the pathogenic RH strain of *T. gondii* and were able to prevent its multiplication in lungs, liver, spleen, and the subcutaneous infection site. The expression of tissue immunity and the production of toxoplasma-immune lymphokines appear to be time-related events.

Toxoplasma gondii actively invades and grows within many types of host cells. Although severe cell-destructive infections leading to symptoms of pneumonia, myocarditis, hepatitis, encephalitis, retinochoroiditis, etc., are medically important, asymptomatic infections are much more numerous and also are of biological and immunological interest. Such infections maintain toxoplasmas in nature and in populations of animals and humans that may have been selected for their ability to develop immunity.

How this anti-toxoplasma immunity is mediated has been the subject of many studies (for a review, see reference 10). There is some agreement on the major importance of cell-mediated immunity and the subsidiary importance of antibody to protection (13). The roles of oxygen intermediates in macrophages and gamma interferon have recently been studied in vitro (23, 27, 30, 31). However, because they are specifically induced but nonspecifically expressed, they cannot account for the immune response against *T. gondii* which is both induced and expressed specifically (11). Immune lymphocytes have been shown to convey specific toxoplasma immunity to mice and hamsters (2, 15, 16, 18, 19, 22, 24). Because, unlike *Leishmania*, *T. gondii* parasitizes mainly nonmacrophages in vivo (so-called somatic cells), we became interested in studying protective immunity in these tissue cells. Chinchilla and Frenkel (7) demonstrated that lymphocytes from immune donors can protect kidney cells and fibroblasts and began to characterize the mediator secreted by lymphocytes conferring specific protective immunity on hamster cells in culture. Subsequently, these authors described a similar specific mediator in mice and showed that cellular immunity was strain restricted (8).

In this third study, we focus on the secretion of both specific and nonspecific protective activity. We identified the latter after refining our measurements of protection by using [^{3}H]uracil incorporation to assess the inhibition of the multiplication of toxoplasmas within infected cells. This technique, initially described by Pfefferkorn and Pfefferkorn (26) and developed by McLeod and Remington (22), quantitates the multiplication of toxoplasmas in approximately 10^5 host cells on a cover slip instead of the 500 to 1,000 host cells counted visually and does so objectively. We knew from earlier work (7) that similar numbers of organisms entered normal cells, or those exposed to immune lymphocytes or supernatants, and that the last two prevented multiplication of toxoplasmas. We therefore wanted to confirm the specificity of protection by lymphocytes and their supernatants against *T. gondii* by using this more sensitive technique and to quantitate nonspecific protection by the related but immunologically distinct *Besnoitia jellisoni* (11). The molecular weight of the specific mediator was confirmed to be between 3,000 and 5,000, but we also found nonspecific lymphokine activity above the 43,000-molecular-weight region. We showed the secretion of mediator to be temporally related to the appearance of protective immunity in intact hamsters and demonstrated the fate of challenge organisms injected 2, 4, and 6 days after immunization with a vaccine candidate of *T. gondii*.

MATERIALS AND METHODS

Animals. Adult female outbred Golden Syrian hamsters were obtained from SASCO, Inc., Omaha, Nebr. Adult male and female PD-4 inbred hamsters were purchased from Charles River Breeding Laboratories, Inc., Wilmington,

* Corresponding author.
† Present address: Facultad de Microbiologia, Universidad de Costa Rica, San Jose, Costa Rica.

Mass., and bred in the Animal Care Facilities at Kansas University Medical Center. Female mice (CF-1) from SASCO were also used throughout these studies. Mice weighed 20 to 25 g each, and hamsters weighed 60 to 80 g each, except when used for primary kidney culture, for which weanling hamsters (30 to 40 g) were used. Animals were isolated and checked serologically and immunologically for absence of antibody and immunity to *T. gondii* and *B. jellisoni*.

Infections. Tachyzoites of the RH, T-45, and ts-4 strains of *T. gondii* and the cystless A strain of *B. jellisoni* were used for immunization of animals and for measuring the immune response in vitro as previously described (7). The ts-4 strain of *T. gondii*, kindly provided by E. Pfefferkorn, Department of Microbiology, Dartmouth University School of Medicine, was maintained in human skin fibroblast cultures at 35°C by serial passage every 3 to 4 days (9). The other strains were passaged in mice (7).

Immunization, challenge, and infection. Hamsters were infected subcutaneously with 10^4 to 10^5 tachyzoites of either *T. gondii* RH or *B. jellisoni* A. Since both microorganisms give rise to fatal infections in hamsters (15), immunization required the prophylactic use of sodium sulfamerazine, 90 mg/dl, in drinking water for 2 weeks, beginning 2 days after infection (7). Immunization with the low-virulence ts-4 vaccine candidate did not require administration of chemoprophylaxis. Hamsters were challenged subcutaneously with the homologous organism 4 weeks after infection. Controls died within 2 weeks; however, when no illness or death occurred among the immunized hamsters, the challenged survivors were considered immune and used as lymphocyte donors (11, 15).

Human skin fibroblast. Monolayers were grown in a medium composed of RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) plus 10% heat-inactivated (60°C, 60 min) fetal calf serum and 100 U of penicillin-streptomycin per μg per ml. This medium is referred to as medium A. The cultures were incubated at 35°C in 25-cm^2 polystyrene tissue culture flasks (Corning Glass Works, Corning, N.Y.). The medium A was replaced once weekly, and the cultures were split 1:3 when confluent. Human skin fibroblasts were kindly provided by E. R. Pfefferkorn. Fibroblast cultures were infected at a ratio of 1 organism to 10 cultured cells in 100 μl of medium A.

Primary hamster kidney cultures. Weanling hamsters were used as cell donors, and the cells were harvested and grown as previously described (7).

Preparation of tachyzoite antigen. Counted suspensions of tachyzoites were centrifuged at 275 × *g*, and the supernatant was discarded. The pellet was suspended in distilled water (10 μl per 10^7 organisms) and subjected to 8 to 10 cycles of freezing and thawing. These freeze-thawed preparations were diluted to give a final concentration equivalent to 10^6 organisms per ml of medium A.

Preparation of lymphocytes. Techniques for preparation of lymphocytes and their supernatants have been described previously (7). Briefly, peritoneal exudate cells were harvested 5 days after casein-antigen stimulation by washing the peritoneal cavity. Cells were macrophage depleted by adherence to plastic petri dishes. These preparations usually contained 95 to 98% lymphocytes, 3 to 5% macrophages, and <1% polymorphonuclear leukocytes as determined by morphology; 90 to 95% of lymphocytes failed to stain with 0.1% toluidine blue, and they were added to target cells in a 1:1 ratio unless otherwise stated.

Preparation of mediator from immune lymphocytes. Adherent cell-depleted lymphocytes (1×10^7 to 2×10^7/ml) from the peritoneal exudates of immune hamsters were incubated for 24 h at 37°C under 5% CO_2 in medium A containing 10^6 freeze-killed homologous organisms per ml. After incubation, lymphocyte cultures were centrifuged at 1,400 × *g* for 20 min at 4°C. The supernatant was tested for antibody in the Sabin-Feldman dye test (12). Few supernatants contained antibody, and only supernatants free of antibody were stored at −20°C until used. Control supernatants were prepared as described above with lymphocytes obtained from uninfected hamsters and exposed to toxoplasma antigen.

Study of immune components. To study the effects of immune or normal lymphocytes on kidney cells, medium A was removed from culture by aspiration. Approximately 10^5 macrophage-depleted lymphocytes were added to each cover slip in a final volume of 100 μl of medium A to provide a 1:1 lymphocyte-to-kidney-cell ratio. To some cover slips, 100 μl of the specific freeze-thawed antigen suspension (10^6 organisms per ml) was added. If no antigen was added, cultures received 100 μl of medium A.

To study lymphocyte supernatants, medium A was removed from culture, and kidney cells were overlaid with 200 μl of undiluted lymphocyte supernatant. In all experiments, some kidney cell cultures receiving medium A were used as controls. As an additional control and to study the effect of antigen on microorganism multiplication, some cells received 200 μl of antigen. After incubation for 24 h, the lymphocyte or mediator-treated kidney cell cultures were infected with 10^5 organisms in a final volume of 100 μl of medium A (approximately 1 organism per 10 kidney cells).

Assessment of multiplication by uracil incorporation. The multiplication rate of toxoplasma or besnoitia in hamster primary kidney cell monolayers was measured by the incorporation of [^{3}H]uracil into acid-precipitable nucleic acid. This precursor is specifically incorporated by the parasite and can be metabolized because toxoplasmas, and apparently besnoitias, contain uracil phosphoribosyl transferase, while host cells lack this enzyme and do not metabolize uracil (22, 26). Infected kidney cells were given a 4-h [^{3}H]uracil pulse with 3 μCi of [5,6-^{3}H]uracil (40-60 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 30 μl of medium A. The [^{3}H]uracil was added during the last 4 h of a 24-h infection period (37°C, 5% CO_2). Thereafter, the radioactive medium A was aspirated and discarded; the monolayer was dissolved in 2 ml of 1% ice-cold sodium dodecyl sulfate (ICN Pharmaceuticals Inc., Plainview, N.Y.) containing 100 μg of unlabeled uracil (ICN) per ml. After a 30-min incubation at room temperature, the nucleic acids were precipitated with 0.5 ml of ice-cold 3 N tricholoracetic acid. Nucleic acid-bound radioactivity was harvested by filtration of the suspension through a glass fiber filter (type A/E; Gelman Sciences, Inc., Ann Arbor, Mich.). Filters in glass scintillation vials were moistened with 150 μl of distilled water, and 1 ml of a tissue solubilizer (Research Products International Corp., Mount Prospect, Ill.) was added; after 30 min, 10 ml of scintillation fluid (Omnifluor; New England Nuclear Corp., Boston, Mass.) in a toluene base was added. Vials were counted in a beta scintillation counter for 10 min. Results were given in total counts per minute, based on the mean counts of at least three identically treated samples. The percent infection of groups treated with immune cells or mediator was estimated by comparison with infected control cultures that received only medium A by using the following formula. Percentage infection = (immune total counts × 100)/control total counts.

Chromatography. Gel filtration of lymphocyte superna-

TABLE 1. Development of toxoplasma antibody and immunity to challenge in hamsters following vaccination with *T. gondii* T-45[a]

Day of measurement after vaccination	Antibody titer (dye test)	Mean day of death (% survival)	Supernatant activity (% infection)
0	<2	8.2 ± 0.4 (0)	93 ± 20
2	<2	9.5 ± 0.8 (0)	115 ± 53
4	<2	9.5 ± 1.4 (0)	ND[b]
6	<2	10.5 ± 0.5 (66)	82 ± 18
8	8	–[c] (100)	ND
10	2,000	42 ± 3.5 (50)	33 ± 50
15	8,000	– (100)	36 ± 8
20	65,000	– (100)	29 ± 21
25	ND	– (100)	ND
30	65,000	– (100)	38 ± 25
35	32,000	– (100)	ND
40	32,000	39 (83)	31 ± 7

[a] The lymphokine activity of peritoneal lymphocytes obtained at eight time intervals was measured in culture.
[b] ND, Not done.
[c] –, All six hamsters survived 60 days.

tants was carried out as described previously (7). Some fractions were pooled and dialyzed against 100 volumes of distilled water with continuous mixing for 24 h at 4°C. The dialysis bags used (Spectrapor; Spectrum Medical Industries, Inc., Los Angeles, Calif.) had a cutoff at molecular weight 3,000 or 12,000. The dialyzed fractions were frozen, lyophilized, sterilized by passage through a 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.), and assayed for immune activity on kidney cells infected with either *T. gondii* or *B. jellisoni*.

Onset of *T. gondii* lymphokine production during genesis of immune response. *T. gondii* T-45 was given as a single subcutaneous dose of 10^2 organisms to groups of five to six hamsters at day zero. At stated time intervals, we studied the ability of peritoneal exudate lymphocytes to produce toxoplasma lymphokines and the antibody titer and resistance of the hamsters to *T. gondii* RH challenge (Table 1).

Fate of *T. gondii* in normal and immune hamsters. Groups of hamsters vaccinated with strain ts-4 were challenged after 2, 4, and 6 days with *T. gondii* RH. After challenge, one hamster per vaccinated group was killed every day for eight consecutive days. Tissue samples (0.1 g) from lung, spleen, and liver were individually ground with Alundum and suspended in 0.85% saline solution, and the tissue suspensions were inoculated intraperitoneally into two mice (half the suspension in each).

The number of toxoplasmas in the tissue samples was estimated by comparison of the day of death of subinoculated mice with a titration curve relating inocula and survival time (Fig. 1A) (15). The number of parasites in immune hamsters was compared with the number of organisms in nonimmune matched controls.

Statistical evaluation. Student's *t* test was used for the comparison of differences between counts. *P* values of ≤ 0.05 were considered significant.

RESULTS

Fate of *T. gondii* in vaccinated hamsters during immunogenesis. Concentrations of *T. gondii* RH in tissues from hamsters challenged 2 days after vaccination increased similarly to those in unvaccinated hamsters. However, in lung, liver, spleen, and the site of subcutaneous inoculation (data not shown) in hamsters challenged 4 days after vaccination, *T. gondii* organisms continued to multiply for only 4 to 5 days, and then the number declined. When animals were challenged 6 days after vaccination, small numbers of the organisms could be found during the 10 days after challenge (Fig. 1B to D). A comparison of the concentration of *T. gondii* organisms in the different organs showed that liver was the first to clear the toxoplasmic infection (Fig. 1C), and vaccinated hamsters were not capable of completely clearing toxoplasmas from tissues during the period studied (Fig. 1B, C, and D).

Elaboration of anti-toxoplasma activity during immunogenesis. Antitoxoplasma activity in lymphocyte supernatants was tested by exposing primary hamster kidney cells in vitro for 24 h and infecting them with toxoplasma tachyzoites. Multiplication of toxoplasmas was measured by [^{3}H]uracil incorporation. Antitoxoplasma activity was found 10, 15, 20, 30, and 40 days after *T. gondii* T-45 vaccination (Table 1) and, in a similar experiment (not shown), as early as 7 days after vaccination. Antibody was detected 8 days after vaccination. Although nonimmune hamsters died from pneumonia after 8 days of infection, some of the vaccinated hamsters survived when challenged after 6 and 8 days and most developed immunity between 10 and 16 days after vaccination. However, a few died from encephalitis at later times.

Determination of the optimal amount of toxoplasma antigen. Peritoneal lymphocytes from toxoplasma-immune hamsters in the presence of homologous antigens were able to protect kidney cells against toxoplasma infection (Fig. 2). Peritoneal lymphocytes from normal or besnoitia-immune animals conferred little or no protection with 10^4 to 10^7 lysed toxoplasmas as antigen; however, protection was seen with 10^8 lysed organisms alone (data not shown) or with toxoplasma-immune, besnoitia-immune, or normal lymphocytes. To avoid this antigen effect, only 2×10^6 lysed toxoplasmas per ml were used in all subsequent experiments with immune lymphocytes or supernatants.

Optimal lymphocyte-to-kidney-cell ratio. Lymphocytes from toxoplasma-immune hamsters were added at ratios of 0.1:1, 1:1, and 5:1 to kidney cells growing on cover clips. Inhibition of the growth of toxoplasmas was proportional to the number of immune lymphocytes added, as long as toxoplasma antigen was also present (Fig. 3).

Secretion time of toxoplasma mediator. The antigen contact time required for toxoplasma immune lymphocytes to produce antitoxoplasma activity was studied by harvesting the supernatant fluids of replicate cultures at intervals. The multiplication of toxoplasmas was progressively inhibited at longer incubation times (Fig. 4). Supernatants harvested after 18 to 24 h of incubation showed the greatest inhibition.

Exposure time for toxoplasma mediator. Using 24-h lymphocyte supernatants, we studied the contact time necessary for maximal protection of kidney cells. Lymphocyte supernatants were applied for increasing periods in a staggered fashion and then removed by aspiration. Thereafter, the kidney cells were rinsed with medium A and infected with aliquants of the same toxoplasma suspension. A sorption time of 18 to 24 h was required to significantly inhibit growth of toxoplasmas (Fig. 5).

Microorganism specificity of protection by immune lymphocytes and supernatants. The multiplication of both toxoplasmas and besnoitias was inhibited maximally (to 12 to 16%) by homologous immune lymphocytes (Table 2). Partial inhibition (to 46 to 52%) was found with the heterologous immune lymphocytes. Normal lymphocytes did not confer any protection, irrespective of whether homologous antigen was used. Similar results were found in five different exper-

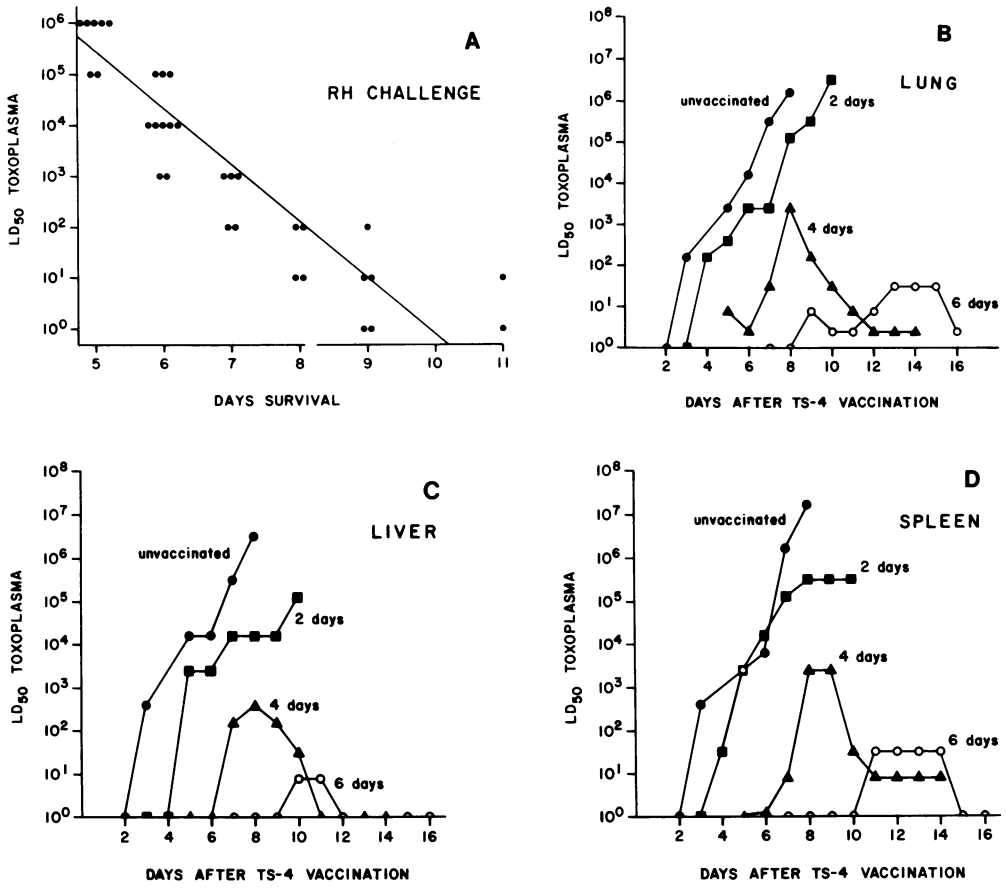

FIG. 1. Enumeration of *T. gondii* RH organisms in tissues of control hamsters and of vaccinated (*T. gondii* ts-4) hamsters challenged on day 2, 4, or 6. Hamster tissues were inoculated intraperitoneally into mice; the survival time of these mice was translated into a quantity by comparison with a titration performed contemporaneously (A). Calculated numbers of toxoplasmas in lung (B), liver (C), and spleen (D) are shown.

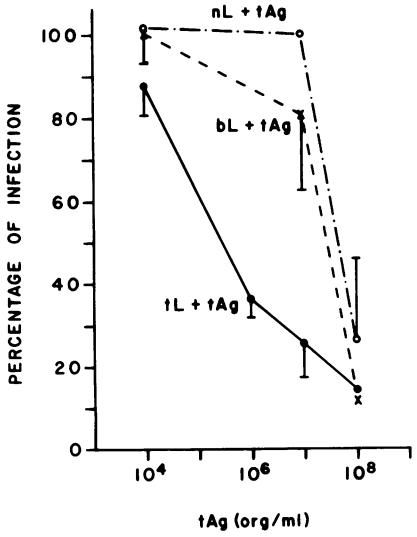

FIG. 2. Effects of different concentrations of toxoplasma antigen on toxoplasma infection of kidney cells treated with normal or immune lymphocytes. All lymphocyte concentrations were 2 × 10^5/ml. Abbreviations: n, normal; t, toxoplasma; b, besnoitia; L, immune lymphocyte; Ag, antigen.

iments (data not shown). Both besnoitia and toxoplasma supernatants showed pronounced activity against the homologous infection and partial activity against the heterologous agent (Table 2).

Effects of dialysis on immune lymphocyte supernatants. Supernatants of toxoplasma-immune lymphocytes were dialyzed through molecular weight 3,000 or 12,000 exclusion membranes. Toxoplasma inhibitory activity was not altered after 3,000-molecular-weight dialysis, and therefore bioactivity was associated with molecules of molecular weight greater than 3,000 (Table 3). As in previous experiments, supernatants from toxoplasma-immune lymphocytes showed more protection against toxoplasmas than against besnoitias. However, after molecular-weight-12,000 dialysis, the toxoplasma percent infection increased from 38 ± 25% to 81 ± 7% (P = 0.05), whereas the nonspecific besnoitia-inhibitory activity remained unchanged at 57 ± 9% and 53 ± 4% (Table 3).

Effect of pH 2 treatment on the specific antimicrobial activity. A supernatant from toxoplasma-immune lymphocytes that showed similar inhibitory activity against *T. gondii* and *B. jellisoni* lost this antimicrobial activity after exposure to pH 2 (Table 4); no conclusion can be made with regard to the specific inhibitory activity.

Chromatography of supernatants from immune lymphocytes. Toxoplasma-immune supernatants and medium A exhibited three peaks after chromatography on Sephadex G-50 (Fig. 6). Only fractions I and II were tested for

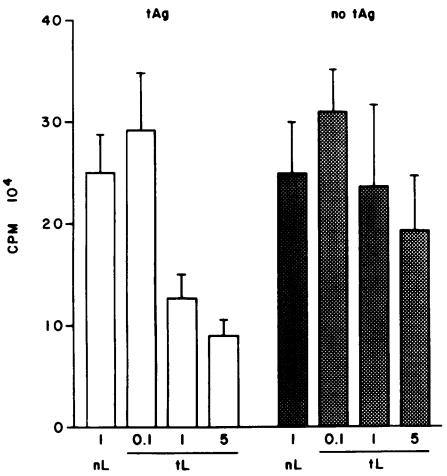

FIG. 3. Determination of optimal lymphocyte: kidney cell ratio, with and without specific antigen (2×10^6 lysed organisms). Numbers of toxoplasmas in kidney cells were measured by uptake of [^{3}H]uracil. Abbreviations: n, normal; t, Toxoplasma; b, Besnoitia; L, immune lymphocytes.

antitoxoplasma activity (fractions that contained most of the proteins). Fraction I contained activity against both *T. gondii* and *B. jellisoni* (Table 5). On the other hand, toxoplasma-immune supernatant fraction II inhibited *T. gondii* only (11,883 ± 1,247 versus 21,185 ± 2,190 for medium A; $P < 0.01$) and did not inhibit *B. jellisoni* growth (6,234 ± 130 versus 6,332 ± 1,305). Similar results were obtained in a second experiment (data not shown). Peak III had previously been shown to have no inhibitory activity against either *T. gondii* or *B. jellisoni* (7).

From a calibration curve of the Sephadex G-50 column with ovalbumin, cytochrome *c*, and vitamin B_{12}, we estimated the molecular weight of fraction I to be higher than 43,000 and that of fraction II to be 3,000 to 5,000.

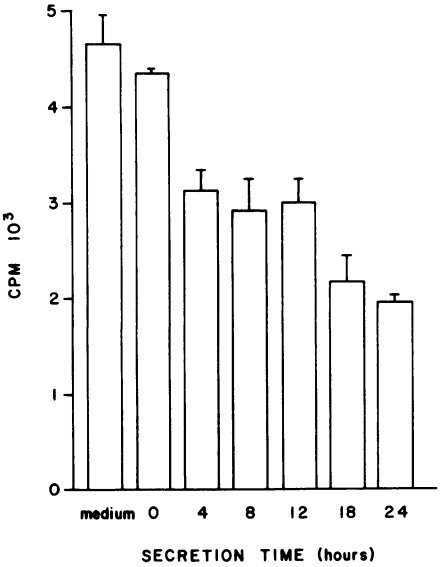

FIG. 4. Determination of inhibitory activity present in supernatants of toxoplasma-immune lymphocytes incubated with toxoplasma antigen for various times. [^{3}H]uracil incorporation by toxoplasma-infected kidney cells after 24 h is shown.

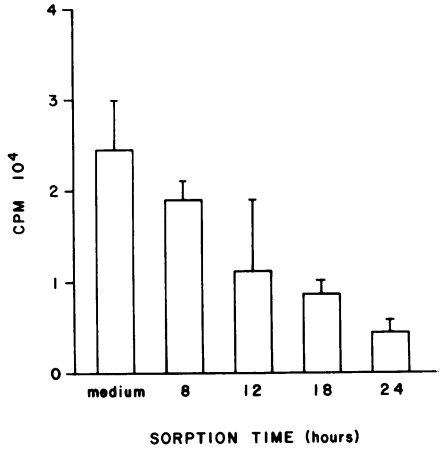

FIG. 5. Expression of inhibitory activity from supernatants of immune lymphocytes against toxoplasma after various periods of exposure to kidney cells. [^{3}H]uracil incorporation by toxoplasma-infected kidney cells after 24 h is shown.

DISCUSSION

Soluble mediators of toxoplasma immunity have been identified in immune supernatants from human peripheral blood cells (5), and in peritoneal exudate and in spleens of mice (29, 30) and hamsters (7). The lymphocyte supernatants from mice and humans protected macrophages and monocytes, and those derived from hamsters and mice also protected kidney cells and fibroblasts (7, 8).

All these supernatants inhibited the multiplication of toxoplasmas in host cells. Although most known mediators are nonspecific, certain mediators of toxoplasma immunity from hamsters and mice were known to be specific (7, 8). Because immunity against *Toxoplasma* is specific in vivo (11), we attribute considerable importance to the demonstration of specific activity by a putative mediator of protective immunity in vitro.

Conventionally, mediators or lymphokines are prepared from supernatants of a heterogeneous lymphoid cell population (1, 14). When lymphocytes from animals chronically infected with *T. gondii* were incubated in the presence of the specific antigen, they produced and released into the culture medium a variety of biologically active substances, such as macrophage migration inhibitory factor (14), interleukin 2 (4), gamma interferon (27, 30), and toxoplasma mediator (7, 8).

To assess parasite specificity of the activity in the lymphocyte supernatants, at least three conditions must be met. First, a microorganism specificity control is required. In the present experiments, *B. jellisoni* was used for this purpose. Although *T. gondii* and *B. jellisoni* are morphologically and biologically closely related and have some antigenic similarities, there is no significant cross-immunity between them (11). A second requirement is the selection of a host cell in which nonspecific inhibitory mechanisms are least pronounced. For this reason, macrophages were avoided when specificity was studied. Activated macrophages are known to kill intracellular organisms nonspecifically by release of oxygen metabolites (23). This oxygen burst is induced by gamma interferon (25), a lymphokine that we suspected to be

TABLE 2. Test for microorganism specificity of protection[a]

Source of lymphocytes and antigen[b]	[^{3}H]uracil incorporation (cpm ± SD) (% infection ± SD)	
	T. gondii	*B. jellisoni*
t L + t Ag	**8,298 ± 2,657 (16 ± 32)**[c]	14,872 ± 1,191 (52 ± 8)
b L + b Ag	23,855 ± 4,858 (46 + 20)	**3,546 ± 833 (12 ± 23)**
n L + t Ag	50,309 ± 21,723 (96 ± 43)	36,504 ± 6,769 (127 ± 18)
n L + b Ag	59,831 ± 7,534 (114 ± 12)	25,194 ± 7,056 (88 ± 28)
None	52,219 ± 18,493 (100 ± 35)	28,755 ± 4,366 (100 ± 15)
Supernatant		
t L + t Ag	**5,810 ± 581 (11 ± 10)**	38,328 ± 2,663 (62 ± 7)
b L + b Ag	23,317 ± 623 (46 ± 3)	**11,727 ± 2,112 (19 ± 18)**
None	50,991 ± 9,243 (100 ± 18)	61,900 ± 18,140 (100 ± 30)

[a] Normal, toxoplasma- or besnoitia-immune lymphocytes and specific antigen or immune supernatants were placed in contact with kidney cells and infected with specific and nonspecific organisms.
[b] Abbreviations: n, normal; t, toxoplasma; b, besnoitia; L, immune lymphocyte; Ag, antigen; None, medium. A only.
[c] Bold-faced type identifies homologous combinations.

TABLE 3. Inhibitory activity of toxoplasma-immune supernatant against *T. gondii* and *B. jellisoni* before and after 3,000- and 12,000-molecular-weight dialysis

Treatment	Activity (cpm ± SD) (% infection ± SD)[a]		
	T. gondii (12,000)	*B. jellisoni* (12,000)	*T. gondii* (3,000)
Before dialysis			
Supernatant	1,393 ± 352 (38 ± 25)d	3,059 ± 289 (57 ± 9)e	3,177 ± 1,263 (23 ± 40)c
Medium A only	3,609 ± 623 (100 ± 17)	6,182 ± 437 (100 ± 7)	13,969 ± 6,654 (100 ± 48)
After dialysis			
Supernatant	2,100 ± 179 (81 ± 9)a	2,497 ± 89 (53 ± 4)b	2,702 ± 1,179 (16 ± 44)c
Medium A only	2,592 ± 320 (100 ± 12)	4,746 ± 1,624 (100 ± 34)	16,470 ± 4,676 (100 ± 28)

[a] Significance compared with control: a, not significant; b, $P = 0.03$; c, $P = 0.02$; d, $P = 0.01$; e, $P = 0.001$.

present in toxoplasma immune supernatant. Thirdly, the number of organisms must be measured sensitively and in a consistently reliable manner. For this reason, we used uracil incorporation into the entire *T. gondii* and *B. jellisoni* population on the cover slip instead of microscopic counts of the organisms in only 500 to 1,000 host cells.

Previous work suggested that lymphocyte suspensions from toxoplasma-immune hamsters and their supernatants were essentially parasite specific (7, 8), although in some experiments there was some effect on the heterologous agent. Besnoitia immune lymphocytes were shown to reduce the growth of toxoplasmas by one-half in vitro (15); however, supernatants from besnoitia-immune lymphocytes had no effect on toxoplasma growth (7). This was interpreted as indicating some unidirectional cross-protection.

While working to establish the uracil evaluation technique, we noted a degree of reciprocal cross-protection both with immune lymphocytes and with their supernatants (Table 2).

This was further corroborated in a series of experiments in which specific (toxoplasma) and nonspecifically (besnoitia) immune lymphocytes were employed. When comparable

TABLE 4. Effects of exposure to pH 2 on the [^{3}H]uracil incorporation by *T. gondii* or *B. jellisoni* in kidney cells treated with toxoplasma-immune supernatant

Exposure to pH 2	Activity (cpm ± SD) (% infection ± SD)[a]	
	T. gondii	*B. jellisoni*
Before		
Supernatant	1,393 ± 432 (39 ± 31)*	3,059 ± 354 (49 ± 12)*
Medium A	3,609 ± 764 (100 ± 21)	6,182 ± 535 (100 ± 9)
After		
Supernatant	2,295 ± 263 (72 ± 11)**	3,669 ± 476 (81 ± 13)†
Medium A	3,184 ± 529 (100 ± 17)	4,503 ± 474 (100 ± 11)

[a] Significance compared with medium A control: *, $P < 0.01$, †, $P < 0.05$, **, not significant.

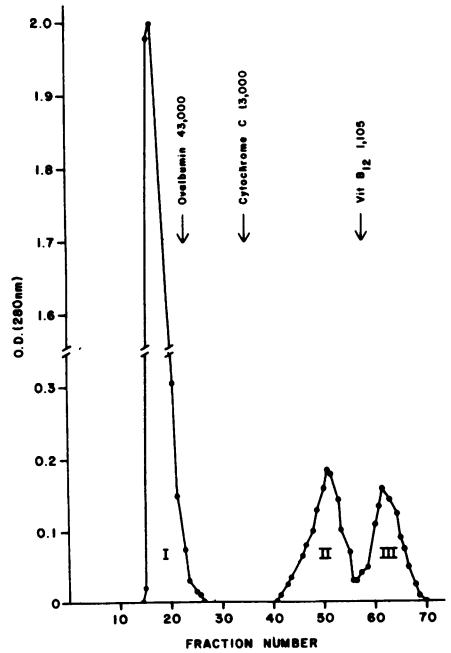

FIG. 6. Profile of lymphocyte supernatant in medium 199–10%, heat-inactivated fetal calf serum–penicillin–streptomycin eluted from a Sephadex G-50 column. Peaks I, II, and III are labeled.

TABLE 5. Specific and nonspecific inhibition by chromatographic fractions I and II of toxoplasma-immune supernatant on growth of *T. gondii* and *B. jellisoni*

Treatment	Activity (cpm ± SD) (% infection ± SD)[a]	
	T. gondii	*B. jellisoni*
Fraction I		
Supernatant	3,322 ± 695 (32 ± 21)*	964 ± 171 (20 ± 18)*
Medium A control	10,309 ± 1,328 (100 ± 13)	4,846 ± 787 (100 ± 16)
Fraction II		
Supernatant	11,883 ± 1,247 (56 ± 10)*	6,332 ± 1,305 (102 ± 21)†
Medium A control	21,185 ± 2,683 (100 ± 13)	6,234 ± 130 (100 ± 2)

[a] Significance compared with control: *, $P < 0.01$; †, not significant.

numbers were used, immune lymphocytes were more effective in controlling growth of the homologous organism. Similar results were obtained with supernatants of committed lymphocytes which were consistently more effective against the homologous organisms than against the heterologous infection.

The activity responsible for the specific protection by toxoplasma or besnoitia mediators was of low molecular weight, as shown by dialysis and Sephadex G-50 chromatography (Tables 3 and 5). Microorganism-specific protection was retained after 3,000-molecular-weight dialysis but was lost after 12,000-molecular-weight dialysis (Table 3). After chromatography, the specific protection was recovered in fraction II (Table 5), with a peak of approximately 3,800 (Fig. 6). This toxoplasma mediator corresponds to that of molecular weight 4,000 to 5,000 described previously (7).

It is known that gamma interferon is present in toxoplasma supernatants (27, 30), and that this is a parasite-nonspecific lymphokine. Therefore, our next step was to investigate whether gamma interferon might be responsible for the nonspecific protection. Because no antiserum against hamster gamma interferon was available, it was necessary to use indirect methods. The activity responsible for the nonspecific protection had a high molecular weight, was retained after 12,000-molecular-weight dialysis (Table 3), and was recovered in the chromatographic exclusion fraction (molecular weight, >43,000) (Table 5; Fig. 2). The nonspecific protection factor also appeared to be sensitive to pH 2 (Table 4), a defining characteristic for gamma interferon (32).

McCabe et al. (20) have shown that the injection of gamma interferon can prolong the time to death of toxoplasma-infected mice. However, in only one experiment did gamma interferon completely protect the mice against death. Shirahata and Shimizu (30) and Sakurai et al. (27) identified gamma interferon in the lymphoid cells of immune mice. Whether our nonspecific protective factor is indeed gamma interferon awaits further study.

Table 6 gives a comparison of toxoplasma mediator with transfer factor, antibody, and certain known lymphokines. Although toxoplasma mediator has some characteristics in common with transfer factor (molecular weight and antigen specificity), there are some differences also. Toxoplasma mediator appears to be strain restricted in mice and hamsters (7, 8), whereas transfer factor is said not to have such restrictions (6, 17). Transfer factor probably acts through interaction with lymphocytes (3); however, Toxoplasma mediator interacts directly with the parasitized host cells, conveying to them specific immune characteristics (Table 2). Toxoplasma mediator was shown to be species restricted (7). Although transfer factor was initially described as species restricted, (17), some nonrestricted transfer factor has also been described (6).

Is mediator production in vitro correlated with immunity in vivo? Hamsters injected intraperitoneally with anti-besnoitia supernatant showed delayed time to death after infection with 10 to 100 organisms when compared with animals receiving antiserum or normal serum (7). This suggested that this activity could be part of the protective immune response. In the present study, the production of antitoxoplasma activity and development of immunity to *T. gondii* RH challenge were parallel events (Table 1). Antitoxoplasma activity was first detected 7 and 10 days after vaccination, a time at which more than 50% of the hamsters survived challenge with *T. gondii* RH. Furthermore, all hamsters that resisted challenge produced antitoxoplasma activity. Although in these experiments, no attempts were made to differentiate between toxoplasma mediator and gamma interferon, the production of either lymphokine requires the appearance of toxoplasma-committed lymphocytes.

The expression of tissue immunity correlated temporally with the appearance of lymphocytes capable of producing toxoplasma mediator. The number of toxoplasmas in hamsters declined starting at day 8 in liver, spleen, and lung tissue (Fig. 1) and at the infection site of hamsters challenged 4 and 6 days after vaccination. Likewise, antibody appeared in 8 days after vaccination (Table 1). Although intracellular toxoplasmas are protected from antibody (12) in an active progressive infection, tachyzoites are exposed to antitoxoplasma antibodies when the host cell disintegrates. In the

TABLE 6. Comparison of attributes of toxoplasma mediator with those of antibody and certain other lymphokines

Factor	Specificity			Mol wt	Strain restriction
	Induction	Expression	Host		
Antibody	+	+	−	150,000	−
Alpha interferon	+	−	+	18,000–20,000	−
Gamma interferon	+	−	+	15,000	−
Transfer factor	+	+	−	3,500–12,000	−
Toxoplasma mediator	+	+	+	3,000–5,000	+

presence of complement, this antibody activates the classical complement pathway, causing cytolysis of the parasite (28). Despite these effects, passively transferred antibodies are not sufficient to protect most animals against primary *T. gondii* (13). The present data therefore suggest a positive correlation between the rate of appearance of toxoplasma mediator and the expression of tissue immunity, while antibodies could also have contributed to parasite elimination. Although the ts-4 vaccine candidate does not persist (9), some of the challenge imoculum does (Fig. 1). This is characteristic of natural and experimental *Toxoplasma* infection, with *Toxoplasma* immunity best described as infection immunity or of premunition type (10).

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