

CELL-MEDIATED IMMUNITY AGAINST BESNOITIA AND TOXOPLASMA IN SPECIFICALLY AND CROSS-IMMUNIZED HAMSTERS AND IN CULTURES*

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The advent of immunosuppressive therapy has increased the need to understand the compromised defense mechanisms which normally cope with latent infections. These generally intracellular infections progress more or less out of contact with antibody. Committed lymphoid cells can adoptively transfer immunity against many of these, including listeriosis (1), lymphocytic choriomeningitis (2), ectromelia (3), equine herpes virus 2 (4), *Histoplasma capsulatum* (5), *Toxoplasma gondii*, and *Besnoitia jellisoni* (6). Analysis of the roles of antibodies, lymphocytes, and macrophages in the intact animals can be critically and more quantitatively pursued in cell culture.

In contrast with the much studied mouse listeriosis model which displays an evanescent cellular infection and immunity (7), chronic hamster besnoitiosis permits the study of host mechanisms controlling latent infections such as those caused by cytomegalovirus, herpes virus, and *Toxoplasma* in man (4). Furthermore, hamsters infected with *Besnoitia* or *Toxoplasma* offer a means to separate humoral, cellular, specific, and nonspecific effects (8), shown by homospecific and heterospecific immunizations and challenges with these related parasites.

Toxoplasma and *Besnoitia* are obligate intracellular protozoa with similar morphology and which give rise to similar lesions in hamsters (9). Sulfadiazine therapy inhibits both parasites (6), providing a means for control of experimental infection for immunization with living, virulent strains. No cross-reactivity is seen between these parasites with the dye test of Sabin and Feldman (10), and immunization with one parasite does not protect against challenge with the other (8, 11). Organisms persist in immunized hosts to sustain a premunition, and as mentioned above this specific immunity may be adoptively transferred to normal recipients with lymphoid cells (6). Immunoglobulins inactivate extracellular organisms with the cooperation of serum components, collectively called accessory factor (10). Even high levels of antibodies offer only slight protection, as measured in acute infections (4) and in established in-

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fection when cellular components are depressed with corticosteroids (12). Although elicited, interferon also appears ineffective against *Besnoitia* and *Toxoplasma* (8, 13, 14).

In the present study we wish to elucidate the effective cellular components and their specificity in the course of immune surveillance (premunition) and after challenge with these pathogens.

Materials and Methods

Animals.—Hamsters were female golden LVG/LAK obtained from Lakeview Hamster Colony, Newfield, N. J. Mice were female Carworth-Webster-derived stock supplied by Mid-Continent Research Animals, Inc., Shawnee, Kans.

Immunization.—Hamsters received subcutaneous (sc) inocula of virulent tachyzoites (rapidly dividing forms, trophozoites) either of *Toxoplasma gondii*, RH strain, or of a cystless strain of *Besnoitia jellisoni* as previously described (4, 6). Sulfadiazine chemoprophylaxis was provided for 3 wk and hamsters were challenged after the 4th wk. Survivors were considered homologously immune and placed in experimental groups 3 wk after challenge.

Challenges.—Inocula for immunization and challenge contained 10^4 organisms by Neubauer Hemacytometer count. Inocula for challenges were further characterized by titration in mice. Survival times were graphed for each dilution (e.g. Fig. 3) and the LD_{50} determined by the proportional parts method of Reed and Muench (15). The ID_{50} equaled the LD_{50} for these *Besnoitia* and *Toxoplasma* strains because surviving mice neither developed antibody nor survived subsequent challenge.

Antisera.—Ascitic serum from chronically infected hamsters was prepared by a method modified from Takemoto and Habel (16) using freeze-killed organisms and Freund's complete adjuvant. Hamsters produced about 15 ml of ascitic fluid every week, having titers of 1:2,000 or greater as determined by modified Sabin-Feldman dye test (4). The suitability of fresh hamster sera or cell-free exudates to serve as accessory factor (AF)¹ was assayed in comparison with human AF.

Preparation of Hamster Cell Cultures.—

Fibroblasts: Fibroblasts from 9-day embryos were prepared and grown to confluency in Medium 199 (Hanks's balanced salt solution) with $NaHCO_3$, 5% HIHS (Heat-inactivated horse serum; 56°C, 30 min) and 100 U penicillin, 100 μ g streptomycin and 1 μ g amphotericin B per milliliter in Leighton tubes at pH 7.4 and 37°C.

Peritoneal exudate cells (PEC): Peritoneal exudate cells were stimulated with 6 ml of 0.5% sodium caseinate (practical grade, Matheson) autoclaved in saline. Immunized hamsters received in addition 10^6 freeze-thaw disrupted homologous organisms obtained from hamsters in the inoculum. 4-5 days later cells were harvested by washing the peritoneal cavity with 6 ml NCTC 109 (17) + 20% HIHS + antibiotics with 60 U of heparin (without preservative). Differential cell counts revealed 75-80% large mononuclear phagocytes, 20-25% small mononuclear cells, and 1-2% polymorphonuclear leukocytes. Suspensions were washed twice at 4°C by sedimentation (100 g) and adjusted to 10^6 nucleated cells per 0.3 ml. Six 22 × 22 mm cover slips were acid-cleaned, heat-sterilized, and placed in polyethylene Petri dishes (100 × 15 mm, Fisher Scientific Co., Pittsburgh, Pa.). Each cover slip was overlaid with 0.3 ml of the washed cell suspension and incubated for 2 h at 37°C in an atmosphere of 5% CO_2 in air. Following adherence of macrophages, various manipulations with cell washing or fluid aspiration and reconstitution were carried out.

¹ *Abbreviations used in this paper:* AF, accessory factor; HIHS, heat-inactivated horse serum; MIF, migration inhibitory factor; and PEC, peritoneal exudate cells.

Lymphocytes: Lymphocytes were also derived from PEC suspensions. Washed cells were adjusted to 10^6 /ml and 10 ml dispensed into each Petri dish. After an adherence period of 1 h at 37°C in an atmosphere of 5% CO₂ in air, the nonadherent cells were resuspended by swirling and decanted into a second dish. The first adherent monolayer was washed with medium and suspended cells also decanted into the second dish. After three washings, the nonadherent population was composed of 95–98% lymphocytes which were 90–95% viable by toluidine blue exclusion. These suspensions were adjusted to 10^6 /0.3 ml for use in reconstitution experiments of 5×10^6 /0.25 ml in neutralization experiments.

Infection

Besnoitia tachyzoite kinetics were followed in the hamster peritoneum, embryo fibroblasts, and peritoneal macrophages.

Intraperitoneal Infection.—Intraperitoneal infection with Besnoitia was carried out in five groups of animals: (a) not immunized; (b) passively protected with 8.0 ml of anti-Besnoitia serum (1:512 dye test titer) given sc in several sites the day of infection; (c) immune to Toxoplasma; (d) Toxoplasma-immune with passive anti-Besnoitia serum; and (e) Besnoitia-immune. Each hamster received an i.p. inoculum, containing a suspension of 10^5 LD₅₀ Besnoitia. At various intervals after infection, 6.0 ml of the harvest medium [75% NCTC 109 (17) + 25% HHIS + antibiotics + heparin] was injected into the peritoneum of an individual hamster from each group. A portion of the aspirated peritoneal fluid was assayed for LD₅₀ by mouse titration. A second portion was dispensed on slides, dried, fixed, and assayed for cell and Besnoitia counts at harvest. A third portion was cultured to assay the extension of Besnoitia growth kinetics in vitro.

Confluent Embryo Fibroblasts.—Confluent embryo fibroblasts were infected with 10^5 Besnoitia tachyzoites per Leighton tube. After various intervals, cover slips were removed and examined for Besnoitia.

Peritoneal Cells.—

Effects of sera: Peritoneal cells were permitted to adhere to cover slips for 1 h. Without removing nonadherent cells, the fluid volume was reduced from each cover slip by careful aspiration from one edge. Infectious mouse-harvested Besnoitia maintained at 4°C was sedimented (150 g) and suspended in either anti-Besnoitia serum (1:256), anti-Toxoplasma serum (1:256), or normal serum (<1:2 for both), all heat-inactivated (56°C for 30 min) as 20% with NCTC 109. Suspensions were adjusted to 10^5 organisms in 0.2-ml vol and dispensed on the cover slips. 2 h were allowed for penetration, followed by rinses designed to wash free cells and organisms from the attached monolayer. Cultures were then incubated in fresh medium containing the respective serum. Cover slips were examined for intracellular Besnoitia up to 24 h after infection.

Cross-infection: Adherent and nonadherent peritoneal cells were prepared from Besnoitia- and Toxoplasma-immune hamsters as before. These monolayers were infected with either 10^5 mouse-derived Toxoplasma or Besnoitia per cover slip. Cover slips were removed at intervals and inspected for intramacrophage parasites.

Lymphocyte neutralization: Peritoneal lymphocytes were prepared from Besnoitia- or Toxoplasma-immune or nonimmune hamsters by adherence separation as before and adjusted to 5×10^6 /0.25 ml in medium 199. Another reaction mixture consisted of unseparated suspensions of lymphocytes and macrophages from Besnoitia-immune animals, which were suspended in excess fluid volume to prevent infection of cells. The cells were incubated in tubes with 0.25 ml suspension of 10^5 target Besnoitia tachyzoites for various periods of time up to 6 h at 37°C in an atmosphere of 5% CO₂ in air. For a positive control resembling the dye test, 10^5 Besnoitia were added in human AF to hamster anti-Besnoitia antibody in a 1:16 dilution to the 0.5 ml reaction mixture. At the end of incubation the cells and organisms were diluted to a 1 ml vol, and 0.2 ml portions were injected into four mice to determine the LD₅₀.

By comparing the average survival of the mice against a titration curve of the original suspension, the remaining organisms in terms of LD₅₀ were extrapolated.

Lymphocyte-macrophage combination: After 3 h incubation, peritoneal adherent cells from nonimmune, Besnoitia-, or Toxoplasma-immune hamsters were washed free of nonadherent cells with the direct spray of medium by syringe and needle. Lymphocytes from the three groups of animals were used to reconstitute the adherent monolayers. Approximately 10⁶ lymphocytes were added to each cover slip monolayer to provide an approximate 1:1 lymphocyte:macrophage ratio. The cells settled for 30 min after which fluid volume over the cover slip was reduced by aspiration. Cultures were then infected with 10⁵ Besnoitia on each cover slip. Following 1 h for infection, all nonattached cells or engulfed organisms were washed free and infected cover slips transferred to new dishes with fresh medium. Incubation continued for 24 h with cover slips removed at intervals for inspection.

Measurement and Analysis of in Vitro Assays.—At each interval of incubation in various experiments, cover slips were removed from Petri dishes or Leighton tubes, dipped twice in physiologic saline, fixed in Helly's solution (Zenker-formol), stained with May-Grünwald-Giemsa by the method of Jacobson and Webb (18), and mounted for microscopic examination. The coverslips were coded and counted by one investigator with a fixed method of scanning (19). Generation times in fibroblasts and peritoneal cells were calculated from cover slip counts allowing for error due to multiple infection (20).

The data were expressed as (a) the number of intracellular parasites per 100 infected macrophages, or (b) the number of intracellular parasites per 100 macrophages of the total population.

The number of macrophages between replicate cover slips did not differ significantly at the 5% level by analysis of variance.

An analysis of variance and multiple comparisons were performed on data according to the methods of Winer (21).

RESULTS

Since pathogenicity of Besnoitia and Toxoplasma has been considered to relate to multiplication rates (20) rather than toxins (22), we examined the growth of these parasites in cells of various immune status.

Besnoitia Multiplication in Hamster Fibroblasts (Table I).—Intracellular growth started with a lag phase followed by exponential replication (Table I). At 6 h many Besnoitia had rounded up in their vacuoles, demonstrating nuclear budding preceding division. Most had completed the first division at 10 h. Clones of four organisms appeared in approximately 40% of the infected cells at 16 h. The mean generation time during exponential growth was 6.3 h. Many of the distended fibroblasts, bearing 8–16 organisms, began lysing after 29 h. A similar, somewhat more rapid dynamics has been described for RH Toxoplasma in fibroblasts by Jones et al. (23).

Besnoitia Multiplication in Peritoneal Macrophages from Immunized and Nonimmunized Hamsters (Figs. 1 and 2).—The generation time of Besnoitia in normal macrophages was 12.8 h, in Toxoplasma-immune 12.8 h, but in Besnoitia-immune 38.5 h, equivalent to a 2- and 3-fold delay.

Nevertheless, in the Besnoitia-immune culture, a few cells were found harboring doublets by 8 h, clones of four by 16 h, and clones of eight by 24 h. Thus, cells in these populations varied in their ability to inhibit parasite growth. By

considering prevalence in the overall culture and counting the number of remaining organisms and all macrophages, digestion was noted as early as 2 h in the *Besnoitia*-immune cells. Deformed organisms became indistinguishable at 8 h and absent at 16 h. At this later interval, 50–60 times more organisms could be counted in the two other groups than in the *Besnoitia*-immune macrophages.

Macrophages from nonimmune hamsters appeared fusiform, maintained contact inhibition, and became heavily parasitized (Fig. 2, *a*). Macrophages from *Toxoplasma*-immune animals were also fusiform with increased numbers of vacuoles and reduced contact inhibition (Fig. 2, *b*). *Besnoitia*-immune phagocytes were quite vacuolated, lower in contact inhibition, with far fewer parasite numbers (Fig. 2, *c*). In some *Besnoitia*-immune cultures which displayed intense anti-*Besnoitia* activity, giant cells formed (Fig. 2, *d*).

TABLE I
Multiplication of Besnoitia jellisoni Strain A in Hamster Embryo Fibroblasts

H postinfection in culture	No. <i>Besnoitia</i> in infected fibroblast
<i>h</i>	\pm <i>SD</i>
1	1.21 \pm 0.06
3	1.18 \pm 0.11
5	1.25 \pm 0.27
7	1.38 \pm 0.13
10	2.10 \pm 0.56
15	3.32 \pm 0.81
20	4.38 \pm 0.41
24	6.88 \pm 1.15
26	9.19 \pm 0.36
28	10.68 \pm 1.11
29	10.78 \pm 0.98
30	lysis

A minimum of 400 cells were counted per interval.

Fate of Besnoitia in the Peritoneum (Figs. 3 and 4).—A linear dose-response relationship between the day of mouse death and 10-fold dilutions of *Besnoitia* inocula (Fig. 3) allowed us to follow parasite numbers in the challenged peritoneum of hamsters (Fig. 4).

Each animal in five groups received 10^5 LD₅₀ *Besnoitia* i.p. *Besnoitia*-immune animals significantly reduced parasite numbers over a 5-day period (<0.01 level *t* test). *Toxoplasma*-immune and nonimmune hamsters, even with passively administered anti-*Besnoitia* serum, were unable to limit *Besnoitia* growth, and subsequently died. A degree of nonspecific resistance was observed in the *Toxoplasma*-immune animals equivalent to that afforded by specific antibody injections in nonimmune hamsters. Incomplete ability in recovery of inoculated parasites from the peritoneum at 1 h was attributed to dilution, and at 8 h was due to parasite penetration into serosal cells lining the peritoneal cavity. While

in specifically immune animals recoverable parasite numbers remained static between 24 and 72 h, and then began to drop, numbers continued to climb after 8 h in all other groups.

Intraperitoneal Inflammatory Response (Fig. 5).—Parasite numbers increased in the two groups not immunized against *Besnoitia*, and declined in immune hamsters. Lymphoid and polymorphonuclear leukocytes initially rose sharply in all groups, but then dropped to be followed by a substantial lymphocyte rise

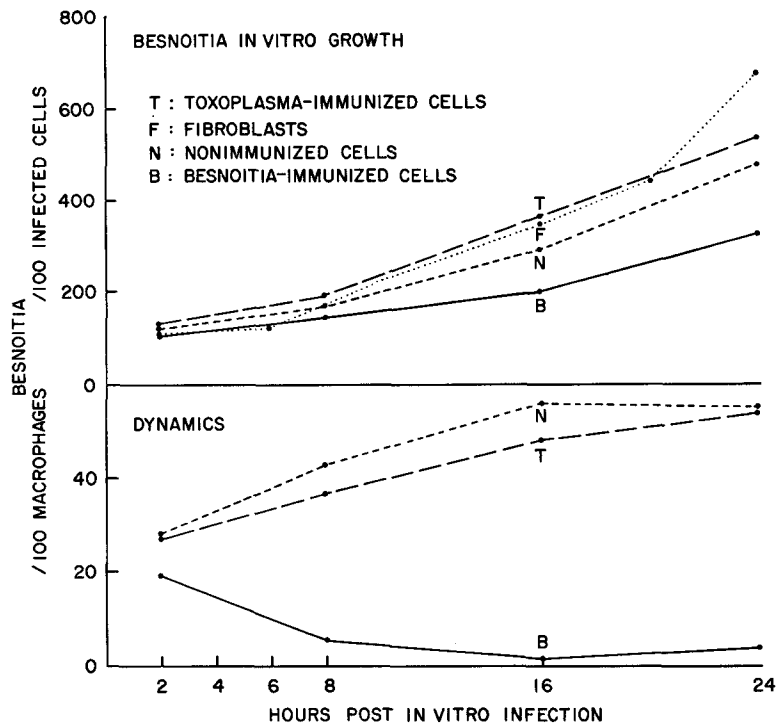


FIG. 1. Curves describing the growth and dynamics of *Besnoitia jellisoni* in cultured macrophages from nonimmune controls (N), Toxoplasma-immune hamsters (T), and Besnoitia-immune hamsters (B). Infected hamster embryo fibroblasts (F) provided a control. Means of four (SD not included).

only in the Besnoitia-immune group (Fig. 5, *a-c*). Macrophage numbers rose in the groups not immunized against *Besnoitia*, but dropped in the Besnoitia-immune peritoneum just after infection. This may have been due to a specific disappearance reaction of macrophages since a few macrophage clusters (or aggregations) were observed on smears (Fig. 5, *c*). The Besnoitia-immune animals also showed an increase of lymphocytes and macrophages between 48 and 120 h, accompanied by a steady decline of *Besnoitia* numbers.

Assay for Accessory Factor in Hamster Serum and Peritoneal Fluid (Table

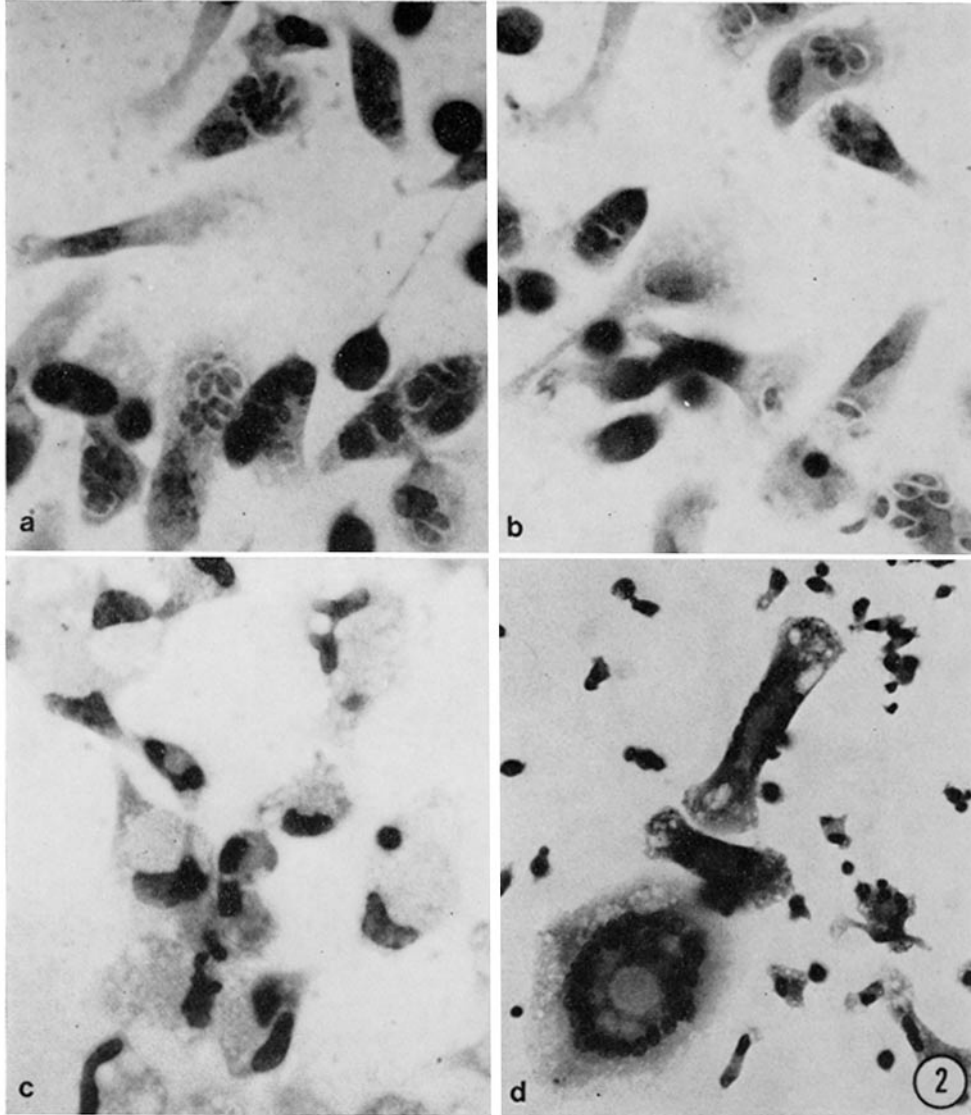


FIG. 2. Cells in culture. (a) 24 h *Besnoitia*-infected peritoneal phagocytes from nonimmune hamsters. ($\times 800$) (b) 24 h *Besnoitia*-infected peritoneal phagocytes from *Toxoplasma*-immune hamsters. ($\times 800$) (c) 24 h *Besnoitia*-infected peritoneal phagocytes from *Besnoitia*-immune hamsters, stimulated i.p. 4 days before harvest with killed antigen and sodium caseinate. ($\times 800$) (d) 24 h *Besnoitia*-infected giant cells from *Besnoitia*-immune hamsters, stimulated i.p. 5 days before harvest with killed antigen and sodium caseinate. ($\times 275$)

II).—The passively administered antisera had been heat-inactivated (56°C for 30 min) before administration to hamsters with i.p. infections. We therefore wanted to determine whether complement-associated AF was available in hamster sera and peritoneal fluids to account for the infection-delaying effects with specific passive antibody. A modified in vitro dye test procedure was performed comparing hamster sera and peritoneal fluid with human plasma containing AF. Live *Toxoplasma* tachyzoites served as indicators. It was found that hamsters do indeed have AF in serum and cell-free exudate, but at approximately half the levels seen in human serum (Table II, tests 1–4).

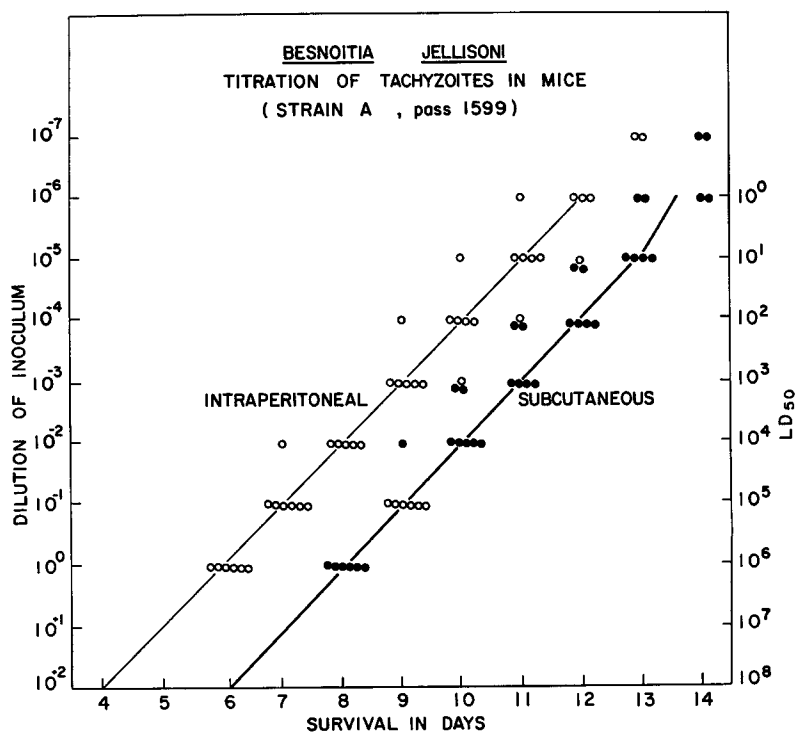


FIG. 3. Titration plots of the linear dose-response relationships between log lethal dose killing 50% (LD_{50}) of *Besnoitia jellisoni* strain A (pass 1599) via i.p. and s.c. routes and mouse survival in days. Six mice per dilution.

Effect of Antisera on Besnoitia Growth in Peritoneal Macrophages Infected in Vitro (Fig. 6).—Peritoneal cell cultures from *Besnoitia*-immune hamsters provided little support for *Besnoitia* growth (Fig. 6). Organisms ingested by macrophages following exposure to antiserum were irregular in shape and had darkly staining intranuclear bodies. Peritoneal cells from nonspecifically immune and from nonimmune hamsters supported the growth of *Besnoitia*. Those

growing in *Toxoplasma*-immune cells on the whole showed a slightly reduced growth rate compared with those in nonimmune cells. In each instance, the addition of *Besnoitia* antiserum lowered growth rates most and *Toxoplasma* antiserum was second in comparison with nonimmune serum.

Cross-Challenge of Cell Cultures (Fig. 7).—Duplicate cultures were prepared from *Besnoitia*-immune, *Toxoplasma*-immune or nonimmune hamsters and infected with either *Toxoplasma* or *Besnoitia* organisms. After 24 h, the homologous organisms were more efficiently suppressed in growth, with only a minor delay in growth in heterologously immune cultures (Fig. 7, *b* and *c*). Cells from *Besnoitia*-immune hamsters in this experiment appeared to provide

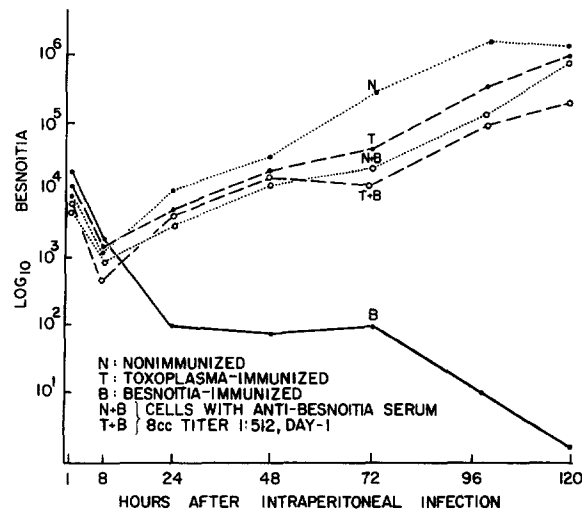


FIG. 4. Fate of *Besnoitia* in the peritoneum of hamsters following administration of 10^5 LD₅₀ *Besnoitia* tachyzoites. The animals were nonimmune controls (N), *Toxoplasma*-immune (T), *Besnoitia*-immune (B), and non- and *Toxoplasma*-immune with 8 cc passively administered anti-*Besnoitia* serum at 1:512 titer given on day 1. One hamster and six mice per interval.

more nonspecific resistance to *Toxoplasma* growth than cells from *Toxoplasma*-immune hamsters to the growth of *Besnoitia*.

Viability of Besnoitia Cultured with Cells or Serum Components (Fig. 8).—Peritoneal lymphocytes only, from either normal, *Toxoplasma*-, or *Besnoitia*-immune hamsters were compared with a macrophage-lymphocyte combination from *Besnoitia*-immune hamsters, and with anti-*Besnoitia* serum and AF when incubated with free *Besnoitia* tachyzoites. It was found that *Besnoitia*-immune lymphocytes under these conditions proved no more detrimental to the free *Besnoitia* than other lymphocytes (Fig. 8). Even lymphocyte-macrophage combinations from specifically immune donors were ineffective when phagocyto-

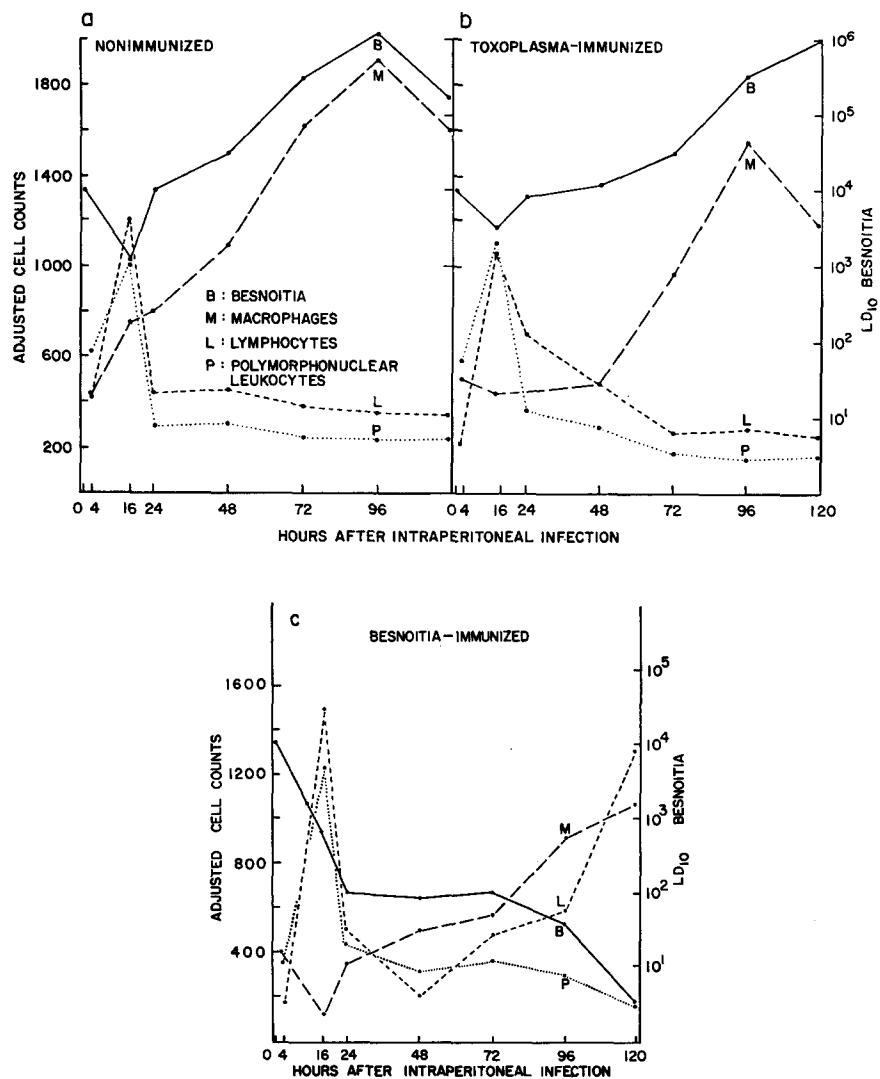


FIG. 5. Free cell and *Besnoitia* dynamics in the hamster peritoneum following administration of 10^5 LD_{50} *Besnoitia* tachyzoites. (a) Nonimmune controls; (b) *Toxoplasma*-immune hamsters; and (c) *Besnoitia*-immune hamsters. Cell types enumerated include: macrophages (M), lymphocytes (L), polymorphonuclear leukocytes (P), and *Besnoitia* tachyzoites (B). Each point represents counts of 50 fields of air-dried cells stained with Giemsa at $\times 400$.

sis was prevented by an excess of fluid. On the other hand, specific antibody and AF reduced the numbers by several logs over the 6 h exposure.

Besnoitia Growth in Macrophages Reconstituted with Lymphocytes (Figs. 9 and 10).—Nine culture combinations of macrophages and lymphocytes, from

TABLE II
Assay of Accessory Factor in Hamsters

Test	Antibody	Accessory factor		Modified toxoplasma
		<i>ml</i>		
1 Rabbit 1:128 (H 56°C)		0.1 Hamster serum		15
2 Rabbit " "		0.3 Hamster serum		85
3 Rabbit " "		0.3 Hamster exudate		80
4 Rabbit " "		0.1 Human plasma		85
5 Hamster 1:1024 (H 56°C)		0.1 Hamster serum		75
6 Hamster " "		0.1 Human plasma		85
7 Hamster <1:2		0.1 Human plasma		5

Complete reaction mixtures: 0.1 ml antibody solution, designated volume of accessory factor and 0.1 ml saline suspension of *Toxoplasma* tachyzoites. Percent modified organisms determined by lack of staining with alkaline methylene blue (10).

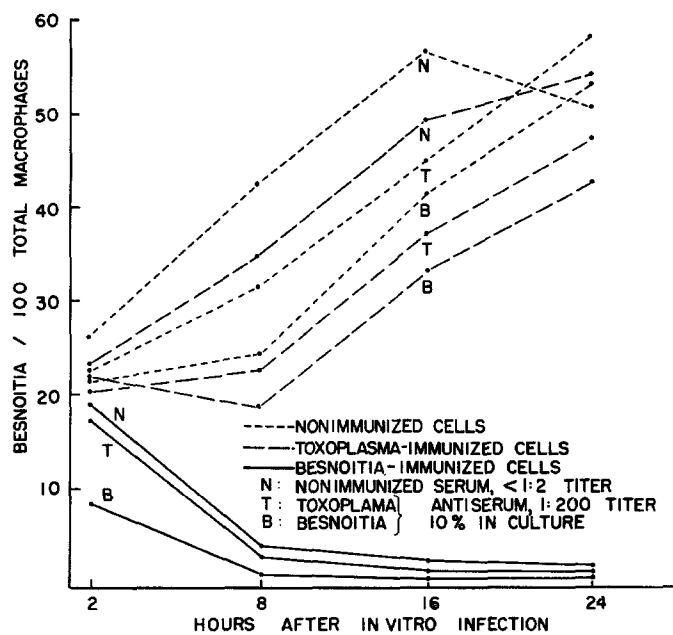


FIG. 6. Effect of antisera on in vitro *Besnoitia* dynamics. Cultures were cells from non-immune controls (---), *Toxoplasma*-immune (- -) and *Besnoitia*-immune (—). Inactivated antisera were added to the cultures, suspending the *Besnoitia* tachyzoites. Sera sources were normal hamster sera (N), *Toxoplasma* (T), and *Besnoitia* (B) providing a 1:200 titer in the medium. Each point represents six counts.

the three sources, were infected with *Besnoitia* and examined at intervals (Fig. 9). After 16–24 h, it was evident that the *Besnoitia*-immune lymphocytes improved the ability of macrophages from nonimmune or *Toxoplasma*-immune hamsters to deal with *Besnoitia* in vitro infections.

These differences were clarified in terms of significant contrasts by analysis of variance on the 24 h raw data. A confidence interval for significant differences between any pair of mean values was constructed from the nine combinations. Significant contrasts divided the combinations into two exclusive groups, and a significant multiple contrast was revealed between the means of the two

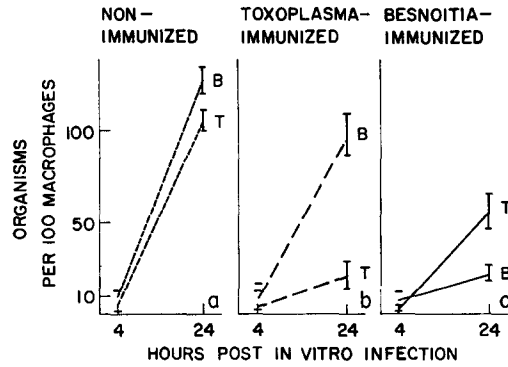


FIG. 7. Cross-challenge in cell culture. Besnoitia (B) and Toxoplasma (T) tachyzoites were used to infect peritoneal cell cultures from nonimmune, Toxoplasma-immune and Besnoitia-immune hamsters. Mean of six \pm SD.

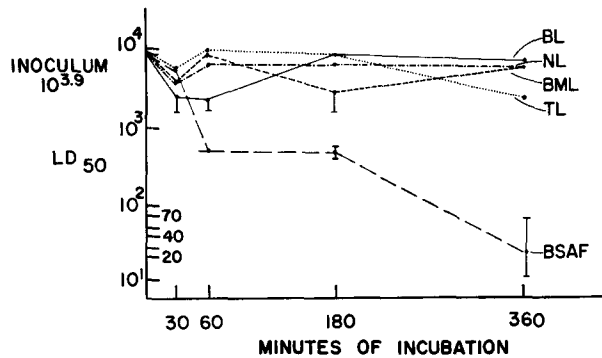


FIG. 8. Neutralization test against free Besnoitia tachyzoites with lymphocytes from nonimmune (NL), Toxoplasma-immune (TL), and Besnoitia-immune (BL) hamsters. Macrophages and lymphocytes from Besnoitia-immune hamsters (BML) and anti-Besnoitia serum (1:20) and accessory factor (BSAF). Each point represents two tubes and eight titration mice.

groups (Fig. 10). Interaction analysis between the lymphocyte classes and three macrophage classes on Besnoitia counts is also shown by graphic interpretation of the 24 h results. These indicate that (a) lymphocytes from Besnoitia-immune hamsters strongly reduced numbers of Besnoitia in macrophages, irrespective of origin; (b) lymphocytes from Toxoplasma-immune hamsters demon-

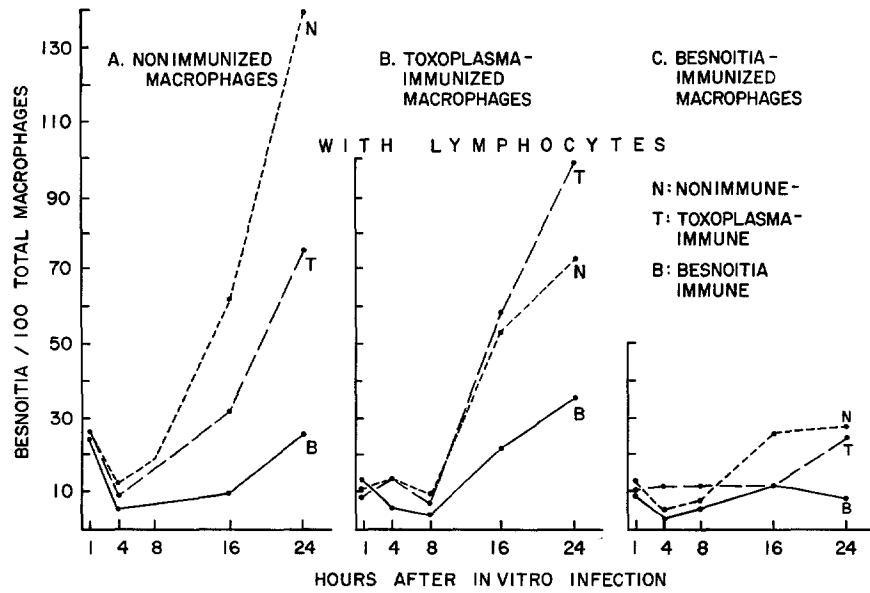


FIG. 9. Effect of macrophage-lymphocyte co-cultivation on Besnoitia growth. Nine groups were formed with B, T, and N macrophages and B (—), T (— · —), and N (---) lymphocytes all infected with Besnoitia. Each point represents two slides and six counts each.

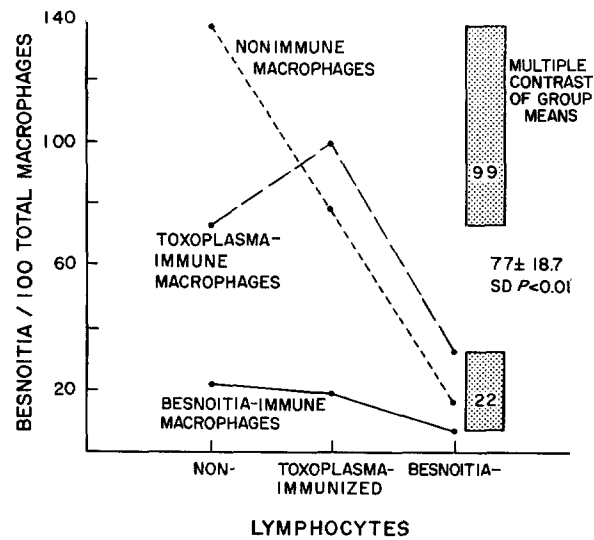


FIG. 10. Interaction analysis of macrophage-lymphocyte co-cultivation on Besnoitia growth at 24 h. Multiple contrast was calculated from analysis of variance data (see text).

strated an intermediate but not significantly different effect from that of non-immune lymphocytes; (c) adherent monocytes from *Besnoitia*-immune hamsters had been specifically instructed to reduce *Besnoitia* before harvest or during adherence; and (d) specific recognition could not be washed from these cells, and could not be overridden by heterospecific lymphocytes.

Giant Cell Aggregation.—Giant cells (Fig. 2, *d*) with 15–150 nuclei formed only in cultures of peritoneal exudates from *Besnoitia*-immune hamsters stimulated or challenged for 5 days with live or dead *Besnoitia* organisms. The resulting type of cell fusion was not seen in casein-stimulated peritoneal cultures of *Toxoplasma*-immune hamsters challenged with *Besnoitia*, although lowered contact inhibition was common.

Separation of Cellular Immunity from a Lymphokine Active on Macrophages.—Formation of giant cells was examined as a possible indicator of a lymphokine-mediated activity. Cell-free supernatants of 24–48 h infected cultures containing giant cells were layered over nonimmune macrophage cultures for 24, 48, and 72 h. These cultures became activated, showed lower contact inhibition, and formed a few small giant cells (15–40 nuclei). However, when these cultures were infected with *Besnoitia* at the end of the three supernatant stimulation intervals, giant cells and individual macrophages supported *Besnoitia* growth as did unstimulated nonimmune cultures.

DISCUSSION

Demonstration of immunity against besnoitiosis and toxoplasmosis by cellular transfer (6) suggested a study of the interaction of these organisms with macrophages and lymphocytes *in vitro*. In advance we wanted to determine the maximal protection of humoral antibodies under the experimental conditions employed, since it was realized that specific antibody could be synthesized in cell recipients and in culture. However, anti-*Besnoitia* serum administered passively to nonimmune or *Toxoplasma*-immune animals offered only a minor delay in parasite growth and host death (Fig. 4), confirming earlier observations (12, 24, 25).

A heat-labile complement-like “accessory factor” has generally been considered necessary for the lysis of antibody-exposed extracellular *Toxoplasma* or *Besnoitia*. Since accessory factor is believed to be absent in mice (26), we wanted to know if its absence in hamsters accounted for the poor passive protection. We found that, on the contrary, accessory factor was available in hamster fluids at about one-half of the human serum levels (Table II), and in mice at about one-fourth human serum levels (unpublished observations).

The inferior protection of humoral antibody in intact hamsters was apparently neither due to lack of accessory factor nor ineffectiveness of heat-inactivated antisera, but due to short periods of extracellular exposure. When the organisms were prevented from entering cells, antibody with accessory factor was fully active (Fig. 8). However, under ordinary circumstances of infection both

organisms can leave and reenter host cells in less than a minute (27, 28). Therefore, the limited efficacy of antibody is not surprising, nor its inability to prevent lethal infection in hamsters.

Committed lymphocytes which are available in immunized animals or are transferred to unprotected animals presumably migrate via the bloodstream into the sites of infection where they interact with microbes and release a variety of biologically active molecules (29). However, when peritoneal lymphocytes from immunized hamsters were incubated with free *Besnoitia*, they did not neutralize extracellular parasites under conditions in which specific antibody and accessory factor were effective (Fig. 8). Apparently lymphokine is not directly cytotoxic for extracellular parasites.

On the other hand, similarly sensitized lymphocytes specifically instructed macrophages in which organisms usually multiplied abundantly, to limit growth or destroy *Besnoitia* *in vitro* (Fig. 9). Thus, lymphocytes assume the role of immune mediators, expressing their effect via the macrophage in the present system.

An increasing number of lymphocytes entered the peritoneum of specifically immune and challenged hamsters at a time when parasite numbers were diminishing. The high lymphocyte-to-macrophage ratio (approximately 1:1) by the 5th day after challenge suggests effective mediator interaction with effector macrophages only in the immune group. In the other group, the number of macrophages also increased, but the number of lymphocytes decreased. Koster and McGregor (29) have reported that effector lymphocytes are released from lymphoid organs of *Listeria*-immune rats, circulate briefly, and enter sites of inflammation such as the casein-stimulated peritoneum, where they may be collected for adoptive transfer of immunity. Contrary to previous reports with unstimulated exudates (6), we have been able to adoptively protect hamsters against *Besnoitia* with peritoneal exudates induced with antigen and caseinate. These peritoneal cells were the active agents in the present study.

The final reduction of *Besnoitia* in the hamster peritoneum within 5 days (Figs. 4 and 5, *c*) resembles the second-set response and disappearance of allogeneic tumor cells 5 days after challenge in mice (30). Reduction and disappearance of infected fibroblasts in cultures harvested 72 h after *i.p.* challenge of hamsters, suggests that lymphocytes may be selectively cytotoxic to *Besnoitia*-infected host cells or that they can limit the growth of organisms within fibroblasts as commonly observed in the first 48 h.

Although macrophages alone, or macrophages and lymphocytes together, were unable to neutralize extracellular *Besnoitia* where conditions were not conducive for phagocytosis (Fig. 8), they were effective against intracellular *Besnoitia* (Figs. 1, 6, 7, and 9). Macrophages could be instructed as late as the periods of adherence and infection in cell culture to control the parasites (Fig. 9). In another study (31), nonadherent cells were required to remain with adherent macrophages for at least 3 h to mediate the inhibition of intracellular

growth of *Histoplasma*. Since we allowed 2–3 h adherence and incubation periods in the present study, close contact of cells may have permitted specific instruction. After adherence, repeated washing to remove nonadherent cells diminished but did not remove specificity. This is congruous with observations (32) that multiple washes did not abolish specific recognition of adherent cells and non-specific listericidal capacity of activated macrophages.

Although nonspecific macrophage activation accompanied microbicidal expression, the activation per se was not sufficient to account for homologous suppression. Macrophages from *Toxoplasma*-immune, and from casein-stimulated nonimmune hamsters also appeared morphologically activated in culture without displaying improved microbicidal effects on *Besnoitia* (Fig. 2 *b*). Pre-incubation with homologously immune lymphocytes conferred immunity to macrophages (Figs. 2 *c* and 9).

Specific immunity at reinfection greatly surpasses the resistance observed by the activation of macrophages following heterologous immunization. For example, specifically immunized hamsters were protected against 10^6 – 10^7 LD₅₀ of *Besnoitia* while cross-challenge with *Toxoplasma* protects against less than 10 LD₅₀ (8). Further, spleen and lymph node cells from immune animals adoptively protected against 10^4 LD₅₀ of *Besnoitia* in nonimmune recipients with no cross-resistance to *Toxoplasma* or sensitized with BCG (6). Specificity was also found in the peritoneum, where 10^4 *Besnoitia* as a challenge dose diminished to about 10 LD₅₀ in immune hamsters, while other groups were unable to contain the growth, and about 10^5 – 10^6 LD₅₀ were recovered after 5 days (Fig. 4). Based on quantitative culture assays, a specific threefold reduction and/or delay of parasite growth was found in peritoneal cell cultures after 24 h (Fig. 1, *b*). Cross-challenge further emphasized the magnitude of specific over nonspecific expression (Fig. 7). In fact, the increased unilateral cross-protection of *Besnoitia* against *Toxoplasma* suggests cross-immunity rather than nonspecific expression by activated macrophages, to be operative here.

Some investigators have emphasized the nonspecific resistance displayed against some intracellular pathogens. Cellular cross-resistance has been studied in mice infected with *Besnoitia* or *Toxoplasma* which were challenged with unrelated organisms (33) and has been observed in cell culture using *Listeria* as a measure of resistance (34). However, *Listeria* is so vulnerable to activated macrophages that it may not be suitable to measure the higher levels of immunity required in more persistent infections.

The present study utilized highly virulent *Toxoplasma* and *Besnoitia* lines to provide a maximal test of the immune system. Results gave compelling evidence that even a sustained ongoing response against one latent infection does not protect against another even though the immune mechanisms are similar.

The mechanism by which lymphocytes stimulate macrophages to express specific microbicidal effects remains to be discovered. Immunospecific mediators may be involved in complement-independent cell-mediated immunity. Pre-

vious work suggests that informational RNA (35, 36) and transfer factor (37) may specifically instruct or recruit nonsensitized lymphocytes to express cell-mediated immunity, and therefore act on macrophages only indirectly.

Lymphokines have rarely been demonstrated to act synergistically in the presence of an eliciting antigen (38). A more common lymphokine effect was observed with the reduction of recoverable macrophages following i.p. challenge which probably represents a macrophage disappearance reaction (Fig. 5, *c*). This reaction has been correlated with specific cellular hypersensitivity (39), as mediated by migration inhibitory factor, or MIF (40). However, in terms of immune protection, this lymphokine is probably of only minor importance since the ultimate removal of *Besnoitia* from the immunized hamster peritoneum required more than 5 days, while the disappearance reaction had subsided by 24 h (Figs. 4 and 5, *c*). In addition, anti-*Besnoitia* suppression seen in immune cultures was not transferred with a probable lymphokine detected as macrophage-fusing activity in immune culture supernatants.

That direct microbicidal enhancing activity was inseparable from MIF was claimed (32, 34) and then rejected when MIF-containing supernatants did not produce microbicidal effects in normal cultured macrophages (41). Youmans and collaborators (42–44) have been able to separate MIF from another soluble factor which inhibits intracellular mycobacterial growth, and presumably derived from two subsets of thymus-derived lymphocytes: one affecting delayed hypersensitivity, another microbicidal function (42).

Specific microbicidal information was transferred with immunized lymphocytes to nonimmune macrophage cultures in the present study, but not with their supernatants. Activation and even giant cell aggregation were produced by the latter without significantly reducing susceptibility to parasite overgrowth. These findings suggest that a lymphokine mediates the giant cell formation, a hypersensitivity reaction, but that this type of activation and fusion alone did not aid in besnoitocidal properties. It seems reasonable to assume that a nonspecific lymphokine would be a poor candidate for a protective *in vivo* mediator, when an immune animal is unable to thwart the secondary heterologous organism (8), and where in culture cross-resistance is much inferior to immunity (Fig. 7).

Cytophilic antibody produced by the mediator lymphocytes may be a better way to explain the specificity of macrophage instruction. Such antibody could account for the retained specificity by repeatedly washed macrophages, but not for the increased parasite killing by these cells, unless this antibody increases the fusion of lysosomes to phagocytic vacuoles which does not usually occur when vacuoles contain living *Toxoplasma* (45). Some evidence exists that a vacuolophilic antibody may penetrate intact cells and bind to parasite vacuoles (46).

Contact language or the adherence properties of mediating cells may be an even more attractive explanation for the macrophages' antiparasite expression.

Intracellular bridges have been observed between them and antigenically-stimulated lymphocytes (47). Lymphocyte-macrophage clusters appear to play an important role in immune responses as shown by *in vitro* (48) and *in vivo* studies (49). Cell cultures from immune hamsters presently investigated displayed an intimate lymphocyte-macrophage contact (Fig. 2, *c*). This suggests that a substance may be secreted or bound to the surface of antigenically-stimulated lymphocytes that specifically arms macrophages (50). This activity could be transferred by a very avid cytophilic antibody or labile mediator acting either at short range (51) or into the target cell (52), or by transferable receptor material (53) as some sort of contact language. Of interest to the present study is the fact that MIF has been shown to remove cell surface material on peritoneal exudate cells from sensitized guinea pigs (54). This surface material seems to maintain a contact inhibition between free exudate cells which is lost in the presence of lymphocytes or their mediators. Cells entering the inflamed peritoneum are without this coat and may aggregate *in vitro*. It is tempting to suggest that without cell contact inhibition and with intimate membrane contact, the type and efficiency of information exchange may be increased.

SUMMARY

The capacity of hamster peritoneal cell populations to control viability and growth of *Besnoitia* and *Toxoplasma* organisms was assessed *in vivo* and *in vitro*. Immunized hamsters reduced the homologous organisms 100- to 10,000-fold over a 5-day period, but the heterologous infection increased 100- to 1,000-fold in numbers, similar as in the nonimmune controls. Passively administered antibody was ineffective although lytic cofactors were supplied by hamsters.

In cultures, peritoneal cells from *Besnoitia*-immune hamsters delayed the growth of homologous parasites to an average of 38.5 h per division; however, in *Toxoplasma*-immune and nonimmune cells, *Besnoitia* divided every 12.8 h. Specificity of immunity was pronounced against both infections. With cross-infections, *Toxoplasma*-immune cultures did not effectively delay *Besnoitia* growth; however, *Besnoitia*-immune cultures reduced *Toxoplasma* growth by one-half. Co-cultivation experiments demonstrated that specifically committed lymphocytes could instruct macrophages to reduce the homologous organism 10-fold, whereas heterologous organisms were reduced only 2-fold.

Lymphocyte supernatants initiated hypersensitivity as indicated by macrophage activation and giant cell formation in culture. However, these supernatants did not transfer infection immunity. Lymphokines could account for the hypersensitivity phenomena, but cell-mediated infection immunity in this model required close lymphocyte-macrophage proximity.

These studies indicate that a number of distinct processes including delayed hypersensitivity, macrophage activation, and specific cellular immunity are acting simultaneously during latent *Besnoitia* infection of hamsters. All three processes are mediated by lymphoid cells and appear to be specifically

induced. Although activated macrophages develop some heightened nonspecific capabilities, these were several orders of magnitude below the specific effects.

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