ASSESSMENT IN VITRO OF IMMUNITY AGAINST

TOXOPLASMA GONDII*

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Acquired immunity to intracellular microbes is known to be in many instances primarily dependent on cellular agencies (1), involving cooperation between lymphocytes and macrophages (2). Lymphocytes respond to previously experienced antigens, and somehow induce macrophages to be more effective in dealing with intracellular organisms. The macrophage response to these lymphocytes or lymphocyte products appears to be nonspecific in terms of the increased immunity expressed (3). Studies on the mechanisms involved in this cellular immunity have proceeded along several lines, including identification of the involved lymphocyte population as T cells (4), partial characterization of the products of stimulated lymphocytes (5), and definition of some parameters of macrophage morphology and physiology that correlate with enhanced effectiveness against microbes (6).

Use of in vitro techniques offers the opportunity to more precisely define the specific determinants and variables in this complex cellular response. It has, however, not always been easy to demonstrate the enhanced antimicrobial activity of activated macrophages in vitro. Success has been reported in models employing mycobacteria (7) and listeria (8), but technical problems may contribute to difficulties in interpreting results obtained with this type of in vitro system.

We report here our studies in vitro on immunity to the coccidian protozoan Toxoplasma gondii. Use of toxoplasmas circumvents some of the technical problems referred to above. Toxoplasmas cannot multiply extracellularly, and studies on their intracellular fate thus are not complicated by continued uptake of parasites growing in the medium, or by the need for antibiotics to control extracellular growth. Toxoplasmas are sufficiently large organisms to exhibit well developed morphologic features visible by phase-contrast microscopy, allowing by direct observation accurate assessment of the fate of the organisms, and measurement of their intracellular generation time. We have used the mouse as an appropriate host to evaluate acquired cellular immunity to toxoplasmosis for a number of reasons, but one clear advantage is the availability of a resident

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Abbreviations used in this paper: HIFCS, heat-inactivated fetal calf serum; S-F, Sabin-Feldman.

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peritoneal cell population which can be collected without resorting to irritative techniques for producing an exudate. Furthermore, chronic toxoplasma infection, and cellular immunity to this parasite, can be reproducibly induced in mice by subcutaneous injection of toxoplasma cysts.

Materials and Methods

Animals and Cells. 25-50 g CFW male mice were used for all experiments. A colony of toxoplasma-infected mice was maintained by injecting the mice with brain tissue containing toxoplasma cysts from mice previously infected with the PeC strain. The brain was mildly emulsified using two or three strokes with the pestle of a tissue homogenizer, and the homogenate was diluted in minimum essential medium (MEM) to a concentration of 10 cysts (1,000-10,000 organisms)/ml. 0.2 ml was injected into the left thigh. Peritoneal cells were studied from these animals at various times after infection. Cells were collected from untreated control animals and the immunized animals by lavage of the unstimulated peritoneal cavity with heparinized phosphate-buffered saline. Cells were suspended in MEM and 20% heat-inactivated fetal calf serum (HIFCS) and allowed to adhere to glass cover slips in Falcon dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for 1 h as previously described (9). Nonadherent cells were separated in those experiments where this was desired by washing twice with MEM and collecting the cell-rich wash.

Organisms. The RH strain of T. gondii was maintained by passage every 3 or 4 days in the peritoneal cavity of mice (9). At the time of harvesting, peritoneal exudate collected on the 3rd day of intraperitoneal infection was diluted in 2-3 ml of MEM, and centrifuged at 30 g for 5 min to remove leukocytes and clumps of material. The toxoplasmas were then pelleted from the supernate by centrifuging at 340 g for 10 min. The pellet was resuspended in MEM and 20% HIFCS and the volume adjusted to yield a toxoplasma concentration of 1 x 10⁶/ml (95% viable by trypan blue exclusion test). This toxoplasma suspension was used to overlay cells in culture during in vitro challenge experiments.

The Pe strain of T. gondii was obtained from Dr. Anne Kimball, Cornell University Medical College, New York. The strain had been isolated from the myocardium of an adult human being. It was selected because it displayed low virulence for mice. Two substrains of this organism were derived. One was rendered more virulent for mice (PeV) by rapid intraperitoneal passage in mice every 5-7 days for 2 mo. 10⁶ toxoplasmas of this PeV strain killed normal mice in 10-14 days. This substrain (PeV) was used as the challenge to document immunity against toxoplasma in mice. The second substrain (PeC, relatively attenuated in virulence for mice) was maintained as a chronic infection in mice by passage of toxoplasma brain cysts every 2-3 mo, and was used to produce the toxoplasma-infected and immune mouse colony described above.

Preparation of Antigens. Toxoplasma antigen was prepared by collecting RH strain of T. gondii 4 days after intraperitoneal infection of mice. The peritoneal exudate was diluted in approximately equal volumes of MEM, centrifuged at 30 g for 5 min, the supernate centrifuged at 340 g for 10 min, and the toxoplasma pellet resuspended in saline to a concentration of about 10⁶/ml. This was frozen-thawed six times (acetone-dry ice to 37°C water bath), then centrifuged at 1,300 g for 20 min. The supernate was then centrifuged at 10,000 g for 1 h. The amount of protein in the final supernate was determined by the Lowry method using a lysozyme standard. The total protein content was correlated with the quantity of toxoplasma complement (C)-fixing antigen. For all experiments 20 μg of toxoplasma antigen/ml of final medium was added to each dish, a quantity adequate for full C-fixing activity in the presence of toxoplasma-positive antisera.

Antisera. Mouse, rabbit, and human sera were tested for the presence of antibodies against toxoplasmas using the Sabin-Feldman (S-F) dye test modified for microdilution techniques as routinely performed in these laboratories (10). Sera were heat inactivated and added to culture dishes in a concentration of greater than 200 (S-F) antibody U/ml of final medium. Antitoxoplasma antibody was used to treat toxoplasmas or macrophages before combining them in culture, or after macrophage infection, depending on the experimental design.

In Vitro Culture Techniques. Macrophages: Adherent peritoneal cells (primarily macrophages) were separated from nonadherent cells by washing cover slips 1 h after plating the peritoneal cells. In
experiments in which as pure a macrophage population as possible was desired the cover slips were washed eight times. Usually, four washes were employed to remove nonadherent cells. Adherent peritoneal exudate cells (approximately $5 \times 10^6$/cover slip) were overlayed with fresh MEM and 20% HIFCS for 24 h before infection with toxoplasmas to allow for adequate spreading. Longer culture periods were occasionally used as indicated in the Results section.

**Lymphocytes.** Peritoneal nonadherent cells were either cultured with the adherent population, or removed and used to overlay different adherent populations, or removed and maintained separated from macrophages depending on the experiment. Antigen (20 µg toxoplasma protein/ml) was added to some of these cells. The cell cultures were then incubated for 24 h. This time was found optimal for induction of inhibition of toxoplasma multiplication in immune macrophages. In other experiments the media from toxoplasma antigen-lymphocyte interaction were collected and used for study of influence on growth of toxoplasmas in macrophage cultures.

Spleen lymphocytes were collected by teasing the spleen gently with forceps in Hanks' balanced salt solution. This mixture was decanted into a 15 ml Falcon plastic tube and the clumps were allowed to settle. The spleen cells in suspension were transferred to another tube and centrifuged at 150 g for 5 min. The pellet was resuspended in 0.83% NH₄Cl to lyse red blood cells. Lymphoid cells were centrifuged again and resuspended in MEM. These cells were found to be 85–95% viable by trypan blue exclusion. Spleen cells used for immune supernatant preparations were suspended to a concentration of $5 \times 10^6$ cells/1.5 ml final medium/dish. Nonadherent spleen cells were also used to document lymphocyte transformation to toxoplasma antigen according to published procedures (11), except that $1 \times 10^6$ cells were used per well instead of $2 \times 10^6$.

**Infection of Cultured Cells.** After cultivation in vitro for 24 h the macrophage or macrophage-lymphocyte mixtures were washed four times with MEM. 1 ml of the toxoplasma-rich suspension ($1 \times 10^6$ toxoplasmas/ml/dish) in MEM—20% HIFCS was then added to the macrophages for 30 min. The fluid was then removed, the cover slips were washed twice, and fresh MEM—20% HIFCS was added. The plates were incubated at 37°C in 5% CO₂/air. Several types of observations were made on toxoplasma-infected cells as previously described (9). Some macrophage monolayers were fixed 3 h after infection to determine the presence of living and degenerating parasites. In other experiments the infection was allowed to proceed for up to 30 h. At various times cover slips were removed, fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.4, and examined under phase-contrast microscopy. These infected cells were monitored for morphology of toxoplasmas and macrophages. Numerous experiments documented that *T. gondii* began to divide synchronously within cytoplasmic vacuoles after a resting period of 6–8 h. Thereafter the number of toxoplasmas per vacuole doubled every 5 h. When more than 16 toxoplasmas were present in a macrophage, the cell tended to round and detach from the cover slip, or to rupture. The generation time was calculated from the plot on semilogarithmic graphs of toxoplasmas per vacuole vs. time.

**Microscopy.** Phase-contrast microscopy was done on glutaraldehyde-fixed specimens using a Zeiss microscope (Carl Zeiss, Inc., New York) with a 63 x planapochr diopter objective or a 100 x phase oil immersion objective. Specimens for electron microscopy were fixed as described previously (9). Thin sections were stained with lead and uranyl solutions and examined with a Siemens Elmiskop I (Siemens Corp., Medical Industrial Div., Iselin, N. J.) at 80 kV using a 50 µg objective aperture.

### Results

**Alterations in Mice Infected with Toxoplasmas Attenuated in Virulence.** After infection with cysts of the Pec strain a number of changes occurred in the mice. Between 8 days and 20 days after infection the average spleen weight of immunized mice rapidly increased from 200 to 650 mg. The large spleens persisted for a variable period, but in general by 90 days they had returned to normal. During the period of splenic hyperplasia there occurred in the spleen a marked increase in the number of glass adherent cells, which had the characteristics of normal mouse spleen macrophages (12). Toxoplasmas were not identified in histologic sections of spleen stained by Giemsa or hematoxylin and eosin.
Toxoplasma antibody appeared in the mouse plasma by 26 days after infection, reached peak titers by 32 days, and remained elevated throughout the life of the animal. Toxoplasma cysts in the brains of the infected mice could first be detected by light microscopy 23 days after infection, and remained present throughout the life of the animal.

These infected mice were tested for immunity by challenge with an intraperitoneal inoculation of 10⁶ organisms of the mouse virulent, PeV strain of T. gondii at 5 days, 11 days, 44 days, and 320 days after infection. This dose and route of PeV T. gondii consistently killed all normal mice, whereas some of the infected mice survived when challenged 11 days after infection-immunity, and all survived at later challenge times. These chronically infected mice therefore demonstrated striking immunity, and were used as “immunized” animals in the experiments described below.

**Effects of Antibodies to Toxoplasma on Survival and Growth of these Organisms In Vitro.** One of the changes in the immunized mice correlated in general with their immunity to challenge with virulent toxoplasmas was the appearance of serum antibody detectable by the S-F dye test. Although previous work had indicated the predominant role of cellular changes, rather than antibody, in acquired immunity to this organism (13), it was nevertheless of interest to examine the effects of antibody on toxoplasmas and on their interactions with various cells. As previously demonstrated in many laboratories, fresh antiserum against toxoplasmas exerted a lethal action, whereas heat-inactivated antisera did not cause morphologic changes in the parasite. Toxoplasmas readily gain entry by the phagocytic route into macrophages or fibroblasts in the absence of antibody (9). Toxoplasmas exposed to heat-inactivated antitoxoplasma antibody, human or mouse, lost their ability to enter fibroblasts or HeLa cells, but were rapidly ingested by macrophages. The fate of these antibody-treated toxoplasmas in macrophages was however altered markedly. In the absence of antibody exposure, approximately half the toxoplasmas entering mouse macrophages in vitro are rapidly killed in phagolysosomes, whereas the other half of the organisms survive and multiply in phagocytic vacuoles that do not acquire lysosomal factors (14). In contrast, more than 90% of the toxoplasmas exposed to antibody were rapidly killed, and studies with thorotrast or acid phosphatase showed that these organisms resided in phagolysosomes. Antibody exposure thus apparently blocked the ability of some of the parasites to alter the phagocytic vacuole in such a way as to prevent fusion with lysosomes.

Pretreatment of the host cells, but not of the toxoplasmas with antibody, followed by washing and infection did not lead to detectable alterations; the survival and growth of the organisms was the same as in a system not exposed to antibody. Furthermore addition of antitoxoplasma antibody to the culture immediately after infection exerted no effect on the generation time in macrophages, indicating that intracellular surviving organisms were not available to the added antibody.

**Multiplication of Toxoplasmas within Macrophages from Normal and Immunized Mice.** We proceeded, then, to observations on possible cellular immunity to toxoplasmas demonstrable in vitro, i.e., altered growth of the organisms in cells from immunized animals in the absence of added antibody. The generation
time of toxoplasmas in macrophages taken from mice longer than 2 wk after immunization was markedly prolonged (Table I). In these experiments both peritoneal lymphocytes and macrophages were maintained in culture for 24 h in the presence of toxoplasma antigen before the monolayers were washed; these immune adherent cells were then infected with toxoplasmas. At 18 h there was minimal division of toxoplasmas in macrophages from immunized mice (mean of 1.3 ± 0.3 toxoplasmas/vacuole), compared to a mean of 5.2 ± 0.5 parasites/vacuole in macrophages from normal mice. Calculated generation times were 28 h in cells from immunized mice compared to 5 h in controls.

**Table I**

<table>
<thead>
<tr>
<th>Source of macrophages</th>
<th>No. of toxoplasmas per vacuole after infection</th>
<th>Calculated generation time*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>10 h</td>
</tr>
<tr>
<td>Toxoplasma immune mice</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Normal mice</td>
<td>1.0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Macrophages were cultured in vitro for 24 h with peritoneal lymphocytes and 20 μg/ml of toxoplasma antigen before infection.

*Calculated by the slope on semilogarithmic graph.

$ Indicates the mean and one standard deviation of 20 experiments.

Under phase-contrast microscopy, at 3 h after infection the same percentage of degenerating toxoplasmas (approximately 50%) was seen in these cells from immune mice as has previously been described in cells from normal mice (9). At time points beyond 3 h, only healthy appearing oval, relatively phase-lucent parasites could be identified, suggesting that the low number of toxoplasmas per vacuole in macrophages from immune mice did not reflect persistence of damaged organisms, or killing and digestion of the organisms over a more prolonged period than in controls. Furthermore, the percent of macrophages infected with toxoplasmas was the same in cells from normal and immune animals during the observation period, indicating no striking difference in initial uptake into immune or normal cells. Toxoplasmas in vacuoles of macrophages from immune mice appeared the same as toxoplasmas within macrophages of normal mice by every parameter, except that they did not divide. The similarity in morphology of the nondividing parasites in immune macrophages and the dividing organisms in normal macrophages was confirmed by electron microscopy of cultures 24 h after infection. Fig. 1 demonstrates the electron microscopic appearance of dividing toxoplasmas in normal cells. The ultrastructure of the parasites is well preserved. The macrophage vacuole is surrounded by mitochondria and endoplasmic reticulum, and contains microvillous structures as described previously (14). Fig. 2 shows a single toxoplasma in a macrophage from an immune animal 24 h after infection of the macrophage in vitro. The parasite appears morphologically normal, and the macrophage vacuoles and surrounding structures are similar to those seen in the normal macrophage.
FIG. 1. An electron photomicrograph of a macrophage fixed 24 h after toxoplasma infection. The rosette of four parasites in the center vacuole indicates two divisions. The vacuole is filled with microvilli arising from the vacuolar membrane. The vacuole is surrounded in most regions by endoplasmic reticulum or mitochondria. (× 15,000).

FIG. 2. An electron photomicrograph of a macrophage from a toxoplasma immune animal 24 h after infection in vitro with toxoplasmas. The toxoplasma shows normal morphology but has not multiplied. The vacuole, surrounding mitochondria and endoplasmic reticulum, and microvilli are indistinguishable from those seen in the normal infected macrophage. (× 19,500).
Requirements for Lymphocytes and for Toxoplasma Antigen for Induction in Macrophages of the Ability to Suppress Toxoplasma Multiplication, and the Variation in these Requirements with Time after Immunization. The ability of macrophages to inhibit toxoplasma multiplication was found to vary depending on the cell population or products present, and on the number of days after immunization that the peritoneal cells were collected. In each experiment the peritoneal cells were allowed to incubate in vitro for 24 h before challenge with toxoplasmas, with or without nonadherent lymphoid cells, and with or without added toxoplasma antigen. All monolayers were then washed free of nonadherent cells and antigen before infection of the macrophages with virulent toxoplasmas. Table II shows the results as toxoplasmas per vacuole at 18 h after in vitro challenge. When no toxoplasma antigen was added during the prechallenge incubation, by 16 days a significant inhibition of toxoplasma multiplication was seen, and this inhibition became maximal at approximately a month after immunization. By 63 days after immunization, however, this effect was largely gone. In one experiment almost 1 yr after immunization no inhibition of toxoplasma multiplication was seen. Inhibition of toxoplasma multiplication was observed in the absence of added toxoplasma antigen even when all nonadherent cells were removed before the prechallenge incubation (second column) in one experiment 19 days after immunization, but no significant inhibition under these conditions was observed longer than 49 days after immunization.

More impressive toxoplasma inhibition was observed when the peritoneal cells

Table II

<table>
<thead>
<tr>
<th>Multiplication of Toxoplasmas in Macrophages Challenged after 24 h of Cultivation with Lymphocytes and/or Toxoplasma Antigen Related to Time after Immunization with Avirulent Toxoplasmas</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prechallenge incubation conditions</strong></td>
</tr>
<tr>
<td>No. of days after immunization</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>16</td>
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<tr>
<td>19</td>
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<tr>
<td>26</td>
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<td>33</td>
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<td>35</td>
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<td>49</td>
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<tr>
<td>63</td>
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<tr>
<td>75</td>
</tr>
<tr>
<td>77</td>
</tr>
<tr>
<td>84</td>
</tr>
<tr>
<td>323</td>
</tr>
</tbody>
</table>

*Mean number of *T. gondii* per vacuole 18 h after in vitro challenge.
were exposed during the prechallenge incubation to toxoplasma antigen. By 16
days after immunization maximum inhibition of toxoplasma growth was evident
(1.3 toxoplasmas/vacuole), and this inhibition could be demonstrated for nearly
a year after immunization. Removal of nonadherent lymphoid cells before the
prechallenge incubation did not alter the development in the macrophages of the
capacity to inhibit toxoplasma multiplication in cells collected up to 2 mo after
immunization, but thereafter toxoplasma inhibition required the presence of
lymphoid cells as well as toxoplasma antigen during the prechallenge incubation.
It is not clear whether toxoplasma antigen had a direct effect on enhancing
inhibition of toxoplasma multiplication in the macrophages during the period
less than 2 mo after immunization, or whether a small number of contaminating
lymphocytes that might be present even after extensive washing was playing a
role in the phenomenon.

Spleen lymphocytes from immunized mice when cultured 3 days in RPMI and
20% fresh human serum containing 50 µg/ml of toxoplasma antigen, then exposed
to [3H]thymidine for 6 h showed a significantly enhanced proliferative response.
Lymphocytes from immune mice incorporated 6.1 ± 2.0 × 10³ cpm of
[3H]thymidine; whereas lymphocytes from immune mice not exposed to toxo-
plasma antigen incorporated 1.6 ± 0.5 × 10³ cpm, and lymphocytes from normal
mice cultured in antigen incorporated 2.0 ± 1.0 × 10³ cpm.

Further Characterization of the Lymphocyte-Antigen Effect on
Macrophages. To evaluate further the effects of lymphocytes and toxoplasma
antigen under various conditions, washed macrophages from normal or immune
animals were overlaid with various lymphoid cells and antigens. After 24 h
incubation, the lymphoid cells and antigen were removed and the immune status
of the washed macrophages was assessed. Table III shows a summary of these
experiments. Inhibition of toxoplasma multiplication was seen when either
spleen or peritoneal lymphocytes and toxoplasma antigen were placed over
macrophages from toxoplasma immune mice for 24 h before challenge. No
significant inhibition was observed when lymphocytes from immune animals
were placed over normal macrophages, even when three times the lymphocyte
number found to be effective with macrophages from immune animals was
employed. No inhibitory effect of lymphocytes from immune mice on toxoplasma
multiplication in fibroblasts could be demonstrated. The absence of effects of the
lymphoid cells from immune mice and antigen on toxoplasmas in normal
macrophages and fibroblasts make it unlikely that the inhibitory phenomenon
was due to interferon-like activity (15).

Further studies were done on the time relationships of in vitro prechallenge
cultivation of lymphocytes, toxoplasma antigen, and macrophages required for
acquisition of the capacity of the macrophages to suppress multiplication of
toxoplasmas in vitro. These studies were done by adding toxoplasma antigen to
mixtures of peritoneal cells in vitro at varying times before washing the cover
slips and infecting with toxoplasmas. Table IV shows the results. When antigen
was added 6 and 12 h before infection some inhibition of toxoplasma multiplica-
tion was seen, but 18-24 h of exposure to antigen led to development of maximal
inhibitory activity. Macrophages cultured with lymphoid cells and toxoplasma
antigen longer than 24 h in vitro lost their ability to inhibit toxoplasma
Multiplication of Toxoplasmas in Cells from Various Sources Cultivated for 24 h In Vitro with Different Lymphocyte Populations and 20 μg/ml of Toxoplasma Antigen

<table>
<thead>
<tr>
<th>Cells in prechallenge 24-h incubation</th>
<th>Toxoplasma per vacuole 18 h after infection of washed macrophages or fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent cells</td>
<td>Nonadherent cells</td>
</tr>
<tr>
<td>Macrophages from toxoplasma immune mice</td>
<td>Peritoneal from toxoplasma immune mice</td>
</tr>
<tr>
<td>Macrophages from toxoplasma immune mice</td>
<td>Spleen from toxoplasma immune mice</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>Peritoneal from normal mice</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>Peritoneal from toxoplasma immune mice</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>(3x) peritoneal from toxoplasma immune mice*</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>Spleen from toxoplasma immune mice</td>
</tr>
<tr>
<td>Fibroblasts (L-cells)</td>
<td>Peritoneal from toxoplasma immune mice</td>
</tr>
</tbody>
</table>

* 1.5 × 10⁸ nonadherent cells/dish rather than 5 × 10⁸.

Multiplication. When lymphocytes and antigen were added after toxoplasma infection, no inhibition of multiplication was observed.

Effects on Toxoplasma Multiplication in Macrophages of Supernates of Immune Lymphocyte-Toxoplasma Antigen Interactions. When lymphocytes from immunized animals (2–3 mo after immunization) were cultured with 20 μg/ml of toxoplasma antigen for 24 h, the medium overlying these cells was found to be highly effective in inducing the inhibition of toxoplasma multiplication in macrophages from animals immunized more than 3 mo previously. As is seen in Table V, the characteristics of the inhibitory activity were the same as those seen with the lymphocytes, i.e. marked inhibition of toxoplasma growth was induced in macrophages from immunized, but not in macrophages from normal animals. Active supernates could be generated by toxoplasma antigen and spleen cells as well as peritoneal lymphoid cells. Significant toxoplasma inhibition could not be induced in macrophages from normal mice or in fibroblasts with spleen cell numbers 20 times larger than those effective with macrophages from immunized animals.

The lack of effects of supernates on normal cells, and also the loss of an effect when immune macrophages were cultured in vitro for more than 24 h suggested the possibility that the effect was a complex one requiring the conjoint action of at least two factors, one released from immune lymphoid cells on exposure to...
antigen, the other present on macrophages from immune but not normal animals and lost on prolonged culture (perhaps cytophilic antibody). Therefore, normal macrophages, or long-term (72 h) cultured macrophages from immunized animals were first incubated with heat-inactivated immune serum and then exposed to active supernates; no induction of the capacity to inhibit toxoplasma growth was seen in these instances.

Animals may be immunized against *T. gondii* by inoculation of avirulent strains of the organism, or by treating animals infected with virulent strains with antiprotozoal drugs during the early stages of the infection. In either case a chronic asymptomatic systemic infection develops, which renders the animal immune to subsequent challenge with virulent toxoplasmas. This immunity cannot be induced using killed vaccines. It can be passively transferred to nonimmune animals by spleen or lymph node cells but not by serum (13). The protection induced by such transfer is immunologically specific (13), which is consistent with our present understanding of thymus-dependent lymphocytes and their role in cellular immune mechanisms (2, 16). Macrophages from immunized animals have been described as “activated,” and they have displayed enhanced ability to control in vitro infection of toxoplasmas as well as of immunologically unrelated organisms (17, 18). A clinical correlate of these observations is the fact that toxoplasmosis in humans is a particularly severe disease in those with depressed cellular immune mechanisms in spite of high antitoxoplasma antibody titers. The infection and the immune response to toxoplasmas does not involve significant granuloma formation, in contrast to many other intracellular agents. The characteristic evolution of toxoplasma infection leads to encystment, after which the organisms remain viable in tissues for long periods of time, without inciting any significant inflammatory response. A typical delayed hypersensitivity reaction, however, does occur in chronically

**Table IV**

*Multiplication of Toxoplasmas in Macrophages Depending on Time and Duration of Exposure of Macrophages to Immune Lymphocytes and Toxoplasma Antigen*

<table>
<thead>
<tr>
<th>Duration of in vitro prechallenge mixture of immune lymphocytes, macrophages and toxoplasma antigen</th>
<th>Toxoplasmas per vacuole in macrophages 18 h after washing and challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no antigen)</td>
<td>4.0</td>
</tr>
<tr>
<td>2 h</td>
<td>3.9</td>
</tr>
<tr>
<td>6 h</td>
<td>2.9</td>
</tr>
<tr>
<td>12 h</td>
<td>1.7</td>
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<tr>
<td>18 h</td>
<td>1.0</td>
</tr>
<tr>
<td>21 h</td>
<td>1.0</td>
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<tr>
<td>24 h</td>
<td>1.2</td>
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<tr>
<td>48 h</td>
<td>2.5</td>
</tr>
<tr>
<td>72 h</td>
<td>4.8</td>
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</tbody>
</table>
infected animals in response to subcutaneous injection of toxoplasma antigens.

The observation that toxoplasmas enter phagolysosomes and are destroyed when they have been pretreated with antitoxoplasma antibody is consistent with similar results found for other obligate intracellular organisms such as vaccinia virus (19) and rickettsia (20). Of interest, Mauel has reported that leishmania do not appear to respond to antibody in this manner (21). We have no information about the mechanism by which the antibody alters the toxoplasmas, rendering them incapable of “turning off” the lysosomal system locally. The lack of effect of antibody added after infection in altering viable toxoplasmas in phagocytic vacuoles is probably due to the inability of the antibody to reach this site, since these phagocytic vacuoles are rendered incapable of fusing with other pinocytic and phagocytic vacuoles, as well as lysosomes.

Antibody might well contribute to host resistance to toxoplasmosis by acting on extracellular organisms released at the time of rupture of infected cells. The action on extracellular toxoplasmas might be a direct lethal one in conjunction with available C₃, or might be an indirect one in which the organisms are rendered incapable of surviving in macrophages, or incapable of entering nonphagocytic cells. These effects probably come into play in some circumstances, but the

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**Table V**

*Multiplication of Toxoplasmas in Cells from Various Sources Treated for 24 h with Supernates of Either Peritoneal or Spleen Immune Lymphocytes and Toxoplasma Antigen*

<table>
<thead>
<tr>
<th>Adherent cell</th>
<th>Supernate of 24-h incubation of toxoplasma antigen and lymphoid cells from immune mice</th>
<th>Toxoplasma per vacuole 18 h after in vitro challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages from toxoplasma immune mice</td>
<td>Peritoneal 5 x 10⁵</td>
<td>1.4</td>
</tr>
<tr>
<td>Macrophages from toxoplasma immune mice</td>
<td>Spleen 5 x 10⁵</td>
<td>1.3</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>Peritoneal 1 x 10⁶</td>
<td>5.1</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>Spleen 5 x 10⁶</td>
<td>4.9</td>
</tr>
<tr>
<td>Macrophages from normal mice</td>
<td>Spleen 1 x 10⁷</td>
<td>4.4</td>
</tr>
<tr>
<td>Fibroblasts (L cells)</td>
<td>Spleen 2 x 10⁷</td>
<td>3.4</td>
</tr>
<tr>
<td>Macrophages from normal mice after culture in 10% heat-inactivated immune serum</td>
<td>Peritoneal 5 x 10⁵</td>
<td>4.1</td>
</tr>
<tr>
<td>Macrophages from immune mice 72 h in vitro and after culture in 10% heat-inactivated immune serum</td>
<td>Peritoneal 5 x 10⁵</td>
<td>3.8</td>
</tr>
</tbody>
</table>
available evidence indicates that the major mechanism involved in protection against toxoplasmosis is cellular immunity (13, 22).

A quantitative increase in macrophage-mediated antimicrobial response in vitro associated with acquisition of cellular immunity by the host has been demonstrated previously in other systems. For example, an increased listericidal action in vitro of “activated” macrophages has been shown in a number of laboratories (8, 17). The assay for killing of listeria appears to be very sensitive (even showing increased activity associated with immunization with unrelated protein antigens [8]), but there remain some uncertainties in interpretation related to variables such as extracellular multiplication, effects of antibiotics, microbe viability, and phagocyte cytotoxicity. Also, indirect methods such as staining or colony counting are necessary, making quantitation somewhat unreliable. Recently, Mauel has described the use of leishmania species for evaluating cellular immune responses (21). These organisms do not survive for more than 72 h in vitro in normal cells, so that the manifestation of cellular immunity in vitro required studies on enhanced rate of destruction, not arrest or reversal of growth of the organisms intracellularly. Similar difficulties have limited the use of etromelia virus as a probe of cellular immunity in in vitro models (23). Inhibition of mycobacterial multiplication (7) as well as certain fungi (24) by immune macrophages has been shown, but with these organisms there may be problems in quantitation similar to those noted with listeria.

The experiments described here in the mouse, and those recently described by others using mice (17, 22), hamsters (25), and humans (26, 27) have confirmed the value of the protozoan, T. gondii, for such in vitro quantitative studies. The technique for determining generation times of toxoplasmas in vitro has been known for some time. It has been used to document the relationship between generation time and animal virulence among various strains of toxoplasmas (28). In vitro studies have also been used to document metabolic requirements of toxoplasmas and their inhibition by various substances (29). Multiplication of this organism can be influenced by many factors. For instance, in HeLa cells the generation time of toxoplasmas is 27 h when cells are maintained in 2% human serum for 3 days before infection, but only 7 h when 20% serum is used. Raising the incubation temperature from 37°C to 40°C shortens the generation time in mouse macrophages from 5 h to approximately 4 h. Various conditions unfavorable for host cell metabolism in vitro prolong the generation time of toxoplasmas (unpublished observations). This body of past experience led us to utilize rate of toxoplasma multiplication (generation time) in macrophages as a method in searching for subtle changes associated with the immune state. Mean generation times in normal macrophages were found to be so consistent that a single time point 18 h after infection could be used to compare cultures under different conditions. Lycke and Lund in their early experiments of toxoplasmas in cell culture found similar reproducibility (30). In addition to these reproducible generation times, toxoplasmas offer many other advantages in relation to quantitative studies of intracellular growth rate. The organisms do not survive or grow extracellularly, so that no antibiotic need be used. Continuing phagocytosis of extracellular organisms is not a factor provided that the end point selected for
counting is earlier than the time of cell rupture due to intracellular growth. Furthermore, the assessment of numbers of toxoplasmas per cell is made readily and reliably by direct microscopic examination.

There have been relatively few studies directed at the kinetics of the cellular immune response in vitro against intracellular microbes. Those working with *Listeria monocytogenes* infections have been aware of the "transient" nature of the cellular immune state, demonstrable 4 days after inoculation of living listeria, but gone by a few weeks after infection (2). In mycobacterial infections, immunity is usually life long though the degree of cutaneous delayed hypersensitivity may be variable (31). In the case of toxoplasmosis, immunity, skin test reactivity, and serum antibodies persist for life. These differences are related in part to the duration that microbes remain viable in the host (1). Most studies of macrophage function in vitro have been done a few weeks after immunization with living microbes or other booster antigenic challenge. These studies have documented antimicrobial macrophage activity during this period. In the experiments described in this paper we noted some inhibition of toxoplasma multiplication without in vitro exposure to lymphocytes or antigen in macrophages collected from 2 wk to 2 mo after infection. After this period macrophages required exposure to immune lymphocytes and toxoplasma antigen in order to develop full toxoplasma inhibitory activity. Probably the capacity of macrophages collected early after the immunizing infection reflects exposure of the phagocytes to immune lymphoid cells and to toxoplasma antigens in vivo. This period correlates with the duration of splenomegaly and increased number of spleen macrophages in infected mice. For the study of induction of immunity in macrophages by products of the interaction between immune lymphocyte and toxoplasma antigen, it was thus necessary to use macrophages collected 3 mo or longer after immunization, a time at which the cells demonstrated little or no inhibitory activity without exposure to these products.

Preliminary studies have shown that macrophages collected from toxoplasma immunized mice and maintained in culture for 24 h are more active in converting \([1-\text{C}^1\text{C}]\text{glucose}\) to \(\text{CO}_2\) in the resting state than are normal macrophages. This is true in the case of cells collected longer than 3 mo after immunization. Apparently, even long after immunization against toxoplasmas, the macrophages remain "perturbable" and readily responsive to induction of an immune state.

During these studies it was noted that peritoneal macrophages from normal (unimmunized) mice did not acquire the ability to inhibit toxoplasma multiplication in vitro after 24 h of cultivation with toxoplasma immune lymphoid cells and toxoplasma antigen. Others, employing in vitro models designed to study cellular immune responses (8, 18, 27, 32) have shown that the inhibitory or microbicidal activity could be conferred on "normal" macrophages by products of lymphoid cells from immune animals. Possible explanations for the failure of induction of immunity in normal macrophages in the toxoplasma system include the following: (a) Perhaps macrophages must be stimulated in some way before they can respond to the toxoplasma lymphokines. The observation that macrophages from mice immunized against toxoplasmas are metabolically more active in the resting state than unstimulated mouse peritoneal macrophages is consistent with this view; and (b) perhaps immunologically specific cytophilic antibody (33) or other cytophilic factors (34) on the macrophages are necessary in
addition to immune lymphocyte products for complete expression of the inhibitory activity. Our studies showing that even macrophages from immunized animals lose responsiveness to lymphokines in vitro during 2–3 days of cultivation is consistent with such a hypothesis, since membrane turnover might well result in the loss of such cytophilic substances.

The experiments described here have shown that a lymphocyte product generated by cultivation of immune lymphocytes and toxoplasma antigen for 24 h in vitro can induce the inhibitory effect on toxoplasmas within certain macrophages. Characterization of this lymphocyte product is now underway; preliminary results indicate that it is stable to freezing at −70°C for several months, and stable to 56°C for 30 min; it is inactivated by heated mouse plasma and by trypsin. The product is released under circumstances which cause a lymphocyte proliferative response as measured by [³H]thymidine incorporation.

The nature of the macrophage alteration responsible for the inhibition of toxoplasma growth remains to be defined. Electron microscopy of toxoplasmas in these “immune” macrophages has demonstrated the parasites to be intact, without evidence of morphological degenerative changes 24 h after infection, and without evidence of lysosomal fusion with the vacuole containing the parasite, using a thorotrast lysosomal label as previously described (14). Others have reported degeneration of toxoplasmas (as documented by alteration of staining characteristics) within macrophages of the immune animal (26). We have not extended our studies to determine the ultimate fate of these slowly dividing or nondividing organisms. They might degenerate eventually, or they might evolve into a toxoplasma cyst, a hallmark of the immune state in toxoplasma infection. In any event the initial cellular immune response appears to be an inhibition of toxoplasma multiplication, rather than a toxoplasmacidal one. Similar initial changes have also been suggested by studies in vitro of mycobacteria (32, 35) and histoplasma (24) in immune cells.

The lack of specificity in the effector arm of cellular immunity, i.e. the increased macrophage microbicidal activity, was first described by Mackaness (36), and by Elberg et al. (37) over a decade ago. Our observations on specificity in cellular immunity to toxoplasmosis are not yet complete, but we have observed that macrophages from BCG-immunized animals placed in culture with purified protein derivative of tuberculin and TB-immune lymphocytes do not inhibit toxoplasma growth, nor do macrophages harvested after intravenous Listeria infection, nor macrophages taken from peritoneal cavities stimulated by mineral oil or endotoxin. These findings are consistent with other observations recently reported indicating that control of toxoplasma infection by nonspecific activation may be somewhat more difficult than control of listeria infection (15, 25). In any event, further studies are required in order to establish the physiological alterations induced in macrophages in acquired cellular immunity, and to answer questions about possible differences in these alterations in the various experimental systems.

Summary

Studies have been made on humoral and cellular immune responses in mice immunized with an attenuated strain of Toxoplasma gondii. Heat-inactivated
antitoxoplasma serum did not cause morphologic changes in the organisms, but did markedly influence their interactions with host cells. Toxoplasmas exposed to antibody were no longer capable of entering fibroblasts or HeLa cells. They were readily engulfed by macrophages, but the antibody treatment strikingly altered the intracellular fate of the parasites leading to killing and digestion of the toxoplasmas in phagolysosomes. Addition of antitoxoplasma antibody immediately after infection of macrophages in vitro had no effect on intracellular multiplication of the organism.

The division time of virulent toxoplasmas in mouse peritoneal macrophages in vitro was markedly prolonged in cells from immunized mice. During the first 2–3 mo after immunization, the macrophages harvested from the peritoneal cavity demonstrated this cellular immunity directly; thereafter exposure of the macrophages to immune lymphocytes and toxoplasma antigen, or to supernates from such an interaction was required for induction of the maximal capacity to inhibit growth of toxoplasmas. Induction of the alteration in macrophages by the lymphocyte product was detectable in 6 h and maximal at 18–24 h.

Cultivation in vitro of macrophages from immunized animals for periods longer than 48 h rendered the cells nonresponsive to the immune lymphocyte-toxoplasma product. Macrophages from the peritoneal cavities of normal, nonimmunized mice were also incapable of developing the capacity to inhibit growth of toxoplasmas in response to this product. The nonresponsiveness of normal macrophages, or of macrophages cultured for several days in vitro was not changed by exposure of the cells to antitoxoplasma serum.

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References


