MACROPHAGE RESPONSES TO TOXOPLASMA ANTIGENS IN VITRO: A POSSIBLE ROLE IN INFLAMMATORY LESIONS IN TOXOPLASMOsis

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Abstract. Toxoplasma antigen and Toxoplasma immune complex were shown to induce increased production and release of acid hydrolases from macrophage cell line P388D in a concentration-dependent manner. Antigen concentrations of 10–50 μg/ml gave a 2–4-fold increase in the activities of acid proteinase, acid phosphatase, and phospholipase A2 compared with control cells without antigen. Results were similar for immune complex concentrations of 30–80 μg/ml compared with controls. No significant lactate dehydrogenase activity was detected in the culture medium, indicating that enzyme release was selective and not due to cell death. These results suggest that increased release of acid hydrolases may play a role in the inflammatory lesions observed in Toxoplasma encephalitis.

Clinical manifestations of human Toxoplasma gondii infections include lymphadenopathy, myocarditis, choroidoretinitis, and encephalitis. Neeley and Conley reported a 5–7-fold increase in the number of mononuclear inflammatory cells in mice infected with T. gondii. Studies of Toxoplasma encephalitis by Frenkel and Escajadillo, Luft and others, Luft and Remington, Conley and others, and Snyder showed substantial inflammatory responses to T. gondii consisting mostly of mononuclear cells. The natural history of Toxoplasma infection in the mouse includes invasion of brain parenchyma, the induction of cellular inflammatory infiltrates following cyst rupture, and release of Toxoplasma antigens.

The importance of interferon-gamma (IFN-γ) in preventing reactivation of Toxoplasma infection has been well documented in several laboratories. Although other cytokines have been shown to confer resistance to Toxoplasma infection, studies by Jones and others and Deckert-Schluter and others have suggested that the rupture of Toxoplasma cysts may be due to deficiency of IFN-γ production by the host cells, resulting in the release of antigenic components that may activate mononuclear cells. Such activation may result in the synthesis and secretion of hydrolytic enzymes that may in turn rupture additional cysts. Antigens released from ruptured cysts may also participate in inducing acute and chronic inflammatory responses, including the accompanying tissue damage. The present study was designed to examine the ability of Toxoplasma antigens and Toxoplasma immune complex to induce synthesis and release of acid hydrolases, (acid proteinase, acid phosphatase, and phospholipase A2) by peritoneal exudate cells and a macrophage cell line.

MATERIALS AND METHODS

Cell culture. Macrophage cell line P388D was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium that was supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin-streptomycin (Gibco, Grand Island, NY).

Peritoneal exudate cells. Thioglycollate-elicited peritoneal exudate cells (PEC) were harvested by lavage from 10 week-old C3H/HeN mice with Hanks’ balanced salt solution (HBSS) without Ca2+ or Mg2+ and supplemented with 5% fetal bovine serum and 5 U/ml of heparin. The suspension, enriched for macrophages by adherence in culture dishes, was incubated for 3–4 hr at 37°C and washed with HBSS.

Preparation of Toxoplasma tachyzoites. Toxoplasma gondii (ts-4 strain, ATCC) was seeded in human foreskin fibroblast (ATCC) culture and maintained in Dulbecco’s minimum essential medium supplemented with 3% fetal bovine serum, 1% L-glutamine and, 2% penicillin-streptomycin. The culture was incubated at 35°C without CO2 for 5–10 days. Harvested tachyzoites and fibroblasts were passed through a 27-gauge needle and a 3-micron polycarbonate filter to release intracellular parasites. Viability of the parasites was determined by tripan blue exclusion.

Preparation of Toxoplasma antigens. Purified Toxoplasma tachyzoites were adjusted to a concentration of 2 × 10⁸ organisms/ml in phosphate-buffered saline (PBS), pH 7.2, and disrupted by sonication (Heat System Cell Disruptor, Rochester, NY). Microscopic examination revealed no intact cells. Sonicates were clarified by centrifugation at 3,500 × g for 15 min and the supernatant was retained as crude antigens. Sonicates containing 10–50 μg/ml of protein or 30–80 μg/ml of protein or soluble Toxoplasma antigen (200 μg/ml) was added to 5 ml of the heated immune serum and kept overnight at 4°C. The reaction mixture was centrifuged at 5,000 × g for 30 min and the resulting precipitate was washed 3 times in 0.15 M saline. The precipitate was suspended to the original volume and the protein content was measured as already described. The resulting immune complex was used for macrophage activation.

In vitro activation of macrophages. Purified murine PEC and macrophage cell line P388D (ATCC) were seeded at a concentration of 2 × 10⁶ cells/dish. The cells were separately activated with crude, soluble, or particulate fractions of Toxoplasma sonicates containing 10–50 μg/ml of protein or with Toxoplasma immune complex at a concentration of 30–80 μg/ml. Cells treated with crude antigens were incubated...
for 8 hr at 37°C in 5% CO₂ and 95% air to determine the kinetics of enzyme release. The cells treated with soluble or particulate antigens or with immune complexes were incubated for 24 hr. The culture media were then removed, cleared by centrifugation and stored at −20°C until assayed for acid phosphatase, acid proteinase, and phospholipase A₂. Adherent cells were washed 3 times in warm PBS (37°C) and removed with sterile rubber policeman into 2 ml of PBS. The cells were disrupted by sonication for 30 seconds at 10-30% power, with 3 bursts under ice. They were centrifuged at 2,000 x g for 15 min to remove any unbroken cells. The supernatant was then assayed for the above listed three enzymes.

**Enzyme assays.** Intracellular and extracellular activities of acid proteinase were determined by a modification of the method described by McLaughlin and Faubert.¹⁹ One hundred fifty microliters of culture supernatant or cellular extract, 150 μl each of 1 M formate buffer (pH 3.0), and deionized water were added to 150 μl of 2% dialyzed ox hemoglobin (Sigma Chemical Co., St. Louis, MO) as substrate. The reaction was allowed to proceed for 2 hr at 37°C and terminated by addition of 0.9 ml of 6% trichloroacetic acid (Sigma Chemical Co.). The tubes were centrifuged at 10,000 x g for 15 min and the resulting supernatant was read at 280 nm with a Gilford 250 spectrophotometer (Gilford Instruments, Oberlin, OH) for the presence of tyrosine as indicator of acid proteinase activity. Acid phosphatase activity was measured by a modified method of Farooqui and others.²⁰ Two hundred microliters of 15 mM p-nitrophenyl phosphate dissolved in acetic acid buffer were added to 150 μl of acetate buffer (pH 4.5) in a reaction vessel. One hundred microliters of supernatant or cellular extract adjusted to contain 30 μg/ml of protein were added to the mixture and incubated at 37°C for 15 min. The reaction was terminated by the addition of 2.5 ml of 1 N sodium hydroxide. The p-nitrophenol released by the enzyme was measured at 410 nm with a Gilford 250 spectrophotometer. Phospholipase A₂ was assayed by the method of Kasuken and Vanha-Pertulä;¹³ with a slight modification. The substrate was prepared by pipetting purified egg lecithin dissolved in ethanol into a beaker and evaporating the ethanol to dryness. An appropriate volume of 100 mM Tris-HCl buffer (pH 7.9) or 100 mM acetate buffer (pH 4.5) was added to yield 5 mM lecithin. Triton X-100, calcium chloride, and sodium dodecyl sulfate (SDS) were added in sequence to yield concentra-

<table>
<thead>
<tr>
<th>Acid phosphatase (nM nitrophenyl/min)</th>
<th>Acid proteinase (nM tyrosine/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ts-4 Antigen (μg/ml)</td>
<td>Total (±SEM)</td>
</tr>
<tr>
<td>Control</td>
<td>67 (±12)</td>
</tr>
<tr>
<td>10</td>
<td>194 (±21)</td>
</tr>
<tr>
<td>20</td>
<td>233 (±18)</td>
</tr>
<tr>
<td>30</td>
<td>233 (±26)</td>
</tr>
<tr>
<td>40</td>
<td>213 (±15)</td>
</tr>
<tr>
<td>50</td>
<td>194 (±17)</td>
</tr>
</tbody>
</table>

* Peritoneal exudate cells were exposed to various concentrations of Toxoplasma antigens for 24 hr. Total and released enzymes in the medium were assayed. Data are the mean ± SEM of three separate experiments.

- **Acid phosphatase and acid proteinase activities in culture supernatant of peritoneal exudate cells exposed to Toxoplasma (ts-4) antigen**

**Table 1**

- **Acid phosphatase and acid proteinase activities in culture supernatant of the P388D cell line exposed to Toxoplasma (ts-4) antigen**

**Table 2**

- Effects of Toxoplasma sonicate on macrophage. Macrophage cell line P388D and PEC were exposed for 24 hr to various concentrations of T. gondii (ts4 strain) sonicate containing 10–50 μg/ml of protein. As shown in Table 1, exposure to 40 μg/ml of sonicate resulted in induction of 213 nM of acid phosphatase and 746 nM of acid proteinase in PEC, respectively. In the P388D cell line, while 40 μg/ml of Toxoplasma antigen induced total maximum acid phosphatase activity of 233 nM, significant synthesis (1,216 nM) of acid proteinase was observed with 20 μg/ml of Toxoplasma antigen (Table 2). There were also significant differences in the extracellular release of acid proteinase and acid phosphatase between the PEC and the P388D cell line. Forty micrograms of Toxoplasma antigen yielded 56% extracellular activity of acid phosphatase in P388D cells in comparison with 26% in PEC (Tables 1 and 2). As shown in Table 3, calcium-dependent phospholipase A synthesis and extracellular release in P388D cells were dependent on antigen concentration and ranged from a minimum of 201 nM to

- **Statistical analysis.** The results represent the means of three separate experiments, each of which was run in triplicate. Statistical analyses were performed using the Student’s t-test.

**RESULTS**

- **Effects of Toxoplasma sonicate on macrophage.** Macrophage cell line P388D and PEC were exposed for 24 hr to various concentrations of T. gondii (ts4 strain) sonicate containing 10–50 μg/ml of protein. As shown in Table 1, exposure to 40 μg/ml of sonicate resulted in induction of 213 nM of acid phosphatase and 746 nM of acid proteinase in PEC, respectively. In the P388D cell line, while 40 μg/ml of Toxoplasma antigen induced total maximum acid phosphatase activity of 233 nM, significant synthesis (1,216 nM) of acid proteinase was observed with 20 μg/ml of Toxoplasma antigen (Table 2). There were also significant differences in the extracellular release of acid proteinase and acid phosphatase between the PEC and the P388D cell line. Forty micrograms of Toxoplasma antigen yielded 56% extracellular activity of acid phosphatase in P388D cells in comparison with 26% in PEC (Tables 1 and 2). As shown in Table 3, calcium-dependent phospholipase A synthesis and extracellular release in P388D cells were dependent on antigen concentration and ranged from a minimum of 201 nM to
Phospholipase A₂ activities in culture supernatant of the P388D cell line exposed to Toxoplasma (ts-4) antigen*  

<table>
<thead>
<tr>
<th>Ts-4 Antigen (µg/ml)</th>
<th>Phospholipase A (nM free fatty acids/min)</th>
<th>% Extracellular activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Total (± SEM)</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>96 (±13)</td>
<td>23</td>
</tr>
<tr>
<td>20</td>
<td>201 (±12)</td>
<td>26</td>
</tr>
<tr>
<td>30</td>
<td>264 (±52)</td>
<td>28</td>
</tr>
<tr>
<td>40</td>
<td>306 (±12)</td>
<td>36</td>
</tr>
<tr>
<td>50</td>
<td>316 (±20)</td>
<td>33</td>
</tr>
</tbody>
</table>

* The P388D cell line were exposed to various concentrations of Toxoplasma antigens for 24 hr. Total and released enzymes in the medium were assayed. Data are the mean ± SEM of three separate experiments.

Kinetics of enzyme synthesis and release induced by Toxoplasma sonicate. The time course of synthesis and extracellular release of enzymes by P388D cell line after exposure to 40 µg/ml of sonicate were also studied. As illustrated in Figure 1, a 3–4-fold increased release of acid proteinase began within 1 hr of exposure compared with untreated cells. The data presented in Figure 2 demonstrate that the total acid phosphatase present in stimulated cells showed a 3-fold increase after 2 hr of incubation. This increase was maintained for an additional 2 hr, followed by a decrease at 6 hr. There was a renewed and steady increase in extracellular release of enzyme that continued beyond 8 hr of incubation. Additional data (not indicated in Figure 2) obtained from the experiment show that the increase continued up to 12 hr of incubation. Such an increase suggests de novo synthesis of the enzyme. When calcium-dependent and calcium-independent phospholipase A activities were measured, it was observed (Figure 3) that there was an initial 5-fold increased extracellular release of calcium-dependent phospholipase A after 30 min of incubation. This was followed by another significant increase in the release of the enzyme between the fourth and sixth hour. The parallel increase in the total activity of the enzyme may suggest continuous synthesis of calcium dependent PLA. The total experimental activity was not similarly increased at 6 hr because at that point, the cells were probably releasing the enzymes without concomitant synthesis. It is important to note that the pattern of enzyme synthesis and release may vary from one enzyme to another. It should therefore not be surprising that the pattern of release and synthesis of calcium-dependent phospholipase A is not the same as the other enzymes. Figure 4 shows the effect of Toxoplasma sonicate on the synthesis and release of calcium-independent phospholipase A₂, with significant difference between the control and experimental cells after 1 hr and between 4 and 6 hr of incubation.

Effects of Toxoplasma immune complex. Parasite-specific immune complexes formed during the course of an infection are known to activate mononuclear cells to synthesize and release cytokines. As shown in Table 4, 80 µg/ml of the Toxoplasma immune complex induced about a 12-fold increase in total acid phosphatase activity ($P < 0.01$) along with a greater than 4-fold parallel extracellular release.

![Figure 1](image1.png)

**Figure 1.** Kinetics of synthesis and extracellular release of acid proteinase from macrophage cell line P388D in response to 40 µg/ml of Toxoplasma crude antigen over an 8-hr period. Cells exposed to antigen were incubated at 37°C and enzymes were assayed using supernatant for extracellular release and both supernatant and cellular enzyme for total synthesis at each time period. Control results represent cells that were not exposed to antigens. Each point represents the mean ± SEM of 3 separate experiments.
Figure 2. Kinetics and extracellular release of acid phosphatase from macrophage cell line P388D in response to 40 μg/ml of *Toxoplasma* crude antigen over an 8-hr period. Cells exposed to antigen were incubated at 37°C and enzymes were assayed using supernatant for extracellular release and both supernatant and cellular enzymes for total synthesis at each time period. Control results represent cells that were not exposed to antigens. Each point represents the mean ± SEM of 3 separate experiments.

Figure 3. Kinetics and extracellular release of calcium-dependent phospholipase A2 from macrophage cell line P388D in response to 40 μg/ml of *Toxoplasma* crude antigen over an 8-hr period. Cells exposed to antigen were incubated at 37°C and enzymes were assayed using supernatant for extracellular release and both supernatant and cellular enzymes for total synthesis at each time period. Control results represent cells that were not exposed to antigens. Each point represents the mean ± SEM of 3 separate experiments.
of the enzyme (15% versus 66%). However, the same immune complex concentration induced only a 2-fold increase in total acid proteinase activity ($P < 0.01$), along with an extracellular enzyme release of 67%. The rates of synthesis of total calcium-dependent and calcium-independent phospholipases were significantly higher in activated cells than in control cells. As shown in Table 5, both calcium-dependent and calcium-independent phospholipase synthesis increased about 2-fold higher in activated cells than in untreated cells. While most of the calcium-dependent phospholipase A$_2$ was retained intracellularly, 55% of the total activity of calcium-dependent phospholipase A$_2$ was released into the medium. The data presented in Tables 2–5 indicate significant increases of enzyme activities (acid proteinase, acid phosphatase, and phospholipase A$_2$) both for non-complexed (Tables 2 and 3) and Toxoplasma-complexed antigens (Tables 4 and 5). However, the increase in induced activities for similar concentrations of complexed and non-complexed antigens varied for each of the enzymes. For instance, 30 $\mu$g/ml of Toxoplasma antigen resulted in total enzyme activities of 146 nM of $p$-nitrophenol/min of acid phosphatase, 1,061 nM of tyrosine/min of acid proteinase, and 264 nM of free fatty acids/min of phospholipase A$_2$. On the other hand, 30 $\mu$g/ml of Toxoplasma immune complex gave 434 nM of $p$-nitrophenol/min of acid phosphatase, 435 nM of tyrosine/min of acid proteinase, and 205 nM of phospholipase A$_2$.

**DISCUSSION**

The ability of activated macrophages to synthesize and release lysosomal hydrolases$^{23-25}$ and cytokines$^{22,26}$ has been demonstrated. The results of this investigation show that

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**TABLE 4**

Synthesis and secretion of acid phosphatase and acid proteinase by the P388D cell line in response to Toxoplasma immune complex*

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Acid phosphatase (nM $p$-nitrophenol/min)</th>
<th>Acid proteinase (nM tyrosine/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (± SEM)</td>
<td>% Activities in supernatant</td>
</tr>
<tr>
<td>Control</td>
<td>60 (±9)</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>434 (±93)</td>
<td>29</td>
</tr>
<tr>
<td>80</td>
<td>813 (±70)</td>
<td>66</td>
</tr>
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</table>

* Toxoplasma immune complex was used to activate two million macrophages of cell line P388D over a period of 24 hr. Total and percent released enzymes into the medium were assayed. Data are the mean ± SEM of three separate experiments.

**TABLE 5**

Synthesis and secretion of calcium-dependent and calcium-independent phospholipase A$_{21}$ (PLA) of P388D cells in response to Toxoplasma immune complex*

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Enzyme activities (nM free fatty acid/min)</th>
<th>Ca$^{2+}$-dependent PLA</th>
<th>Ca$^{2+}$-independent PLA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (± SEM)</td>
<td>% in medium</td>
<td>Total (± SEM)</td>
</tr>
<tr>
<td>Control</td>
<td>93 (±10)</td>
<td>ND†</td>
<td>224 (±26)</td>
</tr>
<tr>
<td>30</td>
<td>205 (±26)</td>
<td>18</td>
<td>525 (±55)</td>
</tr>
<tr>
<td>80</td>
<td>318 (±40)</td>
<td>6</td>
<td>578 (±27)</td>
</tr>
</tbody>
</table>

* Toxoplasma immune complex was used to activate $2 \times 10^6$ macrophages of cell line P388D over a period of 24 hr. Total and percent released enzymes into the medium were assayed. Data are the mean ± SEM of three separate experiments.
† ND = less than 0.1% detected.
when PEC and macrophage cell line P388D were exposed to Toxoplasma antigen, the activated cells synthesized and released significant amounts of acid proteinase, acid phosphatase, and phospholipase A₂. Toxoplasma immune complexes formed in the course of an infection have also been shown to participate in the activation of mononuclear phagocytes. In this investigation, mouse PEC and the P388D cell line showed low level release of all enzymes studied in the absence of exogenous stimulators. This indicates that these enzymes are released as part of normal physiologic processes of the cells with no loss of cell viability. When the cells were cultured for 24 hr with various concentrations of Toxoplasma antigens (10⁻⁵ μg/ml), they remained viable as indicated by several criteria. These included adherence of cells to the surface of culture dish and retention of a cytoplasmic enzyme marker such as LDH.

The natural host response to Toxoplasma infection is often a granulomatous reaction with an admixture of lymphocytes, plasma cells, and macrophages. Studies have shown that in recrudescence toxoplasmosis, Toxoplasma tachyzoites or antigens were associated with microglial nodules that were not found in the adjacent parenchyma. The presence of mononuclear cells and the availability of antigens provide a favorable condition for activation of macrophages to synthesize and release enzymes. Enzyme induction and release were demonstrated in this study with macrophage P388D cell line being activated with Toxoplasma antigens in vitro. These enzymes have a variety of proteolytic activities, including proteinases that are active in the acid and neutral pH ranges. The synthesis and release of various hydrolytic enzymes and leukotrienes by macrophages and the characteristics of their activators have also been studied. However, this study represents the first attempt to determine the ability of Toxoplasma antigens to induce the synthesis and release of enzymes by macrophages. Phospholipase A₂ has been shown to participate in inflammatory processes. Its induction and release by Toxoplasma antigen as well as by Toxoplasma immune complex would suggest a similar role in the pathologic manifestation of recrudescence toxoplasmosis. It is not known if phospholipase A₂ released was due to a direct effect of Toxoplasma antigens or due to mediators induced by the antigens. It is possible that the PLA₂ released by activated macrophages may participate in an inflammatory response by hydrolyzing available phospholipids to release pro-inflammatory fatty acids. The correlation between the presence of tumor necrosis factor and significant concentrations of phospholipase A₂ in certain parasitic infections has been described. Although other studies have conclusively demonstrated that resistance to Toxoplasma encephalitis is mediated by IFN-γ production, we are currently investigating the concomitant or sequential induction of cytokines and mononuclear hydrolyses by Toxoplasma antigens and immune complexes.

The acid proteinase released by Toxoplasma-activated macrophages may also participate in the inflammatory response by cleaving available complement components C3 or C5 into their active fragments C3a and C3b or C5a and C5b. Some evidence for this was provided in our findings that partially purified proteinase, released in response to Toxoplasma antigens, was able to cleave complement component C5 into fragments that were chemotactic to macrophages (Tackey R, Kassim O, unpublished data). It is therefore relevant to note that complement components C3, C3a, and C5a are cleaved by cysteine proteinases of Entamoeba histolytica.

Macrophages (human and rat alveolar and peritoneal) possess oxygen-dependent and oxygen-independent anti-microbial activities, both of which are active in unstimulated and activated cells. Studies by Catterall and others and Chang and Pechere demonstrated that intracellular killing of T. gondii by human and rat alveolar and peritoneal macrophages was by a non-oxidative mechanism, with no oxidative burst being triggered in these cells. Catteral and others also investigated the roles of oxygen scavengers and found that these metabolites had no effect on the toxoplasmicidal activities of human and rat macrophages. This suggests that the intracellular killing of T. gondii in these cells does not involve toxic oxygen metabolites.

In a separate study, Israeli and others exposed human pelvic macrophages to the RH strain of T. gondii. Their results consistently showed that greater than 90% of infected macrophages contained or carried only remnants of T. gondii by 2–6 hr postinfection, suggesting that the intracellular killing most likely involved enzymatic hydrolase degradation. Our study has provided evidence for such enzymatic degradation with the induction of macrophage hydrolases (phospholipase A₂, acid phosphatase, and acid proteinase) from Toxoplasma antigen activation. Our demonstrated extracellular release of the enzymes, also from Toxoplasma antigen activation, would further suggest a role in the killing of brain parenchyma cells and the subsequent development of necrosis that is a frequent feature of Toxoplasma encephalitis.

In conclusion, this study has demonstrated increased synthesis and release of acid proteinase, acid phosphatase, and phospholipase A₂ from peritoneal exudate cells and the macrophage cell line P388D in a concentration-dependent manner to Toxoplasma antigens and Toxoplasma immune complex. The release of these enzymes suggests that they may possibly play a role in the pathogenesis of recrudescence toxoplasmosis, as exemplified in Toxoplasma encephalitis.

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REFERENCES


