Toxoplasma gondii Triggers Granulocyte-Dependent Cytokine-Mediated Lethal Shock in D-Galactosamine-Sensitized Mice

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Infection with the intracellular protozoan Toxoplasma gondii is characterized by an acute proliferative stage, during which infective tachyzoites invade and replicate within a wide variety of host cells, and a chronic slow growing phase consisting of parasite encystment within tissues of the brain and muscle (36). Although infection is usually innocuous, in immunocompromised hosts encysted parasites can reactivate, leading to uncontrolled tachyzoite proliferation, tissue damage, and death (42, 43, 47).

Previous studies employing cytokine depletion, monoclonal antibody (MAb)-mediated depletion, and, more recently, gene knockout mice have established the importance of type 1 cytokines, such as gamma interferon (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) in control of experimental toxoplasmosis (7, 11, 56). Absence of any one of these proinflammatory mediators results in increased mortality during infection as a result of uncontrolled tachyzoite growth. The parasite itself is remarkably effective at stimulating pro-inflammatory cytokines (16, 52). In these animals, time to death early mortality associated with abnormally high levels of inflammatory cytokines (16, 52). In these animals, time to death is prolonged by depletion of CD4+ cells. Similarly, oral infection of genetically susceptible C57BL/6 mice results in gut-associated, IFN-γ-mediated necrosis mediated by CD4+ T lymphocytes (40). Nevertheless, while these elegant studies establish that cytokine-mediated pathology can occur during disease progression, determination of the precise mechanisms involved has been problematic. Thus, inflammatory mediators potentially inducing detrimental host pathology are simultaneously required to halt tachyzoite growth and multiplication, preventing host death from massive parasitemia and associated tissue destruction.

We employed the hepatotoxic compound D-galactosamine (D-Gal) so that we could evaluate the parasite’s ability to induce inflammatory pathology in the absence of a host requirement to control infection. Under normal conditions, mice are relatively resistant to inflammatory cytokine-mediated toxic shock, but intraperitoneal (i.p.) administration of D-Gal induces exquisite sensitivity to overproduction of inflammatory mediators. Thus, injection of D-Gal in conjunction with microbial products such as bacterial lipopolysaccharides (LPS) and superantigens results in rapid mortality, which is believed to be the result of TNF-mediated injury to the liver (10, 25, 38, 49, 51). In the case of LPS, lethality occurs independently of T lymphocytes, but for the staphylococcal superantigens, T lymphocytes are required, since severe combined immunodeficiency (SCID) mice are resistant to D-Gal and superantigen (10, 46, 48, 49).

The precise mechanism by which D-Gal exerts its sensitizing effects is not known, but the compound specifically targets the liver, where it induces metabolic changes in hepatocytes. Thus, levels of liver UDP-galactosamine derivatives rapidly accumulate following D-Gal injection, resulting in depletion of the free nucleotide, leading in turn to widespread cessation in biosynthesis of hepatocyte macromolecules such as RNA, proteins,
and glycoproteins (5, 32). Transcriptional arrest results in increased sensitivity to liver cell death mediated through TNF-α-induced apoptosis (39).

As we report here, i.p. injection of freeze-thawed tachyzoites (FTZ) of strain RH or live ts-4 (an attenuated parasite strain) triggers rapid death when coadministered with D-Gal. Lethality is a result of parasite-induced production of TNF, IFN-γ, and IL-12, as revealed by MAb depletion experiments, and is associated with catastrophic liver damage. Production of nitric oxide (NO) is also involved in the pathology of the response, since in vivo inhibition of NO with aminoguanidine (AG) renders mice resistant to D-Gal plus parasite antigen (Ag) toxicity. Finally, while the response occurs independently of T lymphocytes, antibody-mediated depletion of cells bearing the granulocyte-associated marker GR-1 rescues animals from the lethal effect of D-Gal and Ag coadministration. Our results provide a striking demonstration that T. gondii possesses the capability of inducing granulocyte-dependent inflammatory cytokine pathology, and they provide a convenient experimental framework for dissection of the response.

MATERIALS AND METHODS

Mice. C57BL/6 and C57BL/6.scid female mice (6 to 8 weeks of age) were obtained from Taconic Farms Inc. (Germantown, N.Y.), C3H/HeJ (LPS-hyperresponsive) and C3H/HeOuJ (LPS-responsive) female mice (6 to 8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, Maine). C57BL/6 IL-5−/− mice, kindly provided by E. Pearce, Cornell University, were obtained from offspring of a previously described breeding colony (35). The animals were housed under specific-pathogen-free conditions in the College of Veterinary Medicine animal facility at Cornell University.

Parasites and Ag. Tachyzoites of strain RH and attenuated mutant ts-4 (53) were maintained on human foreskin fibroblast monolayers in Dulbecco’s modified Eagle medium (GIBCO-BRL, Gaithersburg, Md.), 1% fetal calf serum stored in aliquots at −70°C; responsive) female mice (6 to 8 weeks of age) were obtained from Jackson Laboratory, Bar Harbor, Maine.

Fibroblast extract (FBE) was prepared by scraping of uninfected monolayers, sonicating, dialyzing, and filtering exactly as described for STAg preparations. The LPS content of Ag preparations was determined by the amebocyte assay (Limulus) (50.9%; SMC, 6.1%) after i.p. administration of FTZ (5 × 10⁴). FTZ were thawed immediately prior to experiments. To prepare soluble tachyzoite antigen (STA), RH strain tachyzoites were sonicated in the presence of protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.2 μM aprotinin, 1 μM leupeptin, 1 mM EDTA), dialyzed into PBS, and then centrifuged for 1 h at 10,000 × g (6). The resulting supernatants were filtered through a 0.2-μm-pore-size membrane (Corning Costar Corp., Cambridge, Mass.), absolute 0.2 M of MAb GK1.5 6 and 3 days prior to D-Gal and FTZ treatment. In vivo MAb-mediated depletion. MAbs XT22.11 (anti-mouse TNF), XMG6 (anti-mouse IFN-γ), and C17.8 (anti-IL-12; kindly provided by M. Wysocka and G. Trinchieri) were generated as hybridoma supernatants or ascites and purified by passage over protein G-Sepharose (Pharmacia Biotech Inc., Piscataway, N.J.) (45). Each eluted MAb was dialedyzed into PBS, concentrated to 1 mg/ml by a Centriprep 10 concentrator (Amicon), and stored in aliquots at −70°C. Production of inducible NO was blocked by administration of AG (Sigma Chemical Co.) as described elsewhere (20). CD4+ T lymphocytes were depleted from C3H/HeJ mice by i.p. administration of 0.5 mg of MAB GK1.5 6 and 3 days prior to i.p. and FTZ treatment. In vivo depletion of asialo-GM-1-1 positive cells was achieved by i.p. injection of 0.1 ml of anti-asialo-GM-1 24 and 18 h before challenge with D-Gal and T. gondii Ag. Finally, neutrophils and eosinophils bearing the GR-1 surface marker were eliminated by injection of 0.5 mg of RB6-8C5 MAb i.p. 24 and 18 h prior to i.p. and parasite Ag administration. Examination of peritoneal exudate cells from RB6-8C5-treated animals confirmed the specificity of depletion (polymorphonuclear cells: 6% polymorphonuclear leukocytes [PMN]; 2%, large mononuclear cells [LMC], 92%; small mononuclear cells [SMC], 5%) relative to control rat Ig-treated mice (PMN, 25%; LMC, 70.9%; SMC, 6.1%) after i.p. administration of FTZ (5 × 10⁴).

RESULTS

Production of NO. NO was measured by a two-site enzyme-linked immunosorbent assay (ELISA) as described previously (13) using plate-bound MAb HB170 (anti-IFN-γ), a rabbit polyclonal anti-mouse IFN-γ, and peroxidase-conjugated donkey anti-rabbit immunoglobulin (Ig) (Jackson Immune Research Laboratories, West Grove, Pa.). Sample absorbances (405 nm) were measured on a Microplate Bio Kinetics Reader (Bio-Tek Instruments, Inc., Winooski, Vt.) and compared to know amounts of recombinant IFN-γ standard (Genzyme Corp.).

Histopathology. Tissues were fixed in 10% formalin immediately following CO2 asphyxiation of the mice. For the liver, a small sample was cut in transverse section prior to fixation. The samples were embedded in paraffin, sliced into sections (approximately 2 μm thick), and stained with hematoxylin and eosin by the Cornell University Veterinary Medicine Histopathology Laboratory.

In vivo MAB-mediated depletion. MAbs XT22.11 (anti-mouse TNF), XMG6 (anti-mouse IFN-γ), and C17.8 (anti-IL-12; kindly provided by M. Wysocka and G. Trinchieri) were generated as hybridoma supernatants or ascites and purified by passage over protein G-Sepharose (Pharmacia Biotech Inc., Piscataway, N.J.) (45). Each eluted MAb was dialedyzed into PBS, concentrated to 1 mg/ml by a Centriprep 10 concentrator (Amicon), and stored in aliquots at −70°C. Production of inducible NO was blocked by administration of AG (Sigma Chemical Co.) as described elsewhere (20).

Cytokine depletion was accomplished by i.p. injection of 0.5 ml of MAB or control normal rat Ig 2 h prior to injection of D-Gal and parasite Ag. Mortality and morbidity were monitored over the subsequent 24 h.

CD4+ T lymphocytes were depleted from C3H/HeJ mice by i.p. administration of 0.5 mg of MAB GK1.5 6 and 3 days prior to i.p. and FTZ treatment. In vivo depletion of asialo-GM-1-1 positive cells was achieved by i.p. injection of 0.1 ml of anti-asialo-GM-1 24 and 18 h before challenge with D-Gal and T. gondii Ag. Finally, neutrophils and eosinophils bearing the GR-1 surface marker were eliminated by injection of 0.5 mg of RB6-8C5 MAb i.p. 24 and 18 h prior to i.p. and parasite Ag administration. Examination of peritoneal exudate cells from RB6-8C5-treated animals confirmed the specificity of depletion (polymorphonuclear leukocytes [PMN], 2%; large mononuclear cells [LMC], 92%; small mononuclear cells [SMC], 5%) relative to control rat Ig-treated mice (PMN, 25%; LMC, 70.9%; SMC, 6.1%) after i.p. administration of FTZ (5 × 10⁴).

FACS analysis. Fluorescence-activated cell sorter (FACS) analysis was performed on splenocytes from GK1.5-treated C3H/HeJ mice. Splenocyte stained with CD4 and fluorescein isothiocyanate (PharMingen, San Diego, Calif.) and stained on a FACS (Scalibur) (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.) The CD4+ T-lymphocyte population comprised less than 1.1% following GK1.5 treatment.

Statistical analysis. A Wilcoxon signed rank test was employed to assign statistical significance to mortality associated with groups of mice undergoing treatment with MAB. Experiments were performed on a minimum of two independent occasions.
similar toxic properties. As shown in Table 1, while neither results.

experimental system to study the phenomenon further.

to dissect the response. Therefore, we sought a surrogate ex-

portant component of acute infection, the simultaneous need

result suggested that TNF-mediated pathology may be an im-

relative to that of mice receiving control rat Ig. Although this

resulted in a slight, but significant, delay in onset of mortality

symptoms of illness were just beginning to become apparent)

administration of anti-TNF MAb at day 6 postinfection (when

FIG. 1. Administration of anti-TNF MAb during late acute T. gondii infec-

tions delays onset of death. C3H/HeJ mice were lethally infected by i.p. injection

of 100 strain RH tachyzoites. On day 6 postinjection, six mice were administered

a single 0.5-μg injection of either control rat IgG or MAH XT22.11 (rat anti-TNF

MABs). Statistical significance was determined by using a Wilcoxon signed ranked

test ($P < 0.006$). This experiment was repeated twice with essentially identical results.

RESULTS

Production of TNF contributes to host death during acute RH infection. Since inflammatory cytokines contribute to patho-

logy in genetically susceptible hosts infected with low-viru-

lence parasite strain ME49 (16, 40, 52), we initially questioned

whether similar pathology could account for some of the man-

ifestations of illness in mice undergoing acute infection with

the highly virulent parasite strain RH. As shown in Fig. 1,

administration of anti-TNF MAB at day 6 postinfection (when

symptoms of illness were just beginning to become apparent)

resulted in a slight, but significant, delay in onset of mortality

relative to that of mice receiving control rat Ig. Although this

result suggested that TNF-mediated pathology may be an im-

portant component of acute infection, the simultaneous need

for this cytokine to control parasite growth limited our ability

to dissect the response. Therefore, we sought a surrogate ex-

perimental system to study the phenomenon further.

Parasite lysate induces rapid lethality in mice coinjected with d-Gal. Administration of the hepatotoxic d-Gal results in

increased susceptibility to bacterial toxins such as LPS. This takes the form of a lethal, TNF-α-mediated shock response.

Therefore, we sought to determine if T. gondii Ag displayed similar toxic properties. As shown in Table 1, while neither

d-Gal nor STAg alone induced any ill effect at any point after

injection, when administered together, animals became sick

and died 12 to 24 h postinjection. As little as 40 μg of STAg

induced 100% lethality when administered with d-Gal (Table

1). Animals became sick at approximately 8 h postinjection,

with illness characterized by piloerection of the fur, hunching,

and shivering. The same effect, including lethality, was ob-

served when tachyzoites of the avirulent mutant, ts-4, were

administered with d-Gal (Table 1).

Lethality of parasite lysate cannot be attributed to endo-
toxin contamination. Since lethality induced by parasite Ag

d-Gal grossly resembled that induced by LPS, we were con-

cerned that our extracts may have contained bacterial endo-
toxin, which could be responsible for the profound effects

shown in Table 1. Accordingly, extracts were assayed for bac-
terial endotoxin by the highly sensitive Limulus amebocyte

assay. The results of the test revealed background levels of

endotoxin in our Ag preparations ($<1.9$ EU/mg of protein). In

addition, FBE prepared in exactly the same manner as STAg

was completely nontoxic when administered with d-Gal (Table

2).

More importantly, we compared LPS-responsive (C3H/

HeOuJ) and LPS-nonresponsive (C3H/HeJ) mouse strains

for sensitivity to parasite Ag (both FTZ and STAg) and d-Gal.

As shown in Table 3, the T. gondii Ag preparations were lethal

in both LPS-responsive and LPS-nonresponsive mouse strains.

This contrasted with administration of LPS plus d-Gal, which

was lethal only in C3H/HeOuJ animals. Together, our data

strongly suggest that the lethal effects observed stem from the

activity of a parasite molecule(s), rather than being attribut-
able to endotoxin contamination. Nevertheless, to avoid arti-
facts resulting from potential LPS contamination, the experi-

ments described below were performed with C3H/HeJ mice, ex-

cept where explicitly stated otherwise.

d-Gal plus FTZ injection, as well as acute RH infection,

results in major liver damage and high levels of NO in serum.

Administration of d-Gal and FTZ resulted in damage to the

eriver, as measured by appearance of the liver-associated en-

zymes GOT and GPT in sera 4 to 8 h following injection

(Fig. 2A and B). Damage to the liver was dependent upon

coinjection of d-Gal and FTZ, as well as acute RH infection,

for sensitivity to parasite Ag (both FTZ and STAg) and d-Gal.

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able to endotoxin contamination. Nevertheless, to avoid arti-
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ments described below were performed with C3H/HeJ mice, ex-

cept where explicitly stated otherwise.

**TABLE 1**. T. gondii triggers rapid lethality in d-Gal-sensitized mice

<table>
<thead>
<tr>
<th>d-Gal*</th>
<th>STAg (μg)</th>
<th>ts-4 tachyzoites</th>
<th>Mortalityb (no. dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>200</td>
<td>10⁷</td>
<td>0/3</td>
</tr>
<tr>
<td>+</td>
<td>40</td>
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<td>3/3</td>
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<td>+</td>
<td>8</td>
<td></td>
<td>2/3</td>
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<tr>
<td>+</td>
<td>1.6</td>
<td></td>
<td>0/3</td>
</tr>
<tr>
<td>+</td>
<td>0.3</td>
<td></td>
<td>0/3</td>
</tr>
</tbody>
</table>

* d-Gal (20 mg) was injected (+) into groups of three C57BL/6 animals with or without simultaneous i.p. administration of either STAg or tachyzoites of the attenuated strain ts-4, or was not administered (−).

b Lethality measured 12 h postinjection.

The final mouse succumbed between 12 and 24 h postinjection.

**TABLE 2**. Lethal effect of T. gondii in d-Gal-sensitized mice is not attributable to a factor present in FBE

<table>
<thead>
<tr>
<th>d-Gal*</th>
<th>FBE (μg)</th>
<th>ts-4 tachyzoites</th>
<th>Mortalityb (no. dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>0/3</td>
<td>2 × 10⁷</td>
<td>0/3</td>
</tr>
<tr>
<td>+</td>
<td>2 × 10⁷</td>
<td></td>
<td>2/3</td>
</tr>
<tr>
<td>+</td>
<td>200</td>
<td></td>
<td>0/3</td>
</tr>
<tr>
<td>+</td>
<td>40</td>
<td></td>
<td>0/3</td>
</tr>
<tr>
<td>+</td>
<td>8</td>
<td></td>
<td>0/3</td>
</tr>
</tbody>
</table>

* d-Gal (20 mg) was injected i.p. into groups of three C57BL/6 mice in the presence or absence of FBE or ts-4.

b Mortality at 12 h.

c The final mouse displayed gross morbidity at 12 h and was subsequently euthanized.
NO in serum. This response was characterized by steadily rising NO levels, first measurable at 4 h postinjection and continuing until death (Fig. 3). In addition, animals with lethal acute toxoplasmosis possessed high levels of NO circulating in the serum (Fig. 3).

Kinetics of the plasma cytokine response in mice injected with d-Gal plus FTZ. The rising levels of NO and parallel appearance of liver enzymes in the sera of lethally injected mice were suggestive of an uncontrolled inflammatory cytokine response. Accordingly, we measured TNF-α, IL-12, and IFN-γ levels in plasma of animals injected with d-Gal plus FTZ (Fig. 4). As shown, a distinct and highly reproducible pattern of cytokine production occurred following injection. Plasma TNF-α levels peaked 60 min following injection, followed by a peak IL-12 response occurring at 4 h and steadily rising levels of IFN-γ, continuing until the death of the animals. Similar TNF-α kinetics have been reported after administration of d-Gal and bacterial toxins (25, 48, 49). In addition, we found that administration of FTZ alone resulted in cytokine profiles virtually identical to those shown in Fig. 4 and that d-Gal in the absence of parasite Ag failed to induce cytokine production (data not shown). This is consistent with models of low-dose endotoxin shock, which require a sensitizing agent such as d-Gal, since mice are relatively resistant to this type of toxicity (10).

Death induced by d-Gal plus FTZ is mediated by TNF, IL-12, IFN-γ, and NO. We next examined whether some, or none, or both, or none of these mediators were required for lethality. For this purpose, we injected mice with d-Gal plus FTZ without the corresponding Ag. We found that injection of d-Gal alone was lethal, but injection of FTZ alone was not (data not shown). This is consistent with models of low-dose endotoxin shock, which require a sensitizing agent such as d-Gal, since mice are relatively resistant to this type of toxicity (10).

### Table 3

<table>
<thead>
<tr>
<th>d-Gal</th>
<th>FTZ</th>
<th>STAg</th>
<th>LPS</th>
<th>Mortality (no. dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>0/3 3/3</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>3/3 3/3</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0/3 0/3</td>
</tr>
</tbody>
</table>

a 20 mg of d-Gal with or without the indicated Ag coinjected i.p.
b $3 \times 10^7$ freeze-thawed strain RH tachyzoites.
c 150 μg.
d 1 μg.
e Mortality 24 h after injection.

### Figures

**Fig. 2.** Appearance of the liver-associated enzymes GOT and GPT in the sera of C3H/HeJ mice injected with d-Gal plus FTZ, as well as animals undergoing acute infection. (A and B) Mice were administered d-Gal (20 mg) plus FTZ ($5 \times 10^7$), and at the indicated times, sera were collected and GOT (A) and GPT (B) levels were measured. In this experiment, each point is represented by an independent group of animals ($n = 3$). (C and D) A group of mice was lethally infected (100 RH tachyzoites i.p.), and then, on day 6, further experimental groups were administered d-Gal alone or in combination with FTZ. On day 7, serum was collected from all of the animals, and GOT (C) and GPT (D) levels were determined. See Materials and Methods for details of enzyme measurements.
all, of the proinflammatory cytokines induced by the parasite were involved in the lethality of D-Gal and FTZ. Injection of depleting MAbs specific for TNF, IL-12, and IFN-γ rescued mice from the lethal effect of D-Gal and parasite Ag (Table 4). Animals treated in this manner not only survived, but failed to show any signs of sickness associated with the response. In contrast, mice injected with control rat Ig were susceptible to D-Gal plus FTZ lethality.

The high levels of NO appearing in the sera of mice treated with D-Gal plus Ag suggested that this effector molecule may contribute to the pathology of the response. To address this issue, mice were treated with the compound AG in order to block the ability to produce inducible NO. As shown in Table 4, mice subjected to this protocol were completely resistant to D-Gal plus FTZ-induced lethality. These results show that T. gondii-triggered TNF, IL-12, and IFN-γ responses result in death of the animals and suggest that the effector molecule NO plays a crucial role in the response.

Cells bearing the granulocyte-associated marker GR-1 mediate the lethal effect of D-Gal plus FTZ. To determine which cell types were required for D-Gal and parasite Ag lethality, T- and B-lymphocyte-deficient SCID mice were initially tested for susceptibility. As shown in Table 5, the immunodeficient C57Bl/6.scid strain was susceptible to T. gondii-induced lethality, a result indicating that the toxicity of D-Gal and parasite Ag is a T- and B-lymphocyte-independent effect.

To further investigate the cell type involved, in vivo MAb depletions were performed. Interestingly, we found that administration of a rat MAb specific for the granulocyte marker GR-1 rescued mice from the effects of D-Gal plus FTZ, as did injection of a rabbit anti-asialo-GM-1 antiserum which recognizes a glycolipid moiety expressed on NK cells and at low levels on granulocytes (Table 6). As expected, injection of control rat Ig and normal rabbit serum failed to alter the outcome of injection of D-Gal plus FTZ.

We next examined liver pathology in anti-GR-1- and control MAb-treated, as well as RH-infected, animals. As shown in Fig. 5A, injection of D-Gal plus FTZ with control Ig induced severe liver damage, consisting of severe hepatic cord dissociation, severe diffuse hepatic necroses, and hemorrhage throughout the liver. In striking contrast, animals receiving anti-GR-1 MAb displayed intact hepatic architecture and an absence of hemorrhage (Fig. 5B). Although livers from this group (Fig. 5B) appeared essentially normal (Fig. 5D), occasional necrotic hepatocytes were observed in the former. We also examined livers from mice with lethal acute infection. In this case, liver damage did not appear to extend throughout the organ as in animals receiving D-Gal plus FTZ. Nevertheless, hepatic cord dissociation and multiple foci of severe hepatic necrosis were observed (Fig. 5C). In addition, severe necrosis of the liver capsule was apparent in these sections.

Although GR-1 is expressed by both neutrophils and eosinophils, we consider it unlikely that the latter cell type is involved in the response because IL-5 knockout animals, which...
granulocytes. The latter molecular events would lead to neutrophil sequestration and transmigration in the liver. Subsequent neutrophil-mediated tissue eosinophilia (35), retain sensitivity to D-Gal plus FTZ, and NO.

**DISCUSSION**

The results of this study demonstrate that *T. gondii* possesses the capability of inducing lethal cytokine shock in D-Gal-sensitized mice. The response is marked by a rapid burst of TNF-α in serum followed by the appearance of IL-12, IFN-γ, and NO. Blocking of any one of these mediators with MAb or, for NO, AG rescues animals from the lethal outcome of injection of D-Gal plus *T. gondii* Ag. Administration of the combination of D-Gal and parasite extract also induces the appearance of liver-associated enzymes in the serum, a hallmark of hepatic damage. Interestingly, while the response is not *T* lymphocytes dependent, cells bearing the granulocyte marker GR-1 appear to be centrally involved, since their removal with depleting MAb rescues animals from lethality, a result implying that granulocytes serve as a source of the cytokine in this model (3, 39). Our data, which support the concept for a critical role of TNF-α, also show that depletion of IFN-γ, IL-12, and NO allows animals to survive. Therefore, lethality in the D-Gal experimental model is likely to be a complex process involving several mediators. Our laboratory is currently focusing effort on elucidating the pathways leading to death.

Perhaps the most striking aspect of our data regarding D-Gal- and parasite Ag-induced toxicity is that a GR-1-specific depleting MAb rescues animals from lethality, a result implicating granulocytes in pathogenesis of the response. Granulocytes have also been linked to LPS-induced liver damage (29, 34). Several recent reports indicate that granulocytes display protective activity during acute *T. gondii* infection (55, 57). Our data, in general, provide strong support for the concept that cells of this lineage play an important role during *T. gondii* infection and that, in the D-Gal system, they function as mediators of disease.

We do not at present know the functional role of neutrophils in our system. RB6-8C5-treated mice displayed an approximately 50% reduction in subsequent appearance of serum TNF-α (data not shown), raising the possibility that parasite Ag induces rapid release of the latter cytokine from granulocytes. Indeed, granulocytes are known to be capable of TNF-α secretion in response to stimuli such as LPS (3). An alternative model is that TNF-α induces upregulation of adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 in the liver and CD11b/CD18 on neutrophils. The latter molecular events would lead to neutrophil sequestration and transmigration in the liver. Subsequent organ damage could occur through production of reactive oxygen intermediates or other granulocyte mediators (8, 29).

In addition to the data presented here, several other recent reports demonstrate cytokine pathology during *T. gondii* infection. IL-10 knockout mice succumb during acute infection with the low-virulence ME49 strain, and death is associated with...
overproduction of inflammatory cytokines, presumably due to the absence of the downregulatory activity of IL-10 (16, 52). Evidence in support of this concept comes from the finding that MAb depletion of either IL-12 or IFN-γ results in delayed mortality in IL-10 knockout animals. Similarly, lethal oral infection of the C57BL/6 mouse strain results in IFN-γ-mediated gut pathology, and MAb depletion of the latter cytokine prolongs time to death in these animals (40). Interestingly, septic shock due to toxoplasmosis has also been reported for AIDS patients (41).

In each of the murine model studies described above, T or CD4+ lymphocytes were shown to play a role in mediating pathology, as shown by delayed mortality in SCID mice and in animals depleted of CD4+ cells with MAb. Our results show that T. gondii can also induce lethal pathology in the absence of the T-cell compartment, since both SCID mice and anti-CD4- treated mice remain susceptible to D-Gal plus FTZ administration. While the persistence of mortality in T-cell-deficient IL-10 knockout mice (16, 52) and orally infected C57BL/6 mice (40) may in part be attributable to uncontrolled parasite growth and dissemination, our data suggest that T-cell-independent inflammatory cytokine pathology may also contribute to death in these cases.

The role of NO in promoting endotoxemia is complex. The latter chemical mediator has been implicated in promoting lethal vasodilation and hypotension induced by LPS (22). Nevertheless, NO also possesses immunosuppressive properties and as such has been reported to play a role in downregulating overproduction of inflammatory cytokines during lethal shock induced by staphylococcal superantigens (9). In models of endotoxemia employing high doses of LPS, inducible nitric oxide synthase (iNOS) knockout mice have been reported to possess a resistant phenotype, although another report concluded that these knockout animals were indistinguishable from wild-type counterparts under the same conditions (37, 44). In low-dose models in which animals are primed with Propionibacterium acnes followed by LPS, iNOS gene inactivation has been reported to be without effect (44). These and other data suggest that low-dose and high-dose endotoxemia models are mechanistically distinct (24, 44).

For T. gondii-induced toxicity in D-Gal-sensitized mice, inhibition of NO with AG rendered mice resistant to subsequent lethality. This result was somewhat unexpected on the basis of models of low-dose endotoxemia induced by LPS, which do not appear to involve NO as a crucial factor. We are currently further exploring how T. gondii and LPS differ in activation of pathways leading to death.

Infection with a sufficiently low infectious dose of parasite strain ME49 allows survival of acute infection and establishment of chronic disease. In this case, production of NO appears to play a protective role during chronic infection, as determined by an increased cyst number in AG-treated mice (26). Furthermore, in the same model, iNOS knockout mice survive acute infection but succumb during the persistent stage of disease (59). Our data suggest that overproduction of NO may be involved in pathogenesis of lethal acute infection.

The effect of T. gondii Ag appears similar to that of LPS administration in D-Gal-sensitized mice, in that both treatments result in rapid, T-cell-independent, lethal cytokine shock (24, 49). Both LPS and T. gondii Ag induce macrophage acti-
vation in vitro, leading to inflammatory cytokine production. Therefore, it seems likely that the parasite factor(s) responsible for the in vivo effects reported here is identical to that inducing inflammatory cytokine production in cultures of murine macrophages (14, 23). While the T. gondii molecule triggering the inflammatory cytokine cascade has yet to be identified, related studies with Plasmodium falciparum and Trypanosoma cruzi suggest that specific protozoan glycolipid conjugates possess macrophage-activating capability (2, 58).

The cytokines IFN-γ, TNF, and IL-12 are crucial in the protective response to T. gondii (4, 12, 15, 27, 30, 33, 61, 62). Our data show that the parasite can stimulate production of lethally high levels of these same cytokines in T-gal-sensitized mice. To our knowledge, this study represents the first direct demonstration that T. gondii possesses the inherent capability of inducing cytokine toxicity in a T-cell-independent, granulo-cyte-dependent fashion.

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