Decreased Resistance of B Cell-Deficient Mice to Infection with *Toxoplasma gondii* Despite Unimpaired Expression of IFN-γ, TNF-α, and Inducible Nitric Oxide Synthase

Hoi Kang, Jack S. Remington and Yasuhiro Suzuki

*J Immunol* 2000; 164:2629-2634; doi: 10.4049/jimmunol.164.5.2629

http://www.jimmunol.org/content/164/5/2629
Decreased Resistance of B Cell-Deficient Mice to Infection with Toxoplasma gondii Despite Unimpaired Expression of IFN-γ, TNF-α, and Inducible Nitric Oxide Synthase

Hoil Kang, Jack S. Remington, and Yasuhiro Suzuki

The role of B cells in resistance against Toxoplasma gondii was studied using B cell-deficient (μMT) mice. Following peroral infection with 10 cysts of the ME49 strain, all μMT mice survived the acute stage of the infection but died between 3 and 4 wk after infection. In contrast, all control mice were alive at 8 wk after infection. At the stage during which μMT animals succumbed to the infection, parasite replication and pathology were most evident in their brains; small numbers of tachyzoites were also detectable in their lungs. Significantly greater numbers of T. gondii cysts and areas of inflammation associated with tachyzoites were observed in brains of μMT than in control mice. Large areas of necrosis associated with numerous tachyzoites were observed only in brains of μMT mice. Anti-T. gondii IgG Abs were detected only in sera of control mice, whereas similar levels of IFN-γ were detected in sera of both strains of mice. Amounts of mRNA for IFN-γ, IL-10, and inducible NO synthase in the brain did not differ between infected μMT and control mice. Expression of mRNA for TNF-α was increased in brains of μMT mice. Administration of polyclonal rabbit anti-T. gondii IgG Ab prevented early mortality and pathology associated with tachyzoites in the brain in the infected μMT mice. These results indicate that B cells play an important role, most likely through their production of specific Abs, in resistance to persistent active (tachyzoite) infection with T. gondii in mice, especially in the brain and lung. The Journal of Immunology, 2000, 164: 2629–2634.

Cell-mediated immunity is critical for host resistance against acute acquired infection with T. gondii (1–4) and against development of toxoplasmonic encephalitis during the chronic stage of the infection (5–9). Although infection with T. gondii also stimulates humoral immunity, the role of Abs in resistance against T. gondii remains unclear. Previous studies have demonstrated that administration of anti-T. gondii polyclonal (10, 11) or mAbs (12, 13) administered before infection resulted in reduction in mortality or prolonged time to death in mice. However, these studies did not address whether the Ab response during the natural course of the immune response following infection plays a protective role against this organism.

Frenkel and Taylor (14) previously examined the effects of B cell depletion on toxoplasmosis in mice by treatment with anti-μ Ab. From their results, they suggested that Ab may not completely deplete B cell populations in vivo, and although serum IgM is reduced to undetectable levels, IgG is generally readily detectable, albeit at levels 10- to 1000-fold lower than normal (17). To elucidate whether B cells play a protective role in resistance against infection with T. gondii, additional studies using alternative approaches to deplete B lymphocytes are needed.

B cell-deficient (μMT) mice have recently been generated by disruption of one of the membrane exons of the μ-chain gene (18). These animals have no detectable B cells or circulating Ab, yet display normal development of the T lymphocyte compartment (18). Moreover, previous studies have been shown that μMT mice have normal Ag-presenting function for priming of CD4+ T cells to most soluble Ags as well as unimpaired CD8+ T lymphocyte response (19–21). Thus, μMT mice appear to be a suitable model to analyze the role of B cells in host resistance to T. gondii infection. In the present study, we examined susceptibility of μMT mice to peroral infection with T. gondii. These mice suffered early mortality associated with continuous proliferation of tachyzoites in their brains and lungs in the absence of Ab responses to the parasite but with unimpaired expression of IFN-γ, TNF-α, and inducible NO synthase (iNOS).

Materials and Methods

Mice

Female Swiss-Webster (Taconic Farms, Germantown, NY), C57BL/6-background B cell-deficient (μMT) (The Jackson Laboratory, Bar Harbor, ME), and control C57BL/6 mice (The Jackson Laboratory) were 6–8 wk old when used.

1 Abbreviation used in this paper: iNOS, inducible NO synthase.
Infection with T. gondii

Cysts of the ME49 strain were obtained from brains of chronically infected Swiss-Webster mice as described previously (22). μMT and control mice were infected with 10 cysts perorally by gavage.

Histopathology

At 25 days after infection, mice were euthanized by asphyxiation with CO2. Their brains, lungs, hearts, livers, spleens, kidneys, and small and large intestines were removed and immediately fixed in a solution containing 10% Formalin, 70% ethanol, and 5% acetic acid. Two to four 5-μm-thick sections (50- to 100-μm distance between sections) of the organs from each mouse were stained with hematoxylin and eosin. Immunoperoxidase staining using rabbit IgG Ab against either tachyzoite-specific SAG2 or bradyzoite-specific BAG1 were used for detection of tachyzoites and cysts, respectively (23, 24). The specificity of these Abs were described previously (25, 26). Sections stained with hematoxylin and eosin were evaluated for inflammatory changes, and sections stained by the immunoperoxidase method were evaluated for the number of T. gondii cysts and areas associated with tachyzoites.

Detection of mRNA for cytokines and tachyzoite-specific SAG1

RNA was isolated from brains of infected μMT and control mice by using RNA Stat60 (Tel-Test, Friendswood, TX) by following the commercial insert. cDNA was synthesized using the RNA as described previously (22, 27). PCR for cytokines was performed with 5 μl of either a 1:10 dilution or 1:50 dilution of the original cDNA reaction mixture with a Geneamp 9700 thermocycler (Perkin-Elmer, Emeryville, CA) using 30 cycles to produce the amount of DNA within a linear range as described previously (22, 27). This number of cycles was determined in preliminary studies using different amounts of cDNA of the sample. Specific primers for β-actin, IFN-γ, TNF-α, IL-10 (Clontech, Palo Alto, CA), and iNOS (28) designed to span at least one intron allowed differentiation of amplified target DNA derived from either cDNA or genomic DNA in the PCR. PCR for SAG1 was performed using 1 μl of the original cDNA reaction mixture, as described by Gazzinelli et al. (28). For each gene product, the number of cycles was determined experimentally and was defined as that number of cycles that would achieve a detectable concentration that was well below the saturating conditions. In these conditions, 3-fold differences in cDNA concentrations showed clear differences in the amounts of PCR products.

Detection of PCR products

Ten-microliter aliquots of the final PCR mixtures were electrophoresed at 100 V for 1 h on a 1.5% agarose gel and denatured (22, 27). The DNA was then transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA) by standard blotting procedures (29) and UV cross-linked. Oligonucleotide probes for β-actin, IFN-γ, TNF-α, IL-10 (Clontech), and iNOS (26), which hybridize to the PCR products wholly within the region amplified by the primers, were end labeled as described for the 3'-end labeling and signal amplification system for a FluorImager (Amersham, Little Chalfont, England), and hybridization was detected by scanning of the membranes with a FluorImager Storm 860 (Molecular Dynamics, Sunnyvale, CA) as described previously (30). Quantification of mRNA was performed by densitometry analysis with the FluorImager and normalized to the β-actin level.

Serum levels of IFN-γ

Mice were bled at 25 days after infection and the concentrations of IFN-γ in their sera were measured by ELISA using mAbs against IFN-γ (R4–6A2 as capture, XMG 1.2 as secondary) obtained from PharMingen (San Diego, CA).

Toxoplasma Ab

Toxoplasma Ab titers in sera of animals obtained at 25 days after infection were measured by the Sabin-Feldman dye test (31).

Flow cytometry

Spleen cells were obtained from μMT and control mice before and 23 days after infection. A total of 1 × 10^6 of spleen cells was pretreated on ice for 10 min with 10 μl of a predetermined optimal concentration of anti-FcγRII/III receptors (2.4G2) to block non-Ag-specific binding of Abs to the FcγRII/III receptors. Thereafter, the cells were incubated on ice for 30 min with 10 μl of optimal concentrations of FITC-conjugated anti-CD8 mAb (53-6.7) and PE-conjugated anti-CD4 mAb (RM4-5), or PE-conjugated anti-NK1.1 mAb (PK136). The mAbs were obtained from PharMingen. Analysis of stained cells was performed with a FACScan (Becton Dickinson, Mountain View, CA). Dead cells were gated out on the basis of propidium iodide staining.

Administration of anti-T. gondii IgG Ab

T. gondii-specific IgG Abs were purified by using a protein A column from sera of rabbits that had been infected i.p. with 50 cysts of the ME49 strain 5 wk earlier. μMT mice were injected i.p. with 0.12 ml (0.8 mg of protein) of the purified IgG solution (dye test titer, ≥1:16,000) every 3 days beginning at 2 days after infection for 40 days. Control mice were injected with the same amounts of normal rabbit IgG purified from seronegative rabbits in the same manner.

Statistical analysis

Levels of significance for differences between groups of mice were determined by Student’s t or Mann-Whitney U test. Mann-Whitney U test was applied when SDs were significantly different between groups tested. Differences which provided p < 0.05 were considered to be significant.

Results

Effect of T. gondii infection on mortality in B cell-deficient and control mice

Following infection with 10 cysts of the ME49 strain, μMT mice all died between 21 and 28 days after infection (Fig. 1). In contrast, all control mice were alive at 59 days after infection, and thereafter began dying; all died by 85 days after infection (p = 0.0007, Fig. 1).

Histological changes in the organs of infected μMT mice

Because of the remarkable difference in mortality between infected μMT and control mice, we examined their brains, hearts, lungs, livers, kidneys, and small and large intestines at 25 days after infection (when some μMT mice had already died). The most remarkable histological changes were observed in brains of μMT mice. Significantly greater numbers of areas associated with T. gondii tachyzoites were observed in brains of μMT than in control mice (p = 0.0001, Fig. 2B). In μMT mice, there were many small areas of acute focal inflammation with the parasites (Fig. 3A). Large areas of necrosis of brain tissue associated with numerous tachyzoites were observed in μMT but not in control mice (Fig. 3B). Numbers of cysts were also significantly greater in brains of μMT than in control mice (p = 0.019, Fig. 2A). These facts indicate that continuous proliferation of tachyzoites and formation of greater numbers of cysts occur in brains of μMT mice following infection.

Small numbers of tachyzoites were observed in the lungs in 50% of the μMT mice examined (n = 10). T. gondii cysts were also observed in lungs of two of these mice (Fig. 3C).

FIGURE 1. Mortality in μMT and control mice following infection with T. gondii. μMT and control mice were infected perorally with 10 cysts of the ME49 strain. The data are representative of two separate experiments performed.

2630 ROLE OF B CELLS IN RESISTANCE TO T. gondii

Downloaded from http://www.jimmunol.org/ by guest on November 17, 2017
tachyzoites nor cysts were detectable in lungs of control mice (n = 7). Despite these differences in the presence of the parasite between μMT and control mice, infiltration of inflammatory cells in the lungs was similar in both groups.

Although the tachyzoites were not detected, mild infiltration of inflammatory cells was observed in the liver and heart of both μMT and control mice. Neither the parasite nor inflammatory responses were observed in the kidneys or intestines of μMT mice or controls.

Tachyzoite-specific SAG1 mRNA in brains of infected mice
At 25 days after infection, amounts of tachyzoite-specific mRNA encoding SAG1 in the total RNA fractions were measured by reverse transcripting the RNA, followed by amplification of SAG1-specific cDNA using PCR (RT-PCR). Significantly greater amounts of SAG1 mRNA were detected in total RNA obtained from brains of infected μMT mice than control mice (p = 0.0002), although there were variations between individual μMT mice (Fig. 4).

Levels of mRNA for cytokines and iNOS in brains of infected mice
It was recently reported that spleen cells from Chlamydia trachomatis-infected B cell-deficient mice failed to produce Th1-related (IFN-γ) or Th2-related (IL-6 and IL-10) cytokines after C. trachomatis-specific in vitro restimulation (16). Since an IFN-γ-mediated immune response has been shown to be critical for resistance against development of toxoplasmic encephalitis (5–9, 32), we examined whether expression of IFN-γ, TNF-α, and iNOS is impaired in brains of μMT mice infected with T. gondii. We also examined expression of IL-10 in brains of these mice because this cytokine has been reported to inhibit T. gondii microbicidal activity of macrophages activated by IFN-γ (33). Amount of mRNA for the cytokines and iNOS were measured by RT-PCR in the total RNA fractions obtained from brains of mice at 25 days after infection. The amounts of mRNA for IFN-γ, IL-10, and iNOS did not differ between these mice (Fig. 4). The amounts of mRNA for TNF-α were significantly greater in μMT than in control mice (TNF-α:β-actin ratio, 0.191 ± 0.081 vs 0.052 ± 0.013, p = 0.0021) (Fig. 4). In uninfected μMT or control mice, the amounts

FIGURE 3. Histological changes and detection of T. gondii in the brain and lung of infected μMT mice. Mice were infected perorally with 10 cysts of the ME49 strain, and histological study was performed 25 days later. A, An area of acute focal inflammation associated with tachyzoites in the brain. B, A large area of necrosis of brain tissue associated with tachyzoites. C, A cyst observed in the lung. Sections were stained by immunoperoxidase stain using rabbit IgG Abs against (A and B) tachyzoite-specific SAG2 or (C) bradyzoite-specific BAG1 (arrowheads indicate A and B T. gondii tachyzoites and C a cyst). The experiment was performed twice, and there were three to six mice in each group in each experiment.
of mRNA for each of these molecules were very low and mostly undetectable (ratios to β-actin were below 0.001).

IFN-γ levels in sera of infected μMT and control mice

To compare systemic IFN-γ production between μMT and control mice, we measured serum levels of this cytokine at 25 days after infection. Levels of IFN-γ did not differ between these mice (1.95 ± 0.54 ng/ml in controls (n = 7) vs. 2.35 ± 0.53 ng/ml in μMT (n = 9)). The results were reproducible in two separate experiments.

Toxoplasma Ab titers

Toxoplasma dye test Ab titers were examined in sera obtained at 25 days after infection. Whereas high Ab titers (dye test titers ranged from 1:1024 to 1:4096) were observed in sera of control mice, they were undetectable in sera of μMT animals.

Changes in relative percentages of T cell subsets and NK cells in the spleen in μMT and control mice following infection

We examined relative percentages of T cell subsets and NK cells in the spleen of μMT and control mice by flow cytometry before and 23 days after infection. Relative percentages of CD8+ T cells significantly increased following infection in both μMT (from 37.6 ± 4.0% to 50.1 ± 4.9%; p = 0.014) and control mice (from 12.0 ± 0.5% to 18.3 ± 4.1%; p = 0.032). In contrast, relative percentages of NK cells significantly decreased following infection in both μMT (from 8.3 ± 1.7% to 2.9 ± 0.8%; p = 0.0015) and control mice (from 3.3 ± 0.4% to 1.1 ± 0.4%; p = 0.0006). Relative percentages of CD4+ T cells did not differ before and after infection in both groups of animals (data not shown). Because of the absence of B cells in μMT mice, relative percentages of T cells and NK cells were higher in μMT than in control mice.

Passive transfer of anti-T. gondii IgG to infected μMT mice

To examine whether the cause of early death in infected μMT mice was due to the absence of Ab responses, we treated these mice with rabbit anti-T. gondii IgG Abs every 3 days beginning 2 days after infection. Control μMT mice were treated with normal rabbit IgG in the same manner. All control μMT mice died from 21 to 25 days after infection (Fig. 5). μMT mice treated with anti-T. gondii IgG Abs were all alive at the end of observation period (42 days after infection) (Fig. 5). Histological studies were performed on brains and lungs of μMT animals in both groups at 23 days after infection. Significantly fewer numbers of T. gondii cysts and inflammatory areas associated with tachyzoites were observed in brains of mice treated with anti-T. gondii IgG Abs than those treated with normal IgG (p = 0.017 for either the number of cysts or inflammatory areas) (Fig. 6). Small numbers of tachyzoites were detected in lungs of animals only treated with normal IgG (data not shown). Toxoplasma Abs were detected in sera of mice treated with anti-T. gondii IgG Abs (dye test titer at 1:2048) but not in those treated with normal IgG.

Discussion

The results described above reveal that mice deficient in B cells were able to survive the acute stage of infection with T. gondii but succumbed to the parasite 3–4 wk after infection. In contrast, all control mice were still alive 8 wk after infection. As expected,
Toxoplasma Abs were detectable in sera of infected control but not of μMT mice. Adoptive transfer of anti-T. gondii IgG Abs to infected μMT mice prevented their early mortality. Thus, production of Abs against T. gondii by B cells was critical for prevention of mortality in mice during the chronic stage of infection. This is the first evidence for the importance of B cells in host resistance to T. gondii during the natural course of infection.

Histological study revealed large numbers of tachyzoites in brains of μMT mice but not of control mice at the time when μMT mice had begun to die. Tachyzoites were also demonstrable in the lung of only μMT mice but the numbers of the organisms in their lungs were markedly fewer than in their brains. These results indicate that B cells, most likely Ab production by these cells, are important for preventing persistence of tachyzoite replication in the brain and lung in mice during the late stage of the infection.

This is further supported by the evidence that treatment with anti-T. gondii IgG Abs markedly decreased the parasite load in these organs. Since tachyzoites were not detectable in hearts, livers, kidneys, or intestines of the μMT mice when they had begun to die, the mechanism of host defense against T. gondii appears to differ in different organs.

In addition to greater tachyzoite load in brains and lungs of μMT mice, we observed differences in the numbers of T. gondii cysts in these organs. Markedly and significantly greater numbers of cysts were detected in brains of μMT than in control mice. In addition, cysts were detectable only in lungs of μMT mice although the numbers of cysts were small. In our previous experience, formation of T. gondii cysts in the lung is unusual. Persistent proliferation of tachyzoites in the brain and lung of μMT mice may have contributed to formation of greater numbers of cysts in these organs.

Frenkel and Taylor (14) previously addressed the role of B cells in resistance against T. gondii by examining the occurrence of reactivation of latent infection in anti-μ Ab-treated mice infected with a virulent strain and treated with sulfadiazine. They observed mortality associated with toxoplastic pneumonia and/or encephalitis in chronically infected anti-μ-treated animals after discontinuation of sulfadiazine treatment. In the present study, persistence of tachyzoite proliferation was observed in brains and lungs of μMT mice during the late stage of the primary infection. Thus, Ab appears to play a critical role in prevention of proliferation of tachyzoites in these organs in both primary infection and reactivation of latent infection. In addition to the brain and lung, myocarditis was also reported in the anti-μ-treated mice (14). However, this pathology was not evident in μMT mice in the present study. This difference may be due to the side effects of treatment with large amounts of xenogenic anti-μ Ab on the immune system in the previous study, the presence or absence of sulfadiazine treatment, differences in virulence of the parasite and mouse strains, and/or different infection conditions (primary vs reactivation).

Despite the absence of humoral immunity, we observed significant increases in relative percentages of CD8+ T cells and decreases in relative percentages of NK cells in spleens of both μMT and control mice following infection. We also observed unimpaired IFN-γ expression in T. gondii-infected μMT mice. Cellular immunity mediated by IFN-γ (5, 6, 32), TNF-α (7, 8, 27, 28), and iNOS (9, 34) have been reported to be critical for resistance against development of toxoplastic encephalitis in mice. Although infected μMT mice developed severe encephalitis, expression of these molecules in their brains were similar to or higher than those in control mice. Serum levels of IFN-γ were also similar in both groups of mice. In addition, expression of IL-10 in the brain did not differ between μMT and control mice. These results further support the likelihood that absence of Ab production is a major factor in the reduced resistance to T. gondii in these mice.

Eperon et al. (35) recently reported that following infection with Neospora caninum, μMT mice developed focal necrotic cerebral lesions which were absent in control mice. In their studies, IFN-γ production by spleen cells was lower in μMT than in control mice during the early stage of infection; however, it did not differ between these animals during the later stage when cerebral pathology was observed in μMT mice (35). Yang and Brunham (16) recently reported that production of both Th1- and Th2-type cytokines were suppressed in μMT mice following C. trachomatis infection. In infection with lymphocytic choriomeningitis virus, μMT mice were found to have a CD4 helper defect (36). A regulatory role of B cells on T cell responses may differ depending on infectious agents which stimulate the immune system, the stages of infection, and subsets of T cells.

In the presence of specific Abs, T. gondii tachyzoites are rapidly lysed by activation of complement through the classical pathway in vitro (37, 38), although the parasites are resistant to complement in the absence of Abs (39). This Ab-dependent complement-mediated killing may play an important role in resistance of mice to the infection. Abs also appear to play a protective role in collaboration with macrophages. Macrophages have been shown to kill Ab-coated tachyzoites in vitro (40, 41). Since the present study revealed persistent proliferation of tachyzoites in brains and lungs of μMT mice, Ab coating of the parasite to allow for phagocytosis and killing by resident macrophages (microglia and alveolar macrophages) in the brain and lung may be an important protective mechanism. In addition, Ab may inhibit intracellular proliferation of tachyzoites. Mineo and Kasper (42) reported that treatment of tachyzoites with a mAb to T. gondii in the absence of complement did not inhibit either attachment or invasion of the parasite into human fibroblasts but inhibited their intracellular proliferation.

It has been well documented that IFN-γ-mediated cellular immunity is required for survival of mice during both the acute and chronic stages of the infection with T. gondii (1–9). The results of the present study indicate the importance of the B cell Ab response for prevention of persistent proliferation of tachyzoites in the brain and lung during the chronic stage of infection. Thus, humoral and cellular immunity act in concert in host resistance against T. gondii. The role of each immune response appears to differ depending on the stage of the infection and its anatomical location.

Acknowledgments

We thank Dr. Fausto Araujo for providing rabbit anti-T. gondii serum and Edgar K. Gufwoli, Hanna Lee, and Pauline Chu for excellent technical assistance with histological studies. We also thank the serology laboratory of Palo Alto Medical Foundation for performing the Sabin-Feldman dye test.

References


