The temperature-sensitive mutants of *Toxoplasma gondii* and ocular toxoplasmosis

Fangli Lu\(\textsuperscript{a,b,}\textsuperscript{*},\textsuperscript{1}\), Shiguang Huang\(\textsuperscript{b,1}\), Lloyd H. Kasper\(\textsuperscript{c}\)

\(\textsuperscript{a}\) Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, PR China
\(\textsuperscript{b}\) Jinan University School of Medicine, Guangzhou, Guangdong 510632, PR China
\(\textsuperscript{c}\) Department of Medicine and Microbiology, Dartmouth Medical School, Lebanon, NH 03756, USA

**A B S T R A C T**

The risk of blindness caused by ocular toxoplasmosis supports efforts to improve our understanding for control of this disease. In this study, the involvement of CD8\(^+\), CD4\(^+\), B cell, and IL-10 gene in the immune response of primary ocular infection with the temperature-sensitive mutant (ts-4) of the RH *Toxoplasma gondii* strain, and in the protective immunity of ocular ts-4 vaccination and challenge with RH strain was investigated in murine models utilizing inbred C57BL/6 mice-deficient in CD4\(^+\), CD8\(^+\), B cells (\(\mu\)MT), or IL-10 gene. Compared to naive mice, all WT and mutant mice had different degree of ocular pathological changes after ts-4 ocular infection, in which both CD8 KO and IL-10 KO mice showed the most severe ocular lesions. Immunized by ts-4 intracameral (i.c.) inoculation, all mutant mice had partially decreased vaccine-induced resistance associated with increased ocular parasite burdens after RH strain challenge. A significant increase of the percentages of B cells and CD8\(^+\) T cells in the draining lymph nodes were observed in WT and IL-10 KO mice after either infection or challenge. The levels of specific anti-toxoplasma IgG in both eye fluid and serum from all the mice were significantly increased after ts-4 i.c. immunization, except \(\mu\)MT mice. These results suggest that the avirulent ts-4 of *T. gondii* inoculated intracameral can induce both ocular pathology and ocular protective immunity; CD4\(^+\), CD8\(^+\), B cell, and IL-10 gene are all necessary to the vaccine-induced resistance to ocular challenge by virulent RH strain, in which CD8\(^+\) T cells are the most important component.

\(\textcopyright\) 2008 Elsevier Ltd. All rights reserved.

1. Introduction

*Toxoplasma gondii* is a protozoan parasite that infects up to a third of the world’s population [1], and *T. gondii* infection is an important cause of central nervous system and ocular disease both in immunocompromised and in certain immunocompetent populations. Ocular toxoplasmosis (OT), a progressive and recurring disease that can threaten visual function, is the most common cause of human retinocchoroiditis worldwide [2,3]. The prevention of acquired and congenital infections of *T. gondii* is very important in controlling OT [4]. Although toxoplasma-mediated host cell lysis is probably a principal cause of tissue destruction in immunodeficiency states, hypersensitivity and inflammatory responses may be the major cause in otherwise immunosufficient individuals [5]. Thus, immune prevention of OT should be a more effective control strategy. Ts-4 is a temperature-sensitive mutant of the RH *T. gondii* strain, which does not form tissue cysts and is avirulent for immunocompetent mice [6]. Infection with the ts-4 strain of *T. gondii* induces strong protection against rechallenge with virulent toxoplasma in mice [7]. However, little is known about the safety and protective immunity of ts-4 by intraocular infection, the ocular immune characterization induced by the ts-4 and the immune response necessary to protect mice from ocular challenge need to be evaluated. The present study was designed to better understand the specific role of CD4\(^+\) T cell, CD8\(^+\) T cell, B cell, and IL-10 responses in the immunopathogenesis of primary ocular ts-4 infection and in the immunity induced by ts-4 vaccination against ocular toxoplasma challenge by using experimental murine models-deficient in CD4\(^+\), CD8\(^+\) T cells, and B cells, and lacking a functional IL-10 gene.

2. Materials and methods

2.1. Parasites

The temperature-sensitive mutant of RH *T. gondii* strain (ts-4; kindly provided by Elmer Pfefferkorn, Dartmouth Medical...
School, Lebanon, NH) and the RH strain tachyzoites engineered to constitutively express green fluorescent protein (GFP; RH-GFP [kindly provided by John Boothroyd, Stanford University, Stanford, CA]) were used in the present study. They were maintained by continuous passage in human fibroblasts grown in Dulbecco modified Eagle medium (catalog no. 11965; Gibco, Grand Island, NY) supplemented with 10% newborn calf serum plus antibiotics.

2.2. Mice

A breeding pair of CD8 knockout (KO) mice on a C57BL/6 background was kindly provided by TW Mak (Amgen Institute, Toronto, Ontario, Canada). Age-matched (7- to 9-week-old) and sex-matched C57BL/6 wild-type (WT), CD4 KO, B cell-deficient (μMT), and IL-10 KO mice of the same genetic background were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were bred under specific-pathogen-free conditions at the Animal Research Facility at Dartmouth Medical School.

2.3. Immunization and eye inoculation

Mice were immunized by intracamerally (i.c.) injection of 2 × 10⁴ ts-4 strain tachyzoites into the right eye; the left eye of WT, CD4 KO, CD8 KO, μMT, and IL-10 KO mice was challenged by ocular inoculation of 10² RH strain tachyzoites at 28 days postimmunization. Primary infection of naive mice was performed by ocular inoculation with 10² ts-4 strain tachyzoites into the left eye. For observation of survival rate, mice were infected by intraperitoneal (i.p.) injection with 10⁵ ts-4 tachyzoites of T. gondii. Eye inoculation was performed as previously described [8]. Briefly, mice were anaesthetized with ketamine hydrochloride (40 mg/kg) and xylazine (5 mg/kg) by i.p. injection. After the leaking aqueous fluid was blotted on the right eye, a 5-μl parafl”lel injection with 10² ts-4 tachyzoites of T. gondii. The eye tissue was prepared for inflammatory changes. Pathological changes were scored on a scale of 0 (normal) to 4 according to the method of Hu et al. [8] as follows: 0, normal histology; 1, mild inflammation without necrosis; 2, obvious inflammation without necrosis; 3, strong inflammation with necrosis; 4, strong necrosis in whole eye section.

2.4. Eye parasite burden

Parasite numbers in the eye tissue of the strains of mice were quantified [9]. The challenged eyes were isolated at 11 days after ts-4 i.c. vaccination and challenge with 10⁵ RH-GFP tachyzoites. The eye tissue was prepared for inflammatory changes. Pathological changes were scored on a scale of 0 (normal) to 4 according to the method of Hu et al. [8] as follows: 0, normal histology; 1, mild inflammation without necrosis; 2, obvious inflammation without necrosis; 3, strong inflammation with necrosis; 4, strong necrosis in whole eye section.

2.5. Histopathology

At 11 days after oculc primary ts-4 infection or RH strain challenge, mice were sacrificed by CO₂ asphyxiation, and harvested eyes were immediately fixed in 10% buffered formaldehyde (Polyscience, Warrington, PA). Then, 5-μm sections (with a 50- or 100-μm distance between sections) of the eye tissue from each mouse were stained with hematoxylin and eosin and evaluated for inflammatory changes. Pathological changes were scored on a scale of 0 (normal) to 4 according to the method of Hu et al. [8] as follows: 0, normal histology; 1, mild inflammation without necrosis; 2, obvious inflammation without necrosis; 3, strong inflammation with necrosis; 4, strong necrosis in whole eye section.

2.6. Confocal laser scanning microscopy

Five-micrometer thick sections (50- or 100-μm distance between sections) of paraffin wax-embedded eye tissue from each mouse vaccinated and challenged with 100 RH-GFP tachyzoites at 11 days were visualized by using a MRC-1024 confocal scanning laser microscope (Bio-Rad, Hercules, CA) equipped with a Zeiss Axiophot microscope (Oberkochen, Germany), a 40× plan neofluar 1.3 numerical aperture objective lens, and a 15-mW krypton/argon laser. GFP was detected with a 525 DF32 band-pass filter.

2.7. The weight, cell number, and phenotypic analysis of cervical lymph nodes (CLNs)

CLNs from mice infected or challenged were excised at 11 days postinoculation, degreased in PBS at 4°C, and then immediately weighed. CD3/CD16 monoclonal antibodies (mAbs), FITC- or phosphatidylethanolamine (PE)-labeled mAbs against CD3, CD4, CD8, CD19, and isotype-specific control Ig (BD PharMingen, San Diego, CA) were used. Cells (1 × 10⁶) from CLNs were stained with the above mAb and analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA).

2.8. Anti-toxoplasma antibody levels in serum and aqueous fluid

Approximately 10–15 μl of aqueous fluid was withdrawn by using a 27.5-gauge needle via a limbal paracentesis from each naïve mouse and from the left eye of each mouse immunized i.c. with ts-4 at 28 days. Serum from each mouse was withdrawn at the same time. The fluid and serum were stored at −70°C until use. Purified RH parasites (5 × 10⁴/well) were placed in microtitr plates (Nunc-Immuno Plate; Nunc, Roskilde, Denmark), dried overnight, blocked with 5% bovine serum albumin–phosphate-buffered saline (PBS) (Sigma, St. Louis, MO), and washed in PBS (pH 7.2)–0.05% Tween 20 (Bio-Rad). Antisera and aqueous fluid were incubated for 2 h at 37°C. Plates were washed and supplemented with a peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG; whole molecule, 1/40,000; Sigma) for 1 h. After a wash step, tetramethyl benzidine substrate in H₂O₂ (Kirkegaard Perry, Gaithersburg, MD) was used for development. The reaction was stopped 30 min later by the addition of 2N H₂SO₄. OD values were measured at 450 nm with an automatic microplate reader (model 550; Bio-Rad).

2.9. Statistical analysis

Data were analyzed by using the Student’s t-test or the Wilcoxon signed rank test. P values of <0.05 were considered statistically significant.
Fig. 2. Ocular pathology of mice. Panels: infected (A) and challenged C57BL/6 WT mouse (B), infected (C) and challenged μMT mouse (D), infected (E) and challenged CD4 KO mouse (F), infected (G) and challenged CD8 KO mouse (H), and infected (I) and challenged IL-10 KO mouse (J). Mouse A, C, E, G, and I was at 11 days post-primary ocular inoculation with $10^3$ ts-4 tachyzoites of *T. gondii*. Mouse B, D, F, H, and J was at 11 days post-ocular challenge; those mice were immunized by i.c. injection of $2 \times 10^4$ ts-4 strain tachyzoites in the right eye previously, and then challenged by ocular injection with $10^4$ RH tachyzoites of *T. gondii* in the left eye. There were four mice per group, and data are representative of those from two experiments. Magnification, 4×; H&E stain.
3. Results

3.1. Ts-4 infection was lethal for CD8 KO mice

After i.p. inoculation of \(10^5\) ts-4 tachyzoites, all WT mice survived, but about half of the CD8 KO mice died around 2 weeks postinfection and only a few survived for up to 43 days postinfection (Fig. 1), and bloody-ascitis or ascritis contained a large amount of tachyzoites were observed in the CD8 KO mice. In contrast, mice depleted of CD4+ T cells, B cells, or IL-10 gene survived ts-4 infection for at least 54 days during the observation of this study. However, when \(10^6\) or \(2 \times 10^4\) ts-4 tachyzoites were i.c. injected, all the above-mentioned mice including CD8 KO mice survived more than 54 days during the experimental observation period (data not shown).

3.2. Ts-4 could induce ocular lesion

Histological changes (Fig. 2) and inflammatory scores (Table 1) in the eye tissues of genetically deficient and control mice were compared after primary intracocular infection with \(10^6\) ts-4 avirulent parasite strain tachyzoites at 11 days. After primary ocular ts-4 infection, obvious inflammation was observed in the eye tissue of WT mice (Fig. 2A). In contrast, obvious inflammation and necrosis were observed in the eye tissue of \(\mu\)MT mice (Fig. 2C) \((P = 0.043)\); marked inflammation and necrosis were observed in the eye tissue of CD8 KO mice (Fig. 2G) \((P = 0.028)\); strong inflammation and necrosis were observed in the eye tissue of IL-10 KO mice (Fig. 2I) \((P = 0.019)\); however, only mild histological evidence of ocular inflammation in CD4 KO mice (Fig. 2E) \((P = 0.068)\).

3.3. Increased ocular pathogenesis in immunocompromised mice after challenge

There were complete immune protections against ocular infection in WT mice by i.e. immunization with the ts-4 strain tachyzoites in one eye and followed by ocular challenge with \(10^2\) RH strain tachyzoites in another eye. Compared to WT mice (Fig. 2B), at 11 days post-ocular challenge, obvious inflammation and necrosis were observed in the eye tissue of \(\mu\)MT mice (Fig. 2D); marked inflammation and necrosis were observed in the eye tissue of CD8 KO (Fig. 2H) and IL-10 KO (Fig. 2J) mice; whereas only mild histological evidence of ocular inflammation in CD4 KO mice (Fig. 2F).

3.4. Increased ocular parasite burden in immunocompromised mice after challenge

Parasite proliferation in the eye tissues from mutant and control mice by ts-4 i.e. immunization in the right eye and then challenge with \(10^2\) RH-GFP tachyzoites in the left eye was evaluated by confocal microscopy. As shown in Fig. 3, compared to primary RH infection in controls (Fig. 3A), few parasites were seen in the eye tissues of WT (Fig. 3B) and IL10 KO (Fig. 3F) mice; whereas an increased parasite burden was observed in the eye tissues of CD4 KO (Fig. 3D) and CD8 KO (Fig. 3E) mice, and a marked increased parasite burden was observed in the eye tissues of \(\mu\)MT mice (Fig. 3C) at 11 days post-ocular challenge. The results were in correspondence with the ocular parasite burdens quantified after 72 h culture of eye tissues (Fig. 4), i.e. the numbers of tachyzoites in the eye tissues of experimental mice calculated after incubating with monolayers of human fibroblasts at 37 °C for 72 h. Compared to WT mice, \(\mu\)MT mice showed the greatest parasite burden in their eye tissues \((10^6/ml) (P < 0.01)\); parasite burden from the eye tissues of either CD8 KO or CD4 KO mice was also increased \((P < 0.01)\) after ts-4 vaccination and challenge. However, the parasite burden from IL-10 KO mice was similar to that of the WT mice \((P > 0.05)\).

3.5. Anti-toxoplasma antibody level

Compared to naive control mice, at day 28 after ts-4 i.e. immunization in the right eye, the levels of IgG in eye fluid from the left eye were significantly increased in WT, IL-10 KO, CD8 KO, and CD4 KO mice \((P < 0.05)\); the levels of IgG in serum were also significantly increased in the above-mentioned WT \((P < 0.01)\), IL-10 KO \((P < 0.01)\), CD8 KO \((P < 0.01)\), and CD4 KO \((P < 0.05)\) mice. However, anti-toxoplasma IgG antibody level was not detectable in both the serum and eye fluid of \(\mu\)MT mice postimmunization with ts-4 (Fig. 5).

3.6. Phenotypic analysis

Because markedly increased weight/size of the draining CLNs were observed in infected or challenged mice vs. naive mice (Fig. 6A), the total cell numbers of the draining CLNs from different groups were compared after both infection and challenge. The results showed that, the cell numbers were similar between the WT and IL-10 KO mice; while there were significant lower cell numbers in the CLNs of CD4 KO, CD8 KO, and \(\mu\)MT mice, compared to WT mice (Fig. 6B). The percentages of CD3+, CD4+ and CD8+ T cells, and B cells in CLNs at 11 days post-primary ocular infection with ts-4 or ts-4 immunization and challenge with RH tachyzoites were analyzed by flow cytometry. As shown in Fig. 6C, compared to naive control mice, a significant increase of the percentage of B cells and a significant decrease of the CD4+/CD8+ T cell ratio were observed in both infected and challenged WT or IL-10 KO mice.

4. Discussion

Ts-4 is a temperature-sensitive mutant of the RH T. gondii strain. We used C57BL/6 background mouse models-deficient in CD4+ T cell, CD8+ T cell, B cell, and IL-10 gene to investigate the roles of these cells and gene in the development of pathogenesis and immunity after either primary ocular ts-4 inoculation or ocular ts-4 vaccination and then challenged with RH strain tachyzoites of T. gondii. Our data indicate that ts-4 can induce ocular pathogenesis in both immune complete and immunocompromised mice; immunization of ts-4 by ocular route can protect ocular toxoplasma challenge, CD4+, CD8+ T cells, B cells, and IL-10 gene are all essential in resistance to ocular primary ts-4 infection or ts-4 immunization and followed by virulent RH challenge, in which CD8+ T cells are the most important component.

It has been reported that ts-4 infection is lethal for T cell-deficient nude mice [10]. Mice depleted of both CD4+ and CD8+...
Fig. 3. Parasites with green fluorescence in the eye tissue of mice at 11 days postchallenge with 10^2 RH-GFP tachyzoites of *T. gondii*. Panels: infected (A) and challenged C57BL/6 WT mouse (B), challenged μMT mouse (C), challenged CD4 KO mouse (D), challenged CD8 KO mouse (E), and challenged IL-10 KO mouse (F). There were four mice per group, and data are representative of those from two experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

T cells die from ts-4 infection [11]. Our results demonstrated that mice only deficient CD8^+ T cells died from 1 × 10^5 ts-4 i.p. infection at early infective time. Ts-4 has been reported to be nonpersisting in mice [10]; no viable organisms were presented beyond 14 days after inoculation in adult mice [12] and no *T. gondii* of ts-4 strain was observed in or isolated from the tissues of weaned pigs inoculated subcutaneously or intravenously with 5 × 10^5 ts-4 tachyzoites [13]. In this study, our results demonstrated that ts-4 was persistent in CD8 KO mice; before death, a large amount of tachyzoites were observed in the ascites from the CD8 KO mice after i.p. infection. It has been reported that resistance to primary ts-4 infection requires early production of IFN-γ and followed by later requirement of CD4^+ and CD8^+ T cells [11]. The limiting persistence of ts-4 is related to the immunologic response [10]. Our results indicate that CD8^+ T cells are needed by mice to survive acute ts-4 infection. However, we also observed that CD8 KO mice could survive up to 54 days by ocular infection of 10^2 or 2 × 10^4 ts-4 (data not shown). Prior studies from this group suggest that the route of toxoplasma infection markedly influences the survival of mice [14]. Whether the susceptibility or disease severity is dependent on the route of infection or the quantity of ts-4 parasites infected remains to be determined.
Ts-4 mutant can cause disease in young nursing mice but is avirulent for adult mice [12]. Pigs vaccinated subcutaneous or intravenous with the ts-4 mutant develop no clinical signs of disease and no microscopic lesions in the tissues [13,15]. In this study, we found that there were different degree of pathological changes in the eye tissues of both immune-sufficient WT mice and immunocompromised mice-deficient in B cells, CD8 T cell, and IL-10 gene after primary ocular ts-4 infection. Compared to WT mice, µMT mice had obviousocular lesion, and both IL-10 KO and CD8 KO mice had even severe lesions in their eyes; whereas CD4 KO mice had only mild ocular inflammation. Our data indicate that the ts-4 can cause ocular pathogenesis in both immune intact and immunocompromised mice. Consistent with our previous report [16], IL-10 affects the outcome of murine ocular infection with T. gondii, downregulates immune-mediated inflammation, and prevents immunopathogenesis of acute toxoplasmosis. CD8+ T cells have been demonstrated to be critical to the protective immunity by the ts-4 [17]. Although ts-4 has been widely used to vaccinate animals against virulent T. gondii, our results indicate that the ts-4 can induce ocular pathogenesis.

Mice vaccinated with a live ts-4 T. gondii develop complete resistance to subsequent challenge with a highly virulent Toxoplasma strain (RH), which is dependent on IFN-γ synthesis [5]. Our previous findings demonstrated that toxoplasma-sensitive (C57BL/6) and -less sensitive or -resistant (BALB/c and CBA/J) mice vaccinated i.p. with ts-4 develop complete protection to ocular RH infection [14]. In the present study, we found that ocular immunization with the ts-4 in one eye could protect another eye against virulent RH strain of T. gondii challenge in mice, whereas our results demonstrated that mice-deficient in CD8+ T cells developed severe ocular pathogenesis with increased ocular parasite load after RH challenge. In contrast, mice-deficient in CD4+ T cells had mild inflammation after either the primary ts-4 vaccination or challenge, associated with increased parasite load after challenge. Compared to naive mice, although there were markedly increased weight of the draining CLNs from all the mice after infection or challenge, there were significant lower total cell numbers in the CLNs of either CD8 KO or CD4 KO mice after both infection and challenge. Phenotypic analysis showed that CD4+/CD8+ T cell ratio was decreased in CLNs from WT and IL-10 KO mice after either ocular ts-4 infection or RH challenge. Increased CD8+ T cells in efferent lymph node have also been
observed after inoculation with S48 strain of T. gondii, a live vaccine in sheep [18]. CD8+ T lymphocytes can lyse extracellular T. gondii tachyzoites and T. gondii-infected target cells in both humans and mice in vitro [19,20]. CD8+ T cells play a central role for the resolution of acute toxoplasmosis by participating in endogenous IFN-γ production [21], and vaccinated mice depleted of CD8+ T cells are more susceptible to subsequent challenge [22]. Mice-deficient in CD4+ T cell increases the susceptibility to primary peroral T. gondii infection, impairs the ability to be successfully vaccinated [23], and results in a significant increase in the number of brain cysts in vaccinated mice [24]. Treatment with the combination of anti-CD4 and anti-CD8 monoclonal antibodies results in an increase in cyst numbers in the ocular lesion [25]. CD8+ T cells and IFN-γ are known to be important components of resistance to T. gondii. Our previous findings demonstrate that T cells are the major response cells in the eyes of challenged mice [14]. The results from this study, together with our previously published findings [9,16], i.e. mice vaccinated i.p. with the live ts-4 vaccine develop complete resistance to subsequent ocular challenge with a highly virulent RH T. gondii strain, further demonstrated that the major effector cells in the eyes of challenged mice are CD8+ T cells and this immunity is dependent on IFN-γ synthesis. It has been reported that neutralization of either IFN-γ or CD4+ T cells during peroral infection prevents severe necrosis of the ilea and acute mortality [26]. Our results demonstrate that both CD8+ and CD4+ T cells are essential in complete protective immunity against ocular T. gondii challenge and involve in limiting toxoplasma proliferation. Although our results indicate that the ts-4 vaccination by ocular route can induce complete protection, the safety of the route to give the live vaccine should be considered.

In this study, the highest parasite burden associated with severe ocular lesions was observed in the eye tissue of μMT mice after ocular ts-4 vaccination and RH challenge. We also observed that the percentages of B cells in CLNs were increased after both ocular ts-4 infection and RH challenge, and the IgG levels in both eye fluid and serum were increased after ts-4 i.e. immunization. In rabbits with primary OT, specific IgG reaches detectable levels in the inoculated eyes between 5 and 15 days after inoculation and persists both locally and in the serum [27]. Aside from being the precursors of the Ab-secreting cells, B cells are engaged in other immune functions such as Ag presentation to T cells or cytokine production. It has been reported that B cells are required for resistance to T. gondii in mice vaccinated with ts-4 and later challenged with highly virulent tachyzoites; the antibodies produced by B cells block the infection of host cells by tachyzoites [28]. Toxoplasma-specific IgG antibodies play a critical role in prevention of proliferation of tachyzoites in brains and lungs in both primary infection and reactivation of latent infection [29], and specific IgM play an important role in limiting systemic dissemination of tachyzoites during early acute T. gondii infection [30]. B cells, once stimulated, produce a wide range of polarizing cytokines; IFN-γ-producing B cells have been identified in mice infected with T. gondii [31]. Using the Th1 response induced by the infection of T. gondii, Menard et al. [32] demonstrated that B cells can enhance CD4+ and CD8+ T cell responses via a TNF-α-mediated mechanism. Our results indicate that B cells are necessary for vaccine-mediated protection against ocular challenge with virulent RH T. gondii, and the potential role for their antibody production is in limiting for parasite burden.

In conclusion, the live ts-4 vaccine can be used for the prevention of OT in animals, when given by an appropriate manner to enable the induction of protective immune responses. The results from this study have extended our understanding of the characterization of ts-4 and further confirm some of the previous findings about ts-4 by us and others.

Acknowledgments

We thank Kenneth Orndorff and Alice Givan for excellent technical assistance with confocal scanning laser microscopy and flow cytometry. We are grateful to Michael E. Grigg and John C. Boythood for providing RH–GF parasites.

This work was supported by grant from the NIH to L.H.K. (AI19613), grant from the Guangdong Provincial Natural Science Foundation of China to F.L. (06021302), and grant from the Returned Overseas Chinese Scholars, State Education Ministry, China to F.L. ([2006]331).

References


