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Sex-Determined Resistance to *Toxoplasma gondii* Is Associated with Temporal Differences in Cytokine Production

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Examination of a wide range of inbred mice of diverse genetic backgrounds and major histocompatibility complex haplotypes revealed a dramatic difference in the susceptibilities of males and females to *Toxoplasma gondii* infection. Female mice were found to be more susceptible to acute infection, as determined by higher mortality levels, than male mice, while those female mice surviving to have chronic infections harbored more cysts in their brains than did surviving males. This phenomenon was therefore investigated in greater depth immunologically in the BALB/K mouse, a strain showing moderate susceptibility to infection with *T. gondii*. Plasma tumor necrosis factor alpha (TNF-α) levels were elevated in both male and female BALB/K mice on days 8 and 10 postinfection, but not thereafter, with males producing significantly higher levels than females. However, it was not until day 12 postinfection that the first deaths occurred, and these were among female mice, indicating that TNF-α production was not responsible for mortality. In vitro examination of *T. gondii*-specific T-cell proliferative responses from day 15 postinfection onwards revealed significantly higher stimulation indices in male mice than in their female counterparts. This difference was most apparent in splenocyte cultures initiated at day 15 postinfection, where complete suppression of proliferation was noted in the splenocytes from female mice but not from male mice. Analysis of tissue culture supernatants from these cultures revealed distinct differences in the kinetics of production as well as the quantities of gamma interferon (IFN-γ) and interleukin 10 (IL-10) produced. Spleen cells from male mice produced higher levels of IFN-γ in the early stages of infection than those from female mice. IFN-γ levels were highest in the supernatants from male splenocyte cultures initiated at day 15 postinfection. Similar levels of IFN-γ were not obtained from the supernatants of female splenocyte cultures until day 22 postinfection. IL-10 production, on the other hand, peaked at maximal levels in the cell cultures from both sexes initiated at day 22 postinfection. These results suggest that, in male mice, a rapid response to infection with high levels of TNF-α and IFN-γ helps to control parasite multiplication, after which IL-10 production may be important in down regulating these potentially harmful inflammatory mediators. The failure of female mice to respond quickly in terms of T-cell proliferation and IFN-γ production compared with their male counterparts may account for their poor survival rates and higher cyst burdens.

Sexual dimorphism in susceptibility to many human diseases, including those caused by parasitic protozoans, has been observed (2). Although social or epidemiological mechanisms may account for some of these gender-associated differences (8), hormone-influenced immunological mechanisms, many of which can be recreated in laboratory models of disease, would appear to be the paramount determining feature (2). In general, females tend to exhibit better humoral immunity than males, who exhibit greater levels of cellular immunity (6).

While to our knowledge there are no reports of sex-influenced susceptibility to *Toxoplasma gondii* in humans, numerous observations with laboratory animals have indicated that females are more susceptible than males to this parasite. Thus, female mice have been shown to develop more intense inflammatory lesions in their brains than male mice following *T. gondii* infection (31), and estrogen administration has been demonstrated to increase mortality in both male and female gonadectomized mice (30) and guinea pigs (29). However, despite a dramatic increase in our understanding of the protective immune response and immunopathological processes involved in toxoplasmosis, the immunological mechanisms responsible for the observed sexual dimorphism in response to this disease have not been extensively studied. It has, for example, recently been established that early resistance to *T. gondii* infection is dependent on the innate immune response associated with the interaction of macrophages and NK cells (17, 25). T cells, however, particularly $CD8^+$ T cells (7, 39, 47) but also, perhaps, the Th1 $CD4^+$ T-cell subset (18) working together in synergy to produce gamma interferon (IFN-γ), constitute the adaptive immune response leading to survival and long-term protection. Conversely, it has been suggested that Th2 cells and their products, such as interleukin 4 (IL-4) (26) and IL-10 (18), may be involved in disease exacerbation. Significantly, not only have estrogen receptors been detected in mature $CD8^+$ T cells (11) but estrogen has been shown to modify the activity of NK cells (16, 45), increase the ability of macrophages to phagocytose, and influence IFN-γ production (22). It is hardly surprising, therefore, that gender-associated differences in susceptibility to *T. gondii* have been identified.

During the present study, we have confirmed with a number of mouse strains the earlier observations that female mice are more susceptible to *T. gondii* infection than males. In addition, by comparing the evolution of infection in BALB/K mice both in vivo and in vitro, we have identified clear differences in the kinetics and magnitude of T-cell responses and the production of tumor necrosis factor alpha (TNF-α), IFN-γ, and IL-10 between the sexes.

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MATERIALS AND METHODS

Mice. Male and female BALB/K, C57BL/10ScSn (B10), and B6×129 mice, bred and maintained at the University of Strathclyde under conventional conditions, were used when they were 8 to 12 weeks of age. The mice were age matched, and groups of at least 4, but often as many as 18, were used in each experiment as stated.

***T. gondii* and infections.** The avirulent RRA (Beverley) strain of *T. gondii* maintained in this laboratory by continual passage of infective brain homogenate in outbred Strathclyde Albino mice was used for all infections. After calculation of cyst concentrations in brain homogenates of mice infected 17 to 21 weeks previously, experimental infections consisting of 20 cysts in phosphate-buffered saline (PBS) were administered orally by gavage or intraperitoneal injection as stated. The virulent RH strain of *T. gondii* grown in the peritonea of cotton rats was used for preparation of soluble tachyzoite antigen (STAg) as previously described (41).

Monitoring infections. The mice were monitored for mortality daily, and in some experiments mice were weighed at regular intervals. At the end of each experiment, a group of mice were killed and their brain cyst burdens were assessed by microscopic examination of a 30-μl sample from a 1-ml brain homogenate from each mouse. The total brain cyst burden was then calculated for each mouse (39).

T-cell proliferation assays and quantification of IL-10 and IFN-γ production. T-cell proliferations were carried out as previously described (42). Groups of four male and four female BALB/K mice, uninfected or infected with *T. gondii* 15, 22, and 29 days previously, were killed, and their spleens were removed aseptically and placed in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.05 mM β-mercaptoethanol (Gibco, Paisley, United Kingdom). The spleens were gently teased apart, and the resulting cell suspension was centrifuged for 5 min at 200 × *g*. The supernatant was decanted, and the erythrocytes were lysed by resuspension of the pellet in 3 ml of Boyle's solution (0.17 M Tris, 0.6 M ammonium chloride; BDH Ltd., Dorset, United Kingdom) and incubation for 3 min at 37°C. After centrifugation and two washes in RPMI 1640, the cells were resuspended in RPMI 1640 and viable cells excluding trypan blue were counted in a hemocytometer. Cell suspensions were adjusted to 5×10^6 cells per ml, and aliquots of 100 μl containing 5×10^5 cells were added to the wells of a 96-well flat-bottomed tissue culture plate (Costar, Cambridge, Mass.) which contained 100 μl of STAg per well at concentrations of 200, 100, 50, and 25 μg/ml or RPMI 1640 alone in triplicate. The cultures were incubated for 60 h at 37°C in 5% CO_2, after which 100 μl of supernatant was carefully removed from each well and stored at −70°C for measurement of IL-10 and IFN-γ levels. The wells were topped up with 100 μl of RPMI 1640 containing the appropriate concentration of STAg and 0.25 μCi of tritiated thymidine (specific activity, 35 Ci/mmol; ICN/Flow, High Wycombe, United Kingdom). Following a further 12-h incubation at 37°C, the cells were harvested onto filter paper (ICN/Flow) with a cell harvester (Skatronas, Lier, Norway). Thymidine incorporation was measured by liquid scintillation on a β-counter (Pharmacia LKB Biotech, Milton Keynes, United Kingdom), using 1 ml of Optiscint (Pharmacia Biosystems) added to the filter discs in vials (Hughes and Hughes, Somerset, United Kingdom), and counted for 3 min each.

IL-10 and IFN-γ assays. IL-10 and IFN-γ levels were measured in the culture supernatants of nonstimulated cells and in those stimulated with 100 μg of STAg per ml by capture enzyme-linked immunosorbent assay (ELISA). The culture supernatants were assayed in duplicate undiluted and at 1/16 and 1/40 dilutions for IFN-γ and undiluted for IL-10. Microtiter plates were coated overnight at 4°C with capture antibody (rat anti-mouse IL-10, clone JES5-2A5, or rat anti-mouse IFN-γ, clone R4-6A2; Cambridge Bioscience, Cambridge, United Kingdom) in PBS, pH 9.0. After three washes in PBS, pH 7.0, containing 0.05% Tween 20 (Sigma, Poole, United Kingdom), the plates were blocked with PBS, pH 7.0, containing 10% fetal calf serum for 1 h. Samples were applied in duplicate at the dilutions noted above along with standards consisting of murine recombinant IL-10 (0 to 10 pg/ml) or IFN-γ (0 to 7,000 pg/ml) (Cambridge Bioscience) and incubated at 37°C for 2 h before a further three washes. Biotinylated detecting antibody (rat anti-mouse IL-10, clone SXC-1, or rat anti-mouse IFN-γ, clone XMG1.2; Cambridge Bioscience) was added in PBS, pH 7.0, containing 10% fetal calf serum, and the plates were incubated for 45 min before a further three washes. Streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Ala.) was then added to each well (0.5 μg/ml) for 30 min before a final 3 washes and development in substrate consisting of *p*-nitrophenylphosphate in glycine buffer. After a 2-h incubation, absorbances were measured at 405 nm on a Titertek Multiscan plate reader. Cytokine concentrations were determined from a standard curve (the regression coefficient was always greater than $r = 0.98$).

Measurement of plasma TNF-α levels. Plasma samples were obtained from groups of five male and five female BALB/K mice before infection and at 2-day intervals postinfection by collecting blood via the tail vein into heparinized capillary tubes and separating the plasma portion by centrifugation. TNF-α levels in plasma, diluted 1/10 in PBS, were measured by capture ELISA as described above. The capture antibody used was rat anti-mouse TNF-α, clone MP6-XT22, and the biotinylated detecting antibody was rat anti-mouse TNF-α, clone MP6-XT3 (Cambridge Bioscience). The standards consisted of recombinant TNF-α (0 to 20 ng/ml) (Genzyme, Cambridge, United Kingdom). TNF-α concentrations were determined from a standard curve (the regression coefficient was always greater than $r = 0.98$).

Statistics. Statistical analyses were performed with the Mann-Whitney U test.

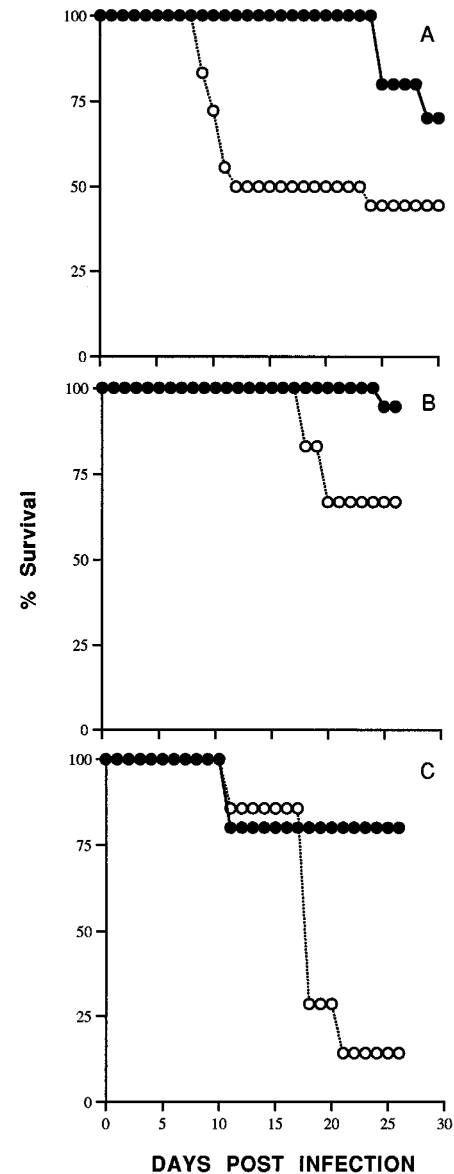

FIG. 1. The percents survival of male (●) and female (○) B6×129 and B10 mice infected with 20 *T. gondii* cysts (RRA strain). B6×129 mice were infected orally (A) (male, $n = 10$; female, $n = 18$), and B10 mice were infected either intraperitoneally (B) (male, $n = 17$; female, $n = 6$) or orally (C) (male, $n = 5$; female, $n = 7$).

RESULTS

Comparison of male and female susceptibilities to *T. gondii* infection in BALB/K, C57BL/10ScSn (B10) and B6×129 mice. Female mice of all the strains examined were more susceptible, as determined by higher mortality levels, than their male counterparts following infection with *T. gondii* (Fig. 1). Among B6×129 female mice infected orally, the mortality level was as high as 76.9%, compared with only 16.7% among males of the same strain ($P = 0.029$). Similarly, female B10 mice also had a greater level of mortality than male B10 mice whether they

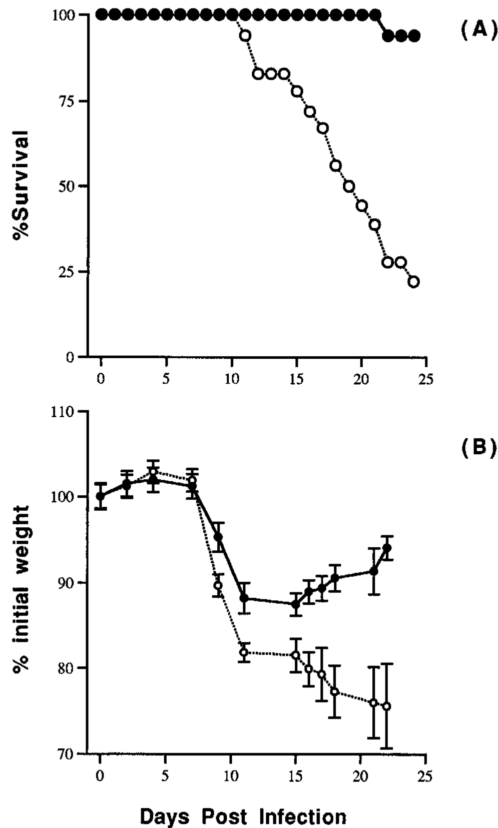

FIG. 2. The percents survival (A) and weight change (B) of male (●) and female (○) BALB/K mice infected orally with 20 *T. gondii* cysts (RRA strain). Groups of 17 male and 18 female mice were monitored in this experiment.

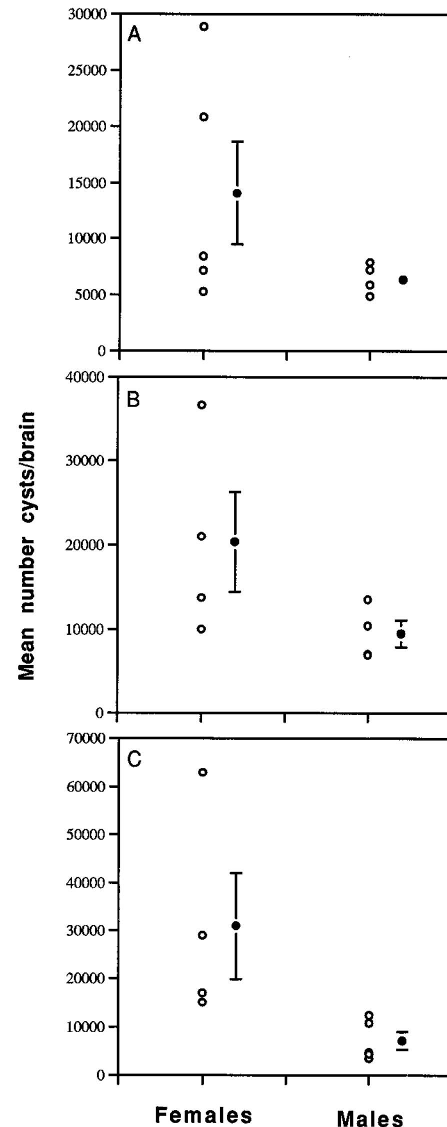

FIG. 3. Comparison of brain cyst burdens in male and female B6×129, B10, and BALB/K mice infected with 20 *T. gondii* cysts (RRA strain). B6×129 (A) and BALB/K (C) mice were infected orally, whereas B10 mice were infected intraperitoneally (B). Open circles represent values for individual mice, and closed circles (and error bars) represent mean cyst counts (± standard errors [SE]) of the groups. The brains of at least four, but when possible five, mice were examined for each group. Female B10 mice infected by the oral route did not survive acute infection.

were infected orally (females, 85.7%; males, 20% [$P = 0.023$]) or intraperitoneally (females, 33.3%; males, 5.8% [$P = 0.121$]).

For BALB/K female mice infected by the oral route, 77.8% mortality was recorded during the period of study. In contrast, only 5.9% ($P = 0.0001$) of their male counterparts died during the study (Fig. 2A). Following infection, both male and female BALB/K mice gained a small amount of weight until day 8 postinfection, after which a decrease in weight was observed for both sexes although this was significantly greater in the females by day 10 ($P = 0.0013$). Male mice continued to lose weight until day 16 postinfection, when an overall decrease of 12.5% was recorded. After day 16, male mice began to regain weight. Female mice continued to lose weight throughout the study, eventually losing as much as 25% of their original weights (Fig. 2B).

At necroscopy, female B6×129 mice infected by the oral route were found to have more cysts in their brains than male mice of the same strain (male, 6,333 ± 533; female, 14,053 ± 4,600 [$P < 0.1$]) (Fig. 3A). This pattern was also observed with BALB/K mice (male, 7,147 ± 1,846; female, 30,967 ± 11,087 [$P < 0.025$]) (Fig. 3C). This observation was independent of the route of infection, as female B10 mice infected by the intraperitoneal route were found to have more brain cysts than their male counterparts infected by the same route (male, 9,500 ± 1,550; female, 20,350 ± 5,910 [$P < 0.1$]) (Fig. 3B).

Plasma TNF-α levels in male and female BALB/K mice. Preinfection plasma samples and plasma samples taken from male and female mice at 2-day intervals postinfection were assayed for TNF-α (Fig. 4). No TNF-α was detected in preinfection samples or in those taken on days 2, 4, and 6 from either male or female mice. TNF-α was, however, detected in samples from both sexes taken on days 8 and 10 postinfection. At one of these time points male mice had significantly more TNF-α in their plasma than the more-susceptible female mice (day 8: male, 38 ± 17.5 ng/ml; female, 14 ± 5.8 ng/ml [$P < 0.375$] and day 10: male, 56 ± 16.2 ng/ml; female, 11 ± 5.1 ng/ml [$P < 0.025$]). TNF-α had declined to nondetectable levels by day 12 postinfection in both male and female mice.

Comparison of T-cell proliferative responses in male and female BALB/K mice infected orally with *T. gondii*. The ability of spleen cells from male and female BALB/K mice to proliferate in the presence of STAg was examined in noninfected mice and at days 15, 22, and 29 postinfection (Fig. 5). There

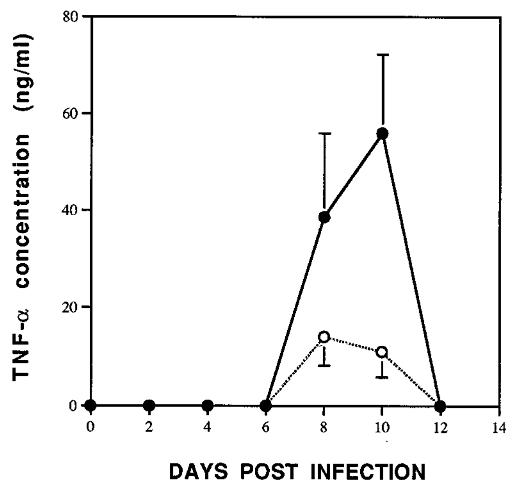

FIG. 4. Comparison of TNF-α levels in the plasma of male (●) and female (○) BALB/K mice following oral infection with 20 *T. gondii* cysts (RRA strain). Results from five male and five female mice are mean cytokine concentrations per milliliter ± SE.

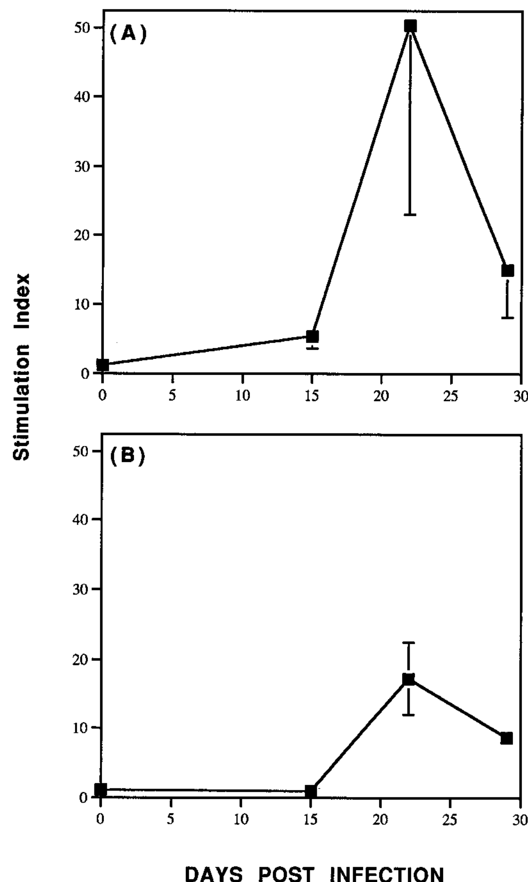

FIG. 5. Comparison of STAg-specific proliferation of spleen cells cultured from male (A) and female (B) BALB/K mice at various times postinfection. The mice were infected with 20 *T. gondii* cysts (RRA strain) by the oral route. Results from groups of four male and four female mice are mean SI ± SE.

were no significant differences in the stimulation indices (SI) of lymphocytes obtained from noninfected male and female mice (day 0). Fifteen days after infection, a significant difference in lymphocyte proliferation between male and female mice (male SI, 5.42 ± 1.76; female SI, 0.95 ± 0.26 [$P < 0.025$]) was observed. By day 22 postinfection, the male SI had increased to 50.35 ± 27.23 and the female SI had increased to 17.24 ± 5.22 before falling to 14.95 ± 6.78 and 8.66 ± 0.82, respectively, by day 29 postinfection, at which time there was no significant difference in response.

Comparison of IL-10 and IFN-γ production by STAg-stimulated spleen cells from male and female BALB/K mice infected orally with *T. gondii*. The culture supernatants from male and female spleen cells used in the proliferation assays described above were assayed for IFN-γ and IL-10 (Fig. 6). Unstimulated spleen cells produced negligible quantities of IFN-γ and IL-10 at all time points examined (results not shown). Cell culture supernatants from infected male BALB/K mice were found to contain maximum levels of IFN-γ 15 days postinfection (156.71 ± 17.01 ng/ml), which was significantly more than that detected in the culture supernatants from female mice at this time (82.36 ± 20.77 ng/ml [$P < 0.025$]). By days 22 and 29 postinfection IFN-γ levels had declined in male splenocyte cultures (106.95 ± 22.29 ng/ml and 57.14 ± 19.44 ng/ml, respectively). IL-10 levels peaked later on day 22 postinfection (6.03 ± 0.59 ng/ml), before falling to 2.31 ± 0.53 ng/ml on day 29 postinfection in these mice. In contrast, the greatest levels of IFN-γ (152.99 ± 26.04 ng/ml) were detected in the culture supernatants from female mice on day 22 postinfection, which also coincided with peak levels of IL-10 in these cultures (5.60 ± 0.41 ng/ml).

DISCUSSION

Previous studies have demonstrated that *T. gondii* infections induce more intense inflammatory lesions in the brains of female mice than in male mice (31). In the present study, we have confirmed the previous authors' observations and demonstrated that female mice from a wide range of strains also exhibited greater levels of mortality and morbidity and developed more cysts in their brains than their male counterparts. As males have been shown to have enhanced NK cell activity compared with females (45) and generally higher levels of cell-mediated immunity (2), these results were not entirely surprising. Furthermore, detailed examination of immunological functions and responses in male and female BALB/K mice at various stages of disease progression revealed profound differences in the magnitude and kinetics of T-cell responses and timing of cytokine production.

Although work from this laboratory as well as that of others has demonstrated a protective role for TNF-α during toxoplasmosis (5, 27), there also exists some evidence that overproduction of TNF-α may be detrimental by increasing mortality (4). In this study, however, we detected significantly more TNF-α in the plasma of the relatively more-resistant male mice than in the plasma of the susceptible female mice, supporting a host protective role for TNF-α. As with a previous study (5), significant TNF-α production preceded the earliest deaths and no TNF-α could be detected during that period of acute infection when fatalities were occurring in both sexes but primarily in females. Nevertheless, immunological events occurring at early time points could ultimately influence mortality during the later stages of infection. TNF-α production is probably an indicator of the recently described innate immune response involving the interaction of NK cells and macrophages (17, 25). The general consensus of opinion suggests that IL-12 production from macrophages stimulates NK cells to produce IFN-γ, which in turn can activate macrophages to kill *T. gondii* by a

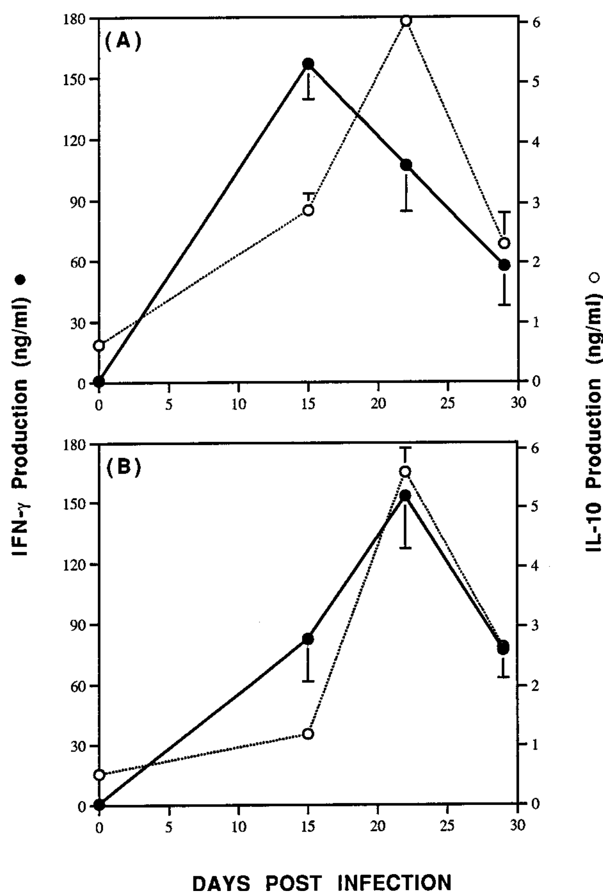

FIG. 6. Comparison of IFN-γ and IL-10 levels in the supernatants of male (A) and female (B) splenocyte cultures as described in the legend to Fig. 5. Splenocyte culture supernatants from groups of four mice at each time point were examined, and results are mean cytokine concentrations per milliliter ± SE.

combination of oxygen-dependent and -independent mechanisms (24, 33, 40).

The level of mortality observed primarily among female mice coincided around day 15 with a period when their T cells failed to proliferate in response to STAg (indeed, SIs from male splenocyte cultures were higher than those for female mice for all time points examined). At day 15 postinfection, lymphocytes from male mice not only demonstrated significant Ag-specific proliferation but produced maximum levels of the host protective cytokine IFN-γ. IFN-γ has been demonstrated to be necessary for the survival of mice during the acute stages of infection (36, 48), and it is thought to exert its effects through activating macrophages to kill parasites through oxygen-dependent (24) and L-arginine-dependent (33) mechanisms or tryptophan starvation (40). The observed delay compared with male mice in the ability of spleen cells from female mice to proliferate and produce IFN-γ in response to STAg may be a further significant factor, contributing to the greater disease susceptibility of female mice.

Interestingly, the maximum levels of IFN-γ in the female splenocyte cultures were not obtained until day 22 postinfection (corresponding to those levels of IFN-γ obtained from male splenocyte cultures on day 15), at which time IL-10 reached maximum but equivalent levels in both the male and female splenocyte cultures. IL-10 has been shown to be capable of down regulating the production and effects of IFN-γ (15), such as the induction of nitric oxide (19) and TNF-α (21, 35). Several publications have recently demonstrated the importance of nitric oxide in the control of toxoplasmosis (19, 33), an effect that can be ablated by IL-10 (19). Therefore, the concomitant maximum production of IFN-γ and IL-10 from STAg-stimulated splenocytes from female mice may be another factor contributing to their disease susceptibility.

IL-10 production may not be wholly detrimental, as this cytokine may have an important role to play in regulating the inflammatory response in toxoplasmosis. The anti-inflammatory effects of IL-10 are now well documented, and its presence during resolution of disease has been demonstrated in many disease models, including experimental autoimmune encephalomyelitis (28) and toxoplasmic encephalitis (9). Under these circumstances, IL-10 appears to be beneficial to the host and serves to control the production of potentially dangerous inflammatory mediators. There is evidence that cytokines such as IFN-γ which are usually associated with host protection (36) may also be detrimental to the infected host. Indeed, McLeod et al. have observed that spleen cell cultures from strains of mice genetically susceptible to acute mortality produced significantly more IFN-γ when stimulated with *T. gondii* Ag in vitro than cells derived from genetically resistant strains (37). Similarly, under certain circumstances IFN-γ has been demonstrated to induce mortality in mice infected with *Plasmodium berghei* (50) or *Plasmodium vinckei* (32), and as a consequence, it has been postulated that at least some of the pathology observed in human and murine models of malaria infection can be attributed to the overproduction of Th1-derived cytokines such as IFN-γ, TNF-β, and IL-2 (49). Both IFN-γ and IL-2 can induce TNF-α production by macrophages (14). The IFN-γ produced by these cells may act in synergy with TNF-α and/or TNF-β to activate macrophages to produce reactive oxygen intermediates (24) and reactive nitrogen intermediates (46), both of which have been shown to be effective in the control of *T. gondii* infection (24, 33, 46) and many other intracellular parasites (23, 38, 43). However, these potentially protective molecules may be toxic to the host if produced in large quantities (10). It is also significant that those mice expressing the highest levels of Vβ5 are those most responsive to *T. gondii* superantigen-driven IFN-γ production and those most susceptible as measured by mortality (12). Experimental evidence shows that recombinant IL-10 can prevent experimental autoimmune encephalomyelitis in rats (44) and reduce the release of TNF-α and IFN-γ, preventing lethality in experimental endotoxemia (21, 35). It would, therefore, appear likely that the timing of IL-10 versus IFN-γ production is of paramount importance in the control of disease. As maximum IL-10 production from male splenocyte cultures stimulated with STAg occurs at a later postinfection time than maximum IFN-γ production, it may serve to control overproduction of potentially dangerous inflammation following disease control. As maximal IFN-γ production, but not IL-10 production, in equivalent female splenocyte cultures develops more slowly, this may allow increased parasite multiplication and ultimately increased pathology compared with the case for males.

The results of the present study imply that sex hormones may be an important factor in deciding the outcome of infection with *T. gondii*, and previous studies with guinea pigs and mice have demonstrated that altering estrogen levels by administering synthetic hormone can profoundly influence disease (29, 30). This has implications for human disease; congenital toxoplasmosis is frequently mild to asymptomatic, and as a consequence it is often undiagnosed in neonates. However, in many of these cases disease becomes apparent later in life, frequently during adolescence when dramatic hormonal

changes are taking place. This would indicate that changes in hormone levels during adolescence could be a major contributing factor to the reactivation of previously asymptomatic congenitally acquired disease.

It has also recently been shown that pregnancy is associated with localized Th2 cell activity at the maternal-fetal interface, allowing the successful alloengraftment of the trophoblast (34). This IL-4-, IL-5-, and IL-10-dependent local down regulation of cell-mediated immunity as a result of hormone-influenced Th2 expansion also has implications with regard to the incidence and severity of congenital toxoplasmosis. For example, in mice, the transmission of *T. gondii* vertically from generation to generation (3) (although this is undoubtedly mouse strain dependent [41]) may be a result of hormone-influenced local tissue cyst reactivation. Significantly, there have also been a few reported cases of apparently immunocompetent chronically infected humans giving birth to congenitally infected children, in the absence of reinfection (13). Conversely, an acute *T. gondii* infection during pregnancy may disrupt the delicate hormone-influenced maternal-fetal immunological balance in favor of antiparasitic proinflammatory abortogenic cytokines such as IFN-γ and TNF-α.

The endocrinological influences on immunity, as demonstrated in the present study, therefore have a profound effect on the course and severity of murine *T. gondii* infection. Whether hormonal influences can also have an effect on the severity of human *T. gondii* infection remains, at present, unstudied.

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