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γδ T cells contribute to immunity against the liver stages of malaria in αβ T-cell-deficient mice

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ABSTRACT **The functional role of γδ T cells (expressing the γδ heterodimeric T-cell receptor for antigen) in infectious diseases remains largely unknown. We have therefore attempted to define the possible role of these T cells in the immune response against the various developmental stages of malaria parasites. For this purpose, we monitored the immune response and the development of liver and blood stages of *Plasmodium yoelii*, a rodent malaria parasite, in immunized and nonimmunized αβ T-cell-deficient and γδ T-cell-deficient mice. Immunization of αβ T-cell-deficient mice with irradiated sporozoites induced an immune response that significantly inhibited the development of the parasite's liver stages. This inhibitory immune response was abolished by an antibody-mediated transient *in vivo* depletion of γδ T cells. Two γδ T-cell clones were derived from malaria-immunized αβ T-cell-deficient mice. The adoptive transfer of one of these γδ T-cell clones to normal mice inhibited the development of liver stages, following sporozoite inoculation. These results provide evidence for γδ T-cell-mediated protective immunity against parasites, in the absence of αβ T cells. As for the blood phase of the infection, both normal mice and γδ T-cell-deficient mice cleared the blood stages of the nonlethal strain of *P. yoelii*, while αβ T-cell-deficient mice failed to control the parasitemia.**

Lymphocytes expressing the γδ T-cell antigen receptor (TCR) are a relatively recently defined T-cell subset, and their functional role, as part of the immune system, remains largely unknown. The mechanisms of antigen recognition of γδ T cells as well as, in many instances, their antigen specificity have yet to be defined. Some experimental evidence suggests that these cells, which often exhibit reactivity with mycobacterial antigen, may participate and have a role in controlling microbial infections (1).

Recent reports indicate that human γδ T cells, bearing the $V_\gamma 9V_\delta 2$ TCR, expand preferentially after incubation with extracts of blood stages of *Plasmodium falciparum* (2, 3). It has also been shown that γδ T cells are expanded, *in vivo*, during the experimental infection of mice with *Leishmania major* and that the depletion of γδ T cells results in the development of larger cutaneous lesions (4). However, the antiparasite activity of these γδ T cells has not been further elucidated.

A series of earlier studies have provided clear evidence that $CD4^+$ and $CD8^+$ αβ T cells play a major role in the protective immune response against liver and erythrocytic stages of the rodent malaria parasite *Plasmodium yoelii* (5–7). In the present paper, we investigate the immunity against liver stages and blood stages of malaria in mutant mice lacking αβ or γδ T cells. These mutant mice were generated by introducing germ-line mutations in either the TCR α-chain or TCR β-chain genes (αβ T-cell-deficient mice) (8) or the TCR δ-chain gene (γδ T-cell-deficient mice) (9).

MATERIALS AND METHODS

Mutant αβ T-Cell-Deficient and γδ T-Cell-Deficient Mice. The mice used in these studies were 6–10 weeks old. (129/Sv × C57BL/6)F_1 mice were purchased from The Jackson Laboratories. Mutant mice used were described previously (8, 9). The background is (129/Sv × C57BL/6) for TCR α mutant (α −/−) mice and (129/Ola × C57BL/6) for TCR β mutant (β −/−) and TCR δ mutant (δ −/−) mice. All of these mice are of the $H\text{-}2^{b/b}$ haplotype. They were housed in autoclaved cages and given autoclaved food and water.

Parasites. *P. yoelii* (17X NL strain) was maintained as described (10). Parasites were injected into the tail vein and parasitemia was determined by microscopic examination of Giemsa-stained thin blood smears, obtained daily, from the 3rd to the 32nd day after inoculation.

Immunization and Challenge. Mice were subjected daily to the bites of γ-irradiated (1.5×10^4 rads; 1 rad = 0.01 Gy), malaria-infected *Anopheles stephensi* mosquitoes (IM), for a period of 2 weeks. Mice exposed to the bites of irradiated noninfected mosquitoes (NM) were used as control. Five days after the last exposure to mosquitoes, the mice were challenged intravenously with 3×10^5 viable sporozoites.

Indirect Immunofluorescence Assay. Sera of the immunized mice were obtained just prior to their challenge with sporozoites, and their antibody titers measured by using sporozoites of *P. yoelii* in an indirect immunofluorescence assay (11). Briefly, *P. yoelii* sporozoites were placed on multispot glass slides, fixed with 2% glutaraldehyde, and stored at −20°C. After 1 hr of incubation with the sera, diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, the slides were washed with PBS and incubated for 1 hr with fluorescein isothiocyanate-labeled, affinity-purified, goat anti-mouse immunoglobulin (Kirkegaard & Perry Laboratories). The slides were then washed and mounted in PBS containing 50% (vol/vol) glycerol and 1% (wt/vol) *p*-phenylenediamine to reduce bleaching.

Quantification of *P. yoelii* rRNA in the Liver of Infected Mice. The amount of parasite rRNA present in the liver of individual mice was measured as described (12). Briefly, total RNA was isolated from the livers of mice sacrificed 42 hr after they had been injected intravenously with 3×10^5 sporozoites of *P. yoelii*. RNA was prepared by the method of Chomczynski and Sacchi (13). One-tenth of the whole liver RNA was precipitated with 2-propanol. The RNA pellet was dissolved in 10 mM Tris·HCl, pH 8.0/1 mM EDTA and denatured at 65°C in 20× SSC (1× SSC = 150 mM NaCl/15

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Abbreviations: TCR, T-cell antigen receptor; mAb, monoclonal antibody; MHC, major histocompatibility complex; PRBC, parasitized red blood cell; HSP, heat shock protein.

mM sodium citrate, pH 7.0) plus 37% (vol/vol) formaldehyde, 1:1 (vol/vol). This preparation was diluted 1:45 and 0.2-ml samples were blotted onto nylon membranes. The RNA was fixed to the filters by UV crosslinking. Hybridization was performed overnight at 42°C in 5× SSPE (1× SSPE = 180 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.7)/1% SDS with heparin at 500 μg/ml and containing three ^{32}P-labeled oligonucleotide probes (10^6 cpm/ml; specific activity, $>2 \times 10^8$ cpm/μg). The probes have been described (12). For comparison purpose, a standard curve was prepared by using serial dilutions of purified RNA from a known number of *P. yoelii*-infected erythrocytes, blotted under the same conditions.

***In Vivo* Depletion of γ/δ T Cells.** Culture supernatants of the hybridoma producing hamster IgG monoclonal antibody (mAb) against pan-TCR δ-chain (GL3) (14) were collected and affinity-purified (Affi-Gel protein A MAPS II kit; Bio-Rad). Sporozoite-immunized $\alpha\beta$ T-cell-deficient mice (α $-/-$) were injected intraperitoneally, on two consecutive days, with 1 mg of purified GL3 mAb. As controls, one group of mice was injected with an equivalent amount of normal hamster immunoglobulin (Accurate Chemicals), while another group did not receive any treatment. The efficacy of the γ/δ T-cell depletion of the mice was estimated by two-color flow cytometry analysis of peripheral blood lymphocytes and drained lymph node cells, using anti-TCR δ mAb conjugated with phycoerythrin and anti-CD3-ε mAb conjugated with fluorescein isothiocyanate (15). Five days after the treatment, the mice were challenged with 3×10^5 sporozoites, and the amount of parasite rRNA in the liver was measured 42 hr later.

Generation of Murine γ/δ T-Cell Clones. $\gamma\delta$ T-cell clones 291-H4 and 219-1 were derived from the spleen cells of two individual sporozoite-immunized $\alpha\beta$ T-cell-deficient mice (α $-/-$). The splenocytes were stimulated once with irradiated (3×10^3 rads) major histocompatibility complex (MHC)-matched (*H-2^b*) normal spleen cells that had been exposed to an extract of *P. yoelii* parasitized red blood cells (PRBCs), as a potential abundant source of parasite antigen. The cloning of T cells was performed by the limiting dilution technique (16), using as antigen-presenting cells irradiated MHC-matched spleen cells that had been exposed to PRBC extract. Once established, the clones were restimulated biweekly with irradiated normal spleen cells that had been exposed to an extract of *P. yoelii* PRBCs. The culture medium was RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% supernatant from EL-4 T lymphoma cells as a source of interleukin 2 and interleukin 4.

Adoptive Transfer of γ/δ T-Cell Clones. γ/δ T-cell clones were harvested 10–14 days after restimulation. Ten million cells of the respective T-cell clones or normal spleen cells from α $-/-$ mice were centrifuged and resuspended in RPMI 1640 medium containing recombinant human interleukin 2 (kindly provided by Hoffmann–La Roche) at 2×10^3 units/ml, immediately before transfer. A total of 0.5 ml of the cell suspension was injected intravenously into each mouse. All mice were challenged with 3×10^5 sporozoites of *P. yoelii*, 4 hr after the cell transfer.

Proliferative Response of the Cloned γ/δ T Cells. Two weeks after stimulation, 3×10^3 cloned T cells were incubated with 10^6 irradiated spleen cells of (129/Sv × C57BL/6)F_1 mice in the presence of various concentrations of purified recombinant 65-kDa heat shock protein (HSP65) from *Mycobacterium bovis*. (This recombinant mycobacterial HSP65 was kindly provided to us by Jan D. A. van Embden, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.) The proliferative response was assessed three days later by measuring the incorporation of [^{3}H]thymidine.

Effect of Anti-TCR-δ mAb on the Proliferative Response of Clone 291-H4. Cells from clone 291-H4 were preincubated for 30 min with various concentrations of Fab fragments of either anti-TCR δ mAb or normal hamster immunoglobulin. These cells were then incubated with antigen-presenting cells and

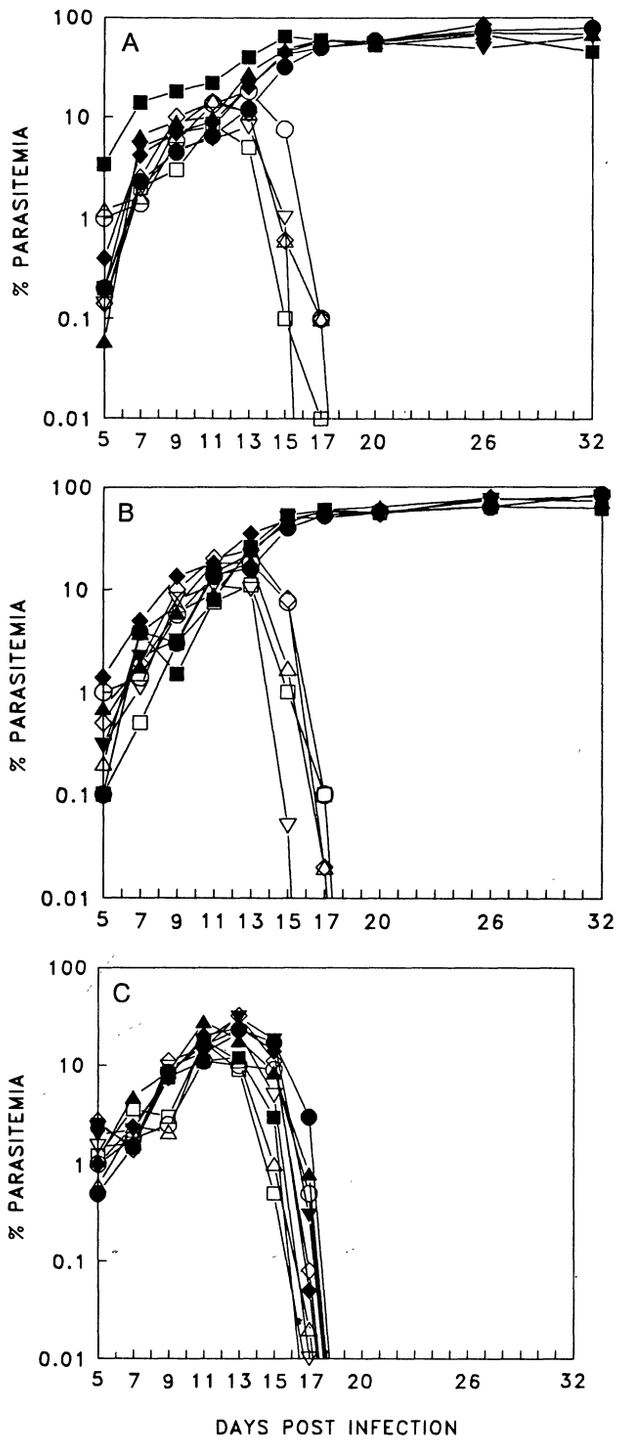

FIG. 1. Course of the blood phase of *P. yoelii* infection (curve of parasitemia) in homozygous (filled symbols) TCR α mutant (α $-/-$) (*A*), TCR β mutant (β $-/-$) (*B*), and TCR δ mutant (δ $-/-$) mice (*C*) and their respective heterozygous (α $+/-$, β $+/-$, δ $+/-$) littermates (open symbols). The various symbols identify individual mice. All mice were infected by intravenous injection of 10^3 live sporozoites of *P. yoelii* (17X NL strain). Thin blood smears were prepared on the days indicated, stained by Giemsa reagent, and examined by light microscopy to determine the level (%) of parasitemia.

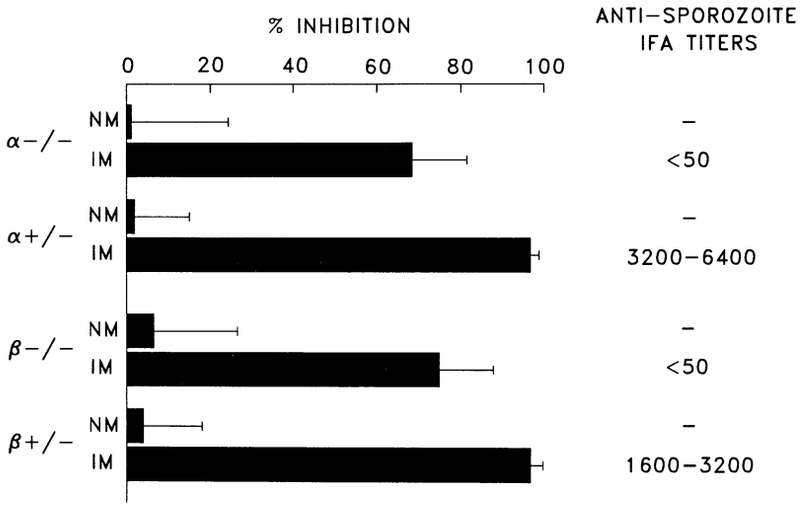

FIG. 2. Inhibition of liver-stage development and anti-sporozoite antibody immunofluorescence assay (IFA) titers of αβ T-cell-deficient mice immunized by exposure to the bites of malaria-infected irradiated (IM) or noninfected mosquitoes (NM). The percent inhibition of liver-stage development was calculated based on the amount of plasmodial rRNA present in the liver of nonimmunized sporozoite-injected control mice. One hundred percent inhibition corresponds to the absence of detectable parasite rRNA in the liver of sporozoite-injected mice. The results are expressed as the mean values ± SD obtained in groups of four mice.

M. bovis recombinant HSP 65 (2 μg/ml), as described above. Fab fragments were obtained by papain digestion of the immunoglobulins (17).

RESULTS AND DISCUSSION

Groups of homozygous TCR α mutant (α −/−), TCR β mutant (β −/−), and TCR δ mutant (δ −/−) mice and their respective heterozygous (+/−) littermates were all infected with viable *P. yoelii* (17X NL strain) sporozoites. The course of the erythrocytic phase of the infection was strikingly different among these different groups of mice. αβ T-cell-deficient mice, α −/− (Fig. 1*A*) and β −/− (Fig. 1*B*), developed progressive parasitemia and died 26–40 days after inoculation. In contrast, their heterozygous littermates (α +/− and β +/−), which develop normal αβ T cells, cleared the blood-stage parasites 16–18 days after sporozoite inoculation and survived the infection. In contrast to αβ T-cell-deficient mice, TCR δ mutant (δ −/−) mice recovered from parasitemia on days 16–18 of infection, as did their respective heterozygous controls (Fig. 1*C*). Earlier studies had shown that clearance of *P. yoelii* blood stages in immunologically intact mice is mediated by $CD4^+$ T cells (5). The present results confirm those earlier observations regarding the requirement for αβ T cells for the elimination of infected red blood cells and suggest that γδ T cells do not play an essential role during the blood phase of the *P. yoelii* infection.

To determine whether αβ T-cell-deficient mice (α −/− or β −/−) could mount an immune response against preerythrocytic forms—namely, sporozoites and/or liver stages of this parasite—the mice were immunized by the natural route of infection, the bites of irradiated sporozoite-infected *Anopheles* mosquitoes. As controls, groups of mice were exposed to the bites of noninfected mosquitoes. The heterozygous mice (α +/− or β +/−) developed significant titers of anti-sporozoite antibodies, as determined by an indirect immunofluorescence assay, while very low levels of or no specific antibodies were detected in the sera of immunized homozygous αβ T-cell-deficient mice (α −/− or β −/−) (Fig. 2).

Sporozoite-immunized αβ T-cell-deficient mice (α −/− or β −/−) and their respective heterozygous littermates were challenged with viable *P. yoelii* sporozoites, and the development of liver stages was evaluated by measuring plasmodial rRNA (12). As expected, parasite development in the liver of sporozoite-immunized heterozygous mice was either abolished or strongly inhibited. Most importantly, sporozoite-immunized αβ T-cell-deficient mice also mounted an immune response that significantly inhibited the development of intrahepatocytic parasites (Fig. 2). In all mice exposed to the bites of noninfected mosquitoes, there was no inhibition of development of the liver stages. These findings suggested that γδ T cells might be responsible for the decrease in liver stages observed in these αβ T-cell-deficient mice.

To further define the possible involvement of γδ T cells, sporozoite-immunized αβ T-cell-deficient mice (α −/−) were transiently depleted *in vivo* of γδ T cells, by injection of a

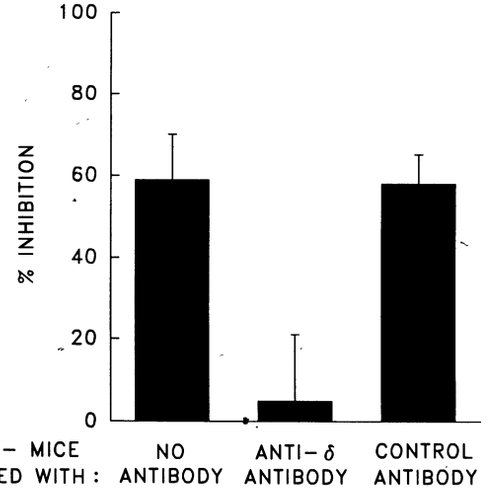

FIG. 3. Reversal of inhibition of liver-stage development in sporozoite-immunized αβ T-cell-deficient mice (α −/−) after transient *in vivo* depletion of γδ T cells by anti-TCR δ mAb. Sporozoite-immunized α −/− mice were injected intraperitoneally with either purified anti-TCR δ mAb (GL3) or an equivalent amount of normal hamster immunoglobulin. Five days after the treatment, mice were challenged with 3×10^5 sporozoites. Forty-two hours later the amount of parasite rRNA present in the liver was measured. The results shown represent those obtained in one of two similar experiments. The results are expressed as the mean values ± SD obtained in groups of four mice.

mAb (GL3) specific for all TCR δ chains (14). This treatment, as determined by flow cytometry analysis, reduced by 92% the number of γδ T cells present in their lymph nodes (data not shown). The injection of this anti-TCR δ antibody into sporozoite-immunized α −/− mice abrogated the inhibition of liver stages induced by their immunization (Fig. 3). Taken together, these findings strongly suggest that γδ T cells play a major role in the antiparasite activity observed in sporozoite-immunized αβ T-cell-deficient mice.

We derived two T-cell clones, 291-H4 and 219-1, from the spleen cells of sporozoite-immunized αβ T-cell-deficient mice (α −/−) by the limiting dilution technique (16). These T-cell clones express TCR δ and CD3 ε (Fig. 4*A*) and are negative for NK1.1, CD4, CD8, and TCR β (data not shown). To determine whether these γδ T-cell clones could interfere with the development of the parasites, these clones were transferred to normal mice, which were inoculated 4 hr later with viable sporozoites. The adoptive transfer of 291-H4 T cells resulted in significant inhibition of parasite development (Fig. 4*B*). In four independent experiments, this inhibition varied from 46% to 67%. Under the same conditions, the adoptive transfer of either the γδ T-cell clone 219-1 or spleen cells from nonimmune α −/− mice (negative control) did not affect parasite development (Fig. 4*B*).

T cells from γδ clones 291-H4 and 219-1 proliferated *in vitro* upon incubation with irradiated spleen cells. Incubation of these γδ T cells with crude parasite extracts obtained from

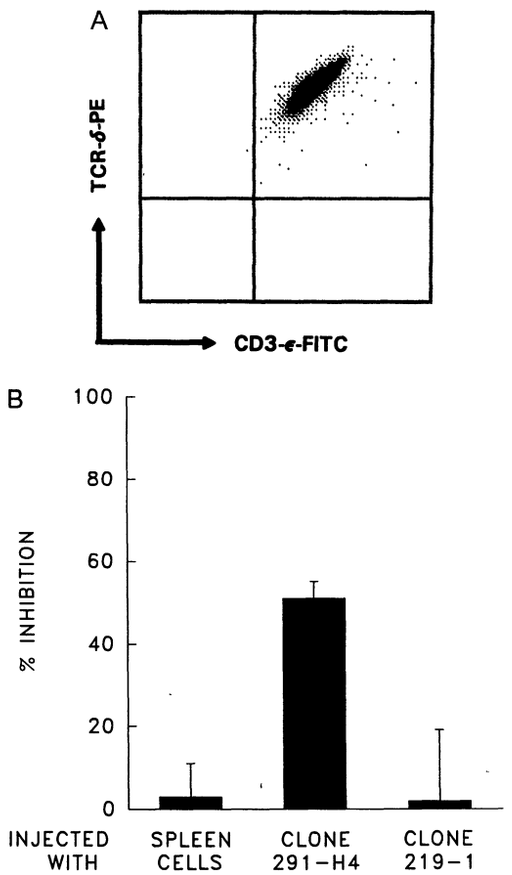

FIG. 4. γδ T-cell clones and their antiplasmodial activity. (*A*) Two-color flow cytometric analysis of the γδ T-cell clone 291-H4: staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 ε mAb (2C11) (horizontally) and phycoerythrin (PE)-conjugated anti-TCR δ mAb (GL3) (vertically). (*B*) *In vivo* antiplasmodial activity of passively transferred γδ T-cell clones 291-H4 and 219-1, derived from sporozoite-immunized TCR α mutant mice. Ten million cells of the respective T-cell clones or spleen cells from αβ T-cell-deficient (α −/−) nonimmunized mice were adoptively transferred to naive (129/Sv × C57BL/6)F_1 mice by tail vein injection. Four hours later, all mice were challenged with 3×10^5 *P. yoelii* sporozoites, and after 42 hr, the amount of *P. yoelii* rRNA present in the liver of these mice, and the percentage of inhibition, was determined. Each group consisted of four mice.

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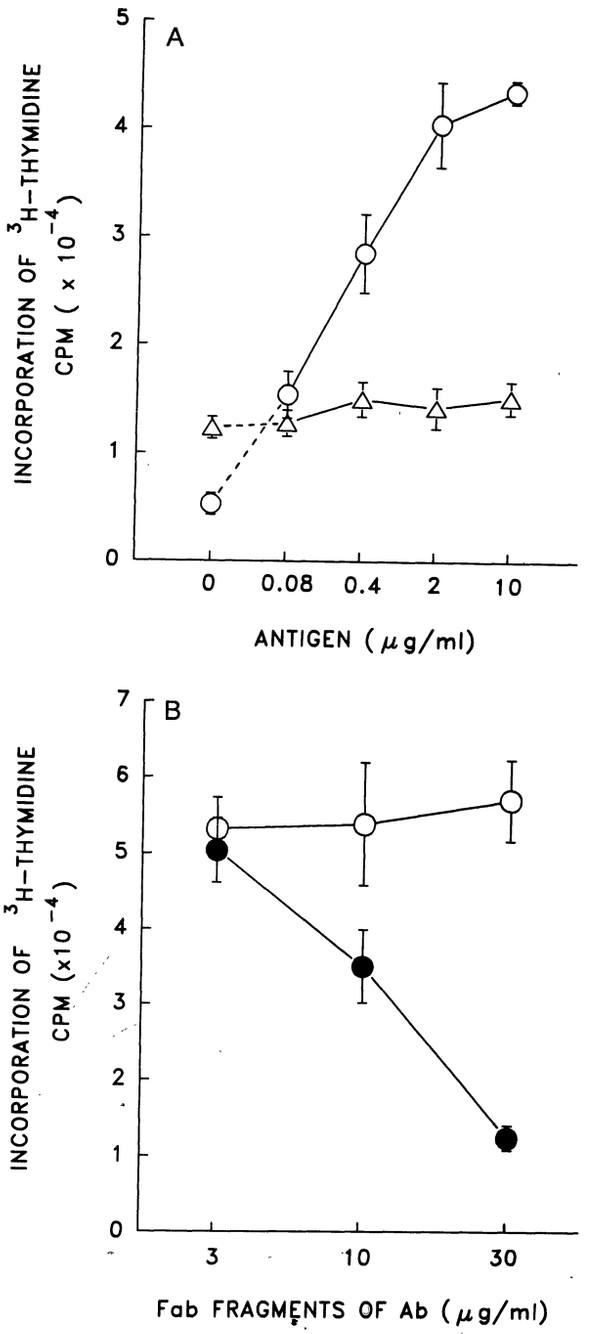

FIG. 5. Proliferative response of γδ T-cell clones to *M. bovis* HSP65. (*A*) Proliferative response ([^{3}H]thymidine incorporation) of protective clone 291-H4 (○) and nonprotective clone 219-1 (△) in the presence and absence of recombinant *M. bovis* HSP65. Three thousand cells of clone 291-H4 or 219-1 were incubated with irradiated spleen cells (10^6) from (129/Sv × C57BL/6)F_1 mice, and with various concentrations of purified recombinant *M. bovis* HSP65. The proliferative response was assessed 3 days later by measuring the incorporation of [^{3}H]thymidine. (*B*) Inhibition of the proliferative response of clone 291-H4 to *M. bovis* HSP65 by preincubation of these T cells with various concentrations of Fab fragments of either anti-TCR-δ mAb (●) or normal hamster immunoglobulin (○). These cells were then cultured with antigen-presenting cells and recombinant *M. bovis* HSP65 (2 μg/ml).

PRBCs failed to enhance the proliferation of these clones (data not shown).

The proliferative activity of the protective clone 291-H4, but not that of clone 219-1, was greatly enhanced when these cells were incubated with purified recombinant *M. bovis* HSP65 (Fig. 5*A*). This proliferative response of clone 291-H4 was inhibited by incubation with Fab fragments of a mAb directed against TCR δ (Fig. 5*B*). The proliferative activity of clone 291-H4, induced by HSP65, does not appear to be restricted by classic class I or class II MHC molecules, since a similar degree of proliferation occurred when spleen cells derived from either BALB/c (*H-2*^{d}) or A/J (*H-2*^{a}) mice were used as antigen-presenting cells.

Since HSP65 is a highly conserved stress protein with considerable sequence homologies to mammalian HSP60 (18–22), our findings suggest that γδ T cells from clone 291-H4 may recognize self-stress proteins expressed by parasitized hepatocytes. A role for γδ T cells in the recognition and elimination of cells being subjected to stress and expressing HSPs had been proposed earlier (23–26). Alternatively, the possibility remains that an HSP60 homolog may be expressed in sporozoites and/or liver stages of malaria parasites, although the presence of HSP60 in malaria parasites remains to be demonstrated. Further investigation is required to determine whether the observed antiplasmodial activity of γδ T cells is mostly mediated by the recognition of self-stress protein(s) or of parasite-specific antigens.

This study has shown that γδ T cells can mediate a protective immune response against a parasitic agent in the absence of αβ T cells. Murine γδ T-cell clones displaying antimicrobial activity will certainly be instrumental in defining the type of antigens recognized by these cells, the nature of their antigen-presenting molecules, and the effector mechanisms by which γδ T cells exert their antimicrobial activity.

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