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Characterization of Protective T Cells in the Acquired Response to *Leishmania donovani* in Genetically Determined Cure (*H-2*^{b}) and Noncure (*H-2*^{d}) Mouse Strains

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The response to reinfection with Ethiopian *Leishmania donovani* was evaluated in genetically determined noncure (*H-2*^{d}) B10.D2 mice that are able to resolve infection due to sublethal irradiation pretreatment after inoculation with a low parasite dose and in C57BL/10 mice that demonstrate the genetically determined cure (*H-2*^{b}) response to *L. donovani*. It was found that after resolution of primary infection, C57BL/10 (cure) mice and sublethally irradiated B10.D2 (noncure) mice were resistant to rechallenge with *L. donovani*. Noncure mice inoculated with a low dose of amastigotes were not, however, solidly immune to reinfection. Adoptive-cell transfer experiments were then done to determine the T-cell subset that was associated with resistance to reinfection, and thus the development of immunity, in sublethally irradiated B10.D2 noncure mice and in C57BL/10 cure mice. T-cell-enriched preparations from spleens of immune donors were treated with subset-specific antibodies and complement prior to adoptive transfer in unprimed recipients. The results of the adoptive transfer experiments provide evidence that the genetically determined cure (*H-2*^{b}) response in C57BL/10 mice and the cure response in genetically determined noncure (*H-2*^{d}) B10.D2 mice brought about by sublethal irradiation pretreatment are mediated primarily by an L3T4$^+$ Lyt-2$^-$ T cell.

During active infection with *Leishmania donovani* in humans, cell-mediated immunity measured by delayed-type hypersensitivity (2, 13, 14, 16, 27), lymphocyte proliferation (9, 13, 14, 35), and the production of gamma interferon and interleukin-2 (8) in response to leishmanial antigen is absent or reduced. After spontaneous or drug-induced cure, these parasite-specific responses recover (8, 9, 13, 14, 16, 27), indicating that healing of visceral leishmaniasis in humans is dependent primarily on the development of cell-mediated immunity.

Mice inoculated with *L. donovani* demonstrate a range of host responses (3, 6, 11, 30) that reflects the broad spectrum of clinical disease described in humans (1, 7, 26). Studies in mice have established the association between the resolution of *L. donovani* infection and the development of cell-mediated immunity. Resolution of infection has been shown to correlate with the development of parasite-specific delayed-type hypersensitivity responses (11, 28, 30), the proliferation of spleen cells in vitro (24), and the generation of macrophage-activating lymphokines (22) and gamma interferon (23). Furthermore, protective immunity can be passively transferred to naive recipients with T-cell-enriched preparations (21, 28, 31), but not with serum or B-cell-enriched preparations (28), from mice that have resolved infection.

What is greatly lacking at present, however, is knowledge of the T-cell subsets that are mediating the resolution of *L. donovani* infection in mice. With the objective of addressing this issue, the effect of depletion of T-cell subsets with subset-specific antibodies and complement on the adoptive transfer of immunity with T-cell-enriched preparations from immune donor mice was examined in unprimed recipients. The T cell associated with the development of protective immunity was characterized in two acquired responses to Ethiopian *L. donovani*: the genetically determined (*H-2*^{b}) cure response in C57BL/10 mice (3) and the cure response in genetically determined (*H-2*^{d}) noncure mice (3) brought about by sublethal irradiation pretreatment (34). The response to reinfection with *L. donovani* was also evaluated in these models and in noncure mice that are able to resolve infection after inoculation with a low parasite dose.

MATERIALS AND METHODS

Mice. Female B10.D2/nSnJ and B10.D2/oSnJ (B10.D2) mice and male and female C57BL/10SnJ (C57BL/10) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. B10.D2/oSn mice were also bred in our facilities from breeding pairs that were originally obtained from Jackson Laboratories. In one experiment, mice of the C57BL/10ScSn (C57BL/10) strain were obtained from Olac (1976) Ltd., Oxon, England. Mice were 6 to 8 weeks of age at the start of all experiments.

The B10.D2/n strain was used in the original study that described the *H-2*^{d} noncure response to *L. donovani* (3). In our hands, the B10.D2/o substrain, which also possesses the *H-2*^{d} haplotype, demonstrates both the noncure phenotype (3) and the irradiation-induced prophylaxis (34) that is shown by the B10.D2/n strain (O. M. Ulczak and P. A. L. Kongshavn, manuscript submitted). The only known difference between the B10.D2/o and the B10.D2/n strains is an allelic difference at the *Hc* locus which determines the presence or absence of the C5 component of complement in the serum. In the B10.D2/n strain, C5 is present, whereas in the B10.D2/o strain, C5 is absent (12, 25).

Parasite. The Ethiopian L82 (known also as the LV9) strain of *Leishmania donovani* used was maintained in our laboratory for animal inoculation by hamster passage. Mice were inoculated intravenously with amastigotes (2×10^5, 8.5×10^5, 5×10^6, or 1×10^7 to 2×10^7) in 0.2 ml of medium,

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and parasite loads were determined in the liver and in some cases also in the spleen. The methods of amastigote preparation from hamster spleen, necropsy of mice, and determination of parasite loads from Giemsa-stained impression smears have been described previously (6).

Sublethal irradiation. Only mice of the B10.D2 strain were sublethally irradiated. Mice received 550 rads of whole-body gamma irradiation from a Gammacell 40 (cesium 137 source) machine (Atomic Energy of Canada Limited) at a dose rate of 145 rads/minute. Mice that were used either as cell donors in adoptive transfers or as experimental (previously infected) mice in the rechallenge experiments were irradiated approximately 2 to 4 h prior to inoculation with amastigotes. Control (previously uninfected) mice used in the rechallenge experiments were irradiated at the same time as experimental mice. Recipients of spleen cells from B10.D2 donors were irradiated from 8 to 10 h prior to adoptive transfer.

Preparation of T-cell-enriched spleen cells. Cells from immune B10.D2 donors were obtained from irradiated mice that were infected 100 days previously with 10^6 *L. donovani*. Impression smears prepared from a sample of three mice on the day of adoptive transfer indicated that the liver parasite load in Leishman Donovan units (LDU) determined 15 days after infection (log LDU, 1.86 ± 0.36) had been resolved to levels below the limits of resolution of the impression smear technique, as indicated by the absence of amastigotes.

Cells from immune C57BL/10 donors were obtained from mice that were infected either 85 days previously with 2×10^7 amastigotes, 95 days previously with 10^7 amastigotes, or 100 days previously with 1.3×10^7 amastigotes. In donors infected with 2×10^7 *L. donovani*, the liver parasite load had been reduced by 91% when compared to the liver parasite load determined 15 days after infection (from log LDU of 3.66 ± 0.02 to log LDU of 0.34 ± 0.34). Among donors infected with approximately 10^7 amastigotes, at 95 days the liver parasite load had been reduced by 83% (log LDU, 0.52 ± 0.26 versus day 15 log LDU, 2.98 ± 0.07). At 100 days, the day 15 parasite load in donors (log LDU, 3.29 ± 0.01) had been resolved, as indicated by the absence of amastigotes in impression smears. Cells were also prepared from age-matched uninfected C57BL/10 donor mice.

Spleens were removed aseptically and cut into fragments in RPMI 1640 or medium 199 supplemented with 5% heat-inactivated (56°C, 30 min) fetal calf serum (Flow Laboratories, Inc., McLean, Va.), 10 mM HEPES (*N*-2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid) (Flow Laboratories), 2 mM L-glutamine (Gibco Laboratories, Grand Island, N.Y.), 1 mM sodium pyruvate (Gibco Laboratories), 0.1 mM nonessential amino acids, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (Gibco Laboratories). The spleen fragments were pressed gently through a large (80)-mesh screen and then through a finer (120)-mesh screen. The cell suspension was allowed to settle for 5 min to remove large clumps of cells and debris by sedimentation. Erythrocytes in the supernatant were lysed with ammonium chloride. After two washes at 250 × *g* for 10 min in supplemented medium, the cell suspension was enriched for T cells by one passage through nylon wool columns according to the method described by Mishell and Shiigi (19).

Depletion of T cells and T-cell subsets with antisera and complement. Further treatment of the T-cell-enriched spleen preparations from donor mice with antisera and complement was done in RPMI 1640 medium supplemented with 0.3% bovine serum albumin (Fraction V, Gibco Laboratories), and 25 mM HEPES. The T-cell-enriched preparation from donor mice was divided into aliquots and pelleted by centrifugation at 250 × *g* for 10 min prior to treatment.

The cell pellets from C57BL/10 donors were suspended at a concentration of 10^7 viable cells/ml in medium alone or in medium containing either anti-Thy-1 (1/40 dilution; rabbit anti-mouse brain-associated anti-T-cell serum, CL 2005; Cedarlane Laboratories, Hornby, Ontario, Canada), anti-L3T4 (1/5 dilution; rat monoclonal immunoglobulin G 2b; GK1.5 hybridoma supernatant collected after 10 days in culture in Dulbecco modified Eagle medium containing 5% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate; TIB 207; American Type Culture Collection, Rockville, Md.), or anti-Lyt-2.2 (1/500 dilution; mouse monoclonal immunoglobulin M; ascites, CL 8922A; Cedarlane Laboratories) antibodies and incubated at 4°C for 45 min. The aliquots were then centrifuged at 250 × *g* for 10 min and suspended at 10^7 cells/ml in rabbit serum as a source of complement (1/10 dilution; Low Tox M rabbit serum, CL3051; Cedarlane Laboratories). After incubation for 60 min in a water bath set at 37°C, the cytotoxicity in the presence of trypan blue was determined to be 59% after treatment with anti-Thy-1, 24% after treatment with anti-L3T4, and 39% after treatment with anti-Lyt-2.2 antibodies.

The cells from B10.D2 donors were treated with medium alone or medium containing various antisera at a concentration of 2.5×10^7 cells/ml. The protocols used for treatment with medium alone, anti-Thy-1 and anti-Lyt-2.2 antibodies, and complement were identical to those described for cells from C57BL/10 donors. Treatment with anti-L3T4 antibody, either alone or in combination with anti-Lyt-2.2 antibody, was done in two cycles in the following manner. Cells were incubated at 4°C for 30 min in medium containing anti-L3T4 or anti-L3T4 together with anti-Lyt-2.2 antibodies. After incubation, the cells were pelleted, suspended in complement, and incubated at 37°C for 30 min. The cells were washed once, and the treatments with antisera and complement were repeated. Cytotoxicity was determined to be 65% after treatment with anti-Thy-1, 69% after anti-L3T4, 14% after anti-Lyt-2.2, and 82% after the combined treatments with anti-L3T4 and anti-Lyt-2.2 antibodies.

After treatment, cells were washed twice in supplemented medium at 250 × *g* for 10 min and inoculated intravenously into mice in a volume of 0.2 ml.

Rechallenge protocol. C57BL/10 mice were infected with a primary inoculum of 10^7 amastigotes. The infected (experimental) mice together with age-matched previously uninfected (control) mice were rechallenged 145 days after primary infection. At the time of rechallenge, the primary infection had been resolved in the experimental group, as indicated by the absence of amastigotes in impression smears. The rechallenge inoculum contained 1.8×10^7 amastigotes.

B10.D2 mice were irradiated and inoculated with 8.5×10^5 amastigotes. The experimental mice together with irradiated control mice were rechallenged 80 days after primary infection. At the time of rechallenge, the day 15 liver parasite load in the primary infection (log LDU, 2.34 ± 0.03) had been reduced by 78% (to log LDU of 0.52 ± 0.48) in the experimental group. In a second experiment, B10.D2 mice were unirradiated but were inoculated with either 2×10^5 or 1×10^7 amastigotes. These experimental mice together with control mice were challenged 80 days after primary infection. At this time, the liver parasite load determined 15 days after primary infection with 2×10^5 amastigotes had been reduced from $\log_{10}$ LDU of 1.92 ± 0.04 to $\log_{10}$ LDU of 0.38 ± 0.22. Parasite loads in mice receiving 10^7 amastigotes in

the primary inoculum were unresolved ($\log_{10}$ LDU, 3.41 ± 0.11) when compared to the parasite load determined at 15 days after infection ($\log_{10}$ LDU, 2.83 ± 0.05). All B10.D2 mice were rechallenged with 1.4×10^7 amastigotes.

All experimental and control mice were necropsied 2 h after rechallenge to determine the initial uptake of amastigotes in the secondary inoculum and at 15, 30, or 55 days after rechallenge. Parasite burdens were determined in the liver and spleen.

Adoptive cell transfer protocol. Unprimed C57BL/10 or B10.D2 recipients received 9×10^6, 1×10^7, or 4.6×10^7 T-cell-enriched spleen cells from immune syngeneic donors or cell preparations from these donors that had been treated with antisera and complement. One day after cell transfer, all recipients and control mice (receiving no cells or receiving cells from uninfected mice) were challenged with 5×10^6 or 1×10^7 amastigotes. Parasite burdens were determined in the livers at 15, 30, 40, 50, 85, or 130 days after infection.

Statistical analysis. The Student's *t* test was used to determine the statistical significance of differences between parasite loads of various experimental and control groups of mice.

RESULTS

Resistance to reinfection. One method by which the presence of immunity can be ascertained in animals that have resolved infection is to rechallenge the animal with a homologous parasite. The resulting parasite load is then compared with that in normal mice that have been infected at the time of rechallenge. Such experiments were done to establish whether C57BL/10 (*H-2*^{b}) and B10.D2 (*H-2*^{d}) mice that are able to control *L. donovani* infection (thereby demonstrating a cure phenotype) were indeed immune.

In the first study, the response to reinfection was evaluated in genetically determined noncure (*H-2*^{d}) B10.D2 mice that were able to control *L. donovani* infection due to sublethal irradiation pretreatment. Mice were sublethally irradiated and inoculated with 8.5×10^5 amastigotes. Sublethal irradiation enabled the noncure mice to control infection (Fig. 1A), as reported earlier (34), in this case at 50 ($P < 0.05$) and 80 ($P < 0.05$) days after infection. These experimental mice together with control normal mice were then challenged with 1.4×10^7 amastigotes 80 days after primary infection. The results in Fig. 1B indicate that the experimental group had significantly lower parasite loads in the spleen at 55 days ($P < 0.001$) and in the liver at 15 ($P < 0.001$) and 55 ($P < 0.005$) days after reinfection when compared to the values in control mice. The uptake of amastigotes in the rechallenge inoculum was also slightly, but significantly, lower in the livers of the experimental group than in the control group, as indicated by the lower parasite load determined 2 h after rechallenge ($P < 0.05$). Similar results were obtained in a subsequent experiment in which irradiated B10.D2 mice were rechallenged at 90 days, after resolution of primary infection. In this case amastigotes were no longer detected in impression smears prepared from mice at 50 and 90 days after primary infection. After rechallenge, liver parasite loads in experimental mice were lower at 15 and 30 days after reinfection than in control mice. In that experiment, the initial uptake of amastigotes in the rechallenge inoculum was identical in both groups (data not shown). These results indicated that noncure B10.D2 mice that were able to control *L. donovani* infection due to prior treatment with sublethal irradiation were immune to reinfection with the same parasite.

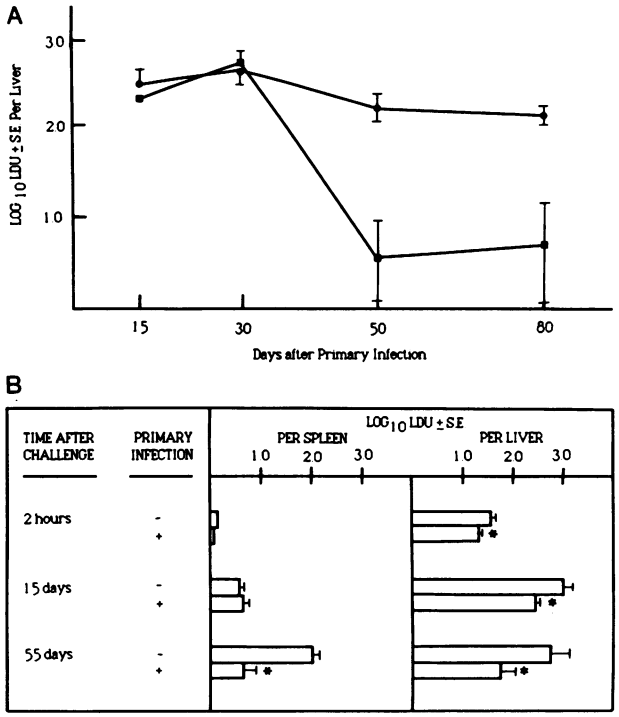

FIG. 1. Resistance to rechallenge with *L. donovani* in previously infected irradiated B10.D2 mice. (A) The course of primary infection with 8.5×10^5 amastigotes in irradiated (■) mice that were subsequently rechallenged at 80 days after infection and in unirradiated mice (●) that were inoculated with the same parasite dose. (B) Spleen and liver parasite loads after challenge with 1.4×10^7 amastigotes in experimental irradiated mice that were infected 80 days previously with 8.5×10^5 amastigotes and in the age-matched control mice that were irradiated and previously uninfected. Each value represents the mean parasite load ± standard error determined from three mice. Asterisks indicate a significantly lower mean parasite load in the experimental group than in the control group.

In a second rechallenge experiment, mice of the same strain were not irradiated but were infected with a low primary dose of 2×10^5 *L. donovani*. Infection resulting from the low inoculum was shown to be resolved both in the present study (Fig. 2A) and in a previous study by Ulczak and Blackwell (34). Unirradiated B10.D2 mice inoculated with 10^7 amastigotes and demonstrating a noncure response (3) were included as a negative control (Fig. 2A). Mice from both previously infected groups together with normal mice were rechallenged with 1.4×10^7 amastigotes 80 days after primary infection.

Throughout the course of the rechallenge infection (Fig. 2B), B10.D2 mice expressing a noncure response to primary infection with *L. donovani* demonstrated parasite loads that were either higher than ($P < 0.005$ to $P < 0.001$) or not significantly different from those in control previously uninfected mice. Unexpectedly, mice that had previously resolved an inoculation of 2×10^5 amastigotes demonstrated a small but significant reduction of parasite load only at 15 days after reinfection in the liver ($P < 0.001$). At all other times, the parasite load in the experimental group was either significantly higher than ($P < 0.025$ to $P < 0.01$) or similar to that in control mice. These results indicated that despite apparent resolution of parasite load in *H-2*^{d} mice inoculated with a low dose of amastigotes, solid immunity is not present, since these mice are largely comparable to those

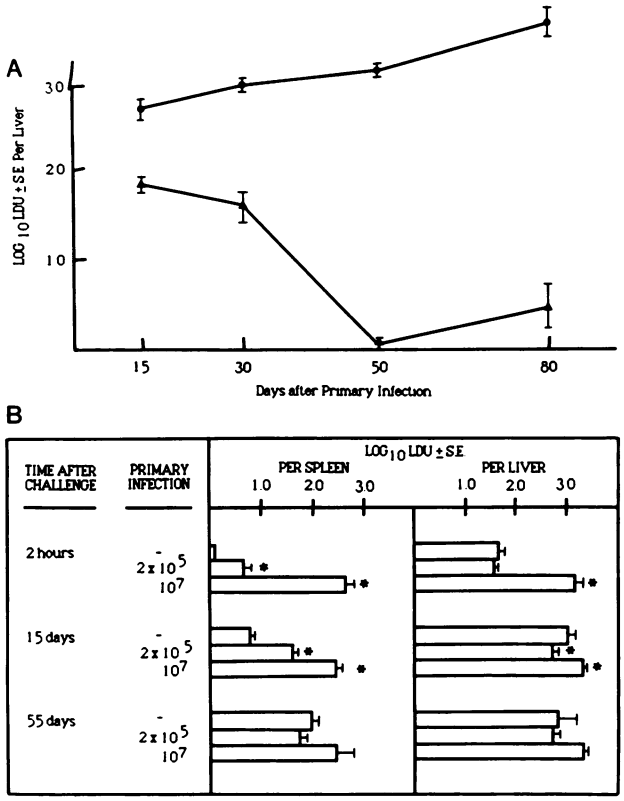

FIG. 2. Resistance to rechallenge with *L. donovani* in previously infected unirradiated B10.D2 mice. (A) The course of primary infection with 2×10^5 (▲) and 1×10^7 (●) amastigotes in mice that were subsequently rechallenged at 80 days after infection. (B) Spleen and liver parasite loads after challenge with 1.4×10^7 amastigotes in mice that were infected 80 days previously with 2×10^5 or 1×10^7 *L. donovani* and in age-matched control mice of the same strain that were previously uninfected. Each value represents the mean parasite load ± standard error determined from three mice. Asterisks indicate a significantly higher mean parasite load in the experimental group than in the control group.

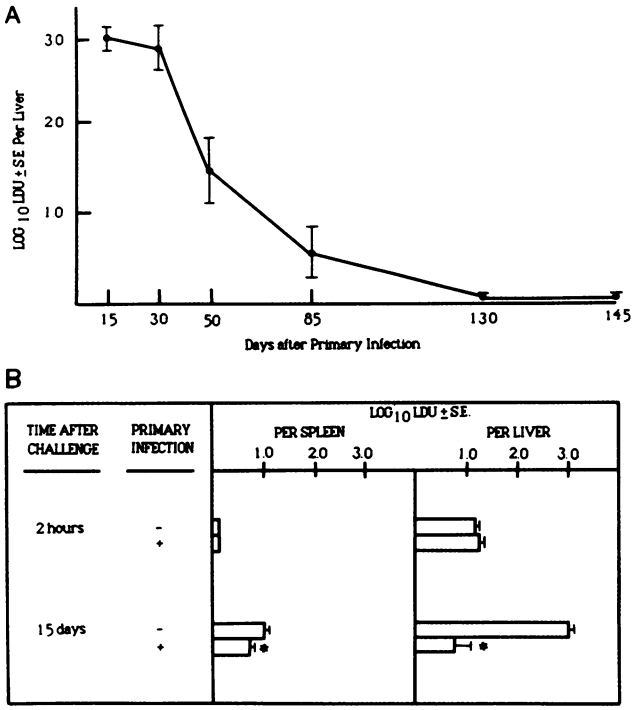

FIG. 3. Resistance to rechallenge with *L. donovani* in previously infected C57BL/10 mice. (A) The course of primary infection with 10^7 amastigotes in mice that were subsequently rechallenged at 145 days after infection. (B) Spleen and liver parasite loads after challenge with 1.8×10^7 amastigotes in experimental mice that were infected 145 days previously with 10^7 *L. donovani* and in age-matched control mice of the same strain that were previously uninfected. Each value represents the mean parasite load ± standard error determined from three mice. The asterisk indicates a significantly lower mean parasite load in the experimental group than in the control group.

demonstrating a noncure response in their ability to resist reinfection with *L. donovani*.

In the final experiment in this series, genetically determined cure (*H-2*^{b}) C57BL/10 mice (3) were rechallenged with 1.8×10^7 amastigotes 145 days after primary infection with 10^7 amastigotes. At the time of rechallenge, the primary infection had been resolved, as indicated by the absence of amastigotes in impression smears prepared from the infected mice (Fig. 3A). Cured C57BL/10 mice were clearly resistant to reinfection with *L. donovani*, as shown by the greatly reduced liver parasite loads at 15 days after rechallenge in these mice in comparison with those in control mice (Fig. 3B; $P < 0.001$). In the spleen, only a small reduction of parasite load was observed in the experimental group ($P < 0.02$). No differences in the initial uptake of amastigotes in the rechallenge inoculum were evident between mice of both groups, as shown by the identical parasite loads in both groups determined 2 h after reinfection.

Transfer of protection with cells from cured (*H-2*^{b}) C57BL/10 mice. Two separate experiments were done to identify the cell population that was responsible for allowing cured C57BL/10 mice to resist reinfection with *L. donovani*. Accordingly, 10^7 T-cell-enriched spleen cells from C57BL/10 mice infected 85 days previously with 2×10^7 *L. donovani* (Fig. 4A) or 100 days previously with 1.3×10^7 *L. donovani* (Fig. 4B) were transferred into unprimed syngeneic recipients. The parasite load after challenge with 5×10^6 amastigotes in the recipients was then compared with that in unprimed control mice receiving 10^7 T-cell-enriched spleen cells from age-matched normal uninfected donors (Fig. 4A) or unprimed control mice receiving no cells (Fig. 4B). In both experiments, protection was transferred with the T-cell-enriched spleen preparation, as demonstrated by the significant reduction of parasite load in recipients at various times after infection ($P < 0.05$ to $P < 0.01$).

The T-cell population associated with protective immunity was then characterized according to the surface phenotype in similar experiments in which the T-cell-enriched spleen cell preparation from immune donors was depleted of T cells or T-cell subsets by treatment with subset-specific antisera and complement prior to adoptive transfer. A representative experiment is shown in Fig. 5. In this case, unprimed recipients received 4.6×10^7 T-cell-enriched spleen cells from C57BL/10 donors infected 95 days previously with 10^7 *L. donovani*, or the cells remaining after treatment of the T-cell-enriched preparation with various antisera and complement. All the recipient mice and control mice receiving no cells were challenged with 5×10^6 amastigotes. Parasite loads were determined in the liver 40 days after challenge. Significant protection was transferred with the T-cell-enriched preparation from the cured C57BL/10 mice ($P <$

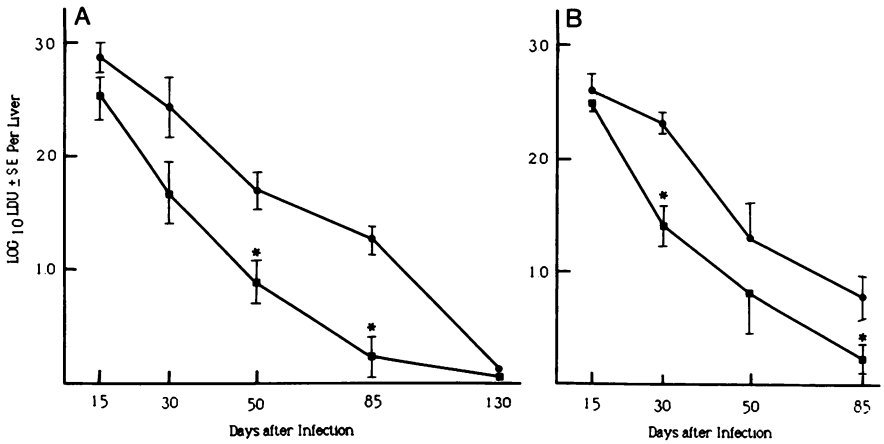

FIG. 4. The effect of adoptive transfer of T-cell-enriched spleen cells from immune C57BL/10 mice on the liver parasite load in unprimed recipients. (A) Unprimed recipients received 10^7 T-cell-enriched spleen cells from mice that were infected 85 days previously with 2×10^7 *L. donovani* (■) or from age-matched uninfected normal mice (●). (B) Unprimed recipients received 10^7 T-cell-enriched spleen cells from donor mice infected 100 days previously with 1.3×10^7 *L. donovani* (■). Control mice received no cells (●). In both experiments, all mice were challenged with 5×10^6 amastigotes one day after adoptive transfer. Each point represents the mean parasite load ± standard error determined from 2 to 4 mice. Asterisks indicate significantly lower mean parasite loads in the group receiving cells from immune donors.

0.05). Transfer of protection was further abrogated by prior treatment of the T-cell-enriched population with either anti-Thy-1 ($P < 0.05$) or anti-L3T4 ($P < 0.01$) antibodies plus complement. Removal of Lyt-2^+ T cells, on the other hand, did not alter the protective capacity of the transferred cells. These results indicated that a Thy-1^+ L3T4^+ Lyt-2^- cell was primarily associated with the genetically determined cure (*H-*2^b) response to Ethiopian *L. donovani* in C57BL/10 mice.

Transfer of protection with cells from cured (*H-*2^d) B10.D2 mice. The final experiments were designed to identify the cell population that was associated with the ability of sublethally irradiated noncure (*H-*2^d) B10.D2 mice to resist reinfection with *L. donovani*. Earlier studies had shown that potent T suppressor cells were present as early as 30 days after infection with 5×10^6 amastigotes in unirradiated B10.D2/n (*H-*2^d) mice (5). The recipients of spleen cells from immune B10.D2 donors were, therefore, sublethally irradiated to remove precursors of T suppressor cells and minimize any regulation by T suppressor cells of the protection transferred by cells.

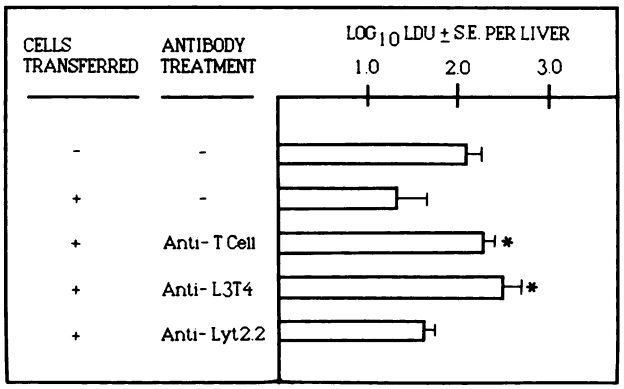

FIG. 5. The effect of depletion of T cells and T-cell subsets on the adoptive transfer of immunity with T-cell-enriched spleen cells from immune C57BL/10 mice in unprimed recipients. Each value represents the mean parasite load ± standard error determined from 3 to 5 mice. Asterisks indicate a significantly higher mean parasite load in the particular recipient group in comparison with that in the recipient group receiving untreated spleen cells.

In preliminary experiments, T-cell-enriched spleen cells, alone, from immune B10.D2 donors conferred protective immunity in unprimed recipients. In those experiments, the transfer of protection was more pronounced at 15 days than at 30 days after challenge (data not shown). To identify the T-cell population from immune B10.D2 donors that was responsible for the transfer of protective immunity in unprimed recipients, immune donor cells were treated with various subset-specific antisera and complement prior to adoptive transfer. An additional recipient group was also included to determine whether or not cooperation between the two major T-cell subsets was vital in the development of protective immunity to *L. donovani*. Accordingly, the mice of this group received a T-cell-enriched preparation that was depleted of both L3T4^+ and Lyt-2^+ cells. The degree of protective immunity that developed in this group was then compared with that in recipients receiving a cell preparation depleted of only L3T4^+ or Lyt-2^+ cells. Figure 6 shows the results of one such experiment in which unprimed recipients received 9×10^6 T-cell-enriched spleen cells from immune B10.D2 donors infected 100 days previously with 10^6 *L. donovani*, or the T-cell-enriched preparation that remained after depletion of T cells and various T-cell subsets. Recipients and control irradiated and unirradiated mice receiving no cells were challenged with 10^7 amastigotes. Parasite loads were determined in the liver 15 days after challenge. In this particular experiment, sublethal irradiation alone had some prophylactic effect against infection, which was already evident at 15 days, as indicated by the lower parasite load in the irradiated control mice receiving no cells ($P < 0.001$). The T-cell-enriched spleen preparation from immune B10.D2 donors transferred potent protection ($P < 0.001$). The parasite load in recipient mice was reduced by almost one log LDU when compared with the parasite load in control mice receiving no cells. Transfer of protection was further abrogated by prior treatment of the immune spleen cells with either anti-Thy-1 ($P < 0.001$) or anti-L3T4 ($P < 0.001$) antibodies and complement, showing that an L3T4^+ T cell was primarily responsible for the transfer of protection

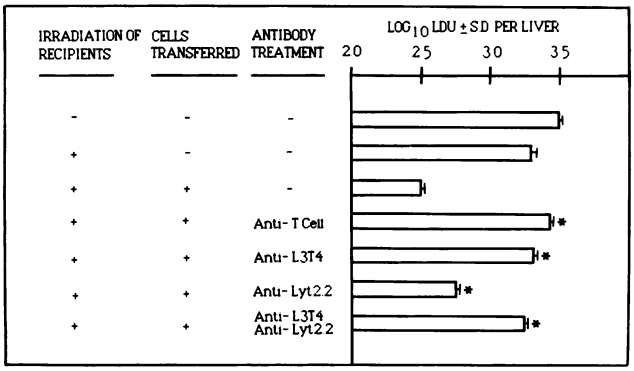

FIG. 6. The effect of depletion of T cells and T-cell subsets on the adoptive transfer of immunity with T-cell-enriched spleen cells from immune B10.D2 mice in unprimed recipients. Each value represents the mean parasite load ± standard deviation determined from 4 to 5 mice. Asterisks indicate a significantly higher mean parasite load in the particular recipient group in comparison with that in the recipient group receiving untreated spleen cells.

in unprimed recipients. Treatment of the cells with anti-Lyt-2.2 antibodies and complement slightly but significantly reduced the transfer of immunity ($P < 0.01$), suggesting a minor role for Lyt-2$^+$ cells in the cure response to Ethiopian *L. donovani* in irradiated B10.D2 mice. This finding is supported by the observation that the removal of all the T cells with anti-T-cell antiserum and complement resulted in a greater liver parasite load in the recipients than did removal of L3T4$^+$ cells alone ($P < 0.01$). However, parasite loads in the recipients of cells depleted of L3T4$^+$ and Lyt-2$^+$ cells was not greater than that in recipients depleted only of L3T4$^+$ cells. Taken together, these results indicated that the T cell primarily associated with the irradiation-induced cure response in noncure (*H-2^d*) B10.D2 mice possessed the L3T4$^+$ Lyt-2$^-$ phenotype.

DISCUSSION

In the present investigation, the development of immunity was examined in two cure models of Ethiopian *Leishmania donovani* on a B10 genetic background. In genetically determined cure (*H-2^b*) C57BL/10 mice, infection was resolved spontaneously (3), whereas in genetically determined noncure (*H-2^d*) B10.D2 mice (3), infection was resolved due to sublethal irradiation pretreatment (34). The development of immunity in both models was evaluated on the basis of (i) resistance to reinfection with a homologous parasite and (ii) adoptive cell transfer of immunity to challenge with a homologous parasite in unprimed recipients.

The results of the reinfection experiments established that after recovery from primary infection, C57BL/10 cure and irradiated B10.D2 noncure mice were resistant to reinfection with, and thus immune to, *L. donovani*. Resistance to rechallenge in a genetically determined noncuring mouse strain that is able to resolve *L. donovani* infection due to prior manipulation (such as irradiation) has not been examined previously. Resistance to reinfection has, however, been determined before in other mouse strains, namely C57BL/6 (that have the *H-2^b* haplotype of C57BL/10 mice [28]) and BALB/c (21) that spontaneously resolve infection with Sudanese strains of *L. donovani*. In those studies, mice were rechallenged during (42 days postinfection [28]) or after (6 to 18 months postinfection [21]) resolution of primary infection and, as in the present study with C57BL/10 mice, the primary inoculum was similar to the secondary inoculum (5×10^6 [28]; 1×10^7 [21]). If the development of immunity after rechallenge in the previously infected (experimental) groups is expressed as $\log_{10}$ LDU of protection, calculated as the difference between the mean $\log_{10}$ LDU of the control, previously uninfected group and the mean $\log_{10}$ LDU of the experimental group, the units of protection observed in the liver 12 to 14 days after rechallenge were determined to be 0.91 $\log_{10}$ LDU in BALB/c mice (21) and 1.17 $\log_{10}$ LDU in C57BL/6 mice (28). The protection in C57BL/10 mice in the present study at the same time after rechallenge was slightly lower, 0.73 $\log_{10}$ LDU (Fig. 3). In the case of the irradiated noncure mice in the present study, protection was even less pronounced at 15 days after reinfection, 0.52 $\log_{10}$ LDU (Fig. 1B). Immunity, however, continued to develop in these mice, as indicated by the doubling in units of protection at 55 days after rechallenge (from 0.52 to 1.09). In view of the range of protection observed after rechallenge in the mouse strains used in the present and earlier work, it appears that the level of immunity that develops may be determined by the infecting strain of *L. donovani* and the host strain that becomes infected.

Additional parameters were examined in this study during reinfection, namely the initial uptake of amastigotes in the rechallenge inoculum (indicated by the parasite load determined 2 h after rechallenge) and the parasite load developing in the spleen after reinfection. It was found that small but significant protection to rechallenge may be present as early as 2 h after reinfection (0.27 $\log_{10}$ LDU of protection; Fig. 1B). Examination of splenic parasite loads after rechallenge indicated that the kinetics of immunity in the spleen may differ from that observed in the liver, at least as shown by the course of infection in irradiated noncure mice (Fig. 1B). In that case, although significant protection was evident in the liver as early as 2 h after reinfection, in the spleen protection was observed only much later, at 55 days after reinfection.

The results of the adoptive transfer experiments demonstrated that T cells were primarily responsible for mediating resistance to reinfection with Ethiopian *L. donovani* in immune C57BL/10 and irradiated B10.D2 mice. This was shown by experiments in which T-cell-enriched spleen preparations, alone, from immune donors transferred immunity to challenge with homologous parasites in unprimed recipients, and transfer of immunity was abrogated after removal of the Thy-1$^+$ cell population (Figs. 4, 5, and 6). These results are in agreement with those of previous studies that examined the adoptive transfer of immunity to another (Sudanese) strain of the parasite in other mouse strains that resolved infection spontaneously (21, 28). The adoptive transfer of immunity to *L. donovani* with T cells, shown here and in the earlier work, is also consistent with the results of other studies that reported higher parasite loads in athymic mice and T-cell-deprived mice (thymectomized, lethally irradiated, and reconstituted with T-cell-depleted syngeneic bone marrow cells) of the curing BALB/c (33) and C57BL/6 (32) strains, respectively.

A major finding of the present investigation is that the T-cell population associated with the adoptive transfer of the cure response to Ethiopian *L. donovani* is primarily an L3T4$^+$ Lyt-2$^-$ cell. The same phenotype was shared by the T cells that mediate the *H-2^b* cure response in C57BL/10 mice (Fig. 5) and the cure response in irradiated *H-2^d* noncure B10.D2 mice (Fig. 6). In the present investigation there was some evidence that Lyt-2$^+$ cells may contribute to the resolution of visceral infection, at least in the cure response in irradiated B10.D2 mice. This was suggested by

the somewhat less efficient resolution of parasite load in unprimed recipients of Lyt-2$^+$ T-cell-depleted preparations, in comparison to recipients of unfractionated cell preparations (Fig. 6). The parasite load in unprimed recipients that received cells depleted only of L3T4$^+$ cells was, however, identical to that in recipients of cells depleted of both L3T4$^+$ and Lyt-2$^+$ subpopulations (Fig. 6), suggesting that Lyt-2$^+$ cells played only a very minor role relative to L3T4$^+$ cells in the control of Ethiopian *L. donovani* infection in vivo, at least as demonstrated by adoptive transfer. The transfer of acquired immunity to *L. donovani*, primarily with the L3T4$^+$ T-cell subpopulation shown here, is not entirely in agreement with the findings of a recent study in which the adoptive transfer of immunity to Sudanese *L. donovani* was examined in athymic (*nu/nu*) recipients of the BALB/c strain (33). Athymic mice were unable to control infection with *L. donovani*, in contrast to euthymic (*nu/+*) mice of the same strain. In that case, L3T4$^+$-cell-enriched spleen preparations, alone, from infected euthymic mice failed to restore the resolution of visceral infection in athymic recipients. Only recipients that were reconstituted with unfractionated spleen cells containing both L3T4$^+$ and Lyt-2$^+$ cells were able to regain the ability to control infection. In the same study, further evidence in support of a dual role for L3T4$^+$ and Lyt-2$^+$ cells in the control of Sudanese *L. donovani* infection was shown by experiments in which euthymic mice were treated with T-cell subset-specific antibodies during infection. Mice that were treated with either anti-L3T4 or anti-Lyt-2 antibodies during the course of infection demonstrated higher liver parasite loads 8 weeks after infection than control mice treated with irrelevant antibody or medium did (33). The results of two other recent studies, on the other hand, have suggested a major role for Lyt-2$^+$ cells in the control of visceral leishmaniasis. Lyt-2$^+$ cells were identified by immunochemical methods as the main population of T cells in the livers of euthymic mice during resolution of infection (18). Limiting dilution analysis of the adoptive transfer of antigen-specific delayed-type hypersensitivity in unprimed recipients demonstrated that the T cells present in the livers of B10 mice during resolution of parasite load were exclusively of the Lyt-2$^+$ phenotype (15). The discrepancy in the results obtained by us and those of others may perhaps be explained by differences in the experimental methods, adoptive transfer models (recipient and donor), and strains of parasite used in the various studies. It is of interest to note that synergy between T-cell populations in the resolution of Sudanese *L. donovani* infection was suggested in a much earlier study by Skov and Twohy (32). This was shown by limiting dilution experiments in which constant minimal numbers of lymph node cells combined with graded numbers of thymocytes, or vice versa, conferred greater restoration of the cure response in T-cell-deprived C57BL/6 mice than expected from the additive effect of both cell types acting independently.

The present investigation provides direct proof for the existence in noncure B10.D2 mice of T cells that are capable of mediating protective immunity to *L. donovani*. The presence of protective cells was suggested earlier by experiments that showed a significant reduction in, and in some cases resolution of, liver parasite loads in noncure mice treated with sublethal irradiation or cyclophosphamide prior to infection (34). It would be of interest to examine further the immunological functions mediated by the L3T4$^+$ T-cell population that contribute to the resolution of disease in vivo in irradiated B10.D2 mice and also in C57BL/10 mice. At least two functions were reported previously for L3T4$^+$ cells, namely the production of gamma interferon (33) and interleukin-2 (17) in response to antigen. It is likely that these functions will be attributed also to the protective L3T4$^+$ cells in the present cure models, in view of earlier work that correlated the resolution of *L. donovani* infection with the production of antigen-specific interleukin-2 (23) and interferon-gamma (23, 33).

In view of the present findings and those reported previously, it appears thus far that the noncure response in B10.D2 mice is the final outcome of an interaction between T cells that mediate protective immunity (34; Fig. 6) and T suppressor cells that regulate the activity of these cells. T suppressor cells were identified previously in unirradiated B10.D2 mice by adoptive transfers in which cells from noncure mice overcame the resolution of disease in unprimed irradiated recipients that were challenged with *L. donovani* (5). Further support for interactions between regulatory T cells and T cells mediating immunity in the noncure response comes from a recent study by Blackwell and Roberts in which B10.D2/n mice were treated in vivo during *L. donovani* infection with monoclonal antibodies directed against I-region-determined molecules (4). Treatment of B10.D2/n mice with one antibody (anti-I-A) exacerbated disease, whereas treatment with the other (anti-I-E) enhanced the resolution of parasite load. Thus, both protective immunity and suppression of this immunity were identified in infected B10.D2 mice expressing the noncure phenotype. The present investigation has demonstrated that the cell that mediates protective immunity in irradiated B10.D2 mice shares the same L3T4$^+$, Lyt-1$^+$ phenotype as the cell that suppresses the resolution of disease in the noncure response (5). Evidence from recent studies has suggested that antigen-specific mouse helper T-cell clones with the L3T4$^+$ phenotype may be divided into two major groups, designated T_H1 and T_H2, distinguished functionally and by differences in lymphokine secretion profiles (10, 20). In a very recent study with L3T4$^+$ antigen-specific T-cell lines obtained from mice immunized with *Leishmania major*, the T-cell line that transferred protective immunity in mice demonstrated the T_H1 property of interleukin-2 and gamma interferon secretion, whereas the nonprotective line secreted interleukin-4 and interleukin-5, characteristic of T_H2 (29). It is possible, therefore, that the *H-2^d* noncure response in our results may be explained as the outcome of an interaction between protective T_H1 and regulatory T_H2 subsets that are induced during infection.

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