INFECTION AND IMMUNITY, June 1994, p. 2575–2581
0019-9567/94/$04.00+0

Vol. 62, No. 6

Expansion of Gamma Interferon-Producing CD8$^+$ T Cells following Secondary Infection of Mice Immune to *Leishmania major*

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Received 18 January 1994/Returned for modification 14 March 1994/Accepted 30 March 1994

Reinfection of immune mice with *Leishmania major* elicits a secondary gamma interferon (IFN-γ) response to which specific CD8$^+$ T cells are essential. We have shown previously that specific CD8$^+$ T cells from reinfected immune mice release substantially higher levels of IFN-γ, a cytokine essential for the efficient activation of parasitized macrophages to kill intracellular *L. major*. By using an ELISPOT assay, which allows the detection of IFN-γ production by individual cells, it is shown here that this elevated IFN-γ response is the result of an increase of up to 50-fold in the frequency of parasite-specific CD8$^+$ T lymphocytes in the spleens and draining lymph nodes of both immune reinfected CBA and BALB/c mice. This observation is additional evidence of the role that CD8$^+$ T cells play in immunity to reinfection with *L. major*.

Protozoans of the genus *Leishmania* are obligate intracellular parasites in their vertebrate hosts and replicate exclusively within the acidified phagolysosomes of macrophages. The genus *Leishmania* includes many species which produce a wide range of clinical diseases, from self-healing cutaneous to uncontrolled diffuse cutaneous disease, from mild to destructive mucosal disease, and from subclinical to fatal visceral disease. The spectrum of clinical manifestations of human leishmaniases can be mimicked in mice of different inbred strains following infection with *Leishmania major*, one of the causes of human cutaneous leishmaniasis. The majority of inbred strains of mice, such as CBA and C57BL/6, are resistant to infection, in that they develop only small lesions that resolve spontaneously, leaving the animal immune to reinfection. In contrast, mice from a few strains such as BALB/c are susceptible to infection and develop severe cutaneous lesions at the site of parasite inoculation and are not immune to reinfection (1, 13).

In a paradigmatic comparison, the outcome of infection, healing or uncontrolled progressive disease, is thought to be determined by the interactions of parasitized macrophages, T lymphocytes, and the resultant cytokine milieu. Because of its ability to activate macrophages to a microbicidal state, gamma interferon (IFN-γ) is one of the key lymphokines accounting for the host's ability to control infection with *L. major* (2, 37, 40). In cutaneous and visceral infections, there is evidence that IFN-γ plays a crucial role in the host's ability to control the parasite load. It is clear that CD4$^+$ T cells and the IFN-γ that they produce significantly contribute to the resolution of cutaneous lesions resulting from a primary infection with *L. major* (17, 18). CD8$^+$ T cells, however, are also important in mediating resistance to reinfection with *L. major* and *Leishmania donovani* (25, 27, 29).

We have shown recently that reinfection of healed *L. major*-immune mice triggers a secondary IFN-γ response to which CD8$^+$ T cells contribute. Indeed, following reinfection, CD8$^+$ T cells present in the lymphoid tissues of immune mice produced significantly higher levels of IFN-γ upon specific stimulation in vitro than those from immune but unchallenged mice (26). In the present report, the frequency of IFN-γ-producing CD8$^+$ T cells was estimated by an ELISPOT assay to determine whether this increased production of IFN-γ by CD8$^+$ T cells from immune reinfected mice was the result of the release of higher levels of IFN-γ by individual cells or the result of an expansion of IFN-γ-producing CD8$^+$ T cells. The results show that reinfection of immune mice with *L. major* leads to a rapid increase in the number of parasite-specific IFN-γ-producing CD8$^+$ T cells. Following this expansion, they transiently recirculate in the blood, the compartment from which they are presumably recruited in the reinfected site.

MATERIALS AND METHODS

Mice. Adult CBA/J and BALB/c mice were obtained either from the animal colony maintained at the Institut Pasteur, Paris, France, from IFFA-Credo, Saint Germain-sur-l'Arbresle, France, or from HARLAN, OLAC, Zeist, The Netherlands.

Parasites. *L. major* LV 39 (MRHO/SU/59/P strain) was maintained in the virulent state by monthly passage in mice. Parasites isolated from the skin lesions of infected mice were grown at 26°C in Dulbecco's modified Eagle's medium over rabbit blood agar (20).

Infections and monitoring of lesions. Primary infection with 2×10^6 stationary-phase *L. major* promastigotes in a volume of 50 μl was performed by subcutaneous injection into the left hind footpad. Lesion development was monitored weekly by measuring the increase in footpad thickness (the infected footpad compared with the uninfected contralateral footpad). Secondary infections were initiated only after complete resolution of the primary lesions, with 2×10^6 stationary-phase *L. major* promastigotes in 50 μl injected subcutaneously into the contralateral footpad.

MAbs. Rat monoclonal antibody (MAb) GK 1.5 (immunoglobulin G2b [IgG2b]; anti-CD4) (10) and MAb 3.168.8.1 (31M) (IgM; anti-CD8) (33) were kindly provided by F. W. Fitch, University of Chicago, Chicago, Ill. Rat MAb H35.17.2 (IgG2b; anti-CD8) (31) was kindly supplied by M. Pierres, Institut National de la Santé et de la Recherche Médicale/

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Centre National de la Recherche Scientifique, Marseille, France. Rat MAb AN-18.17.24 (anti-mouse IFN-γ) was a gift of S. Landolfo, University of Torino, Torino, Italy (32). Rat MAb R4-6A2 (IgG1; anti-mouse IFN-γ) (36) was obtained by G. Spitalny. Rat MAb RL 172.4 (IgM; anti-CD4) (6) was a gift of H. R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland. Hamster MAb 145.2C11 (anti-CD3) (16) was also obtained from the Ludwig Institute for Cancer Research.

Induction of resistance to *L. major* infection in otherwise susceptible BALB/c mice by treatment with anti-CD4 MAb in vivo. Anti-CD4 MAb GK 1.5 (600 μg) was injected intraperitoneally over a period of 24 h in 1 ml of phosphate-buffered saline (PBS) into susceptible BALB/c mice within the first 12 days of infection (27).

T-cell-subset depletion. Single-cell suspensions (5×10^7 cells ml^{-1}) were incubated with a 1/10 dilution of hybridoma culture supernatant containing monoclonal IgM directed against CD4 (RL 172.4) or CD8 (3.168.8.1 [31M]) T-cell surface antigens and a 1/10 dilution of a Low-Tox rabbit complement (Cedarlane, Hornby, Ontario, Canada). Control cell suspensions were treated with complement only. The T-cell-subset-depleted suspensions were washed three times before use, and the efficacy of killing was determined by fluorescence-activated cell sorter (FACS) analysis.

Two-color fluorescence staining and FACS analysis. Samples of 10^6 cells were stained with a mixture of phycoerythrin-labelled anti-CD4 MAb (GK 1.5; Becton Dickinson, Mountain View, Calif.) and 5-(4,6-dichlorotriazinyl)aminofluorescein-conjugated anti-CD8 MAb (H35.17.2). Samples were analyzed with a flow microfluorometer (FACS II; Becton Dickinson) gated by a combination of narrow-angle forward light scatter and perpendicular light scatter to exclude nonviable cells (21).

Isolation of mononuclear cells from blood samples. Blood samples were collected from the retro-orbital sinus. After the blood samples were mixed with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered RPMI 1640 medium containing 20 U of heparin ml^{-1}, 3 ml of the diluted blood sample was carefully overlaid on 3 ml of Ficoll-Paque (Pharmacia Fine Chemicals) in a 15-ml centrifuge tube. Cells were then centrifuged at 1,200 × *g* at 18°C for 30 min. Mononuclear cells were aspirated from the middle layer with a Pasteur pipette and were washed twice, once at 18°C and then at 4°C in 20 mM HEPES-buffered RPMI 1640 medium (7).

Lymphocyte cultures. Unseparated or T-cell-subset-depleted spleen and lymph node cell suspensions (5×10^6 ml^{-1}) were stimulated in the presence or absence of 4×10^6 live *L. major* promastigotes ml^{-1} (rendered unable to replicate by 2- to 5-min irradiation with UV) or 30 U of recombinant mouse interleukin-2 (IL-2) per ml at 37°C and 7% CO_2 in 24-well Costar plates in a final volume of 1 ml per well. Recombinant mouse IL-2 produced by X63Ag8-653 plasmacytoma cells was a gift from F. Melchers, Basel Institute for Immunology, Basel, Switzerland (14). Dulbecco's modified Eagle's medium (Seromed, Berlin, Federal Republic of Germany) supplemented with 5% heat-inactivated fetal calf serum (Seromed), L-asparagine (36 mg $liter^{-1}$), L-glutamine (216 mg $liter^{-1}$), L-arginine (200 mg $liter^{-1}$), 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} was used as the culture medium. Cells were cultured overnight (16 to 20 h), centrifuged, resuspended in fresh medium (5×10^6 ml^{-1}), and transferred to 96-well microtiter plates for the ELISPOT assay (see below).

Detection of IFN-γ-producing T cells with the ELISPOT assay. The basic ELISPOT assay method devised by Sedgwick and Holt (34) and Czerkinsky et al. (8) has been modified to detect IFN-γ-secreting cells by using a pair of anti-IFN-γ MAbs described by Slade and Langhorne (35). Microtiter plates (96 well) (Maxisorp; Nunc) were coated overnight with 5 μg of affinity-purified rat anti-mouse IFN-γ MAb R4-6A2 ml^{-1} in PBS, pH 7.2, at 4°C. Free binding sites were blocked with 1% (wt/vol) bovine serum albumin (Fluka, Buchs, Switzerland) for 2 h at 37°C. Plates were then washed three times with PBS containing 0.01% Tween 20, and the cells cultured overnight (see above) were added at a starting concentration of 5×10^6 cells ml^{-1}. From each group of cultured cells, 8 serial twofold dilutions were prepared in duplicate in a final volume of 0.1 ml and incubated for 4 h at 37°C and 7% CO_2. The specific IFN-γ-producing cells were removed by three washes, and biotin-labeled purified rat anti-mouse IFN-γ MAb AN-18.17.24 at 1 μg ml^{-1} in PBS containing 1% bovine serum albumin was added to each well and incubated overnight at 4°C. After three more washes, the site of cytokine secretion was revealed by the addition of alkaline phosphatase-conjugated streptavidin (Boehringer, Mannheim, Federal Republic of Germany), diluted 10^{-3} from stock in PBS, for 2 h. After three more washes, the enzyme reaction was developed with 5-bromo-4-chloro-3-indolylphosphate at 1 mg ml^{-1} in 2-amino-2-methyl-1-propanol buffer (1 M) (pH 10.25) for a maximum of 2 h at 37°C. The plates were then washed with ultrapure water and dried, and the blue spots were counted microscopically. The colored spots reveal areas of secretion by single cells; the number of spots was used to assess the frequency of IFN-γ-producing cells in murine lymphoid tissues (38).

For all cells used in these experiments, the number of T cells present in the culture was determined by FACS analysis at the time of stimulation and the frequency of IFN-γ-producing T cells was estimated by dividing the number of T cells put in culture by the number of spots. For comparison, the results are also expressed as number of spots present per 10^5 T cells. The relationship between the number of cells per well and the number of spots was linear, and *y* intercept was near the origin.

DTH. The delayed-type hypersensitivity (DTH) response was used to demonstrate the presence of sensitized T cells in immune mice in vivo. DTH was determined 24 h after reinfection by subcutaneous injection of 2×10^6 stationary-phase *L. major* promastigotes into the contralateral footpads of immune BALB/c and CBA mice by measuring the degree of footpad swelling with a dial gauge calliper (Kröplin, Schlüchtern, Federal Republic of Germany).

Estimation of the frequency of *L. major*-specific T cells capable of mediating specific DTH reaction. *Leishmania*-reactive T cells are detectable in vivo through their ability to locally transfer a transient inflammatory reaction known as DTH. This effector function was used to estimate the frequency of *Leishmania*-reactive T cells. Briefly, three different dilutions of mononuclear blood cells from immune or immune, reinfected mice were coinjected in the hind footpads of 10 to 12 syngeneic recipients in the presence or absence of 2×10^6 *L. major* promastigotes. Unsensitized lymphoid cells or those sensitized to an unrelated antigen (sheep erythrocytes) were used as controls (data not shown). The frequencies of *Leishmania*-specific DTH-competent T cells were estimated as previously described (24).

Parasite burden. The number of living *L. major* in infected tissues was determined by the parasite limiting dilution assay described by Titus et al. (41). Briefly, different dilutions of infected-tissue homogenates were distributed in wells of microtiter plates containing rabbit blood agar slants. After 10 to 14 days of incubation at 26°C, the wells containing growing promastigotes were identified by microscopic examination.

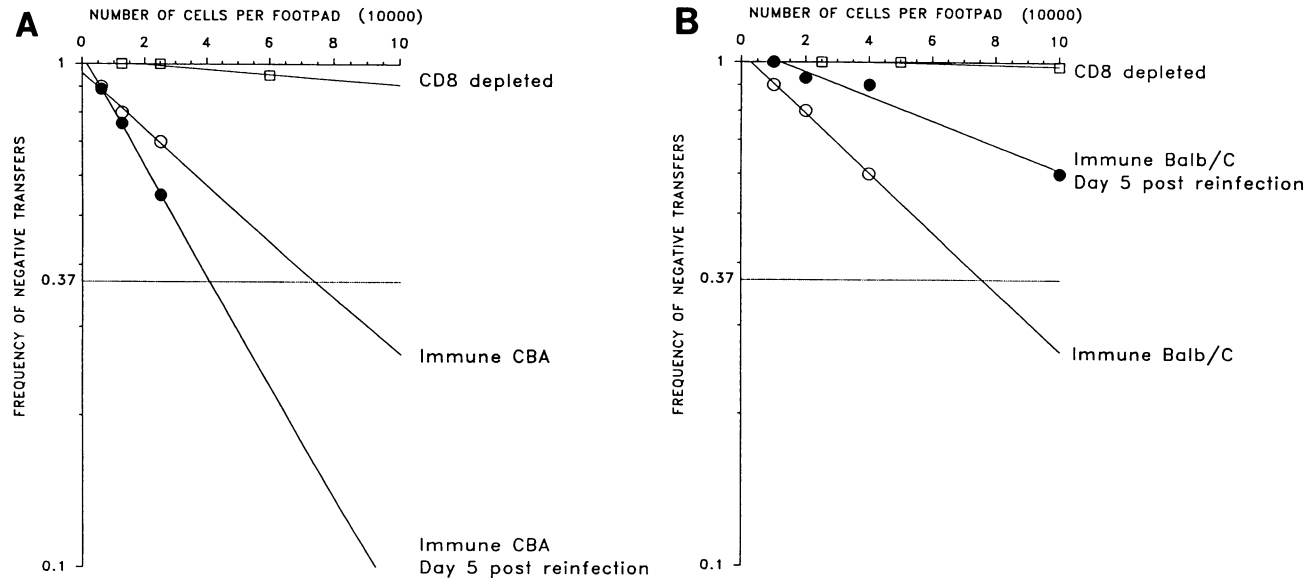

FIG. 1. Frequencies of recirculating *Leishmania*-reactive T cells, capable of transferring DTH reactions, in blood samples from immune mice before and after secondary infection. Mononuclear cells were prepared from diluted blood samples centrifuged on a Ficoll-Hypaque cushion. Once washed and serially diluted, each mononuclear cell dilution was mixed with live stationary-phase *L. major* promastigotes, and the resulting suspension was injected into one footpad of syngeneic naive mice (12 to 20 mice per cell dilution). Sixteen to 18 h later, negative DTH transfers were scored and frequency estimation of T cells able to transfer DTH locally in the presence of live *Leishmania* was calculated by the minimal chi-squared method defined by Taswell. Before transfer, blood mononuclear cells were treated with complement alone (control) or with either MAb 172 IgM (anti-CD4) or 31M IgM (anti-CD8) MAb plus complement. As shown in panels A and B, once depleted of CD8$^+$ T cells, no *Leishmania*-reactive T cells are detectable in the blood mononuclear cell suspension.

The frequency of *L. major* recovered from footpads where the cutaneous lesions occurred was determined by minimum χ^2 analysis applied to the Poisson distribution.

RESULTS

***Leishmania*-reactive T cells belonging to the CD8$^+$ T-cell subset recirculate in mice which have resolved a primary infection with *L. major*.** The local adoptive transfer of a DTH reaction can be used to detect and estimate the presence and frequency of antigen-specific recirculating T cells in vivo. Such an assay allows detection of antigen-specific T cells through their ability to be reactivated by antigen-presenting cells present in the skin, the optimal microenvironment for multiplication of *L. major*. Therefore, this assay was chosen, using live *L. major* promastigotes as a recall antigen. The frequency of *Leishmania*-reactive T cells and their phenotypes were determined in blood samples from mice which had resolved a primary lesion induced by *L. major*. The results in Fig. 1 show that both in resistant CBA mice (Fig. 1A) that had spontaneously resolved their primary cutaneous lesions and in susceptible BALB/c mice (Fig. 1B) that were rendered resistant by injection of 600 μg of anti-CD4 MAb early during infection (28), *Leishmania*-reactive T cells are indeed present in the blood. Interestingly, they all belong to the CD8$^+$ T-cell subset.

Five days after reinfection of these mice, similar frequencies of parasite-reactive CD8$^+$ T cells circulating in the blood were observed (Fig. 1). Considering that the only *Leishmania*-reactive T cells we could detect in circulation with this assay were CD8$^+$ and since we have found previously that in immune reinfected mice, CD8$^+$ T cells contribute to the secondary IFN-γ response, it was deemed essential to determine the number of CD8$^+$ T cells able to produce IFN-γ in the spleens and draining lymph nodes of immune mice after challenge with virulent promastigotes.

Reinfection of immune mice results in an expansion of IFN-γ-producing T cells in spleens and lymph nodes. An ELISPOT assay, which allows the detection of IFN-γ production by single cells, was used in order to assess directly the expansion of *Leishmania*-specific T cells that were able to release IFN-γ following reinfection. The validity of this assay for the determination of the frequency of cells was first confirmed by determining whether the number of IFN-γ-producing cells within an activated lymphocyte population was directly proportional to the number of cells. As shown in Fig. 2, upon stimulation with anti-CD3 MAb, the number of IFN-γ-producing cells increased linearly with the number of cells. By using this method, it was possible to confirm the presence of parasite-reactive T cells that were able to produce IFN-γ in blood samples from BALB/c and CBA mice which had resolved their primary lesions (Table 1).

The ELISPOT assay was used to determine whether the secondary IFN-γ response observed in reinfected, immune BALB/c and CBA mice was the result of an expansion of parasite-specific IFN-γ-producing T cells. Results shown in Fig. 3 demonstrate that in immune CBA mice, the number of T cells producing IFN-γ in response to *L. major* is increased 13 times in the draining lymph nodes and three times in the spleen 5 days after reinfection (Fig. 3A). This expansion was less pronounced in the lymphoid organs of immune BALB/c mice at the same time after reinfection (Fig. 3B). Similar results were obtained in three independent experiments.

Reinfection of immune mice results in increased numbers of parasite-specific IFN-γ-producing CD8$^+$ T cells. The increased frequency of parasite-specific IFN-γ-producing T cells in lymphoid organs of reinfected mice (Fig. 3), together with

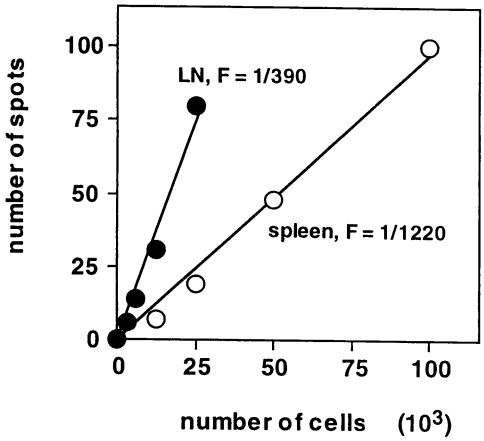

FIG. 2. The number of IFN-γ-producing cells in the ELISPOT assay is proportional to the number of cells. Susceptible BALB/c mice were infected subcutaneously in the hind footpad with 2×10^6 virulent *L. major* promastigotes. Popliteal lymph node (LN) and spleen cells were removed 40 days after infection. Unseparated cells (10^7) from both organs were stimulated overnight with 10 μg of affinity-purified anti-CD3 MAb (145.2C11) ml^{-1}, centrifuged, resuspended in fresh medium (5×10^6 ml^{-1}), and the IFN-γ-secreting cells were determined by the ELISPOT assay, as described in Materials and Methods. The number of spots were determined microscopically. The difference between replicate cultures was less than 10%. F, frequency.

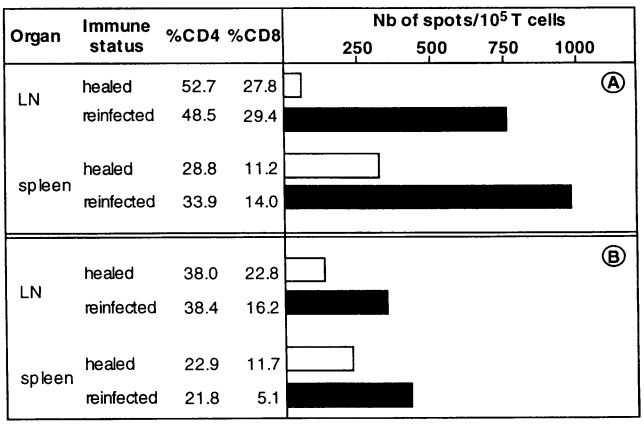

FIG. 3. Reinfection of immune mice results in increased numbers of antigen-specific T lymphocytes producing IFN-γ. The frequencies of IFN-γ-secreting T cells in the lymphoid organs of healed, immune CBA and BALB/c mice were determined before (open bars) and 5 days after reinfection (filled bars) with *L. major* promastigotes. Draining lymph node (LN) or spleen cells (10^7) were stimulated overnight with 4×10^6 live *L. major* promastigotes ml^{-1} (rendered replication-incompetent by 2- to 5-min irradiation with UV), centrifuged, resuspended in fresh medium (5×10^6 ml^{-1}), and the IFN-γ-secreting cells were determined by the ELISPOT assay. The difference between replicate cultures was less than 10%. In unstimulated control cultures, it was not possible to determine frequencies. The number of T cells ($CD4^+$ and $CD8^+$) was determined by FACS analysis at the time of stimulation. The number (Nb) of spots determined in immune and reinfected CBA mice (A) and BALB/c mice (B) is shown. Before reinfection, the number of viable parasites detected in the footpads of healed, immune CBA mice and healed experimentally immune BALB/c mice were 10 and 3, respectively. After reinfection with 2×10^6 virulent *L. major* promastigotes, the DTH response, as measured by footpad swelling, of reinfected CBA mice was 1.08 ± 0.05 mm and that of reinfected BALB/c mice was 1.07 ± 0.1 mm 24 h. Five days after reinfection into the contralateral footpad, the challenged site contained 2.8×10^4 parasites in CBA mice and 2.3×10^5 live parasites in BALB/c mice, as determined by parasite limiting dilution analysis.

the previous demonstration that $CD8^+$ T cells contribute to the enhanced level of IFN-γ released upon reinfection of immune mice (26), prompted us to assess whether the higher IFN-γ production by $CD8^+$ T cells also resulted from an expansion of T cells from this subset following reinfection. Freshly isolated spleen and lymph node cells obtained from immune mice 5 days after reinfection were depleted of $CD4^+$ T cells before stimulation with live *L. major* promastigotes and IFN-γ spot number determination. In all experiments, the percentages of $CD4^+$ and $CD8^+$ cells were determined by FACS analysis before and after removal of $CD4^+$ T cells. The results in Fig. 4A show that 5 days after reinfection of immune CBA mice, the number of IFN-γ-producing $CD8^+$ T cells in the draining lymph nodes was increased 50-fold (compared to the number of IFN-γ-producing $CD8^+$ T cells before reinfection). In immune BALB/c mice, a similar expansion of IFN-γ-producing $CD8^+$ T cells was observed in the draining lymph nodes 5 days after reinfection (Fig. 4B). This expansion of $CD8^+$ T cells was also observed in the spleens of both immune CBA and BALB/c mice 5 days after infectious challenge. Similar results were obtained in three independent experiments.

TABLE 1. Frequencies of IFN-γ-producing cells in blood samples from *L. major*-immune mice[a]

Source of cells	Stimulation of cells	% T cells		Frequency of IFN-γ-producing cells	No. of spots/10^5 cells
		$CD4^+$	$CD8^+$		
Immune CBA mice	APC[b] + *L. major*	58.5	14.7	1/1,005	100
	APC			ND[c]	ND
	L. major			ND	ND
Immune BALB/c mice	APC + *L. major*	60.8	13.1	1/1,610	62
	APC			ND	ND
	L. major			ND	ND

[a] After complete resolution of the primary lesions, mononuclear cells (1.5×10^6 ml^{-1}) isolated from diluted blood samples from *L. major*-immune CBA ($n = 11$) and BALB/c ($n = 20$) mice were stimulated overnight with 4×10^6 live UV-inactivated *L. major* promastigotes ml^{-1} in the presence of 5×10^6 irradiated syngeneic spleen cells ml^{-1}. Cultures of mononuclear cells stimulated with either parasites or irradiated antigen-presenting cells alone were used as controls, and the frequency of IFN-γ-producing cells was determined by the ELISPOT assay. The number of $CD4^+$ and $CD8^+$ T cells in the blood-derived cell suspensions was determined by FACS analysis at the time of stimulation. The difference between replicate cultures was less than 10%.

[b] APC, antigen-presenting cells.

[c] ND, not detectable.

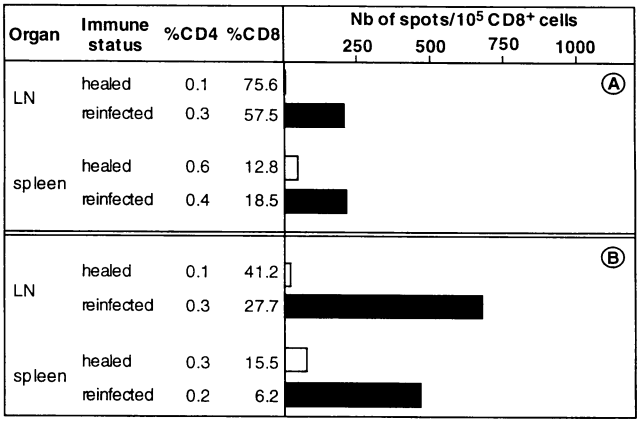

FIG. 4. Reinfection of immune mice results in increased numbers of parasite-specific IFN-γ-producing CD8+ T cells. The frequency of IFN-γ-secreting CD8+ T cells in lymphoid organs of healed, immune CBA and BALB/c mice was determined before (open bars) and 5 days after reinfection (filled bars) with *L. major* promastigotes. CD8+ T cells were obtained by treatment of spleen or lymph node (LN) cell suspensions with anti-CD4 MAb (172.4) and complement. After the killing of CD4+ T cells, the surviving lymph node or spleen cells (10^7) were stimulated overnight with 4×10^6 live UV-inactivated *L. major* promastigotes ml^{-1} and 30 U of recombinant mouse IL-2 ml^{-1}. Cells were collected, centrifuged, resuspended in fresh medium (5×10^6 ml^{-1}), and the IFN-γ-secreting cells were determined by the ELISPOT assay. The frequency was estimated as described in Materials and Methods, and the data are expressed as the number of spots per 10^5 CD8+ T cells. The difference between replicate cultures was less than 10%. In control cultures, containing either IL-2 alone or antigen alone, no frequency could be determined. The efficacy of the elimination of CD4+ T cells and the numbers of CD8+ T cells were determined by FACS analysis at the time of stimulation. The number (Nb) of spots determined in immune and reinfected CBA mice (A) and BALB/c mice (B) is shown. For details on the DTH response and parasite load of these mice, see the legend to Fig. 3.

DISCUSSION

In the present study, the expansion of parasite-specific IFN-γ-producing T cells in lymphoid organs of immune reinfected mice was determined by an IFN-γ-specific ELISPOT assay, which allows the detection of cytokine secretion at the single cell level (39) and thus the evaluation of the relative proportion of cells in a given population that specifically produce IFN-γ. By using this technique, it was first shown that the frequency of *Leishmania*-specific T cells able to produce IFN-γ was higher in the lymphoid organs of both strains of immune mice 5 days after reinfection (Fig. 3). Moreover, the number of IFN-γ-producing CD8+ T cells in the draining lymph nodes as well as in the spleens was significantly higher in immune mice from both strains at this time after infectious challenge (Fig. 4). Thus, these data show that the elevated production of IFN-γ elicited in both immune CBA and experimentally immune BALB/c mice after a secondary challenge with *L. major* is due to an increase in the frequency of responding memory cells, including CD8+ T cells in both lymph nodes and spleens. Although some contribution of other cells, such as double negative α/β+ T cells, γ/δ+ T cells, or NK cells, to the observed IFN-γ response cannot be formally excluded, the antigen dependence of the response makes it unlikely that NK cells or γ/δ+ cells are involved to a significant degree (26). The cell depletion experiments shown in Fig. 1 indicate that the responding cells are CD8+ T cells.

CD8+ T cells and IFN-γ are both involved in the secondary response to *L. major* infection in vivo, since neutralization of endogenous IFN-γ and depletion of CD8+ T cells at the time of reinfection results in a clear reduction of the DTH response (26). *Leishmania*-specific T cells, able to mediate DTH, circulate in the blood of experimentally immune BALB/c mice (27). The frequency of recirculating parasite-reactive T lymphocytes was estimated in vivo by a very sensitive limiting dilution approach (24), which allows the detection of single antigen-specific, DTH-competent T cells. The local adoptive transfer of *Leishmania*-specific DTH was used to determine whether the recall of immunological memory by secondary infectious challenge of immune mice increases the frequency of *Leishmania*-specific cells circulating in the blood. In blood samples from both immune and reinfected mice of the two strains, similar frequencies of *Leishmania*-reactive T cells were found (Fig. 1). Interestingly, and in agreement with our previous results (27), all the DTH-competent effector cells circulating in the blood belong to the CD8+ T-cell subset (Fig. 1). There is relatively little known concerning the recirculation properties of CD8+ memory T cells. The unmodified frequency of DTH-mediating cells in the bloodstream 5 days after reinfection could be due to rapid homing and sequestration of such cells to the lesion (22).

Implicit in the data presented here is that CD8+ T cells, able to recognize *Leishmania*-derived peptides in context with major histocompatibility complex (MHC) class I molecules, are primed during infection and can subsequently be recalled by reinfection of immune mice with *L. major*. This obligate intracellular parasite of the mammalian macrophage has never been shown to exit from the phagolysosome and would thus be considered as exogenous antigen, unlikely to be presented in the context of MHC class I. Although the segregation of endogenous and exogenous antigens into the class I and class II pathways of antigen presentation is generally accepted, many exceptions to this rigid separation have been reported (9, 15, 19, 30, 43).

In experimental leishmaniasis, parasite-specific CD8+ T cells have not been shown to lyse infected target cells directly; rather, they contribute to host defense via the cytokine network. In addition to its ability to activate parasitized macrophages (2), IFN-γ, secreted by specifically activated cells, also influences the cytokine milieu where immune effectors are induced and express their functions (11, 12). Cross-regulation between CD4+ and CD8+ T cells via cytokines may decisively influence the nature of the anti-*Leishmania* immune response and thus the outcome of infection. For example, the specific stimulation of MHC class II-restricted CD4+ cells could result in the release of IL-2 necessary for the activation of CD8+ T cells. Indeed, our previous experiments (26) have shown that exogenous IL-2 is required for optimal stimulation of parasite-specific CD8+ T cells in vitro. One might also speculate that the IFN-γ released by antigen-specific CD8+ T cells, with the help of cytokines provided by CD4+ T cells, could deviate the immune response away from the antibody-producing humoral arm toward a cell-mediated response (5, 42) by favoring the outgrowth of IFN-γ-secreting CD4+ Th1 cells (23).

Although the contribution of *Leishmania*-specific CD8+ T cells to immunity to reinfection has been demonstrated (25, 26), their importance during a primary infection is not well characterized. CD8+ T cells might well be active in the early phase of the infection, inhibiting the induction of IgG response and simultaneously favoring the expression of a strong cell-mediated response, as proposed in the theory of immune class regulation elaborated by Bretscher (3–5).

In conclusion, the enhanced levels of IFN-γ released during a memory response to *L. major* by antigen-specific CD8+ T

cells from the lymph nodes and spleen cells of immune, reinfected mice are due to an expansion of those cells in the lymphoid organs. A more detailed knowledge of the kinetics of appearance and recirculation behavior of antigen-specific $CD8^+$ T cells in secondary *Leishmania* infection is essential for the understanding of their role in vivo.

ACKNOWLEDGMENTS

This work was supported by grants from the Swiss National Science Foundation (32/28872.90), from ARC (6369), and from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) to G. Milon.

We thank Kristin Swihart for critical reading of the manuscript and Robert Etges for many helpful discussions.

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