INFECTION AND IMMUNITY, July 1997, p. 2837–2845 Vol. 65, No. 7
0019-9567/97/$04.00+0

An In Vitro Model for Infection with *Leishmania major* That Mimics the Immune Response in Mice

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Received 3 February 1997/Returned for modification 5 March 1997/Accepted 18 April 1997

By using a primary in vitro response specific for *Leishmania major*, normal T cells from resistant CBA/CaH-T6J and susceptible BALB/c mice commit to a Th1 and a Th2 response, respectively. Since commitment occurred, we measured the production of gamma interferon (IFN-γ), interleukin-1 (IL-1), IL-2, IL-4, IL-5, IL-10, and IL-12, prostaglandin E_2 (PGE_2), transforming growth factor β (TGF-β), and nitric oxide in the first 7 days of the response to identify factors that are critical for Th1 and Th2 development. While cells from resistant CBA mice produced more IFN-γ, IL-10, and nitric oxide, cells from susceptible BALB/c mice produced more IL-1α, IL-5, PGE_2, and TGF-β. Although substantial amounts of IL-12 were detected, IL-12 did not associate with either Th1 or Th2 development. We did not anticipate that cells from resistant CBA mice would make more IL-10 in vitro. However, this also occurred in vivo since CBA mice produced substantial amounts of IL-10 following infection with *L. major*. Moreover, adding anti-IL-10 to primary in vitro responses enhanced production of IFN-γ and nitric oxide by cells from CBA and BALB/c mice. Therefore, IL-10 cannot be regarded as a cytokine that associates with susceptibility to infection with *L. major*. Finally, the data presented here suggest that a collection of factors that can be produced by accessory cells influence Th commitment (e.g., IL-1, PGE_2, and TGF-β favor Th2 development).

Since the discovery of CD4 T cells, one of the major focuses of immunology has been to elucidate the steps that lead to priming of the cells. With the description of the CD4 subsets Th1 and Th2, it became clear that cytokines play a key role in this priming. Th1 and Th2 cells produce distinct panels of cytokines: for example, Th1 cells secrete gamma interferon (IFN-γ) and interleukin-2 (IL-2), and Th2 cells secrete IL-4 and IL-5 (37). Cytokines can cross-regulate each others' production, and this influences the activities of Th1 and Th2 cells. Moreover, other cells of the immune system, such as macrophages (Mϕ), also produce cytokines and factors (e.g., IL-10, IL-12, prostaglandin E_2 [PGE_2], and transforming growth factor β [TGF-β]) that can influence the activities of T cells.

Experimental murine cutaneous leishmaniasis induced by *Leishmania major* is one of the best-studied models in which selective activation of Th1 or Th2 cells occurs (reviewed in reference 59). In resistant mice (C57BL/6 is the most commonly used strain), IFN-γ is the principal mediator of resistance to *L. major* due to its ability to activate Mϕ to destroy the parasite (38, 39, 44, 58), and treating mice with a neutralizing anti-IFN-γ antibody exacerbates the course of infection by promoting the outgrowth of Th2 cells (6, 48). In susceptible mice (BALB/c), IL-4 can block the ability of IFN-γ to activate Mϕ to destroy *Leishmania* (25, 27), and treating mice with a neutralizing anti-IL-4 antibody allows the animals to cure their infection by promoting the outgrowth of Th1 cells (10, 43). Importantly, to be effective, either antibody treatment must be initiated within the first week of infection, suggesting that events occurring early in the priming of CD4 T cells lead to commitment to either Th1 or Th2.

To elucidate the mechanism(s) that leads to selective priming of Th1 and Th2 cells, one needs to determine the factors that cause naive T cells to commit to the Th1 or Th2 lineage. However, in almost all cases, the frequency of a given T-cell antigen specificity is low in a naive experimental animal. As a result, lymphoid tissue from a naive animal will normally not respond to stimulation with antigen in vitro. This fact forced workers to analyze immune responses in intact animals. However, since it is probable that multiple factors contribute to early priming and commitment of CD4 cells to either the Th1 or Th2 phenotype, defining all of these factors in vivo would be quite difficult. Therefore, an in vitro model for infection would be useful. Recently, we described a primary in vitro (PIV) assay system that yields large numbers of *L. major*-specific, CD4, major histocompatibility complex class II-restricted T cells from lymphoid tissues of naive BALB/c or C57 mice (51). The cell surface phenotype and restriction of these PIV T cells are identical to those of the T cells that respond in mice infected with *L. major.*

We also determined the cytokines that these PIV-derived T cells produced when they were restimulated after 7 days of priming. We found that PIV T cells derived from BALB/c and C57 produced nearly identical amounts of IL-2, IL-4, and IFN-γ (51). This observation would predict that although the C57 mouse is used as a resistant model for infection with *L. major*, the mouse may be nearly as susceptible to infection with *L. major* as the BALB/c mouse is. In fact, this is the case. Regarding parasite burden in cutaneous lesions of *L. major*, Scharton and Scott (45) showed that C57 mice have parasite burdens almost equivalent to those of BALB/c mice. Moreover, Behin et al. (5) showed that when challenged with large doses of *L. major* ($>5 \times 10^6$), cutaneous lesions on C57 mice could resemble those on BALB/c mice (i.e., become chronic and ulcerated). On the other hand, Behin et al. (5) also showed that mice of the C3H or CBA background were highly resistant in that they developed only small lesions that healed rapidly even when the mice were challenged with a dose of *L. major* as

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high as 20×10^6. In addition, Scharton and Scott (45) showed that parasite burdens in C3H and CBA mice were low.

Therefore, in this study we examined cytokine production in the PIV response with cells from susceptible BALB/c and highly resistant CBA/CaH-T6J mice. In addition, for purposes of comparison to previous work of ourselves and others, we examined cytokine production in the PIV response with cells from C57 mice. Cytokine production by normal T cells from BALB/c and CBA mice differed greatly in the PIV system. Cells from BALB/c mice produced approximately 10-fold more IL-4 than CBA cells (i.e., BALB/c cells were Th2-like), and CBA cells produced approximately 10-fold more IFN-γ than BALB/c cells (i.e., CBA cells were Th1-like). Moreover, this cytokine response was identical to the response that BALB/c and CBA mice produced when infected with *L. major*. Therefore, since the PIV response was a valid in vitro correlate of *L. major* infection in mice, we measured the secretion of 10 factors in the PIV response (IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-10, IL-12, PGE_2, TGF-β, and nitric oxide [NO]) during the first 7 days of the response. This would be the time when commitment to Th1 or Th2 would occur; thus, this information would indicate the type of environment that leads to the development of a Th1 or Th2 response.

MATERIALS AND METHODS

Mice. Young adult, naive female mice were used. BALB/c and C57BL/6 mice were purchased from the National Cancer Institute (Frederick, Md.). CBA/CaH-T6J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine).

Parasites. Stationary-phase promastigotes of *L. major* (a clone of the LV39 [also known as LRC-L227 or R/SU/59/Neal P] isolate [31]) were used. Parasites were maintained as described previously (31).

PIV response for *L. major*. The assay was performed as previously described with slight modifications (51). Briefly, single-cell suspensions of splenocytes were prepared in Dulbecco's modified Eagle medium (51) supplemented with 5×10^{-5} M 2-mercaptoethanol and 0.5% normal mouse serum. Splenocytes were plated at 10^7 cells per well in 24-well plates with 10^6 promastigotes. At 1, 2, 4, and 7 days of culture, cell-free supernatants were harvested (the supernatants of five replicate wells were pooled for each time point), and the supernatants were tested for the production of cytokines, PGE_2, and nitrite (see below). For cytokine analysis after restimulation of PIV cells, T-cell blasts were isolated from 7-day PIV cultures on Percoll gradients (51). The blast cells were restimulated at 10^6 cells per well in 24-well plates in the presence of 10^6 promastigotes and 5×10^6 syngeneic irradiated spleen cells (900 rads) as a source of fresh antigen-presenting cells (APC). After 48 h, cell-free supernatants were collected and tested for IL-4 and IFN-γ production by enzyme-linked immunosorbent assays (ELISA; see below).

Analysis of infection in vivo. Animals were injected subcutaneously (s.c.) in one hind footpad with 2×10^6 *L. major* promastigotes. Resulting cutaneous lesions were monitored by determining the thickness of the infected footpad with a Digimatic caliper (Mitutoyo, Japan) and comparing this to the thickness of the uninfected contralateral control footpad. To determine cytokine production in vivo, draining popliteal and inguinal lymph nodes were removed after 3 and/or 7 days of infection. Single-cell suspensions were prepared as described above. Cells (5×10^6 per well in 1 ml of suspension) were cultured in 24-well plates in the absence or presence of 10^6 parasites. Cell-free supernatants were collected at 48 h of culture and analyzed for their content of IL-4, IL-10, or IFN-γ by ELISA (see below).

Cytokine, NO, and PGE_2 assays and anticytokines. Levels of cytokines were determined by sandwich ELISAs with published protocols (8, 52) or manufacturer's directions.

The sources of recombinant standards for the ELISAs were as follows: recombinant murine IFN-γ (rmIFN-γ), Genzyme (Cambridge, Mass.); rmIL-1α, Genzyme; rmIL-2, Pharmingen (San Diego, Calif.); rmIL-4, a gift of A. Glasebrook (Eli Lilly, Indianapolis, Ind.); rmIL-5, R & D Systems (Minneapolis, Minn.); rmIL-10, Pharmingen; rmIL-12, a gift of J. Sypek (Genetics Institute, Cambridge, Mass.); and recombinant human TGF-β1, R & D.

The sources of capture and detection antibodies for the ELISAs were as follows: IFN-γ, Pharmingen; IL-1α, Genzyme; IL-2, Pharmingen; IL-4, Pharmingen; IL-5, Pharmingen; and IL-10, Pharmingen.

The IL-12 ELISA was constructed with polyclonal sheep anti-mouse IL-12 (protein G purified, a gift of J. Sypek, Genetics Institute). This antiserum was used as the source of the capture antibody, and a biotinylated form of the antibody was also used as the detection antibody. The ELISA was developed with avidin-peroxidase (Sigma, St. Louis, Mo.).

The TGF-β ELISA used a mouse anti-bovine TGF-β1, -β2, and -β3 (Genzyme) as the capture antibody and a chicken anti-human TGF-β (R & D) as the detection antibody. The assay was developed with peroxidase-conjugated, affinity-purified rabbit anti-chicken immunoglobulin Y (Jackson Immuno Research Labs, West Grove, Pa.). Supernatants analyzed for TGF-β were acid treated before use in the ELISA to activate the TGF-β present in the supernatants. Acid treatment was performed according to directions provided by R & D Systems. TGF-β was not detected in supernatants that were not acid treated.

NO production was determined by measuring nitrite in supernatants by using the Griess reagent as described previously (19). PGE_2 was assayed as described elsewhere (60).

In all the ELISA, NO, and PGE_2 assays described above, samples were assayed in quadruplicate. The detection limits for the ELISAs and assay systems used were as follows: IL-1α, 4 pg/ml; IL-2, 40 pg/ml; IL-4, 10 pg/ml; IL-5, 10 pg/ml; IL-10, 50 pg/ml; IL-12, 50 pg/ml; IFN-γ, 1 U/ml; TGF-β, 40 pg/ml; NO, 1 μM; and PGE_2, 10 pg/ml. When supernatants from PIV responses were measured for cytokine levels, NO, or PGE_2, background responses (i.e., supernatants from cultures which were not stimulated with *L. major*) averaged between 0 and 39.6% of the signals obtained with supernatants from cultures stimulated with *L. major* with one exception: the PGE_2 levels were identical in unstimulated and stimulated cultures.

In some assays, the effect of adding a neutralizing anti-IL-10 antibody (5 μg/ml; JES5-2A5; Pharmingen) to the PIV system was assessed.

Statistical analyses. Data obtained with the PIV response were analyzed statistically to determine whether any differences observed were significant. Thus, for example, the levels of IFN-γ produced in PIV cultures with BALB/c, C57, or CBA cells were analyzed to determine whether the three strains of mice produced significantly different amounts of the cytokine. As might be expected, from PIV response to PIV response, the absolute levels of IFN-γ produced by the three strains varied, which was true for all the mediators studied. However, the relative differences in the levels of production of all the mediators studied was consistent from experiment to experiment. Therefore, the data of all replicate experiments with a given cytokine or molecule were analyzed with a Wilcoxon signed-rank nonparametric analysis program (StatView 4.02; Abacus Concepts, Berkeley, Calif.). Differences were considered significant if $P \leq 0.05$.

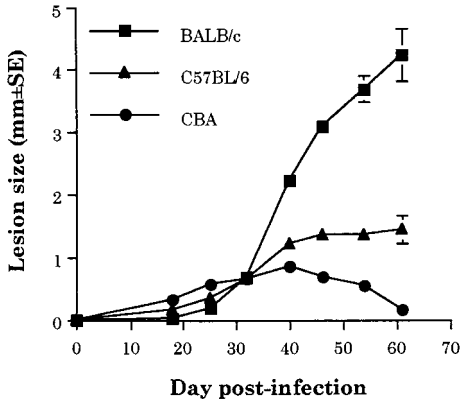

FIG. 1. Comparison of the responses of different mouse strains to *L. major* in vivo. Groups of five BALB/c, C57BL/6, or CBA/CaH-T6J mice were infected with 2×10^6 stationary-phase promastigotes s.c. in one hind footpad. Lesion development was monitored as detailed in Materials and Methods until the severity of BALB/c lesions necessitated sacrificing the animals. SE, standard error of the mean.

RESULTS

Comparing the responses of different mouse strains to *L. major* in vivo and in the PIV response. We selected three mouse strains that vary in their relative resistance to infection with *L. major* (Fig. 1). When infected with 2×10^6 *L. major* promastigotes, BALB/c mice were susceptible and developed uncontrolled cutaneous lesions that became necrotic and ulcerated. In contrast, CBA/CaH-T6J mice healed their lesions rapidly. C57BL/6 mice healed their lesions more slowly and still bore cutaneous lesions at the end of the 2-month observation period (Fig. 1). In general, we find that C57 mice infected with 2×10^6 *L. major* promastigotes take 80 to 100 days to resolve *L. major* lesions.

We next tested the behavior of spleen cells from each of

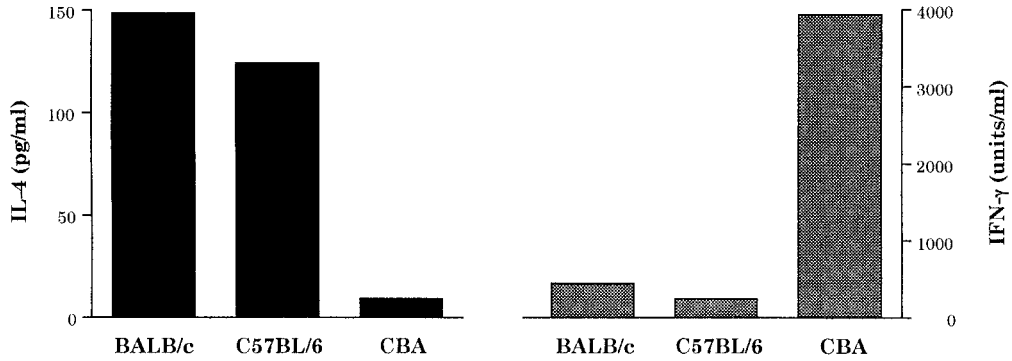

FIG. 2. IL-4 and IFN-γ production by PIV cells restimulated with *L. major* in vitro. T-cell blasts from BALB/c, C57, and CBA PIV responses were isolated after 7 days of priming and restimulated with *L. major* as described in Materials and Methods. After 48 h, supernatants were harvested and assayed by ELISA for IL-4 or IFN-γ. Results shown are from one representative experiment of three. Similar results were obtained with supernatants collected at 24 and 72 h; however, no IL-4 could be detected in 72-h supernatants. In cultures that did not receive *L. major*, cytokine signals were 5- to 10-fold less than in cultures that were restimulated with *L. major*.

these mouse strains in the PIV response. Parallel PIV cultures of each strain were developed for 7 days. The T-cell blasts were recovered and restimulated as described in Materials and Methods. The supernatants of these cultures were then tested for the presence of IL-4 and IFN-γ (Fig. 2). Consistent with our previous experience, in the PIV response (51) BALB/c and C57 mice secreted almost identical amounts of IL-4 and IFN-γ. In contrast, CBA PIV T cells secreted low levels of IL-4 but high levels of IFN-γ.

Next, we determined whether BALB/c, CBA, and C57 mice responded with the same cytokine response in vivo when infected with *L. major* as they did in the PIV response. These mice were infected with 2×10^6 *L. major* promastigotes, and at day 7 of infection, the lymph nodes draining the developing lesion (popliteal and inguinal) were harvested, processed, and stimulated as described in Materials and Methods. Lymph node cells (LNC) from infected BALB/c mice made large amounts of IL-4, C57 cells made smaller but still substantial amounts of the cytokine, while IL-4 could not be detected in CBA cultures. In contrast, CBA mice made large amounts of IFN-γ, while LNC from both BALB/c and C57 made small amounts of the cytokine (Fig. 3). Therefore, the behavior of the three mouse strains in the PIV response mirrored their reaction to infection with *L. major* (compare Fig. 2 and 3).

These data clearly show that the PIV response mirrors infection with *L. major* in vivo: BALB/c mice were Th2-like both in the PIV response and in vivo, while CBA mice were Th1-like. Therefore, since the PIV response is a valid in vitro correlate of *L. major* infection in mice, we next measured the levels of production of a broad panel of cytokines and other relevant factors (e.g., PGE_2) produced in the first 7 days of the PIV response to elucidate which factors were associated with commitment to Th1 or Th2.

Production of Th1- and Th2-associated cytokines in the first 7 days of the PIV response. PIV supernatants were assayed for IFN-γ and IL-2 as indicators of a Th1 response and for IL-4 and IL-5 as indicators of a Th2 response. Splenocytes from CBA (highly resistant) mice produced ever-increasing amounts of IFN-γ such that by day 7 of the PIV response, CBA cultures contained seven to eight times more IFN-γ than either C57 or susceptible BALB/c PIV cultures (Fig. 4). To determine whether these differences were statistically significant, we analyzed the day 7 results by a Wilcoxon signed-rank nonparametric analysis (see Materials and Methods). The day 7 results of seven replicate experiments were examined. This revealed that although BALB/c and C57 responses were not different from each other ($P = 0.398$), the IFN-γ response of CBA cells was significantly higher than that of C57 or BALB/c ($P \leq 0.02$). We could not at any time detect IL-2 in the supernatants of any of the PIV responses during the first 7 days of priming. How-

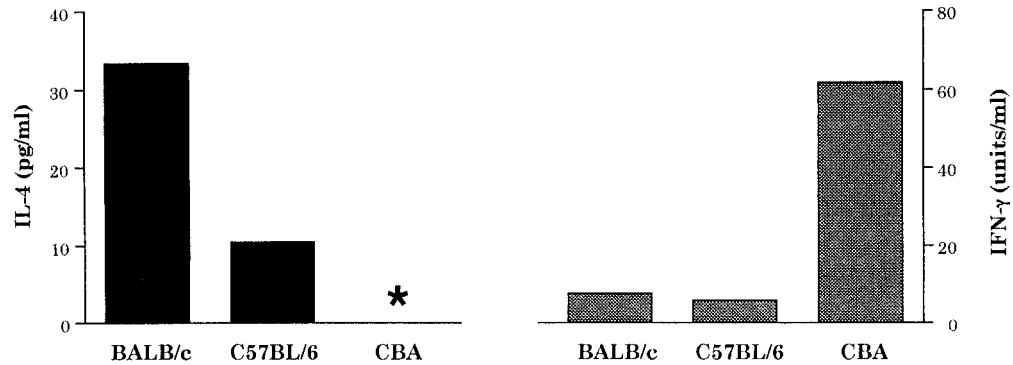

FIG. 3. IL-4 and IFN-γ production by LNC from *L. major*-infected mice restimulated with the parasite in vitro. Groups of five BALB/c, C57, or CBA mice were infected with 2×10^6 stationary-phase promastigotes s.c. in one hind footpad. One week later the draining popliteal and inguinal LNC were harvested and restimulated as described in Materials and Methods. As with the results presented in Fig. 2, maximal cytokine responses were achieved after 48 h of stimulation; in cultures that did not receive *L. major*, cytokine signals were 5- to 10-fold less than in cultures that were restimulated with *L. major*. The asterisk indicates that no IL-4 was detected in cultures of CBA LNC restimulated with *L. major*. The results shown are representative of four replicate experiments.

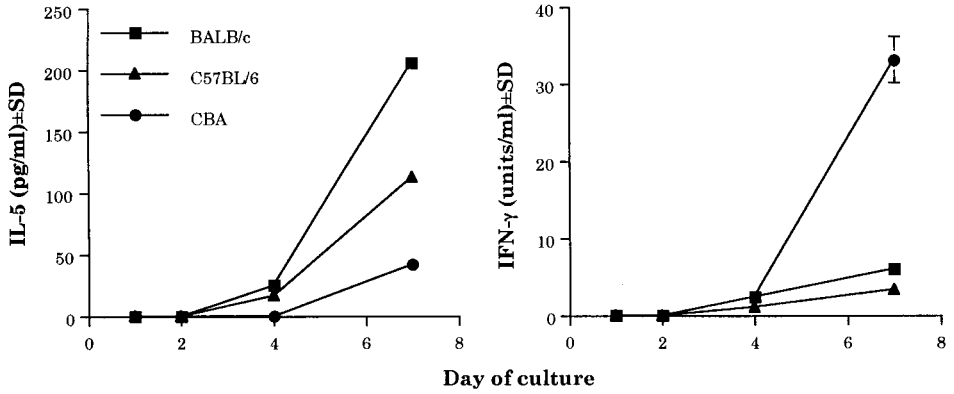

FIG. 4. Production of Th1- and Th2-associated cytokines in the first 7 days of the PIV response. Aliquots of the supernatants of BALB/c, C57, and CBA PIV cultures were collected at days 1, 2, 4, and 7 of priming and assayed by ELISA for IFN-γ or IL-5 as described in Materials and Methods. Data presented are from one representative experiment of seven. SD, standard deviation.

ever, in a previous study (51), we found that if PIV-derived cells were collected after 7 days of priming and were restimulated with *L. major*, they produced substantial amounts of IL-2.

In contrast to the results obtained with IFN-γ, BALB/c cells produced approximately twice as much IL-5 as C57 cells and six times more IL-5 than CBA cells (day 7, Fig. 4). We analyzed the day 7 results of seven replicate experiments with IL-5 by the Wilcoxon signed-rank statistical approach and found that levels of production of IL-5 by the three strains of mice were all significantly different from each other ($0.01 \leq P \leq 0.05$). We could not at any time detect IL-4 in the supernatants of any of the PIV responses during the first 7 days of priming. However, if PIV-derived cells are collected after 7 days of priming and are restimulated with *L. major*, they do produce substantial amounts of IL-4 (Fig. 2) (51).

Taken together, these results indicate that CBA cells committed to a Th1 phenotype and BALB/c cells committed to a Th2 phenotype within the first 7 days of the PIV response.

Production of Mϕ-associated cytokines and factors in the first 7 days of the PIV response. Mϕ and other APC play an important role in leishmaniasis. *L. major* is engulfed by Mϕ and transforms into its amastigote form within the phagolysosomes of Mϕ. In addition, Langerhans cells, which are present in the skin where *L. major* produces disease, also phagocytize *L. major* (35; reviewed in reference 59). Moreover, infection with *L. major* can markedly affect the functions of Mϕ. For example, Mϕ infected with *L. major* secrete large amounts of IL-1α (8, 11), and IL-1α has been shown to be a costimulatory factor for Th2 cells (28, 62). In addition, infection with *Leishmania* can affect the ability of Mϕ to activate T cells: the parasite can augment Th2 activation but inhibit Th1 activation (8, 17, 29, 41). Therefore, we examined the production of several factors that can be produced by Mϕ.

(i) IL-1α. We measured production of IL-1α in the first 7 days of a PIV response with BALB/c, C57, and CBA splenocytes. A BALB/c (susceptible) PIV response generated more IL-1α than either a C57 or CBA response (Fig. 5). When the IL-1α production of BALB/c cells was compared to that of CBA and C57 cells, there was a significant difference ($P \leq 0.05$).

(ii) PGE_2. PGE_2 favors the development of Th2 responses (40). A BALB/c (susceptible) PIV response generated more PGE_2 than either a C57 or a CBA response (Fig. 5). When we analyzed the results statistically, we found that the levels of production of PGE_2 by the three strains of mice were all significantly different from each other ($P \leq 0.05$), except at day 7 of the response when PGE_2 production by BALB/c and C57 cells did not differ.

(iii) IL-12 and TGF-β. Both IL-12 and TGF-β have marked effects on the course of leishmaniasis in mice. For example,

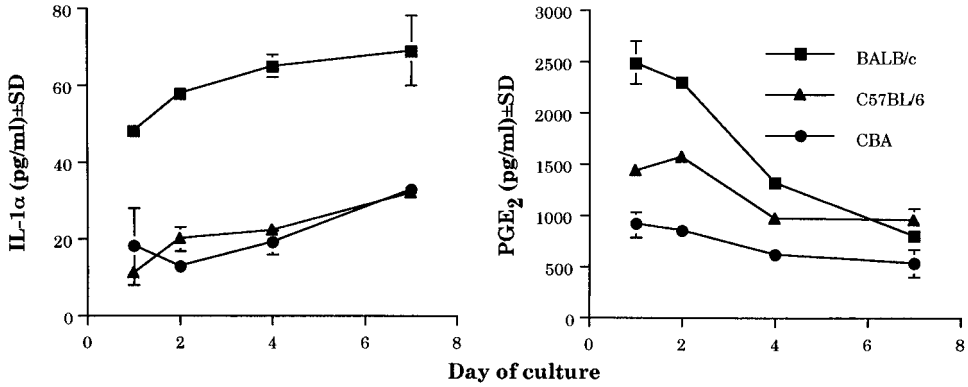

FIG. 5. Production of IL-1α and PGE_2 in the first 7 days of the PIV response. Aliquots of the supernatants of BALB/c, C57, and CBA PIV cultures were collected at days 1, 2, 4, and 7 of priming and assayed for IL-1α or PGE_2. The data shown for IL-1α are from one representative experiment of five, and the data shown for PGE_2 are from one representative experiment of six. SD, standard deviation.

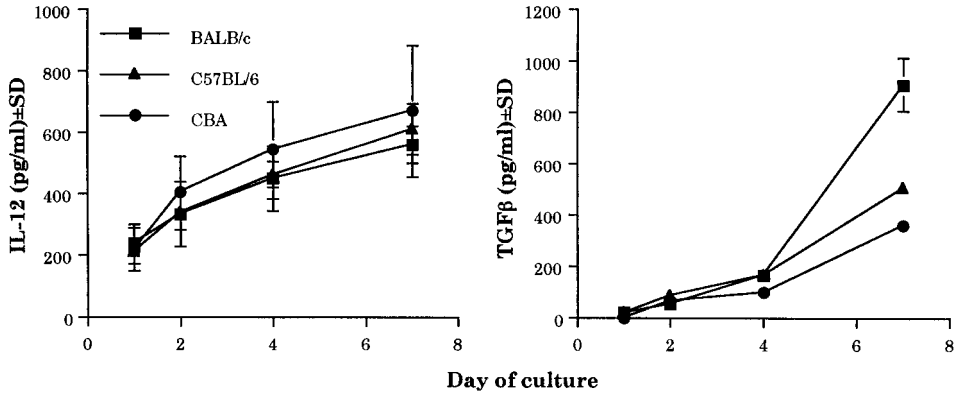

FIG. 6. Production of IL-12 and TGF-β in the first 7 days of the PIV response. Aliquots of the supernatants of BALB/c, C57, and CBA PIV cultures were assayed by ELISA for IL-12 and TGF-β. For IL-12, the data shown are the averages of the results of three independent experiments. For TGF-β, the data presented are for one representative experiment of six. The basal level of TGF-β present in the normal mouse serum used to perform the PIV assays was subtracted from the values presented in the figure. SD, standard deviation.

infusing IL-12 into susceptible BALB/c mice promotes a Th1 response and allows the mice to heal an infection with *L. major* (2, 21, 56), while TGF-β is deleterious in leishmaniasis (4, 55). Therefore, we measured the production of these cytokines in the PIV response.

Levels of IL-12 increased with time in the supernatants of BALB/c, C57, and CBA splenocyte PIV cultures, but there was no difference in the levels of IL-12 produced by the three strains of mice (Fig. 6). Statistical analysis bore out this conclusion since at no time were the IL-12 responses of the three mouse strains significantly different.

In contrast, TGF-β was initially produced at relatively low levels by all three strains of mice, but eventually (by day 7 of the assay) the highest level of TGF-β was produced by BALB/c PIV cells, less was produced by C57 cells, and the least was produced by CBA cells (Fig. 6). Statistical analyses of day 7 results revealed that BALB/c cells produced significantly higher levels of TGF-β than either CBA or C57 cells ($P \leq 0.03$).

(iv) NO. NO is critical for killing of *L. major* in activated Mϕ (18, 26, 33) and correlates with resistance to *L. major* in infected mice (55). Therefore, we measured NO production in BALB/c, C57, and CBA PIV cultures. There was a direct correlation between NO production and resistance to infection with *L. major*. CBA mice produced the most NO, followed by C57 and BALB/c (compare Fig. 1 and 7). Moreover, statistical analyses of day 7 results revealed that both CBA and C57 cells produced significantly more NO than BALB/c cells ($P \leq 0.02$).

(v) IL-10. IL-10, a cytokine secreted by many cell types including Mϕ, affects the immune response and, in particular, inhibits Th1 responses (36). Therefore, we investigated its production in the PIV system. Supernatants of BALB/c, C57, and CBA PIV cultures contained detectable amounts of IL-10 (Fig. 8). Surprisingly, supernatants from cultures of highly resistant CBA splenocytes had higher levels of this cytokine than either susceptible BALB/c or C57 cultures, particularly at day 7 of the response (Fig. 8). Indeed, when the day 7 results were analyzed statistically, the responses of BALB/c and C57 did not differ ($P = 0.2367$), but the IL-10 response of CBA cells was significantly higher than that of either BALB/c or C57 ($P \leq 0.02$).

Since we did not expect that CBA cells would produce IL-10 in the PIV response, we measured the production of IL-10 in vivo in resistant CBA and susceptible BALB/c mice infected with *L. major*. At 3 and 7 days after infection with 2×10^6 *L. major* promastigotes, the LNC draining the developing lesion were harvested and restimulated in vitro as described in Materials and Methods. Both CBA and BALB/c mice produced IL-10 in response to infection with *L. major* (Fig. 8); indeed, LNC from infected CBA mice produced more IL-10 than cells from infected BALB/c mice. Therefore, the PIV system correctly predicted that IL-10 would be produced in mice infected with *L. major*, not only in susceptible BALB/c mice but also in highly resistant CBA mice.

Taken together, these results suggest that IL-10 plays an important regulatory role in both susceptible and resistant mice infected with *L. major*, most likely through its ability to control Th1 development and those molecules associated with Th1 responses (e.g., IFN-γ and NO). To test this hypothesis, we determined the effect of adding a neutralizing anti-IL-10 antibody at the inception of PIV responses initiated with either BALB/c or CBA cells. Anti-IL-10 antibody augmented the production of both IFN-γ and NO by BALB/c or CBA cells (Fig. 9), suggesting that IL-10 indeed can control Th1 responses in both strains of mice following infection with *L. major*.

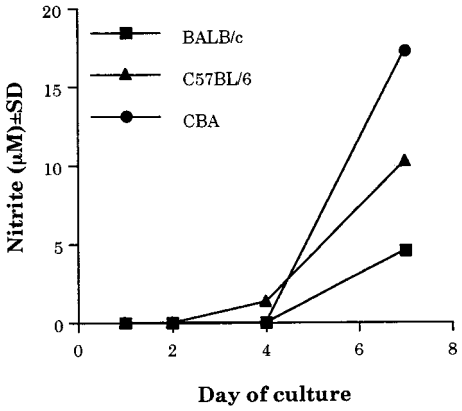

FIG. 7. Production of NO in the first 7 days of the PIV response. The supernatants of BALB/c, C57, and CBA PIV cultures were assayed for the presence of nitrite by using the Griess reagent. The data presented are from one representative experiment of seven. SD, standard deviation.

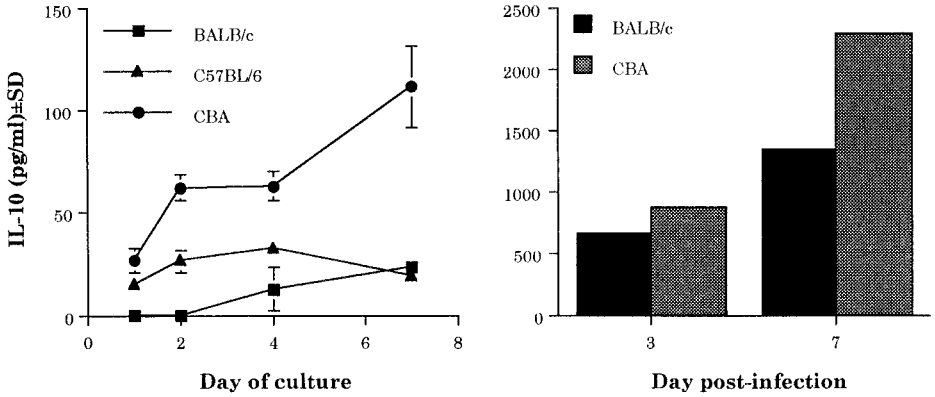

FIG. 8. Production of IL-10 in the first 7 days of the PIV response and by mice infected with *L. major*. (Left) Aliquots of the supernatants of BALB/c, C57, and CBA PIV cultures were collected and assayed by ELISA for IL-10. Data presented are from one representative experiment of five. (Right) Draining LNC were harvested after 3 and 7 days of infection from BALB/c and CBA mice, and the cells were restimulated with *L. major* as described in Materials and Methods. After 48 h, supernatants were collected and assayed for IL-10. In cultures that did not receive *L. major*, IL-10 was not detected. The results shown are from one representative experiment out of seven.

DISCUSSION

In this report, we characterized an in vitro correlate for *L. major* infection in mice. We found that spleen cells from susceptible BALB/c mice produced large amounts of the Th2 cytokines IL-4 and IL-5 and small amounts of the Th1 cytokine IFN-γ (Fig. 2 and 4). This response is identical to the response of BALB/c mice infected with *L. major* (Fig. 3) (reviewed in reference 59). In contrast, spleen cells from resistant CBA mice produced large amounts of the Th1 cytokine IFN-γ and very small to undetectable amounts of IL-4 and IL-5 (Fig. 2 and 4). Again, this is identical to the response of CBA mice infected with *L. major* (Fig. 3).

Since the PIV system mimics the in vivo response to *L. major*, it offers an in vitro correlate for infection with *L. major*. Moreover, we feel that it is superior to T-cell receptor transgenic systems for analyzing the immune response to *L. major*. T-cell receptor transgenic mice are genetically manipulated animals with an atypical immune system consisting of ≥90% T cells that recognize one epitope of a single model antigen that is not leishmanial. In contrast, *L. major* is a complex dividing antigen that profoundly affects the activities of APC such as Mϕ. For example, the parasite can impair signal transduction within the cell (14) and scavenge oxygen radicals (9). Model antigens do not have these effects on APC; moreover, model antigens reside in endosomes while *L. major* resides in phagolysosomes. Further, the advantage of the PIV system is that commitment to Th1 and Th2 occurs without added cytokines or anticytokines (Fig. 2 and 4); adding cytokines is commonly required for Th commitment to occur in T-cell receptor transgenic systems (for examples, see references 30 and 63).

C57 mice are often used as a resistant model for infection with *L. major*. Therefore, it is interesting that compared to CBA spleen cells, cells from C57 mice produced substantial amounts of IL-4 and IL-5 and small amounts of IFN-γ in the PIV system (Fig. 2 and 4). Moreover, the same cytokine profile was observed in CBA and C57 mice infected with *L. major* (Fig. 3). These observations suggest that C57 mice might not be highly resistant to infection with *L. major*, and as mentioned in the introduction, this is true (5, 45). In addition, consistent with the results obtained here with the PIV system, the work of Reiner et al. showed that BALB/c and C57 mice produce similar amounts of IL-4 and IFN-γ in the first few days of infection with *L. major* (42). Finally, the work of Scharton-Kersten and Scott (46) showed that in the first few days of infection with *L. major*, C57 mice produce considerably less IFN-γ and more IL-4 than C3H mice. C3H and CBA mice are both highly resistant to infection with *L. major* (5, 45). These results suggest that although the C57 mouse ultimately resists infection with *L. major*, it takes considerably longer to commit to a resistant Th1 phenotype than do strains such as C3H or

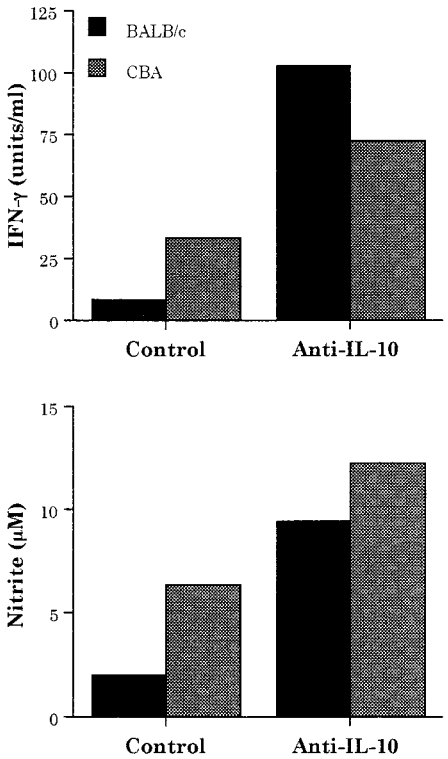

FIG. 9. Anti-IL-10 enhances production of IFN-γ and NO in the PIV response. A neutralizing anti-IL-10 antibody (JES5-2A5) was added (5 μg/ml, final concentration) at the inception of a BALB/c or CBA PIV response. After 7 days of incubation, the effect of this antibody on IFN-γ and NO secretion was determined by measuring the levels of these factors in the culture supernatants. The results shown are from one representative experiment out of five.

CBA. Thus, C3H or CBA may be more appropriate models for studying Th1 development in experimental cutaneous leishmaniasis induced by *L. major.*

Since CBA spleen cells clearly committed to Th1 and BALB/c committed to Th2 in the PIV response, we measured the secretion of several mediators that were produced in the first 7 days of the response, a time when commitment to Th1 or Th2 occurs. These cytokines were selected since they have relevance to either leishmaniasis or to Th1 and Th2 priming.

We first monitored the production of a Th1-specific cytokine, IFN-γ, and a Th2-specific cytokine, IL-5. We were unable to monitor IL-2 as a Th1-specific marker or IL-4 as a Th2-specific marker. Although these cytokines are detected when PIV-derived T cells are restimulated after 7 days of culture (Fig. 2) (51), neither was detected in the supernatants of PIV cultures during the initial 7 days of priming.

We found that spleen cells from resistant CBA mice produced significantly larger amounts of IFN-γ and smaller amounts of IL-5, while BALB/c cells produced significantly smaller amounts of IFN-γ and larger amounts of IL-5 (Fig. 4). This result agrees with other data presented here (Fig. 1 to 3) and with results reported by others (45–48).

The exact role of IL-1α in the differentiation of T cells is uncertain. Some studies with T-cell clones suggested that IL-1α favors the expansion of Th2 cells (28, 62), but studies using an in vitro priming system indicated that IL-1α was not necessary to generate a Th2 response (49). In the *L. major* model, it has been reported that infected Mϕ produce high levels of IL-1α, and that as a result, infecting Mϕ with *L. major* favors the outgrowth of Th2 T-cell clones (8, 11). This is consistent with the observation here that susceptible BALB/c cells produce significantly more IL-1α in the PIV response (Fig. 5). However, treating mice with an anti-IL-1α receptor antibody decreases lesion size in mice infected with *L. major* but does not affect either the immune response to, or the rate of clearance of, *L. major* by the treated animals (57).

PGE_2 is an inhibitor of inflammation and can favor the outgrowth of Th2 cells (40). The exact role of PGE_2 in leishmaniasis is not clear. Although PGE_2 appears to play a detrimental role in susceptible BALB/c mice infected with *L. major* (16, 34), the molecule can enhance parasite killing in vitro by Mϕ from resistant mice (32). In the PIV response, susceptible BALB/c mice produced significantly more PGE_2 than resistant CBA mice (Fig. 5), suggesting that increased production of this molecule by susceptible mice is partly responsible for Th2 development in the animals.

In *L. major*-infected mice, IL-12 can play a beneficial role (2, 21, 56); however, treating resistant mice with an anti-IL-12 antibody does not necessarily prevent the mice from healing (20, 47, 56). There is inconsistency in the literature regarding the amounts of IL-12 produced by BALB/c, C57, and highly resistant mice, such as C3H, following infection with *L. major*. Although substantial amounts of IL-12 are produced in vitro by LNC harvested from mice infected with *L. major* for 1 or 7 days (47), little IL-12 mRNA is detected in the same period (42). However, whether low or high levels of IL-12 are made, BALB/c, C57, and C3H mice produce equivalent amounts of the cytokine following infection with *L. major* (42, 47), observations that agree with the results presented here (Fig. 6).

TGF-β was initially found to play a detrimental role in mice infected with *Leishmania amazonensis* or *Leishmania braziliensis* (4). Recently, TGF-β was shown to play a detrimental role in mice infected with *L. major* as well (47, 55). Indeed, immunohistochemical analysis of lesions of *L. major* showed that NO associated with resistance and TGF-β associated with susceptibility (55). Again, the PIV response mimicked the in vivo response to infection with *L. major*, since spleen cells from resistant CBA mice produced significantly more NO while susceptible BALB/c mice produced significantly more TGF-β (Fig. 6 and 7). Thus, it is possible that TGF-β (which inhibits NO synthesis [15, 61]) produced in a BALB/c PIV response is responsible for inhibiting NO production, while in a CBA PIV response the lower level of TGF-β in conjunction with high levels of IFN-γ leads to high NO production.

The fact that spleen cells from CBA mice produced significantly higher levels of IL-10 in the PIV response was unexpected. IL-10 was initially characterized as a molecule capable of inhibiting IFN-γ production by Th1 cells (36); thus, IL-10 is usually associated with Th2 responses. In addition, IL-10 mRNA is expressed by BALB/c mice following infection with *L. major* but not by C57 mice (22).

Because IL-10 production by CBA mice in the PIV response was unexpected, we determined whether these mice made IL-10 when they were infected with *L. major*. CBA mice produced more IL-10 than infected BALB/c mice (Fig. 8). Possibly, IL-10 is produced by CBA mice to prevent overproduction of IFN-γ in response to infection with *L. major*. Uncontrolled Th1 responses can lead to tissue destruction and autoimmunity (3, 23, 54), and it has been proposed that autoimmunity may contribute to the pathology associated with chronic parasitic diseases such as leishmaniasis (1).

Alternatively, since it has been reported that IL-10 enhances NO production in Mϕ activated with IFN-γ and tumor necrosis factor α in vitro (13), it is possible that IL-10 plays a protective role in CBA mice. However, unlike treating with an anti-IL-4 antibody, treating *L. major*-infected mice with an anti-IL-10 antibody does not prevent the mice from curing the infection (12). Thus, although IL-10 may play an important regulatory role in leishmaniasis, there appears to be another redundant mechanism(s) that compensates for its absence.

It has been hypothesized that Th commitment in mice infected with *L. major* is determined by the cytokines produced in the first week of infection, during which T-cell priming occurs. Specifically, early production of IFN-γ and IL-12 leads to Th1 development, while early production of IL-4 leads to Th2 development (2, 6, 10, 21, 43, 48, 56). However, it is difficult to reconcile this hypothesis with the observations that (i) susceptible and resistant mice both produce a mixture of IL-2, IL-4, and IFN-γ in the first week of infection with *L. major* (42); (ii) susceptible and resistant mice produce equivalent amounts of IL-12 following infection with *L. major* (42, 47); and (iii) a cellular source for early IL-4 production in susceptible mice has not been identified (7, 24). The data presented here offer an alternative or additional mechanism by which Th commitment might be influenced. T cells are activated by accessory cells and APCs which present *L. major* antigens and deliver multiple signals which influence T-cell activation. We show here that in a PIV response, cells from susceptible BALB/c mice produce more IL-1, PGE_2, and TGF-β than cells from resistant mice. IL-1, PGE_2, and TGF-β can be produced by APCs such as Mϕ. In addition, IL-1 and PGE_2 favor Th2 development, and TGF-β deactivates Mϕ. Therefore, it is possible that a collection of factors (e.g., IL-1, PGE_2, TGF-β, and probably other unidentified factors) which can be produced by APCs help bring about commitment to Th2 in susceptible BALB/c mice. This hypothesis is supported by experiments that showed that the environment (i.e., APCs, etc.) in which *L. major*-specific T cells are primed can dictate Th commitment (52). In addition, IFN-α, which is produced by APCs and favors the development of a Th1 response, plays a beneficial role in mice infected with *L. major* (53).

In conclusion, a PIV response which utilizes CBA and

BALB/c mice appears to mirror the immune response that these mice mount when they are infected with *L. major*. The advantage of the system is that it is an in vitro response. As such, it is more easily controlled and manipulated than comparable in vivo experiments. For example, if commitment to Th1 and Th2 is dictated by the activities of APCs, it would be very difficult to determine the relative contribution of various APCs to activation of Th1 and Th2 cells in vivo. However, using the PIV response, we have recently performed such experiments. Briefly, Mϕ, B cells, and dendritic cells all play a role in the priming of *L. major*-specific cells. Dendritic cells were the most potent at priming T cells, followed closely by B cells and finally Mϕ. In addition, Mϕ favored Th1 development while B cells favored Th2 development (50). We hope that the PIV response will continue to enhance our understanding of Th1 and Th2 activation and the mechanisms of resistance to *Leishmania.*

ACKNOWLEDGMENTS

This work was supported by NIH grants AI 22532 and 29955. M.B.P.S. was supported by a fellowship from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brazil.

We thank Andrea Cooper (Colorado State University) for the TGF-β ELISA protocol and Matt Paulson, Monica Estay, and Julie Bleyenberg for technical support. We thank Abul Abbas (Harvard Medical School), Joe Sypek (Genetics Institute), and Cindy Theodos (Tufts University) for critically reading the manuscript.

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Editor: J. M. Mansfield