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Effects of Essential Fatty Acid Deficiency on Prostaglandin E_2 Production and Cell-Mediated Immunity in a Mouse Model of Leprosy

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Results from animal and in vitro studies suggest that essential fatty acid (EFA) deficiency enhances cell-mediated immunity by reducing production of prostaglandins with immunosuppressive actions. However, direct experimental evidence that EFA deficiency enhances T-lymphocyte function in vivo has not been obtained. In this study, athymic (*nu*/*nu*) mice were infected in the footpads with *Mycobacterium leprae* and fed a linoleic acid-free diet. These mice, and infected *nu*/*nu* mice on control diets, were given an adoptive transfer of *M. leprae*-primed, T-cell-enriched lymphocytes. After 2 weeks, *M. leprae* bacilli were harvested from the recipient mice and bacterial viability was determined by the BACTEC system. *M. leprae* recovered from recipient mice fed control diets displayed little reduction in metabolic activity. In contrast, *M. leprae* from recipient mice fed the EFA-deficient (EFAD) diet exhibited markedly reduced viability. In vitro, donor cells from *M. leprae*-primed mice secreted elevated levels of gamma interferon upon exposure to the bacilli. These cells also exhibited an enhanced proliferative response, which was reduced by exogenous prostaglandin E_2 (PGE_2). In addition, *M. leprae*-infected granuloma macrophages (Mϕ) from EFAD recipient *nu*/*nu* mice secreted significantly less PGE_2 than granuloma Mϕ from mice on control diets. These data suggest that enhanced levels of Mϕ-generated PGE_2, induced by *M. leprae* or its constituents, could act as an endogenous negative modulator of the immune response occurring in the microenvironment of the lepromatous granuloma.

Mycobacterium leprae is an obligate intracellular pathogen and the causative agent of leprosy, a disease characterized by a spectrum of clinical and immunological manifestations (33). Clinical leprosy ranges from multibacillary, lepromatous disease, where there is little cell-mediated immunity (CMI) but a potent antibody response and enormous numbers of *M. leprae* bacilli in the lesions, to paucibacillary tuberculoid leprosy, where there is intense delayed-type hypersensitivity to *M. leprae* antigens, low levels of specific antibodies, and few bacilli in the skin. Leprosy affords the immunologist the opportunity to study immune regulation and the interplay of T cells and macrophages (Mϕ) in a specific, nonfatal, chronic immunodeficiency disease.

The importance of the T lymphocyte in host resistance to leprosy is apparent in experimental infection of athymic *nu*/*nu* mice, where *M. leprae* growth is unrestricted (6, 7, 18). Adoptive transfers of immune cells into conventional, athymic, and SCID mice have been utilized in leprosy research for the evaluation of *M. leprae* immunogenicity, reversal reactions, and vaccine development (8, 22, 39). In human lepromatous disease, a number of mechanisms have been offered to explain the characteristic *M. leprae*-specific T-cell anergy, including clonal deletion of *M. leprae*-specific T cells (14) and induction of suppressor T cells (24, 28, 29).

At the level of Mϕ, production of suppressor factors such as interleukin 10, transforming growth factor β, and prostaglandin E_2 (PGE_2) by infected Mϕ may underlie the inability of these host cells to cope with the leprosy bacillus (32, 35, 37, 46, 52). Mϕ isolated from the *nu*/*nu* mouse footpad are gorged with *M. leprae*; however, while retaining many of their normal phenotypic characteristics, these *M. leprae*-infected Mϕ are refractory to gamma interferon (IFN-γ)-induced activation (43) and generate high levels of PGE_2 ex vivo (44). IFN-γ-refractory Mϕ can be generated in vitro as well by infection with a large number of viable *M. leprae* bacilli, and PGE_2 plays a crucial role in the induction of this defect (45).

PGE_2 is synthesized from arachidonic acid (AA) [20:4(n-6)], one of the major phospholipids found in cell membranes, via the cyclooxygenase (COX) pathway. AA is generated from linoleic acid, a plant-derived, essential fatty acid (EFA). Feeding mice a defined diet containing all necessary nutrients except linoleic acid greatly reduces the levels of AA in the tissues (4, 17), thus depriving COX of its substrate. Moreover, linoleic acid deprivation causes the normal AA component of the cellular membrane phospholipids to be replaced by an eicosatrienoic acid [20:3(n-9)]. This alternate phospholipid not only is not a substrate for COX-mediated biosynthesis of PGE_2 but also is a competitive inhibitor of this reaction (4).

The purpose of the present studies was to evaluate the regulatory role of PGE_2 in the immune response to *M. leprae*. Using adoptive transfer of primed T cells into *M. leprae*-infected *nu*/*nu* mice, we demonstrated that CMI, resulting in killing of the bacilli, is enhanced in an EFA-deficient (EFAD) environment.

MATERIALS AND METHODS

Mice. *nu*/*nu* mice [(BALB/c AN BOM) *nu*/*nu* DF] were obtained from Harlan Sprague-Dawley, Inc., Indianapolis, Ind., and were housed under pathogen-free conditions in laminar-flow animal isolators in sterile cages in a temperature-controlled (78 to 80°C) environment. Heterozygous (*nu*/+) mice ([SCH (BALB/c/ANE/+ dF)]; Harlan Sprague-Dawley) were housed under standard laboratory animal housing conditions.

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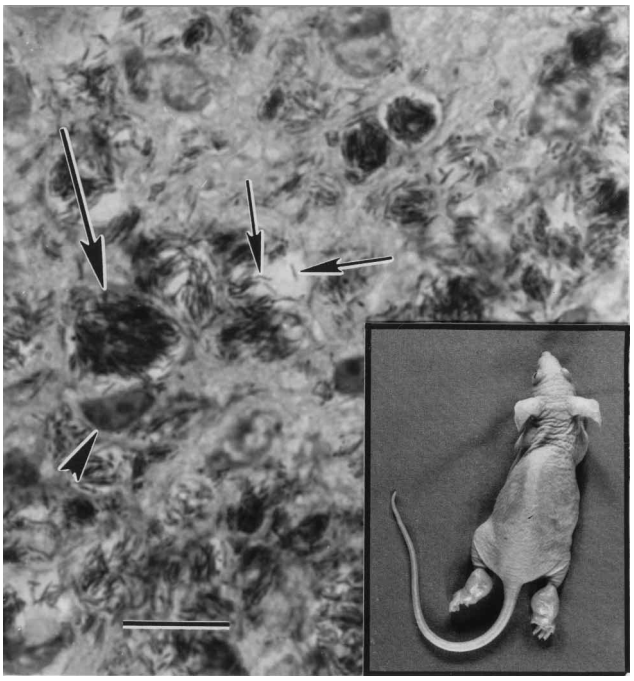

FIG. 1. Histological section of footpad tissue visualized with Fite's acid-fast stain. Mϕ (a nucleus is indicated by the arrowhead) contain large globuli packed with bacilli (large arrow). Small arrows point out individual *M. leprae* bacilli. Bar = 10 μm; Magnification, ×1,362. (Inset) *nu/nu* mouse with enlarged footpads, approximately 6 months after infection with *M. leprae.*

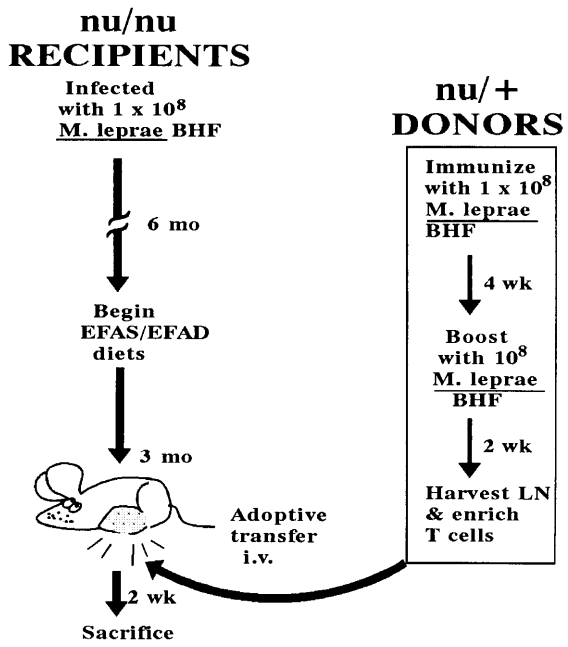

FIG. 2. Protocol for the adoptive transfer of T-cell-enriched cell samples into EFAS and EFAD *nu/nu* mice. LN, lymph nodes.

Cultivation and maintenance of *M. leprae*. *M. leprae* was maintained in continuous passage in *nu/nu* mice at 9-month intervals by inoculation into both hind footpads (BHF) with 10^8 freshly harvested bacilli as previously described (45).

Modulation of AA metabolism in vivo. Groups of *M. leprae*-infected *nu/nu* mice were fed an EFAD diet consisting of casein (20.0%) (Purina Mills, Inc., Richmond, Ind.), Alphacel (4.0%) (ICN Biomedicals, Inc., Costa Mesa, Calif.), mineral mix (3.5%) (ICN), choline chloride (0.1%) (ICN), cornstarch (36.4%) (ICN), sucrose (30.0%) (LSU Food Stores, Baton Rouge, La.), vitamin mix (1.0%) (Purina), and coconut oil (5.0%) (ICN). Control mice were fed either mouse chow (Teklad LM-485) (Harlan Teklad Laboratory, Madison, Wis.) or an EFA-sufficient (EFAS) control diet, which was identical to the EFAD diet except that it contained safflower oil (5.0%) (Purina), a source of linoleic acid, rather than coconut oil.

Fatty acid analysis. After the mice were kept for 12 weeks on the various diets, the liver fatty acid contents from representative mice (eight per diet) were analyzed. Total tissue lipids were extracted from the liver by the method of Folch et al. (12). The fatty acid composition of the methyl esters was determined by gas-liquid chromatography with a model 5890A gas chromatograph (Hewlett-Packard) as described previously (5). A fused silica capillary column (SP2380; Supelco Inc., Bellefonte, Pa.), 60 by 0.25 mm with a 0.2-μm layer of *bis*-cyanopropyl-phenylsilosan, and a splitless mode of injection were used. Individual fatty acid methyl esters were identified by comparing their resolution times with those of a standard fatty acid methyl ester mixture (Nu Check Prep).

Isolation of granuloma Mϕ. *M. leprae*-infected granuloma Mϕ were isolated from the footpads of *nu/nu* mice with a solution of 15 U of collagenase (Sigma Chemical Co., St. Louis, Mo.)/ml and 25 μg of DNase (Sigma)/ml in RPMI 1640 (GIBCO, Grand Island, N.Y.) without serum as previously described (43). The cells were cultured at 2×10^6/well on four-chamber LabTek slides (Miles Scientific, Naperville, Ill.) in complete medium (RPMI 1640 containing 25 mM HEPES [GIBCO], 0.2% $NaHCO_3$ [GIBCO], 2 mM glutamine [Irvine Scientific, Santa Ana, Calif.], and 100 μg of ampicillin [Sigma]/ml) with 2% autologous mouse serum. Supernatants were collected after 24 h of incubation. PGE_2 and thromboxane B_2 (TXB_2) levels were determined by radioimmunoassay as described previously (17).

Adoptive transfer. At 6 months postinfection, the footpads of *M. leprae*-infected *nu/nu* mice are greatly enlarged (Fig. 1, inset) and contain 10^{10} or more bacilli per footpad. Histopathologically (Fig. 1), the infected footpad becomes a huge foreign-body-type Mϕ granuloma containing cells which are packed with bacilli. At this time, these mice were either retained on standard mouse chow or placed on an EFAS or EFAD diet (Fig. 2). After 3 additional months, they served as recipients for adoptive transfer. Primed T cells for adoptive transfer were obtained from heterozygous *nu/+* donor mice inoculated in BHF with 5×10^7 freshly harvested, viable *M. leprae* bacilli and given a booster 4 weeks later in the same manner. Two weeks after the second inoculation, the popliteal lymph nodes were excised, minced, and pressed through a tissue sieve. In most experiments, primed lymph node cells were washed and passed over a goat anti-mouse immunoglobulin Immulon affinity column (Biotecx Laboratories, Inc., Houston, Tex.). In one experiment, a T-cell-enriched population was obtained by elution over a MACs supermagnet after prelabelling of the cells with colloidal superparamagnetic microbeads conjugated with rat anti-mouse B-cell 220 monoclonal antibody (Miltenyi Biotec, Inc., Sunnyvale, Calif.). The *M. leprae*-primed, T-cell-enriched donor population consisted of 77.3% ± 11.4% $CD3^+$, 55.1% ± 5.6% $CD4^+$, 23.2% ± 0.8% $CD8^+$, and 0.7% ± 0.3% immunoglobulin-positive cells (means ± standard deviations). We could not obtain enough T-cell-enriched cell samples from naive lymph nodes to do adoptive-transfer experiments, considering the low number of T cells present and the inevitable losses which occur in the purification procedure, so a T-cell-enriched population obtained from the spleens of naive, uninfected *nu/+* mice and purified in the same manner served as control donor cells. The naive spleen T-cell-enriched donor population consisted of 68.5% ± 20.9% $CD3^+$, 52.2% ± 12.0% $CD4^+$, 19.4% ± 1.5% $CD8^+$, and 2.5% ± 0.7% immunoglobulin-positive cells. Naive and primed T cells were injected intravenously (i.v.) into the *M. leprae*-infected *nu/nu* mice on the various diets at a concentration of 17×10^6 cells/mouse.

Determination of the viability of *M. leprae* harvested from the footpads of recipient *nu/nu* mice. Two weeks after adoptive transfer, the recipient mice were exsanguinated. Tissue was aseptically removed from one footpad, weighed, and disrupted by shaking with glass beads in a Mickle homogenizer (Brinkman, Gomshall, Surrey, United Kingdom). The acid-fast bacilli (AFB) were enumerated (41) and inoculated into the BACTEC 460 system (Becton Dickinson and Co., Towson, Md.). In early experiments BACTEC vials were inoculated solely with a dose of 10^7 AFB per vial. In later experiments separate BACTEC vials were inoculated with 10^7 and 10^8 AFB. The vials were gassed with 2.5% O_2–10% CO_2–balance N_2, incubated at 33°C, and read weekly for 4 weeks (13). BACTEC 460 results are expressed as mean growth indices (GI; 100 GI units = 0.025 μCi of $^{14}CO_2$ generated from [^{14}C]palmitic acid) of quadruplicate samples from each mouse. Tissue from the other footpad was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with Fite's acid-fast stain.

[^{3}H]thymidine incorporation assay. Naive and primed donor cells were isolated from *nu/+* mice and transferred to the wells of 96-well plates (Becton Dickinson Labware, Lincoln Park, N.J.) at 2×10^5 cells/well in 200 μl of complete medium containing 10^{-5} M 2-mercaptoethanol and 10% fetal bovine serum (HyClone, Logan, Utah). The cells were incubated in the presence or absence of 10^7 *M. leprae* bacilli and 10^{-6} M PGE_2 (Sigma) for 6 days. Eighteen hours prior to harvest, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.)/ml was added per well. The cells were then harvested onto filter paper (Cambridge Technology, Inc., Watertown, Mass.), dried, transferred to vials containing 10 ml of scintillation fluid (ready to use for nonaqueous samples;

Fisher Scientific, Houston, Tex.), and counted (Beckman Instruments, Inc., Fullerton, Calif.).

Measurement of IFN-γ. Naive and primed donor cells were transferred at 2×10^5 cells/well in 200 μl of complete medium containing 10^{-5} M 2-mercaptoethanol and 10% fetal bovine serum and stimulated with 10^7 *M. leprae* bacilli for 96 h. The levels of IFN-γ in the supernatants were determined by enzyme-linked immunosorbent assay (Genzyme, Cambridge, Mass.).

Statistical analysis. BACTEC data were analyzed as factorial arrangements of treatments with diet (EFAS and EFAD), treatment by transfer (naive and primed T cells), and diet-transfer interaction as effects in the model in an analysis of variance by using the SAS statistical package (GLM and TTEST procedures). Response variables were the original (raw) GI values, the natural log-transformed GI values, and the ranked values of both the original data and the log-transformed data. Tukey's Studentized range test was used for individual mean comparisons of main effects. Least-square means were analyzed for interaction effects, and all pairwise comparisons were conducted with Bonferroni *t* tests. *t* tests were conducted for the same response variables with respect to the chow diet in comparing transfer levels of T cells (no transfer and primed-T-cell transfers). Other data were analyzed by a two-tailed parametric Student *t* test. All differences were considered significant at a P value of <0.05.

RESULTS

Evaluation of donor cells. To ensure that the primed donor cells were responsive to *M. leprae*, cells from *nu*/+ donor mice were evaluated in vitro by two different criteria. First, they were examined for the production of IFN-γ, a potent Mϕ-activating cytokine, in response to *M. leprae*. Naive donor cells produced virtually no IFN-γ (<0.125 ng/ml), whereas primed donor cells produced elevated levels of IFN-γ (16.9 ± 0.1 ng/ml). Second, these cells were examined for [^{3}H]thymidine uptake. Naive donor cells incorporated low levels of [^{3}H]thymidine ($2.5 \times 10^3 \pm 1.3 \times 10^3$ cpm), and *M. leprae* induced a nonspecific increase ($8.2 \times 10^3 \pm 1.2 \times 10^3$ cpm). Primed donor cells, on the other hand, demonstrated an elevated baseline response ($36.7 \times 10^3 \pm 19.2 \times 10^3$ cpm). In these cells, the addition of *M. leprae* in vitro did not significantly increase uptake of [^{3}H]thymidine ($41.4 \times 10^3 \pm 13.9 \times 10^3$ cpm), indicating that these cells are already maximally stimulated, probably due to the persistence of *M. leprae* in the sensitized lymph nodes (data not shown). The addition of exogenous PGE_2 caused an inhibition of [^{3}H]thymidine incorporation in response to *M. leprae* in both naive ($1.3 \times 10^3 \pm 1.1 \times 10^3$ cpm) and primed ($12.0 \times 10^3 \pm 4.6 \times 10^3$ cpm) donor cells ($P < 0.05$). These data demonstrate that PGE_2 can downregulate a T-cell response to *M. leprae.*

Adoptive transfer of T cells into *nu*/*nu* mice. Figure 3 shows the metabolic activity of *M. leprae* isolated from infected, chow-fed *nu*/*nu* recipients of primed T cells. It is clear from these data that a BACTEC inoculum of 10^7 bacilli (Fig. 3A) is suboptimal for obtaining peak GI readings of 999 within 4 weeks. Under these conditions there was no evidence of an effect of adoptive transfer on the metabolism of *M. leprae* from mice fed standard chow ($P = 0.4647$). In Fig. 3B, which depicts the results obtained with BACTEC vials inoculated with 10^8 *M. leprae* bacilli, GI levels peaked out for all mice receiving no T cells. Here again, adoptive transfer of primed T cells had little effect on the metabolic activity of the bacilli ($P = 0.1201$).

Dietary regulation of AA levels. In order to modify the production of potentially immunosuppressive PGE_2 generated in response to *M. leprae* infection, the *nu*/*nu* recipient mice were fed a diet deficient in the EFA linoleic acid. To ensure that EFA deficiency was attained, mice which had been fed an EFAS or EFAD diet were evaluated for fatty acid content in liver tissue (Table 1). Mice fed the EFAD diet showed a reduction in all n-6 fatty acids examined compared to mice fed the EFAS diet. To determine EFA deficiency, the 20:3(n-9)/20:4(n-6) ratio was calculated; a ratio of less than 0.4 denotes that the minimum dietary requirement for linoleic acid has been met (15). In EFAS mice ($n = 8$) the 20:3(n-9)/20:4(n-6) ratio was 0.025 ± 0.029, whereas in EFAD mice ($n = 8$) the ratio was 1.020 ± 0.117. These findings indicate that the EFAD diet markedly suppressed ($P < 0.0001$) the levels of AA in the *nu*/*nu* recipient mice.

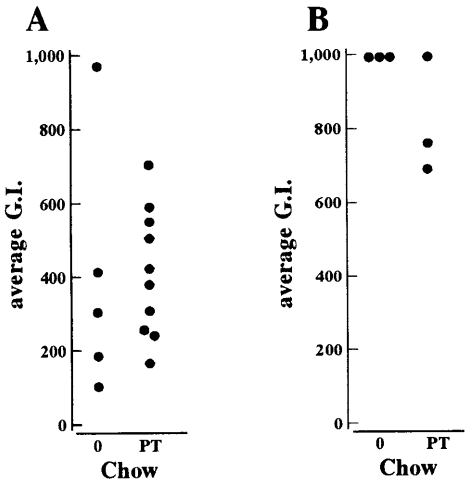

FIG. 3. *M. leprae*-infected *nu*/*nu* mice, fed standard mouse chow, were given either no adoptive transfer (0) or an i.v. injection of T-cell-enriched cell samples isolated from the lymph nodes of *M. leprae*-infected mice (PT [primed T cells]). Two weeks after adoptive transfer, *M. leprae* bacilli were harvested from the footpads and their viability was determined by the BACTEC 460 system. Four separate adoptive-transfer experiments were done. Each datum point represents the GI reading for an individual mouse. (A) Results obtained with BACTEC vials inoculated with 10^7 *M. leprae* bacilli. $P = 0.4647$ for chow-0 values versus chow-PT values. (B) Results obtained with BACTEC vials inoculated with 10^8 *M. leprae* bacilli. $P = 0.1201$ for chow-0 values versus chow-PT values.

Production of PGE_2 by granuloma Mϕ is regulated by dietary EFA. Because the *M. leprae*-gorged Mϕ of the *nu*/*nu* footpad granuloma produce large amounts of PGE_2 (44), it was important to ascertain that PGE_2 levels at this site were modified by diet. *M. leprae*-infected footpad granuloma Mϕ isolated from EFAD mice secreted significantly less PGE_2 ($P = 0.028$) than granuloma Mϕ from control mice (Table 2). These findings show that dietary EFA deprivation abrogates PGE_2 production at the site of infection in these mice. Com-

TABLE 1. Fatty acid compositions of liver phospholipids

Fatty acid	wt% of total identified fatty acid in mice fed the following diet[a]	
	EFAS	EFAD
16:0	28.31 ± 4.15	25.81 ± 3.50
16:1(n-7)	2.28 ± 0.72	4.89 ± 1.18
18:0	12.02 ± 2.37	6.87 ± 1.32
18:1(n-9) T[b]	16.53 ± 4.15	39.57 ± 5.77
18:1(n-9) C[c]	3.66 ± 1.17	7.34 ± 2.44
18:2(n-6)	19.43 ± 1.71	3.75 ± 0.82
20:1(n-9)	0.56 ± 0.13	1.83 ± 0.61
20:3(n-9)	0.28 ± 0.29	3.99 ± 1.18[d]
20:3(n-6)	1.03 ± 0.22	0.61 ± 0.13
20:4(n-6)	12.35 ± 2.06	3.93 ± 1.09[d]
22:4(n-6)	0.63 ± 0.22	0.10 ± 0.06
22:5(n-6)	2.38 ± 1.03	0.52 ± 0.14
22:6(n-3)	0.53 ± 0.12	0.80 ± 0.17

[a] Values are percentages of total fatty acid in liver phospholipids by weight (means ± standard deviations). Eight mice per diet were evaluated in each case.
[b] T, *trans*.
[c] C, *cis*.
[d] EFAS diet versus EFAD diet; $P < 0.0001$.

TABLE 2. PGE_2 and TXB_2 production by granuloma and peritoneal Mϕ harvested from *M. leprae*-infected *nu/nu* mice[a]

Mϕ	Diet	PGE_2 (ng/ml)	TXB_2 (ng/ml)
Granuloma	Control	11.05 ± 2.44	1.49 ± 0.98
	EFAD	0.96 ± 0.12[b]	1.16 ± 1.02
Peritoneal	Control	0.44 ± 0.07[c]	3.79 ± 1.48
	EFAD	0.20 ± 0.04[c]	2.26 ± 1.29

[a] *nu/nu* mice were infected in BHF with 10^8 *M. leprae* bacilli. At 7 months postinfection, groups of mice were either continued on mouse chow (control) or fed an EFAD diet. Mϕ were harvested 2 months later. Values are means ± standard deviations obtained with duplicate wells. Data are from one of two independent experiments. Values have been corrected for background levels: the medium contained 0.23 ng of PGE_2/ml and 9.66 ng of TXB_2/ml.

[b] Control diet versus EFAD diet; $P < 0.03$.

[c] Granuloma Mϕ versus peritoneal Mϕ in mice on the same diet; $P < 0.03$.

pared to background levels, no PGE_2 was produced by the peritoneal Mϕ isolated from either control or EFAD mice. In addition, these samples were also analyzed for the presence of a second COX product of AA, TXB_2. Levels of TXB_2 production were very low, and no difference between granuloma Mϕ from EFAS mice and those from EFAD mice was seen. TXB_2 levels were higher in peritoneal Mϕ, but there was no significant difference between the EFAD and control groups.

Effect of dietary EFA on the fate of *M. leprae* in infected *nu/nu* recipients of T cells. As shown in Fig. 2, *M. leprae*-infected *nu/nu* mice were fed either an EFAS or an EFAD diet. After approximately 3 months, groups of these mice received T-cell-enriched samples of 17×10^6 cells from naive mice or from *M. leprae*-primed mice. Two weeks later the footpads were harvested for histopathology and BACTEC studies.

Figure 4 shows the metabolic activity of *M. leprae* isolated from infected EFAS or EFAD *nu/nu* recipients of primed T cells. Figure 4A depicts results obtained with BACTEC vials inoculated with 10^7 AFB. For mice fed the EFAS diet, the scatter of GI points does not demonstrate a difference between recipients of naive T cells and recipients of primed T cells ($P = 0.9075$). However, in mice fed the EFAD diet, the difference between recipients of naive T cells and of primed T cells is apparent. The metabolism of *M. leprae*, derived from mice fed the EFAD diet and inoculated with primed T cells, was markedly reduced in all but one of eight mice ($P = 0.0020$). Therefore, induction of CMI by primed T cells, measured as the degree of killing of *M. leprae* in the recipient, was significantly more effective in *nu/nu* mice fed an EFAD diet than in those fed an EFAS diet ($P = 0.0182$). Figure 4B, which depicts the results obtained with BACTEC vials inoculated with 10^8 bacilli, demonstrates that *M. leprae* bacilli from EFAS mice which were recipients of naive T cells were metabolically active as shown by peak GI values in five of eight mice and exhibited definite metabolic activity in the remaining three mice. Likewise, *M. leprae* from most mice which were fed EFAS diets and which received primed T cells exhibited peak metabolic activity ($P = 0.2240$). Interestingly, in three of eight EFAS mice which received primed T cells, the *M. leprae* bacilli were metabolically suppressed, suggesting effective adoptive transfer of CMI. In EFAD mice which received either no cells or naive cells, the metabolism of *M. leprae* was not affected, indicating that the EFAD diet itself had no adverse effect on the viability of the bacilli in the *nu/nu* mouse footpad. In marked contrast, in 9 of 11 EFAD mice which were recipients of primed T cells, the metabolism of *M. leprae* was almost totally suppressed ($P = 0.0001$). These data again show that transfer of primed T cells results in greater *M. leprae* inhibition in EFAD mice than in

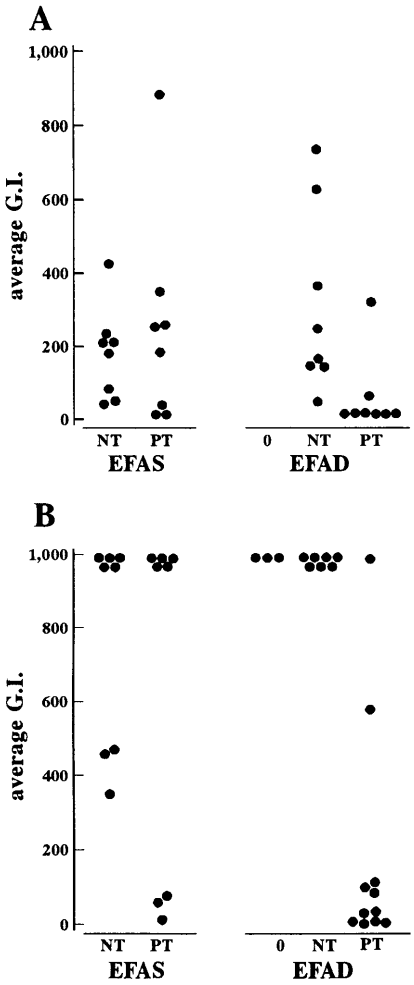

FIG. 4. *M. leprae*-infected *nu/nu* mice, fed an EFAS or an EFAD diet, were given i.v. injections of T-cell-enriched cell samples isolated from the spleens of uninfected mice (NT [naive T cells]) or from the lymph nodes of *M. leprae*-infected mice (PT [primed T cells]). Two weeks after adoptive transfer, *M. leprae* bacilli were harvested from the footpads and their viability was determined by the BACTEC 460 system. Three separate adoptive-transfer experiments were done. 0, no adoptive transfer. Each datum point represents the GI reading for an individual mouse. (A) Results obtained with BACTEC vials inoculated with 10^7 *M. leprae* bacilli. Statistical significances were as follows: EFAS-NT versus EFAS-PT, $P = 0.9075$; EFAD-NT versus EFAD-PT, $P = 0.0020$; EFAS-PT versus EFAD-PT, $P = 0.0182$. (B) Results obtained with BACTEC vials inoculated with 10^8 *M. leprae* bacilli. Statistical significances were as follows: EFAS-NT versus EFAS-PT, $P = 0.2240$; EFAD-NT versus EFAD-PT, $P = 0.0001$; EFAS-PT versus EFAD-PT, $P = 0.0058$.

EFAS mice ($P = 0.0058$). There was no significant difference in the number of *M. leprae* bacilli per gram of tissue in any of the footpads tested, regardless of the diet of the recipient mice or of the source of the donor cells (mean ± standard error of the mean = $2.14 \times 10^{10} \pm 0.21 \times 10^{10}$ *M. leprae* bacilli/g of footpad tissue; $n = 41$).

DISCUSSION

The major COX-derived metabolite of AA produced by activated Mϕ is PGE_2. It is generally recognized that PGE_2 possesses immunosuppressive actions and serves as a negative regulator of the immune response (16, 20). Previous in vivo studies have shown that EFA deficiency, which reduces PGE_2 production, accentuates the severity of experimental allergic encephalomyelitis, a T-cell-dependent autoimmune inflamma-

tory disease of the central nervous system in rats (48). The severity of the disease was ameliorated by supplementation with EFA, and treatment of the animals with indomethacin, a pharmacologic inhibitor of COX, abrogated the improvement resulting from EFA supplementation. Others have demonstrated accelerated skin allograft rejection in mice fed an EFAD diet, and oral supplementation with linoleic acid prolonged survival of the skin allograft (25). Another group has shown that EFAD mice infected with *Trypanosoma cruzi* exhibited reduced parasitemia and increased survival compared to control animals (36). Each of these observations suggests that EFA deficiency potentiates CMI. In the present study, we provide experimental in vivo evidence that EFA deficiency is crucial to the successful induction of CMI by adoptively transferred T lymphocytes in an athymic mouse model of leprosy.

Our previous work identified PGE_2 produced by *M. leprae*-infected Mϕ as a likely candidate for the immunosuppressive agent in this model (44). Mϕ activation requires two signals: a priming signal, with IFN-γ being the major activating factor (27), and a triggering signal generally provided by lipopolysaccharide or tumor necrosis factor alpha (30, 42, 51). Activated murine Mϕ efficiently exert a deleterious effect on *M. leprae* via an IFN-γ–lipopolysaccharide-induced, L-arginine-dependent production of nitric oxide (1). However, Mϕ infected in vitro with high numbers of bacilli are refractory to these activation signals (45). In *nu/nu* mice, the defective response is a localized phenomenon, being restricted to the *M. leprae*-gorged Mϕ of the granulomatous footpad (43). Peritoneal Mϕ from these same animals are responsive to IFN-γ.

Coinciding with this inability to become activated by IFN-γ, infection of Mϕ with large numbers of viable *M. leprae* bacilli results in a burst of PGE_2 production (45). Exogenous PGE_2 mimics this downregulatory effect in normal Mϕ, and inclusion of indomethacin reverses the effects of *M. leprae* infection on the Mϕ. Mϕ from *nu/nu* mouse footpad granulomas similarly produce large amounts of PGE_2 in culture (44), although little PGE_2 is generated by peritoneal Mϕ from these same mice, again indicating a localized effect.

The ability of PGE_2 to abate a T-cell response has been reported for many in vitro systems (3, 10, 26, 38), including those involving mycobacteria (2, 9, 11, 23, 34, 49, 53). Our demonstration of a reduction in T-cell responsiveness in the presence of PGE_2 is especially intriguing in light of the fact that the adoptive transfer of primed-T-cell-enriched samples of immune cells into our *M. leprae*-infected *nu/nu* mice fed a diet of standard mouse chow resulted in little demonstrable inhibition of *M. leprae* metabolism.

Therefore, an amalgamation of our in vivo and ex vivo observations led us to hypothesize that sufficient amounts of PGE_2 are produced locally in the lepromatous footpad to downregulate the immune competence of an adoptively transferred, highly activated T-cell population. Even if this T-cell population retains its helper functions, the *M. leprae*-gorged Mϕ in the footpad are refractory to the activating signals of IFN-γ. The footpad granuloma of the *M. leprae*-infected *nu/nu* mouse is a very dynamic lesion; 15 to 20% of the infected cells are less than 1 week old (19). The new monocytes could also be downregulated by elevated PGE_2 levels as they migrate into the footpad, although our in vitro studies suggest that there is a delay of 24 h before exogenous PGE_2 renders the Mϕ unresponsive to activation (45). Therefore, the elimination of local PGE_2 production in the *M. leprae*-infected *nu/nu* footpad should allow a protective response to be initiated by adoptively transferred *M. leprae*-primed T cells.

Because *nu/nu* mice do not readily lend themselves to excessive handling and repeated injections, we chose to regulate PGE_2 production by dietary, rather than pharmacologic, means. EFA deprivation has different effects on the AA contents of different tissues (21). Over the course of several weeks, there was a reduction of AA in the hepatic lipids. More importantly, however, this diet was capable of reducing PGE_2 production at the site of infection, i.e., the lepromatous footpad. Furthermore, the long division time of the intracellular leprosy bacillus, 2 weeks (40), and the resulting protracted development of the lepromatous granuloma in *nu/nu* mice make this an ideal model to allow the demonstration of subtle modulating influences, such as those elicited in this study.

Clearance of the bacilli from the lepromatous footpad was not demonstrated, even in EFAD mice which received primed T cells. This is not surprising since it may take months to years of antimycobacterial treatment before clearance of bacilli from the lesions of patients with lepromatous leprosy is observed. In 1962, Waters and Rees (50) introduced a morphological index (MI) based on the solid and beaded carbolfuchsin staining characteristics of *M. leprae*. Following effective chemotherapy, the MI decreases much sooner than clearance of the bacilli occurs. For clinical purposes in human leprosy, the MI is employed only in smears of bacteria. However, in sectioned and stained preparations of footpad tissue from control and experimental *nu/nu* mice, differences in the morphology of the bacteria were discernible. *M. leprae* bacilli from the footpads of EFAS mice which were recipients of primed T cells were more solidly stained, while those from EFAD mice which were recipients of primed T cells exhibited a more beaded appearance. These subjective differences were agreed upon by three different observers in a blinded fashion. The highly subjective nature of this technique, however, reinforces the value of the recently described BACTEC system for the rapid and quantitative determination of the viability of the uncultivable leprosy bacillus (13, 31).

There are, of course, many metabolic products into which AA may be converted, including an assortment of prostaglandins and thromboxanes and a variety of hydroxyeicosatetraenoic acids (47). The involvement of these metabolites in immune functions is an area of active and expanding interest. Although TXB_2 was not generated by footpad granuloma Mϕ, it cannot be ruled out that some of the other products of AA metabolism may play important roles in the regulation of the immune response in leprosy. Likewise, even though the EFAS and EFAD diets were isocaloric and nutritionally identical except for the fatty acid present, the possibility of diet-induced changes in membrane fluidity, adherence, or phagocytic activity in the Mϕ also cannot be excluded. Nevertheless, the correlation of PGE_2 production with the downregulation of both T-cell and Mϕ (44, 45) functions coupled with the restoration of immune functions when PGE_2 production is reduced in vivo with an EFAD diet or in vitro with indomethacin (44, 45) is striking. While having little influence on cells in the rest of the body, induction of enhanced Mϕ PGE_2 production by *M. leprae* or its constituents in the microenvironment of the lepromatous granuloma could affect considerably the function of the afflicted and surrounding cells, both T cells and Mϕ, resulting in a localized immune deficiency in the vicinity of the bacilli.

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