

## Immune Reactivity to Fractionated *Leishmania aethiopia* Antigens during Active Human Infection

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Fractionated antigen preparations of *Leishmania aethiopia* parasites were used to stimulate the peripheral blood lymphocytes of patients with active cutaneous leishmaniasis. In assays measuring lymphocyte proliferation, 9 of 10 patients with similar clinical presentations of infection responded in a similar pattern to the fractionated antigens. Marked proliferation was observed in response to antigen fractions with molecular masses of 43 to 36, 33 to 27, and less than 22 kDa. The induction of relatively high levels of gamma interferon (IFN- $\gamma$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) was also observed in responses to these same three antigen fractions. In contrast, the proliferative, IFN- $\gamma$ , and TNF- $\alpha$  responses of patient lymphocytes to antigens with a molecular mass greater than 60 kDa were uniformly low. The results of this study suggest that the antigens of *Leishmania* parasites, which are recognized by T cells in patients with active cutaneous leishmaniasis, may be partitioned in the lower-molecular-mass antigenic determinants associated with whole-parasite preparations. The observed association between antigen-induced proliferation and IFN- $\gamma$  and TNF- $\alpha$  production may be indicative of potential disease-limiting immune effector activities which have developed during infection.

*Leishmania aethiopia* is the causative agent of cutaneous leishmaniasis in the highlands of Ethiopia and the infection can present in either of two clinical forms (4). The majority of cases occur as a localized cutaneous leishmaniasis (LCL) in which a single lesion develops at the site of infection. Left untreated, these lesions will heal over a period of time ranging from 3 months to more than a year. The rarer form of *L. aethiopia* infection is diffuse cutaneous leishmaniasis (DCL) in which multiple lesions develop over the face, trunk, and extremities. Histopathological studies have shown that few parasites are observable in the lesions of LCL patients while each of the lesions of DCL patients contains many organisms (4). While the biological basis for the differences between the development of LCL and DCL remains unknown, it has been suggested that both host factors and differences in parasite pathogenicity may play an important role in determining the outcome of infection (1, 2, 4, 5).

Previous studies have shown differences in the abilities of LCL and DCL patient lymphocytes to respond to *L. aethiopia* antigens (1, 2, 5, 23). LCL patient lymphocytes exhibit significant proliferation (1, 2, 23) and interleukin-2 and gamma interferon (IFN- $\gamma$ ) production in response to whole-cell lysates of *L. aethiopia* in vitro (2) and react by delayed hypersensitivity skin testing in vivo (5). In contrast, DCL patient lymphocytes are unable to respond to *L. aethiopia* antigens in vitro by assays measuring proliferation and interleukin-2 and IFN- $\gamma$  expression or skin test reactions (1, 2, 5, 23). It is therefore believed that a dysfunction in

antigen-specific immune effector activities may contribute to the pathogenesis associated with the DCL form of infection.

Little is known about the molecular identities of the parasite antigens which are responsible for inducing disease-limiting immune reactivity following *L. aethiopia* infection. Previous studies in experimental models of *Leishmania* infection have associated IFN- $\gamma$  production with the killing of intracellular parasites, such as *Leishmania* spp., and recovery from infection (10, 15, 16). Production of the cytokine tumor necrosis factor alpha (TNF- $\alpha$ ) has also been shown to be induced during microbial infections, and TNF- $\alpha$  may act synergistically with IFN- $\gamma$  to reduce intracellular parasitism (9). The identification of the specific *Leishmania* antigens which induce the expression of IFN- $\gamma$  and TNF- $\alpha$  would be of value in elucidating the mechanisms of immune resistance which control parasite growth and in developing strategies of immune intervention for the treatment of cutaneous leishmaniasis.

In a recent study, Melby et al. (13) reported on the patterns of proliferative and IFN- $\gamma$  responses by patient lymphocytes to *Leishmania* antigens fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. A heterogeneous proliferative response to a wide variety of antigens with molecular masses of less than 100 kDa was observed in the lymphocytes of cured or healing LCL patients. The IFN- $\gamma$  responses of the cured LCL patients to the same fractions of antigens were also heterogeneous and were associated with proliferation in 25 to 85% of the observed responses.

In this study we have observed 10 LCL patients with active *L. aethiopia* infection and their peripheral blood lymphocyte responses to parasite antigen fractions. In 9 of the 10 patients tested, stimulation of lymphocytes in vitro with three distinct antigen fractions with molecular masses of less than 45 kDa induced both proliferation and the concomitant expression of the cytokines IFN- $\gamma$  and TNF- $\alpha$ .

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## MATERIALS AND METHODS

**Study subjects.** Ten patients, aged 15 to 68 years, with active LCL who attended the dermatology clinic of the All Africa Leprosy Rehabilitation and Training Center Hospital in Addis Ababa, Ethiopia, were included in the study. The patients were diagnosed by clinical manifestations and histopathological examination and/or parasite culture from skin biopsy specimens (1, 2). Clinically there were no distinguishable differences between the study patients, and all presented with singular lesions. Two of the patients were culture negative but histopathologically confirmed to have leishmaniasis by the finding of Leishman-Donovan bodies in the biopsy specimen. Eight healthy individuals were also included in the study; six had no history of exposure to leishmaniasis and two were laboratory personnel involved in leishmaniasis research projects.

**Preparation and culture of parasites.** *L. aethiopica* isolate 1627/87 was grown from stocks of parasites in the collection of Armauer Hansen Research Institute. The strain was isolated from a skin lesion of an Ethiopian patient with LCL and was confirmed to be *L. aethiopica* by DNA hybridization with an *L. aethiopica*-specific kDNA probe (11a). Promastigotes were grown at 25°C in RPMI 1640 medium (Flow, Irvine, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml as previously described (2). Promastigotes in stationary phase were harvested by centrifugation at 3,000 rpm for 10 min and washed twice in phosphate-buffered saline (PBS). Washed promastigotes were resuspended in PBS containing 1.2 mM phenylmethylsulfonyl fluoride (Sigma) and kept frozen at -70°C. This preparation was referred to as whole *L. aethiopica* antigen.

**One-dimensional immunoblots.** SDS-PAGE was performed under reducing conditions with 10% polyacrylamide and 2.6% cross-linking, using vertical slab gels (160 by 180 by 1.5 mm). Antigen samples were boiled in a water bath for 5 min in sample buffer (0.5 M Tris HCl [pH 6.8], 25% glycerol, 4% SDS, 2.5% 2-mercaptoethanol), cooled, and spun prior to loading. Electrophoresis was stopped when the dye front had migrated 8 cm in the resolving gel. Molecular mass markers (Bio-Rad) were run on the same gel and were used to extrapolate the molecular masses of the *Leishmania* antigens. The proteins were then electrotransferred to nitrocellulose (NC) paper with a pore size of 0.2 µm (Sartorius) in transfer buffer (370 mM glycine, 30 mM Tris HCl [pH 8.3]) for 75 min at 42 V at 4°C, using a Bio-Rad Trans-Blot apparatus as previously described (14). The NC paper was then cut into 4-mm-wide horizontal strips for use in T-cell stimulation assays or 7-mm strips for immunoblotting with patient sera (11). For T-cell immunoblotting, sterilization was effected by overnight incubation with 0.1% sodium azide in PBS. The NC strips were then washed with sterile PBS and cut into squares (4 by 4 mm) and transferred to triplicate wells of a microtiter plate. As a control, 40 µg of whole *L. aethiopica* antigen was spotted onto NC squares (4 by 4 mm). Immunoblotting with sera as well as immunoperoxidase staining of blots was performed as previously described (14).

**Isolation and culture of PMC.** Peripheral blood mononuclear cells (PMC) were obtained by venipuncture, followed by defibrination with glass beads and separation on a Ficoll gradient. The cells were washed three times in RPMI 1640 (Flow), and 200-µl samples containing  $4 \times 10^5$  PMC were added to the inner 60 wells of flat-bottomed 96-well micro-

titer plates (Falcon) which contained RPMI 1640 plus either antigens, phytohemagglutinin (2 µg/ml; Difco, Detroit, Mich.), or 10% normal human serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2 mM glutamine (GIBCO, Grand Island, N.Y.). The cultures were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 6 days, at which time [<sup>3</sup>H]thymidine (1 µCi) (Amersham, Buckinghamshire, United Kingdom) was added to each well. After an additional 18 h of incubation, the cells were harvested with a Skatron (Lierbyen, Norway) harvester onto filter paper, allowed to dry, and then prepared for liquid scintillation counting in an LKB Rackbeta counter. Results are presented as mean counts per minute present in stimulated cultures after the subtraction of counts per minute present in PMC cultured without antigen. The stimulation index (SI) is simply the counts per minute of antigen-stimulated cultures/counts per minute of control cultures without added antigen.

**IFN-γ and TNF-α assays.** Supernatants from triplicate cell cultures stimulated as described above were collected after 6 days of incubation, pooled, and stored at -70°C until assayed. IFN-γ and TNF-α activities in each supernatant pool were separately measured by enzyme-linked immunosorbent assay kits purchased from the Holland Biotechnology bv., Leiden, The Netherlands, and T Cell Sciences, Inc., Cambridge, Mass., respectively, and used per manufacturer's instructions.

**Statistical analysis.** The Student *t* test in two-tailed comparisons was used to determine significant differences between the test groups within the same assay. Regression analysis was used to compare the significance of differences between assays.

## RESULTS

**Proliferative responses to whole and fractionated *L. aethiopica* antigens.** Ten patients with active LCL were tested to determine the pattern of specific antigen recognition by PMC in proliferation assays. A strong proliferative response to whole *L. aethiopica* antigen preparations (Table 1) was observed in all of the 10 patients studied, with a mean stimulation of  $73,920 \pm 6,845$  cpm and a mean SI of  $55.6 \pm 24.06$ . A similar assay on the PMC of five healthy control subjects to the same antigen preparation showed significantly lower stimulation of  $18,790 \pm 4,581$  cpm (SI =  $22.5 \pm 18.5$ ,  $P < 0.005$  [data not shown]).

To identify the molecular masses of the antigens that stimulated the proliferation of PMC, *L. aethiopica* antigen preparations were separated by SDS-PAGE and transferred onto NC filter sheets. Proliferative assays were then performed with PMC incubated with each of the 20 individual NC-bound fractions of the separated *L. aethiopica* antigens. For molecular mass comparisons, an SDS-polyacrylamide gel containing *L. aethiopica* promastigotes separated similarly (see above) and stained for total protein is shown in Fig. 1. The SIs of the 10 patients to all antigen fractions are shown in Table 1. The PMC from 9 of the 10 LCL patients (patients 1 to 9) showed greater proliferative responses to the low-molecular-mass fractions with molecular masses of 43 to 36 (fractions 11 and 12), 33 to 27 (fractions 14 to 16), and less than 24 (fractions 18 to 20) kDa. A representative panel of observed proliferative responses from four individual patients with active LCL (Fig. 2A, B, D, and E) and two healthy controls (Fig. 2C and F) is shown. Some individual variation in the magnitude of the specific responses was seen between the infected subjects. However, when analyzed individually and in relation to the responses to all 20 frac-

TABLE 1. SI in proliferation assays to unfractionated and fractionated *L. aethiopicum* antigens

Stimulant	SI of patient:									
	1	2	3	4	5	6	7	8	9	10
Unfractionated <sup>a</sup>	66.2	90.4	33.3	21.4	34.9	82.9	21.7	11.7	31.8	32.4
Antigen fraction <sup>b</sup>										
1	12.8	8.4	2.7	2.0	1.9	10.5	2.8	2.5	7.6	4.6
2	16.9	7.4	2.5	0.9	2.2	7.9	2.8	2.0	6.0	4.7
3	9.0	7.6	2.0	1.4	3.5	7.9	1.4	2.0	5.1	6.1
4	9.0	7.4	2.4	1.5	1.9	13.2	2.1	2.4	3.8	11.3
5	8.6	7.2	2.0	1.3	1.7	15.8	1.8	2.5	4.4	16.9
6	8.6	9.6	1.5	1.9	1.9	16.0	2.1	2.0	5.7	6.6
7	10.0	7.2	1.6	1.5	2.9	7.9	2.1	2.6	3.2	8.7
8	13.1	9.6	2.0	1.6	1.9	9.2	3.9	3.5	8.9	12.1
9	13.1	11.7	3.0	1.7	11.5	10.5	3.2	2.0	8.2	9.6
10	11.7	13.8	6.7	1.8	13.5	7.9	7.9	2.6	12.3	6.9
11	26.9	19.7	24.2	4.6	12.5	32.9	9.3	6.0	8.9	2.4
12	15.9	19.7	13.3	0.9	10.3	11.8	16.4	5.3	7.6	4.4
13	17.2	13.8	15.8	1.3	15.4	15.1	10.0	2.8	10.8	8.6
14	26.9	22.9	21.2	3.2	18.6	22.4	14.3	3.9	11.4	14.3
15	42.1	21.1	36.4	1.8	17.6	92.1	5.7	6.8	18.0	12.6
16	43.0	12.0	40.0	1.7	13.8	22.5	3.2	3.6	10.1	15.6
17	15.2	14.9	9.7	2.1	16.7	55.3	6.8	5.0	7.6	18.7
18	16.0	21.6	24.2	3.0	16.0	52.6	10.7	4.5	8.9	24.3
19	43.2	25.5	30.3	3.2	19.2	21.1	11.4	5.7	13.9	13.9
20	20.0	25.0	27.3	4.6	27.2	14.5	23.6	7.7	8.9	25.8
Phytohemagglutinin	24.6	24.0	15.8	29.7	30.7	33.2	21.7	11.7	31.8	32.4

<sup>a</sup> Whole *L. aethiopicum* promastigotes.

<sup>b</sup> T-cell immunoblot fraction number.

tions, each of the patients had elevated proliferative activity against the same three low-molecular-mass antigen fractions. None of the healthy controls tested showed significant proliferative responses to the three antigen fractions recognized by the patients with active infection.

**Comparison of patient antibody and cellular recognition profiles.** Each of the study subjects was also tested for antibody reactivity against the same *L. aethiopicum* blots that were used to prepare antigens for the proliferative assays. At the top of each panel in Fig. 2 is shown the reactivity pattern of the serum immunoglobulins from the respective patient or controls on a conventional immunoblot. Each of the LCL patients showed antibody reactivity to antigens with molecular masses of greater than 50 kDa and in particular to

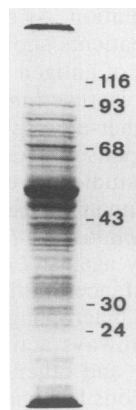


FIG. 1. Profile of *L. aethiopicum* proteins separated by SDS-PAGE. Relative positions of molecular mass standards on SDS-PAGE are indicated to the right of the gel.

antigens with molecular masses of 50, 55, 70, and 72 kDa. As such, significant antibody reactivity was not observed against the same antigens which stimulated lymphoproliferation.

**IFN- $\gamma$  and TNF- $\alpha$  responses to fractionated antigens.** The production of IFN- $\gamma$  and TNF- $\alpha$  by PMC cultures stimulated with each of the 20 *Leishmania* antigen fractions was measured in two LCL patients with representative profiles of proliferation to the antigen fractions (Fig. 3 [patient 2 in Table 1]; Fig. 4 [patient 3 in Table 1]). Figure 3A and 4A show IFN- $\gamma$  production. Three peaks of IFN- $\gamma$  production were observed to the fractions with molecular masses of 43 to 36, 33 to 27, and 22 kDa which overlapped with the three peaks of lymphoproliferation (Fig. 3C and 4C). There was no detectable IFN- $\gamma$  production induced by the NC-bound antigens with molecular masses of greater than 45 kDa. The direct comparisons of the responses to each of the fractions tested showed a strong correlation between IFN- $\gamma$  production and lymphoproliferation ( $r = 0.884$ ,  $P < 0.001$  [Fig. 3];  $r = 0.908$ ,  $P < 0.001$  [Fig. 4]).

The supernatants from the cultures of PMC stimulated with NC-bound *L. aethiopicum* fractions were further tested to determine the levels of TNF- $\alpha$  induced in the antigen-driven responses. The patterns of TNF- $\alpha$  production (Fig. 3B and 4B) in response to antigen were similar to the lymphoproliferative and IFN- $\gamma$  responses described above. There was little measurable TNF- $\alpha$  production induced by NC squares with antigens with molecular masses of greater than 45 kDa. In the lower-molecular-mass range, three peaks of TNF- $\alpha$  production were again observed in positions overlapping with the peaks observed in the lymphoproliferative (Fig. 3C and 4C) and IFN- $\gamma$  (Fig. 3A and 4A) curves. The responses of the two representative patients to all 20 antigen fractions showed significant correlation between TNF- $\alpha$  production and proliferation ( $r = 0.871$ ,  $P < 0.001$

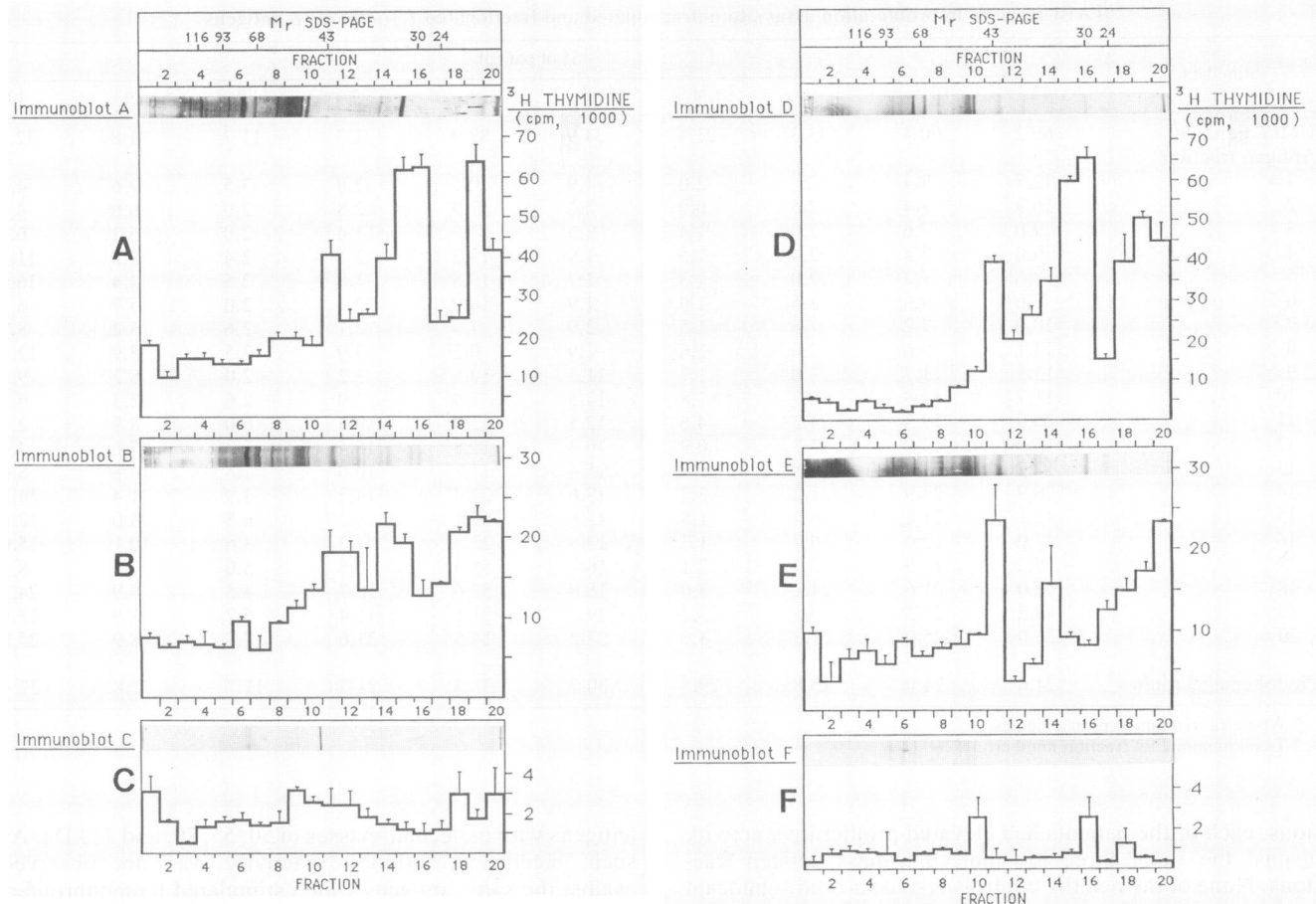


FIG. 2. Profiles of lymphoproliferation and immunoblot reactivities of patients and controls. The individual lymphoproliferative responses of four representative patients (patient 1 [A], patient 2 [B], patient 3 [D], and patient 4 [E]) and two healthy nonimmune individuals (C and F) to 20 fractions of the *L. aethiops* antigens are shown (data taken from Table 1 and plotted). Line bars indicate standard errors of the means for triplicate determinations. At the top of each panel is the immunoblot showing the subject's antibody reactivity to the equivalent antigens contained in the respective fraction. Relative positions of molecular mass standards on SDS-PAGE are shown.

[Fig. 3B and C];  $r = 0.869$ ,  $P < 0.001$  [Fig. 4B and C]) and the expression of IFN- $\gamma$  ( $r = 0.814$ ,  $P < 0.001$  [Fig. 3A and B];  $r = 0.755$ ,  $P < 0.001$  [Fig. 4A and B]).

#### DISCUSSION

In this study we have investigated the immunoreactivities of polypeptide antigens purified from *L. aethiops* parasites with peripheral blood lymphocytes and antibodies of patients with active cutaneous leishmaniasis. The antigens used in this study were separated from whole-cell lysates of *L. aethiops* promastigotes by preparative electrophoresis which resolved into 20 fractions with molecular masses ranging from 200 to less than 22 kDa. In assays measuring the cellular responses of 10 patients with LCL, we observed significant proliferative responses by cells stimulated in vitro by the low-molecular-mass fractions of the antigens tested. Three fractions with molecular masses of 43 to 36, 33 to 27, and less than 22 kDa were found to be potent activators of T-cell activity in 9 of the 10 patients tested. There was no apparent difference in the clinical presentation of leishmaniasis in the patient who did not respond and the nine patients who showed responsiveness. All nine of the responding patients showed very similar and reproducible patterns of strong lymphoproliferation in response to all three of these

fractions. In further study, we observed that the supernatants from the mononuclear cell cultures stimulated with these same three fractions also contained high levels of IFN- $\gamma$  and TNF- $\alpha$ .

A direct comparison of the antigen specificity of patient T cells and antibodies showed differences in the two respective patterns of antigen recognition. As observed in our previous study (14), each of the patients showed some differences in their respective patterns of antigen recognition on immunoblots. The most intensive reactions with antibodies were observed against the higher-molecular-mass antigens which did not induce T-cell responses. This finding is similar to the results of our previous studies investigating the T-cell and antibody responses of human subjects against another intracellular pathogen, *Mycobacterium leprae* (7, 19).

The immune reactivity against *Leishmania* parasite antigens which confers resistance to infection is mediated by T cells which likely induce macrophages to destroy intracellular parasites through pathways activated by IFN- $\gamma$  (6, 15). The results of our study and others (2, 12, 24, 25) suggest that during active cutaneous leishmaniasis, specific antigen-driven stimulation may induce several coupled immune effector activities, which are associated with the expression of IFN- $\gamma$ . In this study we have shown that the stimulation of

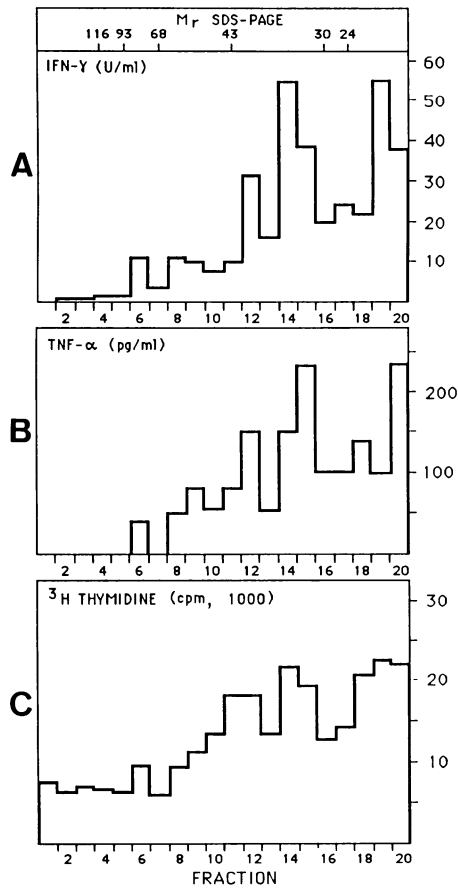


FIG. 3. Comparison of IFN- $\gamma$ , TNF- $\alpha$ , and proliferative responses of patient peripheral blood monocytes stimulated with *Leishmania* antigen fractions. The responses of a representative LCL patient (patient 2 from Table 1) to the 20 fractions of *Leishmania* antigens are shown. (A) IFN- $\gamma$  activity; (B) TNF- $\alpha$  activity; (C) proliferation response. Relative positions of molecular mass standards on SDS-PAGE are shown.

patient lymphocytes with three low-molecular-mass fractions of *L. aethiopic* antigens which induced the expression of IFN- $\gamma$  also resulted in the proliferative expansion of antigen-reactive cells and the expression of TNF- $\alpha$ .

A recent study using methodology similar to ours (13) has reported on the specific responses of subjects with past or healing visceral leishmaniasis (*Leishmania donovani*), LCL (*Leishmania braziliensis*, *Leishmania major*), or mucocutaneous leishmaniasis (*L. braziliensis*) to fractionated *Leishmania* antigens. This study also showed that in general, strong proliferative responses were associated with strong IFN- $\gamma$  responses. Their results differ from ours in that the responses of their subjects were distributed over a broad spectrum of the fractions tested. Patients with active cutaneous leishmaniasis most likely differ from healed or recovering patients in their antigen burden and precursor frequencies of responding cells. As such, the patterns of responsiveness observed in the two studies could be related to the immune and clinical status of the subjects tested. However, while Melby et al. (13) could not detect obvious immunodominant antigens such as we have described, antigens with lower molecular masses (10 to 20 kDa) were more active in the induction of lymphoproliferation than higher-molecular-mass determinants in patients recovering from

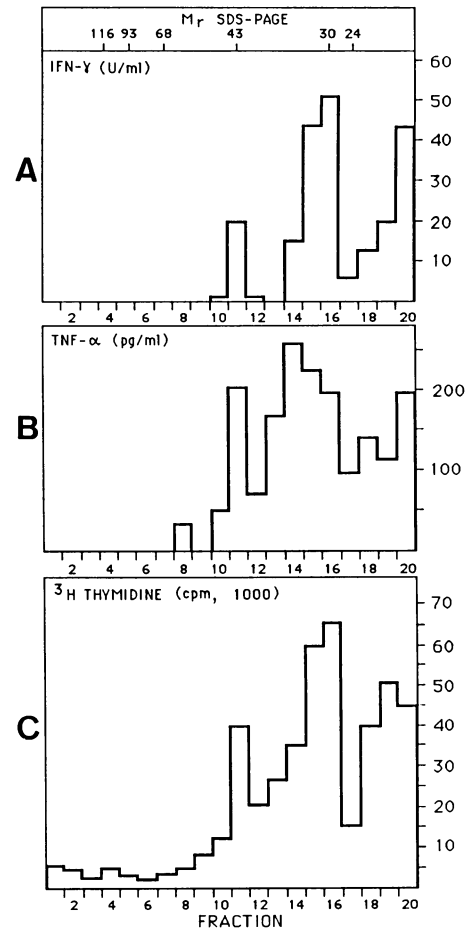


FIG. 4. Comparison of IFN- $\gamma$ , TNF- $\alpha$ , and proliferative responses of patient peripheral blood monocytes stimulated with *Leishmania* antigen fractions. The responses of a representative LCL patient (patient 3 from Table 1) to the 20 fractions of *Leishmania* antigens are shown. (A) IFN- $\gamma$  activity; (B) TNF- $\alpha$  activity; (C) proliferation response. Relative positions of molecular mass standards on SDS-PAGE are shown.

visceral leishmaniasis. Studies using experimental models of murine cutaneous leishmaniasis have also demonstrated that immune T cells are stimulated by low-molecular-mass antigens. T cells from BALB/c mice infected with *L. major* were shown to respond with proliferation and IFN- $\gamma$  production to antigens with relative molecular masses in the range of 8 to 18, 20 to 30, and 40 to 50 kDa (24). The results of our study of the stimulation of human patient T cells with *L. aethiopic* antigens show marked similarities to these observations.

The presence of TNF- $\alpha$  in the culture supernatants with high amounts of IFN- $\gamma$  is concordant with earlier observations that IFN- $\gamma$  enhances the production of TNF- $\alpha$  (18). The enhancing effect of IFN- $\gamma$  on TNF- $\alpha$  production has also been observed during antigen stimulation by *Mycobacterium tuberculosis* (22) and *Plasmodium* parasites (3). This finding may also be of importance in understanding the role of lymphokines in protection against *Leishmania* parasites. While TNF- $\alpha$  has been reported to play a role in the host defense against a variety of intracellular parasites and bacterial pathogens (8, 9, 17, 27, 29, 30), the role of TNF- $\alpha$  in the protective response against *Leishmania* parasites is as yet unclear. We have recently shown that elevated levels of

TNF- $\alpha$  are present in the sera of *L. aethiopica*-infected patients with the DCL form of disease and to a lesser extent in patients with LCL (20). Other studies have also found similar high levels of TNF- $\alpha$  in sera from patients with visceral leishmaniasis, malaria, and trypanosomiasis (26).

Recent reports give evidence that in addition to IFN- $\gamma$ , other lymphokines are also needed to achieve macrophage activation to kill intracellular *L. major* (16). IFN- $\gamma$  and TNF- $\alpha$  act synergistically in the induction of HLA class II molecules (21), monocyte differentiation (28), and induction of macrophages with tumoricidal activities (9). Further, a recent study has demonstrated that IFN- $\gamma$ - and TNF- $\alpha$ -activated macrophages kill intracellular amastigotes in the experimental model of leishmaniasis (3a). Our results show the induction of IFN- $\gamma$  and TNF- $\alpha$  production by the same fractions of *L. aethiopica* antigens. Currently, studies to further elaborate the collaborative action of IFN- $\gamma$  and TNF- $\alpha$  in the activation of human macrophages to eliminate intracellular *Leishmania* parasites are in progress in our laboratory.

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