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Identification of Antigens Recognized by T Cells in Human Leishmaniasis: Analysis of T-Cell Clones by Immunoblotting

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Immunity in human leishmaniasis is mediated by sensitized T lymphocytes; however, the antigens involved in eliciting this immunity have not been defined. We describe the generation of human T-lymphocyte clones derived from two patients with healed leishmaniasis. By use of one- and two-dimensional cellular immunoblotting techniques, we directly identified the parasite antigens recognized by these clones. To our knowledge, these are the first leishmanial antigens identified to which CD4$^+$, gamma interferon-producing T cells from immune individuals have been shown to respond, and the strategy may be of general use for the identification of antigens involved in immunity in this disease.

The intracellular parasite of the genus *Leishmania* causes cutaneous, mucosal, and visceral disease in humans and is a considerable public health problem worldwide. Cutaneous ulceration is usually localized and heals spontaneously or with specific chemotherapy (2, 26). Mucosal involvement is a rare complication of metastatic spread to the nasal or oropharyngeal mucosa from a primary skin lesion (which may have previously healed) and is most commonly caused by members of the *Leishmania brasiliensis* complex (16). These patients develop destructive, disfiguring mucosal lesions which may require extensive surgical debridement, in addition to chemotherapy, to effect a cure. Visceral leishmaniasis, which is caused by *Leishmania donovani*, is characterized by chronic fever, wasting, peripheral blood cytopenias, and massive hepatosplenomegaly and may lead to death without specific chemotherapy (1, 10). In areas endemic for visceral leishmaniasis, there appears to be a significant population of individuals who have had inapparent or self-resolving infection with *L. donovani* and are presumably resistant to visceral disease (1, 10, 25).

The cellular immune response in human leishmaniasis is responsible for the control and resolution of infection (12, 17, 23). Patients with localized cutaneous or mucosal disease generally have a strong cellular immune response to either homologous or heterologous leishmanial antigens, as indicated by a positive delayed-type hypersensitivity skin test or in vitro antigen-specific lymphocyte proliferation (2, 4, 26). In contrast, in subjects with visceral leishmaniasis there is little evidence of a cellular immune response against parasite antigens until cure is accomplished (3, 7, 9, 24, 25). Subclinical or self-resolving *L. donovani* infections appear to elicit an anti-leishmanial cell-mediated immune response (1, 10, 25).

There is extensive epidemiological and limited human experimental data that suggest that recovery from leishmanial infection, with the development of a parasite-specific cellular immune response, results in resistance to reinfection (6, 15, 20). The nature of the parasite antigens involved in eliciting the cellular immune response (and protective immunity) in human infections is unknown. In previous studies we have shown that the T-cell response in human infections is remarkably heterogeneous and that individual antigens differ in their ability to elicit gamma interferon (IFN-γ) production (17a). In the murine model, control or exacerbation of cutaneous disease appears to be associated with different subsets of CD4$^+$ T cells; the subset which produces IFN-γ in response to antigenic stimuli correlates with a protective response (8, 27). Therefore, we employed CD4$^+$; IFN-γ producing human T-lymphocyte clones (which to our knowledge have not been described previously) derived from individuals with healed leishmaniasis to further define the leishmanial antigens which elicit this T-cell response. By using one- and two-dimensional cellular immunoblotting techniques (14, 30; Melby et al., in press), the antigen specificity of these clones could be determined. This strategy of direct identification of T-cell antigens from within a complex antigen pool has great potential for the isolation of the antigens that are involved in immunity to leishmanial infection.

MATERIALS AND METHODS

Subjects. Two subjects with past leishmaniasis were studied. One subject with mucosal leishmaniasis developed extensive nasal mucosal involvement years after resolution of a primary skin lesion. *Leishmania* parasites, presumably *L. braziliensis braziliensis*, were isolated from the nasal lesion (but failed to grow axenically), and the patient was effectively treated by surgical debridement and with pentavalent antimony therapy. Five years prior to this study, the second subject developed a localized inflammatory nodule at the site of an accidental laboratory inoculation with *L. donovani*; there was no evidence of visceral involvement in this subject. The nodule was excised surgically, *L. donovani* was cultured from the tissue, and no further therapy was given, and he has remained free of any evidence of leishmaniasis.

Mononuclear cells. Peripheral blood mononuclear cells (PBMNCs) were isolated by centrifugation of heparinized blood over lymphocyte separation medium (Organon Teknika Corp., Durham, N.C.), washed in RPMI 1640 medium, and stored frozen in liquid nitrogen. Prior to use the cells were rapidly thawed, washed in RPMI 1640 medium, and suspended in complete culture medium containing RPMI 1640 medium with 10% human type AB-negative serum (single donor) or 7.5% autologous plasma, 2 mM glutamine,

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12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and 50 μg of gentamicin per ml.

Antigens. Soluble *L. donovani* antigen (SLDA) was prepared from promastigotes from an Indian strain of *L. donovani* (Mongi strain; National Institutes of Health) by the method of Scott et al. (28). The antigen was used in cell culture at a final concentration of 20 μg/ml.

T-lymphocyte clones. PBMNCs from the two subjects studied were stimulated with SLDA in complete culture medium for 1 week; lymphoblasts were harvested over lymphocyte separation medium and cloned by limiting dilution (seeded at 10, 3, 1, and 0.3 T cells per well) with SLDA and 10^5 irradiated autologous PBMNCs. The percentage of positive wells was consistent with an independent Poisson distribution, and the probability of clonality was approximately 86 and 98% for the MM and SH clones, respectively. After 8 days of culture, cells from microscopically positive wells were expanded either by weekly stimulation with SLDA, 5% human interleukin-2 (IL-2; 640 U/ml; Electro-Nucleonics, Inc., Silver Spring, Md.), and irradiated autologous PBMNCs or by weekly stimulation with 2.5 μg of phytohemagglutinin per ml–5% IL-2–irradiated allogeneic PBMNCs. Cells were rested in the absence of antigen and IL-2 for 3 to 5 days prior to use in antigen stimulation assays. The surface phenotype of the T-cell clones was determined by using a fluorescence activated cell sorter and anti-$CD8^+$, anti-$CD4^+$, and anti-$CD3^+$ monoclonal antibodies (anti-Leu-2, anti-Leu-3, and anti-Leu-4, respectively; Becton Dickinson, Mountain View, Calif.) and an irrelevant control monoclonal antibody.

Antigen stimulation assay. Proliferation and IFN-γ production of the T-lymphocyte clones (TLCs) was measured by culturing 2×10^4 to 3×10^4 TLCs with 1×10^5 autologous irradiated PBMNCs with or without SLDA in 200 μl of complete medium for 3 to 4 days (quadruplicate wells in the standard proliferation assay). Cells were harvested for 18 h after the addition of 1 μCi of [^{3}H]thymidine, and the incorporated ^{3}H was measured by beta-emission spectroscopy. The IFN-γ activity of 3- to 4-day TLC culture supernatants (pooled from quadruplicate wells of the standard proliferation assay) was measured by using a cytopathic effect inhibition assay with vesicular stomatitis virus in WISH cells (Biofluids Inc., Rockville, Md.). IFN-γ activity is expressed as the log titer, and in immunoblot studies it was considered positive if it was greater than or equal to 2.5 times that of the control wells. Because the individual two-dimensional immunoblots contained no replicate wells, statistical analysis could not be done.

One-dimensional immunoblots. One-dimensional immunoblotting studies were done by using a modification of the method of Lamb and Young (14, 30), as described previously (Melby et al., in press). SLDA (300 μg) was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions by using a 7.5 to 15.0% gradient or a 12.5% acrylamide gel (13). The gel was washed extensively in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol), and the separated proteins were transferred to nitrocellulose (NC; pore size, 0.1 μm; Schleicher & Schuell, Inc., Keene, N. H.) (29). The NC was then handled aseptically, washed in sterile phosphate-buffered saline with 100 μg of gentamicin per ml, and overlaid on a 96-well flat-bottom tissue culture plate so that the horizontal bands of transferred protein would run parallel to the short dimension of the plate. NC disks were then punched into individual wells by using a sterile 6-mm skin biopsy punch (Baker-Cummins, Division of Key Pharmaceuticals, Inc., Miami, Fla.) in such a way that all of the NC-bound separated antigen was incorporated into the plate, with 24 different fractions represented in quadruplicate wells. Additional disks of blank NC were punched into control wells. A total of 2×10^4 to 3×10^4 TLCs were cocultured with 1×10^5 to 2×10^5 autologous irradiated PBMNCs in 200 μl of complete medium in wells containing either electrophoretically fractionated, NC-bound antigen or blank NC. For those TLCs that appeared to be dependent on IL-2 for growth, the medium was supplemented with 2.5% IL-2. Cells were cultured for 5 days and harvested as described above.

Two-dimensional immunoblots. Two-dimensional gel electrophoresis was done by the method of Hochstrasser et al. (11). The isoelectric focusing was done on a tube gel (1.5 mm by 60 mm) for 1,600 V-h by using a mini-gel electrophoresis apparatus (Hoefer Scientific Instruments, Inc.). Multiple gels from the same electrophoresis run showed an identical pattern of separation following silver staining. Immunoblots were prepared as described previously (Melby et al., in press) by two-dimensional electrophoresis of 25 to 50 μg of SLDA, and the gels were washed and blotted onto NC as described above for the one-dimensional immunoblots. The nitrocellulose was washed in phosphate-buffered saline and then stained with 0.2% Ponceau S in 3% trichloroacetic acid, which allowed for the visualization of 20 to 30 protein spots. The stained NC was then overlaid on a 96-well culture plate, the location of the stained antigens was marked on the cover of the plate, and individual NC disks were punched into the wells. Cells were cultured and harvested as noted above. By using a small-format two-dimensional gel, the entire two-dimensional immunoblot could be incorporated into two 96-well plates, minimizing the number of cells needed for a single assay. The location of the stained antigens marked on the plate cover could be correlated with a replicate gel that was stained by the method of Oakley et al. (21). In this way an individual antigen which induced a proliferative response from a TLC could be localized to a particular well of the culture plate, and its position could be identified on the stained replicate gel.

RESULTS

Electrophoretic separation of antigens. One-dimensional SDS-PAGE of SLDA under reducing conditions resulted in the separation of multiple proteins, the majority of which were in the range of 10 to 80 kilodaltons (kDa) (Fig. 1). Two-dimensional gel electrophoresis further resolved SLDA into several hundred discrete proteins that could be visualized by silver staining. Most of the proteins migrated within a pH range of 5.0 to 6.5 (Fig. 1).

Generation of TLCs. T-cell clones were generated by limiting dilution following 1 week of stimulation of PBMNCs with SLDA. The clones were expanded by stimulation with antigen, IL-2, and irradiated autologous PBMNCs or phytohemagglutinin, IL-2, and irradiated allogeneic PBMNCs. The latter method proved to be quite effective and reduced the number of cells needed from the subjects studied. All of the clones were of the $CD4^+$ phenotype and proliferated and produced IFN-γ upon stimulation with SLDA (in the absence of IL-2). Antigen-specific responses were tested numerous times, and results of a representative experiment are shown in Table 1. The level of IFN-γ production by clone SH3 may not be significantly more than control levels (statistical analysis could not be done because the IFN-γ was measured in supernatants pooled from replicate wells). The

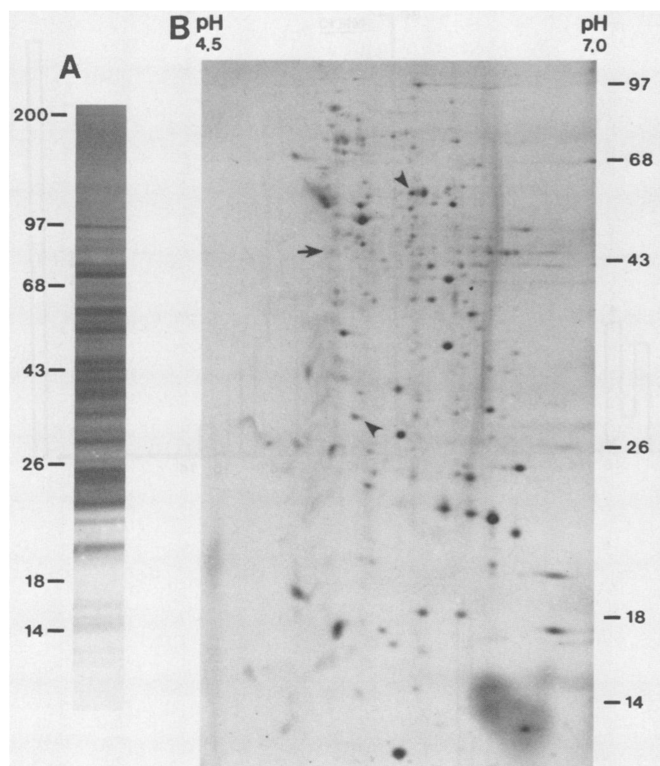

FIG. 1. Separation of soluble SLDAs by one- and two-dimensional electrophoresis. SLDAs were separated by 12.5% SDS-PAGE (300 μg) (A) and in two dimensions (25 μg) (B). Gels were silver stained, and the corresponding molecular weight markers (in thousands) are shown for each gel. The regions of the gel corresponding to the peak proliferative responses of the two clones analyzed by two-dimensional immunoblotting are shown by the arrow (clone SH1) and arrowheads (clone MM10) (see Fig. 3).

clones proliferated much less in response to medium alone, and there was no greater response to medium containing fetal bovine serum, a possible contaminating protein in SLDA (data not shown). A period of rest in the absence of antigen and IL-2 was required to obtain optimal antigen-stimulated proliferative responses, and these responses could then be enhanced by the addition of exogenous IL-2. Clones MM10 and SH1 were highly reactive and were most easily expanded, so they were studied most extensively.

Antigen specificity of TLCs. The antigen specificity of six of the TLCs was analyzed by one-dimensional immunoblotting. SLDA was separated by one-dimensional SDS-PAGE (Fig. 1) and transferred to NC, and TLC proliferation to the NC-bound antigen fractions was measured. These TLCs proliferated in response to a restricted number of the SLDA fractions (Fig. 2). In most instances a peak proliferative response within a single fraction was associated with responses of a lesser magnitude in adjacent fractions. Thus, it appeared that there was some dispersion of antigen determinants during the separation or blotting procedures. Clones MM6 and MM7, which were obtained from the subject with cured mucosal leishmaniasis, each showed a discrete peak of proliferation to antigens with approximate M_rs of 43 and 20 kDa, respectively. To achieve optimal proliferative responses under the immunoblotting conditions, these clones required supplementation with exogenous IL-2 (32 U/ml). A third clone (MM10) from this patient, which demonstrated IL-2-independent growth characteristics, showed a proliferative response to two discrete areas of the immunoblot corresponding to antigens with approximate M_rs of 60 and 26 kDa. Immunoblot analyses of subclones of MM10 showed identical patterns of response (data not shown). Whether this bimodal response was due to the presence of cross-reactive epitopes or degradation of antigen during preparation or electrophoresis is uncertain. Supplementation with exogenous IL-2 enhanced but did not alter the pattern of the response (data not shown).

TLCs generated from a patient with past cutaneous *L. donovani* infection were also analyzed by immunoblotting. A highly reactive clone, SH1, recognized an antigen at approximately 43 kDa, and clone SH3 had a small, but significant, response to an antigen at 90 kDa. A bimodal proliferative response to antigens of 43 and 25 kDa was seen with clone SH2.

Two-dimensional immunoblotting studies of clones MM10 and SH1 allowed for identification of the antigens to which these clones responded with considerably greater resolution (see arrow and arrowheads in Fig. 1). Clone MM10 again exhibited a bimodal response to antigens with approximate M_rs of 60 and 28 kDa and an approximate pI of 5.7. Each of these responses was associated with significant IFN-γ production (Fig. 3). Clone SH1 showed a proliferative response with IFN-γ production to an antigen with an M_r of 43 kDa and a pI of approximately 5.2. As in the one-dimensional immunoblot studies, the peak proliferative response of each clone was associated with responses of a lesser magnitude in adjacent wells, suggesting some dispersion of antigenic sites. Clone SH1 also responded to an antigen with a very low M_r migrating at the gel dye front (<10 kDa), which probably represented stimulation by small degradation products (which would not be visualized by silver staining) of the 43-kDa antigen. A similar proliferative response to low-M_r antigens running in the gel dye front was also seen in the one-dimensional immunoblot studies of several of the clones. Since $CD4^+$ T cells recognized antigens which were denatured and enzymatically processed by accessory cells, one would expect that these low-M_r degradation fragments could stimulate T-cell proliferation.

TABLE 1. Proliferative and IFN-γ responses of *Leishmania* reactive human T-cell clones

TLC	[^{3}H]thymidine incorporation (cpm ± SD)		Log IFN-γ titer	
	Medium	SLDA	Medium	SLDA
MM6	917 ± 65	43,865 ± 8,990	<0.7	2.8
MM7	106 ± 39	15,172 ± 1,140	<0.7	2.4
MM10	905 ± 248	63,885 ± 8,108	<0.7	3.0
SH1	4,241 ± 2,620	44,157 ± 810	<0.7	1.7
SH2	3,393 ± 1,326	44,313 ± 3,649	<0.7	1.4
SH3	757 ± 274	17,017 ± 2,383	<0.7	1.0

DISCUSSION

Control and resolution of leishmanial infection are mediated by cellular immune mechanisms; therefore, the identification of antigens involved in eliciting T-cell responses is of critical importance. Using $CD4^+$, IFN-γ-producing T-cell clones derived from individuals with past leishmanial infection, we identified individual antigens which elicited a cellular immune response from within a complex pool of parasite proteins. This strategy may be of use, then, in the identification of protective antigens in subjects with this disease.

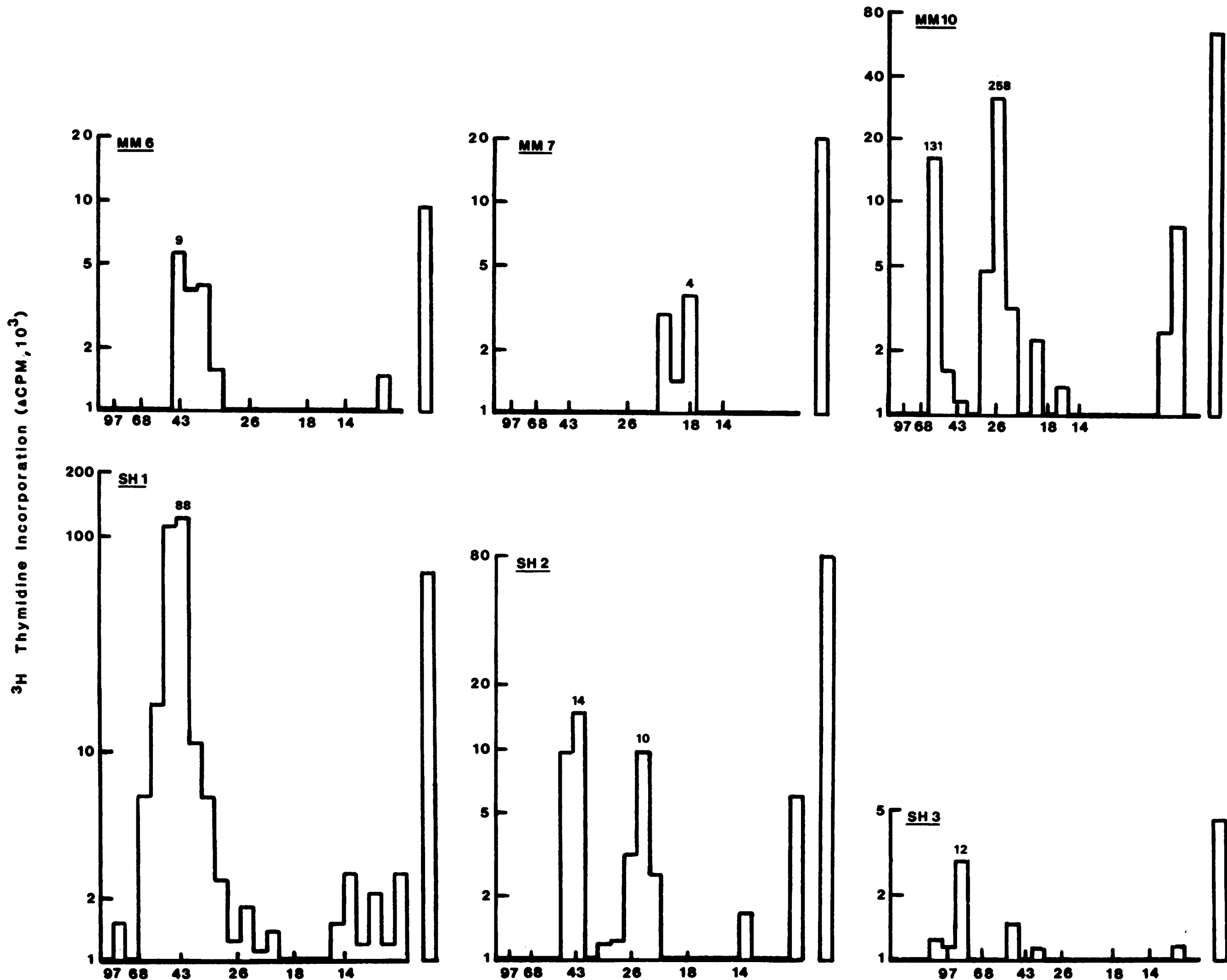

FIG. 2. Analysis of the antigen specificities of six *L. donovani*-reactive human TLCs by one-dimensional immunoblotting. The proliferative responses to the 24 fractions are expressed as the difference in mean counts per minute (ΔCPM; mean counts per minute in experimental wells − mean counts per minute in control wells) and are shown on the vertical axis (logarithmic scale). The 24 fractions with reference molecular weights (in thousands) are presented on the horizontal axis. Stimulation indices (mean counts per minute in experimental wells/means counts per minute in control wells) of some fractions are shown as the numbers above the proliferative response of that fraction. The single column to the right of each graph represents the proliferative response of the clone to unfractionated, NC-bound antigens. In the MM6 and MM7 immunoblots, IL-2 was supplemented in experimental and control wells at a final concentration of 32 U/ml.

By one-dimensional immunoblot analysis, the antigen specificity of six different clones was determined. In each case a peak proliferative response could be associated with an antigen or antigens of a particular molecular weight. Considering the complexity of the parasite soluble antigen pool and the level of resolution attained by one-dimensional separation techniques, isolation of a particular antigen to which a clone responds, either by use of antibody reagents or direct amino acid sequence analysis, would be difficult. Therefore, we adapted the immunoblotting method to include the separation of antigens by two-dimensional electrophoresis, which resolved SLDA into more than 200 discrete proteins that were visible on a silver-stained two-dimensional gel, and allowed for further definition of the antigen specificities of two of the T-cell clones. The proliferative responses of these clones could be localized to a very small number of proteins on the two-dimensional gels.

These *L. donovani*-reactive human TLCs were all of the $CD4^+$ phenotype. The cloning method used only a single round of stimulation of PBMNCs with antigen, followed by limiting dilution in order to obtain clones most representative of the repertoire of *L. donovani*-specific T cells present in the immune host. One would expect the selection of $CD4^+$ cells by cloning in the absence of exogenous IL-2. *L. donovani* antigens were used to generate clones from individuals with past homologous or heterologous leishmanial infections. Because of the T-cell unresponsiveness in patients with visceral leishmaniasis, we undertook an approach using T cells from an individual with a self-limiting *L. donovani* infection and cross-reactive T cells from an individual with past *L. brasiliensis* infection. T-cell reactivity in patients with cutaneous leishmaniasis to heterologous antigens is extensive (Melby et al., in press; unpublished data).

Each of the clones had the capacity to proliferate, and all but one showed significant IFN-γ production upon stimulation with whole soluble antigen, and IFN-γ production was

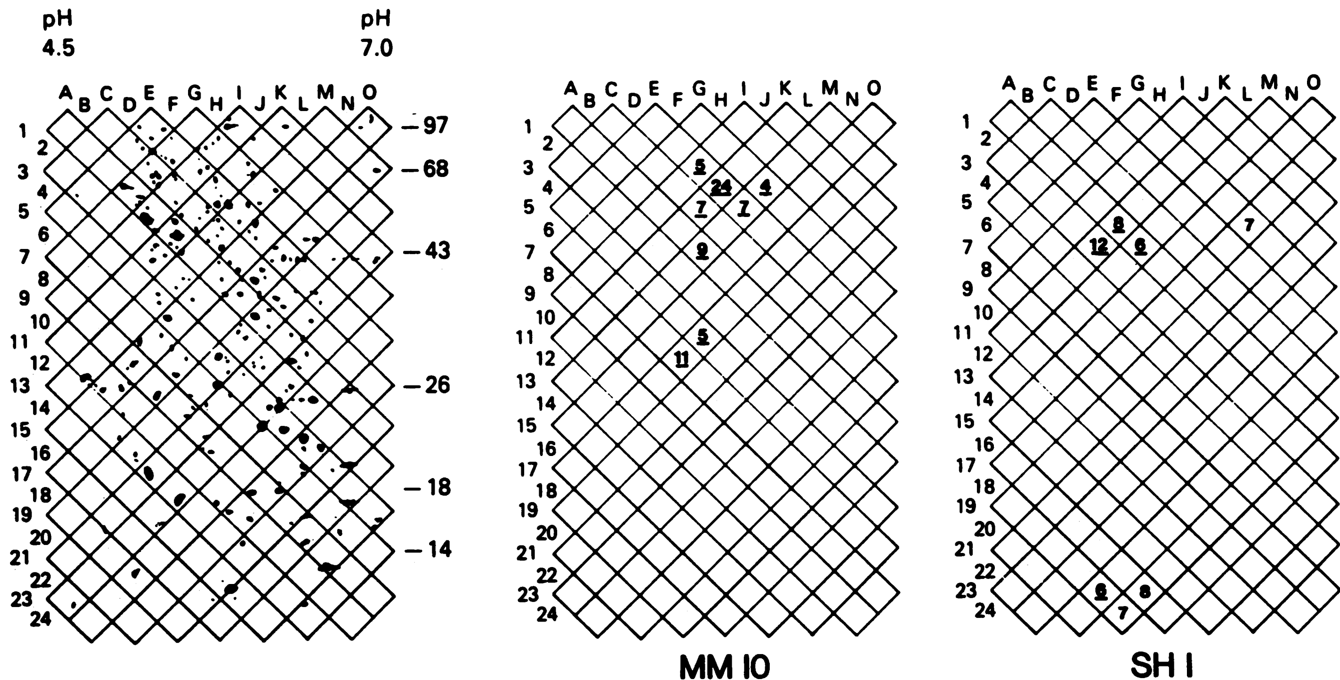

FIG. 3. Analysis of the antigen specificities of two *L. donovani*-reactive TLCs by two-dimensional immunoblotting. On the left is a schematic localization of individual antigens (from a replicate silver-stained gel) within the wells of the two-dimensional immunoblot culture plate. The proliferative responses of clones MM10 and SH1 are shown as stimulation indices recorded within the wells of the plate. Stimulation indices of <4.0 were not recorded. Underscoring of the stimulation index number indicates that significant IFN-γ production was associated with that proliferative response. Each clone was analyzed at least twice with identical results; the results shown are from the same experiment with identical replicate two-dimensional gels. Clone MM10 (control, 2,146 cpm; log IFN-γ titer, 0.9; experimental range, 9,240 to 52,310 cpm; log IFN-γ titer, 1.4 to 2.1) and clone SH1 (control, 1,180 cpm; log IFN-γ titer, <0.7; experimental range, 6,535 to 14,786 cpm; log IFN-γ titer, 1.6 to 2.0) were tested.

associated with the proliferative responses seen in the immunoblot analyses. In experimental models, different $CD4^+$ T-lymphocyte subsets have been associated with both the control and the exacerbation of cutaneous infection, and the subset which is involved in disease control produces IFN-γ upon antigenic stimulation (8, 27). Genetically resistant mice can also be rendered susceptible by anti-IFN-γ treatment (M. Belosevic, D. S. Finbloom, P. H. Van der Meide, and C. A. Nacy, Abstr. Annu. Meet. Am. Soc. Trop. Med. Hyg. 1988, p. 146). In vitro, IFN-γ has been shown to induce macrophage activation and killing of intracellular *Leishmania* spp. (5, 18, 19). IFN-γ produced by antigen-stimulated lymphocytes from patients with cutaneous and mucosal leishmaniasis can also activate human macrophages to kill intracellular organisms (2, 22). Thus, antigens which elicit a T-cell response and production of IFN-γ would likely contribute, at least in part, to the control and resolution of leishmanial infections in humans and may be involved in protection against reinfection. Thus, the preliminary identification of antigens which stimulate TLCs derived from individuals with healed leishmaniasis has great potential for the further characterization of T-cell antigens in subjects with this disease. The approach should also be applicable to the identification of specific antigens recognized by T-cell clones generated in response to heterogeneous or ill-defined antigens in other microbial, tumor, or autoimmune systems.

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