Clin. exp. Immunol. (1990) **80**, 77–82

Parasite antigens recognized by patients with cutaneous leishmaniasis

C. L. JAFFE, R. SHOR*, H. TRAU* & J. H. PASSWELL* *Department of Biophysics–MacArthur Center for Molecular Biology of Tropical Diseases, Weizmann Institute of Science, Rehovot and *Samuel Jared Kushnick Paediatric Immunology Laboratory, Chaim Sheba Medical Center, Tel Hashomer, Israel*

(Accepted for publication 6 November 1989)

SUMMARY

Humoral and cell-mediated responses to crude and purified parasite antigens were examined in patients with active cutaneous leishmaniasis caused by *Leishmania major*. The patients had serum antibody titres against parasite lysates ranging from 1/500 to 1/10 000 and recognized multiple components by Western blotting with molecular weights between 5000 and >200 000. Several components, particularly at 5 and 50 kD, were recognized by most of the patients. The lymphoproliferative responses to two pure antigens, promastigote surface protease and lipophosphoglycan, both considered potential candidates for the development of a human vaccine were measured. No response to promastigote surface protease was observed even at 10 μg/ml; however, weak proliferation to lipophosphoglycan was consistently present. T cell blots examining peripheral blood lymphocyte proliferation, showed antigen-specific responses to a 72–82 kD component in promastigote lysates.

Keywords *Leishmania major* lipophosphoglycan parasite surface protease lymphocyte proliferation

INTRODUCTION

Leishmaniasis comprises a spectrum of different diseases found all over the world. The parasite is transmitted by the sandfly vector to a suitable host as the promastigote, where it invades host macrophages, transforms and multiplies as the intracellular amastigote. Cutaneous, mucocutaneous and visceral leishmaniasis represent the major clinical groupings, although other forms of disease are occasionally observed (Peters & Killick-Kendrick, 1987). The mildest form of leishmaniasis is the cutaneous form (CL), which frequently presents as a localized chronic granulomatous lesion at the site of the sandfly bite. Left untreated, these lesions often ulcerate and eventually resolve leaving scarring. Cure is believed to impart long-lasting immunity to reinfection by the same species of parasite (Peters & Killick-Kendrick, 1987). For these reasons leishmanization, the controlled injection of virulent promastigotes, has been practiced in high-risk areas of the USSR and Israel, and is currently employed in Iran (Jaffe & Greenblatt, 1990). However, experimental evidence in both animal models and humans suggests that it should be possible to develop a killed vaccine, and perhaps eventually a defined vaccine (Jaffe & Greenblatt, 1990). This disease, caused by *L. major*, is presently the focus of a World Health Organization (WHO) special program to develop an effective vaccine.

Correspondence: Dr Charles L. Jaffe, Department of Biophysics, Weizmann Institute of Science, Rehovot 76100, Israel.

Crude, partially purified and pure parasite antigens have been shown to protect mice against a challenge by parasites which cause CL (Jaffe & Greenblatt, 1990). Among the pure antigens which have been suggested as putative candidate molecules are the promastigote surface protease (PSP), the lipophosphoglycan (LPG) and M2 (Handman & Mitchell, 1985; Champsi & McMahon-Pratt, 1988; Russell & Alexander, 1988); however almost nothing is known regarding the immune response of patients with active disease to these antigens. An understanding of the humoral and cellular immune responses to the various parasite antigens during both active disease and upon cure is neccessary to the eventual development of vaccine for CL. These studies represent an initial attempt to define such relevant antigens.

MATERIALS AND METHODS

Human serum and cells

Serum and peripheral blood mononuclear cells (PBMC) were obtained from patients with nodular or nodular-ulcerating lesions typical of CL. Tissue biopsy samples were obtained from the lesion margins for histological staining and parasite cultures. Healthy laboratory personnel with no history of leishmaniasis were used as negative controls.

Lymphocyte proliferation

The mononuclear cells were separated by density centrifugation on Ficoll–Hypaque and resuspended for lymphocyte prolifer-

Table 1. Response to *Leishmania* antigens in 15 patients with cutaneous leishmaniasis

Antigen	Dose (μg)	Stimulation index (±s.e.m.) Patient	Control
Particulate	1	6·12±2·22	0·79±0·45
	5	9·82±3·93	1·27±0·07
	10	8·93±3·38	0·38*
Soluble	1	6·20±2·18	0·85±0·42
	5	11·60±4·09	1·16±0·78
	10	10·30±3·90	0·50*
WIS-2·86	1	11·74±5·60	0·83±0·58
	5	13·98±7·09	1·60±1·60
	10	15·12±7·68	1·71±1·70
LPG	0·1	2·77±0·59	0·59*
	0·5	2·18±0·46	0·33±0·11
	1·0	1·20±0·26	0·10*
PG	0·5	1·38±0·22	0·81±0·07
	1·0	2·05±0·19	0·53*
PSP†	0·5	1·12±0·19	ND
	2·0	1·00±0·19	ND

* Only one healthy control was examined at this concentration.

† Several patients were also examined at 0·25, 5 and 10 μg.

ND, not determined.

ation assays in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics. Proliferative assays were carried out in either U-bottomed 96-well tissue culture plates or flat-bottomed plates for T cell-blots (Falcon Labware, Oxnard, CA). Antigens were added to 1×10^5 mononuclear cells at the concentrations indicated in Table 1. For the T cell Western blots, 2×10^5 mononuclear cells were added to microtitre plates already containing the antigen bound to nitrocellulose paper discs, prepared as described below. The antigen-stimulated cultures were incubated for 96 h at 37°C and then pulsed for 24 h with ^{3}H-thymidine. The cells were harvested and the radioactivity incorporated by the cells was analysed. Results are presented as a stimulation index derived by dividing the ct/min incorporated into the test cultures by the ct/min incorporated by the untreated cultures. All variables were tested in either triplicate or quadruplicate wells.

Parasite antigens

Crude promastigote membranes or lysates used in radioimmunosorbent assays (RIA), Western blotting or lymphocyte proliferation were prepared from local isolates of *L. major* (MHOM/IL/79/Perlstein or MHOM/IL/86/WIS-2). Promastigotes were grown in Schneider's *Drosophila* medium containing 10% FCS and antibiotics. Membrane antigen for RIA and Western blotting was prepared as previously described (Jaffe & McMahon-Pratt, 1983). In brief, 10^{10} promastigotes were washed twice with PBS and resuspended in 20 mM Tris-HCl (pH 7·3) containing 40 mM NaCl, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide and 1 μg/ml leupeptin (35 ml) at 0°C. The cells were equilibrated at 1500 psi with N_2 for 10 min at 4°C and lysed by nitrogen cavitation in a Cell Disruption Bomb (Parr Instrument Co., Moine, IL). The homogenate was centrifuged at 4350 *g* (10 min) and the pellet discarded. The supernatant was further fractionated by centrifugation at 39 000 *g* (30 min) and the pellet containing the crude membrane fraction obtained. Crude antigens for lymphocyte proliferation were made from promastigotes (WIS-2.86) by quickly freeze–thawing (three times) in sterile PBS and sonicating for 30 sec in a bath sonicator (Passwell *et al.*, 1987). This antigen was further fractionated by centrifugation for 1 h at 39 000 *g* into a particulate and soluble antigen. Protein concentration was determined using the BioRad protein reagent. Antigens were stored aliquoted and frozen at −70°C until use. The hydrophilic form of *L. major* PSP was prepared according to Bouvier, Etges & Bordier (1985) and kindly provided to us by Drs C. Bordier and D. Rivier from the University of Lausanne, Switzerland. LPG from *L. major* and the released form, phosphoglycan (PG) (Handman & Mitchell, 1985), was a gift from Dr E. Handman at the Walter and Eliza Hall Institute of Medical Research, Australia.

Determination of specific antibody titres against *L. major* was carried out as previously described (Passwell *et al.*, 1987) using a RIA on microtitre plates coated with promastigote membrane antigen.

Antibody and T cell blots

Western blots for antibody binding were carried out on *L. major* membranes separated on 10% SDS–PAGE and transferred to nitrocellulose paper (0·45 μm) (Lammeli, 1970; Towbin, Staehelin & Gordon, 1979). The paper was quenched with 0·3% Tween 20 in PBS and incubated for 2 h at room temperature with the various human sera (1/1000 dilution 0·3% FCS/PBS). Following several washes with 0·05% Tween 20 in PBS the strips were probed with ^{125}I-Labelled sheep F(ab')$_2$ fragment anti-human IgG (0·2 μCi/ml, Amersham International Amersham, UK) washed, dried and exposed to Kodak X-Omat film for autoradiography. T cell Western blots (Young & Lamb, 1986) were accomplished as follows: following transfer of the promastigote lysates separated by gel electrophoresis to nitrocellulose paper, as above, the blots were washed extensively with sterile PBS (overnight with four 500-ml changes) and once with RPMI 1640. The paper was then incubated for 30 min with 3% FCS/PBS and overlayed on a sterile 96-well microtitre plate. Discs were punched out into the wells below using a sterile 6-mm tissue biopsy punch. The mononuclear cells were added to the antigen and the lymphocyte proliferation assay completed as described above.

RESULTS

The anti-parasite antibodies titres for the fifteen patients examined by RIA ranged between 1/500 to 1/10 000(Fig. 1a). The titre for several of these patients was measured 1 year later, at which time they had completely healed and showed titers < 1/500. The promastigote antigens recognized by serum antibodies from all 15 subjects were determined by Western blotting (Fig. 1b). No simple relation is seen between antibody titre and the intensity of reaction or number of bands recognized by Western blotting, even though the same antigen was employed for both procedures. The first two lanes (a and b) were incubated with

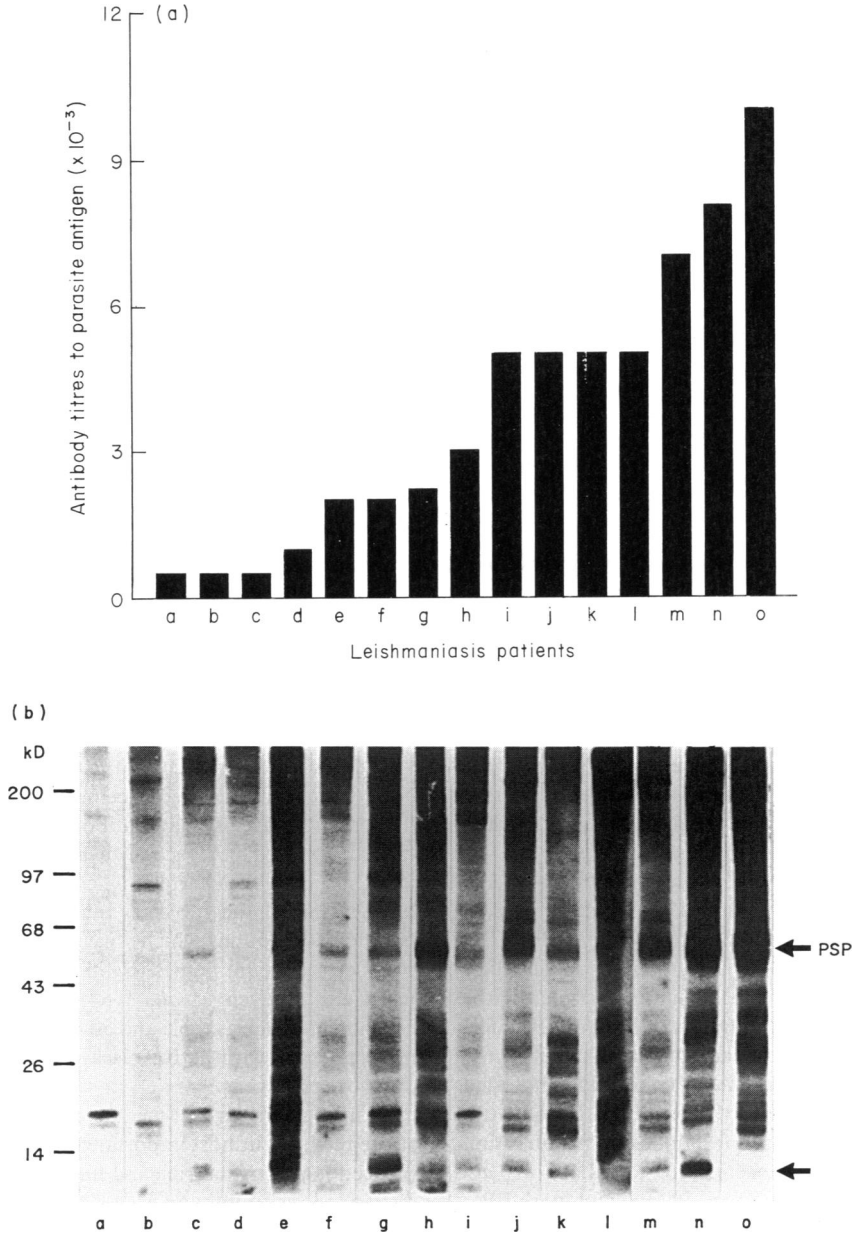

Fig. 1. Identification of *Leishmania major* antigens recognized by anti-parasite antibodies in cutaneous leishmaniasis patient serum. (a) Serum titres of anti-leishmanial antibodies on promastigote membranes. Healthy control serum are labelled a and b. (b) Western blot on promastigote antigen of human sera. Patient serum are arranged in the same order as panel a with increasing antibody titres from left to right (lane c to o, 1/500 to 1/10 000, respectively). Reactions with serum from healthy controls are shown lanes a and b.

healthy control sera from an endemic area. These sera recognize bands with molecular weights of about 16, 18, 90, 165 and >200 000. The two low molecular weight bands are also strongly recognized by all of the patient sera. In addition to these bands, approximately 13 leishmanial components ranging from the gel front to >200 000 kD are specifically recognized by the patient sera. Almost all of these sera (12/13) recognized a band at 50 kD which probably corresponds to the PSP. PSP migrates with a molecular weight of about 50 000 in nonreduced gels. The intensity of reaction against the 50-kD band tends to directly parallel the increasing anti-leishmanial serum titre. A second band of about 5000 is also recognized by a majority of the patient sera (10/13). Additional antigens in the range 20–43, 68, 82 and 172 kD were recognized by multiple patient sera.

Whole parasite lysates were screened for their ability to induce antigen -specific T cell proliferation. In Fig. 2 the proliferation induced by different molecular weight fractions of a SDS–PAGE transferred to nitrocellulose paper are plotted for two patients with active disease (Fig. 2a, b) and a healthy control (Fig. 2c). The molecular fraction inducing the greatest stimulation for both patients migrates at about 72–82 kD. The stimulation for these two fractions ranges from 3·2 to 4·9-fold

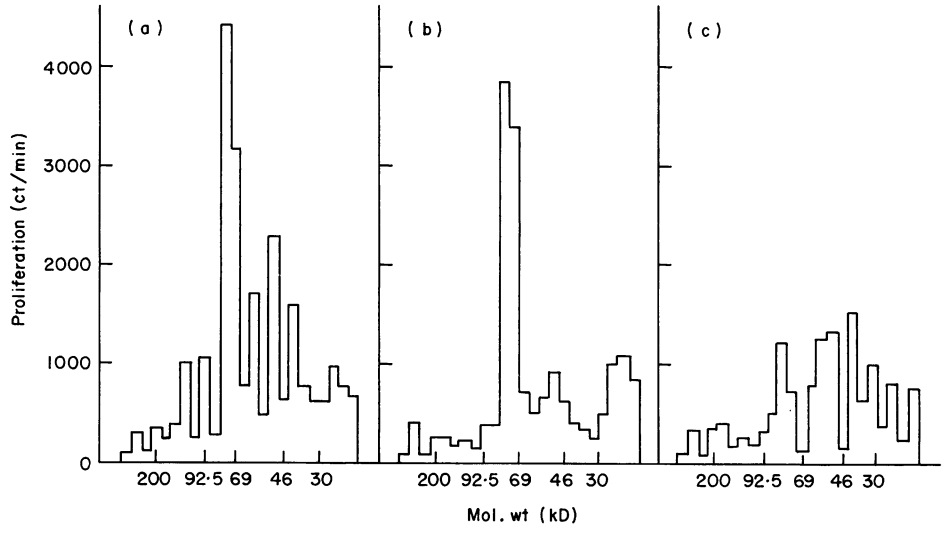

Fig. 2. Analysis of leishmanial antigen-specific pheripheral blood lymphocytes proliferation by T cell western blotting. (a) Patient with cutaneous leishmaniasis; (b) patient with cutaneous leishmaniasis; and (c) healthy control subject.

the proliferation induced by the same fractions with the normal control. Two additional fractions of about 50–55 kD and 60–65 kD also induced stimulation 1·8- and 2·5-fold, respectively, above the normal control. The fraction at 50–55kD coincides with the major 50 kD antigen recognized by most serum antibodies.

The response of patients' PMBC to crude antigens and to several purified leishmanial antigens was compared in parallel (Table 1). The pure antigens utilized were PSP and LPG. The secreted form of LPG, PG, was also examined. The best proliferation for the soluble and particulate fractions of the crude promastigote antigen was obtained at 5 μg/ml. Proliferation induced by the whole lysates had not peaked at 10 μg/ml, the highest concentration examined. At all concentrations tested the lysed promastigotes stimulated the patients PBMC better than either of the two crude antigen fractions. No proliferation was observed with PSP at any concentration tested up to 10 μg/ml. However, a weak stimulation of the PMBC with both LPG and PG was noted. Interestingly, increasing concentrations of LPG appeared to inhibit both the patient and control proliferative responses to this antigen. The best proliferation with LPG was obtained at 0·1 μg/ml—the lowest concentration examined. For the PG the proliferation was better at higher concentrations.

DISCUSSION

Extensive progress has been made towards understanding cutaneous leishmaniasis in model systems (Muller *et al.*, 1989); however, relatively little information is available on the immunology of this disease in humans. It appears that lesion resolution is probably dependent on the recruitment of appropriate T cells into the lesion which activate the infected mononuclear cells to kill intracellular amastigotes (Modlin *et al.*, 1985; Passwell, Shor & Shoham, 1986; Huszar *et al.*, 1987). Interferon-gamma (IFN-γ) is one lymphokine which has been shown to induce leishmanicidal activity *in vitro* and is probably involved in this process (Passwell, Shor & Shoham, 1986).

Less is known regarding the relevant parasite antigens for development of a defined vaccine in humans. In this paper we demonstrate that serum antibodies from patients with CL caused by *L. major* recognize a wide repertoire of parasite antigens. This is not surprising, considering the variable genetic background of the subjects. Inbred mouse strains of diverse genetic backgrounds which vary in their resistance to *L. major* produce serum antibodies to different parasite antigens during infection (Nguyen *et al.*, 1984). However, specific parasite components such as the 5 and 50 kD antigens are recognized by most patients with active disease and might prove useful as diagnostic antigens.

An appropriate cell-mediated response has been demonstrated to be of utmost importance in both the resolution of and protection against murine and human CL (Muller *et al.*, 1989). Active lesions contain an excess of T suppressor/cytotoxic cells (Passwell *et al.*, 1986; Huszar *et al.*, 1987). Secretion of lymphokines such as IFN-γ or IL-2 by such cells are believed to be responsible for eventual healing of these lesions (Passwell *et al.*, 1986; Muller *et al.*, 1989). Using T cell blots, Melby & Sacks (1987) have shown that PMBC from patients with CL react with antigen fractions, 10–40 kD, of soluble promastigote antigen from *L. major*. A preliminary report (Conceicao-Silva *et al.*, 1987) using a soluble antigen from *L. brazilliensis* showed that patients with mucocutaneous leishmaniasis react with three separate antigen fractions of >50 kD. Using a total parasite extract of *L. major* which is probably more representative of the total antigenic repertoire seen by the host, a major stimulatory fraction of 73–82 kD was identified in this study.

Obviously, there is no evidence from such limited experiments that the antigens on the blots recognized by proliferating cells from patients with acute disease are involved in the induction of a protective response. This will require additional characterization of the parasite antigens, including protection studies in model systems and of the proliferating cells, including

phenotypic and functional studies. While it has been shown in mice that T helper cells ($L3T4^+$) are capable of inducing both protection and exacerbation of the disease (Muller *et al.*, 1989), the recent division of these cells into TH1 and TH2 subsets, based on lymphokine secretion (Mosmann & Coffman, 1987), may provide an answer to these contradictory results. Protective parasite specific T cells belong to the TH1 subset which secrete IFN-γ and IL-2. The excerbating T cells presumably belong to the TH2 subset which secrete IL-4. However, one must be aware that phenotypic analysis and *in vitro* functional studies of the T cells do not necessarily correlate with *in vivo* protection studies following cell transfer. Experiments have shown that a $L3T4^+$ T cell line which secretes IFN-γ and exhibits leishmaniacidal activity *in vitro* causes excerbation of the disease when transferred to mice (Muller *et al.*, 1989). However, the analysis of patients responses to specific leishmanial antigens by these techniques over the course of the disease, before and after cure, should prove useful in initially screening leishmanial antigens for further vaccine studies.

Finally we examined the T cell proliferation to two putative vaccine candidates, PSP and LPG (Handman & Mitchell, 1985; Russell & Alexander, 1988; Jaffe & Greenblatt, 1990). PSP is a major membrane glycoprotein present in promastigotes of all *Leishmania* species. Evidence suggests that it is found in both stages of the parasite; however, no proliferation to PSP was observed at any concentration examined. These experiments were carried out using the hydrophilic form of PSP from which the lipid anchor has been removed by treatment with an inositol-specific phospholipase C. Either form appears to be an equally good immunogen in mice (Rivier *et al.*, 1989). When tested in mice immunized with hydrophilic-PSP, this form was shown to be about 100-fold less effective than the amphiphilic form of PSP in stimulating lymph node T cells. Optimal stimulation was obtained with hydrophilic-PSP at 3–12 μg/ml (Rivier *et al.*, 1989), similar to the concentration range utilized in these studies. While our results suggest that PSP is not a T cell antigen in active patients, one cannot rule out the possibility that patients with active disease preferentially respond to the amphiphilic form of this molecule or that T cell proliferation to PSP coincides with cure. Either hypothesis might explain the proliferation observed at 50 kD in the T cell Western blot.

The second antigen examined, LPG, is an abundant membrane carbohydrate present in all species of *Leishmania*. In the released form, PG, the lipid anchor is absent, cleaved by inositol-specific phospholipase C. These molecules, LPG/PG, show structural variation between species of *Leishmania* and are present in both parasite stages. Either form of this antigen, LPG or PG, specifically stimulated the PMBC to proliferate. LPG was able to stimulate cells at antigen concentrations at least 10-fold less than PG. This may be due to the presence of a lipid moiety which could conceivably promote the efficiency of antigen presentation.

Future research directed towards defining important leishmanial antigens for vaccine development should focus on longitudinal studies which compare the responses in active disease and following cure. Since experiments using murine models have shown that an antigenic specific proliferation alone can not identify protective antigens (Muller *et al.*, 1989), it will be necessary to characterize both the antigens inducing specific T cell responses and the lymphokines they produce. Hopefully, this approach will lead to the rational development of a defined vaccine for cutaneous leishmaniasis.

ACKNOWLEDGMENTS

This work was supported in part by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and the John and Catherine T. MacArthur Foundation.

REFERENCES

Bouvier, J., Etges, R.J. & Bordier C. (1985) Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes *J. biol. Chem.* **260,** 15504.

Champsi, J. & McMahon-Pratt, D. (1988) Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infect. Immun.* **52,** 3272.

Conceicao-Silva, F., Scharfstein, J., Hendriks, J., Cysne, L., Mendonca, S.C.F. & Coutinho, S.G. (1987) Recognition of various *Leishmania braziliensis braziliensis* antigen fractions by homogeneous T cell populations derived from peripheral blood of cutaneous and mucosal leishmaniasis patients. *Mem. Inst. Oswaldo Cruz Rio,* **82,** 118.

Handman, E. & Mitchell, G.F. (1985) Immunization with *Leishmania* receptor for macrophages protects mice against cutaneous leishmaniasis. *Proc. natl Acad Sci. USA*, **82,** 5910.

Huszar, M., Shor, R., Trau, H., Gazit, E. & Passwell, J.H. (1987) The T-cell phenotypes in the lesion of patients with cutaneous leishmaniasis. *Clin. exp. Dermatol.* **12,** 103.

Jaffe, C.L. & Greenblatt, C.L. (1990) Reflections on vaccine development against the intracellular parasite, *Leishmania*. In *Vaccines and Immunotherapy*. (ed. by S. J. Cryz) Pergamon Press, London. (In press).

Jaffe, C.L. & McMahon-Pratt, D. (1983) Monoclonal antibodies specific for *Leishmania tropica*. I. Characterization of antigens associated with stage- and species-specific determinants. *J. Immunol.* **131,** 1987.

Lammeli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680.

Melby, P.C. & Sacks, D.L. (1987) Analysis of the hetrogeneity of T-cell responses in human leishmaniasis. *Ann. Inst. Pasteur. Immunol.* **138,** 768.

Modlin, R.L., Tapia, F.J., Bloom, B.R., Gallinoto, M.E., Castes, M., Rondon, A.J., Rea, T.H. & Convit, J. (1985) *In situ* characterization of the cellular immune response in American cutaneous leishmaniasis. *Clin. exp. Immunol.* **60,** 241.

Mosmann, T.R. & Coffman, R.L. (1987) Two types of mouse helper T cell clone. Implications for immune regulation. *Immunol. Today*, **8,** 223.

Muller, I., Pedrazzini, T., Farrell, J.P. & Louis, J. (1989) T-cell responses and immunity to experimental infection with *Leishmania major. Annu. Rev. Immunol.* **7,** 561.

Nguyen, T.V., Mauel, J., Etges, R.F., Bouvier, J. & Bordier, C. (1984) Immunogenic proteins of *Leishmania major* during mouse infection. *Parasite Immunol.* **6,** 265.

Passwell, J.H., Shor, R. & Shoham J. (1986) The enhancing effect of interferon-beta and gamma on the killing of *Leishmania tropica major* in human mononuclear phagocytes *in vitro. J. Immunol.* **136,** 3062.

Passwell, J.H., Shor, R., Trau, H., Shoham, J. & Jaffe, C.L. (1987) Antigen-stimulated lymphokines from patients with cutaneous leishmaniasis induce monocyte killing of *Leishmania major* intracellular amastigotes. *J. Immunol.* **139,** 4208.

Peters, W. & Killick-Kendrick, R. (1987) *The Leishmaniasis in Biology and Medicine* p.941. Academic Press, London.

Rivier, D., Didisheim, S., Etges, R., Bordier, C. & Mauel J. (1989) Studies on antigenicity of amphiphilic and hydrophilic forms of

Leishmania major promastigote surface protease in the mouse. In *NATO ASI Series. Leishmaniasis: The First Centenary New Strategies for Control* (ed. by D. T. Hart) p. 423. Plenum Press, London.

RUSSELL, D.G. & ALEXANDER, J. (1988) Effective immunization against cutaneous leishmaniasis wtih defined membrane antigens reconstituted into liposomes. *J. Immunol.* **140,** 1274.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. *Proc. natl Acad. Sci. USA*, **76,** 4350.

YOUNG, D.B. & LAMB, J.R. (1986) T lymphocytes respond to solid-phase antigen: a novel approach to the molecular analysis of cellular immunity. *Immunology*, **59,** 167.