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Stage-Specific, Strain-Specific, and Cross-Reactive Antigens of *Leishmania* Species Identified by Monoclonal Antibodies

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The fusion of NS-1 myeloma cells with spleen cells from mice chronically infected with *Leishmania tropica* resulted in nine clones of hybridomas producing monospecific antibodies to membrane antigens of *L. tropica*. One of the antibodies (L-5-85) bound specifically to the promastigote form of the parasite, and the remaining eight recognized antigens shared by the promastigote and amastigote of *L. tropica*. Four of the antibodies (L-5-16, L-5-34, L-5-44, and L-2-3F11) detected parasite antigens on the surface of *L. tropica*-infected macrophages. Common antigens shared by *L. tropica*, *L. mexicana*, and *L. donovani* were identified as well as one antigen apparent on most *Leishmania* spp. and present also in *Crithidia fasciculata*. Two monoclonal antibodies (L-5-27 and L-5-41) were found to bind only to strains of *L. tropica* from simple cutaneous leishmaniasis. A special property shared by these two antibodies was the inhibition of parasite growth in macrophages in vitro.

Leishmania tropica, the protozoan causing cutaneous leishmaniasis in humans, is a trypanosomatid flagellate transmitted by blood-sucking phlebotomine sandflies. The parasites exist in the insect vector as extracellular motile promastigotes. Forms of similar morphology also occur when parasites are grown in cell-free media at 26 to 28°C. In mammalian hosts, they parasitize macrophages in the form of intracellular nonmotile amastigotes, living in phagolysosomal vacuoles.

A severe limitation in the study of some major problems in leishmaniasis has been a lack of characterized parasite antigens. Problems such as the taxonomy of *Leishmania* spp., the question of antigens expressed by the different life cycle stages of the parasite, and the characterization of *Leishmania* spp. antigens displayed on infected macrophages could be only partially approached with polyvalent antisera (1, 4, 5).

The usefulness of antibodies as probes for the analysis of parasite antigens has been limited by the variability of the immune response, the extreme heterogeneity of antibodies, and the inability to prepare pure preparations of antigens. The introduction by Köhler and Milstein (8) of a method for producing large amounts of monoclonal antibodies of defined specificity makes available a potentially valuable tool for serological analysis of parasite antigens. For this reason we fused mouse NS-1 myeloma cells with spleen cells from mice chronically infected with *L. tropica* in an attempt to produce monoclonal antibodies to antigens of *L. tropica*. We have been able to generate for the first time homogeneous, monospecific antibodies to membrane antigens of *L. tropica*. These antibodies distinguish between various species of *Leishmania*, between different strains of *L. tropica* causing different disease states in humans, and between amastigotes and promastigotes of *L. tropica*. They also detect *L. tropica* antigens displayed on infected macrophages. Most importantly, two of the antibodies promote killing of *L. tropica* in macrophages in vitro.

MATERIALS AND METHODS

Cells. The parental cell line used was the NS-1 variant of the P3 (MOPC-21) line (8). Cells of the NS-1 line are resistant to azaguanine and do not synthesize the MOPC-21 γ heavy chain. Although NS-1 synthesizes the MOPC-21 κ chain, it is not secreted. NS-1-derived hybrid cell lines secreting the normal parental spleen cell immunoglobulin also secrete hybrid molecules containing the MOPC-21 κ chain.

Parasites. *Leishmania* spp. promastigotes obtained from the World Health Organization Reference Center for Leishmaniasis, the Department of Protozoology, Hadassah Medical School in Jerusalem, Israel, were grown at 26°C in RPMI 1640 medium (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal calf serum (GIBCO, Auckland, New Zealand) (Table 1).

Immunization and fusion. BALB/c female mice 7 weeks of age were infected intradermally with 5×10^6 *L. tropica* (LRC-L137) promastigotes. Forty days later, when the mice were displaying large open ulcers at the injection site, they were boosted intraperitoneally with 5×10^7 heat-killed *L. tropica* promastigotes. Three days later, the spleens were removed, and the

TABLE 1. Strains of *Leishmania* spp. used in this study

WHO[a] no.	Name	Country of origin	Host	Disease
LRC-L287	*L. tropica*	Israel	Human	Oriental sore
LRC-L137	*L. tropica*	Israel	Human	Oriental sore
LRC-L38	*L. tropica*	Turkestan	Human	Oriental sore
LRC-L251	*L. tropica*	Israel	Human	Oriental sore
LRC-L32	*L. tropica*	Iraq	Human	Leishmaniasis recidiva
LRC-L286	*L. tropica*	Iraq	Human	Leishmaniasis recidiva
LRC-L285	*Leishmania* sp.[b]	Dominican Republic	Human	Diffuse cutaneous leishmaniasis
LRC L94	*L. mexicana*	British Honduras	Human	Cutaneous leishmaniasis
LRC-L52	*L. donovani*	India	Human	Visceral leishmaniasis
LRC-L144	*L. enriettii*	Brazil	Guinea pig	

[a] WHO, World Health Organization.
[b] Species undetermined (Schnur, personal communication).

spleen cells were suspended in serum-free RPMI 1640. The methods for cell fusion were as previously described (6–8). In brief, spleen cells (1.5×10^8) were mixed with NS-1 cells (1.5×10^8) and pelleted by centrifugation at $200 \times g$ for 10 min. The cells were fused by suspending the pellet in RPMI 1640 containing 50% (wt/vol) polyethylene glycol (BDH Chemicals Ltd., Poole, England). The cells were washed, suspended in RPMI 1640 with 10% fetal bovine serum (Flow Laboratories), and cultured in 96-well microculture plates (Costar, Cambridge, Mass.). Approximately 10^6 cells were cultured in each well.

Growth of antibody-producing cell hybrids. Growth of antibody-producing cell hybrids was done as previously described (6), except that the cells were placed in HAT medium immediately after the fusion. Cloning was performed by limiting dilution in microculture plates with 10^6 mouse thymocyte feeder cells per well. After cloning, cells from cultures expressing the highest antibody production were expanded into larger cultures. For storage, 10^6 cells were frozen in 0.5 ml of fetal bovine serum containing 10% dimethyl sulfoxide and placed in liquid nitrogen. Cells (2×10^6 to 5×10^6) from each clone were injected subcutaneously or intraperitoneally into BALB/c mice irradiated with 400 R, and tumors (hybridomas) appeared within 10 to 20 days. The mice were bled every few days, and sera were pooled and stored at −70°C until used.

Antibody assays of culture supernatants. Reactivity against *L. tropica* membrane antigens was measured by a solid-phase radioimmunoassay (RIA) by the method of Stocker and Heusser (15) and adapted for detection of *Toxoplasma gondii* membrane antigens by Handman and Remington (6) and for *Leishmania* spp. by McMahon-Pratt and David (10). In brief, 5×10^6 intact promastigotes in 50 μl of phosphate-buffered saline (PBS), pH 7.3, were dispensed into each well of a 96-well polyvinyl chloride microtiter plate (Cooke Laboratory Products, Alexandria, Va). The plates were centrifuged at $200 \times g$ for 5 min and dipped for 5 min in a beaker containing 0.25% glutaraldehyde in cold PBS (pH 7.3). The plates were then washed three times in PBS and incubated with 1% bovine serum albumin in PBS (RIA buffer) (Miles Laboratories, Inc., Elkhart, Ind.) for 60 min at room temperature. Antigen-coated wells were reacted with 30 μl of culture supernatant for 60 min at room temperature, washed in RIA buffer, and incubated for an additional 60 min at room temperature with 50 μl of ^{125}I-labeled protein A (Pharmacia Fine Chemicals, Uppsala, Sweden; 40 μCi/μg, 10,000 cpm per well). The plates were washed three times in RIA buffer and air dried, and the wells were cut and counted in a gamma counter. A polyvalent rabbit anti-*L. tropica* promastigote serum was used as a positive control (5).

Indirect fluorescent antibody test. The indirect fluorescent antibody test was performed on *L. tropica* promastigotes and amastigotes as previously described (6). Promastigotes cultured in RPMI medium with 10% fetal bovine serum were washed three times in PBS and adjusted to a concentration of 2×10^7 to 5×10^7/ml; a volume of 50 μl of this suspension was distributed to each well of a round-bottomed microtiter tray. The trays were centrifuged at $200 \times g$ for 5 min, and the fluid was removed and replaced with 30 μl of the diluted sera or culture supernatants to be tested. After 30 min of incubation at 4°C the parasites were washed in PBS and suspended for another 30 min in the various fluorescein isothiocyanate-conjugated reagents. The fluorescein isothiocyanate-conjugated rabbit anti-mouse $F(ab)_2$, immunoglobulin G1 (IgG1), IgG2a, and IgG3 were prepared as described by Handman and Remington (6) and were found to be specific for the heavy chain by double diffusion in agar and by immunofluorescence. An affinity-purified sheep anti-mouse IgG2b was a generous gift of J. W. Goding of this institute.

For immunofluorescence on *L. tropica*-infected macrophages, mouse peritoneal macrophages were cultured on cover slips in 24-well Costar trays. The cells (2×10^6/ml) were allowed to adhere at 37°C in an atmosphere of 10% CO_2 for 1 h. They were then washed to remove nonadherent cells, and *L. tropica* promastigotes were added at a ratio of two parasites per cell. After 48 h 60 to 80% of cells were infected as determined by counting 1,000 cells in a Giemsa-stained culture. The cell monolayers were washed in warm PBS and fixed in situ in 2% Formalin in PBS. Cells were thoroughly washed in PBS and incubated with dilutions of the test sera for 30 min at 37°C and then with fluorescein isothiocyanate-conjugated reagents for an additional 30 min at 37°C. When living infected macrophages were tested, the assay was performed at 4°C.

***L. tropica* infection of mouse peritoneal macrophages.** Resident peritoneal macrophages were harvested from

TABLE 2. Properties of monoclonal antibodies to *L. tropica* membrane antigens

Monoclonal antibody	Isotype	Reactivity with *L. tropica*		
		Promastigote	Amastigote	Infected macrophage
L-5-16	IgG3	+	+	+
L-5-27	IgG2b	+	+	−
L-5-85	IgG2a	+	−	−
L-5-28	IgG3	+	+	−
L-5-34	IgG3	+	+	+
L-5-41	IgG2b	+	+	−
L-5-44	IgG2a	+	+	+
L-2-3F11	IgG2a	+	+	+
L-2-4B5	IgG2a	+	+	−

BALB/c mice and seeded on cover slips as described above. Before infection of macrophage monolayers, promastigotes were washed in PBS (pH 7.3) and treated for 20 min on ice with 1 mg of IgG of each of the monoclonal antibodies per ml. Then the promastigotes were centrifuged, the fluid was removed, and the parasites were suspended in McCoy medium (Flow Laboratories) with 10% fetal bovine serum and incubated at 37°C for 4, 8, 16, or 24 h. At each time point, cover slips were washed vigorously with warm PBS, fixed in methanol, and stained with Giemsa.

Protease treatment of promastigotes. *L. tropica* promastigotes were treated with pronase or subtilisin (100 μg/ml or 1 mg/ml; Sigma Chemical Co., Saint Louis, Mo.) at room temperature for 20 min. They were then washed and examined for binding to the monoclonal antibodies in the RIA described above.

Sodium metaperiodate treatment of promastigotes. *L. tropica* promastigotes were treated with 20 mM periodate on ice in the dark, washed, and assayed in the RIA as described above. Parasite viability as assessed by staining with ethidium bromide and acridine orange (5, 14) was not markedly reduced by treatment with proteases or periodate.

Purification of hybridoma antibodies. Purification of the IgG fraction from serum of hybridoma-bearing mice was done on protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) (11).

RESULTS

Antibodies that react with membrane antigens of promastigotes are produced in *L. tropica* LRC-L137-infected BALB/c mice and are readily detected 1 month after infection (13). Spleen cells of *L. tropica*-infected BALB/c mice on day 40 of infection were fused with NS-1 myeloma cells. Hybridomas producing antibodies to promastigote membrane antigens were detected in a solid-phase RIA by using intact glutaraldehyde-fixed promastigotes of *L. tropica* LRC-L137. These antibodies bound to intact promastigotes at levels 4- to 10-fold greater than the blank value obtained with the culture supernatant from the control parental mouse myeloma (data not shown). Nine stable, independently arising clones with the highest binding activity were injected into BALB/c mice that had been irradiated with 400 R 1 day before.

When run on two-dimensional gel electrophoresis these antibodies showed the typical heavy and light chain composition of monoclonal antibodies (12) (data not shown).

Characterization of monoclonal antibodies to *L. tropica* promastigotes. We examined the monoclonal antibodies with respect to their isotype and their target antigens. Using antisera specific for the heavy chains of mouse IgG in an IFA test, we demonstrated that all antibodies belonged to the IgG isotypes IgG2a, IgG2b, and IgG3 (Table 2).

Monoclonal antibodies identified by screening on intact promastigotes showed a clear specificity for membrane antigens. They bound to the membrane of living promastigotes, producing bright fluorescence in the IFA test. Immunofluorescence on amastigotes of *L. tropica* showed that most of the antibodies recognized antigens shared by the two life-cycle stages of the parasite.

The exception was L-5-85, an IgG2a antibody that detected a promastigote-specific determinant (Table 2). Four of the monoclonal antibodies (L-5-16, L-5-34, L-5-44, and L-2-3F11) also detected parasite antigens expressed on the surface of *L. tropica*-infected macrophages as determined by the IFA test.

Nature of the target antigens. Treatment of *L. tropica* promastigotes with low concentrations of sodium periodate decreased the binding of L-5-85 and L-5-41 in the RIA by 65%. This suggests that the determinants recognized by L-5-85 and L-5-41 are carbohydrate or that carbohydrate plays a role in the structure of the antigen molecule. The binding of L-5-16, L-5-27, L-5-28, and L-5-34 was inhibited by treatment of the parasites with 1 mg of pronase per ml, but not by treatment with 100 μg of pronase per ml. The binding was not completely abolished, but reduced by 50 to 64%. Treatment of the promastigotes with 1 mg of subtilisin per ml reduced the binding of L-5-41, L-2-3F11, and L-2-4B5 by 57 to 60%. Treatment of the parasites with periodate before pronase decreased the binding of L-5-44 by 60%. Pronase, subtilisin, or sodium metaperiodate alone had no effect on the binding of L-5-44 to treated parasites. The data summarized in Table 3 indicate that treatment of promastigotes with L-5-27 or with L-5-41 abolished their capacity to multiply within macrophages in vitro and indeed promoted parasite killing. In macrophages infected with untreated promastigotes or promastigotes treated with other anti-*L. tropica* monoclonal antibodies there was an increased number of parasites per 100 cells over a 24-h period. Continued infection by extracellular promastigotes as well as proliferation of amasti-

TABLE 3. Survival of *L. tropica* treated with specific monoclonal antibodies in normal mouse macrophages

Antibody used for treatment	No. of parasites in 100 cells[a] at time:			
	4 h	8 h	16 h	24 h
	45 ± 4.5	69 ± 6.1	176 ± 6.6	124 ± 5.4
NS-1	56 ± 3.6	80 ± 4.0	84 ± 3.6	130 ± 4.7
Chronically infected BALB/c	73 ± 2.6	81 ± 4.3	89 ± 2.7	110 ± 5.6
L-5-16	56 ± 1.6	86 ± 3.8	139 ± 7.9	120 ± 4.8
L-5-27	62 ± 3.4	35 ± 1.8	6 ± 1.4	3 ± 0.9
L-5-85	63 ± 4.3	89 ± 3.4	168 ± 8.9	135 ± 2.6
L-5-28	42 ± 2.6	56 ± 5.3	143 ± 8.2	178 ± 4.5
L-5-34	38 ± 3.2	86 ± 2.9	150 ± 6.3	162 ± 4.5
L-5-41	59 ± 2.7	26 ± 4.7	3 ± 1.35	5 ± 3.6
L-5-44	70 ± 6.5	96 ± 4.1	116 ± 3.4	136 ± 5.1
L-2-3F11	63 ± 2.6	91 ± 3.4	86 ± 1.5	130 ± 4.7
L-2-4B5	81 ± 9.6	105 ± 6.4	102 ± 4.8	135 ± 3.2

[a] Mean and standard error of 1,000 cells counted on duplicate cover slips.

gotes could have contributed to the increase in intracellular parasites. However, under the same conditions, in macrophages infected with promastigotes treated with L-5-27 or L-5-41 there was a decrease in the number of intracellular parasites. Treatment of already parasitized macrophages with the monoclonal antibodies had no effect on parasite survival in macrophages. Killing of antibody-treated parasites was evident as early as 8 h after infection, suggesting a possible interference with the transformation of the promastigote to the intracellular amastigote form. It is of interest that L-5-27 seemed to be directed to a protein determinant shared by the promastigote and amastigote, whereas L-5-41 seemed to detect a carbohydrate determinant which is shared by the amastigote and promastigote. However, both antibodies were similar functionally and may interfere with critical steps in the parasite metabolism which allow its survival within the macrophage. The monoclonal antibodies had no effect on the proliferation of *L. tropica* promastigotes in RPMI 1640 medium. However, preliminary studies showed that in the presence of complement L-5-16, L-2-3F11, and L-2-4B5 were cytotoxic to promastigotes.

Detection of isolate-specific and cross-reactive antigens of *Leishmania* spp. Table 4 is a summary of the reactivity of the *L. tropica* monoclonal antibodies on a panel of *Leishmania* strains and isolates of *L. tropica* from patients with various disease patterns of cutaneous leishmaniasis. Two anti-*L. tropica* monoclonal antibodies (L-5-27 and L-5-41) were specific for *L. tropica* isolated from simple cutaneous leishmaniasis and did not bind to *L. tropica* isolated from cases of leishmaniasis recidiva or to *Leishmania* spp. from diffuse cutaneous leishmaniasis; neither did they bind to other strains of *Leishmania*. These antibodies promoted killing of *L. tropica* parasites in macrophages in vitro. L-5-85 seemed to recognize a determinant shared by the *L. tropica* from simple cutaneous leishmaniasis

TABLE 4. Detection of strain-specific and cross-reactive antigens of *Leishmania* spp.

Monoclonal antibody	Reactivity with *Leishmania* strain:										
	L. tropica						*Leishmania* spp. L285[b]	*L. mexicana* L94	*L. donovani* L52	*L. enriettii* L144	*C. fasciculata*
	L137	L287	L251	L38	L32[a]	L286[a]					
L-5-16	+	+	+	+	+	+	+	–	–	+	–
L-5-27	+	+	+	+	–	–	–	–	–	–	–
L-5-85	+	+	+	+	–	–	–	–	–	+	–
L-5-28	+	+	+	+	–	–	–	+	+	+	–
L-5-34	+	+	+	+	–	–	–	+	+	+	–
L-5-41	+	+	+	+	–	–	–	–	–	–	–
L-5-44	+	+	+	+	–	–	+	–	–	+	–
L-2-3F11	+	+	+	+	+	+	–	+	+	+	+
L-2-4B5	+	+	+	+	–	–	–	–	+	–	+

[a] From a patient with leishmaniasis recidiva.

[b] From a patient with diffuse cutaneous leishmaniasis, species undetermined (Schnur, personal communication).

and *L. enriettii* which causes in guinea pigs, a disease pattern similar to human cutaneous leishmaniasis. L-5-28 and L-5-34 detected *Leishmania* cross-reacting antigens present in *L. tropica, L. mexicana, L. donovani*, and *L. enriettii*. The antibody L-2-3F11 may recognize an ancestral determinant present in most *Leishmania* spp. and even in *Crithidia fasciculata*; the exception being a strain of *Leishmania* sp. from a case of diffuse cutaneous leishmaniasis, that did not react with this antibody.

DISCUSSION

The results presented above characterize certain properties of a series of monoclonal antibodies to membrane antigens of *L. tropica*. Supernatants from all cell cultures were screened for binding to *L. tropica* in a solid-phase RIA with glutaraldehyde-fixed promastigotes. With this assay it was anticipated that all selected antibodies would be directed to membrane antigens (10–15). Formal proof was obtained by indirect fluorescence on living promastigotes. The staining was bright and unequivocally on the parasite surface. Treatment of the parasites with proteases caused markedly decreased binding of most monoclonal antibodies, supporting the idea that they were surface protein antigens. Protease treatment did not completely abolish binding of the antibodies, even at a concentration of 1 mg/ml. Our previous studies have shown that *L. tropica* membrane proteins are resistant to proteolysis by papain, chymotrypsin, and staphylococcal V8 protease (5). Sodium metaperiodate treatment of the parasites caused reduced binding of L-5-41 and L-5-85, suggesting that the target antigens are carbohydrate or that carbohydrate plays a role in the structure of the antigen molecule (16) and in the expression of the target epitope. L-5-41 may be situated on a glycoprotein since subtilisin treatment also reduced the binding of L-5-41 to *L. tropica* promastigotes. It was of interest that pronase, subtilisin, or periodate treatment of the parasite had no effect on the binding of L-5-44. However, treatment of the promastigotes with periodate followed by pronase reduced the binding by 60%, suggesting that carbohydrate may protect the protein determinants from proteolysis (16).

Most antibodies recognized antigens shared by the amastigote and promastigote forms of the parasite. The parental spleen cells used for fusion came from chronically infected BALB/c mice harboring amastigotes, but originally injected with promastigotes. The mice were injected with promastigotes before the fusion experiment, and it is possible that proliferation of B-cell clones recognizing shared antigens was boosted. The screening for the antibody-producing clones was done on *L. tropica* promastigotes which restricted the detection of antibodies to promastigote antigens. However, only one monoclonal antibody (L-5-85) recognized a promastigote-specific epitope.

We have shown that periodate treatment of promastigotes decreased the binding of L-5-85, suggesting the target antigen of L-5-85 is a carbohydrate. It would be interesting to investigate whether its presence on the promastigote is important for the parasite location in sand fly guts, since it has been suggested that sugars on the parasite attach it to the gut wall (2).

Four of the *L. tropica* monoclonal antibodies (L-5-16, L-5-34, L-5-44, and L-2-3F11) detected parasite antigens displayed on infected macrophages. They should allow a more detailed characterization of the *L. tropica* antigens expressed on the infected macrophage. This may be essential for the study of antigen presentation in leishmaniasis in mice resistant or susceptible to disease (4). Characterization and isolation of these antigens may also provide an answer to the question of T-cell recognition of antigen in these animals in the induction and effector phases of the immune response (9a).

Two antibodies L-5-27 and L-5-41 were functionally active in vitro, promoting killing of pretreated parasites in macrophages. They had no effect on promastigotes grown in culture in the presence of the antibodies. The mechanism of killing is not clear, but since the effect could be observed as early as 8 h after infection of macrophages with treated promastigotes, the antibody could interfere with the transformation of the promastigotes to amastigotes. Untransformed promastigotes are more prone to digestion within the macrophage (9). These two antibodies were specific for *L. tropica* isolated from patients with simple cutaneous leishmaniasis and did not bind to *L. tropica* from patients with leishmaniasis recidiva or diffuse cutaneous leishmaniasis. L-5-27 and L-5-41 may be useful as immunodiagnostic reagents distinguishing between relatively innocuous *L. tropica* and isolates associated with more virulent disease states. The rest of the antibodies recognized antigenic determinants shared by various *Leishmania* strains. The antibody L-2-3F11 may recognize an ancestral determinant present in most *Leishmania* spp. and even in the primitive flagellate *C. fasciculata*.

Three major unresolved problems in *Leishmania* spp. research may be approached by using these and other monoclonal antibodies (10): the question of the taxonomy of the *Leishmania* spp. and the inability to distinguish at primary isolation between relatively innocuous species and species that cause severe disease in man, the problem of parasite differentiation from

the flagellate in the vector to the obligatory intracellular form living in the macrophage, and finally the identification and characterization of parasite antigens displayed on *Leishmania* spp.-infected macrophages (1, 3, 4). The use of monoclonal antibodies should allow the study of the origin and the location of the *Leishmania* spp. antigens on the macrophage membrane as well as the characterization of these antigens in terms of their relationship to host histocompatibility antigens involved in antigen presentation.

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LITERATURE CITED

1. **Berman, J. D., and D. M. Dwyer.** 1981. Expression of *Leishmania* antigen on the surface membrane of infected human macrophages *in vitro*. Clin. Exp. Immunol. **44**:342–348.
2. **Dwyer, D. M., S. G. Langreth, and N. K. Dwyer.** 1974. Evidence for a polysaccharide surface coat in the developmental stages of *Leishmania donovani*: a fine structure cytochemical study. Z. Parasitenkd. **43**:227–249.
3. **Farah, F. S., S. A. Sarura, and N. Nuvaysi-Sothi.** 1975. The role of the macrophage in cutaneous leishmaniasis. Immunology **29**:755–758.
4. **Handman, E., R. Ceredig, and G. F. Mitchell.** 1979. Murine cutaneous leishmaniasis: disease patterns in intact and nude mice of various genotypes and examination of some differences between normal and infected macrophages. Aust. J. Exp. Biol. Med. Sci. **57**:9–29.
5. **Handman, E., G. F. Mitchell, and J. W. Goding.** 1981. Identification and characterization of protein antigens of *Leishmania tropica* isolates. J. Immunol. **126**:508–512.
6. **Handman, E., and J. S. Remington.** 1980. Serological and immunochemical characterization of monoclonal antibodies to *Toxoplasma gondii*. Immunol. 40:579–588.
7. **Köhler, G., S. C. Howe, and C. Milstein.** 1976. Fusion between immunoglobulin secreting and non-secreting myeloma cell lines. Eur. J. Immunol. **6**:292–295.
8. **Köhler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
9. **Lewis, D. H., and W. Peters.** 1977. The resistance of intracellular *Leishmania* parasites to digestion by lysosomal enzymes. Ann. Trop. Med. Parasitol. **71**:295–312.

9a. **Louis, J. A., R. H. Zubler, S. G. Coutinho, G. Lima, R. Behin, J. Mavel, and H. D. Engers.** 1982. The *in vitro* generation and functional analysis of murine T cell populations and clones specific for a protozoan parasite, *Leishmania tropica*. Immunol. Rev. **61**:215–243.

10. **McMahon-Pratt, D., and J. R. David.** 1981. Monoclonal antibodies that distinguish between New World species of leishmania. Nature (London) **291**:581–583.
11. **Oi, V. T., and L. A. Herzenberg.** 1980. Immunoglobulin producing hybrid cell lines, p. 351–372. *In* B. B. Mishell, and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman, San Francisco.
12. **Oi, V. T., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg.** 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. Curr. Top. Microbiol. Immunol. **81**:115–129.
13. **Olobo, J. O., E. Handman, J. M. Curtis, and G. F. Mitchell.** 1980. Antibodies to *Leishmania tropica* promastigotes during infection in mice of various genotypes. Aust. J. Exp. Biol. Med. Sci. **58**:595–601.
14. **Parks, D. R., V. M. Bryan, V. T. Oi, and L. A. Herzenberg.** 1979. Antigen specific identification and cloning of hybridomas with a fluorescence activated cell sorter. Proc. Natl. Acad. Sci. U.S.A. **76**:1962–1966.
15. **Stocker, J. W., and C. H. Heusser.** 1979. Methods for binding cells to plastic: application to a solid-phase radioimmunoassay for cell-surface antigens. J. Immunol. Methods **26**:87–95.
16. **Wilson, I. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3A° resolution. Nature (London) **289**:366–373.