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The effect of *Leishmania tropica* on stimulation of lymphocytes with phytohaemagglutinin

F. S. FARAH *Department of Medicine and W.H.O.–Immunology Research and Training Center, American University of Beirut, Beirut, Lebanon*

S. LAZARY & A. DE WECK *Institut für klinische Immunologie, Inselspital Bern, Bern, Switzerland*

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Summary. Cell-mediated immune (CMI) responses are important in the immunity against leishmanial infections. However, infection persists in the presence of CMI for unknown reasons. Evidence is presented that *L. tropica* and its products are capable of inhibition of the stimulation of lymphocytes by PHA. This inhibition is dose dependent, and not dependent on competition for nutrients in the medium, nor on neutralization of PHA. The inhibition is observed on the lymphocytes of species susceptible to leishmanial infection, and not operative in resistant species. The mechanisms of the lymphocyte suppression are discussed.

INTRODUCTION

There is a growing interest in the study of the immune response in leishmanial infections, particularly in infection by *L. tropica*, which produces cutaneous leishmaniasis. Among other findings, it has been suggested repeatedly that cell-mediated immune responses play an important role in this disease (Shaw and Voller, 1968; Bryceson, 1969; Bryceson, Bray, Wolstencroft and Dumonde, 1970; Bryceson and Turk, 1971; Blewett, Kadivar and Soulsby, 1971; Preston, Carter, Leuchars, Davies and Dumonde, 1972; Bryceson, Preston, Bray and Dumonde, 1972; Skov and Twohy, 1974b). The observations made in experimental infections in animals (Bryceson *et al.*, 1970; Bryceson and Turk, 1971, Blewett *et al.*, 1971; Preston *et al.*, 1971; Shaw *et al.*, 1968) and in some clinical forms of human disease such as diffuse cutaneous leishmaniasis (DCL) (Bryceson, 1969, 1972a, b) support this concept.

An important parameter in the study of CMI is the response of lymphocytes to stimulation either specifically with antigens or non-specifically with for example phytohaemagglutinin (PHA). The latter is regarded as generally indicative of the functional state of T lymphocytes. However, the experimental conditions under which stimulation (specific or non-specific) is performed may also influence the lymphocyte response. For instance, the relative concentration of macrophages in the test system is important in determining whether stimulation will be facilitated or inhibited (Keller, 1975). Similarly, the antigens used for the detection and measurement of CMI may themselves, in various ways produce profound influence(s) on lymphocyte responses in general, as has been shown with certain strains of mycoplasma (Roberts, 1972).

In the course of our studies on the immunology of

Correspondence: Dr F. S. Farah, Institute for Clinical Immunology Inselspital, Bern, Switzerland.

cutaneous leishmaniasis, it became apparent that the effects of *L. tropica* on the lymphocytes of some species need to be defined. In this report we shall describe experiments which show that *L. tropica* depresses PHA stimulation, as measured by [^{3}H]-thymidine incorporation, of normal lymphocytes of mice and guinea-pigs.

MATERIALS AND METHODS

L. tropica

Cultures of *L. tropica* were supplied by the W.H.O.–Immunology Research and Training Centre at the American University of Beirut (AUB), Beirut, Lebanon. The organism was originally cultured from lesions of patients suffering from cutaneous leishmaniasis, seen at the Dermatology Clinics of AUB. Other cultures were kindly supplied by Dr J. Mauel (W.H.O.–Immunology Research and Training Center, Lausanne, Switzerland). The organism was maintained on NNN media overlayed with enriched Eagle's medium, and harvested on the 7–14th day of culture by centrifugation at 2500 r.p.m. for 15 min, and washed twice in Hanks's balanced salt solution (HBSS) before use.

Killed organisms were prepared by heating washed *L. tropica* in a boiling water bath for 10 min or by repeated freezing and thawing. The parasites killed in these two ways maintained an intact cell membrane as judged by direct light microscopy and immunofluorescent staining.

Soluble antigens were prepared by homogenization of the parasite in saline, using a Sorvall homogenizer, for 10 min at maximum speed. The homogenate was centrifuged at 3000 r.p.m. for 30 min and the precipitate discarded. The protein content of the antigen solution was determined according to the method of Lowry, Rosebrough, Farr and Randall (1951).

Lymphocytes

Lymphocytes were obtained from normal CBA mice and from normal strain 2 guinea-pigs. Mouse lymphocytes were collected aseptically from the pooled spleens of three to four animals about 10–15 weeks old. The spleens were first cut into small pieces, minced with forceps and fragments were allowed to settle for a few minutes in HBSS in conical centrifuge tubes. The supernatant cell suspension was then centrifuged at 1400 r.p.m. for 10 min at room temperature, the pellet washed twice in HBSS and resuspended in RPMI 1640 containing 5 per cent heat-inactivated foetal calf serum (HI-FCS). The cells were counted in a haemocytometer after dilution in 0·05 per cent trypan blue.

Guinea-pig lymphocytes were collected from the spleen or cervical lymph nodes of normal non-stimulated guinea-pigs about 6 months old. The tissues were freed from fat, cut into small pieces and forced through a steel wire mesh. The cells were washed, resuspended and counted as above.

Lymphocytes were cultured in triplicate in Falcon tissue culture tubes in 1·0 ml RPMI 1640 containing 5 per cent HI-FCS, 100 u of penicillin and 100 μg streptomycin per ml, and buffered with 0·04 M HEPES buffer. The cultures were maintained at 37° in a humidified incubator. One microcurie of tritiated thymidine per ml of culture was added on the 4th day and the cells were harvested 6–18 h later according to the method of Sörensen, Anderson and Guisse (1969).

Lymphocyte stimulation was induced with PHA-P (Difco) added to the final concentration of 20 μg/ml culture (1/1000). The degree of stimulation of lymphocytes was indicated by the incorporation of tritiated thymidine, measured by liquid scintillation in a Beckman Scintillation Counter model LS-330 or LS-133. The results were expressed as c.p.m. per culture and for the purposes of comparison, as stimulation index, SI = (average c.p.m. of test culture)/(average c.p.m. of control culture).

RESULTS

(1) The effects of *L. tropica* on PHA stimulation of mouse and guinea-pig lymphocytes

Table 1 shows the results of a typical experiment in which mouse and guinea-pig spleen cells were cultured in the presence of varying amounts of living *L. tropica*, and were simultaneously stimulated with PHA. In all these experiments, *L. tropica* was added immediately prior to the addition of PHA. Fig. 1 summarizes additional data from several other experiments.

Clearly, *L. tropica* suppresses the PHA stimulation of the cells of both species. The degree of suppression is dose dependent; increasing suppression is observed with the addition of higher doses of parasite.

PHA appears to remain effective for stimulation of

Table 1. Suppression of PHA stimulation of guinea-pig and mouse spleen lymphocytes with *L. tropica*

Guinea-pig*			Mouse		
L. tropica ($\times 10^6$)	PHA (20 μg/ml)	c.p.m. $\pm$ s.e.m.†	*L.t.*	PHA	c.p.m. $\pm$ s.e.m.†
—	—	726 $\pm$ 45	—	—	9815 $\pm$ 928
—	+	36,896 $\pm$ 2047	—	+	53,184 $\pm$ 2487
1·8	+	19,138 $\pm$ 2123	1·25	+	42,574 $\pm$ 10,378
4·9	+	8208 $\pm$ 438	2·5	+	18,117 $\pm$ 5826
7·4	+	6465 $\pm$ 320	4·0	+	27,063 $\pm$ 4542
10	+	4206 $\pm$ 160	8·0	+	11,489 $\pm$ 3392
5	—	578 $\pm$ 161	2·5	—	7103 $\pm$ 1086

* $3{\cdot}0 \times 10^6$ cells per culture of 1·0 ml.
† c.p.m. Average of triplicate cultures $\pm$ standard error of mean.

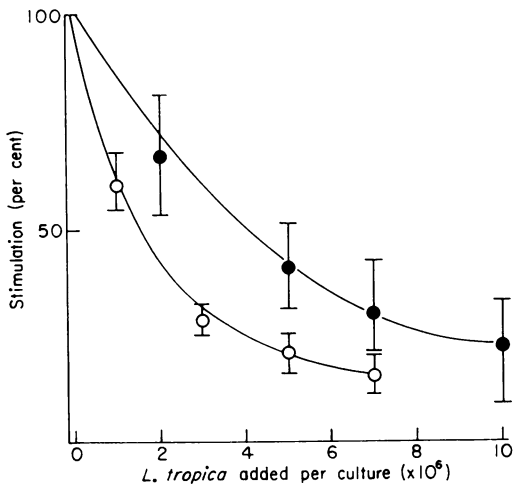

Figure 1. Suppression of PHA stimulation of guinea-pig (●) (average of three experiments) and mouse (○) (average of five experiments) spleen cell cultures by living *L. tropica*. The vertical bars represent s.e.m.

Table 2. Stimulation of guinea-pig lymph node cells with PHA absorbed with *L. tropica**

Cells stimulated with:†	Response‡	Index
—	929 $\pm$ 338	
PHA	38,917 $\pm$ 4362	44·9
PHA absorbed with $0{\cdot}5 \times 10^6$ live *L.t.*	34,850 $\pm$ 164	37·5
PHA absorbed with 10×10^6 live *L.t.*	39,898 $\pm$ 3202	43·0
PHA absorbed with $0{\cdot}5 \pm 10^6$ dead *L.t.*	41,481 $\pm$ 5106	44·7
PHA absorbed with 10×10^6 dead *L.t.*	45,114 $\pm$ 2819	48·6

* Similar results were obtained with mouse spleen cells.
† $3{\cdot}0 \times 10^6$ cells were used per culture. Final concentrations of PHA is 20 μg/ml of culture.
‡ c.p.m. Average of triplicate cultures $\pm$ standard error of mean.

lymphocytes in the presence of *L. tropica*. It is not adsorbed by the organism. When PHA is incubated with *L. tropica* overnight at 37°, and the organisms removed by centrifugation, the supernatant retains its full capacity to stimulate lymphocytes as shown in Table 2.

(2) Does *L. tropica* compete with lymphocytes for nutrients in the culture medium?

When two living organisms, in this instance the lymphocyte and the parasite, coexist in culture, due consideration must be given to possible competition between them for the nutrients available in the limited culture medium. Table 3 shows that if competition does occur it is not sufficient to explain the observed decrease in lymphocyte proliferation. Fresh culture medium was added daily (Table 3B) and in another instance the medium was replaced after the first 24 h of culture with fresh medium (Table 3C). PHA stimulation was similarly suppressed in the two cases and in both the responses were similar to those observed in the untreated cultures (Table 3A). Futhermore, killed *L. tropica* produced

Table 3. Effect of replenishing culture on suppression of PHA stimulation of lymphocytes* (guinea-pig lymph node cells)

L. tropica ($\times 10^6$)	PHA (20 μg/ml)	A† (average c.p.m.)	B† (average c.p.m.)	C† (average c.p.m.)
—	−	430	3408	1987
—	+	48,792	150,719	160,718
1·1	+	37,169	95,837	78,080
3·4	+	23,879	68,302	70,470
4·5	+	22,560	49,505	137,235
6·8	+	13,607	37,835	40,544
9·0	+	12,807	36,081	24,786

* 2×10^6 cells were used per culture of 1·0 ml.

† A = regular culture; B = 0·5 ml of medium added daily; C = after the first 24 h, the culture medium was replaced by fresh medium.

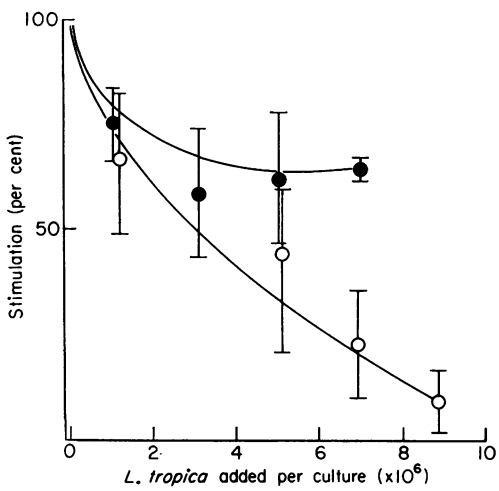

Figure 2. Suppression of PHA stimulation of guinea-pig (●) and mouse (○) spleen cell cultures by killed *L. tropica*. The points on the curves represent the average of three experiments and the vertical bars the s.e.m.

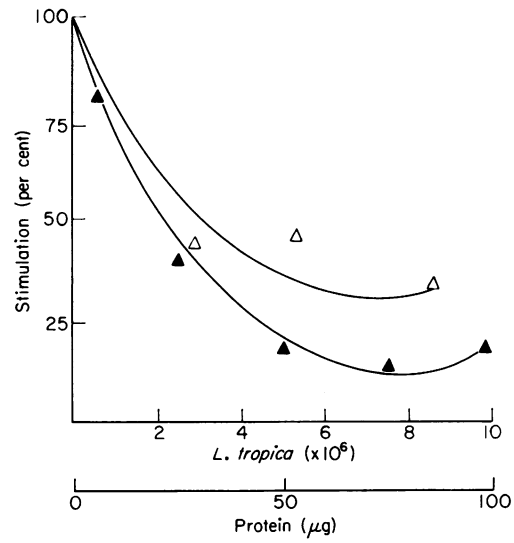

Figure 3. Effect of soluble antigen (0·5 mg protein/ml) on PHA stimulation of guinea-pig lymph node cells. Similar results were obtained with guinea-pig and mouse spleen cells. *L. tropica* (△); soluble antigen (▲).

the same inhibition of mouse and guinea-pig spleen cells (Fig. 2), although the effect appears to be much less marked on guinea-pig than on mouse cells.

(3) The inhibitory effects of soluble antigen

Soluble antigen added in increasing amounts to the cultures of lymphocytes, produced suppression of PHA stimulation of guinea-pig lymph node cells (Fig. 3).

(4) Effect of *L. tropica* on lymphocytes of other species

Lymphocytes obtained from cow peripheral blood and pig lymph nodes were cultured in the presence of increasing amounts of *L. tropica*. No suppression of stimulation of the cells of these two species was noted (Table 4).

Table 4. Effect of *L. tropica* on lymphocytes of other species

L. tropica ($\times 10^6$)	PHA (20 μg/ml)	Cow* (c.p.m. $\pm$ s.e.m.)	Pig† (c.p.m. $\pm$ s.e.m.)
—	−	3051 $\pm$ 524	648 $\pm$ 42
—	+	322,807 $\pm$ 17,147	104,551 $\pm$ 4023
2·0	+	333,389 $\pm$ 10,594	109,836 $\pm$ 2114
5·1	+	366,546 $\pm$ 16,180	119,933 $\pm$ 1187
7·4	+	357,319 $\pm$ 2998	133,024 $\pm$ 14,092
10·2	+	383,770 $\pm$ 15,689	114,385 $\pm$ 5475
5·1	−	12,178 $\pm$ 2193	729 $\pm$ 164

* $1{\cdot}2 \times 10^6$ lymphocytes per culture (1·0 ml).
† $3{\cdot}0 \times 10^6$ lymphocytes per culture (1·0 ml).

DISCUSSION

Cell-mediated immune (CMI) responses play an important role in the immunology of cutaneous leishmaniasis. It has been shown that immunosuppression of CMI in animals aggravates the infection (Bryceson *et al.*, 1972; Preston *et al.*, 1972; Shaw and Voller, 1968). It has also been postulated that diffuse cutaneous leishmaniasis is associated with depression of CMI, perhaps even with a selective defect (albeit temporary) in the response to leishmania antigens (Bryceson, 1972a, b). However, the exact role played by CMI in the control of the human infection remains somewhat vague and ill defined. One needs only to recall that the self healing oriental sore lasts for many months after both antibody-mediated immunity (AbMI) and CMI are well established. Healing occurs at a later stage, possibly involving more complex mechanisms than hitherto assumed. Furthermore, the findings of chronic (non-healing) cutaneous lesions and of relapsing (recidiva) lesions in the presence of well-defined AbMI and CMI further underlines the complexity of the infection. Among the many questions raised is one dealing with the effects of the organisms themselves on the activity of lymphocytes, the key cells in the immune response. Is the organism capable in some general or specific way, of suppressing the reaction of lymphocytes, and therefore furthering its own survival in the host?

The phenomenon of suppression of lymphocyte reactivity has already been described for viruses and for mycoplasma (Roberts, 1972). In the case of the latter, the suppression was related to competition for essential nutrients in the culture medium. The results reported in this communication show that *L. tropica* undoubtedly suppresses the ability of lymphocytes of both guinea-pigs and mice to be stimulated by PHA. The suppression is apparently not dependent on nutritional depletion nor is it due to the binding of PHA by *L. tropica*, thereby making it unavailable for the stimulation of the lymphocytes. Furthermore, our results show no evidence of primary toxic effect of *L. tropica* on the lymphocytes of the various species studied, since the thymidine uptake by control cells cultured in the presence of *L. tropica* is similar to the control cells cultured alone. Also, PHA stimulation of cow and pig cells was not at all depressed in the presence of the organism.

Similarly, suppression was obtained with soluble antigen. The antigens obtained by homogenization are mostly surface antigens and are not distinguishable from antigens prepared by solubilizing the parasite surface with detergents (F. S. Farah, unpublished data). The fact that soluble antigens, like the living organism, depress lymphocytes reactions in both animal species in a similar manner may suggest a common mechanism dependent upon the same or similar substance(s). Such substance(s) could either be present on the surface of the parasite or be produced by it. This substance(s) could act on the lymphocyte membrane leading to the suppression of the cell's ability to be stimulated by PHA. An alternative possibility is interference with lymphocyte metabolism.

Killed *L. tropica* produces a strong inhibition of the PHA response of lymphocytes of the CBA mouse, and a lesser one of guinea-pig spleen cells. Indeed, no effect (and even possibly mild stimulation) of the lymph node cells of the guinea-pig has been observed in preliminary experiments (unpublished data). CBA mice are susceptible to infection with *L. tropica* whereas guinea-pigs are resistant although they do become infected with *L. enriettii*. It was therefore of interest to examine the effects of *L. tropica* on the activity of lymphocytes of other species not susceptible to leishmanial infection. Table 4 shows that for the cow and pig which to our knowledge are not susceptible to leishmanial infection, no inhibition occurs.

The mechanism of inhibition of lymphocyte proliferation is not yet known. Mouse cells may be more susceptible to the suppressant (found in small amounts in the dead parasite) than the cells of the guinea-pig, because the number of membrane surface

sites (receptors) for leishmania products differs in the two species. Binding of leishmania organisms (or soluble products) could sterically interfere with interaction between PHA and lymphocyte membranes. It may be relevant that the living parasite and soluble antigen also produce inhibition of stimulation by allogeneic cells (in mixed lymphocyte culture in mice) and by PPD in lymphocytes from BCG-sensitized guinea-pigs (F. S. Farah, unpublished data). Whether inhibition of stimulation by the organisms or their products is due to interference with membrane signals or through blockade of intracellular processes remains to be investigated.

It has been shown (Opitz, Niethammer, Lemke, Flad and Huget, 1975) that activated macrophages release a factor that inhibits thymidine uptake by PHA-stimulated lymphocytes. Such a mechanism must also be explored, and work is in progress to find out whether supernatants of parasitized macrophages are operative in the system described. If macrophages are active in this respect it would add another area of activity to those already defined for the macrophage in leishmanial infections (Farah, Nuwayri-Salti and Samra, 1975).

Whatever the nature of the suppression observed *in vitro*, it is intriguing to consider that in the clinical situation the organism might possibly be responsible for similar depression of lymphocyte reactions at/or around the local lesion, either directly, or by soluble antigens secreted by the intracellular parasite. Such a mechanism would favour maintenance or initiation of infection. It would also offer an explanation for the prolonged nature of the illness and of the chronic and relapsing lesions, even when an adequate immune response is present in the patient. The hypothesis might also help explain the reappearance of *Leishmania*-specific CMI with anti-parasite treatment and recovery of patients suffering from disseminated cutaneous leishmaniasis (DCL) and kalazar. The cell-mediated immunity developed by parasitized patients may be rendered ineffective if large numbers of parasites with local immunosuppressive activity are still present in the lesions.

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REFERENCES

Blewett T.M., Kadivar M.H. & Soulsby E.J.L. (1972) Cutaneous leishmaniasis in the guinea pig. Delayed hypersensitivity, lymphocyte stimulation and inhibition of macrophage migration. *Amer. J. trop. Med. Hyg.* **20,** 546.

Bryceson A.D.M. (1969) Diffuse cutaneous leishmaniasis in Ethiopia. I. Clinical and histological features of the disease. *Trans. roy. Soc. trop. Med. Hyg.* **63,** 708.

Bryceson A.D.M. (1972a) Diffuse cutaneous leishmaniasis in Ethiopia. III. Immunological studies. *Trans. roy. Soc. trop. Med. Hyg.* **63,** 380.

Bryceson A.D.M. (1972b) Diffuse cutaneous leishmaniasis in Ethiopia. IV. Pathogenesis of diffuse leishmaniasis. *Trans. Roy. Soc. trop. Med. Hyg.* **64,** 378.

Bryceson A.D.M., Bray R.S., Wolstencroft R.A. & Dumonde, D.C. (1970) Immunity in cutaneous leishmaniasis of the guinea pig. *Clin. exp. Immunol.* **7,** 301.

Bryceson A.D.M., Preston P.M., Bray R.S. & Dumonde D.C. (1972) Experimental cutaneous leishmaniasis. II. Effects of immunosuppression and antigenic competition on the course of the infection with *Leishmania enriettii* in the guinea pig. *Clin. exp. Immunol.* **10,** 305.

Bryceson A.D.M. & Turk J.L. (1971) The effect of prolonged treatment with antilymphocyte serum on the course of infection with BCG and *Leishmania enriettii* in the guinea pig. *J. Path.* **104,** 153.

Farah F.S., Nuwayri-Salti N., Samra S.H. (1975) The role of the macrophage in cutaneous leishmaniasis. *Immunology*, **29,** 755.

Keller P. (1975) Major changes in lymphocyte proliferation evoked by activated macrophages. *Cell. Immunol.* **17,** 542.

Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. (1951) Protein measurement with Folin phenol reagent. *J. biol. Chem.* **193,** 265.

Opitz H.G., Niethammer D., Lemke H., Flad H.D. & Huget R. (1975) Inhibition of ^{3}H-thymidine incorporation of lymphocytes by a soluble factor from macrophages. *Cell. Immunol.* **16,** 379.

Preston P.M., Carter R.L., Leuchars E., Davies A.J.J. & Dumonde D.C. (1972) Experimental cutaneous leishmaniasis. III. Effects of thymectomy on the course of infection of CBA mice with leishmania tropica. *Clin. exp. Immunol.* **10,** 337.

Roberts D.H. (1972) Inhibition of lymphocyte transformation induced by phytohemagglutinin with porcine mycoplasma. *Brit. vet. J.* **128,** 585.

Shaw J.J. & Voller A. (1968) Observations on the susceptibility of white mice to infection with *Leishmania mexicana* following whole body X-irradiation. *Ann. trop. Med. Parasit.* **62,** 174.

Skov C.B. & Twohy D.W. (1974a) Cellular immunity to *Leishmania donovani*. I. The effect of T-cell depletion on resistance to *L. donovani* in mice. *J. Immunol.* **113,** 2004.

Skov C.B. & Twohy, D.W. (1974b) Cellular immunity to *Leishmania donovani*. II. Evidence for synergy between thymocytes and lymph node cells in reconstitution of acquired resistance to *L. donovani*. *J. Immunol.* **113,** 2012.

Sörensen S.F., Anderson V. & Gisse, J. (1969) Rapid method for quantitation of incorporation of 3H-thymidine by lymphocytes *in vitro*. *Acta path. microbiol. scand.* **15,** 508.