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Immunosuppression in Kenyan visceral leishmaniasis

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SUMMARY

Cell-mediated immune responses were evaluated in 15 patients with active visceral leishmaniasis from Masinga location in eastern Kenya where the disease is endemic. Age and sex matched controls were selected from a village school in the same area. *In vivo* studies were carried out by skin testing with leishmanin, tuberculin, streptococcal and candida antigens. Lymphocyte blastogenic transformation to the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) and the antigens purified protein derivative (PPD), streptokinase–streptodornase (SKSD) and leishmanial antigen (LA) was studied *in vitro*. The results showed that immunosuppression in visceral leishmaniasis in Kenya was both specific and non-specific. In the majority of patients there was complete anergy to all antigens *in vivo* and *in vitro*. The suppression of responses to mitogens was less marked. Recovery of non-specific responses preceded the development of specific immunity. In a small number of patients (23%) immune unresponsiveness to leishmanial antigens persisted 1 year after parasitological cure.

INTRODUCTION

Cell-mediated immunity in visceral leishmaniasis is known to be suppressed, as measured by the leishmanin skin test which is negative in active disease. With recovery after treatment, patients develop a positive skin reaction and resist experimental challenge with *L. donovani* (Manson-Bahr, 1959). These observations suggest that during active visceral leishmaniasis the host has a specific defect in mediating an effective cellular immune response against *L. donovani*. The specificity, extent and duration of this immune defect have not, however, been characterized. The present study was designed to evaluate systematically the immunosuppression associated with visceral leishmaniasis in Kenya by studying delayed skin hypersensitivity and lymphocyte blastogenic responses to mitogens and antigens at various stages of the disease.

MATERIALS AND METHODS

Study population. Thirty individuals from Masinga location in eastern Kenya where visceral leishmaniasis is endemic were investigated. These comprised (i) 15 patients admitted to the Clinical Research Centre, Kenyatta National Hospital, Nairobi and (ii) 15 leishmanin negative normal individuals of the same age group (5–18 years) selected from a village school. A third group of 53 individuals were skin tested only. Active visceral leishmaniasis was diagnosed by the detection of Leishmania in direct smears and cultures from splenic aspirates. Skin tests and lymphocyte studies

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were carried out on all 15 controls and on 10 patients before treatment and at $1\frac{1}{2}$, 3, 6 and 12 months from the end of treatment. Five additional patients were included from the $1\frac{1}{2}$ month follow-up onwards. Attendance at each follow-up examination was between 80–100%.

Preparation of antigens and mitogens. L. donovani isolated from a Kenyan patient was cultured in RPMI 1640 with 20% fetal calf serum. Aqueous extracts of promastigotes were prepared by four cycles of freezing and thawing of washed parasites suspended in 10 times their volume of distilled water. The disrupted promastigotes were removed by centrifugation at 2,500*g* for 20 min. The supernatant was sterilized by passage through a 0·22 μm filter (Millipore Corporation, Bedford, Massachusetts, USA) and stored at −20°C until used. Protein concentration was determined by the method of Lowry *et al.* (1951). The antigen (LA) was used at a final concentration of 50 μg/ml which was found to give optimal stimulation. Leishmanin (LMN) for skin testing was prepared from a suspension of washed *L. donovani* promastigotes at 5×10^6 parasites per ml of 0·5% phenol saline.

Purified protein derivate (PPD) was obtained from the Ministry of Agriculture Fisheries and Foods, Central Veterinary Laboratory, Weybridge, Surrey, England. Streptokinase–streptodornase (SKSD) (Lederle Laboratories, Cyanamide Co., West Germany) was dialysed to remove preservatives and diluted in phosphate-buffered saline (PBS). Lyophilized phytohaemagglutinin HA 15 (PHA) (Wellcome Reagents Ltd., Beckenham, Kent, England) was reconstituted with 5 ml of distilled water. Concanavalin A (Con A) (Sigma Chemical Co., St Louis, Missouri, USA) was reconstituted in PBS. Final concentrations of antigens and mitogens inducing optimal thymidine incorporation were PPD (10–100 μg/ml), SKSD (100–200 units/ml), PHA (1/100 dilution) and Con A (10–30 μg/ml).

Lymphocyte culture and assay of ^{3}H-thymidine incorporation. Blood from control individuals were collected in Kivaa village, 160 km by road from Nairobi. Fifteen millilitres of venous blood was mixed with 3 ml of 10% EDTA (May & Baker, Dagenham, Essex, England)–4·5% dextran (D-5251, Sigma Chemical Co.) and red cells were allowed to sediment at ambient temperature for 45 min. The leucocyte rich plasma was transferred into sterile centrifuge tubes and transported back to Nairobi for initiation of cultures the same day. Blood was similarly obtained from patients at the Clinical Research Centre, Nairobi.

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque sedimentation (Pharmacia Inc., Piscataway, New Jersey, USA) of the leucocyte rich plasma from all individuals (Böyum, 1968). The cells were washed three times with Hank's balanced salt solution (KC Biological Inc., Lenexa, Kansas, USA) and suspended at $1{\cdot}5 \times 10^6$ cells/ml in RPMI 1640 (KC Biological Inc.) containing 10% heat-inactivated pooled human serum supplemented with penicillin 100 units/ml, streptomycin 100 μg/ml (Gibco, Grand Island, New York, USA) and L-glutamine 2 mM/dl (Flow Laboratories, Rockville, Maryland, USA). One hundred microlitres of cell suspension and 10 μl of mitogen or antigen were put in microtitre plate wells in quadruplicate (Sterilin, Teddington, Middlesex, England). For mitogen-induced proliferation, cultures were performed in flat bottomed plates and incubation was continued for 3 days in a 5% CO_2, humidified incubator. For antigen-induced proliferation, round bottomed plates were used and incubation was continued for 5 days. Half a microcurie of methyl-tritiated thymidine (sp. act. 24 Ci/mmole, Amersham International Ltd., Amersham, Buckinghamshire, England) was added to each well 18 hr before harvesting. Cells were collected onto glass fibre filter paper discs and washed by a semi-automated harvester (Flow Laboratories, Norway). The dried discs were placed in 1 ml of scintillation fluid (Buhler, 1962) and ^{3}H content measured in a PRIAS CLD/400 liquid scintillation counter (Hewlett Packard, Downers Grove, Illinois, USA). Data were expressed as mean c.p.m. of quadruplicate wells ±1 s.d. or Δ c.p.m. = mean c.p.m. in antigen stimulated cultures minus c.p.m. in control cultures. Student's *t*-test for unpaired samples was used to determine the significance of observed differences.

Skin tests. After blood was taken for lymphocyte studies, individuals were skin tested intradermally with 0·1 ml of the following antigens: leishmanin 5×10^5 promastigotes (Clinical Research Centre, Nairobi, Kenya); tuberculin, 1 TU (National Public Health Laboratories, Nairobi, Kenya); SKSD, 10 units (Lederle Laboratories, Cyanamide Co.) and *Candida albicans* antigens (DHS test; 1%, Bencard, Brentford, Middlesex, England). The size of induration was measured in two diameters after 48 hr by the ball point pen technique (Sokal, 1975). A positive

reaction was taken as a palpable area of induration 4 mm or more in diameter (Bates, Suen & Tranum, 1979). Chi-square test was used to compare rates of skin test positivity.

RESULTS

Study population

The clinical and laboratory features of the 15 patients on admission are summarized in Table 1. All patients were febrile but no secondary infections were detected. In addition to anaemia and leucopaenia, hypoalbuminaemia was a common finding. Globulin levels were markedly elevated. The response to antimony therapy (10 mg Sb/kg × 31 doses) was prompt and all the patients were parasitologically cured at the end of the treatment period. With recovery there was a return of the haematological and biochemical parameters toward normal. Three of the 15 patients developed intercurrent infections during therapy—pneumococcal pneumonia, herpes zoster and measles.

Skin tests

The results of delayed hypersensitivity reactions to intradermal injection of LMN, PPD, SKSD and candida are shown in Table 2. Two patients had PPD responses measuring 12 mm and 16 mm in diameter. There was no clinical or radiological evidence of active tuberculosis in either of them. Unlike the controls, all patients were unresponsive to SKSD and candida.

Mitogen-induced lymphocyte proliferation

PBMC from patients with active visceral leishmaniasis had significantly lower responses to PHA as compared to those from normal controls ($P < 0{\cdot}0001$; Fig. 1). The mean responses to Con A were not significantly different in the two groups ($P > 0{\cdot}10$).

Table 1. Clinical and laboratory features of 15 patients with visceral leishmaniasis: mean value (range)

M:F	9:6
Age	10·8 years (5–18)
Duration of symptoms	9 months (6–12)
Splenic axis	13 cm (8–21)
Haemoglobin	8·6 g/dl (5·1–13·3)
Leucocyte count	$3{\cdot}6 \times 10^9$/l (1·6–4·7)
Lymphocyte count	$1{\cdot}8 \times 10^9$/l (0·85–2·4)
Albumin	26 g/l (17–40)
Globulin	69 g/l (48–78)

Table 2. Numbers (and percentages) of patients with positive delayed hypersensitivity skin test (≥4 mm induration) to four antigens before treatment

	LMN	PPD	SKSD	Candida
Patients $n=15$	0	2(13)	0	0
Controls $n=68$	0	37(54)	49(72)	26(38)
P* values	—	<0·01	<0·0005	<0·01
*x^2	n.a.	6·76	23·49	6·67

n.a. = not applicable.

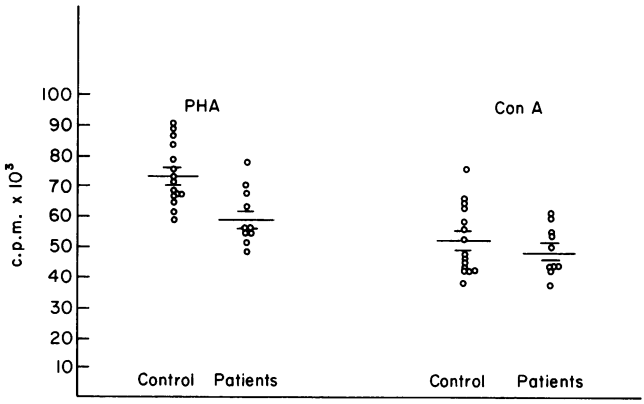

Fig. 1. Mitogen-induced thymidine incorporation in peripheral blood mononuclear cells of patients with active visceral leishmaniasis as compared to control population. Cross bars represent mean c.p.m. ± s.e.m.

Antigen-induced lymphocyte proliferation

PBMC from patients with active visceral leishmaniasis failed to respond to leishmanial antigen, PPD and SKSD with the exception of the two tuberculin positive patients whose cells proliferated *in vitro* in response to PPD (Table 3). These two patients developed significantly greater cellular responses as measured by size of induration and by ^{3}H-thymidine uptake 6 weeks after therapy, suggesting that the initial responses had also been suppressed.

Duration of immunosuppression

The time course for the recovery of delayed skin hypersensitivity to various antigens is shown in Table 4. Normal responses to PPD, SKSD and candida were observed in some patients as early as 6 weeks after therapy and reached control frequencies by 3 months (PPD, candida) and 1 year (SKSD). These results correlated with *in vitro* lymphocyte proliferative responses to PHA and the same antigens in individuals and patients as a group (Table 5). On the other hand, responsiveness to

Table 3. Antigen-induced thymidine incorporation in peripheral blood mononuclear cells of patients with active visceral leishmaniasis and controls

	Patients ($n=10$)	Controls‡ ($n=15$)
LA	78 ± 33	438 ± 107
PPD	218 ± 63* (8)	10,617 ± 1,650
	12,831 ± 2,019† (2)	
SKSD	340 ± 189	6,880 ± 1,288

Values indicate mean Δ c.p.m. ± s.e.m. Unstimulated counts were 1,122 ± 158 and 1,439 ± 199 for patients and controls respectively.

* Tuberculin negative patients.

† Tuberculin positive patients.

‡ Number of controls with positive delayed hypersensitivity skin tests to PPD and SKSD was nine and 11 respectively.

Table 4. Positive delayed hypersensitivity skin test responses before treatment and at each follow-up examination

	LMN	PPD	SKSD	Candida
Patients:				
Pre-Rx ($n=15$)	0	2	0	0
6 weeks ($n=14$)	0	6	1	3
3 months ($n=12$)	4	6	4	4
6 months ($n=15$)	8	10	9	8
1 year ($n=13$)	10	8	9	9

Table 5. Recovery of *in vitro* lymphocyte proliferative responses to mitogen and non-specific antigens

	PHA	PPD	SKSD
Controls ($n=15$)	73,340±2,618*	10,617±1,650†	6,880±1,288‡
Time after Rx:			
6 weeks ($n=14$)	62,684±5,619	8,264±2,848	1,134±430
3 months ($n=12$)	67,685±5,504	7,682±2,751	1,532±495
6 months ($n=15$)	71,633±5,786	10,834±2,824	3,235±668
1 year ($n=13$)	72,034±2,480	11,663±2,848	7,049±1,045

* Mean c.p.m. ± s.e.m.; †‡ mean Δ c.p.m. ± s.e.m.

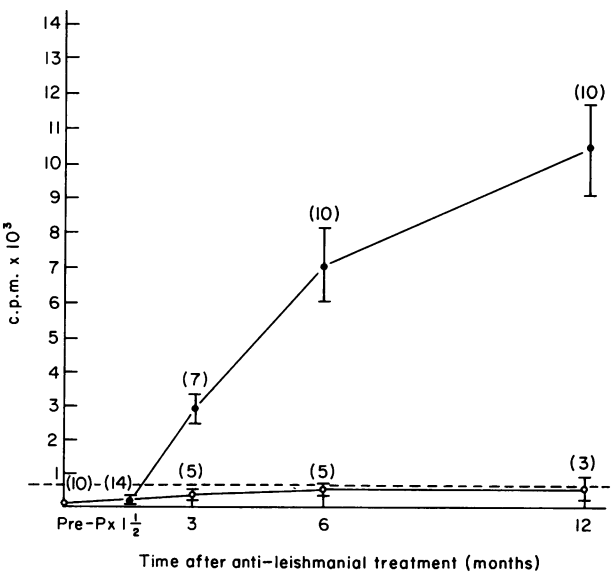

Fig. 2. Development of *in vitro* responsiveness to leishmanial antigen after treatment. At each follow-up examination the mean Δ c.p.m. ± s.e.m. and the number of patients tested are indicated.

leishmanin developed in two distinct patterns (Fig. 2). The majority of patients (77%) acquired leishmanin positivity gradually between 3 and 12 months. A smaller number (23%) remained anergic to leishmanin even at the end of 12 months.

DISCUSSION

Few studies have been carried out on the immunosuppression of human visceral leishmaniasis, and the existing data are inconsistent. In one patient with visceral leishmaniasis acquired in Greece, Wyler, Weinbaum & Harrod (1979) reported that peripheral blood lymphocytes proliferated in response to leishmanial antigen *in vitro* in spite of a negative leishmanin skin stest. The *in vitro* responses to Con A and SKSD were normal. Studying 14 Brazilian patients *in vitro* alone, Carvalho, Teixeira & Johnson (1981) characterized the immunosuppression of South American visceral leishmaniasis as being specific as well as short lived. This was in contrast to the findings of Levy & Mendes (1981) who demonstrated impaired delayed cutaneous hypersensitivity to leishmanin and other unrelated antigens in 10 Brazilian patients. In India, Ghose *et al.* (1979) found 15 patients with visceral leishmaniasis to be either non-responsive or only minimally responsive to PHA. In another Indian study tuberculin and DNCB skin tests were negative in six of seven patients with active visceral leishmaniasis (Aikat *et al.*, 1979). Migration inhibition responses to BCG were also impaired.

In the present study the cellular immune responses in human visceral leishmaniasis in Kenya were assessed by skin testing and lymphocyte transformation. Immunosuppression was present in all 15 patients at the time of diagnosis. Suppression was both specific and non-specific. There was complete absence of responses to leishmanial antigen. Suppression of responses to the non-specific antigens was less consistent, but often as profound. Reduction of mitogenic responses was much less marked. Recovery of the non-specific responses preceded specific recovery, suggesting that more than one mechanism of suppression was involved.

The element of non-specific suppression is profound, resulting in almost complete ablation of response to the three antigens used. Responses *in vitro* correlated with skin test results, indicating that the phenomenon was not due merely to deficient inflammation. Despite this, mitogenic responses were remarkably little affected. This combination of events differs from the situation in diffuse cutaneous leishmaniasis in which the suppression is entirely specific (Bryceson, 1970; Petersen *et al.*, 1982) but is similar to that reported in measles (Whittle *et al.*, 1973) and malnutrition (Greenwood & Whittle, 1981). In a separate study in which patients with visceral leishmaniasis were compared with appropriate controls, they were found to be undernourished by the following criteria: mid-arm circumference reduced by 2 cm, mean weight gain of 25% final body weight after 5 weeks hospitalization and skin or hair changes typical of malnutrition in 30% of patients (Gachii, personal communication). Nutritional oedema, however, was rare, despite a mean serum albumin level of 2·6 g/dl. Poor nutritional status, therefore, could certainly contribute to the initially observed immunosuppression. However, as restoration of nutrition was generally achieved by the end of hospitalization, malnutrition could not explain the persistence of both specific and non-specific immunosuppression beyond that period of time. In malnutrition, as in measles, humoral factors have been held partly responsible for the immunosuppression. Our preliminary studies have shown that serum factors have a minor role in the immunosuppression of Kenyan visceral leishmaniasis. The nature of the humoral factors have not been identified. Londner *et al.* (1981) reported decreased response to PHA, PPD and LA when PBMC were cultured in the presence of purified excreted factor from leishmanial promastigotes. Whether similar factors are present in sufficient quantity and concentration in patients with visceral leishmaniasis is unknown. In the immunosuppression of malnutrition and measles, the absence of a low molecular weight serum compound (Beatty & Ďowdle, 1979) or the presence of immune complexes (Greenwood & Whittle, 1981) have been implicated. The latter is present in high titre in patients with visceral leishmaniasis (Bowry, personal communication).

Because patients usually present months after the onset of symptoms, it is impossible to know whether there has been an initial immune response to *L. donovani* which then became suppressed, as

has been reported in animal models (Ho, Ellner & Reiner, 1981) and might be the case in man (Cahill, 1964). The persistence of specific unresponsiveness suggests either a prolonged depletion of specific responder T cells or the presence of a long lived suppressor cell population maintained perhaps by a persistent antigen drive. Reduction in the total number of circulating T lymphocytes as well as depletion of small lymphocytes in the spleen and lymph nodes have been demonstrated in visceral leishmaniasis (Musumeci *et al.*, 1981; Rezai *et al.*, 1978; Veress *et al.*, 1977). Since a positive correlation exists between the number of Tu cells and the PBMC response to PHA and Con A (Victorino & Hodgson, 1980), it is possible that a reduction in these cells may contribute to defects in cell-mediated immune responses in visceral leishmaniasis. Furthermore, selective recruitment of antigen specific reactive lymphocytes to secondary lymphoid tissues may occur (Basten *et al.*, 1982). In visceral leishmaniasis, where the spleen and liver are usually grossly enlarged, there might also be some degree of non-specific trapping to account for the general immune unresponsiveness as measured from PBMC. On the other hand, generation of suppressor cells by Con A has been found to be greater in patients with visceral leishmaniasis than in normal individuals in Kenya, suggesting an increase in suppressor cell activity in these patients (Koech & Allison, 1980). The prolonged specific immunosuppression in visceral leishmaniasis may indicate an antigen specific suppressor cell activity superimposed upon a general T lymphocyte hyporeactivity to other antigens and mitogens.

It is not clear how long the antigens persist after treatment of visceral leishmaniasis. Dead Leishmania are rapidly digested. After conventional treatment with sodium stibogluconate in a dose of 10 mg/kg daily for 30 days, 30% of Kenyan patients still have parasites in splenic aspirates (Anabwani, personal communication). Here, as elsewhere (Tuckman, 1949), these residual parasites are said not to be of clinical importance as almost all patients continue to recover satisfactorily. Nevertheless it might be that more vigorous treatment in terms of dose or frequency of administration which eliminates parasites more efficiently might reverse the immune defect more quickly. This could be of importance in preventing relapses and the acquisition of drug resistance.

In this context, the patients who failed to acquire specific responsiveness within 1 year are of interest. They resemble patients with diffuse cutaneous leishmaniasis (Bryceson, 1969) yet they remain well one year after treatment and have not relapsed, suggesting that they may have acquired some other effective defence mechanism. In Kenya, relapse of visceral leishmaniasis, or possibly reinfection, may occur as late as 3 years after treatment (Wijiers, 1971). Long term follow-up will be necessary to determine if these patients are more liable to relapse or are more susceptible to reinfection. They also re-pose the old question: is the unresponsiveness genetically determined or induced by the parasite?

Differences between the results of this study and those cited suggest the possibility that the effects of visceral leishmaniasis on the immune response may not be the same in different parts of the world. These differences could reflect variation in the severity of disease, or in human populations or in the capacities of different strains of parasites to induce or suppress responsiveness to differing degrees, and thus produce a spectrum of disease as occurs in cutaneous leishmaniasis (Bryceson, 1975).

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