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Cell-Mediated Immune Response in Indian Kala Azar and Post-Kala Azar Dermal Leishmaniasis

J. P. HALDAR,[1] SANDHYA GHOSE,[2] K. C. SAHA,[3] AND A. C. GHOSE[1]*

Division of Immunology, National Institute of Cholera and Enteric Diseases (Indian Council of Medical Research), Calcutta-700 010,[1] and Department of Medicine, Medical College[2] and Department of Dermatology, School of Tropical Medicine,[3] Calcutta-700 073, India

Received 29 June 1983/Accepted 18 August 1983

Cell-mediated immune (CMI) response in 16 Indian kala azar (KA) and 12 post-kala azar dermal leishmaniasis (PKADL) patients was studied in detail by in vitro lymphocyte transformation experiments and by in vivo skin testing. Peripheral blood lymphocytes of active KA patients failed to be stimulated by leishmania antigen. On the other hand, lymphocytes from a majority of the active KA patients could be stimulated by phytohemagglutinin. Active KA patients also failed to show delayed type hypersensitivity reaction to leishmanin, although 72% of them showed delayed type hypersensitivity to a purified protein derivative of tuberculin. Longitudinal studies indicated that antigen-specific CMI response usually appeared in treated KA patients after 12 to 20 weeks of antileishmanial drug therapy, although individual variations were noted. CMI response in PKADL patients was variable as about two-thirds of them showed positive sensitization to leishmania antigen in either in vivo or in vitro tests. Usually, patients with newly acquired PKADL exhibited better CMI response than those with chronic PKADL. However, lymphocytes from all of these patients could be stimulated normally by phytohemagglutinin. Results presented in this study show an impairment of CMI response in active KA which appears to be more specific to leishmania than generalized in nature. Moreover, restoration of specific T-cell responsiveness was aided by antileishmanial drug therapy which resulted in the reduction of antigenic load by parasite destruction and a concomitant decrease in circulating antibody levels, particularly that of the immunoglobulin G class. We suggest that the protection afforded by specific CMI response against *Leishmania donovani* infection may not be absolute and probably depends on other host-related factors leading to parasite destruction and patient recovery.

Kala azar (KA) is the visceral form of leishmaniasis and is caused by the intracellular parasite *Leishmania donovani*. The disease is usually fatal if untreated, but KA patients, once cured, are immune to the viscerotropic form of the disease (18). However, some KA patients may develop post-kala azar dermal leishmaniasis (PKADL) characterized by the formation of nonulcerative dermal lesions (4). The apparent change in the viscerotropic properties of *L. donovani* to dermatotropism is believed to be induced by the immunoregulatory mechanism in cured KA patients (1).

Humoral immune response in active KA is marked by the production of specific and nonspecific circulating antibodies (5, 11), although their protective role has yet to be established (20, 26). Antileishmanial antibodies are also found in the sera of patients with newly acquired PKADL and those with chronic PKADL with persistent skin lesions (12). The importance of cell-mediated immunity in KA and PKADL was documented in earlier in vivo studies with leishmanin (16, 19, 23). Recently, correlative in vitro studies have been reported with South American (7) and African (13) visceral leishmaniasis (VL) cases. However, limited information is available on the cell-mediated immune (CMI) status in Indian KA and PKADL.

In the present study, we attempted to define the CMI status of a limited number of Indian KA patients followed-up longitudinally during and after the period of treatment. Furthermore, alterations in their serum antileishmanial antibody titers were followed-up to evaluate the role of humoral antibodies in the modulation of CMI response. Additionally, CMI responses in some newly infected as well as chronic PKADL pa-

tients have been determined, and the results are discussed within the framework of our knowledge of the immunology of leishmaniasis.

MATERIALS AND METHODS

Patients. Sixteen KA patients were chosen for this study. They were admitted to the Medical College Hospital, Calcutta, for treatment. Diagnosis was made after careful clinical examination and was confirmed by the demonstration of amastigotes in bone marrow smears. All of these patients were suffering from fever for variable periods (2 to 6 months) and had enlarged spleens (6 to 11 cm below the costal margin) and livers (2 to 6 cm below the costal margin). Marked anemia (6.5 to 12.1 g% hemoglobin) and leukopenia (2,300 to 4,900 leukocytes per mm^3 of blood) were the characteristic features associated with the disease. Blood from patients with active disease were collected before the beginning of antileishmanial drug therapy. A second blood sample was collected several weeks after treatment. Some of these patients were followed-up as outpatients.

Blood samples were also collected from 12 PKADL patients attending the Dermatology Clinic of the School of Tropical Medicine, Calcutta, and the Calcutta Metropolitan Development Authority Polyclinic. Clinical diagnosis was confirmed by the demonstration of amastigotes in skin smears. Six of these patients had relatively newly developed PKADL as they had signs of the disease for a period ranging from a few months to about 1 year. The remaining six patients had chronic PKADL and had suffered from the disease for the last 8 to 30 years. Clinical features associated with both groups of patients were described earlier (12). Blood samples were also collected from apparently normal individuals (controls) living in Calcutta, a nonendemic area for KA.

Preparation of leishmania antigen. The parasite *L. donovani* was isolated from the bone marrow culture of a KA patient. The strain was cultivated in a liquid culture medium (21) to yield promastigotes which were subsequently used for the leishmanin skin test and for the preparation of soluble leishmania antigen. The protein content of the soluble antigen was determined by the method of Lowry et al. (15). Detailed methodology for the antigen preparation was described earlier (11). The same preparation of leishmania antigen was used in the lymphocyte transformation and antibody assay experiments.

Delayed hypersensitivity reaction in vivo. KA and PKADL patients were tested in vivo for the delayed type hypersensitivity (DTH) reaction to intradermal injection of leishmanin. For this test, 0.2 ml of leishmanin reagent (containing 10^8 *L. donovani* promastigotes per ml of 0.5% phenol-saline) was injected intradermally into the volar surface of the forearm of the patients, and their skin reactions were recorded after 48 h. An induration area of ≥5 mm in diameter indicated a positive reaction.

KA patients were also tested to determine their reactivity to intradermal injection of 0.1 ml (1 tuberculin unit) of purified protein derivative (PPD) of tuberculin (BCG Vaccine Laboratory, Madras, India). An induration area ≥10 mm in diameter was considered a positive reaction.

Lymphocyte transformation test in vitro. Peripheral blood was obtained from patients and normal controls by vein puncture and collected in heparinized glass tubes. Lymphocytes were isolated by the Ficoll-Hypaque density gradient centrifugation method (3). Cells were washed and finally suspended in RPMI 1640 with bicarbonate (Flow Laboratories) supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM L-glutamine, streptomycin (100 μg/ml), and penicillin (100 U/ml).

Lymphocytes (5×10^5 cells) were cultured in screw-capped siliconized tubes (Pyrex) in the presence of different doses (1, 10, and 50 μg) of leishmania antigen and 10% inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). Unstimulated cultures with fetal calf serum (but without antigen) were also run simultaneously. Lymphocyte cultures, in duplicate, were incubated at 37°C for 120 h, after which 1 μCi of [^{3}H]thymidine (specific activity, 52 Ci/mmol) (The Radiochemical Centre, Amersham, England) was added. Cells were incubated for another 24 h and harvested by the trichloroacetic acid precipitation and centrifugation method. Radioactive incorporation was measured in a liquid scintillation counter (Electronic Corp. of India Ltd.). The lymphocyte transformation index (LTI) was expressed as: counts per 100 s for stimulated culture/counts per 100 s for unstimulated culture.

Lymphocytes (5×10^5 cells) were also cultured in the presence of different doses (2.5 and 10 μg) of phytohemagglutinin (PHA) (Phytohemagglutinin P; Difco Laboratories, Detroit, Mich.) and inactivated AB serum (10%). Unstimulated culture contained AB serum but not PHA. Lymphocyte cultures, in duplicate, were incubated (37°C) for 48 h, followed by the addition of 1μCi of [^{3}H]thymidine. Cultures were incubated for another 24 h and harvested. Radioactivity was measured, and the results were expressed as described above.

Determination of antibody titers by the micro-enzyme-linked immunosorbent assay. Immunoglobulin G and immunoglobulin M class specific antileishmanial antibody titers in active and treated KA sera were determined by the micro-enzyme-linked immunosorbent assay method as described earlier (11).

Statistical analysis. Results were analyzed by Student's *t* test unless mentioned otherwise.

RESULTS

DTH skin reaction to leishmanin in KA. DTH skin reaction was found to be absent in all of the 16 active KA patients but was present in 7 out of 12 of the treated patients examined (induration diameter 5 to 15 mm). It should be mentioned here that KA patients usually showed positive reaction to leishmanin after 12 to 20 weeks of treatment. Patients skin tested before this period failed to show a positive reaction. Leishmanin skin tests were also carried out in 10 normal controls, all of whom showed negative reactions.

DTH skin reaction to PPD in active KA. Sixteen active KA patients were tested for their reactivity to intradermal injection of PPD. Of the 16, 12 (72%) demonstrated positive DTH reac-

tions (≥10-mm induration). Out of 48 normal controls tested, 32 (66%) showed positive reactivity to PPD.

Leishmania antigen-induced lymphocyte transformation in KA. Leishmania antigen-induced transformation experiments were carried out with the lymphocytes of nine KA patients during the active phase of infection and after treatment. Results obtained with each patient are presented in Table 1 along with those of normal controls. Spleen sizes and circulating antibody titers of the respective patients, determined before and after treatment, are also presented for comparison. Lymphocytes of active KA patients failed to be stimulated when cultured in the presence of leishmania antigen. On the other hand, lymphocytes of a majority of these patients could be stimulated to various extents by the same antigen after treatment with antileishmanial drug therapy. Positive transformation (LTI, >2) were usually obtained after 12 to 20 weeks of treatment. This correlated well ($P < 0.05$, as determined by product-moment correlation) with the reduction of their spleen sizes. Furthermore, treated patients also showed considerable reduction of their antibody titers (particularly of immunoglobulin G class) during this period. The lymphoproliferative response in vitro in treated patients showed reasonably good agreement with the results of the leishmanin skin test in vivo, although no significant correlation ($P > 0.05$, as determined by Spearman's rank correlation) between the magnitudes of these responses could be noted. On statistical analysis, the treated KA group showed significantly higher ($0.01 < P < 0.05$) sensitization to leishmania antigen compared with those of active KA and normal controls. No significant difference ($P > 0.05$), however, could be noted between all these groups with respect to the radioactive counts incorporated in their unstimulated lymphocyte cultures.

PHA-induced lymphocyte transformation in KA. Results of the PHA-induced lymphocyte transformation experiments with active and treated KA patients as well as normal controls

TABLE 1. Results of leishmania antigen-induced transformation experiments with lymphocytes from active and treated KA patients and normal controls

Patient no.	Age (yr)/ sex	Period[a] (wk)	Spleen size (cm)[b]	Radioactivity of unstimulated culture (counts/100 s)	LTI of stimulated culture at the following dose of leishmania antigen (μg):			Antibody titer[c] (reciprocal)	
					1	10	50	IgG[d]	IgM
1	10/F	Active	9	310	0.36	0.69	0.62	3,200	100
		Treated (20)	2	233	6.4	16.0	27.3	800	100
2	10/M	Active	6	306	1.01	0.84	1.1	3,200	200
		Treated (3)	2	192	3.6	2.0	1.2	3,200	200
3	25/M	Active	7	146	0.84	0.87	0.84	3,200	400
		Treated (11)	3	424	1.0	1.0	1.0	1,600	400
4	30/M	Active	6	206	0.59	1.27	ND[e]	1,600	100
		Treated (4)	3	435	1.6	1.5	ND	400	50
5	45/M	Active	6	397	0.31	0.61	ND	12,800	800
		Treated (19)	1	231	6.3	19.3	ND	3,200	400
6	22/F	Active	8	380	0.42	0.46	1.5	6,400	400
		Treated (15)	3	262	1.7	1.3	5.0	800	200
7	8/M	Active	11	287	1.06	0.54	ND	1,600	400
		Treated (17)	1	199	13.3	35.3	ND	200	200
8	28/F	Active	8	235	0.86	0.66	0.80	6,400	200
		Treated (15)	2	319	1.7	12.0	1.2	800	100
9	12/M	Active	10	190	1.28	1.92	ND	1,600	400
		Treated (18)	2	292	3.5	7.0	ND	400	200
Mean		Active		273.0 ± 28.5[f]	0.75 ± 0.11	0.87 ± 0.15	0.97 ± 0.15		
		Treated		287.4 ± 30.1[f]	4.30 ± 1.3[g]	10.60 ± 3.8[g]	7.14 ± 5.1		
Normal controls ($n = 9$)				303.8 ± 66.7[f]	1.25 ± 0.18	1.25 ± 0.17	1.14 ± 0.12		

[a] Figure in the parenthesis denotes weeks elapsed since the active phase (zero week) sample collection.
[b] Measured below the costal margin in midclavicular line.
[c] Determined by micro-enzyme-linked immunosorbent assay technique.
[d] IgG, Immunoglobulin G.
[e] ND, Not determined.
[f] Standard error of the mean.
[g] Significant ($0.01 < P < 0.05$) when compared with active KA or normal control groups.

TABLE 2. LTIs of active KA, treated KA, PKADL, and normal controls in presence of PHA

Group	No. studied	LTI (mean ± SEM) with:	
		2.5 μg of PHA	10 μg of PHA
Active KA	16	16.8 ± 4.8[a]	32.9 ± 8.3[a]
Treated KA	11	30.7 ± 8.9[b]	56.6 ± 15.8[b]
PKADL	10	26.2 ± 9.3	
Normal controls	16	29.8 ± 5.1	50.5 ± 5.9

[a] Not significant ($P > 0.05$) when compared with treated KA, PKADL, or normal control groups.
[b] Not significant ($P > 0.05$) when compared with the same 11 patients with active KA by the paired t-test.

are summarized in Table 2. Lymphocytes from subjects belonging to different groups exhibited wide variations in their responsiveness to PHA, although all of them (except two active KA cases) showed positive transformations. It is evident (Table 2) that the mean LTIs of active KA patients were definitely lower than those of treated patients and normal controls at both the PHA doses, although these differences were not statistically significant ($P > 0.05$). The same was true when the results, obtained with the same 11 active and treated KA patients, were analyzed statistically by the paired t-test.

CMI response in PKADL patients to leishmania antigen. CMI response in 12 PKADL (6 newly developed and 6 chronic) patients to leishmania antigen was measured by in vitro and in vivo experiments, and the results are presented in Table 3. It is apparent that eight (five newly developed and three chronic) of the patients showed positive lymphocyte transformation (LTI, >2). Optimum stimulation could be obtained with 10 μg of antigen in most of these experiments. Lymphocytes of recently developed PKADL patients were better stimulated by leishmania antigen than those of chronic patients. Interestingly, three chronic patients, whose lymphocytes were not stimulated in the presence of antigen, remained unresponsive to the antigen even after prolonged antimony therapy.

Leishmanin skin tests were performed with nine (four recently infected and five chronic) PKADL patients, and six (three from each group) of them exhibited positive DTH reactions (Table 3). Patients showing positive reactivity to leishmanin in vivo also showed definite sensitization to leishmania antigen in vitro, and Spearman's rank correlation between the two was statistically significant ($0.01 < P < 0.05$).

PHA-induced lymphocyte transformation in PKADL. Lymphocytes of 10 (6 recently developed and 4 chronic) PKADL patients were tested in vitro for their responsiveness to a 2.5-μg dose of PHA. The results showed that lymphocytes from all of these patients could be stimulated normally by PHA. Mean LTIs of the PKADL group were quite comparable ($P > 0.05$) to those of normal controls (Table 2).

DISCUSSION

The results presented in this study clearly demonstrate an impairment of CMI in active KA. Moreover, this "anergy" of the CMI component appeared to be more specific to leish-

TABLE 3. Results of CMI response in PKADL patients to leishmania antigen

Patient group and no.	Age (yr)/ sex	DTH reaction to leishmanin (mm)	Radioactivity of unstimulated culture (counts/100 s)	LTI of stimulated culture at following dose of leishmania antigen (μg):		
				1	10	50
Recently acquired						
1	20/M	10	379	9.3	9.5	5.0
2	30/M	ND[a]	300	1.9	1.5	1.3
3	8/F	6	171	2.8	5.5	5.6
4	18/M	<5	488	4.5	7.4	1.0
5	23/M	10	240	12.2	15.4	1.3
6	20/M	ND	100	1.7	3.6	2.5
Chronic						
7	46/M	7	304	3.5	3.2	ND
8	40/F	ND	148	1.4	1.0	1.1
9	55/M	<5	473	0.88	1.0	1.0
10	48/M	7	300	14.2	24.5	2.9
11	52/M	<5	382	0.82	0.81	1.0
12	50/M	6	324	2.6	4.3	2.2
Mean			300.8 ± 35.1	4.65 ± 1.33[b]	6.5 ± 2.1[b]	2.26 ± 0.49[b]

[a] ND, Not determined.
[b] Significant ($0.01 < P < 0.05$) when compared with normal controls (Table 1).

mania (Table 1) than generalized in nature as suggested by the results of the DTH skin reaction to PPD and PHA-induced lymphocyte transformation experiments (Table 2). The latter result is somewhat in contrast to those reported in an earlier study (10) in which a definite impairment in the PHA-induced lymphocyte transformation ability of KA patients was noted. In the earlier study, blood samples were collected from patients in the endemic areas of North Bihar, India, and there was a time difference of about 10 to 12 h between the collection, transportation, and processing of the sample for use in the experiment. Ficoll-Hypaque-separated lymphocytes, were found to contain some granulocytes which might have been responsible for the observed impairment. In the present study, blood samples were processed for the experiment within ca. 1 h of collection, and granulocyte contamination was negligible.

The degree and nature of immunosuppression in active VL cases is yet to be established unambiguously. Levy and Mendes (14) and recently, Ho et al. (13) in their studies of VL cases in South America and Africa, respectively, demonstrated profound immunosuppression which was specific to leishmania antigen as well as generalized in nature. In a few Indian KA cases, Aikat et al. (2) noted an absence of DTH response to unrelated antigens, although no data were available with respect to leishmanin. Significant depletion in the number of circulating T-cells (9, 22) and small lymphocytes in the thymus-dependent regions of the spleen and lymph nodes (28) in KA were, in fact, indicative of generalized immunosuppression. In contrast, Wyler et al. (30) demonstrated a positive lymphoproliferative response in vitro to both specific and nonspecific antigens in one patient with active Mediterranean VL. However, the patient showed a negative DTH reaction to leishmanin in the active phase but not when tested after 1 year of treatment. In another study Carvalho et al. (7) noted the lack of leishmania antigen-specific CMI responsiveness in patients with active South American VL. Data presented in our study indicate that the T-cell responsiveness in a majority of the Indian KA patients was not abrogated against PHA and antigens unrelated to leishmania.

The involvement of some humoral factors in immunosuppression in active KA was recently observed in one such patient by Wyler (29). Alternatively, it is possible that *L. donovani* infection in humans stimulates the production of suppressor T-cells for effective suppression of the CMI to leishmania antigen. On the other hand, these cells may not interfere with the generation and activity of helper T-cells which cooperate with the B-cells leading to the production of a high level of antibodies. That the suppressor and helper T-cells might differ in their functional specificity repertoires has been suggested earlier (24).

Carvalho et al. (7) observed that positive lymphocyte responsiveness to antigen was restored in patients with American VL after 2 to 4 weeks of successful chemotherapy. On the other hand, Ho et al. (13) noted that a majority of the treated KA patients in Africa acquired leishmanin positivity within 3 to 12 months. Our data clearly show that CMI response to leishmania antigen was expressed in a majority of the KA patients after 12 to 20 weeks of treatment. Interestingly, restoration of positive CMI response was shown to occur in the parallel with the decrease in spleen size and levels of both specific (Table 1) and nonspecific (J. P. Halder, Ph.D. thesis, University of Calcutta, India, 1982) antibodies in KA patients. Thus, antileishmanial drug therapy aided in parasite destruction, resulting in the reduction of antigenic load with a concomitant decrease in serum antibody levels and subsequent restoration of specific T-cell responsiveness.

DTH reactivity to leishmanin failed to appear in a number of cured KA patients. Similar results were obtained earlier by other workers (19). Again, one-third of the PKADL patients failed to demonstrate positivity in their T-cell responsiveness to leishmania antigen. None of the PKADL patients, however, showed any signs of the viscerotropic form of the disease and were, therefore, immune to KA. Conversely, antigen-specific T-cell responsiveness did not protect a PKADL patient from the dermatotropic form of the disease, likely to be caused by the same parasite. Although cross-reactions between different leishmania species were noted with respect to DTH testing, these could not be correlated with cross-protection (16, 18). Such observations are difficult to interpret as DTH reactivity, once acquired, may persist for a prolonged period (16). The persistence of DTH reaction was also noted in this study in patients cured of KA for many years. Interestingly, one of the KA patients suffered a relapse after about 1 year of apparent recovery, although he had already developed a positive T-cell responsiveness to leishmania antigen. Unfortunately, no information was available regarding the CMI status of this patient at the time of relapse because he was admitted and treated elsewhere. All of the above observations suggest that the protection afforded by specific CMI response (demonstrable by in vitro or in vivo tests) against *L. donovani* infection may not be absolute and probably depends on other host-related factors leading to parasite destruction and patient recovery.

All of the KA patients studied here responded to antileishmanial drug therapy, although individual variations were noted in the degree of their responsiveness to treatment. Interestingly, two of the PKADL patients failed to give a definite history of being treated for KA earlier. It is possible that these patients were spontaneously cured of a weak primary infection (18) and became immune to the viscerotropic, but not the dermatotropic, form of the disease (8). Subclinical exposure to *L. donovani*, and subsequent development of immunity could be demonstrated in persons living in KA endemic areas (6, 19, 25). Reports are already in existence to show that certain individuals may carry a cryptic infection of *L. donovani* without any definite clinical symptoms (17, 27). This information, along with the present results, suggest some variations within humans with respect to susceptibility to *L. donovani* infection, pathogenesis, and subsequent recovery.

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