Exploitation of Parasite Derived Antigen in Therapeutic Success of Human Cutaneous Leishmaniasis in Brazil

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In a complete study in 25 patients with American cutaneous leishmaniasis, caused by Leishmania braziliensis *complex, immunotherapeutic efficacy of parasite derived antigen (94-67 KD) has been compared to antimonial therapy. Additionally, to delineate the mechanism of therapeutic success, microscopical features of immune response in active lesions and healed or non-healed lesions following therapy were analyzed. The results showed that cure rates in immunotherapy and chemotherapy were equal (>83%). The immunohistochemical changes in two therapeutic groups were also largely similar. The analysis of humoral and cellular immune response suggest that appropriate stimulation of T helper cells in the lesion site, in association with one or more cytokines, play a key role in the healing process.*

Key words: human cutaneous leishmaniasis - Brazil - chemotherapy - immunotherapy

Treatment of American cutaneous leishmaniasis (ACL) with pentavalent antimonials, which has been in practice for more than six decades, presents many difficulties (Oster et al. 1985, Chulay et al. 1985). Thus, there is a need for a well tolerated and effective adjunct, or substitute for antimony compounds, in the treatment of ACL.

Encouraging results are now accumulating in treating ACL effectively with a combination of heat killed Leishmania promastigotes and viable bacille Calmette Guérin (BCG) (Convit et al. 1987, 1989). We have also reported recently a successful immunotherapy in human cutaneous leishmaniasis (caused by *Leishmania major* or *Leishmania tropica*) using Leishmania promastigotes derived fractions (Monjour et al. 1991). All the observations indicate that immunological phenomena are critical in the pathogenesis and healing process of human cutaneous leishmaniasis.

Thus, in the present study, the therapeutic potential of *L. b. braziliensis* promastigote derived fraction 2 (LbbF2, 94-67 kD), has been compared to conventional chemotherapy to bring about a cure in ACL. To delineate the mechanism of therapeutic success, microscopical features of immune response in active lesions and healed or non-healed lesions following therapy were analyzed.

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MATERIALS AND METHODS

Patients and study design - Twenty-five ACL patients with typical localized and ulcerative cutaneous lesion (>4 months old) were from an endemic area in the State of Pernambuco, Brazil. Patients were randomly selected and were excluded if they had any acute or chronic disease, if they were pregnant, or if they had received antimonial medication during previous four months. An informed consent was obtained from all patients or their guardians and study was approved by the Ethical Committe of Universidade Federal de Pernambuco, Recife, PE, Brazil. All clinical and therapeutical studies were conducted by two dermatologists (Departamento de Dermatologia, Universidade Federal de Pernambuco, Recife). Parasitological confirmation of clinical diagnosis was based on visualization of parasites in Giemsa-stained smears prepared with the aspirated material from the border of skin lesion, and in tissue imprints from the biopsy. Number of lesions with their location were described; mean size of the lesion was noted by assessing maximum and minimum diameters.

Patients were divided in two groups and subjected to either chemotherapy or immunotherapy. Evolution of macroscopical features of lesion along with the treatment was used as yardstick to

assess the clinical improvement. Lesion size was recorded after completion of therapy. Patients were considered cured when ulcerated lesions healed completely leaving no signs of desquamation on healed surface or borders; absence of parasites was confirmed by histological examination of hematoxylin and eosin stained biopsy section taken from healed area of the skin. The observation period of individual patients varied between one to six months after starting of treatment. Serum antibody response, and microscopical features of CMI were studied before treatment and after cure (four months after treatment in non-healed cases).

Chemotherapy - The patients in the chemotherapy group received 50 mg of Glucantime (Rhodia SA Brazil) per kg body weight in a course of 20 daily intramuscular injections, with a minimum one and maximum three courses at two week intervals between successive courses.

Immunotherapy - The immunotherapeutic agent LbbF2 was obtained by the same method in principle as described previously (Monjour et al. 1988). Briefly, 2 x 10^9 *L.b.braziliensis* promastigotes, harvested at stationary phase after 7-10 days culture in RPMI 1640 (supplemented with 15% heat inactivated fetal calf serum, 1% glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin), were washed in cold phosphate buffered saline (pH 7.4). Parasites were lysed in 2 ml buffer containing 0.5% Nonidet P 40 (v/v), 1% Sodium dodecyl sulfate (w/v),100 IU/ml aprotinin and 2mM phenylmethyl sulphonyl fluoride, pH 7.8. The extract was sonicated on ice, three times for 30 seconds and the soluble lysate electrophoresed under reducing conditions on SDS-PAGE gels along with molecular weight marker run in parallel (Laemmli 1970). The gel portion containing protein of apparent molecular weight of 94-67 kD was electroeluted, dialysed at 2°C against distilled water and immediately lyophilized. Removal of sodium dodecyl sulfate from the preparation was achieved by column chromatography using ion-retardation resin AG 11A8 (Bio-Rad SA France) as described by Kapp and Vinogradov (1987). Finally, protein concentration of the preparation (designated as LbbF2) was measured with BCA reagent (Pierce chemical company, Rockford, USA).

Patients in the immunotherapy group received LbbF2 (without adjuvant) intradermally in the deltoid region with a minimum one and maximum three doses at one month apart. Each dose comprised 50 µg (protein equivalent) of LbbF2 in 0.25 ml of physiological saline.

Assay for antileishmanial antibodies - Measurements of serum antibody levels were performed by indirect immunofluorescence (IIF) with acetone-fixed *L.b.braziliensis* promastigotes as described elsewhere (Monjour et al. 1978).

Immunohistochemical studies - In practice, 4 mm punch biopsy samples of skin were obtained and placed in a cryotube (Nunc Inc. USA) embedded in optimal-cutting-temperature compound and kept in liquid nitrogen till use.

Cryostat sections (6-8 µm) of biopsy material on gelatin coated slides were dried overnight at 37°C, rehydrated in tris-buffered saline (TBS), and incubated with a panel of mouse anti-human monoclonal antibodies followed by incubation with biotinylated horse anti-mouse Ig and then avidin-biotin-peroxidase complexes according to the instructions given in Vectastin ABC kit (Vector laboratories, Burlingame, USA). The reaction product was developed with 3,3' diaminobenzidine (Sigma, USA) at 0.5mg/ml and 0.01% H_2O_2. Sections were counterstained with hematoxylin. Sources of mouse anti-human monoclonal antibodies used to identify cell surface phenotypes in biopsy sections were: "DAKO-T4" for CD4, "DAKO-T8" for CD8 (Dakopatts, Hamburg, FRG); "IOT14" for CD25 (anti-interleukin-2 receptor) and "IOM2" for CD14 (Immunotech SA Marseille, France). For revelation of HLA-DR group of antigen on stained cells (CD14), we performed double staining. Prior to counterstaining, the slides were incubated for 30 min at 37°C with FITC conjugated mouse anti-human HLA-DR, "IOT2a" (Immunotech S.A. Marseille, France) at 1:50. After washing in TBS, the section was mounted in glycine.

Statistical analysis - Results are given as mean + SD. Data were analyzed by unpaired Student's t test and, where necessary, by Chi square Fisher's exact test.

RESULTS

There were 25 patients, 13 under chemotherapy group (Table I) and 12 under immunotherapy group (Table II), having single or multiple lesions, who completed the study. Parasites isolated from lesions of each patient corresponded to *L.b.braziliensis* complex (MHOM/BR/91-LEM 2382).

Clinical and parasitological evolution of lesions - In response to therapy, the cure rates were same 84.6% for chemotherapy and 83.33% for immunotherapy; the difference is insignificant ($P>0.90$, Chi square Fisher's exact test). Skin biopsy of healed lesion in each case was found free of demonstrable parasites. Failure of treatment, as determined clinically and parasitologically four months after completion of therapy, was moderate, being recorded in two of 13 (15.38%) receiving chemotherapy and two of 12 (16.66%) receiving immunotherapy.

Quantity of therapeutic agents was varied with individual patients in both groups (Tables I, II) and determined by periodic assessment of clinical improvement of the lesion. For therapeutic success, the patients with chemotherapy

TABLE I

Clinical and parasitological evaluation in ACL in response to chemotherapy

Patient no.	Age, sex	No. of lesions, sites	Lesion size[a] cm	No. of courses of glucantime	Lesion size 1 mth post-treatment cm	Clinical evaluation		Parasite in skin biopsy[b]
						Clinical outcome	Months after treatment	
1	35, F	1, Leg	1.9	3	1.1	Healed	1	-
2	27, M	1, Leg	4.7	3	3.7	Not healed	4[c]	+
3	12, M	1, Leg	2.5	2	2.0	Healed	1	-
4	15, F	1, Knee	4.0	1	3.4	Healed	1.5	-
5	52, M	1, Neck	4.7	3	4.0	Not healed	4[c]	+
6	42, F	1, Leg	1.5	1	1.0	Healed	1	-
7	12, M	1, Leg	3.7	2	3.0	Healed	1	-
8	28, M	1, Face	4.5	2	3.5	Healed	2	-
9	7, F	2, Nose	1.7	2	1.3	Healed	1	-
10	4, F	1, Finger	1.6	2	1.2	Healed	1	-
11	10, M	2, Arms	1.5	2	0.9	Healed	1	-
12	13, F	2, Wrist	3.1	2	2.3	Healed	1	-
13	10, F	7, Arms, legs, ear	1.0	1	0.8	Healed	1	-

[a]: lesions were ulcerative in nature. Size represents mean of maximum and minimum diameters of single lesion. In case of multiple lesions, median size was considered for calculation. Chemotherapeutic protocol is described under Methods.
[b]: - absent; + present
[c]: persistence of lesions four months after completion of treatment was considered failure of treatment.

TABLE II

Clinical and parasitological evaluation in ACL in response to immunotherapy

Patient no.	Age, sex	No. of lesions, sites	Lesion, size[a] cm	No. of doses of LbbF2	Lesion size 1 mth post-treatment cm	Clinical evaluation		Parasite in skin biopsy[b]
						Clinical outcome	Months after treatment	
1	18, M	1, Back	1.6	3	1.5	Healed	2.5	-
2	45,M	8, Arms	0.9	1	0.6	Healed	1.5	-
3	30, M	1, Leg	1.5	3	1.7	Healed	2	-
4	21, M	1, Leg	2.8	3	5.0	Not healed	4[c]	+
5	31, M	9, Arms, legs neck, ear	1.0	2	0.5	Healed	2	-
6	35, M	7, Arms, legs	4.8	1	4.0	Healed	2.5	-
7	27, M	1, Leg	4.0	3	5.0	Not healed	4[c]	+
8	21, M	1, Leg	3.5	2	4.0	Healed	2.5	-
9	41, F	1, Arm	1.5	1	1.3	Healed	1.5	-
10	33, M	1, Arm	4.7	3	3.0	Healed	3	-
11	30, M	1, Arm	6.0	3	5.7	Healed	3	-
12	25, M	1, Face	5.5	3	5.0	Healed	3	-

[a]: lesions were ulcerative in nature. Size represents mean of maximum and minimum diameters. In case of multiple lesions, median size was considered for calculation. LbbF2 = *Leishmania b. braziliensis* fraction (94-67 kD), the immunotherapic agent. Therapeutic protocol is described under Methods.
[b]: absent; + present.
[c]: persistence of lesions four months after completion of treatment was considered failure of treatment.

TABLE III

Immunohistochemical findings in ACL in response to chemotherapy and immunotherapy

		% of cells stained in skin biopsy[a]				
		Pre-treatment (n=25)	Post-treatment			
			Healed		Non-healed	
Cell phenotype in dermis	Antigen		Chemotherapy (n=11)	Immunotherapy (n=10)	Chemotherapy (n=2)	Immunotherapy (n=2)
T Lymphocytes	CD4	15 ± 5	49 ± 9[b]	45 ± 7[b]	17 ± 4	20 ± 3
	CD8	35 ± 5	17 ± 3[b]	20 ± 2[b]	40 ± 7	38 ± 2
Macrophages	CD14	43 ± 4	31 ± 3[b]	35 ± 5[b]	44 ± 6	40 ± 7
	HLA-DR	5 ± 2[c]	12 ± 2[b,c]	15 ± 3[b]	13 ± 2[c]	9 ± 1
Cells with IL-2 receptor	CD25	0	0	6 ± 2	0	1 ± 0.7

[a]: data are mean + SD
[b]: significant difference from pre-treatment value (P<0.01)
[c]: difference between pre-treatment and post-treatment chemotherapy group, values is significant (P<0.01 student's test)

received average 36.3 doses (range 20-60) of glucantime whereas those with immunotherapy received 2.2 doses in average (range 1-3) of LbbF2. It is noteworthy that patients with multiple lesion (>2) were healed with less quantity of therapeutic agents. Among three such patients in immunotherapy group, two required only one dose of LbbF2 and other one required two doses; the sole patient with seven lesions in chemotherapy group required one course of glucantime. The average time taken for healing the lesion with chemotherapy and immunotherapy was 2.81 and 4.45 months respectively after starting of treatment. Only patients in the chemotherapy group reported slight side effects (bone and muscle pain and fever).

Antibody formation - All sera of active ACL patients in both therapeutic groups reacted with *L.b.braziliensis* promastigotes (IIF titer range 100-400) and antibody level remained unchanged even after cure (data not shown).

Immunohistochemistry - Cellular phenotypes in the dermis of active lesions before treatment and healed or non-healed lesions after treatment are shown in Table III.

The infiltration of cellular phenotypes in lesions cured by chemotherapy or immunotherapy was found largely similar. In active lesions, $CD8^+$ lymphocytes predominated; $CD4^+$ cells were in the minority and scattered among the $CD8^+$ cells. In healed lesions, $CD8^+$ cells were in minority and a prominent influx of $CD4^+$ phenotypes was evident. The quantitative change in CD4/CD8 positive cells between active and healed lesions was always significant (P<0.01). $CD\ 14^+$ staining on dermal macrophages decreased significantly (P< 0.01) after healing of the lesions, but HLA-DR expression on these cells was increased (P< 0.01). The expression of light chain of the IL-2 receptor (Tac) on lymphocytes was not encountered in active lesions. After treatment, expression of IL-2 receptor was found on a moderate number of lymphoid cells (6 +2) in healed lesions which received immunotherapy but not chemotherapy. Some cells (1+0.7) expressing IL-2 receptor were also found in non-healed lesions receiving immunotherapy.

DISCUSSION

The results indicate that in localized ACL immunotherapy, with *L.b. braziliensis* promastigote derived antigen, is effective. Although average treatment time and healing time in our immunotherapy were markedly longer than those in chemotherapy, clinical improvement was clear and easily distinguishable among different patients. As would be expected with immunotherapy, the lesions might get bigger before the immune reaction took over. Indeed, whereas none of the chemotherapy group's lesions enlarged, four of the immunotherapy group's did.

We were able to decide the number of doses required for healing the lesions; some patients being cured with a single dose and others requiring two or three, and interestingly, patients with multiple lesions were healed with comparatively less quantity of therapeutic agents than those with single lesion. It is unlikely that healing of cases

observed in the present study was spontaneous. Cutaneous leishmaniasis from endemic area in the State of Pernambuco, never cures spontaneously; moreover, before starting of treatment, skin biopsy samples of active lesion did not show any histological indication towards self-healing. As cure rates in the two therapeutic groups are equal, immunotherapy could be a good alternative to conventional chemotherapy in localized ACL.

The mechanism of therapeutic success following therapy was analyzed. Level of antileishmania antibodies, as observed in our study, does not suggest that humoral immune response participates actively in the host parasite interaction to influence the clinical events and cure of the disease.

At mononuclear cell level of active lesions there was a predominance of $CD8^+$ lymphocytes over $CD4^+$ cells. The relationship of CD4-CD8 cell population becomes inverse when the lesions are healed following therapy; whereas same pretreatment relationship is maintained in non-healed but treated lesions which seem to develop a chronicity. Intralesional multiplication of amastigotes seems to be associated with excess suppressor phenotypes at tissue level, and parasites are ultimately eliminated when helper/inducer phenotypes predominate.

In the present study, increased population of CD14 positive macrophage in the dermis of active lesion and their decrease after healing were recorded. Yet, infiltration of histocytes/macrophages has also been found to favour the proliferation of parasites at the site of leishmania infection (Gutierrez et al. 1991). It is noteworthy that HLA-DR expression on macrophage at tissue level in our cases was significantly increased along with the healing. Since the only known inducer of HLA-DR on macrophages is IFN-γ (Unanue & Allen 1987) we suspect a localized secretion of IFN-γ by activated lymphocytes during the healing process.

The immunohistochemical changes in the two therapeutic groups were found largely similar, so how the *Leishmania* derived fraction antigen brought about a healing? It seems that specific antigen strongly induces the stimulatory activity of helper T cell subset to the appropriate level to carry out activation of macrophage and thereby elimination of parasites to achieve the therapeutic success. This is further supported by the fact that IL-2 receptor cells (Tac) was increased pronouncedly in healed lesion of immunotherapy group. Because the expression of the IL-2 receptor is dependent on the availability of IFN-γ and IL-2 such phenomenon indicates the interaction of cells and cytokines involved in the development of effective CMI response at tissue level of human cutaneous leishmaniasis.

The analysis presented here provides an insight into the specific requirements for protective immunity against leishmanial infection. Further, therapeutic efficacy of our promastigote derived antigen reemphasize that immunotherapy could turn out to be useful device in the treatment of leishmanial disease.

REFERENCES

Chulay JD, Spencer HC, Mugambi M 1985. Electrocardiographic changes during treatment of leishmaniasis with pentavalent antimony (Sodium stibogluconate). *Am J Trop Med Hyg 34:* 702-709.

Convit J, Castellanos PL, Rondon A, Pinardi ME, Ulrich M, Castes M, Bloom B, Garcia L 1987. Immunotherapy versus chemotherapy in localized cutaneous leishmaniasis. *Lancet 1:* 401-405.

Convit J, Castellanos PL,Ulrich M, Castes M, Rondon A, Pinardi ME, Rodriquez N, Bloom BR, Bretana A 1989. Immunotherapy of localized, intermediate,and diffused forms of American cutaneous leishmaniasis. *J. Inf Dis 160:* 104-114.

Gutierrez Y, Salinas GH, Palma G, Valderrama LB, Santrich CV, Saraiva NG 1991. Correlation between histopathology, immune response, clinical presentation, and evolution in *Leishmania braziliensis* infection. *Am J Trop Med Hyg 45:* 281-289.

Kapp OH, Vinogradov SN 1987. Removal of sodium dodecyl sulfate from proteins. *Anal Biochem 91:* 230-235.

Laemmli UK 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (London) 227:* 680-685.

Monjour L, Mille C, Druilhe P, Gentilini M 1978. Application de l'immuno-électro-diffusion sur membrane d'acétate de cellulose au diagnostic de la leishmaniose viscérale humaine et canine. *Ann Soc Belge Méd Trop 58:* 293-300.

Monjour L, Vouldoukis I, Ogunkolade BW, Hetzel C, Ichen M, Frommel D 1988. Vaccination and treatment trials against murine leishmaniasis with semi-purified Leishmania antigens. *Trans R Soc Trop Med Hyg 82:* 412-415.

Monjour L, Mansouri P, Cubas AC, Rolland-Burger L, Barzegari M, Dowlati Y 1991. Immunotherapy as treatment of cutaneous leishmaniasis. *J Inf Dis 164:* 1244-1245.

Oster CN, Chulay JD, Hendricks LD, Pamplin CL, Ballou WR Berman JD, Takafuji ET, Tramont EC, Canfield CJ 1985. American cutaneous leishmaniasis: a comparison of three sodium stibogluconate treatment schedules. *Am J Trop Med Hyg 34:* 856-860.

Unanue ER, Allen PM 1987. The basis for the immunoregulatory role of macrophage and other accessory cells. *Science 236:* 551-557.