Cell Mediated and Humoral Immune responses to parasites in patients with Cutaneous lesions caused by *L. donovani senso lato* in Lebanon

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INTRODUCTION

Dermal lesions caused by members of the *Leishmania donovani* complex are not a frequent d

especially in the eastern Mediterranean region. These parasites are more viscerotropic, in t

of the world, , causing lethal infantile Kala-Azar, and a less severe systemic infection in adult

latter cases they cause variable degrees of immune suppression which in some individuals agg

illnesses considered usually not grave or terminal. Furthermore the cutaneous infection is

countries forming the Mediterranean basin, caused by members of the *Leishmania tropica* an

complexes.

immune reactions of the host to the classically recognized Old World strains of this par

ther the viscerotropic or dermotropic strains, have been exhaustively studied. In short in

of infection, systemic (by *infantum and/ donnavani senso lato*) or dermal (by *major* or *tro*

arms of the immune system may be evoked, although the humoral is certainly more consis

uced than the cell mediated one. Effectively antibodies to parasite antigens form in infectio

member of either the dermotropic or viscerotropic strains of *Leishmania* .

Since the cutaneous form is self healing (with no treatment), typically the humoral respo short lasting and in moderate titers (1-6). On the other hand cell mediated immunity (the de type response) develops within 2-3 months from the appearance of the skin lesion and several years thereafter.

In the visceral form, and, even if the patient is under treatment, the parasite number c *donnovani* family rises acutely in blood after the onset of disease, remain elevated for some and then drop gradually thereafter to disappear almost totally about a year or so after the of disease (7-10).

In this type of infection delayed type hypersensitivity, if it forms, takes much longer to appe 10).

These observations were consistent enough for different researchers to use them as diagnost tools (11-13) and for tracking these disorders epidemiologically (14-21). The antigens used we from different parasite preparations and extracts (27-33) and they all proved adequate.

On the other hand *L. donovani* causing a cutaneous lesion is certainly not a common finding.

In this study to better understand the way the immune system reacts to attacks by this spe
strain and in the absence of overt visceral invasion, we decided to study the expressio
mmunity in both its forms in a number of these patients with concurrent clinical disease.

For detection of antibodies we used two types of antigen preparations: Whole parasite lysates
reference strain IPTI (30), and rk39, a highly conserved sequence found in both the Old and
World parasite species all of which cause visceral leishmaniasis (*L. donovani*, *L. infantum* an
***chagasi*).**

The presence of elevated levels of antibodies to both type antigens was confirmed in 7/18 studied patients. In addition two more sera reacted with only one of the two antigens use determine delayed hypersensitivity we used an extract from one of our isolates. The identity used isolate had been revealed in a previous study by enzyme electrophoresis (34). We con the identity of our stabilates by analyzing them electrophoretically. This method in addi molecular identification techniques were used on different isolates obtained from the p under study. Probes for sequences coding for K39 gene yielded the final proof.

We concluded that the immune system in patients with dermal leishman caused by a strain of parasite known to be viscerotropic responds in line with disorder it normally causes in the host i.e.kala-azar. To reach a be understanding of the pathophysiology of these parasites we recommend tha similar cases the human host immune reactions always be determined.

TERIALS AND METHODS

nts and study groups.

his study was performed on 22 subjects, 18 patients with skin lesions, and 4 with no apparent sease.

he patients were referred to our laboratory from the American University of Beirut Medical C hey had lesions typical of cutaneous leishmaniasis. They were confirmed by histologic examina skin biopsies stained with a modified Romanovsky (Wright-Giemsa) stain (35). In order to is d characterize the parasites, a portion of the lesion biopsy was inoculated for culture on a mo NN medium (36), supplemented with a 15-30% heat inactivated fetal bovine serum (HIFBS) a tibiotics (GIBCO laboratories, Grand Island, USA).

arasites from only eight of the patients (group A), were isolated and successfully propagated in

There were no isolates from the other 10 cases (group B). To verify whether this peculiar dermotropic strain exhibits clinically distinguishing features, we devised a questionnaire to record the detailed biographic data on each patient.

Subject 19 had recovered about two months before she entered the study from a disorder suspected to have been visceral leishmaniasis. She had been bed ridden in a community hospital for close to three months at the end of which she returned home undiagnosed. Her symptoms had mildly improved. She complained from the outset of low-grade fever and hepato-splenomegaly (documented by history physical exam and ultrasound).

Finally we included in this study three subjects (20-22) who were symptom free and had a negative past history. They had a strongly positive leishmanin Skin Test (ST) defined by an induration of 1.2-1.4 cm in diameter at 48 hours using antigens obtained from one of our isolates (supplied by Corixa inc. Seatle Washington State).

ologic analysis. The sera from the 22 subjects were found devoid of anti *Leishmania major* ant

en screened by ELISA. Two preparations of *Lmajor* antigens were tested namely a lysate wh

arated, and soluble proteins fractionated from the lysate.

the present study anti-*L.donovani* antibodies were detected by micro-ELISA technique (2

d for antigens whole lysate of *L. donovani* parasites and rk39. As mentioned earlier, rk3

ombinant polypeptide. It represents the protein product of a high repeat genomic segment typ

***donovani* strain of parasite. Qu J.Q. *et al*. (31) have demonstrated that it is both a sensiti**

cific test with no cross-reactivity with antibodies to antigens present in leishmaniases cases

strains that belong to other parasite complexes. This part of the study was carried out at

oratories (Seattle, WA, USA).

- **The procedure was described in a previous work. In short, the plates were coated overni**

  4ºC with 10ng lysate protein/well and in other plates 25 ng of rk39/well, in coating buffe

  sera with antibodies were used at 1:50 dilution.

- **For both the lysate and rk39 antigens, the absorbency read at 405 nm. To make sure tha**

  finding is reliable the cutoff point was taken at five SD from the value of the mean obtain

  ten Caucasian North American control subjects.

- **It was 0.15 for the whole lysate and 0.075 for rk39. All patients up to number 19 (inclu**

  were skin tested using the same product that detected the three subjects included in our st

  numbers 20-22.

rasite Cultures and Genomic DNA Preparation

Parasites from lesion biopsies were grown to late log phase (2-7 x 10^7/ ml) in medium 199 with

HIFBS. Genomic DNA was prepared from five (a-e) isolates, four of which belonged to pa

whose sera were included among the ELISA tested samples (3, 8,9,10).

We had no available serum for the fifth tested isolate. We included in addition, reference s

belonging to *Leishmania infantum* (IPTI); *Leishmania chagasi* (MHOM/BR/82/BA-

Leishmania tropica (MHOM/SA/91/WR1063C); *Leishmania major* (Friedland) and *Leish*

amazonensis (IFLA/BR/67/PH8). They were cultured in axenic media and genomic DNA extr

as described by Burns *et al*. (29)

Southern Blot Analysis.

- **Genomic DNA (2.5 ug) from *Leishmania* isolates (a, b, c, d and e) were digested with re enzymes Sal I and Pst I or with Pst I alone. The same was applied on reference strains o of *L. infantum, L. chagasi, L. tropica, L. major,* and *L. amazonensis*. DNA was separated electrophoresis, to be analyzed by Southern Blott.**
- **Blotts were probed with radio-labeled [-^{32} P]dCTP) DNA inserts containing the (1.2 kb fragment (repetitive domain) of the k39 clone of *L. chagasi* (29) or with the full length c insert (0.8kb) of the *L. major* sequence LmSP1. The blots were washed to a final stringe 0.2 x SSC at 65^{0}C for 30 min. and analyzed by autoradiography.**

SULTS

able 1 summarizes the pertinent biographic features of the study group.

hey were: 11 males and 11 females; age between seven years and seventy, occupation varied
oth genders the younger age group was students. The majority of adult females were housewi
ut with partial occupation in the fields during summer, exclusively confined to daytime.

he adult males were farmers, shopkeepers or teachers mostly in the same locality as t
welling address.

ABLE 1: Patient's pertinent biodata

itials	Patients #	Sex M/F	Age (years)	Geographic locality	Diagnosis	Treatment Before Diagnosis
t	1	M	N. A.	N.A.	CL	Antibiotics
IaFa	2	M	7	Tripoli	CL	Antibiotics & Antifungal
aYa	3	M	25	Beirut	CL	Antibiotics
aAm	4	M	53	Jbeil	CL	Antibiotics & Glucantime 1.5g
hAK	5	M	32	Bikaa	CL	Antibiotics
IaAw	6	F	60	Akkar	CL	Antibiotics
IaMr	7	F	34	Beirut	CL	Antibiotics
aAb	8	F	9	Beirut	CL	Antibiotics
lYa	9	M	8	Beirut	CL	Antibiotics
IaFa	10	M	28	Saida	CL	Antibiotics
aAl	11	M	21	Syria	CL	Antibiotics & Fucidin 2%
aBa	12	F	56	Tripoli	CL	Antibiotics
IaDi	13	M	20	Syria	CL	Antibiotics
IoBa	14	M	30	Akkar	CL	Antibiotics
IaHS	15	F	50	Akkar	CL	Antibiotics
IaYe	16	F	65	Tripoli	CL	Antibiotics
IuKa	17	M	49	Syria	CL	Antibiotics
IaTa	18	F	70	Beirut	CL	Antibiotics
aUY	19	F	38	Akkar	KA(?)	Antibiotics
lAt	20	F	35	Akkar	LT+ve	___
aSh	21	F	40	Akkar	LT+ve	___
aFa	22	F	35	Akkar	LT+ve	___

M: Male; F: Female; N.A.: not available
CL: Cutaneous Leishmaniasis; KA: Kala-Azar; LT+ve: Leishmanin Test Positive

- **Table 2 summarizes the features of the skin lesions in the patients (18 cases) plus the results of the cultures and of ELISA testing. Four of the positive cultures were found in a previous study to belong to *L. donovani* (34).**
- **Number, size, age and clinical appearance of skin lesions in each case is correlated with the presence of antibodies detected by ELISA.**
- **Most of the skin lesions were single (only 4 had 2 or more lesions). The commonest site for lesions was the face. There was no relationship among any of the biographic data of our patients, and any of the parameters describing the lesions and the titer of anti-*Leishmania* antibody.**

TABLE 2 Results of Elisa Readings (Absorbance at 405 nM). Positive results are > 0.15 for Whole Lysate of L.donovani (L) and > 0.075 for rK39 (See text for details)

	A		B		+Skin test		Kala Azar	
Patient Number	L	rK39	L	rK39	L	rK39	L	rK39
1	0.069	0.043						
2	Patient number 2 had no serum available but was found to have antibodies in a previous study although the titer was not determined.							
3	0.070	0.006						
4			0.33*	0.006				
5	0.081	0.042						
6			0.078	0.049				
7			0.173*	0.026				
8	0.120*	0.014						
9	0.264*	0.251*						
10	0.530*	0.387*						
11			0.347*	0.199*				
12			0.236*	0.340*				
13	0.320*	0.165*						
14			0.048*	0.058				
15			0.069	0.031				
16			0.047	0.019				
17			0.277*	0.356*				
18			0.188	0.206				
19							0.140*	0.041
20					0.060	0.018		
21					0.039	0.005		
22					0.077	0.085*		
TOTAL	4+ve	3+ve	6+ve	4+ve		1+ve	1+ve	

+skin positive: positive Monte-Negro with extract from one of our isolates

A: positive cultures; B: negative cultures

3 represents the actual readings on the ELISA trays with antigens from lysates of whole paras

ecific recombinant protein rK39. None of our probands had a significant skin reaction to our

skin testing.

erification of the isolates' identity and characterization of the molecular conservation of DNA

nces between the Lebanese isolates and other *Leishmania* species confirmed earlier findings b

).

esults by Southern blot of the analyses of isolates (a, b, c, d and e) and several reference proma

s probed with a DNA fragment comprising the repetitive domain of the *L. chagasi* gene seque

dized strongly the LcKin gene sequences. Using the same technique genomic DNA (2.5 .mu.g/l

L. chagasi digested with Bam HI, Hind III and Psi I or Psi I digested DNA from L. amazonesis

iensis, L. guyanesis, L. donovani, L. infantum, L. major, and T. cruzi .

TABLE 3 - Correlation between physical characteristics of the lesions and Culture/ELISA results

		Lesions									
		Site			Size(cm)						
PT#	Lesions#	1st	2nd	3rd	1st	2nd	3rd	Description	Duration	Culture/ELISA Results	
1	1	Face			1x0.7			Missing	3 months	+ve/-ve	
2	1	Face			1.5x1.3			DH	4 months	+ve/+ve	
3	1	L.Ear			3x1.5			DH	1 month	+ve/-ve	
4	2	L. Elbow	R. Elbow		5x6	4x6		WU & Scab	3-4months	-ve/+ve	
5	1	L.Knee			2.5x1			WU	13 days	+ve/-ve	
6	1	L.Cheek			5x3			DH	2 months	-ve/-ve	
7	2	Forehead	L.Cheek		3x2			Missing	4 months	-ve/-ve	
8	1	Chin			1x0.5			P	3 months	+ve/-ve	
9	1	Face			1.5			WU	4-5months	+ve/+ve	
10	1	Face			1x1.2			P	4months	+ve/+ve	
11	1	L.Temple			4.5x2.5			WU	16 months	-ve/+ve	
12	3	R.Arm	R.Arm	R.Arm	5x3	3x1.5	1x1	P	5-9 months	+ve/+ve	
13	1	R.Cheek			1x1			DH	6 months	-ve/+ve	
14	1	R.Hand			2.2x1.5			Both	7 months	-ve/-ve	
15	1	R.Hand			2.8x3			DH	3 months	-ve/-ve	
16	1	Face			2.5x2			DH	Few days to Several months	-ve/-ve	
17	8	Face 4	R.Arm 3	L.Arm 1	All between 0.5x0.5 and 6x5.5			All varieties	6 months	+ve/+ve	
18	1	Face			1x1			DH		-ve/+ve	

PT: Patient Number

#: Number of lesions

L: Left; R:Right

DH: Dry Hyperkeratotic; WU:Wet Ulcer; P:Papule

The blots were probed with a 2.4 kb Hind III fragment from the LcKin homology domain w the 915 bp repetitive insert of K39, to Sal I and Pst I restriction fragments in the Lebanese isolates. Since the same probe was previously shown to hybridize strongly with *L. donovani* genomic sequences (28), the results would suggest that the Lebanese isolates are indeed most closely related to *L. chagasi* and *L. donovani.* The K39 probe showed a significantly weaker hybridizing signal to Pst I restriction fragments of *L. infantum, L. tropica, L. major* and *L. amazonensis* suggesting less conservation of the repeat sequence in these species.

- **All the above and following studies were carried out at Corixa GSK . Sal I (3.7 and 6.0 and three Pst I (4.0, 3.0 and 0.9 kb) hybridizing bands were detected in all of the Leba isolates.**
- **By comparison, the K39 probe hybridized to two Pst I fragments, with sizes correspon closely to the (4.0 and 3.0 kb), species detected in all of the Lebanese isolates.**
- **A major distinction between the Lebanese isolates and *L. chagasi* is the absence of the (0.9 Pst I species in *L. chagasi* indicative of the presence of a second copy or polymorphis restriction sites of K39.**

- **Duplicate blot (control) probed with a *L. major* derived cDNA fragment, LmSP1. LmSP1 hybridiz strongly to a conserved DNA sequence of all *Leishmania spp*. tested.**

- **The Lebanese isolates showed identical hybridization patterns for both the Sal I and Pst I restricti fragments.**

- **In addition, the two Pst I hybridizing fragments (1.4 and 3.0 kb) were indistinguishable between t Lebanese isolates and those of *L chagasi.***

ussion

e literature is replete with studies on the immune reactions to the established Old World

rmotropic strains of *Leishmania* (*tropica* and *major)* at different times in the course of infectio

nce little is known about how the immune system in the human reacts to an invasive parasite s

***donovani* when it causes limited pathology instead of the expected devastating systemic disord**

cided to investigate the type of immunity induced in this situation.

the present study, we used molecular probes, to confirm that the most common *Leishmania* pa

cause cutaneous disease in Lebanon is a strain that belongs to the *L. donovani* complex.

- **Hence we investigated both the humoral and the cell mediated responses in our patie established that cutaneous leishmaniasis caused by either one of the dermotropic occasionally induces a short lasting humoral response with moderate levels of antibodi active lesion stage; the appropriate response in these cases is almost invariably a long la mediated immunity.**

- **On the other hand the immune reaction elicited by the invasion of the viscerotropic strains parasites (causing Kala-azar) is well known to be principally humoral peaking at the heig parasitemia.**

- **A cell mediated reaction may or may not develop in visceralized cases. When it does, it usually after recovery and with some delay.**

se observations stood the test of time and both parameters, humoral response and cell
nunity constituted tools for surveillance studies. In our case 50% of our patients had eleva
hmania (IPTI antigen) antibodies.

s was further corroborated by the presence of anti-rk39 antibodies in the sera of these patien
could conclude that in situations of unconventional association between parasite strain and
ase the immune reaction is dictated by the strain of the organism rather than by the pat
dition produced in the host.

ether this is a reflection of moderation in the virulence and the invasiveness of the parasite is
ecially that we successfully isolated parasites from the blood of some of these patients wh
comitant organo-megaly. On the other hand according to the literature rk39 antibodies
sent during concurrent active visceral leishmaniasis.

egitimate question is whether these patients have a low degree visceral disease amountin

oclinical infection, as such occurrences are drawing more attention (36-37). Then the c

order is deceptively limited to the skin and the subclinical invasion may be, depressing the in

ctions.

better evaluate the response of the immune system to unconventional pathology by specific s

***Leishmania* parasites we recommend that in these patients blood cultures be obtained ar**

mune response be always determined.

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