

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

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INTRODUCTION

Dermal lesions caused by members of the *Leishmania donovani* complex are not a frequent disease, especially in the eastern Mediterranean region. These parasites are more viscerotropic, in the rest of the world, causing lethal infantile Kala-Azar, and a less severe systemic infection in adults. In the latter cases they cause variable degrees of immune suppression which in some individuals aggravates other illnesses considered usually not grave or terminal. Furthermore the cutaneous infection is common in the countries forming the Mediterranean basin, caused by members of the *Leishmania tropica* and *Leishmania major* complexes.



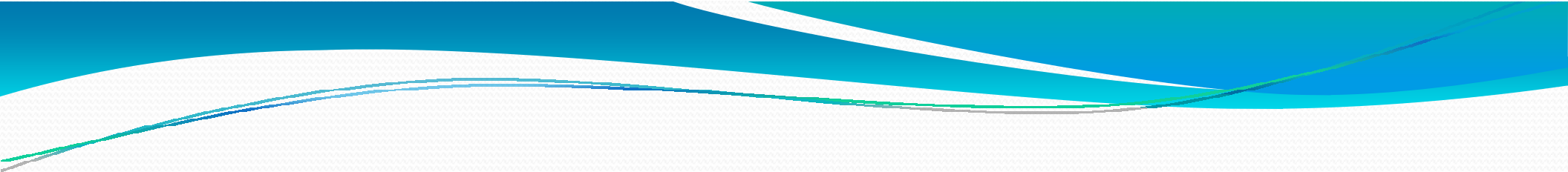
immune reactions of the host to the classically recognized Old World strains of this parasite, whether the viscerotropic or dermatropic strains, have been exhaustively studied. In short in cases of infection, systemic (by *infantum and/ donnavani senso lato*) or dermal (by *major or tropica*) arms of the immune system may be evoked, although the humoral is certainly more consistently evoked than the cell mediated one. Effectively antibodies to parasite antigens form in infection with either member of either the dermatropic or viscerotropic strains of *Leishmania* .



Since the cutaneous form is self healing (with no treatment), typically the humoral response is short lasting and in moderate titers (1-6). On the other hand cell mediated immunity (the delayed 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 of *donnovani* family rises acutely in blood after the onset of disease, remain elevated for some time and then drop gradually thereafter to disappear almost totally about a year or so after the onset of disease (7-10).

In this type of infection delayed type hypersensitivity, if it forms, takes much longer to appear (7-10).



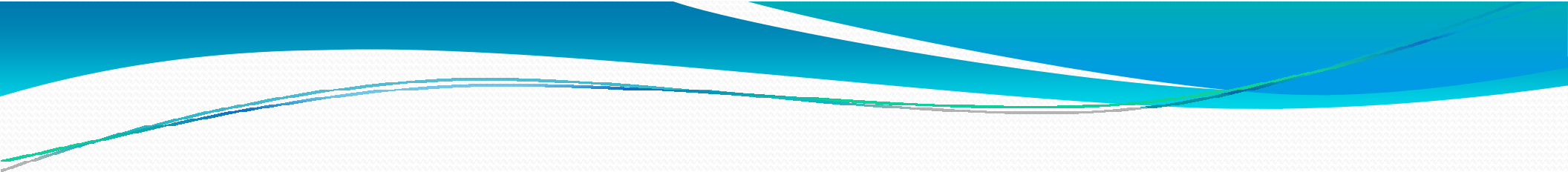
These observations were consistent enough for different researchers to use them as diagnostic tools (11-13) and for tracking these disorders epidemiologically (14-21). The antigens used were from different parasite preparations and extracts (27-33) and they all proved adequate.



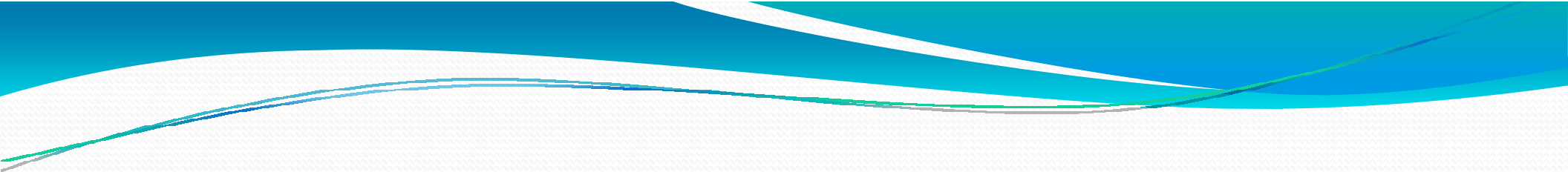
On the other hand *L. donovani* causing a cutaneous lesion is certainly not a common finding.

In this study to better understand the way the immune system reacts to attacks by this specific strain and in the absence of overt visceral invasion, we decided to study the expression of the immune community 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 of the reference strain IPTI (30), and rk39, a highly conserved sequence found in both the Old and New World parasite species all of which cause visceral leishmaniasis (*L. donovani*, *L. infantum* and *L. 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 used to determine delayed hypersensitivity we used an extract from one of our isolates. The identity of the used isolate had been revealed in a previous study by enzyme electrophoresis (34). We confirmed the identity of our stabilates by analyzing them electrophoretically. This method in addition molecular identification techniques were used on different isolates obtained from the patients under study. Probes for sequences coding for K39 gene yielded the final proof.



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



MATERIALS AND METHODS

Patients and study groups.

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

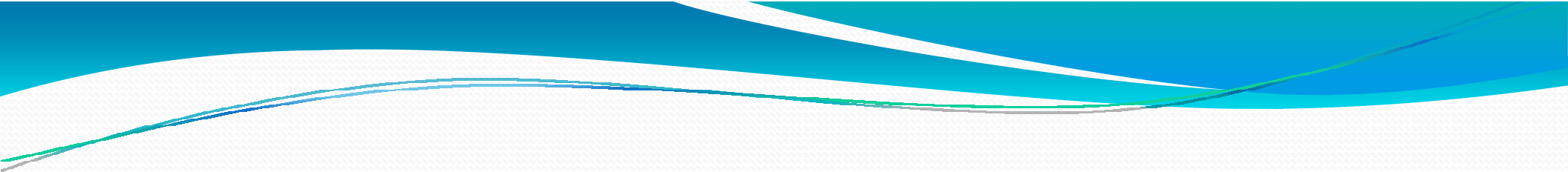
The patients were referred to our laboratory from the American University of Beirut Medical Center. They had lesions typical of cutaneous leishmaniasis. They were confirmed by histologic examination of skin biopsies stained with a modified Romanovsky (Wright-Giemsa) stain (35). In order to isolate and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

Parasites 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 dermatropic 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. Seattle Washington State).



ologic analysis. The sera from the 22 subjects were found devoid of anti *Leishmania major* antibodies. The sera were then screened by ELISA. Two preparations of *Lmajor* antigens were tested namely a lysate which was prepared, and soluble proteins fractionated from the lysate.

In the present study anti-*L.donovani* antibodies were detected by micro-ELISA technique (2) using whole lysate of *L. donovani* parasites and rk39. As mentioned earlier, rk39 is a recombinant polypeptide. It represents the protein product of a high repeat genomic segment type 1 of the *L. donovani* strain of parasite. Qu J.Q. *et al.* (31) have demonstrated that it is both a sensitive and specific test with no cross-reactivity with antibodies to antigens present in leishmaniases cases caused by strains that belong to other parasite complexes. This part of the study was carried out at the University of Washington Laboratories (Seattle, WA, USA).

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- **The procedure was described in a previous work. In short, the plates were coated overnight at 4°C with 10ng lysate protein/well and in other plates 25 ng of rk39/well, in coating buffer. 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 the finding is reliable the cutoff point was taken at five SD from the value of the mean obtained from 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 (including numbers 19-22) were skin tested using the same product that detected the three subjects included in our study with numbers 20-22.**



Parasite Cultures and Genomic DNA Preparation

Parasites from lesion biopsies were grown to late log phase ($2-7 \times 10^7$ / ml) in medium 199 with 10% FIFBS. Genomic DNA was prepared from five (a-e) isolates, four of which belonged to patients 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 strains belonging to *Leishmania infantum* (IPTI); *Leishmania chagasi* (MHOM/BR/82/BA-1); *Leishmania tropica* (MHOM/SA/91/WR1063C); *Leishmania major* (Friedland) and *Leishmania amazonensis* (IFLA/BR/67/PH8). They were cultured in axenic media and genomic DNA extracted 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 restriction enzymes Sal I and Pst I or with Pst I alone. The same was applied on reference strains of *L. infantum*, *L. chagasi*, *L. tropica*, *L. major*, and *L. amazonensis*. DNA was separated by agarose gel electrophoresis, to be analyzed by Southern Blot.**
- **Blots were probed with radio-labeled [³²P]dCTP) DNA inserts containing the (1.2 kb) repetitive domain fragment (repetitive domain) of the k39 clone of *L. chagasi* (29) or with the full length cDNA insert (0.8kb) of the *L. major* sequence LmSP1. The blots were washed to a final stringency of 0.2 x SSC at 65°C for 30 min. and analyzed by autoradiography.**



RESULTS

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

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

The adult males were farmers, shopkeepers or teachers mostly in the same locality as the dwelling address.

TABLE 1: Patient's pertinent biodata

Initials	Patients #	Sex M/F	Age (years)	Geographic locality	Diagnosis	Treatment Before Diagnosis
t	1	M	N. A.	N.A.	CL	Antibiotics
aFa	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
aAw	6	F	60	Akkar	CL	Antibiotics
aMr	7	F	34	Beirut	CL	Antibiotics
aAb	8	F	9	Beirut	CL	Antibiotics
lYa	9	M	8	Beirut	CL	Antibiotics
aFa	10	M	28	Saida	CL	Antibiotics
aAl	11	M	21	Syria	CL	Antibiotics & Fucidin 2%
aBa	12	F	56	Tripoli	CL	Antibiotics
aDi	13	M	20	Syria	CL	Antibiotics
oBa	14	M	30	Akkar	CL	Antibiotics
aHS	15	F	50	Akkar	CL	Antibiotics
aYe	16	F	65	Tripoli	CL	Antibiotics
uKa	17	M	49	Syria	CL	Antibiotics
aTa	18	F	70	Beirut	CL	Antibiotics
aUY	19	F	38	Akkar	KA(?)	Antibiotics
lAt	20	F	35	Akkar	LT+ve	—
Sh	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


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- **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)

Patient Number	A		B		+Skin test		Kala Azar	
	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 parasite and specific recombinant protein rK39. None of our probands had a significant skin reaction to our skin testing.

Verification of the isolates' identity and characterization of the molecular conservation of DNA sequences between the Lebanese isolates and other *Leishmania* species confirmed earlier findings by (1998).

Results by Southern blot of the analyses of isolates (a, b, c, d and e) and several reference promastigotes were probed with a DNA fragment comprising the repetitive domain of the *L. chagasi* gene sequence. The results showed that the Lebanese isolates hybridized strongly the LcKin gene sequences. Using the same technique genomic DNA (2.5 µg/µl) from *L. chagasi* digested with Bam HI, Hind III and Psi I or Psi I digested DNA from *L. amazonensis*, *L. infantum*, *L. major*, *L. donovani*, *L. infantum*, *L. major*, and *T. cruzi*.

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

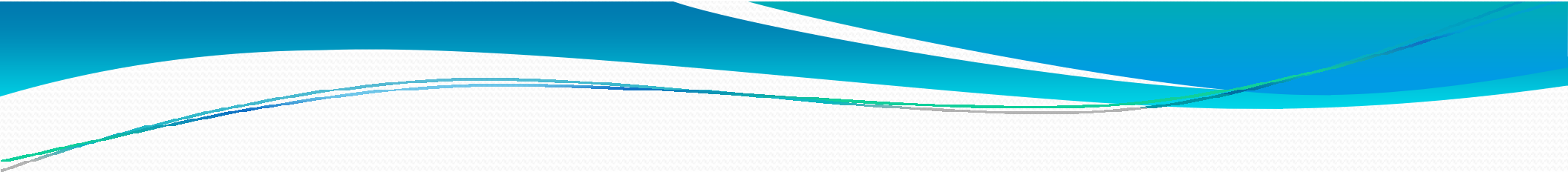
PT#	Lesions#	Site			Size(cm)			Description	Duration	Culture/ELISA Results
		1 st	2 nd	3 rd	1 st	2 nd	3 rd			
1	1	Face			1x0.7			Missing	3 months	+ve/-ve
2	1	Face			1.5x1.3			DH	4 months	+ve/+ve
3	1	L.Ear			3x1.5			DH	1 month	+ve/-ve
4	2	L. Elbow	R. Elbow		5x6	4x6		WU & Scab	3-4months	-ve/+ve
5	1	L.Knee			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

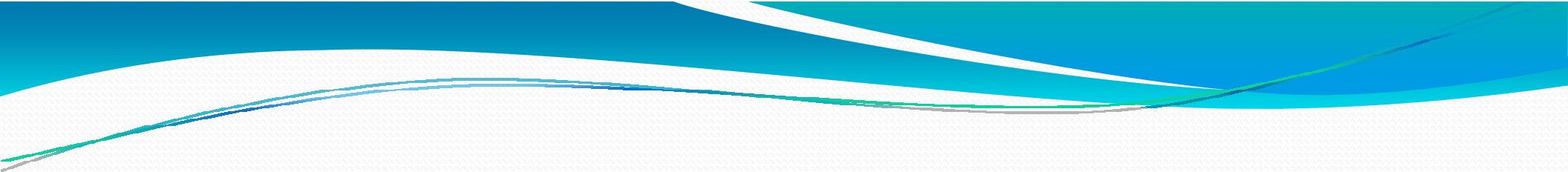
L: Left; R:Right

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 with 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.

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

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- Duplicate blot (control) probed with a *L. major* derived cDNA fragment, LmSP1. LmSP1 hybridized 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 restriction fragments.
 - In addition, the two Pst I hybridizing fragments (1.4 and 3.0 kb) were indistinguishable between Lebanese isolates and those of *L. chagasi*.




mission

The literature is replete with studies on the immune reactions to the established Old World promastigote strains of *Leishmania* (*tropica* and *major*) at different times in the course of infection.

Since little is known about how the immune system in the human reacts to an invasive parasite such as *L. donovani* when it causes limited pathology instead of the expected devastating systemic disorder, we decided to investigate the type of immunity induced in this situation.


In the present study, we used molecular probes, to confirm that the most common *Leishmania* parasite to 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 patients. It has been established that cutaneous leishmaniasis caused by either one of the dermatotropic strains occasionally induces a short lasting humoral response with moderate levels of antibodies during the active lesion stage; the appropriate response in these cases is almost invariably a long lasting cell mediated immunity.

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


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



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

s was further corroborated by the presence of anti-rk39 antibodies in the sera of these patients.
could conclude that in situations of unconventional association between parasite strain and
case the immune reaction is dictated by the strain of the organism rather than by the patient's
condition produced in the host.

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



legitimate question is whether these patients have a low degree visceral disease amounting to subclinical infection, as such occurrences are drawing more attention (36-37). Then the order is deceptively limited to the skin and the subclinical invasion may be, depressing the infections.

better evaluate the response of the immune system to unconventional pathology by specific studies. For *Leishmania* parasites we recommend that in these patients blood cultures be obtained and the immune response be always determined.



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