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Research Article

Cell mediated and humoral immune responses to parasites in patients with cutaneous leishmaniasis caused by L. donovani senso lato in Lebanon

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Abstract

The countries included in the geographic "Fertile Crescent" of the Near East are Syria, Iraq, Jordan, Palestine, Lebanon and Israel. Cutaneous leishmaniasis is one of the major infections endemic in this region caused mostly by *L.tropica,* and sometimes by *L. major*, while Kala azar, the systemic infection is caused by *L. donovani infantum/ chagasi*). The latter presents as a generalized systemic infection, lethal in children less than three year old, becoming milder as the age of the infected subjects is greater. It becomes definitely hypo-endemic in mature subjects in Lebanon. During the last few years many investigators reported patients with skin lesions caused by *L.donovani.* Soon after, it was realized that this unusual situation, is prevalent on the strip of plains and hills bordering the Mediterranean Basin. The fact that this invasive strain, instead of invading all lymphatic tissues leading to their eventual malfunction, can be stopped at the skin, creating an unusual situation. This must have important implications. These observations led us to investigate which of the immune system reactions succeeded in limiting the invasiveness of this strain of *Leishmania* parasites. We studied both arms of the immune system, hoping that once determined, it may be amplified enough to reduce the cutaneous lesion from a skin sore to total resistance by the skin to this parasite. With this objective we tested the humoral response measuring anti-*leishmania* antibodies in the sera of these subjects, applying more than one technique. To assess the T cells mediated response we used intradermal (Monte Negro) test with the appropriate antigens. For both types of testing we had the necessary controls. Finally we used Southern blot for detection of any change in the genomic bases of our isolates hoping that once determined the genetic modification can be amplified to produce a weak enough strain which can circulate live in tissues conferring immunity rather than clinical disease.

Keywords: *Leishmania donovani, rk39, cutaneous Leishmaniasis, anti-leishmania antibodies, Monte Negro skin testing.*

Introduction

The "Near East" is composed mainly of Syria, Iraq, Jordan, Lebanon, Palestine and Israel. This entire region is endemic for Leishmaniasis in both of its clinical forms, the cutaneous and the systemic [1-4]. The cutaneous infection is caused mainly by *L.tropica*, and less frequently *L.major,* forming many types of skin lesions, from a simple boil to large ulcers [1-4]. This type of infection seemed to be on the increase in this entire area at different rates depending on the region (e.g. Aleppo and Latakia in Syria, and in some parts of Iraq) [4-8]. The systemic infection (kala-azar) is caused by *L.donovani,* which apparently is the same as *infantum* in the countries bordering the Mediterranean, referred to as *chagasi* in north and subtropical Africa, and *donovani* in the lands spreading from the "Near East" to the western borders of China [1-4]. Systemic leishmaniasis presents classically as a generalized systemic infection in which the parasite in circulating lymph occupies and destroys monocytes and B lymphocytes [1-4]. It causes a lethal infection in children younger than three years [9] but, in endemic zones, symptoms become fewer, less specific and in some cases minimal as the parasite infects older subjects. In endemic areas, the majority of the infected subjects with clinically detectable or with sub-clinical infection are aged 20-50 years. Most patients present with few non-specific, though chronic symptoms, such as chronic low-grade fever generalized poor health, and lack of energy often leading to classifying the case as immune deficient [10]. Others may not complain of any symptom constituting thus a silent reservoir [10-12] which may spread *Leishmania* parasites, not only through blood and other organ donation, but also like HIV by needle sharing [13] or via contact with any of the body fluids such as saliva and semen [14], furthermore, subjects from non-endemic zones, fail to limit to the skin, a dermotropic strain, such as *L.tropica,* developing instead an overwhelming systemic infection [15]. All of these observations and findings suggest that populations living in endemic regions acquire "passively" or at least unaware, some resistance to the development of overt clinical illness. Hence, it seems we are aware of no more than the tip of an iceberg of infected people [16-19]. In this report, we are describing patients with skin lesions caused by *L.donovani*, a situation certainly not common. It was noted earlier in two patients on the Mediterranean slopes of the Pyrenees in south of France as well as in Lebanon causing both types of infections cutaneous and systemic [20,21]. We are confirming this situation, in the current study, after collecting our data over close to a decade. We decided to identify the way the immune system responds in these subjects, hoping to identify the factors that lead to limiting to the skin, a parasite which usually crosses this first barrier unrestricted, and invades through lymph the entire body. With this objective we evaluated the (B) and (T)) cell responses in our patients, and compared the results with those obtained by colleagues using similar techniques, on patients with skin lesions produced by one of the dermotropic strains (*L.tropica, L.major, L.bresiliensis*) [22-28] and also the same parameters (B lymphocyte responses) in patients with systemic leishmaniasis due to

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one of the *L.donovani* species [29-34] and also the cell mediated (T lymphocytes) response [35].

Materials and Methods

Patients and study groups.

This study was performed on 22 subjects, referred to our laboratory at the American University of Beirut. Eighteen had skin sores. The remaining four carried no skin lesions. Patient no 19 had a systemic disorder for which she was hospitalized. We included her because we diagnosed proved she had Kala Azar. Her history goes back three months ago during which she was bed-ridden in a community hospital. She was discharged home undiagnosed. Her symptoms had mildly improved. Her main complaints were all along low- grade fever, hepato-splenomegaly (documented by physical exam, and ultrasound), a mild anemia that was refractory to all treatment, and a moderately elevated proportion of monocytes (10-13%). Finally we included as well, three subjects (20-22) who were symptom free and had a negative past history, but 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. To verify whether these patients with *L.donovani* cutaneous infection, had any special clinical features that may distinguish them from others with skin lesions, produced by *L. tropica* or *L.major,* we had each patient fill out a questionnaire which covered all vital data as well as the current and past addresses (including travel). Biopsies from every lesion were stained with a modified Romanovsky (Wright-Giemsa) stain [36] A portion of the lesion biopsy was inoculated on modified NNN [37] medium with 25-30% Heat inactivated fetal bovine serum (HIFBS) and antibiotics (GIBCO laboratories, Grand island, USA), or else injected into the animal model (syngeneic Balb/c mice) and once the animal became sick it was sacrificed, and the parasites isolated in large numbers (from lymphatic organs and part of the collected parasites cryopreserved). The identity of our isolates was always re-confirmed by iso-enzyme electrophoresis run with reference strains. In addition, the identity of a few of the isolated samples from this focus was determined, using molecular technics as well [38,39].

Antigens and response by B and T lymphocytes

In all cases (1-19) the humoral response to *Leishmani*a antigens was evaluated. For *L.major* we used, two types of antigen preparations: Lysate of whole promastigotes (un-separated), and the soluble protein fraction obtained from these lysates. For *L. tropica* antibody detection we used a lysate of entire parasites (promastigote forms) obtained from cultures of isolates well identified as reference strains. Finally to assess anti- *L.donovani* antibodies we used lysates of entire *L. donovani* parasites propagated *in-vitro* and a polypeptide encoded by a DNA sequence referred to as k39 as mentioned earlier. Effectively it was confirmed in both the Old and New World parasite species that cause visceral leishmaniasis (*L. donovani*, *L. infantum* and *L. chagasi)* using micro-ELISA technique. A few of our parasite sample were studied at Corixa Laboratories (Seattle WA, USA) under the supervision of late Dr. Yasir Skeiky, The rest were all carried out in our laboratory, at the American University of Beirut and finalized later at the Chronic Care Center, The procedure followed was the one described earlier, in short, the plates were coated overnight at 4°C with the appropriate antigen (10ng lysate protein/well and in other plates 25 ng of rk39/ well) in coating buffer, the sera with antibodies were used at 1:100 dilution. For all lysates and rk39 antigen, the absorbency was read at 405 nm. To make sure that the findings are reliable the cutoff point was taken at five SD from the value of the mean obtained on ten Caucasian North American control subjects who never travelled to endemic areas. It was 0.15 for whole lysates and 0.075 for rk39.

Antigens and response by cell mediated immunity

All patients, 1-19, were skin tested using the same product that detected the three subjects included in our study as positive controls (subjects number 20-22).

Genomic DNA Preparation, for Southern Blot Analysis.

Parasites obtained from lesion biopsies were grown to late log phase (2-7 x 10^7/ ml) in the medium mentioned above. Genomic DNA was prepared from five (a-e) isolates, four of which belonged to patients whose sera were included among the ELISA tested samples. 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 methods were applied on reference strains of each of *Leishmania infantum* (IPTI); *Leishmania chagasi* (MHOM/BR/82/ BA-2,C1 *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 above, *L. infantum, L. chagasi, L. tropica, L. major,* and *L.amazonensis.* DNA was separated by gel electrophoresis, to be analyzed by Southern Blot. Blots were probed with radio-labeled -^{32}P] dCTP) DNA inserts containing the (1.2 kb Pst 1 fragment (repetitive domain) of the k39 clone of *L. chagasi* or with the full length cDNA insert (0.8kb) of the *L. major* LmSP1. The blots were washed to a final stringency of 0.2 x SSC at 65°C for 30 min. and analyzed by autoradiography.

Ethical Clearance

This study was checked and approved by the IRB of the American University of Beirut IRB, accepted by IRB at the Chronic Care Center.

Results

The patients salient items in the biodata are summarized in Table 1. As mentioned the patients came from different parts of Lebanon, but mainly from the north Tripoli and Akkar region, a (rural region), in northern Lebanon as previously reported [40]. In both genders the younger age group were students, the majority of adult females were housewives (helping occasionally in the fields). The majority of the adult males were farmers, a few were shopkeepers or teachers mostly in the same locale. Most of the skin lesions were single (only 4 patients had 2 or more lesions), they varied in size, and shape (from a 3-5 cm button like lesion with an oozing crater, to very small flat macular lesions <3mm in diameter), to a dry scab around (4x8cm), to an extended flat excoriated wet lesion spread over an area of about 10 x 5 cm.etc.). The most common site for the lesions was the face. There was no obvious relationship between any of the biographic data of our patients, and any of the parameters that described the lesions or the titer of anti-*Leishmania* antibodies. Table 2 summarizes all the data clinically relevant on every subject, and the presence of antibodies only to *L.infantum* strain and rK39 in every candidate, since as mentioned above none of the patients'sera reacted to *L. major* and the response to *L.tropica* was equivocal in two samples only. Effectively as mentioned above ELISA results revealed no anti-*Leishmania major* and insignificant response to *L.tropica*. In contrast antibodies to antigens (IPTI and rK39) were elevated in the majority of our subjects. Effectively 10/18 of the tested subjects had elevated anti-IPTI antibody; their majority had anti-rK39 antibody as well. The patient with Kala Azar had only anti-IPTI. None of the features of the skin lesions, their number per person, their pathologic aspect such as appearance, size, age, and location on the body, correlated with the presence and level of antibodies, detected and measured by ELISA. In addition the success in obtaining the parasites in cultures and also the identity of the parasite all failed to correlate with any clinical feature of the lesions nor with the age of any. Finally, all the techniques used to classify the parasites (direct and indirect) agreed that the parasites

Table1: Patient's pertinent biodata.

Initials	Patient #	Gender M/F	Age (years)	Geograp. Locality	Diagnosis	Treatment before diagnosis
Pt	1	M	N. A.	N.A.	CL	antibiotics
HaFa	2	M	7	Tripoli	CL	antibiotics & antifungal
FaYa	3	M	25	Beirut	CL	antibiotics
JaAm	4	M	53	Jbeil	CL	antibiotics &
KhAK	5	M	32	Bikaa	CL	1.5g Glucantime
MaAw	6	F	60	Akkar	CL	antibiotic
NaMr	7	F	34	Beirut	CL	antibiotic
YaAb	8	F	9	Beirut	CL	antibiotic
AlYa	9	M	8	Beirut	CL	antibiotic
MaFa	10	M	28	Saida	CL	antibiotics
BaAl	11	M	21	Syria δ	CL	antibiotics
FaBa	12	F	56	Tripoli	CL	antibiotics
HaDi	13	M	20	Syria δ	CL	antibiotics
MoBa	14	M	30	Akkar	CL	antibiotics
HaHS	15	F	50	Akkar	CL	antibiotics
HaYe	16	F	65	Tripoli	CL	antibiotics&
Ka	17	M	49	Syria δ	CL	antifungal
MaTa	18	F	70	Beirut	CL	Fucidin 2%
SaUY	19	F	38	Akkar	KA(?)	Antibiotics
AlAt	20	F	35	Akkar	LT+ve	Antibiotics
JaSh	21	F	40	Akkar	LT+ve	Antibiotics
KaFa	22	F	35	Akkar	LT+ve	

M: Male; F: Female; N.A.: not available; δ: Truck drivers between Akkar (north Lebanon) and Latakia in Syria .CL: Cutaneous Leishmaniasis; KA: Kala-azar; LT+ve: Leishmanin skin test positive.

belong to *L.* infantum *senso lato* [40]. Table 3 represents the actual readings on the ELISA trays for components of *L. donovani* entire lysate, and the specific recombinant protein rK39. Finally none of our probands (with active skin lesions and under treatment for kala azar), had a significant skin reaction to Monte-Negro skin testing. The verification of the isolates' identity and characterization of the molecular conservation of DNA sequences between the Lebanese isolates and other *Leishmania* isolates concorded with our earlier findings [17]. 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 in restriction sites of K39. 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 the Lebanese isolates and those of *L chagasi.*

Discussion and Conclusion

Little is known about the immune system reaction in humans to invasive parasites such as *L.donovani* when they cause limited pathology instead of the expected systemic disorder. In this study we investigated both types of immune responses to a limited skin infection by this parasite. We used molecular probes, to confirm that the most common *Leishmania* parasite to cause cutaneous disease in Lebanon is a strain that belongs to one of the varieties of the *L. donovani* complex (/*L .infantum/chagasi*). The results revealed that more than 50% of our patients had elevated anti-*Leishmania donovani* (IPTI and rK39 antigens) antibodies. Whereas cell mediated immunity to a mixture of *L. donvani and rk39 antigens* was negative in all patients with skin lesions and the patient with kala azar. Whether this is a reflection of moderation in the virulence and the invasiveness of the parasite is not clear, especially that we successfully isolated parasites from the blood of some of these patients who had only border line anemia but no organomegaly and were found to have humoral response to this parasite. On the other hand, according to the literature anti-rk39 *antibodies* are only present during an active visceral leishmaniasis. The question is whether these patients have a low grade sub-clinical visceral infection in parallel paralyzing T cell reaction.All of this implies that limiting the treatment to the skin lesion leaves a risk of increasing the size of the human reservoir, in addition to the classical canine reservoir. Considering how much more mobile humans are this overlooked reservoir, is often mistakenly believed to be safe after curing the dermal infection. Patients with scars of *leishmania* on the skin from the Mediterranean littoral, including our area (Lebanon, Latakia, the French Riviera, Mediterranean Pyrenees in France, Alpes maritimes, etc.). should be avoided as blood donors unless thoroughly tested or treated. Furthermore these subjects have a reduced immunity, which exposes them to a number of infectious agents and also to neoplasm. Finally to prove cure the conventional tests used are certainly insufficient in this situation, therefore from our experience we recommend the following additional tests:

1. Blood cultures on the appropriate media to be kept at room temperature (22-24 C) for at least 6-8 weeks.
2. A modification of the skin widow technique which in our experience is highly successful in recovering Leishmania (manuscript in preparation)
3. Intradermal injection of lysed buffy coat cells (from a suspected patient) in syngeneic Balb /c mice (strain susceptible to *L. donovani*) to observe for several weeks after which the smashed spleen WBC are studied To conclude, the immune system in patients with dermal leishmaniasis caused by a viscerotropic strain of parasite reacts in the same manner as it does in the systemic infection caused by such parasites. To reach a better understanding of the patho-physiology of these parasites we recommend that in similar cases the human host immune reactions be always determined.

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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).

Groups	**A**		**B**		**+Skin test**		**Kala Azar**	
Patient Number	L	rK39	L	rK39	L	rK39	L	rK39
1	0.069	0.043						
2								
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	

A: Patients with positive Leishmania cultures; B: Patients with negative cultures.
+Skin Test: patients included because of positive Monte-Negro test with extract from one of our isolates.
Patient with KA: female we histologically diagnosed to have Kala azar.

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Table 3: Correlation between physical characteristics of the lesions and Culture/ELISA results Lesions

		Site			Size(cm)			Description	Duration	Culture/ELISA Results
PT#	Lesions#	1st	2nd	3rd	1st	2nd	3rd			
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			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			1.5x2			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.Cheek		1.4 x0.6			P	9 months	-ve/+ve
13	1				6x5.5			DH	5 months	+ve/+ve
14	1	R.Hand			3x1.5			Both	6 months	-ve/+ve
15	1	R.Hand			5x3			DH	7 months	-ve/-ve
16	1	Face			1x1			DH	3 months	-ve/-ve
17	8	Face 4			2.2x1.5 2.8x3 2.5x2			All varieties	Few days to Several months	+ve/+ve
18	1	Face			1x1			DH	6 months	-ve/+ve

PT: Patient Number
#: lesion Number
L: Left; R:Right
DH: Dry Hyperkeratotic; WU:Wet Ulcer; P:Papule

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