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Evaluation of Antigens for the Serodiagnosis of Kala-azar and Oriental Sores by means of the Indirect Immunofluorescence Antibody Test (IFAT)*

Summary: Antigens and corresponding sera were collected from travellers with leishmaniasis returning to Germany from different endemic areas of the old world. The antigenicity of these *Leishmania* strains, which were maintained in Syrian hamsters, was compared by indirect immunofluorescence (IFAT). Antigenicity was demonstrated by antibody titres in 18 sera from 11 patients. The amastogotic stages of nine strains of *Leishmania donovani* and four strains of *Leishmania tropica* were compared with each other and with the culture forms of insect flagellates (*Strigomonas oncopelti* and *Leptomonas ctenocephali*). Eighteen sera from 11 patients were available for antibody determination with these antigens. The maximal antibody titres in a single serum varied considerably depending on which antigen was used for the test. High antibody levels could only be maintained when *Leishmania donovani* was employed as the antigen, but considerable differences also occurred between the different strains of this species. The other antigens were weaker. No differences in antigenicity between amastigotes and promastigotes of the same strain were observed. It is important to select suitable antigens. Low titres may be of doubtful specificity and are a poor baseline for the fall in titre which is an essential index of effective treatment.

Zusammenfassung: *Die Bedeutung der Antigenaktivität bei der Serodiagnose des Kala-Azar und der Orientbeule mittels der indirekten Immunfluoreszenz (IFAT).* Wir sammelten Parasiten und Seren von Reisenden, die aus verschiedenen endemischen Gebieten der Alten Welt mit einer Leishmaniasis nach Deutschland zurückkehrten. Die Antigenaktivitäten der isolierten und fortlaufend in Goldhamstern gehaltenen *Leishmania*-Stämme wurden im indirekten Immunofluoreszenztest (IFAT) verglichen. Die Antigenität wurde an Hand von Antikörpertitern in 18 Serumproben von 11 Patienten bewiesen. Neun Stämme des *Leishmania donovani*-Komplexes und vier *Leishmania tropica*-Isolate wurden in ihrem amastigoten Stadium miteinander verglichen. Hinzu kamen zwei Insekten-Flagellaten als Kulturformen: *Strigomonas oncopelti* und *Leptomonas ctenocephali*. 18 Serumproben von 11 Patienten standen für die Antikörperbestimmung mit diesen Antigenen zur Verfügung. Die maximalen Titerhöhen variierten in ein- und derselben Antiserumprobe zum Teil erheblich, je nachdem, welches Antigen für den Test benutzt wurde. Hohe Antikörpertiter konnten nur erhalten werden, wenn *Leishmania donovani* als Antigen vorlag, es ergaben sich aber auch zwischen den einzelnen Stämmen dieser Leishmaniaart erhebliche Unterschiede in der Antigenaktivität. Antigene anderer Art erwiesen sich als wenig wirksam. Zwischen amastigoten und promastigoten Entwicklungsformen eines *Leishmania donovani*-Stammes konnten keine Unterschiede in der Antigenaktivität erkannt werden. Für den Nachweis möglichst hoher Antikörpertiter im IFAT ist die Auswahl geeigneter Antigene von ausschlaggebender Bedeutung. Niedrige Titer erschweren deren Beurteilung als spezifisch und sind eine schlechte Ausgangsposition für die Beobachtung des obligatorischen Titerabfalles nach erfolgreicher Therapie.

Introduction

Human leishmaniasis is not endemic to Germany since climatic conditions prevent the occurrence and survival of adequate arthropod vectors. The disease is therefore of minor interest as far as public health is concerned (1). However, imported cases, mainly from countries around the Mediterranean Sea, do create an increasing individual medical problem. In particular, more children are becoming infected as a result of tourism (2). Antigens and antisera were collected from tourists and employees returning from infected areas with different stages of the two clinical types of old world leishmaniasis. The reason for this was to gain experience in and give recommendations for diagnosis in our local situation which certainly does not differ significantly from that in neighbouring countries. Since the direct identification of the causative agent is often difficult even for experts, the culture of the parasite and the development of serodiagnostic methods are of particular importance (3). The serodiagnostic method of choice, at least in our geographic region, was the indirect immunofluorescent antibody test (4, 5, 6, 7). The quality of the antigens employed in serodiagnostic procedures is more decisive for success than other factors. We therefore investigated different antigen preparations

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using the indirect immunofluorescent antibody test in order to select a particular antigen which produces the most significant and reproducible titres with a spectrum of appropriate antisera. Three questions were to be answered: Does an autologous combination between antigen and antisera give higher titres than a heterologous combination? Does the amastigote stage of an antigen produce different titres with a specific antiserum than the promastigote stage of the same *Leishmania* strain? What is the antigenic activity of culture forms of lower trypanosomatids as compared to specific *Leishmania* antigens?

Material and Methods

The parasitic strains used as antigens were isolated and maintained as amastigotes in Syrian hamsters. Nine different leishmanial isolates of the *L. donovani* complex were available as antigens. These originated from different places in almost the entire Eurasian endemic area, i. e. from India and Nepal in the Far East, the Western Mediterranean islands and Tuscany in North Italy to Kenya in the Tropics. All parasites were human isolates with one exception coming from a dog in Greece. Three isolates (India I, Sudan and Kenya) were received as old laboratory strains. The parasites were cultured *in vivo* continuously for between a few months and 26 years. The virulence and

Table 1: Antigen-dependent titre difference in the IFAT in different human *Leishmania* infections.

	Patients' sera	Antigens					
		L. donovani 9 strains		*L. tropica* 4 strains		*Strigomonas oncopelti*	*Leptomonas ctenocephal*
		Antigen-dependent titre variation	Average	Antigen-dependent titre variation	Average	–	–
	Control serum +	2048–8192	4096	256–4096	1024	64	64
	Control serum Ø	Ø	Ø	Ø	Ø	Ø	Ø
Kala-azar	1. T.T., Spain	1024–4096	2048	32–2048	128	128	64
	2a. Ph.v.R., Italy (Rome?)	16–256	64	Ø–64	32	32	32
	2b. Ph.v.R., Italy (Rome?)	32–2048	256	Ø–128	32	64	64
	2c. Ph.v.R., Italy (Rome?)	16–128	64	64	64	16	32
	3a. E.W., Elba I. (Italy)	64–256	128	64-128	128	64	32
	3b. E.W., Elba I. (Italy)	64–256	128	Ø–64	32	32	32
	4a. M.G., Elba II. (Italy)	64–512	256	32–512	128	16	64
	4b. M.G., Elba II. (Italy)	32–128	64	Ø–32	16	Ø	32
	5. T.D., Saudi Arabia	64–256	128	Ø–128	32	16	16
	6a. K.A., Abu Dhabi	128–2048	512	128–256	128	32	32
	6b. K.A., Abu Dhabi	256–2048	512	256–512	512	32	64
	6c. K.A., Abu Dhabi	512–2048	1024	128–512	256	32	64
	7a. M.St., India	2048–8192	4096	Ø–2048	256	256	128
	7b. M.St., India	512–4096	2048	32–512	512	512	128
Oriental sores	8. W.O., Malta	Ø–128	32	Ø–64	16	Ø	Ø
	9. M.B., Saudi Arabia I	Ø–32	16	Ø–16	Ø	Ø	Ø
	10. K.S., Saudi Arabia II	Ø	Ø	Ø	Ø	16	16
	11. S.A., Iran	64–256	123	Ø–512	128	32	32

reproductive capacity of the strains did not change after adaptation to hamsters. Two strains originated in India and one each in Southern Sudan, Kenya, Abu Dhabi, Malta and Greece; two isolates were from the Isle of Elba (Italy). Three of the four *L. tropica* (major) strains, isolated from human Oriental sores and reared three to five years in the underlip of hamsters, came from Saudi Arabia and the fourth from Tunisia. Culture forms of *Strigomonas oncopelti* (strain Silvio) and *Leptomonas ctenocephali* (strain FNH) completed the range of antigens investigated. The latter strain came from the gut of a German cat flea. Fresh slide preparations of all antigens were used for the test. These preparations were of approximately equal density. They were fixed in methanol for 5 min after air-drying. Culture forms were produced in the simple diphasic medium proposed in 1979 by *Aljeboory* (8) from the *L. donovani* strain India I, which was maintained for more than a quarter of a century in golden hamsters. These were then compared with the amastigotes from the spleen of carrier animals. Eighteen serum samples from 11 patients with parasitologically confirmed leishmaniasis (seven kala-azars, four Oriental sores) were tested by means of indirect immunofluorescence (IFAT) for leishmanial antibody titres using the 15 different antigens. In the test, the fixed antigen was first incubated with the patients' sera and then with antihuman-globulin, each for 1 h at 37° C. The conjugated antihuman IgG was supplied by the Institut Pasteur, Paris, and diluted 1:30. Counter staining was carried out with Evans blue at 1:5000.

Results

The results of our comparison of the different antigens investigated is shown in Table 1. The *L.donovani* isolates are apparently more suitable for the demonstration of significantly high antibody titres than are the antigens of the other species. However, differences in the antigenicity of individual *L. donovani* strains were evident since different antibody titres occurred with the same serum. The antibody titres from one serum sample differed to a remarkable degree in some cases (three to six titre steps), evidently depending on the antigenicity of the *Leishmania* strain used as the antigen. The so-called *Leishmania* species obviously contains a spectrum of strong, weak and unreliable antigens, culminating in a medium range antigenicity for *L. donovani*. Differences in antigenic activity do not depend on the number of artificial needle-borne passages through laboratory animals. Our *L. donovani* strain with the strongest antigenicity as amastigotes was tested with the same antiserum with corresponding flagellated cultural stage. There were no evident differences between the titres from 1:16 to 1:512 when amastigote antigen preparations were compared.

In five cases of kala-azar, the corresponding antigens were tested with their own antisera. In only one of these cases did this autologous reaction result in a maximum titre (Table 2). In the six other cases, the heterologous antigens of the same species had a comparable or even better antigenicity than the parasite which had originally stimulated the respective antibody production in the patient. The four *L. tropica* sera reacted more strongly with the *L. donovani* antigen than with the homologous combination. Only when a serum contained high antibody levels against *L. donovani* antigen were lower titre reactions against *L. tropica* also observed. Otherwise, only uncharacteristic titres appeared which were indistinguishable from non-specific reactions, and could not therefore support a diagnosis. The range in antigenic activity seems to be as wide for *L. tropica* as it is for the other species. One strain proved to be weaker than *Leptomonas ctenocephali* and the antigenicity of another was similar to that of *L. donovani*. In general, testing with non-specific antigens in *L. donovani* infections resulted in significantly lower serum titres than those obtained with the homologous antigen-antibody reaction. The low serum antibody titres obtained in tests using the two heterologous

Table 2: Titre maxima obtained in kala-azar sera using autologous and heterologus. *L. donovani* antigens.

				Antigens								
	No.	Name	Geographical origin	Elba I	India II	Abu Dhabi	Elba II	Malta	Sudan	Athens	India I	Kenya
Patients' sera	3645/77	E. W.	Elba I	256	128	128	128	64	64	128	256	128
	3719/77	E. W.	Elba I	64	64	64	64	64	64	256	128	128
	486/80	M. St.	India II	4096	4096	2048	4096	4096	8192	4096	8192	4096
	484/80	M. St.	India II	4096	4096	512	4096	2048	1024	512	4096	4096
	2647/77	K. A.	Abu Dhabi	1024	1024	2048	1024	512	512	1024	1024	1024
	2647/79	K. A.	Abu Dhabi	256	512	2048	256	256	256	512	2048	512
Kala azar	1803/79	M. G.	Elba II	32	32	64	32	32	32	32	128	32
	229/79	M. G.	Elba II	64	256	128	256	512	64	256	256	256
	562	W. O.	Malta	32	16	∅	16	32	16	32	32	128

insect flagellates as antigens proved to be almost identical to and not fundamentally different from those obtained with some *L. tropica* antigens.

Discussion

Although experience has shown that low serotitres can bring an infection into evidence, an immunological test should also be as sensitive as possible in order to demonstrate antibodies in high titres in a patient's serum. An immunological test should be as specific as possible to avoid unspecific reactions. Sensitivity and specificity of a test should allow the detection of declining titres after successful treatment. The close antigenic relationship between all lower trypanosomatids seems to induce a certain generosity in the use of heterologous antigens in diagnostic tests for human leishmaniasis (9, 10, 11). This, however, is only justified if high antibody levels are present or suspected and if homologous antigens are not available. Generally, antigens with an optimal antigenicity should be selected. It remains to be seen whether mixtures of different antigens will have real advantages over high quality single strain antigens. As *Bray* (12) pointed out in 1976, more standardization in testing is desirable with respect to the antigen used in the IFAT.

Promastigote culture forms are usually used and proposed as antigens in immunological research. Antibodies are, however, always produced in response to growing amastigotes (13, 14). Differences, at least in the IFAT active surface-antigens, have been described between the two developmental stages (15). Precautions against the use of amastigotes produced *in vivo* as antigens seem to be justified since it is suspected that the native parasites are either not clean enough or that they consist of antigen-antibody complexes which are not useful for immunological tests. They are also difficult to manage in large quantities. According to previous statements (13), our comparison does not confirm this aspect. The substitution certainly seems immaterial to the outcome of the test (4). We were not able to detect any notable difference in the antigenicity of amastigote and promastigote stages of the same *Leishmania* strain as described by *Bray* and *Lainson* (16). Preparing the antigens properly seems to be of more importance. According to Iranian experts (17, 18), only fresh preparations should be used.

It is known that in most cases uncomplicated old world dermal leishmaniasis or Oriental sores do not produce reliable titres of circulating antibodies, at least not when such diagnostic aid is required. A current explanation holds the poor challenge of the immune system responsible. Experience shows that titres usually rise when the lymphatic system becomes involved in the clinical course of dermal leishmaniasis, which supports this explanation. However, independent of a patient's immune response and his ability to control the infection, *Leishmania* parasites isolated from Oriental sores are generally less antigenic than *L. donovani.*

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