Monoclonal Antibodies for the Identification of New World *Leishmania* Species

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Monoclonal antibodies specific for selected species complexes of Leishmania *have been employed for the characterization of several representative strains of* Leishmania *isolated from different hosts and localities in the Americas. In the past 15 years, data have been accumulated concerning (i) the specificities of a number of these monoclonal antibodies and (ii) the antigenic variation (level of the expressed antigenic determinants) occurring among New World* Leishmania *species or strain variants as recognized by the monoclonal antibodies. This report is an attempt to summarize in brief the data accumulated to date on these points and to indicate the directions for future applications of these specific monoclonal antibodies for identification of leishmanial isolates.*

Key words: *Leishmania* - serodemes - monoclonal antibodies - radioimmune binding assay - immunotaxonomy

Monoclonal antibodies have been employed extensively for the identification of *Leishmania* species, development of diagnostic tests, investigation of molecules associated with parasite virulence and/or pathogenicity, and in the characterization of defined leishmanial antigens that should be able to produce immunoprotection against human leishmaniasis following vaccination (reviewed in Grimaldi & Tesh 1993).

Parasitic protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) are a biologically diverse group of microorganisms (Lainson & Shaw 1987). Taxonomic studies of *Leishmania* isolates from the New World indicate tremendous diversity within this genus (Cupolillo et al. 1994). A number of new *Leishmania* species have been described recently from sylvan areas of the Neotropics (Silveira et al. 1987, Lainson et al. 1989, Lainson & Shaw 1989, Kreutzer et al. 1991, Grimaldi et al. 1992, Yoshida et al. 1993). Some other taxonomically distinct taxa, isolated from wild mammals and vectors in the Brazilian Amazon Region, have also been described as unnamed new species (Lainson & Shaw 1987, Grimaldi et al. 1989, 1991). Much of this recent information on the epidemiology of the various New World *Leishmania* has been made through the application of techniques employing specific monoclonal antobodies as well as other molecular criteria for identifying and classifying leishmanial field isolates in comparison with standard reference strains (Saravia et al. 1985, Grimaldi et al. 1987, 1989, 1991, 1992, Aguilar et al. 1989, Yoshida et al. 1990, Barral et al. 1991, Darce et al. 1991, Falqueto et al. 1991, Hashiguchi et al. 1991, Kreutzer et al. 1991, Ponce et al. 1991, Bonfante-Garrido et al. 1992).

In order to determine a crosspanel of monoclonal antibodies suitable for parasite (species) identification, the WHO Special Programme for Research and Training on Tropical Diseases (TDR), the Pan American Health Organization (PAHO) and the Academia de Ciencias de America Latina, jointly organized two meetings in Washington, D.C. (February 1993) and Cali, Colombia (December 1993). Based upon the double blinded testing of *Leishmania* monoclonal antibodies (99), fourteen monoclonal antibodies were selected and recommended for general use in the identification of *Leishmania* species. Here we discuss the reactivities of some of these antibodies with *Leishmania* group- and species-specific antigens.

MATERIALS AND METHODS

Serodeme analysis using monoclonal antibodies - The monoclonals that have been used for characterization and identification of leishmanial isolates are listed elsewhere (Grimaldi et al. 1987, 1991, Hashiguchi et al. 1991, Kreutzer et al. 1991, Bonfante-Garrido et al. 1992). These monoclonals that distinguish both New World and Old World species of *Leishmania* were produced as reported previously (McMahon-Pratt & David 1981, McMahon-Pratt et al. 1982, 1985, 1986, Jaffe & McMahon-Pratt 1983, Jaffe et al. 1984, Pan & McMahon-Pratt 1988) according to the method of Kohler and Milstein (1975) as modified by Kennett et al. (1978). Characterization of the

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Leishmania was performed with an indirect radioimmune binding assay (RIA) using whole parasite lysates as antigen. The technique has been described in detail before (Grimaldi et al. 1987). A subsample of the leishmanial stocks were also analyzed by indirect immunofluorescence (McMahon-Pratt et al. 1986) or ELISA assays (Jaffe & McMahon-Pratt 1987) using the monoclonal antibodies.

Leishmania - Over the past 15 years, we have collected and characterized, by species-specific monoclonal antibodies and RIA, a large panel of leishmanial parasites. The results with specific monoclonal antibodies on both the identification and classification of leishmanial parasites were also confirmed, using in parallel isoenzyme electrophoresis (including numerical zymotaxonomic analyses) and other molecular techniques such as analysis of restriction enzyme digestion patterns of kinetoplast DNA and molecular karyotypes (Grimaldi et al. 1991, 1992, Hashiguchi et al. 1991, Kreutzer et al. 1991, Bonfante-Garrido et al. 1992, Yoshida et al. 1993, Cupolillo et al. 1994). *Leishmania* promastigotes were cultured in Schneider's *Drosophila* Medium (Gibco, Grand Island, NY) (Hendricks et al. 1978) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA) at 24°C. Parasites in the log phase of growth were harvested by centrifugation (1,500 x g for 10 min, at 4°C) and washed twice in phosphate-buffered saline (PBS), pH 7.3. The final pellet was used for preparation of samples for parasite characterization using monoclonal antibodies (Grimaldi et al. 1987).

RESULTS AND DISCUSSION

Some preliminary results on the reactivity of monoclonal antibodies derived for selected species complexes of *Leishmania* (McMahon-Pratt et al. 1982, 1985, 1986, Jaffe & McMahon-Pratt 1983, Jaffe et al. 1984, Shaw et al. 1986, Pan & McMahon-Pratt 1988, Hanham et al. 1990) were confirmed in our recent studies using a large sample (1,500 *Leishmania* stocks, isolated from humans and a variety of mammalian and sandfly hosts) from different localities in the New World. Included are results of our work as well as data from other recently published studies (Grimaldi et al. 1987, 1989, 1991, Aguilar et al. 1989, Barral et al. 1991, Darce et al. 1991, Falqueto et al. 1991, Hashiguchi et al. 1991, Kreutzer et al. 1991, Ponce et al. 1991, Bonfante-Garrido et al. 1992).

Qualitatively, the reactivity of the monoclonal antibodies did not show any variation related to time in culture, culture media, or parasite virulence (Grimaldi et al. 1987). However, variation in the sensitivity of the test may occur due to the type of screening assay used (i.e., immunofluorescence, RIA or the ELISA technique). As an exemple, the *L. donovani* group-specific epitope recognized by monoclonal antibody D2, using RIA (Jaffe et al. 1984, Grimaldi et al. 1987) or ELISA, is weakly detected by IFA. However, as indicated in previous studies (McMahon-Pratt et al. 1986, Grimaldi et al. 1987, 1991, Barral et al. 1991), independent of the origin of *Leishmania* stock (i.e., host species involved or the clinical state of the infection) or of geographic area of isolation, some of the monoclonal antibodies showed a high and consistent qualitative specificity at a species level. In these analyses (see Table), the following monoclonals were the most specific: D2 (LXXVIII, 2E5-A8) for *L. (L.) chagasi*; B11 (VII-5G3-F3) for *L. (V.) panamensis*; B18 (XIV-2A5-A10) for *L. (V.) braziliensis*; M3 (IX-5H9-C10) for *L. (L.) amazonensis*; and V1 (CLXXVI-3C11-F14) for *L. (L.) venezuelensis*. However, significant differences between the reactivity patterns with specific monoclonal antibodies could be observed among stocks from certain species complexes of *Leishmania* from distinct endemic areas. These differences can be related with strain variation in the level of expression of certain antigenic determinants, as recognized by some of the monoclonal antibodies. For instance, Venezuelan isolates of *L. (V.) braziliensis* showed a distinct profile (Bonfante-Garrido et al. 1992) when they were compared with the same parasite species which circulates in Bolivia, Brazil or Colombia (Barral et al. 1991, Grimaldi et al. 1991, Grimaldi & McMahon-Pratt, unpublished data). Also, the species-specific epitope recognized by monoclonal antibody B19 (XLIV-5A2-B9) (Grimaldi et al. 1987) could not be detected in some variant strains of *L. (V.) guyanensis* (Grimaldi et al. 1991), indicating that they had lost the epitope. In addition, some *L. (V.) braziliensis* isolates from the Brazilian Amazon Region (Grimaldi et al. 1991), as well as other variant strains of this parasite from Bolivia and Peru (Grimaldi & McMahon-Pratt, unpublished data) did not react with the specific monoclonal antibody B16 (XIII-3E6-B11) that identify this species (Shaw et al. 1986, McMahon-Pratt et al. 1986, Grimaldi et al. 1987, Barral et al. 1991). Furthermore, although naturally occurring hybrid parasites *(L. (V.) braziliensis* x *L. (V.) guyanensis)* from Venezuela reacted with the specific monoclonal antibodies (B16 and B18) for *L. (V.) braziliensis* (Bonfante-Garrido et al. 1992, Grimaldi & McMahon-Pratt, unpublished data), conflicting results were obtained when the *L. (V.) braziliensis* x *L. (V.) panamensis* hybrids from Nicaragua were analyzed by the same method (Darce et al. 1991). Indeed, these monoclonals as

well as the *L. (V.) panamensis*-specific monoclonal antibody (B11) did not react with the later hybrid isolates, indicating that they had lost the epitopes specific to both the parental species (Momen et al. 1993). On the other hand, numerical analysis of the enzymic profiles of *L. (L.) venezuelensis* isolates showed that this species was phenetically closely related to the WHO *L. (L.) mexicana* reference strain. However, these strains did not react with any of the monoclonal antibodies group-specific for *L. mexicana* complex parasites, other than the species-specific (V1) monoclonal antibody (Bonfante-Garrido et al. 1992).

As aforementioned, not all known species of *Leishmania* are recognized by a distinct/specific monoclonal antibody (Grimaldi et al. 1987, 1991, 1992, Kreutzer et al. 1991). However, the patterns observed with the less specific monoclonals (as defined by either qualitative or quantitative reactions with the expressed antigens) are indicative of these species. For example, monoclonal antibodies B3 (VI-4D10-D12) and B12 (XIII-3H6-A12) were found to be useful in the confirmation of *L. (V.) braziliensis* from Argentina, Bolivia, Brazil, Colombia, Nicaragua and Peru (Grimaldi et al. 1987, Barral et al. 1991, Grimaldi & McMahon-Pratt, unpublished data) or *L. (V.) panamensis* from Colombia, Costa Rica, Ecuador, Honduras and Nicaragua (Grimaldi et al., 1987 Grimaldi & McMahon-Pratt, unpublished data). In contrast, these monoclonals were not reactive in RIA tests with stocks of *L. (V.) guyanensis, L. (V.) shawi, L. (V.) lainsoni, L. (V.) colombiensis* or *L. (V.) equatorensis* regardless of geographic origin (Grimaldi et al., 1991, 1992, Kreutzer et al. 1991, Bonfante-Garrido et al. 1992, Grimaldi & McMahon-Pratt, unpublished data). In addition, although B4 (VI-2 A5-A4) crossreacted with *L. (V.) panamensis, L. (V.) colombiensis* and *L. (V.) equatorensis* (Kreutzer et al. 1991, Grimaldi et al. 1992), the former species could be easily distinguished using in conjunction the more specific monoclonal antibody (B11). Moreover, certain of the quantitative antigenic variations occurring betwen groups or species often exceed that detected within each of these taxonomic groups (Grimaldi et al. 1987).

There are other unusual features about leishmaniases of the New World. Although American cutaneous leishmaniasis is usually caused by parasite species belonging to the *L. braziliensis* or *L. mexicana* complex (Lainson & Shaw 1987, Grimaldi et al. 1989), a few cases of the disease from Brazil (Momen et al. 1985) and Ecuador (Hashiguchi et al. 1991) have been associated with a parasite similar to the Old World *L. (L.) major.* Interestingly, these *L. major*-like parasites cross-reacted with several monoclonal antibodies (T1, XLVI-5B8-A8; T2, XLVI-4H12-C2; T3, XLVI-5A5-D4; T4, LXVIII-1A4-G1; and T8, LXVII-3E12-F8) (Momen et al. 1985, Hashiguchi et al. 1991) produced against members of the *L. major* or *L. tropica* complex (Jaffe & McMahon-Pratt 1983). In addition, our experience would indicate the existence of a number of other leishmanial parasites circulating in the Americas [e.g., *L. (V.) colombiensis; L. (L.) equatorensis; and L. (L.) venezuelensis*] that also cross-reacted with the *L. major* species-specific monoclonals (Kreutzer et al. 1991, Bonfante-Garrido et al. 1992, Grimaldi et al. 1992). Work is now in progress to better define the phylogenetic relationship between these parasites and Old World *L. (L.) major* strains. Whatever the explanation for the existence of these *L. major* related parasites, the results point to caution for all researchers working with New World *Leishmania* isolates. We recommend that when classifying these parasites, reference strains of Old World species as well as the *L. major*-specific monoclonals (e.g., the monoclonal T1, XIX-2D8-D7) be included for comparison.

Several monoclonal antibodies (D-2, B-4, B-5, B-7, B-16, B-19, M-3, M-7, P-9, T-9) analyzed in this study were selected by the WHO Workshops and recommended for general use in the identification of *Leishmania* species. The analyses of strains brought by the participants of the Cali Workshop also pointed strongly to the need for the incorporation of additional monoclonals (e.g., B-3, B-11, B-12, B-18, V-1) in an expanded crosspanel. We should mention that a free "Monoclonal antibody kit" for diagnosis/identification of *Leishmania* species, consisting of lyophilized aliquots (100 µl) of the monoclonal antibodies (titers 10^{-4} to 10^{-6}) will be available soon; as part of the kit, a description of methods (immunofluorescence and the ELISA technique) will also be provided. Requests for the kit will be made to Dr F Modabber, World Health Organization, Geneva, Switzerland. A formal request form indicating the potential application and resources available for analyses will be requested by WHO.

In conclusion, problems related to the differentiation and identification of some leishmanial parasites were encountered using serodeme analysis with specific monoclonal antibodies, as well as when those samples were analyzed by isoenzyme characterization (Grimaldi et al. 1987, 1989, 1991). Some of these isolates represent additional new species (Grimaldi et al. 1991, 1992, Kreutzer et al. 1991) or hybrid parasites (Darce et al. 1991, Bonfante-Garrido et al. 1992) and further investigation with new monoclonal antibodies is recommended in these situations. A comparison of the

TABLE

Radioimmune binding assay results, employing *Leishmania* species-or group-specific monoclonal antibodies, with representative strains of New World *Leishmania* species[a]

Species[b]	Stock Code	Country of Origin	Monoclonal antibodies[c]											
			CR	D2	B3	B4	B11	B12	B16	B18	B19	M3	M7	V1
L.chagasi	MHOM/BR/74/PP75	Brazil, Bahia	9.2	8.4										
L.chagasi	MCAN/BR/84/C17382	Brazil, Ceará	8.9	8.3										
L.chagasi	MCAN/BR/82/CCG3	Brazil, R. Janeiro	8.7	11.3										
L.chagasi	MHOM/HN/85/H-25	Honduras	8.0	10.6										
L.braziliensis	MHOM/BR/75/M2903	Brazil, Pará	14.3		76.4			21.0	26.7	26.5				
L.braziliensis	MHOM/BR/81/ALG	Brazil, R. Janeiro	13.6		48.6			32.5	31.5	28.6				
L.braziliensis	MHOM/PE/00/LH-15	Peru	10.4		26.4			24.8	13.8	16.8				
L.braziliensis	MHOM/VE/76/H-9	Venezuela	9.6					25.7	16.6	15.9				
L.braziliensis[d]	ICAR/BR/86/IM2978	Brazil, Rondônia	6.6		5.3			32.0		8.1				
L.braziliensis[e]	MHOM/BR/88/IM3482	Brazil, Amazonas	15.0					15.1		4.2				
L.braziliensis[f]	MHOM/BR/88/IM3483	Brazil, Amazonas	5.2					5.0		4.0				
L.panamensis	MHOM/PA/71/LS94	Panama	4.0		25.0	8.4	6.7	8.9						
L.panamensis	MHOM/CR/78/ICMRT72	Costa Rica	6.5		18.8	12.6	17.0	9.2						
L.panamensis	MHOM/HN/79/INC-4	Honduras	5.2		17.7	11.2	13.3	6.8						
L.panamensis	MHOM/EC/87/G-07	Ecuador	7.8		32.9	30.3	27.8	7.5						
L.guyanensis	MHOM/BR/75/M4147	Brazil, Pará	14.4								15.0			
L.guyanensis	MHOM/BR/88/IM3471	Brazil, Amazonas	4.4											
L.shawi	MCEB/BR/84/M8408	Brazil, Pará	12.0											
L.naiffi	MDAS/BR/78/M5169	Brazil, Pará	11.8					6.2						
L.lainsoni	MHOM/BR/81/M6424	Brazil, Pará	13.9											
L.colombiensis	IHAR/CO/85/CL500	Colombia	9.4											
L.equatorensis	MHOM/EC/82/Lsp1	Ecuador	11.3			18.4								
L. amazonensis	MDID/BR/87/IM3217	Brazil, Amazonas	8.5									8.6	4.6	
L.amazonensis	MPOT/EC/87/G-03	Ecuador, Los Rios	6.0									11.3	11.2	
L.amazonensis	MTAM/EC/87/G-04	Ecuador, Bolivar	5.9									12.6	9.9	
L.amazonensis	MHOM/VE/72/L44	Venezuela	7.8									12.0		
L.mexicana	MNYC/BZ/62/M379	Belize, Cayo	7.5										26.3	
L.mexicana	HOM/MX/83/VADYCV	Mexico	6.7										5.4	
L.mexicana	MHOM/EC/88/Pautel	Ecuador, Azuay	7.8										6.6	
L.pifanoi	MHOM/VE/60/LtRod	Venezuela	5.8											
L.venezuelensis	MHOM/VE/74/PM-H3	Venezuela, Lara	4.0											6.2
L.aristidesi	MORY/PA/68/GML	Panama	13.4											
L.foratinnii	MDID/BR/77/Conch.	Brazil, São Paulo	10.0											

a: n numbers shown are the ratios (counts/min with test preparations) / (counts/min with control preparations); see text. Values >3 (not printed) were classed as positive

b: identification also established by isoenzyme analysis

c: prepared from the following hybridoma clones: CR, G2D10 (this is a cross-reactive monoclonal antibody used as positive control throughout the experiments); D2, LXXVIII-2E5-A8; B3, VI-4D10-D12; B4, VI-2A5-A4; B11, VII-II-5G3-F3; B12, XIII-3H6-A12; B16, XIII-3E6-B11; B18, XIV-2A5-A10; B19, XLIV-5A2-B9; M3, IX-5H9-C10; and M7, LXVIII-1D7-B8

d-f: classified by numerical zymotaxonomical analysis as distinct zymodemes (IOC-32, IOC-33 and IOC-35, respectively) or strain variants of this parasite species (Cupolillo et al. 1994)

discriminatory ability of the two typing methods for *Leishmania* using Simpson's index of diversity showed that serodeme analysis is more discriminating, even though the zymodeme analysis produced more groups (Cupolillo et al. 1993). The continual discovery of new leishmanial species in tropical America is, in part, a reflection of the increasingly sophisticated methods for parasite differentiation. However, it also indicates that there has been a much greater evolutionary divergence among this parasite group in the New World, compared to the Old World.

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