Journal of Cellular Biochemistry 24:103–112 (1984)
Molecular Biology of Host-Parasite Interactions 135–144

Characterization of Isolates and Clones of Leishmania by Analysis of Kinetoplast DNA

Terry W. Spithill, Raelene J. Grumont, and Graham F. Mitchell

Laboratory of Immunoparasitology, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

The genetic characterization of pathogenic isolates of Leishmania was attempted by analysis of the molecular properties of kinetoplast DNA (kDNA) minicircles. Unit minicircle size is not conserved during speciation of Leishmania since the minicircles of strains and clones of L t major are smaller (700 bp) than those found in certain strains of L mexicana ssp (820 bp), L donovani (850 bp) or L t tropica (900 bp). Schizodeme analysis of minicircles reveals a high degree of sequence divergence in kDNA of Leishmania with the degree of microheterogeneity varying between species. This sequence divergence allows the discrimination of species, strains, and clones of Leishmania into schizodemes. Southern blot hybridization experiments reveal that at high stringency overall minicircle sequence homology is conserved among clones and strains of one species (L t major) but not between different species. This property of minicircle DNA permits the use of kDNA probes as a species-specific diagnostic test for the identification of unknown Leishmania isolates. The properties of kDNA from an L t tropica strain LRC-L32 (a "recidiva" organism) are so diverged from those of L t major strains as to support the classification [22,23] of L t tropica and L t major as separate species of Leishmania rather than subspecies of L tropica.

Key words: kinetoplast DNA, schizodeme analysis, minicircles, Southern blot hybridization, cell-dot blot hybridization, Leishmania, species, strain, clone characterization

The protozoan parasites of the genus Leishmania comprise a group of morphologically similar organisms which cause a range of disease patterns in man, and this clinical heterogeneity is thought to be determined by genetic heterogeneity in both the parasite and the infected host populations [1,2]. A genetic characterization of Leishmania is needed to determine the genetic diversity within the genus—ie to quantitate the number of species and subspecies, to determine the relatedness of isolates causing different disease states, and to study the heterogeneity of primary field isolates by the genetic comparison of clones.

Received April 5, 1983; accepted October 4, 1983.

© 1984 Alan R. Liss, Inc.

Restriction endonuclease fingerprinting of kinetoplast DNA (kDNA) has been proposed as a general method for classifying and distinguishing isolates and clones of kinetoplastid protozoa including Leishmania at the genotype level [3–5]. This approach is possible because Leishmania are members of the order Kinetoplastida, a group of protozoa possessing an unusual mitochondrial DNA structure termed kDNA [6–8]. In Leishmania, kDNA consists of two molecular components catenated into a network: the maxicircle DNA, a 30-kb circular DNA molecule which encodes mitochondrial genes [9,10] present at about 50 copies per cell, and the minicircle DNA, a 0.7- 0.9-kb circular DNA molecule which has no known coding function, present at about 10,000 copies per cell [9,11–13]. Previous work on sequence relationships among kDNA minicircles from different genera has shown that a kDNA network consists of a population of minicircles of different sequence and that the degree of sequence heterogeneity varies among different genera [6–8]. The occurrence of sequence heterogeneity within the major component of kDNA has been exploited as a genotypic marker to compare strains and clones of T cruzi [3,4,14], T brucei [7,15,16], Crithidia [5,19], cutaneous isolates of Leishmania [11,12], and other trypanosomatids [5,14].

We have initiated a genetic characterization of Leishmania isolates pathogenic to man (L tropica major, L tropica tropica, L donovani, L mexicana ssp) by studying the properties of isolated kDNA. The results indicate that extensive sequence divergence is occurring within kDNA from different Leishmania species, and this has allowed us to show that Leishmania species and subspecies responsible for different clinical phenotypes can be readily distinguished by kDNA analysis. In contrast, overall kDNA sequence is conserved within one species (L t major) and this permits the identification of related clones and strains of a species and forms the basis of a rapid diagnostic test for identifying unknown isolates of Leishmania.

MATERIALS AND METHODS

Parasites

Parasites were obtained from the WHO Reference Center for Leishmaniasis, Jerusalem, Israel (Table I). The parasites were maintained at 26°C in N.N.N. medium [17] or RPMI 1640 medium supplemented with Hepes buffer (25 mM, pH 6.75) and fetal calf serum (10% v/v). Large-scale cultures for DNA isolations were grown in 3–5 liters of RPMI 1640 medium either as spinner cultures or in P flasks.

Isolation and Analysis of Kinetoplast DNA

Isolation, restriction endonuclease digestion, and agarose or acrylamide gel analysis of kinetoplast DNA was performed as described [9,17]. For Southern blots, the agarose gel was treated as follows: 2 × 15 min in 0.25 M HCl, 90 min in 1 M NaOH, 120 min in 3 M NaCl, 1 M Tris-HCl, pH 7.5; 20 min in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na Citrate, pH 7.0). Blotting was performed for 16 hr with 20 × SSC as buffer. The nitrocellulose filter blot was rinsed in 2 × SSC and baked in vacuo at 80°C for 3 hr. Hybridization of nick-translated kDNA probes to DNA on the nitroicellulose filter was performed as described using 1 × 10^6 cpm probe/ml [17]. The filter was washed at high stringency in 0.2 × SSC, 0.1% SDS at 65°C for 4 hr (corresponding to $t_m - 12$ for minicircles of 46% GC) and autoradiography was performed at −80°C with intensifying screens using preflashed film.

TABLE I. Strains of Leishmania Studied

WHO-LRC Number	Other designations	Species[a]	Origin	Disease
L137	WR260, LV561	L t major	Man, Israel, 1967	Cutaneous
L137/7/V121[b]		L t major	Mice, Australia, 1981	Cutaneous
L251		L t major	Man, Israel, 1979	Cutaneous
L38	LV39, P	L t major	Wild animal, USSR, 1959	Cutaneous
L32	LV142	L t tropica (minor)	Man, Iraq, 1965	Recidivans
L94		L mexicana[c] ssp	Man, Belize, 1958	Cutaneous
L52	LV557, WR352, SC23	L donovani	Man, India, 1954	Visceral

[a]L t major and L t tropica (minor) are considered by many workers to be separate species—viz L major and L tropica, respectively [22]—although the trinomial designation is still in use.
[b]For further description of this clone, see reference 17.
[c]Strain LRC-L94 was isolated from a simple cutaneous lesion and is probably L mexicana mexicana, but this is not certain (Dr L. Schnur, personal communication).

Dot Blot Hybridization

Promastigotes (10^4–10^6 cells in 2 μl volume) were dotted onto nitrocellulose filters, dried in air and processed [18]. Hybridization to kDNA probes was performed as described [17] using 1×10^6 cpm of probe/ml and the filters were washed at high stringency as described above.

RESULTS

Kinetoplast DNA was isolated from various strains of different Leishmania species (L donovani, L52; L mexicana ssp, L94; L t tropica, L32), several strains of L t major (L137, L38, L251), and one clone derived from L t major strain L137 (clone 121) which has previously been shown to be virulent in mice (Table I) [17]. When digested with endonuclease EcoRI, unit length minicircles of different sizes are released from the kDNA networks and are resolved by low-voltage agarose gel electrophoresis into a single major DNA band (Fig. 1). It is evident that clones and strains of L t major possess the same size minicircle (700 bp) which is smaller than that found in L mexicana ssp (820 bp), L donovani (850 bp), or L t tropica (900 bp). Thus, unit minicircle size is conserved within several strains of one species of Leishmania (L t major) but has diverged considerably in particular strains of other species. In results to be presented elsewhere [28] we have recently found that the EcoRI cut minicircles actually consist of a population of molecules which can be resolved under different electrophoresis conditions.

A more detailed comparison of isolated kDNAs was obtained by fine-structure fingerprint or schizodeme analysis of endonuclease Taq 1 digests of kDNAs fractionated by gradient acrylamide gel electrophoresis (Fig. 2). With these digests, sequence heterogeneity in minicircle DNA is revealed since the network DNA is not totally digested and the fragments that are released are resolved into nonequimolar bands, the sum of the molecular weights of which is several times the size of the unit length minicircle. This sequence heterogeneity is more pronounced in kDNAs from strains

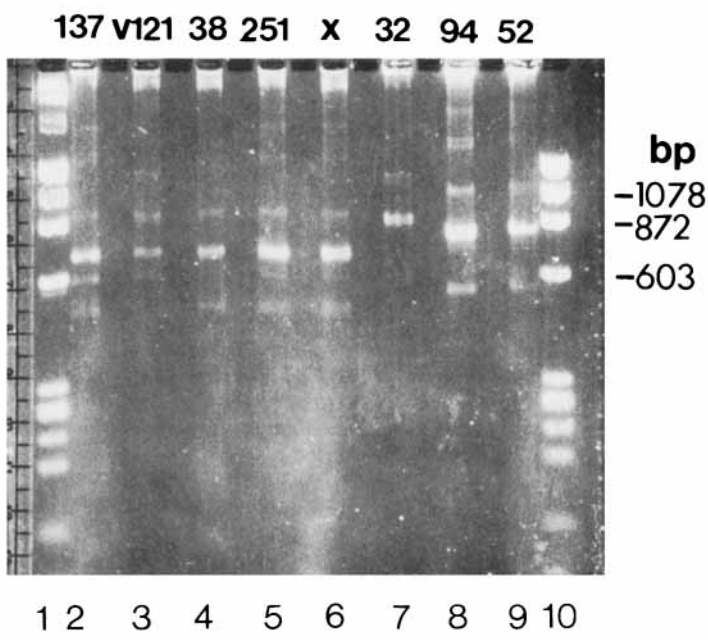

Fig. 1. Agarose gel electrophoresis of EcoRI digests of kDNAs from various species of Leishmania. DNA fragments were fractionated in a 2% agarose gel at low voltage. Lanes 1, 10) Hind III digest of λ DNA and Hae III digest of ØX174 DNA. Lanes 2–9) kDNAs from strains L137; virulent clone L137/7/V121; L38; L251; X = kDNA from a stock originally designated L285 which now types as L251 (see Discussion); L32; L94; L52, respectively. Molecular weight in base pairs (bp) indicated on the right.

of L t major, where there are about 21–30 major sequence classes released by Taq I compared with about 4–8 different major sequence classes in kDNAs of other Leishmania species (legend, Fig. 2). It is clear that schizodeme analysis of kDNA distinguishes the various species and even strains of one species (L t major). In addition, schizodeme analysis of kDNA from clones of L t major strain L137 has previously shown that these clones are all closely related based on the very similar fingerprints obtained [17]. These results demonstrate that extensive sequence divergence is occurring within minicircles of various Leishmania species.

Schizodeme analysis provides information on the extent of microheterogeneity with respect to particular restriction endonuclease recognition sites. To study overall sequence homologies within minicircles, kDNAs from the various species were digested with EcoRI, fractionated on agarose gels (Fig. 3), and blotted onto nitrocellulose filters. The blots were hybridized to nick-translated probes made with total kDNA from various strains of different species (L137, L32, L94, or L52), and hybrids stable to a high stringency wash (0.2 × SSC, 65°C, 4 hr) were detected by autoradiography. As shown in Figure 3, each kDNA probe hybridized specifically only to minicircles from the same species, with the L t major L137 probe sharing sequence homologies with another L t major strain L251. These results clearly demonstrate that during speciation of Leishmania, minicircle sequences have diverged to such an extent that there is no conservation of homologous sequences of sufficient length to give stable hybrids at high stringency. In contrast, different strains of L t

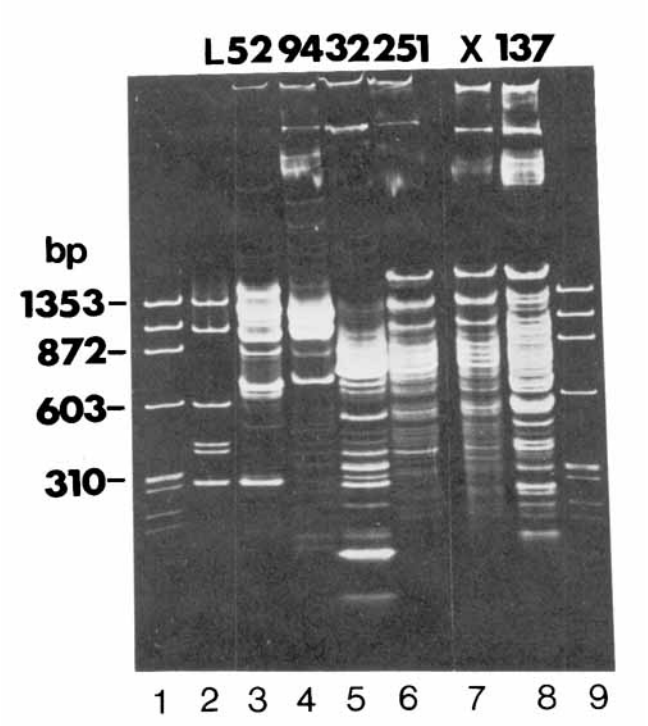

Fig. 2. Gradient acrylamide gel electrophoresis of Taq I digests of kDNAs from various species of Leishmania. DNA fragments were fractionated in a 3.5–10% acrylamide gel. Lanes 1,9) Hae III digest of ØX174 DNA. Lane 2) PstI/TaqI digest of pBR322 DNA. Lanes 3–8) kDNAs from strains L52, L94, L32, L251, X (see Fig. 1), L137, respectively. Molecular weights in bp indicated on the left. The number of major sequences classes was derived from the sum of the molecular weights of the major DNA fragments and the size of the unit length minicircle for each strain. Since minicircle size is greater in acrylamide gels than agarose gels two values for the number of major sequence classes are calculated: L137, 21–27; L251, 23–30; X, 23–30; L32, 5–7; L94, 4–5; L52, 6–8. These values are underestimates of the total number of sequence classes in these strains since minor classes released by Taq I are visible only when longer exposures are used and Taq I does not cut all minicircles in the networks.

major have conserved highly homologous minicircle sequences, and the same is true for minicircles of the various clones of strain L137 [17].

This distinctive species-specific nature of the cross hybridization with total kDNA probes suggested that such hybridization to kDNA in whole cells may form the basis of a diagnostic test for Leishmania [18]. To test this, promastigotes of the various species of Leishmania were dotted directly onto nitrocellulose filters, dried, lysed in situ and the liberated kDNA hybridized to nick-translated probes made from total kDNA. At high stringency, the hybridization of a kDNA probe from L t tropica L32 was highly specific with no crosshybridization to DNA from other species (Fig. 4). The kDNA probe from L donovani L52 was species-specific at 10^5 cells/dot although some cross hybridization to DNA from other L t major strains was evident at 10^6 cells/dot (Fig. 4). The kDNA probe from L t major L137 clearly recognized the homologous cell dot as well as other strains of L t major, but also showed a degree of crosshydridization with DNA from L donovani L52 (Fig. 4). In all cases, however, the homologous hybridization of DNA in cell dots with the different kDNA

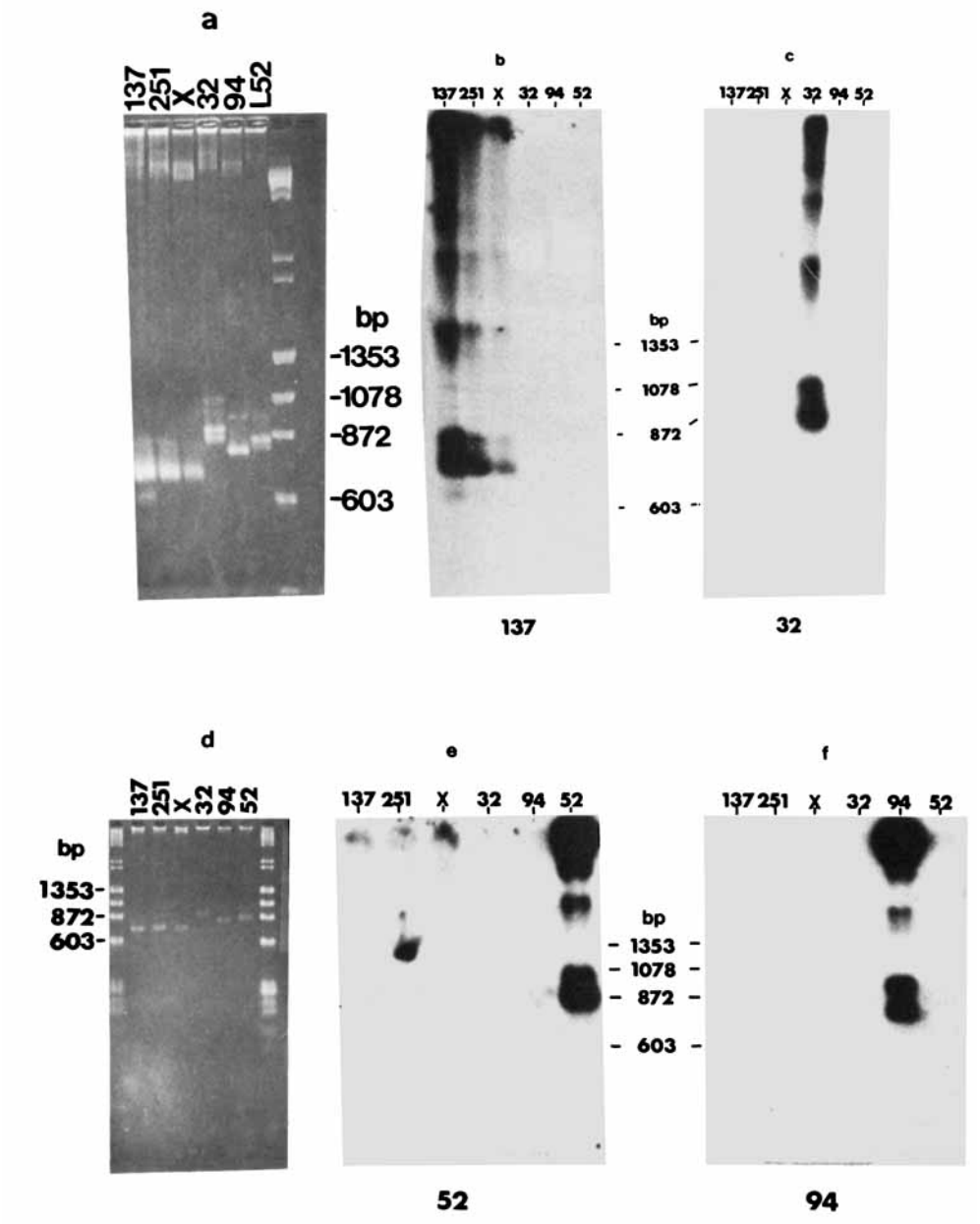

Fig. 3. Southern blot analysis of sequence homologies between kDNAs from different species of Leishmania. EcoRI digests of kDNAs were fractionated in 2% agarose gels at high voltage (panels a, d) and the DNA blots were hybridized to kDNA probes from strains L137 (b), L32 (c), L52 (e), and L94 (f). Hybrids stable at high stringency were detected by autoradiography. The source of kDNA in each lane is indicated above the figure (X; see legend, Fig. 1); the probe used is indicated below the figure. Molecular weights in bp.

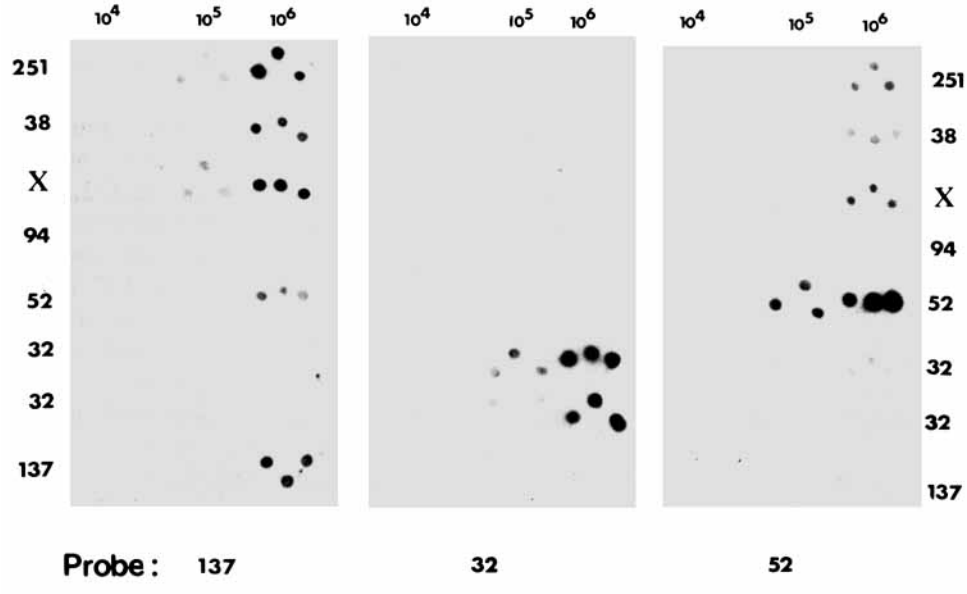

Fig. 4. Dot blot hybridization of kDNA probes to DNA from promastigotes of Leishmania. Promastigotes (10^4–10^6 cells) were dotted in triplicate onto three nitrocellulose filters, processed, and hybridized to kDNA probes from strains L137, L32, or L52 as indicated. Hybrids stable at high stringency were detected by autoradiography for 2 hr. The source of cells is shown beside the figure (X; see legend, Fig. 1).

probes was readily detectable after autoradiography for 2 hr and, with longer exposures, specific detection of homologous cells was possible to a level of 10^4 cells/dot. These results suggests that a species-specific diagnostic test for the identification of unknown Leishmania isolates is possible using kDNA probes.

DISCUSSION

The genetic characterization of clones, strains, and species of Leishmania has been attempted by employing the molecular properties of kDNA minicircles as genotypic markers. The analysis of kDNA from different species of Leishmania has shown that extensive sequence divergence has occurred in minicircles during speciation such that minicircles from different species no longer cross-hybridize under stringent conditions and even differ in unit length size. This is true for species from the Old World causing different disease phenotypes (L t major, L t tropica, L donovani) or species from the Old or New World causing a similar cutaneous disease (L t major, L mexicana ssp). Similarly, it has recently been shown that minicircles from L braziliensis ssp and L mexicana ssp [18], as well as two other strains of L t tropica and L t major [12], do not cross-hybridize under moderately stringent conditions. Thus, species-specific hybridization between minicircles appears to be a general property of kDNA from Leishmania and is a useful marker for discriminating dif-

ferent species. Variation in minicircle size may also be a general characteristic of species of Leishmania, but the analysis of kDNA from other strains of these species is needed to prove this. Note, however, that the sizes of the minicircles in other strains of L t major (700 bp) and L m mexicana (800 bp) [11,12] are similar to our results.

The results presented here raise serious doubts concerning the classification of L t tropica strain L32 as a subspecies of L tropica presumptively related to L t major and support the classification of L t tropica and L t major as separate species [22,23]. The kDNA of strain L32 differs from that of L t major strains in unit minicircle size and number of major minicircle sequence classes, and exhibits no detectable overall sequence homology with kDNA of L t major. Thus, L t tropica strain L32 is as diverged from L t major as L donovani or L mexicana ssp. These properties are in contrast to those exhibited by kDNAs from subspecies of L braziliensis [18] and C fasciculata [19], which do share intraspecific sequence homology. Subspecies of C fasciculata [20] and T brucei [16] also share minicircle size. Previous work using other biochemical markers has also supported the separation of L t tropica and L t major into separate species [24–27]. The work reported here provides additional genotypic evidence for this taxonomic separation.

The schizodeme analysis of kDNA has revealed that different species of Leishmania exhibit different degrees of microheterogeneity in minicircles, the number of major sequence classes released from networks with endonuclease Taq I varying from about four to eight for L donovani, L mexicana ssp, and L t tropica to about 21 to 30 for L t major. However, the total number of sequence classes released by Taq I (including minor classes) is more comparable, being about 24–35 for L t major and 16–25 for the other species. It appears then that kDNA networks in L t major predominantly consist of at least 20–30 different sequence classes (unique minicircles) present at high copy number whereas networks in the other species consist of a few (4–8) dominant sequence classes present at high copy number as well as several minor sequence classes present in much lower abundance. This degree of heterogeneity is greater than that seen with kDNA from L tarentolae [13], comparable to that observed with kDNA from C fasciculata [6,7,19] but very much less than the 200–300 sequence classes observed in kDNA from T brucei [6–8].

In contrast to the situation with kDNA from different species, the properties of kDNA from clones and strains of one species do indeed permit the identification of close relationships. Clones and strains of L t major share unit minicircle size, exhibit extensive overall sequence homology within minicircle DNA, and can be distinguished only by differences in their kDNA fingerprints. In the case of the various clones, these differences were minor, suggesting that all the clones were very closely related and that the avirulent clones were genetic variants derived recently from the virulent clone [17]. These results suggest that crosshybridization between kDNAs is a valid marker for grouping strains of one species of Leishmania. This is also true for strains and subspecies of L braziliensis [18]. In addition, the detection of overall sequence homologies between kDNAs has recently been used to establish relationships between reference strains and unknown field isolates of Leishmania [11,12].

The conservation of minicircle sequence homology among clones and strains within a single species of Leishmania, together with the complete lack of such homology at high stringency between different species, provided an approach to developing

a rapid diagnostic test for classifying unknown field isolates of Leishmania [18]. In situ hybridization of total kDNA probes to DNA released from promastigotes dotted onto nitrocellulose filters appears to be sufficiently discriminating to allow the identification of different Leishmania species at a cell concentration of at least 10^4/dot. Our results show that dot hybridization has the potential to distinguish the L t major group of parasites from the pathogenic L donovani and potentially pathogenic L t tropica groups, but the analysis of other strains of these species is needed to prove this. Optimization of the cell dot hybridization procedure by the use of highly purified kDNA or cloned minicircle sequences as probes should provide a specific, sensitive, and rapid diagnostic test. Similar experiments with various subspecies of the New World species L braziliensis and L mexicana have previously shown that these species can also be distinguished by dot hybridization with kDNA probes and that L mexicana amazonensis and L t major kDNAs exhibit very weak crosshybridization in cell dots [18].

One question that needs to be addressed concerns the problem of whether classifications based on comparisons of a mitochondrial DNA such as kDNA genuinely reflect relatedness at the genomic DNA level. This appears to be true with T brucei stocks [16]. Our preliminary results comparing the structure of restriction endonuclease sites around ribosomal RNA genes in Southern blots of genomic DNA from Leishmania indicate that the clones of L t major strain L137 all share a similar fragment pattern which is different from the unique patterns exhibited by L donovani and L t tropica. It therefore appears that close relationships among Leishmania determined by analysis of kDNA minicircles may indeed reflect similarities in genomic DNA sequences.

Finally, we wish to clarify our data on isolate LRC-L285, a diffuse cutaneous isolate of unknown identity. We originally suggested (Abstract, these proceedings) that L285 was an L t major isolate based on the ability of kDNA from our L285 stock to hybridize to kDNA from strain L137 at high stringency. It now seems certain that our stock of L285 has been contaminated with L t major strain L251. Our original cultures of L285 yielded kDNA, which gave a unique fingerprint with endonuclease Hpa II and exhibited a unit minicircle size of about 800 bp. Different kDNA fingerprints have been obtained with kDNAs from later cultures that are very similar to fingerprints of L251 (Fig. 2). These kDNAs exhibit a smaller minicircle size (700 bp, Fig. 1) and exhibit extensive sequence homology with kDNA from L t major strain L137 (Figs. 3, 4). We infer that our current stock of L285 is in fact L251. Therefore, we cannot make a definitive statement regarding the identity of L285. Our results do emphasize the need for extreme caution in culturing stocks of Leishmania and also show the efficacy of using schizodeme analysis to identify unknown isolates of Leishmania [cf 21].

ACKNOWLEDGMENTS

This work was supported by the Australian National Health and Medical Research Council, the Rockefeller Foundation Great Neglected Diseases Program, and the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

REFERENCES

1. Belehu A, Louis JA, Pugin P, Miescher PA: In Miescher PA, Mueller-Eberhardt HJ, Krause R (eds): "Immunopathology of Parasitic Diseases." Berlin: Springer-Verlag, 1980.
2. Mauel J, Behin R: In Levandowsky M, Hutner SH (eds): "Biochemistry and Physiology of Protozoa." New York: Academic Press, 1981, Vol 4, p 385.
3. Mattei DM, Goldenberg S, Morel C, Azevedo HP, Roitman I: FEBS Lett 74:264, 1977.
4. Morel C, Chiari E, Plessmann Camargo E, Mattei DM, Romanha AJ, Simpson L: Proc Natl Acad Sci USA 77:6810, 1980.
5. Plessmann Camargo E, Mattei DM, Barbieri CL, Morel CM: J Protozool 29:251, 1982.
6. Borst P, Hoeijmakers JHJ: Plasmid 2:20, 1979.
7. Borst P, Hoeijmakers JHJ: In Cummings D, Borst P, Dawid I, Weissman S, Fox CF (eds): "Extrachromosomal DNA." New York: Academic Press, 1979.
8. Englund PT, Hajduk SL, Marini JC: Annu Rev Biochem 51:695, 1982.
9. Simpson L: Proc Natl Acad Sci USA 76:1585, 1979.
10. Simpson L, Spithill TW, Simpson AM: Mol Biochem Parasitol 6:253, 1982.
11. Arnot DE, Barker DC: Mol Biochem Parasitol 3:47, 1981.
12. Barker DC, Arnot DE, Butcher J: In Chance ML, Walton BC (eds): "Biochemical Characterization of Leishmania." Geneva: WHO, 1982.
13. Challberg SS, Englund PT: J Mol Biol 138:447, 1980.
14. Frasch ACC, Goijman SG, Cazzulo JJ, Stoppani AOM: Mol Biochem Parasitol 4:163, 1981.
15. Borst P, Fase-Fowler F, Hoeijmakers JHJ, Frasch ACC: Biochim Biophys Acta 610:197, 1980.
16. Borst P, Fase-Fowler F, Gibson WC: Mol Biochem Parasitol 3:117, 1981.
17. Handman E, Hocking RE, Mitchell GF, Spithill TW: Mol Biochem Parasitol 7:111, 1983.
18. Wirth DF, McMahon Pratt D: Proc Natl Acad Sci USA 79:6999, 1982.
19. Hoeijmakers JHJ, Borst P: Plasmid 7:210, 1982.
20. Hoeijmakers JHJ, Schoutsen B, Borst P: Plasmid 7:199, 1982.
21. Goncalves AM, Chiari E, Deane MP, Carneiro M, Romanha AJ, Morel CM: In Newton BA (ed): "Application of Biochemical and Molecular Biology Techniques to Problems of Parasite and Vector Identification." Geneva: WHO, 1982 (in press).
22. Bray RS: Annu Rev Micro 28:189, 1974.
23. Bray RS, Ashford RW, Bray MA: Trans R Soc Trop Med Hyg 67:345, 1973.
24. Chance ML, Peters W, Shchory L: Ann Trop Med Parasitol 68:307, 1974.
25. Gardener PJ, Chance ML, Peters W: Ann Trop Med Parasitol 68:317, 1974.
26. Lanotte G, Rioux JA, Maazoun R, Pasteur N, Pratlong F, Lepart J: Ann Parasitol Hum Comp 56:575, 1981.
27. Schottelius J: Z Parasitenkd 66:237, 1982.
28. Spithill TW, Grumont RJ: Mol Biochem Parasitol, 1984 (in press).