Elsevier

MOLBIO 01193

Chromosome size and number polymorphisms in *Leishmania infantum* suggest amplification/deletion and possible genetic exchange

Michel Pagès[1], Patrick Bastien[2], Francisco Veas[2], Valérie Rossi[1], Michel Bellis[1], Patrick Wincker[1], Jean-Antoine Rioux[2] and Gérard Roizès[1]

[1]*Centre de Recherche de Biochimie Macromoléculaire du CNRS et Unité 249 de l'INSERM, Montpellier, France and* [2]*Laboratoire d'Ecologie Médicale et Pathologie Parasitaire, CNRS Faculté de Médecine, Montpellier, France*

(Received 13 January 1989; accepted 30 March 1989)

We have studied the molecular karyotypes of 21 strains and 14 clones of *Leishmania infantum* using pulsed field gel electrophoresis (PFGE). We detected a high degree of polymorphism within this species, with 'strain-specific' patterns for most isolates, even within a restricted endemic area. Variations relate to both the size of chromosomes (270–2600 kb) and their number, which can vary from 24 to 31 between closely related isolates. This polymorphism does not correlate with isoenzyme analysis. Small size variations between homologous chromosomes of different strains are suggestive of DNA amplification/deletion events. Strains are also shown to be multiclonal, with slight differences between most clones, but with a predominant clone concealing the others in PFGE analysis. The analysis of these data leads to the hypothesis of occasional genetic exchange by nuclear fusion in *Leishmania*, as recently shown in the related protozoan *Trypanosoma brucei*.

Key words: Chromosomal rearrangement; Genetic exchange; *Leishmania*; Molecular karyotype; Pulsed field gel electrophoresis

Introduction

The protozoan parasite *Leishmania infantum* is the causative agent of anthropo-zoonotic visceral/cutaneous leishmaniasis, an important public health problem in the Mediterranean basin, Africa and Latin America. Like other trypanosomatids, it undergoes a complex life cycle, here involving transmission by a sandfly vector to man or a canidae reservoir, in which it penetrates histiocytes and macrophage cells, leading to a fatal disease. The *L. infantum* complex has been well characterized by epidemiological [1] and biochemical [2] criteria. Isoenzyme analysis has made it possible to distinguish isoenzyme variants, mostly cutaneous and benign, from the main zymodeme (MON-1 or LON-49), mainly responsible for visceral disease over an extensive area [3,4]. However, if the epidemiology and life cycle of the parasite are being elucidated, the basic genetics remain obscure, e.g., the existence of a sexual and/or genetic exchange, the ploidy, the genome structure. Due to the absence of chromosome condensation during mitotic division, the chromosomal structure and organization of the genome have not been studied until recently. The advent of techniques allowing separation of large DNA molecules, i.e., pulsed field gel electrophoresis (PFGE) [5] and orthogonal field alternation gel electrophoresis (OFAGE) [6] has lead many workers to explore this field with various protozoa. Van der Ploeg et al. [7] and Gibson et al. [8,9] first demonstrated variations in chromosome size and chromosomal distribution of selected genes between several species of kinetoplastid parasites by PFGE. Other workers [10–12] have shown molecular karyotype polymorphisms between a wide variety of *Leishmania* species and between isolates of the same species from well

Correspondence address: Michel Pagès, Centre de Recherche de Biochimie Macromoléculaire du CNRS et Unité 249 de l'INSERM, Boite Postale 5051, F-34033 Montpellier Cedex, France.

Abbreviations: PFGE, pulsed field gel electrophoresis; OFAGE, orthogonal field alternation gel electrophoresis.

0166-6851/89/$03.50 © 1989 Elsevier Science Publishers B.V. (Biomedical Division)

ORSTOM Fonds Documentaire
N° : 31.355 ex1
Cote : B
22 FEV. 1991

separated geographical areas. The significance of this polymorphism remains unknown.

Here, we analyse the molecular karyotypes of 35 strains and clones of *L. infantum* (Table I) by PFGE. We report a high degree of polymorphism within this sole complex in a restricted endemic area. This polymorphism does not correlate with isoenzymic variation. It affects both the size and the number of chromosomal bands. Analysis of the results leads to a hypothesis of unusual genetic exchange in these organisms.

Materials and Methods

Parasites. The 21 strains of *L. infantum* examined are listed in Table I, as well as 14 clones issued

TABLE I

List of *L. infantum* strains examined

Strain	W.H.O. code	Zymodeme MON-	Host	Infection	Clone of	Place of isolation
LEM75	MHOM/FR/78/LEM75	1	*Homo sapiens*	VL	–	France (Hérault)
LEM1163	MHOM/FR/87/LEM1163-CLO	1	–	–	LEM75	France (LEM)
LEM189	MHOM/FR/80/LEM189	11	*H. sapiens*	CL	–	France (Pyrénées-Or.)
LEM1135	MHOM/FR/87/LEM1135-CLO	11	–	–	LEM189	France (LEM)
LEM1136	MHOM/FR/87/LEM1136-CLO	11	–	–	LEM189	France (LEM)
LEM1137	MHOM/FR/87/LEM1137-CLO	11	–	–	LEM189	France (LEM)
LEM236	MHOM/IT/79/ISS7	27	*H. sapiens*	VL	–	Italy (Arola)
LEM244	MCAN/FR/81/LEM244	1	*Canis familiaris*	VL	–	France (Pyrénées-Or.)
LEM1279	MCAN/FR/87/LEM1279-CLO	1	–	–	LEM244	France (LEM)
LEM1280	MCAN/FR/87/LEM1280-CLO	1	–	–	LEM244	France (LEM)
LEM1281	MCAN/FR/87/LEM1281-CLO	1	–	–	LEM244	France (LEM)
LEM1282	MCAN/FR/87/LEM1282-CLO	1	–	–	LEM244	France (LEM)
LEM1283	MCAN/FR/87/LEM1283-CLO	1	–	–	LEM244	France (LEM)
LEM250	MCAN/FR/81/LEM250	1	*C. familiaris*	VL	–	France (Pyrénées-Or.)
LEM251	MCAN/FR/81/LEM251	1	*C. familiaris*	VL	–	France (Pyrénées-Or.)
LEM1284	MCAN/FR/87/LEM1284-CLO	1	–	–	LEM251	France (LEM)
LEM267	MCAN/FR/81/LEM267	1	*C. familiaris*	VL	–	France (Pyrénées-Or.)
LEM272	MCAN/FR/81/LEM272	1	*C. familiaris*	VL	–	France (Pyrénées-Or.)
LEM274	MCAN/FR/81/LEM274	1	*C. familiaris*	VL	–	France (Pyrénées-Or.)
LEM307	MHOM/ES/81/LEM307	29	*H. sapiens*	CL	–	Spain (Catalonia)
LEM1100	MHOM/FR/87/LEM1100-CLO	29	–	–	LEM307	France (LEM)
LEM332	MHOM/FR/82/LEM332	1	*H. sapiens*	VL	–	France (Hérault)
LEM356	MHOM/FR/82/LEM356	33	*H. sapiens*	CL	–	France (Pyrénées-Or.)
LEM400	MHOM/FR/82/LEM400	29	*H. sapiens*	CL	–	France (Pyrénées-Or.)
LEM417	MHOM/DZ/82/LIPA59	24	*H. sapiens*	CL	–	Algeria (El Asnam)
LEM448	MHOM/FR/83/LEM448	29	*H. sapiens*	CL	–	France (Pyrénées-Or.)
LEM458	MHOM/ES/83/BCN2	28	*H. sapiens*	CL	–	Spain (Catalonia)
LEM1138	MHOM/FR/87/LEM1138-CLO	28	–	–	LEM458	France (LEM)
LEM1139	MHOM/FR/87/LEM1139-CLO	28	–	–	LEM458	France (LEM)
LEM1140	MHOM/FR/87/LEM1140-CLO	28	–	–	LEM458	France (LEM)
LEM511	MCAN/DZ/83/LIPA121	79	*C. familiaris*	VL	–	Algeria (Kabylie)
LEM538	MHOM/FR/84/LEM538	34	*H. sapiens*	CL	–	France (Pyrénées-Or.)
LEM622	MHOM/CF/00/LEM622	1	*H. sapiens*	VL	–	Center Africa Rep.
LEM765	MHOM/FR/85/LEM765	1	*H. sapiens*	VL	–	France (Corsica)
LEM1099	0000/FR/87/LEM1099	1	–	–	–	–

LEM, Laboratoire d'Ecologie Médicale (Montpellier); Zymodeme, population defined by a same isoenzyme profile (here, 15 enzymes). MON, code name for Montpellier (where these strains were characterized) as opposed for instance to LON for London zymodemes. The vast majority of strains isolated in de Mediterranean Basin are zymodeme MON-1 (LON-49). VL, visceral leishmaniasis; CL, cutaneous leishmaniasis. The strains from France (Pyrénées Orientales) and Spain (Catalonia) are from the same restricted endemic area.

from 6 of these strains. All strains and clones were checked for isoenzymes prior to our study (15 enzymes). Parasites were grown at 24°C in NNN medium, then passed to brain heart infusion/blood agar (Gibco) up to a final concentration of 7×10^8 ml^{-1}. Cloning of individual parasites was carried out using the 'hanging-drop' method [13].

DNA preparation and electrophoretic technique. Preparation of intact chromosomal DNA was as described [14]. The PFGE apparatus used has been described previously [15]. PFGE was carried out at 150 V for 72–96 h, with 1.5% agarose gels, using various combinations of pulse frequencies ranging from 40 to 150 s. for each gel. Yeast *(Saccharomyces cerevisiae)* chromosomes were used as size markers. Gels were stained with ethidium bromide, photographed in ultraviolet transillumination, and transferred onto nylon filters (Hybond N, Amersham) by alkaline transfer, according to the manufacturer's instructions.

Cloning of Leishmania DNA sequences. DNA from *L. infantum* LEM75 was digested with *Pst*I, and the resulting fragments were ligated into plasmid vector Bluescript (Stratagene). The recombinant plasmids were transformed into *Escherichia coli* TG1 by standard techniques [16]. 100 colonies harbouring recombinant plasmids were chosen at random and a 'mini-preparation' of DNA was performed [16]. Single or low-copy DNA fragments were selected by hybridisation using radiolabelled total DNA from LEM75 as a probe.

Hybridisation. Insert DNA probe was separated from plasmid DNA by *Pst*I digestion and electrophoresis, then radiolabelled as published [17]. Hybridisation/dehybridisation of nylon filters were performed according to the manufacturer's instructions. Filters were washed to a stringency of 2 × SSPE, 0.1% SDS at 65°C for 15–30 min.

Results

Analysis of the molecular karyotype of L. infantum strain LEM189

Since it was impossible to delineate a common karyotype for all the strains of the *L. infantum* complex, the clearly resolved karyotypes of strain LEM189 and clone LEM1136 (which are strictly identical) were selected as a reference (Figs. 1 and 3).

Chromosome number and genome size. Twenty-two non-stoicheiometrically staining bands are clearly individualized. Four have a 'double' staining intensity: Nos. 6, 8, 12 and 21. If each single and each 'double' band represented 1 and 2 chromosomes, respectively, the number of chromosomes in this strain would be 26: seven large chromosomes of 2600–1500 kb, 14 intermediate chromosomes of 1000–550 kb and five small ones of 550–300 kb. Note that the largest chromosomes are less then 2600 kb (yeast chromosome 12); this is true for all *L. infantum* strains examined, as well as for most Old and New World complexes (unpublished data). The minimum size of the haploid genome of this strain would then be about 26 Mb, i.e., 0.03 pg. This value is much less than the 0.12 pg DNA content/cell reported for *L. donovani* s.st. [18]. This observation leaves space for a diploidy or aneuploidy hypothesis (see Discussion).

Do Leishmania strains have a polyclonal structure? Isoenzymic data (i.e. the extreme scarcity of multiple banding patterns) [19], as well as the great stability of PFGE karyotypes of cloned strains maintained in culture in vitro over long periods (up to 155 days; see also ref. 11), suggest that *Leishmania* strains are homogeneous.

We have cloned our reference strain LEM189 and compared the molecular karyotypes of three of these clones: great homogeneity is noted between clones, and the karyotype of clone LEM1136 and the 'parent' strain are indistinguishable (Fig. 1); in view of the polymorphism existing elsewhere (see next section), this indicates a close kinship between the strain and the clones. However, a 1700-kb band absent from the parent strain and from the other clones is seen in clone 1137. This additional band was still present after four months of in vitro culture of this clone.

Data from 14 clones issued from six strains indicate that the presence of such an extra band (different for each case) in the karyotype of clones

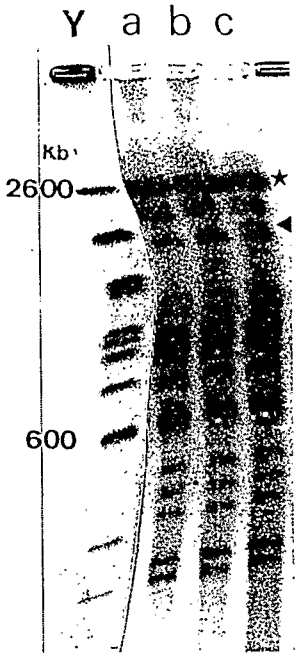

Fig. 1. Size estimation of chromosomes of *L. infantum* by pulsed field gel electrophoresis. Strain LEM189 (a) compared to clones LEM1136 (b) and LEM1137 (c). The arrow shows an extra chromosome present in LEM1137 only. Y, yeast DNA (*S. cerevisiae*). The pulse frequencies were 120 s for 40 h and 60 s for 40 h. The 1.5% agarose gel was run at 150 V, at a temperature of 15°C, stained with ethidium bromide and photographed under ultraviolet transillumination. The high-molecular-weight compression zone (star) can readily be resolved into 3 bands using a 150 s pulse time.

compared with the 'parent' strain is common: five out of 14 clones.

Two hypotheses can explain the origin of this extra chromosome/fragment; it could be the result of a chromosomal rearrangement following cloning and culture (as suggested in ref. 20 for *Trypanosoma brucei*); or it could pre-exist in a clone present in a lower proportion in the original strain. When the karyotypes of the strain and the clone differ (by this single band), all the other bands are identical in size and staining intensity. It therefore seems unlikely that this fragment results from another chromosome by rearrangement. Moreover, within this hypothesis, the frequency of this additional band in the clones contrasts with the total lack of rearrangements shown during prolonged subcultures. Therefore, we prefer the hypothesis of a 'polyclonal' structure of *Leishmania* strains, where a predominant clone would conceal closely related variant clones in PFGE analysis. The karyotypes of the strains cloned so far are representative of one clone, and not of a mixture of different clones.

Polymorphism of molecular karyotypes in L. infantum

PFGE analysis of 12 *L. infantum* strains collected in various geographical areas (Southern France, Corsica, Italy, Spain, Algeria and Central Africa) reveals a striking polymorphism, within zymodeme MON-1 (Fig. 2) as well as between different zymodemes (MON-1, 11 and 34; Fig. 3).

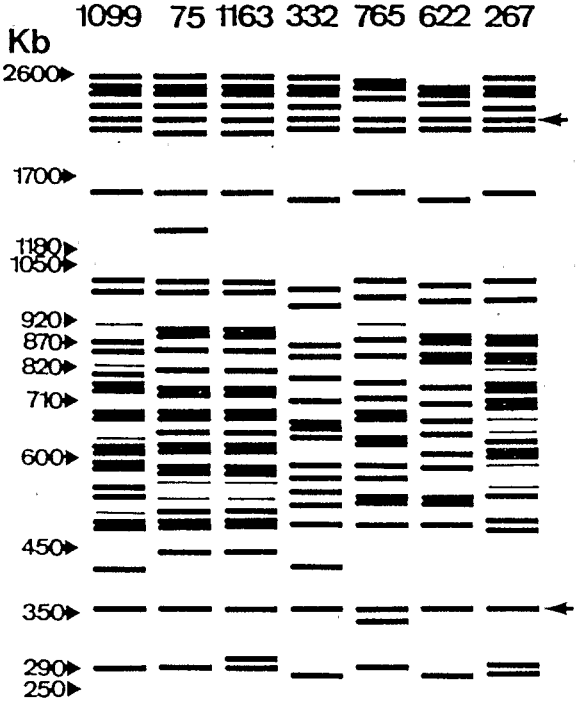

Fig. 2. Diagrammatic representation of the karyotypes of 6 strains and 1 clone of *L. infantum* zymodeme MON-1. The 250–2600-kb vertical scale is drawn from yeast DNA: it is not arithmetic and actually represents what is seen on several gels. The chromosomal bands of strain LEM75 were numbered from 1 to 24 in increasing size order. The karyotypes of LEM75 and its clone LEM1163 are very similar except that band No. 18 is missing from the clone; on the opposite, the clone has an extra small chromosome in second position. Only 2 bands are identical in all the strains (Nos. 2 and 21 of strain LEM75; arrows).

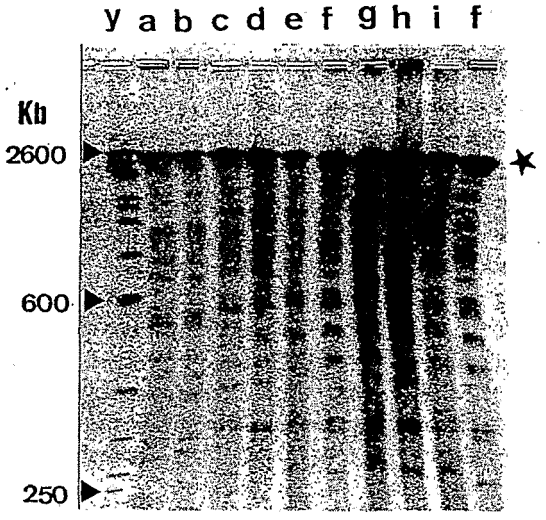

Fig. 3. Comparison of pulsed field gel electrophoresis karyotypes of several strains originating from a restricted endemic area. Photograph of an ethidium bromide-stained gel; pulse frequencies were 100 s/15 h. 80 s/14 h and 60 s/33 h. Y, *S. cerevisiae*; (a) LEM356; (b) LEM1136; (c) LEM250; (d) LEM272; (e) LEM538; (f) LEM244; (g) LEM251; (h) LEM267; (i) LEM274. Canine isolates (c,d,f,g,h,i) have complex and polymorphic karyotypes despite belonging to the same zymodeme (MON-1). In lanes a, b, d, e, f and h, the compression zone (star) represents 5 distinct chromosomal bands.

This is particularly obvious in the varying sizes and intensities of chromosomal bands, but it also affects the number of chromosomes (Figs. 2 and 3). The number of bands varies from 19 to 24, with four to seven 'double' bands, e.g., between LEM622 and LEM1163: thus, one can estimate that the number of chromosomes varies from 24 to 31 between all the strains examined. There is just as much polymorphism between different cutaneous isozymic variants (not shown). Therefore, it seems difficult to assign karyotypic features to a zymodeme or to a cutaneous/visceral phenotype.

Study of a restricted endemic area in the Pyrenees (Southern France)

In view of the karyotype polymorphism between strains from different geographical areas (whatever the zymodeme is), we investigated further the possibility of grouping neighbouring strains according to common karyotypic features.

We thus studied the molecular karyotype of 13 strains originating from a well-characterised restricted endemic area (Table I). Five strains (LEM189, 356, 400, 448 and 307) showed high karyotypic homogeneity, with 16 to 21 shared bands, although they belong to three different zymodemes (Fig. 3a,b). Note that these zymodemes differ by only three alleles of isozyme NP1. Variation between these strains (i.e., 4–5 bands) is only slightly higher than between clones from the same strains (0–2 bands). On the other hand, the eight other strains (zymodemes MON-1, 28 and 34) exhibit a surprising degree of polymorphism in their karyotypes (Fig. 3) and show 'complex' PFGE patterns, i.e., comprising more than 25 bands, with many poorly individualised bands and several 'double' bands. These 'complex' patterns cannot be due to a mixture of clones, since several clones issued from two of these strains (LEM244 and 251) exhibit karyotypes indistinguishable from that of the parent strain.

Finally, Southern blot hybridisations of the PFGE patterns of three of these strains (LEM244, 251 and 538) with two 'chromosome-specific' genomic DNA probes (see Materials and Methods) resulted in hybridisation to two neighbouring chromosomes for each probe, instead of one single chromosome for all the other strains examined (Fig. 4f and g). Note that this double band, present in strain L244 and clone L1281, is absent from clone L1282 (not shown). Therefore, these 'complex' patterns could be due to the presence of several pairs of homologous chro-

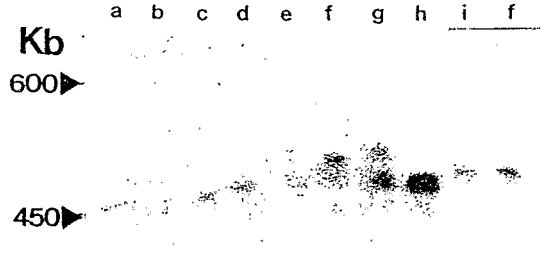

Fig. 4. Southern hybridisation analysis showing the chromosomal locations of a random genomic DNA probe. Strains as in Fig. 4. DNA probe isa106 hybridises with one 510-kb chromosome in LEM1136 (b); with chromosomes of neighbouring sizes (± 50 kb) in (a) LEM356; (c) LEM250; (d) LEM272; (e) LEM538; (h) LEM267; (i) LEM274; and with two in (f) LEM244; and (g) LEM251.

nosomes of different sizes; this again suggests neuploidy in *Leishmania*, at least for some trains.

Chromosome size polymorphism involves DNA amplification/deletion events

Two mechanisms can be considered to explain he chromosome size polymorphism within *L. infantum*: interchromosomal rearrangements such as translocation, or intrachromosomal events such as DNA sequence amplification/deletion.

Twenty-two single or low-copy DNA probes cloned at random from *L. infantum* whole genomic DNA were hybridised onto Southern blots of PFGE gels of 19 *L. infantum* strains. 14 chromosomes, from 300 to 2600 kb in size, were each hybridised by 1 to 4 distinct marker probes. These probes always hybridised to one chromosome (except for the three strains mentioned in the previous section), and for each probe, the hybridised chromosomes differed in size by only ±50 kb between the different strains (Fig. 4). Moreover, when two probes hybridise to the same chromosome in one strain, they are also associated on a single chromosome in the other strains.

This suggests homology between the hybridised chromosomes; these appear to exhibit only small size variations among different strains of the same complex. If large-scale interchromosomal exchanges occurred, a scattering of DNA probes over the whole karyotype pattern should be observed. This is not the case for the 22 probes examined. Therefore, it seems reasonable to propose that the chromosome size variations are mostly due to DNA sequence amplification/deletion events.

Discussion

In this study, the molecular karyotype of 21 strains and 14 clones of the *L. infantum* complex were analysed by PFGE. A high degree of polymorphism was shown, affecting not only the chromosome size but also their number. This polymorphism is as important within the main visceral zymodeme as between strains of different zymodemes. A low degree of intra-strain polymorphism is shown: a number of clones issuing from the same strains exhibited karyotypes slightly different from the 'parent' strain in one or two specific chromosomes. This suggests that *Leishmania* strains have an unusual polyclonal structure, with one clone predominant in PFGE analysis, and a close 'kinship' between clones. On the other hand, chromosome-specific DNA probes were found to hybridise on chromosomes differing in size by only ±50 kb between the different strains. Lastly, the study of a restricted transmission area in Southern France revealed (i) a high degree of karyotype homology between five strains belonging to three different zymodemes; and (ii) important polymorphism between the eight other strains, even between strains of the same zymodeme (MON-1). Thus, no correlation could be found between phenotype (zymodeme) and molecular karyotype.

This work raises the following points. (1) Since the sizes of homologous chromosomes vary little between strains of the same complex, the chromosome size polymorphism is probably due to intrachromosomal events like DNA sequence addition/deletion, rather than to interchromosomal rearrangements like translocation. Similar observations have been made for *Trypanosoma cruzi* [9] and *Plasmodium falciparum* [21]. In view of their amplification mode (unequal crossing-over), the tandem repeat DNA sequences might be best suited to such events.

(2) Variations in the chromosome numbers between biologically very close *Leishmania* strains raise the questions of the parasite's ploidy, of clonal evolution and of the existence of genetic exchange.

Leishmania have a clonal reproduction by binary fission, and neither genetic exchange nor any morphologically distinguishable sexual cells have ever been found. However, there are some arguments for the presence of occasional genetic exchange in these parasites [19,22,23].

Difficulties were encountered when trying to interpret the results shown here within the scope of an exclusively clonal development. Indeed, to explain how several clones from a same strain can differ by only one additional chromosome (the rest of the karyotype being indistinguishable) within the above hypothesis, one has to allow that a chromosome duplication has occurred followed by a size modification (by addition/deletion). This

event would lead to aneuploidy, with homologous chromosomes having different sizes; but it seems extremely unlikely. Moreover, to generate the high degree of polymorphism we observed between closely related strains, this type of complex event (duplication + addition/deletion) would have to occur very frequently during the parasite evolution. And with such a genome reorganisation leading to modifications in chromosome number, one would obviously expect interchromosomal translocation events as well, which we did not observe (see Results). Finally, it has been shown experimentally that there is great stability of the karyotype during strictly clonal development of a strain without exchange opportunities, in culture, in the sandfly vector (unpublished data) or in the mammal host [11].

These considerations lead up to examine the question of genetic exchange in *Leishmania* in the light of recent experimental crossing experiments carried out with *T. brucei* and *P. falciparum* in the insect vector. With *T. brucei* [24], the resulting hybrids possess 50% more nuclear DNA than the parents, which seems to be due to the addition of some chromosomal material from the two parents [20]. This DNA excess could be deleted during successive mitotic divisions in the mammalian host [25]. Similar experiments carried out between different *P. falciparum* clones produced hybrids which also exhibit new non-parental karyotypes [26]. Lastly, variations in the DNA content of up to 40% have been reported between clones of *T. cruzi* [27].

Although these facts have not been demonstrated in *Leishmania*, this model is consistent with our results. Indeed, the fusion of nuclear material within a synkaryon, followed by progressive partial deletions, could explain the chromosome size and number variations between biologically very close isolates from the same focus, as well as the DNA content variations (estimated as 15–20%, from our results). In other respects, the presence of several clones with slightly different karyotypes within the same isolate could represent the final stage of this process of 'simplification' from a complex hybrid karyotype, the additional chromosomes being eliminated.

Again, this interpretation suggests aneuploidy in these parasites. Spithill and Samaras [28] strongly suggested a 'polysomy for one chromosome in *L. major* (see also ref. 29), and Kemp et al. [30] a 'partial' diploidy for certain chromosomes in *P. falciparum*. Our results support the same hypothesis particularly in the fact that two chromosome-specific DNA probes for 16 strains examined each gave a double hybridization for three strains having a 'complex' karyotype (Fig. 4).

Conclusion. This study suggests the occurrence of occasional genetic exchange in *Leishmania*. Although Mendelian sexuality seems precluded, parasexuality remains a possibility. Such events, as well as the absence of morphologically distinguishable sexual cells, have been demonstrated in ciliates [31]. Multinucleate forms of *L. mexicana* have been reported in the sandfly midgut [32] and occasional merogony occurs in *T. lewisi* [31].

On the other hand, the striking polymorphism between karyotypes of clinically, epidemiologically and biochemically very close *L. infantum* strains stresses the need to study the heterogeneity of multiple strains of the same complex before stating the interest of PFGE for taxonomical use. However, this technique is certainly a powerful tool for the investigation of the genome structure and dynamics of *Leishmania*. Such work is in progress.

Acknowledgements

We thank Mmes. A. Martini and C. Rouquairol for technical assistance in parasite maintenance, Dr. F. Pratlong and Mlle. C. Durand for isoenzymic identification of the strains, and Drs. M. Taminh and C. Blaineau for stimulating discussions.

References

1 Rioux, J.A. (1977) Ecologie des Leishmanioses (Int. Coll. CNRS, Montpellier, 18–24 August, 1974), Editions CNRS, Paris.

2 Moreno, G., Rioux, J.A., Lanotte, G., Pratlong, F. and Serres, E. (1986) Le complexe *Leishmania donovani* s.l.

Analyse enzymatique et traitement numérique. Individualisation du complexe *Leishmania infantum*. In: (Rioux, J.A., ed.), *Leishmania*: Taxonomy and Phylogeny (Int. Coll. CNRS/INSERM, 2–6 July, 1984), pp. 105–117, Institut Méditérannéen d'Epidémiologie, Montpellier.

3 Rioux, J.A., Lanotte, G., Pratlong, F., Dereure, J., Jarry, D.M., Moreno, G., Killick-Kendrick, R., Périères, J., Guilvard, E., Belmonte, A. and Portus, M. (1985) La Leishmaniose cutanée dans le sud-est de la France. Résultats d'une enquête éco-épidémiologique dans les Pyrénées orientales. Med. Mal. Inf. 11, 650–656.

4 Pozio, E., Gramiccia, M., Gradoni, L. and Amerio, P. (1985) Isolation of the agent causing cutaneous leishmaniasis in Italy and its visceralization in inbred hamsters. Trans. R. Soc. Trop. Med. Hyg. 79, 260–261.

5 Schwartz, D.C. and Cantor, C.R. (1984) Separation of yeast chromosome sized DNAs by pulsed field gradient gel electrophoresis. Cell 37, 67–75.

6 Carle, G.F. and Olson, M.V. (1984) Separation of chromosomal DNA molecules from yeast by orthogonal field alteration gel electrophoresis. Nucleic Acids Res. 12, 5647–5664.

7 Van der Ploeg, L.H.T., Cornelissen, A.W.C.A., Barry, J.D. and Borst, P. (1984) The chromosomes of Kinetoplastida. EMBO J. 3, 3109–3115.

8 Gibson, W.C., Osinga, K., Michels, P.A.M. and Borst, P. (1985) Trypanosomes of subgenus Trypanozoon are diploid for housekeeping genes. Mol. Biochem. Parasitol. 16, 231–242.

9 Gibson, W.C. and Miles, M.A. (1986) The karyotype and ploidy of *Trypanosoma cruzi*. EMBO J. 5, 1299–1305.

10 Comeau, A.M., Miller, S.I. and Wirth, D.F. (1986) Chromosome location of four genes in *Leishmania*. Mol. Biochem. Parasitol. 21, 161–169.

11 Giannini, S.H., Schittini, M., Keithly, J.S., Warburton, P.W., Cantor, C.R. and Van der Ploeg, L.H.T. (1986) Karyotype analysis of Leishmania species and its use in classification and diagnosis. Science 232, 762–765.

12 Bishop, R.P. and Miles, M.A. (1987) Chromosome size polymorphisms of *Leishmania donovani*. Mol. Biochem. Parasitol. 24, 263–272.

13 Bastien, P. and Wahba, M. (In Press) A simplification of cloning *Leishmania*. Ann. Trop. Med. Parasitol.

14 Van der Ploeg, L.H.T., Schwarz, D.C., Cantor, C.R. and Borst, P. (1984) Antigenic variation in *Trypanosoma brucei* analysed by electrophoretic separation of chromosome-sized DNA molecules. Cell 37, 77–84.

15 Bellis, M., Pagès, M. and Roizès, G. (1987) Electrophorèse de l'ADN en champ pulsé. Rev. Inst. Pasteur (Lyon) 20, 45–55.

16 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

17 Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6–13.

18 Leon, W., Fouts, D.L. and Manning, J. (1978) Sequence arrangement of the 16S and 26S rDNA genes in the pathogenic haemoflagellate *Leishmania donovani*. Nucleic Acids Res. 5, 491–504.

19 Rioux, J.A., Lanotte, G., Maazoun, R. and Pasteur, N. (1985) L'electrophorèse des enzymes dans le genre *Leishmania* Ross, 1903. Parassitologia 27, 141–156.

20 Wells, J.M., Prospero, T.D., Jenni, L. and Le Page, R.W.F. (1987) DNA contents and molecular karyotypes of hybrid *Trypanosoma brucei*. Mol. Biochem. Parasitol. 24, 103–116.

21 Corcoran, A.M., Forsyth, K.P., Bianco, A.C., Brown, G.V. and Kemp, D.J. (1986) Chromosome size polymorphisms in *Plasmodium falciparum* can involve deletions and are frequent in natural parasite population. Cell 44, 161–169.

22 Maazoun, R., Lanotte, G., Rioux, J.A., Pasteur, N., Rioux, J.A., Killick-Kendrick, R. and Pratlong, F. (1981) Signification du polymorphisme enzymatique chez les Leishmanies. Ann. Parasitol. Hum. Comp. 56, 467–475.

23 Tait, A. (1983) Sexual processes in the kinetoplastida. Parasitology 86, 29–5723.

24 Jenni, L., Marti, S., Schweizer, J., Betschart, B., Le Page, R.W.F., Wells, J.M., Tait, A., Paindavoine, P., Pays, E. and Steinert, M. (1986) Hybrid formation between African trypanosomes during cyclical transmission. Nature 322, 173–175.

25 Paindavoine, P., Zampetti-Bosseler, F., Pays, E., Schweizer, J., Guyaux, M., Jenni, L. and Steinert, M. (1986) Trypanosome hybrids generated in tse-tse flies by nuclear fusion. EMBO J. 5, 3631–3636.

26 Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R. and Carter, R. (1987) Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science 236, 1661–1666.

27 Dvorak, J.A., Hall, T.E., Crane, M.S.J., Engel, J.C., McDaniel, J.P. and Uriegas, R. (1982) *Trypanosoma cruzi*: Flow cytometric analysis. Analysis of total DNA/organism by means of mithramycin-induced fluorescence. J. Protozool. 29, 430–437.

28 Spithill, T.W. and Samaras, N. (1987) Genomic organization, chromosomal location and transcription of dispersed and repeated tubulin genes in *Leishmania major*. Mol. Biochem. Parasitol. 24, 23–37.

29 Iovannisci, D. and Beverley, S.M. (1989) Structural alterations of chromosome 2 in *Leishmania major* as evidence for diploidy, including spontaneous amplification of the mini-exon array. Mol. Biochem. Parasitol. 34, 177–188.

30 Kemp, D.J., Corcoran, L.M., Coppel, R.L., Stahl, H.D., Bianco, A.E., Brown, G.V. and Anders, R.F. (1985) Size variation in chromosomes from independently cultured isolates of *Plasmodium falciparum*. Nature 315, 347–350.

31 De Puytorac, P., Grain, J. and Mignot, J.P. (1987) Précis de protistologie, Editions Boubée, Paris.

32 Lainson, R. and Shaw, J.J. (1986) The genus *Leishmania* Ross, 1903. Speculations on evolution and speciation. In: *Leishmania*: Taxonomy and Phylogeny (Int. Coll. CNRS/INSERM, 2–6 July, 1984) (Rioux, J.A., ed.), pp. 241–245, Institut Méditérranéen d'Epidémiologie, Montpellier.