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

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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 canid 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 chromosomal 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...
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 1) 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 1, as well as 14 clones issued from 6 of these checked for isozymes. Para medium, the inoculation of 7 x 10^7 parasites was first method [13].

DNA preparation Preparation described [1] was described as an ethidium bromide transilluminators (Hybon, according

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Results

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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 $7 \times 10^8$ 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 *PstI*, 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 *PstI* 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-stoichiometrically 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 than 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.s. [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 subbed field gel electrophoresis. Strain LEM119 (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.

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 zymodesmes (MON-1, 11 and 34; Fig. 3).

![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).](image)

![Comparative karyotypes of several strains. Photographs and intensities were taken of gel fractions of **(a)** LEM272, **(b)** LEM267, **(c)** LEM259, **(d)** LEM259, **(e)** LEM267.](image)

This is partly due to the fact that the numbers with four to six chromosomes are relatively rare and the polymorphism affects the numbers of bands.

**Study of a restriction fragment length polymorphism (Southern France)**

In view of the possible presence of homologous bands in different strains, we have studied the restriction pattern of a number of strains.
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 differentzymodemes (Fig. 3a,b). Note that thesezymodemes 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 356) 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 chromosomes.

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

![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.

![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 (h); with chromosomes of neighbouring sizes (± 50 kb) in (a) LEM356; (c) LEM1250; (d) LEM272; (e) LEM538; (h) LEM267; (f) LEM274; and with two in (f) LEM244; and (g) LEM251.](image-url)
Chromosome size polymorphism involves DNA amplification/deletion events

Two mechanisms can be considered to explain the 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 gous chromosomes seems extremal the high degree of clones. In such a case, the insect vector. hybridisation, parents, which some chromosomes [20]. This is in agreement with [25]. Similar between different hybrids which genotypes [26]. I tent of up to 6 clones of *T. cr:*

Although the strain is Leishmania, with our results in material with a progressive partia monosome size androgically very well as well as the 11 as 15-20%, fr ploidy, the presence of the present karyotypes.

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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, parasisexuality 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.

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References


Key words: Tryp

Introduction

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Note: Nucleotid- been submitted sion number 20-Abbreviations: eioe; ETAR1-repertoire; normal human; senni; s. senani; Antwerp try zoon antigen tyi test; cDNA, saline sodium c human serum in of Tropical Med

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