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CHARACTERIZATION OF PATHOGENIC TRYPANOSOMATIDAE BY RESTRICTION ENDONUCLEASE FINGERPRINTING OF KINETOPLAST DNA MINICIRCLES

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Abstract. A simple protocol was developed for the routine preparation of a kinetoplast DNA fraction from trypanosomatids. The digestion of this DNA with selected restriction endonucleases, followed by the electrophoretic analysis of the fragments on polyacrylamide gradient gels, yielded characteristic patterns that could be used for the intrinsic characterization of stocks (populations derived by serial passage in vivo and/or in vitro from a primary isolation, without any implication of homogeneity or characterization), strains (sets of populations originating from a group of trypanosomes of a given species or subspecies present at a given time in a given host or culture, and defined by the possession of one or more designated characters), and clones (trypanosomes derived from a single individual by binary fission) of certain pathogenic hemoflagellates.

The characterization of the parasites of the family Trypanosomatidae, which includes trypanosomes and leishmanias pathogenic to man and domestic animals, has until recently relied mainly on their biology and morphology and, when applicable, also on the clinical picture together with the epidemiology and geographic distribution. Although the use and the compilation of such data are of course essential, the complexity of the subject justifies the search for alternative methods for studying these organisms. The problems in the characterization and in the nomenclature of Kinetoplastida in general, and of trypanosomes and leishmanias in particular, have been discussed.[1–6]

Lumsden has proposed the use of both intrinsic (related to the organism itself) and extrinsic (involving the response of other components to the presence of the organism) characters to study parasite populations.[3,4,7] Recently, various methods have been used or proposed for the intrinsic characterization of trypanosomatids: *a*) fine structure of the kinetoplast as revealed by electron microscopy;[8] *b*) isoenzyme constitution;[9,10] *c*) buoyant density determination of nuclear and kinetoplast DNA (kDNA);[2,5,11] *d*) restriction endonuclease fingerprinting of kDNA;[12,13] e) agglutination reactions by various lectins;[14,15] *f*) protein typing by disc electrophoresis;[16] *g*) characterization of the enzymes for the metabolism of arginine and ornithine;[17] *h*) analysis of ^{131}I-labeled surface proteins;[18] and *i*) nutritional requirements.[19] Some methods can discriminate organisms even at the strain level (isoenzyme constitution, restriction fingerprinting of kDNA). Others are applicable only for discriminating at the species or genus level; for instance, electron microscopy of the kinetoplast, agglutination reactions, protein typing by disc electrophoresis and immunoelectrophoresis all failed to reveal any differences among several strains of *Trypanosoma cruzi*.[8,15,16,20,21]

The biochemical methods for parasite characterization have been divided by Newton into two main categories:[2] those that are concerned with the cell phenotype (e.g., isoenzyme analysis), and those that investigate the cell genotype (e.g., DNA buoyant density studies).

The applicability of the methods for genotype characterization to parasites has in general been rather limited by the costly equipment and specialized techniques needed. However, the recent advances in the field of DNA biochemistry, some of which have been reviewed in this symposium, make it feasible to develop new and simple ways for their application to the biochemical characterization of parasites.

We would like to report here some of our results on the characterization of pathogenic hemoflagellates by restriction endonuclease fingerprinting of kDNA minicircles. We have recently developed a simple and efficient protocol for the routine preparation of a kDNA fraction from *Trypanosoma cruzi,* the causative agent of Chagas' disease (Morel, Chiari, Camargo, Mattei, Romanha and Simpson, in press). This method also proved useful for the preparation of kDNA from several other trypanosomatids, including *Leishmania*

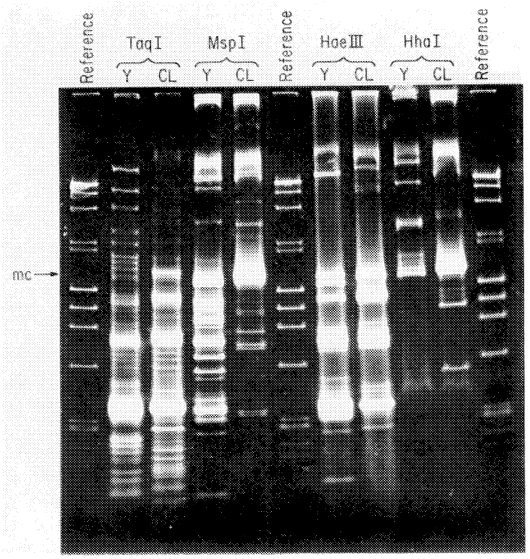

FIGURE 1. Acrylamide gradient gel electrophoresis comparison of kDNA digests from the Y and CL 'polar' strains of *T. cruzi* by *Taq* I, *Msp* I, *Hae* III, and *Hha* I. Reference DNA is a mixture of λ DNA digested with HindIII and ØX174-RF DNA digested with *Hae* III.

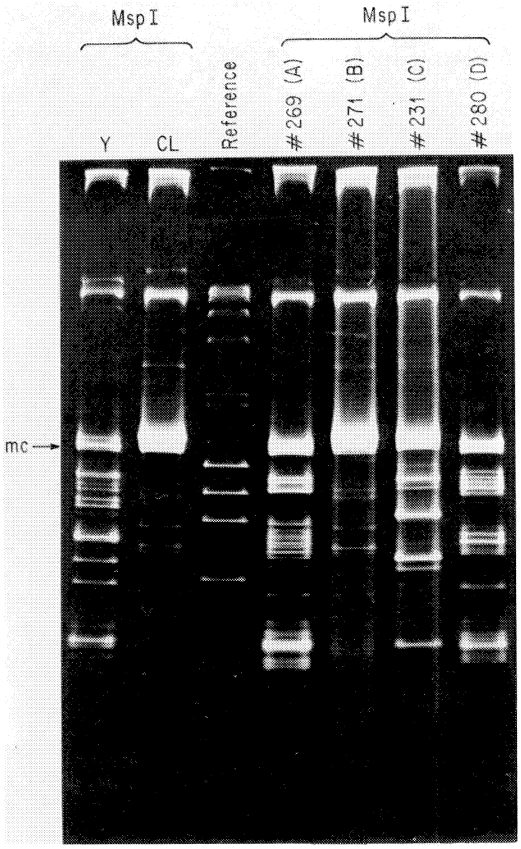

FIGURE 2. Acrylamide gradient gel electrophoresis comparison of *Msp* I kDNA digests from the Y and CL strains and *T. cruzi* stocks #269, #271, #231 and #280 from human cases of Chagas' disease. The four human stocks represent the four zymodeme groups of *T. cruzi* described by Romanha et al.[23,24] The experimental details on the isolation of *T. cruzi* from humans by blood culture are given elsewhere (Morel et al., in press).

(unpublished). This kDNA fraction can be digested with various restriction endonucleases and the resulting fragments efficiently analysed on polyacrylamide gradient gels. If the nuclease used introduces frequent cuts in the minicircle population of the kDNA under study, the restriction patterns obtained are characteristic of the particular organism being analysed and can be used for comparative purposes; we have introduced the term "schizodeme" to denote the populations of cells displaying similar DNA restriction patterns (Morel et al., in press).

Figure 1 shows the profiles obtained upon digestion with different restriction nucleases of the kDNA fraction from the Y and CL 'polar' strains of *T. cruzi*.[22] By varying the nuclease used one can go from a digestion that yields only a small number of fragments (e.g., *Hha* I) or many fragments of minicircles (*e.g., Taq* I). Quantitative as well as qualitative differences between the restriction profiles are readily visible for the four enzymes used.

Figure 2 shows that this method for *T. cruzi* characterization can be used for stocks derived from hemoculture from human cases of Chagas' disease. In this experiment, the above-mentioned Y and CL strains and four human isolates of *T. cruzi* which represent the four zymodeme groups of Romanha and coworkers[23,24] are compared by *Msp* I restriction fingerprinting. It is clear that not only the 'polar' strains but also the *T. cruzi* stocks from human origin can be discriminated and characterized by this method.

One of the arguments raised against the possible use of restriction fingerprints from minicircles for the characterization of hemoflagellates was that the sequence evolution of minicircles would be too rapid and the restriction profiles would not be a stable and reliable character.[25] In addition to other controls (Morel et al., in press), Figure 3 shows that this is not the case for the strains we analysed. In this experiment we made a compar-

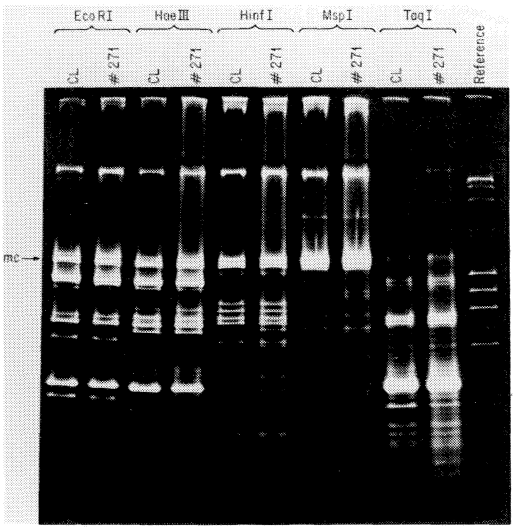

FIGURE 3. Acrylamide gradient gel electrophoresis comparison of *Eco*RI, *Hae* III, *Hin*fI, *Msp* I, and *Taq* I kDNA digests from the CL strain and human stock #271.

ison of the restriction fingerprints from strain CL (isolated from a vector in the South of Brazil) and human stock #271 (isolated from a patient in Southeast Brazil) produced by five different endonucleases. It is clear that the digests are very similar if not identical with all enzymes tested; this result shows that the rate of kDNA sequence evolution is not so rapid as to make the restriction profiles meaningless.

The above-mentioned results have been extended to other *T. cruzi* stocks and clones (Morel et al., in press). It was shown that this method confirmed and extended the results obtained by isoenzyme characterization, and that it can be adapted for use in clinical laboratories. Recently we obtained evidence that it also can be used for the characterization of several insect trypanosomatids and pathogenic *Leishmania* (Morel, Camargo, Roitman, Mattei, Grimaldi, Lima, Lopes and Simpson, unpublished). We think that this new tool will become increasingly useful for the characterization of the organisms of the order Kinetoplastida, particularly for those that are pathogenic to man and his domestic animals.

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DISCUSSION

Question by Nina Agabian: Do you attribute changes in the redistribution pattern upon cell cloning to an original mixed infection, or do you believe there is a correlation between epimastigote-trypomastigote conversion and kDNA rearrangement?

Answer: The changes in restriction profiles observed until now upon cell cloning are entirely due to the presence of a mixed population composed of two homogeneous subpopulations. The proportion of trypomastigotes in culture did not change the restriction patterns. For instance, one clonal culture (CL 14) with 2.4% or 60% trypomastigotes displayed the same restriction profile.

Question by Nina Agabian: Have you done a secondary cloning of one of these species?

Answer: Yes. In collaboration with Egler Chiari and coworkers we have analyzed three subclones of each of four primary clones. In no case could a further modification of the restriction fingerprints be detected. However, in one case it was shown that the primary cloning step that had not been successful for the same two initial subpopulations still coexisted in the "cloned" culture. Thus, restriction fingerprinting of kDNA can in fact help the monitoring of cell cloning experiments with *T. cruzi* cultures.

Question by Kenneth Stuart: Is the correlation

between minicircle fragment patterns and the epimastigote-to-trypomastigote transformation correlated with changes in mitochondrial respiratory enzymes?

Answer: We have not investigated this point. However, as it is known that, in contrast to the situation of the *T. brucei* subgroup, there are no qualitative differences in energy metabolism among the various *T. cruzi* forms, we think that there will be no correlation among these phenomena.

Question by John David: Have you looked at *Leishmania* organisms such as *L. braziliensis* or *L. mexicana*? Can you tell the difference between these? Is there a difference between *Leishmania* and *T. cruzi* restriction fingerprints?

Answer: We have already started a collaboration with the laboratories of Drs. Roitman, Cuba-Cuba, Grimaldi and Mayrink in Brazil in order to investigate the applicability of this technique to the genus *Leishmania*. However, the number of samples investigated until now is small and the fact that we are doing the analysis blindly for most stocks prevents us from making any definite generalizations at the moment. However, the results obtained in our lab by Lima and Lopes allow us to say that: 1) classification of the *Leishmania* samples into various subgroups according to their kDNA restriction fingerprints ("schizodemes," Morel et al., submitted) is possible and can be done easily; 2) the restriction profiles are completely different from those of *T. cruzi*; 3) the restriction profiles seem also to be stable intrinsic characters, for they did not change after 1 year in culture or in animals; 4) of the restriction endonucleases tested until now, *Msp* I (an isoschizomer of *Hpa* II) and *BspRI* (an isoschizomer of *Hae* III) seem to be very good for the restriction typing of *Leishmania*.

Question by Piet Borst: We have used *Taq* I and *Mbo* II digests of *T. brucei* kDNA for strain identification and we also found that the digestion patterns are sufficiently stable to use them as the most discriminating tag for distinguishing strains. Two questions: 1) We cannot deduce phylogenetic relations from minicircle digests of *T. brucei*. Can you? 2) If there is genetic exchange in *T. cruzi*, and if inheritance of kDNA is uniparental, kDNA sequences would not run parallel with properties determined by nuclear genes. Any evidence for this?

Answer: I am very happy to hear that your recent results with *T. brucei* also point to the stability of restriction digest patterns of kDNA minicircles, for some experiments by Leon et al.[25] had raised some questions about this point in *T. cruzi*. Answering your questions: 1) I agree it would be very difficult, if not impossible, to deduce phylogenetic relationships by restriction digest of minicircles. Their heterogeneity precludes any quantitative analysis of the possible differences or similarities, making it impossible to apply to their digests some of the recent mathematical methods for estimating genetic variations by restriction endonuclease analysis. I believe that DNA sequencing of selected maxicircle genes (e.g., rRNA genes) will be a much more powerful approach for investigating phylogenetic relationships; it would have the additional advantage of also making it possible to look at the position of the Trypanosomatidae among Kinetoplastida and also in relation to other organisms that do not have a kinetoplast DNA and hence do not possess minicircles. In relation to your second question, although I think it touches a very interesting point, that is, the possibility of genetic exchange in trypanosomes, I am afraid I cannot now bring any contribution to it. Although since the original description of *T. cruzi* by Chagas in 1909 the possibility of a sexual cycle in this parasite has been hypothesized, this phenomenon has not yet been proved. The fact that we can now easily identify clonal populations by restriction digests or isoenzyme analysis will probably help to devise some experiments to investigate the possibility of genetic exchange. Miles[10] quoted some unpublished results showing that he could get no evidence for genetic interaction when mixtures of *T. cruzi* zymodemes 1 and 2 were cyclically transmitted. We could speculate by analysis of the fact that we could isolate two different schizodemes from a given *T. cruzi* strain by cell cloning, but that would of course be premature. So I would conclude by saying that we do not have at the moment any evidence for genetic exchange or "maternal" inheritance of kDNA, but that restriction fingerprinting identification of clonal populations might help to devise meaningful experiments to investigate this hypothesis.