Recurrent lesions in human *Leishmania braziliensis* infection—reactivation or reinfection?

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Strains of *Leishmania braziliensis* subspecies isolated from initial and recurrent lesions in 24 patients from the Pacific coast of Colombia were examined for distinguishing polymorphisms by enzyme electrophoresis, restriction endonuclease analysis of kDNA, and molecular karyotyping of nuclear DNA. Recurrent strains from 12 patients (50%) were identical to the initially infecting strain by all methods of characterisation. Phenotypic and genotypic identity, together with clinical data, support endogenous reactivation as the mechanisms of recurrent disease in these 12 patients. 5 of the 24 (22%) recurrent strains differed from the initial strain by all methods. The remaining 7 strain pairs, not separated by enzyme polymorphisms, showed differing schizodeme and/or karyotype profiles. Patients whose recurrent lesions were caused by strains different from those causing the initial lesions had a significantly longer disease-free interval than patients whose lesions were caused by identical strains. Recurrent lesions occurred further from initial lesions in the former than in the latter group. Exogenous reinfection is the most plausible explanation for recurrences due to disparate organisms. These findings have important implications for both treatment evaluation and vaccination strategies for American tegumentary leishmaniasis.

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Introduction

Human tegumentary disease caused by *Leishmania braziliensis* is distinguished from other leishmaniases by its chronic, latent, and metastatic behaviour. Recurrent lesions are a troublesome but intriguing feature of this illness. Typical scars are present at the time of diagnosis of active lesions in many patients with *L braziliensis* subspecies infection[1-3] and provide an approximate index of recurrent leishmaniasis. Recurrent disease is important because of the potential for mucosal involvement and the difficulty and greater cost of treating that disease manifestation.

The two most likely mechanisms of recurrent leishmaniasis are reactivation of persistent infection and exogenous reinfection. Reactivation implies long-term survival of the parasite despite initial healing of lesions. If it is possible for the parasite to persist in sensitised immunocompetent individuals, can it be eradicated from the human host? Assessment of treatment outcomes requires the ability to distinguish the two mechanisms, because reactivation would indicate treatment failure, reinfection would not. Evidence of exogenous reinfection, on the other hand, has implications for vaccine development since it would show that naturally acquired infection does not uniformly confer lasting protection from subsequent infection or disease. An understanding of the mechanisms underlying recurrence of tegumentary leishmaniasis is essential, since both treatment efficacy and acquired resistance to *L braziliensis* subspecies must ultimately be assessed in terms of their effect on the reduction of morbidity.

There is no reliable means to establish whether the primary leishmanial parasite has been eliminated from a human host. We therefore investigated whether reactivation and reinfection might be discriminated by high resolution methods to distinguish sequential isolates—enzyme electrophoresis, kDNA restriction fragment electrophoresis, and molecular karyotyping by pulse field gradient gel electrophoresis (PFGE). These methods, together with clinical information and epidemiological history, have provided new insights into the natural history of recurrent disease in human *L braziliensis* subspecies infection.

Patients and methods

Follow-up examination of previously diagnosed and treated patients allowed the isolation of leishmania from initial and recurrent cutaneous lesions in 24 patients from the Pacific coast region of Colombia. All participants gave written informed consent. The 24 cases studied are among 71 recurrences observed in 472 parasitologically diagnosed patients followed to date. Only patients from whom primary and recurrent strains were available were included in the analysis.

Tissue samples were obtained by needle aspiration and punch biopsy and inoculated into culture media.[4] Leishmania were cloned by means of the colony formation technique.[5] For biochemical analysis leishmania strains were propagated by passage from diphasic Senekjie's medium to liquid Schneider's medium that contained 10% heat-inactivated fetal calf serum, 1000 U/ml penicillin, 1000 μg/ml streptomycin, and 1% L-glutamine (Gibco). Cultures were harvested after 4 days at 27°C. Leishmania strains designated by the World Health Organisation (WHO) as reference organisms[6] were used in the comparative characterisation of strains isolated from patients with recurrent lesions—*L braziliensis panamensis* MHOM/PA/1971/LS94, *L braziliensis braziliensis* MHOM/BR/1975/M2903, and *L braziliensis guyanensis* MHOM/BR/1975/M4147.

For enzyme electrophoresis extracts of leishmania were prepared and stored in liquid nitrogen.[7] Starch gel electrophoresis,[8]

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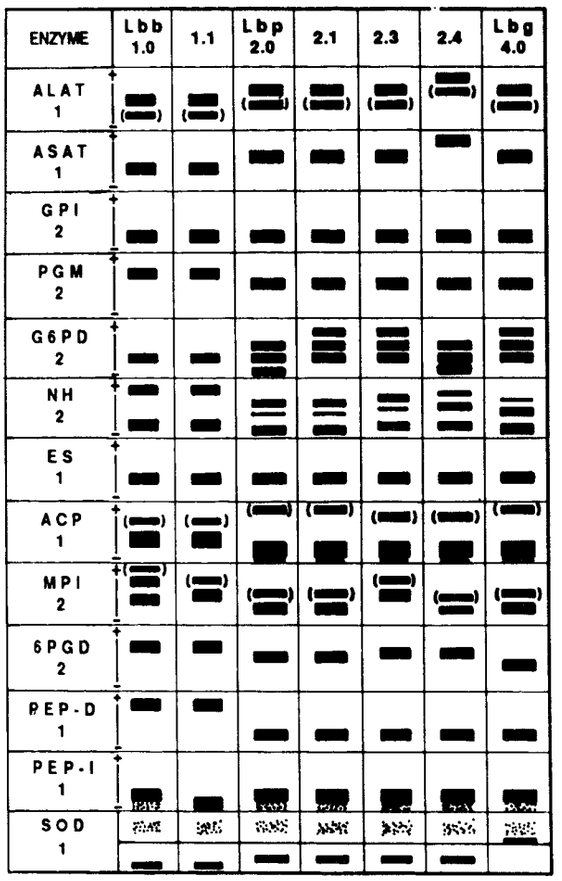

Fig 1—Summary of enzyme polymorphisms distinguishing zymodemes represented by recurrent strain pairs and WHO reference strains for *L braziliensis braziliensis (Lbb), L braziliensis panamensis (Lbp),* and *L braziliensis guyanensis (Lbg).*

ALAT = alanine aminotransferase; ASAT = aspartate aminotransferase; GPI = glucose phosphate isomerase; PGM = phosphoglucomutase; G6PD = glucose-6-phosphate dehydrogenase; NH = nucleoside hydrolase; ES = B-esterase; ACP = acid phosphatase; MPI = mannose phosphate isomerase; 6PGD = 6-phosphogluconate dehydrogenase; PEP-D = proline iminopeptidase; PEP-1 = aminopeptidase; SOD = superoxide dismutase.

development of enzyme activity,[9-11] and cellulose acetate electrophoresis were carried out by published methods. Thirteen enzymes were examined.[7]

Washed promastigotes were submitted to kDNA extraction.[12] Based on restriction enzyme sites observed in minicircle sequences during preliminary studies, restriction banding profiles generated by digestion with the restriction enzymes *Bce* 243 (Fiocruz) and *Sau* 3AI (Stratagene) were selected for analysis of strain heterogeneity. Restriction fragments were separated by vertical electrophoresis in 5–10% polyacrylamide gels, and profiles were revealed by silver staining.[12] Details of the methods used for karyotype analysis by PFGE and by transverse alternating field electrophoresis (TAFE) have been described elsewhere.[13]

Clinical characteristics of patients with recurrences due to identical and different strains were compared by the Mann-Whitney rank sum test (continuous variables) or Fisher's exact test (binomial variables).

Results

Primary and recurrent strains belonged to four zymodemes: three (2·1, 2·3, 2·4) were identified as *L braziliensis panamensis*, based on similarity to the enzyme profile of WHO reference strain MHOM/PA/1971/LS94 (zymodeme 2·0) (fig 1) and reactivity with monoclonal antibody VII5G3G3 (B11)[14] (specific for an epitope of *L braziliensis panamensis*); the fourth (1·1) was identified as *L braziliensis braziliensis*, based on similarity to the enzyme profile of the WHO reference strain MHOM/BR/1975/M2903 (zymodeme 1·0). The primary and recurrent isolates had identical enzyme profiles in 19 of the 24 pairs of strains. The 24 recurrences involved sequential lesions due to *L braziliensis panamensis* in all but 1 case, in which the initially diagnosed lesion was caused by *L braziliensis panamensis* and the recurrent lesion by *L braziliensis braziliensis*. The enzyme polymorphisms distinguishing the zymodemes represented by the 5 non-identical pairs of strains are shown schematically in fig 1.

TABLE I—ENZYME, SCHIZODEME, AND KARYOTYPE ANALYSIS OF RECURRENT STRAINS AND CLONES

Strain code	Zymodeme	Schizodeme	Karyotype
2238 *vs* 2238R	I (2·4)	I	I
2387 *vs* 2387R	I (2·4)	I	I
2407 *vs* 2407R	I (2·4)	I	I
2509 *vs* 2509R	I (2·3)	I	I
1135 *vs* 1135R	I (2·4)	I	I
2025 *vs* 2025R	I (2·4)	I	I
1–4–3 *vs* 1–4–3R	I (2·4)	I	I
2406 *vs* 2406R	I (2·3)	I	I
2426 *vs* 2426R	I (2·3)	I	NA
2326 *vs* 2326R	I (2·4)	I	NA
2169 *vs* 2169R	I (2·4)	I	NA
2179 *vs* 2179R	I (2·4)	I	NA
1244 trunk *vs* nose R_1	I (2·4)	I	NA
1244 face *vs* nose R_1	I (2·4)	I	NA
1244 trunk *vs* finger R2	D (2·4, 2·1)	D	D
1244 face *vs* finger R2	D (2·4, 2·1)	D	D
1150 *vs* 1150R	I (2·1)	D	NA
2124 *vs* 2124R	I (2·3)	D	D
2124 *vs* clones (5)	I (2·3)	I	NA
2249 *vs* 2249R	I (2·3)	D	D
2249 *vs* clones (5)	I (2·3)	I	I
2170 *vs* 2170R	I (2·4)	D	D
2170 *vs* clones (3)	I (2·4)	1/3 D	I
2539 *vs* 2539R	I (2·3)	D	NA
2322 *vs* 2322R	I (2·4)	D	NA
1300 *vs* 1300R	I (2·1)	D	NA
2485 neck *vs* 2485R	D (2·3, 2·4)	D	D
2485 neck *vs* clones (5)	I (2·3)	I	I
2485 foot *vs* 2485R	D (2·3, 2·4)	D	D
2485 foot *vs* clones (10)	I (2·3)	D	1/10 D
2053 *vs* 2053R	D (2·3, 1·1)	D	NA
2309 *vs* arm R	D (2·3, 2·4)	D	D
2309 *vs* face R	D (2·3, 2·4)	D	D
2421 *vs* 2421 R	D (2·3, 2·4)	D	D

R = recurrent strain; I = identical: D = disparate; NA = not analysed

Restriction endonuclease fragment profiles of kDNA were identical in 12 of the 24 pairs of strains (table I). The five pairs non-identical by enzyme polymorphisms were also non-identical by schizodeme analysis. Seven other pairs of strains showed microheterogeneity in their kDNA restriction profiles.

Karyotypes of 32 strains from primary and recurrent lesions of 15 patients were examined by TAFE and PFGE. 20 unique karyotypes were discerned among the 32 strains. The results accorded with those of restriction endonuclease analysis of kDNA. Subspecies, zymodeme, and schizodeme distinctions of the strains were corroborated by chromosomal size polymorphisms, but karyotyping showed differences not evident by isoenzyme analyses (table I).

10 of the 24 strains isolated from initial lesions were cloned. Isoenzyme typing did not show clonal heterogeneity, even in strains from patients whose subsequent lesions were caused by leishmania of a different zymodeme. Karyotype and schizodeme analyses yielded evidence of clonal diversity in 1 of 3 and 2 of 10 strains examined, respectively. Results of schizodeme and karyotype studies of clones were not uniformly concordant.

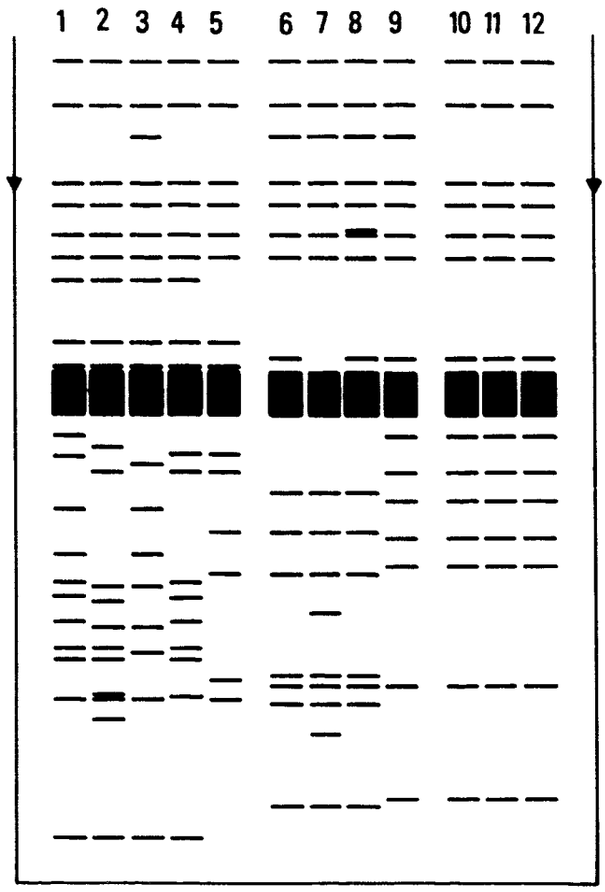

Fig 2—Schematic representation of microheterogeneity in kDNA restriction profile with *Bce* 243 or *Sau* 3AI.

Columns 1–5 = strain 2485: 1 = initial strain; 2 = clone 3; 3 = clone 9; 4 = clone 11; 5 = recurrent strain.
Columns 6–9 = strain 2170: 6 = initial strain; 7 = clone 1; 8 = clone 2; 9 = recurrent strain.
Columns 10–12 = strain 2238: 10 = inital strain; 11 = clone 1; 12 = recurrent strain.

Clones 1, 2, and 3 of strain 2170 and the initial strain had identical karyotypes, whereas the restriction profile of clone 1 was slightly different (fig 2). Clones 3, 9, 10, and 11 of strain 2485, isolated from a lesion on the foot, each presented distinct restriction fragment profiles, yet only clone 9 showed differences in karyotype. In all of the few cases in which variant clones from the initial isolate were detected, the recurrent strains were different from any of the clones derived from the initial strain. Notably, the schizodeme pattern of strain 2485 (foot) was a partial composite of individual clone patterns. The schizodeme pattern of strain 2485 (neck) and its clones was distinct from that of the strain isolated from the patient's foot at the same time.

TABLE II—CLINICAL CHARACTERISTICS OF PATIENTS WITH DISPARATE AND IDENTICAL STRAINS ISOLATED FROM RECURRENT LESIONS (CONTINUOUS VARIABLES)

—	Identical		Disparate	
	Median(n)	Range	Median (n)	Range
Time (mo)				
Evolution of initial lesion	2·0 (11)	0·5–30·0	2·5 (12)	1·3–6·0
Between initial and recurrent lesions	5·5 (12)	1–28	19·5* (12)	6–60
Treatment received for initial lesion (total mg/kg antimony)	390 (11)	137–752	205 (11)	0–889
Montenegro (mm)	3·8 (8)	0–18·5	10·2 (12)	4·0–17·0
IFAT (reciprocal titre)	16 (12)	4–32	16 (11)	4–128
Lymphocyte transformation (net CPM × 10^3)	8·39 (8)	0–71·3	7·19 (6)	0·26–42·2
Size (mm)				
Initial lesion	23 (12)	10–120	23 (12)	4–55
Recurrent lesion	11 (12)	5–120	12 (11)	5–20
Number of lesions				
Initial	2·5 (12)	1–4	3·0 (11)	1–9
Recurrent	1 (11)	1–6	1 (12)	1–2

*Mann-Whitney test $p < 0.01$
IFAT = immunofluorescent antibody titre; CPM = counts per min.

TABLE III—CLINICAL CHARACTERISTICS OF PATIENTS WITH DISPARATE AND IDENTICAL STRAINS ISOLATED FROM RECURRENT LESIONS (CATEGORICAL VARIABLES)

—	No/total (%)		p†
	Identical	Disparate	
*Recurrent lesion close to initial lesion**	9/12 (75%)	2/12 (17%)	0·006
Ulceration present			
Initial lesion	9/12 (75%)	9/12 (75%)	0·679
Recurrent lesion	6/11 (55%)	5/12 (42%)	0·421
Typical scar or history of prior lesion	2/12 (17%)	3/12 (25%)	0·500
Adenopathy at initial diagnosis	1/9 (11%)	2/9 (22%)	0·500

*Same site as or within 5 cm of original lesion
†Fisher's exact test.

The clinical characteristics of patients are summarised in tables II and III. Patients from whom identical strains were isolated had had shorter disease-free times between initial and recurrent lesions, showed less vigorous cutaneous delayed hypersensitivity to intradermal application of leishmanin at initial diagnosis, and received more antimony treatment than patients whose lesions were caused by different strains (table II). Recurrent lesions caused by strains identical to the initial strain were also more likely to be located close to the initial lesion (table III).

Discussion

Recurrent leishmaniasis is a seldom recognised and poorly understood cause of morbidity.[15–17] The most severe manifestation, mucosal disease, has generally been described in association with healed cutaneous lesions caused by *L braziliensis braziliensis* and has been attributed to spread of persistent infection.[2,18,19] Systematic examination of parasitologically diagnosed patients shows evidence of prior leishmanial lesions in many presenting with active disease.[16,18,20,21] At the time of first diagnosis, 5 of the 24 patients in this study had scars compatible with healed leishmanial lesions. Therefore, their recurrences probably represented at least a third episode.

Case-reports of tegumentary leishmaniasis occurring long after exposure have provided anecdotal evidence of persistence of leishmania infection in human beings.[22] The diagnosis of visceral leishmaniasis in patients with human immunodeficiency virus infection corroborates the occurrence of persistent inapparent infection by various leishmania species.[23–25]

Previous reports on the characterisation of recurrent strains have each described single cases[15,26–28] but have provided evidence that recurrent disease is not unique to the *L braziliensis* complex. For example, three strains representing three zymodemes of *L tropica* were isolated over 14 years from a patient with "leishmaniasis recidivans", suggesting either that the original infection had been mixed or that reinfection had occurred.[27] However, the finding of

the identical schizodeme for *L tropica* strains isolated from lesions in the same patient occurring 14 years apart[28] supports the stability of restriction profiles in persistent infections.

Reactivation is the most likely mechanism of recurrent lesions involving genotypically and phenotypically identical, sequentially isolated strains. Schizodeme and karyotype profiles are strain-specific,[13,29] and it is unlikely that infection could have occurred twice with the same strains in 12 of 24 cases. Clinical data also support reactivation. The total amount of antimony treatment received was higher and the cutaneous delayed hypersensitivity response to leishmania antigen was lower in this group of patients at the time of first diagnosis, suggesting a low cell-mediated immune response as a possible risk factor for relapse and reiterating different mechanisms of recurrent disease.

5 of the 24 strain pairs differed at all levels of biochemical discrimination. The number of enzyme polymorphisms in these strains was similar to that distinguishing recognised subspecies of the braziliensis complex. We know of no direct evidence for the simultaneous natural inoculation of leishmania of different subspecies or zymodemes. Therefore independent infections are the most plausible explanation for those cases of recurrence.

7 other strain pairs of the same zymodeme were distinguished on the basis of chromosome size polymorphisms and/or restriction fragment length polymorphisms of kDNA. To examine whether these disparate recurrent strains represented clonal populations present in the initial strain rather than new infection, clones were propagated from 10 of 24 initially isolated strains. Evidence of clonal heterogeneity was found in only 2 of the 10 strains examined. In no case did the organisms isolated from recurrent lesions show a restriction pattern or molecular karyotype identical to clones derived from the initial strain.

Results of karyotype analyses concurred with the categorisation of paired strains based on enzyme polymorphisms and restriction fragment length polymorphism profiles of kDNA. Concordance of nuclear and extranuclear DNA polymorphisms at the level of strains is interesting, since kDNA differences are thought to have little biological significance. These observations raise the possibility of parallel evolution of nuclear and extranuclear DNA and the interaction of these elements. Available evidence indicates that karyotype is a stable characteristic; the karyotype of *L major* clones did not change over 4–5 years,[13] which is longer than the maximum time to recurrence in our patients. Furthermore, since karyotypes of these disparate strains differed by a minimum of three DNA bands, the rate of chromosomal mutation would need to be extraordinarily high. Because of the stability of kDNA restriction profiles reported in uncloned and cloned populations,[30] it is unlikely that the observed heterogeneity arose during infection. It is also unlikely that antimony treatment induced karyotypic variants, since the patients who had received the highest amount of antimony treatment were those with identical karyotypes in the primary and recurrent isolates; this finding argues against the induction of novel karyotypes by antimony treatment.

The clinical features and the findings that 5 of 24 recurrences showed polymorphic gene products and that 12 involved organisms that were distinguishable at the levels of nuclear and/or extranuclear DNA suggest that exogenous reinfection with closely related organisms occurs and is a mechanism of recurrent disease. Immunity acquired though natural infection with *L braziliensis* subspecies in these 12 patients did not protect against subsequent clinically apparent infections with a closely related organism. The finding that reinfection by closely related leishmania causes a large proportion of recurrent disease is important to strategies of immunoprophylaxis.

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Pancreatic islet transplantation after upper abdominal exenteration and liver replacement

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Nine patients who became diabetic after upper-abdominal exenteration and liver transplantation were given pancreatic islet-cell grafts obtained from the liver donor (eight cases), a third-party donor (one), or both (four). Two patients were diabetic when they died of infections after 48 and 109 days, as was a third patient who died of tumour recurrence after 178 days. The other 6 are alive 101–186 days postoperatively, and five are insulin-free or on insulin only during night-time parenteral alimentation. C-peptide increased 1·7 to 3·3 fold in response to intravenous glucose in these five patients who have had glycosylated haemoglobin in the high normal range. However, the kinetics of the C-peptide responses to intravenous glucose in all eight patients tested revealed an absent first-phase release and a delayed peak response consistent with transplantation and/or engraftment of a suboptimal islet cell mass. The longest survivor, who requires neither parenteral alimentation nor insulin, is the first unequivocal example of successful clinical islet-cell transplantation.

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Introduction

Long-term reversal of hyperglycaemia following islet transplantation has been reported in animal models of diabetes,[1] but not in patients,[2–5] though Scharp's group in St Louis[5] has lately reported one encouraging case to an American Diabetes Association meeting (see *JAMA* 1990; **264:** 427). Clinical failure has been attributed to poor harvesting and organ preservation techniques, inadequate procedures for isolating and purifying islets, and failure to control islet-directed immune destruction. However, improvements in technique[6,7] have lately increased the yield and purity of functionally competent islet grafts,[2,5,8–10] and the potent new immunosuppressive agent FK 506 has made control of rejection easier.[11,12] Using these advances, we have given islet allografts to patients rendered apancreatic by upper-abdominal exenteration.[13,14]

Patients and methods

Patients

Nine patients aged 8-58 years underwent upper-abdominal exenteration for tumours too extensive to be removed with less drastic procedures. Liver, pancreas, spleen, stomach, duodenum, proximal jejunum, and terminal ileum and, in three cases, the ascending and transverse colon were removed.[13,14] A cadaveric liver orthotopic allograft was done[14] and the graft portal vein was anastomosed to the recipient superior mesenteric vein. Arterialisation was from the recipient aorta or coeliac axis (fig 1). A 14G catheter with a heparin lock was placed in a superior mesenteric venous tributary. Bowel continuity was re-established and biliary drainage was via a choledochojejunostomy (fig 1).

Organ procurement

The cadaveric donors were the same ABO type as the recipient except for the second pancreas donor (type O) for patient 8, who was

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