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From population to genome: ecogenetics of *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana*

By J. C. DUJARDIN*, A. L. BAÑULS†, K. VICTOIR*†, S. DE DONCKER*, J. AREVALO‡, A. LLANOS-CUENTAS‡, M. TIBAYRENC† and D. LE RAY*

**Laboratory of Protozoology, Institute of Tropical Medicine 'Prince Leopold', 155 Nationalestraat, B-2000 Antwerpen, Belgium*
†Génétique Moléculaire des Parasites et Vecteurs, UMR CNRS/ORSTOM 9926, ORSTOM, BP 5045, F-34.032 Montpellier Cedex, France
‡Instituto de Medicina Tropical 'Alexander von Humboldt', Universidad Peruana Cayetano Heredia, AP 5045, Lima 100, Peru

The size polymorphism of nine chromosomes, recognized by specific probes, was analysed in populations of *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana* from various Peruvian biogeographical units. Interpretation of the polymorphism, by statistical and phenetic methods, led to the identification of five consensus (α- and β-tubulin) and four variable chromosomes. The dynamics of the variable chromosomes were studied. The promoter role of the environment on their polymorphism was indicated by: (1) the discrimination of *L. braziliensis* (forest) and *L. peruviana* (Andes) by the size of the chromosome containing the gp63 genes; and (2) the fact that, within *L. peruviana*, the polymorphism of the variable chromosomes revealed a strong eco-geographical structuring of parasite populations, accompanied by increasing chromosomal dissimilarity along a cline from north to south. The adaptative significance of the polymorphism of the variable chromosomes was suggested by: (1) a correlation between chromosomal polymorphism and phenotype variability (lesion type in patients and virulence *in vitro*); and (2) the association between the decrease in size of the gp63-containing chromosome from *L. braziliensis* to *L. peruviana*, and a rearrangement of the gp63 genes, probably accompanied by a decrease in their copy number. As chromosomal variation was shown to be more dependant on eco-geographical differences than isoenzymatic variation, chromosome variation and enzyme variation probably differ in adaptative significance.

Extensive diversity occurs amongst natural populations of *Leishmania*. This diversity is expressed at various levels, including the parasites' macro-ecology (biotope) and micro-ecology (the ecological niche formed by the parasites' respective hosts), the type of pathology they produce and their biology (e.g. the type of development in the vector). Although some of this diversity is attributable to extrinsic factors, much of it must be intrinsic. Various characters have therefore been studied, particularly since the massive diffusion of molecular biology, to assess genetic variation in the genus *Leishmania*. These include isoenzyme variation, considered the reference standard for genetic characterization of *Leishmania* (Anon., 1990) and, after the development of pulsed-field gel electrophoresis (PFGE: Schwartz *et al.*, 1983), molecular karyotype. This recently acquired ability to study karyotypes is conceptually important. Chromosomal characters do not reflect genetic variation (changes in the DNA sequences themselves) but genomic variation (changes in the arrangement of DNA sequences), which has considerable significance (Wilson *et al.*, 1974).

About 100 chromosomes, occasionally with additional elements such as circular episomes or small nucleic acids, form the *Leishmania* karyotype (Galindo and Ramirez Ochoa, 1989; Lighthall and Giannini, 1992). The 'normal' karyotype has an important plasticity, both

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in terms of chromosomal size (Lighthall and Giannini, 1992) and number (Cruz *et al.*, 1993). The present paper focuses on the variation in chromosomal size and particularly its possible significance at the population level.

INTERPRETATION OF CHROMOSOMAL SIZE POLYMORPHISM

The methods used for successful, comprehensive analysis of chromosomal size polymorphism must minimize the effects of small size variation caused by technical artifacts, such as inhomogeneity in the electrical fields used in PFGE and variation in the quantity of migrating DNA (Lai *et al.*, 1989), or by biological phenomena such as amplification/deletion of the telomeres (Bernards *et al.*, 1983). One way of achieving this is to base the phenetic analysis on a chromosomal size difference index (CSDI) and then consider only clustering above a certain threshold as significant (Dujardin *et al.*, 1995*b*). Statistical analysis of the chromosomal size distribution can also be used to reveal one or more modes of normal distribution and criteria such as size-conservation (coefficient of size-variation within each mode <5%; Lighthall and Giannini, 1992) and size-specificity (the mean size specific for a group of organisms; Dujardin *et al.*, 1993*a*) can then be introduced. Chromosomes can be classified into three categories (Lighthall and Giannini, 1992): consensus (conserved throughout the genus or subgenera, containing house-keeping genes); variable (conserved within a species or geographic population) and hypervariable (varying from one isolate to the other).

SIGNIFICANCE OF THE SIZE POLYMORPHISM

The most important function of a chromosome is to preserve the linkage between particular genes (Macgregor, 1982). What, therefore, is the significance of chromosome size polymorphism and the related genetic/genomic variation caused by sexual recombination, mutation and sorting by drift or selection?

The results of isoenzyme and random amplified polymorphic DNA (RAPD) studies indicate that sexual recombination is either absent or very rare in natural populations of *Leishmania* (Tibayrenc *et al.*, 1990; Bañuls, 1993). Some chromosomal size polymorphism, including the presence of heteromorphic chromosomes in the same stock (Dujardin *et al.*, 1995*a*) and the random association between variants of different chromosomes (Blaineau *et al.*, 1992), has been attributed to it. However, the apparent contradictions between the results of studies using different types of genetic markers remain to be solved and the frequency and innovative impact of sexual recombination among *Leishmania* populations has still largely to be determined.

Although 'genomic' mutations have been studied at the level of the mechanisms responsible [mostly amplification/deletion of repetitive sequences (see Lighthall and Giannini, 1992) but also DNA recombination (Liu *et al.*, 1992)], as far as we are aware their sorting in natural populations has never been investigated. The karyotype (of any organism) is probably the product of selection for gene arrangement and the size and number of chromosomes, and is therefore a means of achieving an adaptative phenotype (Macgregor, 1982). The possible influence of selection on the karyotype polymorphism of *Leishmania* has therefore been explored.

PERUVIAN *LEISHMANIA* SPP.: A MODEL FOR STUDYING THE ADAPTATIVE SIGNIFICANCE OF CHROMOSOMAL SIZE VARIATION

The adaptative significance of chromosomal size variation in *Leishmania* could best be investigated in natural populations of the parasite which: (1) belong to one, single or two, closely related species; (2) are encountered in ecologically varied conditions and (3) can be characterized by different phenotypes. All three of these criteria are met by populations of two *Leishmania* species (*L. peruviana* and

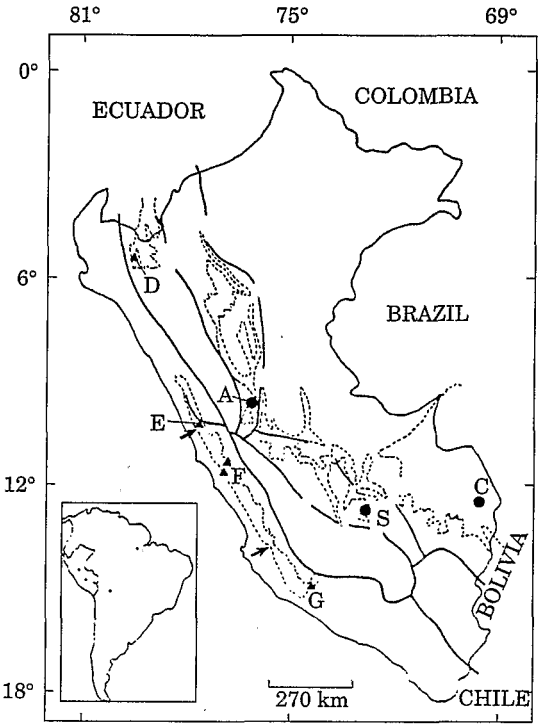

Fig. 1. Maps of Peru and South America (insert), showing the origin of the *Leishmania peruviana* (▲) and *L. braziliensis* (●) isolates (Dujardin *et al.*, 1995*b*; published with the permission of Cambridge University Press). (~), Main Andean mountain ranges; (– – –), limits of the biogeographical units (BGU; Lamas, 1982); A, Huallaga/Huanuco; B, Chanchamayo/Santa Ana; C, Inambari; D, Huancabamba; E, Surco-North; F, Surco-Centre; G, Surco-South; (→), borders between the three Surco BGU.

L. braziliensis) in Peru. Both of these species are endemic, belong to the same subgenus (*Viannia*) and are genetically very close, only being distinguishable at one enzyme locus (Arana *et al.*, 1990; Guerrini, 1993). *Leishmania peruviana* is found on the western slopes of the Andes and in inter-Andean valleys. It is responsible for the disease known locally as *uta*, characterized by cutaneous lesions of varying severity which are never associated with mucosal metastasis (unpubl. obs.). *Leishmania braziliensis* is endemic in the Amazonian forest and is responsible for primary cutaneous lesions which are more severe than those of *uta* and which, in about 10% of cases (Llanos-Cuentas, 1991), lead to severe mucosal metastasis (*espundia*).

The territory in which either or both species are endemic is geographically subdivided by numerous natural barriers (e.g. the Andes between forest and coast, deserts and valleys on the coast and western slopes of the Andes and large rivers in the forest) and is, ecologically, one of the richest in the World (Lamas, 1982). Although Peru has been divided into 48 biogeographical units (BGU) based on the endemic butterfly species in each area (Lamas, 1982), the butterfly distributions seem to reflect more general eco-geographical differences between the units. Assuming that the factors involved in the distribution of butterflies might also be responsible for the structuring of populations of other organisms, we have sampled *L. braziliensis* (11 isolates) and *L. peruviana* (37 isolates) in various BGU (Fig. 1) and investigated the karyotype of the parasites using nine chromosomes recognized by specific probes.

CHROMOSOMAL SIZE CONSERVATION AND VARIATION IN *L. BRAZILIENSIS* AND *L. PERUVIANA*

Although the five chromosomes bearing β-tubulin genes (of 295, 555, 760 and 1640 kb) or α-tubulin genes (of 700 kb) were conserved throughout the whole sample (Dujardin, 1995), the other four chromosomes were variable. The chromosome recognized by probe pLb-134Sp (Dujardin *et al.*, 1993*a*) showed a conserved pattern specific to *L. braziliensis* (700 kb) and *L. peruviana* (610 kb). Within *L. peruviana*, the size variability of two other chromosomes (recognized by probes pLb-22 and pLb-168) indicated different karyotypic populations or karyodemes (Dujardin *et al.*, 1993*b*). There was less polymorphism in the four variable chromosomes of *L. braziliensis*, as revealed by phenetic analysis: the largest CSDI for *L. braziliensis* and *L. peruviana* were 325 and 694 kb, respectively (Fig. 2; Dujardin *et al.*, 1995*b*). Populations of *L. peruviana* were selected to test our adaptative hypothesis further because of their relatively high polymorphism and the eco-geographical heterogeneity of the Andes in which they occur.

LEISHMANIA PERUVIANA: ECO-GEOGRAPHICAL STRUCTURING AS KARYODEMES

When the polymorphism of the four variable chromosomes was analysed by principal component analysis, *L. peruviana* populations were found to cluster according to their BGU of origin, revealing a strong eco-geographical structuring (Fig. 3; Dujardin *et al.*, 1993*b*). Such structuring was also apparent in the chromosomal dissimilarity of *L. braziliensis* isolates, which increased from north to south in a chromosomal cline (Fig. 2; Dujardin *et al.*, 1995*b*). Although this might be the result of genetic drift (as the insulated nature of the Andean valleys where *L. peruviana* is endemic favours the formation of small, relatively isolated populations of parasites and, consequently, genetic drift), strong selective factors, such as those imposed by the vector, are also probably involved. At least three different vector species are encountered in the different BGU sampled (Dujardin *et al.*, 1993*b*; Perez *et al.*, 1994; A. Caceres, P. Villaseca, R. Inga, M. Lopez, J. Arevalo and A. Llanos-Cuentas, unpubl. obs.). Lainson and Shaw (1987) thought that the sandfly vectors were involved in the diversification of leishmanial populations and the specific relationships between parasites and vectors have recently been attributed to the lipophosphoglycan on the parasite surface (Pimenta *et al.*, 1994).

The strong link between the polymorphism of the variable chromosomes and eco-geographical differences was highlighted by a study of the isoenzymes of the same *L. peruviana* populations (Bañuls, 1993). Correlations were calculated between karyotype divergence (measured by CSDI on the four variable chromosomes) or enzymatic variation (measured by Jaccard distance on 16 loci) and eco-geographical differences (the distance separating the different foci). Eco-geographical differences were found to explain only 8% of the total enzymatic variation but most (62·3%) of the total karyotype divergence (Dujardin, 1995). The mechanisms promoting variability in the two sets of characters are therefore probably different, possibly at the level of selective pressure.

ASSOCIATION BETWEEN CHROMOSOMAL AND PHENOTYPIC VARIABILITY

If the variability of the four chromosomes studied does have an adaptative significance, it should be possible to associate it with some phenotypic effects. Such an association has been indicated (see Table) by preliminary, indirect observations of patients living in the areas where the parasites were isolated (Dujardin, 1995; A. Llanos-Cuentas and C. Davies, unpubl. obs.), as well as by direct observations *in vitro* (Dujardin, 1995). All parasites with a pLb-134Sp chromosome of 700 kb (i.e. *L. braziliensis*), for example, can cause *espundia*, whereas all parasites with the smaller, 610-kb chromosome (i.e. *L. peruviana*) cannot. Within *L. peruviana*, all parasites with a large, 1300-kb pLb-22 chromosome and a small,

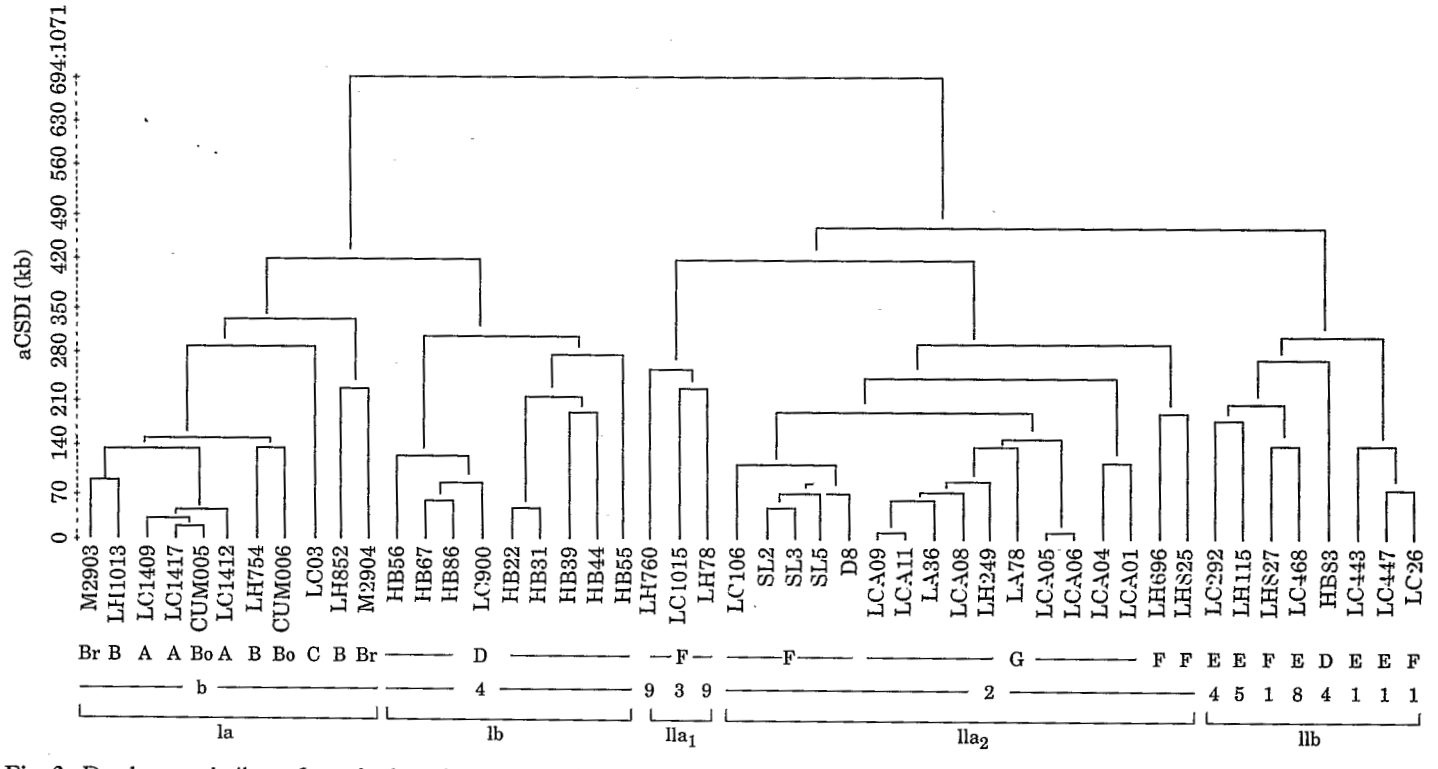

Fig. 2. Dendrogram built-up from absolute chromosomal size-difference indices (aCSDI). Under the dendrogram are given, from top to bottom: the isolate code, its geographical origin [Br, Brazil; Bo, Bolivia; A–G, BGU (see legend to Fig. 1)], its species (b, *L. braziliensis*) or *L. peruviana* karyodeme (1–9), and the cluster to which it belongs (Ia, Ib, IIa_1, IIa_2 or IIb). From Dujardin *et al.* (1995*b*) and published with the permission of Cambridge University Press.

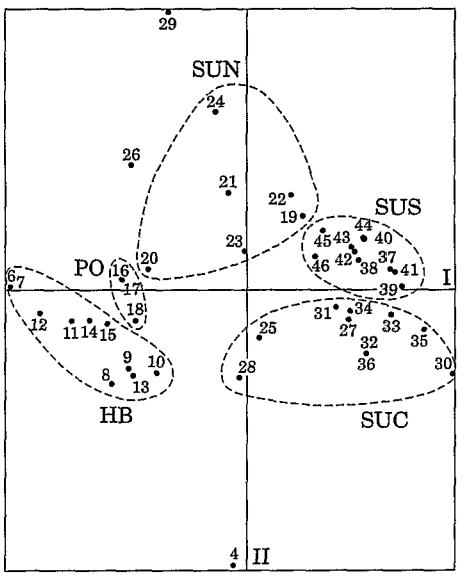

Fig. 3. Principal component analysis of the sizes of chromosomes hybridized by the four probes pLb-134Sp, -134Sg, -22 and -168. Main axes are responsible for 57·6% (I) and 23·3% (II) of the total variability. Clustering of *L. peruviana* isolates by BGU is observed. From Dujardin *et al.* (1993*b*) and published with the permission of the Liverpool School of Tropical Medicine.

640-kb pLb-168 one were isolated in the BGU where patients show the most numerous and largest cutaneous lesions (i.e. Huancabamba, in northern Peru; Fig. 1), and they were the most virulent, in terms of growth characteristics, *in vitro*. In comparison, parasites with a smaller (1150-kb) pLb-22 chromosome and/or a larger (700-kb) pLb-168 one, found in southern BGU (Surco; Fig. 1), where patients show the least numerous and smallest lesions, were less virulent.

Once such links between chromosomal polymorphism and phenotypic variability had been loosely established, the next step was to see if genes coding for key functions were present on and rearranged in the chromosomal variants.

REARRANGEMENT OF REPETITIVE GENES CODING FOR KEY FUNCTIONS

Analysis of chromosomal genetic content was initiated with the pLb-134Sp chromosome. The size of this chromosome decreases from *L. braziliensis* to *L. peruviana* (by about 90 kb, on average) and this may be related to the sequence recognized by the pLb-134Sp probe, as the hybridization intensity with this probe often follows a similar trend. Sequencing revealed that this probe recognized a highly conserved portion of the gp63 genes and restriction fragment length polymorphism (RFLP) analysis showed that there were dramatic rearrangements of these genes between the two species (Fig. 4; Dujardin *et al.*, 1994*a*),

TABLE

*Association between eco-geography, genotype (mean size of chromosome hybridizing with the respective probe) and phenotype**

Eco-geography	*Probe and (recognized genotype)*	*Phenotype*
Forest	pLb-134Sp (700 kb)	Potential *espundia* in patients
Andes	pLb-134Sp (610 kb)	Unable to produce *espundia*
Huancabamba	pLb-22 (>1300 kb) and pLb-168 (640 kb)	Numerous, large lesions in patients; high multiplication rate *in vitro*
Surco-North and -Centre	pLb-22 (<1300 kb) and/ or pLb-168 (700 kb)	Few, small lesions
Surco-South	pLb-22 (1150 kb) and pLb-168 (700 kb)	Low multiplication rate *in vitro*

*From Dujardin (1995). Phenotypes were based on indirect observations of patients living in the areas where the parasites were isolated (A. Llanos-Cuentas and C. Davies, unpubl. obs.) or of multiplication of the parasites *in vitro*.

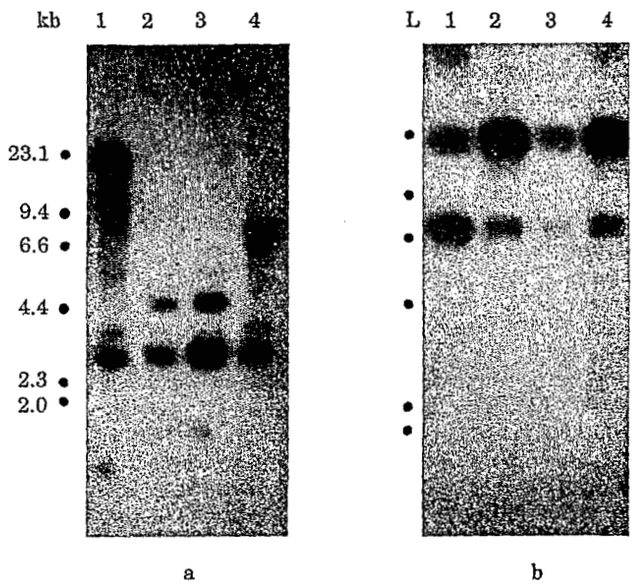

Fig. 4. Polymorphic pattern of genomic DNA from *L. braziliensis* M2903 (lane 1), *L. guyanensis* M5378 (2) and LEM 669 (3) and *L. peruviana* LC26 (4), restricted with *Sal*I (a) or *Bgl*I (b) and hybridized with pLb-134Sp (gp63). L, λ/*Hin*dIII ladder. From Dujardin *et al.* (1994) and published with the permission of the Liverpool School of Tropical Medicine.

probably associated with a decrease in copy number from *L. braziliensis* to *L. peruviana* (Victoir *et al.*, 1995). The repetitive nature of the gp63 genes [organized in a tandem array of more than 22 copies in *L. guyanensis* (Steinkraus *et al.*, 1993)] might favour recombination by unequal cross-overs. Given the functional importance of gp63 (see Medina-Acosta *et al.*, 1993), it is obvious that any rearrangement of gp63 genes, if it affects their functionality (e.g. by gene dosage or position effect), could easily affect parasite phenotype.

CONCLUSIONS

In the present *L. braziliensis–L. peruviana* model, the suggested cause-effect link between rearrangement of gp63 genes and phenotypic variation needs to be confirmed by further experimental, clinical and epidemiological studies. At this stage, however, all the results support a coherent hypothesis of adaptative chromosomal variability in natural populations of Peruvian *Leishmania*. Some chromosomes may be predisposed to rearrangement because they contain many repetitive sequences. If these repetitive sequences correspond to functionally important genes, rearrangement of their host chromosome will sometimes lead to a phenotypic variant with a selective advantage or disadvantage, such as a difference in virulence. The high level of ecological diversity in Peru strongly favours selection of the variants with advantageous phenotypes.

The general value of this adaptative hypothesis should be addressed. Firstly, within the *L. braziliensis–L. peruviana* context, can the size variation of the three other chromosomes recognized as variable be explained by this hypothesis, and what is the reason for the conservatism in the five consensus chromosomes? Secondly, the adaptative hypothesis should be tested in other *Leishmania* models, where other populations assigned to the same species are encountered under different ecological conditions (such as Old World *L. infantum* and New World *L. chagasi*) or are responsible for variable

pathologies (e.g. dermotropic and viscerotropic *L. infantum*). Finally, as the vectors constitute the interface between the environment and the parasites, the genetics of the vector populations should be studied in parallel with that of the parasite populations.

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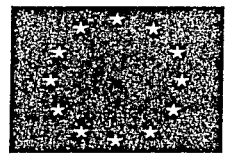

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