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Numerical immunotaxonomy of *Leishmania*

I. DIFFERENTIATION OF FOUR STRAINS OF *LEISHMANIA* BY SEROLOGICAL TESTS

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Summary. This paper describes the application of numerical methods to the arrangement of four leishmanial strains according to their reactivity and cross-reactivity in tests of parasite agglutination, indirect immunofluorescence and passive cutaneous anaphylaxis with antisera prepared by immunization or infection of rabbits, guinea-pigs and mice. Using corresponding pools of animal sera as test 'reagents' the antigenic reactivity of the four leishmanial strains (*L. enriettii, L. tropica major, L. aethiopica* and *L. mexicana amazonensis*) was scaled by reference to end-point serum titres; and antigenic relationships between individual strain pairs were expressed as mean similarity coefficients, giving equal weight to the results of the different serological tests. Overall analysis of the results revealed that *L. mexicana amazonensis* and *L. tropica major* were the two most closely related strains, clustering with an overall similarity coefficient of 89 per cent, whereas coefficients of similarity between other strain combinations fell between 75 and 80 per cent. Although different sera had different discriminatory capacity for the leishmanial strains, two combinations of serum reagent and test system yielded relationships between the four strains that most closely approximated to the overall values. These were: (a) immunofluorescence tests with mouse antisera; and (b) agglutination tests with selected rabbit antisera. The results illustrate the use of a number of immunological parameters in relating micro-organisms of a given genus, and reveal a serological classification of the four leishmanial strains at variance with their geographical origin.

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INTRODUCTION

Classification of *Leishmania* is based largely upon the organotropism of the parasite, upon the clinico-pathological and epidemiological features of human leishmaniasis (Turk and Bryceson, 1971) and upon serological reactions with rabbit antisera (Noguchi, 1926; Adler, 1964). All these approaches have given rise to conceptual and technical difficulties. Thus, parasite organotropism may be an unreliable criterion since *Leishmania* are not in every case located exclusively in the skin or viscera (Manson-Bahr, 1959; Hoogstraal and Heynemann, 1969). Likewise, the use of clinico-pathological and epidemiological criteria for major classification purposes is limited by overlap of the principal forms of clinical leishmaniasis and by the difficulty of transmitting similar

clinical disease to experimental animals (Adler and Zuckerman, 1948; Manson-Bahr, 1964; Mata, Ruiz, Gaxa and Morales, 1968; Preston and Dumonde, 1975). The use of single serological tests to classify leishmanial variants reveals numerous cross-reactions within the genus (Bray and Lainson, 1965, 1966, 1967) and with other flagellate protozoa (Camargo and Rebonato, 1964). It would seem therefore that the use of a single serological test or of a very few clinical or physiological criteria has not yielded a classification of leishmanial variants which has proved of general epidemiological or pathogenetic significance. A wide variety of immunological phenomena are now known to be involved in the host response to leishmania (Bryceson, Bray, Wolstencroft and Dumonde, 1970) and opportunity therefore arises to exploit these for classification purposes.

In the present work we have sought to develop a numerical approach to the immunotaxonomy of *Leishmania*, by measuring the reactivity of four selected leishmanial variants with different components of the immunological response of infected and immunized hosts, and by giving equal weight (Adanson, 1763) to the results of different tests. The approach consists of evaluating the proportion of immunologically reactive features that the different organisms have in common, in order to express their relationships as indices of similarity, as in bacterial taxonomy (Sneath, 1962). We have compared the capacity of three laboratory animal species (rabbit, guinea-pig and mouse) to react with the organisms by examining the specificity of humoral and cell-mediated immunological responses to infection and to artificial immunization; and, where appropriate, by determining the extent of the resulting cross-protection. In this paper we describe the use of three humoral test systems (parasite agglutination, immunofluorescence, and passive cutaneous anaphylaxis) in evaluating the relationships between four leishmanial strains. In subsequent papers we describe the strain relationships by tests of immediate and delayed hypersensitivity, by *in vitro* tests of cell-mediated immunity, and by the extent of resistance to infection following cross-immunization.

MATERIALS AND METHODS

Experimental design

Four *Leishmania* strains (*L. enriettii*, *L. tropica major*, *L. aethiopica* and *L. mexicana amazonensis*) were chosen for this study. *L. enriettii* and a Russian strain of *L. tropica* (*L. tropica major*) were known to produce self-healing cutaneous leishmaniasis in guinea-pigs and CBA mice respectively where many features of their immunological response had been established in earlier work (Bryceson *et al.*, 1970; Preston, Carter, Leuchars, Davies and Dumonde, 1972). An Ethiopian strain of leishmania (now designated *L. aethiopica*) and a strain of *L. mexicana* were introduced into the study because it was felt that in their origin from the Old and New World they might resemble respectively the organisms *L. tropica major* and *L. enriettii* in their immunological reactivity. Preliminary experiments with these four strains showed that cutaneous infection in guinea-pigs could only be achieved with *L. enriettii* and that CBA mice could only be infected cutaneously with *L. mexicana* and *L. tropica major*. Accordingly, sera were obtained from guinea-pigs infected with *L. enriettii* and from CBA mice infected with *L. tropica major* and *L. mexicana amazonensis* as models of 'natural' host–parasite relations. Sera were also obtained from rabbits, guinea-pigs and CBA mice immunized with killed organisms of all four strains. The experiments consisted of comparing the reactivity and cross-reactivity of antigenic preparations of these organisms with the homologous and heterologous sera viewed as 'test reagents'. By evaluating the ability of these 'reagents' to discriminate the four organisms in agglutination, immunofluorescence and passive cutaneous anaphylaxis, indices of similarity between the organisms were obtained.

Organisms

The origins and past histories, as far as they are known, of the materials used are given briefly below together with the stabilate designations of the materials most nearly related to them and at present in the cryobank of the Department of Medical Protozoology, London School of Hygiene and Tropical Medicine.

(i) *Leishmania enriettii*. Isolated 1945 from naturally infected laboratory guinea-pig at Curitiba, Parana State, Brazil (Muniz and Medina, 1948); 1946–56 passaged in guinea-pigs and NNN medium (University of Sao Paulo, Brazil; J. O. Coutinho, A. D. F. Amaral); in NNN medium (Tulane University, New Orleans, U.S.A.; A. D'Alessandro, J. G. Yaeger); in guinea-pigs (Wellcome Research Laboratories, Beckenham, England; R. A. Neal); in

NNN medium (LSHTM, London; R. S. Bray). Stabilate LUMP 826 (20.12.72).

(ii) *Leishmania tropica major.* Isolated January 1959 from *Rhombomys opimus*, Karakul, Bokhara, Uzbekistan, USSR; passaged in 4N medium (S. D. Moshkovsky); in 4N medium (Wellcome Research Laboratories, Beckenham, England; Neal 1964); in mouse and 4N medium (LSHTM, London; P. G. Sargeaunt). Stabilate LUMP 567 (18 May 1971).

(iii) *Leishmania aethiopica.* Isolated 17.2.72 from *Phlebotomus longipes*, Kutaber, Ethiopa (Bray *et al.*, 1973); passaged in 4N medium (Addis Ababa; R. W. Ashford); in 4N medium (LSTM, Liverpool, England, designated Strain L 123; P. J. Gardener); in 4N (LSHTM, London; V. C. L. C. Wilson). Stabilate LUMP 729 (13.7.72).

(iv) *Leishmania mexicana amazonensis.* Isolated 1971 from *Proechimys guyanensis*, Utinga Forest, Belem, Para, Brazil, passaged in hamsters (Wellcome Parasitology Unit, Belem; R. Lainson and J. J. Shaw); passaged in hamsters and 4N (LSHTM, London; V. C. L. C. Wilson). Stabilate LUMP 736 (18 July 1972).

Cryopreservation principles and methods were those described by Lumsden (1972) and Lumsden, Herbert and McNeillage (1973).

Maintenance of strains and preparation of immunizing antigens

Organisms were cultured at 25° in Hanks's balanced salt solution overlying NNN medium using rabbit blood in Roux flasks. Strains were maintained by passage in culture every 5–7 days and where appropriate by passage in guinea-pigs (*L. enriettii*) and CBA mice (*L. tropica major* and *L. mexicana amazonensis*). To prepare sonicated promastigotes for artificial immunization, organisms were harvested from 500 ml volumes of medium and were washed three times by centrifugation at 3000 *g* for 15 min using 500 ml quantities of phosphate-buffered saline pH 7·2 (PBS). After resuspension in a small volume of PBS, promastigotes were counted with the aid of a haemocytometer and the concentration adjusted to $5–8 \times 10^9$/ml. Sonication was undertaken at 0–4° with an MSE ultrasonic disintegrator set at 6 kc/s, for 3 30 s exposures. Disintegrated promastigotes were stored in PBS at −70° until required.

Immunization of rabbits, guinea-pigs and mice

Rabbits of the old English breed were immunized in duplicate or triplicate, intravenously and intramuscularly. The intravenous dose was 2×10^9 disintegrated promastigotes at weekly intervals for 3 weeks; the rabbits were bled 14 days after the last injection. The intramuscular dose consisted of 3×10^9 disintegrated promastigotes suspended in 0·5 ml PBS and emulsified with 0·5 ml of Freund's complete adjuvant (FCA) (Difco Laboratories) injected into several sites, followed after 4 weeks with a further injection of 3×10^9 disintegrated promastigotes emulsified with Freund's incomplete adjuvant (FIA:Difco); the rabbits were bled 20 days after the last injection. Guinea-pigs of the outbred Hartley strain weighing about 350 g were immunized in groups of three to four intraperitoneally and intramuscularly/subcutaneously. The course of intraperitoneal immunization consisted of four injections of $2·8 \times 10^8$ disintegrated promastigotes at weekly intervals; the animals were bled 7 days after the last injection. For intramuscular/subcutaneous immunization 8×10^8 disintegrated promastigotes were suspended in 0·25 ml Hanks's BSS, emulsified with 0·25 ml FCA, and injected into the hind foot pads and hind leg muscles; 3 weeks later animals were boosted intramuscularly with 5×10^8 disintegrated promastigotes emulsified in FIA and they were bled after a further 3 weeks. Groups of 6-week-old CBA mice were immunized intraperitoneally by three injections of 1×10^7 disintegrated promastigotes at weekly intervals followed by a fourth injection of 2×10^9 disintegrated promastigotes; these animals were bled 1 week after the last injection. Mice were also immunized intramuscularly: 10^8 disintegrated promastigotes in 0·2 ml PBS were emulsified with 0·2 ml FCA and injected into the muscles of the hind legs, followed 2 weeks later by 2×10^8 disintegrated promastigotes emulsified with FIA; these mice were bled 2 weeks after the last injection.

In previous work we have shown that injection of guinea-pigs with FCA alone does not result in the development of circulating antibody to *L. enriettii*, as judged by haemagglutination, passive cutaneous anaphylaxis (Bryceson *et al.*, 1970) or immunofluorescence (Radwanski, Bryceson, Preston and Dumonde, 1974); nor does FCA alone induce resistance to *L. enriettii* or the development of delayed hypersensitivity responses in guinea-pigs (Bryceson *et al.*, 1970, 1972). For these reasons we considered that immunization of animals with FCA-emulsions of leishmanial organisms would not interfere with the specificity of corresponding antibody production.

Infection of guinea-pigs and mice

Infection of guinea-pigs with *L. enriettii* was initiated from 4-week-old nasal lesions by injecting 10^6 amastigotes subcutaneously into the tip of the nose (see Bryceson *et al.*, 1970); sera were obtained 6 weeks after infection. CBA mice were found susceptible to *L. tropica major* (Preston *et al.*, 1972) or to *L. mexicana amazonensis*, but not to *L. enriettii* and *L. aethiopica*. Infections were produced by intradermal inoculation in the shaven rump of 10^6 amastigotes from 3–4-week-old lesions or by 10^6 promastigotes (Preston *et al.*, 1972); sera were obtained 12 weeks after infection.

Serological methods

Parasite agglutination. This was accomplished with formalinized trypsinized organisms after the method used for *Trypanosoma cruzi* epimastigotes by Vattuone and Yanovsky (1971). Twenty volumes of 0·4 per cent trypsin in PBS (pH 7·7) was mixed with one volume of washed packed promastigotes and the suspension was incubated at 37° for 45 mins. The organisms were then washed four times in PBS (pH 7·2) and suspended in 0·4 per cent formaldehyde in PBS (pH 7·2). The suspension was filtered through a sintered glass filter (porosity 15–40 μm) to remove any remaining clumped organisms, and finally standardized on a spectrophotometer at wavelength 555 nm to an optical density reading of 0·50. These suspensions were stored at 4° for several months without apparent loss of agglutinating reactivity. Agglutination tests were done in MRC pattern perspex trays by mixing 0·2 ml volumes of the formalinized promastigotes with 0·2 ml serial dilutions of test sera in PBS (pH 7·2). Trays were kept for 2 h at room temperature and 4° overnight. Results were read by low power microscopic examination of individual well contents pipetted onto slides; agglutination was graded on a four point scale (−, +, + +, + + +). The agglutination titre was taken as the highest (2-fold) serial dilution of test serum which gave appreciable agglutination (+) when compared with a control suspension of organisms incubated under the same conditions with added PBS.

Immunofluorescence. Cultured promastigotes were washed thrice in PBS (pH 7·2), resuspended at a concentration of about 10^7/ml and using a Pasteur pipette small drops were placed on grease-free PTFE-coated 12 spot microscope slides (C. A. Hendley and Co., Essex). Excess fluid was sucked back, the slides were fan dried and stored at −70°. Just before use the slides were removed from −70° and immediately fan dried. Indirect immunofluorescence was undertaken on unfixed smears by a standard 'sandwich' technique (see Preston *et al.*, 1972). The middle layer consisted of serial two-fold dilutions of rabbit, guinea-pig or mouse sera, allowed to react for 30 min at room temperature in a moist chamber, and washed for 30 min, with constant agitation, in four changes of PBS (pH 7·2). The top layer consisted of the appropriate, diluted (1:20–1:40) fluorescein isothiocyanate (FITC)-conjugated antiglobulin, i.e. sheep anti-rabbit, swine, anti-guinea-pig or guinea-pig anti-mouse (Nordic Diagnostics) containing 0·1 per cent Evans Blue, poured on the slides, left for 30 min and washed as above. The slides were mounted with aqueous Polarfluor mountant B (Polaron Equipment), and examined either immediately or within 24 h on a Zeiss Photomicroscope II equipped with a ×25 objective and a dry, dark field condenser. Lighting was provided by a HBO 200 mercury vapour lamp with the use of BG 38 and BG 12 exciter filters and 50/44 barrier filters. Controls consisted of preparations exposed to dilutions of serum of unsensitized animals, a known positive serum, and promastigotes exposed to FITC-conjugated antiglobulins alone.

Immunofluorescence was graded visually on a four point scale (−, +, + +, + + +). Serum titres were recorded as the highest serial (two-fold) dilution of serum which gave appreciable (+) immunofluorescence compared to control preparations.

Passive cutaneous anaphylaxis. This was done in guinea-pigs using dilutions of rabbit and guinea-pig serum intradermally and soluble leishmanial antigen intravenously. Soluble antigen ('PSA': Bryceson *et al.*, 1970) was prepared by urea extraction of promastigotes as previously described. For the PCA test, 0·1 ml volumes of diluted sera were injected intradermally into the shaved flanks of Hartley strain guinea-pigs weighing about 450 g. Two test sera, of which one was always homologous to the antigen to be injected intravenously, were titrated on groups of five guinea-pigs. Six serum dilutions, in two rows of three, were conveniently injected on either flank, and injection sites were randomized in individual guinea-pigs. Two control sites were also marked out on each guinea-pig. The negative control was 0·1 ml Hanks's BSS and the positive control was 0·1 ml of a standard dilution of rabbit anti-bovine gamma-globulin

BGG) previously shown to produce a small area of dye leakage after the injection of 2 mg of the corresponding antigen. After a skin-fixation period of 24 h a mixture of 2 mg protein soluble antigen (PSA), 2 mg BGG Fraction II (Miles Laboratories Inc., Kamkakee, U.S.A.) in Hanks's BSS, and 0·5 ml of 1 per cent Evans blue was injected intravenously. Five guinea-pigs were used for each titration and readings were only taken from those which responded appropriately to both the positive and negative controls. PCA titres were recorded 30 min after the intravenous injection, as the reciprocal of the highest dilution of antiserum causing appreciable dye-leakage as compared to the negative controls.

Pooling and storage of 'reagent' sera

Sera were accordingly pooled as 'reagents' from correspondingly immunized or infected animals. For example, rabbits were immunized intravenously in four sets of three animals with the four separate organisms, and immunized intramuscularly in two sets of two animals with *L. enriettii* and *L. tropica major*. These sixteen animals therefore yielded six serological reagents derived from two different immunizing procedures (i.e. intravenous and intramuscular injection of sonicated organisms); and the serum pools were divided into 0·5 ml aliquots and stored at −20° for the duration of the study. Altogether twenty pools of sera (rabbit, I–VI; guinea-pig, 1–6; mouse, A–H) were designated as working reagents for this study and stored in aliquots under identical conditions.

Scaling of results

Using the various pools of animal sera as 'test reagents' the antigenic reactivity of the four leishmanial variants was expressed as the end-point titres of homologous and heterologous immune sera from the infected and artificially immunized animals. The following six-point scale was constructed to grade the strength of these reactions in the three test systems employed:

End-point titres of homologous or heterologous sera from infected or artificially immunized animals	Scale value assigned to strength of reaction
0–1:10	1
1:20–1:40	2
1:80–1:160	3
1:320–1:640	4
1:1280–1:2560	5
1:5120–1:10,240	6

A titre of 1:10 was assigned the lowest value of 1 due to the possibility that low titres could represent nonspecific reactivity. From a titre of 1:20 every four-fold increase was considered one step in scale value, thus arriving at a value of 6 for the highest titres (1:5120 and 1:10,240) encountered in this study. By this means, appropriate scale-values for the end-points of homologous and heterologous reactions were entered beside the tabulated results of serum pool titres (Tables 1–7). These scale values were converted to lie between zero and unity so that the antigenic relationships between given pairs of strains could be expressed as similarity coefficients.

Calculation of similarity coefficients between leishmanial strains

For any single test system, the antigenic relationship between pairs of strains was revealed by their cross-reactions with a given serological reagent. To calculate similarity coefficients between strains, scale values were first rescaled to fall in the range zero to unity as follows:

$$\text{rescaled value} = \frac{\text{actual scale value} - \text{lowest scale value}}{\text{highest scale value} - \text{lowest scale value}}.$$

For example, using a rabbit antiserum against *L. enriettii* (pool I) in a direct agglutination test (see Table 1), the titres obtained with *L. enriettii* (1:1280) and *L. tropica* (1:320) were assigned scale values of 5 and 4 respectively. When rescaled on a 'zero to unity' basis, these figures gave the quotients $\frac{5-1}{6-1}$ (0·8) and $\frac{4-1}{6-1}$ (0·6) respectively for grading the reactivity of *L. enriettii* and *L. tropica* (designated strains 'a' and 'b' respectively: see Tables 1–2) with the antiserum reagent (pool I). The numerical difference between these re-scaled values ($\Delta ab = 0{\cdot}2$) was then recorded (Table 2); and the degree of similarity was then scored by subtracting this difference (0·2) from unity (i.e. $1 - 0{\cdot}2 = 0{\cdot}8$). When multiplied by 100 this score yielded a single similarity coefficient (i.e. 80 per cent) between the two strains (*L. enriettii* and *L. tropica*) for that combination of serological reagent (antiserum pool I) and test system (direct agglutination). This percentage figure was entered on the right-hand side of the table of results for corresponding reagent: test combinations (Pool I, ab = 80 per cent, Table 2).

Using a given serum reagent (e.g. Pool I) in a given test (e.g. direct agglutination) the relative reactivity of all four leishmanial strains was recorded as a set of six similarity coefficients for individual pairs of

strain combinations (e.g. ab to cd, Table 2). Sets of these similarity coefficients were then assembled (see Table 2) corresponding to the sets of sera from a single animal species (e.g. rabbit) tested by a single serological method (e.g. direct agglutination); mean coefficients for the six leishmanial strain combinations were then calculated for that serological test situation (e.g. agglutination with all rabbit antisera, Table 2).

Altogether twenty-one serum pools from rabbits, guinea-pigs and mice were examined by tests of agglutination and passive cutaneous anaphylaxis (rabbit and guinea-pig sera) and by immunofluorescence (with sera from all three animal species). When all the sets of similarity coefficients were assembled, a mean similarity coefficient was evaluated for each serum pool in respect of its ability to react by all three serological methods with a given pair of leishmanial strains. For example, serum I gave similarity coefficients between strains 'a' and 'b' of 80 per cent in agglutination (Table 2), 60 per cent in immunofluorescence (Table 4) and 100 per cent in PCA (Table 7), yielding a mean similarity coefficient of 80 per cent for that serum in relating strains 'a' and 'b'. Average values of these similarity coefficients were then calculated for the three separate groups of rabbit, guinea-pig and mouse sera; and from these data overall similarity coefficients were obtained based on 'animal species' (see Table 8).

The data were also analysed with reference to 'serological test' (Table 8). Three sets of average similarity coefficients were obtained from the testing (Tables 1–7) of serum 'reagents' by agglutination, by immunofluorescence, or by cutaneous anaphylaxis; and from these data overall similarity coefficients were obtained based on 'serological test' (Table 8). The experimental design was asymmetrical because mouse sera did not react appreciably by direct agglutination and by passive cutaneous anaphylaxis. Despite the experimental asymmetry the overall similarity coefficients calculated for 'serological test' and for 'animal species' were in close agreement.

By inspection of the results, combinations of serological reagents, test systems and animal species were sought which yielded similarity coefficients for the six pairs of strain combinations that approximated most closely to the overall similarity coefficients obtained as described. By this means it was possible to indicate which procedures appeared to be of best taxonomic value in estimating relationships between the four leishmanial strains.

Discriminatory capacity of serological reagents

The supplementary question arose of whether a positive identification of these four strains could have been made by the use of certain of these serological reagents in specified test systems. Accordingly, a numerical procedure was devised whereby the preferential activity of a given serological reagent with the homologous organism was expressed as an index by reference to the extent of serological cross-reactivity with the three heterologous organisms. Calculation of this 'discriminatory index' was done as follows.

For any single test system (i.e. agglutination, immunofluorescence or PCA) the maximum possible discrimination afforded by a given serum was taken as a set of four numbers 6:(1,1,1) where the first number (i.e. 6) was the maximum scale-value possible for a homologous reaction, and the other three numbers (i.e. 1,1,1) were scale-values assigned for minimal reactivity in the heterologous reactions. On this basis, zero discrimination would be recorded when the titres (and hence the scale values) given by the heterologous reactions were identical to that given by the homologous reaction, e.g. 3:(3,3,3); (as in Table 5, serum pool 7). Convenient handling of these sets of numbers termed 'scale notation' in Tables 1–7) is illustrated in these examples by the quotients $\frac{6}{1}$ (= 6·0) and $\frac{3}{3}$ (= 1·0) respectively where the numerator corresponds to the homologous scale-value and the denominator refers to the arithmetic mean of the three heterologous scale-values. In the separate test systems, quotients obtained for the 'reagent' sera therefore fell between 1·0 and 6·0; and individual quotients (q) were therefore rescaled to fall between 0 and 100 per cent by means of the fraction $\frac{q-1}{6-1} \times 100$ per cent. The term 'discriminatory index' (DI) was given to the percentages thus obtained which described conveniently the relative capacity of a serological reagent to discriminate positively for its homologous organism in the given test procedure. In this paper, no negative discriminatory indices were recorded; and inspection of the results (see Table 9) indicated that to be of discriminatory value, a serum was required to have an index (DI) of greater than 20 per cent.

RESULTS

Direct parasite agglutination tests

Rabbit antisera

Six serum pools to serve as 'reagents' (I–VI) were

Table 1. Anti-leishmanial agglutination titres of pooled sera from groups of immunized rabbits

Source and description of serum pool reagent		Titres (and scale values)† of serum pools against the four *Leishmania*				Characteristics of serum pools	
Pool no.	Immunization of rabbits*	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	Scale notation†	Discriminatory index‡ (per cent)
I	*L. enrietti* (i.v.: 14 days)						
	Serum titres:	1280 (5)	320 (4)	640 (4)	80 (3)	5:(4,4,3)	7·2
	Rescaled values§	0·8	0·6	0·6	0·4		
II	*L. tropica* (i.v.: 14 days)						
	Serum titres:	10 (1)	1280 (5)	320 (4)	20 (2)	5:(1,4,2)	22·8
	Rescaled values§	0·0	0·8	0·6	0·2		
III	*L. mexicana* (i.v.: 14 days)						
	Serum titres:	320 (4)	1280 (5)	5120 (6)	80 (3)	6:(4,5,3)	10
	Rescaled values§	0·6	0·8	1·0	0·4		
IV	*L. aethiopica* (i.v.: 14 days)						
	Serum titres:	640 (4)	160 (3)	160 (3)	2560 (5)	5:(4,3,3)	10
	Rescaled values§	0·6	0·4	0·4	0·8		
V	*L. enriettii* (i.m.: 20 days)						
	Serum titres:	5120 (6)	2560 (5)	1280 (5)	20 (2)	6:(5,5,2)	10
	Rescaled values§	1·0	0·8	0·8	0·2		
VI	*L. tropica* (i.m.: 20 days)						
	Serum titres:	80 (3)	2560 (5)	1280 (5)	160 (3)	5:(3,5,3)	7·2
	Rescaled values§	0·4	0·8	0·8	0·4		

* Immunization: i.m., intramuscular (with Freund's adjuvants); i.v. intravenous (without adjuvants); sera taken at 14 or 20 days after last immunizing injection.

† For scaling notation and rescaling of serum titres see 'Materials and Methods'.

‡ For definition of discriminatory index (of sera) see 'Materials and Methods'.

§ Rescaled values are used in Table 2 for calculation of similarity coefficients between leishmanial strains (denoted (a)–(d) in Table 2).

made from sixteen rabbits in groups of two or three as indicated in Table 1 which shows agglutination titres obtained against all four organisms. The agglutination titres against homologous and heterologous leishmania were expressed on the scale 1–6 (see Materials and Methods) and then rescaled to fall between zero and unity (Table 1). The numerical differences (Δ) between the rescaled values for pairs of the *Leishmania* strains were recorded for agglutination reactions with all six serum pools (I–VI) and from these data the mean similarity coefficients were obtained (Table 2). This analysis revealed mean values between 57 and 93 per cent (Table 2); on this basis the most related strains were *L. tropica major* and *L. mexicana amazonensis*; and the greatest strain differences were between these two strains and *L. aethiopica*. *L. enriettii* emerged with approximately similar relationships (67–70 per cent) to the other three organisms.

Inspection of the discriminatory capacity of the six serum pools in these tests (Table 1) revealed only one serum (pool II) whose discriminatory index was greater than 20 per cent. This serum, produced by intravenous immunization with *L. tropica major*, was the only rabbit serum pool which would positively identify its homologous organism by agglutination reactions with the four strains.

Guinea-pig antisera

Guinea-pigs were immunized without adjuvant by the intraperitoneal route and with adjuvant by the intramuscular route. The antisera produced by intraperitoneal immunization had no agglutinating ability (serum pools 5 and 6, Table 3). Titres for numerical evaluation were recorded with pools of sera produced by intramuscular immunization with antigen–adjuvant emulsions, obtained 23 days after boosting (pools 1–4, Table 3). These sera (pools 1–4) gave generally lower homologous and heterologous titres

Table 2. Calculation of similarity coefficients between pairs of *Leishmania* based upon cross-agglutination test with rabbit antisera

	Rescaled values (see Table 1) of serum pool titres against:				Numerical differences (Δ) between rescaled values for pairs of *Leishmania*						Similarity coefficients [(1 − Δ) × 100%] relating pairs of *Leishmania*					
	L. enriettii (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	Δ_{ab}	Δ_{ac}	Δ_{ad}	Δ_{bc}	Δ_{bd}	Δ_{cd}	ab	ac	ad	bc	bd	cd
I Anti-*enriettii* (i.v.)	0·8	0·6	0·6	0·4	0·2	0·2	0·4	0·0	0·2	0·2	80	80	60	100	80	80
II Anti-*tropica* (i.v.)	0·0	0·8	0·6	0·2	0·8	0·6	0·2	0·2	0·6	0·4	20	40	80	80	40	60
III Anti-*mexicana* (i.v.)	0·6	0·8	1·0	0·4	0·2	0·4	0·2	0·2	0·4	0·6	80	60	80	80	60	40
IV Anti-*aethiopica* (i.v.)	0·6	0·4	0·4	0·8	0·2	0·2	0·2	0·0	0·4	0·4	80	80	80	100	60	60
V Anti-*enriettii* (i.m.)	1·0	0·8	0·8	0·2	0·2	0·2	0·8	0·0	0·6	0·6	80	80	20	100	40	40
VI Anti-*tropica* (i.m.)	0·4	0·8	0·8	0·4	0·4	0·4	0·0	0·0	0·4	0·4	80	60	100	100	60	60
Mean similarity coefficients between pairs of strains based upon cross-agglutination with all six rabbit serum pools											66·7	66·7	70·0	93·3	56·7	56·7

Table 3. Anti-leishmanial agglutination titres of sera from groups of infected and artificially immunized guinea-pigs

Description of serum pool reagent		Titres (and scale values)* of serum pools against the four *Leishmania*				Similarity coefficients						Characteristics of serum pools	
Pool no.	Immunization	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	ab	ac	ad	bd	bc	cd	Scale notation*	Discriminatory index (per cent)
1	*L. enriettii* (i.m.: 3 weeks)												
	Serum titres	80 (3)	< 10 (1)	< 10 (1)	< 10 (1)	60	60	60	100	100	100	3:(1,1,1)	40·0
	Rescaled values	0·4	0·0	0·0	0·0								
2	*L. tropica* (i.m.: 3 weeks)												
	Serum titres	< 10 (1)	640 (1)	< 10 (1)	10 (1)	40	100	100	40	40	100	4:(1,1,1)	60·0
	Rescaled values	0·0	0·6	0·0	0·0								
3	*L. mexicana* (i.m.: 3 weeks)												
	Serum titres	160 (3)	320 (4)	640 (4)	80 (3)	80	80	100	100	80	80	4:(3,4,3)	4·0
	Rescaled values	0·4	0·6	0·6	0·4								
4	*L. aethiopica* (i.m.: 3 weeks)												
	Serum titres	1280 (5)	80 (3)	80 (3)	1280 (5)	60	60	100	100	60	60	5:(5,3,3)	9·2
	Rescaled values	0·8	0·4	0·4	0·8								
5*	*L. enriettii* (i.p.: 1 week)					Means							
	Serum titres	10	< 10	< 10	< 10	60	75	90	85	70	85		
	Rescaled values												
6*	*L. tropica* (i.p.: 1 week)												
	Serum titres	< 10	< 10	< 10	< 10								
	Rescaled values												
7*	Nasal lesion 6 weeks after infection with 1×10^6 am†												
	Serum titres	10	40	(0)	(0)								
	Rescaled values												

Immunization of guinea-pigs: i.m., intramuscular (with adjuvants); i.p., intraperitoneal (without adjuvants). Sera taken 1 or 3 weeks after last immunizing injection. For other abbreviations see Tables 1 and 2.

* Scale notations and discriminatory indices for serum pools 5–7 are not evaluated because of their low agglutination titres.

† am = Amastigotes of *L. enriettii*.

than the rabbit sera. Calculation of the similarity coefficients between the six strain combinations, based upon serum pools 1–4, revealed mean values between 60 and 90 per cent. On this basis the most related strains were *L. enriettii* and *L. aethiopica* (mean similarity coefficient 90 per cent; Table 3).

In contrast to the rabbit sera, guinea-pig anti-*enriettii* (pool 1) and anti-*tropica* (pool 2) were species specific in respect of the four strains examined and gave discriminatory indices (DI) of 40 and 30 per cent respectively. Antiserum against *L. mexicana* (DI:4·0 per cent) cross-reacted most strongly with *L. tropica major*, whilst antiserum against *L. aethiopica* (DI:7·2 per cent) cross-reacted up to its homologous titre with *L. enriettii*. Post-infection sera (pool 7) had agglutination titres too low for consideration as taxonomic reagents.

Conclusions

Although the rabbit and guinea-pig sera gave different arrangements of the four leishmanial strains it was of interest to take the results together in the evaluation of strain similarities by sero-agglutination. On this basis the most distantly related strain pairs were *L. enriettii:L. tropica major*, and *L. tropica major:L. aethiopica* (average similarity coefficients: 63·3 per cent). The two most closely related strains were *L. tropica major* and *L. mexicana* (average similarity coefficient: 89·2 per cent).

As the guinea-pig serum pools 1 (anti-*enriettii*) and 2 (anti-*tropica*) reacted exclusively with the homologous organisms (DI:40 and 60 per cent respectively), identification of these two organisms could be made with certainty by the use of these two serum pools. Given this identification, *L. mexicana* and *L. aethiopica* could then be differentiated by means of rabbit serum pools III and V, even though the discriminatory capacity for all four strains was low (DI:10 per cent). It was of interest that antibody produced during the only 'natural' infection (i.e. *L. enriettii* in the guinea-pig) had agglutination titres too low for discriminatory or taxonomic purposes, despite the fact that high immunofluorescence titres were observed (Table 5).

Immunofluorescence reactions of rabbit, guinea-pig and mouse antisera with unfixed promastigotes

Tables 4 and 5 present immunofluorescence titres of serum pools I–VI (rabbit) and 1–7 (guinea-pig) with the four leishmanial strains; and Table 6 presents the immunofluorescence titres obtained with eight serum pools (A–H) derived by artificial immunization and infection of CBA mice with the organisms. The results show that highest titres were obtained with some rabbit sera and that guinea-pig and mouse sera raised by intraperitoneal immunization and by infection had immunofluorescence titres of sufficient strength for use in this study. In contrast to the agglutination results, immunofluorescence analysis revealed that antisera raised in the different animal species gave very similar ranking between mean similarity coefficients of strain pair combinations. By immunofluorescence, the two most related strains were *L. tropica major* and *L. mexicana*; and the most distant were *L. enriettii* and *L. aethiopica*. However, relationships between *L. enriettii* and both *L. tropica major* and *L. mexicana* (78 and 76 per cent) were of the same order as the relationships between these two strains and *L. aethiopica* (77 and 81 per cent respectively).

As judged by the discriminatory index, all rabbit sera were poor at differentiating between the four leishmanial strains by immunofluorescence. However, adjuvant-immunization of both mouse and guinea-pig with *L. enriettii* produced sera (pools A and 1) of high discriminatory value (60 per cent; 40 per cent) for this organism, which could therefore be identified with certainty. The additional use of mouse sera E and F (DI:25 per cent) with sera A and 1 would therefore lead to the positive identification of *L. enriettii* and *L. tropica* by immunofluorescence. Differentiation of *L. mexicana* from *L. aethiopica* could then be made by using the rabbit serum III and guinea-pig serum 3; but the identification of *L. aethiopica* by immunofluorescence could only be made by exclusion.

Passive cutaneous anaphylaxis (PCA)

Table 7 presents data obtained with PCA reactions undertaken in guinea-pig skin using (intradermally) all six serum pools (I–VI) from the sets of immunized rabbits, two of the serum pools (3 and 4) from the sets of immunized guinea-pigs; and using urea-extracted soluble antigen (PSA) from the four leishmanial strains (intravenously, after 24 h skin fixation of antibody). No PCA reactions were given by the mouse antisera when tested in guinea-pig skin; and the PCA-reactivity of guinea-pig serum pools 1, 2, 5, 6 and 7 was too low to be considered.

Table 4. Anti-leishmanial immunofluorescence titres of serum pools from immunized rabbits

Source and description of serum pool reagent		Titres (and scale values)* of serum pools against the four *Leishmania*				Similarity coefficients						Characteristics of serum pools	
Pool no.	Immunization (see also Table 2)	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	ab	ac	ad	bc	bd	cd	Scale notation*	Discriminatory index† (per cent)
I	*L. enriettii* (i.v.: 14 days)												
	Serum titres	1280 (5)	80 (3)	160 (3)	80 (3)	60	60	60	100	100	100	5:(3,3,3)	13.2
	Rescaled values	0·8	0·4	0·4	(0·4)								
II	*L. tropica* (i.v.: 14 days)												
	Serum titres	320 (4)	320 (4)	80 (3)	10 (1)	100	80	40	80	40	60	4:(4,3,1)	10·0
	Rescaled values	0·6	0·6	0·4	0·0								
III	*L. mexicana* (i.v.: 14 days)												
	Serum titres	320 (4)	640 (4)	2560 (5)	320 (4)	100	80	100	80	100	80	5:(4,4,4)	5·0
	Rescaled values	0·6	0·6	0·8	0·6								
IV	*L. aethiopica* (i.v.: 14 days)												
	Serum titres	160 (3)	160 (3)	320 (4)	320 (4)	100	80	80	80	80	100	4:(3,3,4)	4·0
	Rescaled values	0·4	0·4	0·6	0·6								
V	*L. enriettii* (i.m.: 20 days)												
	Serum titres	1280 (5)	160 (3)	160 (3)	80 (3)	60	60	60	100	100	100	5:(3,3,3)	13·2
	Rescaled values	0·8	0·4	0·4	0·4								
VI	*L. tropica* (i.m.: 20 days)												
	Serum titres	1280 (5)	1280 (5)	1280 (5)	80 (3)	100	100	60	100	60	60	5:(5,5,3)	3·0
	Rescaled values	0·8	0·8	0·8	0·4								
					Means:	86·7	76·7	67·7	90·0	80·0	83·3		

* For scaling, notation and rescaling of serum titres see Table 1 and Materials and Methods section.

† For definition of discriminatory index (of sera) see Materials and Methods section; other abbreviations as in Tables 1–3.

Table 5. Anti-leishmanial immunofluorescence titres of serum pools from infected and artificially immunized guinea-pigs

Description of serum pool reagent		Titres (and scale values)* of serum pools against the four *Leishmania*				Similarity coefficients						Characteristics of serum pools	
Pool no.	Immunization	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	ab	ac	ad	bc	bd	cd	Scale notation	Discriminatory index (per cent)
1	*L. enrietti* (i.m.: 3 weeks)												
	Serum titres	640 (4)	10 (1)	40 (2)	10 (1)	40	60	40	80	100	80	4:(1,2,1)	40
	Rescaled values	0·6	0·0	0·2	0·0								
2	*L. tropica* (i.m.: 3 weeks)												
	Serum titres	80 (3)	640 (4)	320 (4)	20 (2)	80	80	80	100	60	60	4:(3,4,2)	6·6
	Rescaled values	0·4	0·6	0·6	0·2								
3	*L. mexicana* (i.m.: 3 weeks)												
	Serum titres	80 (3)	640 (4)	640 (4)	10 (1)	80	80	60	100	40	40	4:(3,4,1)	10
	Rescaled values	0·4	0·6	0·6	0·0								
4	*L. aethiopica* (i.m.: 3 weeks)												
	Serum titres	160 (3)	160 (3)	80 (3)	320 (4)	100	100	80	100	80	80	4:(3,3,3)	6·6
	Rescaled values	0·4	0·4	0·4	0·6								
5	*L. enriettii* (i.p.: 1 week)												
	Serum titres	80 (3)	10 (1)	40 (2)	20 (2)	60	80	80	80	80	100	3:(1,2,2)	16
	Rescaled values	0·4	0·0	0·2	0·2								
6	*L. tropica* (i.p.: 1 week)												
	Serum titres	10 (1)	160 (3)	40 (2)	20 (2)	60	80	80	80	80	100	3:(1,2,2)	16
	Rescaled values	0·0	0·4	0·2	0·2								
7	Nasal lesion following *L. enriettii* infection with 1×10^6 am (6 weeks)												
	Serum titres	160 (3)	160 (3)	80 (3)	80 (3)	100	100	100	100	100	100	3:(3,3,3)	0
	Rescaled values	0·4	0·4	0·4	0·4								
					Means:	74·3	82·3	74·3	91·4	77·1	80·0		

* For scaling notation and other abbreviations see previous tables.

Table 6. Anti-leishmanial immunofluorescence titres of serum pools from groups of infected and artificially immunized mice

Description of serum pool reagent		Titres (and scale values) of serum pools against the four *Leishmania*				Similarity coefficients						Characteristics of serum pools	
Pool no.	Immunization	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	ab	ac	ad	bc	bd	cd	Scale notation	Discriminatory index (per cent)
A	*L. enriettii* (i.m.: 2 weeks)												
	Serum titres	320 (4)	10 (1)	< 10 (1)	< 10 (1)	40	40	40	100	100	100	4:(1,1,1)	60·0
	Rescaled values	0·6	0·0	0·0	0·0								
B	*L. tropica* (i.m.: 2 weeks)												
	Serum titres	10 (1)	160 (3)	80 (3)	20 (2)	60	60	80	100	80	80	3:(1,3,2)	10·0
	Rescaled values	0·0	0·4	0·4	0·2								
C	*L. mexicana* (i.m.: 2 weeks)												
	Serum titres	40 (2)	80 (3)	80 (3)	40 (2)	80	80	100	100	80	80	3:(2,3,2)	5·6
	Rescaled values	0·2	0·4	0·4	0·2								
D	*L. aethiopica* (i.m.: 2 weeks)												
	Serum titres	160 (3)	80 (3)	40 (2)	320 (4)	100	80	80	80	80	60	4:(3,3,2)	10·0
	Rescaled values	0·4	0·4	0·2	0·6								
E	*L. enriettii* (i.p.: 1 week)												
	Serum titres	160 (3)	20 (2)	10 (1)	10 (1)	80	60	60	80	80	100	3:(2,1,1)	25·0
	Rescaled values	0·4	0·2	0·0	0·0								
F	*L. tropica* (i.p.: 1 week)												
	Serum titres	< 10 (1)	80 (3)	40 (2)	10 (1)	60	80	100	80	60	80	3:(1,2,1)	25·0
	Rescaled values	0·0	0·4	0·2	0·0								
G	Rump lesion following infection with 10^6 (am) *L. tropica* (12 weeks)												
	Serum titres	160 (3)	320 (4)	160 (3)	320 (4)	80	80	100	100	80	80	4:(3,4,3)	4·0
	Rescaled values	0·4	0·6	0·6	0·4								
H	As (G) but infected with *L. mexicana* (12 weeks)												
	Serum titres	320 (4)	320 (4)	320 (4)	40 (2)	100	100	60	100	60	60	4:(4,4,2)	4·0
	Rescaled values	0·6	0·6	0·6	0·2								
					Means:	75·0	72·5	77·5	92·5	77·5	80·0		

Table 7. Passive cutaneous anaphylaxis titres of sera from *Leishmania*-immunized rabbits and guinea-pigs

Description of serum pool reagents		Titres (and scale values) of serum pools against the four *Leishmania*				Similarity coefficients						Characteristics of serum pools	
Pool no.	Immunization	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)	ab	ac	ad	bc	bc	cd	Scale notation	Discriminatory index (per cent)
Rabbit sera													
I	*L. enriettii* (i.v.: 14 days)												
	Serum titres	2560 (5)	2560 (5)	2560 (5)	2560 (5)	100	100	100	100	100	100	5:(5,5,5)	0
	Rescaled values	0·8	0·8	0·8	0·8								
II	*L. tropica* (i.v.: 14 days)												
	Serum titres	20 (2)	2560 (5)	80 (3)	640 (4)	40	80	60	60	80	80	5:(2,3,4)	13·2
	Rescaled values	0·2	0·8	0·4	0·6								
III	*L. mexicana* (i.v.: 14 days)												
	Serum titres	80 (3)	20 (2)	1280 (5)	20 (2)	80	60	80	40	100	40	5:(3,2,2)	22·8
	Rescaled values	0·4	0·2	0·8	0·2								
IV	*L. aethiopica* (i.v.: 14 days)												
	Serum titres	40 (2)	20 (2)	160 (3)	320 (4)	100	80	60	80	60	80	4:(2,2,3)	14·2
	Rescaled values	0·2	0·2	0·4	0·6								
V	*L. enriettii* (i.m.: 3 weeks)												
	Serum titres	2560 (5)	2560 (5)	2560 (5)	2560 (5)	100	100	100	100	100	100	5:(5,5,5)	0
	Rescaled values	0·8	0·8	0·8	0·8								
VI	*L. tropica* (i.m.: 3 weeks)												
	Serum titres	640 (4)	2560 (5)	160 (3)	2560 (5)	80	80	80	60	100	60	5:(4,3,5)	5·0
	Rescaled values	0·6	0·8	0·4	0·8								
					Means:	83·3	83·3	80·0	73·3	90·0	76·7		
Guinea-pig sera													
3	*L. mexicana* (i.m.: 3 weeks)												
	Serum titres	640 (4)	640 (4)	640 (4)	20 (2)	100	100	60	100	60	60	4:(4,4,2)	4·0
	Rescaled values	0·6	0·6	0·6	0·2								
4	*L. aethiopica* (i.m.: 3 weeks)												
	Serum titres	40 (2)	40 (2)	20 (2)	40 (2)	100	100	100	100	100	100	2:(2,2,2)	0
	Rescaled values	0·2	0·2	0·2	0·2								
					Means:	100	100	80	100	80	80		

Table 8. Evaluation of overall similarity between pairs of Leishmanial strains based on the reactivity of sera from three immunized animal species studied in three test systems

	Similarity coefficients relating strain pairs of *Leishmania*												
	Average values from serological reactions of different animal species							Average values from sera tested by different serological methods					
	ab	ac	ad	bc	bd	cd		ab	ac	ad	bc	bd	cd
Rabbit	78·9	75·6	72·2	85·6	75·6	72·2	Agglutination	63·3	70·8	80·0	89·2	63·3	70·8
Guinea-pig	71·9	83·3	80·9	88·6	78·6	87·1	Immunofluorescence	78·6	77·3	72·8	91·3	78·2	81·1
Mouse	75·0	72·5	77·5	92·5	77·5	80·0	PCA	91·7	91·7	80·0	86·7	85·0	78·3
	Overall similarity coefficients ('animal species')							Overall similarity coefficients ('serological tests')					
	75·3	77·1	76·9	88·9	77·2	79·8		77·9	79·9	77·6	89·0	75·5	76·8
	±3·5	±5·6	±4·4	±3·5	±1·5	±7·5		±14·2	±10·6	±4·1	±2·3	±11·1	±5·3
	t-tests (*P*-values) of paired differences from 'bc'							*t*-tests (*P*-values) of paired differences from 'bc'					
	<0·005	<0·025	<0·0125	—	<0·005	<0·1		<0·15	<0·15	<0·01	—	<0·05	<0·01
	Strain pair differences (see Fig. 1B) (per cent)							Strain pair differences (see Fig. 1A) (per cent)					
	24·7	21·9	23·1	11·1	22·8	20·2		22·1	20·0	22·4	11·0	24·5	23·2

The low resolving power of antisera in PCA led to clustering of strains as revealed by inter-strain similarity coefficients (Table 7). In detail moreover, a different ranking of inter-strain differences was revealed by antisera raised in the two animal species (rabbit and guinea-pig).

One rabbit serum (anti-*mexicana*) proved to have significant discriminatory activity for the homologous organism (pool III: DI = 22·8 per cent); but apart from this serum, none of the others emerged as of discriminatory value by the PCA test. On this basis a positive identification of *L. mexicana* would be possible given the four strains and the rabbit serum reagent III in PCA testing.

Comparison of groups of results

Table 8 gives the overall similarities between pairs of strains based upon data derived from all serological tests given by sera from the three different animal species; and upon data derived from the behaviour of all animal sera tested by the three different serological methods. By both 'animal species' and 'serological test', very similar overall relationships were obtained between corresponding strain pairs.

By both methods of evaluation, the most closely related strains were *L. tropica* and *L. mexicana* (combination bc: similarity coefficients 88·9 and 89·0 per cent respectively), whereas lesser degrees of similarity were obtained between other strain pair combinations (75–80 per cent). As judged by Student's *t*-tests (Table 8) the relationship between strains 'b' and 'c' was significantly closer than most other strain combinations, whether the overall data were evaluated in terms of 'serological tests' or 'animal species'. Similarity coefficients for the strain pair combinations represented by the symbols ab, ac, ad, bd, cd were significantly different from one another (Table 8).

To represent strain differences diagramatically, the 'taxonomic distances' between strain pairs were evaluated by subtracting from 100 their overall similarity coefficients. Individual strains were then arranged spatially at the four apices of a tetrahedron, whose several edges corresponded respectively in length to the numerical values of these strain differences derived from consideration of serological 'test systems' (Fig. 1A). A similar tetrahedron was constructed to represent strain differences derived from consideration of the reactivity of 'animal species' sera (Fig. 1B). These two tetrahedra looked very similar.

By inspection of sets of similarity coefficients from different serological tests it was evident that two sets of serum reagent : test systems yielded relationships between the four strains that most closely approximated to the overall values. In order of diminishing approximation these were: (i) immunofluorescence results obtained with all eight mouse serum pools (Fig. 1C); and (ii) agglutination results obtained with intravenously immunized rabbit sera (Fig. 1D). This latter set of relationships was of particular interest to evaluate since intravenously-immunized rabbit sera were the first to be employed in leishmanial immunotaxonomy by Adler (1964) using a test system dominated by sero-agglutination.

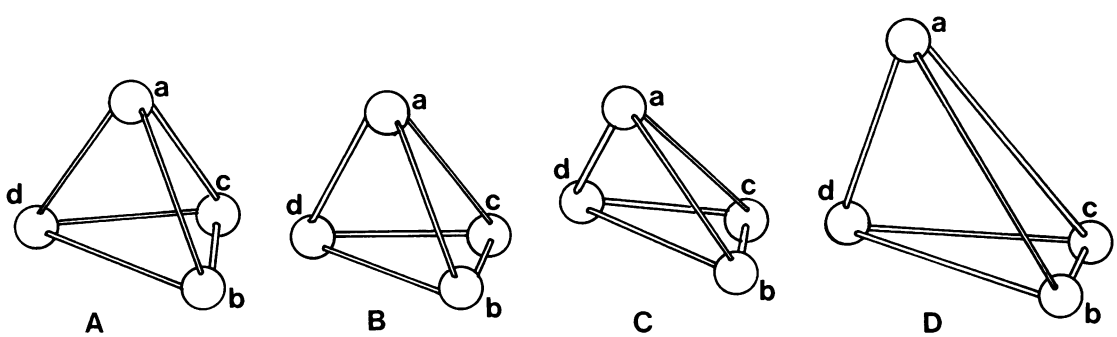

Figure 1. Diagrammatic representation of 'taxonomic distances' between the four leishmanial strains (a–d) as revealed by: (A) averaging values obtained from all serum pools tested by the different serological methods; (B) averaging values obtained from the serological reactions of the three different animal species; (C) averaging values obtained from eight mouse serum pools tested by immunofluorescence; and (D) averaging values obtained from four serum pools from intravenously-immunized rabbits tested by direct agglutination ('Adler-type'). Strain designations: (a), *L. enrietii*; (b) *L. tropica major*; (c) *L. mexicana amazonensis*; and (d) *L. aethiopica*. By all four sets of criteria, strains (b) and (c) were the most closely related.

Table 9. Display of serological titres of most discriminatory antiserum pools

Description of serum pool			Corresponding titres† against *Leishmania*			
Code no. and test system	Method of immunization	Discriminatory index* (per cent)	*L. enriettii* (a)	*L. tropica* (b)	*L. mexicana* (c)	*L. aethiopica* (d)
A (Immunofl.)	Mouse anti-*enriettii* (i.m.)	60·0	**320**	10	< 10	< 10
2 (Agg.)	Guinea pig anti-*tropica* (i.m.)	60·0	< 10	**640**	< 10	10
1 (Agg.)	Guinea pig anti-*enriettii* (i.m.)	40·0	**80**	< 10	< 10	< 10
1 (Immunofl.)		40·0	**640**	< 10	< 40	< 10
E (Immunofl.)	Mouse anti-*enriettii* (i.p.)	25·0	**160**	20	10	10
F (Immunofl.)	Mouse anti-*tropica* (i.p.)	25·0	< 10	80	40	10
II (Agg)	Rabbit anti-*tropica* (i.v.)	22·8	10	**1280**	320	20
III (PCA)	Rabbit anti-*mexicana* (i.v.)	22·8	80	20	**1280**	20

* Discriminatory index evaluated as in Tables 1 and 3–7. Other abbreviations as in previous Tables.

† Serum titres (expressed as reciprocal) evaluated by immunofluorescence (Immunofl.) or agglutination (Agg.) or cutaneous anaphylaxis (PCA). Titres with homologous antisera are in bold type.

Only seven serum pool reagents had discriminatory indices of greater than 20 per cent in eight selected test systems (Table 9). It was of interest that five of these seven sera were derived by artificial immunization of mice or guinea-pigs with *L. enriettii* or *L. tropica*. Inspection of Table 9 revealed that positive identification of *L. enriettii*, *L. tropica* and *L. mexicana*, from the set of four organisms, could be made by means of the three serum pools A, 2 and III in tests of immunofluorescence, agglutination, and passive cutaneous anaphylaxis respectively. By exclusion, the fourth strain (*L. aethiopica*) could be identified, although no antiserum pool raised against it had any acceptable discriminatory capacity.

DISCUSSION

The study of clinical and experimental leishmaniasis has revealed that a wide variety of immunological responses are generated following cutaneous infection with leishmania and following immunization with leishmanial antigens. Thus geographically different leishmanial isolates differ in their infectivity for man or for a given experimental animal; and animal species differ in their susceptibility or resistance to experimental infection with a given isolate. Present classification divides *Leishmania* into about ten main species according to their epidemiological, morphological and physiological features (Bryceson *et al.*, 1970; Lainson & Shaw, 1972) but fails to predict with regularity the form of host response associated with natural or deliberate infection. The question therefore arises of whether components of the immunological response would provide a more appropriate basis for the arrangement of strains in terms of their significance in the spectrum of clinical leishmaniasis. In this paper we have assessed the similarities and differences between four such isolates by their cross-reactivity in tests of agglutination, immunofluorescence, and passive cutaneous anaphylaxis, with twenty-one different pools of immune sera generated by immunization and infection of rabbits, guinea-pigs and mice. The relationships between individual strains were evaluated by their extent of overall cross-reactivity; and in grading serological reactions on a numerical scale, the relationships between the different strains were expressible by similarity coefficients, as in bacterial taxonomy (Sneath, 1962; Matossian-Rogers, 1972).

Although the arrangement of strains differed widely from one serological reaction to another (Tables 1–7), the average results yielded by all

rabbit, guinea-pig and mouse sera were very similar (Table 8). Thus the two most closely related strains were *L. mexicana amazonensis* (termed strain 'c') and *L. tropica major* (termed strain 'b'), with an overall similarity coefficient of 89 per cent; all other five strain relationships yielded overall similarity coefficients of between 75 and 80 per cent (Table 8). The detailed differences in strain arrangements given by an individual serological reagent in the three test systems (Tables 1–7) reflect the different biological and immunochemical requirements of agglutination, immunofluorescence and PCA test systems as well as differences in the representation of complex parasite antigens offered to react with antibody in the separate tests. On this basis sera were prepared by immunization with and without Freund's complete adjuvant, and where appropriate, by infection, so that different serum pools from a given animal species would contain a different representation of antibody class, affinity and immunochemical reactivity. By averaging the results of serological tests it was hoped to 'iron out' these expected variations whilst at the same time making use of the differences in immunogenicity and antigenic reactivity implied by these different immunization and test procedures.

The choice of leishmanial isolates from this initial work was governed by several features. Firstly, much recent information was available concerning the immunological response of albino guinea pigs to the *L. enriettii* isolate and of CBA mice to the *L. tropica major* isolate; and these isolates had been maintained in our laboratories for some years without apparent change in pathogenicity for their respective animal hosts. *L. aethiopica* was chosen as a second representative of Old World *Leishmania*; for it was anticipated that this strain would be more closely related to *L. tropica major* than to *L. enriettii* (a New World isolate). Likewise, a strain of *L. mexicana amazonensis* was chosen for its geographical relation to *L. enriettii*. In fact, the geographical relationships were not reflected by overall serological reactivity; thus *L. enriettii*, *L. tropica major* and *L. aethiopica* emerged with similar relationships (75–80 per cent similarity) and *L. mexicana amazonensis* was '90 per cent similar' to *L. tropica major* (Fig. 1). None of the strains were infective in rabbits; only *L. enriettii* was infective for guinea pigs; and only *L. mexicana amazonensis* and *L. tropica major* were infective for CBA mice. Although these overall serological relationships did not correspond with geographical source (i.e. Old World or New World origin) there appeared to be some relation to patterns of rodent infectivity.

The question of strain identification is clearly a different matter from those taxonomic enquiries which seek to establish relationships between strains. However, eight of the twenty-one serum pools emerged as possible reagents for identification of one or more of these leishmanial strains if they were presented as 'unknowns'. A simple formula was devised whereby the resolving power of a particular antiserum in these tests could be expressed. The term 'discriminatory index' was used to illustrate the sort of criterion by which a serum of diagnostic utility might be judged. Although a modified agglutination test (Adler, 1964) has been proposed for the sero-differentiation of leishmanial strains, only one antiserum produced by the Adler procedure (intravenous immunization of rabbits with killed organisms) had any appreciable discriminatory capacity for identifying a homologous organism in that serological test (serum pool II, Table 9; discriminatory index, 22·8 per cent). Against this background it would be of interest to examine these four strains with regard to biochemical characters such as DNA buoyant density and isoenzyme variation (see Lumsden, 1974).

The use of a number of immunological test systems in arriving at an index of similarity between strains of a given micro-organism illustrates the general principles of numerical taxonomy. In subsequent papers we describe the use of tests of immediate and delayed hypersensitivity, of cell mediated immunity *in vitro*, and of cross-resistance produced by immunization, as an extension of this taxonomic approach.

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