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The Genus *Leishmania*

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The systematic position of the so-called " species " of Leishmania *is examined and an attempt made to determine their phylogenetic relationships. The morphology of the organisms as seen by light- and electron-microscopy is described; neither method provides useful criteria for the determination of species. The behaviour of the parasites in insect and in vertebrate hosts offers a better method of classification. In this way, the species may be divided into 4 main groups, comprising the mammalian species involving man, the distinctive species* L. enriettii *in the guinea-pig, those infecting lizards, and species apparently in various stages of evolution in phlebotomines. The so-called " human " group is divided into visceral forms (originating chiefly in wild canidae) and cutaneous forms (probably of rodent origin). The named species of the former group include* L. donovani *and* L. infantum. *The cutaneous species include* L. tropica tropica (=minor), L. tropica major, L. brasiliensis, L. peruana, L. guyanensis, *and* L. mexicana. L. pifanoi *is probably not a distinct species but represents various forms as modified by the failure of cell-mediated immunity in the host. Leishmanial infections can be identified first by ascertaining the geographical area where the infection was acquired, and then by more or less complicated laboratory investigations including characteristics in culture, serological tests, the response of special hosts in terms of symptomatology, and the behaviour of the parasite in the phlebotomine host. No test is infallible, and an effective simple test is urgently needed. The preservation of* Leishmania *strains is an important research procedure and a method for conserving parasites by lyophilization is described briefly.*

Flagellates of the family Trypanosomatidae fall into two natural groups. The more primitive, in which the parasite is confined to invertebrates, includes the genera *Leptomonas*, *Crithidia*, *Blastocrithidia*, and *Herpetomonas*. The more advanced, which has both vertebrate and invertebrate hosts, includes the genera *Leishmania* and *Trypanosoma* (now divided into special sections and subgenera).

A feature of parasites belonging to the second group is that they change their form during the course of development; thus, a species of *Leishmania* assumes a " leptomonad " morphology in the insect host, and species of *Trypanosoma* become " crithidial " " leptomonad ", or even " leishmanial " at certain times during their life cycles. This terminology is illogical and has often proved to be confusing. Hoare & Wallace (1966) recently introduced some new terms to indicate the various developmental stages and it is to be hoped that they will become widely adopted. The uniform Greek etymology of these terms makes their meaning easily apparent. The new terms relate to the position of the flagellum or mastigote (=whip) and are as follows:

(1) " amastigote ", equivalent to the former " leishmanial stage " (no external flagellum present);

(2) " promastigote ", equivalent to the former " leptomonad stage " (the flagellum arises near a kinetoplast in front of the nucleus and emerges at the anterior end);

(3) " opisthomastigote ", equivalent to the former " herpetomonad " (the flagellum arises near a kinetoplast behind the nucleus and emerges at the anterior end; there is no undulating membrane);

(4) " epimastigote ", equivalent to the former " crithidial stage " (the flagellum arises near a juxtanuclear kinetoplast and emerges at the anterior end; an undulating membrane is present);

(5) " trypomastigote ", equivalent to the former " trypanosome " (the flagellum arises near a posterior

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kinetoplast and emerges at the anterior end; an undulating membrane is present); and

(6) " choanomastigote ", equivalent to the peculiar " barleycorn form " of the purely insect genus *Crithidia* (the flagellum arises anterior to the nucleus and emerges through a wide funnel-shaped reservoir).

Moškovskij has suggested that these terms could be conveniently shortened to " amast ", " promast ", etc.

EVOLUTION AND PHYLOGENETIC RELATIONSHIPS

The phylogeny of the family Trypanosomatidae is clearly revealed by studying the ontogenetic changes that occur in the life cycle of the various genera. Probably all have arisen from insect flagellates; the ancestral form of *Leishmania* was *Leptomonas*, an organism living solely in the invertebrate host and transmitted by the ingestion of resistant forms expelled with the excreta of the host.

Hoare (1948) traced the subsequent course of events by assuming that the flagellates became adapted to life in a vertebrate host, at first, presumably, by the animal swallowing an infected insect, the flagellates being set free and multiplying in the intestine; later by infection through the posterior station, as in *Trypanosoma cruzi*. Thus, the earliest example is found today in *Leishmania chamaeleonis*, confined to the intestine and cloaca of chameleons and still in the promastigote form. Next, the blood becomes invaded by small numbers of promastigotes, as in *Leishmania henrici* of iguanas, or by large numbers, as in *L. hemidactyli* of geckos. If a sandfly bites such a lizard, taking up infected blood, cyclical development now occurs in the phlebotomine.

The parasite is still largely in the promastigote stage in the lizard host but a further step may be taken by *Leishmania adleri* of *Latastia longicauda*, a lizard in Kenya. This parasite, when inoculated into the skin of man, assumes the amastigote form for the first time. Presumably some such event took place also in nature, and the mammalian genus *Leishmania* arose. The parasites became phagocytosed by cells of the lymphoid–macrophage system, where they succeeded in establishing themselves, and multiplication occurred. Certain species of Phlebotomini took up the infection from the skin of the animal and development of the organism in the promastigote form occurred in the gut of the insect, eventually with anterior migration to the mouth-parts. It seems probable that *Leishmania adleri* itself has undergone this evolutionary step, since anterior migration in the insect (*Sergentomyia clydei*) appears to occur (Heisch, 1958). V'jukov has suggested that an alternative path in evolution might be that a carnivorous animal (of the family Canidae, for example) devours a lizard infected with promastigotes, which are set free in the gut and enter reticulo-endothelial cells in various organs and tissues.

The main process of evolution apparently ceases at this point. Deane (1948), however, suggested that further development may have occurred in the common parasite of sloths, *Endotrypanum schaudinni*, which lives in the erythrocytes of these animals. He found amastigotes in the organs of infected sloths and thought that they might be the developmental stages of the parasite in the blood. It is interesting to note that Shaw (1964) produced strong evidence that *E. schaudinni* is transmitted by species of *Phlebotomus* in Panama, thus confirming Deane's hypothesis. Shaw also proved that in culture *E. schaudinni* grew into promastigotes rather than epimastigotes and moreover appeared to be serologically very similar to species of *Leishmania*.

Speciation of the human parasites has been profuse in Central and South America, possibly as the result of the great variety of sylvatic vectors that exist in the New World, and perhaps also the multiplicity of vertebrate hosts. The zoogeography of leishmaniasis in the Old and New Worlds is totally different; no doubt a forest environment is more conducive to the production of mutations than the barren steppes of the Old World with their much more limited fauna. On the other hand, species of *Leishmania* in lizards are much more common in the Old World than in the New; in fact, few reliable records exist of saurian species in the latter. Certainly, cultures of blood taken from lizards in Central America, Venezuela, and Brazil have always proved to be negative for promastigotes, though the presence of the epimastigote stages of a trypanosome may be observed.

Moškovskij has expressed the view that the more contrasting the climatic conditions, the more variable the physicogeographical conditions (i.e., landscape), and the more prolific the hosts, the greater will be the diversification of the agent and of the clinical types of the disease.

MORPHOLOGY

The morphological characters of all species of the genus *Leishmania*, in both the amastigote

and promastigote forms, are remarkably similar, and with the exception of *Leishmania enriettii* it is practically impossible to differentiate between them visually.

Although small, its length being 2–5 μm, the amastigote is highly distinctive. It is usually an ovoid body having one blunt and one more attenuated end, but the shape varies considerably. The organism may sometimes be spherical or may be drawn out into a torpedo shape, the latter being more often seen in the species confined to the skin and mucous membranes.

The diagnostic characters are the nucleus and the kinetoplast, which are invariably present. After wet fixation and haematoxylin or Feulgen staining, the true structure of the nucleus may be observed; the fine nuclear membrane surrounds a clear space, in the centre of which lies the karyosome. With Romanowsky-stained dried films, or in sections, the nucleus appears to be solid or may be flattened into a semicircle.

The kinetoplast (variously, and incorrectly, termed " parabasal body ", " kinetonucleus ", " accessory nucleus ", etc.) is a rod-shaped body, often lying at right angles to the nucleus and staining very intensely. Sometimes, a fine line may be seen running from the vicinity of the kinetoplast to the edge of the parasite; this represents the " rhizoplast " or rudiment of the flagellum. The structure of all these bodies can be satisfactorily elucidated only by the examination of thin sections by electron microscopy. A small dot, or blepharoplast, is described as occurring near the kinetoplast; it is actually the basal body or centriole from which the flagellum arises. The blepharoplast is usually invisible by light microscopy and was wrongly thought by early workers to be a component of the kinetoplast.

Akinetoplastic forms are sometimes seen in rapidly growing lesions in animals or, less frequently, in man. However, in N.N.N. medium the abnormality disappears, and probably the organelle merely becomes lost in a degeneration of the parasite caused by excessive growth and inadequate nutrition.

A flagellar vacuole exists and is said to lie near the basal granule and kinetoplast, although its exact relationships, e.g., its connexion with the pit through which the flagellum emerges, are uncertain. Adler et al. (1966) were convinced that the vacuole increases in size when the flagellum is growing and that if such development were inhibited by immune serum (see below, Adler's test) the vacuole could grow to an immense size (up to 18 μm in diameter in the syncytia). Adler et al. (*op. cit.*) suggested that this structure had an " osmoregulating " function, and that its contents are periodically emptied into the reservoir or pit. Obviously more work on all these organelles is necessary. The cytoplasm of the amastigote sometimes contains a large vacuole and volutin granules may be present.

The amastigote multiplies by binary fission and dividing forms are often seen in heavy infections. The kinetoplast, together with the basal granule, divides first, and almost simultaneous division of the nucleus follows. Various mitotic phenomena have been observed, but the small size of the chromosomes has led to some diversity of opinion regarding their number (3–8); certainly, mitotic figures are easily demonstrable with the Feulgen technique in the spleen of mice heavily infected with the amastigote stages of the *upsilon* strain of *Trypanosoma cruzi*. Multiplication of the promastigote stage of *Leishmania* proceeds in a similar manner but by longitudinal binary fission, the organism splitting from the anterior end.

The morphology of the promastigote varies according to the stage of growth in the fly; briefly, there is progressive elongation of the body (to about 20 μm), the free flagellum begins to emerge from beyond the basal plate and, after some hours, the flagellum (also about 20 μm in length) becomes fully functional. Adler (1964) stated that its function not only relates to locomotion but also to attachment to the gut wall by the " expanded distal end ". There is, however, no ultramicroscopic evidence of this fixation.

Multiplication of the human species of *Leishmania* occurs in the alimentary tract of suitable species of phlebotomine flies that have fed on an infected individual. Within 6–8 hours, division has begun, leading to the production of small, stumpy promastigotes; rapid multiplication continues, some flagellates clinging to the mucosal cells with their flagella, others forming rosettes in the lumen of the midgut. Within 5 days, anterior migration occurs and at the time of the next meal (blood or fruit juice), the promastigotes are almost blocking the proventriculus. This meal does not hinder the forward migration of the flagellates, which accumulate in the pharynx and mouth-parts of the insects about the ninth or tenth day after the original feed. During the course of this migration the flagellates

become longer, although the metacyclic promastigotes are thin and active.

The multiplication of lizard species of *Leishmania* also occurs in *Phlebotomus*, but the migration of the developmental forms usually proceeds posteriorly and is sometimes accompanied by a reversion to amastigote forms. Observations have largely been made on wild-caught flies and the presence in such specimens of trypanosome infections, or even of species of *Leptomonas*, may be most confusing. Petrišćeva has drawn attention to the fact that a sterile environment is required for the proper development of promastigotes in the sandfly gut; if bacteria are present, the development of the parasite is inhibited. This, however, has not been the experience of Brazilian workers (Coelho, 1966).

Further remarks on the behaviour of *Leishmania* spp. in the insect host are given below (pp. 485 et seqq.).

ULTRASTRUCTURE

The amastigote

Garnham & Bird (1962) described in detail the fine structure of the amastigote stages of *L. mexicana*, as follows. The general features of these organisms are similar to those of other flagellates belonging to the family, particularly with regard to the flagellar structure. The flagellum arises from a short blepharoplast or cylinder containing 9 double peripheral fibrils and ending in a plate from which the axoneme itself begins, and by which it leaves the body in an invagination of the cytoplasm. In the amastigote stage the external part of the flagellum of course stops short at the surface of the organism. The blepharoplast lies on the margin of the so-called " flagellar vacuole " and alongside it. The kinetoplast, an organelle plainly visible under light-microscopy, is seen under higher magnification to be a body of unexpected complexity. The portion visible under light-microscopy is represented by an electron-dense granular band with a distinct and regular fibrillar pattern, lying inside an enormous body totally invisible under lower magnifications. This body occupies a large part of the cytoplasm of the *Leishmania*; it possesses a double membrane, tubes, and villi typical of a protozoal mitochondrion. The shape is irregular, which makes it appear as two or more fragments in sections. Different species of *Leishmania* present this appearance, and although the mitochondrion appears larger in *Leishmania mexicana* than in *Leishmania donovani*, it is very large in the latter also. This body is obviously a single giant mitochondrion, the denser part of which becomes visible under the light microscope as the kinetoplast. In Giemsa-stained smears it may be represented by a large vacuole often prominent in *Leishmania mexicana* infections. The mitochondrial origin of the kinetoplast in other flagellates has also been indicated by other workers (e.g., Clark & Wallace, 1960).

The amastigote body is surrounded by a double membrane, and immediately below the inner membrane lies a row of microtubules numbering between 130 and 200. There is very little evidence that a connexion exists between these subpellicular fibrils and the blepharoplast, as was stated by Rudzinska & Vickerman (1968) to be the case in trypanosomes. The diameter of the fibril is 20 nm, which may be compared with that of *Bodo* where the fibril measures 25 nm. The nucleus has no obvious membrane, perhaps because active division is taking place, but it contains lighter and darker portions.

Sanyal & Sen Gupta (1967) have given an account of the fine structure of *Leishmania donovani* as seen in sections of a dermal leishmanoid. They point out that there are small morphological differences between the organism they describe and *Leishmania mexicana*. *Leishmania donovani* is smaller and possesses fewer subpellicular fibrils (100 instead of 200); there is a giant mitochondrion, but more than one may be present. The flagellar vacuole was clearly visible in their preparations as a sac invaginated by the locomotory apparatus and it was accompanied by lateral vacuoles.

The ultrastructure of *Leishmania mexicana* has also been studied by Tay (1966).

The promastigote

Observations have been made by various authors on the fine structure of the promastigote stage of human species of *Leishmania*. The earlier work showed the typical flagellar bundle type of construction, with the 9 double peripheral fibrils in the basal granule (so-called " kinetosome ") and 9+2 in the flagellum itself. The sheath enclosing the flagellum is thought to be an invagination of the pellicle of the organism. Rudzinska et al. (1964) later confirmed this work, although they pointed out that there were 2 unit membranes enclosing the flagellum in the amastigote stage but only a single one in the promastigote; also the nucleus of the former contains 2 prominent nucleoli but there is only 1 in the nucleus of the latter. These workers

emphasized the changes that take place in the kinetoplast during the transformation; the structure is said to become extensively enlarged, developing branches that are mitochondrial in nature and which may split up into new mitochondria.

Avakjan has reported interesting research on the ultrastructure of the amastigote form of *Leishmania tropica major* (Ashkhabad strain) grown in a culture of chicken fibroblasts. He found that, on penetration of a cell by the promastigote, the flagellum is lost after 20 hours. The kinetoplast (i.e., mitochondrion) elongates as a long sleeve to the opposite end and the Golgi apparatus and flagellar elements persist, but later there is gross degeneration of the host cells, presumably because of an incorrect host–parasite relationship.

In more detail, the following observations were made by Avakjan at the Gamaleja Institute for Epidemiology and Microbiology, Moscow.

" The body of the promastigote is covered by a smooth, 3-layered membrane 10–12 nm thick, consisting of 2 osmiophilic layers each 25 nm thick separated by an osmiophilic layer 5 nm thick. Very close to the internal osmiophilic membrane are the tubular structures and high-resolution photomicrographs show the fibrils that connect the cytoplasmic membranes with these tubular structures. In cross-section the tubules have the appearance of double rings, their diameter in *Leishmania tropica* being 25 nm and the distance between their centres 35–40 nm. In the wall of the tubular structures are 9 longitudinal threads, each with a diameter of 3–4 nm. In oblique sections the structures resemble the tubes that are situated over the whole surface of the cell. The tubules lie at a strictly regular distance from each other and stretch from the flagellum to the opposite side of the body. It is possible that they simultaneously fulfil a skeletal function and also allow the body to make elastic movements.

" The endoplasmic reticulum of the promastigotes is not very developed; nevertheless, it is easy to establish the presence of links between endoplasmic canals, cisterns, and flattened bubbles, which lie close to the cytoplasmic membrane. Osmiophilic granules are situated on the membranes of the endoplasmic reticulum. In form and size (10–12 nm) they are identical to the ribosomes; their ribonucleoproteid nature can be detected with the aid of luminescent microscopy by staining parallel preparations with acridine orange. The cisterns of the endoplasmic reticulum, and the larger vacuoles, are often filled with homogeneous or finely granular material, the nature of which is unknown.

" The Golgi apparatus is usually found between the nucleus and the kinetoplast; it has a characteristic structure and consists of a complex of canals, flattened pouches, and cisterns, which, in cross-sections, seems closely similar to membranes.

" The nucleus takes up a great part of the cell body. Depending on the level of the section and the functional condition of the cell, the nucleus can have various forms, sizes, and structures. There is a double membrane, each lamina being 7.5 nm thick; granules corresponding to ribosomes are found on the external surface. Around the nucleus there is a light perinuclear zone 14–15 nm long; the membrane of the nucleus is porous, the diameter of the pores being 60–80 nm. The blepharoplast consists of 2 parts, distal and proximal, which differ in structure. In the proximal part of the blepharoplast there are 9 bundles of fibrils, each consisting of 3 threads. In cross-section, the fibrils are situated under the membrane in strict order. There are no fibrils in the centre, which is filled with loose material; the central fibrils appear in the distal part, i.e., at the transition of the blepharoplast into the flagellum. How the central fibrils of the flagellum appear is not yet known.

" The flagellum possesses 9 double peripheral fibrils, plus 2 centrally situated ones (formula, 18+2). The membrane of the cell passes directly over the flagellum, covering it like a sheath. The diameter of the fibrils is 22–25 nm. In longitudinal section, the fibrils are seen to pass along the flagellum ".

An essential element, lacking from these electron-microscopic observations, is a study of the structure of promastigotes in the insect itself, rather than in culture. The function of the mitochondrion in other flagellates differs greatly in the vertebrate and invertebrate host, and the very unusual structure of the mitochondrion in the amastigote, where it occupies a large part of the cell body and contains the specialized kinetoplastic portion, should be investigated further.

SPECIES OF *LEISHMANIA*

The essential similarity of the morphology of *Leishmania* throughout the animal kingdom presents a problem to the taxonomist. There are two points of view; at one extreme it is considered that all the " strains " should be relegated to a single species (i.e., to *Leishmania donovani*, as having priority

over later names), at the other extreme, it is felt that separate specific status should be given to each " variety ". The former treatment appeals to the protozoologist as taxonomically sound; the latter is preferred by the practical worker who likes to have a distinctive label available for the organisms with which he is concerned.

Hoare (1956) attempted to solve the problem by the creation of " nosodemes ", i.e., strains associated with a particular clinical entity, of " serodemes " to categorize infections with well-marked immunological differences, and of "xenodemes" for organisms found in special vertebrate or invertebrate hosts. However, this suggestion has not yet received general recognition.

The introduction of a trinomial system could be justified but it would entail the use of the term *Leishmania donovani donovani* and other subspecies, and would in general be unacceptable because the trivial name " *donovani* " is inevitably associated with the visceral disease. It thus appears to be simpler and less cumbersome to adopt a binomial system (with one exception; see *Leishmania tropica*, below).

The above-mentioned considerations apply to all species of *Leishmania*, but are especially applicable to the forms found in man. The classification of species into " human " forms is undesirable because all the organisms are either of direct animal origin or were zoonotic in the past. Nevertheless, the classical terminology includes the three so-called human species, *Leishmania donovani*, *Leishmania tropica*, and *Leishmania brasiliensis*, and lies between the two extremes discussed above. Many workers would prefer to retain this terminology, particularly if the use of *Leishmania tropica* could be extended to certain New World infections. Moreover, Moškovskij has emphasized that importations of Old World species into the New World have undoubtedly occurred, with local adaptation and possibly the emergence of new strains and " species " (see also Chaffee, 1963).

For ease of reference, and in line with the recommendations of the late Professor S. Adler, liberal separation of the organisms on a binomial system has been adopted in this article. It is acknowledged, however, that infinite series of gradations exist between strains and that it is sometimes difficult to arrive at a firm identification. The application of molecular and mathematical taxonomy may resolve the problem in the future.

Various attempts have been made to stabilize the nomenclature of *Leishmania* species, including the classifications of Kirk (1949) and of Nicoli (1963). The latter suggested that a phylogenetic solution is likely to be the most satisfactory, and that it should be based upon the systematic position of the specific phlebotomine vectors with which the organism is associated. Nicoli also favoured a trinomial system for *Leishmania donovani* (e.g., *Leishmania donovani sinensis*, *infantum*, *chagasi*, and *archibaldi*) and Pessoâ (1963) used this system.

The species fall into the following four main groups:

(1) mamalian species: (*a*) in wild Canidae (and final hosts in dog and man), (*b*) in Rodentia (and final host in dog and man);

(2) the special guinea-pig species *Leishmania enriettii*;

(3) lizard species at various stages of evolution; and

(4) species in process of evolution in Phlebotomini.

Mammalian species

These are the species of *Leishmania* that secondarily affect man and dogs. In general, the visceral disease in man has, or had, a probable origin in jackals and foxes, while the cutaneous disease derives from a great variety of wild rodents. The dog acts as a liaison reservoir for both forms, though not in all situations (e.g., there is a 25% infection rate in dogs in some parts of the Mediterranean area and in Peru but none in India and Central America).

The *visceral* species are as follows:

(1) *Leishmania donovani*, present in persons of all ages;

(2) *Leishmania infantum*; chiefly present in children;

(3) *Leishmania chagasi*, usually regarded as a synonym of *Leishmania donovani*; and

(4) unnamed species found in Sudan and East Africa; although minor differences exist, the causative organism is best regarded as *Leishmania donovani*.

Lysenko has expressed the belief that kala azar probably had its origin in an enzootic from an ancient source in central Asia, becoming endemic in rural zones of the same region and secondarily affecting dogs; the endemic spread into towns in Transcaucasia, etc., and around the Mediterranean, where it was accompanied by the canine disease,

but the endemic in India was not associated with infection in dogs. Maruašvili extended this idea by explaining that two forms of the visceral disease exist—namely:

(1) an anthroponotic form, with prevalence of post-kala-azar dermal leishmanoids, caused by *Leishmania donovani* in India and by *Leishmania donovani* var. *archibaldi* in tropical Africa, and

(2) a zoonotic form in the USSR, the Mediterranean area, and Latin America, affecting children but with few or no dermal leishmanoids (*Leishmania infantum*).

The cutaneous species of *Leishmania* are as follows.

(1) *Leishmania tropica tropica* (=*minor*), the causative organism of the classical form of Borovskij's disease (urban).

(2) *Leishmania tropica major*, the causative organism of the zoonotic form of oriental sore, with a much more limited distribution (rural).

Although these two forms of oriental sore can be divided roughly into "dry" and "wet", respectively, there are many intermediate forms. Demina has found it possible to differentiate between the two organisms by the great difference in size and shape and by the failure of *Leishmania tropica tropica* (=*minor*) to infect mice on inoculation into the peritoneum. It is possible that in the southern republics of the USSR, the two strains exist together, *major* conferring an immunity against infections of *minor*, which is thereby largely suppressed in the parasite population. If conditions for *major* are unsuitable, then *minor* can manifest itself, and a "substitution" follows.

(3) *Leishmania brasiliensis*, the cause of *espundia*.

(4) *Leishmania peruana*, the cause of *uta*.

(5) *Leishmania pifanoi*, the cause of leishmaniasis tegumentaria diffusa in Venezuela. This disease represents a manifestation of the host's inability to react to the infection. Instead of a generalized sensitization, the cells are incapable of resisting the parasite, which therefore fulminates in a diffuse pattern; such cases fail to give a positive Montenegro reaction and the blood may contain parasites. This explanation is confirmed by the fact that the anergic form of the disease is widely distributed, though rare, in north-eastern Brazil (*Leishmania brasiliensis*) Venezuela ("*Leishmania pifanoi*"), Mexico (*Leishmania mexicana*), Ethiopia and the Sudan (*Leishmania tropica*). The disease is accompanied by an extremely heavy parasitization of the lesion in contrast to the situation in post-kala-azar dermal leishmanoid, in which organisms are scanty and the condition has a different origin. The name "*Leishmania pifanoi* Medina" should therefore probably be abandoned. This form of leishmaniasis has now been interpreted (Garnham & Humphrey, 1969) in terms of the failure of cell-mediated immunity, and studies by Bray & Bryceson (1969) are elucidating the nature of the phenomenon *in vitro*.

(6) *Leishmania guyanensis*, the cause of *pian-bois*; there may also be related species farther north and west of French Guiana.

(7) *Leishmania mexicana*, the cause of Chiclero's ulcer.

In addition to these fairly well defined organisms, there are a number of strains of unknown affinities isolated from rodents. In Belém, Brazil, Lainson & Shaw (1969) have found strains unassociated with human disease. It is interesting to note that, experimentally, strains isolated from animals often appear to have a low virulence for man. In Brazil, Kenya, Panama, and the Sudan, certain animal strains may be undergoing speciation and are, as yet, poorly adapted to a human host. Work on these problems is at present being carried out in Brazil and Ethiopia by parasitology units of the Wellcome Foundation under the direction of Dr R. Lainson and Professor R. S. Bray, respectively, and similar work is also being undertaken in the USSR.

Leishmania enriettii

This is one of the few, if not the only, species of *Leishmania* that has a clearly defined taxonomic position. This is on account of its size, which is almost double that of the human Leishmanias; moreover, the flagellar vacuole is larger and the kinetoplast is unusually thin. The only susceptible animal is the guinea-pig (*Cavia porcellus*), from which *Leishmania enriettii* was originally isolated in Brazil. Cultures of *Leishmania enriettii* quickly lose their infectivity to the vertebrate host and the organism usually disappears fairly soon from lesions; thus the infective strain is easily lost.

Lizard species

As stated above, species of *Leishmania* in reptiles are almost entirely confined to the Old World, where they have been studied from early in the century. Intensive studies, including work both on the transmission of strains by sandflies and on

Leishmania in lizards *

Species of *Leishmania*	Vertebrate host	Involvement of organs [a]	Suspected *Phlebotomus* host	Region
chamaeleonis	*Chamaeleon pumilus*, etc.	Cloaca		South Africa, etc.
agamae	*Agama stellio, A. sanguinolenta*	Blood	*papatasi, caucasicus arpaklensis*	Eastern Mediterranean, Turkmenian SSR
ceramodactyli	*Ceramodactylus doriae*	Blood	*papatasi, caucasicus arpaklensis*	Eastern Mediterranean, Turkmenian SSR
gymnodactyli	*Gymnodactylus caspius*	Blood	*papatasi, caucasicus arpaklensis*	Turkmenian SSR
zmeevi	*Eremias intermedia grammica*	Blood	*papatasi, arpaklensis*	Turkmenian SSR
hemidactyli	*Hemidactylus brooki*	Blood	?	India, Assam
tarentolae	*Tarentola mauritanica*	Blood, spleen	*minutus*	North Africa, Malta
adleri	*Latastia longicaudata*	Blood, skin	*clydei*	Kenya
unnamed	*Alsophylax pipiens*	Blood, liver	*Sergentomyia* sp.	Mainland China
henrici	*Anolis* sp.	Blood, cloaca	?	Martinique

* Adapted from Saf'janova (1966).

[a] Parasites are usually in promastigote form but occasionally are amastigote, even in blood.

their serological relationships, have been carried out in recent years by a number of Soviet investigators (e.g., Belova, 1964, 1965, 1971). All strains of promastigotes isolated from reptiles were found to be closely allied to each other, and for this reason Saf'janova and V'jukov doubt the validity of certain named species. Now that a large number of strains (including some also from snakes) has been isolated in the USSR, it is desirable that this research should be continued to confirm their relationships with other species of *Leishmania* from lizards in Africa, India, and the Middle East.

The accompanying table summarizes present information about species of *Leishmania* in lizards. Several names may be synonymous. Little work has been done on the morphology of these organisms, and the amastigote stages have never been described in detail. Avakjan, however, has pointed out a striking difference in the ultrastructure of the reptilian and mammalian species. In the former, the subpellicular microtubules lie 58–67 nm apart; in the latter, these structures are only 35–42 nm apart.

In rare instances, some of the lizard species may be found in the cloaca or intestine, but mostly the parasite is present in the occult state in the blood.

Species confined to invertebrates

The discovery of promastigotes in the hind-gut or rectum of *Phlebotomus* spp. always raises the question of their origin. An interesting example was found at the Gorgas Memorial Institute, Panama (1966), in the posterior half of the hind-gut and on the rectal ampullae of a local species of *Phlebotomus*. Growth led to a palisade formation of stumpy, flattened promastigotes, which failed to infect animals. The organism may therefore have been a true *Leptomonas*. An earlier example is *Leishmania papatasi*, first seen by Wenyon in Algeria in 1912, and described by Laveran & Franchini (1920).

IDENTIFICATION OF SPECIES

For general purposes in human medicine, it is enough to determine the geographical origin of a given strain of *Leishmania* and the disease with which it is associated. In a few places — e.g., Panama and the adjacent part of South America — however, two or more species may be present simultaneously and identification becomes more difficult. An attempt may be made to define the zoological position of a species of *Leishmania* by applying certain criteria that will give an indication of the affinities of the organism. The methods are largely research procedures and entail access to a reference centre for strains of *Leishmania*.

There is thus a need for a new and simple laboratory test for the identification of species; at

present, the specific reactions are both cumbersome and difficult to carry out. The fluorescent antibody test is an excellent method for the diagnosis of visceral leishmaniasis in places where Chagas' disease is absent, or for the cutaneous forms if the organism has been isolated and can be used as an antigen with selected immune sera from hamsters. Hitherto, this test has been used solely for the diagnosis of human cases but its use might be extended to observations on the relationships and affinities of strains of *Leishmania* isolated from lizards, *Phlebotomus*, or rodents. It will be recalled that this test is in general group-specific rather than species-specific; for example, all malaria parasites from primates exhibit cross-reactivity, but the primate group does not cross-react with rodent or avian infections. It may be found that the test shows similar group-specificity for *Leishmania.*

The following summary outlines the criteria to be used in the identification of *Leishmania* species.

Morphology

Although *Leishmania* assumes different forms in the course of its developmental cycle in vertebrate and invertebrate hosts, neither the mature nor the promastigote stages exhibit any marked structural differences in the various species, though *Leishmania enriettii* is twice the size of the others. The submicroscopic details are only now being studied and it is probable that they will be applicable to developmental stage or generic characters, rather than to specific features.

Characteristics in culture

Adler & Theodor (1931) showed that *Leishmania tropica* and *Leishmania infantum* grow differently on glycerol and agar plates, but little use has been made of this criterion, which needs further investigation. *Leishmania guyanensis* may be distinguished from other strains since it cannot be maintained in culture media containing rabbit blood; if rat blood is substituted, however, growth flourishes (Bray & Munford, 1967). These authors have also stated that in tissue culture some species of *Leishmania* have special temperature requirements, which may serve as diagnostic criteria.

Serology

Agglutination, precipitin, and complement-fixation tests have all been tried for differentiating species of *Leishmania* but have been largely discarded. On the other hand, Adler (1964) claimed to have obtained consistent success with certain serological reactions, by means of which he was able to identify and classify the different species. The method involves the introduction of immune sera into cultures of the organism. Bray & El Nahal (1966) used haemagglutination reactions and obtained consistently good results. Immunodiffusion in agar gel has also proved useful for the differentiation of strains, particularly at the Gorgas Memorial Laboratory for isolations of the parasite from man, animals, and species of *Phlebotomus.*

Response of vertebrate hosts

The " nosodemes " of Hoare indicate how the different forms of leishmaniasis may be classified, and in the human host the various species may be distinguished according to the tissue attacked. This criterion is not absolutely reliable since *Leishmania donovani* infection may stop short at the skin or lymph glands, and fail to reach the viscera. *Leishmania tropica* may invade mucous membranes, *Leishmania " pifanoi "* may enter the blood stream (though such infections may result from contamination of needles used to pierce infected skin), and *Leishmania brasiliensis* is sometimes confined to the skin. The response in animals is even more erratic since the " cutaneous " species quite often enter the bone marrow and internal organs of dogs and hamsters. The pathological changes in the vertebrate are, as a rule, uniform and indistinguishable, but *Leishmania mexicana* gives rise to extraordinary tumours with a characteristic histology. The significance of this finding in specific identification is still uncertain, though it is apparently absent in *Leishmania donovani* infections.

Response of invertebrate hosts

Adler & Theodor (1931) have shown that complete development of the parasite, with invasion of the mouth-parts, occurs only when the appropriate vector is involved. Thus, *Leishmania tropica* will undergo the complete cycle in *Phlebotomus papatasi*, while *Leishmania infantum* will remain largely confined to the midgut of this species of sandfly. The adaptation of the host to the parasite may thus be an important clue to the taxonomic position of the latter, but is unlikely to provide a useful practical means of differentiation. Recent work by Coelho (1966) in Brazil and by Belova & Saf'janova (1963) in the USSR has indicated the scope of the problem.

Specific host

Leishmania adleri is confined to *Latastia*, and *Leishmania enriettii* to guineapigs; apart from these examples and a few others in reptiles, the mammalian forms are fairly nonspecific in their hosts and the infection often takes the form of a widespread zoonosis. This character is therefore useless for distinguishing the more important species. Perhaps, however, the avirulent strains of *Leishmania donovani* discovered by Heisch (1958) in ground-squirrels and gerbils in Kenya are examples of early differentiation into new species.

Reaction to drugs

Since kala azar in different regions exhibits different responses to antimonial compounds, some observers consider that the Indian and tropical African forms, for example, are caused by different species of *Leishmania*.

To summarize, only two of the above criteria—serology and vertebrate response—are of consistent practical importance today; four—the vertebrate host, the invertebrate host, response to drugs, and characteristics in culture—provide limited information; while one criterion—ultrastructural morphology—may perhaps be applicable in the future. The serological reactions are considered in more detail below.

SEROLOGY

The gross changes in the composition of the blood proteins have long been recognized as a useful guide to the diagnosis of visceral leishmaniasis, and Napier's formaldehyde test is useful as a screening measure in surveys of populations in endemic zones. A positive reaction plus splenomegaly and leucopenia is almost pathognomonic of the disease, although specific confirmation is, of course, required.

Complement-fixation and agglutination tests were used by the early workers for the diagnosis of the disease and even in attempts to differentiate the various species, but agglutination tests in particular gave perplexing results and these methods have, in general, been abandoned.

Two new reactions have been introduced and are claimed to be reliable and uniform. These are (1) the growth of cultures in the presence of dilutions of homologous and heterologous antisera (Adler's phenomenon), and (2) haemagglutination (as used by Bray). The latter test is still in course of development but it appears to give highly specific results. Gel-diffusion is useful for antigen analysis, and the fluorescent antibody test can detect early cases of kala azar.

The test devised by Adler (1964) entails first the preparation of immune sera by the repeated inoculation of promastigotes from cultures into rabbits. Some rabbits give a poor antibody response and it is essential for the successful application of this test to inoculate a number of rabbits and then to select sera with high titres. The specific antisera are added in various concentrations to culture tubes of Locke's serum-agar, which are subsequently inoculated with rich cultures of the organism to be tested. The cultures are examined every 3–4 days in order to observe the characteristic effects, which are as follows.

(1) Growth of an organism with its homologous antiserum is accompanied by the formation of syncytia (the organisms failing to separate although nuclear division continues, while flagellar development is inhibited).

(2) In higher dilutions, immature flagella may emerge from the surface of the syncytium.

(3) Clumped colonies of active flagellates are formed at still higher dilutions.

(4) The final titre (which may reach 1/20 000 or even 1/50 000) is the one below which all the flagellates remain free.

In heterologous systems, only effects (3) or (4) may be observed in the lower dilutions and this forms the basis for distinguishing species. Thus, Adler was able to separate *Leishmania infantum* from *Leishmania donovani*, and to differentiate the latter from *Leishmania brasiliensis*, *Leishmania mexicana*, and *Leishmania tropica*. The two subspecies of *Leishmania tropica* (*major* and *minor*) can also be identified in this way. It is interesting to note that *Leishmania adleri* reacts to a certain extent with antisera prepared from human species, though in general the reptilian species differ markedly from the mammalian species in their serology.

Saf'janova has reported Adler's phenomenon to be very promising for the identification of *Leishmania* species. However, in simultaneous tests with a large number of strains, qualitative comparison of their growth characteristics in media with immune sera did not always permit clear evaluation of the relationships, the extent of the effects depending on the individual properties of strains and on the titres of immune sera affecting them. Therefore she developed a method for the quantitative eva-

luation of the phenomenon, in which the results are expressed as a single value (Saf'janova, 1966). The evaluation is based on 3 characteristics of the growth of promastigotes in the presence of immune sera: (1) the highest serum dilution at which syncytia or colonies (immobile or poorly motile) are observed; (2) the lowest serum dilution at which free motile organisms of normal form are observed; and (3) the serum dilution at which the number of free motile organisms approaches that in the control (the control being a culture of the strain in a normal nutrient medium without immune serum). Using serum dilutions from 1 : 5 to 1 : 280, the effect of immune serum on the growth of strains is evaluated by assigning a score from 1 to 9 to each of the serum dilutions noted above. The extent of the antigenic relationships between strains is then evaluated by adding the three scores. Initially, the score is determined for each serum in tests with the homologous strain characterizing the serum titre. If, in tests with heterologous strains, a score is obtained that approximates to that characterizing the serum titre, this indicates an antigenic relation between the strain under test and that against which the antiserum was prepared. The final conclusion on the extent of the relationship between strains is reached by carrying out cross-tests for purposes of comparison.

Further investigations on the immunological relationships of the various species are now being carried out by the Wellcome Parasitology Unit in Belém. The results may be expected to throw new light on the specific status of the New World forms. In this context, it may be stated here that Garnham (1965) erroneously quoted the late Professor Adler as believing that *Leishmania mexicana* is closely related to the species found in Panama; this is not the case.

IMMUNITY

The subject of immunity to *Leishmania* is discussed in detail by Heyneman (1971). However, the identification of species and strains may be facilitated by a study of cross-immunity reactions, hypersensitivity tests (e.g., Montenegro reaction), etc., if it is borne in mind that specificity is often slight and that a positive skin reaction is not necessarily an indication of immunity.

PRESERVATION OF STRAINS

Strains of *Leishmania* can be maintained indefinitely by placing either cultural forms or infected tissue in liquid nitrogen. The material is mixed with glycerol to a final concentration of 10% and frozen slowly at first, i.e., the temperature is allowed to fall at the rate of 1 degC/min until it reaches −20°C, then rapidly). The technique is widely used and has been described in detail by Allain (1964).

Serebrjakov has reported success in conserving the promastigote form of *Leishmania* by lyophilization, the technique being as follows.

" Two strains of *Leishmania tropica major*, isolated from *Phlebotomus papatasi* and from a man, were used. Both strains had typical properties of *Leishmania tropica major* and were highly virulent for albino mice, in which 100% infection was obtained.

" The culture was grown on N.N.N. medium enriched with 0.2% peptone solution in flasks at 26°C for 9 days. The suspension, containing about 50 million actively moving promastigotes per 1 ml, was concentrated in a centrifuge for 1 minute at 1 000 rev/min. Under these conditions, it was possible to obtain a homogeneous, and rather loose, deposit of organisms, which maintained their original morphological properties and good motility. The deposit was resuspended in a small amount of saline or peptone water at pH 7.1 and either rabbit serum or glycerol (5–10%) was added in a ratio of 1 : 1. At different times from 1 to 22 hours following centrifugation the suspension was poured into 5-ml ampoules in 1-ml amounts and subjected to lyophilization. Microscopic examination of the suspension carried out immediately before lyophilization showed no morphological changes in the organisms.

" The lyophilization of leishmanial cultures was carried out in a portable drying assembly, designed by K. A. Jusupov and V. A. Vošte, consisting of a special glass chamber, a filter, a revolving McLeod compression vacuum gauge and a BH-461 vacuum pump. The ampoules were submerged in a mixture of solid carbon dioxide and 96% ethyl alcohol at −78°C and the contents were frozen at a cooling rate of 3 degC/s. After freezing, the ampoules were connected with the drying assembly, in which an effective vacuum (0.1 mm Hg) was developed in 4–5 minutes and became stabilized after 1.5–2 hours at 5.10^{-2} mm Hg. The whole process of drying (20–30 ampoules) lasted 9–16 hours in different experiments. For the first 3–4 hours of drying, the ampoules, filled with lyophilized material, remained submerged in a cooling mixture (ice and common salt) at −10°C. Afterwards, when the free water had been removed from the lyophilized material, the cooling was stopped and drying was completed at room

temperature. It was assumed that drying was finished after a short period of heating (by submerging the ampoules in water at 33–35°C), as indicated by a noticeable vacuum drop. During the whole period of drying, the condenser was filled with the cooling mixture, which was replaced as it evaporated. After drying, the ampoules were soldered in vacuum and stored in a common refrigerator at 4–6°C.

" In a number of experiments, the lyophilization of leishmanial cultures prepared in the same way was carried out in a flask filled with 10–20 ml of the same suspension. The freezing was maintained by submerging the flask in the mixture of solid carbon dioxide and ethyl alcohol. The freezing process was hastened by rotation of the flask, which was accompanied by the formation of thin layers of frozen material on the walls and bottom of the flask. Both the vacuum and the temperature maintained in the flasks were the same as in the ampoules but the time of drying was reduced to 6–12 hours.

The culture dried with serum supplement was a tablet-shaped, porous, agglomerated mass whereas that dried with glycerol was in the form of a film stuck tightly to the bottom of the ampoule. In both cases, the dry culture could easily be dissolved in distilled and peptone water within a minute.

After periods of storage up to 15 months, the ampoule content was resuspended in distilled water and the morphology and virulence of the lyophilized culture were studied, the morphology being examined in a crushed drop after 10 minutes, 2 hours, 16 hours, and 40 hours. The lyophilized culture preparations obtained after short exposures (10 minutes or 2 hours) in distilled water displayed single, polymorphous, wrinkled promastigotes 2–3 times smaller in size than usual, with and without flagella, and with limited motility. The preparations obtained after exposures of 15 or 40 hours in distilled water displayed living, actively motile organisms, well contoured and having clearly visible nuclei and kinetoplasts. Such cells were present in both types of preparation, i.e., those dried with glycerol and those dried with serum.

" In order to determine the virulence of the lyophilized cultures, mice and golden hamsters were inoculated at different periods within 15 months after lyophilization. The material was resuspended in 1.5 ml of distilled water and incubated in a thermostat at 24°C for 40 hours; motile promastigotes could be observed in the preparations of this culture. Each mouse was inoculated with 0.05 ml into the skin of the ear, and was examined every 10 days.

" By the tenth day, at least half of the animals had developed characteristic infiltrations in the skin of the concha, and *Leishmania* were found in smears taken from some animals. During the next 5 months, ulceration did not develop in the affected part; however, the ears of all animals became deformed and distinct vessel hyperaemia was noted. The animals that appeared normal on the 10th day of observation showed no pathological manifestations during the following 5 months.

" The inoculation of golden hamsters with lyophilized culture of *Leishmania tropica major* was carried out after 1 month of storage. The material was introduced into intradermal pockets made in the ears of the test animals and 1 in 4 of these animals developed an ear lesion."

RÉSUMÉ

LE GENRE *LEISHMANIA*

L'auteur examine la position, sur le plan de la systématique, de ce qu'il est convenu d'appeler les espèces de *Leishmania* et s'efforce d'établir les liens phylogénétiques qui les unissent. Grâce à la microscopie optique et électronique, on peut étudier la morphologie et l'ultrastructure de ces organismes, mais aucune de ces méthodes ne fournit de critères permettant de différencier les espèces. Aussi est-il préférable de fonder les essais de classification sur le comportement des parasites chez l'insecte vecteur et chez l'hôte vertébré. On aboutit de la sorte à répartir les espèces de *Leishmania* en quatre groupes principaux: espèces parasites des mammifères, y compris l'homme; *L. enriettii*, espèce aux caractères morphologiques bien définis qui infecte le cobaye; espèces parasites des lézards; espèces dont on observe divers stades évolutifs chez les phlébotominés.

Parmi les formes « humaines », on distingue les formes viscérales, transmises à l'homme généralement à partir de canidés sauvages comme le chacal et le renard, et les formes cutanées, dont l'origine doit probablement être cherchée chez des rongeurs sauvages. Les espèces responsables des formes viscérales sont *L. donovani* et *L. infantum*; celles qui causent les formes cutanées sont *L. tropica tropica (= minor)*, *L. tropica major*, *L. brasiliensis*,

L. peruana, *L. guyanensis* et *L. mexicana*. Quant à *L. pifanoi*, elle ne correspond probablement pas à une espèce distincte, mais est à l'origine de certaines formes de leishmaniose cutanée dont les aspects particuliers semblent résulter d'une défaillance de l'immunité à support cellulaire chez l'hôte.

Il est généralement possible d'identifier une infection leishmanienne d'après l'origine géographique de la contamination. On peut aussi recourir à toute une série d'investigations de laboratoire plus ou moins compliquées: mise en culture du parasite, épreuves sérologiques, inoculation à des animaux réceptifs et étude du comportement de l'agent infectieux chez l'invertébré. Aucun de ces procédés n'est infaillible, et il apparaît nécessaire de mettre au point une épreuve simple, pratique et fiable. La conservation des souches de *Leishmania* présente un intérêt considérable pour la recherche et une technique de lyophilisation est brièvement décrite.

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