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ULTRASTRUCTURAL COMPARISON OF PROMASTIGOTE FLAGELLATES (LEPTOMONADS) OF WILD-CAUGHT PANAMANIAN *PHLEBOTOMUS**

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ABSTRACT: The electron-dense DNA in the kinetoplast of *Leishmania* is in the form of a narrow band of rather uniform anteroposteriorly directed fibers. In one strain of flagellates isolated from *Phlebotomus sanguinarius* in Panama the DNA of the kinetoplast appeared in irregular anteroposteriorly directed fibers and masses after OsO_4 fixation. After Ryter-Kellenberger fixation the DNA appeared as very fine fibrils that filled the kinetoplast. Because of this difference, as well as differences in gross morphology and in growth pattern in the sandfly host it is concluded that this strain is not *Leishmania* but is probably *Crithidia* sp.

The close similarity between the insect stages (promastigote, Hoare and Wallace, 1966) of *Leishmania* and monoxenous insect parasites of the genus *Leptomonas* and related genera has been noted ever since they were first studied. No generic morphological criterion for promastigotes of *Leishmania* is known and infectivity tests with laboratory mammals provide the only certain means of differentiation though immunodiffusion tests are promising. Clark and Wallace (1960) found that the ultrastructure of the kinetoplast showed differences between monoxenous insect parasites of the genera *Herpetomonas* and *Crithidia* and the heteroxenous *Trypanosoma* and *Leishmania*.

In the search for naturally infected *Phlebotomus* sandflies the staff of the Leishmaniasis Project at the Gorgas Memorial Laboratory, Panama, dissected over 7,000 wild-caught females during the years 1961 through 1966 (Johnson, McConnell, and Hertig, 1963; McConnell, 1963; later unpublished data). Over 500 natural infections with leptomonad flagellates were found in seven species of sandflies from various localities. Pure cultures were obtained over 100 times. Of the 19 sandfly strains tested in hamsters, five resulted in infections indistinguishable from those produced by Panamanian human strains (*Leishmania braziliensis sensu lato*). Cultures from Panama were brought to the University of Minnesota where electron microscope studies were conducted. This paper presents a comparison of seven strains isolated from wild-caught sandflies and one from a human cutaneous lesion.

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MATERIALS AND METHODS

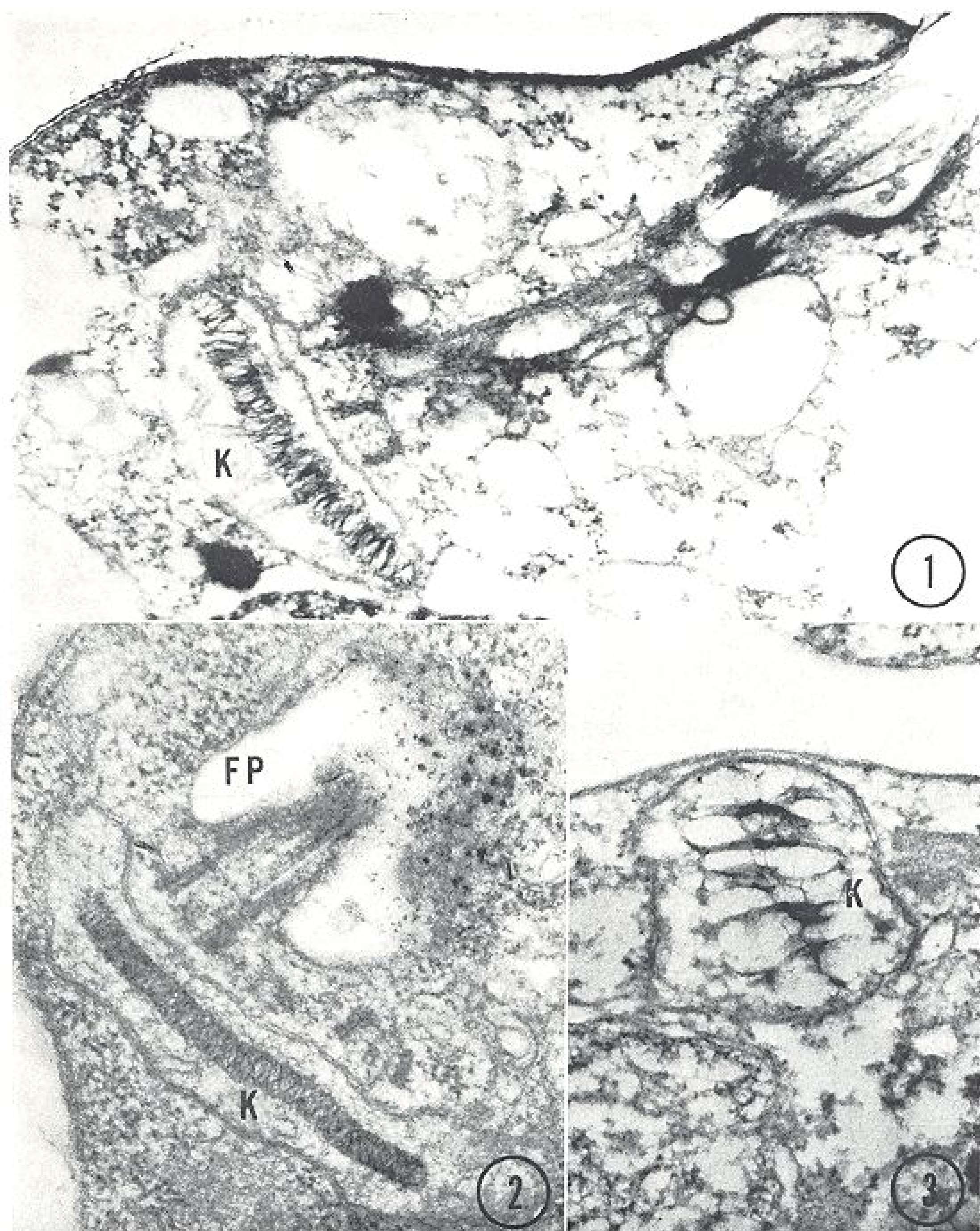
The methods of isolation, culture, and animal inoculation in Panama were given by McConnell (1963). At the University of Minnesota the cultures were maintained on Diamond and Herman's (1954) neopeptone medium modified as follows:

Bacto Neopeptone	2 g
NaCl	0.6 g
Water	100 ml

This constitutes the broth which forms the overlay and the base for the agar. For the agar base 2 g of agar were added to 100 ml of broth which was tubed in 5-ml quantities in screw-cap culture tubes and autoclaved. To each tube of melted agar, cooled to 50 C, 0.5 ml of rabbit or human blood was added. Rabbit blood medium was used for all strains except two (5003 and 6445), which grew better on human blood medium. The tubes were cooled in slants and 0.5 ml of autoclaved broth overlay was added to each. Cultures were incubated at 21 C and transferred every 2 weeks.

Microscopic studies were made on dried smears stained with Giemsa's stain and on living organisms using the dark phase oil immersion lens.

For electron microscopy the liquid overlay from two 7-day cultures was centrifuged and the organisms fixed for 1 hr in cold 3.1% glutaraldehyde in phosphate buffer at pH 7.4 (Pease, 1964). At each subsequent change the tube was centrifuged, the fluid poured off, the next fluid added, and the pellet broken up very gently. Following fixation the material was treated with glutaraldehyde buffer, 5 min, cold R-K fixative (1% OsO, buffered to pH 6.2 with veronal acetate and containing 0.1 M $CaCl_2$, Ryter et al., 1958) for 1 hr, two rinses in R-K buffer, 0.5% uranyl acetate in R-K buffer 1 hr at room temperature, and two rinses in R-K buffer. The final suspension was mixed with melted 2% agar which was allowed to harden.



FIGURES 1–3. Electron micrographs of kinetoplast regions of flagellates isolated from sandflies. **1, 2.** DNA in compact band, characteristic of leishmanial ultrastructure of kinetoplast. **3.** DNA clumped. Figure 1. Strain 2195, OsO$_4$ fixation. Figure 2. Strain 6408, R-K fixation. Figure 3. Strain 6346, OsO$_4$ fixation. 64,000 ×. Abbreviations (all figures): FP, flagellar pocket; K, kinetoplast; M, mitochondrion.

TABLE I. *Comparison of ultrastructure of leptomonads from wild-caught* Phlebotomus *and a human case of cutaneous leishmaniasis in Panama.*

Strain	Source, place, date	Hamster infected	Schneider-Hertig immunodiffusion group no.	Kinetoplast ultrastructure
Sandfly strains				
2195	*Phlebotomus trapidoi* F. & H. Quebrada Bonita 9 July 1961	Yes	Uncertain	Leishmanial
5003	*P. trapidoi* F. & H. Almirante 29 Nov. 1961	No	II	Leishmanial
6103	*P. ylephiletor* F. & H. Cerro Campana 6 Sept. 1962	Yes	I	Leishmanial
6217	*P. trapidoi* Quebrada Bonita 4 Oct. 1962	Yes (Inoculated directly with sandfly gut)	I	Leishmanial
6346	*P. sanguinarius* F. & H. Quebrada Bonita 4 Oct. 1962	No	Uncertain	Fine-fibrous (Unusual morphology and growth pattern noted on original dissection)
6408	*P. trapidoi* Almirante 8 Oct. 1962	No (Only original sandfly gut inoculated)	Uncertain	Leishmanial
6445	*P. gomezi* Nitz. Quebrada Bonita 19 Nov. 1962	Yes (After 18 mos. in culture)	II	Leishmanial
Human strain				
Monteza	13 Dec. 1960 Probably contracted in Bocas del Toro Prov.	Yes	I	Leishmanial

Blocks of agar containing organisms were dehydrated in acetone and embedded in vestopal (Ryter and Kellenberger, 1958).

Some preparations (referred to below as treated with OsO_4 fixation) were fixed without the special features of the Ryter-Kellenberger (R-K) fixation. The procedure was as above except that there was no $CaCl_2$ in the fixative, the pH of the OsO_4 fixative was 8.3, and the postfixation bath in uranyl acetate was omitted. In either case sections were cut on the Porter-Blum Mt 1 microtome, stained with 2% uranyl acetate, and examined with an RCA EMU 3D electron microscope at 50 kv at original magnification of 16,000 and enlarged thereafter photographically.

The histories of the seven sandfly cultures and the one human strain studied are presented in Table I. Experimental infection of hamsters is significant and identifies the organism definitely as *Leishmania* sp. The negative results with animal inoculations are not conclusive since the conditions of the experiments varied from strain to strain and in some cases the amount of material inoculated or the number of attempts was minimal.

These eight strains were among the 16 strains (8 human, 8 sandfly) studied by the agar gel diffusion method by Schneider and Hertig (1966). They found two immunologically distinct groups of Panamanian strains, each of which contained both human and sandfly strains. Human strains from other countries formed still other groups. Of the seven sandfly strains dealt with in this paper, four were evenly divided between Groups I and II, while three (2195, 6346, and 6408) were listed as "uncertain."

RESULTS

In all strains studied the kinetoplast, when seen in a longitudinal section of the organism, appears as an oval to oblong sac composed of two unit membranes. The inner membrane is invaginated here and there into the kinetoplast to form cristae. In occasional sections (Fig. 4) an extension of the kinetoplast to the mitochondrion was seen.

In seven of the eight strains [one strain of human origin (Monteza), four sandfly strains which infected hamsters (2195, 6103, 6217, 6445), and two which did not (5003, 6408)], the kinetoplast is of the same general type. The shape, as seen in longitudinal sections of

the organism (Figs. 1, 2) is a transversely elongate, narrow oval. The DNA band is compact, sharply delimited at its anterior and posterior margins, and is composed of relatively coarse and uniform fibrils. Between preparations fixed with OsO_4 (Fig. 1) and by the R-K fixation (Fig. 2) there is little difference. This is the type regarded as characteristic of *Leishmania*.

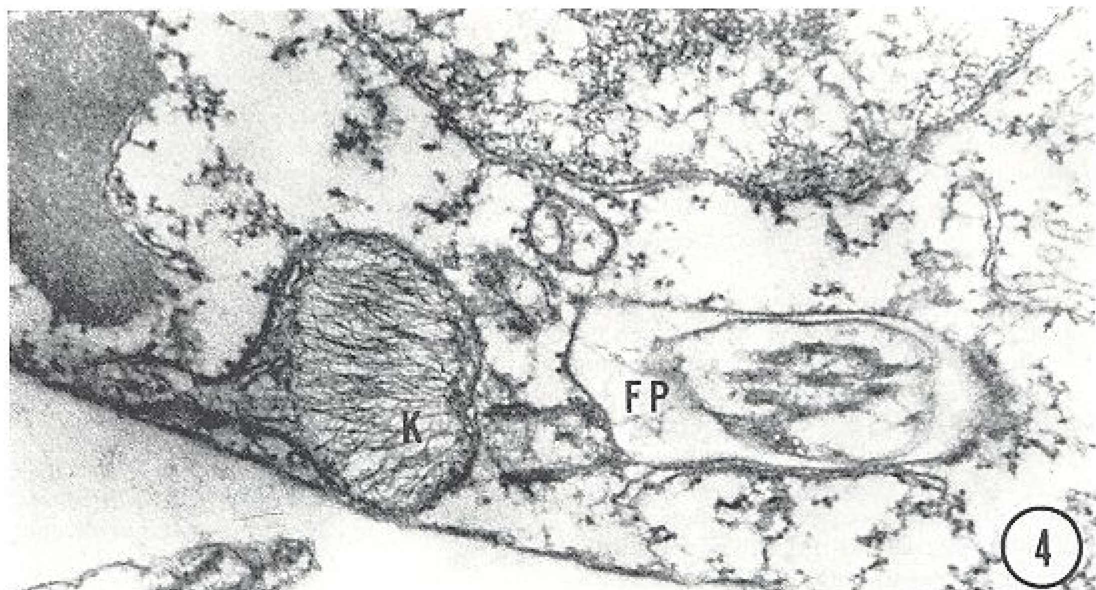
Strain 6346 differs in a number of respects from the others. At the time of dissection of the original insect host it was noted that the flagellates were unlike those of any other natural infection in that they were relatively thick with rounded ends. Furthermore, the growth pattern in the sandfly gut was unusual. Panamanian strains of whatever origin differ in their growth patterns from those repeatedly observed by workers with Old World kala azar and oriental sore, where development takes place at the "anterior station," i.e., in the anterior part of the midgut, or cardia, with at times growth forward into the pharynx or even mouthparts. This pattern is also exhibited by Panamanian strains but the hindgut is also involved, particularly the thin-walled portion just posterior to the Malpighian tubules. This short section of the hindgut, roughly triangular in optical section, has been called the "hind-triangle" by the Panama group. In naturally infected sandflies the hind-triangle is almost invariably a site of attachment of the flagellates, with few or none elsewhere in the hindgut. This pattern is also found in sandflies fed on hamster lesions of both human and sandfly strains (Johnson et al., 1963) and in sandflies artificially fed with cultures (Hertig and McConnell, 1963). Strain 6346 was unique among the 500-odd natural infections in that the flagellates avoided the hind-triangle but were abundant throughout the rest of the hindgut. Cultures were fed artificially to both sexes of two species of laboratory-reared sandflies, *P. gomezi* and *P. sanguinarius* (M.H., unpublished data). The original growth pattern was reproduced in all specimens, with flagellates abundant and attached throughout the posterior part of the hindgut and rectal ampulla, with few or none in the hind-triangle, and none at all in the midgut or foregut.

The structure of the kinetoplast of strain 6346 is entirely different from the others. The outline in longitudinal sections is subcircular with the anteroposterior axis very little shorter than the transverse axis. After OsO_4 fixation the DNA is in very irregular fibers and masses which are generally anteroposteriorly oriented but have no uniformity in thickness (Fig. 3). After R-K fixation the DNA appears as fine anteroposteriorly directed fibers that occupy most of the space inside the kinetoplast (Figs. 4, 5).

DISCUSSION

While *Phlebotomus* of many species have been examined in the search for flagellate parasites in many parts of the world no monoxenous insect flagellates have been definitely identified. The vast majority of the protozoa found have been identified as *Leishmania* spp. *Bodo* sp. was reported from *P. minutus* by Shortt (1925). Three species of *Trypanosoma* have been found to be transmitted by *Phlebotomus*. *T. platydactyli* Catouillard of a gecko is transmitted by *P. parroti* Adler and Theodor (Adler and Theodor, 1935), *T. bocagei* França of a toad by *P. squamirostris* Newstead (Feng and Chung, 1940), and *T. phyllotis* Herrer of the rodent, *Phyllotis*, by *P. noguchii* Shannon (Herrer, 1942). Other epimastigotes (Hoare and Wallace, 1966) which are presumably *Trypanosoma* (though they might be *Blastocrithidia*) were found by Lewis and Minter (1960) and by Hoogstraal and Deitlin (1964) in *P. garnhami* Heisch et al. Strangways-Dixon and Lainson (1962) found two *Phlebotomus* in British Honduras with flagellate infections which they thought might have been *Leptomonas* or *Herpetomonas*. Sherlock and Pessôa (1966) reported flagellates in *P. micropygus* Mangabeira which they did not believe were *Leishmania*. Their figures are highly suggestive of *Trypanosoma*. In the Panama studies presumed trypanosomes were found in *P. vespertilionis* Fairchild and Hertig, *P. trinidadensis* Newstead, *P. trapidoi*, F. and H., and *P. ylephiletor* F. and H. (Johnson, McConnell, and Hertig, 1963; McConnell and Correa, 1964).

The lack of records of *Leptomonas*, *Herpetomonas*, or *Crithidia* from *Phlebotomus*, with the possible exception of the British Honduras record cited above, is remarkable in view of their relative frequency in other Diptera, especially the mosquitoes. This is possibly due



FIGURES 4, 5. Electron micrographs of kinetoplast region of strain 6346, R-K fixation, showing fine fibrillar DNA commonly found in monoxenous promastigotes from insects, in this case probably *Crithidia*. 4. Beginning of mitochondrial extension. 64,000 ×.

to the great difficulty in distinguishing these genera from the promastigote (leptomonad) stages of *Leishmania*.

Although certain microscopic differences between strain 6346 and the *Leishmania* strains isolated from sandflies were noted these were not in themselves sufficient evidence of its nonleishmanial nature as a considerable degree of variation among the leishmanias was noted. But when added to this, the distinctive growth pattern in the hindgut of the insect host and the ultrastructure of the kinetoplast show that this is not *Leishmania* but that it belongs to one of the genera of insect flagellates, probably *Crithidia*.

Insect flagellates should occur more or less equally in males and females. All of the promastigote or leptomonad infections in over 7,000 dissected *Phlebotomus* were found in females. This is a strong indication that these flagellates were *Leishmania*, with the single exception recognized at dissection as unusual. Indeed, in all cases where the results of various tests were significant, these flagellates exhibited leishmanial structure or behavior or both. Leaving out of consideration the same exceptional flagellate, it may be noted that while the history of the various sandfly strains used in the present study differed as to infection of hamsters and immunological relationships, all exhibited leishmanial ultrastructure of the kinetoplast. That the single nonleishmanial infection found was in a female rather than in one of the 262 males dissected has no significance. The extreme rarity of this presumed *Crithidia* raises the question as to how it could maintain itself if *Phlebotomus* should be its natural host. This need not be the case as Adler and Theodor (1930) infected *P. papatasi* with four of Noguchi's cultures (Noguchi and Tilden, 1926) then known as *Herpetomonas oncopelti* (two strains), *H. lygaeorum*, and *H. muscidarum*. Wallace (1966) reviewed the confused history and nomenclature of these cultures but their exact identities are less important in the present context than the fact that flagellates from nonphlebotomine sources did infect *Phlebotomus*.

In view of the lack of clear-cut microscopic criteria for differentiating cultured *Leishmania* from promastigote insect flagellates another criterion would be very useful. The available evidence strongly supports the view that this purpose is served by the characteristics of the DNA band in the kinetoplasts as seen with the electron microscope after R-K fixation.

The picture in *Leishmania* as found in this study agrees essentially with many published descriptions (see Rudzinska, D'Alesandro, and Trager (1964); Garnham and Byrd, 1962 and Mühlpfordt, 1964). There are not as many studies of the kinetoplasts of monoxenous trypanosomatids but the micrographs of Clark and Wallace (1960), Indurkar (1965), Kusel, Moore, and Weber (1967), and unpublished pictures in our laboratory show that the fibrous DNA band after R-K fixation and the irregularly clumped fibers after OsO_4 fixation are common among the insect flagellates.

The difference in structure of the kinetoplast may well be related to the difference in life histories. Rudzinska, D'Alesandro, and Trager (1964) show that the kinetoplast plays a role in the transformation from the leishmanial (amastigote) stage of *Leishmania donovani* in the vertebrate host to the promastigote stage in culture. Schulz and MacClure (1961) and others have shown that *Trypanosoma cruzi*, which has an amastigote intracellular stage, has a kinetoplast with a compact DNA band in all stages. But the literature does not present a consistent picture of the nature of the kinetoplast DNA in other species of *Trypanosoma*, so we cannot generalize on this genus at present. The Ryter-Kellenberger fixation is particularly effective in preserving the fine fibrillar nature of the DNA in the prokaryote nucleus which lacks histone. Further comparative studies on the form of the DNA and on associated proteins in the kinetoplasts should be made.

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