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Leptomonads of Wild-Caught Panamanian *Phlebotomus*: Culture and Animal Inoculation[1]

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Cultures were made from wild-caught *Phlebotomus* sandflies found to be naturally infected with leptomonad flagellates. Pure cultures were obtained from 89 of 218 infected sandflies (40% of those cultured).

Hamsters were inoculated with 13 of these strains. Two strains produced lesions comparable with those following inoculation of cultures of local human strains of *Leishmania braziliensis, s. lat.*

Cultures of the sandfly strains exhibited typical leptomonad morphology but also usually contained some greatly elongated forms. The sandfly flagellates have not yet been identified and their source is unknown, but available evidence indicates that these infections are acquired through a blood meal from a mammalian host.

In the course of studies on the transmission problem of cutaneous leishmaniasis in Panama, many wild-caught *Phlebotomus* sandflies were dissected and examined in the search for natural infections of leptomonad flagellates, as reported in the preceding paper (Johnson, *et al.*, 1963). A large number of such natural infections was found (416 out of 4861 female sandflies of the more common man-biting species). These sandflies came from several areas where the human disease, caused by the local strain of *Leishmania braziliensis, s. lat.*, is endemic. Cultures were made from a number of the dissections containing flagellates, and some strains obtained in pure culture were inoculated into hamsters.

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Materials and Methods

In preparation for dissection, several sandflies at a time (immobilized by refrigeration at 4°C) were shaken vigorously in a vial of saline to remove scales and any loose extraneous material. They were then transferred to a drop of saline on a glass slide, where all appendages were removed. Finally, a single specimen, minus its appendages, was placed in a drop of saline on a cover slip and was dissected by severing the neck as close to the head as possible and then withdrawing the gut from the tip of the abdomen. (By pulling gently, the terminal segments of the abdomen can be torn from the rest of the body accompanied by the entire mid- and hindgut.) For examination the cover slip containing the dissection was inverted over a depression slide and sealed with saline.

Following microscopic examination, certain of the dissections observed to contain flagellates had the terminal segments of the abdomen removed and placed in phenol for eventual determination as to species. The

gut was then transferred with a glass capillary to a blood agar slant. Several drops of sterile saline were usually added at this point to provide adequate moisture.

Sterile technique was used throughout, within the limitations imposed by the nature of the dissections. The inoculated tubes were rubber stoppered to prevent evaporation and incubated at 22°C or at a room temperature of approximately 25°C. At irregular intervals the cultures were examined microscopically, and those showing growth were transferred to fresh media for routine handling. No culture, unless contaminated, was discarded as negative until it had been held for approximately 6 weeks.

The medium for isolation and maintenance of hemoflagellates is prepared as follows: To each 100 ml of distilled water is added 2.5 gm Difco Bacto-Beef and the mixture is boiled and filtered through filter paper. This is followed by addition of 2.0 gm Difco Neopeptone, 0.5 gm NaCl, and 2.0 gm agar. The pH is adjusted to 7.2–7.4 before steam sterilization of the basic medium. To this stock medium is added 15% defibrinated rabbit blood plus penicillin (500 units per milliliter) and streptomycin (1.0 mg per milliliter).

Results

With this technique 89 strains of flagellates were isolated in pure culture from naturally infected *Phlebotomus* during the 1961 calendar year. All of the isolations came from the 5 most common man-biting species. These were: *trapidoi*, 56; *ylephiletor*, 16; *sanguinarius*, 9; *gomezi*, 4; and *panamensis*, 4. At times there was considerable variation in the proportion of successful isolations, but the over-all success rate was 40%, or 89 isolations from the 218 dissections which were cultured.

Culture forms of 13 of the isolates in the first or second transfer were inoculated intradermally into the dorsum of the snout of golden hamsters. Eight of the strains were from sandflies collected at Quebrada Bonita, near the Transisthmian Highway in central Panama, and five from those sandflies taken at Almirante, on the Caribbean slope in northwestern Panama. With two exceptions, a *gomezi* and a *sanguinarius* collected at Quebrada Bonita, all were from *trapidoi*. Two strains, both from *trapidoi* collected at Quebrada Bonita, have produced nonulcerated swellings rich in L-D bodies that are typical of those produced experimentally with local human strains. In one case lesions developed within a month after inoculation, while in the other there was an incubation period of 11 months.

The establishment of only two infections from 13 strains is in sharp contrast to the results routinely obtained in hamsters inoculated with cultures of local human strains. In the latter case we are able to establish an infection in almost 100% of hamsters inoculated intradermally in the nose with a heavy inoculum (growth from two or more slants). Apparently the majority of the isolates from sandflies is not infective for hamsters, although it should be pointed out that historically the establishment of infections with *L. braziliensis* in animal hosts has been difficult, and the factors involved are not well understood. For instance, in our work in Panama we have never succeeded in infecting the spiny rat, *Proechimys*, with strains of *Leishmania* originally isolated from that rodent, although the same strains are infective for hamsters. At least one strain has remained so after 4 years in culture.

Each of the isolates from wild-caught sandflies which has been subjected to a sufficiently careful examination has been found to contain some flagellates, apparently leptomonads, greatly elongated at the posterior end (Figs. 2–9). (Unfortunately, the strain which produced a hamster infection so promptly was lost before the presence of long forms in these isolates had been noted; the other strain had long forms.) The number of the elongated forms rises with the age of the

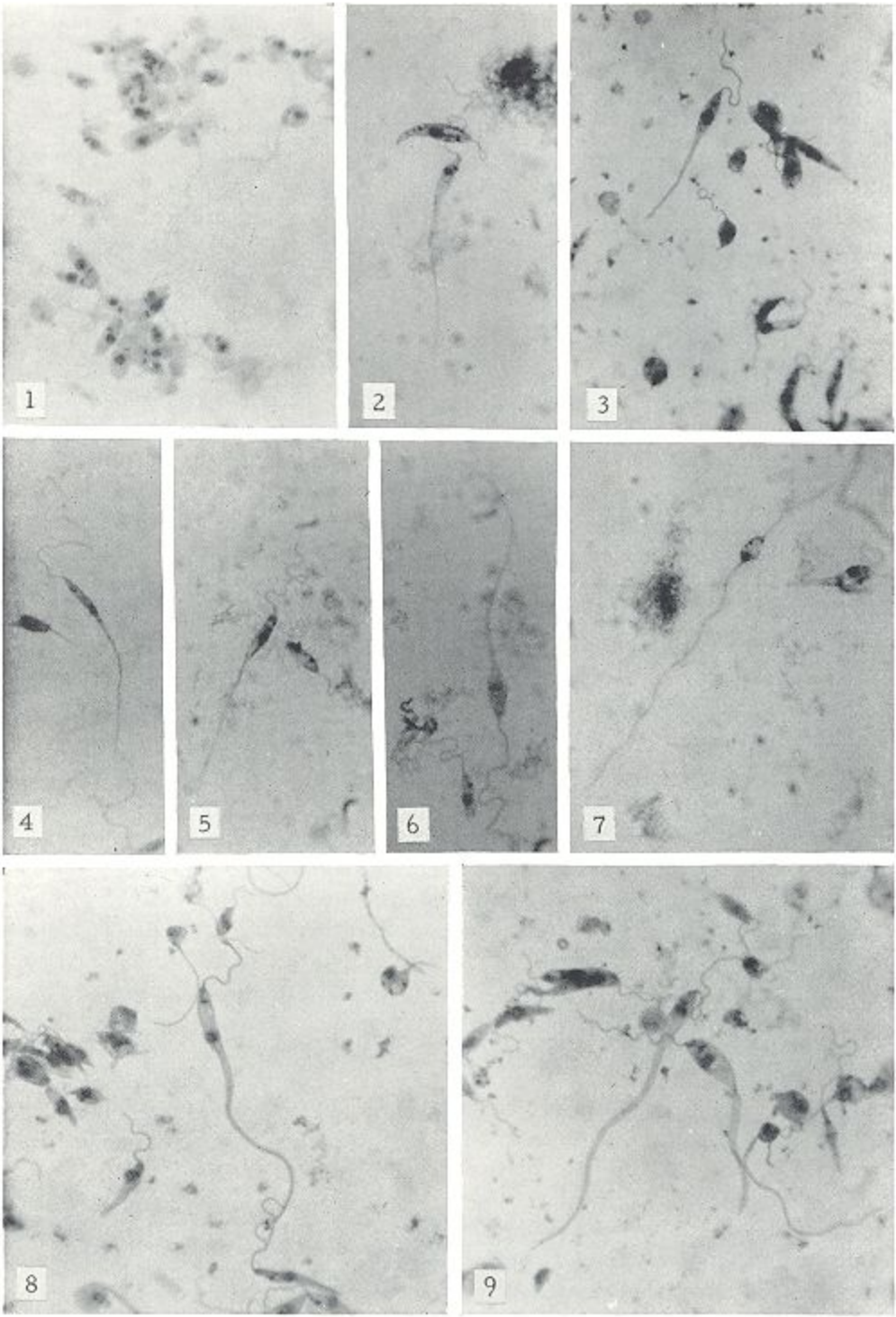

FIG. 1. *Leishmania* isolated from spiny rat, *Proechimys*; no elongate forms ever noted, in contrast to sandfly flagellate strains. Giemsa stain; × 920.

FIGS. 2–9. Leptomonads isolated from wild-caught *Phlebotomus* sandflies, illustrating elongate as well as "normal" cultural forms. There are represented three strains cultured from *P. trapidoi*, collected in two widely separated areas of Panama. Giemsa stain; × 920.

culture. Fresh isolates, and cultures recently transferred, may contain relatively few of these individuals. In one instance, however, an elongate leptomonad was observed in the hindgut of a *P. trapidoi*. All intermediate degrees of elongation appear in culture and were at first overlooked as part of the variation which can be expected in cultures of *Leishmania*. We have never, however, encountered individuals so extremely elongated in *Leishmania* from other sources. In culture the strains from sandflies survive longer without transfer and with less moisture present than is characteristic of strains from Panamanian human cases.

Morphologically, the flagellates from culture appear to be typical leptomonads, with the kinetoplast located well anterior to the nucleus, a free flagellum, and with no suggestion of an undulating membrane. The ratio of the distance from the nucleus to the anterior end of the body relative to the distance from the nucleus to the posterior end varies from approximately 1:1 in nonelongate leptomonads to 1:12 in very long specimens. This indicates that elongation is due to differential growth of the posterior portion of the flagellate and is not a proportional over-all increase in length. The ratio in culture forms from other sources remains approximately 1:1. The nonelongate forms in the sandfly cultures are indistinguishable from the leptomonads found in strains isolated from human cases.

Slightly less elongate leptomonads were figured for *Leptomonas ctenocephali* by Tyzzer and Walker (1919) in a comparative study of that organism and *Leishmania infantum*. They considered the presence of long forms in cultures of *Leptomonas ctenocephali* to be one of the distinguishing characteristics of the two species. Wenyon (1926), in a discussion of similar forms in *Herpetomonas mirabilis*, suggested that elongation may be an abnormal condition resulting from retardation of nuclear division.

It is uncertain whether there is any relationship between the isolates containing long forms and human leishmaniasis. Antigen was prepared from two of the strains with long forms and, using the agar-diffusion precipitin technique, was tested against antiserum produced in rabbits against a Panamanian human strain of *Leishmania*. A precipitin band was produced between the antigen from the human strain and the antiserum (control), but not between the antiserum and the antigens from the sandfly strains. The complete lack of reaction in the latter case suggests that the human strain and at least these two sandfly isolates are different.

Discussion

It seems clear that at least the two strains which produced hamster lesions can properly be considered to belong to the genus *Leishmania*. (The characteristics justifying inclusion in this genus are: (1) leptomonad morphology; (2) the possession of both invertebrate and vertebrate hosts; and (3) the production in the latter of an intracellular infection with typical L-D bodies.) It is possible that the other strains represent a species of *Leptomonas* or *Phytomonas*, but whatever may turn out to be the final identification, there is evidence suggesting that all the flagellate infections of wild-caught sandflies were obtained through a blood meal from an animal host. No infections were found in any of 262 male *Phlebotomus* taken in catches which yielded a number of females of the same species containing flagellates (Johnson *et al.*, 1962, 1963). If the parasites are *Leptomonas* or *Phytomonas*, acquired by the sandflies as either larvae or adults, the males could be expected to have the same opportunity as the females to acquire the infection. Additionally, flagellates have never been observed in *Phlebotomus* reared in the laboratory from wild-caught stock.

It is also noteworthy that the natural infections follow the same pattern as those

experimentally produced with local human strains of *Leishmania*. Although we have not attempted the artificial infection of sandflies with strains containing the long leptomonads, it is possible to correlate the various stages of infection observed in wild-caught flies with the progress of infections established by feeding on hamsters inoculated with human strains. In both there appears to be a progressive forward movement of the infection from the point of initial establishment in the hindgut.

While we do not yet know the whole range of natural hosts of the principal man-biting species, the available evidence indicates that they feed chiefly on mammals. Over a period of 5 months daytime collections made weekly in resting places such as tree buttresses and animal burrows yielded about 500 females of the man-biting species, which were dissected in the search for natural infections. These collections were made in one of the study areas (Quebrada Bonita) which had yielded many natural infections and were made at least 60 hours after any collections with horse or man as bait in order to avoid confusion with blood meals of these sources. Red blood was present in 62 females (8 of which were also naturally infected); stained smears were made of the midgut contents; 11 of the smears were unsatisfactory as to erythrocyte morphology; in the other 51, the red cells were nonnucleated, i.e., of mammalian origin. Nucleated red cells were found in *trinidadensis*, a very common buttress-inhabiting species, known to feed on geckos, and in *vexillarius*, a rather uncommon sandfly which has been taken biting man but of otherwise unknown feeding habits. Common man-biting species have been attracted to chicken-baited traps and birds thus can not be excluded. However, the consistently mammalian source of blood in the above series points strongly to a mammal as probable source of the leptomonad infections.

Addendum

After Dr. McConnell left Gorgas Memorial Laboratory (August, 1962) we continued attempts to infect hamsters with flagellates from naturally infected sandflies. By this time all of us, including Dr. McConnell, suspected that heavy anterior infections would be the type most likely to produce infections in hamsters. This feeling was based on the fact that the two previous successes were obtained with cultures from sandflies with heavy anterior infections, together with the assumption that transmission is by the bite of sandflies which have anterior infections with flagellates.

Eleven strains of leptomonad flagellates were recovered from the 24 infected guts which we cultured during September to November, 1962. After varying periods of time flagellates in seven of these strains developed long tails, such as those described by Dr. McConnell.

Another strain, from the hindgut of a *sanguinarius*, has big cigar-shaped leptomonad flagellates of remarkably consistent morphology. It is unlike any other strain we have isolated from sandflies. Inoculation of cultures into the nose of hamsters has not caused infections in these animals.

The remaining three strains, from one *ylephiletor* and two *trapidoi*, have not developed long-tailed forms in the 6 or 7 months they have been under observation. All three were recovered from guts with heavy anterior infections. Second-transfer cultures of the short-tailed strain from *ylephiletor* (collected at Cerro Campana) were inoculated intradermally into the nose of hamsters. Lesions containing L-D bodies developed in a little over 3 weeks and were consistent with those described by Dr. McConnell in other hamsters inoculated with strains from naturally infected sandflies. In the case of the two *trapidoi* the triturated guts themselves were inoculated into the nose of hamsters. Those

inoculated with the gut of a *trapidoi* collected at Quebrada Bonita were negative at 2 months, but a month later lesions containing L-D bodies had developed. Hamsters inoculated with the other *trapidoi*, collected at Almirante, remained negative.

One of the seven long-tailed strains was recovered from an *ylephiletor* female, infected only in the hindgut, and collected at the same time and place as the *ylephiletor* which produced the short-tailed, hamster-infecting strain. Hamsters inoculated with first-transfer cultures of the long-tailed *ylephiletor* strain never developed lesions, nor were L-D bodies demonstrated in smears of tissue aspirated from the nose. This is entirely in keeping with the results obtained by Dr. McConnell and supports our hypothesis that short-tailed strains from heavy anterior infections are necessary for the production of hamster infections.

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