

NATURAL INFECTIONS OF *LEISHMANIA* AND TRYPANOSOMES DEMONSTRATED BY SKIN CULTURE

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ABSTRACT: In the search for potential reservoirs of leishmaniasis, cultures were made from the skin of 81 wild-caught animals, of 17 genera, which showed no lesions or other external signs of infection. *Leptomonad* flagellates were obtained in culture from the skin of six of eight porcupines, *Coendou rothschildi*. These organisms were tested in hamsters and shown to be *Leishmania* sp. Sections of skin of positive porcupines showed L-D bodies in cells of the upper dermis. A few leptomonad flagellates were found in a small proportion of laboratory-reared *Phlebotomus* sandflies fed on infected porcupines. Trypanosomes were cultured from the skin of an opossum, *Didelphis marsupialis*, and of a marmoset, *Saguinus Geoffroyi*.

The search for the reservoir of cutaneous leishmaniasis in Panama has been carried on for a number of years at Gorgas Memorial Laboratory. Natural infections have been demonstrated in the spiny rats, *Proechimys semispinosus* (Tomes) and *Hoplomys gymnurus* Goldman (Ann. Rep. G.M.L. 1957-58), but in both cases only by culture of heart blood. There were no external signs of infection. Various members of the staff had examined by means of cultures and smears a great number of other animals, of at least 35 genera, from various parts of Panama, without isolating *Leishmania* or finding any external leishmanial lesion, until Thatcher et al. (1965) found an inconspicuous lesion containing L-D bodies on the ear of a kinkajou, *Potos flavus* (Schreber).

In Peru it had been found (Herrero, 1948) that dogs naturally or experimentally infected with the *Leishmania* of Peruvian uta had skin lesions which were at best very inconspicuous, showing only slight depigmentation, and often had no external sign of infection. Nevertheless, smears from such lesions or the sites of inoculation often contained L-D bodies which could be revealed by prolonged examination.

MATERIALS AND METHODS

In March 1965 we began the examination of apparently normal skin of a variety of wild-caught Panamanian forest animals by means of stained smears. In one case smears were made

from ample scrapings of sound skin on the rump of a porcupine, *Coendou rothschildi* Thomas. In one smear three L-D bodies were found.

This positive finding was followed by the examination of other porcupines, in the course of which a biopsy-culture technique was developed. This method was based on previous work in Peru (Herrero, to be published elsewhere) in which tissue obtained by biopsy of the mucosa of Peruvian espondia (mucocutaneous leishmaniasis) patients was triturated in saline-plus-antibiotics, refrigerated for 24 hr, and then injected into dogs. *Leishmania* forms were recovered in culture from the developing papule.

The technique finally developed for making cultures from the skin of porcupines and other animals is as follows: (1) After ether anesthesia, suitable areas are prepared by plucking out the spines of porcupines; hair of other animals is clipped and the skin shaved; (2) the skin is swabbed with iodine, followed by ether before the iodine has dried; (3) about 1.5 cm² of skin is removed with a scalpel and dropped into 2.5 ml saline containing 500 units of potassium penicillin and 1 mg streptomycin sulphate per ml of saline; (4) the skin is triturated with scissors as finely as possible; (5) saline and tissue are transferred to a rubber-stoppered test tube and refrigerated at 4 to 6 C for 24 to 72 hr; (6) cultures are made by inoculating each of five or six tubes with 0.3 to 0.4 ml of the saline containing the finer skin particles.

The culture medium is prepared as follows: To 1,000 ml distilled water is added 25.0 g Difco Bacto-beef, which is boiled and filtered through filter paper. Distilled water is added to bring the mixture to its original volume. There is then added 20.0 g Difco Bacto-peptone, 5.0 g NaCl, and 30.0 g Difco Bacto-agar. The pH is adjusted to 7.2 to 7.4 and this stock medium is then autoclaved. Whole rabbit blood (no anticoagulant or defibrination) is added to the melted medium in the proportion of 10 to 13%, and the medium is tubed and slanted.

The above differs from the modification of

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Senekji's medium described by McConnell (1963) and which had been in routine use in this laboratory, chiefly in the omission of antibiotics and in a 50% increase in the amount of agar, which gives a final proportion of about 3% of the stock medium. Previous experience (AH) in the culture of various strains of *Leishmania* and other hemoflagellates had shown that better growth occurs on the firmer agar.

This formula yields a medium with no water of condensation. However, there should be some liquid, which in the case of skin cultures is supplied by the saline suspension; otherwise a few drops of saline should be added. Cultures are incubated at the temperature of an air-conditioned room, 20 to 22 C. The first examination is made at 4 to 6 days, since even in the presence of contamination it may be possible to observe growth of leptomonads before they are overgrown.

RESULTS

Leptomonad flagellates were isolated from six of eight porcupines. Two of the six yielded positive skin cultures on four different occasions, including autopsy, over a period of 5 months; both had been captured 3½ months before the first biopsies, so that the duration of the infection was at least 8½ months.

Two of the positive porcupines were trapped at Quebrada Bonita, a point near the town of Buena Vista on the Transisthmian Highway. The other four positives and both negatives came from Achiote, between Gatun Lake and the Caribbean, on the road to Piña.

Skin biopsies were usually made from two or more sites on the animal at the same time, with other sites chosen on subsequent occasions. Biopsy sites were chiefly rump, thigh, forehead, back, foreleg, or belly. A total of 24 sets of skin cultures (five or six tubes each) were made from the six positive porcupines; 20 sets had at least one positive tube; two were contaminated, two negative. An individual set of skin cultures was rated negative only if at least half of the tubes were negative and uncontaminated. In one case all 18 tubes from three pieces of skin were positive. Each of the six animals had at least two positive biopsies. In one case all six biopsies were positive, as well as five pieces of skin taken from the freshly killed animal, making 11 different sites from which leptomonads were recovered.

Cultures of heart blood were made from all eight porcupines, either soon after capture, at about the time biopsies were made, or, in one

case, soon after accidental death. All were negative for leptomonads. In one case, however, with three negative blood cultures and six positive skin cultures on three occasions, heart blood of the animal immediately after death gave a scanty culture of a blastocritidia-form flagellate, which was also cultured from the liver at autopsy. A leptomonad, however, was cultured from the bone marrow, while spleen and kidney were negative.

Stained smears were made from all the skin biopsies without finding any L-D bodies except in the one previously mentioned. The biopsy-culture method is thus shown to be much more effective as well as less laborious than the examination of smears of apparently normal skin for the presence of *Leishmania*.

Four of the *Coendou* leptomonad strains have been tested in hamsters by intradermal inoculation. They produce an inconspicuous lesion from which L-D bodies may be recovered for at least several months and are thus shown to be *Leishmania*. These strains are under further study.

Other wild-caught animals were examined by the same technique. Cultures were made of two pieces of skin taken from each of the 53 animals, belonging to 16 genera, listed below.

Marsupialia

- 1 *Marmosa robinsoni* Bangs
(brown murine opossum)
- 2 *Caluromys derbianus* Waterhouse
(woolly opossum)
- 4 *Philander opossum* L.
(four-eyed opossum)
- 6 *Didelphis marsupialis* L.
(common opossum)

Carnivora

- 1 *Bassaricyon gabbii* J. A. Allen (olingo)
- 4 *Potos flavus* (Schreber) (kinkajou)

Rodentia

- 9 *Proechimys semispinosus* (Tomes)
(spiny rat)
- 3 *Tylomys panamensis* Gray
(white-tailed rat)
- 2 *Rattus rattus* L. (common rat)
- 1 *Sigmodon hispidus* Say and Ord
(cotton rat)
- 10 *Oryzomys* spp. (rice rat)
- 1 *Hoplomys gymnurus* Goldman
(spiny rat)

3 *Zygodontomys cherriei* J. A. Allen
(cane rat)

Edentata

1 *Choloepus hoffmanni* Peters
(two-toed sloth)

1 *Tamandua tetradactyla* L.
(three-toed anteater)

Primates

4 *Saguinus geoffroyi* Pucheran
(marmoset)

No leptomonads were isolated. Cultures of 46 of the 53 animals were negative; five animals (one each of *Caluromys*, *Didelphis*, *Potos*, *Proechimys*, *Oryzomys*) had both sets of skin cultures contaminated.

A trypanosome was obtained from one of the six *Didelphis*, a juvenile, in the blood of which the parasite was demonstrated microscopically some weeks later. This organism closely resembles *Trypanosoma cruzi* in morphology and behavior, as shown by the infection of *Rhodnius prolixus* in a xenodiagnostic test, infection of a young white mouse by inoculation of a *Rhodnius* gut, and the infection of hamsters with cultures.

A trypanosome was also cultured from the skin of one of four marmosets, *Saguinus geoffroyi*. No further study of this organism was made.

Treatment of the biopsy tissues with antibiotics greatly reduces bacterial contamination, but fungi remain a hazard. Of the 126 culture tubes made from the skin of the six positive porcupines, 57 had leptomonads, 42 were negative, 27 were contaminated by fungi, and none by bacteria. The total of 683 skin-culture tubes used in this study gave the following results: 57, leptomonads; 6, trypanosomes; 444, negative; 155, contaminated by fungi, 21 by bacteria. Cultures of heart blood were only rarely contaminated.

Laboratory-reared sandflies, *Phlebotomus sanguinarius* Fairchild and Hertig, were fed on two positive porcupines. In the gut of one each of 19 and 32 sandflies dissected after 5 and 7 days, respectively, a few motile leptomonad flagellates were seen.

Sections of the skin of three positive porcupines showed L-D bodies in the cells of the upper dermis, with the majority parasitizing the elements contiguous with the basal or germinal layer of the epidermis. For the most

part the parasites were found generally distributed, with very few infected cells per microscopic field and only one or two L-D bodies per cell. There were, however, focal concentrations of infected cells, with some containing up to five or six L-D bodies. The parasites were usually located in a well-defined vacuole in the cytoplasm, regardless of the type of cell.

The host cells most frequently invaded were the fibrocytes, the ordinary connective tissue cells comprising the bulk of the dermis. Parasites were also seen occasionally in the so-called fixed tissue macrophages and the endothelial cells of the capillaries. In none of these cells was there any evidence of alteration aside from the presence of the vacuoles surrounding the L-D bodies. Similarly, there was a complete absence of any edema, cellular infiltration, or other reaction to the presence of the parasites.

Further histopathologic studies and experimental work with *Phlebotomus*, porcupines, other animals and their leptomonads are in progress. In the meantime the role of porcupines as potential reservoirs of leishmaniasis remains an open question. The relation of the porcupine *Leishmania* to other Panamanian strains which have been studied is still to be determined.

The available data indicate that in infected porcupines the parasites (1) are widely distributed in the skin, in cells of the upper dermis, over most, perhaps all, of the body; (2) are readily cultured from skin, but (3) cannot be demonstrated by culture of the heart blood of the living animal, although (4) they have been cultured occasionally from the spleen, liver, or bone marrow at autopsy; in any case (5) they are accessible in very limited numbers to *Phlebotomus* sandflies in the act of feeding.

The biopsy-culture technique provides an effective means of revealing the presence of *Leishmania* in skin which shows no sign of infection. An incidental by-product of the skin cultures is the isolation of trypanosomes (which in most cases could be revealed more readily by working directly with peripheral blood). An important feature of the method is the prolonged exposure of the tissues to antibiotics before cultures are made, rather than relying

on antibiotics incorporated in the culture medium.

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