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COMPARATIVE STUDIES OF HERPETOMONADS AND LEISHMANIAS.

II. Differentiation of the Organisms by Serological Reactions and Fermentation Tests.

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Rogers, who first cultivated the parasite of kala-azar[1] and found that under cultural conditions it acquired a flagellum and became morphologically indistinguishable from the herpetomonads so widely distributed in nature as parasites of insects and plants, suggested that the organism might pass the flagellated stage of its life history in some blood-sucking insect.[2] The theory proved an extremely difficult one to substantiate, however. The extremely slow development of kala-azar and of other infections due to leishmanias, and the lack of susceptible animals, both contribute to the difficulty of carrying out transmission experiments. The fact that blood-sucking insects which have been allowed to feed upon patients with kala-azar or oriental sore are found on subsequent dissection to contain flagellates does not justify the conclusion that the flagellates are leishmanias, because the latter cannot be distinguished on morphological grounds from the flagellates occurring in many insects. Even when the insects used have been bred in the laboratory, and one may reasonably assume that they are free from flagellate infection, it is essential to be able to demonstrate in some way that the strain isolated from the insect after feeding is the same as that obtained from the patient. The injection into animals of herpetomonads derived from insects and plants[3] has not given results which are sufficiently striking or constant to be considered convincing evidence of the pathogenicity of the inocu-

[1] Rogers, L., *Lancet*, 1904, lxxxii, 215.

[2] Rogers, L., *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 284.

[3] Discussed in Part I of this report.[7]

lated organisms; and the interpretation of the findings in such instances is complicated by the presence of natural flagellate infections in the animals employed (*e.g.*, mice, rats, amphibians, and birds).

The immunological properties of the leishmanias and herpetomonads have been very little studied. The writer,[4] in 1924, showed that at least three species of leishmanias could be distinguished by serological tests. Kligler[5] has also found that *L. brasiliensis* and *L. infantum* are immunologically distinct from one another and from *L. tropica*, while different strains of *L. tropica* are serologically identical. Wagener and Koch[6] recently carried out comparative serological tests with cultures of four strains of *Leishmania* and one strain of *Herpetomonas ctenocephali*, the latter a flagellate of the dog flea, which has been regarded as a possible vector of canine leishmaniasis. The anti-leishmania sera of these authors, which were prepared with killed cultures, had no effect upon *H. ctenocephali* but affected equally the different species of leishmanias.

Investigation of the chemical changes which may take place in various carbohydrate-containing culture media has not so far been utilized for the differentiation of species of *Herpetomonas*. Kligler[5] attempted to distinguish leishmanias by this means but was unable to draw any definite conclusion from his study with a small series of strains.

As a first step in the application of immunological and biochemical methods to the differentiation of flagellate species, a number of strains of herpetomonads have been isolated from various sources and compared in serological and fermenting properties with one another and with the leishmanias. Twelve strains in all have been cultivated as described in Part I of this report.[7] Two additional flagellate strains, *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*, furnished by courtesy of Dr. E. E. Tyzzer, were included in the study. The leishmania strains were the same as those I have employed previ-

[4] Noguchi, H., *Proc. Internat. Conf. Health Prob. Trop. America*, Kingston, Jamaica, British West Indies, July 22 to August 1, 1924, published in Boston, 1924, p. 455.

[5] Kligler, I. J., *Tr. Roy. Soc. Med.*, 1925, xix, 330.

[6] Wagener, E. H., and Koch, D. A., *Univ. Calif. Pub. Zool.*, 1926, xxviii, 365.

[7] Noguchi, H., and Tilden, E. B., *J. Exp. Med.*, 1926, xliv, 307.

ously for immunological investigation.[4] Comparison of the biological characteristics of these eighteen strains of flagellates brought to light a number of interesting facts.

Serological Differentiation of Herpetomonads and Leishmanias.

Rabbits were immunized against *Herpetomonas oncopelti*, *H. lygæorum*, *H. culicidarum*, *H. muscidarum*, *H. media*, *H. parva*, *Leishmania tropica*, *L. brasiliensis*, *L. infantum*, and *L. donovani* by intravenous injections of 1 to 2 cc. of rich living cultures at 4 day intervals on four successive occasions. The animals were bled 9 days after the last inoculation. The action of the specific immune sera thus prepared was tested upon the homologous and heterologous strains by agglutination and by complement fixation.

For the agglutination test, 0.05 cc. of immune serum was added to 1 cc. of a saline suspension of culture. A drop of the mixture was examined immediately with the dark-field microscope and again after 30 minutes and after 18 and 24 hours. Gross examination of the tubes was carried out as well. Control tests with normal rabbit serum were made in each instance. The presence in the suspension under test of rosettes or agglomerated masses did not interfere with the observations, since the changes brought about by specific agglutination are easily to be recognized. Under the dark-field microscope both flagellum and cytoplasm are seen to be profoundly affected by the specific immune serum. The organisms become sluggish or motionless, the bodies swollen and uneven in contour, the flagella twisted irregularly and adherent to whatever comes in contact with them, and finally the bodies are broken up. With a strong specific serum the gross findings are similarly striking. Within 30 minutes the turbid suspension becomes granular and begins to sediment and after 18 hours the supernatant fluid is clear, and there is a compact whitish sediment at the bottom of the tube.

For test of the specific complement-fixing power of the sera, the anticomplementary titer of a given antigen was first determined and half the anticomplementary dose employed. The immune serum was inactivated by heating at 55°C. for 30 minutes, and the amount used was 0.1 cc. in a total volume of 1 cc., 0.9 per cent saline being the diluent. The quantity of guinea pig serum, employed as complement, was 0.1 cc. of a 40 per cent dilution, and of anti-sheep amboceptor 2 hemolytic units. Incubation for fixation (30 minutes) and subsequent hemolysis (30 minutes) was carried out at 37°C.

The results of the agglutination and complement fixation tests are recorded in Tables I and II. The striking phenomena observed were as follows:

1. The strong reactions of the flagellates with their homologous sera. These were clear-cut and unmistakable.

TABLE I.

Agglutination Tests.

Source of flagellate.	Antisera.										
	Anti-*oncopelti* (strain from *O. fasciatus*).	Anti-*lygæorum* (strain from *L. kalmii* No. 2).	Anti-*culicidarum* (strain from *Anopheles quadrimaculatus*).	Anti-*muscidarum*.	Anti-*media*.	Anti-*parva*.	Anti-*tropica*.	Anti-*brasiliensis*.	Anti-*infantum*.	Anti-*donovani*.	Normal rabbit serum.
O. fasciatus	++++	+	−	−	−	−	−	−	−	−	−
Oncopeltus sp.?	++++	+	−	−	−	−	−	−	−	−	−
L. kalmii No. 1	++++	+	−	−	−	−	−	−	−	−	−
" " " 2	+	++++	−	−	−	−	−	−	−	−	−
A. quadrimaculatus	−	−	++++	−	−	−	−	−	−	−	−
C. pipiens	−	−	++++	−	−	−	−	−	−	−	−
M. domestica	−	−	−	++++	−	−	−	−	−	−	−
Calliphora sp.? No. 1	−	−	−	−	++++	−	−	−	−	−	−
" " " 2	−	−	−	−	−	++++	−	−	−	−	−
A. syriaca No. 46	+	++++	−	−	−	−	−	−	−	−	−
" " " 213	++++	++	−	−	−	−	−	−	−	−	−
" *nivea*	++++	+	−	−	−	−	−	−	−	−	−
Oriental sore.											
L. tropica	−	−	−	−	−	−	++++	+	+	−	−
Espundia.											
L. brasiliensis	−	−	−	−	−	−	−	++++	−	−	−
Kala-azar.											
L. infantum	−	−	−	−	−	−	+	−	++++	+	−
" *donovani*	−	−	−	−	−	−	−	−	++	++++	−
Ctenocephalus canis.											
H. ctenocephali	−	−	−	−	−	−	−	−	−	−	−
Rana pipiens.											
T. rotatorium	−	−	−	−	−	−	−	−	−	−	−

++++, rapid, complete precipitation, agglutination involving both cytoplasm and flagella. +, definite effect on flagella, which are twisted and adhere to one another.

TABLE II.

Complement Fixation Tests.

Source of flagellate.	Antisera.										
	Anti-*oncopelti* (strain from *O. fasciatus*).	Anti-*lygæorum* (strain from *L. kalmii* No. 2).	Anti-*culicidarum* (strain from *Anopheles quadrimaculatus*).	Anti-*muscidarum*.	Anti-*media*.	Anti-*parva*.	Anti-*tropica*.	Anti-*brasiliensis*.	Anti-*infantum*.	Anti-*donovani*.	Normal rabbit serum.
O. fasciatus	++++	++	−	−	−	−	−	−	−	−	−
Oncopeltus sp.?	++++	++	−	−	−	−	−	−	−	−	−
L. kalmii No. 1	++++	++	−	−	−	−	−	−	−	−	−
" " " 2	++	++++	−	−	−	−	−	−	−	−	−
A. quadrimaculatus	−	−	++++	−	−	−	−	−	−	−	−
C. pipiens	−	−	++++	−	−	−	−	−	−	−	−
M. domestica	−	−	−	++++	−	−	−	−	−	−	−
Calliphora sp.? No. 1	−	−	−	−	++++	−	−	−	−	−	−
" " " 2	−	−	−	−	−	++++	−	−	−	−	−
A. syriaca No. 46	++	++++	−	−	−	−	−	−	−	−	−
" " " 213	++++	++	−	−	−	−	−	−	−	−	−
" *nivea*	++++	++	−	−	−	−	−	−	−	−	−
Oriental sore.											
L. tropica	−	−	−	−	−	−	++++	−	+	−	−
Espundia.											
L. brasiliensis	−	−	−	−	−	−	−	++++	−	−	−
Kala-azar.											
L. infantum	−	−	−	−	−	−	+	−	++++	++	−
" *donovani*	−	−	−	−	−	−	−	−	++	++++	−
Ctenocephalus canis.											
H. ctenocephali	−	−	−	−	−	−	−	−	−	−	−
Rana pipiens.											
T. rotatorium	−	−	−	−	−	−	−	−	−	−	−

++++, complete inhibition of hemolysis. +++, 25 per cent hemolysis. ++, 50 per cent hemolysis. +, 75 per cent hemolysis. −, complete hemolysis.

2. The absolute indifference to anti-leishmania immune sera of the herpetomonad flagellates and of *T. rotatorium*, and *vice versa*, an indifference showing that no serological affinity exists between the leishmanias and the other flagellates studied.

3. The reciprocal reactions which took place among the different strains of herpetomonads derived from milkweeds and the insects feeding on the latex of these plants. For example, the anti-*oncopelti* serum had an equally strong effect upon cultures isolated from *Oncopeltus fasciatus*, *Lygæus kalmii* No. 1, the Peruvian species of *Oncopeltus*, *Asclepias syriaca* No. 213, and *Asclepias nivea;* while the anti-*lygæorum* serum had the same effect upon the culture derived from *Asclepias syriaca* No. 46 as upon the homologous strain. If these serological reactions may be regarded as indicating species specificity of herpetomonads, we may conclude that *Herpetomonas oncopelti* was present in three insects and two plants and *H. lygæorum* in one insect and one plant, or, viewing the matter from another point of view, that the insect, *Lygæus kalmii*, may harbor either *H. oncopelti* or *H. lygæorum*, as may also the plant, *Asclepias syriaca*.

4. The serological independence of three strains of herpetomonads derived from flies (a house fly and two bluebottle flies).

5. The serological identity of the two strains isolated from mosquitoes, one from *Anopheles* (adult) and the other from *Culex* (larva).

6. The inability of heterologous immune sera to affect *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*.

The reactions among the leishmanias were similar to those previously reported, *L. tropica*, *L. brasiliensis*, and *L. donovani* being serologically distinct, and *L. infantum* related to *L. donovani*.

Fermentation of Carbohydrates by Herpetomonads and Leishmanias.

Leptospira medium, to which litmus had been added as an indicator of acid formation, and which contained the carbohydrate in a concentration of 1 per cent, was employed. All the flagellates grew luxuriantly on this medium at room temperature. The tubes were allowed to stand for 18 days before the final results were recorded, in order to permit the fermentation to reach the maximum point. Some of the sugars were split slowly. Table III shows the degree of acid formation, indicative of carbohydrate cleavage, observed in the various media.

TABLE III.

Fermentation Tests.

Results read after 18 days at 20°C.

Source of flagellate.	Carbohydrate in the medium.																
	Glucose.	Levulose.	Mannose.	Saccharose.	Raffinose.	Inulin.	Galactose.	Maltose.	Salicin.	Xylose.	Mannitol.	Dextrin.	Arabinose.	Amygdalin.	Lactose.	Dulcitol.	Rhamnose.
O. fasciatus	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	–	–	–	–
Oncopeltus sp.?	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	–	–	–	–
L. kalmii No. 1	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	–	–	–	–
" " " 2	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A. quadrimaculatus	++++	++++	++++	++++	++++	++++	+++	+++	++++	+	+++	+	–	++++	–	–	–
C. pipiens	++++	++++	++++	++++	++++	++++	+++	+++	++++	+	+++	+	–	++++	–	–	–
M. domestica	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	–	+	–	–
Calliphora sp.? No. 1	++++	++++	++++	++++	++	++++	+	–	–	–	–	–	–	–	–	–	–
" " " 2	++++	++++	++++	++++	++	–	++	–	–	–	–	–	–	–	–	–	–
A. syriaca No. 46	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
" " " 213	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	–	–	–	–
" *nivea*	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	+	–	–	–	–
Oriental sore.																	
L. tropica	+++	+++	+++	+++	++	+?	–	–	–	–	–	–	–	–	–	–	–
Espundia.																	
L. brasiliensis	+++	+++	++	+++	++	–	–	–	–	–	–	–	–	–	–	–	–
Kala-azar.																	
L. infantum	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–
" *donovani*	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–	–
Ctenocephalus canis.																	
H. ctenocephali	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Rana pipiens.																	
T. rotatorium	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

++++, deep red, turbid (coagulation of serum); +++, deep red, serum clear; ++, markedly red, serum clear; +, pink, serum clear;—, no change in original bluish color.

The great variability in the fermentative faculty of the different flagellates is at once apparent. Some fermented practically all of the carbohydrates tested, others only a few, and still others none at all. *H. oncopelti* attacked 13 of the 17 carbohydrates tested, *H. lygæorum* only 3. *H. culicidarum* affected 13 carbohydrates, *H. muscidarum* 14, and each had a distinguishing feature, the former fermenting amygdalin but not lactose, the latter slightly attacking lactose but not affecting amygdalin. All the leishmania strains fermented glucose, levulose, mannose, saccharose, and raffinose, and *L. tropica* affected inulin slightly. *H. parva*, the strain from *Calliphora* No. 2, fermented the same sugars as the leishmanias, and galactose in addition. *H. media*, from *Calliphora* No. 1, also resembled the leishmanias but differed from them, and from *H. parva*, in fermenting inulin energetically. It affected galactose only slightly. Only dulcitol and rhamnose were unaffected by any of the flagellates, and of the 18 strains of flagellates tested, *H. ctenocephali* and *T. rotatorium* were the only ones which did not ferment any of the carbohydrates. *H. muscidarum* was the only flagellate to ferment lactose, and arabinose was affected only by *H. muscidarum* and *H. oncopelti*. Amygdalin was fermented only by *H. culicidarum*.

Whether the observed differences will persist during prolonged cultivation remains to be seen.

DISCUSSION.

The classification adopted for the species of *Herpetomonas* isolated in the course of the present investigation requires some comment, in view of the fact that heretofore the custom has been to regard the flagellates found in a given host as specific for that host and to name each strain in a way that indicates its source. If this custom had been rigidly followed, however, it would have been necessary to select several names for organisms which in cultural characteristics, in immunological properties, and in biochemical activity behave in the same way, as, for example, the strains isolated from *Oncopeltus fasciatus*, the Peruvian species of *Oncopeltus*, *Lygæus kalmii* No. 1, *Asclepias syriaca* No. 213, and *Asclepias nivea*. We already had evidence that the strain in *A. nivea* was the same as that in *O. fasciatus*, since the healthy *nivea* plants had been experimentally infected by the feeding

of specimens of *O. fasciatus* known by feces examination to harbor a *Herpetomonas*.[8] That this same species of flagellate should be present in the normal plant host of *O. fasciatus* (*Asclepias syriaca*) was to have been expected. Nor is it surprising that the same organism should be isolated from another insect which feeds on *A. syriaca*, namely, *Lygæus kalmii*. The infection of this insect could have taken place through ingestion either of infected plant latex or of infected dejecta of *O. fasciatus* deposited on the plant. The ingestion of feces by the bugs has been observed during the course of the experiments, and the insects also have been seen with proboscis inserted into the abdominal cavity of other bugs which were dead or moribund. All the facts, therefore, justify us in regarding these four strains as the same species. The isolation of the same flagellate from the Peruvian species of *Oncopeltus* is perhaps less readily explainable, though not remarkable.

Similarly the facts support the conclusion that the strains isolated from *Lygæus kalmii* No. 2 and *Asclepias syriaca* No. 46 constitute another species. While these two strains showed a slight group reaction toward the anti-*oncopelti* immune serum, they differed greatly from *H. oncopelti* in their action on carbohydrates, fermenting only three sugars instead of thirteen. They average slightly smaller in size, both under natural conditions and in culture, though the morphological differences alone would probably not enable one to discriminate the species. That *Lygæus kalmii* should harbor two types of *Herpetomonas* is not remarkable, and that its plant host should have become infected with both of them is wholly reasonable.

The striking morphological differences among some of the strains under discussion, as they occur in nature, appear to be due to the environment, since they disappear on cultivation under the same conditions. The typical herpetomonad of the plant latex has a flat, ribbon-like body, usually with one or two twists, the cytoplasm is clear, almost hyaline, and the flagellum short—about half the length of the body—and rather straight. The body of the herpetomonad

[8] These Haitian milkweeds, as already stated in Part I of this report, were presented by Dr. Francis O. Holmes, of the Boyce Thompson Institute for Plant Research, Yonkers, N. Y., who had infected them by allowing infected specimens of *Oncopeltus fasciatus* to feed upon the seed pods.

seen in the insect gut is usually cylindrical in appearance and is filled with volutin granules; the flagellum is as long as the body or longer, and rather flexible. The flagellates seen in the latex of *A. nivea*, though known to have come from *O. fasciatus*, are charactersitic of the plant type, and the same is true of *H. oncopelti* and *H. lygæorum* as they occur in *A. syriaca*.

In the case of the flagellates isolated from *Calliphora*, we were confronted with the alternative of identifying two strains, distinct morphologically, serologically, and in biochemical activity, with *H. calliphoræ*, or of choosing new names for them. The former course was manifestly unreasonable. The names *H. media* and *H. parva* were selected for these organisms because of the morphological differences between them, but there are differences as well in biological characters whereby the organisms may be identified at any time. We chose a new name also for the strain isolated from *Musca domestica*, because it appeared hardly legitimate to assign to *Herpetomonas muscæ domesticæ* the characteristics which this particular strain had shown.

The flagellates isolated from mosquitoes came from two different host genera, *Anopheles* and *Culex*, but could not be differentiated morphologically, culturally, or in biochemical properties. The name selected for the organism represented by these two strains was *Herpetomonas culicidarum*, which indicates its occurrence in two genera of the family Culicidæ and denotes also the specific properties by which it may be differentiated from the other flagellates of the series, and perhaps also from other flagellates of mosquitoes.

SUMMARY.

Serological reactions and fermentation tests have been employed in the present investigation as a means of differentiating various strains of herpetomonads from one another as well as from leishmanias. The twelve strains of herpetomonads isolated from insects and plants all proved to be serologically unrelated to any of the leishmanias, and were distinguishable from them by the manner in which they affected various carbohydrates.

Three of the strains of herpetomonads tested had been isolated from milkweeds (*Asclepias syriaca* and *A. nivea*) and four from bugs which feed on the latices of these plants (*Oncopeltus fasciatus*, *Oncopeltus sp.?* from Peru, and *Lygæus kalmii*). When tested for their serological

and carbohydrate-fermenting properties, however, the seven strains proved to be of two kinds only, one represented by the strain first isolated from *Oncopeltus fasciatus* and hence named *H. oncopelti*, the other by *H. lygæorum*, so named because it was first isolated from *Lygæus kalmii*. Serologically there was a certain degree of group reaction among the flagellates of these two types, but in their action upon carbohydrates they were entirely different, *H. oncopelti* splitting thirteen carbohydrates, *H. lygæorum* only three.

Three strains of herpetomonads isolated from flies proved to be distinct both in serological properties and in their action upon carbohydrates. One, derived from the house fly, and called *H. muscidarum*, was able to ferment most of the carbohydrates tested, including lactose which was not affected by any of the other strains. The other two, isolated from bluebottle flies, behaved much the same as the leishmanias with regard to carbohydrate fermentation, attacking five of the same sugars. One of them fermented galactose in addition, the other both galactose and inulin.

Two strains from mosquitoes (*Anopheles* and *Culex*) behaved identically in serological reactions and also in fermentation tests. They are regarded as one species and have been named *H. culicidarum*. This organism ferments thirteen sugars, including amygdalin which no other organism of the series attacks.

One of the most striking phenomena observed was the entire lack of fermentative faculty on the part of *Herpetomonas ctenocephali* and *Trypanosoma rotatorium*. Neither of these organisms was affected by any of the immune sera prepared with other flagellates.

The serological specificity of *Leishmania tropica*, *L. brasiliensis*, and *L. donovani*, and the close relation between *L. donovani* and *L. infantum* were confirmed in the present study. These organisms could not, however, be differentiated by fermentation tests.

The data presented suggest that the biological characteristics of flagellates of the *Herpetomonas* group may be utilized with advantage for identification of a species which occurs in different environments and for separation of different species when they are found in the same environment. If the leishmania parasites pass the flagellated or herpetomonad stage of their life history in some invertebrate host, it may be possible by tests of the sort described to distinguish them from the non-pathogenic herpetomonads which are so widely distributed among insects and plants.