

Immunodiffusion Reactions of Panamanian *Leishmania*¹

Curt R. Schneider and Marshall Hertig -

Gorgas Memorial Laboratory, Panama, Republic of Panama

(Submitted for publication, 8 April 1965)

SCHNEIDER, C. R., AND HERTIG, M. 1966. Immunodiffusion reactions of Panamanian *Leishmania*. *Experimental Parasitology* 18, 25-34. The agar gel diffusion test was utilized in an effort to distinguish five human strains and eight wild-caught *Phlebotomus* sandfly strains of Panamanian leptomonads plus three human strains from other countries. Concentrated and purified antigens were prepared from cultured organisms by ether extraction, saline extraction, and dialysis. Antisera were prepared in rabbits by using quantified inocula.

At least two different immunological groups of human leishmanias have thus been recognized. The members of each group share a number of antigens with each other. There may also be intergroup reactions but they are weaker and usually limited to a single line of precipitate.

Within a group, the strains were not limited to a particular geographical location but originated in widely separated sites in the Republic of Panama. Each group contains strains pathogenic for man.

Cutaneous leishmaniasis has a widespread distribution in the Republic of Panama. The disease is better understood clinically than epidemiologically and many of the factors which influence its distribution, including the question of vertebrate reservoirs and the ecology of the insect hosts are still obscure. In nature the infection is undoubtedly transmitted by the bites of infected *Phlebotomus* sandflies.

Preliminary trials with culture forms utilizing immunodiffusion techniques have suggested that strain differences within the species *Leishmania braziliensis sensu lato* could be recognized (McConnell, 1963). This lead has been followed up in the present work. The technique of agar gel diffusion has been utilized with antigens derived from culture leptomonads and antisera obtained by inoculating rabbits with culture material.

¹ The work reported here was supported in part by a research grant (AI-01251) from the National Institute of Allergy and Infectious Diseases, N.I.H., U.S.P.H.S.

MATERIALS AND METHODS

Culture methods. Organisms were maintained on Senekjic's medium (1939, 1943). Transfers were made at 2-week intervals. Material sufficient for use as antigen was obtained by culturing the organisms in 125-ml or 250-ml screw-cap Erlenmeyer flasks. The bottom of a flask was layered with medium and allowed to solidify. The water of condensation in which the organisms grow was augmented by the addition of sterile physiological saline to a depth of about 2 mm. Cultures were routinely kept in a constant-temperature chamber at 25°C.

Preparation of antigen. All strains do not thrive equally well in this medium and certain of them can be characterized as "slow-growers." Quantities of organisms used in diffusion experiments and in immunizing rabbits were standardized by the following procedure: At peak growth, organisms were harvested, washed three times and suspended in sterile saline. A count was then made in a Spencer Bright-Line hemocytometer by using the

method described by Chang and Negherbon (1947a). Counted suspensions were placed in ampoules, in 1 or 2 cc amounts, quick-frozen in a mixture of dry-ice and ethyl alcohol, and lyophilized. The quantity of fluid introduced and the count were recorded on the ampoule; thus the number of organisms in the dried contents of any ampoule could always be determined.

Antigen for use in the gel diffusion test was prepared according to a method modified from one described Chaffee (1963) in a personal letter to one of us (CRS). Lyophilized organisms were weighed. A total of 50 mg of leptomonad powder was ground for 10 minutes in 5 cc of absolute (di-ethyl) ether in a tissue grinder suspended in an ice-water bath. The ground organisms were then transferred to a cold centrifuge tube and spun down, the ether decanted and replaced by 1 ml of saline and 1 ml of distilled water. The resulting suspension was again ground for 10 minutes. The supernatant was dialyzed overnight against pH 7.4 phosphate buffer saline and then used directly in the test.

Preparation of antisera. The contents of an ampoule of lyophilized organisms were resuspended in an appropriate amount of distilled water. Inocula of approximately 150 to 250 million organisms were injected intravenously, or rarely intraperitoneally, every 3 or 4 days for a period of 4 weeks. Two rabbits were immunized to each antigen. Rabbits were bled by cardiac puncture 7 days after the final injection. Antisera to identical strains were not pooled but were tested separately and the reading from the strongest reactor was recorded. Sera were stored at -10 to -20°C .

Diffusion technique. A micro version of the Ouchterlony test was employed (Crowle, 1961) in which reagents are fed to the agar through small wells drilled in a plastic matrix or template. The template is a 1-inch square of "Plexiglas," 1/8 inch thick. It has a central well with four others equally spaced at a dis-

tance of approximately 5 mm center-to-center. The wells are 9/64 inch (approximately 3.57 mm) in diameter on the upper side and narrow to 1/16 inch (approximately 1.59 mm) at the bottom as they open onto the agar.

The test slide is prepared as follows: Double layers of plastic electrician's tape applied across the slide so as to leave a central area about 20 mm wide, serve to support the template about 0.5 mm above the surface of the glass. Approximately 0.3 ml (4 or 5 drops) of hot agar is then applied with a capillary pipette to fill this space and the template is lowered into position. The reagent is added from a 0.5 ml tuberculin syringe, using a 22-gage needle from which the bevelled tip has been ground away.

The prime advantage of miniaturization of the technique is the saving in reagent. At the same time, finished reactions can be photographed and then strained, dried, and stored permanently with more convenience than is possible with reactions carried out in Petri dishes. Photographs were taken (Figs. 1 and 2) with a Leitz "Focalslide" apparatus. "Darkfield" illumination was achieved by supporting the slide above a hollow cone of light from an electronic flash reflector.

Strains of Leishmania. In the current work, the named strains were derived from human infections. The Panamanian strains of human origin, with one exception, were isolated by members of the Gorgas Memorial Laboratory staff. The sandfly strains (identified by "WC" numbers) were obtained from wild-caught *Phlebotomus*, dissections of which were performed in this laboratory by Drs. P. T. Johnson and E. McConnell. They have been cited in a previous publication concerned with the occurrence of natural insect infections on the Isthmus (Johnson *et al.* 1963).

The majority of the strains, both human and sandfly, in the following list have produced experimental lesions in hamsters. Six of the eight sandfly strains were among those referred to by McConnell (1963) as infective

FIGS. 1 and 2. Micro diffusion reactions with reagents derived from leptomonads: antigens in central depot, antisera in peripheral depots. $\times 3.5$. Strains: B = Batista, G = Guatemala, Mo = Monteza, Mx = Mexicana, U = Uta, V = Vega, VH = Van Horn.

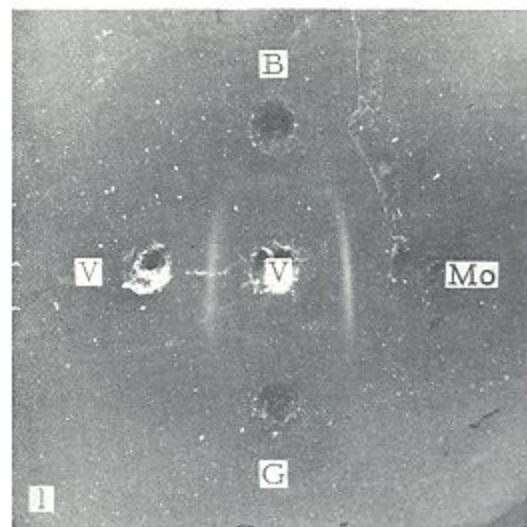


FIG. 1. Single band reaction of identity between Vega antigen and Vega, Batista and Monteza antisera.

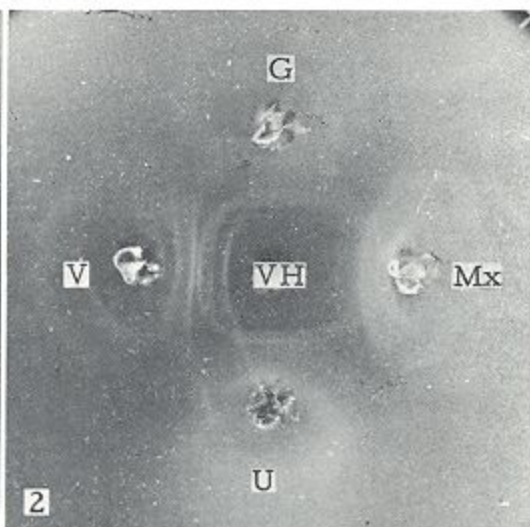


FIG. 2. Five distinct bands of precipitate between Van Horn antigen and Vega antiserum, two of the bands continuous with those of Guatemala and Uta antisera.

or otherwise. However, none of the cultures of sandfly strains used in the present work had been derived from hamster passage.

Batista. Isolated from a Panamanian patient from Buena Vista on the Transisthmian Highway, on 8 November 1960. The culture employed in this work was passed through a hamster and re-isolated on 3 September 1962.

Monteza. Isolated from a Panamanian patient on 13 December 1960. The infection was probably contracted on the Changuinola River in Bocas del Toro Province. The current culture was re-isolated from a hamster on 3 September 1962.

Vega. Isolated from a Panamanian patient from Chepo, about 30 miles east of Panama City, on 19 January 1960. The culture was passed through a hamster and re-isolated on 7 November 1962.

SH-24. Isolated from a case of leishmaniasis contracted in Panama and diagnosed in London. Culture received on 29 December 1963, through the courtesy of Professor

P.C.C. Garnham of the London School of Hygiene and Tropical Medicine. The infection was probably contracted in Achioté, Colon Province. It has not infected a hamster to date.

Van Horn. Isolated from a Panamanian patient on 8 April 1964. The infection was probably contracted in Achioté, Colon Province. Hamsters have been infected.

Guatemala. Isolated from a patient in Guatemala on 5 September 1959. The culture was presented to Dr. McConnell by Public Health officials in Guatemala City, 12 April 1960. The original culture tube was labeled "*Leishmania tropica*" together with the patient's name and date. Hamsters have been infected.

Mexicana. This strain was originally brought from British Honduras to London and was made available to us through the courtesy of Professor Garnham who identified it as *Leishmania mexicana*. The current culture was re-isolated from a hamster on 20 June 1963.

Uta. Strain L-12. Isolated in 1944 from a case of Peruvian uta and maintained only in culture since then. Dr. A. Herrero presented the strain to Dr. McConnell in June, 1961. It has not infected hamsters.

WC-2195. Isolated from *Phlebotomus trapidoi* caught at Quebrada Bonita on 9 July 1961, and dissected on 12 July 1961. It has infected hamsters (McConnell, 1963).

WC-5003. Isolated from *P. trapidoi* taken at Almirante (Bocas del Toro Province) on 29 November 1961. Dissected 1 December 1961. It has not infected hamsters.

WC-6103. Isolated from *P. ylephiletor* caught on Cerro Campana, 6 September 1962. Dissected 8 September 1962. It has infected hamsters (McConnell, 1963).

WC-6217. Isolated from *P. trapidoi* caught at Quebrada Bonita on 4 October 1962. Dissected 8 October 1962. Hamsters were infected by inoculation of triturated sandfly gut (McConnell, 1963).

WC-6346. Isolated from *P. sanguinarius* caught at Quebrada Bonita on 4 October 1962. Dissected 8 October 1962. It has not infected hamsters. This strain was noted in the original dissection as being morphologically unlike any other strain isolated from sandflies (McConnell, 1963).

WC-6408. Isolated from *P. trapidoi* caught at Almirante, 8 October 1962. Dissected 10 October 1962. A hamster inoculated with the triturated gut of the sandfly was negative (McConnell, 1963). There have been no inoculations with cultures.

WC-6445. Isolated from *P. gomezi* caught at Quebrada Bonita on 19 November 1962. Dissected 20 November 1962. This is one of the seven "long-tail" strains mentioned by McConnell (1963). It was not tested in hamsters until immunodiffusion results made this desirable 18 months later; hamsters infected. The "long-tail" feature was not noted upon reisolation in culture from the hamster.

WC-7258. Isolated from *P. sanguinarius* caught at Mandinga (Colon Province) on 22

May 1964. Dissected 24 May 1964. It has not infected hamsters.

RESULTS

Homologous reactions were obtained in all cases. However, certain antisera were consistently weak; the reactions involving them required a long time to develop (up to 1 week, refilling the wells at least once) and were usually limited to a single line of precipitate.

Antigen prepared according to Chaffee's (1963) method had a high coefficient of diffusion so that many of the visible precipitate bands occurred close to, or even in a circle around, the antiserum depot. That such reactions represented true antigen-antibody complexes and not artifacts of technique was indicated by the fact that they were never seen around all of the antiserum depots on a single slide.

Because of variation in antisera, it is occasionally possible to observe an apparently anomalous reaction in which a heterologous system provided a strong visible reaction while an adjacent homologous system appeared non-reactive or weak.

With regard to heterologous reactions, a glance at the tabulated data (Table I) shows certain apparent relationships. It must be cautioned, however, that the symbol (—) (no reaction) may not always indicate the absence of a relationship but merely a weak antiserum. The symbols (|...|) in the table, which indicate the maximum number of precipitate bands noted in a reaction, do not carry any connotation of the relative strength of the reaction but only its occurrence. For example, the homologous reaction of Monteza antigen and antiserum repeatedly revealed three lines of precipitate. But it was very weak and difficult to detect except when photographed in a dark field with an electronic flash as a light source. Other antisera were stronger; two of them, against Mexicana and WC-6445, reacted with most or all of the other antigens. In two cases

TABLE I
*Immordiffusion Reactions of Human and Sandfly Leptomonads**
 (All sandfly strains from naturally infected, wild-caught Panamanian *Phlebotomus*)

Antiserum / Antigen	Group I			Group II			"Mexicana" group		Uncertain group			Human Peru			
	Human origin Panama			Human Panama			Human group		Sandfly						
Batista	Monteza	Vega	Van Horn	WC-6103	WC-6217	SH-24	WC-5003	WC-6445	WC-7258	Guatemala	Mexicana	WC-2198	WC-6346	WC-6408	Uta
Monteza															
Vega															
Van Horn															
WC-6103															
WC-6217															
SH-24															
WC-5003															
WC-6445															
WC-7258															
Guatemala															
Mexicana															
WC-2198															
WC-6346															
WC-6408															
Uta															

* (-), No visible reaction; (---), ++++, |||||. Each line represents one band of precipitate; number of lines indicates maximum number of bands observed, not relative intensity.

(WC-7258 antigen versus WC-6445 antiserum and Van Horn antigen versus Vega antiserum) as many as five different lines of precipitate could be distinguished.

Reactions of identity were occasionally seen. These are discussed below. For the most part they were not noted. It is felt that their absence is less significant than their presence, especially when there is a suspicion (as here) that certain of the cultures are antigenically depleted because of prolonged *in vitro* maintenance.

Three Panamanian strains of human origin (i.e., Batista, Monteza, and Vega) repeatedly cross-reacted, although not strongly, indicating at least one shared antigen. None of these strains produced strong antisera. When tested on the same slide, a single-line reaction of identity was seen (Fig. 1). This is particularly interesting in view of the different sites of origin of these strains. The Batista strain came from Buena Vista on the Transisthmian Highway, in Colon Province, very close to the Panama Canal, whereas the Monteza strain was isolated from a native of the Province of Chiriqui and the infection was probably contracted on the Changuinola River, in the Province of Bocas del Toro. The third strain, Vega, was isolated from a resident of Chepo at the eastern end of the Province of Panama.

Another Panamanian strain isolated from a human patient (SH-24) did not react with these three. SH-24 reacted weakly with Mexicana and Guatemala. However, it produced strong and definite reactions of identity when paired with antisera to WC-5003 and WC-6445 and we have therefore thought of these three strains as constituting a second group, separate and apart from the first, or "Batista," group.

The reactions of the Van Horn strain proved to be weak, both with regard to antigen and antiserum, although it has been in culture a relatively short time. Its strongest heterologous reactions were with Vega. When Van Horn antigen was tested against Vega

antiserum, five distinct precipitate lines resulted, only two of which could be identified as group reactions (being continuous with lines produced by adjacent Uta and Guatemala antisera) (Fig. 2). Van Horn also reacted, although faintly, with WC-6103, but did not show significant reactivity against any of the strains of Group II ("SH-24"). Although Van Horn antigen did not react with Batista antiserum, a transposition of these reagents (Batista antigen versus Van Horn antiserum) produced two distinct precipitate bands. Thus we believe that Van Horn can be included in Group I ("Batista").

It is of interest that two of the human leishmanias which are distinguishable antigenically (Van Horn and SH-24) originated in a single locality in Panama, i.e., Achioté, near the Caribbean shore.

Mexicana and Guatemala apparently share at least four rather strong antigens. The strains do not, however, seem to be identical. Mexicana produced the stronger antiserum as indicated by its readiness to react in some degree with all of the other strains tested. The Guatemala strain produced, on the other hand, a weak antiserum. With the exception of the reaction with WC-7258 antigen, its heterologous reactions were limited to faint lines of precipitate curved closely around the antiserum depot.

WC-6217 from Quebrada Bonita reacts strongly with the Batista, Monteza and Vega antisera and clearly belongs in Group I. WC-6103 may represent a more antigenically exhausted version of WC-6217. These two have been in culture for about the same length of time and they clearly share two antigens. WC-6103 reacts only weakly with Monteza and Vega and not at all with Batista, but it shows no tendency to react with SH-24 either, and the reactions with WC-5003 and WC-6445 are extremely faint, so much so that we believe it belongs with the "Batista" group.

WC-6408 reacted with almost all other antigens, but very weakly. In most cases the reaction took the form of a single, barely

visible line of precipitate curved around the antibody depot. We do not feel that it can be assigned as yet to any of the other groups.

Likewise, WC-2195 antigen reacts but weakly and then only with the strongest of the antisera, e.g., Mexicana, WC-6445. It has been in culture for more than 3 years and its antigenicity may have been impoverished with time. Its antiserum reacts, although the reactions are very faint, with some members of all of the above-mentioned groups and thus it cannot be clearly assigned to any of them.

WC-6346 differs markedly from other strains in several respects. In culture the organisms are sausage-shaped and highly refractile when compared with other strains. The pattern of motility is also distinctive, the organisms describing considerably more erratic paths of movement than do the other leptomonads. Moreover, there is a tendency in old cultures (10 days and more) for progressive motion as such to cease although flagellar movements continue active, a phenomenon which has not been seen in other cultures. Thus, this strain can easily be separated on morphological grounds alone from all others currently being carried in this laboratory. WC-6346 antigen reacted with Batista and Monteza but not with Vega. Some degree of reaction with Mexicana and with SH-24 was noted and faint reactions occurred with WC-6103, WC-6408, and WC-6445. WC-6346 antiserum, however, appeared decidedly weak and reacted strongly only with the homologous antigen. It thus seems that WC-6346 shares certain antigens with a number of other strains which are themselves not too closely related (although it is understood that these degrees of "relationship" are relative). WC-6346 has not infected a hamster.

WC-7258, of rather recent isolation, reacts with both the "Batista" group and the "SH-24" group. However, its reactions, as antigen, seem to be strongest with regard to the latter, and it is tentatively included here in the "SH-24" group.

The Uta strain, from Peru, shares an anti-

gen with Mexicana and SH-24 and with four of the sandfly strains. In view of the fact that this strain has been in continuous cultivation for more than 20 years it is to be assumed that important antigens have been lost with time. In the present work, the Uta strain can be associated with none of the others on a clear-cut basis.

Thus certain groups became apparent in the present work (Table II), although the

TABLE II
Immunological Groupings of Culture Strains of Panamanian and other Leptomonads

Panamanian strains		
Group I ("Batista")	Group II ("SH-24")	Uncertain
Batista	SH-24	WC-2195
Monteza	WC-5003	WC-6346
Vega	WC-6445	WC-6408
Van Horn WC-6103 WC-6217	WC-7258	
Other strains "Mexicana" group		
Mexicana		
Guatemala		
Peruvian Uta		

technique did not permit uniformly distinct separations. The three human strains, Batista, Monteza and Vega, form one group, to which may be added WC-6217 and probably WC-6103. Another human Panamanian strain, SH-24, and two sandfly strains (WC-5003, and WC-6445) demonstrate a strong affinity for each other and there seems to be little question of their essential identity as a second group. WC-7258 probably belongs with them.

Guatemala and Mexicana are paired. The latter has already been separated from *L. braziliensis* in the literature, first as *L. tropica mexicana* Biagi and then as *L. mexicana* Garnham. Some of the Panamanian strains share antigens with them but none seems to be identical with them.

Three sandfly strains (WC-2195, WC-6346, and WC-6408) cannot be assigned to either

group on the basis of the data reported here. One of these (WC-6346) may represent something quite different from *L. braziliensis*.

A Peruvian strain from a case of uta has not been clearly related to any of the other leishmanias tested.

DISCUSSION

Three strains of origin geographically distant from Panama were included in the present study (Uta, Mexicana, and Guatemala). The others represent Panamanian leishmanias which have not been separable from other geographic strains of *L. braziliensis sensu lato* by any previous criterion.

The species of *Leishmania* have been based on clinical and sometimes geographical grounds. Kirk (1949) has shown that the different clinical types of human infection correspond with biological differences in the parasite and suggests that the classical species are, therefore, taxonomically valid. But it is apparent that immunological evidence, if clear-cut and repeatable, can add weight to the matter.

Although many attempts have been made to differentiate the leishmanias serologically (Noguchi, 1926; Kligler, 1925; Wagener and Koch, 1926; Ray, 1929; Das Gupta, 1930; Franchini and Pirami, 1930; Laurinsich, 1931; Zdrodowski and Woskressenski, 1931; Khodukin and Sofiev, 1930; Row, 1931; Fonseca, 1933; Khodukin *et al.*, 1936; da Cunha, 1938; Senekjic and Lewis, 1945; Chang and Negherbon, 1947b; Adler, 1963) the results have not always been in agreement. In general, the sum total of information conveyed by these reports is that the leishmanias probably share a group antigen, at least when they have been freshly isolated in culture; and that the reactions of this group antigen commonly overshadow more specific reactions. There is little consensus regarding the virtues of any given immunological technique. That utilized by Adler (1963) in which immune sera are incorporated in the culture media currently enjoys a well-deserved vogue.

Kirk (1949) has stated that "although single investigators have often obtained clear-cut results, a general survey indicates that there may be marked differences in the behavior of strains from the same type of infection. Workers with extensive experience . . . have found that the relatively clear results originally obtained with only a few strains become obscured when a larger number of strains is studied." He was referring to different species of *Leishmania*, but his remarks apply as readily to the problem of identifying intraspecific strains of *Leishmania braziliensis*.

Two facts emerge from the present data. First, there seem to exist in Panama at least two immunologically distinct strains of *Leishmania* which, in spite of some shared antigens, can be distinguished from each other and grouped. Both "groups" contain members derived from human infections.

Second, these groups are not associated with any specific locality within the Republic of Panama. Members of one group have been found with origins as far apart as Bocas del Toro (Monteza) and Chepo (Vega). Nor is there any readily apparent correlation between sandfly strains from either group with any one species of *Phlebotomus*. In Group I ("Batista"), the two wild-caught strains came from *trapidoi* and *ylephiletor*; in Group II ("SH-24"), the three wild-caught strains were found in *trapidoi*, *gomezi* and *sanguinari*.

Certain of the strains are better antibody inducers than others. In the present program inocula were carefully quantified and the resulting antisera proved extremely variable in their response to homologous antigens. Individual variation in the rabbits was checked by inoculating 2 rabbits with each strain. It has been reported that all *Leishmania* have a similar antigenic constitution when newly isolated (da Cunha, 1942) but that prolonged cultivation without animal passage results in a loss of some antigens. Thus, it may be fallacious to insist on comparing strains which have been carried *in vitro* for a long period

(e.g., Uta) with recently isolated strains (e.g., Van Horn and WC-7258).

Leishmanias are reported to possess two distinct antigen types, each able to stimulate characteristic agglutinins in rabbits (da Cunha, 1938). One is a flagellar antigen, present in small quantities, the other is a somatic antigen. Adler (1962) reported that Garnham's *L. mexicana* (the strain used in the present experiments) and a strain of *L. diffusa* shared a considerable number of somatic antigens but had little or no flagellar antigens in common. In the present work, certain strains produced strongly reactive antisera (e.g., Mexicana and WC-6445). It is not unreasonable to interpret this broad reactivity, which could be demonstrated against most or all of the antigens used in the experiments, as due to somatic antigens.

ACKNOWLEDGMENT

The excellent technical assistance of Mrs. Paula Wiest Fábrega is gratefully acknowledged by the authors.

REFERENCES

- ADLER, S. 1962. Approaches to research in leishmaniasis. *Scientific Reports of the Istituto Superiore di Sanità* **2**, 143-150.
- ADLER, S. 1963. A serological comparison between a *Leishmania* from *Phlebotomus martini* and strains from kala azar acquired in Kenya. *Israel Journal of Experimental Medicine* **11**, 31-34.
- CHAPPEE, E. F. 1963. Personal communication.
- CHANG, S. L., AND NEGHERBON, W. O. 1947a. Studies on haemoflagellates. II. A study of the growth rates of *Leishmania donovani*, *L. brasiliensis*, *L. tropica*, and *Trypanosoma cruzi* in culture. *Journal of Infectious Diseases* **60**, 172-184.
- CHANG, S. L., AND NEGHERBON, W. O. 1947b. Studies on hemoflagellates. III. The specificity of serological reactions of *Leishmania donovani*, *L. brasiliensis*, *L. tropica*, and *Trypanosoma cruzi*. *Journal of Infectious Diseases* **61**, 209-227.
- CROWLE, A. J. 1961. "Immunitdiffusion." Academic Press, New York.
- DA CUNHA, A. M. 1938. Sur l'agglutination des *Leishmania*. Agglutination flagellaire et agglutination somatique. *Comptes Rendus de la Société de Biologie* **128**, 209-211.
- DA CUNHA, A. M. 1942. A soro-aglutinação das leishmanias. *Memorias do Instituto Oswaldo Cruz* **37**, 35-76.
- DAS GUPTA, B. M. 1930. On the differentiation of *Leishmania tropica* from the parasite of dermal leishmanoid. *Indian Journal of Medical Research* **18**, 105-108.
- DA FONSECA, F. 1933. Differentiation of flagellates of the genus *Leishmania* Ross, 1903, by the lytic action of specific sera. *The American Journal of Tropical Medicine* **13**, 113-126.
- FRANCHINI, G., AND PIRAMI, E. 1930. Ricerche sierologiche sulle leishmaniosi, sui flagellati degli isetti e sull'*Herpetomonas* del gecko (*H. tarentolae*). *Archivio Italiano di Scienze Mediche Coloniali*, **11**, 666-669.
- JOHNSON, P. T., MCCONNELL, E., AND HERTIG, M. 1963. Natural infections of leptomonad flagellates in Panamanian *Phlebotomus sandflies*. *Experimental Parasitology* **14**, 107-122.
- KHODUKIN, N. I., AND SOFIEV, M. S. 1930. Zur Frage der Identität von *Leishmania donovani* und *Leishmania canis*. *Archiv für Schiffs- und Tropen-Hygiene* **34**, 369-382.
- KHODUKIN, N. I., SOFIEV, M. S., AND KEVORKOV, N. P. 1936. Sur l'identification des différentes souches de leishmanioses. Problème de l'agent pathogène des leishmanioses. *Annales de l'Institut Pasteur, Paris* **57**, 487-515.
- KIRK, R. 1949. The differentiation and nomenclature of *Leishmania*. *Parasitology* **39**, 263-273.
- KLICGLER, I. J. 1925. The cultural and serological relationship of *Leishmania*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **19**, 330-335.
- LAURINSICH, A. 1931. Ricerche sull' agglutinazioni di varie specie di *Leishmania*. *La Pediatria*, (Naples) **39**, 345-350.
- MCCONNELL, E. 1963. Leptomonads of wild-caught Panamanian *Phlebotomus*: culture and animal inoculation. *Experimental Parasitology*, **14**, 123-128.
- NOGUCHI, H. 1926. Comparative studies of the herpetomonads and leishmanias. *Journal of Experimental Medicine* **44**, 327-337.
- RAY, J. C. 1929. Serologische Untersuchungen bei Leishmanien. *Archiv für Schiffs- und Tropen-Hygiene* **33**, 598-602.
- ROW, R. 1931. Agglutination in leishmaniasis. *Indian Journal of Medical Research* **19**, 641-655.
- SENEKJIE, H. A. 1939. Studies on the culture of *Leishmania tropica*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **33**, 267-269.
- SENEKJIE, H. A. 1943. Biochemical reactions, cultural characteristics and growth requirements of *Trypanosoma cruzi*. *American Journal of Tropical Medicine* **23**, 523-531.
- SENEKJIE, H. A. AND LEWIS, R. A. 1945. *In vitro*

effects of specific immune blood on certain blood and tissue flagellates. *Proceedings of the Society for Experimental Biology and Medicine* **59**, 185-188.

WAGNER, E. H., AND KOCH, D. A. 1926. The biological relationships of *Leishmania* and certain

herpetomonads. *University of California Publications in Zoology* **28**, 365-388.

ZDRODOWSKI, P., AND WOSKRESSENSKI, B. 1931. Sur la sérologie comparée du groupe de leishmanies d'origine humaine et canine. *Bulletin de la Société de Pathologie Exotique* **24**, 37-41.