THE DEVELOPMENT OF LEISHMANIA DONOVANI IN VITRO AT 37°C.

Effects of the Kind of Serum

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Plate 11

(Received for publication, September 16, 1952)

The protozoan parasite *Leishmania donovani*, which causes human visceral leishmaniasis, exists in nature in two rather different forms: (*a*) an elongate, flagellated motile form, the leptomonad, which occurs within the lumen of the alimentary tract of sandflies of the genus *Phlebotomus*; (*b*) a smaller, aflagellate, non-motile, round or ovoid form, the leishmania, which occurs intracellularly especially within the reticulo-endothelial cells of the vertebrate host (1, 2). If either stage is placed in any of a variety of culture media containing serum and hemoglobin and incubated at temperatures of 22–30°C., the flagellated motile forms develop in abundance (2–6). They can be kept going by appropriate subcultures for long periods of time. However, if either stage, or an actively growing culture, is placed in any of the same culture media and incubated at 37°C., no development occurs and the organisms present die within a day or two (7, 4). The leishmania form, which, in nature, grows intracellularly at 37°C., has not been obtained in culture except with surviving host cells (8, 9).

In the course of experiments directed toward the extracellular cultivation of this intracellular stage, there has been observed a limited development at 37°C. of a form of *L. donovani* which may be considered intermediate, both morphologically and physiologically, between the leishmania and the leptomonad forms.

Materials and Methods

Maintenance of the Parasites.—The strain of *L. donovani*, originally isolated in Khartoum, was obtained in infected hamsters (*Cricetus auratus*) through the kindness of Dr. L. A. Stauber of Rutgers University (10). It was maintained in hamsters by passage at intervals of approximately 6 weeks. Material from the spleen of an infected animal was suspended in nutrient broth and inoculated intraperitoneally in a dose containing the equivalent of 30 to 60 mg. spleen per 100 gm. body weight. The hamsters weighed 60 to 100 gm. at the time of infection. They generally continued to gain weight during the first 3 to 4 weeks after inoculation. Loss in weight became apparent by the 5th or 6th week, except in a few individuals which survived the infection for a long time.

Several cultural strains of leptomonad forms, derived from infected hamster spleens,

were maintained at 28°C. Of a number of different media tried, the biphasic medium described by Chang (5) proved to be most convenient. The solid portion of this medium was prepared exactly as described by Chang, placed in 2.5 ml. amounts per test tube, and autoclaved. The tubes were sealed with parafilm and stored in a refrigerator. In the liquid portion, it was found necessary to increase the amount of horse hemoglobin solution. Hence to each 100 ml. of sterile solution of salts, glucose, bacto-tryptone, and Lilly liver extract 343 were added aseptically 15 ml. of horse serum and 25 ml. of a horse hemoglobin solution. The latter was prepared by laking 1 part of settled horse red blood cells with 2 parts of sterile distilled water. The mixture was left several days in a refrigerator, after which time it could be cleared by centrifuging. The complete solution was stored in tubes in a refrigerator. For use, the solid medium was melted and allowed to resolidify at a slant. 2.5 ml. of the liquid medium was added to each slant. Each completed tube was inoculated with one or two drops of a week-old culture and incubated at 28°C. in a slanted position so as to permit maximum exposure to the air (5). Such cultures showed within 4 or 5 days many leptomonads, with numerous rosettes and masses of actively multiplying forms. 5-day-old cultures contained approximately 50,000 organisms per c.mm. The cultures began to deteriorate after the 7th day. Three different strains of leptomonads, tested after 3 months of cultivation, produced infection in hamsters.

Preparation of the Parasite Suspensions.—Suspensions of leishmanias were prepared from infected hamsters which had been inoculated 6 to 9 weeks previously. The hamster was anesthetized lightly with ether and was decapitated. The spleen was removed aseptically to a small sterile Petri dish in which it was weighed. It was then cut into fragments and ground in a glass tissue grinder with 4 ml. of nutrient broth to each 0.6 to 1.0 gm. of spleen. The suspension was centrifuged for 5 minutes in the cold at a low speed (about 500 R.P.M.). This gave a sediment of the coarser tissue particles and most of the red cells, and a supernatant which contained many leishmanias and fine granules. The supernatant was centrifuged for ½ hour in the cold at 2000 R.P.M. to give a white sediment containing chiefly leishmanias (with sometimes a very small red "button" of red cells at the bottom of the tube) and a turbid reddish supernatant containing the fine tissue granules. This supernatant was discarded and the residue was resuspended in nutrient broth at the rate of 4 to 5 ml. broth per 0.1 ml. of white sediment. Such preparations contained little extraneous material other than small numbers of erythrocytes. Their content of leishmanias was about 50,000 to 100,000 per c.mm. Similar but more extensive differential centrifugation methods have been previously used by others for the preparation of leishmanial suspensions (11, 12).

Suspensions of leptomonads were prepared by centrifuging the fluid portion of 4- or 5-day-old cultures for ½ hour in the cold at 2000 R.P.M. and resuspending the sediment in nutrient broth. The suspensions contained about 30,000 to 60,000 leptomonads per c.mm.

The number of leishmanias or leptomonads in a suspension was determined by mixing 0.2 ml. of the suspension with 0.2 ml. of citrated human or rabbit blood of known red cell content. A film of the mixture was prepared and stained with Giemsa stain. The parasites per 1000 or 10,000 red cells were counted. The determinations had an error of about 25 per cent, which was not too great for the purpose for which they were used.

The Culture Media.—Extracts of human or rabbit erythrocytes were prepared in a diluent (solution K-2) of the composition shown in Table I. The solutions were prepared in water redistilled in a pyrex glass still. All the stock solutions were sterilized by autoclaving except the solution of $NaHCO_3$, which was passed through a Selas porcelain filter. The final mixture showed a faint turbidity which appeared following the addition of stock solution 5.

Rabbit blood was obtained by cardiac puncture and was defibrinated by shaking with glass beads. The blood was centrifuged, usually in 20 ml. amounts, and the serum was drawn off. The red cell mass was frozen in a dry-ice–alcohol mixture and stored for 1 to several

days in a dry-ice box. Human blood in acid-citrate-dextrose preservative was obtained through the courtesy of the blood banks of the New York Hospital and the Memorial Hospital, New York, and stored in a refrigerator. For use, 20 ml. amounts were placed in centrifuge tubes and centrifuged. The supernatant plasma was removed and the cells were resuspended in about one and one-half times their volume of solution K-2. They were again centrifuged, the supernatant fluid was discarded, and the cells were frozen in the same manner as the rabbit cells. To prepare the extracts, both types of blood cells were thawed, and the hemolyzed mass suspended in twice its volume of solution K-2. The mixture was centrifuged for an hour at 3000 R.P.M. and the upper two-thirds of the liquid used. This treatment removed any intact cells present, but it did not remove the bulk of the red cell ghosts. The final preparation had a pH of about 7.2.

TABLE I

Composition of Solution (K-2) Used in Preparing Erythrocyte Extracts

To prepare 100 ml. of solution the indicated amounts of sterile stock solution were added in the order shown to a flask that had been provided with 84 ml. of distilled water and autoclaved.

Stock solutions			Final mixture	
No.	Material	Concentration	Amount of stock solution	Concentration
		gm./100 ml.	*ml./100 ml.*	*mg./100 ml.*
1	NaCl	6.600	5.0	330.0
	KCl	8.800		440.0
2	$NaH_2PO_4.H_2O$	0.552	2.5	13.8
	K_2HPO_4	6.272		156.8
3	$NaHCO_3$	5.000	1.8	90.0
7	Glucose	5.000	5.0	250.0
4	$CaCl_2$	0.261	1.5	3.9
5	$MnSo_4.4H_2O$	0.220	2.0	4.4

Rabbit, hamster, and guinea pig serum, and one sample of human serum, were prepared in the usual way. Most of the human serum used was pooled, ampouled material purchased from Microbiological Associates, Inc., Bethesda.

The Cultures.—These were prepared in 50 ml. Erlenmeyer flasks equipped with a rubber stopper bearing gas inlet and outlet tubes plugged with cotton. Each flask contained 4 ml. of fluid, consisting ordinarily of 3 ml. of red cell extract, 0.6 ml. of serum, and 0.4 ml. of the suspension of leishmanias or leptomonads. The flasks were held in an incubator at 37–38°C. and a slow current of 95 per cent air with 5 per cent CO_2 was passed through them. The gas mixture was passed usually through two flasks in series, and never through more than three. In some experiments with leptomonads, 25 ml. flasks were used which received half the quantities and were merely plugged with cotton. Some flasks of this type were incubated at 28°C.

Most of the larger flasks incubated at 37–38°C. were followed over a period of 4 days or

longer. The culture fluid was changed every 2nd day in the following way. The contents of the flask were transferred to a graduated 15 ml. centrifuge tube and centrifuged for 20 minutes in the cold at 2000 R.P.M. The supernatant liquid was drawn off to the 0.4 ml. mark. The residue was resuspended in the small amount of remaining supernatant. A small drop of this suspension was used for the preparation of a wet mount and a dry film. The bulk of it was returned to the culture flask which had in the meantime been provided with fresh culture medium, ordinarily 3 ml. of red cell extract, 0.6 ml. of serum, and 0.4 ml. of nutrient broth.

The wet mount was examined immediately with a phase contrast oil immersion lens, while the film was fixed in methyl alcohol and stained in Giemsa stain. If a count was desired, 0.1 or 0.2 ml. of the resuspended material was mixed in a small tube with an equal volume of blood of known red cell content, and a stained film of the mixture was prepared.

In some experiments the viability of the forms present in a culture flask was tested by one of the following methods: (*a*) inoculation of a drop or two to a tube of NNN medium[1] prepared with rabbit blood and incubated at 28°C.; (*b*) incubation of the entire flask contents at 28°C.; (*c*) incubation at 28°C. of a hanging drop preparation made from the resuspended residue after centrifugation of the contents of the flask. The third method was most satisfactory and showed motile leptomonads within 1 or 2 days, if any surviving parasites were present at the time of preparation.

Since the forms which developed in culture at 37°C. were usually beginning to deteriorate after the 4th day, most of the experiments have been terminated after only 4 days. The condition of the parasites in the different media being tested was compared especially on the 2nd and 4th days.

RESULTS

The Course of Development and the Morphology of the Cultural Forms Appearing after Incubation at 37°C.

The appearance and initial multiplication at 37°C. of a form intermediate between the leishmania and leptomonad forms were first noted in flasks containing rabbit erythrocyte extract in solution K-2 and human serum, and inoculated with a suspension of leishmanias prepared in the manner described in the previous section. In such preparations, or better in similar preparations with human erythrocyte extract, some intermediate forms could sometimes be seen after 1 day of incubation and always after 2 days. Usually they were more numerous by the 4th day. The extent of transformation of the leishmanias into intermediate forms, and the extent of multiplication of the latter, varied greatly with the particular sample of pooled human serum or of hamster serum. With the most favorable sera, motile leptomonads as well as intermediate forms appeared in the cultures at 37°C. The extent of the variation is illustrated by the experiment shown in Table II. In flasks such as Nos. 1 and 3 of Table II the extent of multiplication was considerable. Thus in flask 1 there were present originally 11,900 leishmanias per c.mm. After 4 days the

[1] NNN medium contained 0.9 per cent NaCl and 1.5 per cent agar in distilled water. It was tubed in amounts of 6 ml. and autoclaved. For use, the medium was melted, and cooled to about 50°C., and 2 ml. of defibrinated rabbit blood was added to each tube. The contents of the tube were mixed and allowed to solidify at a slant.

total concentration of organisms was 52,500 per c.mm., of which about 90 per cent were intermediate forms, 6 per cent leishmanias, and 4 per cent flagellated leptomonads.

Beginning on the 5th day, all forms decreased slowly in numbers, even though fresh medium was provided every other day, or daily in some experiments. Although most of the flasks were discontinued at 4 days, in two separate experiments flasks with intermediate forms in the medium of human red cell extract and human serum were kept going for 10 and 11 days respectively. At these times viable forms were still present, as shown by the appearance of motile leptomonads in hanging drop preparations of material removed from the cultures and incubated at 28°C. In these experiments and in similar ones of

TABLE II

Effect of the Sample of Serum on the Development of the Intermediate Forms from Leishmania Incubated at 37°C.

Each flask contained 3 ml. of human red cell extract, 0.6 ml. of the indicated serum, and 0.4 ml. of a suspension in nutrient broth containing 119,000 leishmanias per c.mm. A current of 95 per cent air with 5 per cent CO_2 was passed through the flasks.

Flask	Serum	Observation at 2 days
1	Normal hamster A	Very many rosettes and large clusters of intermediate forms, as well as some motile leptomonads
2	Normal hamster B	Numerous intermediate forms. No motile leptomonads
3	Pooled human serum sample A	Very many rosettes and large clusters of intermediate forms, as well as some motile leptomonads
4	Human serum from one individual	A few intermediate forms
5	Pooled human serum sample B	A few intermediate forms

shorter duration, it was found that the better the development and multiplication of the intermediate forms during the first 4 days, the better was the survival of the cultures as shown by the appearance of leptomonads following incubation at 28°C.

When leptomonads from cultures maintained at 28°C. were placed at 37°C. in the medium most favorable for development of the intermediate forms, some retained their typical leptomonad form and motility even after 4 days. A majority, however, became non-motile and had either a short stumpy flagellum or none at all. Forms of the latter type resembled some of the larger intermediate forms.

The morphology of the intermediate forms, and for comparison, that of leishmanias and leptomonads, have been studied in fresh preparations examined with the phase contrast microscope, as well as in the usual films stained with Giemsa stain.

The appearance of leptomonads and of leishmanias in stained preparations has been repeatedly described (13). Fig. 6 shows a cluster of chiefly leptomonad forms which were present after 2 days of incubation at 37°C. of a culture initiated with a suspension of leishmanias. It is evident that they do not differ from the usual leptomonads observed in cultures at 28°C. A few intermediate forms can be seen in Fig. 6, and two rosettes of these forms are illustrated in Figs. 4 and 5. Fig. 4 shows also a typical leishmania. During the stage of active multiplication, the intermediate forms were chiefly somewhat elongate, ovoid, and pointed at one or both ends. Their cytoplasm stained blue with Giemsa stain and contained a number of small unstained vacuoles. The reddish purple nucleus was usually situated toward the posterior end of the cell. In the anterior portion, the very dark purple rod-shaped kinetoplast generally subtended a pink area, which corresponded to the similarly stained material from which the flagellum originates in leptomonads, and to the small eosinophilic vacuole often visible in leishmanias. Only rarely however, was there any indication of a protruding short stubby flagellum. Dividing forms were numerous, the most common being forms with one nucleus and a dividing kinetoplast or two kinetoplasts.

The measurement of 25 non-dividing individuals of each of the three forms seen in a stained preparation from a 2-day-old culture gave the following sizes, in microns: leishmanias 1.9 to 4.5 (average, 2.9) by 1.3 to 2.9 (average, 1.7); intermediates 4.8 to 7.6 (average, 5.9) by 2.1 to 6.0 (average, 3.3); leptomonads (without flagellum) 8 to 15 (average, 11.6) by 1.6 to 3.2 (average, 2.4).

In cultures over 4 days old, and in younger cultures in which the transformation and development were poor, the intermediate forms were larger and more nearly round than the actively growing intermediate forms, and their cytoplasm was generally more vacuolated. Such forms were also more likely to show a protruding stubby flagellum.

Living leptomonads examined with phase contrast showed the flagellum very clearly and had cytoplasm of an even, medium density with small dark granules and clear, circular inclusions.

In some individuals the nucleus could be seen as a clear, circular area with a relatively large, centrally located darker structure, the karyosome (14). The nucleus of intermediate forms had a similar appearance (note certain individuals in Figs. 2 and 3). The cytoplasm of intermediate forms (Figs. 2 and 3) was generally darker in the smaller individuals and brighter in the larger ones. The latter also had larger and more numerous refractile inclusions. The material anterior to the kinetoplast could sometimes be seen as a clear area. This is shown in Fig. 3 by the rather round intermediate form at the top of the cluster, which also shows the axoneme of the flagellum within the clear structure and a very short protruding flagellar stub. The cytoplasm of degenerate individuals had a characteristically different appearance from that of individuals in good condition.

Leishmanias seen with phase contrast within the living spleen cells of infected hamsters (Fig. 1), or freshly extruded from such cells, were very similar, except for their smaller size range, to the smaller, darker, intermediate forms which developed *in vitro*. It was especially interesting to find in preparations of infected spleen cells certain larger organisms which could not have been distinguished from the actively growing smaller intermediate forms seen in the

best cultures. One such organism is illustrated at the upper left corner of the infected cell shown in Fig. 1.

Cultural Conditions Affecting Survival and Development of Intermediate Forms and Leptomonads

The variable results obtained with different batches of human serum have already been indicated. Nevertheless, by including appropriate controls in each experiment, it has been possible to study a few of the cultural conditions affecting the development of the parasites at 37°C.

The Red Cell Extract and the Serum.—Human red cell extract has given somewhat better results than rabbit red cell extract, and both of these have given much better results than duck red cell extract. These differences in the effectiveness of the red cell extracts may be merely reflections of differences in the effects of the corresponding sera. Duck serum rapidly destroyed both the leishmania and leptomonad forms at room temperatures. Rabbit serum had no such effect (indeed, it has often been used as a constituent of the culture medium for leptomonads), but at 37–38°C. few or no intermediate forms ever developed from suspensions of leishmanias in the presence of rabbit serum. Moreover, leptomonads placed in a medium of either human or rabbit erythrocyte extract plus rabbit serum and incubated at 37–38°C. were usually dead within 1 or 2 days. Such results were obtained in several experiments with serum from several rabbits and with old, inactivated serum as well as with fresh serum.

The serum of a single guinea pig, tested in one experiment, likewise did not support the production of any intermediate forms. The sera of two hamsters infected with *L. donovani* and inoculated 3 and 4 months previously, when tested with human erythrocyte extract, showed no intermediate forms even after 4 days, whereas in the presence of three different samples of normal hamster serum intermediate forms appeared within a day and increased as with human serum. Normal hamster serum supported the survival of leptomonads at 37–38°C. for at least 4 days, just as did human serum.

In flasks containing 0.6 ml. of pooled human serum, the intermediate forms developed as well with human red cell extract diluted with an equal volume of solution K-2 as with the full strength extract. If no red cell extract were present, viable forms could not be demonstrated even after only 4 days at 37–38°C.

In the absence of added human serum very few intermediate forms appeared, but their development was about as good with only 0.3 ml. of human serum as with the usual 0.6 ml.

The Inhibitory Effect of Nicotinamide.—In the course of trials of certain supplements, it was observed that the incorporation into solution K-2 of a solution containing ascorbic acid, glutathione, and nicotinamide in amounts

giving final concentrations of 1 mg., 100 mg., and 400 mg. per 100 ml. respectively, inhibited almost completely the appearance of the intermediate forms.

TABLE III

The Effect of Nicotinamide on the Development of Leishmanias and Leptomonads in Vitro at 37°C.

Flasks 1 to 12 were 50 ml. Erlenmeyer flasks and received a current of 95 per cent air with 5 per cent CO_2. Each flask contained 2.8 ml. human red cell extract, 0.6 ml. pooled human serum, and 0.2 ml. of either water or a solution which gave the indicated final concentration of supplement. All except 11 and 12 received fresh medium on day 2. After day 2 for 11 and 12, and day 4 for 1 to 10, the cultures were incubated at 28°C. and examined for the appearance of leptomonads.

Flasks 1 to 8 were each inoculated with 0.4 ml. of a suspension in nutrient broth containing 150,000 leishmanias per c.mm.

Flasks 9 to 12 were each inoculated with 0.4 ml. of a suspension in nutrient broth containing 42,000 leptomonads per c.mm.

Flasks	Form	Supplements	Concentration	Observations on day		Appearance of leptomonads after 5 days' incubation at 28°C.
				2	4	
			mg./100 ml.			
1, 2	Leishmania	None	—	Intermediates present	Intermediates present	+
3, 4	Leishmania	Nicotinamide	400	Intermediates absent	Intermediates absent	0
5, 6	Leishmania	Glutathione	100	Intermediates present	Intermediates present	+
7, 8	Leishmania	Ascorbic acid Glutathione Nicotinamide	1 100 400	One intermediate noted in flask 8	Intermediates absent	0
9, 10	Leptomonad	None	—	Motile leptomonads and intermediates	Intermediates present	+
11, 12	Leptomonad	Ascorbic acid Glutathione Nicotinamide	1 100 400	Degenerate forms only	—	0

Tests of the glutathione and nicotinamide separately showed that while the glutathione had no effect or possibly a slight favorable effect, the nicotinamide[2]

[2] Two samples of nicotinamide purchased approximately 2 years apart from Nutritional Biochemicals Corporation, Cleveland, were tested.

had the inhibitory effect of the mixture. Both the mixture and nicotinamide itself brought about the complete death of leptomonads after 2 days of incubation at 37°C., but they had much less effect on leptomonads incubated at 28°C. Tables III and IV show the results obtained in an experiment of this kind. It is to be noted that nicotinamide, in the presence of added glutathione and ascorbic acid, delayed but did not prevent the transformation at 28°C. of leishmanias into leptomonads. A deleterious effect of the supplement on lep-

TABLE IV

The Effect of Nicotinamide on the Development of Leishmanias and Leptomonads in Vitro at 28°C.

Flasks 13 to 20 were 25 ml. Erlenmeyer flasks plugged with cotton and exposed to the air. Each contained 0.3 ml. pooled human serum and either 1.5 ml. human red cell extract or 1.4 ml. of this extract plus 0.1 ml. of solution to give the indicated final concentrations of supplements.

The flasks were inoculated with 0.2 ml. of the same leishmania or leptomonad suspensions used for the experiment shown in Table III.

Flasks	Form	Supplements	Concentration	Observations on day	
				1	4
			mg./100 ml.		
13, 14	Leishmania	None	—	Numerous active leptomonads	Abundant active leptomonads
15, 16	Leishmania	Ascorbic acid Glutathione Nicotinamide	1 100 400	Leishmania and a few transitional forms but none motile	Numerous active leptomonads
17, 18	Leptomonad	None	—	Numerous active leptomonads	Numerous active leptomonads
19, 20	Leptomonad	Ascorbic acid Glutathione Nicotinamide	1 100 400	Numerous active leptomonads	Fewer leptomonads than in flasks 17, 18

tomonads at 28°C. was not noticeable until the 4th day (see also Table V). In a test of the effect of three concentrations of nicotinamide on leptomonads incubated for 2 days at 37–38°C. in the human red cell extract–human serum medium, complete degeneration of the leptomonads was observed only at the highest concentration of 400 mg. per 100 ml. At the lower concentrations of 200 and 80 mg. per 100 ml., the proportion of motile forms was decreased and of degenerate forms increased, as compared to the controls.

An experiment with isonicotinic acid hydrazide,[3] a substance so effective against tubercle bacilli (15), showed that this substance was not much more

[3] Nydrazid, Squibb and Co., New York.

active *in vitro* against *L. donovani* than nicotinamide. Nydrazid at 400 mg. per 100 ml. caused the red cell extract to turn brown. This effect was much less at a concentration of 40 mg. per 100 ml. At the latter concentration the nydrazid had almost the same effect in preventing the development of leptomonads at 37°C. as did nicotinamide at 400 mg. per 100 ml. At 8 mg. per 100 ml. nydrazid had no effect. Unlike nicotinamide, nydrazid at 40 mg. per 100 ml. had a marked toxic effect on leptomonads at 28°C. as well as at 37°C. (Table V).

TABLE V

Effects of Nicotinamide and Isonicotinic Acid Hydrazide on the Development of Leptomonads at 28°C.

Each flask contained 1.5 ml. human red cell extract (except flasks 3 and 4 which contained 1.4 ml.), 0.3 ml. human serum, and the indicated concentrations of supplements. The flasks were inoculated with 0.2 ml. of a suspension in nutrient broth of 35,000 leptomonads per c.mm.

Flasks	Supplements	Concentration	No. of motile leptomonads seen in a 1 min. examination of a wet mount prepared with a 5 mm. loop of culture material	
			On day 2	On day 4
		mg./100 ml.		
1	None	—	25	44
2			26	46
3	Nicotinamide	400	12	7
4			18	10
5	Isonicotinic acid hydrazide	40	6	0
6			4	1
7	Isonicotinic acid hydrazide	8	23	31
8			12	35

DISCUSSION

The forms intermediate between leishmanias and leptomonads described in the present paper resemble closely the "curious formations" seen by Hawking (9) in some of his tissue cultures of *L. donovani*, which were incubated at 37°C. He noted the appearance in certain infected tissue cultures over 16 days old of extracellular masses of pyriform parasites intermediate between the leishmanial and the elongated leptomonad stage. It is probable that similar if not identical forms were seen by Wenyon (13) in an exceptional case in which the spleen of an infected dog contained leptomonads as well as leishmanias of a "particularly large size and varied shape." The unusual infection in this

animal must have resembled the infections obtained in tissue cultures, where leptomonads appear and multiply extracellularly at a temperature of 37°C., which is lethal to them in non-living culture media (8, 9). True leishmanial forms in such tissue cultures multiply intracellularly.

Some cultures of leptomonads in ordinary media at 28°C. show during the phase of active multiplication groups of aflagellate forms somewhat resembling the intermediate forms. Such aflagellate forms predominate in cultures at 28°C. of certain strains of *Leishmania infantum* (3).

By the use of red cell extract with human or hamster serum it has now been possible to obtain *in vitro* and in the absence of host cells the development of intermediate forms and leptomonads at 37°C., a type of development previously observed only in the presence of surviving tissue cells. The results already obtained indicate the greater specificity and the more exacting requirements of these forms at 37°C. than of the leptomonads grown at 28°C. Even with the media which supported a rapid initial multiplication of intermediate forms, their continuous cultivation has not yet been possible. One is reminded of the more exacting nutritional requirements of certain bacteria when grown at higher temperatures (16, 17). The failure of the truly intracellular forms, the leishmanias, to multiply as such under the cultural conditions used suggests that they have still more exacting environmental requirements.

SUMMARY

Suspensions of leishmanias from the spleen of hamsters infected with *Leishmania donovani* were placed in culture flasks and incubated at 37°C. In a medium of human erythrocyte extract and human serum there appeared within a day or two aflagellate forms resembling leishmanias but larger, as well as other aflagellate forms more nearly resembling rounded leptomonads. These intermediate forms multiplied during the first 4 days of culture. They then slowly died off, despite frequent renewal of the culture medium. Sometimes a small proportion of motile, typical leptomonads also appeared in such cultures. Leptomonads from cultures maintained at 28°C., when placed in the human red cell extract–human serum medium and incubated at 37°C., survived at least 4 days. For both types of effect, human serum could be replaced by normal hamster serum but not by rabbit serum.

Nicotinamide, added to the human red cell extract–human serum medium at a concentration of 400 mg. per 100 ml., completely prevented the development of intermediate forms from leishmanias and brought about the rapid death of leptomonads at 37°C.

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EXPLANATION OF PLATE 11

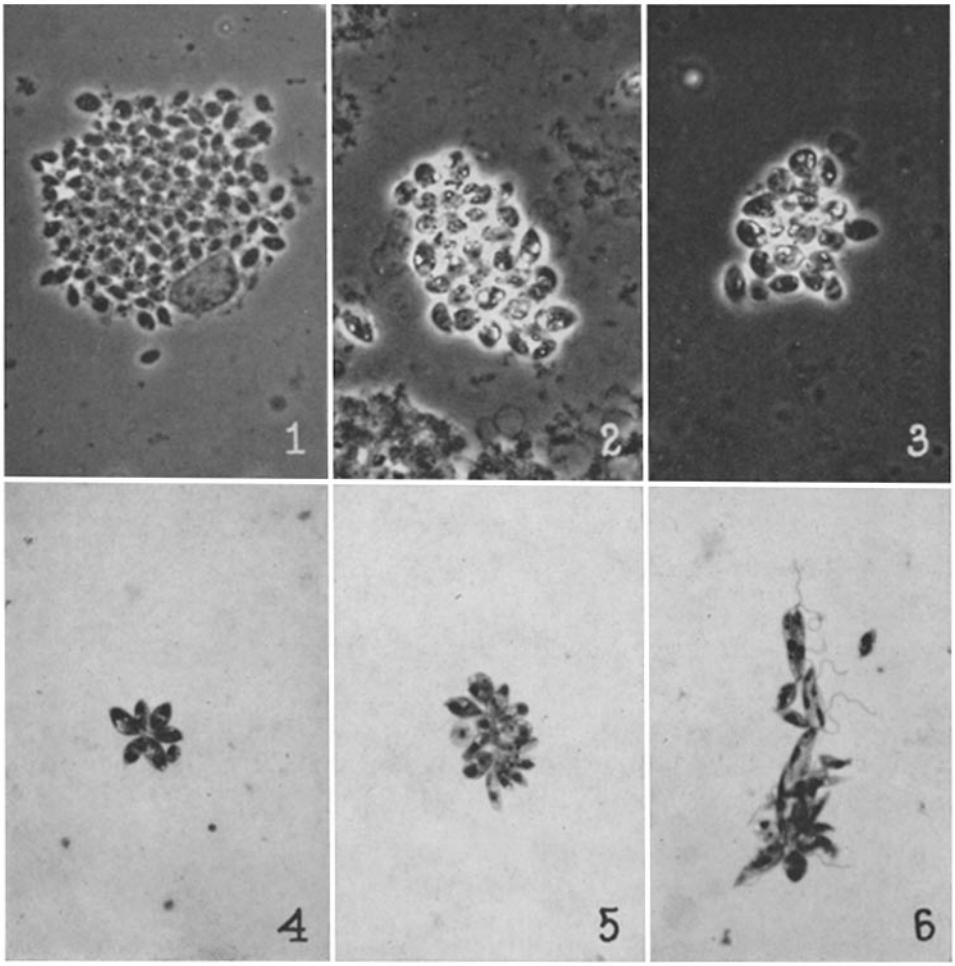
FIGS. 1 to 3, photographs of living material as seen with phase-contrast. Figs. 4–6, photographs from films stained with Giemsa stain. All × 776.

FIG. 1. A cell full of leishmanias, from the spleen of an infected hamster. Note several larger forms at the periphery.

FIGS. 2 and 3. Clusters of growing intermediate forms from a 2 day culture at 37°C. in human erythrocyte extract—human serum medium.

FIGS. 4 and 5. Like Figs. 2 and 3, but from another culture. Note the typical leishmania in Fig. 4.

FIG. 6. A cluster of flagellated leptomonads, with some intermediate forms, from a third 2-day culture at 37°C. in human erythrocyte extract—human serum medium.



(Trager: *Leishmania donovani in vitro* at 37°C.)