Ann. Parasitol. Hum. Comp.,
1988 63, nº 2, pp. 134-145.

OBSERVATIONS ON THE DEVELOPMENT OF *LEISHMANIA (L.) CHAGASI* CUNHA AND CHAGAS IN THE MIDGUT OF THE SANDFLY VECTOR *LUTZOMYIA LONGIPALPIS* (LUTZ AND NEIVA)

R. LAINSON, J. J. SHAW

SUMMARY. Previous observations have shown that forms of *Leishmania*, infective to hamsters, are present in the midgut of experimentally infected sandflies from 15-221 hours after the infective bloodmeal. In a continuation of these studies, stained smears of the midgut contents of *Lutzomyia longipalpis* infected with *Leishmania (L.) chagasi*, made at intervals between 15-120 hours, revealed two lines of parasite development. One of these is the direct transformation of small, non-dividing amastigotes into very small promastigotes, which are considered to represent the « infective » or « metacyclic » flagellates involved in subsequent transmission of the parasite. The other stems from enlarged, highly vacuolated amastigotes which undergo at least two divisions before giving rise to large, elongate and non-dividing promastigotes. These are thought to represent the non-infective forms of the parasite seen in *in vitro* cultures, and their function remains speculative.

Key-words: *Leishmania chagasi*. Development in sandfly. Infective promastigotes. Non infective promastigotes.

Observations sur le développement de *Leishmania (L.) chagasi* Cunha et Chagas dans l'estomac du phlébotome vecteur *Lutzomyia longipalpis* (Lutz et Neiva).

RÉSUMÉ. Des expériences préliminaires ont montré que des formes de *Leishmania*, pathogènes pour le hamster, sont présentes dans l'estomac de phlébotomes 15 à 221 heures après le repas infectant. Lors d'études complémentaires, des frottis colorés de contenus stomacaux de *Lutzomyia longipalpis* infectés par *Leishmania (L.) chagasi*, réalisés séquentiellement entre 15 et 120 heures après le repas, ont permis de mettre en évidence deux modalités de développement du parasite. La première montre la transformation directe et sans division, d'amastigotes petits en promastigotes très petits qui représentent probablement les flagellés « infectants » ou « métacycliques » impliqués dans la transmission du parasite. Selon l'autre modalité, des amastigotes agrandis et fortement vacuolés subissent au moins deux divisions avant de donner des promastigotes grands et allongés cessant de se diviser. Ces derniers dont la fonction reste obscure, sont supposés représenter les formes non infectantes du parasite présentes dans les cultures *in vitro*.

Mots-clés : *Leishmania chagasi*. Développement chez le phlébotome. Promastigotes infectants. Promastigotes non infectants.

The Wellcome Parasitology Unit, Instituto Evandro Chagas, Fúndaçao Serviços de Saúde Pública, 66.001 Belém, Pará, Brazil.

Accepté le 21 mars 1988.

Introduction

Shortt *et al.* (1926), working with *Leishmania (L.) donovani* in *Phlebotomus argentipes*, Adler and Theodor (1930) with *L. (L.) infantum* in *P. perniciosus*, noted very short, active promastigotes in the cibarium and proboscis of the infected flies, and considered them as the infective forms at « ... the end point of this cycle in the sandfly... » (Adler and Theodor, 1931). A number of other workers have remarked on similar forms of other species of *Leishmania* in a variety of naturally or experimentally infected sandflies (Killick-Kendrick, 1979, for review), and the opinion developed that the presence ot these « metacyclic » promastigotes in the proboscis was probably prerequisite to transmission (Killick-Kendrick, 1979, 1986). The hypothesis gained support by the demonstration of morphologically similar flagellates in blood-agar cultures of *Leishmania*, and Sacks and colleagues found that infectivity of the parasites increased throughout the log phase to a maximum at the stationary phase in cultures. The « infective forms » were successfully separated by agglutinating the log phase forms out with peanut lectin, and the two forms were differentiated by their respective resistance to complement (Franke *et al.*, 1985), and on surface antigens and carbohydrates (Sacks *et al.*, 1985). In the sandfly gut, Sacks and Perkins (1985) showed that an increasing number of infective forms were demonstrable in *Lutzomyia longipalpis* experimentally infected with *Leishmania (L.) amazonensis*, from « ... as early as 3 days... » after the infective bloodmeal.

Although Shortt and Swaminath (1928) had earlier suggested that transmission might be due to the regurgitation of a mass of flagellates blocking the cibarium and pharynx of the sandfly, transmission by way of metacyclic forms from the proboscis has remained the hypothesis most widely accepted. Interest in the « regurgitation » theory was revived, however, when Lainson *et al.* (1977) observed that although experimental transmission of *L. (L.) chagasi* by the bite of *Lu. longipalpis* was achieved on days 7, 11, 14 and 17 after the infective bloodmeal, « ... promastigotes were detected in the probosces of very few flies, and *only* after 14 days... » It was suggested that possibly there was « ... a surge of parasites forward from the pharynx, into the mouthparts, during the feeding process ».

More recently, discussion of these findings in relation to the mechanism of transmission (Killick-Kendrick, 1986) prompted us to study the infectivity of *Leishmania* in the sandfly in greater detail, and it was shown (Lainson *et al.*, 1987) that stages of *L. (L.) amazonensis* capable of infecting hamsters were present in experimentally infected *Lutzomyia flaviscutellata* at 15, 25, 40, 49, 70, 96 and 120 hours after the infective feed. Similarly, infective stages of *L. (L.) chagasi* were demonstrated in *Lu. longipalpis* examined at 38, 50, 63, 87, 110, 135, 171 and 221 hours following the infective bloodmeal. It was concluded that « ... at least *some* of the flagellates in the sandfly possess the characters of infectivity after a very short sojourn in the insect host — as early as 15 hours », and that « Possibly these parasites have never lost this since the amastigote stage ».

During these and subsequent studies, stained smears of the midgut contents were made from a number of *Lu. longipalpis* infected with *L. (L.) chagasi*, at variable times after the infective bloodmeal. We record, here, some observations on the different forms of the parasite encountered in this material.

Materials and methods

The parasite strains used were ILON/BR/84/M8188, from a naturally infected specimen of *Lu. longipalpis* (Lainson *et al.*, 1985), and MCER/BR/81/M6445, from a fox, *Cerdocyon thous* (Silveira *et al.*, 1982).

Methods of infecting the sandflies through a chick membrane on infected hamster spleen, and the subsequent dissection of the infected flies, were as previously described (Lainson *et al.*, 1987).

Observations at 15, 24, 36, 43, 60, 72, 90 and 120 hours were in each case made on 6 flies : three infected with strain M8188, and three with M6445. No observations were made on midgut development beyond 5 days, and no attempts were made to study subsequent development in the anterior parts of the gut such as the cibarium, pharynx and proboscis.

Smears of infected midguts were fixed in aqueous Bouin's fluid and stained by a modified Giemsa method (Lainson, 1958). Parasites were measured using a Zeiss « Morphomat 10 », and all measurements are given in μm.

Results

No significant differences were noted in the development of the two strains of *L. (L.) chagasi* in *Lu. longipalpis*, and all the following observations follow examination of the sandflies infected with strain M8188.

I — Midgut smears from sandflies 15 hours after the infective bloodmeal *(figs. 1-8; fig. 23).*

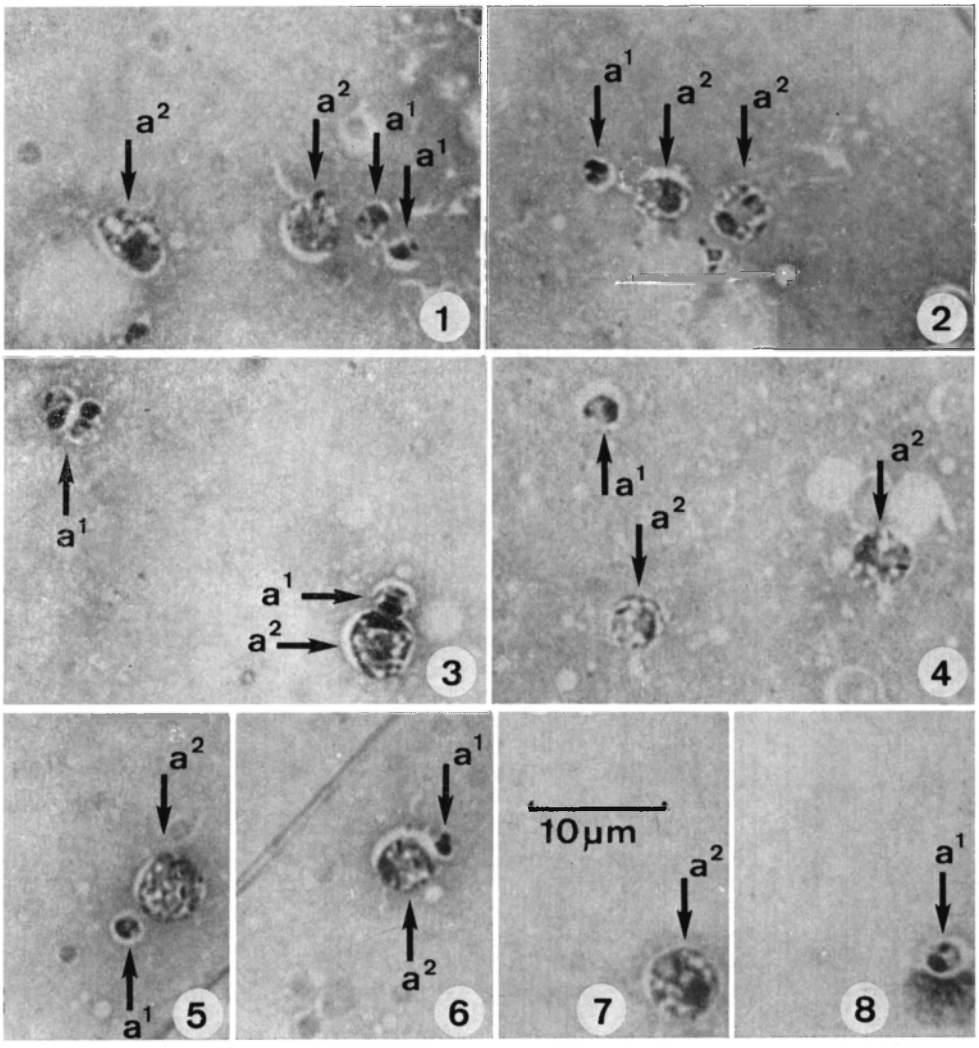
Round to oval amastigotes were abundant in all the smears examined. They showed no evidence of division or flagellar extrusion at this stage, but were clearly of *two types.*

a) *Small, undivided amastigotes (a^1, in figs. 1-6, 8 and 23).*

These were in the *minority* (28.0 % of 1,087 parasites counted in 100 randomly selected fields under the × 100 oil immersion lens). Morphologically they appeared virtually *unchanged* from the amastigotes seen in the vertebrate tissues (e. g. the

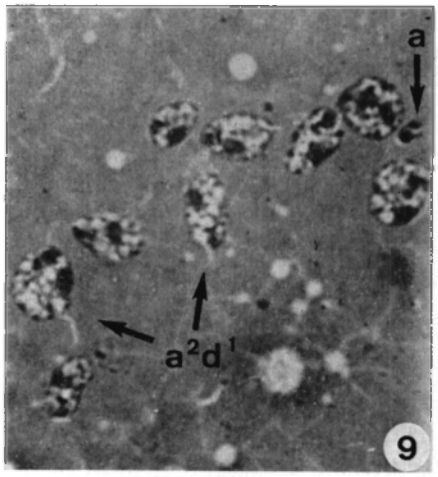
splenic material on which the sandflies were fed). The cytoplasm was rarely vacuolated, and both the nucleus and kinetoplast were compact and deeply stained.

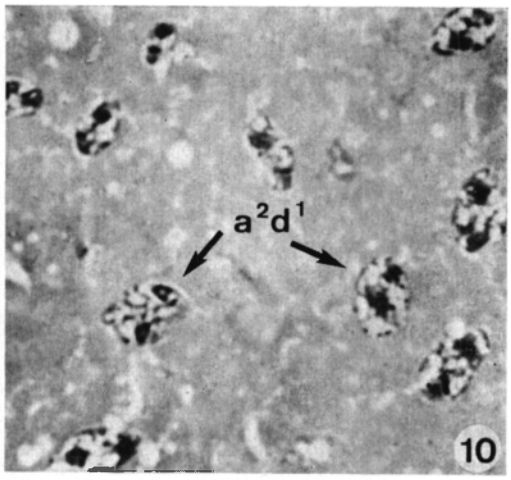
The parasites averaged 2.28 ± 0.40 × 1.85 ± 0.39, for 30 measured, with a range of 1.5 × 1.5 to 3.0 × 3.0.

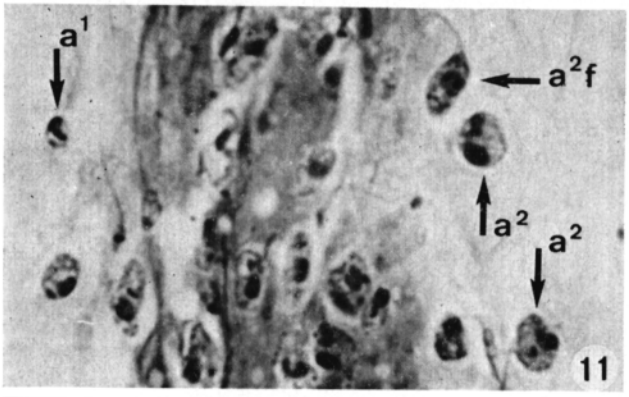


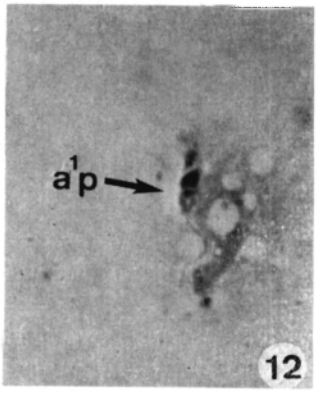
FIGS. 1-8. — Early development of *Leishmania (L.) chagasi* in the midgut of the sandfly *Lutzomyia longipalpis*.

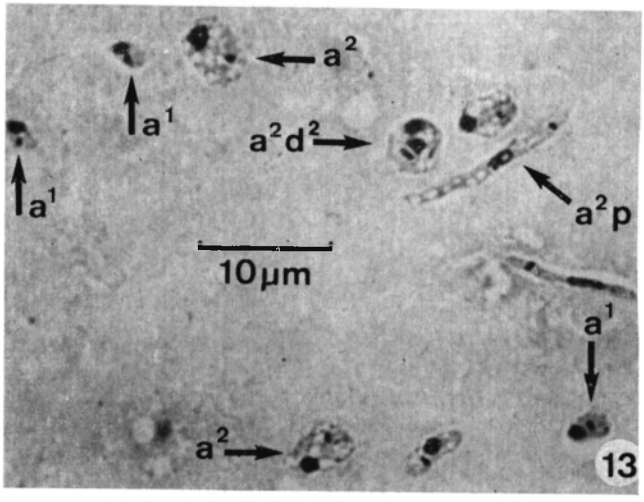
Figs. 1-8. Smear of midgut contents 15 hours after the infective bloodmeal, showing two types of amastigotes: small, non-dividing amastigotes *(a^1)* giving rise to small promastigotes (a^1p in subsequent figures), and enlarged forms *(a^2)* which undergo division before giving rise to large promastigotes (a^2p in subsequent figures).

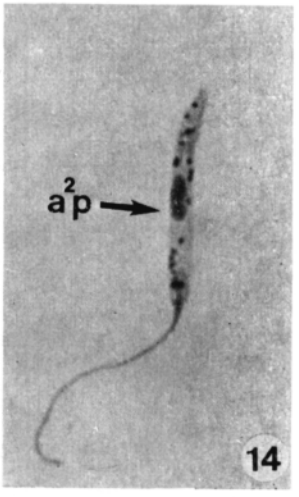

a
a²d¹
9


a²d¹
10


a¹
a²f
a²
a²
11


a¹p
12


a²
a¹
a²d²
a¹
a²p
10µm
a¹
a²
13


a²p
14

b) *Large, undivided amastigotes (a^2, in figs. 1-7 and 23).*

These were in the *majority* (approximately 72 %) and, in sharp contrast, were substantially *enlarged.* Characteristically they possessed a highly vacuolated and poorly stained cytoplasm, a diffuse, pale nucleus and a delicate, rod-shaped kinetoplast. In general their outlines were indistinct. For 30 organisms measured, they averaged $4.55 \pm 0.66 \times 3.8 \pm 0.70$, with a range of 4.0×3.0 to 6.0×5.0.

Throughout the rest of this paper, the terms a^1 and a^2 are used for the small and large amastigotes. The various parasite forms derived from these are then indicated by the addition of d^1 and d^2, for primary and secondary division forms ; *f*, for forms with a developing flagellum ; and *p*, for promastigotes.

II — MIDGUT SMEARS FROM SANDFLIES 24 HOURS AFTER THE INFECTIVE BLOOD-MEAL *(figs. 9, 10 and 23).*

Once again, the parasites fell into two distinct groups :

a) *The small, non-dividing amastigote line (a^1, in figs. 9 and 23).*

At 24 hours these remained unchanged.

b) *The large, dividing amastigote line.*

At this time these parasites had increased in size to an average of $6.3 \pm 1.01 \times 4.1 \pm 0.58$, and their cytoplasm was even more markedly vacuolated. In addition, a high proportion were in the process of division *(a^2d^1)*, with the paired nuclei now of a more compact and deeply staining nature. The kinetoplast of these binucleate forms was most often still in the form of a single, delicate rod, but was clearly initiating division in some of the organisms. On some occasions these dividing forms showed a very short, free, rudimentary flagellum (about 3 μm long), which suggests that at least *some* of the a^2 type amastigotes were destined to undergo only one division prior to forming promastigotes.

III — MIDGUT SMEARS FROM SANDFLIES 36 HOURS AFTER THE INFECTIVE BLOOD-MEAL *(figs. 11, 12 and 23).*

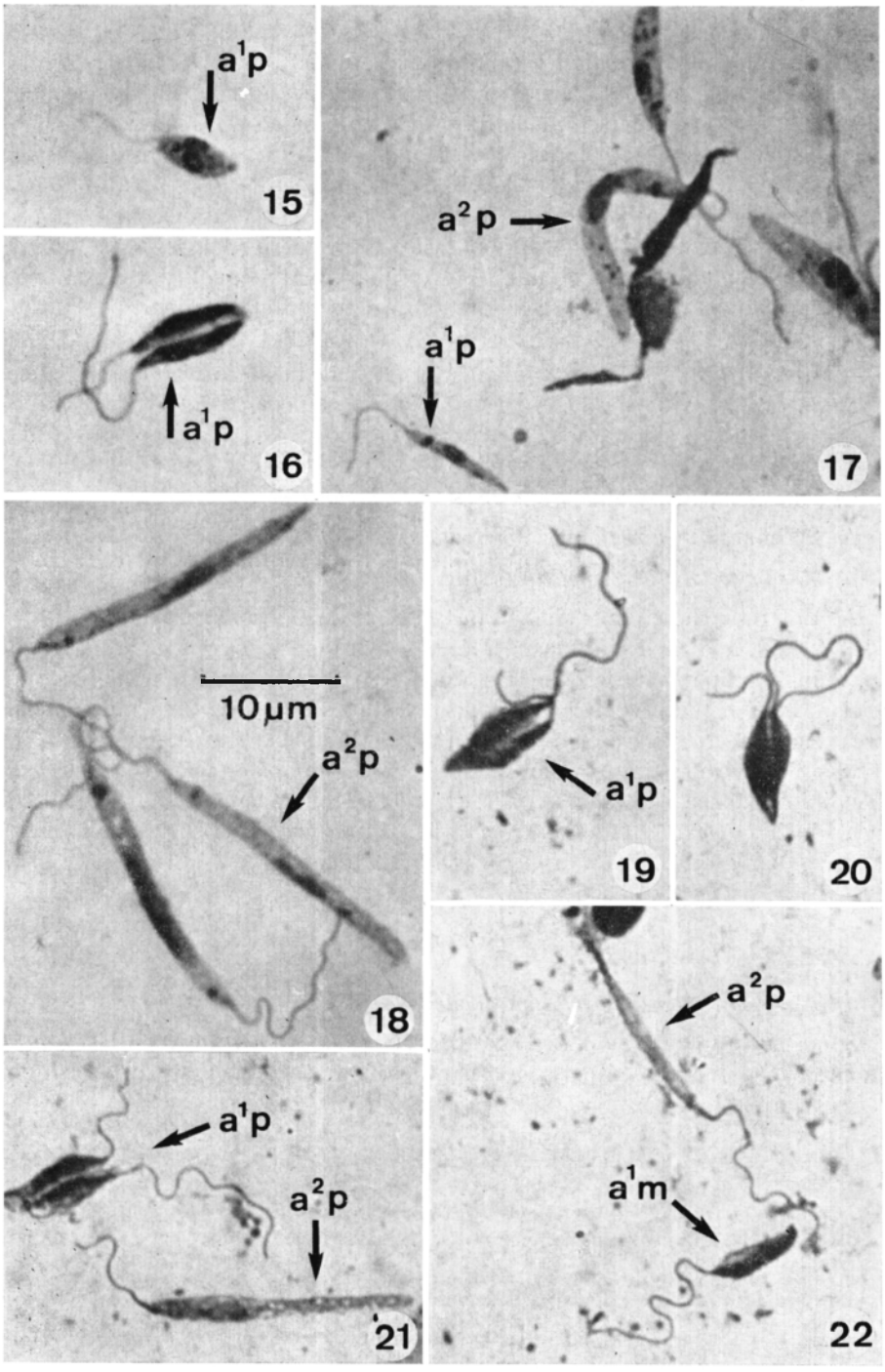
a) *The small, non-dividing amastigote line.*

Some tiny, unchanged amastigote forms *(fig. 11, a^1)* were still present at 36 hours in the midgut contents. On the other hand, some had clearly transformed

---

FIGS. 9-14. — Development of *L. (L.) chagasi* in the midgut of *Lu. longipalpis.*

Figs. 9 and 10. At 24 hours: with small, non-divinding amastigotes *(a^1)* and very numerous large, dividing amastigotes *(a^2d^1)*. Figs. 11 and 12. At 36 hours: with non-dividing small amastigotes *(a^1)* developing directly into small promastigotes *(a^1p)*; and large amastigotes *(a^2)*, some of which are producing a free flagellum *(a^2f)*. Fig. 13. At 43 hours: large amastigotes *(a^2)* are still undergoing division *(a^2d^2)* and others are developing into large, elongated promastigotes *(a^2p)*. Small, non-dividing amastigotes are still present *(a^1)*. Fig. 14. Smear of midgut at 60 hours: large, elongating promastigote *(a^2p)*.


a¹p
15
a¹p
16
a²p
a¹p
17
10µm
a²p
18
a¹p
19
20
a¹p
a²p
21
a²p
a¹m
22

into small promastigotes, measuring little over 10.0 μm including the free flagellum *(fig. 12, a^1p)*. No evidence could be found that the small amastigotes divide prior to transformation into the flagellate form.

b) *The large, dividing amastigote line.*

The variety of parasite forms found in the smears at this time are thought to be derived from the large, dividing amastigote line, by virtue ot their relatively bulky and vacuolated appearance. They included rounded or oval, uninucleated bodies *(fig. 11, a^2)* and stoutly ovoid forms with a developing flagellum *(fig. 11, a^2f)*.

IV — MIDGUT SMEARS FROM SANDFLIES 43 HOURS AFTER THE INFECTIVE BLOOD-MEAL *(figs. 13 and 23)*.

a) *The small, non-dividing amastigote line.*

Seemingly unchanged, small *(a^1)* amastigotes were still present, with an increasing number of small promastigotes *(fig. 23, a^1p)*.

b) *The large, dividing amastigote line.*

The presence of large dividing amastigotes at 43 hours *(fig. 13, a^2d^2)*, together with other, uninucleate forms *(a^2)* strongly suggests that a *second* division takes place about this time.

Large, elongated promastigotes, with a relatively well developed flagellum and a highly vacuolated cytoplasm *(fig. 13, a^2p)*, are believed to be derived from the large, dividing amastigote line by elongation of the parasites and development of the free flagellum *(fig. 11, a^2f)*.

V — MIDGUT SMEARS FROM SANDFLIES 60 HOURS AFTER THE INFECTIVE BLOOD-MEAL *(figs. 14-17)*.

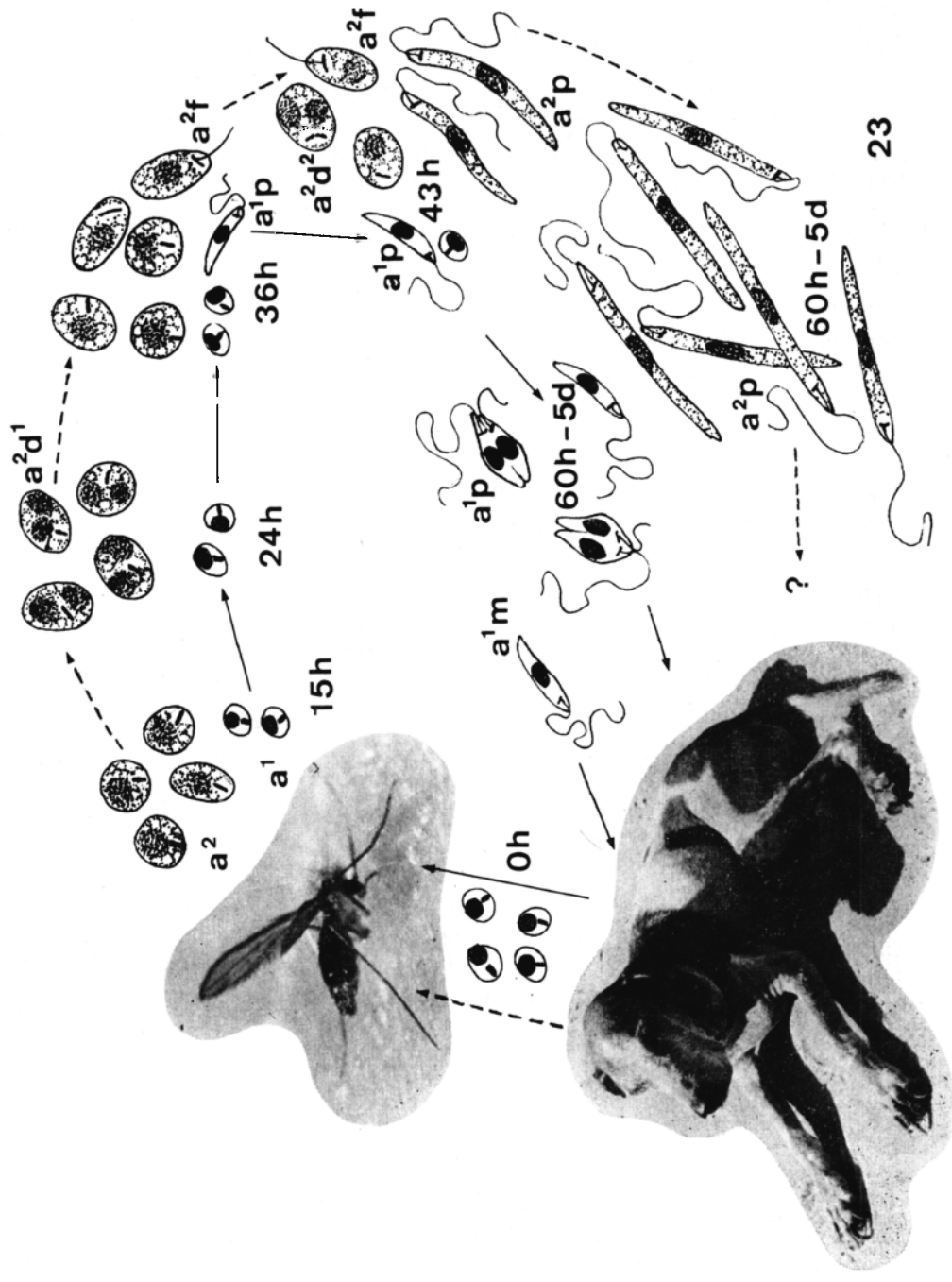
a) *The small, non-dividing amastigote line.*

The small, undivided amastigotes seen in earlier stages of the infection were seemingly now absent, and their place taken by increasing numbers of small, dividing promastigotes *(figs. 15-17, a^1p)*. These we consider to be dividing forms of the small promastigotes *(fig. 12)*, which have arisen *directly* from the small, non-dividing line of amastigotes *(a^1)*. The dividing flagellates are characterized by their « barley-corn » appearance — due to the longitudinal divisional cleft in their cytoplasm. The parasites are devoid of conspicuous vacuoles, and stain

---

FIGS. 15-22. — Development of *L. (L.) chagasi* in the midgut of *Lu. longipalpis*.

Figs. 15-17. At 60 hours: small, dividing promastigotes *(a^1p)*, considered to be derived from the a^1 and a^2 lines of amastigotes, respectively. Fig. 18. At 90 hours: large promastigotes *(a^2p)*. Figs. 19-22. At 5 days, showing actively dividing, small promastigotes *(a^1p)* producingwhat are considered to be the infective metacyclic promastigotes *(a^1m)*, and large (non-infective?) promastigotes *(a^2p)*.


a²
a¹
15h
24h
36h
43h
60h-5d
a²d¹
a²f
a¹p
a²d²
a¹m
Oh
?
23

so densely that the nuclei and kinetoplasts become difficult to distinguish in black and white photographs. Prior to separation of the daughter organisms, the flagellum of one is very much longer than that of the other *(figs. 16, 19, 20, 21* and *23).*

b) *The large, dividing amastigote line.*

Smears at 60 hours showed very abundant, large and elongated promastigotes *(figs. 14, 17* and *23 a^2p),* although these had not yet reached the proportions they assumed later in the infection (e. g. *fig. 18*). They appeared to be undergoing *no* division at this stage of the infection.

VI — MIDGUT SMEARS FROM SANDFLIES 90-120 HOURS AFTER THE INFECTIVE BLOODMEAL *(figs. 18-23).*

This was the limit of the present observations. The smears at this stage showed very numerous, highly elongated and *non-dividing promastigotes,* and much less frequent *small, dividing promastigotes* (in the proportion of 4 : 1, for 100 parasites examined). The larger flagellates are considered as the end product of the a^2 line amastigotes, and the smaller ones as deriving from the small a^1 amastigotes.

Discussion

Attempts to study the early development of *Leishmania* species in their sandfly vectors are usually made difficult by the small number of amastigotes ingested with the insect's bloodmeal. Nonetheless, Shortt (1928) found division stages of *L. (L.) donovani,* « ... representing multiplication before the flagellate condition... », in *Phlebotomus argentipes,* 24 hours after the infective bloodmeal: and Strangways-Dixon and Lainson (1966) described enlargement and division of the amastigotes of *L. (L.) mexicana,* in the midgut contents of sandflies, within 24 hours of feeding them on infected hamsters.

In the present study, the technique of feeding *Lu. longipalpis* on suspensions of heavily infected hamster spleen, through a chick-skin membrane, has overcome

FIG. 23. — Early development of *Leishmania (L.) chagasi* in the midgut of the sandfly *Lutzomyia longipalpis.* Amastigotes are ingested (0 h) and, by 15 hours, are of two morphological types: small, seemingly unchanged forms *(a^1),* and enlarged, highly vacuolated parasites *(a^2).* By 24 hours, the small forms remain unchanged, but the large amastigotes are now in active division *(a^2d^1).* At about 36 hours, some of the small amastigotes have transformed into tiny promastigotes *(a^1p),* and a few of the large ones show signs of a developing flagellum *(a^2f).* By 43 hours, transformation of the small amastigotes has continued, and a second division of at least some of the large amastigotes occurs *(a^2d^2).* Progeny of the a^2 amastigotes are now in the process of developing into large, elongate promastigotes *(a^2f-a^2p).* From 60 hours to 5 days, the midgut is packed with the large, *non-dividing* promastigotes *(a^2p),* interspersed with a smaller number of small, *dividing* flagellates *(a^1p)* which give rise to the small promastigotes *(a^1m).* The latter are presumed to represent the infective « metacyclic » parasites responsible for transmission: the role and fate of the large promastigotes remains uncertain.

the problem of scanty parasites in the bloodmeal and enabled confirmation of these author's observations. Furthermore, the presence of enlarged, dividing amastigotes in the midgut as late as 43 hours after the infective feed suggests that at least *two* divisions of some amastigotes may take place before the flagellate condition is assumed.

The infectivity of midgut contents to hamsters as early as 15-24 hours after the sandfly's infective bloodmeal (Lainson *et al.*, 1987) is presumably due to the small, seemingly unchanged amastigotes *(a^1)*. Between 36-43 hours these give rise *directly* to small promastigotes *(a^1p)*, and the infectivity of the gut contents at this time is probably due to both these and any remaining, untransformed a^1 amastigotes. By 3-5 days, transformation appears to have been completed, and infectivity almost certainly now depends on the promastigote forms a^1p. These tiny flagellates have all the morphological characteristics of the « infective » or « metacyclic » promastigotes encountered by earlier workers in the anterior station of the sandfly gut (Killick-Kendrick, 1986).

By virtue of the greater abundance and active division of the enlarged, a^2 line of amastigotes, the large, elongated promastigotes they produce greatly outnumber the small « infective » flagellates *(by about 4 : 1)*. It is particularly interesting that a prolonged search of smears failed to produce any evidence of division of the large a^2p promastigotes.

While one cannot entirely disclude the possibility that some of the large a^2 amastigotes, seen early on the infection, might also be infective to hamsters, we feel this unlikely in view of the profound physiological changes which must be taking place during their active division. Whether or not the large, elongated a^2p promastigotes derived from them are infective is equally doubtful. If (as we suspect) they are not, it remains to indicate their function. We presume that they represent the non-infective forms described in *in vitro* cultures by Sacks *et al.* (1985). Perhaps they play some role in establishing certain physiological conditions within the sandfly's gut which are important for the development of the metacyclic promastigotes.

Were genetic exchange an established feature of the genus *Leishmania*, it would be tempting to suggest that the small and large amastigote lines, and the correspondingly small and large promastigotes they produce, represent two morphologically distinct forms of the parasite involved in this process. Further studies are underway in attempts to determine their nature and respective roles in the mechanism of transmission.

ACKNOWLEDGEMENTS. — These studies were carried out under the auspices of the Wellcome Trust London, and the Instituto Evandro Chagas of the Fundaçao Serviços de Saúde Pública do Brasil. We are indebted to Iorlando da Rocha Barata and Antonio Julio de Oliveira Monteiro for technical assistance.

REFERENCES

ADLER S., THEODOR O. : The exit of *Leishmania infantum* from the proboscis of *Phlebotomus perniciosus*. *Nature*, London, 1930, *126*, 883.

ADLEB S., THEODOR O. : Investigations on Mediterranean Kala azar I-V. *Proc. Roy. Soc.*, London, Series B., 1931, *108*, 447-502.

FRANKE E. D., MCGREEVY P. B., KATZ S. P., SACKS D. L. : Growth cycle-dependent generation of complement-resistant *Leishmania* promastigotes. *J. Immunol.*, 1985, *134*, 2713-2718.

KILLICK-KENDRICK R. : Biology of *Leishmania* in phlebotomine sandflies, pp. 395-460. *In:* W. H. R. LUMSDEN and D. A. EVANS, *Biology of the Kinetoplastida*, 1979, vol. 2, *Academic Press*, London.

KILLICK-KENDRICK R. : The transmission of leishmaniasis by the bite of the sandfly. *J. Roy. Army Med. Corp.*, 1986, *132*, 134-140.

LAINSON R. : Observations on the development and nature of pseudocysts and cysts of *Toxoplasma gondii*. *Trans. R. Soc. trop. Med. Hyg.*, 1958, *52*, 396-407.

LAINSON R., RYAN L., SHAW J. J. : Infective stages of *Leishmania* in the sandly vector and some observations on the mechanism of transmission. *Mem. Inst. Oswaldo Cruz*, 1987, *82*, 421-424.

LAINSON R., SHAW J. J., RYAN L., RIBEIRO R. S. M., SILVEIRA F. T. : Leishmaniasis in Brazil. XXI. Visceral leishmaniasis in the Amazon Region and further observations on the role of *Lutzomyia longipalpis* (Lutz and Neiva, 1912) as the vector. *Trans. Roy. Soc. trop. Med. Hyg.*, 1985, *79*, 223-226.

LAINSON R., WARD R. D., SHAW J. J. : Experimental transmission of *Leishmania chagasi*, the causative agent of neotropical visceral leishmaniasis, by the sandfly *Lutzomyia longipalpis* (Lutz and Neiva). *Nature*, London, 1977, *266* (5603), 628-630.

SACKS D. L., HEINY S., SHER A. : Identification of cell surface carbohydrate and antigenic changes beetween noninfective and infective developmental stages of *Leishmania major* promastigotes. *J. Immunol.*, 1985, *135*, 564-569.

SACKS D. L., PERKINS P. V. : Development of infective stage *Leishmania* promastigotes in the sandfly vector and some observations on the mechanism of transmission. *Am. J. trop. Med. Hyg.*, 1985, *34*, 456-459.

SHORTT H. E. : The life history of *Leishmania donovani* in its insect and mammalian hosts. *Trans. 7th Cong. Far Eastern Ass. trop. Med.*, December, 1927, vol. III, Calcutta : Thascker's Press, 12-18.

SHORTT H. E., SWAMINATH C. S. : The method of feeding of *Phlebotomus argentipes* with relation to its bearing on the transmission of kala azar. *Ind. J. Med. Res.*, 1928, *15*, 827-836.

SHORTT H. E., BARRAUD P. J., CRAIGHEAD A. C. : The lige-history and morphology of *Herpetomonas donovani* in the sandfly *Phlebotomus argentipes*. *Ind. J. Med. Res.*, 1926, *13*, 947-959.

SILVEIRA F. T., LAINSON R., SHAW J. J., POVOA M. M. : Leishmaniasis in Brazil. XVIII. Further evidence incriminating the fox *Cerdocyon thous* (L) as a reservoir of Amazonian visceral leishmaniasis. *Trans. Roy. Soc. trop. Med. Hyg.*, 1982, *76*, 830-832.

STRANGWAYS-DIXON J., LAINSON R. : The epidemiology of dermal leishmaniasis in British Honduras. Part. III. The transmission of *Leishmania mexicana* to man by *Phlebotomus pessoanus*, with observations on the development of the parasite in different species of *Phlebotomus*. *Trans. Roy. Soc. Med. Hyg.*, 1966, *60*, 192-207.