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ON THE LIFE CYCLE OF TRYPANOSOMES OF THE *LEWISI* GROUP AND THEIR RELATIONSHIPS TO OTHER MAMMALIAN TRYPANOSOMES

Maria Paumgartten DEANE (1)

SUMMARY

The Author discusses some phases in the life cycle of *Trypanosoma cruzi* and related species.

It is suggested that the cycle of all mammalian trypanosomes follows a similar pattern and that, in comparing species, one should realize that morphology is not always an indication of the competence of each stage. Several studies have been made with no consideration for the fact that the trypomastigote is a non-dividing form among species of the *lewisi* group and the physiological differences between them and the dividing trypomastigotes of the *brucei* group cannot be taken only as specific differences. On the other hand, polymorphism among species of the *lewisi* group has been generally overlooked and yet it may have the same significance it apparently has among the *brucei* trypanosomes.

Most of the characters proposed as distinctive between groups of mammalian trypanosomes should be re-evaluated. The Author prefers to keep the name "*lewisi* group" for species such as *cruzi*, *lewisi*, *rangeli* and others — that may be pathogenic, polymorphic and transmitted through the bite of the insect vector — but that do not multiply primarily as trypomastigotes in the bloodstream of the vertebrate host.

INTRODUCTION

According to VICKERMAN [49] the life cycle of Protozoa can be vizualized as "a series of morphogenetic responses on the part of competent cells to certain environmental changes". This is a most interesting and stimulating approach for the study of mammalian trypanosomes. Among these protozoa, morphogenetic responses give rise to the stages known since a long time by their appearance under the light microscope. But we are all aware, of course, that those are only some of the most obvious aspects of morphogenesis, and that we must think of this rather as a series of metabolic changes not always translated in evident morphological differences.

Changes being regulated through genetic mechanisms, they obviously represent specific responses to external stimuli. The interplaying of all factors and mechanisms involved poses complex problems of interpretation and explains why in repeated experiments results are so often inconsistent. This is especially true when we study populations of cells grown in such variable and ever changing environments as the orga-

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nisms of the vertebrate and invertebrate hosts and tubes with undefined culture media.

For some time we have been investigating the factors involved in transformation among trypanosomes of the *lewisi* group. In attempting to interpret our findings in the light of other Authors' work we came to the conclusion that many things do not seem to fit well within the current ideas about the cycle of species of this group and about their relationships to other mammalian trypanosomes.

In this paper we just wish to go over some of the major phases of the cycle of the *lewisi* group of trypanosomes, inserting our own observations where they seem relevant and advancing a few hypotheses on competence and transformation of morphological stages. For these we will use the nomenclature proposed by HOARE & WALLACE [26] which has obvious advantages over the old one. We will keep the name "trypomastigote" for the blood trypanosomes and refer to the metacyclic trypanosome as "metatrypomastigote".

MULTIPLICATION AND DIFFERENTIATION IN THE VERTEBRATE HOST

Characteristically the trypomastigote is a non-dividing stage in all species of the *lewisi* group. However, division in this stage has been occasionally seen for several species [20, 22, 30]. Working with *Trypanosoma conorhini*, we have never found any sign of division among trypomastigotes in blood smears, but we saw a few of them dividing in the peritoneal cavity of inoculated mice and many more in cultures maintained at 37°C [13, 14].

An explanation can readily be offered for these findings without doing away with the concept that the trypomastigote is a non-dividing form, if we admit that sometimes transformation occurs before the division initiated in a previous stage is completed, or vice-versa. This is probably true, but it might be worth to investigate, as it has been done with the toad trypanosome *T. mega* [44], if in the *lewisi* group all forms in this stage are actually unable to go through the synthetizing activities that must precede cellular division.

Anyway, it is a known fact that species of this group do not usually divide as trypomastigotes in the bloodstream of the vertebrate.

For *T. cruzi* multiplication occurs at the well known intracellular amastigote stage.

In *T. lewisi* the multiplication forms found in the bloodstream of the rat are also well known, and they usually appear as promastigotes or epimastigotes undergoing binary or multiple fission. However, as emphasized by ORMEROD [34], the initial reproductive stages of *T. lewisi*, although described many years ago, have been generally overlooked. They may be found within visceral capillaries chiefly in the kidneys, first as rounded forms of various sizes, with a variable number of nuclei and kinetoplasts and without a free flagellum, and later as rosettes of small promastigotes or epimastigotes.

Similar forms have been described in the capillaries of various organs by CARPANO [9] for *T. theileri*, by DAVIS [12] for *T. zapi*, by GREWAL [21] for *T. nabiasi*, and we have found them for *T. conorhini* and *T. rangeli*. They are not easily detected because they are delicate, stain faintly and may be very sparsely distributed. They were more abundant in animals treated with corticosteroids and they appeared after inoculation not only of blood forms of *T. lewisi*, but also of culture forms of this species, *T. conorhini* and *T. rangeli*.

These findings suggest that an initial reproductive phase in visceral capillaries is a common occurrence among species of the *lewisi* group of trypanosomes. This would explain: a) why multiplication forms are unknown for so many species of the group studied only in blood smears [22, 24, 51], and, b) the lack of correlation between the proportion of dividing forms found in the peripheral blood and the heavy parasitemias produced in experimental infections with some species [12, 35].

We might even speculate that such a phase could have preceded the adaptation of *T. cruzi* to intracellular multiplication. MUNIZ & FREITAS [33] were able to observe part of the cycle in a medium without cells incubated at 37°C and, indeed, the intracellular localization of *T. cruzi* may be not essential from the point of view of the me-

tabolism of the parasite, as possibly it is not for the amastigote stage of species of the genus *Leishmania* [29].

We could then admit that: a) because the bloodstream was not an adequate medium for multiplication of these trypanosomes, they succeeded when they became adapted to places in the organism of the vertebrate where they could find a certain protection; b) these locations could have been first the lumen of visceral capillaries and later, for some species such as the *cruzi*-like, the capillary endothelial cells and eventually other cells, and, c) because of the especial conditions in these locations, they were changed into rounded aflagellate forms and started multiplying. A free flagellum and an eloganted body became again necessary when the parasite had to migrate to colonize elsewhere in the organism of the vertebrate, or to be accessible to the invertebrate host.

It is possible that the main differences between groups of mammalian trypanosomes originated at this point, that is, at the way they adapted for multiplication in the vertebrate and, as we see it, this is also the main distinction between the two major groups of HOARE [24]. The morphological stage in which multiplication occurs is probably dependent on the environment where it occurs and we should accept that the multiplication phase of trypanosomes in the vertebrate includes all forms that are competent to multiply, whatever their appearance and whether they multiply in the bloodstream or elsewhere. We think this is an important concept when we want to compare physiology of species.

It is through multiplication that the parasite tries its survival in the vertebrate host and its survival depends in part on the immune responses of the latter. The *lewisi* group of trypanosomes of the New World offer a whole gamut of adaptation to vertebrates. Species of both the *lewisi* and the *cruzi* types are very common among small wild animals, especially rodents and marsupials. In the wild host the infection is usually detected by the finding of a few trypomastigotes and/or through xenodiagnosis in the case of *cruzi*, *rangeli* and *conorhini* species. The infection seems to be of a chronic nature and parasitemia has been observed to rise after splenectomy [12]. This means that multiplication keeps going on at low levels despite the immune responses of the host, and that the trypomastigote stage keeps appearing in the peripheral blood.

As suggested by DAVIS [12], *T. lewisi* may be less adapted to the rat than those wild species to their natural hosts. If this be true it is unfortunate, since the species has been generally and widely taken as a model for the group. In infections of the laboratory rat by *T. lewisi*, multiplication is intense, with invasion of the bloodstream by dividing forms and by trypomastigotes of different sizes and shapes. Through the use of corticosteroids and other means that interfere with the production of antibodies, multiplication may be intensified and massive parasitemia may result in death of the host [34, 35, 40]. If infection is allowed to follow its normal course, reproduction of the parasite is rapidly checked, dividing forms disappear from the peripheral blood and eventually the trypomastigote population becomes monomorphic and finally also disappears.

This sequence of morphogenetic events has been interpreted solely on the basis of the immune reaction of the host. As it is well known, TALIAFERRO [45, 46] postulated the production of an antibody with specific mitosis inhibiting properties which he called "ablastin". As the theory goes, once their reproductive period is stopped by ablastin, the parasite simply enters in a process of aging.

ORMEROD [34] has already discussed some facts that do not seem to fit in with this theory and we shall come back to them in relation to the competence of *T. lewisi* trypomastigote stages. For the moment we wish only to comment on the course of infections in corticosteroids treated rats. We found, as did PATTON & CLARK [35], that the increase of parasitemia that eventually led to death of the animals was not proportional to the increase in the number of dividing forms in the peripheral blood, and this may be explained by the previously mentioned fact that multiplication of *T. lewisi* occurs primarily in the capillaries. We also verified that, although the flagellate population remains polymorphic until the animals die, the so-called "adult" trypomastigotes keep appearing throughout the infection. This should not be expected if the latter stage

in normal infections simply represented young forms grown older because their ability to multiply has been arrested through antibody action. If this were the case, once the antibody production is checked and, therefore, multiplication goes on unchecked, the population should remain permanently young.

It must be clear that we are not discussing the existence or qualities of ablastin. The point we want to bring into discussion is the general idea that successive populations of *T. lewisi* in the peripheral blood of the host represent successive stages in a process of senescence that is precipitated by the action of some factor interfering with their reproductive capacities. The way these facts are interpreted has a great bearing on the assumptions over the relationships of the hemoflagellates and are very important for studies on morphogenesis. If we want to study transformation we want to know which is transforming into which, how and why.

As an alternative we propose the hypothesis that somewhere during multiplication, among all mammalian trypanosomes, a phase differentiates that represents a dead end for the parasite in the vertebrate host, as much as the metatrypomastigote represents a dead end in the invertebrate. Both are competent to initiate the alternate part of the cycle, but they are able to multiply again only after de-differentiation, under stimuli not present in the same environment where they were produced. The undifferentiated stages, in both the vertebrate and the invertebrate, proceed to multiply continuously and give rise to differentiated cells, until their growth is checked by some factor.

This would explain many things, including the observation that "adult" *T. lewisi* are unable to synthetize nucleic acids [46]. Also, the hypothesis is more conforming to a general rule in life histories of digenetic parasites: through multiplication the parasite survives in a given host, but it must guarantee its survival as a species by being prepared to develop in the alternate host.

POLYMORPHISM

For trypanosomes of the *brucei* group polymorphism is usually emphasized as a distinctive character and they are frequently referred to as "the polymorphic trypanosomes". According to several Authors [5, 6, 36, 49, 52, 53], the slender form in the *brucei* group is adapted to life and multiplication in the vertebrate while the stumpy trypomastigote is the one competent to initiate the cycle in the invertebrate. The slender form does not differentiate and eventually degenerates in the mid gut of the tsetse fly [36]. Structural and physiological differences have been found by VICKERMAN [49] between the two stages.

On the other hand, a definite polymorphism of the trypomastigote stage of *T. cruzi* is recognized since the species was first described, in 1909 [10]. Slender, broad and intermediate forms can be distinguished by several morphological characters [16, 42]. The polymorphism in *T. cruzi* has been variously interpreted and the subject was reviewed by SILVA & CAMARGO [42]. In the first detailed description of the cycle of *T. cruzi* in the invertebrate host, DIAS [17] mentioned that not all the ingested trypomastigotes develop, but some remain as such for many days in the midgut of the insect. According to SILVA [41], slender forms remain unaltered for several days and finally degenerate in a medium where the broad trypomastigotes readily differentiate into epimastigotes, thus starting the normal cycle in culture and, also, in the insect midgut it is the slender forms that do not differentiate.

A great polymorphism has been also recognized for *T. lewisi*, even if only the trypomastigote stage is considered. Some of these trypomastigotes, for instance, the early small ones, could be those that are competent to start new colonies in other places of the vertebrate organism. In infections by *T. lewisi* multiplication is completely checked in a relatively short time [11]. As a result, the monomorphic final population competent to infect the invertebrate, being not replenished, should be exhausted, due to antibodies, or old age, or both. When a mixed population is inoculated in the peritoneum of clean rats, the "adult" trypomastigotes appear almost immediately in the peripheral blood. Here the population remains monomorphic for some time until the polymorphic flagellates begin to appear. The lag phase seems to depend on the proportion of "contaminant" reproductive stages

in the inoculum and we interpret this as an indication that the "adult" trypomastigote, being not competent no infect the vertebrate, simply survives for some time in the blood of the inoculated animal.

A similar situation occurs with the rat species *T. conorhini* in the laboratory mouse. The infection is very light, short lived and dies out completely as demonstrated by negative xenodiagnosis with triatomid bugs. The final population of trypomastigotes is monomorphic and proved to be entirely incapable of initiating infection in new mice. On the other hand it readily differentiates in culture media.

Things may be different with species of both the *lewisi* and the *cruzi* types in their natural hosts, as already indicated, and various degrees of adaptation may produce bloodstream populations that are more or less mixed as far as the competence of their elements is concerned.

MULTIPLICATION IN THE INVERTEBRATE HOST

Among trypanosomes of the *lewisi* group multiplication in the invertebrate host and in the usual culture media occurs chiefly by binary fission in the epimastigote stage. There is no proof up to now that trypanosomes dispose of any means for genetic transfer and some interesting experiments were recently made with drug resistant strains of *T. cruzi* in the invertebrate host, with negative results [4].

However, we think that the question should be considered still open to investigation and we call attention to a reproductive phase we described in cultures of *T. conorhini* and labelled "cyst-like-bodies" [15]. Similar forms were found by IRALU [28] and later by ourselves in cultures of *T. cruzi*. As we interpret them, they result from the fusion of epimastigotes, followed by internal reorganization and division of the organelles and redistribution of these among the daughter cells that are formed, perhaps by internal budding. The process seems too complicated to be meaningless and we suggest that redistribution of nuclei and kinetoplasts originated from different mother epimastigotes might have genetic significance.

FACTORS INVOLVED IN MORPHOGENESIS

The environmental factors that influence transformation of the flagellates both in their vertebrate and invertebrate hosts are still little understood and most of the available information is found in recent reviews [27, 48].

We do not know if antibodies have any morphogenetic action on individual flagellates, but they certainly do influence populations through selective destruction of sensitive elements. Since, as already stated [1], differentiation begins when the cell begins to synthetize a new protein, transforming cells may be immune to antibodies produced against undifferentiated cells. This is well illustrated by studies on the "relapse variants" of the *brucei* trypanosomes [8] and the monomorphic aspect of the population at the end of infection by *T. lewisi* and *T. conorhini* may be exactly the result of selection by antibodies. Indeed, it has been shown that "young" populations of *T. lewisi* are susceptible to antisera that do not destroy the "adult" forms [34], and antigenic differences between the two were actually demonstrated by the Ouchterlony technique [18].

Antibodies have been demonstrated to have some effect on the reproduction of the flagellates in cultures [2, 3] but there is no evidence that they interfere with transformation or multiplication in the invertebrate host.

In studying morphogenesis in *T. conorhini* we found that temperature and an unknown factor present in fresh red cells seem to be important for the *in vitro* transformation of the trypomastigote into epimastigote [14]. Within a temperature range of 36-38°C the normal cycle in culture is not initiated and most of the trypomastigotes keep a healthy aspect for several days. In temperatures of 31-34°C it looks as if the flagellates are undecided which way to go and they finally die without undergoing complete differentiation. In temperatures of 25-28°C differentiation soon begins and, in some cultures, within 4 hours more than 70% of the flagellates are already in the epimastigote stage, many showing signs of division. Within 8 hours differentiation may be complete, as judged by observation of fresh and stained material.

On the other hand, in cultures where more than 90% of the flagellates were already epimastigotes or transition forms, judging by the position of the kinetoplast, the population returned to the blood type trypomastigote if thereafter incubated at 37°C. This happened if transformation was allowed to progress only for some time, because, after 8 hours at the lower temperatures, incubation at 37°C resulted in death of all forms without any further transformation, within 24 hours (unpublished data). These results show that differentiation of trypomastigote into epimastigote is a reversible phenomenon if it has not proceeded beyond a certain point and that within a short period the flagellates have a sort of ambivalence as to their competence to go one way or the other.

This dual competence seems to exist in other phases of the cycle and the intermediate form among trypomastigotes may be one of these ambivalent stages.

STRUCTURAL AND PHYSIOLOGICAL BASIS OF MORPHOGENESIS

On the meaning of competence of trypanosomes to transform and what is transformation in physiological and structural terms, some progress has been made recently through the use of the electronmicroscope and the correlation of these data with those obtained by cytochemical and biochemical methods. Of especial interest are the findings on the structure of the kinetoplast, its relationship to the rest of the chondriome of the cell and its possible communication with the nucleus during its migrations back and forth in the cell [32, 43, 48, 49].

The structural relationship of the kinetoplast to the rest of the chondriome is undoubtful and its regulating action on the synthesis of mitochondrial enzymes among KINETOPLASTIDA should be expected [27, 32, 43, 48, 49]. However, some generalizations that have been made about those enzymes in mammalian trypanosomes seem, to us, unjustified.

Indeed, it has been widely hypothetized [19, 31, 37, 38, 48, 49, 50] that *T. lewisi* and *T. cruzi* differ from species of the *brucei* group in that the latter switch respiratory systems when passing from the vertebrate to the invertebrate host and vice-versa, while the former keep depending on mitochondrial respiratory enzymes throughout their cycles. Since the synthesis of these enzymes is regulated by the kinetoplastic DNA, species of the *lewisi* group would be unable to survive in either host with an impaired kinetoplast, while for species of the *brucei* group the organelle is necessary only for the cycle in the invertebrate. According to VICKERMAN [49, 50], in the *brucei* group transformation from the slender to the stumpy trypomastigote includes development of the chondriome and this is part of the structural and physiological basis of the competence of the stumpy form to initiate the cycle in the invertebrate; the opposite, that is, reduction of the chondriome, is part of the preparatory steps that make the metatrypomastigote competent to develop in the vertebrate.

The natural occurrence of dyskinetoplastic mutants among species of the *brucei-evansi* group [23, 25], some previous negative attempts to produce dyskinetoplastic trypomastigotes of *T. cruzi* through the use of drugs [31] and the well known data on the respiratory enzymes of Trypanosomatidae [19, 27, 37, 38] are the basis for the above assumptions.

However, in a paper soon to be published [16] we describe experiments demonstrating that *T. cruzi* made dyskinetoplastic by the action of acriflavine is able to go through most of the steps that characterize its cycle in the mammalian host: penetration into cells, differentiation into amastigotes, multiplication in this stage and differentiation into trypomastigotes. In the usual culture media the dyskinetoplastic epimastigotes may transform into (dyskinetoplastic) metatrypomastigotes, but those that are not competent to differentiate have a limited survival and cannot be subcultured.

These results suggest that a normal kinetoplast is essential for *T. cruzi* only during the part of its cycle which corresponds to development in the invertebrate host. They also suggest that the already discussed polymorphic character of *T. cruzi* trypomastigotes may have the same significance it apparently has among species of the *brucei* group.

Going over the published data on the respiratory enzymes of trypanosomes [27, 38], we have the following criticism to make:

a) To study species that multiply as trypomastigotes in the bloodstream, the flagellates are always collected when parasitemia and, therefore, multiplication are at a maximum.

b) When working with the blood forms of *T. lewisi*, the Authors specify they used the "adult" or monomorphic population, what means that the multiplication stages were ignored.

c) In studies with *T. cruzi* both the intracellular multiplication forms and the polymorphism of the trypomastigotes are ignored.

d) Some workers [19, 37] who used spectrographic methods for cytochromes, either tested only the "adult" or monomorphic stages of *T. lewisi*, or the culture forms of *T. cruzi* and extended the results to "the blood forms of the *lewisi* group".

Great differences should be expected in the metabolism of the various species of trypanosomes but we think that, to be sure which are these differences, we have to be aware of what each stage means in the cycle of the flagellate. In other words, we should compare multiplication forms with multiplication forms and differentiated forms with differentiated forms.

COMMENTS ON THE CLASSIFICATION OF MAMMALIAN TRYPANOSOMES

In sucessive trials to classify mammalian trypanosomes [24], several characters have been proposed that cannot withstand a critical appraisal. Among these we could just mention polymorphism, continuity of multiplication and pathogenicity. If we come to development in the insect host, we have *T. rangeli* that in many respects is more like the *lewisi* group but is transmitted through the salivary glands of triatomid bugs [47]. As for the physiological characters that have been suggested [19] as one more proof of deep differences between groups of trypanosomes, we do not think that they have been properly evaluated.

We do not mean to say, of course, that species do not fall in natural groups. But differences between them are probably more gradual and less schematic than has been supposed.

That is why we still prefer to keep the name "*lewisi* group" as a conveniently non-committal label for the species we have been talking about in this paper. Since definitions must be given we tentatively define it as group of mammalian trypanosomes that do not multiply primarily as trypomastigotes in the bloodstream of the vertebrate host.

Anyway, one should try not to be biased by classifications made before a good knowledge of the organisms involved is achieved. It should be realized that tentative classifications (and definitions) may be very useful as long as they are recognized as subject to change. And this reminds us of a statetment made by SANDON [39] about Protozoa: "Protozoa" — he says — "are not a subkingdom and not a phylum. They are a convenience."

RESUMO

Sôbre o ciclo evolutivo de tripanosomas do grupo lewisi *e suas relações com outros tripanosomas de mamíferos*

Discutem-se algumas fases do ciclo evolutivo do *Trypanosoma cruzi* e espécies afins.

Sugere-se que as transformações por que passam os tripanosomas de mamíferos em seu ciclo evolutivo têm implicações fisiológicas semelhantes para tôdas as espécies, mas que nem sempre a morfologia é indicação da competência de cada estágio.

Ao contrário do grupo *brucei*, as espécies do grupo *lewisi* se multiplicam primàriamente fora da corrente circulatória do vertebrado e sob outras formas que não o tripomastigoto. As diferenças fisiológicas entre tripomastigotos do sangue periférico não podem, portanto, ser interpretadas como caracterizando diferenças entre os dois grupos, visto que essas formas não têm em ambos a mesma competência. Por outro lado, não se tem dado a devida importância ao polimorfismo do grupo *lewisi* — que parece ter a mesma significação fisiológica que lhe é atribuída no grupo *brucei*.

Por julgar necessária a reavaliação dos caractéres propostos nas classificações de tripanosomas, a Autora prefere conservar o

têrmo "grupo *lewisi*" para indicar espécies que, como *cruzi*, *lewisi*, *rangeli* e outras, podem ser patogênicas, polimórficas e transmitidas através da picada do inseto transmissor, mas que não se multiplicam primàriamente sob a forma de tripomastigotos na corrente circulatória do hospedeiro vertebrado.

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