

DIFFERENTIATION OF *HERPETOMONAS MEGASELIAE*: POPULATION AND PHYSIOLOGICAL CHANGES

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Herpetomonas megaseliae (1) was shown to undergo differentiation in culture. The generic character (opisthomastigote) was acquired near the end of the exponential growth phase due to posterior migration of the kinetoplast. Approximately 90% of exponential phase organisms were promastigotes but toward the end of rapid multiplication this percentage dropped to 50% while the relative proportion of opisthomastigotes increased. Physiological changes also occurred in which the organisms became increasingly cyanide-sensitive, anaerobic stimulation of glucose uptake was doubled, and anaerobic acid production was halved as the makeup of the culture population shifted. Differentiation was postulated to involve a metabolic shift from anaerobic to aerobic metabolism.

Herpetomonas megaseliae was described by Daggett *et al.* (1) from the phorid fly *Megaselia scalaris*. In the original description it was noted that in the case of *H. megaseliae*, the *Herpetomonas* generic character, opisthomastigote forms, appeared in culture but disappeared when the organisms were re-introduced into the natural hosts. Thus in culture a differentiation occurred in which the organism acquired its generic character but in the host a de-differentiation occurred in which the acquired character was lost. In a previous paper we discussed the ultrastructural changes accompanying the differentiation process in *H. megaseliae* (2). Differentiation was observed to involve a posterior migration of the kinetoplast, to a post-nuclear position, with concurrent expansion of the flagellar pocket. This paper is intended to complement the ultrastructural observations and describes the population and physiological changes which are associated with cytodifferentiation in this "lower" trypanosomatid flagellate.

METHODS

Stock cultures of *Herpetomonas megaseliae* (ATCC # 30210) were maintained in Mansour's medium with rabbit blood, in 5 ml screw cap tubes (1, 3) and transferred weekly by sterile loop.

Initial growth-population composition studies were done using Grace's Insect Cell Culture Medium (Grand Island Biological) with varying concentrations of lobster hemolymph (LH) and fetal bovine serum (FBS) (also GIBCO). Cultures were initiated by loop transfer or by inoculation

of 4.5×10^7 organisms from either Mansour's medium or Grace's plus 10% LH and 10% FBS. Flagellates were counted by hemocytometer at 24 hr intervals for 7 days and at each counting, culture smears were made, air dried, fixed in absolute methanol and Giemsa stained. In later studies a variety of culture conditions were used, e.g. various LH and FBS concentrations, screw cap tubes, Erlenmeyer flasks of volumes up to one liter, and different blood sources (human and rabbit) and blood concentrations in Mansour's medium. Although growth rates, absolute numbers of organisms at stationary phase, and proportions of the three morphological types in the population varied somewhat with culture conditions, opisthomastigotes appeared in every culture following the rapid growth phase. Thus, it was assumed that differentiation occurred under all culture conditions.

Cultures used for growth, population composition and physiological studies described in this paper were counted at regular intervals and the population composition, determined from stained smears, was expressed as percentages of promastigotes, paramastigotes and opisthomastigotes, as previously described (2).

Physiological studies were conducted on cultures initiated with early log phase organisms and grown in one liter flasks with 200 ml Mansour's medium base plus 30 ml hemolyzed, defibrinated (outdated) human blood. This culture system was used to provide "undifferentiated" flagellates 3 days post-inoculation and "differentiated" flagellates 5 days post-inoculation. Flasks

were prepared and inoculated in pairs. One flask was harvested and used on day 3 and the other on day 5. The proportions of three body types were determined. The flagellates were considered "undifferentiated" if more than 90% of the forms were promastigote and "differentiated" if less than 60% of the forms were promastigote. Cultures were harvested and washed by centrifugation in Locke's solution without glucose, counted by hemocytometer, and diluted to 10^8 flagellates/ml as previously described (4). Oxygen uptake and cyanide inhibition studies were conducted manometrically according to the methods of Umbreit *et al.* (5) and Zeledon and de Monge (6). Anaerobic glycolysis was determined manometrically (5, 7) under an atmosphere of 5% CO_2 -95% N_2 . NaHCO_3 was substituted for KH_2PO_4 in Locke's solution. Results of manometric studies are expressed as per cent differences. In the case of cyanide inhibition studies, results are expressed as

per cent inhibition of O_2 uptake as a function of cyanide concentration. Paired controls without cyanide were used to calculate non-inhibited O_2 uptake rates. In glycolytic studies, results are expressed as per cent stimulation of anaerobic CO_2 released from the bicarbonate incubation medium in the presence of $1.67 \times 10^{-3}\text{M}$ glucose compared to endogenous (control) rate of CO_2 released from the same medium (Fig. 5).

Pasteur effect studies were done on flagellate suspensions prepared as above and incubated aerobically and anaerobically (under continuously flowing atmosphere of 5% CO_2 -95% N_2) for 2 hr. At the end of incubation, organisms were precipitated with $\text{ZnSO}_4 \cdot \text{Ba}(\text{OH})_2$ and the glucose concentration of the incubation medium determined by the Glucostat method (Worthington Biochemical). Glucose uptake was calculated by subtraction of glucose concentration at 2 hr from initial concentration

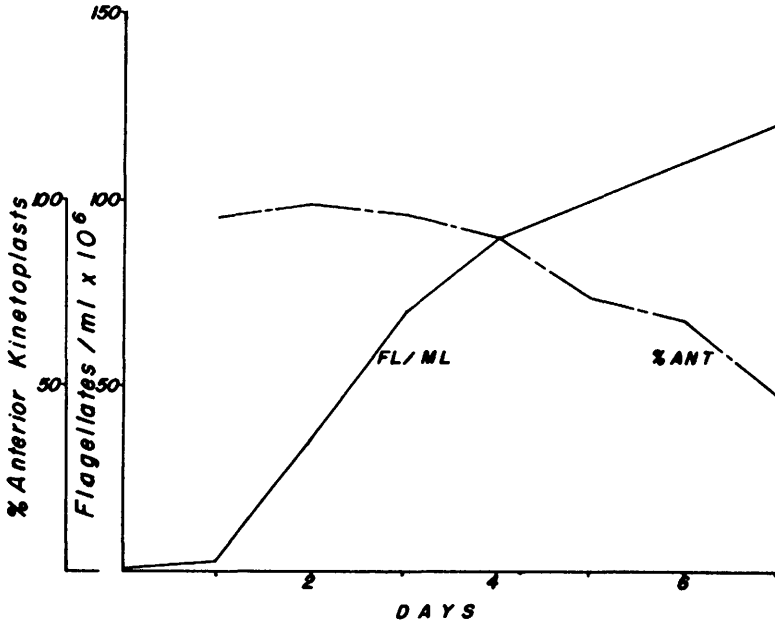


FIGURE 1. Population growth and changes in proportions of promastigotes (% anterior kinetoplasts) in *H. megastelae* cultures in Grace's medium plus 10% LH and 10% FBS.

(1.67×10^{-3}). Results are expressed as percent stimulation of glucose uptake by anaerobiosis (Fig. 5).

Reverse transformation studies were done on differentiated cultures from Mansour's medium in one liter screw cap flasks. Organisms were harvested aseptically by centrifugation and resuspended in fresh Mansour's medium. Smears were made of this suspension at 2-4 hr intervals for 26 hr and organisms were counted by hemocytometer at each interval. Percentages of the three body types in the flagellate suspension were determined as above, but in these studies the number of flagellates inoculated was much higher, relative to the volume of fresh medium, than in the growth studies.

RESULTS

Figure 1 shows population increase and proportionate decrease in promastigote forms when *H. megasteliae* was grown in 25 ml Grace's medium with 10% LH and 10% FBS in 125 ml screw-cap Erlenmeyer flasks. Figure 2 shows similar data, except that the culture medium was Mansour's with 15% (v/v) hemolyzed human blood

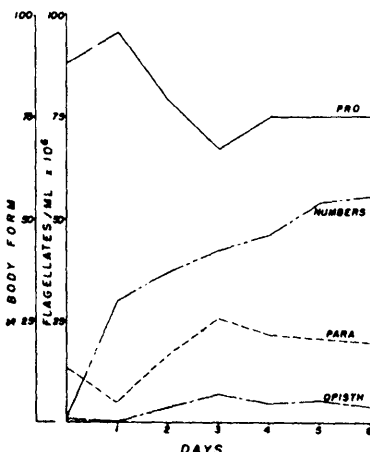


FIGURE 2. Population growth and changes in population composition in *H. megasteliae* cultures in Mansour's medium. "PRO", "PARA" and "OP1STH" = percentage of promastigotes, paramastigotes and opisthomastigotes respectively in the population.

and the proportions of the para- and opisthomastigote forms in the population also are shown. Figure 3 shows the relative distribution of the three body forms in the cultures used for physiological studies at days 3 and 5 post-inoculation.

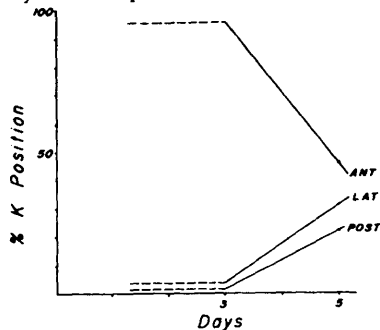


FIGURE 3. Population composition at 3 and 5 days post-inoculation in Mansour's medium in liter flasks. Abbreviations as in Fig. 2.

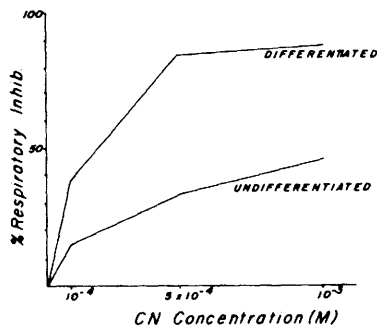


FIGURE 4. Differences in cyanide sensitivities of differentiated and undifferentiated cultures of *H. megasteliae*. Average of 7 experiments, 3-6 individual measurements per point per experiment.

Figure 4 indicates the respiratory response of "differentiated" and "undifferentiated" cultures to various concentrations of KCN in the incubation medium. It is evident from the curves that the respiration of undifferentiated forms was less sensitive to cyanide inhibition than the respiration of differentiated forms.

Figure 5 shows the effect of glucose upon anaerobic and aerobic "glycolysis" by dif-

ferentiated and undifferentiated forms. When flagellates from 3-day cultures were used, anaerobiosis stimulated glucose uptake about 200%; when flagellates from 5-day cultures were used, anaerobiosis stimulated glucose uptake about 400%. Differences in anaerobic acid production also were observed, e.g., with 3-day cultures, glucose stimulated acid production 350%, while with 5-day cultures, glucose stimulated acid production about 175%.

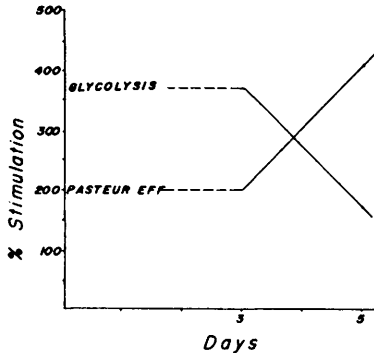


FIGURE 5. Differences in glycolytic stimulation by glucose and anaerobic stimulation of glucose uptake between 3- and 5-day cultures of *H. megastellae*. Average of 4 experiments with duplicate measurements per experiment per point in case of Pasteur effect. Average of 5 experiments with 4 individual determinations per point in case of glycolysis.

Herpetomonas megastellae has been maintained in a variety of culture media throughout 4 years. During this time, dividing flagellates consistently showed kinetoplasts anterior to nuclei. This observation was consistent with studies described by Wenyon (8), and led us to perform the reverse transformation experiments shown in Fig. 6. The data indicate that when differentiated cultures were resuspended in fresh blood-based culture medium, anterior migration of the kinetoplast occurred before an increase in flagellate numbers could be detected. There was an approximate 10 hr interval between the time when anterior kinetoplast migration was virtually completed and the time when an increase in flagellate numbers could be detected.

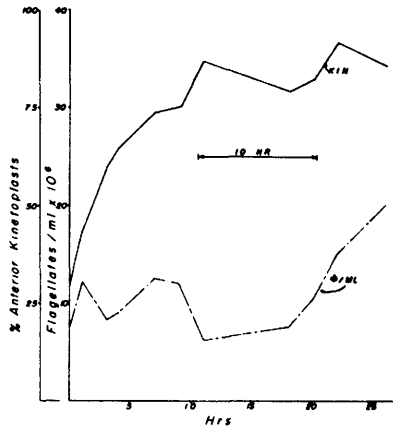


FIGURE 6. Reverse transformation (changes in proportion of promastigote forms) and flagellate numbers during first day after resuspension in fresh Mansour's medium.

DISCUSSION

The data presented here suggest that *Herpetomonas megastellae* development of the generic character (opisthomastigote) is accompanied by a physiological change in which the organisms acquire increased dependence upon aerobic metabolic pathways. It should be emphasized that differentiated cultures consisted of millions of cells of three different types, viz., promastigotes, paramastigotes, and opisthomastigotes, while undifferentiated cultures consisted of millions of cells, predominantly of a single type.

A variety of cytological forms have been recognized for *Herpetomonas* species both *in vitro* and *in vivo* (1, 9, 10, 11). Wenyon (8) implied that the promastigote of *H. muscarum* (= *H. muscae domesticae*) was the dividing form, while differentiation into the opisthomastigote occurred after the end of the multiplication. The results obtained with *H. megastellae* support such an interpretation, but both Fig. 1 and Fig. 2 suggest that opisthomastigote forms appear before the population reaches a peak. Thus differentiation is an individual phenomenon with individual flagellates leaving the cell cycle (perhaps after a pre-programmed number of divisions?) and undergoing a

morphological change in which the kinetoplast migrates posteriorly. The generic character genome in *Herpetomonas* therefore provides some individuals with the capacity to undergo a morphological change after some time in a certain culture situation.

In the case of *H. megaseliae*, the significant observation may not be the fact of differentiation, but rather the events associated with de-differentiation. Figure 6 suggests that de-differentiation must occur before multiplication. This conclusion is consistent with Wenyon's (8) statement and our failure to observe dividing para- and opisthomastigote forms. Although only a portion of the population in a differentiated culture is opisthomastigote, Fig. 6 indicates that virtually the entire population must attain the promastigote condition before a population increase is observed. It is conceivable that even though the entire population is not morphologically differentiated, it may be biochemically differentiated (12). This idea is supported by ultrastructural observations which show that even in paramastigote forms, the Golgi apparatus is inflated in comparison with that of promastigote forms (2). Of particular interest in *H. megaseliae* is the approximate 10 hr lag between attainment of near 100% promastigote by the population and the onset of multiplication by the same population. We interpret this observation as manifestation of a requirement for macromolecular synthesis, re-adjustment of differentiation control mechanisms and/or biochemical de-differentiation prior to mitosis. If requirements for biochemical de-differentiation are found to be common among the Trypanosomatidae, such shifts might be useful in the chemotherapy of those species involved in areas of medical or veterinary concern.

Physiological differences between trypanosomatid life cycle stages have been described for a number of species, however, *H. megaseliae* is the only lower (monogenic) trypanosomatid for which such differences have been reported. The nature of the reported physiological differences between two *H. megaseliae* populations is not unlike that of other, higher, trypanosomatids, however, the direction of change with differentiation in *H. megaseliae* is not entirely like that of other

trypanosomatids. Metabolic shifts in members of the genus *Trypanosoma* are generally toward aerobiosis in the dividing culture forms, but *T. brucei* may pass through a cyanide-insensitive phase, in broth containing relatively high blood-lysate concentration, before establishing itself as a cyanide-sensitive form capable of growth in broth of relatively low blood-lysate concentration (13). Perhaps the curves shown in Fig. 2 were produced by a similar set of events. If analogy exists, the new promastigotes appearing after day 4 may have used "recycled" molecules or newly synthesized molecules; if the latter, then biochemical differentiation may have been more drastic than suspected. In any case, these physiological shifts take their place in the large body of literature describing such shifts in general in trypanosomatid life cycles (7, 13, 14).

Observations on the ultrastructure of *H. megaseliae* complement those of previous workers, on *Leishmania donovani*. In the latter species, transformation from an intracellular amastigote, in the vertebrate host, to a culture promastigote, is accompanied by a metabolic shift to aerobiosis (15, 16). The aerobic *L. donovani* promastigote of course is the metabolic opposite of the relatively anaerobic *H. megaseliae* promastigote; however, ultrastructural changes involved in the anaerobic-aerobic metabolic shift are remarkably similar in the two species. Specifically, there is a change in the nature of the mitochondrial matrix material, in both species, which accompanies the metabolic shift to relative aerobiosis, regardless of the body form involved (2, 17). Rudzinska *et al.* (17) observed a clearing and concurrent aggregation of particles in the mitochondrial matrix accompanying anaerobic to aerobic transformation in *L. donovani*, and Janovy *et al.* (2) observed identical changes in the mitochondrial matrix material of *H. megaseliae* accompanying the equivalent metabolic shift.

Finally, although the structural and metabolic differences between different *H. megaseliae* development stages and those reported for other members of the Trypanosomatidae are similar in kind but perhaps dissimilar in direction, we feel that *H. megaseliae*, because of its ease of culture, and simple life cycle and adaptability to

many culture conditions and environments (1, 18) may be the very tool with which many basic principles of trypanosomatid development biology will be eventually carved out of the ever-growing mass of information about this important family of protozoan parasites.

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