Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 45 (1988)
Heft: 2

Artikel: Requirements of defined cultivation conditions for standard growth of "Leishmania" promastigotes in vitro
Autor: Lemesre, J.L. / Darcy, F. / Kweider, M.
DOI: https://doi.org/10.5169/seals-314063

Acta Tropica *45*, 99–108 (1988)

Centre d'Immunologie et de Biologie Parasitaire, Unité Mixte INSERM U 167 – CNRS 624, Institut Pasteur de Lille, France

Requirements of defined cultivation conditions for standard growth of *Leishmania* promastigotes in vitro

J. L. LEMESRE*, F. DARCY, M. KWEIDER, A. CAPRON, F. SANTORO

Summary

The growth characteristics of *L. chagasi* (MHOM/BR/79/LI01) and *L. braziliensis* (MHOM/BR/72/1670), the causative agents of visceral and muco-cutaneous leishmaniases, respectively, were compared. Inoculum size clearly influences the growth course of both *Leishmania* species, whatever the culture medium used (serum-supplemented media: GLSH or RPMI, and a chemically defined medium: LITR9). Cultures initiated with low concentrations failed to promote cell growth, while typical growth curves were obtained when higher promastigote inocula were used. For all the species tested, the higher the initial density of flagellates in the medium, the shorter were the periods covered by the latent and particularly by the logarithmic growth phases. In contrast, using constant inocula, variations in the volume of the incubation medium did not change the time-course of the different culture phases of either *Leishmania* species, provided that the ratio of incubation medium to total flask volume was comparable. Only cell division time significantly increased with the culture volume. We also determined whether or not the growth characteristics of the promastigotes of *L. chagasi* or *L. braziliensis* could be generalized to other members of the genus. Our results show that, whatever the culture medium used, *L. infantum* behaves in the same way as does *L. chagasi,* whereas *L. panamensis, L. guyanensis, L. mexicana* and *L. amazonensis* display growth patterns similar to that of *L. braziliensis.*

Key words: *Leishmania* promastigotes; growth characteristics; reproducible growth curves; defined cultivation conditions.

* Present address: Office de la Recherche Scientifique et Technique d'Outre-mer (ORSTOM), Centre de Brazzaville, B.P. 181, Brazzaville, République Populaire du Congo

Correspondence: Dr J. L. Lemesre, Centre d'Immunologie et de Biologie Parasitaire, Institut Pasteur, B.P. 245, 1 Rue du Professeur A. Calmette, 59019 Lille Cedex, France

Introduction

Leishmania species have a simple life cycle which includes a flagellate promastigote form in the sandfly vector and a non-flagellate intracellular amastigote stage within the mononuclear phagocytes of vertebrate hosts.

In cell-free medium at 26 °C, the parasites grow and multiply as a promastigote form similar to that found in the vector (Bray, 1974) which remains infective when injected into susceptible hosts (Keithly, 1976). In vitro cultivation of *Leishmania* species opened up new possibilities for the determination of the biochemical and immunological characteristics of these parasites (Schnur et al., 1972; Chance et al., 1974; Kreutzer and Christensen, 1980; McMahon-Pratt et al., 1982). In particular a large range of studies focused on the comparison of surface antigens of various *Leishmania* species and sub-species (Handman and Hocking, 1982; Lepay et al., 1983; Gardiner et al., 1984; Lemesre et al., 1985).

Recent work has shown a sequential development of promastigotes from a noninfective to an infective stage in the case of *L. tropica* and *L. chagasi,* both in vitro (Sacks and Perkins, 1984; Rizvi et al., 1985) and in the sandfly vector (Sacks and Perkins, 1985). In addition, modifications in the expression of some surface components of promastigotes during the course of culture appear to be associated with their infectivity (Sacks et al., 1985; Rizvi et al., 1985; Kweider et al., 1987). These clearly defined changes in the parasite during in vitro differentiation highlights the utmost importance of defined culture conditions in order to obtain reproducible growth curves with well controlled maturation stages.

To further compare the biological behaviour of *L. braziliensis* and *L. chagasi,* parasites responsible for two different pathological manifestations of leishmaniasis (muco-cutaneous and visceral), it was necessary to first standardize the culture conditions of both *Leishmania* species. As a second step, we attempted to determine whether or not the growth characteristics of the promastigotes of *L. chagasi* or *L. braziliensis* could be generalized to other members of the genus *Leishmania.*

Materials and Methods

Parasites. – The following strains of visceral, cutaneous and mucocutaneous *Leishmania* species used throughout this study were generously provided by Dr D. Le Ray (ITMA, Anvers, Belgium) and Dr M. Sadigursky (FIOCRUZ, Salvador de Bahia, Brazil): *L. chagasi* (MHOM/BR/79/LI01), *L. infantum* (MHOM/BL/67/IT263), *L. braziliensis* (MHOM/BR/72/1670), *L. panamensis* (MCHO/PA/00/M4039), *L. guyanensis* (MHOM/BR/78/M5378), *L. mexicana* (MNYC/BZ/62/M379) and *L. amazonensis* (MHOM/VE/76/JAP78). They were maintained in our laboratory by subculture in liquid monophasic media.

Culture media. – GLSH (Glucose, Lactalbumin, Serum and Haemoglobin) developed by Jadin and Le Ray (1969) and RPMI 1640, supplemented with 10% (vol/vol) heat-inactivated bovine serum were used. Crystalline penicillin and streptomycin were added to a final concentration of 50 to 100 μg per milliliter to decrease the risk of bacterial contamination.

A chemically defined culture medium, recently described and called LITR9 (Sadigursky and Brodskyn, 1986) was also used. To basic LIT medium containing liver infusion broth and tryptose, a mixture of RPMI 1640 and Medium 199 was added. This combination permitted high parasite yields.

Cultivation procedure. – The standard incubation temperature was 26±1°C. Cultures of promastigotes of all *Leishmania* species were routinely maintained by bi-weekly passage of mid-log phase parasites into 50 ml of fresh GLSH, RPMI or LITR9 culture media. Initial concentrations of promastigotes were 10^5 flagellates per ml for *L. chagasi* and *L. infantum* and 5.0×10^5 flagellates per ml for the other *Leishmania* species. For the standardization of *L. chagasi* and *L. braziliensis* cultures, variable concentrations of mid-log phase promastigotes (see results) were transferred to 250 ml flasks containing 50 ml of culture medium. Cultivation of mid-log phase promastigotes at a precise density was also performed in 250, 50 or 25 ml calibrated flasks (Gravis, France) containing 50, 10 or 5 ml of culture medium, respectively. Two replicate experiments using three replicate culture flasks per test were carried out for 10^5 and 5.0×10^5 initial concentration in GLSH, RPMI and LITR9 media.

Promastigote counts. – 20 µl of Vortex-homogenized culture samples were mixed with 20 µl of Hanks balanced salt solution (HBSS) containing 0.2% glutaraldehyde (SERVA, Heidelberg, W. Germany). The flagellate concentrations were determined after adequate dilution in HBSS by enumeration of fixed promastigotes in a Thoma-counting chamber at 400× magnification. Simultaneously, Erythrosin B staining was used to differentiate between alive and dead cells. 50 µl promastigote suspension and 50µl 0.4% Erythrosin B staining solution were previously precooled to 4°C and then mixed. After 5 min incubation in ice, one drop was examined at 400× magnification in order to determine the percentage viability (stained promastigotes were nonviable).

Results

The following results were obtained by counting daily the promastigote number of various *Leishmania* species in different culture conditions. Since standard deviations for each test point were below 5% of the corresponding mean values, the data could be compared for significant differences. In parallel, Erythrosin B staining demonstrated that for all species studied the percentage mortality was less than 5% during the exponential growth phase and reached 15 to 20% in the late stationary phase (data not shown).

Fig. 1, which represents the growth curves of *L. chagasi* (A) and *L. braziliensis* (B) promastigotes in GLSH medium, clearly demonstrates the obvious influence of the inoculum on the multiplication of both parasites and in addition significant differences in the growth rates of the two *Leishmania* species. Culture initiated with low concentrations (10^4 *L. chagasi* and 2.0×10^5 *L. braziliensis* parasites per ml) failed to grow, while typical growth curves were obtained when higher promastigote concentrations were used. Cultures initiated with densities ranging from 10^5 to 10^6 organisms per ml for *L. chagasi* (Fig. 1 A) and from 5.0×10^5 to 10^6 parasites per ml for *L. braziliensis* (Fig. 1 B) supported intensive growth. Moreover, the time when the latent phase ended and the point at which the logarithmic growth phase merged into the stationary phase, were also dependent on the original density. The latent phase completely disappeared when inocula of 5.0×10^6 promastigotes and more were used (data not shown). Greater numbers of flagellates in the inocula resulted in shorter latent and logarithmic phases and in a higher number of generations. Indeed, the

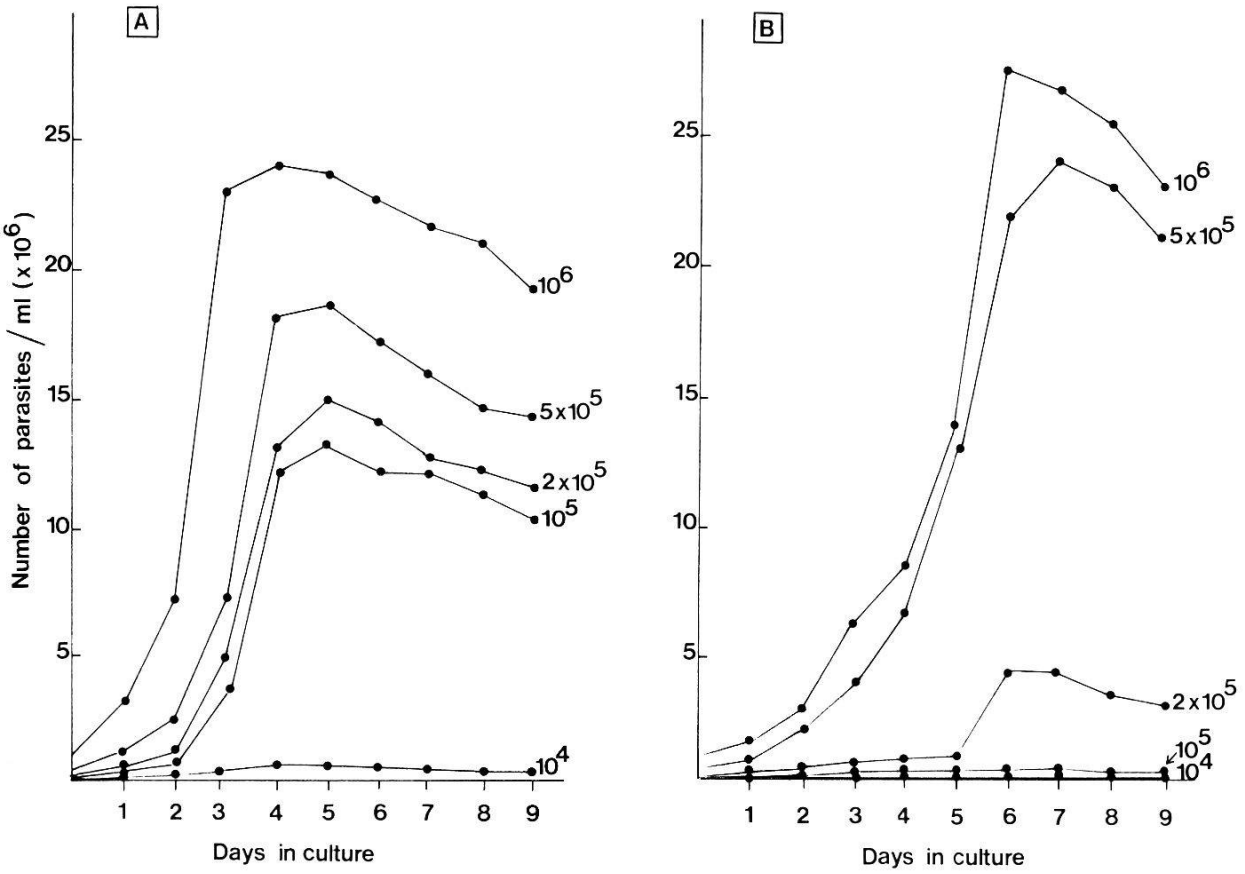

Fig.1. Influence of the initial inoculum of promastigotes on the growth curves of *L. chagasi* (A) and *L. braziliensis* (B) in GLSH-medium. Original concentrations (number of flagellates/ml) are indicated. The volume of incubation medium was constant throughout this study (50 ml).

number of promastigotes (x) at peak growth was greater in cultures started with higher densities of organisms (y). Nevertheless the ratio x/y gradually decreased as y increased (Fig. 1).

Significant differences in the growth rate were also observed between *L. chagasi* and *L. braziliensis* cultures. Fig. 1 shows that with the initial population densities of 5.0×10^5 or 10^6 parasites per ml *L. chagasi* cultures always entered more quickly into the stationary phase than did *L. braziliensis* cultures; nevertheless, the number of parasites at the stationary phase was always lower with *L. chagasi.*

RPMI medium was also used to cultivate these two *Leishmania* species. Of interest is the fact that this medium did not support sustained growth of *L. braziliensis* (data not shown). By contrast, RPMI was able to promote a significant multiplication of *L. chagasi* promastigotes when adequate parasite concentrations were used (Fig. 2). Similar effects of the concentration of the inoculum on the growth of *L. chagasi* were found in RPMI medium when compared to GLSH medium. However, total cell yields were slightly higher in RPMI medium whatever the original population densities used.

The growth curves represented in Fig. 3 show the influence of the original volume of GLSH medium on *L. braziliensis* (A) and *L. chagasi* (B) cultures

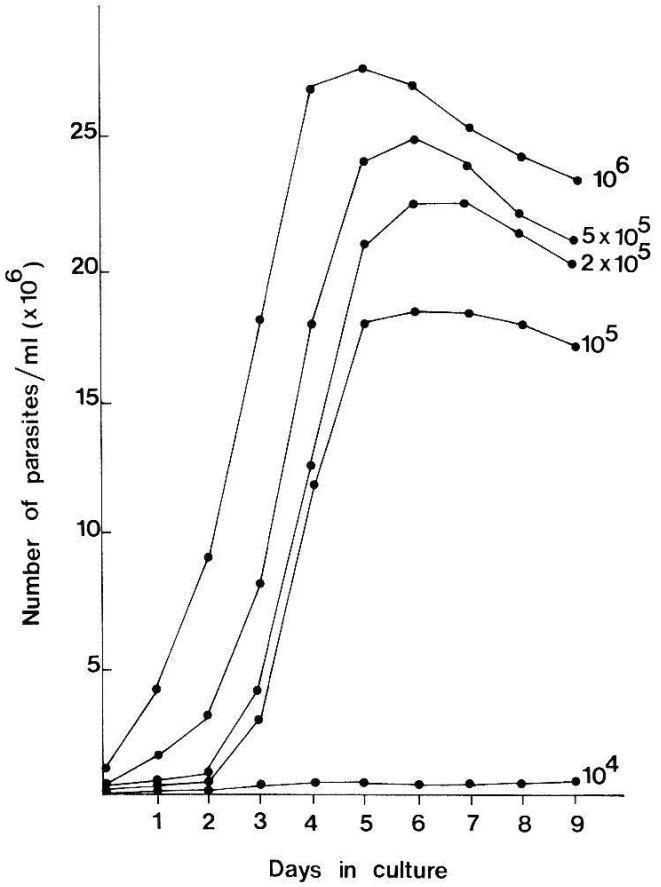

Fig. 2. Effect of the initial inoculum of promastigotes on the growth course of *L. chagasi* in RPMI medium. Original concentrations (number of flagellates/ml) are indicated. The volume of incubation medium was 50 ml.

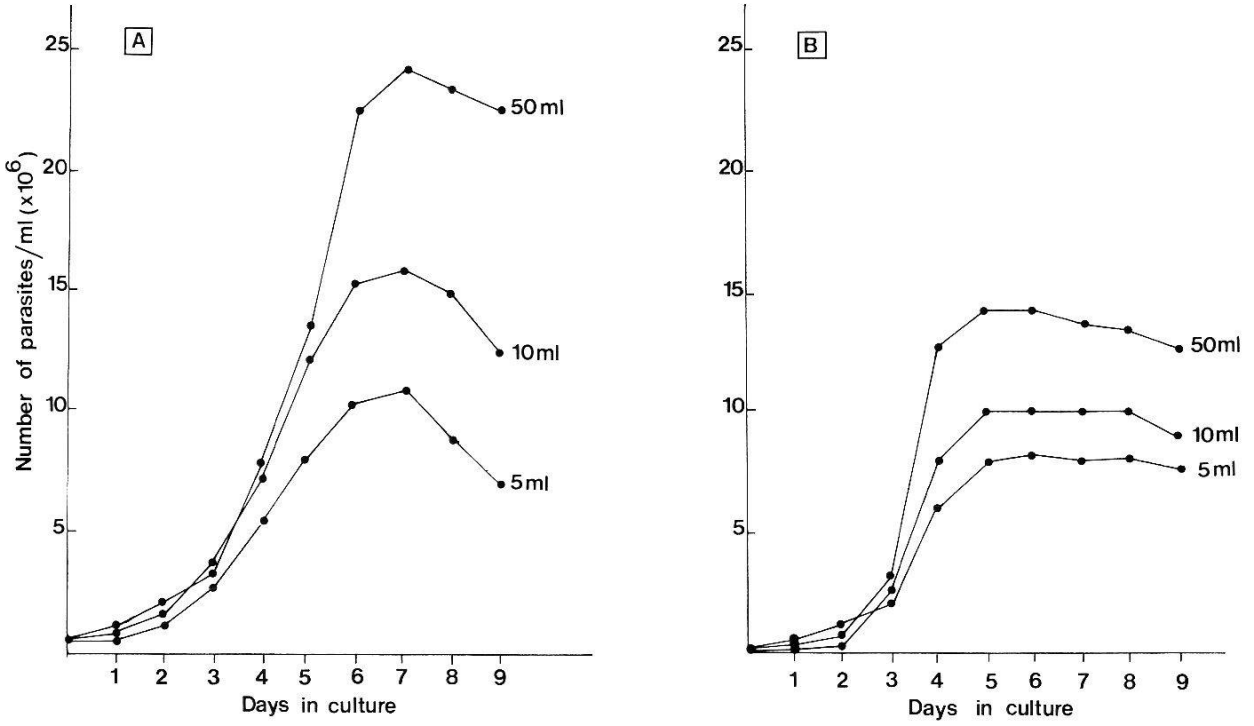

Fig. 3. Influence of the initial volume of incubation medium (GLSH) on the growth rate of *L. braziliensis* (A) and *L. chagasi* (B) culture initiated with constant densities of mid-log phase promastigotes (5.0×10^5 and 10^5 parasites per ml, respectively).

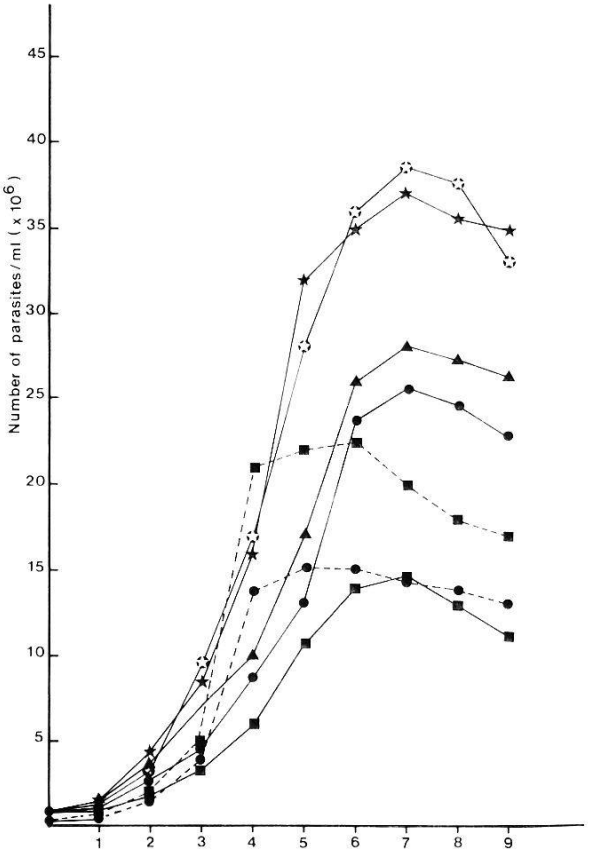

Fig. 4

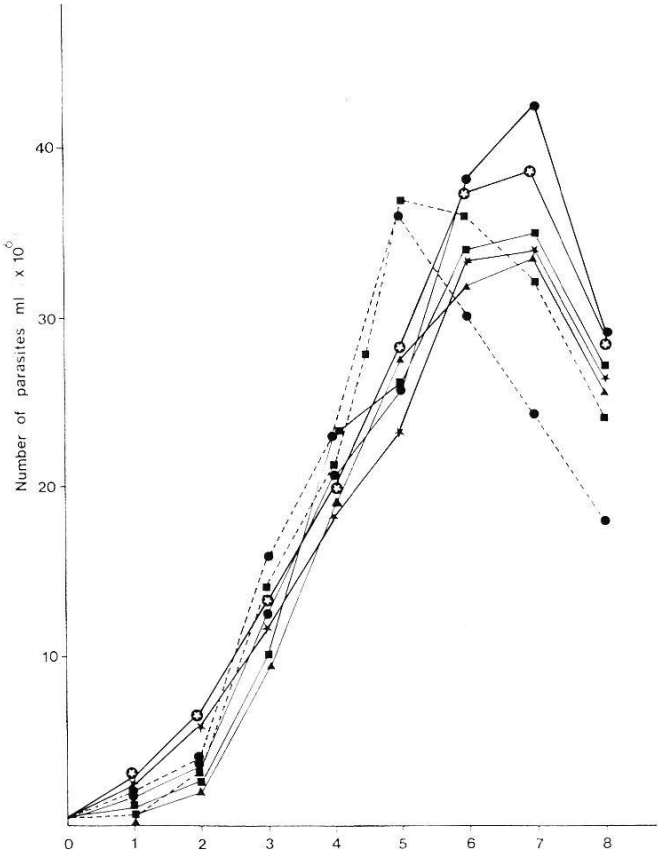

Fig. 5

Fig. 4. Growth patterns of *L. chagasi* (●– – –●), *L. infantum* (■– – –■), *L. braziliensis* (●——●), *L. panamensis* (▲——▲), *L. guyanensis* (■——■), *L. mexicana* (★——★), and *L. amazonensis* (✪——✪) culture in GLSH medium (50 ml) initiated with 10^5 promastigotes per ml for *L. chagasi* and *L. infantum* and 5.0×10^5 flagellates per ml for the other *Leishmania* species.

Fig. 5. Growth patterns of *L. chagasi* (●– – –●), *L. infantum* (■– – –■), *L. braziliensis* (●——●), *L. panamensis* (▲——▲), *L. guyanensis* (■——■), *L. mexicana* (★——★), and *L. amazonensis* (✪——✪) culture in LITR9 medium (50 ml) initiated with 10^5 promastigotes per ml for *L. chagasi* and *L. infantum* and 5.0×10^5 flagellates per ml for the other *Leishmania* species.

initiated with constant densities of mid-log phase promastigotes (5.0×10^5 and 10^5 parasites per ml, respectively). The ratio of culture volume to total flask volume was constant throughout this study (1/5). The general configuration of the culture curves of *Leishmania* species did not change when the volume of incubation medium varied from 5 to 50 ml. Indeed, periods covered by the latent and logarithmic growth phases were similar for each parasite. By contrast, total cell yield significantly increased when the volume of the incubation medium was larger, demonstrating an increase in the speed of promastigote multiplication.

The reported statements concerning the influence of inoculum concentration and incubation volume on *L. chagasi* and *L. braziliensis* growth, established in both GLSH and RPMI media were also demonstrated for the two species in a completely defined medium, LITR9 (data not shown).

The culture curves represented in Fig. 4 and Fig. 5 show the growth characteristics of other visceral and cuteaneous *Leishmania* species in GLSH and LITR9 media, respectively. Initial inocula of 10^5 parasites per ml for *L. chagasi* and *L. infantum*, and 5.0×10^5 flagellates per ml for *L. braziliensis, L. panamensis, L. guyanensis, L. mexicana* and *L. amazonensis* were chosen, since they lead to well-separated growth phases. Two growth patterns issue from these data. The first one is observed with both causative agents of visceral leishmaniasis, where the promastigotes switch from the logarithmic to the stationary phase of growth at about 5 days after the seeding. The second one is obtained with other species. Although the cultures were started with higher original density, the periods covered by the logarithmic growth phase was always longer and parasites entered the stationary phase only at the 7th day. In contrast to data observed with GLSH medium (Fig. 4), the stationary phase was very short when parasites were cultured in LITR9 (Fig. 5).

Discussion

A basic question emerges from recent studies on the importance of the maturation stage of *L. tropica* (Sacks and Perkins, 1984; Sacks et al., 1985), *L. chagasi* (Rizvi et al., 1985), *L. mexicana* (Mallinson and Coombs, 1986), and *L. braziliensis* (Kweider et al., 1987) culture promastigotes on infectivity, surface antigenic expression and enzyme content. Are the characteristics described for the above four species applicable to all other members of the genus? In order to answer this question, as a first step, it is important to determine whether or not different culture conditions exert a noticeable influence on the duration of the different growth phases of *Leishmania* cultures.

In the present work, we compared the growth characteristics of two different *Leishmania* species, *L. chagasi* and *L. braziliensis*, the causative agents of visceral and muco-cutaneous leishmaniases, respectively. This study was performed using various culture media: a new completely defined LITR9 medium (Sadigursky and Brodskyn, 1986) and two serum-supplemented monophasic media, RPMI 1640 and GLSH which is widely used in the cultivation of Old and New World cutaneous and visceral leishmanial organisms (Le Ray, 1974). While *L. chagasi* was capable of sustained in vitro growth in all these media, *L. braziliensis* failed to multiply in RPMI medium. It is worth mentioning that the latter species is difficult to grow in vitro (Le Ray et al., 1973), which explains why very little biochemical and immunological data have been obtained for this parasite.

Our study shows that the original parasite concentration influences the growth curve of both *Leishmania* species whatever medium was used (GLSH, RPMI or LITR9). First, parasites failed to multiply when their initial densities were too low which seems to indicate that growth is dependent on a minimal concentration of some metabolites, either excreted-secreted or processed from

the culture medium by the parasites. Indeed a recent study has demonstrated that media conditioned by *Leishmania* promastigotes could play a key role in their in vitro growth regulation (Lemesre et al., in preparation). In each species, when original flagellate concentrations of 10^6 parasites per ml or more were used, the latent and particularly the logarithmic growth phases were shorter. Moreover, *L. chagasi* multiplication occurred faster than that of *L. braziliensis.* Indeed, with comparable initial densities, there is a difference of about two days between the time at which *L. chagasi* and *L. braziliensis* entered the stationary phase. In contrast, using initial concentrations of 10^5 and 5.0×10^5 parasites per ml for *L. chagasi* and *L. braziliensis,* respectively, variations in the incubation medium volume did not change the time-course of the different culture phases of either *Leishmania* species. Only the rate of multiplication significantly increased with the culture volume. These data are of consequence when mass cultures are required. Indeed, we have found that using the original densities defined above, identical culture courses are obtained when these parasites are grown in 200 ml of GLSH medium (data not shown), provided that the ratio of incubation medium volume to total flask volume is comparable (1/5). This method has enabled us to produce large stocks of well-defined promastigotes from each maturation stage that are required for further studies of the parasites differentiation.

In view of the important differences observed between the culture patterns of *L. chagasi* and *L. braziliensis,* as a second step, we have compared the growth characteristics of other members of the *Leishmania* genus. Interestingly, in our culture conditions, the different species studied fall into only two categories, no intermediate patterns being observed. Indeed, *L. infantum* behaves in the same way as does *L. chagasi,* whereas species causing cutaneous disease display growth patterns similar to that of *L. braziliensis.* It is not yet possible to definitely establish a correlation between the growth characteristics of the *Leishmania* species and their clinical expressions, because the strains studied were adapted to long-term culture and thus do not reflect the characteristics of recently isolated parasites. Whilst Dvorak (1984) has demonstrated a variation in initial growth rate for different *Trypanosoma cruzi* cloned strains, the results presented here clearly show that growth characteristics differentiate *Leishmania* species. In this case, a possible strain effect can be presumed unlikely although this point remains to be tested. This study clearly demonstrates that, for instance, after 5 days, *L. infantum* and *L. chagasi* entered the stationary phase whereas the other species remained in the logarithmic phase. These data suggest that standard conditions for reproducible culture curves should be established in order to discriminate parasites from latent, logarithmic and stationary phases prior to physiological, biochemical and immunological studies depending on the maturation stages of *Leishmania* promastigotes.

All comparative studies on the biological properties of various *Leishmania* species reported before the work of Sacks and Perkins (1984) and Sacks et al.

(1985) were done either with non-defined stages (Schnur et al., 1972; Chance et al., 1974; Ramasamy et al., 1981; Handman et al., 1981; Handman and Hocking, 1982; Lepay et al., 1983) or poorly controlled log-phase promastigotes (Gardiner et al., 1984; Etges et al., 1985; Lemesre et al., 1985). Indeed, investigators have usually considered cultured promastigotes to be biologically uniform populations. The immediate question resulting from the finding of Sacks et al. (1985) is whether the differences previously reported between *Leishmania* species were due to biological changes accompanying promastigote differentiation or relate to genuine species characteristics. In the future, taxonomic classifications of *Leishmania* species based on isoenzyme or comparative surface antigenic studies should take the potential biological heterogeneity of cultured promastigotes into account and should be performed with parasites from standardized cultures with well-defined growth phases.

Acknowledgments

This work received financial support from the CEE [grant TSD-M-010-F(RS)], the Rockfeller Foundation, and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. – We are grateful to Drs R. J. Pierce and D. Afchain for invaluable help in correction of this manuscript. – We also are thankful for the expert technical assistance of Marc Loyens and the secretarial help of Claudine Colson and Marie-France Massard.

Bray R. S.: *Leishmania.* Ann. Rev. Microbiol. *28,* 189–217 (1974).

Chance M. L., Peters W., Shchory L.: Biochemical taxonomy of *Leishmania.* I. Observations on DNA. Ann. trop. Med. Parasit. *68,* 307–316 (1974).

Dvorak J. A.: The natural heterogeneity of *Trypanosoma cruzi:* biological and medical implications. J. cell. Biochem. *24,* 357–371 (1984).

Etges R. J., Bouvier J., Hoffman R., Bordier C.: Evidence that the major surface proteins of three *Leishmania* species are structurally related. Molec. biochem. Parasit. *14,* 141–149 (1985).

Gardiner P. R., Jaffe C. L., Dwyer D. M.: Identification of cross-reactive promastigote cell surface antigens of some leishmanial stocks by ^{125}I labeling and immunoprecipitation. Infect. Immun. *43,* 637–643 (1984).

Handman E., Hocking R. E.: Stage specific, strain-specific and cross-reactive antigens of *Leishmania* identified by monoclonal antibodies. Infect. Immun. *37,* 28–33 (1982).

Handman E., Mitchell G. F., Goding J. W.: Identification and characterization of protein antigens of *Leishmania tropica* isolates. J. Immunol. *126,* 508–512 (1981).

Jadin J. B., Le Ray D.: Acquisitions récentes dans les techniques de culture des trypanosomes africains. Ann. Soc. belge Méd. trop. *49,* 331–340 (1969).

Keithly J. S.: Infectivity of *Leishmania donovani* amastigotes and promastigotes for golden hamsters. J. Protozool. *23,* 244–245 (1976).

Kreutzer R. D., Christensen H. A.: Characterization of *Leishmania* species by isoenzyme electrophoresis. Amer. J. trop. Med. Hyg. *29,* 199–208 (1980).

Kweider M., Lemesre J. L., Darcy F., Kusnierz J. P., Capron A., Santoro F.: Infectivity of *Leishmania braziliensis* promastigotes is dependent on the increasing expression of a 65,000 dalton surface antigen. J. Immunol. *138,* 299–305 (1987).

Lemesre J. L., Rizvi F. S., Afchain D., Sadigursky M., Capron A.: Sub-species specific surface antigens of promastigotes of the *Leishmania donovani* complex. Infect. Immun. *50,* 136–141 (1985).

Lepay D. A., Nogueira N., Cohn Z.: Surface antigens of *Leishmania donovani* promastigotes. J. exp. Med. *157*, 1562–1572 (1983).

Le Ray D.: Structures antigéniques de *Trypanosoma brucei brucei* (Protozoa, Kinetoplastida): analyse immunoélectrophorétique et étude comparative. Doctorat es-Sciences, Louvain 1974.

Le Ray D., Afchain D., Jadin J., Capron A., Yasarol S., Lanotte G., Fameree L.: Diagnostic immunoélectrophorétique de la leishmaniose viscérale à l'aide d'un extrait antigénique hydrosoluble de *Leishmania donovani*. Résultats préliminaires. Ann. Soc. belge Méd. trop. *53*, 31–41 (1973).

Mallinson D. J., Coombs G. H.: Studies on the "Metacyclics" of *Leishmania*. British Society for Parasitology. Spring Meeting, 8–10 April 1986 (Abstract N° 15) (1986).

McMahon-Pratt D., Bennet E., David J. R.: Monoclonal antibodies that distinguish subspecies of *Leishmania braziliensis*. J. Immunol. *129*, 926–927 (1982).

Ramasamy R., Jamnadas H., Mutinga M. J.: Proteins and surface proteins of *Leishmania* promastigotes and their possible relevance to the characterization of strains. Int. J. Parasit. *11*, 387–390 (1981).

Rizvi F. S., Afchain D., Sherlock I., Sadigursky M., Capron A., Santoro F.: Infectivity of *Leishmania* promastigotes is associated with surface antigenic expression. Immunol. Lett. *11*, 317 (1985).

Sacks D. L., Perkins P. V.: Identification of an infective stage of *Leishmania* promastigotes. Science (Wash. D.C.) *223*, 1417–1419 (1984).

Sacks D. L., Perkins P. V.: Development of infective stage of *Leishmania* promastigotes within phlebotomine sandflies. Amer. J. trop. Med. Hyg. *34*, 456–459 (1985).

Sacks D. L., Hieny S., Sher A.: Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania* major promastigotes. J. Immunol. *135*, 564–569 (1985).

Sadigursky M., Brodskyn C. I.: A new liquid medium without blood and serum for culture of hemoflagellates. Amer. J. trop. Med. Hyg. *35*, 942–944 (1986).

Schnur L. F., Zuckerman Z., Greenblatt C. L.: Leishmanial serotypes as distinguished by the gel diffusion of factors excreted in vitro and in vivo. Israel J. med. Sci. *8*, 932–944 (1972).