BHATTACHARYA, Amal, 1938-

BIOLOGY AND PARASITE-HOST CELL RELATIONSHIPS OF LEISHMANIA DONOVANI (PROTOZOA: PROTOMONADIDA).

The University of Nebraska - Lincoln,
Ph.D., 1973
Zoology

BIOLOGY AND PARASITE-HOST CELL RELATIONSHIPS OF *LEISHMANIA DONOVANI* (PROTOZOA: PROTOMONADIDA)

by

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A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebraska-Lincoln

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Department of Zoology

Under the Supervision of Professor John Janovy, Jr.

Lincoln, Nebraska

December, 1973

TITLE

Biology and parasite-host cell relationships of *Leishmania donovani* (Protozoa : Protomonadida)

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GRAD S2002 300 5-68(7)

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ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. John Janovy, Jr. for his advice and guidance throughout the course of this investigation. I express my gratitude to the late Dr. Leslie A. Stauber of Rutgers-The State University, for kindly making the infected animals available to us. I thank Dr. M. R. Banerjee for his advice and partial use of his laboratory facilities. I also thank Mr. Pierre M. Daggett, and Miss Joan Elise Decker for their constant help and cooperation. Especially, to my wife, Anjana, I wish to express my deepest appreciation for her encouragement.

Amal Bhattacharya

INTRODUCTION

Leishmania donovani, the causative agent of human kala-azar (visceral leishmaniasis) has a dimorphic life cycle. The elongate promastigote form with flagellum and anterior kinetoplast inhabits the gut of an insect vector and can be maintained in laboratory at 25 C in a suitable blood based medium. In a mammalian host (near 37 C) promastigotes lose their flagellum and transform into intracellular, rounded, amastigotes. Greenblatt and Glaser (1965), Guttman (1963), Janovy (1967), Lamy (1969), Lemma and Schiller (1964), Simpson (1968), Trager (1953), and Trager and Krassner (1967) have considered temperature shift to be a major triggering factor and transformation inducer in the genus *Leishmania*.

In *L. donovani*, alterations of temperature (20-40 hours after change from 37 C to 27 C) or nutritional factors or both may be the inductive force in the transformation from amastigote to promastigote *in vitro* (Simpson, 1968). This transformation involves a shift to aerobic metabolism and increased respiratory rate (Janovy, 1967; Simpson, 1968). Extracellular cultivation of the amastigote has been attempted at 37 C (Trager, 1953). Limited development of forms morphologically and physiologically "intermediate" between pro- and amastigotes, was reported. During the first 4 days of growth

the "intermediate" forms increased in number to four times the original count and then died in spite of repeated changes of culture medium. Trager (1953) suggested that these organisms might have exacting nutritional requirements, at 37 C, which were not present in the medium and which presumably must be supplied by a mammalian host. However, promastigote to amastigote transformation and the subsequent growth and multiplication of L. donovani in a cell free medium up to 34 C incubation temperature has been shown (Lemma and Schiller, 1964). The necessary studies of ultrastructure were not done to show equivalence with intracellular forms, however, growth at 34 C necessitated a lengthy adaptation through many generations. In L. enrietti, a parasite of guinea-pigs, at culture temperatures above 30 C there was a decline in viability, though there is an initial elevated respiratory capacity (Greenblatt and Glaser, 1965). At 34 C there was a very slow growth and above this point they were capable of one division only (Greenblatt and Glaser, 1965).

The critical question of infectivity of these artificial or "manufactured" amastigotes was not answered satisfactorily in any of these studies. The maintenance of Leishmania promastigotes in culture in blood-based medium is a common procedure in many laboratories. The infectivity for animals may be lost in this type of stock (Stauber, 1966). Unpublished data from our laboratory showed that L. donovani maintained by serial passage through hamster has 4-6 times more infectivity than laboratory stock cultures of the same strain. It was felt

a comparative study of the cell cycle of stock culture (SC) and recent isolate (RI) at 25, 35, 37 C would help to assess the equivalency of the two sources of infective material.

Although amastigotes unmistakably equivalent to those of hamster spleen have not yet been successfully cultured by themselves, by the above methods, infected cell culture work has shown that the amastigotes could be grown *in vitro* in mammalian cells at 32-37 C. *Leishmania enrietti* invaded tissue culture cells at 32-34 C (Castro and Pinto, 1960). Akiyama and Taylor (1970), Herman (1966), Miller and Twohy (1967) showed limited multiplication of amastigotes in infected macrophages in primary hamster and mouse peritoneal exudate cell cultures. Very little attention has been given to the biochemical relationships of the host parasite complex in the mammalian host. The reasons are likely the technical difficulty of cleanly separating amastigotes from host cells and the biological difficulty that parasites begin transforming as soon as they are removed from host cells. There is the additional problem of equivalency of "manufactured" amastigotes to those from spleen, although attempts to produce amastigotes with elevated temperature were in reality attempts to alleviate the first two problems. Amastigotes separated from spleen tissue have been shown not to be the ultrastructural equivalent of *in vivo* forms (Rudzinska, D'Alesandro, and Trager, 1964). Although evidently not yet attempted, the study of these biochemical relationships in

infected cell cultures seems a natural first step or preliminary approach to study of the same relationships in the intact host.

Thus the present study of Leishmania donovani investigates the following problems: (1) cell cycle differences between the less infectious SC and more infectious RI forms, (2) influence of temperature on the cell cycle of SC and RI, and (3) macromolecular interaction between the parasite and the host cell following infection as a possible initial step in creating an environment conducive to parasite growth.

MATERIALS AND METHODS

Stock Cultures and Recent Isolates:

Leishmania donovani, 2S strain, isolated in the Sudan directly from human to animal in November, 1961 (Stauber, 1966) was used. Laboratory stock cultures were maintained at 25 C in 25 x 125 mm screw cap culture tubes containing 2 ml Tanabe's Medium (Janovy, 1967) and loop transferred weekly. Hamster infections were maintained by intraperitoneal inoculation of infected spleen tissue, homogenized in Locke's Solution (Tobie *et al*, 1950), into noninfected hamster. The stock cultures (loop tranferred), and recent isolate (no more than one loop transfer following successful culture of infected spleen) are referred to as SC and RI respectively.

Growth Curves:

Growth curve data were obtained by cell counts in a haemocytometer. Samples were counted every 24 hours in 5 experiments and a mean growth curve was plotted.

Cell Cycle Methods:

All growth curve and cell cycle experiments were started with equal numbers of SC and RI flagellates in equal volumes (3 ml) of medium.

Radioactive precursors of DNA, RNA and protein were used to study the cell cycle of promastigotes, effect of temperature on promastigote macromolecular biosynthesis and interaction between host cell and parasite in vitro. Specific activities of the precursors used were: 1.9 ci/mmole ^{3}H-thymidine (Schwarz/Mann), 19.2 ci/mmole ^{3}H-6-uridine (Schwarz/Mann) and 38 ci/mmole ^{3}H-leucine (Amersham/Searle). In cell cycle studies cultures were initiated with equal numbers of promastigotes in SC and RI material. The cultures were incubated at 25 C for four or five days and then transferred to 25 C, 35 C, 37 C from time of pulsing onwards. The flagellates were pulse labelled for 15 minutes according to procedure of Steinert and Steinert (1962) with 0.5 µci/ml ^{3}H-thymidine. The cells were centrifuged at 1500 rpm for 5 minutes at room temperature and the pellet was resuspended in fresh medium containing a hundredfold concentration of nonradioactive thymidine 10 µgm (.04 µ moles) . Excess thymidine was added to prevent further incorporation of any left over radioactive thymidine during the chase period. Cells were harvested hourly for 24 hours. Cell cycle time was determined by scoring the frequency of labelled dividing forms at different intervals according to the methods of Howard and Pelc (Band and Mohrlok, 1972; Cameron and Jeter, 1970; Howard and Pelc, 1953; Lesher, Fry and Sacher, 1961; Prescott and Stone, 1967; Quastler and Sherman, 1959; Sisken, 1962; Sisken, Morasca and Kirby, 1965; Steinert and Steinert, 1962; Stubblefield, 1968; Van Assel and Steinert, 1971; Young, 1962). The experiment was repeated five times with same protocol.

Growth curves were determined on all control samples. The percentage of dividing forms were determined from control nonlabelled slides in five experiments. The cells were considered to be dividing if they had one nucleus and two kinetoplasts (1N2K), two nuclei and one kinetoplast (2N1K) or two nuclei and two kinetoplasts (2N2K).

The "mitotic" phase (or "D", see below) in all above cases of cell cycle studies, in synchronized and non-synchronized cultures was determined according to the following formula of Stubblefield (1968).

$$\theta = \frac{\ln (1 + \lambda)}{\ln 2}$$

where θ = mean duration of mitosis

λ = mean fraction of mitotic cells

There is a definite limitation in recognizing mitotic phases in the Trypanosomatidae (Cosgrove and Skeen, 1970; Van Assel and Steinert, 1971). The modification made by Steinert and Steinert (1962) and subsequently used by other workers in the field has been used. In cell cycle calculations D (= Division) replaced M (= mitosis) and was considered as the period from the time of complete separation of daughter nuclei or daughter kinetoplasts to the complete separation of daughter cells. Thus some of the actual events of mitosis and of kinetoplast division are included in G_2 (Cosgrove and Skeen, 1970). Accordingly, this procedure was followed.

Synchronization Methods of SC and RI Cells:

In one set of experiments, attempting for synchronization, the SC and RI were treated with 1 x 10^{-6} M 5 Fluorodeoxyuridine (FUdR, Hoffman-La Roche Inc., N. J.). Parasites were washed with fresh medium as described above to remove the inhibitor after 15 hours and pulse labelled in fresh medium with 0.5 µci/ml ^{3}H-thymidine for 15 minutes. The cells were then washed and resuspended in fresh medium without FUdR, cells were harvested at hourly intervals and processed for autoradiography. Dividing form index of treated and untreated cells was scored, plotted as a function of time and FUdR induced cell synchronization was determined by Engelberg method (Engelberg, 1961).

Methods for Determining Macromolecular Changes in Promastigote (SC) due to Elevated Temperature:

To study promastigote metabolism at elevated temperatures, SC was grown at 25 C for 4-5 days. Cultures were transferred at time=0 to 25 C, 25 C, or 37 C and pulse labelled for 15 minutes at time=4, 24, and 48 hours with 0.5 µci/ml of ^{3}H-leucine. Smears were made and cells processed for autoradiography. Four experiments were performed with the same protocol.

Methods for Infected Cell Culture:

Prior to the initiation of labelling and pre-labelling experiments with infected cell cultures, preliminary

experiments were done to ascertain the appropriate species of parasite, volume of culture, initial exudate cell concentrations, initial parasite/host cell ratio, percentage of macrophages in an exudate, and interval of harvesting time. Altogether, 10 independent exploratory exudate cell cultures were performed using L. donovani as the parasite, 7 using L. mexicana (a Garnham strain, see Janovy and Poorman, 1969) as the parasite, and 7 using L. braziliensis (an NIH-CDC strain obtained from the University of Oklahoma Medical School and subsequently discarded) as the parasite. The number of cells, culture volumes, parasite/cell ratios, and harvest and pulsing times were decided upon the results of these experiments. The cultures using L. donovani as parasite and golden hamster macrophage cell, as host below gave maximum survival. In view of the above results L. donovani was used as the parasite and the ratio of 2 parasite/macrophage was followed as a standard procedure for infection.

Peritoneal exudate cells were collected from golden hamsters stimulated intraperitoneally with 3 ml 2 per cent starch suspension in Locke's Solution 24 and 48 hours prior to harvest as previously done by Mauel and Defendi (1971). The method of Akiyama and Taylor (1970) was modified for collection of peritoneal exudate cells. Ten ml sterile McCoy's 5A (modified) Medium with 30% Fetal Bovine Serum (GIBCO) were injected into the peritoneal cavity. The abdomen was gently kneaded and the fluid withdrawn into the original needle and syringe, which were not removed. Exudate cells from more

than one hamster were pooled in siliconized 250 ml screw cap Erlenmeyer flasks and counted by haemocytometer. The concentration of macrophage cells was adjusted to approximately 1.25×10^6 cells/ml by the addition of McCoy's medium. Exudates containing less than 90% macrophage cells were discarded. Two ml of the medium containing macrophage cells were dispensed in siliconized 16 x 125 mm Leighton tubes each containing a 9 x 35 mm coverslip. Promastigotes in 0.5 ml Tanabe's Medium at a ratio of 2 parasites per macrophage cell were introduced into the Leighton tube. Macrophage cell cultures were kept at 35 C.

Infected cell cultures were pulse labelled for one hour with 0.5 µci/ml of ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-leucine separately. Coverslips containing the cells were removed, washed in nonradioactive medium and introduced in fresh medium. Coverslips were removed at 4, 24, 48 hours after incubation and prepared for autoradiography.

Control slides were prepared with only macrophage cells pulse labelled for one hour at 4, 24, 48 hours after establishment of the primary culture. The experiments were done four times.

In prelabelling studies exudate macrophage cultures were pulse labelled for one hour with ^{3}H-uridine or ^{3}H-leucine. Coverslips were washed and the cells incubated in fresh medium. Unlabelled flagellates were added to the macrophage culture previously labelled with ^{3}H-uridine. In the case of leucine

labelled cells, the cultures were incubated for 24 hours prior to the addition of unlabelled flagellates. The experiments were done four times.

In some experiments coverslips from uridine labelled cultures were removed at 24 and 48 hours and divided into 3 groups for the following treatment: (1) untreated (2) DNAse and (3) RNAse. Bovine pancreatic DNAse-I (Sigma) was mixed in a ratio of 0.1 mg/ml in Tris-HCl buffer, 0.01 M, pH 7.2, containing 3.3×10^{-3} M $MgCl_2$. Bovine pancreas RNAse-A (Sigma) was mixed in a ratio of 0.2 mg/ml in Tris-HCl buffer 0.01 M, pH 7.2 (Steinert and Steinert, 1969). The nuclease digestion was carried out by incubating cells at 37 C for 30 min. Coverslips from leucine labelled cell cultures were harvested as above but not subjected to DNAse and RNAse treatment. Three experiments were performed of each experimental protocol.

Promastigotes from 4 to 5 days SC were pulse labelled with 0.5 µci/ml of ^{3}H-uridine or ^{3}H-leucine for 15 minutes. Following the pulse, flagellates were washed by centrifugation and resuspended in fresh medium. These labelled flagellates were introduced into Leighton tubes with exudate cells in a ratio of 2 flagellates and one macrophage. Coverslips were harvested after 4, 5, and 6 hours and processed for autoradiography. Two experiments were performed of the same protocol.

Determination of Macromolecular Biosynthesis by Autoradiography:

The cells used for autoradiography were fixed in absolute methanol (Steinert and Steinert, 1962) for 10 minutes, hydrated,

washed in 5% cold trichloracetic acid for 10 minutes and dehydrated to absolute methanol. For coating with nuclear emulsion the slides were prewarmed in a thermostatically controlled light proof chamber which also contained the emulsion melted at 42-44 C (Barnawell et al., 1970). Eastman Kodak NTB-3 nuclear emulsion was used. Autoradiographs were exposed in the dark at 4 C for two weeks. Sample slides were developed weekly to determine the optimal time of exposure. After two weeks slides were developed using Kodak D-11 developer for two minutes, washed in distilled water for several seconds, fixed in Kodak-acid fixer for six minutes, rinsed in running tap water at 15-20 C for 20 minutes and stained with Giemsa through the emulsion.

Autoradiographs were scored under 1250 magnification and silver grains were counted with an ocular disc grid with 100 μm^2 per square. Photography was done in the same magnification with a green filter. Fifty squares were counted for background and each value in all cases of grain counting. Values are expressed as the mean grain count per square plus or minus standard error.