INFECTION OF PRIMARY AND SERIAL CELL CULTURES WITH LEISHMANIA DONOVANI

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
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INFECTION OF PRIMARY AND SERIAL CELL CULTURES WITH *LEISHMANIA DONOVANI*

By

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INTRODUCTION

Visceral leishmaniasis (kala-azar) occurs along the Mediterranean Coasts and in India, China, the USSR, and Africa. The disease is caused by the protozoan, Leishmania donovani, and is transmitted to man by sandflies of the genus Phlebotomus. Natural hosts other than man include dogs, foxes, and some species of rodents.

There are two stages in the life cycle of L. donovani. Within the digestive tract of the sandfly the parasite exists as an elongate, flagellate form known as a leptomonad. When inoculated into a mammalian host by the bite of the sandfly, these forms are ingested by cells of the reticuloendothelial system, particularly the macrophages of the liver and spleen where they transform into aflagellate intracellular forms called Leishman Donovan (LD) bodies. The parasites multiply rapidly within the cytoplasm of the host cell which is eventually destroyed. Liberated LD bodies then invade other mononuclear cells and continue to proliferate. The infection in man is a wasting disease characterized by such symptoms as progressive anemia, fever, edema, and splenohepatomegaly. Kala-azar is usually fatal if untreated.

The flagellate stage of L. donovani can be continually cultured on blood supplemented media incubated at room temperatures. At 37 C limited survival of transitional forms between leptomonads and LD bodies can be obtained,

but generally LD bodies of L. donovani cannot be cultured in the absence of living tissues (Trager, 1967). Tissue cultures have been successfully employed in the study of many intracellular protozoa but optimal growth has never been obtained for Leishmania in cell cultures (Pipkin, 1960). The purpose of this study is to examine the survival and multiplication of Leishmania donovani in several primary and serial cell culture systems.

LITERATURE REVIEW

Early attempts to cultivate the intracellular stage of Leishmania donovani in tissue culture were largely unsuccessful. Yen and Chung (1934) used explants of embryonic chick brain, heart, liver, and intestine in hanging drop cultures to obtain growth of leptomonads at 20 C, but when these cultures were incubated at 36 C the parasites failed to survive in any form. Gavrilov and Laurencin (1938) infected explants of hamster liver, spleen, and kidney at 37 C with leptomonads. They demonstrated intracellular parasites in the liver explants 9 days later, but were unsuccessful in establishing infections in the other tissues. Pai and Hu (1941) maintained hanging drop cultures of chick, hamster, and human tissues infected with L. donovani at 36 C for up to 7 days. The parasites survived for up to 35 days in slower growing flask cultures.

Later attempts to cultivate L. donovani in tissue culture were more successful. Using techniques developed for the culture of Plasmodium gallinaceum, Hawking (1948) infected explants of hamster spleen grown in plasma clot cultures with LD bodies obtained from infected hamster spleen. Within 24 hours after culture infected macrophages migrated from the explanted tissue, and transmitted the infection to macrophages from normal spleen explants placed nearby. Tchernomoretz (1946) infected plasma clot cultures

of embryonic calf spleen with LD bodies and was able to maintain the infection over several subcultures. More modern tissue culture methods enabled Belle (1958) to cultivate L. donovani in association with monolayered human amnion cells. Frothingham and Lehtimaki (1967) infected human amnion cells with LD bodies and clearly demonstrated numerous intracellular parasites located in small spaces within the cytoplasm. At 36 C these forms persisted for about two weeks before they degenerated.

Established serial cell lines have also been infected with L. donovani. Serial cell cultures of monkey kidney cells can be infected with LD bodies at 37 C as reported by Herman (1968) who used this culture system to examine the effects of Acriflavin on the growth and morphology of LD bodies in culture. Infected cultures were maintained over four serial transfers and were useful only for short term experiments. The invasion of HeLa and J-111 serial cells has been described by Miller and Twohy (1966). HeLa cells were rapidly invaded by leptomonads but failed to support growth of the parasites. J-111 cells were less rapidly infected but supported parasite growth over a 72 hour period. Continuous cultivation of L. donovani in a cell line derived from a dog sarcoma has been accomplished by Lamy et al (1964). More recently Frothingham and Lehtimaki (1969) have reported extended growth of L. donovani in this same cell line which they obtained from Lamy.

The morphology of L. donovani in tissue culture depends in part on the type of serum in the medium and on the temperature of incubation. Many extracellular leptomonads and intermediate forms were noted by Hawking (1948) after 16 days of incubation at 36 C in hamster spleen explants. Similar morphological forms were recorded by Tchernomoretz (1946) and by Belle (1958) in their experiments with tissue cultures incubated at 36 C. Frothingham and Lehtimaki (1967) recorded the development of numerous extracellular flagellates after 2 days of culture at 33 C in human amnion cell cultures. Two weeks after infection they described elongated intracellular forms which sometimes possessed flagella. This intracellular infection persisted for up to 60 days in spite of the morphological changes in the parasites. When the temperature was raised to 36 C the extracellular parasites disappeared and the intracellular forms rounded up into typical LD bodies. At this temperature cultures were maintained as long as 20 days. When 20% horse serum was substituted for fetal bovine serum and the cultures incubated at 33 C the extracellular parasites again disappeared and the intracellular forms degenerated. Similar variations of parasite morphology with temperature change were noted by Frothingham and Lehtimaki (1969) in serial cultures of L. donovani in dog sarcoma cells. At 36 C the parasites multiplied as normal LD bodies embedded singly in the cytoplasm. At 33 C many flagellates developed and the intracellular forms became elongate.

In spite of some long periods of survival only limited multiplication of LD bodies has been achieved in tissue culture. Hawking (1948) and Tchernomoretz (1946) maintained infected cultures only for short time periods with little increase in the number of parasites. Belle (1958) observed an increase in total parasite numbers after 48 and 96 hours of culture; however, most of the parasites were extracellular transitional forms. In cultures incubated at 33 C Frothingham and Lehtimaki (1967) observed an increase in the percent of human amnion cells infected with elongate intracellular parasites over a twenty day period. In addition to intracellular parasites leptomonads were present in the culture medium making it difficult to substantiate intracellular multiplication. Herman (1966) obtained a 3.4 fold increase in the number of intracellular LD bodies grown in hamster peritoneal cells over a seven day period. Similar growth of LD bodies was obtained by Miller and Twohy (1969) using cultured mouse macrophages. These cells rapidly ingested LD bodies, but parasite growth was limited and usually ceased by 72 hours after infection.

An important factor in cultures infected with L. donovani is the relative rate of growth of parasites and host cells. Thus Herman (1968) reported that cultures of monkey kidney cells reproduced at a much faster rate than did the intracellular LD bodies. After each serial transfer of the cultures infected cells decreased in number, and by the fifth subculture infected cells were difficult to find.

In contrast to this, Miller and Twohy (1966) found that an equilibrium had apparently been reached by both parasites and J-111 host cells with respect to their multiplication. Similar results have been obtained by Lamy et al (1964) with L. donovani in dog sarcoma cells described by the authors as histiocytes. Parasites infected approximately 10% of the host cells with an average of 5 parasites per infected cell. The cultures were transferred every three to four weeks and have been maintained for a year without adding new cells or parasites.

MATERIALS AND METHODS

All cell cultures were incubated at 37 C in 16 X 125 mm Leighton tubes, each containing a 9 X 35 mm coverslip. Primary cultures were continuously agitated on a rocker platform operating at 5 to 10 complete cycles per minute. Spleen macrophages were first cultured in a 10% CO_2-90% air atmosphere for 24 hours and then changed to a 5% CO_2-95% air atmosphere for the duration of the experiment. Stock serial cell cultures were maintained without rocking at 37 C in 250 ml glass serum bottles under 10 ml of Eagle's Basal Medium (EM) with 10% Fetal Bovine Serum (FBS). In early experiments with both J-111[1] (human leukemic monocyte) and Hep #2[2] (human epidermoid carcinoma) cell lines, 50 ug per ml of amphotericin B was added to retard fungal contamination. The cultures were transferred by treatment with 0.25% trypsin once a week, or more frequently if the monolayer became dense. Primary cell cultures were maintained in either NCTC 135 synthetic tissue culture medium supplemented with 10% horse serum or 10% FBS; or in Eagle's Minimal Essential Medium (MEM) supplemented with 10% FBS, Eagle's nonessential amino acids, and 100 mM sodium pyruvate, as described by Mishell and Dutton (1967)

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[1]Obtained from Microbiological Associates, Bethesda, Maryland.

[2]Obtained from Dr. W. N. Mack, Michigan State University.

for the culture of spleen cells. All media contained 250 units of penicillin and 250 ug of streptomycin per ml. Sufficient sodium bicarbonate was added to bring the phenol red contained in the medium to a cherry red color (pH 7.4). The nutrient medium was changed every 48 hours, or sooner if the medium became highly acidic.

Peritoneal cells were collected from male or female C 57 Bl/6j inbred mice according to the method of Miller and Twohy (1967 and 1969). Mice were prestimulated with 1 ml of NCTC 135 or 1 ml of Hanks' Balanced Salt Solution daily for two successive days. On the third day 2.5 ml of culture medium without serum was injected into the peritoneal cavity and the exudate withdrawn with a needle and syringe. The pooled cell suspension was centrifuged once at 250 g for 10 minutes, and the cell pellet was resuspended in complete culture medium. Cell numbers were estimated with a Type A Coulter Counter using a 100u aperture at an aperture current setting of 4 and a threshold value of 30. The cells were adjusted to a final concentration of 2×10^6 cells per ml and inoculated in a 1 ml volume into each Leighton tube. In later experiments the cell concentration was estimated from the height of the cell pellet rather than from direct counts on the Coulter Counter.

Spleen cells were harvested according to the method of Mishell and Dutton (1967). Mice were killed by cervical dislocation and the spleens were aseptically removed, placed in a sterile 60 mm petri dish containing a small amount of

MEM with serum, and teased apart with forceps to release the cells. Cell clumps were suspended by force pipetting and the suspension was transferred to a centrifuge tube. Tissue fragments were allowed to settle for 10 minutes in an ice bath before the cells were placed in another centrifuge tube and centrifuged at 250 g for 10 minutes. The cells were resuspended in complete culture medium and added to Leighton tubes. The spleens from four animals were used to inoculate five Leighton tubes.

The 3S strain of Leishmania donovani was maintained by routine passage in 1 to 4 month old hamsters. Animals were killed about one month after an intracardiac injection of 15×10^6 LD bodies, or two months after an intraperitoneal injection of 30×10^6 LD bodies. The spleens were aseptically removed, minced with scissors, and ground with a small volume of NCTC 135 in a Teflon pestle homogenizer. The homogenate was centrifuged at 63 g for 5 minutes to remove tissue particles. The supernatant containing the LD bodies was decanted and centrifuged at 565 g for 20 minutes. The white layer of sediment containing the parasites was resuspended in NCTC 135 and the parasites were counted under the 100X oil immersion objective of a phase microscope in a Petroff-Hausser counting chamber.

Cell cultures were infected by replacing the medium with fresh medium containing about 5 LD bodies for each cell adhered to the coverslip. At appropriate times after infection coverslips were fixed in 5% gluteraldehyde buffered to pH 7

with Sörenson's buffers and stained with May-Grünwald Giemsa, according to the method of Miller and Twohy (1967). The total number of cells, the number of infected cells, and the total number of intracellular LD bodies in 8 to 24, 40X or 100X microscopic fields were determined for each coverslip. The data from the coverslips of each time period were averaged, and three parameters of parasite growth were determined: the number of parasites per infected cell (LD bodies/infected cells), the number of parasites per cell (LD bodies/total cells), and the total number of parasites per coverslip. The survival and growth of intracellular LD bodies were determined by comparing the above parameters for each time period after infection.

RESULTS

Infection of J-111 serial cells

Each of 20 Leighton tubes were inoculated with 1 ml of a cell suspension containing 1 X 10^4 J-111 cells per ml. The cultures were incubated at 37 C for 24 hours before they were inoculated with 5 X 10^4 LD bodies per Leighton tube. Five coverslips were fixed at 24, 48, 72, and 96 hours after addition of the parasites.

One day after exposure to the parasites 19.6% of the host cells contained LD bodies (Table 1). These small rounded intracellular parasites were usually embedded in pairs within the host cell cytoplasm. At 48 hours the percent of infected cells decreased, but the number of parasites per infected cell and the total number of parasites per coverslip increased. The number of parasites decreased significantly at 72 hours, and continued to decrease up to 96 hours when the last series of coverslips were examined. At this time the percent of infected cells was one fourth of the value recorded 24 hours after infection.

Vacuolation of the J-111 cell cytoplasm was rare in both normal and infected cultures. Intracellular LD bodies were closely associated with the host cell cytoplasm and only rarely located within apparent vacuoles. After 24 hours in culture LD bodies frequently appeared to be superimposed on the host cell surface. These parasites were

Table 1. The infection of J-111 serial cells with LD bodies of L. donovani. Data represent the average of eight 100X microscopic fields per coverslip.

Hours after infection	Number of coverslips	Number of host cells	Number of cells infected	Number of parasites	Parasites per infected cell	Percent of cells infected
24	5	97	19	35	1.84	19.6
48	4	118	17	56	3.30	14.4
72	4	164	14	31	2.21	8.5
96	3	141	7	10	1.43	5.0

impossible to classify as either intracellular or extracellular and were therefore not counted. Transformation of LD bodies to leptomonads was not observed at 37 C in cultures inoculated with LD bodies. When leptomonads were used to initiate infection of cell cultures the majority of them transformed into typical LD bodies within 24 hours. The few elongate intracellular and extracellular parasites present at 24 hours were absent at 48 hours. Evidence of parasite degeneration, represented by remnants of parasite nuclei and kinetoplasts, was common in coverslips from later time periods. Only a few infected cells were found to contain more than 5 intracellular LD bodies.

Attempts to serially transfer infected cultures at various time periods after infection were unsuccessful. In one such attempt conducted over a seven day period the number of LD bodies per infected cells dropped to 0.004 by the seventh day of culture.

Infection of Hep #2 serial cells

Hep #2 serial cells were substituted for J-111 cells and infected as described above. Twenty-four hours after inoculation of the LD bodies about 23% of the cells contained small rounded parasites (Table 2). The ratio of the number of parasites to infected cells remained relatively constant over the entire 72 hours as did the percent of infected cells. The total number of intracellular parasites increased at 48 hours. This value decreased at 72 hours but remained slightly above the percent of infected cells recorded 24 hours after infection.

Table 2. The infection of Hep #2 serial cells with LD bodies of L. donovani. Data represent the average of eight 43X microscopic fields per coverslip.

Hours after infection	Number of coverslips	Number of host cells	Number of cells infected	Number of parasites	Parasites per infected cell	Percent of cells infected
24	4	216	49	105	2.14	22.7
48	5	262	73	160	2.19	27.8
72	4	230	53	122	2.30	23.0

Vacuolation was common in both infected and normal Hep #2 serial cells. LD bodies were easily seen within vacuoles of various sizes, and extracellular parasites were rare. As in cultures of J-111 cells, no transformation of LD bodies to leptomonads occurred in cultures incubated at 37 C. Infected cultures were maintained over three serial transfers, but at each subculture the percent of infected cells decreased.

An experiment was conducted to determine the effect of different concentrations of serum on the survival of serial cells and parasites. Twenty-four Leighton tubes were inoculated with Hep #2 cells and allowed to monolayer for 24 hours with EM supplemented with 10% FBS. These cultures were then infected with 5 LD bodies per cell, and incubated in 10% serum at 37 C for another 24 hours. The culture medium was then replaced with fresh medium containing either 5% or 10% FBS. Coverslips were fixed and stained at 24, 48, and 72 hours after infection.

The lower serum concentration did not alter the normal course of infection. Cultures incubated in 10% serum contained a few more parasites per infected cell at 48 hours than did the cultures with 5% serum, but neither serum concentration supported further parasite growth as indicated by the drop in the ratio of parasites to infected cells by 72 hours (Table 3). Similarly the percent of infected cells remained constant for 48 hours in cultures incubated in both serum concentrations, and this value dropped only slightly at 72 hours.

Table 3. The effect of 5% and 10% FBS on the growth of L. donovani in Hep #2 serial cells. Data represent the average of sixteen 100X microscopic fields per coverslip.

Hours after infection	Number of coverslips	% FBS	Number of host cells	Number of cells infected	Number of parasites	Parasites per infected cell	Percent of cells infected
24	5	10	101	21	39	1.85	20.8
48	4	5	96	25	65	2.60	26.0
48	3	10	137	29	84	2.89	21.2
72	2	5	204	28	47	1.68	13.7
72	2	10	215	35	71	2.03	16.3

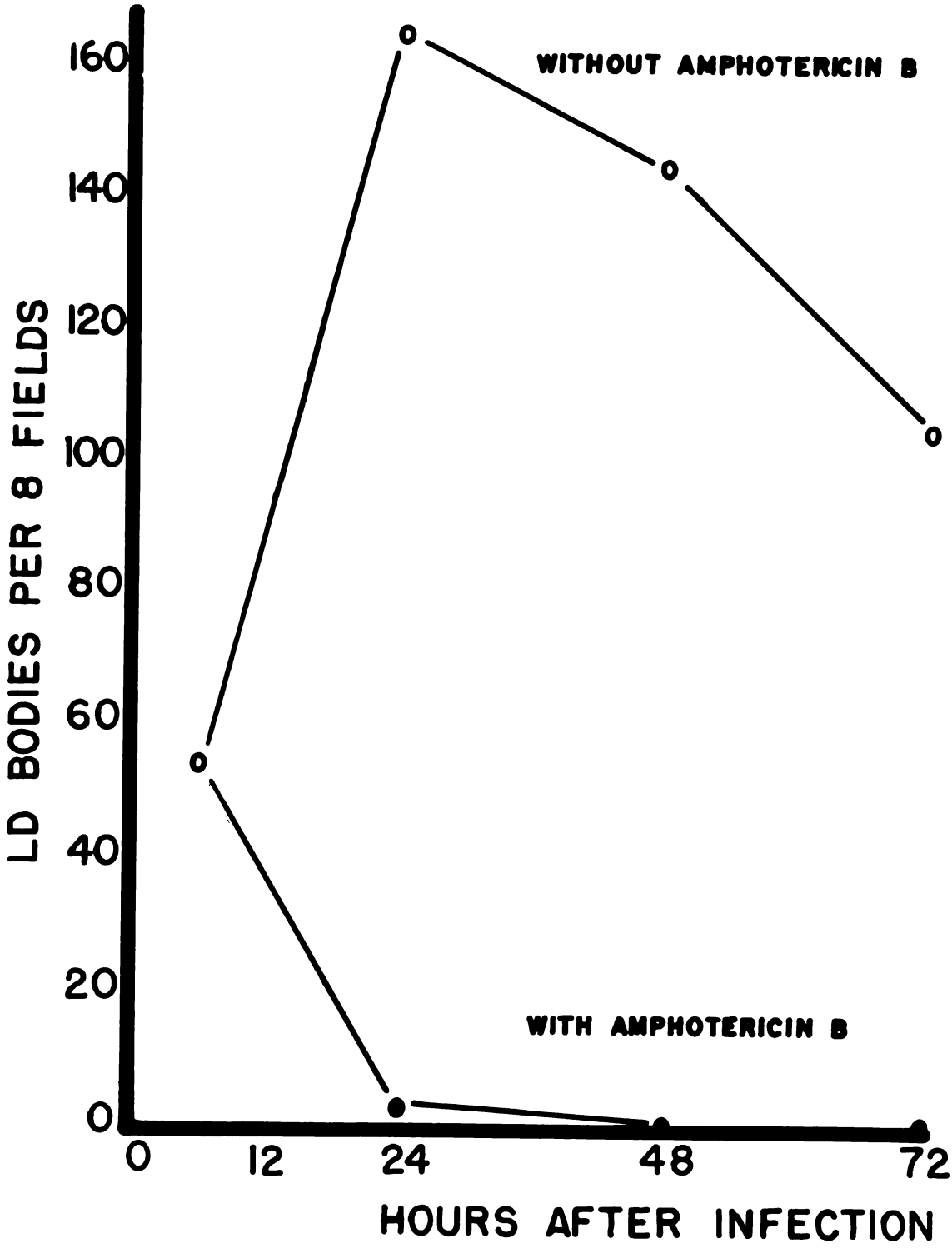
Action of amphotericin B on intracellular LD bodies

Occasionally serial cell cultures became contaminated with yeast. These cultures were either discarded or treated with the antimycotics, Nystatin or amphotericin B. However, when cultures which had been treated with either of these compounds were infected with L. donovani, the results were consistently abnormal. At 48 hours after infection very few intracellular parasites could be found in the stained coverslips, even after careful examination. An experiment was therefore designed to determine the effect of amphotericin B on the survival and growth of LD bodies within cultured mouse peritoneal macrophages.

Normal mouse macrophages were harvested and cultured in Leighton tubes with NCTC 135 and 10% horse serum. One day later the cells were infected with 5 LD bodies per host cell adhered to the coverslip. Six hours later the medium was removed and replaced with fresh medium with or without 50 ug of amphotericin B per ml of medium. Five coverslips from each group were fixed at 6, 24, 48, and 72 hours after infection, and the number of LD bodies in eight 43X fields per coverslip was recorded.

Within 18 hours after addition of amphotericin B a dramatic decrease in the number of intracellular parasites was recorded in the experimental group (Fig 1). Control cultures supported parasite growth for 48 hours, but the number of parasites declined at the 72 hour sample. Cultures treated with amphotericin B were completely free of parasites

FIGURE 1. The effect of amphotericin B on the survival of intracellular LD bodies. Normal mouse peritoneal macrophages were cultured in Leighton tubes and infected 24 hours later with 5 LD bodies per cell. Six hours later half of the cultures were treated with 50 ug of amphotericin B (closed circles) and parasite survival was compared to that obtained in control cultures (open circles). Each point represents the average of 5 coverslips.


WITHOUT AMPHOTERICIN B
WITH AMPHOTERICIN B
LD BODIES PER 8 FIELDS
160
140
120
100
80
60
40
20
0
0
12
24
48
72
HOURS AFTER INFECTION

at 48 hours and also at 72 hours, leaving an apparently normal cell sheet. Degenerated parasites were evident within macrophages of the test group at the 24 hour sample.

Infection of cultured spleen and peritoneal macrophages

Survival of normal mouse spleen cells in culture depended on the type of medium and the type and source of serum. MEM as modified by Mishell and Dutton (1967) was found superior to NCTC 135 and EM for the initiation of cultures. Several sera were examined as the 10% component of MEM including horse, new born calf, human, rabbit, and several lots of FBS. Only a specially screened lot of FBS[1] supported consistant survival of cultured spleen cells. Concentrations of serum other than 10% were not tested for their effect on cells and parasites in culture.

After consistent cell survival was obtained an experiment was conducted to determine the extent of growth of LD bodies of L. donovani within cultured spleen macrophages. Spleens were harvested and cultured in 25 Leighton tubes. The next day the cultures were infected with 5 LD bodies per cell and six hours later the medium was replaced with fresh medium. The medium on all remaining tubes was changed every 24 hours. Coverslips were fixed at 6, 24, 48, 72, and 96 hours after infection.

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[1] Obtained from Dr. H. C. Miller, Roswell Park Memorial Institute, Buffalo, New York.

Table 4. Infection of spleen macrophages in culture with L. donovani. Data represent the average of twenty-four 100X microscopic fields per coverslip.

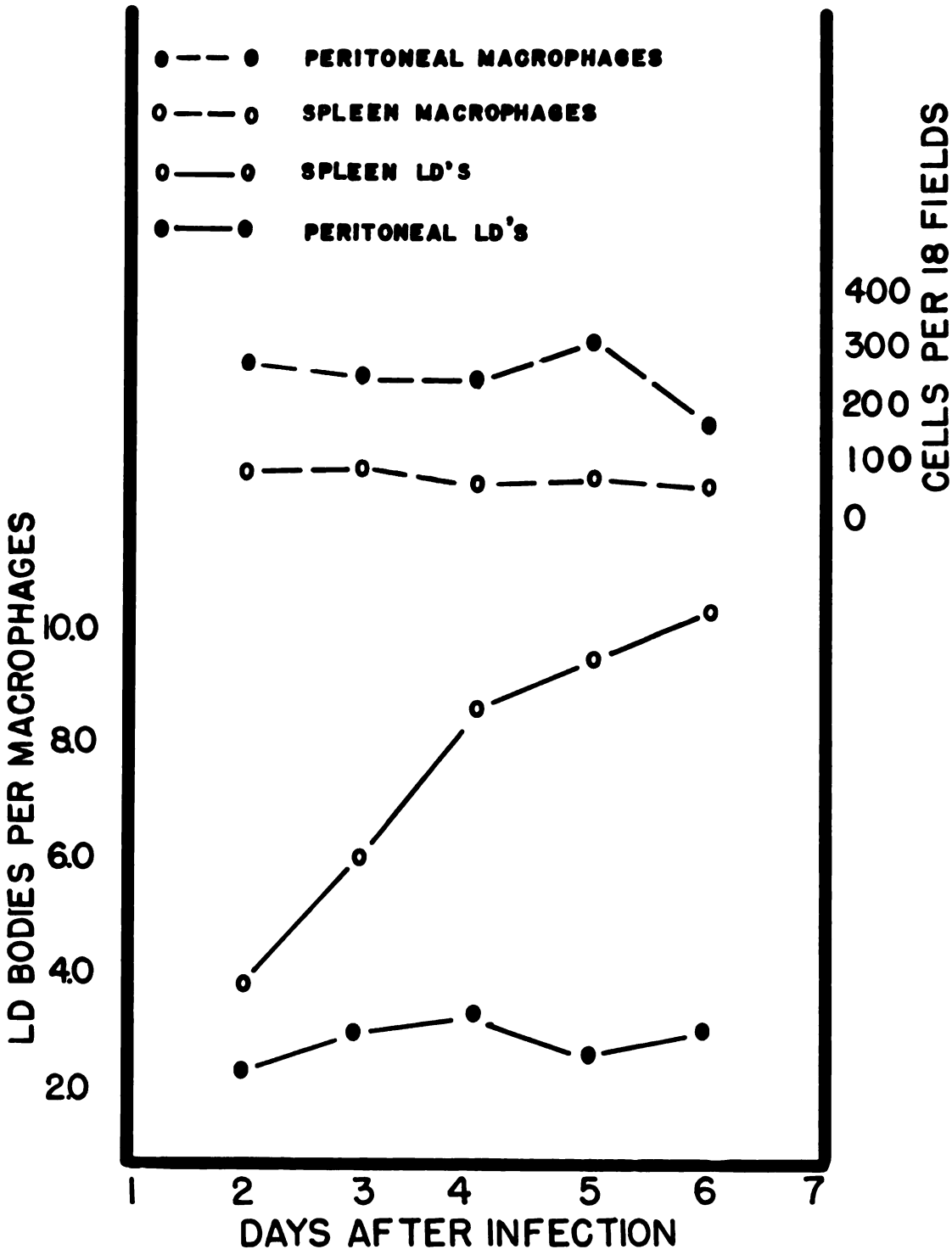
Hours after infection	Number of coverslips	Number of host cells	Number of cells infected	Number of parasites	Parasites per host cell	Percent of cells infected
6	4	169	46	107	0.63	27.2
24	5	74	36	128	1.73	48.7
48	5	62	31	135	2.18	50.0
72	5	50	31	154	3.08	62.0
96	5	39	24	129	3.31	61.5

Spleen macrophages phagocytized the LD bodies and over the 96 hour period there was a 5.2 fold increase in the number of parasites per host cell (Table 4). Although the percent of infected cells increased at 24 hours and remained relatively constant thereafter, the number of host cells and the number of infected cells decreased steadily over the course of the experiment. No significant increase in the total number of parasites was recorded over the 96 hours of the experiment.

Since cultured spleen macrophages seemed to support good growth of the parasites an experiment was conducted to compare the extent of LD proliferation within both spleen and peritoneal macrophages in culture. Unstimulated peritoneal exudates were collected and cultured as described under Materials and Methods. Three hours later spleens were harvested and cultured from the same group of mice. The next day the medium on all tubes was decanted and replaced with fresh medium containing the parasites. Four coverslips from each group were fixed at 48, 72, 96, 120, and 144 hours after infection.

Spleen and peritoneal macrophages aged at different rates in culture. For the initial 24 to 48 hours in culture spleen cells were small round mononuclear cells with an indented nucleus and a high nucleus to cytoplasm volume. These cells were firmly attached to the coverslips, and could not be removed by vigorous agitation.

FIGURE 2. The growth of L. donovani in cultured spleen (open circles) and peritoneal (closed circles) macrophages from the same group of mice. After one day in culture, the cultures were infected with 5 LD bodies per cell. The broken lines show the survival of infected spleen and peritoneal macrophages in culture. Points are the averages of three coverslips.


PERITONEAL MACROPHAGES
SPLEEN MACROPHAGES
SPLEEN LD'S
PERITONEAL LD'S
LD BODIES PER MACROPHAGES
10.0
8.0
6.0
4.0
2.0
CELLS PER 18 FIELDS
400
300
200
100
0
1
2
3
4
5
6
7
DAYS AFTER INFECTION

Peritoneal cells of the same age in culture were already very large cells with ovoid nuclei and numerous pseudopodia extended in all directions. By 72 hours of culture the spleen macrophages grew in size and also extended pseudopodia, becoming indistinguishable from the peritoneal cells by about 96 hours in culture.

More peritoneal cells adhered to the coverslips than did spleen cells throughout the entire experiment. This difference in the number of host cells in each group was compensated for by adjusting the parasite inoculum to 5 LD bodies per cell adhered to the coverslips. At all time periods spleen cells phagocytized more parasites than did the peritoneal macrophages (Figure 2). By 96 hours after infection 73% of the spleen macrophages were parasitized while 34% of the peritoneal cells contained LD bodies. Over the four day period from 48 to 144 hours after infection LD bodies multiplied 4 fold within the spleen macrophages. Only a slight increase in the number of parasites per cell occurred in the peritoneal cells over a 48 hour period. By 96 hours after infection multiplication of parasites within the peritoneal cells ceased while the spleen cells continued to support proliferation of the LD bodies.

An experiment was conducted to determine the effect of adding new spleen cells to cultures previously infected with L. donovani. Each of 52 Leighton tubes was inoculated

with a spleen cell suspension prepared from the spleens of 45 male mice. Each culture was infected with 1 X 10^6 LD bodies and coverslips were fixed at 24, 72, 96, and 120 hours after infection. At 120 hours after infection half of the remaining cultures were inoculated with a spleen cell preparation obtained from the spleens of 5 mice and coverslips were fixed from these cultures and controls at 24, 72, 96, and 120 hours after the addition of the new spleen cells.

Over the first 120 hours after infection a seven fold increase in the number of parasites per cell was recorded (Table 5). One day later, 24 hours after the addition of new cells, the number of parasites per host cell decreased in cultures with and without new cells. It is also important to note the increase in the total number of host cells at this time period in those cultures to which new cells were added. The total number of LD bodies per coverslip also increased over the first 120 hours of culture, but over the next 5 days the number of parasites remained relatively constant in those cultures inoculated with fresh spleen cells while the total number of LD bodies decreased in the control cultures. In contrast to this the percent of infected cells was similar in both control and experimental cultures after the addition of the fresh spleen macrophages.

Table 5. The effect of adding new spleen cells to cultures of spleen macrophages previously infected with L. donovani. New cells (NC) were added 5 days after infection to half of the remaining cultures. Data represent the average of twenty-four 100X microscopic fields per coverslip.

Days after infection	Number of coverslips	Number of spleen cells	Number of cells infected	Number of parasites	Parasites per total cells	Percent of cells infected
1	2	154	23	46	0.30	14.9
3	5	99	44	150	1.52	44.4
4	4	127	55	183	1.44	43.3
5	5	103	53	219	2.13	51.5
6	5	121	40	153	1.26	33.1
6 (NC)	4	174	49	209	1.20	28.2
8	4	127	33	124	0.98	26.0
8 (NC)	5	165	53	211	1.28	32.1
9	3	117	31	117	1.00	26.5
9 (NC)	3	136	45	198	1.46	33.1
10	3	100	16	49	0.49	16.0
10 (NC)	3	167	37	157	0.94	22.2

DISCUSSION

Early work with Leishmania donovani in tissue cultures attempted to elucidate certain aspects of the life cycle of this intracellular parasite. Later efforts were directed toward the replacement of laboratory animals with cell cultures as a source of large numbers of LD bodies. In this work attempts were made to infect J-111 cells because of their monocytic origin and because of previous work with this cell type by Miller and Twohy (1966). Hep #2 cells were used because of their immediate availability.

Both J-111 and Hep #2 cells phagocytized LD bodies to a limited extent. Usually about 20 percent of J-111 cells, and about 30 percent of Hep #2 cells became infected after 24 hours of exposure to parasites. This probably represents a continuous uptake of LD bodies rather than multiplication. Extracellular LD bodies were regularly present in coverslips fixed 24 hours after the addition of parasites. The size and shape of the parasites was similar in both cell lines, but the parasites resided in obvious cytoplasmic vacuoles in the Hep #2 cells and appeared to be embedded directly in the cytoplasm of the J-111 host cells.

The presence or absence of cytoplasmic vacuoles in the J-111 and Hep #2 serial cells is probably a function of the cell type and other cultural conditions, rather than a result of infection with L. donovani. Generally Hep #2 cells contained vacuoles while J-111 cells did not.

Both normal and infected cells of each cell line were consistent in this respect. No evidence was found to correlate host cell vacuolation with the enhanced destruction of intracellular parasites. Usually parasites would survive in the Hep #2 cells in greater numbers and for longer time periods than in the J-111 cells. Although L. donovani can exist in vacuoles and seemingly avoid immediate intracellular digestion, the fate of the individual parasites cannot be followed over extended time periods and the effect of vacuoles on the survival of parasites cannot be determined with certainty.

Both cell lines supported limited survival and probably some multiplication of L. donovani. In Hep #2 cells the increase in the number of parasites 48 hours after infection probably represents parasite multiplication, and the concomitant increase in the number of infected cells could result from the continuous invasion of new host cells by liberated parasites. Occasionally host cells were observed to contain over 20 intracellular parasites. In the J-111 cells the slight increase in the number of LD bodies per infected cell at 48 hours after infection may also represent a limited multiplication of the parasites, but the decrease in the percent of infected cells at this time period may indicate that few new host cells are being invaded. Few host cells were found to contain more than 5 intracellular parasites.

Although some parasite multiplication probably occurred in both cell lines, extended growth was not achieved. Over a 72 hour period the number of parasites per Hep #2 host cells remained relatively constant as did the percent of infected cells. This may indicate that an equilibrium existed between the rates of host cell and parasite multiplication over the 72 hours of the experiment. However a constant level of infected cells could not be maintained after transfer of infected cultures, and the number of infected cells steadily decreased upon each subculture. In the J-111 cultures the parasites survived for about one week. The number of parasites per infected J-111 host cells began to decrease by 72 hours after infection and could not be increased by serial transfer of the infected cultures. Probably the majority of the parasites in both cell lines were destroyed by action of the host cells. Remnants of parasite nuclei and kinetoplasts in the cytoplasm of the host cells indicates the destruction of parasites, but a few LD bodies may have survived and continued to multiply at a low rate relative to the rate of host cell multiplication. Intracellular LD bodies could be found in low numbers up to seven days after infection of J-111 cells and up to 15 days after infection of Hep #2 cells.

Hep #2 cells were cultured with 5% FBS in an attempt to increase the number of intracellular parasites by

decreasing the growth rate of the host cells; however no significant increase in the rate of parasite multiplication was observed in cultures with 5% serum. At 48 hours after infection the number of parasites per infected cell increased to a similar extent in both 10% and 5% FBS, and extensive multiplication was not observed with either concentration of serum. The effects of low serum concentrations on Leishmania in cell cultures has been studied by several investigators. Frothingham and Lehtimaki (1969) found that after 33 days with 5% FBS only 2% of the cultured dog sarcoma host cells were infected with L. mexicana while 27% of the cells grown in 20% FBS were infected. Herman (1968) found that even with the use of a maintenance medium containing 2% serum, LD bodies of L. donovani were steadily diluted on each serial transfer of the monkey kidney cell cultures. Apparently a lower serum concentration does not increase the rate of multiplication of the parasites, and may be deleterious to their maintenance and multiplication.

Miller and Twohy (1966) successfully infected J-111 serial cells in EM supplemented with 10% horse serum with leptomonads of the Khartoum strain of L. donovani, and obtained multiplication of the parasites over a 72 hour period. In the present study multiplication was obtained for 48 hours before the number of parasites began to decrease. In their discussion of the development of tissue

culture systems permitting continuous cultivation of L. donovani under conditions inimical to extracellular forms, Frothingham and Lehtimaki (1967) cite several factors that influence parasite survival and growth. These factors include the clinical, geographical, and laboratory history of the parasites as well as cultural factors such as temperature, composition of the medium, and the type of host cell. Such factors could account for the parasite growth observed in J-111 serial cells by Miller and Twohy and the shorter period of parasite survival recorded in this study.

The low rate of multiplication of LD bodies in both serial cell lines is likely the result of an adverse intracellular environment. Although J-111 serial cells are of monocytic origin, cultures of these cells grow as epithelial cell sheets, morphologically similar to Hep #2 cells. L. donovani might be expected to grow better in a macrophage-like cell type. It is interesting to note that Lamy (1964) describes dog sarcoma cells as histiocytes, and that these cell cultures require serial transfer only once every three to four weeks. Hep #2 and J-111 serial cells are transferred about every four to five days. This rapid rate of multiplication may have the effect of diluting out parasites which may be reproducing at a slow rate. Rapid host cell multiplication may limit the availability of adequate concentrations of vital

metabolites to the parasites, thus limiting the rate of parasite multiplication.

Amphotericin B has been used successfully by Prata (1963) in the treatment of kala-azar. Results from the present study demonstrate the antileishmanial activity of this polyene antimycotic in tissue cultures infected with L. donovani. The mode of action of amphotericin B is probably similar to that of Nystatin, another polyene antimycotic, which is leishmanicidal to leptomonads cultured at room temperature, and is also probably lethal to LD bodies grown at 37 C in tissue culture. Nystatin most likely acts by physically altering certain permeability characteristics of the leptomonad cell membrane thus releasing vital intracellular constituents into the culture medium (Ghosh and Chaterjee, 1961). If a similar mode of action operates on intracellular LD bodies of L. donovani in tissue culture at 37 C, the dramatic drop in the number of intracellular parasites 18 hours after addition of amphotericin B is easily understandable. Herman (1968) has suggested that L. donovani and other intracellular parasites may be very sensitive to changes in permeability. It is doubtful that any viable parasites remained in culture after 72 hours of exposure to amphotericin B. No intact parasites could be found after extensive examination of coverslips and evidence of intracellular digestion was obvious; however the possibility exists that a few parasites might have survived the treatment with amphotericin B.

Spleen macrophages were cultured in an attempt to find a more suitable host cell for the survival and multiplication of L. donovani in vitro than the mouse peritoneal macrophage. In the initial experiment with spleen macrophages survival and growth of the parasites was obtained with a 5 fold increase in the number of parasites per cell over a 96 hour period. The frequent changes of nutrient medium during the experiment probably accounts for the loss of host cells and for the relative stability in the total number of LD bodies from 24 to 96 hours. In a culture system supplemented with 40% horse serum peritoneal cells usually support parasite growth for only 72 hours (Miller and Twohy, 1969). A comparison of the spleen and peritoneal macrophages cultured under similar conditions was therefore conducted.

Changes in the morphology of cultured spleen and peritoneal macrophages during the experiment were similar to those reported by Cohn and Benson (1965). These authors described the formation of large phagocytic cells from initially small mononuclear cells with little cytoplasm. Correlated with the increase in size was the development of phase dense lysosome-like granules, and an increase in the content of acid phosphatase, beta-glucuronidase, and cathepsin. In this study peritoneal cells differentiated approximately 24 to 48 hours before spleen cells of the same age. Multiplication of LD bodies usually ceased by

72 to 96 hours in peritoneal cells while spleen cells supported growth for 120 to 124 hours after infection. Possibly enhanced intracellular digestion of the parasites associated with the maturation of the host cells in culture is responsible for the limitation of the growth of LD bodies in the two cell types.

Although parasite growth was limited in both cell types spleen cells provided a more optimal host cell for proliferation of L. donovani than did the peritoneal macrophages. During the 4 day period from 48 to 124 hours after infection spleen cells supported a 4 fold increase in the number of parasites per cell while the peritoneal cells supported only a slight increase in the ratio of parasites to cells over the first 72 hours after infection. Although the rate of multiplication of L. donovani in cultured spleen cells is greater than that obtained in peritoneal macrophages under similar conditions, it is not as great as the 50 fold increase in the number of LD bodies in hamster liver 8 days after infection reported by Stauber (1955).

Prolonged maintenance of LD bodies was achieved by the addition of new spleen macrophages to previously infected cultures. During the initial 120 hours after infection the increase in the number of parasites per macrophages and in the total number of parasites per coverslip indicates a rapid proliferation of parasites. One day after fresh cells

were added to the cultures the population of host cells significantly increased when compared to the control cultures; however the number of parasites per cell was the same in both control and experimental cultures. Therefore although growth of LD bodies ceased at 120 hours in the control cultures, parasites continued to multiply in cultures with new cells. New cells must have been invaded within 24 hours after the addition of the new cells since the percent of infected cells was similar in both control and experimental cultures while the number of host cells increased in the cultures inoculated with the new cells.

The course of infection of hamsters with L. donovani was followed by Stauber (1955). Impression smears of infected organs were made at various times after infection and the number of parasites was counted under the microscope. A rapid proliferation of the LD bodies in the spleen and liver was recorded in the early days after infection. Later the number of parasites approached a maximum value which was maintained until death of the animal. In the present study the total number of parasites also reached a maximum value after a rapid initial multiplication, and this high number of LD bodies was maintained by the addition of new host cells to the infected cultures. Possibly an equilibrium was reached between the degeneration and multiplication of individual LD bodies.

Parasite degeneration may be due to an adverse intracellular environment within the more differentiated spleen macrophages while the newly added cells may provide a more optimal host cell for parasite growth. The degeneration of parasites in some cells and the multiplication of parasites in other cells can account for the observed results. Cultures without new cells showed a progressive drop in the total number of LD bodies.

SUMMARY

Attempts were made to cultivate Leishmania donovani in serial cell lines of J-111 (human leukemic monocytes) and Hep #2 (human epidermoid carcinoma) cells. Limited multiplication of intracellular LD bodies was obtained in both cell lines; however parasites eventually disappeared from the cultures probably as a result of an inadequate host cell environment.

Peritoneal macrophages from normal mice were cultured to demonstrate the lethal effect of amphotericin B on intracellular LD bodies. Parasites were rapidly eliminated from host cells after 18 hours of exposure to 50 ug of amphotericin B per ml of culture medium. By 48 hours of treatment no LD bodies could be found in cells cultured with amphotericin B while control cultures showed normal growth of parasites. Amphotericin B may produce changes in the permeability of intracellular LD bodies resulting in their rapid destruction by host cells.

Spleen macrophages cultured from normal mice were more optimal host cells for the growth of L. donovani than peritoneal macrophages obtained from the same group of mice. Parasites multiplied more rapidly over a longer time period in spleen macrophages than in peritoneal cells, but parasite growth ceased in spleen macrophages by 120 to 124 hours after infection. The addition of new spleen macrophages infected 120 hours previously with L. donovani

initiated renewed parasite multiplication which ceased 96 hours after the addition of fresh cells. This multiplication may be due to the influx of younger more optimal host cells.

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