INVASION OF CULTURED CELLS BY LEPTOMONADS OF LEISHMANIA DONOVANI

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INVASION OF CULTURED CELLS BY LEPTOMONADS OF *LEISHMANIA DONOVANI*

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INTRODUCTION

Leishmania donovani is the causative agent of visceral leishmaniasis (Kala-azar). This disease occurs in the Far-East, Asia, India, and the Mediterranean. The parasite is transmitted as a fusiform, flagellated leptomonad by the sandfly (Phlebotomus). The flagellates are known to be carried by circulating blood or taken up and carried by monocytes to the spleen and liver where they are found within macrophages and Kupffer cells. Once inside the host cells, the leptomonads rapidly transform into round aflagellar forms referred to as leishmaniform or Leishman Donovan (L.D.) bodies. The L.D. bodies proliferate and cause hyperplasia of the host cells, eventually leaving the cells when the intracellular population averages about 15 parasites. The free L.D. bodies then enter other mononuclear macrophages. Other cell types are less readily infected but in terminal cases, L. donovani can be found within nearly every host cell type. At this point the following symptoms are associated with the cumulative infection: headache, fever, edema, ascites and splenohepatomegaly. If the host is not treated at this point it may die within a few months. Natural hosts other than man include dogs, jackals and foxes.

The following are a few of the many aspects of the mammalian life cycle of L. donovani that are poorly understood: 1) the method that leptomonads use to invade host cells, 2) the host-cell specificity of the parasite, 3) the possible

cellular resistance to the parasite as a function of acquired resistance, 4) factors promoting multiplication of L.D. bodies in the cells and 5) a method of release of the parasites from the host cells.

The main objective of this investigation was to determine the process of cellular invasion by leptomonad forms of L. donovani. Details of the host cell specificity for both infection and multiplication were studied primarily to find a suitable system for observing cellular invasion. Many researchers have assumed that the parasites enter the mononuclear cells by phagocytosis but have shown no concrete evidence to support their assumption. Taliaferro (1962) stated; "If the Leishmania are essentially non-invasive, do they enter the macrophages passively as a result of phagocytosis? If, on the contrary, the Leishmania are highly invasive and possibly protected by being in the macrophage, how does the host tilt the balance in its own favor and recover?"

Mammalian phagocytes are separated into two major groups: the polymorphonuclear phagocytes of the bone marrow, blood and tissues and the mononuclear phagocytes. The latter include monocytes of the blood and macrophages of the tissues and reticulo-endothelial system. The ultrastructure and metabolism of these cell types as well as the process by which phagocytes take up foreign material was reviewed by Hirsch (1965). In summarizing the role of phagocytosis in resistence he notes that phagocytes must first be delivered to the site where foreign material has entered the host. This is followed by

the chemotatic attraction of the phagocyte to the foreign particle which is then either engulfed or repelled depending upon the phagocyte's metabolism, the surface properties of the particle, the presence of opsonins, and the physiological nature of the environment. Engulfment is followed by vacuole formation around the particle, degranulation of lysosomes into the vacuole, and digestion of the particle. This last step does not occur after ingestion of parasites which are able to survive in the intracellular position.

The present study utilized cell culture techniques primarily for the advantages they offer in observation of the response of cells to L. donovani. Regulation of the cell environment and control of the number of infective agents are other advantages offered by the use of the cell culture system.

LITERATURE REVIEW

One of the earliest studies of Leishmania donovani in tissue culture was that of Chung (1934) in which he used hanging drop cultures of ground, infected hamster spleen. When these cells were incubated at 20°C, leptomonads were noted only after 6 days of culture. However, no leptomonads were observed when the cells were incubated at 37°C. In an extension of this investigation, Yen and Chung (1934) attempted to infect hanging drop explant cultures of embryonic chick brain, heart, liver and intestine with L.D. bodies from infected hamster spleen. No intracellular parasites could be found in the cells cultured at 37°C. In some cultures maintained at 20°C, the L.D. bodies transformed to leptomonads but there was no evidence of their multiplication.

Gavrilov and Laurencin (1938) were the first investigators successful in infecting tissue cultures with leptomonads. They cultured explants of hamster liver, spleen and kidney at 37°C. Nine days after inoculation of the cultures with leptomonads they found L.D. bodies in the embryonic liver cells. But, no infection could be established in the other types of explants.

Weinman (1939) reported extracellular proliferation of L.D. bodies of L. tropica in the presence of guinea pig and frog tissue grown on serum-tyrode agar slants at 37°C. He was unsuccessful in obtaining leptomonad survival, infection, or transformation to L.D. bodies on plasma clot tissue cultures of guinea pig tissue. Pipkin (1960), in reviewing Weinman's

work, stated that, "since the leishmaniform organisms were admittedly extracellular, there is room for doubt that they were really leishmaniform organisms at all, but rather, were degenerate leptomonads".

Pai and Hu (1941) attempted to infect chick embryo, human fetus, and hamster spleen tissue cultures at 37°C with leptomonads of L. donovani. Many of the cells in this study contained between 1 and 15 parasites in the cytoplasm but no division of the L.D. bodies occurred within the host cells.

Hawking (1948), using rabbit serum instead of hamster serum in the medium, cultured spleen tissue from hamsters previously infected with L. donovani. After a few days, stained preparations of infected macrophages and monocytes revealed intracellular L.D. bodies that were undergoing binary fission. In older degenerating cultures at 37°C he observed extracellular leptomonads. The investigator suggested that daily examination of the cultures, at which time the temperature dropped from 37°C to 16-20°C, may have provided the necessary stimulus to cause exflagellation. But, the leptomonads also underwent multiplication at 37°C which is very atypical of this form at elevated temperatures, (Lemma and Schiller, 1964).

Tchernomoretz (1946) inoculated explants of calf spleen at 37°C with leptomonads of L. donovani. Between 18 and 24 hours large numbers of L.D. bodies and intermediate stages of the parasite were found both free in the medium and within the cells. In subsequent studies spleens from hamsters infected with L. infantum were explanted onto cultures of rabbit plasma and 10% rabbit serum. In these cultures the

L.D. bodies proliferated and were capable of being subcultured up to 15 days.

Pipkin and Coles (1960) infected explants of newborn Syrian hamster spleen and liver tissues cultured at 37°C with leptomonads of L. braziliensis. Twenty-four hours later intracellular L.D. bodies were found in stained preparations. Normal embryonic mouse liver cells growing in Leighton tubes were exposed to leptomonads from blood agar cultures but no intracellular infections were produced. Also they obtained peritoneal exudate cells from cotton rats that had received intraperitoneal injections of sterile mineral oil. The cells were permitted to grow for 7 to 9 days. After this time they were challanged with leptomonads. Coverglasses that were removed and stained 2 and 3 days later revealed intracellular L.D. bodies in the peritoneal macrophages.

Belle (1958) attempted to infect human epithelial cells monolayered on coverglasses in roller tubes with leptomonads of L. donovani. Forty-eight hours later pleomorphic forms of the parasite were found free in the medium, but none could be found within the cells. At 96 hours a few pear-shaped and ovoid intracellular forms of the parasite were found.

Herman (1964) reported attempts to infect fibroblasts from the peritoneal exudate fluid of hamsters with L.D. bodies of L. donovani. Only a few of these cells appeared to have L.D. bodies when the coverglasses were removed and stained 11 days and 14 days later in spite of massive inoculations used in some experiments.

The goals of the previous investigators were primarily to reproduce the mammalian phase of the life cycle in tissue and cell culture systems. This was attained with various degrees of success through manipulation of cell types, media, and temperatures. As an application of this methodology, Read and Chang (1955) investigated the cytochemical changes of the parasitemia. In this study buffy coat cells from chicken blood were cultured on coverglasses with 50% chicken serum and 50% Gey's salt solution at 37°C. On the fifth day of culture the macrophages were infected with leptomonads of L. donovani. The coverglasses were removed at 24 hour intervals and compared to noninfected control macrophages. The infected cells contained fewer fat particles than the controls, but showed no increase of esterase or alkaline phosphatase activity over that of the uninfected controls.

Herman (1965) used cell cultures to successfully demonstrate fluorescent antibody reactions against intracellular L.D. bodies of L. donovani. The cells were infected in vivo by injecting L.D. bodies from an infected hamster spleen into the peritoneal cavities of uninfected hamsters after previous saline-stimulation. Thirty hours after inoculation the peritoneum was washed with saline and the infected exudate cells were placed into culture.

Only a few investigations have been made on the mode of entry of Leishmania into cells and the succeeding host cell-parasite interactions. Pulvertaft and Hoyle (1960) harvested and cultured monocytes and spleen cells to describe the invasion and intracellular propagation of L. donovani. The

monocytes obtained by a peritoneal wash and cultured in small observation chambers were very active and extruded individual pseudopodia up to 100μ long. These cells became sluggish after 1-2 weeks in culture. Upon inoculation of fresh cell cultures, the attraction between leptomonads and monocytes was described as "reciprocal and instantaneous so that when first observed, many monocytes were already attracted to the parasites". The long extended pseudopodia were described as engulfing the parasite by the posterior end. The time required for the parasites to be taken into the cells varied from a few minutes to 24 hours. In some instances the leptomonads were reported to be killed by the monocytes.

These authors also cultured spleens from infected hamsters in an unsuccessful attempt to show intracellular proliferation of the in vivo established L.D. bodies. At best the parasites survived in these macrophages for 10 days. They attempted to demonstrate exflagellation of the infected cell cultures. This could only be done when the cells were removed from their culture tubes and placed into Adler's medium which is normally used to maintain leptomonads at room temperature.

De Castro and Pinto (1960) presented a preliminary report on the influence of temperature upon the growth of L. enriettii in cell culture. They infected several cell strains growing at 37, 34 and 32°C with leishmaniform parasites obtained from closed lesions of infected patients. Survival and multiplication of the L.D. bodies resulted when the cultures were incubated at 32 and 34°C but not at 37°C. They reported that host

cells were infected by phagocytosis, but no evidence for this process was presented in this abstract.

Invasion and growth of other protozoa and bacteria have received more detailed investigation. Phagocytosis and multiplication of Toxoplasma gondii was observed by Vischer and Suter (1954) in cultures of peritoneal macrophages. By fixing and staining the cells at appropriate intervals after inoculation of the cultured cells with Toxoplasma these investigators observed the following changes: stained preparations made 8 hours after inoculation revealed no intracellular parasites, but at 36 hours the cells contained between 1 and 16 parasites. The host cells with a maximum number of parasites ruptured and the Toxoplasma were subsequently phagocytized by other macrophages where they again proliferated.

Mackaness (1954) infected cultured rabbit macrophages at 37°C with Mycobacterium tuberculosis. He found that virulent strains of M. tuberculosis always survived when engulfed by the macrophages and grew much faster than intracellular attenuated strains.

Cell cultures of primary fetal skin cells parasitized with Brucella abortus (Richardson, 1959) were demonstrated to contain "localized compartments" of brucellae. Electron microscope studies by Karlsbad, Kessel, DePetris and Monaco (1964) showed that brucellae were localized within vacuoles after phagocytosis by peritoneal macrophages in vitro.

Other microorganisms that have been reported to attain the intracellular position by phagocytosis in cell culture

systems include: Mycobacterium leprae (DeSousa, DeAzebedo and De Castro, 1959), Salmonella typhimurium (Furness 1958), Salmonella enteritidis (Mitsuhashi et. al., 1961), Leptospira pomona (Miller and Wilson, 1962), and influenza virus (Boand, Kempf and Hanson, 1959).

The details of the events that occur during phagocytosis were shown in a cinemicrophotographic study by Hirsch (1962) of polymorphonuclear leucocytes from humans, rabbits, and chickens. The films showed granule migration to the vacuole containing the microorganism and the digestion of the vacuole content. Both Bacillus megaterium and zymosan (yeast cell) were used in this study. The investigator proposed that degranulation consisted of fusion of the granule (lysosome) membrane and the leucocyte membrane surrounding the engulfed particle. The granules then discharged their contents into the phagocytic vacuole. This was supported by the following observations from the fast motion pictures: 1) lysis of granules only adjacent to the engulfed particle, 2) increase of vacuole size with continued degranulation, 3) contraction of a clear zone in the cytoplasm towards the vacuole containing the ingested organism immediately following granule lysis and 4) a rim of phase darkening on the engulfed organism's surface following each granule release.

Myrvik, Leade, and Fariss (1961) investigated the occurrence of lysosomes in both alveolar and peritoneal macrophages. The lysozyme content of alveolar macrophages was found to vary between 2000 and 4000 µg per ml of packed macrophages. The

lysozyme content of peritoneal exudates decreased with the increase in time between stimulation and harvesting of the exudate. The decrease in lysozyme content was correlated with the replacement of polymorphonuclear cells by macrophages. Only low levels of lysozyme were demonstrated to be present in peritoneal macrophage granules.

Cohn and Benson (1965) compared mononuclear phagocyte differentiation *in vitro* and *in vivo*. During differentiation in both the peritoneum and in peritoneal exudate cultures, mononuclear phagocytes increased in size and protein content. As the cells became progressively older, an accumulation of phase dense lysozome granules was noted which reacted strongly for acid phosphatase. Cathepsin and β-glucuronidase also increased with macrophage age.

MATERIALS AND METHODS

Both serial and primary lines of cells were used in this study. Stock cultures of the HeLa[1] (human epidermoid carcinoma) and J-111[2] (human leukemic monocyte) serial lines were aseptically maintained at 37°C in 100 ml prescription bottles. Eagle's Basal Medium[3] was used with 10% horse serum for culturing J-111 cells. The cultures were routinely transferred when the monolayer became thick and dense (about 3 days for HeLa cells and 7 days for J-111 cells). The culture transfer techniques, glassware preparation, sterilization, and preparation of medium and solutions described by Merchant, Kahn and Murphy (1965) were followed in this investigation. Leptomonads were prepared for inoculation into Leighton tubes containing monolayered serial cell lines as described later with the primary cells.

Primary macrophages were obtained from hamster peritoneal exudate. Golden hamsters of 40-80 grams were given aseptic, intraperitoneal injections of 1 ml of Hank's balanced salt solution (pH 7.2) 48 and 24 hours prior to collection and 5 ml at the time of collection of the peritoneal fluid. After the last injection of balanced salt solution (BSS), the abdominal regions of the animals were gently kneaded. Five minutes later a 2.5 ml hypodermic syringe was moistened with approximately

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[1]Obtained from Dr. D. M. Schuurmans, February 1965, Michigan Public Health Laboratory, Lansing, Michigan.

[2]Obtained from Dr. D. J. Merchant, July 1965, University of Michigan, Ann Arbor, Michigan.

[3]Microbiological Associates, 4813 Bethesda, Maryland.

100 USP units of heparin. A 19 gauge needle with 3 extra holes filed into the shaft was attached and the peritoneal fluid withdrawn. Usually about 2.5 ml of peritoneal exudate was collected per hamster and treated aseptically. In most experiments the exudate from 5 or 6 hamsters was pooled and centrifuged at 500 rpm for 5 minutes, the supernatant decanted and the cells resuspended in Eagle's medium with 20% horse serum. The centrifugation, decanting and resuspension were repeated as before. The cells were counted in a hemacytometer and adjusted to 5×10^5 cells per ml. One ml of the cell suspension was transferred to each Leighton tube containing a 10 x 35 mm coverglass and 5 ml were used in each serum bottle containing a 18 x 16 mm coverglass. The cells were monolayered and incubated at 37°C with medium changes every 24 hours.

The Khartoum strain[4] and the 3S strain[5] of Leishmania donovani were maintained as leptomonads on modified Novy-MacNeal-Nicolle (NNN) diphasic medium (Lemma and Schiller, 1964). One hundred units of penicillin and 100 µgm of streptomycin were added per ml of BSS overlay. The culture tubes were slanted at a 15° angle and incubated at room temperature (22-24°C). Routine transfers of cultures employing an inoculum of 2×10^5 leptomonads were made every 2 weeks.

The 3S strain was also maintained in infected hamsters. When symptoms of infection appeared in about 2 months, the spleens were removed, ground in a tissue grinder with BSS

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[4]Obtained from Dr. E. L. Schiller, April 1965, Johns Hopkins University, Baltimore, Maryland.

[5]Obtained from Dr. L. Stauber, Rutgers State University, New Jersey.

and the L.D. bodies counted. Approximately 1 x 10^6 L.D. bodies in the spleen suspension were transferred intraperitoneally to each of 5 normal hamsters. To establish growth of this strain *in vitro*, spleens from heavily infected animals (determined by stained impression smears of biopsied material) were ground in a tissue grinder with an equal volume of Hank's BSS. One ml of this suspension was placed into each NNN culture. After 3 days at room temperature (22-24°C) flagellated leptomonads appeared. These reached a maximum population after 10 days. The 3S strain leptomonads were not used in experiments until after 3 transfers in NNN cultures.

The leptomonads in the fluid overlay of the NNN cultures of both the Khartoum and 3S strains of *L. donovani* were aseptically placed into sterile 15 ml conical centrifuge tubes and centrifuged at 1500 rpm for 20 minutes. The supernatant was decanted and the remaining packed leptomonads were resuspended in Eagle's medium. The above steps starting with the centrifugation were repeated. The parasites were counted in a hemacytometer and diluted to the concentration used in each experiment. Leighton tubes containing cells monolayered on coverglasses were inoculated with 1 ml each of the leptomonad preparation.

Coverglasses were removed from Leighton tubes with a bent wire and flushed with BSS. The monolayered cells were fixed in absolute methanol for 5 minutes and stained with Giemsa (1:50) for 8 minutes.

For observing the process by which leptomonads gain entrance into macrophage cells, a chamber was devised that

was similar in principle to the Rose perfusion chamber (Rose, 1954), but which allowed the use of smaller coverglasses. The base of the chamber was a 75 x 25 mm glass microscope slide. At the center of this slide a glass ring (13 mm in diameter and 4 mm in depth) was cemented into place with epoxy cement. Before the glass ring was placed into position, slots were ground into the microscope slide and 22 gauge hypodermic needles inserted and glued below the ring. The needles served as entrance and exit ports for perfusion of the culture media. Latex tubing was attached to each of the hypodermic needles. One of the tubes was inserted into a 20 ml bottle containing medium and the other connected to a hypodermic syringe fitted with an automatic pipette. Sterile vasoline was evenly applied to the open edge of the glass ring. A 18 x 16 mm coverglass with monolayered macrophages was placed on the glass ring with the cell surface down. At routine intervals, fresh sterile medium was drawn into the chamber by the syringe. The chamber was then placed into an Incu-Stage[6] incubator mounted on the microscope and set at 37°C. The cells in the chamber were perfused with fresh medium containing 1 x 10^7 leptomonads, Bacillus megaterium or polystyrene[7] particles per ml and observed both with and without phase contrast. For observing the infection process in the perfusion chamber, leptomonads were prepared by methods identical to those employed for inoculation of Leighton tubes. B. megaterium from nutrient

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[6]Lab-Line Instruments, Inc., 15th and Bloomingdale Aves., Melrose Park, Ill.

[7]Dow Chemical Co., Midland, Michigan.

broth cultures was centrifuged at 2500 rpm for 20 minutes. The supernatant was removed, the bacteria resuspended in BSS, the mixture again centrifuged and the supernatant decanted. This time the bacterial cells were resuspended in Eagle's medium and counted in a hemacytometer. Polystyrene particles were suspended in BSS, centrifuged at 2500 rpm for 5 minutes, resuspended in BSS and again centrifuged. This time the particles were resuspended in the Eagle's medium.

The invasion process was observed over long periods in the perfusion chamber with phase contrast provided by an A.O. microscope equipped with an ortho-illuminator. To increase the amount of light essential for photographic exposure of 1/25 second or less under phase contrast, the monolayered coverglasses were edged with sterile vasoline and placed cell surface down on a glass slide with a drop of medium. Only short periods of observation could be made with these preparations, but more light penetrated the medium. Photographs of the entrance of the above agents into macrophages were made individually but sequentially using Kodak Plus-X film and an Eastman Kodak 35 mm camera.

RESULTS

The initial experiments of this investigation were devised to determine the optimal conditions for examination of the infection process. The first experiment was designed to compare the susceptibility of different cell types to infection with leptomonads of L. donovani.

I. Comparative Infection of J-111 and HeLa Cells.

J-111 cells and HeLa cells were each transferred into 8 Leighton tubes containing coverglasses. Twenty-four hours later they were infected with 10^6 leptomonads per tube (Khartoum strain). In sequence, a coverglass of each cell line was removed, fixed, and stained 1, 2, 3, 5, 8, 24, 48 and 72 hours after exposure to the leptomonads. Counts were made of the cell and parasite populations.

The total number of intracellular L.D. bodies in the J-111 cells increased steadily throughout the experiment (Table I). The percentage of infected J-111 cells also increased rapidly up to 8 hours and less dramatically from 24 to 72 hours. (Table I and Figure IB). The mean number of parasites per parasitized cell fluctuated slightly over the 72 hour period but remained low throughout the experiment. It is also important to note the changes in the total number of J-111 cells throughout the 72 hour period. The increases in the total number of host cells and the total number of intracellular L.D. bodies over the 8 to 72 hour

period were somewhat balanced as shown by the minor changes in the per cent of infected cells. In the HeLa cell cultures the total number of intracellular L.D. bodies per 43X field increased rapidly for the first 2 hours appearing to reach a peak population 5 hours after inoculation (Table I). A gradual but definite decrease in the population was noted after the above initial period of leptomonad infection (Figure IA). The percent of parasitized cells was at its highest level 1 hour after infection decreasing throughout the rest of the experiment. The mean number of intracellular L.D. bodies per parasitized cell also decreased but less dramatically (Table I). The HeLa cell population increased almost tenfold over the course of the experiment.

The above comparison shows that within the first few hours of the experiment the leptomonads have a greater affinity for the HeLa cells than the J-111 cells. Even though initially fewer parasites were found in the J-111 than in the HeLa cells, the organisms in J-111 cells proliferated over the entire 72 hour period, at which time their population exceeded that of the L.D. body population in the HeLa cells. Although the increase in the percentage of infected cells in the first few hours of the experiment was probably due to continuous entrance of the leptomonads into the host cells, the decrease in parasites after 5 hours was evidence that the HeLa cells did not support the growth of the L.D. bodies.

Table I. Comparative Infection of J-111 and HeLa Cells with Leptomonads of L. donovani.

	Time before fixation (hours)	Cells per field[1]	L.D. bodies per field	Percent of cells parasitized[2]	Mean number of L.D. bodies per infected cell
J-111 Cells	1	57.8	0.5	0.8	1.0
	2	62.2	1.9	2.4	1.3
	3	60.7	5.7	5.7	1.6
	5	87.4	9.1	8.7	1.2
	8	108.0	19.4	10.0	1.8
	24	130.9	17.6	12.2	1.1
	48	186.4	32.1	12.3	1.4
	72	251.4	81.9	18.1	1.8
HeLa Cells	1	110.1	21.9	11.7	1.7
	2	306.5	44.6	10.4	1.4
	3	340.7	46.3	9.7	1.4
	5	502.9	70.9	9.4	1.5
	8	537.0	42.1	5.6	1.4
	24	621.5	48.7	4.9	1.6
	48	832.3	34.9	3.5	1.2
	72	1051.1	16.3	1.6	1.0

[1]Based on the average of 10-43X objective fields.

[2]Based on a count of 250 serial cells.

Changes in the population of intracellular L.D. bodies in J-111 and HeLa cells.

Changes in the percentages of infected J-111 and HeLa cells.

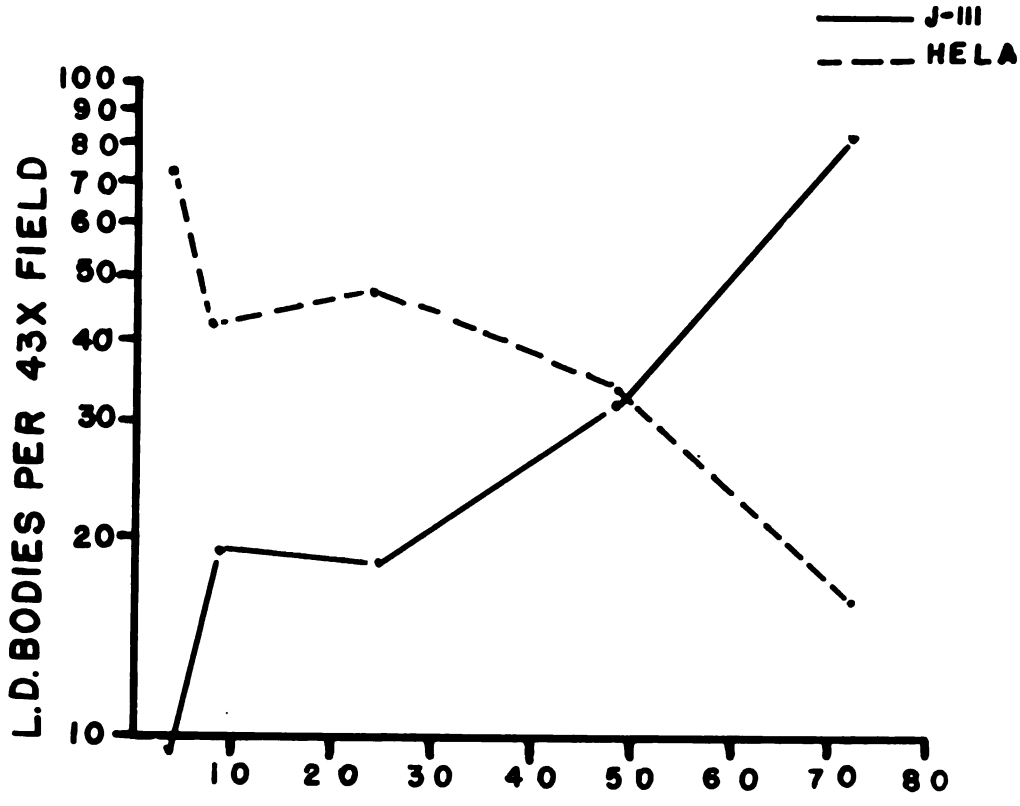

Figure 1A.

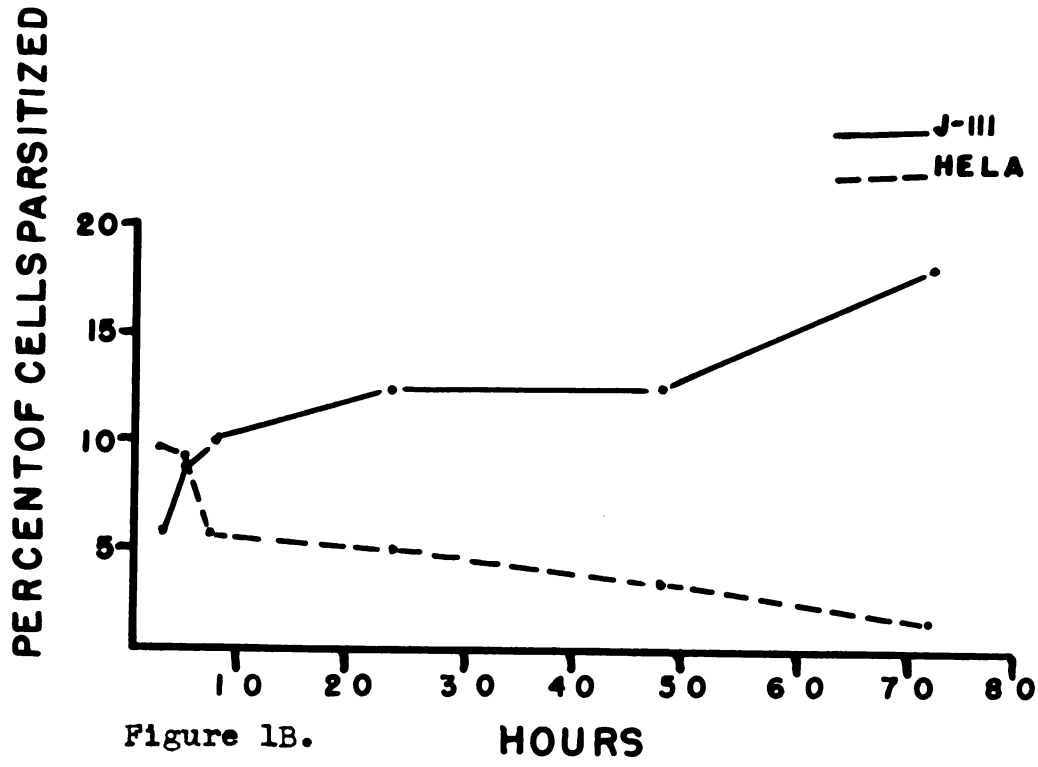

Figure 1B.

Leptomonads exposed to Eagle's medium at 37°C with and without host cells in the medium rounded up and became immotile in a few hours. It is doubtful if these organisms were infective after 5 hours at 37°C. Observations of the stained HeLa cell preparations demonstrated the occasional presence of intracellular, degenerate L.D. bodies which consisted of two granules representing the nucleus and kinetoplast.

II. Susceptibility of Macrophages to Infection with Leptomonads.

Since the previous studies with serial cell lines produced only low incidences of infection and limited growth of L.D. bodies, studies were undertaken using primary macrophages. This type of phagocyte is considered to be the primary site of infection in the mammalian host and would therefore seem to be an ideal cell for in vitro infection and propagation.

Macrophages were harvested from hamster peritoneal fluid and 5×10^5 exudate cells were allowed to monolayer on a coverglass in each of 6 Leighton tubes for 24 hours. Not all of these cells attached to the coverglass. Some were subsequently washed from the tubes with replacement of medium. An inoculum of 2.5×10^6 leptomonads of the Khartoum strain was added to each tube. One coverglass was fixed and stained at each of the following times post inoculation: 1, 2, 3, 4, 5, and 6 hours. Most of the cells in the peritoneal exudate were mononuclear macrophages. Differential counts showed that fibroblasts and polymorphonuclear cells composed only about 10 to 15% of the total cell population. Only on rare

occasion did fibroblasts from these experimental cultures contain an intracellular L.D. body. Parasites were never found within the polymorphonuclear cells.

Both the number of L.D. bodies per 100 macrophages and the percent of infected macrophages increased rapidly during the first two hours after inoculation. More than half of the cells were parasitized in the first hour, but the intracellular L.D. population nearly doubled between the first and second hour of exposure to leptomonads. There was only a slight increase in the total number of parasites, in the percent of infected macrophages, and in the mean number of parasites per macrophage between 2 and 6 hours after inoculation. The macrophage population did not change significantly over the 6 hour period of this investigation.

In an experiment similar to the one previously described, the infectivity of the Khartoum and 3S strains of L. donovani was compared over a 72 hour period. A suspension of 5×10^5 macrophages was added to each of 14 Leighton tubes with enclosed coverglasses. After 24 hours of incubation each tube was inoculated with 1.25×10^6 leptomonads; one series of 7 tubes was inoculated with Khartoum strain and the other series with 3S strain. This gave a ratio of 2.5 leptomonads per macrophage in each tube. One coverslip from each series was removed from the Leighton tube, fixed and stained at each time interval listed in Table III. Over the 72 hour period of this experiment counts showed no increase in the number of macrophages, so 250 macrophages were examined and

Table II. Infection of Primary Macrophages with 2.5 x 10^6 Leptomonads (Khartoum)

Time before fixation (hours)	Number of L.D. bodies (per 100 cells)[1]	Percent of cells parasitized	Mean number of L.D. bodies per infected cell
1	227	66.2	3.4
2	448	89.2	5.0
3	508	96.2	5.3
4	484	83.8	5.8
5	507	89.7	5.7
6	574	97.8	5.9

[1]Based on a count of 250 macrophages.

the number of L.D. bodies per 100 macrophages was used as the population index.

The increase in the number of L.D. bodies up to 6 hours post inoculation was believed to show primarily the rate of initial invasion by leptomonads and, in the 6 to 72 hour period, the reproduction of L.D. bodies and infection of other cells with the bodies. With the Khartoum strain, 100% of the leptomonads were represented by intracellular L.D. bodies 6 hours after inoculation with a total of 272 L.D. bodies or a ratio of 2.7 L.D. bodies per macrophage (Table III). With the 3S strain this ratio was only 1.7 L.D. bodies per macrophage at 6 hours, representing only 60% of the inoculated leptomonads. By 6 hours, leptomonads of the Khartoum strain infected nearly 90% of the macrophages, but leptomonads of the 3S strain infected only 51.9% of the macrophages. There was no significant difference in the number of L.D. bodies per infected macrophage between the two strains.

Between 6 and 72 hours after inoculation the 3S strain L.D. bodies increased rapidly in culture attaining a population of 441 L.D. bodies per 100 macrophages and infecting 93% of the macrophages in the culture at 72 hours. The L.D. bodies of the Khartoum strain showed a more gradual increase (Figure IIA and B). Seventy-two hours after infection the total number of parasites, the percent of macrophages infected and the average number of parasites per infected cell were nearly identical for the two strains. This level of infection may have been a peak parasite population for the macrophage culture system.

This experiment clearly shows that the Khartoum leptomonads had a greater affinity for macrophages than the 3S leptomonads. A comparison of the growth rates of the two strains after initial infection probably was not valid in accessing strain characteristics because the potentiality for further growth of the Khartoum strain may have been limited by the number of macrophages available.

Further support of this *in vitro* study of the two strains was given by the authors experiences of maintaining infections with the strains *in vivo*. Golden hamsters succumb in about 1 to 2½ months when inoculated intraperitoneally with 2×10^6 L.D. bodies of the Khartoum strain of *L. donovani*. With the same size inoculum of the 3S strain, hamsters died in the second or third month.

One other point of interest should be emphasized. In both experiments with macrophages--early in the infection period (½ to 1 hour after exposure to leptomonads), when about only 50-70% of the macrophages were infected--the macrophages each contained over 3 L.D. bodies. If each L.D. body was represented by one invading leptomonad, the infection process was not governed by a random chance contact of leptomonads and macrophages. Random contact and cell invasion would result in a much higher percentage of infected cells at the time the average infected cell contained 3 or more macrophages.

Table III. Comparative Infections of Macrophages with Khartoum and 3S strains.

	Time before fixation (hours)	Number of L.D. bodies (per 100 cells)[1]	Percent of cells parasitized	Mean number of L.D. bodies per infected cell
Khartoum strain	½	124	42.9	2.9
	1	186	60.2	3.1
	3	228	73.8	3.1
	6	272	89.2	3.2
	11	342	95.0	3.6
	24	349	92.1	3.8
	72	452	91.3	4.9
3S	½	111	43.8	2.5
	1	167	52.1	3.2
	3	186	60.0	3.1
	6	173	51.9	3.3
	11	222	58.3	3.8
	24	262	67.1	3.9
	72	441	93.0	4.8

[1]Based on a count of 250 macrophages.

Changes in the percent of cells infected with the Khartoum and the 3S strains.

Changes in the L.D. body populations in macrophages infected with the Khartoum and the 3S strains.

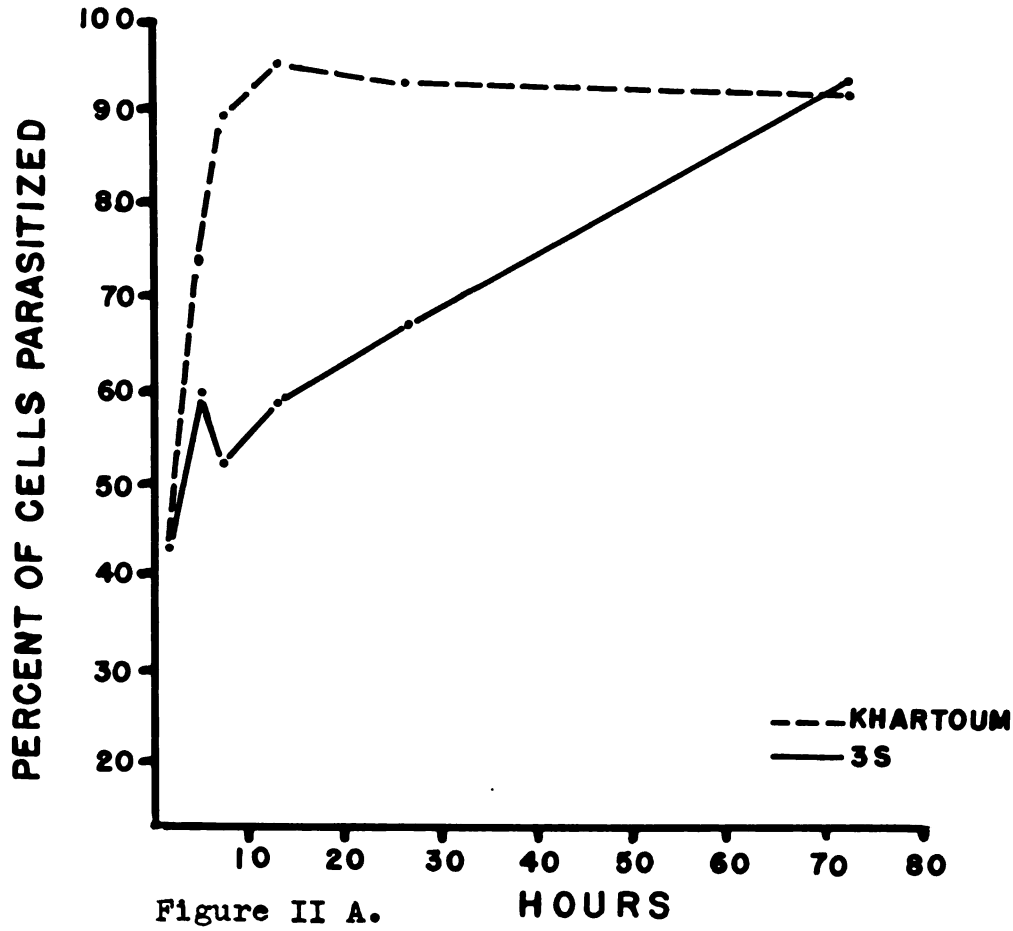

Figure II A.

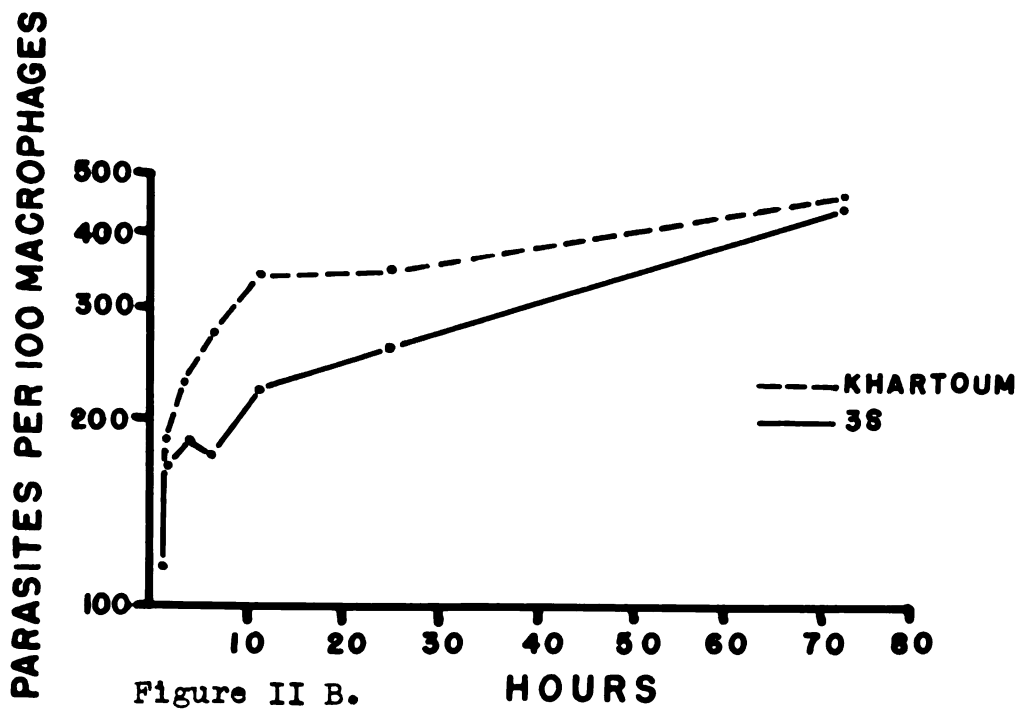

Figure II B.

III. Culture In vitro of In vivo Infected Macrophages.

The above experiments with macrophages were not believed to give valid values on the rate of multiplication of L.D. bodies in primary macrophages. To determine the intracellular growth of L.D. bodies, peritoneal macrophages and their intracellular L.D. bodies from hamsters infected 2 months previously with L. donovani (3S strain) were cultured on coverglasses in Leighton tubes. The animals were necropsied after collecting the macrophages and impression smears were made of the spleen to confirm the abundance of L.D. bodies. The cells in Leighton tubes were fixed at 1, 3, 6, 18, and 30 hours after inoculation into culture. These were then stained and counted.

The total number of intracellular L.D. bodies per 43X field remained relatively constant up to 6 hours post cultivation (Table IV). An appreciable increase in L.D. bodies occurred between 6 and 30 hours. The total number of L.D. bodies and the percentage of infected cells followed similar patterns of increase. In this experiment, as in the preceeding experiments with peritoneal macrophages, the mean number of L.D. bodies per parasitized macrophage remained relatively constant, even during periods of obvious parasite proliferation. Again, with low percentages of the macrophages infected, the average number of parasites per macrophages was exceedingly high. It should be noted that even though the mean number of parasites per infected cell was relatively low and constant, some macrophages contained as

Table IV. Changes in Macrophages *In Vitro* Infected *In Vivo*.

Time before fixation (hours)	Cell Counts per 43X field[1]	Number of L.D. bodies (per 43X field)	Percent of cells parasitized	Mean Number of L.D. per infected cell
1	40.5	26.3	29.0	2.2
3	36.0	24.0	28.8	2.2
6	37.1	25.3	32.5	2.1
18	37.5	45.5	55.1	2.2
30	33.7	56.2	59.6	2.8

[1]Based on the average of 10, 43X fields.

many as 15 L.D. bodies per cell. The maximum generation time of L.D. bodies, calculated between 6 and at 18 hours, was 14.2 hours.

IV. Parasitization of Macrophages of Various Ages In Vitro.

In order to determine the effect of the cultural age of the macrophages upon the infection rate, peritoneal macrophages were harvested from uninfected hamsters and placed into Leighton tubes. These were infected from 2 to 7 days later. Two Leighton tubes with coverglasses of monolayered macrophages were infected at each time interval (Table V) with 2×10^6 leptomonads of the Khartoum strain per tube. Macrophages of one of the tubes of the pair was fixed at 1 hour and the other at 4 hours post inoculation. Results of this experiment were summarized on Table V. The cells fixed at 1 hour post inoculation showed lower infections than those fixed at 4 hours. A marked decrease in the percentage of cells infected was noted with increasing age (Figure III). The mean number of intracellular L.D. bodies per infected cell remained about 3 throughout the duration of this experiment even when less than 50% of the cells were infected. Again this was obviously not a case of random contact and invasion of macrophages.

As the age of the macrophages in culture increased, the morphology of the cells was observed to undergo changes. The stained coverglasses revealed little deviation in the appearance of the cells up to 3 days in culture. These

Table V. Parasitization of Macrophages of Various Ages.

In vitro age of macrophages (days)	Time of exposure to leptomonads (hours)	Percent of cells parasitized	Mean Number of parasites per infected cell
2	1	40.4	3.0
	4	83.2	3.9
4	1	27.2	2.8
	4	56.3	3.6
5	1	13.7	2.0
	4	24.0	3.1
7	1	8.7	2.4
	4	12.2	3.2

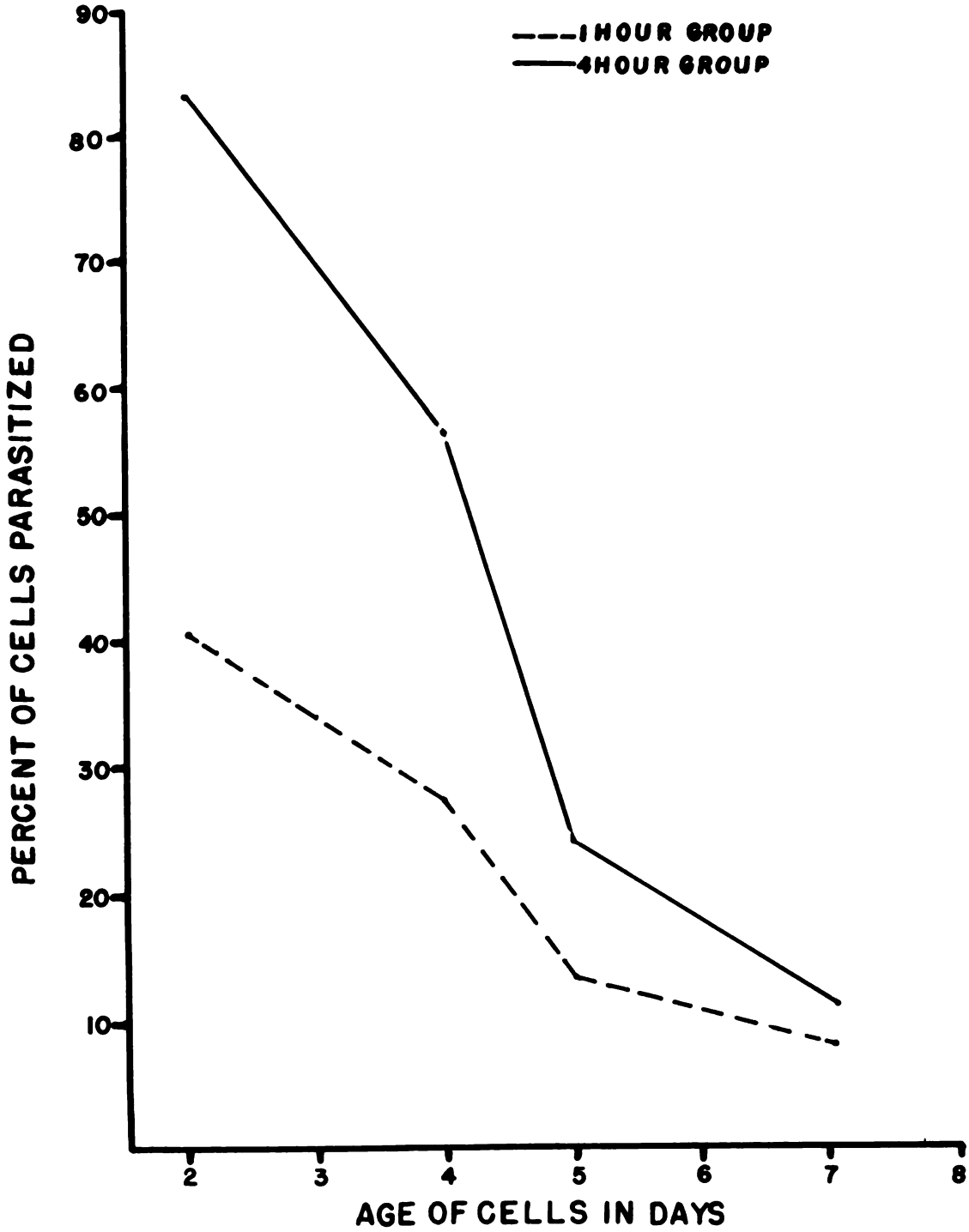

Figure III. Macrophage age vs. percent of macrophages infected.

macrophages were round, about 15-20μ in diameter, had a "kidney" shaped nucleus and extruded pseudopodia. In contrast, macrophages in culture for 4 days or longer were about 30-40μ in diameter and had a large, slightly oval nucleus. In addition, these cells usually contained many large granules.

V. Observations of the Infection of Macrophages with Leptomonads.

To study the behavior of leptomonads entering mononuclear macrophages, primary cell cultures were monolayered and incubated for 6-24 hours after removal from hamster peritonea. These were then transferred to the perfusion chamber for observation. Many of the cells displayed slow, random, amoeboid-like, unidirectional movement over the coverglass of about 5μ per minute. These phagocytes were approximately 15μ in diameter but their rounded form changed frequently to a triangular form with active movement. As the macrophage moved, a short thin sol-like membranous veil was laid down. Both pinocytic activity and cytoplasmic extrusions were observed in some of the cells (Plate I,G).

Leptomonads were perfused into the chamber for study of the infection process. Within about 5 minutes the parasites began to attach by the anterior end of their flagella to the main body or pseudopodial veils of the mononuclear cells (Plates IA, IIA and G). The leptomonads were very active at the time of contact. These parasites appeared to pull at their site of attachment to the macrophage and moved back and forth very rapidly as if trying to free themselves. Everytime the

long flagellum made contact with the macrophage membrane, it adhered at a point more proximal to the parasite cell. Engulfment began either with flagellar attachment (Plates IA, IIA and G) or when the anterior end of the leptomonad was in contact with the macrophage membrane. A thin pseudopodial veil flowed over the leptomonad toward the posterior end of the leptomonad (Plates IC and D, IIB, C, D and H). This envelopment of the leptomonad usually occurred in about 2-5 minutes after initial contact of its flagellum. Many times the flagellum was still visible within the extended pseudopod (Plate I, H and I). The pseudopod containing the parasite was pulled into the main body of the cell and a vacuole was definitely visible around it (Plate II, E). The small lysosome-like granules of the macrophage (previously described by Myrvik, Leake and Fariss, 1961) occasionally migrated up to the vacuole but no release of their contents was noted. After the leptomonad was drawn into the macrophage, the pseudopod slowly retracted behind it (Plate IE, IID). At this point the vacuole disappeared and the parasite rounded up. The flagellum, if still present, was no longer visible. The parasite now had the characteristic shape and size of an L.D. body. The complete process from flagellar attachment to the disappearance of the vacuole required 10-20 minutes.

The above pattern of behavior occurred with the majority of the leptomonads ingested. Degenerate leptomonads described by Pipkin (1960) were occasionally also observed to be engulfed

by the macrophages in a similar sequence of events. Vacuole formation and granule migration were not always observed, probably because the dense granular cytoplasm interfered with optical observation. Rarely, partial engulfment would be followed by cytoplasmic extrusions (Plate IG) of the host cell. These were described by Bessis (1956) as evidence of degeneration of the mononuclear macrophage. When this occurred the ingestion process was terminated. On one occasion two macrophages were observed to struggle over the same leptomonad. One of the cells was finally able to engulf a major portion of the leptomonad and force the other cell to release its hold on the parasite.

Stained preparations of the cells from the perfusion chamber more readily demonstrated intracellular L.D. bodies. Some of these cells were noted to contain as many as 10-15 L.D. bodies.

Macrophages that had been in culture bottles for 96 hours, before being transferred to the perfusion chamber for observation (Plate II, G-I), were very sluggish in movement and were much larger (30-40μ) than the macrophages described above. The leptomonads were taken into these cells as previously described but the time required for the complete process varied from 30-60 minutes in a series of 10 observations.

VI. Phagocytic Events Observed for Bacteria and Polystyrene Particles.

Control studies of the phagocytic events were made to reveal differences between the phagocytosis of the leptomonads

Plate I. The engulfment of leptomonads by macrophages. A-F. Sequential photographs of the complete engulfment process of a leptomonad by a 24 hour old *in vitro* macrophage. G-I. Partial engulfment of leptomonads by macrophages in culture for 24 hours. Note the fine pseudopodial veil with pinocytic vacuoles on the left side of the macrophage and the extruding cytoplasm on the right side (I-G). The flagellum of the leptomonad can be seen within the cytoplasm of the macrophages in I-H.

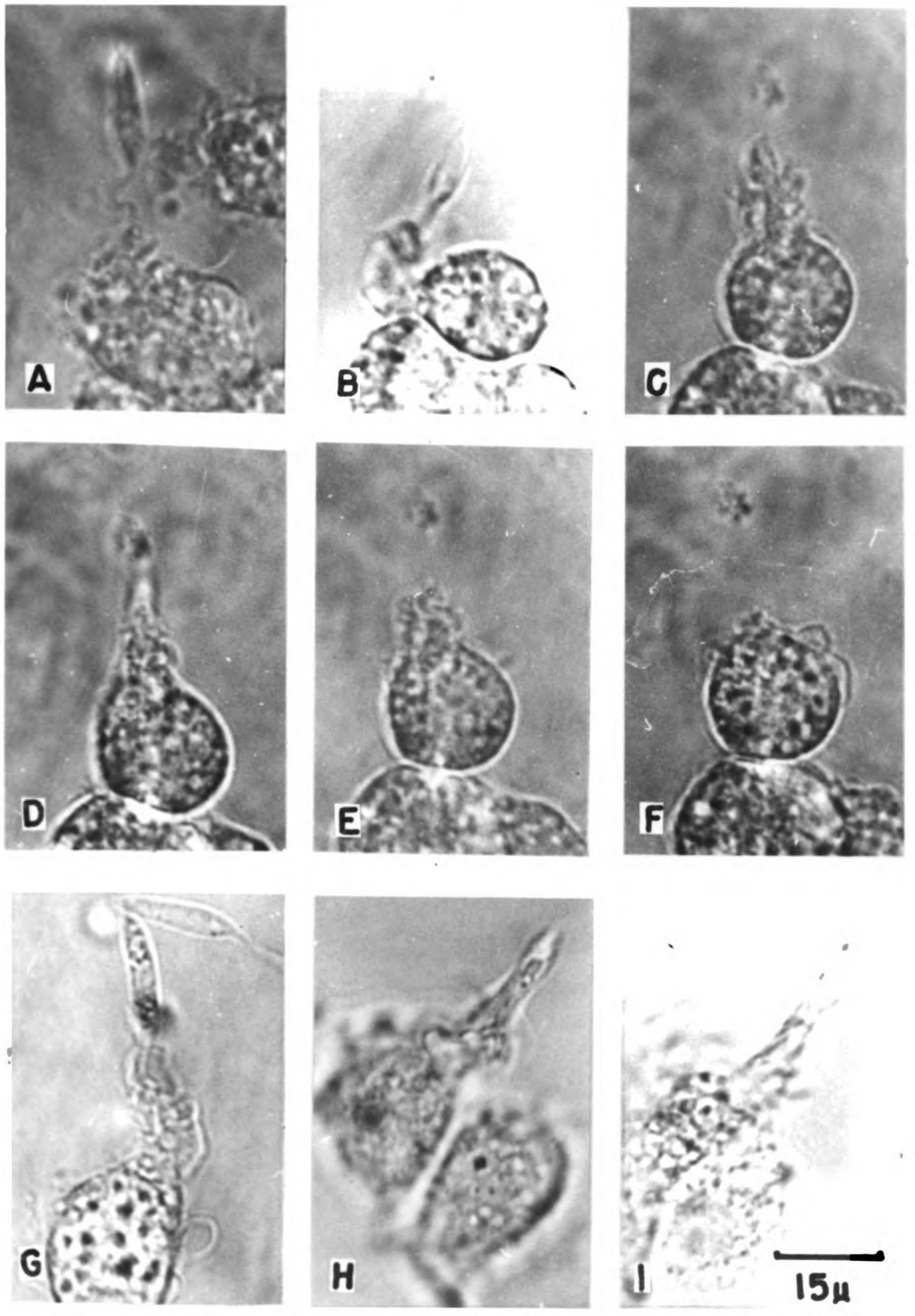
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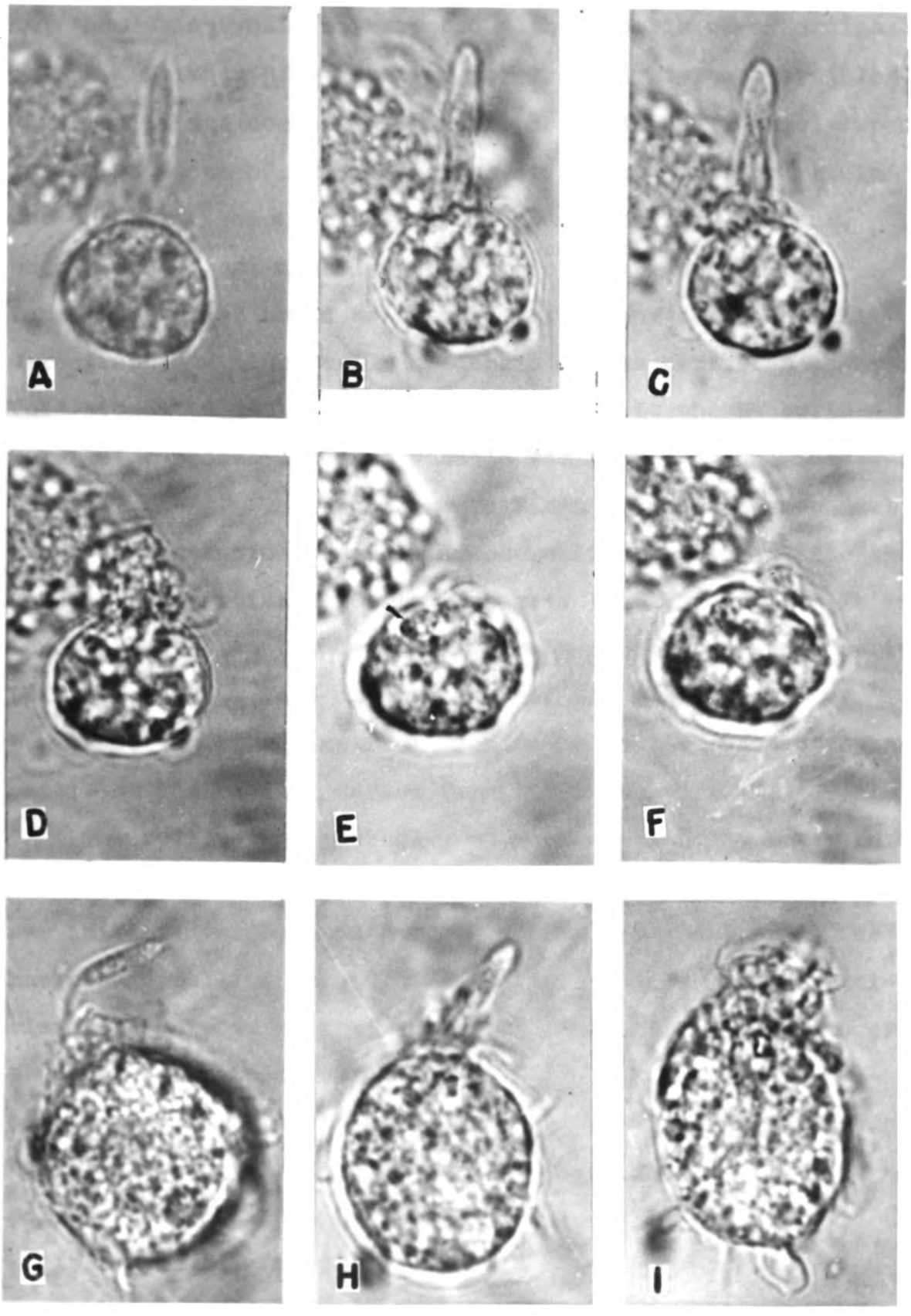
Plate II. The engulfment of leptomonads by 24 and 96 hour old macrophages.

A-F. Sequential engulfment of a leptomonad by a macrophage that has been in culture for 24 hours including flagellar attachment (II-A) and the formation of an intracellular vacuole containing the transitional leptomonad--L.D. body (II-E). G-I. Sequential engulfment of a leptomonad by a macrophage that has been in culture for 96 hours. The magnification is the same as plate I.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and that which would be expected with bacteria or a foreign object. Neither B. megaterium nor the polystyrene particles were motile and contact with macrophages depended solely upon random movement of the phagocytes or slight currents within the chamber. This may have been the reason that an hour, post inoculation, usually passed before entrance of the foreign material could be observed. In many cases bacilli were trapped against cells or debris in the perfusion chamber. This seemed to facilitate their capture. As the bacteria were being phagocytized, a vacuole was formed around them. (Plate I, A-F). The bacilli were taken into the macrophages where degranulation of small lysosome-like particles into the vacuole and partial digestion occurred simultaneously. It is impossible to see the degranulation in the photographic series (Plate I, A-F). In 8 observations of the phagocytosis of B. megaterium, the time from engulfment to the onset of cytopepsis of the bacilli varied from 15-25 minutes.

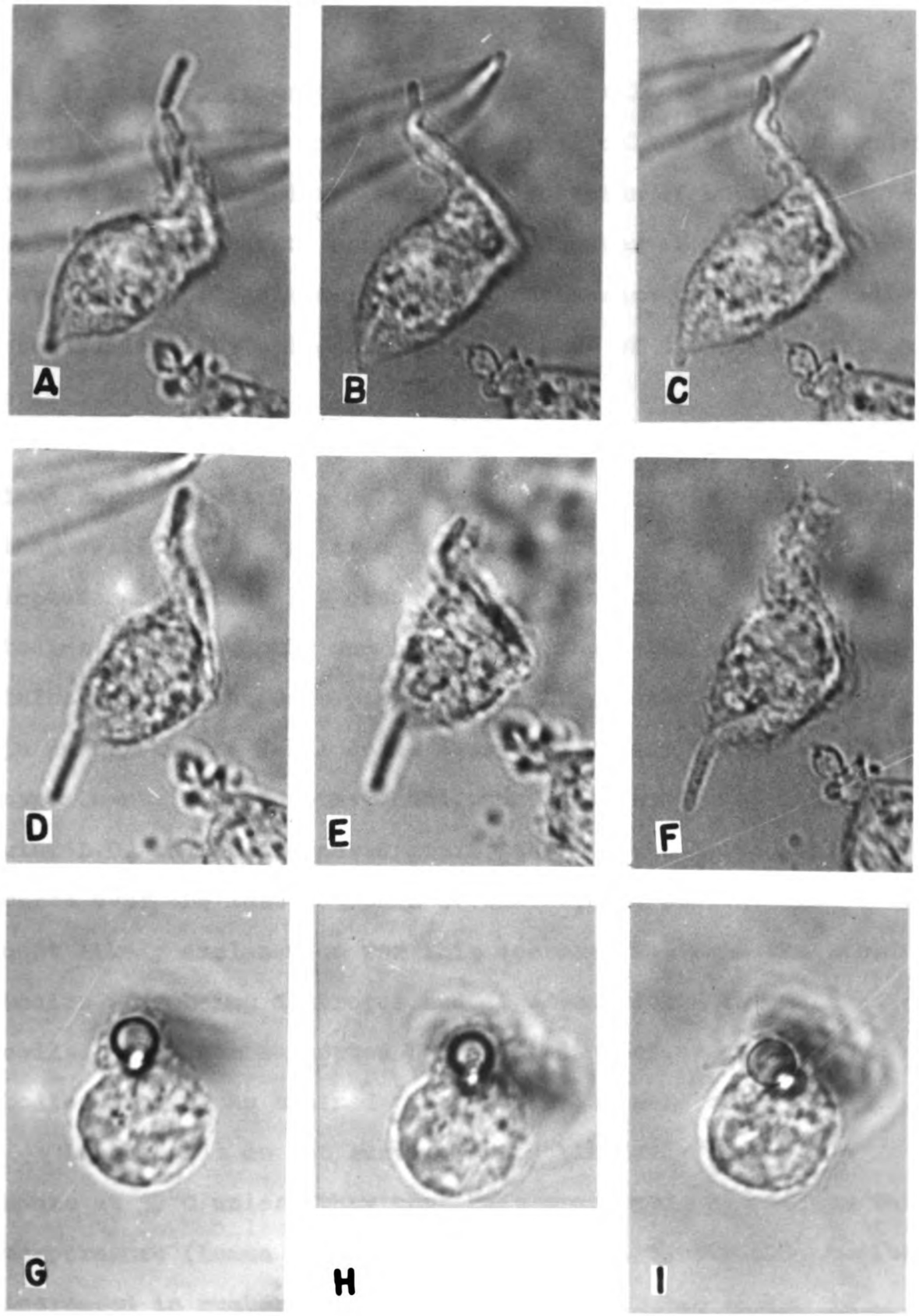
The polystyrene particles were observed to be taken into the cells in much the same manner as the bacilli. Phagocytosis was initiated with the pseudopodial veil (Plate I,G) of the macrophage pulling the particle into the cell. These particles were very refractive (Plate II G-I) and the presence or absence of a vacuole was impossible to confirm. The intracellular granules appeared to be very passive to this foreign body. Quite often the engulfment process was reversed and the polystyrene particles were voided from the macrophages.

In general, the phagocytosis of both the bacilli and the polystyrene differed from that of leptomonads by the

readiness with which the leptomonads attached. Degranulation and partial digestion seen with ingested bacilli did not occur with the leptomonad ingestion. The frequent voiding after ingesting seen with polystyrene particles was not observed with leptomonads or bacilli.

Plate III. Phagocytosis of bacilli and polystyrene particles.

A-F. Sequential phagocytosis of B. megaterium by a 24 hour old macrophage. B-C. The bacilli are first enclosed in a long vacuole (III B & C) and later digested (III D-F) with some of the bacilli pushed through the opposite end of the macrophage while still enclosed by the macrophage membrane. The "kidney" shaped nucleus of the macrophage shows in III F. G-I. Partial phagocytosis of a polystyrene particle. The magnification is the same as plate I.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DISCUSSION

Initially, attempts were made to grow leptomonads in serial cell lines. The use of a serial cell line was intended to overcome the need for periodic isolation of macrophages and make it easier to obtain a large number of cells for investigation. HeLa cells were used because of their immediate availability and J-111 cells because of their monocytic origin.

In vitro infections of both HeLa and J-111 cell lines demonstrated that HeLa cells took up leptomonads much faster than J-111 cells. After a one hour period of exposure to leptomonads, about 10% of the HeLa cells contained L.D. bodies, whereas, only one percent of the J-111 cells were infected over the same period. The L.D. bodies increased in number in the J-111 cells over the 72 hour period of the experiment. On the other hand, the total number of intracellular L.D. bodies decreased appreciably in the HeLa cell cultures over the last 48 hours of the 72 hour period. The most likely explanation for this decrease was that the L.D. bodies were being destroyed and digested within the HeLa cells. This was supported by the finding of degenerate L.D. bodies within the stained preparations of HeLa cells.

Leptomonads do not survive extracellularly for over 5 hours at 37°C unless they have been previously adapted to this temperature (Lemma and Schiller, 1964). But, the L.D. bodies increased in number in the J-111 cells over the entire 72

hour period. The increase of L.D. bodies after the first few hours must be due to intracellular proliferation in the J-111 cells, followed by their release from the infected cells and subsequent entrance into uninfected cells. No attempt was made to observe the phagocytosis of the leptomonads using J-111 or HeLa cells. The Giemsa stained cells examined at 1 and 3 hours post inoculation did show distortions of the cell membrane of the J-111 cells but no pseudopodia were visible. Shepard (1955) has reported that HeLa cells can be induced to phagocytize bacteria. Although J-111 and HeLa cells appear similar with respect to their epitheloid morphology, the intracellular environment of the J-111 cell was probably much more favorable for survival and proliferation of L. donovani. This was not completely surprising since J-111 cells originated from human leukemic monocytes.

The low incidence of infection and the abnormalities encountered, suggested an unfavorable host-parasite relationship. Therefore, serial cell lines were replaced by macrophages in the rest of this investigation.

The peritoneal exudate proved to be an excellent source of phagocytic macrophages. Because these cells survived well in culture and were believed to be the normal host-cell type for leptomonad invasion, they were used exclusively in the remaining studies. Intraperitoneal BSS stimulations were used for the collection of these cells. Rowley (1962) points out that phagocytic cells that are harvested by stimulation with glycogen or oils are not normal, possessing amongst other

differences, more active enzyme systems than normal cells (Cohn and Morse, 1959).

Macrophages from the peritoneal exudate were much more susceptible to L. donovani than J-111 or HeLa cslls. Within 2 hours approximately 90% of the cells were infected with an average of 3 to 4 L.D. bodies per cell. This incidence of infection far exceeded that encountered with either HeLa or J-111 cells. This was expected since Adler (1964) observed that the parasites were first picked up by circulating monocytes and shortly thereafter were found in spleen and liver macrophages.

Since occasional fibroblasts from the peritoneal exudate were found infected, their potential as a host-cell for the parasite merits consideration. Herman (1964) failed to infect peritoneal fibroblasts in vivo. Pulvertaft and Hoyle (1960) were unable to infect fibroblasts from explanted hamster spleens. In a review of the use of tissue culture for propagating protozoa, Pipkin (1960) emphasized that cells undergo drastic changes in vitro. They are further complicated by variations that arise between laboratories in the methods of obtaining cells and preparing media, and also with temperature differences for inoubation. In view of these differences it was not surprising that the attempts to infect the fibroblastic cell, which seemed to be just at the threshold of susceptibility to L. donovani, produced variable results.

Differences were noted in the infectivity of two strains of L. donovani for macrophages maintained in primary culture.

Nearly 90% of the cells inoculated with Khartoum strain of L. donovani were infected after 6 hours, whereas, under identical conditions the 3S strain infected only about 50% of the cells in 6 hours. The increase in L.D. bodies after the first 6 hours, when any remaining extracellular leptomonads in the culture medium would be dead, was due to proliferation of the intracellular parasites. The L.D. bodies of the 3S strain continued to increase while those of the Khartoum strain showed little increase. In 72 hours the populations of the two strains were equal. Possibly the 72 hour population level represents a maximum parasite population attainable in this culture system. This could explain the failure of the Khartoum strain to increase in numbers.

In both of the experiments in which leptomonads were used to infect macrophages, an average of about 3 L.D. bodies per cell were found within macrophages after one hour when only 50-60% of the cells were infected. One hour was not sufficient time for significant L.D. body proliferation, and the L.D. bodies must have represented multiple leptomonad invasions of certain of the macrophages, it was difficult to believe that the remaining cells could have escaped infection because of a lack of chance contact with the leptomonads. Instead it suggested that certain macrophages were more susceptible than others. Possibly the chance contact and uptake of a leptomonad by certain macrophages enhances the subsequent uptake of more leptomonads by these cells. Rowley (1960) has found that phagocytosis of particulate or colloidal materials enhances

subsequent phagocytic and metabolic activity of macrophages. The same phenomenon may exist after the uptake of leptomonads by macrophages.

By culturing peritoneal macrophages from an animal that was previously infected with L. donovani, an attempt was made to determine the early growth rate of the leishmaniform organisms in cell culture without the complications of continued engulfment of leptomonads. There was very little multiplication or invasion of new cells during the first 6 hours that the cells were in culture. This may have represented a period of adjustment to the in vitro culture system. Between 6 and 30 hours, however, L.D. body proliferation did occur. Unfortunately growth rates were too irregular and the number of observations too few for accurate determinations of the growth rate.

The age in vitro of the macrophages proved to be an important factor in their infection by L. donovani. The ability of macrophages to take up leptomonads decreased as the age in vitro of the host cell increased. This decrease in number of parasitized cells with the age of culture can be correlated with the morphological and physiological changes of the macrophages. Cohn and Benson (1965) described these changes as an enlargement of the macrophages accompanied by accumulation of phase-dense granules. The granules were demonstrated to be lysosome-like organelles with acid phosphatase, cathepsin and β-glucuronidase activity. Rowley (1962) believed that the longer the macrophages are in culture, the

more abnormal these cells become. He pointed out that many workers have drawn their conclusions from work with macrophages after several days of in vitro growth and have ignored the first day in vitro. Pulvertaft and Hoyle (1960), studying primary macrophages, reported that the fine, uniform granules gradually increased in size as the cells became older. They described the motion of the cells as sluggish after 1 week in vitro. No attempt was made by these workers to correlate the percentage of infection with the aging process.

Observations of the infectious process in living macrophages revealed that the initial flagellar contact depended primarily on the movement of the leptomonads. The possibility of some trophic stimulus attracting the leptomonad to the monocyte was considered but the observations of leptomonad behavior casted doubt on this possibility. Only when the flagella touched the cells, apparently by chance, was attachment observed. The close proximity of the leptomonad and host cell membranes did not stimulate any obvious signs of a trophic attraction. A study of the structural complement of the flagella and the macrophage cell membrane might reveal the physical basis for attachment. Visually it appeared as though a loss of the flagellar attraction to the host cell membrane would prevent the natural infection process.

Pulvertaft and Hoyle (1960), with the aid of cinemicrographic techniques, observed leptomonads of L. donovani entering "monocytes" in culture posterior end first. These workers called the entrance process invasion, but at no time was it referred to as phagocytosis. Many of the leptomonads that

they observed were dead before or soon after ingestion. The pseudopodia of the monocytes were described as being extended up to 100μ toward the leptomonads. Their descriptions plus illustrations of very large "monocytes" indicated an *in vitro* cell which differed radically from the hamster macrophage employed in this present investigation.

The uptake of *B. megaterium* and the inert polystyrene particles by peritoneal macrophages was similar to the phagocytic process described by Hirsch (1965), and Cohn and Benson (1965). The bacteria and polystyrene particles were used as a control to compare phagocytosis of *L. donovani* with true phagocytosis. The major difference between bacterial phagocytosis and leishmanial phagocytosis was that cytopepsis followed ingestion of the bacteria but not the leptomonads. Shortly after the *B. megaterium* rods were taken into the macrophages, rapid degeneration of the vacuolated rods commenced. On the other hand, after having attained an intracellular position, the leptomonads of *Leishmania* formed a compact round sphere with no evidence of degranulation or digestion. The vacuole that was seen to form around the entering leptomonad disappeared in a very short time. One can only speculate as to how the entering parasite was able to resist digestion on the part of the macrophage and instead utilize this intracellular environment for growth and proliferation. Organisms such as the *B. megaterium* that were destined to be partially or totally digested may have released some "soluble substance" upon being engulfed that stimulated lysosome attraction and

degranulation, as suggested by (Hirsch, 1965). Cohn, Hirsch and Wiener (1963) reviewed evidence indicating that the latent enzymes within lysosomes were activated by intracellular pH changes upon the engulfment of particles. Whatever the mechanism that normally triggers this enzyme activation, after engulfment of L. donovani either enzyme activation did not occur or the parasite membrane was not affected by the enzymes. However, disappearance of the flagellum may have been a result of enzyme action. Rudzinski, D'Alesando and Trager (1962) have demonstrated that the outer surface of the L.D. body consists of two complete membranes. They further noted that only one such membrane surrounds the leptomonad. The association of the L.D. body and its outer membrane was very intimate, in that daughter L.D. bodies also had double membranes. The two membranes remain associated during the division process. In contrast to the L.D. bodies of L. donovani, intracellular bacteria seemed to lack the intimate association between the cell wall and host cell cytoplasm. Brucellae appeared to reside and divide within a well formed vacuole in the cytoplasm (Richardson, 1959 and Karlsbad, Kessel, DePetris and Monaco, 1964). It was quite possible that the outer membrane of the L.D. body came from the original vacuole and that protection of the parasite from digestion is in someway related to the molecular configuration of these two membranes. But, further investigation is necessary to substantiate this hypothesis. It would be interesting to know if there was a fundamental difference in the physiological basis of the association with

the host cell between L.D. bodies of L. donovani and bacteria such as Brucella.

SUMMARY

1. J-111 (originating from human monocytes) and HeLa serial cell lines (from human epidermoid carcinoma) and primary hamster peritoneal macrophages were cultured as monolayers on coverglasses in Leighton tubes with Eagle's medium. Leptomonads of both the Khartoum and 3S strain of L. donovani were obtained from the fluid overlay of modified NNN medium and used to infect the cell cultures. Counts of parasites and parasitized cells were made from stained coverglass preparations. A fabricated Rose-type perfusion chamber was used to observe leptomonad entrance into macrophages.

2. Leptomonads initially infected about 12% of the HeLa cells but were unable to survive and multiply in these cells. Only about 1% of the J-111 cells were initially infected with Leishmania. Since leptomonads die within about 5 hours in these cultures, the progressive increase in intracellular parasites in the J-111 cells was attributed to proliferation of the intracellular leishmaniform (L.D.) stages. Primary macrophages displayed a 60 to 90% incidence of initial infection within 2 hours after inoculation with leptomonads followed by subsequent multiplication of the L.D. bodies. Leptomonads of the Khartoum strain showed a higher incidence of infection than those of the 3S strain.

The infectivity of leptomonads for macrophages decreased with the increased cultural age of these host cells. Twenty-four hour macrophages showed a 40% infection with 1 hour of

exposure to leptomonads and 83% infection after 4 hours exposure. Seven day old macrophages showed only 9% and 12% infection respectively for one and 4 hours of exposure. Distinct morphological changes were correlated with the aging of the macrophages in culture.

3. Observations of the invasion of macrophages in the perfusion chamber revealed that the leptomonads consistently first attached only by their flagella to the unextended cell membrane or extruded pseudopodia of the macrophages. The mobility of the leptomonads greatly increased their chances of making contact with the host cells. This initial contact stimulated the subsequent phagocytosis of the leptomonad by the macrophage. As the leptomonad was taken into the host cell, a vacuole was seen to form around it. This was visible for only a few minutes and then disappeared along with the flagellum of the parasite. By this time the 2 x 15μ parasite had rounded up to approximately a 3μ diameter. The complete ingestion process from flagellar contact to the disappearance of the vacuole lasted 10-20 minutes for 24 hour old macrophages. Phagocytosis was slower with 96 hour macrophages.

To compare leptomonad phagocytosis with bacterial phagocytosis, bacteria were added to cultures of hamster peritoneal macrophages. Engagement by the phagocytes depended on random contact, which was much slower than contact with active leptomonads. Bacteria were taken into vacuoles, and upon release of the lysosome granules, rapidly degenerated within the vacuoles.

4. Leptomonads seemed to have predilection for certain cells. With only about 50% of the macrophages invaded by leptomonads, the infected cells contained an average of about 3 L.D. bodies shortly after inoculation. This suggested that the entrance of leptomonads into a cell enhanced further infection of the same cell.

Observations of leptomonads suggested that, if flagellar attachment to the host cell membrane or pseudopod could be blocked, the natural infection process would not proceed past this point.

Suggestions have been made to explain how the parasites escape digestion to survive and multiply within the host cell cytoplasm.

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