CELLULAR IMMUNITY TO LEISHMANIA DONOVANI

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This is to certify that the

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ABSTRACT

CELLULAR IMMUNITY TO
LEISHMANIA DONOVANI

by Harold C. Miller

Since previous workers had failed to demonstrate protective antibodies against *Leishmania donovani*, an attempt was made to determine if cellular immunity prevails as a mechanism of resistance against leishmaniasis. After recovery from an initial intraperitoneal injection of *Leishmania*, mice demonstrated resistance to parasite proliferation from a challenge infection. To determine macrophage resistance, in vitro, mice were immunized by 1 to 4 injections of live parasites. Peritoneal macrophages from immune and normal mice were cultured and infected with the intracellular form of *L. donovani*. No proliferation of the parasites occurred in immune macrophages, but multiplication was demonstrated in the normal cells over a 72 hour period. Macrophages from mice immunized by either the 3S or Khartoum strain were resistant to challenge, in vitro, by either strain. The cellular immunity was not strain specific. Homologous serum was not essential for the demonstration of macrophage resistance. Serum from immune mice did not inhibit parasite proliferation in normal cells or significantly reduce their survival in macrophages from immune mice. When lymphocytes were harvested from immune animals 2 months after the last superinfection and added to cultures of normal and immune macrophages they did not enhance or lower the level of

macrophage resistance. Several attempts were made to passively transfer cellular immunity to L. donovani using macrophages, their products and components. Normal mice injected with macrophages from immunized mice demonstrated partial protection against a challenge infection as determined by a comparison of parasites in liver impression smears of controls and passively immunized mice. Medium from cultures of immune macrophages conferred leishmanial resistance to normal cells in culture since the parasites failed to multiply in passively immunized macrophages. When this "transfer medium" was incubated with ribonuclease its capacity for passive transfer was lost. RNA extracted from immune macrophages was demonstrated to confer resistance to normal cells in culture. The activity of the nucleic acid was titered. 2.4ug of RNA protected each culture, or the harvest from 1 immune macrophage was sufficient to protect approximately 2½ normal macrophages. RNA was separated by sucrose gradient centrifugation and upon incubation with normal macrophages the "light" RNA (4.7 to 8.8S) was demonstrated to contain the molecular fraction responsible for passive transfer. Immunity to L. donovani was dependent upon cellular factors rather than humoral antibodies and thus resembles the cellular immunity described for a number of intracellular bacteria and Toxoplasma.

CELLULAR IMMUNITY

TO

LEISHMANIA DONOVANI

By

Harold C. Miller

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INTRODUCTION

Leishmania donovani is the causative agent of visceral leishmaniasis or Kala-Azar. This disease occurs in Far-Eastern Asia, India, the Mediterranean area, Africa and South America. For the most part visceral leishmaniasis is endemic in these areas and is transferred to men from sylvatic and domestic mammalian reservoirs by the sandfly (*Phlebotomus*). Humans usually die from clinical diagnosed Kala-Azar unless treated. In eastern India and Pakistan *L. donovani* human epidemics through man to man transmission by the sandfly occur every 15-20 years.

The parasites multiply in the gut of the sandfly as elongate, flagellate forms termed the leptomonad stage. When inoculated into man and other mammals, the leptomonads are phagocytized by cells of the reticuloendothelial system, particularly macrophages. Inside the cell the parasites transform to the leishmaniform stage, which is also called an LD body, before extensive intracellular multiplication occurs. In cutaneous forms of leishmaniasis parasites are localized at the site of inoculation, but in visceral leishmaniasis the organisms spread from cell to cell and to all parts of the body with heavy concentrations in the spleen and liver.

Humans and animals have a long lasting resistance to reinfection after recovery from most forms of leishmaniasis, but the nature of the immunity is not known. Specific antibodies are produced by the host to various stages of the

Leishmania but none of these have been shown to protect the host against infection. Only limited attempts have been made to demonstrate cell associated systems of resistance with leishmaniasis.

The purpose of this investigation was to determine the role of cellular immunity in the host resistance to *L. donovani*. In most of the experiments cell cultures of immune macrophages were used to assay intracellular growth of the parasite as a criterion of cellular immunity. The effects of (1) serum or lymphocytes from normal and immunized animals, (2) possible parasite strain differences in inducing resistance and (3) passive transfer via RNA preparations from cells and medium were tested in this culture system.

LITERATURE REVIEW

A characteristic feature of visceral leishmaniasis is the hyperplasia of the cells of the reticuloendothelial system and hyperglobulinemia. Although specific antibodies are produced by the host to Leishmania they are non-protective and thus confer no immunity. Veira da Cunha et al. (1959) found no correlation between the complement fixation titers and the large amount of gamma-globulin in the serum. They concluded that the hyperglobulinemia was a result of the overproduction of immunologically competent cells producing "nonsense" gamma-globulin. Passive immunization with antibody failed to protect man against infection (D'Alesandro, 1954, and Adler and Adler, 1955). Nevertheless, individuals that survived the disease, with or without treatment, were immune to further infection by L. donovani, Napier (1946) and Prata (1957). Stauber (1963) summarized our limited knowledge of resistance to leishmaniasis by stating, "Concerning humoral aspects of resistance, there is yet no evidence for an antibody basis of acquired resistance to either dermal or visceral leishmaniasis."

Manson-Bahr (1961 and 1963), Adler (1963 and 1964), and Adler and Nelken (1965) cite evidence of cellular reactivity to L. donovani and L. tropica as expressed by an Arthus-like reaction or leishmaniomata (skin nodules). The former investigator demonstrated protection of humans by vaccination with live strains of L. donovani from rodents. Upon challenge with strains of L. donovani isolated from

humans, these subjects resisted infection (Manson-Bahr, 1959). Adler and Nelken (1965) failed to transfer the delayed hypersensitivity reaction to L. donovani by injecting washed leucocytes from the blood of one hypersensitive donor into non-sensitive human recipients. Bray and Lainson (1965) were unsuccessful in similar experiments on humans, monkeys, rabbits and guinea pigs. They did not test the resistance of the recipients to infection. More recently, Boysia (1967) was able to passively transfer delayed hypersensitivity to normal guinea pigs using lymph node cells from L. donovani sensitized guinea pigs. Nevertheless, the role of delayed hypersensitivity in host protection, with the exception of helminth infections, has not been established (Humphrey, 1967).

Cellular immunity has been demonstrated to be an important factor in the host's resistance to a number of intracellular parasites including: Mycobacterium, Brucella, Listeria, Salmonella, Pasteurella, Toxoplasma and Besnoitia. The subject was reviewed by Elberg (1960), Suter and Ramsier (1964), Mackaness and Blanden (1966) and Shands (1967). Cellular immunity can be defined operationally by the following criteria from the evidence reviewed by these authors: 1) It is a form of resistance that is induced only in the presence of live organisms and results in enhanced ability of macrophages to prevent multiplication of intracellular parasites. 2) It can occur in the absence of serum antibodies or be enhanced by their presence. 3) Cellular

immunity can be passively transferred from an immune to a normal host only by monocytic cells or their components. The classical experiments of Lurie (1942) showed the importance of cellular immunity in resistance to tuberculosis. After infecting normal and immune macrophages, in vitro, he implanted the cells into the eye chambers of normal rabbits and guinea pigs. Ten to fourteen days later both bacterial cultures and histological examinations were made of the lesions. Eye chambers containing the immune macrophages were found to have 2 to 20 times fewer bacteria than the chambers receiving normal macrophages. The injection of either immune or normal serum in combination with either immune or normal macrophages into the eye chambers had no additional effect on the bacterial survival. Fong, et al. (1956, 1957 and 1959) cultured macrophages from normal and immune rabbits in the presence of sera from both normal and immune animals. Using the extent of host cell cytopathology when infected with tubercle bacilli as the index of resistance, immune serum was found to be necessary for expression of complete protection of immune macrophages. In the presence of immune serum, normal macrophages were only partially destroyed in comparison with their complete destruction when cultured with normal serum. Suter (1953) found that tubercle bacilli multiplied rapidly in macrophages from normal rabbits or guinea pigs in culture, but that bacterial growth was retarded or completely inhibited in macrophages from immune animals.

Immune serum showed no protection of normal macrophages against infection in culture, nor did it enhance the resistance of immune macrophages. Similar results with Mycobacterium tuberculosis were obtained by Abe (1958) and Berthrong and Hamilton (1958).

Cellular immunity to Brucella was reported by Pomales-Lebron and Stinebring (1957) and Braun et al. (1958) studying guinea pigs immunized with the viable avirulent Brucella abortus organisms, and by Fitzgeorge et al. (1967) who tested resistance in blood macrophages from immunized calves. When cultures of immune macrophages from these animals were infected, in vitro, with a virulent strain, intracellular bacterial multiplication was inhibited and the bacteria were eventually destroyed. In normal macrophages the B. abortus proliferation was not inhibited. Elberg et al. (1957) reported similar results with B. melitensis and Holland and Pickett (1958) broadened the specificity of Brucella immunity when they found that guinea pigs, mice or rats immunized with viable, smooth B. abortus or B. melitensis produced macrophages that resisted either smooth or nonsmooth B. abortus, B. suis or B. melitensis. Brucella was sensitive to serum antibodies when outside of the host cell but was protected inside the cell cytoplasm (Elberg et al., 1957).

Cellular immunity has been demonstrated to Pasteurella tularensis. Rabbits and guinea pigs were immunized with an initial injection of avirulent bacteria followed by an

injection of virulent organisms 3 weeks later (Thorpe and Marcus, 1964a and 1964b). Peritoneal and aveolar macrophages harvested from these immunized hosts destroyed the bacteria used as an in vitro challenge. Serum from the immunized animals did not enhance this effect. Mackaness (1962 and 1963) and Miki and Mackaness (1964) employed Listeria infections in mice to study cellular immunity. The mice that survived an initial infection of 0.5 LD_{50} of L. monocytogenes were immune when challenged with an inoculation of 9 LD_{50} of the organism. Using plaque formation on monolayers of macrophages as a test of immunity, Listeria showed a plaque-producing efficiency of 91.2% on monolayers of normal macrophages and only 0.9% on monolayers of immune macrophages. Cellular immunity was demonstrated as early as 4 days after initial infection and lasted about 3 weeks. Passive administration of immune serum or pre-opsonization of bacteria with immune serum did not alter bacterial growth rates in mouse spleens. Cell-bound antibodies were ruled out as a possible mechanism of immunity since pretreatment of immune macrophages with 2 M urea did not alter the ability of these cells to destroy Listeria (Miki and Mackaness, 1964). Immunity against Listeria also conferred resistance against B. abortus and M. tuberculosis but only homologous organisms were capable of provoking an accelerated recall of acquired cellular immunity (Mackaness, 1962 and 1964). As a further manifestation of the non-specificity of resistance in cellular immunity

systems, Sato et al. (1962) demonstrated that macrophages from mice immunized with live S. enteritidis inhibited the intracellular growth of S. typhimurium, S. choleraesuis and Echerichia coli as well as S. enteritidis. Mice immunized with live S. typhimurium showed a capacity to limit growth of a superinfecting dose of Listeria monocytogenes and visa versa (Blanden et al., 1966).

Passive transfer of cellular immunity was first accomplished by Sever (1960) using macrophages from mice immunized to tuberculosis. By injecting the cells into normal animals a limited degree of resistance was conferred. No increased resistance was seen in animals receiving plasma, spleen homogenate or spleen filtrate from either normal or immunized animals, or macrophages from normal mice. Similar results were obtained by Suter (1961). Fong et al. (1962) passively transferred immunity (i.e. the resistance of histiocytes to degeneration) by inoculations of live macrophages from rabbits immunized to BCG serially through 3 groups of normal rabbits. Lymphocytes were much less efficient in passive transfer than macrophages. They contended that this serial dilution mitigated any possible transfer of non viable antigens and that viable bacteria could not be demonstrated in the macrophage inoculum. If bacteria were in fact absent this experiment provided reasonable evidence that the passively transferred mechanism of resistance underwent replication prior to each transfer. Serum from actively immunized animals was

necessary as a component of their in vitro test of immunity but was inactive in the absence of immune cells. Further studies indicated that the factor responsible for passive transfer was in the ribosomal fraction of macrophage RNA and was inactivated by ribonuclease (RNase) (Fong et al., 1963a and Fong et al., 1963b). The transfer factor proved to be not species specific in that RNA from rabbits passively immunized guinea pigs and mice.

Saito et al. (1962) passively transferred peritoneal macrophages from mice that had received macrophages intravenously from another group of mice immunized with live Salmonella. Macrophages from these serially immunized animals inhibited intracellular growth of virulent S. enteritidis in cell culture. The degree of inhibition was inferior to that displayed by macrophages from actively immunized mice. Although the transferred cells from the donors contained small numbers of viable organisms, the parasites were too few to confer active cellular resistance. Mitsuhashi and Saito (1962) collected medium from cultures of immune macrophages. This transfer medium was centrifuged, filtered and placed on monolayered cultures of normal cells with an equal amount of fresh medium. Transfer medium conferred cellular resistance against S. enteritidis to normal macrophages. Saito and Mitsuhashi (1965a) believed the "transfer agent" in the medium was RNA since its activity was destroyed by incubation with ribonuclease (but not deoxyribonuclease),

because it was not dialyzed through cellophane and since it retained its active state for at least 3 months at -10 C and for 24 hours at 37 C. In another experiment they demonstrated that the ribosomal fraction of immune macrophages could be used to passively transfer cellular immunity to S. enteritidis. Its activity was destroyed by ribonuclease but not deoxyribonuclease or trypsin. The ribosomal fraction of macrophages from mice inoculated with killed vaccine of S. enteritidis was incapable of the passive transfer of cellular immunity. Recently these Japanese workers (Kurashige et al., 1967) have identified a cellular antibody which they believe is responsible for cellular immunity to S. enteritidis. The Australian workers (Jenkin et al., 1964; Rowley et al., 1964 and Turner et al., 1964) and McIntyre et al. (1967) reported cellular immunity to S. typhimurium and S. enteritidis, respectively, to be due to cell bound or associated antibodies. Mitsuhashi et al. (1967) incubated normal macrophages with RNA from cells immune to S. enteritidis and passively transferred the ability of the recipient macrophages to produce "cellular antibody". However, since the peritoneal exudate, which was their source of macrophages, also contains lymphocytes they could not rule out this cell as a possible source of the immunogenic RNA.

Of more pertinent interest to the study of protozoan immunity was the demonstration of cellular immunity to the obligate intracellular parasite, Toxoplasma gondii (Vischer

and Suter, 1954). Cultures of macrophages from mice, rats or guinea pigs immunized by injections with live, attenuated parasites supported only limited parasitic growth and immune serum completely halted intracellular proliferation. Normal cells were readily destroyed by the organisms even in the presence of immune serum. More recently Frenkel (1967) demonstrated that spleen and lymph node cells from animals immunized with Besnoitia jellisoni (another obligate intracellular protozoan closely related to Toxoplasma) or to T. gondii could be used to transfer cellular immunity to normal animals. Specific antiserum to Besnoitia slightly enhanced the protection transferred by the cells. The immunity to both of these protozoa was of the premunition type and was sensitive to hypercorticism. Ruskin and Remington (1968) recently demonstrated that mice infected with Toxoplasma gondii were resistant to intracellular Listeria monocytogenes and Salmonella typhimurium. This protection against lethal infection with either of these pathogens lasted as long as 7 months in the presence of intracellular Toxoplasma parasites. They further showed that mice immunized to Listeria were not protected against Toxoplasma and that interferon did not mediate the resistance observed in the toxoplasma-immunized mice.

MATERIALS AND METHODS

Immunization of mice

All mice employed in this investigation were young, Swiss-Webster females purchased in lots of 100 animals with an average initial weight of 15 to 25 g. Mice for each experiment were randomly segregated into control and experimental groups prior to immunization by infection with parasites. Experimental groups to be immunized via the intraperitoneal route received an initial injection of either 15×10^6 or 30×10^6 viable Leishman Donovan (LD) bodies of Leishmania donovani (3S strain[1]). Additional intraperitoneal immunizations employed 30×10^6 parasites at 20 to 30 day intervals. Mice infected by the intravenous route were given 7.5×10^6 or 15×10^6 LD bodies (3S or Khartoum strain[1]) for the initial immunizing infection and equal numbers for superinfections at 20 to 30 day intervals. The Khartoum strain of this species was used in one experiment to compare strain differences. The course of earlier infections was followed by counting the parasites in liver and spleen impression smears by the method described by Stauber (1958).

Collection and cultivation of macrophages

The general procedure for prestimulation and the collection of peritoneal macrophages was similar to that

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[1]The 3S strain of L. donovani was obtained from Dr. Leslie Stauber, Department of Zoology, Rutgers University and the Khartoum strain from Dr. Paul E. Thompson, Park Davis and Company.

previously described for hamsters (Miller and Twohy, 1967). One ml of Hanks' Balanced Salt Solution (BSS) or medium NCTC 109 was used for each daily intraperitoneal prestimulation. After 2 prestimulations on the third day 2.5 ml of BSS or NCTC 109 were injected into the peritoneal cavity and the cells were withdrawn with a syringe and needle. The peritoneal exudates of all the animals in an experimental group were either pooled or the collection from each 5 mice was combined and tubed separately to restrict the danger of microbial contamination. The cells were centrifuged once at 250 x g and resuspended in Eagle's Basal Medium (EM) or NCTC 109 with supplements (Chang, 1964). Cells were counted in a hemocytometer or with a Model A Coulter Counter using a 100 u aperture at an aperture current setting of 4 and a threshold value of 30. The concentration of cells per ml was adjusted to 2 x 10^6, usually by adding more medium, and 1 ml of the suspension was pipetted into each Leighton tube. Cultures were incubated in a CO_2 incubator at 37 C with a 5% CO_2 - 95% air atmosphere. The EM employed in culture contained either 20% horse or newborn calf serum, or 10% horse and 10% mouse serum; 200 units of penicillin and 200 ug of streptomycin per ml and sufficient 1.4% $NaHCO_3$ to bring the medium to a cherry red color with phenol red indicator (pH 7.2 to 7.4). In later experiments NCTC 109 supplemented with a 10% solution of a 1:5 dilution of bovine embryo extract, 40% horse serum (Chang, 1964), 400 units of penicillin

and 400 ug streptomycin was used instead of EM. When NCTC 109 was employed in culture twenty-four hours after establishment of cells the medium was replaced with fresh NCTC 109 containing 200 units penicillin and 200 ug streptomycin.

Blood was collected from normal and immune mice at the time of decapitation. It was refrigerated overnight, centrifuged and the serum removed and filtered through a 0.45 u Millipore filter. This serum was not inactivated but was used within 24 hours of collection as a 10% component of EM along with 10% horse serum.

Inoculation and maintenance of cultures

Both the 3S and Khartoum strain of L. donovani was maintained by routine passage in 1 to 4 month old hamsters, using 50 x 10^6 parasites for each intraperitoneal injection or 30 x 10^6 LD bodies for each intracardiac injection. The LD bodies were harvested from the spleens one month or longer after intracardiac injections and 2 months or longer after intraperitoneal injections. Infected spleens were aseptically removed, minced with scissors and ground with a small volume of BSS or cell culture medium in a Teflon pestle homogenizer. The homogenate was centrifuged at 63 x g for 5 minutes and the supernatant containing the LD bodies was withdrawn. After the LD bodies were counted in a Petroff-Hausser counting chamber under the oil immersion objective of a phase microscope, the suspension was adjusted to the proper concentration of parasites with medium. For inoculating animals LD bodies were harvested

and counted as described above except that the parasites were suspended in BSS instead of medium. Heat killed parasites were prepared by incubating the above parasite-BSS suspension at 56 C for ½ hour. After heating, the preparation was centrifuged and resuspended in either BSS or culture medium and counted in a Petroff-Hausser counting chamber.

In earlier experiments after 24 hours of growth the medium was replaced on the cell cultures with fresh medium containing the LD bodies. Subsequently over the course of the experiment the medium was replaced daily. In a few experiments the inoculum was added in a 0.2 ml volume of additional fresh medium and the medium changed 24 and 96 hours later. In the incubator the CO_2 generally reduced the pH of the culture so that the phenol red indicator gave an orange color to the medium (pH 7.0).

The macrophages adhering to the cover slips were fixed in glutaraldehyde and stained with May Grünwald Giemsa at various intervals after infection as described by Miller and Twohy (1967). The total number of macrophages, the number of infected cells and the total number of intracellular LD bodies were counted in 5 to 50 - 43X microscopic fields per coverslip. The parasite population was expressed as the average number of LD bodies per cell, based on the total number of macrophages rather than just the infected cells in the fields counted.

Preparation of lymphocytes

Lymphocytes were obtained from popliteal and mesenteric lymph nodes of mice. After aseptic dissection the lymph nodes were probed gently with a pasteur pipette to release the cells into a small volume of BSS where they were mixed by force pipetting and vortexing. Lymphocytes from each 2 animals were pooled into 10 ml of BSS and permitted to stand in a screw-capped 12 ml centrifuge tube for 10 minutes at 10 C to settle clumps of cells. The supernatant was then removed, centrifuged at 250 x g and the cells resuspended in 15 ml of medium and the lymphocytes from each 2 mice dispensed into a 200 ml serum bottle. After 6 and 18 hours of culture at 37 C in a CO_2 incubator the medium from each bottle containing the suspended lymphocytes was transferred to another sterile serum bottle. These 2 decantations separated the lymphocytes from the macrophages and fibroblasts which adhered to the glass. Finally 24 hours after initial culture, the lymphocyte suspension was decanted from the serum bottles and centrifuged at 250 x g. The cells were resuspended in fresh medium and counted both with a hemocytometer and a Coulter Counter with a 100 u aperture (aperture current 4, threshold value 20), adjusted to 1 x 10^6 cells/ml and added to experimental macrophage cultures.

Passive transfer via immune cells

Peritoneal macrophages collected from mice receiving three intravenous injections of parasites and from normal

control mice were washed in BSS and adjusted to 3.5 x 10^6 cells per ml as previously described. Fifty more mice served as recipients for these cells. Twenty of these mice each received an intraperitoneal injection of 1 ml of the immune cell suspension, fifteen received an equal number of normal cells and fifteen mice were given sham intraperitoneal injections of 1 ml of BSS. Fourteen days later four of the mice that received immune macrophages were killed and impression smears of their livers examined for parasites that could have been inoculated with the immunizing macrophages. At this time the remaining animals of all three groups were injected intravenously with 1 x 10^7 LD bodies. At periodic intervals mice were killed and the LD bodies in liver impression smears counted.

Passive transfer of immunity via cell culture medium

Peritoneal macrophages were collected from mice immunized with 3 intravenous injections of 7.5 x 10^6 LD bodies each over 2 months and from comparable normal mice and cultured in serum bottles with EM. Six hours later the medium was withdrawn and centrifuged at 1000 x g to remove lymphocytes and dead cells. Heat killed LD bodies were added to the supernatant to give a final concentration of 2 x 10^6 parasites/ml before the medium was returned to the cells. Twenty-four hours later the medium was removed from both immunized and normal cells, centrifuged again at 1000 x g to remove cells and parasites and passed through a 0.45 u Millipore filter. This

transfer medium (TM) was stored in the refrigerator for 1-4 days before used. Macrophages were collected from another group of normal mice and cultured in Leighton tubes. The TM from both normal and immune macrophages was then diluted 1:1 with fresh medium and added to separate lots of the normal macrophage cultures. TM from immune and normal cells that was incubated with ribonuclease was exposed for 12 hours at 37 C to 10 ug/ml of 5X recrystallized bovine pancreatic enzyme.

Extraction of RNA

Macrophages from mice immunized by 3 intravenous injections of 7.5×10^6 LD bodies per mouse over a 2 month period were cultured in EM for 24 hours in serum bottles using 45×10^6 cells per bottle. Normal macrophages from mice of the same age and weight were handled in an identical manner. Twenty-four hours later medium containing the lymphocytes was decanted and discarded. The cells were washed 4 times with BSS to remove all remaining lymphocytes. Adhering cells were scraped from the glass surface using a rubber policeman. Approximately 4.5×10^8 cells were pooled in either 2 ml of BSS or 0.01 M of Tris pH 7 buffer with 0.3 M sucrose and frozen at -10 C. RNA was extracted from the cells initially using the methods of Delihas and Staehelin (1966). Four ml of Tris buffer and 50 mg of bentonite (prepared by differential centrifugation according to the method of Fraenkel-Conrat

et al., 1961) was added to 2 ml of thawed cell suspension. The mixture was homogenized for 3 minutes using a Potter-Elvehjem tissue grinder with a Teflon pestle rotated at 200 rpm. All operations were carried out in an ice bath at 4 C. Six ml of 76% phenol and 0.1% 8-hydroxyquinoline solution were then added and the mixture homogenized for another 2 minutes. It was then placed on a magnetic stirrer for 1½ hours at room temperature before centrifugation for 20 minutes at 9000 x g and 4 C in a Sorvall RC 2 centrifuge. The aqueous phase was decanted to just above the interface. The remaining interface and phenol phases were combined with a volume of 0.01 M Tris buffer equal to the decanted aqueous layer. This mixture was then stirred, centrifuged and again the aqueous layer removed. The aqueous layers from both extractions were pooled, combined with 2 volumes of cold absolute ethanol and stored overnight at -18 C to precipitate the RNA. The mixture was centrifuged at 9000 x g for 20 minutes at -10 C to precipitate the RNA. Again Tris buffer was added followed by a second precipitation with ethanol and centrifugation. The RNA was then suspended in buffer and assayed for its absorbancy at 260 mu on a Beckman DU. It was assumed that an A_{260} value of 22 equaled 1 mg of RNA/ml (Askonas and Rhodes, 1965). The RNA was stored in ethanol at -18 C and when used was centrifuged and assayed as above after resuspending in cell culture medium.

In the last 3 experiments the RNA extraction procedure was altered slightly by following the methods of Bishop et

al. (1967). The macrophages were suspended in a pH 5.1 sodium acetate buffer containing 0.01 M EDTA and 8 mg/ml of bentonite. To the mixture was added an equal volume of 88% phenol solution. This was homogenized for 1 minute. After the first centrifugation at 9000 x g the aqueous phase was removed and saved and an amount of acetate buffer equal to the volume of aqueous layer was added to the tubes. The mixture was then incubated at 65 C for 6 minutes and rapidly chilled to 4 C. Following centrifugation at 9000 x g for 20 minutes at 4 C the aqueous phase was removed, pooled with the first aqueous layer, an equal volume of 88% phenol added and the mixture stirred at 20 C for 6 minutes. After centrifugation again at 9000 x g the final supernatant was held overnight at -20 C with 2.5 x the supernatant volume of absolute ethanol. The precipitate was collected after centrifugation. The last precipitation procedure was handled as described in the first preparation of RNA.

Sucrose gradient separation

Sucrose gradient tubes were prepared by the method and apparatus described by Martin and Ames (1961). A 20% solution of cold 0.584 M sucrose in 0.05 M Tris-HCl buffer at pH 7.5 was placed in one mixing chamber and a 5% solution of this sucrose mixture was added to the other chamber. After preparing the linear gradient tubes the RNA was suspended in 0.1 ml Tris buffer and layered over each linear sucrose gradient. The tubes were centrifuged for 17 hours

at 39,000 rpm in a Spinco SW-39 swinging bucket rotor of a Spinco Model L centrifuge. Forty-four fractions consisting of 3 drops each were collected manually after puncturing the bottom of the tube. The fractions were analyzed for absorbancy at 260 mu and those containing the RNA were tested in vitro for their ability to passively transfer cellular immunity.

RESULTS

Immunity of mice to reinfection with L. donovani.

A comparison was made of the growth of LD bodies inoculated into normal and previously infected mice. The latter animals had been given an intraperitoneal injection of 30 x 10^6 parasites per mouse. One month later both the normal and infected animals were challenged with an intravenous inoculation of 15 x 10^6 LD bodies. Liver impression smears were made of 4 animals from each group of mice at each interval after inoculation.

Normal and experimental mice had approximately equal numbers of LD bodies 4 days after inoculation (Fig. 1). The previously infected animals supported a less rapid rate of parasite proliferation and showed a lower peak population after challenge than the normal mice. The peak for the superinfected animals could have fallen any time between 8 and 29 days after infection and may have been slightly higher than that obtained at 15 days. Growth of the parasites in the normal hosts was continuous over the course of the experiment and demonstrated a 19 fold increase in population 29 days after inoculation. A comparison of samples from both groups at 8 and 29 days after infection showed them to be significantly different ($P < .05$ and $P < .01$, respectively). Throughout the course of the experiment none of the animals died from infection. Striking spleeno- and hepatomegally was noted in the pre-infected group throughout the experiment, but in the normal

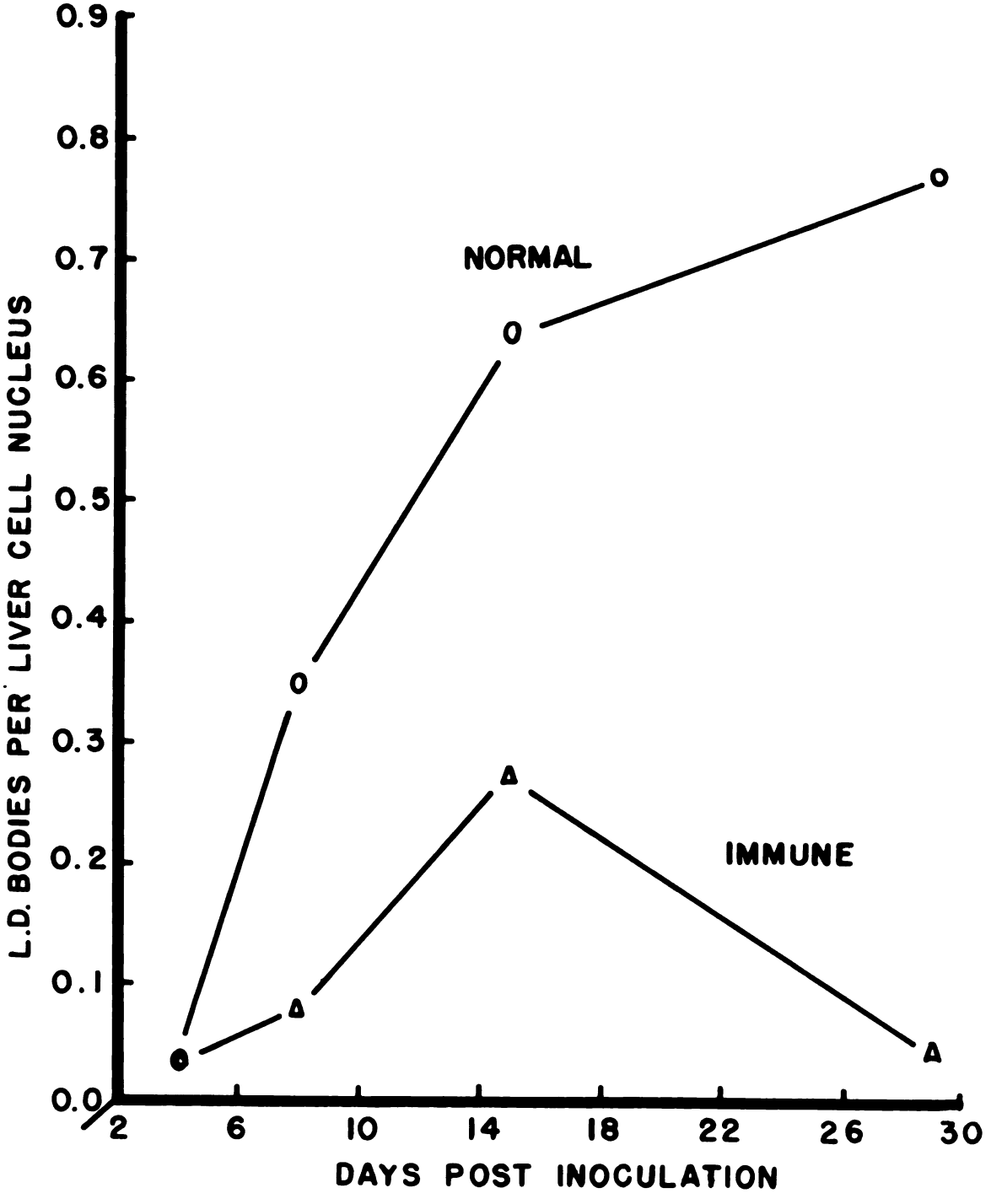
group these changes developed progressively with the course of the infection. This experiment demonstrated that mice develop a degree of immunity to reinfection within 1 month after a single intraperitoneal infection and henceforth will be called "immune" mice.

Parasite survival in macrophages from immune and normal mice

Macrophages from both immune and normal mice survived equally well in culture. Twenty-four hours after introduction into Leighton tube cultures an examination of the monolayer revealed that 10 to 25% of the initial cell inoculum was adhered to the coverslip. A few of the remaining macrophages were on the glass of the Leighton tube, but the majority of the cells did not attach to a glass surface. Very few polymorphonuclear cells were encountered. Lymphocyte to monocyte-like cells constituted a minority of the cell population. True lymphocytes do not adhere to glass and thus are reportedly removed with subsequent medium changes (Chang, 1964). Usually there was a gradual decline in cell numbers over the course of a cultural experiment which is commonly encountered in macrophage cultures (Chang, 1964). LD body multiplication in normal mouse macrophages was limited and usually ceased by 72 to 96 hours after infection.

A series of experiments was designed to compare parasite survival in macrophages cultured from immunized and normal hosts. Macrophages used in the first 3 experiments were harvested from the same two groups of mice at different periods of time after immunization. The immune

FIGURE 1. Comparison of the growth of LD bodies inoculated into normal (circles) and previously infected (triangles) mice. The latter animals were given an intraperitoneal injection of 30×10^6 LD bodies per mouse. One month later both groups received an intravenous challenge of 15×10^6 parasites per mouse. Each point is a mean of impression smear counts from 4 animals.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NORMAL
IMMUNE
2
6
10
14
18
22
26
30
DAYS POST INOCULATION

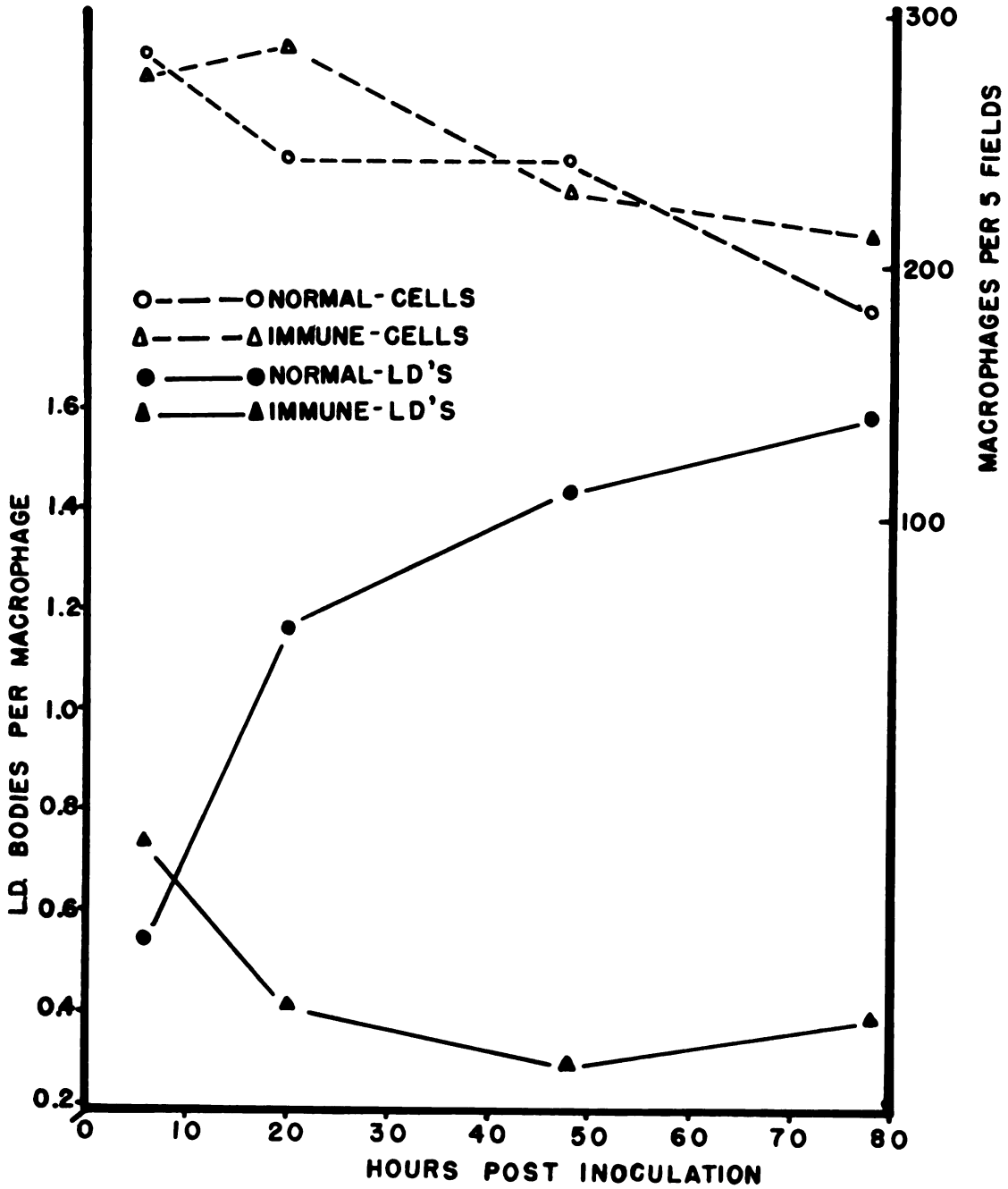
group consisted of 15 mice given two injections of 30 x 10^6 viable LD bodies of L. donovani approximately 5 weeks apart. Another group of 15 mice from the same lot were used as normal controls.

Macrophages from both the immune and control mice were harvested and cultured 4 weeks after the last immunization, or over 2 months after the first injection of parasites. Twenty-four hours later 1 x 10^6 LD bodies (approximately 4 parasites for each macrophage adhering to the coverslip) were inoculated into each Leighton tube. Two tubes from each group were fixed at each time interval after inoculation. The macrophage populations from both immunized and normal hosts (hereafter called immunized and normal macrophages for brevity) declined slightly over the 72 hour period after infection (Fig. 2). Six hours after infection a comparison of the number of LD bodies per cell showed little difference between the immunized and normal cells, but by 24 hours the parasite population increased in normal cells and decreased slightly in the immunized macrophages. The number of LD bodies per cell increased slightly in the normal cells over the next 48 hour period but remained low in the immunized macrophages. Although this difference in parasite numbers in the two macrophage populations was consistent throughout the experiment, there were too few tubes for a statistical comparison at any single time period.

The experiment was repeated 24 days later using the same groups of mice but employing 4 tubes from each group at each time interval (Fig. 3). Again the immunized cells had fewer parasites than the normal macrophages, but a marked difference was only evident 72 hours after infection. A comparison of the mean number of parasites per cell at 72 hours showed a significant statistical difference between the immunized and normal macrophages ($P < .05$ using the student t test).

A third experiment with the same group of mice 10 days later again showed a marked difference in macrophage resistance to LD bodies of L. donovani (Fig. 4). This difference was apparent 24 hours after infection. A similar statistical comparison of the average number of parasites per macrophage in the normal and immunized macrophages revealed a high statistical significance ($P < .01$) for each time interval. When the above three experiments on the same group of mice (Figs. 2, 3 and 4) were compared, the difference in the number of parasites in immunized and normal macrophages showed no clear correlation with the period of time after the last immunization which might be expected if resistance was either increasing or decreasing with time. In addition to these experiments studies on several other groups of mice have given results showing a similar difference in LD body survival in the immune and normal phagocytes. A few experiments have failed

FIGURE 2. The survival of L. donovani in macrophages from immune (solid triangles) and normal (solid circles) mice in culture. The broken lines show the survival of the macrophages from immune (triangles) and normal (circles) mice in culture. The immunized mice were infected with 2 intraperitoneal injections of 30×10^6 LD bodies 36 days apart. The phagocytes were harvested 64 days after the initial infection and the cultures inoculated with 1×10^6 parasites per Leighton tube 1 day later. Each point is the average of two tubes.


300
200
100
MACROPHAGES PER 5 FIELDS
O——O NORMAL-CELLS
Δ——Δ IMMUNE-CELLS
●——● NORMAL-LD'S
▲——▲ IMMUNE-LD'S
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
LD BODIES PER MACROPHAGE
0
10
20
30
40
50
60
70
80
HOURS POST INOCULATION

because either the macrophages or LD bodies did not survive in culture but in none of the experiments have immunized cells ever shown a higher parasite incidence than the normal host cells.

The effect of serum for immune mice

The next experiment was an attempt to compare the relative importance of macrophage associated resistance and antibodies which might be present in immune serum. Forty mice were divided into 2 groups consisting of 20 experimental and 20 control animals. The experimental group was immunized with an initial intraperitoneal injection of 15×10^6 LD bodies followed by 3 injections of 30×10^6 parasites over the next $2\frac{1}{2}$ months. Sera and macrophages were harvested from each group 12 days after the experimental group received their last injection of parasites. The cells were cultured in Leighton tubes and 24 hours after the harvest the culture medium was replaced with fresh medium containing either 10% normal mouse serum or immune mouse serum, 10% horse serum and 5×10^5 LD bodies per ml. The different combinations of sera and macrophages resulted in 4 groups of culture tubes: (1) normal cells with normal serum (NC-NS), (2) normal cells with serum from immune mice (NC-IS), (3) immunized macrophages with normal serum (IC-NS) and (4) immunized macrophages with serum from immune mice (IC-IS). The protocol for fixing and counting parasites was similar to that of the previous experiments.

FIGURE 3. The survival of L. donovani in macrophages from immune and normal mice in culture. The phagocytes were harvested from the same mice used in the last experiment but 24 days later or 38 days after the initial infection. The symbols for each line and the parasite inoculum are the same as in Figure 2. Each point is the average of four tubes.

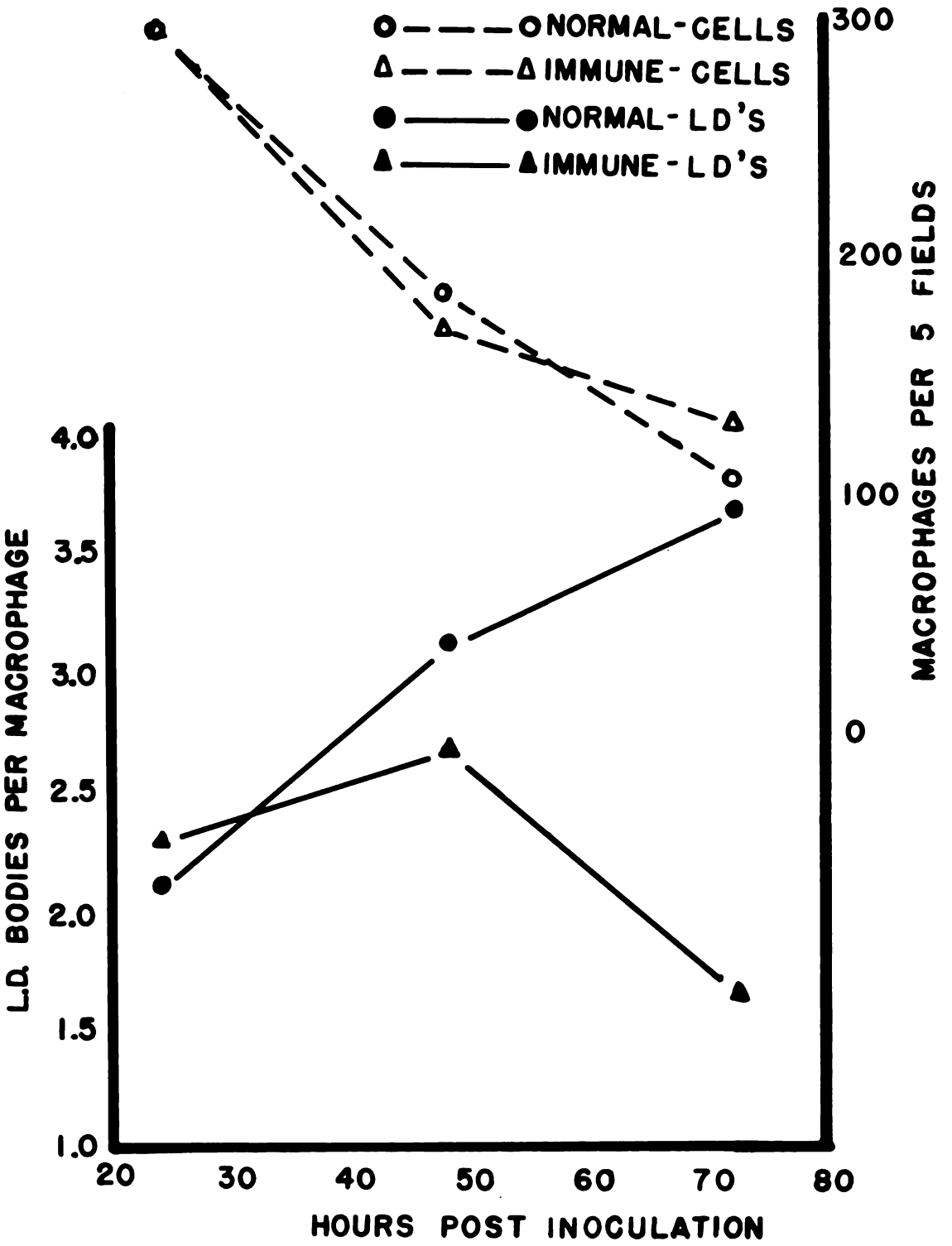
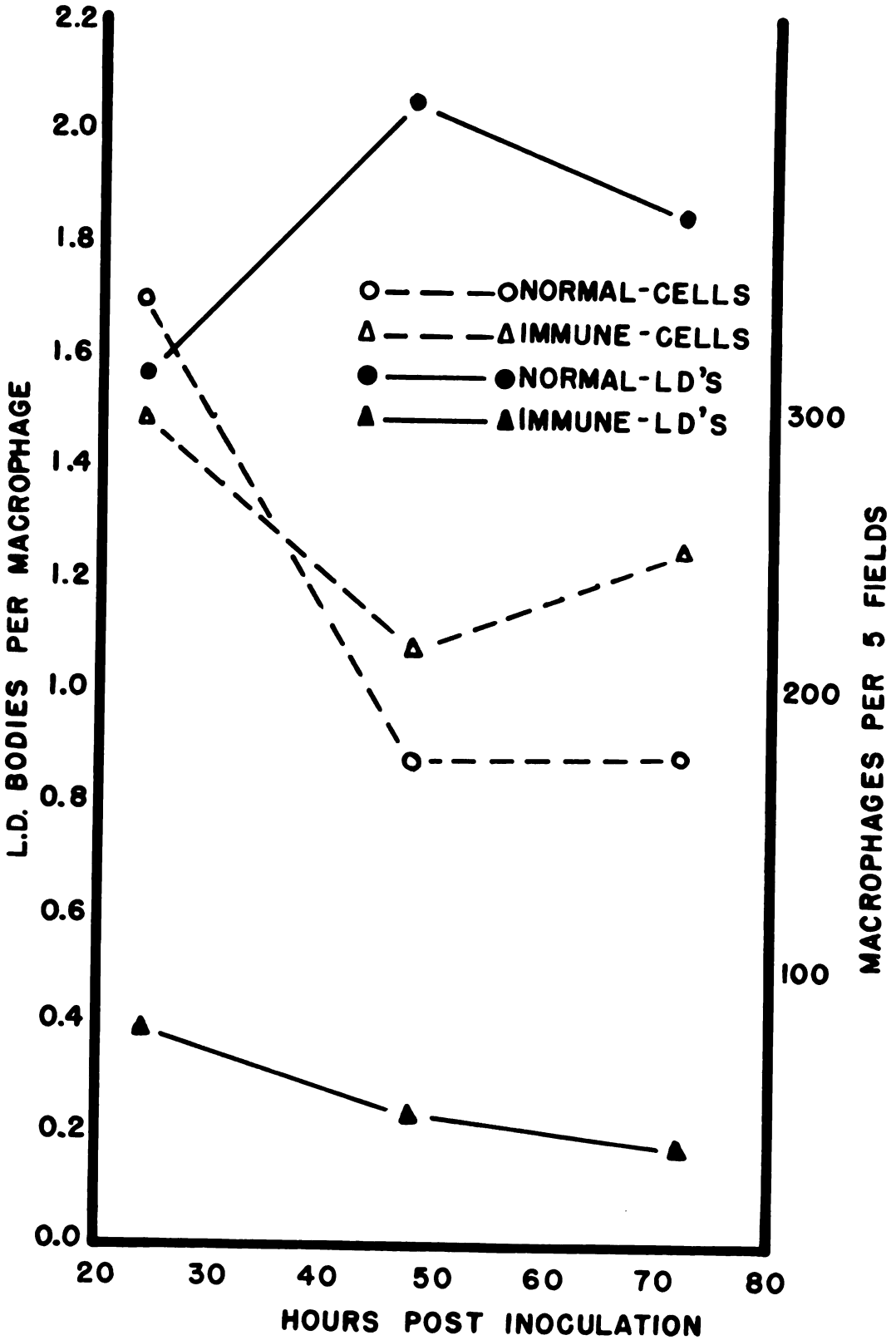

NORMAL-CELLS
IMMUNE-CELLS
NORMAL-LD'S
IMMUNE-LD'S
300
200
100
0
MACROPHAGES PER 5 FIELDS
4.0
3.5
3.0
2.5
2.0
1.5
1.0
L.D. BODIES PER MACROPHAGE
20
30
40
50
60
70
80
HOURS POST INOCULATION

FIGURE 4. The survival of L. donovani in macrophages from immune and normal mice in culture. The phagocytes were harvested from the same mice used in the last 2 experiments but 10 days later or 98 days after the initial infection. The symbols for each line and the parasite inoculum are the same as in Figure 2. Each point is the average of 4 tubes.


2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
L.D. BODIES PER MACROPHAGE
300
200
100
MACROPHAGES PER 5 FIELDS
NORMAL-CELLS
IMMUNE-CELLS
NORMAL-L.D.'S
IMMUNE-L.D.'S
20
30
40
50
60
70
80
HOURS POST INOCULATION

The results of this experiment showed the characteristic difference between survival of parasites in normal and immunized macrophages at both 60 and 72 hours after infection regardless of the source of mouse serum (Fig. 5). More parasites were actually found in normal macrophages exposed to immune serum (NC-IS) than in normal macrophages exposed to normal serum (NC-NS). Parasite destruction in immune macrophages may have been slightly enhanced by immune serum, but the statistical comparison showed no significant difference between groups IC-NS and IC-IS. On the other hand, despite the source of the serum, immunized macrophages showed significantly lower numbers of parasites than normal macrophages ($P < .05$ to .01, Table 1). Unfortunately by 72 hours after infection the cell populations had decreased markedly in many tubes giving less validity to a comparison at that time of culture. It seemed evident, however, that the resistance resided with the macrophage source and that resistance was not dependent upon the immune serum.

Macrophage immunity to different strains of L. donovani

In an effort to study the specificity of the macrophage immunity in cell culture to different strains of the parasites, two groups of 30 mice were immunized, one with the 3S and the other with the Khartoum strain of L. donovani. Both received 2 intravenous injections of 15 x 10^6 LD bodies per mouse 1½ months apart. One month after

FIGURE 5. The effect of immune serum on the survival of L. donovani in macrophages from both immune and normal mice. NC-NS, normal macrophages cultured with normal mouse serum (solid circles); NC-IS, normal macrophages cultured with immune mouse serum (open circles); IC-NS, immune macrophages cultured with normal mouse serum (open triangles); and IC-IS, immune macrophages cultured with immune mouse serum (solid triangles). The initial immunization was 15 x 10^6 parasites followed by 3 injections of 30 x 10^6 LD bodies over the next 78 days; 12 days after the last immunization the macrophages from immune and normal mice were placed in culture and each Leighton tube inoculated with 5 x 10^5 parasites the next day.

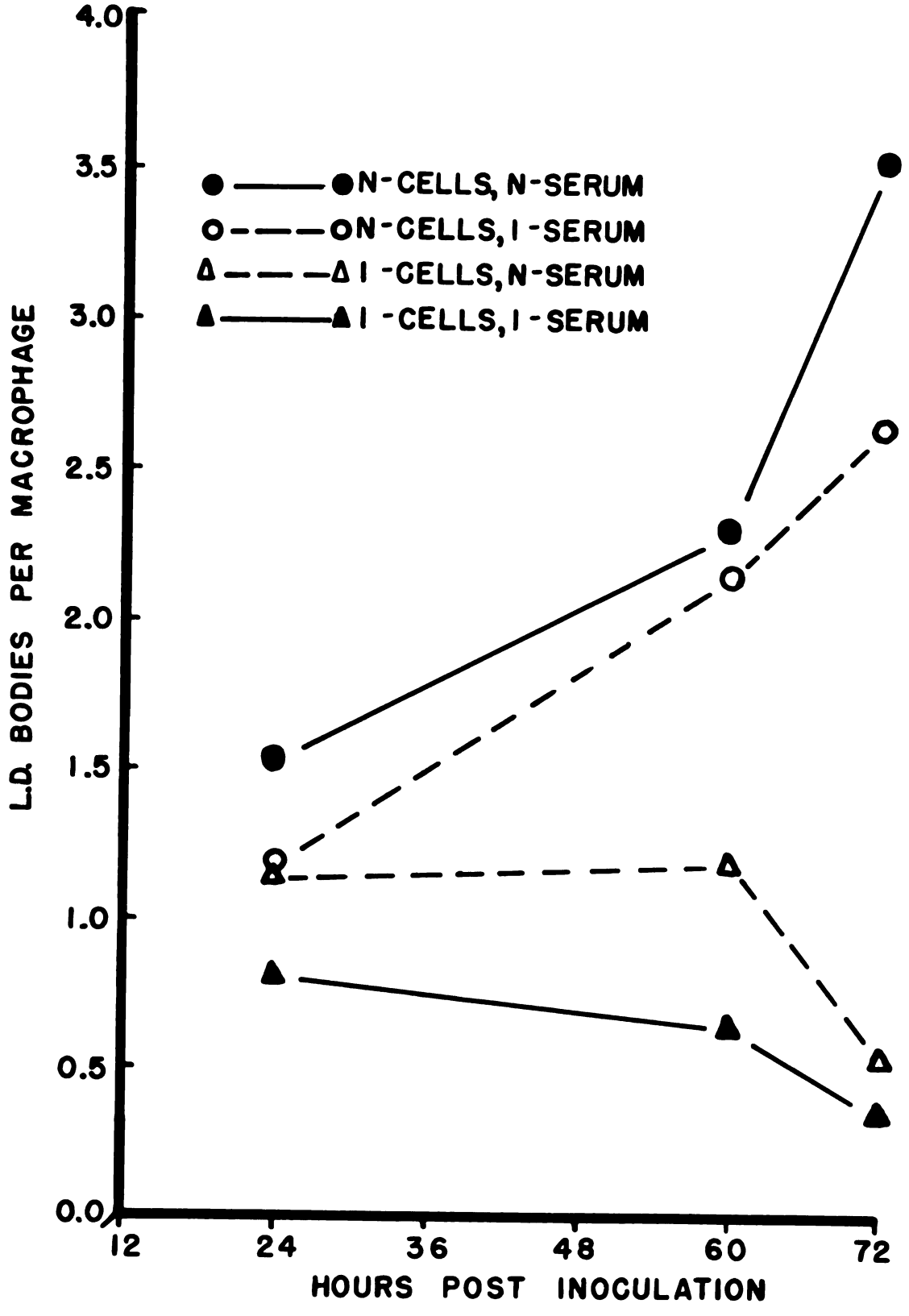

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
L.D. BODIES PER MACROPHAGE
N-CELLS, N-SERUM
N-CELLS, I-SERUM
I-CELLS, N-SERUM
I-CELLS, I-SERUM
12
24
36
48
60
72
HOURS POST INOCULATION

Table 1. The multiple effects of serum from normal and immune mice on intracellular L. donovani in macrophages from normal and immune mice in culture. The number of surviving cultures at each interval, the macrophage population and the statistical probabilities (computed after pooling the standard deviations for each time interval) are listed. NC-NS, normal cells and normal serum; NC-IS, normal cells and immune serum; IC-NS, immune cells and normal serum and IC-IS, immune cells and serum.

Group(s)	24 hours			60 hours			72 hours		
	No. tubes	cells/ 10-43X fields	LD's/cell	No. tubes	cells/ 10-43X fields	LD's/cell	No. tubes	cells/ 10-43X fields	Ld's/cell
NC-NS	3	493	1.19 ± .19*	4	225	2.25 ± .57	3	173	2.65 ± 1.20
IC-IS	4	393	1.54 ± .62	4	241	2.30 ± 1.80	3	136	3.54 ± .81
IC-NS	4	325	1.12 ± .36	3	346	1.18 ± .37	4	64	.55 ± .31
IC-IS	3	272	.72 ± .56	4	329	0.65 ± .22	2	135	.36 ± .06
NC-NS vs. NC-IS		P>.05			P>.05			P>.05	
NC-NS vs. IC-NS		P>.05			P<.05			P<.05	
NC-IS vs. IC-IS		P>.05			P<.01			P<.05	
IC-NS vs. IC-IS		P>.05			P>.05			P>.05	
NC-NS vs. IC-IS		P>.05			P<.01			P<.05	

*Standard deviation

the last injection macrophages were collected and cultured from both groups of immunized mice and from normal control mice. Twenty-four hours later the cultures of macrophages immunized with 3S or Khartoum strains and the normal macrophages from the control group were each divided into 2 sub groups (designated groups I to VI, Table 2): one to be challenged with 3S and the other with the Khartoum strain of parasites.

At 6 and 24 hours post inoculation little change was seen in the LD body population in any of the 6 groups (Table 2), but samples taken 48 and 72 hours after inoculation showed more pronounced changes in the intracellular parasite population. The normal macrophages showed very similar increases in number of LD bodies in samples taken 48 and 72 hours after infection with either parasite strain. The macrophages from mice immunized with the 3S strain of LD bodies showed little change in the intracellular LD population over the course of the experiment irrespective of the strain used to challenge immunity. Macrophages from mice immunized with the Khartoum strain also demonstrated little change in intracellular parasite numbers when challenged with the homologous strain. But, when challenged with the 3S strain there was a period of LD body proliferation as noted at 24 and 48 hours. This was followed by a more precipitous drop in the LD body population. At 72 hours this LD population was similar to that of the other subgroups of immune macrophages. Although

Table 2. A comparison of parasite strain differences with respect to their ability to induce cellular immunity.

Hours post inoculation	Group I 3S immunized 3S challenged	Group II 3S immunized K challenged	Group III K immunized K challenged	Group IV K immunized 3S challenged	Group V Normal 3S infected	Group VI Normal K infected
	LD bodies per macrophage*					
	No. of tubes	No. of tubes	No. of tubes	No. of tubes	No. of tubes	No. of tubes
6	(2) 1.84°±.05*	(2) 1.97±.17	(2) 2.41±.20	(2) 3.36±.24	(2) 2.36±.10	(2) 2.11±.10
24	(3) 2.50 ±.50	(4) 1.95±.93	(4) 2.45±.79	(5) 3.47±.45	(3) 2.84± .28	(4) 2.68±.25
48	(4) 1.77 ±.42	(4) 2.12±.57	(5) 1.73±.20	(4) 4.50±.36	(3) 4.43±1.11	(4) 3.90±.29
72	(4) 1.46 ±.25	(4) 1.81±.65	(4) 1.63±.10	(4) 2.35±.35	(4) 7.91±1.93	(4) 6.80±.98

*Standard deviation

°LD bodies per macrophage

the expression of immunity was delayed in this group, there was essentially no difference between the two parasite strains of L. donovani either in their ability to immunize animals as displayed by their macrophages or in the specificity of macrophage immunity.

The effect of lymphocytes on macrophage resistance

An experimental and a control group of 20 mice each were established. The experimental group received two intravenous injections of 15 x 10^6 parasites per animal over a 1½ month period. Two months after the first immunization lymphocytes and macrophages were collected from both groups. In a four way test lymphocytes from immunized animals were added to 24 hour old cultures of immune macrophages (IL-IM) and normal macrophages (IL-NM); and normal lymphocytes were added to cultures of immune macrophages (NL-IM) and normal macrophages (NL-NM). All lymphocytes were added in a ratio of 4 lymphocytes to each macrophage on the monolayer. Twenty-four hours later the surviving macrophages were counted and the cultures were inoculated with an average of 2 LD bodies per macrophage.. The course of infection was followed over the next 72 hours by the routine methods previously discussed.

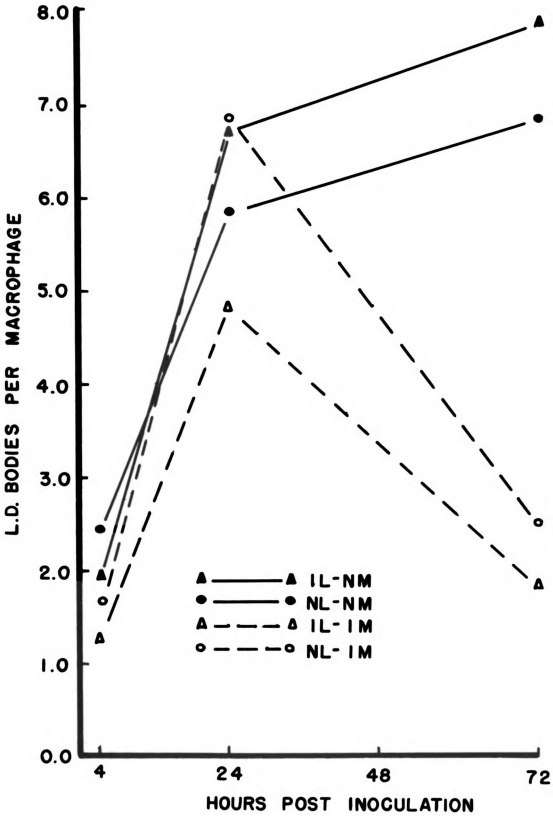
A statistical comparison of intracellular LD's in all groups of macrophages 6 and 24 hours after infection showed no significant difference in the number of parasites present. The results are depicted in Fig. 6. At 72 hours

only the characteristic difference between LD body populations in normal and immune macrophages was noted ($P < .01$). Exposure of the normal macrophages to the immune lymphocytes (IL-NM) slightly enhanced the growth of the parasites in comparison to those exposed to normal lymphocytes (NL-NM) but this increase was not statistically significant. Cells of groups IL-IM demonstrated greater supression of parasite numbers in comparison to those of group NL-IM but again this difference was not statistically significant. Thus lymphocytes taken from immune animals $3\frac{1}{2}$ months after initial infection did not confer significant resistance on normal macrophages or enhance the resistance of immune macrophages.

Stimulation of macrophage resistance with heat killed parasites

Although Saito and Mitsuhashi (1965) have exposed macrophages to heat killed S. enteritidis to enhance the expression of cellular immunity, in vitro, no attempt has been made to quantitatively compare a macrophage's resistance with and without this pretreatment in culture. In an attempt to study the effect of preincubation with heat killed parasites on macrophage resistance one group of mice was immunized with three intravenous injections of 7.5×10^6 parasites over a 2 month period and a second group set up as controls. Macrophages were collected from each group 56 days after the last immunization of the experimental mice and the macrophages from each group of mice

FIGURE 6. The effect of lymphocytes on macrophage resistance. NL-IM, normal lymphocytes cultured with immune macrophages (open triangles). IL-IM, immune lymphocytes cultured with immune macrophages (open circles). IL-NM, immune lymphocytes and normal macrophages (solid triangles). NL-NM, normal lymphocytes and normal macrophages (solid circles). Immune macrophages and lymphocytes were derived from mice immunized with 2 intravenous injections of 15×10^6 LD bodies over a $1\frac{1}{2}$ month period. Two months after immunization the cells were collected and cultured. Each point represents the average of 2 tubes at 6 hours and 4-6 tubes at the remaining intervals.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
L.D. BODIES PER MACROPHAGE
4
24
48
72
HOURS POST INOCULATION
IL-NM
NL-NM
IL-IM
NL-IM

were divided into two aliquots. 4×10^6 heat killed LD bodies were added to one aliquot from each group of mice before the suspensions were inoculated into Leighton tubes. This procedure resulted in four series of cultures. Series I contained macrophages from immune mice exposed to heat killed LD bodies; Series II, unstimulated immune macrophages; Series III, normal macrophages plus dead parasites and Series IV, only normal macrophages. All cultures were inoculated with 5×10^5 live parasites 24 hours later. Four cultures from each series were counted and the parasites per cell determined 6, 24, 72 and 120 hours after infection.

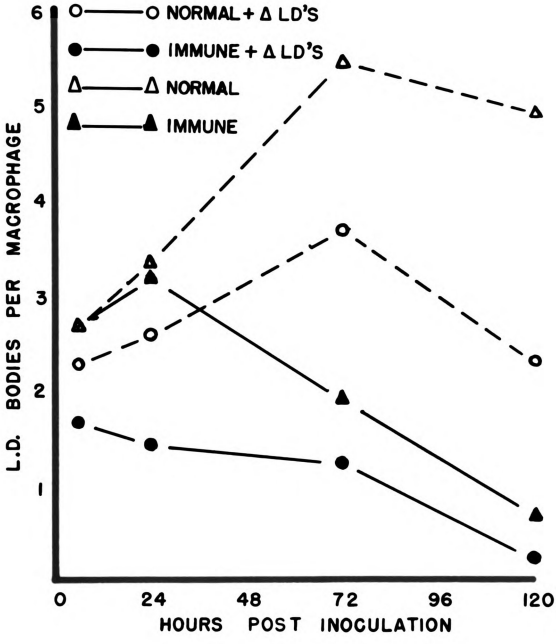
A statistical comparison of the mean number of parasites per cell (Fig. 7) in normal macrophages of Series III and IV cultures showed that the peritoneal cells stimulated with heat killed parasites had significantly lower numbers of parasites per cell 72 and 120 hours after infection than the unstimulated controls ($P < .02$ and .05, respectively). A similar difference was noted in the mean number of parasites per cell in macrophages harvested from the immune mice (Series I and II). Those exposed to the heat killed preparation had fewer intracellular parasites than the unexposed macrophages although a significant statistical difference was seen only at 24 and 120 hours after infection ($P < .05$ and .01, respectively). However, macrophages from immune hosts still displayed significantly lower numbers of intracellular parasites than cells from

normal animals after 72 hours of infection even without prior incubation with the heat killed LD bodies. Thus, the primary effect was still the source of macrophages and not the cultural stimulation of the cells. Coverslips from two cultures of each series were examined just prior to infection or 24 hours after adding dead parasites. No intracellular LD bodies were noted on these slides, eliminating the possibility that parasite counts early in the infection included dead, phagocytized LD bodies. This experiment shows that incubation with heat killed parasites increased the resistance of macrophages from both normal and immune mice to live LD bodies of L. donovani.

The passive transfer of resistance to normal mice

In demonstrations of cellular immunity to M. tuberculosis and S. enteritidis passive transfer of cellular immunity to normal donors has been accomplished via injection of immune macrophages as previously reviewed. To determine if passive immunization could also be brought about using macrophages from host immunized to L. donovani the following experiment was designed. Two groups of donor mice were employed in this study. Group I consisted of 30 mice immunized with 3 intravenous injections of 7.5 x 10^6 LD bodies of L. donovani over a 2 month period. Group II contained 30 normal control mice of the same age and source as group I. Three groups of younger recipient mice were set up after the immunization of group I was

FIGURE 7. Expressions of macrophage resistance after preincubation with heat killed LD bodies in vitro. Series I, immune macrophages incubated with heat killed LD bodies (solid circles). Series II, immune macrophages (open triangles). Series III, normal macrophages incubated with heat killed LD bodies (open circles). Series IV, normal macrophages (solid triangles). The immune macrophages were cultured from mice given 3 intravenous injections of 7.5×10^6 parasites per mouse over a 2 month period. Each point represents a mean of 4 Leighton tubes.


NORMAL + Δ LD'S
IMMUNE + Δ LD'S
NORMAL
IMMUNE
L.D. BODIES PER MACROPHAGE
6
5
4
3
2
1
0
24
48
72
96
120
HOURS POST INOCULATION

complete: Group III, consisting of 20 normal mice, received immune macrophages from donor mice of group I. Group IV, containing 20 mice, received normal macrophages from group II mice. The 15 mice of group V were injected with BSS instead of macrophages.

The peritoneal macrophages were collected from the donor mice (groups I and II) 17 days after the last immunization. The cells were counted and 1 ml of a suspension adjusted to contain 3.5×10^6 cells injected into each mouse of Groups III and IV. One ml of BSS was injected into each mouse of Group V. Two weeks later all mice in groups III, IV and V were challenged with an intravenous injection of 1×10^7 parasites except for 4 animals of groups III and IV which were killed to make spleen and liver impression smears. An exhaustive examination of these smears revealed no parasites. The infections in the remaining animals of all recipient mice were followed by periodically sacrificing the mice in lots of 2, 3 or 4 animals and counting the parasites in impression smears of their livers.

The incidence of parasites in the livers of mice receiving macrophages from immune mice (group III) was considerably lower than that of the mice injected with either normal macrophages or BSS (groups IV and V) in the counts 23 and 33 days after infection (Table 3). The above differences were statistically significant ($P < .05$ to .01). The incidence of infection in the mice stimulated

with normal macrophages and those given BSS (groups IV and V) were not considered significantly different. The parasite populations in these two groups peaked at different periods of time after infection but the interval between peaks could not be ascertained in samples taken only at 23 and 33 days. The maximum number of parasites per cell was almost identical in these two groups. This experiment demonstrated that the macrophages from immune mice did passively confer resistance to normal mice.

Passive transfer of cellular resistance with cell culture medium

Three groups of mice were used for the passive transfer experiment: Group A consisted of 20 mice which were immunized with 3 intravenous injections of 7.5×10^6 parasites over a 2 month period; Group B contained 20 normal control mice, and Group C was made up of 20 mice used as a source for recipient normal macrophages. Macrophages were collected from Groups A and B one month after the last intravenous injection. Transfer medium (TM) was collected from these cells 24 hours after initial culture and stored at 4 C for up to 4 days. Normal recipient macrophages were harvested from mice of Group C and cultured in Leighton tubes. After 6 hours of culture their medium was removed and replaced with a 1:1 dilution of TM and fresh medium. Half of the cultures were incubated with TM collected from immune cells (TMIC) and half with TM from normal macrophages (TMNC). This medium was

Table 3. The effect of passive immunization on the course of infection with L. donovani in mice.

Days after Challenge infection	Group III (immune cells)		Group IV (normal cells)		Group V BSS	
	No. Mice	No. Parasites*	No. Mice	No. Parasites	No. Mice	No. Parasites
5	2	10 ± 5°	2	15 ± 5	2	30 ± 23
15	4	95 ± 93	4	68 ± 30	4	95 ± 103
23	4	128 ± 53	3	477 ± 75	3	275 ± 61
33	4	35 ± 19	3	235 ± 79	3	457 ± 119
43					3	209 ± 97

*Number of parasites per 250 cell nuclei in liver impression smears

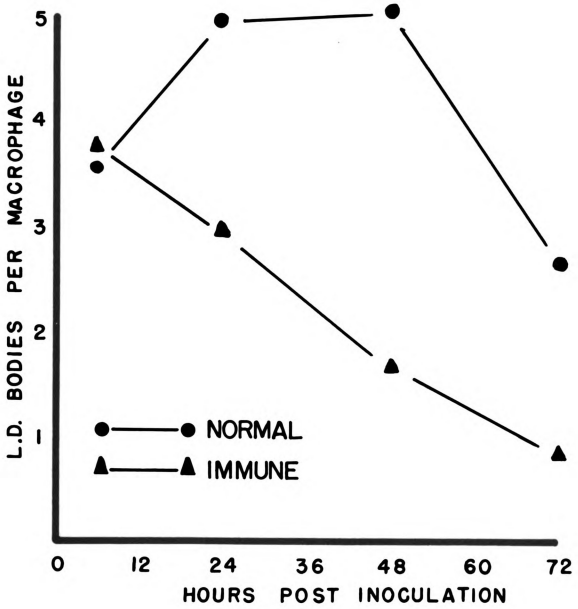
°The standard deviation of the mean

replaced daily for 3 days from the stock supply of either TMIC or TMNC. The TM was then decanted and replaced with fresh EM medium containing an inoculum of 5×10^5 parasites. Coverslips were fixed from each group of cultures over the next 72 hours to count the parasite populations.

The recipient normal macrophages that were incubated with TMNC supported a short period of intracellular proliferation, a plateau and finally a decline in parasite numbers (Fig. 8). In the macrophages incubated with TMIC the parasite population progressively declined for the duration of the experiment. The mean number of parasites per cell between the TMIC and TMNC incubated groups were significantly different at 24, 48, and 72 hours after infection ($P < .01$). Medium from immune macrophages (TMIC) contained some factor responsible for the transfer to normal macrophages of a rapid, pronounced resistance to the parasites.

This experiment was repeated using the same groups of animals 2 months later. Half of the TMIC and TMNC was incubated with ribonuclease. After the macrophages from recipient mice (group C) were cultured 24 hours in Leighton tubes they were divided into 4 groups. Their medium was removed and replaced with a 1:1 ratio of fresh medium and TM from one of 4 sources: TMIC, TMIC-RNase, TMNC and TMNC-RNase. A fourth group consisted of a control receiving only fresh medium. After the last of 3 changes of TM the fresh medium containing 4 LD bodies for each macrophage

FIGURE 8. Passive transfer of cellular resistance with cell culture medium from immune macrophage. Cultures of normal macrophage incubated with medium from immune macrophages or TMIC (solid triangles). Normal macrophage cultures incubated with medium from normal macrophage cultures or TMNC (solid circles). The TMIC was collected from cultures of immune macrophages harvested from mice injected with 3 intravenous injections of 7.5×10^6 parasites per mouse over a 2 month period. The macrophages used as the source of TM were collected one month after the immune group received its last intravenous injection. Each point represents a mean of counts from 4 Leighton tube cultures.


L.D. BODIES PER MACROPHAGE
5
4
3
2
1
NORMAL
IMMUNE
0
12
24
36
48
60
72
HOURS POST INOCULATION

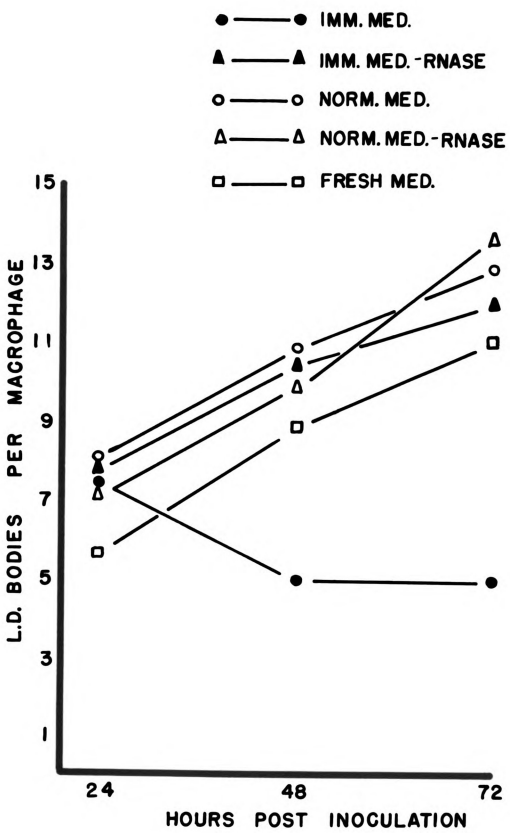
was added to the cultures. Enumeration of the parasites 24 hours later (Fig. 9) showed between 5.5 and 8 LD bodies per macrophage in all 5 groups. By 48 and 72 hours after inoculation with parasites the intracellular parasite population of cultures inoculated with TMIC dropped from about 7.5 to 5 LD bodies per cell, whereas the remaining four groups showed approximately 1.5- to 2-fold increases between 24 and 72 hours post inoculation. The population of parasites incubated with TMIC was statistically different from the other groups 48 and 72 hours after inoculation ($P < .01$ and $P < .05$, respectively). Thus, the factor responsible for passive transfer with cell culture medium must either be RNA or RNA dependent since it was destroyed by ribonuclease.

Passive transfer of cellular immunity by RNA

An RNA factor responsible for passive transfer of cellular immunity to M. tuberculosis and S. enteritidis has been implicated by several investigators. The inactivation of TM by RNase in the former experiment suggested that RNA was responsible for the passive transfer of resistance to L. donovani and prompted the following three experiments.

First an RNA was extracted from immune or normal macrophages to test its ability to passively confer cellular immunity to normal macrophages in cell culture. Normal macrophages to be used as recipient cells were collected, cultured and the Leighton tube cultures divided into 3 groups. After 9 hours of culture the tubes of group I

FIGURE 9. Passive transfer medium incubated with ribonuclease. Medium from immune macrophage culture incubated with ribonuclease before culturing with normal macrophages or TMIC-RNase (solid triangles). Immunogenic medium incubated with normal macrophages without prior RNase treatment or TMIC (solid circles). Normal macrophages cultured with medium from cultures of normal macrophages and tested with ribonuclease or TMNC-RNase (open triangles) and without ribonuclease or TMNC (open circles). Normal macrophages cultured with fresh medium only. TM was derived as in the former experiment. Each point represents a mean of counts from 4 Leighton tube cultures.

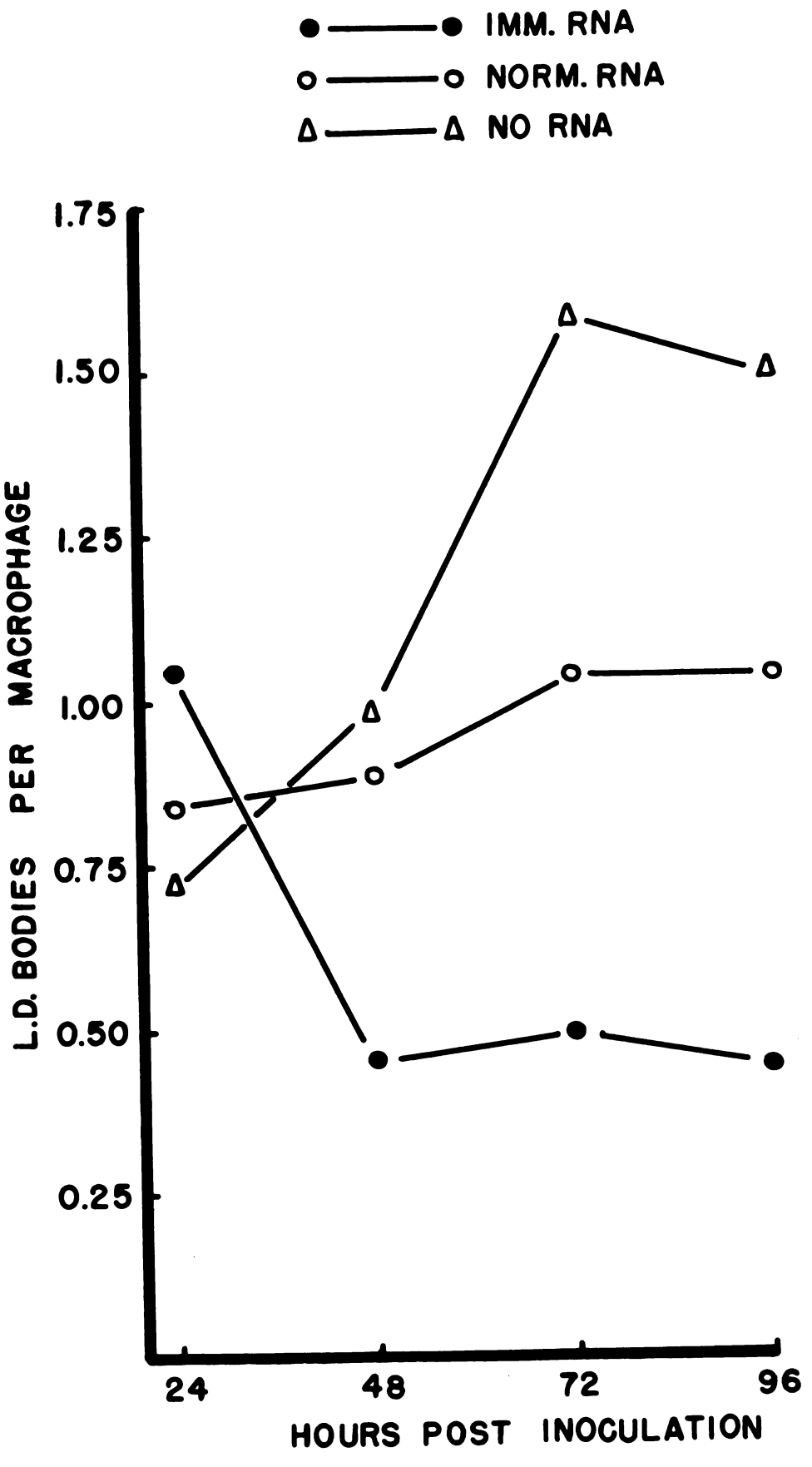

IMM. MED.
IMM. MED.-RNASE
NORM. MED.
NORM. MED.-RNASE
FRESH MED.
L.D. BODIES PER MACROPHAGE
15
13
11
9
7
5
3
1
24
48
72
HOURS POST INOCULATION

were inoculated with 3.2 ug/tube of RNA from immune cells and group II an equal amount of RNA from normal cells suspended in fresh medium. Group III was given fresh medium also but with no RNA. Twenty-four hours later the medium was changed and 7.3 ug of RNA was added to each tube of I and II and fresh medium to the third group. Forty-eight hours later the cells were challenged with 1 parasite per macrophage. Substantial parasite growth was seen in macrophages of group III between 24 and 72 hours post inoculation (Fig. 10). Only slight proliferation occurred in the macrophages of group II treated with normal RNA. Nevertheless the differences between groups II and III were not significant. The intracellular parasites declined in numbers between 24 and 48 hours post inoculation after treatment with RNA extracted from immune cells. No further decrease was seen between 48 and 96 hours. When the number of LD bodies per macrophage in cells of group I were compared with those of the control groups (II and III) the populations were significantly different at 48, 72 and 96 hours after challenge ($P < .01$ to $P < .05$). The ability of RNA from immune macrophages to passively transfer resistance to L. donovani, in vitro, was demonstrated by this experiment.

Titration of immunogenic RNA

The RNA employed in this experiment was extracted from normal and immune mouse macrophages using the procedure of Bishop et al. (1967) which was described in the

FIGURE 10. Passive transfer of cellular immunity by RNA. Group I was normal macrophages incubated with immunogenic RNA before challenge in vitro (open circles). Group II was incubated with normal RNA (solid circles) and Group III without RNA. Groups I and II were incubated with RNA for 48 hours before challenge with approximately 1 parasite per macrophage. Each point represents a mean of counts from 6-8 Leighton tube cultures.


IMM. RNA
NORM. RNA
NO RNA
1.75
1.50
1.25
1.00
0.75
0.50
0.25
L.D. BODIES PER MACROPHAGE
24
48
72
96
HOURS POST INOCULATION

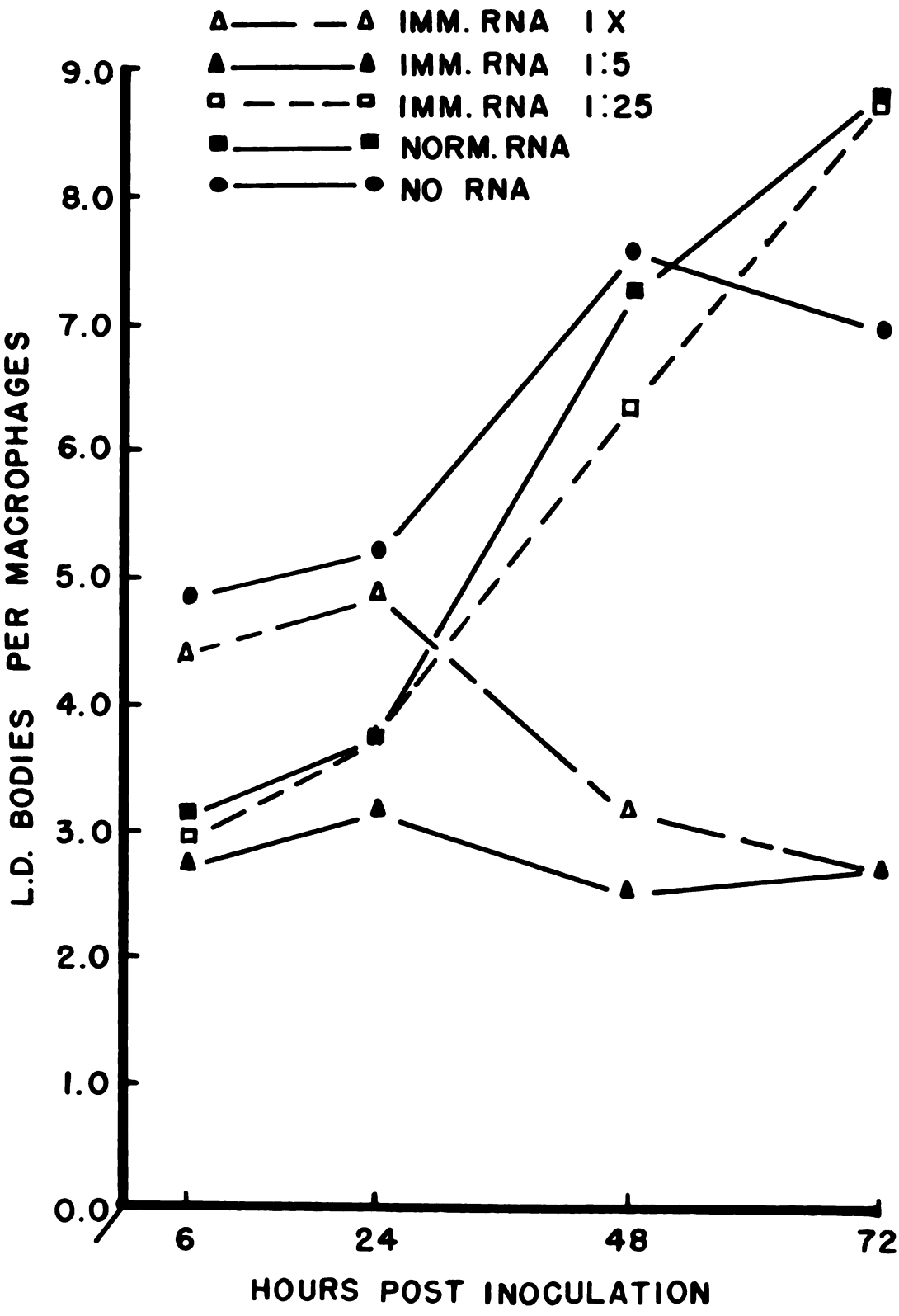
Materials and Methods. The immune mice which served as a source of macrophage RNA were given 2 intravenous injections of 15 x 10^6 LD bodies 1½ months apart. RNA was extracted from these cells 1½ months later. Normal macrophages were cultured in Leighton tubes with NCTC 109 medium plus supplements and 24 hours later randomly divided into 5 groups of 20 Leighton tubes per group. The groups were treated as follows: Group I received 12 ug of immunogenic RNA per ml; group II received a 1:5 dilution of immunogenic RNA or 2.4 ug/ml; group III, a 1:25 dilution of immunogenic RNA or 0.48 ug/ml; group IV was given 12 ug/ml of "normal" RNA and group V received no RNA. Forty-eight hours later the medium was removed and fresh medium containing 4 LD bodies per macrophage was added.

The data on Fig. 11 demonstrated that only groups I and II receiving 12 and 2.4 ug of immunogenic RNA, respectively, per tube showed a statistically significant degree of protection 48 and 72 hours after infection ($P < .01$). The RNA harvested from one immune macrophage could protect on the average 2½ normal recipient macrophages. This experiment demonstrated that RNA from immune macrophages could be diluted 5 times and remain immunogenic but somewhere between the 1:5 and 1:25 dilutions the concentration lost its activity.

Separation and identification of immunogenic RNA

There has been some recent success in the separation of RNA extracted from antigen stimulated macrophages into

FIGURE 11. Titration of immunogenic RNA. Group I received 12 ug of immune RNA per culture tube of normal macrophage (open triangles). Groups II and III received 1:5 and 1:25 dilutions of immunogenic RNA (solid triangles and open squares respectively). Group IV received normal RNA (solid squares) and group V was given no RNA. The immunogenic RNA was derived from macrophages of immune mice given 2 intravenous injections of 15 x 10^6 LD bodies 1½ months apart. The cells were cultured for 48 hours before inoculation with 4 LD bodies per macrophage. Each point represents the mean of counts from 4 Leighton tube cultures.


IMM. RNA 1 X
IMM. RNA 1:5
IMM. RNA 1:25
NORM. RNA
NO RNA
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
L.D. BODIES PER MACROPHAGES
6
24
48
72
HOURS POST INOCULATION

fractions of different sedimentation values. Certain of the fractions have been demonstrated to induce antibody synthesis in lymphocytes. This research is reviewed in the discussion. These results prompted an attempt to identify the RNA fraction responsible for the passive transfer of cellular immunity to L. donovani. RNA employed for separation by sucrose density gradient centrifugation was part of the material collected from the previous experiment.

An analysis of the RNA of the samples collected showed peaks of both the immunogenic and normal RNA 1.53 and 3.28 cm from the meniscus of the sucrose gradient (Fig. 12). Calculation gave S_{20w} values of 4.7 and 10.4, respectively, for two peaks.

The biological activity of the 2 fractions was tested in vitro. Eighty Leighton tube cultures of macrophages from normal mice were divided into 4 groups of 20 tubes each. Cultures of the first group received 7 ug/tube of the 4.7 to 8.8S fractions of immunogenic RNA (Fig. 12). This was termed "light" immunogenic RNA. The second group was given an equal amount of "heavy" immunogenic RNA ($>$10.4S) from samples 1 to 4. A third group received 14 ug/tube of the non-fractionated immunogenic RNA and a fourth group was given 14 ug/tube of pooled heavy and light normal RNA. Forty-eight hours later the medium containing RNA was replaced with medium containing LD bodies adjusted to give 4 parasites per macrophage.

Fig. 13 shows progressive protection against parasite proliferation in cells cultured with both the non-fractionated RNA and the fractionated "light" RNA from immune macrophages. Over the 72 hour course of this experiment the LD bodies grew equally well in cells treated with the pooled fractions of normal RNA and with "heavy" fractions of immune RNA. The immunogenic activity resided in the 4.7 to 8.8S fraction of RNA from immune macrophages.

FIGURE 12. Absorbancy at 260 mu of RNA in fractions separated by density gradient centrifugation at 39,000 rpm for 17 hours. Dotted line represents immune RNA and solid line, normal RNA.

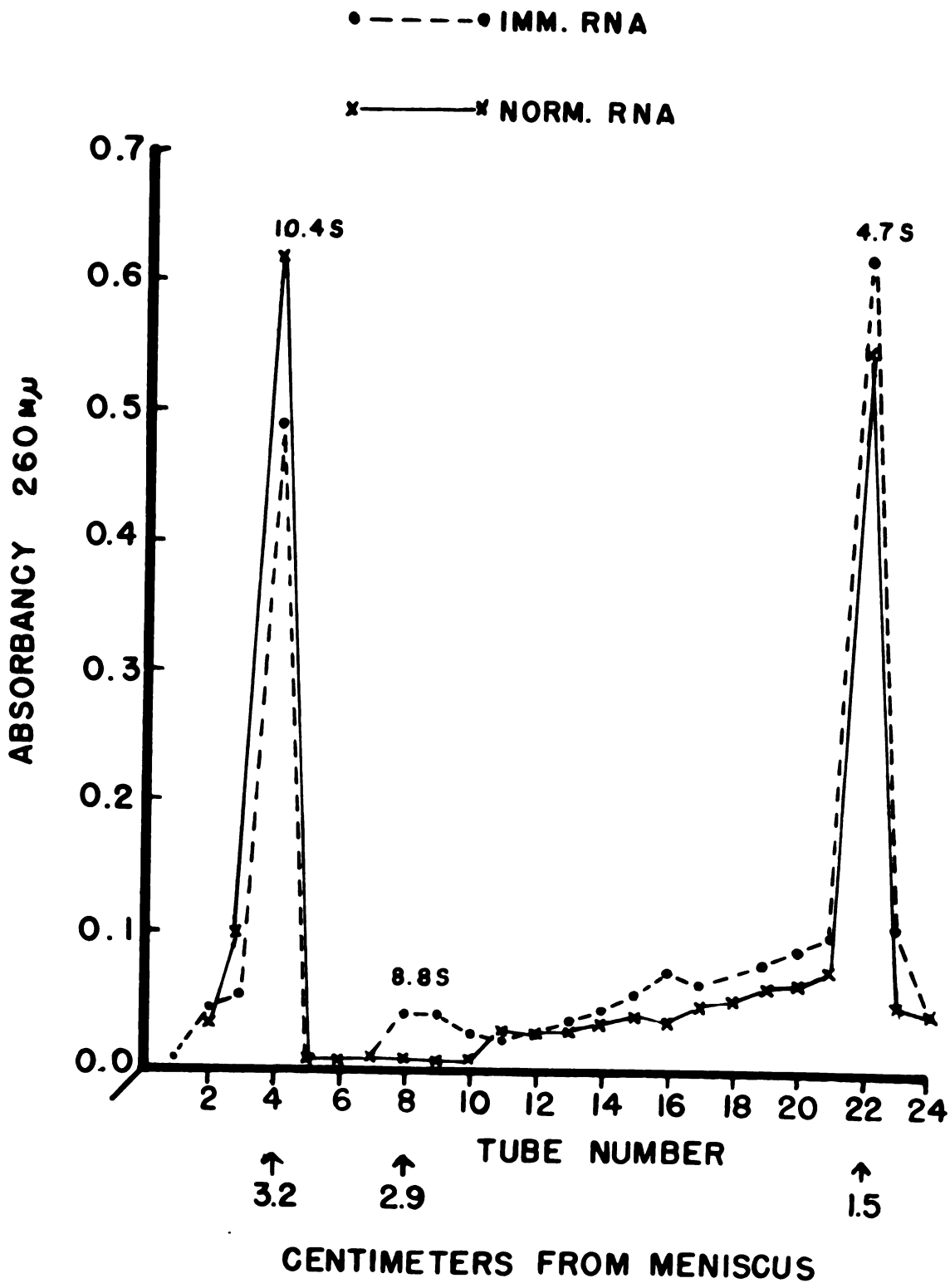
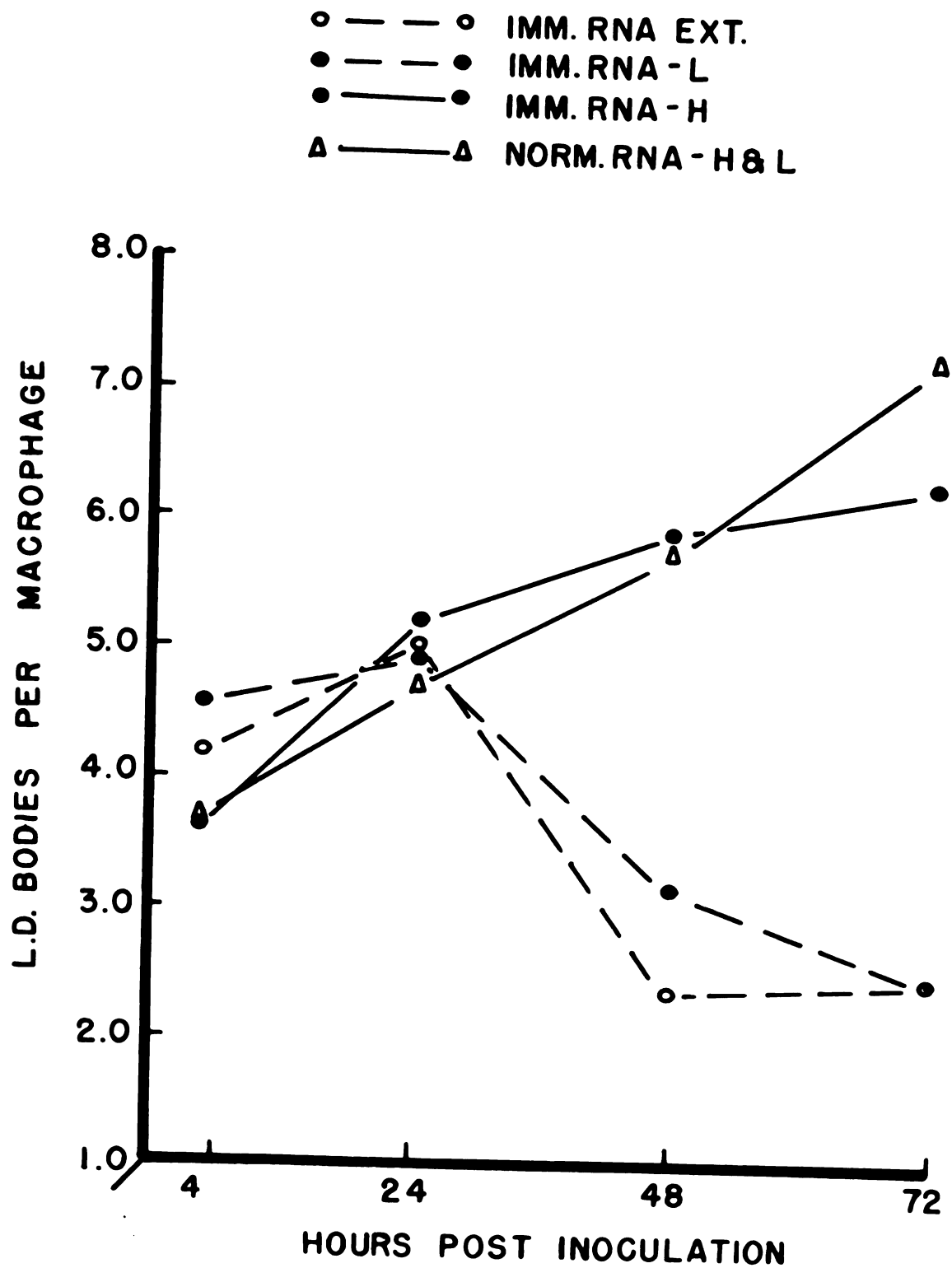

IMM. RNA
NORM. RNA
ABSORBANCY 260mμ
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10.4S
8.8S
4.7S
2
4
6
8
10
12
14
16
18
20
22
24
TUBE NUMBER
3.2
2.9
1.5
CENTIMETERS FROM MENISCUS

FIGURE 13. Separation and identification of immunogenic RNA. Normal macrophages preincubated with non-separated immunogenic RNA (open circles) prior to infection with LD bodies. Cells preincubated with "light" immunogenic RNA (solid circles); with "heavy" immunogenic RNA; with "heavy" and "light" normal RNA. The cells were preincubated with RNA in culture for 48 hours and then infected with about 4 parasites per macrophage. Each point represents means obtained from 6 Leighton tube cultures.


IMM. RNA EXT.
IMM. RNA - L
IMM. RNA - H
NORM. RNA - H & L
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
L.D. BODIES PER MACROPHAGE
4
24
48
72
HOURS POST INOCULATION

DISCUSSION

Mice were selected as experimental hosts for this investigation after considering their level of acquired resistance to leishmaniasis and the characteristics of their macrophages in culture. Stauber (1958) was unable to demonstrate resistance to L. donovani in hamsters or chinchillas. Rats and rabbits seemed to possess a complete innate resistance to the infection, showing no proliferation of the parasites in their macrophages. But the mouse, gerbil and guinea pig possessed an intermediate level of susceptibility. In these latter hosts a resistance developed to the initial infection which eventually reduced the number of parasites. Of these three hosts, mice seemed to be the best choice for this investigation since their macrophages have been cultured in a number of media containing heterologous sera and their cultural characteristics are well known (Chang, 1964). Although rapid proliferation of the parasites in the intact mouse was limited to 15-30 days this initial infection permitted the development of immunity to challenging superinfection. This was demonstrated by Franchino-Cappuccino and Stauber (1959) and Stauber (1962) and confirmed by the first experiment in this investigation.

When LD bodies of L. donovani were added to the cell cultures, the parasites were ingested by macrophages from immune and normal mice since, in most experiments, the

number of parasites in cells from both sources was similar 6 hours after infection. Following initial infection the number of parasites usually increased in the normal macrophages but remained at the initial level or declined in the phagocytes from immune mice. In this way, the macrophages cultured from infected hosts displayed a greater resistance to infection than similar phagocytes from normal hosts. Between experiments there were variations in the initial level of infection, the rate of parasite proliferation in normal cells and the inhibition of growth in immune cells. These variations can probably be attributed to undefined conditions or nutrients of culture which cannot be precisely duplicated between experiments. With such variations quantitative comparisons of the degree of cellular immunity in different experiments conducted at different times are of limited value, even if the same experimental animals are used. Thus it was impossible to determine if this immunity increased or decreased with time after the initial infection.

Circulating antibodies did not seem to play a role in this cellular immunity since serum from immune animals did not significantly decrease the parasite incidence in macrophages from either infected or normal animals. These observations on the effect of immune serum are consistent with the failure of many investigators to demonstrate protective antibodies in animals and humans infected with Leishmania (Stauber, 1963). Factors associated with immune cells were essential for resistance to the intracellular survival of L. donovani.

Although treatment of normal macrophages with heat killed parasites gave some protection against L. donovani proliferation, it did not compare with the resistance displayed by immune macrophages. No proliferation of LD bodies occurred in immune cells with or without preincubation with heat killed LD bodies. Nevertheless the mechanism which permits the immune macrophage to inhibit growth or destroy LD bodies may be more rapidly activated by preincubation with heat killed parasites. There also may be an increased stimulation of the innate microbicidal properties of normal macrophages by this treatment. It must be noted however that intraperitoneal injections of heat killed LD bodies do not induce cellular immunity in mice to Leishmania (Twohy et al., 1968). Dead organisms are also inactive in producing cellular immunity to other microbial agents. For example, Kochan and Rose (1962) compared mouse susceptibility to live tubercle bacilli 3 days after immunization with endotoxin and 10 days after immunization with live or heat killed BCG. Animals injected with endotoxin or heat killed BCG were killed upon challenge with live BCG whereas those immunized with living BCG were resistant to the challenge.

The possibility exists that the immune peritoneal macrophages predominate by a process of selection of normal Leishmania-resistant cells from a heterogeneous population of macrophages, in vitro. Passive transfer experiments with other etiological agents, however, casts considerable

doubt on this explanation of cellular immunity. For example, Saito, et al. (1962) harvested 32_P labeled macrophages from hosts immunized with Salmonella enteritides and injected them intravenously into normal recipient mice. Only a trace of the labeled compound was found in the peritoneal macrophages, but these cells were resistant to infection, in vitro. This indicated the immune cells did not migrate to the peritoneum to elicit the cellular immunity. With Leishmania other evidence suggests that the cells collected by our harvest procedure were not selected for their ability to overcome the parasite by their migration to the peritoneum: (1) There was no evidence of mass migration of cells to the peritoneum in response to stimulation with BSS. Comparative counts of cell harvests from stimulated and unstimulated mice showed no consistent difference in the numbers of peritoneal macrophages present. (2) There is no reason to expect a lower incidence of infected macrophages in the peritoneal cavity than in other organs of the body (Stauber, 1966). (3) The incidence of parasites in the superinfected mice was very low at the time of cell collection, which means that a relatively small proportion of the macrophages could be inhibited from migration by the presence of intracellular parasites.

The results of the passive transfer of cellular immunity to leishmaniasis are consistent with results obtained by others. Unfortunately when mice were passively immunized, the presence of sufficient parasites in the macrophages

from the donor mice to confer an active immunity to the recipient normal mice could not be ruled out. However, this did seem doubtful since extensive microscopic examination of the macrophages that were transferred revealed no parasites. Eliminating parasites from macrophages used for passive transfer of immunity has been a problem to other investigators. Starting with histiocytes (macrophages) from the actively immunized host, Fong et al. (1962) transferred passive resistance serially by macrophage inoculations through 3 groups of normal rabbits. The authors argued that this serial dilution mitigated any carry over of antigens, and viable bacteria could not be demonstrated in the inoculated macrophages.

Passive transfer of resistance to L. donovani with cell culture medium from immune macrophages paralleled the results of Saito and Mitsuhashi (1965) in their studies of transfer of immunity to S. enteritidis. When they treated the transfer medium with deoxyribonuclease, ribonuclease and trypsin, only the ribonuclease was found to inhibit the passive transfer of cellular immunity. The inactivation of the transfer of cellular immunity to L. donovani by incubating the transfer medium with RNase suggested that the factor responsible for transfer was RNA. Later experiments confirmed this contention. The passive transfer of cellular immunity by extracts of macrophage RNA showed that RNA or RNA plus a contaminant conferred upon macrophages a refractivity to parasite proliferation.

Although the mechanism of cellular immunity is for the most part unknown and the ribonucleic acid transfer of cellular immunity against L. donovani is not understood, several possibilities exist as explanations for its induction and action. The RNA may act as a messenger for protein synthesis in the macrophage. Fong et al. (1963) and Saito and Mitsuhashi (1965) have suggested that the RNA may act as primer for the formation of new RNA by the target macrophage. The RNA could then be involved in the synthesis, induction of synthesis or activation of lysosomal enzymes. The evidence for enhanced enzyme activity in immune macrophages is discussed later.

Although cellular immunity to L. donovani and several other pathogens is not dependent upon humoral antibodies, the mechanism of resistance may be a facet of the complex intercellular process leading to the synthesis of circulating antibody. The macrophage is believed to degrade antigen and later transfer RNA or antigen associated RNA to lymphocytes which finally synthesize the antibody (Shands, 1967).

Fishman and Adler (1963) and Fishman et al. (1964) have demonstrated that RNA extracted from T^2 bacteriophage-stimulated macrophages induced cultures of lymph node cells to produce antibody. It was not certain whether the immunogenic properties of this macrophage RNA should be attributed to information transferred by the RNA or to RNA serving as an adjuvant for an antigen-RNA complex.

Friedman et al. (1965) and Askonas and Rhodes (1965) have demonstrated that these RNA preparatives contain antigenic fragments. Immunogenic macrophage RNA from cells that had been exposed to various bacteriophage antigens was found not to be a unique species of nucleic acid (Gottlieb and Glisin, 1967) since it bound equally to DNA from normal and immune macrophages. In contrast to these studies, Raska and Cohen (1967) demonstrated accelerated incorporation of labeled uridine into RNA of macrophages during incubation with sheep RBC's, in vitro. This labeled RNA formed a specific molecular hybrid with mouse DNA not found when normal RNA was used, suggesting the synthesis of new RNA upon antigen stimulation. Also in accord with this Halac et al. (1964) noted a change in base composition of total RNA following pinocytosis of bovine serum albumen but no attempt was made to determine the species of RNA responsible. Bishop et al. (1967) demonstrated RNA synthesis in macrophages treated with sheep RBC's. The majority of the RNA biological activity in inducing lymphocyte synthesis of antibody was identified by sucrose density gradient separation as lying between 6 to 10S values. Pulse-labeling studies showed synthesis of 6 to 10S RNA by macrophages 30 minutes after exposure to the label during the process of phagocytosis of sheep RBC's and 60 minutes following pulse labeling in cells not exposed to antigen.

It is interesting that the demonstration of active RNA in the 4.7 to 8.8S region from <u>Leishmania</u> immunized

macrophages is consistent with the results of Bishop et al. (1967) even though the Leishmania studies involved transfer of cellular immunity and the cited authors were concerned with induction of antibody synthesis. The demonstrations of antibody in macrophages immune to S. enteritidis (Kurashige et al., 1967) and the presence of this antibody in cells immunized by passive transfer with immunogenic RNA (Mitsuhashi et al., 1967) suggests either an analogy or homology between protective humoral antibodies and cellular immunity. The presence of antibody in macrophages must be accepted with caution. Dumonde (1967) has reviewed good circumstantial evidence that peritoneal lymphocytes produce cell bound antibody and delayed hypersensitivity. The cell preparations of Kurashige et al. (1967) and Mitsuhashi et al. (1967) were derived from peritoneal exudate and no attempt was made to exclude lymphocytes. The antibody may not have been of macrophage origin even though the necessity of macrophages has been unequivocally demonstrated for cellular immunity to Salmonella enteritidis.

The importance of macrophage RNA in humoral antibody formation needs further consideration for its possible analogy to cellular immunity.

As another possible explanation for the induction of cellular immunity, Mackaness (1964 and 1967) has proposed that lymphocyte-mediated delayed hypersensitivity may influence the development of cellular immunity. The

foundation of this hypothesis is based on numerous observations of the development of delayed hypersensitivity preceding cellular immunity in infections involving intracellular parasites. Boysia's (1967) demonstration of a delayed hypersensitivity reaction to L. donovani is another example of the association of the two phenomena and suggested work is needed to determine the role of lymphocytes in immunity to L. donovani. The unsuccessful attempt to demonstrate a lymphocyte role in the induction of cellular immunity to Leishmania in the present study may be due to the time at which these cells were collected. If attempts had been made to collect lymphocytes at various times during the expression of delayed hypersensitivity, their role in mediating cellular immunity could have been more critically evaluated. In addition, this study should be carried out with inbred strains of mice so that possible antigenic differences between lymphocytes and macrophages do not give cell reactions that confuse the results. Fong et al. (1961) observed limited passive transfer of cellular immunity to guinea pigs against tubercle bacilli with injections of lymphocytes from immunized hosts, whereas macrophages from these animals gave excellent protection for longer periods of time. Better support of Mackaness's hypothesis comes from the study of Frenkel (1967). He transferred cellular immunity to Besnoita jellisoni and T. gondii to hamsters by injections of lymph node and spleen cells

harvested from infected hosts. Delayed hypersensitivity developed as early as 5 days after infection, whereas cellular immunity could not be passively transferred from infected donor animals until 21 days after infection.

The role of immunogenic RNA, antibody, lymphocytes or delayed hypersensitivity in inducing or mediating cellular immunity remains to be proven. Another obvious possibility is that these phenomena are somehow interrelated and all play a part in the development of cellular immunity. Their action may culminate in an altered physiological response of the macrophage to the parasite.

As a possible mechanism for immune macrophage destruction of invading intracellular parasites the changes in the enhanced physiology of the cell warrant consideration. It has been shown that the immune macrophage has many enhanced metabolic processes (Suter and Hullinger, 1960; Stahelin et al., 1957 and Karnovsky, 1962), and there have been numerous studies of macrophage lysosomal enzymes in attempt to delineate factors involved in cellular immunity (Carson and Dannenberg, 1965; Dannenberg and Bennett, 1963 and 1964; Dannenberg et al., 1963; Mizunoe and Dannenberg, 1965; and Yarborough et al., 1967). The subject was reviewed by Dannenberg (1968). After mild stimulation with mineral oil, lysozyme and acid phosphatase levels were higher in peritoneal macrophages of tuberculosis animals than normal controls. Both normal and infected animals showed similar levels of proteases,

esterases, nonspecific lipase, deoxyribonuclease and ribonuclease (Carson and Dannenberg, 1965 and Dannenberg and Bennett, 1963). Peritoneal macrophages from mice infected with BCG had elevated acid phosphatase, B glucuronidase and cathepsin levels (Saito and Suter, 1965 and Thorbeck and Benacerraf, 1962). Lysosomal enzyme concentrations from macrophages were increased as a result of stimulation with heat killed BCG and the cells more readily destroyed live L. monocytogenes in cell culture (Cohn and Wiener, 1963; Heise et al., 1965 and Mizunoe and Dannenberg, 1965). Macrophages from mice vaccinated with high concentrations of viable BCG showed larger and more lysosomes and increased microbicidal activity against S. typhimurium (Mackaness, 1968). Peritoneal macrophages from mice stimulated by Escherichia coli lipopolysaccharide showed increased levels of acid phosphatase and increased inhibition of S. typhimurium (Auzins and Rowley, 1962). Nevertheless, the prolonged effects of cellular immunity were demonstrated to be induced only by host cell association with live pathogen. When Osawa et al. (1967) implanted diffusion chambers charged with virulent S. enteritidis into the peritonea of mice, this exposure resulted in no protection to the animals even though high titers of agglutinating antibody were formed against the bacteria. Similar results were obtained by Osebold and DiCapus (1968) when they implanted diffusion chambers containing L. monocytogenes into mice.

The development of immunity to any pathogen whether growing in an intracellular or extracellular niche may involve 3 different expressions of resistance, i.e. 1) humoral antibody formation and its many manifestations of immunity, 2) delayed hypersensitivity and 3) cellular immunity. Cell-bound antibodies may or may not play a role in the last two phenomenon. Each of these may be expressed in variable degrees depending on the type of parasite interaction with the host.

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