DELAYED-TYPE HYPERSENSITIVITY AND CELLULAR IMMUNITY TO LEISHMANIA DONOVANI

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DELAYED-TYPE HYPERSENSITIVITY AND CELLULAR IMMUNITY TO *LEISHMANIA DONOVANI*

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INTRODUCTION

The protozoan Leishmania donovani is the parasite responsible for visceral leishmaniasis (also known as Kala-azar or Dum-Dum fever) in susceptible mammals. Transmission of this disease is accomplished by several species of the genus Phlebotomus (sandflies). Favorable climate, altitude and vegetation appears to be essential for sandfly proliferation. This disease occurs in areas of Asia, the Middle East, Africa and in limited frequency in South America. Reservoir hosts include dogs, foxes, gerbils and humans depending in part on the species of Phlebotomus prevalent in an infected area. Without treatment, the disease is usually fatal in humans.

The leishmanial stage (LD bodies) of this parasite is engulfed when the sandfly takes a blood meal from an infected animal. The parasites transform into a flagellate leptomonad stage which multiplies in the midgut. Within three to six days many leptomonads have migrated into the pharynx and proboscis where they are in a position to be inoculated into the skin of an animal during the next blood meal. After introduction of the leptomonads into the skin, macrophages phagocytize the leptomonads where they undergo transformation to the leishmanial stage. With rapid

multiplication of the leishmanial stages, large numbers of cells are destroyed with subsequent neutropenia and anemia usually developing. Monocytic hyperplasia is common in the spleen, liver and bone marrow. The liver and spleen are enlarged and discolored. Histological examination show both enlargement and proliferation of the Kupffer cells of the liver. The macrophages in the greatly enlarged spleen are packed with parasites.

Animals and humans which recover from a primary infection of L. donovani appear to exhibit a resistance of long duration to reinfection.

Since macrophages from immune mice have been shown to be resistant to the intracellular parasite L. donovani (Miller and Twohy, 1969) and delayed hypersensitivity is believed to accompany cellular immunity (Mackaness, 1967), it would be of interest to determine if delayed hypersensitivity to leishmanial antigens can be demonstrated and when in the course of infection it first develops.

The purpose of this investigation was to try to detect a delayed hypersensitivity type of reaction in mice and correlate it with the development of resistance.

LITERATURE REVIEW

The relationship between the immunological phenomena of macrophage resistance (cellular immunity) and delayed-type hypersensitivity (cellular hypersensitivity) has aroused much recent interest. This stems from the fact that intracellular parasites induce a delayed-type hypersensitivity response toward microbial antigens without any known exception (Mackaness, 1967). Both cellular immunity and cellular hypersensitivity have now been demonstrated to Besnoitia, Brucella, Leishmania, Listeria, Mycobacterium, Pasteurella, Salmonella, and Toxoplasma as reviewed by Lawrence (1956), Elberg (1960), Suter and Ramsier (1964), Mackaness and Blanden (1966), Uhr (1966), Boysia (1967), Frenkel (1967), Shands (1967), Turk (1967), Dannenberg (1968), Mackaness (1968) and Miller and Twohy (1969).

Cellular immunity

Acquired cellular immunity implies that macrophages from immune hosts possess a greater capacity to restrict the multiplication of intracellular parasites than phagocytes from normal animals. Only live organisms have been known to induce a state of acquired cellular immunity (Mackaness, 1962, 1967). A significant resistance can not be passively transferred to any of the intracellular

parasites by serum from immune hosts although it may enhance the expression of cellular immunity (Suter and Ramsier, 1964; Miki and Mackaness, 1964; Frenkel, 1967 and Mackaness, 1967, 1968). Cellular immunity was passively transferred to normal recipient hosts using peritoneal macrophages by Sever (1960) and later by Suter (1961), Saito et al. (1962) and Fong et al. (1962). More recently Frenkel (1967) and Mackaness (1968) used sensitized lymph node and spleen cells to transfer cellular immunity to normal animals. Macrophage resistance is not specific for the immunizing organisms since these immune cells inhibit a wide variety of intracellular parasites (Sato et al., 1962; Mackaness, 1964 and Blanden et al., 1966). However, Mackaness (1968) noted that lymphoid cells from mice which were immune to BCG did not passively transfer the non-specific type of macrophage resistance unless there was an immunologically specific interaction with BCG in recipient mice. Cross resistance to Listeria monocytogenes was not observed unless normal mice received both sensitized lymphoid cells and an active BCG infection. Thus the induction process involving the lymphocyte appeared to be specific.

Delayed-type hypersensitivity

Delayed-type hypersensitivity is a specifically provoked and slow evolving cellular reaction to the presence of antigen in a sensitized animal. Intense delayed-type

hypersensitivity reactions to intracellular parasites are best induced by viable organisms (Mackaness, 1968). The only known exception is a discovery by Rich (1951) that dead tubercle bacilli would sensitize guinea pigs. Dannenberg (1968) suggests that the lipopolysaccharide of the tubercle bacillus cell wall acts as a non-specific adjuvant for the establishment of cellular hypersensitivity to tuberculoprotein.

Cellular hypersensitivity sharply contrasts with cellular immunity in that it is highly specific for the infecting parasite (Dannenberg, 1968). Serum from sensitized donors has repeatedly failed to transfer delayed-type hypersensitivity to recipient animals.

A state of cellular hypersensitivity can be passively transferred to normal recipient animals by means of lymphoid cells. This reaction is immunologically specific in that it requires an antigen homologous to that used for immunization to elicit a detectable delayed hypersensitivity response in recipient animals. Passive or adoptive transfer of delayed-type hypersensitivity was analytically reviewed by Bloom and Chase (1967).

Interrelationship between delayed-type hypersensitivity and cellular immunity

Macrophages participate in many events associated with both cellular hypersensitivity and cellular immunity. Macrophages become activated (i.e., new lysosomes and

other organelles as well as an increased concentration of digestive enzymes) by certain antigens (Cohn and Parks, 1967a, 1967b; Dannenberg et al., 1963a, 1963b; and Yarborough et al., 1967). This phagocytic cell is mobilized and may proliferate when exposed to these antigens (Lurie, 1939; Waksman and Matoltsy, 1958; Khoo and Mackaness, 1964 and Spector, 1967). Dannenberg (1968) suggested that the macrophage surviving destruction in the immunological reaction was stimulated by the sensitized lymphocyte into an increased mobilization of its phagocytic process which must be accompanied by a biochemical activation. Mackaness (1967) suggested that since the onset of delayed-type hypersensitivity preceded the development of a detectable cellular immunity, the immunologically specific state of cellular hypersensitivity somehow enhanced the microbicidal capacity of macrophages. When sensitized cells were re-exposed to tubercle bacilli for example, the exaggerated and accelerated inflammatory reaction associated with delayed hypersensitivity may serve to rapidly immobilize and possibly destroy this organism (Suter and Ramsier, 1964).

Mackaness (1962, 1964) and Mackaness and Blanden (1966) have shown that events which induce cellular immunity are dependent upon immunological processes. In summary, these authors have shown that: (1) the tissues of the host must respond immunologically to the infecting parasite before antimicrobial activity can be detected in macrophages in vivo or in vitro, (2) the reacquisition of

resistance in convalescent animals upon reinfection appeared at an accelerated rate, (3) only reinfection with the homologous parasite accelerated the recall of cellular immunity, (4) an injection of either viable or dead parasites into actively infected animals enhanced resistance only if homologous organisms or antigens were used. It appears that the induction of cellular immunity depends in part on the interaction of specifically sensitized tissues with specific antigen.

Once cellular immunity is established, this form of resistance becomes basically non-immunological because the microbicidal power of immune macrophages lacks specificity. Other related or unrelated species of intracellular parasites were completely or partially destroyed by immune cells both *in vitro* and *in vivo* (Mackaness, 1968). Elberg *et al.* (1957) used cultured immune peritoneal macrophages from infected mice to demonstrate complete cross resistance between *Mycobacterium tuberculosis* and *Brucella melitensis*. Similar results were reported by Mackaness (1964) when mice infected with either *Mycobacterium tuberculosis*, *Brucella abortus* or *Listeria monocytogenes* demonstrated for a time antimicrobial resistance to both the specific infecting parasite and the other non-specific intracellular parasites. Blanden *et al.* (1966) infected mice with either *Listeria monocytogenes* or *Salmonella typhimurium* and demonstrated, *in vitro*, that macrophages from both groups of infected animals were equally resistant to *Salmonella typhimurium*.

They further reported that serum or cell bound antibody to Salmonella could not be demonstrated in animals infected with Listeria monocytogenes. Ruskin and Remington (1968) were the first to demonstrate that the infection of mice with the intracellular protozoan Toxoplasma gondii conferred resistance to challenge with Listeria monocytogenes and Salmonella typhimurium. They also reported that mice infected with Listeria monocytogenes resisted lethal Toxoplasma challenge.

Mackaness (1968) refers to Blanden's unpublished results which further relates these two immunological phenomena. Mice infected with 10^4 BCG organisms developed tuberculin sensitivity six to nine days later. Maximum sensitivity was shown twenty-one to twenty-eight days after initial infection. During the infection intravenous challenges with Listeria monocytogenes showed only a minor increase in resistance to this organism. Macrophages from these mice showed no resistance to Salmonella typhimurium, in vitro. If normal mice were infected intravenously with 10^7 BCG organisms, mice not only became hypersensitive to tuberculin but also demonstrated in twelve to fifteen days a non-specific resistance to an intravenous challenge of Listeria monocytogenes. It appeared that the 10^4 BCG dose was only a sensitizing level of infection whereas the 10^7 BCG dose was both a sensitizing and immunizing level of infection. If the lightly infected mice were challenged with an immunizing dose of 10^7 BCG, a maximal level of

resistance to both BCG and Listeria monocytogenes was obtained within one to two days. This occurred only when an intense tuberculin sensitivity was demonstrable at the time of reinfection. These authors suggested that the accelerated acquisition of a non-specific cellular immunity was due to the interaction of specific antigen with previously sensitized tissues.

In another experiment Mackaness (1968) sensitized donor mice with only 10^3 viable Listeria monocytogenes. Eight days later a suspension of purified spleen lymphoid cells was prepared from these donors and injected intravenously into normal recipient mice. A high level of resistance was obtained within twenty-four hours after challenge with Listeria monocytogenes. However, challenge with unrelated BCG conferred no measurable protection. Since cellular hypersensitivity was psssively transferred by spleen lymphoid cells, Mackaness reasoned that the induction of cellular immunity was specific for the infecting organism. He further suggested that non-specific cellular immunity developed in recipient animals was a direct consequence of the more specific cellular hypersensitivity phenomenon.

Other authors view target cell type of destruction as a possible mechanism of resistance. Bray and Bryceson's (1968) failure to demonstrate resistant macrophages from guinea pigs infected with Leishmania enriettii and their report that normal guinea pig macrophages that were

infected in culture were destroyed by lymphocytes from infected guinea pigs suggests that the lymphocyte may at least play a role in resistance. Speel et al. (1968) presented data indicating that mouse spleen cells sensitized to a persistent non-cytocidal mumps virus have the capacity to destroy target cells carrying mumps-virus antigen. They suggested that cell-mediated cytotoxic interactions between lymphocytes and virus infected cells may be an effective means of controlling moderate viral infections in host cells.

MATERIALS AND METHODS

Experimental animals

Swiss-Webster female mice were used in all skin tests for delayed-type hypersensitivity. In experiments designed to show macrophage-target cell type of destruction and the inhibition of macrophage migration in capillary tubes, young inbred C57BL/6J female mice were employed. All mice of similar ages were segregated randomly into experimental and control groups at the start of an experiment.

Experimental organism

All investigations utilized the 3S strain of *Leishmania donovani* that was obtained from Dr. Leslie Stauber, Department of Zoology, Rutgers University. The organism was maintained by routine hamster to hamster passage. Hamsters were infected with either an intraperitoneal (ip) injection of 30×10^6 LD bodies or an intracardial injection of 15×10^6 LD bodies. The animals were sacrificed one to three months later and the heavily parasitized spleen removed aseptically. A spleen was minced into small pieces (3 mm to 5 mm) with sterile scissors and ground in a 15 ml Teflon pestle homogenizer containing 10 ml of tissue culture medium without additives (NCTC 135).

The suspension was transferred to a 12 ml sterile centrifuge tube and centrifuged 5 minutes at 63 x g and the supernatant then centrifuged again at 910 x g for 10 minutes. After discarding the supernatant the white surface phase of the sediment was removed by use of a Pasteur pipette. This layer which contained most of the LD bodies was resuspended in 10 ml of NCTC 135 for a final 10 minute centrifugation at 910 x g. Again the white surface phase was removed and resuspended in 10 ml of NCTC 135. The parasite population was determined with the aid of a Petroff-Hausser counting chamber and a phase microscope. The suspension was adjusted to the desired LD body concentration using NCTC 135.

Immunization of mice

Mice were immunized or sensitized by injecting viable LD bodies into either the peritoneal cavity (ip) or the caudal vein (iv). Depending on the nature of each experiment, the ip injections ranged from 1×10^6 to 30×10^6 LD bodies and iv injections ranged from 5×10^6 to 15×10^6 LD bodies.

Collection, cultivation and inoculation of macrophages in culture

The procedures for prestimulation and collection of macrophages were similar to those described for mice and hamsters (Miller and Twohy, 1967, 1969). Mice were prestimulated by injecting one ml of NCTC 135 into the

peritoneal cavity 48 and 24 hours before harvesting the peritoneal exudates. Peritoneal cells were collected after injecting 2.5 ml of collecting medium (NCTC 135 with 10 units of heparin, 200 units of penicillin and 200 µg of streptomycin per ml) into the peritoneum. The abdomen was gently kneaded and the peritoneal exudate withdrawn with a sterile syringe and needle. The entire exudate from an experimental group of mice was either pooled or the exudates from 5 animals combined in separate tubes. After cooling the exudate to 10 C the cells were centrifuged for 10 minutes at 360 x g and resuspended in 6 ml of complete tissue culture medium (Chang, 1964). The cell population was determined with a Model A Coulter Counter using a threshold value of 60 and a 100 µ aperture at an aperture current setting of 4. After adjusting the cell population to a desired concentration with extra medium (usually 1.5×10^6 to 2.0×10^6 cells per ml), one ml of the suspension was pipetted into each Leighton tube containing a 35 mm by 9 mm coverslip and incubated at 37 C in a CO_2 incubator with an atmosphere of 5% carbon dioxide and 95% air.

The complete medium employed in these investigations contained 50% NCTC 135, 40% inactivated horse serum, 10% beef embryo extract, 200 units of penicillin and 200 µg of streptomycin (Chang, 1964). After the adherence of macrophages to the coverslips in about 4 hours, the medium was usually replaced with fresh medium containing LD

bodies. Another medium change was made about 6 to 24 hours after infection if lymphocytes were added to the culture system.

Preparation of lymph node, spleen and peritoneal lymphocytes

Lymph node and spleen cell suspensions were obtained from normal and infected mice for delayed hypersensitivity studies. Lymph nodes were removed aseptically from the popliteal, inguinal, mesenteric, cervical, brachial or axillary regions, trimmed of surrounding tissues and pooled in 10 ml of collecting medium. After gentle probing with forceps, forced pipetting and vortexing, the suspension was allowed to settle for 5 minutes at 10 C to remove cellular debris. The supernatant was transferred to a 12 ml centrifuge tube and centrifuged 10 minutes at 360 x g. After discarding the supernatant the white upper phase of the sediment was collected and resuspended in fresh, complete NCTC 135 medium.

Since a pure preparation of lymphocytes was desirable, 5 ml of the lymphocyte suspension was dispensed into 30 ml sterile plastic tissue culture flasks and incubated in a 5% carbon dioxide incubator at 37 C. After 6 and 18 hours of culture the suspension from each tissue culture flask was transferred to another culture flask to allow macrophages and fibroblasts to adhere to the surface and to separate the lymphocytes which will not adhere to

plastic or glass. After 24 hours of culture the medium was collected and centrifuged at 360 x g for 10 minutes and the lymphocytes were resuspended in fresh complete medium. The cells were counted with a Model A Coulter Counter (threshold value 30) and adjusted to the desired concentration.

Peritoneal lymphocytes were removed from cultures of peritoneal exudates and recultured to remove adhering cells as described for lymph node and spleen lymphocytes.

Viability test for lymphocytes

In some experiments the ability of viable lymphocytes to exclude the vital stain Trypan Blue was determined (Merchant et al., 1964). This involved mixing 0.5 ml of a cell suspension containing 1 x 10^5 to 2 x 10^5 cells per ml with 0.1 ml of 0.4% Trypan Blue in Balanced Salt Solution. This preparation was allowed to stand 10 minutes at room temperature before the stained and unstained cells were counted in a Petroff-Hausser counting chamber.

Fixation, staining and counting of parasites and macrophages

Cells adhering to the coverslip were fixed in a 6% glutaraldehyde solution and stained with May-Grünwald Giemsa as previously described by Miller and Twohy (1967). The total number of macrophages, the total number of infected macrophages and the total number of intracellular LD bodies were counted in 8 fields per coverslip at 450X.

The average number of LD bodies per cell was based on the total macrophage population counted.

Skin test and capillary migration

To prepare phenol treated parasites as antigens, LD bodies suspended in one ml of NCTC 135 were added to one ml of a 2% phenol solution and incubated with periodic agitation for one hour at 37 C. Following centrifugation at 360 x g the supernatant was discarded and the sediment resuspended with NCTC 135. After a second centrifugation and resuspension the LD body population was determined in a Petroff-Hausser counting chamber and adjusted to the desired concentration.

Heat-killed parasites were prepared by exposing LD bodies suspended in 2 ml of NCTC 135 for 30 minutes to a 56 C water bath. The rest of the procedure was identical to that employed to prepare phenolized organisms.

Freeze-killed parasites were prepared by exposing LD bodies suspended in 3 ml of NCTC 135 to slow freezing at -20 C. The preparation was thawed at room temperature. The freeze-thaw process was repeated two more times and the antigen stored at 10 C.

The skin-testing technique employed was a slight modification of work by Khoo and Mackaness (1964). It involved the injection of 0.03 ml of antigen or control preparations into the base of the hind foot-pad of mice.

Foot-pad thickness was measured using a stereoscopic microscope equipped with an ocular micrometer.

The method employed to measure the migration of peritoneal exudate cells was similar to that described by David (1966). It involved placing peritoneal exudate cells from normal and infected mice into capillary tubes, inserting these tubes into perfusion chambers and adding antigen with the medium. The area of migration of cells out of the capillary tubes onto the chamber glass surface was determined from photographs.

The antigen preparation used in this study consisted of a 1:1:1 mixture of heat-killed, freeze-thawed and live LD bodies totaling 25 x 10^6 live and dead parasites per culture perfusion chamber.

Destruction of infected macrophages by lymphocytes

The general technique of Bray and Bryceson (1968) was followed using L. donovani infections in mice and mouse macrophages. Mice were infected with L. donovani and lymph node, spleen and peritoneal exudate cells were harvested from normal and sensitized animals at various times after infection. Lymphocytes were separated from other cell types as previously described and added in a 5X to 50X ratio to cultures of normal mouse macrophages which had been previously infected with L. donovani. Cells were fixed at various time periods following

lymphocyte addition to determine cell and parasite populations.

Statistical analysis

The student t test was employed to compare means for each experimental group of animals.

RESULTS

Effects of skin testing on mice infected with L. donovani

The foot-pad response to phenol-killed LD bodies was compared in normal and infected mice as a possible test for delayed hypersensitivity to L. donovani. Infected animals received 30 x 10^6 LD bodies ip 7 1/2, 4 1/2 and 2 1/2 months prior to skin testing. Experimental mice were injected in the hind foot-pad with 1 x 10^6 phenol treated LD bodies and controls were given an equal volume of parasite free NCTC 135. No significant difference in foot-pad thickening was noted over a period of 72 hours between infected animals receiving either the treated parasites or medium used to suspend the cells.

These previously sensitized mice were reinfected ip with 30 x 10^6 LD bodies in an attempt to enhance a hypersensitive state. When tested with 5 x 10^6 heat treated organisms 7 and 14 days after reinfection, again no significant difference between experimental and control mice was observed over a period of 72 hours.

Capillary tube migration as a test of delayed hypersensitivity to L. donovani antigens

One group of mice were sensitized with one ip injection of 27 x 10^6 LD bodies and comparable animals

were set aside as controls. The peritoneal exudate cells were harvested from both groups of mice 7 and 21 days after infection and placed in capillary tubes. After 24 hours of exposure to killed and live LD body antigen preparations in culture, the monocytic-phagocytes from both the sensitized and control mice migrated approximately equal distances. Thus no delayed hypersensitivity was detected using this technique.

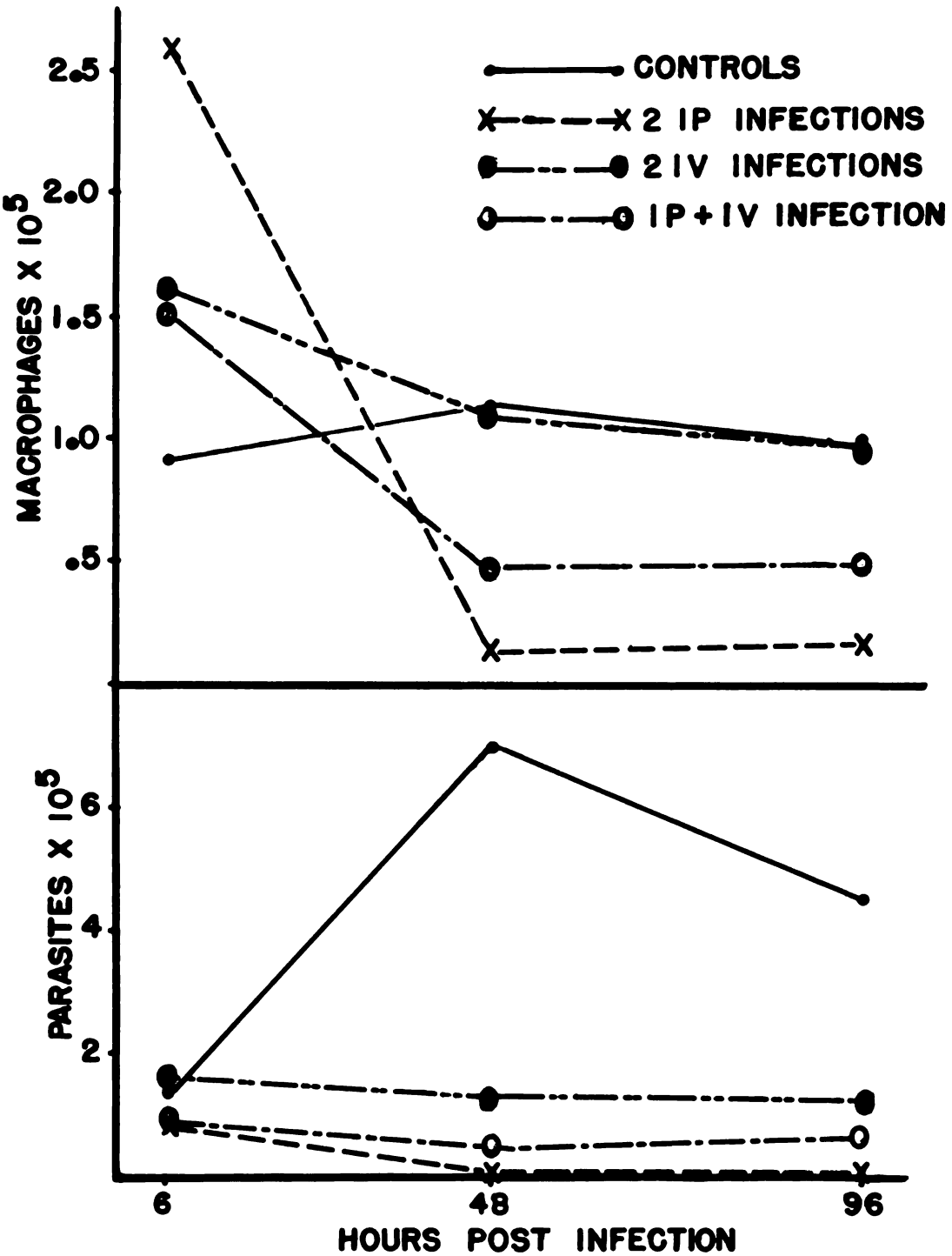
The sensitivity of macrophages from infected mice

It had been observed in this laboratory that macrophages harvested from mice immunized by ip injections often failed to adhere to the coverslip after infection. Mackaness (1962) observed a similar phenomenon in peritoneal macrophages harvested from mice infected with Listeria monocytogenes. From this observation an experiment was designed to test the development of cellular immunity by both the ip and iv route of infection and to follow macrophage survival after infection. Separate groups of mice were infected by one of two routes or combination of routes: (1) 2 ip injections of 30×10^6 parasites, (2) 2 iv injections of 15×10^6 parasites, and (3) 1 ip injection of 30×10^6 plus an iv injection of 15×10^6 LD bodies. The infections were given 33 days apart and peritoneal cells were harvested and cultured 20 days after the last injection and infected 4 hours later with parasites. Very few macrophages from mice receiving either

the ip or ip plus iv injections remained attached to the coverslips 6 hours after infection (Figure 1). Losses were minimal in cultures of phagocytes from mice receiving iv injections and in the cells from uninfected control animals. The number of parasites increased between 6 and 48 hours in the cultures from normal mice and showed little increase in the iv infected mice demonstrating cellular immunity after 2 iv injections. Cell populations were too low to evaluate parasite multiplication in macrophages from either ip infected group.

This experiment was repeated one month later on cells from the same mice. Most of the macrophages from the ip infected mice disappeared from the coverslips while the macrophage loss was less pronounced in the other experimental groups. Phagocytes from iv infected mice demonstrated more resistance to intracellular ID body proliferation than normal infected controls. Since some sensitized lymphocytes would have remained in the culture system in spite of the medium change and could have played a role in the macrophage destruction, attempts were made to induce cell destruction with lymphocytes, <u>in vitro</u>, as a possible manifestation of a delayed hypersensitivity-type of reaction.

Figure 1.--The survival of infected macrophages from mice inoculated by various routes of infection, and the effect of the route of injection on parasite survival in culture. [Mice were given, at a 33 day interval, 2 ip injections of 30×10^6 LD bodies, 2 iv injections of 15×10^6 LD bodies or 1 ip injection of 30×10^6 parasites followed by an iv injection of 15×10^6 LD bodies. Macrophages were harvested 20 days after the last infection.]


CONTROLS
2 IP INFECTIONS
2 IV INFECTIONS
IP + IV INFECTION
MACROPHAGES X 10^5
2.5
2.0
1.5
1.0
.5
PARASITES X 10^5
6
4
2
6
48
96
HOURS POST INFECTION

The effect of sensitized lymph node cells on macrophage and parasite survival

These experiments were designed to compare the effect of normal and sensitized lymphocytes on parasite and macrophage survival in culture. The general procedure of these tests were those reported by Bray and Bryceson (1968). Lymph nodes were removed from mice which had received 2 ip and 1 iv injections (5, 4 and 1 1/2 months respectively prior to cell harvest). Peritoneal cells were harvested from normal mice, cultured and inoculated 4 hours later with parasites. After 24 hours in culture, 5 lymphocytes for each adhering macrophage (hereafter referred to as 5X concentration of lymphocytes for brevity) from either normal or sensitized animals were added to each Leighton tube. Four coverslips per group were fixed at each time interval following lymphocyte addition (Table 1, Exp. 1). There was no significant decline in the macrophage or parasite population at either 6 and 48 hours after adding lymph node cells.

A similar experiment was designed to determine what possible effect a more recent infection and a higher concentration of lymphocytes would have on macrophage and parasite survival. Sensitized lymphocytes were obtained 2 weeks after the last of 2 infections with *L. donovani*. The experiment revealed a general cell loss in all infected cultures including those which received either a 5X or a 25X concentration of normal or sensitized

TABLE 1.--The effect of lymph node cells on macrophage and parasite survival.

Exp. no.	No. of tubes	Parasites inoculated per macrophage	Lymphocytes[a] added per macrophage	% decrease or increase in macrophages up to 48 hours	% decrease or increase in parasites between 6 & 48 hours
1	4	5	5X sensitized	- 13	+ 344
	4	5	5X normal	+ 17	+ 486
	4	0	5X sensitized	+ 38	---
	4	0	5X normal	- 32	---
	4	5	---	- 14	+ 212
2	5	5	5X sensitized	- 60[b]	+ 172
	5	5	5X normal	- 52	+ 62
	5	5	25X sensitized	- 39	+ 18
	5	5	25X normal	- 33	+ 226
	5	5	---	- 12	+ 235
	5	0	---	- 36	---

[a]Sensitized lymphocytes were harvested from mice inoculated with 2 injections of 30 x 10^6 LD bodies ip 6 and 5 months and 15 x 10^6 LD bodies iv 1 1/2 months prior to cell harvest for Experiment 1, and 30 x 10^6 LD bodies ip 34 days and 20 x 10^6 LD bodies iv 12 days prior to cell harvest for Experiment 2.

[b]All macrophage losses in this experiment were determined from 0 to 48 hours.

lymphocytes and the parasite free control cultures (Table 1, Exp. 2). No correlation could be established between the concentration or the source of lymphocytes present and the degree of cell destruction. Again the parasites multiplied in all inoculated groups as evidenced by a total parasite increase at 48 hours. Lymph node cells from sensitized mice did not seem to alter the survival of either the parasites or their host cells.

The effect of sensitized spleen and peritoneal lymphocytes on macrophage and parasite survival

A series of experiments were designed to determine if spleen and peritoneal lymphocytes from sensitized mice would be more effective in the destruction of parasitized macrophages than lymph node cells. All the experimental mice employed received a single infection of parasites by either the ip or iv route. Spleen cells were harvested within 7 to 36 days after infection in an attempt to detect sensitivity if it was transitional in nature. Spleen cells were added at a 25X concentration in all experiments. In Experiment 4 the procedure was varied by adding the LD bodies to the lymphocyte cultures in an attempt to stimulate the lymphocytic response before the mixture of lymphocytes and LD bodies was added to the macrophage cultures. None of the cultures receiving lymphocytes from infected mice showed a significant macrophage loss over that of control cultures where normal lymphocytes or no

lymphocytes were added (Table 2). There was thus no evidence of a delayed hypersensitivity-type of reaction being induced by spleen cells. Neither was there any evidence of cellular immunity since parasite populations were comparable in all groups of cultures.

Peritoneal lymphocytes from these infected mice also failed to effect the survival of either normal macrophages infected with L. donovani or the parasite itself (Table 3).

The failure to repeat Bray and Bryceson's results using cells from mice infected with L. donovani suggested that cell sensitivity of the type responsible for macrophage destruction is not well developed in mice. If this sensitivity is of short duration it might be better detected if less extreme immunization procedures were employed. Therefore, further experiments were designed to determine if a light infection with parasites produces lymphocytes which destroy infected macrophages and if the peritoneal cells from these hosts slough from the monolayer in response to the parasites. Mice were divided into two experimental groups. One group received an ip injection of 1×10^6 LD bodies per animal and the mice of the second group were injected with 5×10^6 LD bodies by the iv route. Another group of mice from the same lot were used as a source of normal macrophages and lymphocytes. Cells from these animals were collected for tests 14, 28 and 42 days following infection.

TABLE 2.--The effect of spleen lymphocytes on macrophage and parasite survival.

Exp. no.	Days after ip or iv infection	No. of tubes	Parasites inoculated per macrophage	Lymphocytes[a] added per macrophage	% decrease or increase in macrophages up to 48 hours	% decrease or increase in parasites between 6 & 48 hours
3	22 days--ip	4	5	25X sensitized	- 34	+ 12
	uninfected	4	5	25X normal	- 45	- 2
	uninfected	4	5	---	+ 7	- 16
4	36 days--ip	5	5	25X sensitized	- 50	+ 16
	uninfected	5	5	25X normal	- 49	- 28
	uninfected	5	5	---	- 34	- 45
5	7 days--iv	5	5	25X sensitized	- 8	+ 55
	uninfected	5	5	25X normal	- 29	+ 61
	uninfected	5	5	---	0	- 18
6	21 days--iv	5	5	25X sensitized	- 16	+ 87
	uninfected	5	5	25X normal	- 19	+ 87
	uninfected	5	5	---	- 3	+ 144

[a]Animals were injected with 30 x 10^6 LD bodies ip and spleen lymphocytes obtained 22 days (Exp. 3) and 36 days (Exp. 4) after infection. Mice infected with 15 x 10^6 LD bodies iv were harvested from 7 days (Exp. 5) and 21 days (Exp. 6) later.

TABLE 3.--The effect of peritoneal lymphocytes on macrophage and parasite survival.

Exp. no.	Days after ip or iv infection	No. of tubes	Parasites inoculated per macrophage	Lymphocytes[a] added per macrophage	% decrease or increase in macrophages up to 48 hours	% decrease or increase in parasites between 6 & 48 hours
5	7 days	5	5	15X sensitized	- 23	- 41
	uninfected	5	5	15X normal	- 2	+ 2
	uninfected	5	5	---	0	- 18
6	21 days	5	5	15X sensitized	- 22	+ 96
	uninfected	5	5	15X normal	- 19	+ 103
	uninfected	5	5	---	- 3	+ 144

[a]Sensitized lymphocytes were obtained from mice injected with 15 x 10^6 LD bodies iv 7 days (Exp. 5) and 21 days (Exp. 6) after infection.

Fourteen days after infection the results showed a stable macrophage population irrespective of the source of macrophages or peritoneal lymphocytes (Tables 4 and 5). The effect of spleen lymphocytes was not tested at this time. Parasite proliferation was similar in all groups. Thus neither cell destruction or cellular immunity was demonstrated 14 days after infection.

The procedure was repeated 28 days after infection. The results were markedly different. Cultures of normal macrophages which received a 30X concentration of peritoneal lymphocytes from ip sensitized mice lost 66 per cent of their macrophages within 8 hours after infection. Cultures which received a similar concentration of peritoneal lymphocytes from iv injected mice lost only 12 per cent of their cells and the controls with 30X normal lymphocytes added lost 9 per cent of their macrophages during this time interval. Between 8 and 60 hours, all remaining macrophages in the cultures to which ip sensitized lymphocytes were added disappeared from the coverslips while other groups suffered only minor losses (Table 8, Exp. 8).

Cultures of peritoneal macrophages harvested from ip injected animals also lost about 85 per cent of their cells by 8 hours after infection (Table 5, Exp. 8) whereas no significant change was noted in populations from the iv injected or the control animals. Between 8 and 60 hours the remaining macrophages from the ip injected animals sloughed from the coverslips while macrophage cultures from

TABLE 4.--The effect of peritoneal and spleen lymphocytes on macrophage and parasite survival.

Exp. no.	Days after ip or iv infection	No. of tubes	Parasites inoculated per macrophage	Lymphocytes[a] added per macrophage	% decrease or increase in macrophage up to 60 hours	% decrease or increase in parasites
7	14 days--ip	5	5	30X sensitized peritoneal	- 8	+ 40
	uninfected	5	5	30X normal peritoneal	+ 9	+ 78
	14 days--iv	5	5	30X sensitized peritoneal	0	+ 53
	uninfected	5	5	---	+ 8	+ 76
8	28 days--ip	5	5	30X sensitized	- 100[b]	---
	uninfected	3	5	30X normal peritoneal	- 16	+ 32
	28 days--iv	5	5	30X sensitized peritoneal	- 28	+ 42
	uninfected	5	5	---	- 11	+ 20
	28 days--ip	5	25	50X sensitized spleen	- 36	+ 51
	uninfected	5	25	50X normal spleen	+ 2	+ 209
	28 days--iv	4	25	50X sensitized spleen	0	+ 209
	uninfected	5	25	---	- 11	+ 20
9	42 days--ip	5	10	15X sensitized peritoneal	- 10	+ 75
	uninfected	5	10	15X normal peritoneal	- 4	+ 209
	42 days--iv	5	10	15X sensitized peritoneal	0	+ 83
	uninfected	5	10	---	- 11	+ 98
	uninfected	5	0	---	- 13	---

[a]Mice infected with either 1 x 10^6 LD bodies ip or 5 x 10^6 LD bodies iv were used as cell sources 14 days (Exp. 7), 28 days (Exp. 8) or 42 days (Exp. 9) after infection.

[b]$p < 0.01$ compared with controls receiving normal peritoneal lymphocytes.

TABLE 5.--The effect of viable LD bodies on macrophages and lymphocytes from sensitized hosts.

Exp. no.	Days after ip or iv infection[a]	No. of tubes	Parasites inoculated per macrophage	% decrease or increase in macrophages up to 60 hours	% decrease or increase in parasites
7	14 days--ip	5	5	+ 2	+ 52
	uninfected	5	5	+ 8	+ 76
	14 days--iv	5	5	+ 5	+ 80
8	28 days--ip	5	5	- 100[b]	--
	uninfected	5	5	- 28	+ 20
	28 days--iv	5	5	- 44	- 60
9	42 days--ip	5	10	- 20	- 72[b]
	uninfected	5	10	- 9	+ 72[b]
	42 days--iv	5	10	- 2	- 23[b]

[a]Sensitized peritoneal cells were harvested from mice infected with 1 x 10^6 LD bodies ip or 5 x 10^6 LD bodies iv 14, 28 and 42 days after infection.

[b]$p < 0.01$ compared with normal controls.

iv injected mice lost a total of 44 per cent of their cells by 60 hours and the control cells decreased by only 28 per cent. Limited growth of LD bodies was observed in normal host cells while macrophages from iv injected mice demonstrated an inhibition of multiplication which is usually associated with cellular immunity. Insufficient cells survived to depict LD body proliferation in cultures from ip infected mice.

The ability of spleen lymphocytes to induce macrophage destruction was also tested. Normal peritoneal macrophages were inoculated 4 hours after culture with 25 LD bodies per adhering macrophage. Spleen lymphocytes from either ip or iv infected mice as well as normal mice were added to appropriate Leighton tubes in a ratio of 50X lymphocytes per adhering macrophage. Between 8 and 60 hours a 36 per cent cell loss was recorded in cultures with sensitized lymphocytes from ip injected mice while the macrophage population from cultures with lymphocytes from normal and iv injected hosts remained unchanged (Table 4, Exp. 8). Macrophages from all 3 groups supported some growth of the parasites.

When this experiment was repeated 42 days after infection, there was no evidence of the destruction of normal macrophages by a 15X concentration of peritoneal lymphocytes from either group of sensitized hosts in response to <u>in vitro</u> infection (Table 4, Exp. 9). Normal macrophages supported the growth of LD bodies showing no

evidence of the passive transfer of resistance by peritoneal lymphocytes from infected mice. After inoculation with 10 LD bodies per adhering macrophage, the phagocytes from both the ip and iv infected mice contained more parasites than cultures from normal mice 6 hours after infection. Whereas LD bodies almost doubled in macrophages from normal mice between 6 and 48 hours after infection, the number of parasites decreased in phagocytes from iv injected animals and declined drastically in macrophages from ip injected mice (Table 5, Exp. 9). Thus a considerable degree of cellular immunity to L. donovani was evident in macrophages from both iv and ip infected mice 42 days after initial infection. No evidence of macrophage destruction was observed in the macrophages from hosts infected by either route of injection.

Lymphocyte viability was checked in most of the latter experiments by the use of trypan blue. Between 73 and 88 per cent of the cells excluded the stain and thus were assumed to be viable prior to their addition to macrophage cultures.

DISCUSSION

The general mechanism involved in the development of cellular immunity to an intracellular parasite is poorly defined. The role of the lymphocyte is the focal point of conflict in the development of macrophage mediated cellular immunity. Mackaness (1967) suggests that the earlier onset of a specific delayed hypersensitivity somehow enhances the destructive power of macrophages towards many intracellular parasites. Bray and Bryceson (1968) showed that lymphocytes from convalescent guinea pigs will destroy normal guinea pig macrophages infected with Leishmania enriettii in culture and inferred this was a possible mechanism of resistance, in vivo. Speel et al. (1968) have demonstrated that sensitized lymphocytes destroyed virus infected epithelial cells and proposed that cytotoxic interactions by sensitized lymphocytes destroyed host cells containing virus and may be a mechanism of host resistance.

Macrophage mediated cellular immunity to L. donovani has only been demonstrated in mice (Miller and Twohy, 1969). They showed that parasites in peritoneal macrophages from infected mice failed to multiply or declined in numbers when compared to the increasing number of parasites in normal macrophages. Since no more immune macrophages than

normal macrophages were lost from the coverslips after infection, the target cell type of destruction inferred by Bray and Bryceson's work using guinea pig hosts would not appear to be a resistance mechanism in mice unless the sensitized lymphocytes would act on the parasite without destroying the host cell. Thus the role of the lymphocyte in macrophage mediated cellular immunity to Leishmania is uncertain.

In order to follow Mackaness's concept of macrophage immunity, delayed hypersensitivity should be present before or concurrent with cellular immunity. A first step in looking for a relationship between delayed hypersensitivity and macrophage mediated cellular immunity to L. donovani is to correlate the two phenomena using mice as experimental hosts.

It appeared from these studies that mice do not develop a pronounced delayed hypersensitivity reaction to L. donovani. When heat or phenol treated LD bodies were injected into the foot-pads of infected mice, no visible erythema or edema was ever observed. Although other species of Leishmania have elicited a delayed-type cutaneous response in certain sensitized animals (rabbits, guinea pigs, dogs, monkeys and humans), such a response has never been reported in mice. Furthermore, positive skin sensitivity tests to L. donovani has been limited to the guinea pig and humans. Stauber (1963) and Adler (1964) reported that there was much variation in skin sensitivity

in infected human hosts depending in part on the infecting strain of L. donovani and the time of skin testing. Thus the host, the strain of parasite and the time of skin testing may play an important part in the overall immunological mechanism which determines delayed-type skin reactivity.

The inhibition of migration of peritoneal exudate cells obtained from sensitized animals when exposed to specific antigen is considered another test of delayed hypersensitivity. When peritoneal exudate cells were employed in this test, the lymphocytes were shown to be cells sensitive to antigen (David et al., 1964; Bloom and Bennett, 1966) and macrophages constituted the migrating cell population (David et al., 1964; David, 1965). Recent work by Bloom and Bennett (1966, 1967) have shown that the inhibition of the migration of macrophages was mediated by sensitized lymphocytes through the liberation of a migration inhibitory factor (MIF) only in the presence of specific antigen. In this study peritoneal exudates were harvested 1 and 3 weeks after mice received a single ip injection and migration of their peritoneal cells from capillary tubes was unaffected by the presence of specific antigen in the cultural medium. At the present time it is uncertain if the test lacks the sensitivity essential for detecting delayed hypersensitivity in mice to L. donovani, or its failure is a result of the transitional

nature of delayed hypersensitivity in the mouse to this parasite.

Another *in vitro* correlate of delayed hypersensitivity is target cell destruction. Lymphocytes from properly immunized animals have been shown to destroy a number of types of target cells when they are exposed to the specific antigen. The cytotoxic effect seems to involve the interaction of sensitized lymphocytes with specific antigen (Ruddle and Waksman, 1968). Both target cell destruction and capillary tube migration are considered manifestations of cellular hypersensitivity because: (1) an immunologically specific interaction occurs between the lymphocyte and the antigen, (2) a substance appears to be released from sensitized lymphocytes, and (3) the delayed cutaneous reactions, *in vivo*, correlate with either the test for the inhibition of macrophage migration or with destruction of target cells by sensitized lymphocytes.

Bray and Bryceson (1968) reported the *in vitro* destruction of normal infected macrophages by lymph node cells obtained from guinea pigs infected with *L. enriettii*. An attempt was made to repeat the results of these authors using cells from mice infected with *L. donovani*. Lymph node cells from mice heavily superinfected over several months and mice infected by the iv route a few weeks before testing failed to specifically destroy infected macrophages. Although some macrophages were lost, this was of a non-specific nature as macrophages from the control group

receiving normal lymphocytes were also partially destroyed. Similar results were obtained in most experiments using spleen cells instead of lymph node cells.

A phenomenon possibly related to the one described by Bray and Bryceson was demonstrated in mice which had received at least one ip infection one month prior to cell harvest. Monolayered peritoneal exudates from mice infected by the ip route or by both the ip and iv routes disappeared from the coverslip surface soon after infection in culture. Macrophages from mice infected twice by the iv route and from normal mice did not demonstrate this reaction. When this experiment was repeated using the same animals about one month later, only cells from ip infected mice disappeared from the coverslips. Macrophages from mice infected by both routes had lost their sensitivity to the parasite. Cells cultured from iv infected mice were more resistant to parasite multiplication than normal cells.

A similar demonstration of the sloughing of cultured macrophages occurred when cells from mice that were lightly infected by one ip injection were harvested 4 weeks later and infected, in vitro with parasites. Normal macrophages infected with LD bodies were destroyed by a 30X concentration of sensitized peritoneal lymphocytes obtained from the ip infected hosts. Spleen lymphocytes from the same mice also induced this cell destruction. Cell losses were heavy and in sharp contrast to that of controls. These few demonstrations of macrophage destruction were similar

in that the phenomenon was first demonstrable about one month after infection and could only be induced with cells from mice infected by the ip route. Macrophages harvested from iv infected mice demonstrated cellular immunity to L. donovani without a significant loss of macrophages. Lymphocytes from iv infected mice did not destroy normal macrophages infected with the parasite.

When high infecting doses of parasites are employed, the development of delayed hypersensitivity could be difficult to demonstrate due to the more rapid establishment of parasites in the host and the rapid onset of cellular immunity. This may be true in mice infected via the iv route since this route of infection results in much higher levels of intracellular parasites in the spleen and liver (Stauber, 1958). If a parasite inoculum is injected by the ip route, however, only a portion of these organisms become established in host tissues. This may prolong the transitional nature of the delayed hypersensitivity state and increase the chances for its detection.

It appeared from these studies that the mouse was a difficult host in which to pursue delayed hypersensitivity studies to L. donovani. Numerous failures of skin, capillary migration and target cell destruction tests suggests that the infecting dose, route of infection and time of testing are critical determinants of a successful demonstration of this transitional phenomenon in mice.

The three tests successfully employed in this investigation contained two identical components, sensitized lymphocytes and antigen in the form of viable LD bodies. In demonstrating the loss of macrophages from ip infected mice, the cells were cultured for 4 hours before culture medium was removed and replaced with LD bodies. Since some sensitized lymphocytes remained in the Leighton tubes despite one medium change, these cells were probably involved in this reaction. However, the few lymphocytes that did remain would have had to be extremely active in the cell destruction process.

It appeared from these studies that delayed hypersensitivity which was demonstrated, *in vitro*, 4 weeks after mice received an ip infection preceded the development of macrophage mediated cellular immunity which was first demonstrated 6 weeks after the initial infection. This would substantiate Mackaness's hypothesis that delayed hypersensitivity occurs before or concomitant with the development of cellular immunity.

Bray and Bryceson's failure to demonstrate resistance macrophages from guinea pigs infected with *L. enriettii* and their report that normal guinea pig macrophages infected in culture were destroyed by lymphocytes from infected guinea pigs suggests that lymphocytes may play a role in resistance. In our host-parasite system normal or immune mouse macrophages were not destroyed when infected in culture. The parasites failed to multiply or decline in

numbers within the immune macrophage and there was no cytopathic effect on the cells. This suggests that resistance in the guinea pig may be from the greater development of the lymphocyte response and a poor induction of resistance in the macrophage. In the mouse the lymphocyte and delayed hypersensitivity phenomenon may be just a transitional stage to the development of macrophage-mediated immunity.

SUMMARY

Mice do not appear to develop a pronounced or lasting delayed hypersensitivity to Leishmania donovani as demonstrated by the failure of skin tests and tests for the in vitro inhibition of macrophage migration from capillary tubes. Peritoneal exudate cells harvested from mice 4 weeks after intraperitoneal infection sloughed off of coverslips after infection, in vitro. Similar cells from intravenously infected mice and normal mice remained adhered to the coverslips. When spleen and peritoneal lymphocytes were harvested from the same mice 4 weeks after intraperitoneal injections and added to cultures of macrophages from normal mice infected with L. donovani, the latter host cells were also lost from the coverslip monolayer. Lymphocytes from mice infected by the intravenous route and from normal mice did not effect host cell survival. This resembled a type of target cell destruction which has been associated with a delayed hypersensitivity. Macrophages cultured from mice injected by the intravenous route showed a loss of intracellular parasites without a significant reduction in host cells 4 and 6 weeks following in vivo infection. Macrophages harvested from mice 6 weeks after intraperitoneal infection demonstrated a similar

parasite loss without a significant reduction in the number of macrophages. Peritoneal and spleen lymphocytes from these mice did not induce the destruction of macrophages from normal mice infected, in vitro.

In mice, the type of delayed hypersensitivity associated with macrophage destruction, in vitro, may be transitional to the development of macrophage immunity. The brief occurrence of this hypersensitive state may be directly related to the route and number of parasites injected since intraperitoneal infections are known to allow fewer parasites to become established in the hosts tissue. This route of infection may prolong the transitional nature of the delayed hypersensitive state over that found in intravenously infected animals and increase its chances for detection.

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