T CELL DEPENDENCE AND SYNERGY IN ACQUIRED IMMUNITY TO LEISHMANIA DONOVANI

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
CLARE B. SKOV
1974

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Ph. D. degree in Microbiology and
Public Health

Major professor

Date June 19, 1974

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ABSTRACT

T CELL DEPENDENCE AND SYNERGY
IN ACQUIRED IMMUNITY TO *LEISHMANIA DONOVANI*

By

Clare B. Skov

Several recent studies have suggested that thymus-dependent (T) immunity is primarily responsible for acquired resistance to *Leishmania*, but the mechanisms of this immunity have not been elucidated. In this investigation, a system was developed such that mice were immunosuppressed and reconstituted to determine if acquired immunity to *L. donovani* in mice was T cell dependent, and if so, whether the mechanism of acquired resistance involved interaction between different populations of T cells.

Inbred C57Bl/6J mice were given various combinations of immunosuppressive treatments and infected intravenously with *L. donovani* amastigotes. Liver-parasites were enumerated during the course of infection from parasite counts on liver impression slides.

In normal mice the parasite population reached its peak about 24 days post infection, after which parasite numbers gradually declined but were still detectable on day 60. The course of infection was not altered in mice which had been thymectomized, lethally irradiated, and reconstituted with syngeneic marrow cells. Moderate suppression of acquired resistance resulted when thymectomized-irradiated mice were reconstituted with marrow cells previously treated with anti-brain associated theta serum, and/or when recipient mice were treated with azathioprine on the same day they

were infected. Splenectomy, either alone or together with irradiation failed to suppress immunity. However, when mice were suppressed by combining splenectomy with all the above treatments, a long-lasting high level of immunosuppression resulted, and the mice so treated were designated as deprived mice.

To determine if T cells were required and/or if T cells interacted synergistically in acquired immunity, deprived mice were infected and reconstituted with thymocytes and/or lymph node cells (LNC). The capacity to resist infection was restored by LNC or thymocytes. LNC were 20-25 times more immunocompetent than thymocytes. Reconstitution by both cell types was inhibited by *in vivo* treatment of recipient mice with azathioprine or anti-brain associated theta serum. "Limiting dilution" experiments showed that constant minimal numbers of LNC combined with graded numbers of thymocytes, or vice versa, conferred greater protection than expected from the additive effect of both cell types acting independently. Cultured peritoneal macrophages from infected convalescing control and reconstituted mice suppressed parasite multiplication. Macrophages from infected deprived mice were unable to prevent parasite multiplication *in vitro*.

The results of this investigation provide convincing evidence that 1) acquired resistance of mice to *L. donovani* is predominantly dependent on thymus-derived lymphocytes, and that 2) the mechanism of cellular immunity to this parasite involves synergistic interaction of different T cell subpopulations and parasiticidal activity by macrophages.

T CELL DEPENDENCE AND SYNERGY IN ACQUIRED IMMUNITY TO *LEISHMANIA DONOVANI*

By

Clare B. Skov

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1974

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the members of my advisory committee, Dr. Virginia H. Mallmann, Dr. Harold C. Miller, Dr. Jeffery F. Williams, and especially my advisor, Dr. Donald W. Twohy, for their critical guidance and encouragement. In addition, I wish to thank Dr. D. E. Schoenhard as representative of the support received from state and federal sources in the form of assistantships and NDEA and NIH training grants.

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INTRODUCTION

Immunologic responses toward infectious or foreign agents and modified self-antigens are classically categorized into two systems; the humoral and the cellular immune systems. Natural activation of one system alone rarely occurs. Depending on the nature of antigen and other factors, either system may be relatively ineffective or predominantly responsible for defense against a particular antigenic agent. The immune response to many antigens may require cooperation between the two systems. Experimental evidence suggests that the interaction of lymphoid cells with antigen and with each other, and their consequent immune activation, usually depends on a variety of cell-derived chemical mediators or lymphokines (62).

Although specific antibodies may be induced, they may be ineffective against intracellular infectious agents including many viruses, bacteria, protozoa, and fungi. Host resistance against these organisms depends primarily on activation of thymus-derived (T) lymphocytes. T cells may recruit and activate other lymphocytes and/or macrophages. Both activated lymphocytes and macrophages may lyse target cells in the absence of antibody. The expression of cellular immunity to infection resides principally in activated macrophages while lymphocytes which lyse target cells directly have been demonstrated in transplantation immunity.

This study was designed to elucidate the cellular aspects of immunity to *Leishmania donovani*. In man, *L. donovani* causes visceral leishmaniasis (kala azar), a zoonotic tropical disease which is usually fatal if untreated. *Leishmania* exist in two morphological forms. The promastigote is a

flagellated form found both in culture and in the gut of its vector, the sandfly (Phlebotomines). This stage of the parasite transforms into an intracellular amastigote (also called the Leishman-Donovan or LD body) after ingestion by a macrophage, the host cell. Two major clinical forms of leishmaniasis exist, cutaneous and visceral leishmaniasis. Other forms of the disease, post-kala-azar dermal leishmaniasis, and metastasizing forms such as mucocutaneous and diffuse cutaneous leishmaniasis occur less frequently. A large range of host responses occur in man. Spontaneous recovery and solid immunity usually occur after a case of the chronic cutaneous disease. Delayed hypersensitivity usually develops but circulating antibodies are absent during the course of cutaneous leishmaniasis. In contrast, the visceral disease usually does not induce delayed hypersensitivity until after healing occurs, but may induce antibodies and cause hepatosplenomegaly, nonspecific hyperglobulinemia, and death without treatment. A small portion of human victims apparently recover spontaneously and successfully treated cases usually result in life-long immunity. However, the mechanisms of this resistance to visceral leishmaniasis are poorly understood.

The purpose of this investigation was to determine both the nature and the role of the lymphoid cells which confer protection against intracellular *L. donovani* in mice. A system was developed in which the capacity of the mice to immunologically eliminate the parasite was significantly suppressed. Immunosuppressed mice were used in reconstitution experiments to determine 1) if normal T cells from the thymus (T1) or peripheral sources (T2) could protect mice against *L. donovani*; 2) if T1 and T2 lymphocytes act synergistically in their protective roles; and 3) if T2 cells from immune convalescing donors are more immunocompetent than cells from non-immune donors.

LITERATURE REVIEW

Cell interactions in immunity. Recognition of the heterogeneous nature of the cellular components of the immune system has been the most significant advance in modern immunology. The mode of expression of the effector branch of the immune response has become the basis for the arbitrary division of immune phenomena into humoral and cellular categories. It is now realized that many immune responses depend on lymphocyte-lymphocyte and/or lymphocyte-macrophage interactions. Many such interactions involve the production of antigen induced lymphokines which regulate the expression of the immune response. Macrophages (93,53), bone marrow derived (B) lymphocytes (115,129), and thymus-derived (T) lymphocytes (6,37,40,49,65) have all been reported to produce soluble mediators. In addition, B-B cell (35), T-macrophage-B cell (23,43,44,87,105), and T-T cell (20,128) interactions have been reported. However, the major role in the immune system's cell interactions has been ascribed to T cells. Immune responses to most antigens are probably thymus dependent. The subject of T cell regulation of B cell responses was recently reviewed by Katz and Benacerraf (62).

Thymus-dependent immune responses. Thymic function and the mechanism of cell interactions are neither simple nor well understood. Thymus-dependency shows great variation in relation to the nature of antigenic stimulus and the organism in which the response takes place. The clearest division of immune function, related to derivation of lymphocytes from distinct primary lymphoid organs, has been observed in birds. Bursectomy of chickens

removes most of the antibody producing potential without altering the ability to reject grafts, which is lost following thymectomy (29,130). In contrast, *in utero* thymectomy of lambs at the end of the first trimester, has little effect on either antibody production (26) or rejection of grafts (27). In man, immunological deficiency diseases have been linked to separate B and T stem-cell defects. Patients with congenital agammaglobulinemia possess thymus-dependent cellular responses whereas children with congenital thymic aplasia produce antibodies to some antigens while having markedly impaired cellular responses (56).

The dichotomy of the immune system among mammals has been most thoroughly investigated in mice. The production of antibodies in mice was first shown to be dependent on T-B cooperation by Claman et al. (23) and Miller et al. (88). Humoral responses to most antigens tested have required T-B collaboration (71). T cells have been shown to be responsible for carrier-molecule recognition as "helper cells" (100,118) while differentiated B cells synthesize hapten specific antibody (34). Different immunoglobulin classes appear to vary in their relative dependency on T cells. Taylor and Wortis (117) and Torrigiani (124) found that IgM and IgG2 production were relatively less thymus-dependent than was IgG1. Although recent reports by Tingle and Shuster (122) and Kagnoff et al. (61) have presented evidence which questions the thymus-independence previously ascribed to brucellin and lipopolysaccharide, some antigens may be thymus-independent. Only the IgM response increased when high doses of a polyvalent hapten-carrier conjugate was injected into thymus-deprived mice (4). And, even in the presence of an intact thymus, polyvalent conjugates and lipopolysaccharides were shown to give rise to higher IgM/IgG ratios than do monovalent conjugates (33,78).

Macrophages may also play a vital role in T-B cooperation. Feldmann

and his collaborators (42,43,44) have proposed that specific and nonspecific factors secreted by T cells mediate antibody synthesis by B cells. In their model, the specific factor, an IgM-antigen polymer, is presented to the cells via macrophage intermediates while T cell derived nonspecific mediator(s) augment B cell proliferation. It has been postulated that two signals are required to trigger B cells. Dukor and Hartman (39) proposed that triggering of antibody-secreting precursor cells involves specific binding of an antigen to the immunoglobulin receptor and the interaction of activated C3 with the B cell's C3 receptor. Schrader (105) presented an *in vitro* model which indicated that the second signal required to trigger B cells is a macrophage product while the T cell product only has an amplifying effect. The "allogeneic effect" provides complementary evidence for the existence of a thymus-dependent amplifier factor (96). The concept of a three cell system is indirectly supported by two other lines of evidence. Although it is still a controversial issue, there is increasing evidence that T cells, like B cells, possess antigen-specific receptors and that these receptors on T cells are immunoglobulin-like or IgM (6,7,39,79,101). In addition, macrophage-derived lymphocyte-activating factors have been reported (53,93).

The regulatory role of T cells is complex and involves a wide range of immune phenomena. T cells have been shown to suppress and/or enhance the activity of both B and T cells (10,20,38,50,52,95,136). T and B cells may both be responsible for immunological memory (89) or rendered tolerant (22). Terman et al. (118) has introduced evidence which suggests that neonatal tolerance may be a result of active suppression. Perhaps a clearer definition of T-B interaction will arise from experiments using hapten-specific tolerant B cells (116) and carrier-specific tolerant T cells (51).

Identification of the specific roles of the thymus and T cells is

central to an understanding of the inductive influences which determine the differentiation and immunocompetence of T cells. In mice, precursors of T and B lymphoid cells apparently originate from marrow stem cells. T cells, after leaving the thymus, may migrate to secondary lymphoid organs or become recirculating peripheral T cells (121, 131). T cells have been identified by their capacity to restore antibody production to sheep erythrocytes and graft rejection in thymectomized mice; they are also identified by their sensitivity to anti-theta or anti-lymphocyte serum (ALS). However, it is not clear whether the T "helper" function in antibody production and T cell functions in cellular immunity are different functions of the same or distinct cells. In addition, the mechanism by which the thymus affects immunocompetence is not well defined. There is good evidence that thymosin, the putative thymic hormone, can replace some of the biological functions assigned to the thymus (54,123,135). Miller et al. (86), Goldstein (55), and Bach and Bach (8) have demonstrated induction of theta antigen and "helper" function in marrow cells via thymic factor(s) *in vitro*. There seems to be much promise in the experimental success in restoration of immunological function with thymic factor(s). Interpretation of these results is still guarded with respect to the mode of action since polyions and other agents also appear to be capable of restoring some responses in T cell deprived mice (36). Kook and Trainin (64) have recently found that thymic factor(s) may exert its effect via cyclic adenosine monophosphate which suggests that thymic factor(s) functions as a hormone (55).

Cellular immunity. T cells are primarily responsible for cell-mediated immune functions. The term cell-mediated immunity has evolved to encompass a broad spectrum of immune phenomena in which propitiation of effector mechanisms, following exposure of the immune system to antigen, usually involves interaction of two or more lymphoid cell types and may be

expressed by either of the two major effector systems. Mechanisms controlling production of antibody include mediation via collaborating lymphoid cells and a well characterized antibody-feedback inhibition system (125). Mechanisms governing cellular immunity have not been elucidated.

Cellular immune reactions include those responses in which the main expression of the effector branch is carried out by either specifically sensitized T cells and/or macrophages which have been armed by T cells. Such expressions of immunity may be made independent of humoral antibody and passively transferred only by sensitized lymphoid cells. The delayed skin response has long been used as an *in vivo* indicator of cellular immunity. Both events are putatively related to or dependent on common mechanisms. Both are apparently initiated by antigenic stimulation of T cell receptors, either directly or via presentation by macrophages, which results in transformation and clonal proliferation of specific antigen reactive cells. Consequent events, although less clear, include the production of soluble mediators, the suppression or recruitment and activation of other cells, and possibly the differentiation of T cells into memory and/or cytotoxic lymphocytes, depending on the microenvironment and the nature of the antigen. In contrast to the above concept, several *in vitro* models have indicated that nonsensitized B cells and macrophages may be cytotoxic to antibody coated erythrocytes (105,126) or tumor cells (63,134). Whether these phenomena occur *in vivo* or play a significant role in cellular immunity has not been established.

Immune rejection of grafts is commonly viewed to be dependent mainly on cellular immunity because adoptive transfer of immunosensitive lymphocytes results in accelerated rejection of allografts (12). The role of humoral antibody in graft rejection has been questioned on the basis of the failure of immune sera to passively accelerate graft rejection (16).

Silverstein and Kraner (108) have demonstrated that allograft rejection can occur in sheep in the absence of serum antibody. However, antibodies are frequently induced by grafts and may play a role in transplantation immunity depending on the circumstances, i.e., the nature of the antigen or presensitization (84). Lance and Batchelor (66) found that treatment of mice with ALS selectively depletes the T cell population and prolongs the survival of skin allografts. Nevertheless, these experiments failed to differentiate between lysis by antibody and T cells because both may be T cell dependent. Several reviews of transplantation immunology have been written (90,102,132).

The analogy between graft and tumor immunology was made evident by Mitchison (91) who showed that rejection of either kind of tissue graft depended on adoptive transfer of specifically sensitized lymphoid cells. It has become evident that similar mechanisms may be involved both in immunological rejection or enhancement of grafts, tumors, and normal tissues (12,24,25,58,97). Existing evidence suggests a role for both humoral and cellular responses in graft, tumor, and autoimmunity (57,58,92); which mechanism predominates may be decided by microenvironmental conditions. Particularly interesting is the fact that *in vivo*, lymphoid cells are apparently "tolerant" to distinct transplantation antigens possessed by both tumor and normal cells, but can express immunological sensitivity to the same cells *in vitro* (11,24,25,58). The existence of a serum "blocking" factor is apparently related to the inability to reject tumors. An analogous serum factor appears to operate in suppression of both *in vivo* and *in vitro* autosensitization (24,25,58,97). T cells may be responsible for homeostatic control as exemplified by the NZB mouse model in which autoimmune disease and associated lymphoid malignancies follow progressive loss of T cell function (47).

Cellular immunity also plays a major role in defending against intracellular parasites. In 1942, Lurie (72) first demonstrated that tubercle bacilli survived less well in macrophages from immunized donors as compared to macrophages from nonimmunized donors. Activated macrophages have been shown to possess enhanced microbicidal or growth inhibiting activity against a variety of intracellular parasites including *Brucella* (41), *Pasterella* (119), *Listeria* (73), *Salmonella* (104), *Toxoplasma* (127), and *Leishmania* (85). Delayed hypersensitivity to tuberculin was first passively transferred with cellular exudates by Chase (21). The first successful transfer of cellular immunity to an intracellular parasite (mycobacteria) was reported by Sever in 1960 (107). During the past decade, adoptive transfer of cellular immunity has been accomplished with cells from animals immunized to a wide variety of intracellular parasites (5,46,77,103,104). Passive transfer of immunity with lymphoid cells, but not with serum, has become the major criterion of cellular immunity.

Two major hypotheses have been proposed to define the nature of acquired cellular resistance to infectious intracellular parasites. From their studies of the cellular basis of immunity to mycobacteria and *Listeria*, Mackaness and his collaborators have advanced the concept of a two stage mechanism of defense (75,76). Mackaness (73,74) found that macrophages were primarily responsible for enhanced microbicidal activity in immune mice, both homologous and heterologous organisms being destroyed. But only homologous organisms were able to evoke recall of the immune mechanism for the enhanced microbicidal activity. The expression of nonspecific immunity, but not delayed hypersensitivity, by adoptively transferred immune cells was also found to require the presence of specific antigen (77). Thus, according to their model, activated macrophages have been shown to be nonspecifically armed and to exhibit increased physical and metabolic activity

(22,32). The first stage of cellular resistance requires the specific sensitization of lymphocytes which respond by transforming, proliferating, and producing nonspecific soluble mediators. Control over intracellular parasites follows consequent activation of macrophages in the effector stage. This interpretation of evidence is consistent with the placement of cellular immunity and delayed hypersensitivity as manifestations of a common first stage immune mechanism.

Alternatively, Youmans and Youmans (133) have fractionated mycobacterial preparations into cell-wall and ribosomal preparations for the purpose of comparing their potential for eliciting specific and nonspecific cellular responses. When coupled with tuberculoprotein, the stable cell-wall fractions were found to elicit transient nonspecific protection together with strong tuberculin hypersensitivity (31). The nonspecific protection to *Listeria* was similarly transient when heat-killed cells were used for vaccine. In contrast, immunization of mice with *Listeria* provided long-lasting protection. Similarly, Collins (28) reported that anamnestic immunity to *Salmonella* was also specifically recalled and that the expression of specific immunity was greater and longer lasting than the nonspecific expression of immunity. The labile ribsomal fraction did not produce tuberculin hypersensitivity but only evoked specific immunity against virulent mycobacteria (30). Circulating antibodies had no apparent role. Thus cellular resistance to tuberculosis was completely dissociated from delayed hypersensitivity. On the basis of their work with mycobacteria, the Youmans proposed the hypothesis that distinct nonspecific (granulomatous) and specific immune responses occur together following immunization with viable attenuated cells while only the nonspecific response is activated by killed cells. If this model is correct, then data involving microbicidal activity of cultured macrophages harvested by methods including

injections of homologous antigen shortly before their harvest should be questioned because under such conditions, one would not be able to distinguish between specific immunity and delayed hypersensitivity related nonspecific immunity.

Acquired immunity to *Listeria* and mycobacteria involves the interaction of lymphocytes and macrophages. The lymphocytes responsible for passive transfer of immunity to *Listeria* were shown by Lane and Unanue (69) to be T cells by their sensitivity to anti-theta serum. T cells were also shown to be responsible for resistance to mycobacteria by North (94) who demonstrated that T cell deficient mice were unable to prevent bacterial growth. However, apparent differences exist in the lymphocytes responsible for adoptive transfer of resistance to these organisms. Cells which convey protection against *Listeria* were found to be radiation-resistant residual T lymphocytes in thymectomized, irradiated, and bone marrow reconstituted (TxIrBM) rats (82) and mice (94). In response to *Listeria*, most of the proliferating cells in the liver of TxIrBM mice were macrophages and their proliferative response was not reconstituted by thymocytes (94). McGregor and Logie (83) found that adoptive transfer of resitance to *Listeria* was dependent upon rapidly proliferating, large lymphocytes, which had a short circulating lifespan, and were no longer detectable by passive transfer of thoracic duct cells eleven days post infection. In contrast, Lefford et al. (70) found that lymphocytes which confer adoptive immunity to tubercle bacilli in rats were initially a population of large cells similar to those described by McGregor and Logie (83). However, the large cells either matured or were replaced by small recirculating, long-lived cells which conferred long-lasting immunity as detected by passive transfer. The capacity of lymphocytes from TxIrBM rats to passively transfer immunity to tubercle bacilli was reconstituted by injection of normal thoracic duct

lymphocytes (82). Similarly, resistance to tuberculosis and the proliferative response of lymphocytes in the livers of TxIrBM mice were reconstituted by thymocytes (94).

Cantor, Asofsky, and their collaborators have investigated the nature and roles of lymphocytes involved in the graft-*vs*-host (GVH) response, a classical model for cellular immunity. They transplanted various populations of parental lymphocytes (i.e., thymocytes and peripheral blood lymphocytes) into neonatal F_1 hybrids, either together or separately, in limiting dilution experiments. Their results demonstrated that both donor cell types caused GVH reactions but that peripheral blood lymphocytes were approximately 20 times more reactive than thymocytes (20). When injected together, the two cell components acted synergistically only when both cell types were allogeneic to the host. The cells in excess in the thymus were apparently precursors of GVH effector cells (T1) while the predominant cell type among peripheral lymphocytes (T2) appeared to amplify the reactivity of the first cell. Tigelaar and Asofsky (120,121) also found T2 cells to be relatively more active in producing lethal runting than splenomegaly, the opposite of T1 activity in GVH reactions. Both cell types were shown to be T cells (19) and to possess different homing potentials (121). Raff and Cantor (99) proposed the hypothesis that distinct cell types may be responsible for initiating different expressions of cellular immunity.

Evidence supporting the functional heterogeneity of T cells is becoming increasingly abundant. Marked differences in the relative responses to phytohemagglutinin and concanavalin A, as well as differences in cell surface markers was demonstrated between central and peripheral T cells by Stobo et al. (113) and Stobo and Paul (112). Stobo et al. (114) also found differences in T cell radiation sensitivity in the spleen. Altman et al. (6) reported a dissociation between lymphocyte proliferation and

production of monocyte chemotactic factor. Similarly, Rocklin (100) dissociated between antigen induced lymphocyte proliferation and the production of MIF (macrophage inhibition factor). Tigelaar and Asofsky (120) described a population of cortisone-resistant thymocytes which had reduced GVH reactivity; these cells could not act synergistically with either normal thymocytes or peripheral lymphocytes. Segal et al. (106) also distinguished between two populations of T cells by differential cortisone sensitivity. Recently, Wagner (128) reported T-T synergy in an *in vitro* allograft system in which T2 cells provided the major source for precursor cells of cytotoxic lymphocytes, while T1 cells acted mainly as amplifier cells. Results obtained by Baker et al. (9,10) indicate that suppressor T cells, possibly T1, limit B cell proliferation while peripheral T cells, probably T2, amplify antibody production by B cells. Finally, the results contained in this dissertation are consistent with the model proposed by Raff and Cantor (99).

Immunity to *Leishmania*. Speciation of *Leishmania* which are infective to man is still based on clinical criteria and therefore is not well defined. This grouping system is inadequate because of the large range in parasite virulence and/or host responses which is reflected by a broad spectrum of clinical forms. Adler (2) has provided the clearest separation of *Leishmania* "species" on the basis of the growth characteristics of promastigotes cultured in immune sera. He was able to distinguish between *L. tropica major* and *L. tropica minor* which cause "wet" and "dry" cutaneous leishmaniasis, respectively, in the old world. He also differentiated between *L. donovani* and *L. tropica* and between agents causing cutaneous disease in America including *L. mexicana, L. braziliensis,* and *L. pifano.*

Although *L. tropica* and *L. braziliensis* may be used to induce antibodies in experimental animals, antibodies cannot be detected in most

cases of cutaneous leishamaniasis. Detection of species-specific antibodies in experimental animals is complicated by cross-reactivity caused by numerous group-specific antibodies. Most of the immunoglobulin in *L. donovani* induced hyperglobulinemia is not specific for the parasite and positive serological findings are not correlated with protection. As expected, promastigotes have additional antigen not shared by amastigotes.

Protective cross-reactivity does occur between certain groups within species. Generally, the more virulent strain confers complete cross-protection while the less virulent strain, even though it induces a positive delayed (montenegro) reaction, may only afford partial or homologous protection.

Examples of cross-immunity are the demonstrations by Lainson and Shaw (68) and Adler and Gunders (1) that *L. t. major* protected against *L. t. minor*. Similarly, rodent strains of *L. donovani* protected man against human strains in experiments done by Manson-Bahr (80,81) and Southgate and Manson-Bahr (109). Success at prophylaxis against visceralization of *L. donovani* may depend on the route of admistration and/or on the preparation of the "vaccine" parasites. Various manipulations of the parasites may decrease or increase virulence (3,110,111).

While protective immunity may be established either following spontaneous or chemotherapeutic cure, it is still not clear whether it is premunitive or sterile immunity. Heyneman (60) proposed an alternative hypothesis that immunity to visceral leishmaniasis resides largely in the skin where cell-bound antibodies, plasma cells, and sensitized lymphocytes are concentrated. As yet, there is no evidence that circulating or cytophilic antibodies provide functional protection. Injection of *L. donovani* into immune people may produce an Arthus reaction (79) but attempts to passively transfer dermal hypersensitivity to cutaneous forms in man have failed (3,

15). By emulsifying promastigotes in adjuvant, Boysia (14) induced delayed hypersensitivity in guinea pigs and passively transferred the delayed response only with lymphoid cells. Both immediate and delayed type reactions probably occur but neither has been shown to signify anything more than an indication of host contact with a wide range of group-specific antigens. Adoptive transfer of protection to *L. donovani* in mice has been reported by Miller and Twohy (85).

Bryceson, Preston, and their colleagues have recently developed two experimental models which suggest that development and maintenance of immunity to *Leishmania* are predominantly thymus-dependent immune responses (17, 18,98). Single cutaneous lesions in guinea pigs infected with *L. enriettii* ulcerate and heal in about three months. During infection, delayed hypersensitivity reactions become positive; they persist following recovery and are passively transferrable via lymphoid cells (17). Following recovery, macrophage migration inhibition, lymphocyte transformation, and lymphokine production by *L. enriettii* sensitive lymphocytes were demonstrated *in vitro*. Guinea pigs immunosuppressed with ALS demonstrated greater susceptibility to infection (18). Unlike the relatively quick healing lesions of controls, the metastasizing lesions in ALS treated animals contained few lymphocytes around heavily infected macrophages and numerous plasma cells.

In the second experimental model, "thymus-deprived" (TxIrBM) mice were infected with *L. t. major* (98). Slow healing occurred in some deprived mice. Furthermore, lesions developed relatively slowly but endured much longer than in controls. Delayed hypersensitivity developed only in those mice with healing lesions. Agglutinating antibody titers were pronounced in both deprived and control mice while comparatively low fluorescent antibody titers existed in deprived mice. The most striking observation from histological sections of lymph nodes from deprived mice was that they were

nearly completely filled with macrophages.

In addition to suggesting that "cell-mediated immunity" plays a major role in immunity to *Leishmania* Garnham and Humphrey (48) also suggest that antibodies may suppress an effective immune response, perhaps much like "blocking factors" found in tumor and autoimmune studies (25,59). Evidence that activated macrophages kill *L. donovani* while normal macrophages do not (85), further supports the concept that cellular immunity plays the major role in protective immunity to *Leishmania*.

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ARTICLE 1

Cellular Immunity to *Leishmania donovani*.

I. The effect of T cell depletion on resistance to *L. donovani* in mice.

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This is Journal Article No. 6790 from the Michigan Agricultural Experiment Station.

[1]This work was supported by Grant No. DADA 17-69C-9135 from the U.S. Army Medical Research and Development Command under sponsorship of the Commission on Parasitic Diseases of the Armed Forces Epidemiology Board, and with support from the Michigan Agricultural Experiment Station. Journal Article No. 6790 from the Michigan Agricultural Experiment Station.

[2]Abbreviations used in this paper: MEM, Minimal Essential Medium; Az, azathioprine; aBAΘ, anti-brain associated theta serum; BM, bone marrow cells; $^{\Theta-}$BM, aBAΘ serum treated bone marrow cells; Tx, thymectomized; Sx, splenectomized; Ir, irradiated; TxIrBM, thymectomized, irradiated, and bone marrow reconstituted mice; TxIr$^{\Theta-}$BM, thymectomized, irradiated, and reconstituted with aBAΘ serum treated marrow cells; TxIr$^{\Theta-}$BMAz, azathioprine treatment of mice previously thymectomized, irradiated, and reconstituted with aBAΘ serum treated marrow cells; TxSxIr$^{\Theta-}$BMAz, mice treated with azathioprine after being thymectomized, splenectomized, irradiated, and reconstituted with aBAΘ serum treated marrow cells; T, thymus-dependent; B, bone marrow derived; NMS, normal mouse serum.

ABSTRACT

C57Bl/6J mice were treated with varied combinations of immunosuppressive treatments and infected intravenously with *Leishmania donovani* amastigotes. In normal mice the parasite population reached its peak about 24 days post infection, after which parasite numbers gradually declined but were still detectable on day 60. The course of infection was not altered in mice which had been thymectomized, lethally irradiated, and reconstituted with syngeneic marrow cells. Moderate suppression of acquired resistance resulted when thymectomized irradiated mice were reconstituted with marrow cells which were previously treated with anti-brain associated theta serum and/or when recipient mice were treated with azathioprine on the same day they were infected. Splenectomy alone or together with irradiation failed to suppress immunity, but a long-lasting high level of immunosuppression resulted when splenectomy was combined with all the above treatments. These results suggest that 1) small numbers of thymus-dependent cells which survive irradiation and thymectomy or which may be present in the bone marrow, may be sufficient to mount an immune response to *L. donovani* in mice, and 2) that T cells play an important role in acquired resistance to *L. donovani* in mice.

INTRODUCTION

In man, visceral leishmaniasis is characterized by nonspecific hyperglobulinemeia, hepatosplenomegaly, and an absence of delayed hypersensitivity (30). Although specific antibodies are produced, evidence for immune protection mediated by humoral mechanisms is lacking. Without treatment the disease is usually fatal, but recovery is generally followed by development of delayed hypersensitivity and long-lasting immunity (35).

The nature of the immunity has not been well defined.

Literature dealing with the cellular nature of immunity to *Leishmania* is scarce. Cutaneous leishmaniasis is characterized by spontaneous recovery accompanied with delayed hypersensitivity but an absence of a humoral response (9,10,11). Boysia (8) successfully transferred delayed hypersensitivity to *L. tropica* using lymph node cells from guinea pigs immunized with promastigotes in adjuvant. Miller and Twohy (34) reported that the efferent branch of immunity to *L. donovani*, like cellular immunity to mycobacteria (42), *Brucella* (18), *Listeria* (27,28), and *Toxoplasma* (4,5) is expressed by macrophages. Two experimental models have recently demonstrated that slow-healing forms of cutaneous leishmaniasis in guinea pigs (12) and mice (37) can be induced by selective immunosuppression of thymus-dependent (T) immune responses. Twohy (unpublished) found C57Bl/6J mice to be a suitable host for investigating the mechanisms of immunity to *L. donovani*.

This investigation was performed to determine 1) whether experimental depletion of the thymus-dependent lymphoid system would inhibit the development of immunity to *L. donovani* in C57Bl/6J mice and 2) what treatments could be routinely employed to eliminate the development of this immunity. It was found that extensive and multiple treatments were required to significantly suppress the expression of immunity to *L. donovani*.

MATERIALS AND METHODS

Experimental design. Inbred female C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor) at approximately 6 to 8 wk of age and maintained on a standard pellet diet and acidified-chlorinated water (22). The mice were used to assess the effects of separate and combined immunosuppressive treatments on the course of *L. donovani* infection.

Control and immunosuppressed mice were infected intravenously (i.v.) through the tail vein with 10 or 20 x 10^6 amastigotes (LD bodies), usually within 24 hr but never later than 48 hr after the harvest. The level of infection was calculated from the number of parasites in liver impression smears according to the method of Stauber (40). Prior to the harvest of livers for each assay, the blood from each group was collected after decapitation. Sera was decanted from the pooled blood, sterilized by filtration, and stored at -20 C.

Parasites. *L. donovani* (3S) amastigotes were maintained by serial intracardial passage in young male hamsters. Amastigotes were asceptically harvested by homogenizing the spleens of infected hamsters in cold NCTC 135 medium, centrifuging the homogenate at 63g for 5 min, and collecting the LD bodies from the supernatant fluid by centrifugation at 360g for 10 min. The pellet containing parasites was resuspended in cold NCTC 135 medium and counted in a Petroff-Hausser counting chamber by phase microscopy. The parasites remained viable in suspension for 3 days at 5 C.

Immunosuppressive procedures. Surgical procedures were performed on mice anesthetized with sodium pentobarbital. Seven to eleven week old mice were thymectomized (Tx) by the method described by Miller (33). Splenectomy (Sx) was performed one to two weeks after thymectomy. Both surgical wounds were closed with sterile autoclips (Clay Adams). About 35 days after thymectomy, the mice were placed into individual restraining chambers and irradiated (Ir) with 1000 R from a ^{60}Co gamma emitter. One day after irradiation, bone marrow (BM) cells were flushed from the femurs and tibias of normal or thymectomized syngeneic donors of the same age. Suspensions of single cells were obtained by progressive gentle passage of the cells through 20 to 27 gauge needles. Marrow cells were suspended in

cold MEM (Minimal Essential Medium) and held on ice until used. In later experiments, marrow cells were pretreated with anti-brain associated theta (aBAΘ) serum plus guinea pig complement (designated theta-negative bone marrow, $^{\Theta-}$BM). Irradiated mice were reconstituted i.v. with 5 x 10^6 viable untreated or aBAΘ serum treated marrow cells; marrow cells and LD bodies were usually injected together. Within 24 hr of the injection of LD bodies, designated groups also received azathioprine (Az).

Azathioprine. Az (Burroughs Wellcome) was dissolved in saline with 1 N NaOH immediately prior to use and 150 to 200 mg/kg of body weight was injected by the intraperitoneal (i.p.) route 18 to 20 hr after infection.

Complement. Immediately before use, lyophilized guinea pig complement (GIBCO) was reconstituted and absorbed with agarose as described by Cohen and Schlesinger (15).

Anti-brain associated theta serum. The protocol used by Golub (21) was used for the production and absorption of aBAΘ serum. Briefly, homogenized mouse or rat brain was emulsified in Freunds' complete adjuvant (BBL) and 0.3 ml was injected subcutaneously at 10 sites in the legs, back, and neck of adult New Zealand White Rabbits. Booster injections were given by the same route two weeks later. The rabbits were bled by cardiac puncture 10 days later and the sera were collected from the blood by standard procedures. The sera were pooled, heat inactivated, and absorbed four times with a mixture of erythrocytes and homogenized livers and kidneys, such that 5 ml of aBAΘ serum was absorbed with tissues from four C57Bl mice. Aliquots of absorbed sera were stored at -20 C.

Cytotoxicity and *in vitro* treatment of bone marrow. The cytotoxicity of aBAΘ serum was tested against thymus and marrow cell suspensions

according to the method described by Golub (21). A 1/16 dilution of absorbed aBAΘ serum completely killed thymocytes (3 x 10^7 cells/ml) and about 40% of the bone marrow cells *in vitro*. The procedure for testing cytotoxicity was modified for the purpose of eliminating T cells from marrow prior to injection into irradiated recipients. In brief, a 1 ml suspension of marrow cells, containing 1 to 3 x 10^7 cells in MEM, was incubated on ice with 0.4 ml of a 1/4 dilution of absorbed aBAΘ serum for 30 min. Two-tenths ml of absorbed complement, diluted 1/5, was added and the mixture was incubated for 60 min at 35 C. Treated marrow cells were pooled, centrifuged for 6 min at 250g, resuspended in cold MEM, and the viability determined by trypan blue exclusion.

Radial immunodiffusion assay. The relative IgG and IgM content of sera from control, suppressed, and normal (uninfected) mice were quantitated by radial immunodiffusion as described by Mancini et al. (29) and Fahey and McKelvey (19). Goat anti-mouse IgG and anti-mouse IgM (heavy chain) were purchased from Miles Laboratory and Cappel Laboratory, respectively.

Histology. Histological sections were fixed in Bouin's Fluid and stained with hematoxylin and eosin.

RESULTS

Effect of thymectomy and irradiation. The course of infection in normal mice was compared to irradiation and thymectomized-irradiated-bone marrow reconstituted (TxIrBM) mice (Fig. 1). The peak of infection in controls was reached between day 21 and 28 post infection. The number of parasites in the spleen and liver had declined by day 60, to levels which were as low or lower than parasite levels on day 3. In some experiments,

peak parasite levels persisted longer in spleens, but generally the course of infection followed a parallel course in both organs. In nonreconstituted mice, irradiation did not affect parasite proliferation until 9 days after irradiation when these mice began dying from irradiation; all mice in this group died by day 14 post irradiation. The course and level of infection in TxIrBM mice was not different from that in non-treated controls.

Attempts to eliminate residual T cells. The above results suggested that if T cells were necessary for the expression of resistance to *L. donovani*, successful elimination of parasites by TxIrBM mice depended on the presence of T cells in the marrow cells used to reconstitute TxIr recipients and/or of residual T cells in the recipients themselves. Attempts were made to eliminate T cells in marrow first by thymectomizing donors, and secondly by treating marrow cells from thymectomized donors with aBAΘ serum ($^{\Theta-}$BM). In some experiments Az was used to eliminate residual T cells in thymectomized and TxIr$^{\Theta-}$BM mice. Reconstitution of TxIr recipients with marrow cells from thymectomized donors failed to reduce resistance. However, moderate suppression of resistance resulted when TxIr mice were reconstituted with $^{\Theta-}$BM, and when Az was used to eliminate residual T cells in thymectomized or TxIr$^{\Theta-}$BM mice (Fig. 2). Az treatment of TxIr$^{\Theta-}$BM mice afforded an apparent advantage over other regimens at 40 days post infection. Regardless of the combination of immunosuppressive measures used, the peak level of infection (day 24) in controls was not significantly different from the level of infection in suppressed groups on the same day. Although not significantly different from each other on day 60, all three immunosuppressed groups were significantly different from the control group ($P<0.05$).

Because the spleen represented a major potential source of residual

T cells in the host, several experiments were performed to assess the effect of splenectomy on the course of infection. Splenectomy, with or without IrBM treatment, did not appreciably suppress the immune elimination of parasites (Fig. 3). However, when combined with $TxIr^{\Theta}$-BMAz treatments (Fig. 4), significant immunosuppression resulted ($P<0.001$). The combined treatment, $TxSxIr^{\Theta}$-BMAz (hereafter designated deprived mice), permitted parasite multiplication up to day 60. Without splenectomy, the other combined treatments delayed the peak of infection from day 24 to about day 40, and the level of infection was significantly higher than in controls ($P<0.01$). Comparison of the slopes of parasite multiplication prior to day 24 (Fig. 1) and in deprived mice from day 40 to day 60, indicates that the rate of proliferation was reduced in deprived mice after day 24.

Effect of suppression on immunoglobulin levels. Results of radial immunodiffusion test used to determine the effects of *Leishmania* infection on the relative amounts of IgG and IgM in control and deprived mice are given in Table 1. Compared to the amounts of IgG and IgM in pooled sera from uninfected normal mice (NMS), infection of control mice resulted in a progressive increase in both IgM and IgG up to day 50 when parasite levels had been greatly reduced. Initially, the amount of IgM in deprived mice was not different form IgM levels in NMS while the amount of IgG was from 71 to 83 percent less than the amount of IgG in NMS.

Two major precipitin bands were observed when NMS and control sera were reacted with goat anti-mouse IgG serum. These bands possibly represented IgG1 and IgG2. The radii of both bands from control sera increased from day 24 to day 50. Sera from deprived mice formed a single band.

Histological observations. In histological sections from the liver, lung, and intestines of a deprived mouse there was a general depletion of lymphoid cells. Histological sections of liver (Fig. 5) had many macrophages containing parasites in the absence of pathological manifestations. LD bodies normally associated with infection were not seen in alveolar macrophages in lung sections.

DISCUSSION

This experiment provides evidence that T cells play an important role in the development of immunity to the intracellular parasite, *L. donovani*, in mice. The results are consistent with those of Bryceson et al. (12) and Preston et al. (37) who found that anti-lymphocyte serum and TxIrBM treatments increased susceptibility to cutaneous leishmaniasis in guinea pigs and mice, respectively. However, in this system, more extensive treatments were required to effectively deplete the T cell system.

The requirement for T cells was not apparent at first because TxIrBM treatment was ineffective in altering the course of *L. donovani* infection in C57Bl mice. Similarly, North (36) observed that resistance to *Listeria* was not altered in TxIrBM mice even though resistance to *Listeria* in mice had previously been shown to be T cell dependent (25). The same treatment was effective in suppressing immune resistance to tuberculosis. However, the mycobacterial growth rate prior to the onset of immunity was greater than the rate of growth in TxIrBM host after the onset of immunity in simultaneously infected controls.

In this study, the pattern of *L. donovani* growth in deprived mice mirrored this trend suggesting that the immune response was incompletely suppressed. Preston et al. (37) also observed that the course of *L. tropica* infection in some TxIrBM mice was prolonged and less acute. There is

no evidence to implicate that humoral mechanisms could account for the apparent residual resistance, however, several lines of evidence have shown that T cells exist both in bone marrow of normal mice and in the spleens of irradiated mice (17,23,41). It is possible that resistance to different organisms may require different types or different quantities of T cells for the induction of immunity.

It was concluded that the incomplete removal of T cells was the major factor contributing to the failure of the TxIrBM treatment to suppress the immune response to *L. donovani*. Additional treatments were therefore implemented to eliminate the potential sources of T cells. Bone marrow donors were thymectomized and marrow cells were treated *in vitro* with aBAθ serum prior to injection into TxIr recipients. In addition, recipient mice were treated with Az on the day of infection. Az has been shown to have a relatively selective affect in inhibiting the immune response to *Listeria* (44), thymidine incorporation in the mixed lymphocyte reaction (1), and T-rosette forming cells (3,4).

Splenectomy was employed because the spleen was presumed to be the most likely source of residual T cells as well as the possible source of a putative primary-lymphoid-organ function (6,13,41). Splenectomy apparently removed residual T cells, the putative primary-lymphoid-organ function, or the procedure produced an environment in which Az was more effective.

An immunosuppressive protocol which extensively removed T cells from both, donor and recipient mice, was required to eliminate the immune response of C57Bl mice to *L. donovani*. The effects of TxIrBM, aBAθ serum, and Az treatments are well established, but the effect of splenectomy on cellular immunity has not been elucidated. Splenectomy is known to increase susceptibility of some animals to malaria, but only delays the early

antibody response to soluble antigens given i.v. (38).

Several lines of evidence suggest that the spleen may play several important roles in cell-mediated immunity. Battisto et al. (6) and Bucsi et al. (13) have observed a deficiency in the T-B cooperative capacity in neonatally splenectomized and congenitally spleenless mice. Ito et al. (24) have suggested that B cells require both a thymic influence and the splenic environment for differentiation into antibody producing cells. It has been postulated that the spleen in rabbits plays an important role in the genesis of effector cells or secretes a hormone which allows progenitor cells to develop (7). Bach et al. (2) found that splenectomy did not modify serum thymic activity in normal mice, but when followed by thymectomy, the effects of thymectomy became apparent much sooner. Relative to cellular immunity, splenectomized animals have been observed to have a more effective response to tumor grafts. Gershon et al. (20) have advanced the premise that splenectomy soon after the transplantation of thymocytes increases graft-*vs*-host reactivity in the splenectomized mouse by removal of spleen seeking suppressor cells. In the second article of this series (39), we reported that immunity to *L. donovani* was reconstituted by thymocytes and/or lymph node cells; thus we concluded that in this study, the major effect of splenectomy was the removal of residual T cells in TxIrBM mice.

The greater suppression of IgG than IgM in deprived mice parallels the findings by Preston et al. (37) that in *L. tropica* infected TxIrBM mice, immunofluorescent antibody titers were reduced while agglutination titers were equal in control and TxIrBM mice. The superimposition or absence of one of the IgG bands in the radial immunodiffusion of sera from deprived mice suggests that one subclass of IgG was reduced more than the other. If so, these observations agree with evidence reported by Torrigiani (43) that mouse IgG1 is relatively more T cell dependent than IgG2 and IgM.

Studies of the adoptive transfer of protection against infection with *Listeria* (31) and mycobacteria (26) indicate that the protective cell populations vary depending on the nature of the infective agent and the time cells are taken during the course of infection. During an acute infection or early phase of a chronic infection, large short-lived noncirculating lymphoid cells passively transfer resistance. In contrast, small long-lived circulating lymphocytes were responsible for protection after three weeks in chronic infections. These observations raise the question as to whether these cells represent distinct T cell populations. Many lines of evidence have recently supported the concept of T cell heterogeneity and T-T synergy (4,5,14,16,20,34,41,46). The second study in this series investigated the possibility that two T cell populations may be involved in the cellular response to *L. donovani*.

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TABLE I

Relative amounts of IgG and IgM in pooled sera from deprived and control mice during the convalescent stage of infection.

	Percentage of IgG and IgM in normal mouse serum[a]					
	Days post infection					
	24		40		50	
	IgG	IgM	IgG	IgM	IgG	IgM
	%	%	%	%	%	%
Deprived	78	96	71	95	83	88
Control	106	107	116	105	127	116

[a]Amounts of immunoglobulin were quantitated by the radial immunodiffusion method and expressed as the percentage of IgG or IgM found in pooled sera from uninfected normal mice. Serum from 5 to 10 mice was pooled for each group; mice with incomplete thymectomy were not excluded from the deprived group. Each serum sample was tested in duplicate.

Figure 1. Growth curve of *L. donovani* in the livers and spleens of control, irradiated (Ir), and thymectomized-irradiated-bone marrow reconstituted (TxIrBM) mice infected i.v. with 20 x 10^6 LD bodies. Each point represents the mean parasite count from 5 mice.

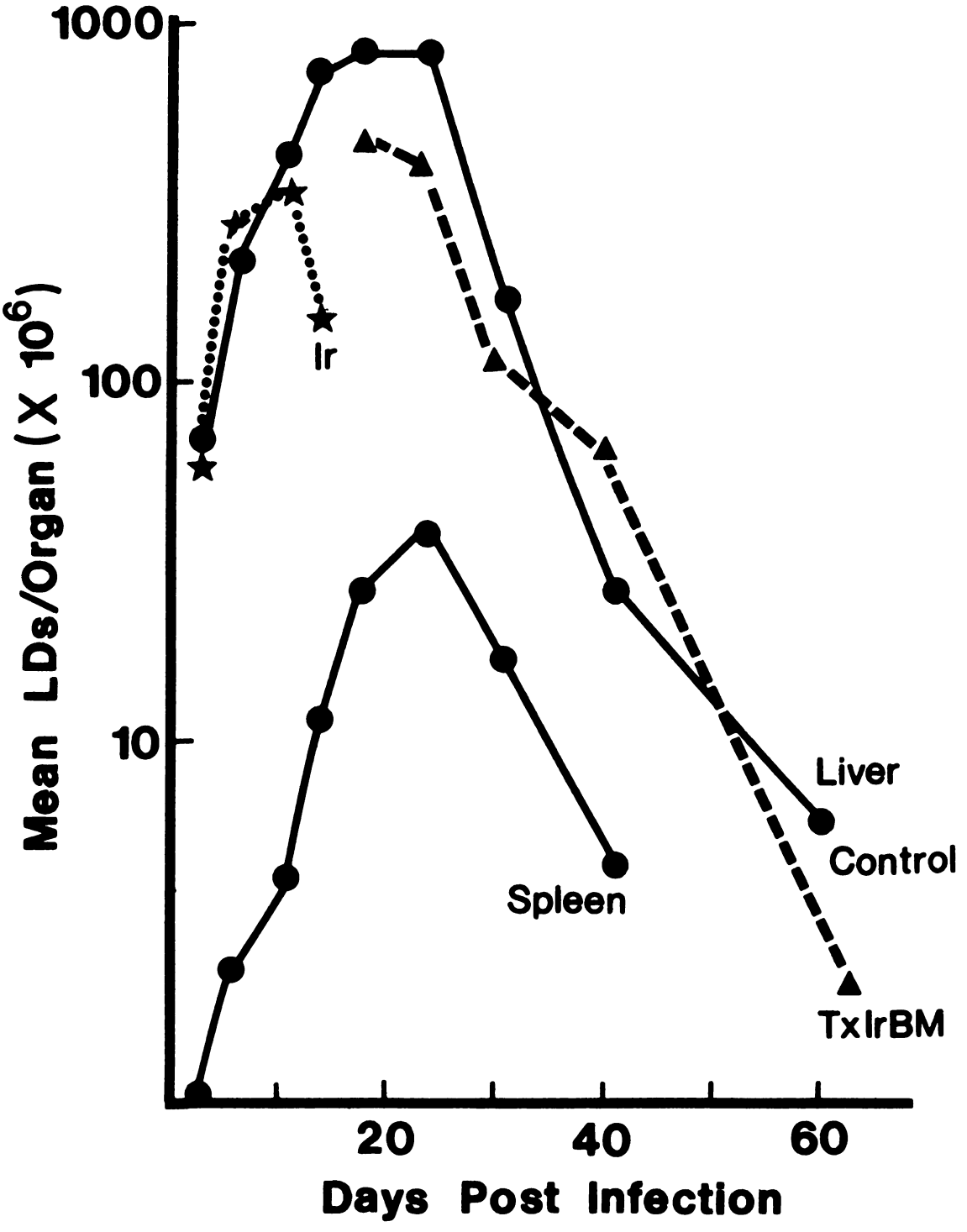
1000
100
10
Mean LDs/Organ (X 10^6)
Ir
Liver
Control
Spleen
TxIrBM
20
40
60
Days Post Infection

Figure 1

Figure 2. Growth curve of *L. donovani* from 24 to 60 days post infection in livers of untreated controls compared to mice receiving various treatments including thymectomy (Tx), irradiation (Ir), reconstitution with theta-negative bone marrow cells ($^{\theta-}$BM), and azathioprine (Az). The mice were infected i.v. with 20 x 10^6 LD bodies. Each point represents the mean number of parasites in livers from 5-8 mice except for group-A on day 40 which represent 3 mice.

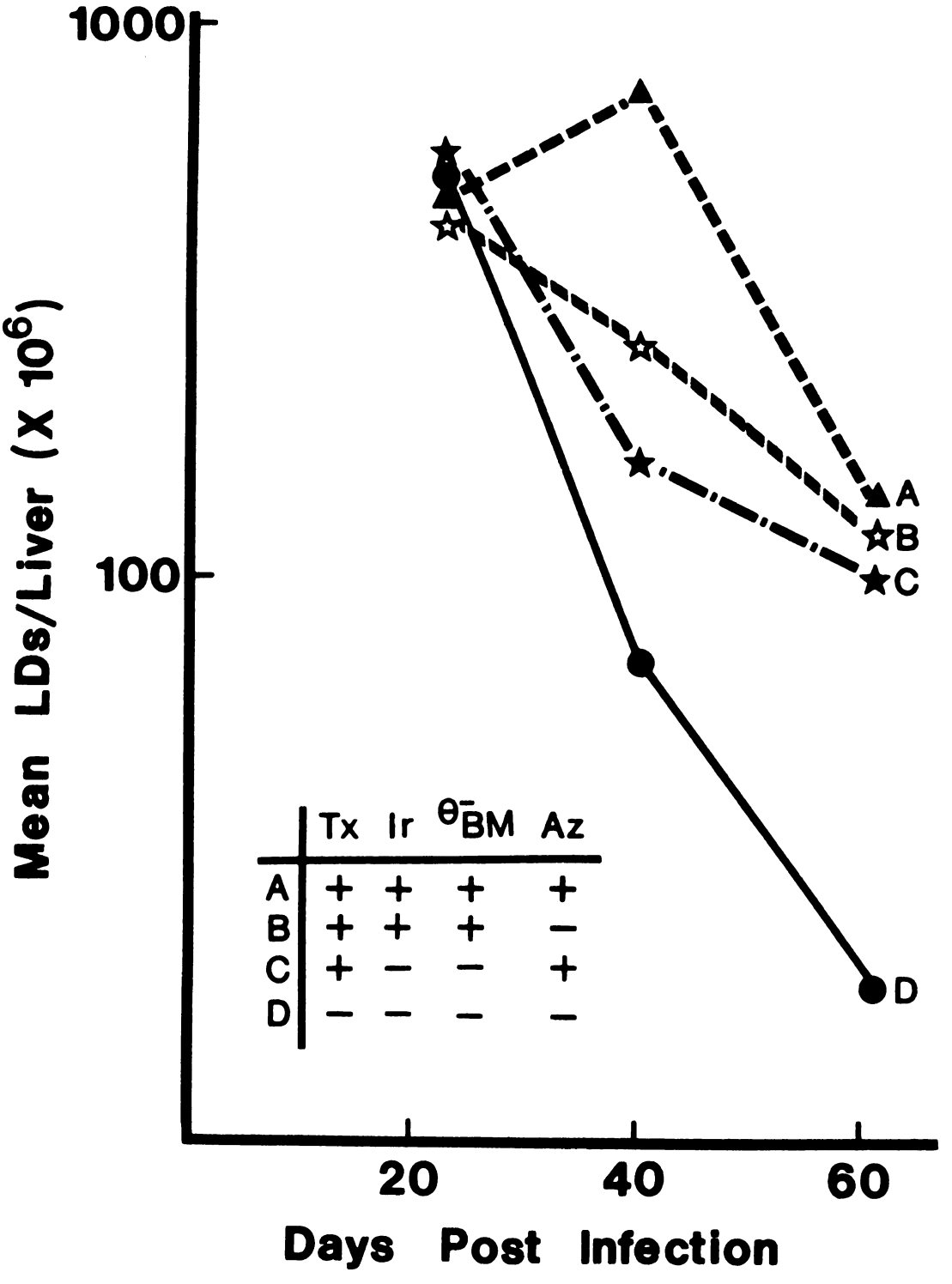
1000
100
Mean LDs/Liver (X 10^6)
A
B
C
D
Tx Ir θ^-_{BM} Az
A + + + +
B + + + −
C + − − +
D − − − −
20
40
60
Days Post Infection

Figure 2

Figure 3. Growth curve of *L. donovani* from day 24 to 40 in control, splenectomized (Sx), and splenectomized-irradiated-bone marrow reconstituted (SxIrBM) mice injected i.v. with 20 x 10^6 LD bodies. Each point represents the mean number of liver-parasite from 5 mice.

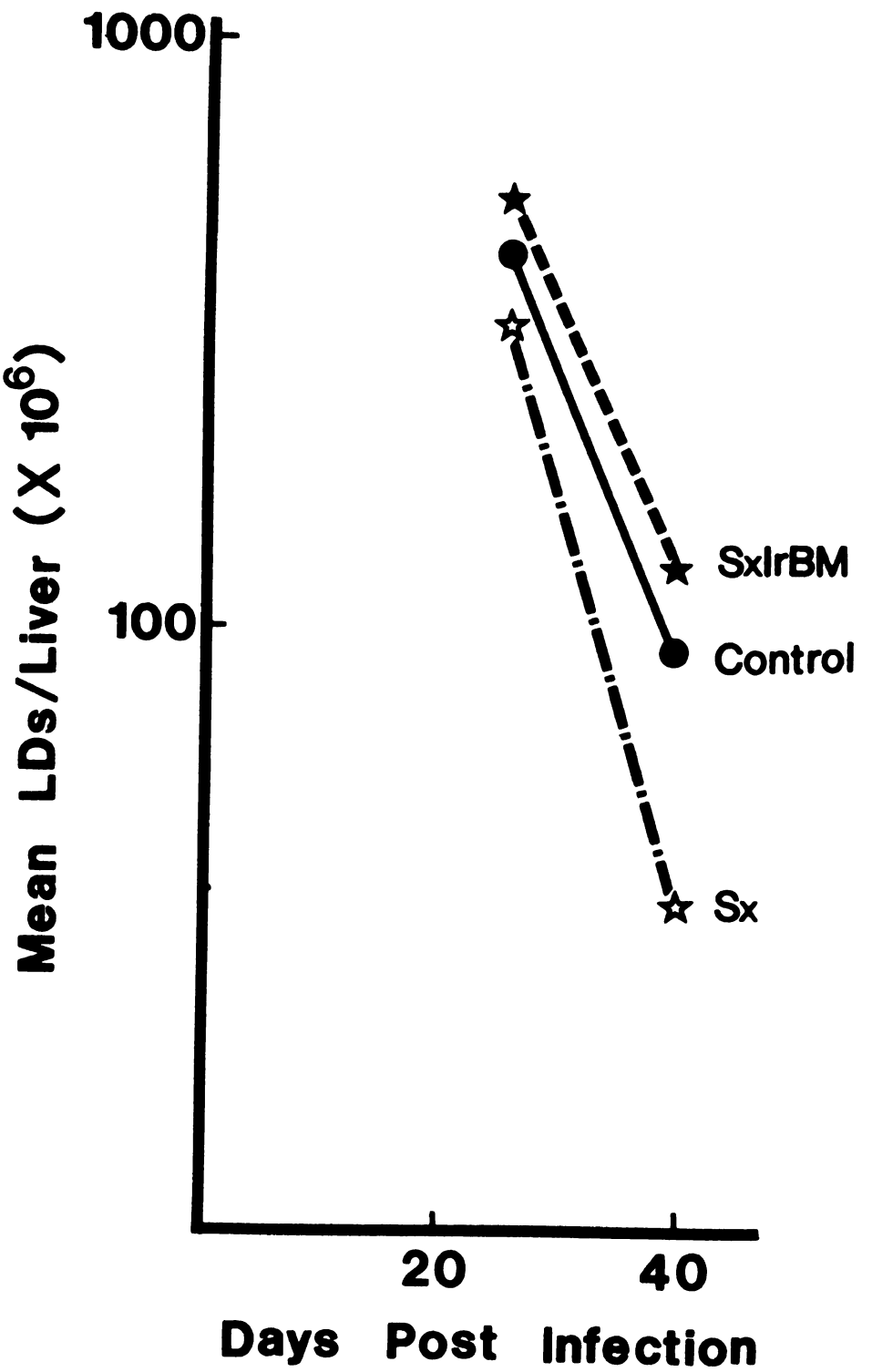
1000
100
Mean LDs/Liver (X 10^6)
SxIrBM
Control
Sx
20
40
Days Post Infection

Figure 3

Figure 4. Growth curve of *L. donovani* from day 24 to 60 in control and deprived mice infected i.v. with 20 x 10^6 LD bodies. Mice were deprived by thymectomy-splenectomy-irradiation-reconstitution with theta-negative bone marrow, and azathioprine ($TxSxIr^{\Theta}$-BMAz). A second group received the same treatment except that splenectomy was omitted ($TxIr^{\Theta}$-BMAz). Each point represents the mean number of liver-parasites from 5-8 mice.

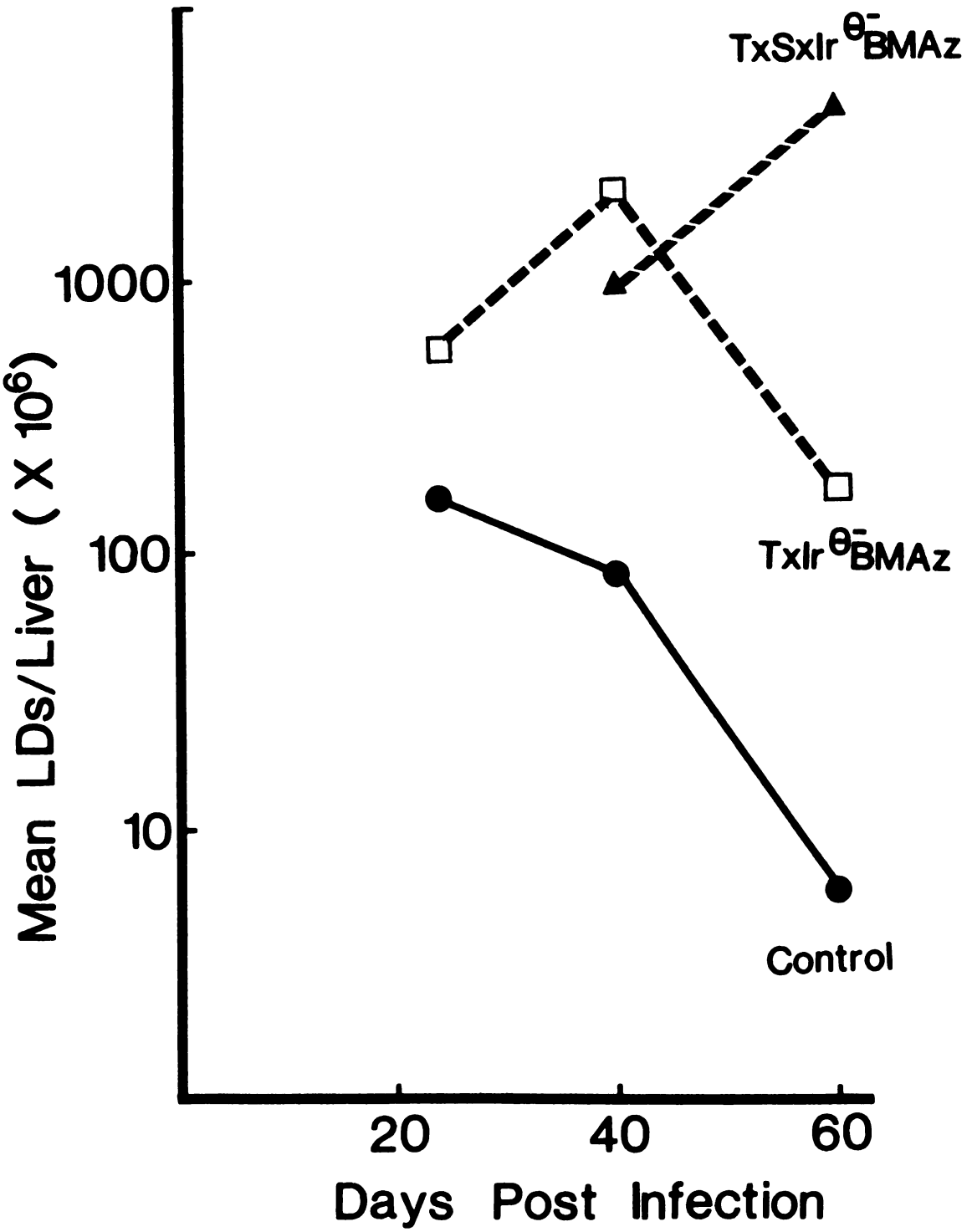
TxSxIr θ⁻BMAz
TxIr θ⁻BMAz
Control
1000
100
10
Mean LDs/Liver (X 10⁶)
20
40
60
Days Post Infection

Figure 4

Figure 5. Liver section from a deprived mouse about 4 wk after infection i.v. with 10 x 10^6 LD bodies. Note the absence of pathological manifestations around macrophages (M) which are filled with amastigotes (LD). H and E. (X 1000 and 2400).

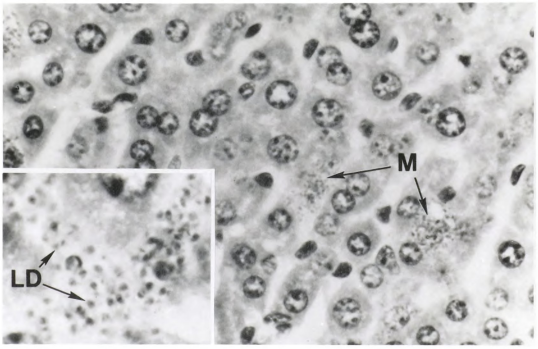

Figure 5

ARTICLE 2

Cellular Immunity to *Leishmania donovani*. II. Evidence for synergy between thymocytes and lymph node cells (T-T) in reconstitution of acquired resistance to *L. donovani* in mice.

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This is Journal Article No. 6791 from the Michigan Agricultural Experiment Station.

[1]This work was supported by Grant No. DADA 17-69C-9135 from the U.S. Army Medical Research and Development Command under sponsorship of the Commission on Parasitic Diseases of the Armed Forces Epidemiology Board, and with support from the Michigan Agricultural Experiment Station. Journal Article No. 6791 from the Michigan Agricultural Experiment Station.

[2]Abbreviations used in this paper: T, thymus-dependent; LNC, lymph node cell; Thym, thymocyte; GVH, graft-*vs*-host; Az, azathioprine; aBAΘ, anti-brain associated theta serum; PEC, peritoneal exudate cells; EDTA, sodium ethylene diaminetetraacetate; i.p., intraperitoneally; i.v., intravenously.

ABSTRACT

C57B1/6J mice were infected with *Leishmania donovani* after a series of treatments that were designed to deplete thymus-dependent (T) cell populations. Deprived mice did not acquire immune resistance to the parasite. The capacity to resist infection was restored by either lymph node cells (LNC) or thymocytes, but 20-25 times more thymocytes than LNC were required. Reconstitution of immunocompetence by both cell types was inhibited by *in vivo* treatment with azathioprine or anti-brain associated theta serum. Limiting dilution experiments showed that constant minimal numbers of LNC combined with graded numbers of thymocytes, or vice versa, conferred greater protection than expected from the additive effect of both cell types acting independently. Cultured peritoneal macrophages, harvested from normal and reconstituted mice 44 days after infection, suppressed parasite multiplication. Macrophages from infected deprived mice were unable to prevent parasite multiplication *in vitro*. These results provide strong evidence that acquired resistance of mice to *L. donovani* involves T-T cell synergy and relies principally on cellular immunity.

INTRODUCTION

Cell-mediated immunity represents a wide range of immune phenomena including induction mechanisms of humoral responses to many antigens as well as the more classically defined cellular immunity. Thymus-dependent (T) cell interactions with other lymphoid cells may be a basic hallmark of the development of the immune response since induction and adoptive transfer of cell-mediated immunity can be reduced or abrogated by T cell depletion.

Cantor and Asofsky (6) recently presented evidence for T1-T2 cell synergy in the graft-*versus*-host (GVH) response, a classical model of cellular immunity. Several lines of evidence support the concept of T cell heterogeneity and T-T cell interaction. Subpopulations of T cells reportedly differ with regard to surface antigens (11), sensitivity to corticosteroids (8) and azathioprine (2), "homing" patterns (24), responsiveness to mitogens (17,22) and their immune function (6,7,22,24,27).

A substantial body of evidence has shown that acquired immunity to many intracellular parasites is initiated and maintained by T cells and activated macrophages. The nature or stage of differentiation of cells capable of passively transferring protection to *Listeria* (12) and mycobacteria (14) varies depending on the infective agent employed and the time during the course of the immune response when donor cells are collected. Corticosteroid treatment reportedly differentiates between cells which confer immunity in *Listeria* infections (18) and graft rejection (8). These differences in cell properties may also be a result of T cell heterogeneity.

Since T cell interaction in cellular immune reactions to infectious intracellular parasites has not been reported, this study was designed to test for T-T cell interaction in cellular immunity against *Leishmania donovani*, an intracellular protozoan parasite. Acquired immunity to leishmaniasis has been reported to be dependent on T cells (4,19) and on activated macrophages (15). This paper reports the results of limiting dilution experiments in which infected deprived mice were reconstituted with thymocytes and/or lymph node cells (LNC). The results are consistent with the evidence of T1-T2 cell synergy found by Cantor and Asofsky (6) and Wagner (27).

MATERIALS AND METHODS

Experimental design. The general techniques and procedures for treatment and maintenance of mice and parasites was previously described (20). Fig. 1 summarizes the basic protocol employed for preparing and reconstituting T cell deprived C57BL/6J mice (Jackson Laboratory, Bar Harbor). In brief, thymectomized, splenectomized, and lethally irradiated mice were reconstituted intravenously (i.v.) with T cell depleted bone marrow cells. Recipient mice were infected with 10 x 10^6 *L. donovani* (3S) amastigotes (LD bodies) and injected intraperitoneally (i.p.) with azathiprine (Az) about 18 hr later. Five days after Az treatment, recipient mice were reconstituted i.p. with graded numbers of thymocytes and/or LNC. To test for synergistic effects, one cell type was injected in graded numbers together with a constant number of the second cell type which was supplied at a level that provided minimal or undetectable reconstitution alone. As previously described (20), liver impression smears were used to assess the number of parasites in control mice at the peak of infection (day 24) and in all groups 16-26 days later.

In vivo effect of Az and anti-brain associated theta (aBAθ) serum. Az and aBAθ serum were prepared as previously described (20) and used to test the T cell dependency of reconstitution. One group of deprived mice was reconstituted with 10^6 thymocytes and a second group was reconstituted with 10^5 LNC. Both major groups were divided into 3 subgroups: the first subgroup in each major group served as untreated controls; the second and third subgroups were injected once with 1 ml of a 1/2 dilution of aBAθ serum or 150 mg/kg body weight/ml of Az, respectively. Both treatments were administered about 20 hr after reconstitution. The number of liver-parasites in treated, untreated reconstitution controls, and deprived mice was compared on day 45.

In vitro correlates of resistance. Peritoneal exudate cells (PEC) were collected from infected mice immediately prior to liver harvests in one experiment. Six mice per group were stimulated by i.p. injections of 5 x 10^6 LD bodies. After 48 hr the PEC donors were killed by cervical dislocation and injected i.p. with 2.5 ml (5 ml to each deprived animal) of a 20 mg% solution of EDTA (sodium ethylene diaminetetraacetate) in 0.85% NaCl, neutralized to pH 7.0 with $NaHCO_3$. The PEC were withdrawn 10 to 20 min later with a syringe and perforated needle. The exudate from each mouse was transferred to a centrifuge tube containing 5 ml of cold NCTC 135 medium, centrifuged at 63g for 10 min, resuspended in 2 ml of complete culture medium (NCTC 135 medium supplemented with 2% bovine embryo extract, 40% horse serum, 400 units of penicillin, and 400 mg of streptomycin per ml), and duplicate 0.2 ml aliquots of each cell suspension were transferred to appropriate chambers of 8-chamber Lab Tek tissue culture slides. After the PEC were incubated at 37 C for 2 hr in a humidified 95% air-5% CO_2 atmosphere, the medium was replaced with fresh complete culture medium containing 1.5 x 10^6 LD bodies per ml and the cultures were reincubated. Culture-chamber slides containing infected adherent macrophages were periodically removed from culture, fixed with 5% glutaraldehyde in phosphate buffer (pH 7), and stained in May-Gruenwald Giemsa. The numbers of macrophages and intracellular LD bodies were counted in 5 microscope fields per chamber to compare the relative potential of macrophages from experimental and control mice to prevent amastigote proliferation *in vitro*.

Reconstitution with cells from immunized donors. In this experiment we compared the potentials of cells from non-immune and immunized donors to reconstitute resistance. Two groups of donors were immunized:

the first group was infected i.v. with 20 x 10^6 LD bodies 30 days before cell transfers; 8 days later, the second group was injected i.v. with 20 x 10^6 LD bodies combined with 5 x 10^7 thymocytes followed by an i.p. injection of 20 x 10^7 thymocytes ("T-boosted"). A third group of normal mice served as donors of non-immune cells. Deprived recipients were divided into 6 groups which were reconstituted with graded numbers of one of the following cell types: 1) normal thymocytes, 2) normal LNC, 3) a mixture of normal thymocytes and LNC, 4) spleen cells from donors immunized 30 days earlier; 5) spleen cells from "T-boosted" donors immunized 22 days earlier, and 6) a mixture of normal thymocytes with LNC from the "T-boosted" immune donors. Liver-parasites were counted 45 days later.

Cell suspensions. Thymuses were excised from 3-4 wk old syngeneic donors, freed of fascia, rinsed in cold MEM (minimal essential medium), and teased into suspension. Cell viability was determined by the trypan blue exclusion method. After the cells were counted in a hemocytometer they were diluted with MEM to the desired concentration. Lymph nodes and spleens were excised from adult mice and handled in a like manner. Cells used for reconstitution were injected i.p. 5 days after recipients were treated with Az as described above.

Statistical methods. Regression lines of the log number of parasites as a function of the number of inoculated lymphoid cells were calculated by the least squares method. The slopes and individual data points were compared by the Student's t test. Extremely variant data, such as data associated with mice which were runted or when complete thymectomy was in doubt, were excluded on the basis of an outlier test.

The parallel line assay was used to compare relative immunocompetency of thymocytes and LNC. The statistical significance of reconstitutive potency of different populations of graded cells or cell combinations, as depicted by regression lines, was evaluated by analysis of covariance.

RESULTS

Reconstitution with thymocytes and LNC. The effect of reconstitution with different numbers of thymocytes or LNC from syngeneic mice was determined using groups of 8-10 deprived mice as recipients for each concentration and cell type employed. The geometric means of the number of parasites found in the livers of each group at 45 days post infection were plotted against the corresponding log number of inoculated cells (Fig. 2). Because of deaths and the exclusion of some data due to incomplete thymectomy, final parasite counts were based on 3-9 mice per group; however, most points represent the means derived from 5 or more mice. The difference between the number of parasites in deprived and control mice was highly significant ($P<0.001$).

On the basis of the parallel line assay, the relative reconstitutive potency of LNC was calculated to be 20-25 times greater than thymocytes. An analysis of covariance showed that the immunocompetence of thymocytes and LNC were significantly different ($P<0.001$). The number of parasites in deprived mice and mice reconstituted by 10^4 LNC was not significantly different, but extrapolation of the regression line shows that equal levels of infection could be expected in deprived mice and mice reconstituted with about 6×10^3 LNC. Reconstitution with 10^5 thymocytes also did not confer measurable immunity by 40-50 days post infection.

Inhibition with Az and aBAθ serum. Approximately 18-22 hr after i.p. injections of either 10^6 thymocytes or 10^5 LNC, either Az or aBAθ serum was administered i.p. The results of this experiment are summarized in Table 1. Treatment of LNC reconstituted mice with Az or aBAθ serum eliminated the protection conferred as compared with untreated mice ($P<0.001$ and $P<0.02$, respectively). Az and aBAθ serum treatment of thymocyte reconstituted mice only partially inhibited the protective effect since both groups had fewer parasites than the deprived group ($P<0.05$ and $P<0.001$, respectively) but more parasites than the untreated mice ($P<0.02$ and $P<0.05$, respectively). The moderate level of reconstitution in untreated mice was not significantly different from the levels of reconstitution by the same number of cells in other experiments.

Synergy among thymocytes and LNC. Fig. 3 shows the results of reconstituting deprived mice with graded numbers of thymocytes mixed in each case with 10^4 LNC. By themselves, 10^4 LNC provide little or no protection. The level of protection conferred by thymocyte-LNC combinations was approximately 10 times greater than the level of protection afforded by thymocytes alone and greatly exceeded the level of protection expected from an additive effect of both cell types acting independently. Although the slopes were not significantly different, the differences in the levels of protection were highly significant ($P<0.01$).

Fig. 4 shows that when a constant, non-protective number of thymocytes (10^5) was inoculated together with graded numbers of LNC, the protection conferred by combinations of both cell types also exceeded that predicted by an additive effect. Reconstitution with 10^4 LNC combined with 10^5 thymocytes conferred significantly greater protection than 10^4 LNC alone ($P<0.01$). The synergistic effect was most evident when fewer than 5×10^4 LNC were inoculated with 10^5 thymocytes. Reconstitution

with higher numbers of LNC in either group resulted in levels of protection which approached those observed in normal mice and differences between groups became insignificant. The slope of the regression line representing reconstitution with this synergistic cell combination was less than the slope of lines representing the effect of LNC alone or the reversed synergistic mixture of cells (Fig. 3).

In vitro response to parasites. In one experiment, PEC were collected prior to liver harvest at 44 days post infection. This experiment was designed to compare parasite survival and/or proliferation in cultured macrophages from infected control, reconstituted, and deprived hosts. For each time interval, duplicate cultures of PEC from each mouse (6 mice per group) were infected with amastigotes at the beginning of the culture period. Fig. 5 shows that a smaller number of macrophages were harvested from deprived and reconstituted animals than from normal controls, but that cells from all except one reconstituted group of mice followed similar survival patterns. The number of parasites in macrophages from control and reconstituted mice decreased during the 44 hr culture period. In contrast, macrophages from deprived mice were unable to prevent parasite multiplication. Differences in the number of parasites in control as compared to deprived cell cultures were highly significant after 24-44 hr in culture ($P<0.001$). The results of parasite death or survival in macrophages were consistent with the results from *in vivo* tests shown in Figs. 2 and 4 where reconstitution with 5×10^4 LNC conferred a moderate level of protection and protection conferred by 5×10^6 thymocytes was equivalent to controls. The *in vitro* response of macrophages to parasites roughly reflected the level of immunocompetence in the hosts as determined from liver impression smears.

Reconstitution with immune cells. This experiment was designed to compare the levels of protection conferred by reconstituting deprived mice with cells from immunized and non-immunized donors. The data was incomplete as a result of the unprecedented death toll which occurred either as a result of *Pseudomonas aeruginosa* infections and/or predisposing trauma and exposure experienced in transit for irradiation treatment.

Spleen cells collected from convalescing donors 4 wk after infection were used as a source of sensitized cells because this source had been shown to have a maximum effect in adoptive transfer experiments (Twohy, unpublished). A second group of donors was "T-boosted" at the time of infection with thymocytes from 26 day old donors in an attempt to increase the capacity of donor cells to adoptively transfer protection. Immune spleen cells (10^5) from donors immunized 4 wk (group-A) and 3 wk (group-C) prior to cell transfers conferred greater protection than 10^5 normal thymocytes, which afforded little or no protection ($P<0.05$ and $P<0.01$, respectively; Fig. 6). Resistance conferred by normal thymocytes (10^5) combined with 10^4 LNC from donors that were "T-boosted" and immunized 3 wk prior to cell transfer (group-D) was greater than the level of resistance given by the same cell combination from non-immune donors (group-B) or from 10^5 thymocytes alone ($P<0.02$ and $P<0.01$, respectively). Both groups of mice which were reconstituted with cells from "T-boosted" donors exhibited a higher level of protection than mice reconstituted with spleen cells from 4 wk immune donors (groups C and D *vs* A; $P<0.02$). The slopes and the relative levels of immunocompetence of non-immune thymocytes and LNC shown in Fig. 6 are consistent with those shown in Fig. 2.

DISCUSSION

The mechanism of acquired resistance to several intracellular protozoan parasites has been shown to be similar to that described for intracellular bacterial parasites. Like resistance to *Listeria* (13) and *Mycobacterium* (23), protection against *Toxoplasma* (25), *Besnoitia* (9), and *Leishmania* (15) are dependent on cellular immunity as determined by the requirement of cells rather than serum for passive transfer of delayed hypersensitivity or acquired resistance, and by the demonstration of parasiticidal behavior of "immune" macrophages *in vitro*. Evidence that resistance to *Leishmania* is T cell dependent was found by Bryceson et al. (4) and Preston et al. (19) who demonstrated that T cell depletion resulted in increased susceptibility to cutaneous leishmaniasis in guinea pigs and mice. Increased susceptibility to visceral leishmaniasis in T cell deprived mice was reported in the preceeding paper (20).

This paper describes the reconstitution of deprived mice with thymocytes and LNC as well as synergistic activity by combinations of the two cell types. Both cell types conferred protection against infection with *L. donovani*. Equivalent levels of protection required approximately 20-25 times more thymocytes than LNC.

The levels of protection which resulted when both cell types were injected together greatly exceeded the levels of protection that were predicted from the additive effects of independently acting populations. It was hypothesized that as the varied population was given in greater numbers, it would account for a greater proportion of the resistance conferred by a mixed population of cells. Thus, if synergistic activity occurred, the level of resistance given by mixed cells was expected to progressively approach the level of resistance which resulted when the varied cell population was administered alone. A flatter dose-response

(regression) curve did result when graded numbers of LNC were injected with 10^5 thymocytes (Fig. 3). However, the slope of the lines representing graded numbers of thymocytes mixed with 10^4 LNC or graded numbers of thymocytes alone were not significantly different (Fig. 2). Perhaps a flatter regression line would have resulted in the second case had varying numbers of thymocytes been mixed with a more limiting number of LNC. Alternatively, if the mechanism of interaction involves two-way communication such that the reactivity of LNC depends on stimulation from thymocytes, which in turn causes LNC to enhance the activity of thymocytes, then the level of enhancement of thymocytes by a constant number of LNC could vary depending on the number of responding thymocytes. Under these conditions, reconstitution with graded numbers of thymocytes, with and without 10^4 LNC, could be expected to result in nearly parallel regression lines as shown in Fig. 3. The flatter dose-response curve observed for reconstitution with graded numbers of LNC mixed with 10^5 thymocytes (Fig. 4) could also be expected under these circumstances since the required signal, or stimulus from thymocytes, would be constant while the enhancing effect would vary with the number of LNC.

Regarding the above hypothetical explanation of the differences in slopes, it is important to remember that neither population of cells used in these experiments were pure. Perhaps spleen-seeking and lymph node-seeking cells represent relatively pure subpopulations of T cells. Tigelaar (24) observed relatively flat dose-response curves when graded numbers of lymph node-seeking or spleen-seeking T cells were tested alone for their ability to provide a GVH reaction in irradiated mice. However, when graded numbers of lymph node-seeking cells and constant numbers of spleen-seeking cells were injected together, the dose-response slopes and the levels of GVH reactivity were much higher and resembled dose-response

curves resulting from injections of normal LNC or spleen cells.

A wide range of host responses are characteristic for *L. donovani*. Human and hamster hosts usually permit unrestricted parasite proliferation without developing immunity. In man, chemotherapy prevents death and is followed by strong but poorly understood immunity. Cotton rats also permit unrestricted multiplication of parasites but apparently suffer no ill effects from high levels of infection. Mice show a wide range of susceptibility, some strains possess complete innate resistance while other strains experience a long course of infection (3). When infected intravenously, C57BL/6J female mice support high levels of infection which are followed by strong immunity (Twohy, unpublished).

Deprived mice have been used in a variety of studies designed to elucidate lymphocyte roles and interactions in the immune response. The immunosuppressed C57BL mouse system used in this investigation proved to be a useful model for demonstrating T-T cell interaction in the rejection of intracellular parasites. However, the interpretation of results in these experiments was complicated by several factors. As has been observed by other investigators (10), a great deal of variability may be inherent with reconstitution of deprived mice with thymocytes. This is especially true with adult thymocyte donors or donors of different ages (17). In these experiments, thymocyte donors were always between 23 and 28 days old. Reconstitution with adult thymocytes was poor and inconsistent. Another variable, runting, occurred occassionally among the groups of reconstituted and deprived mice. In most cases, the runted mice exhibited higher numbers of parasites in their livers than did others in the same group even though their body weight was much lower.

Electron micrographs of intracellular *Leishmania* amastigotes show that two complete membranes surround LD bodies. Putatively, the inner

membrane is the cytoplasmic membrane of the LD body while the outer membrane may be a vacuole-like membrane belonging to the host. Such a membrane may in effect protect the parasite from recognition by the host. Alternatively, the amastigote, either by serial passage in the hamster or by the presence of a remnant outer coat, may acquire xenogeneic histocompatibility antigens which would stimulate a graft rejection type of immune response against the parasite. It seems unlikely that the expression of immunity toward *Leishmania* reported here was a result of graft rejection mechanisms since the xenogeneic antigens would have been significantly diluted by the massive proliferation of the parasites and the immune rejection would have been expected to have occurred much earlier. Furthermore, macrophages from mice which were immunized with hamster spleen were unable to suppress parasite growth *in vitro* while the parasites were killed by macrophages from donors which were immunized with amastigotes from mouse or hamster spleens (Twohy, unpublished).

The relative number and behavior of macrophages harvested from the peritoneum of infected controls was similar to that reported by Miller and Twohy (15). More macrophages were harvested from control than from deprived animals. Parasites did not survive in macrophages from the immune controls and macrophages from deprived mice supported parasite proliferation similar to that seen in macrophages from normal animals (15). In general, the *in vitro* tests correlated well with the pattern of resistance seen *in vivo*. The Youmans hypothesized that cellular immunity can be completely dissociated from delayed hypersensitivity and that nonspecific killing of intracellular parasites by activated macrophages may be an expression of delayed hypersensitivity (26). The observations reported here do not clarify this question. However, the correlation of

the *in vivo* and *in vitro* expressions of immunity indicates that the *in vitro* assessment of immunity in this system was valid. Like the recall of passively transferred delayed hypersensitivity in other systems, specific antigenic stimulation approximately 48 hr prior to PEC harvest was necessary to obtain macrophages with parasiticidal properties.

In the experiment in which the cells used for reconstitution were taken from infected donors, the injection of the donors simultaneously with parasites and thymocytes appeared to enhance the transfer of immunity when compared to donors which were immunized without "T-boosting". In previous passive transfer experiments, *in vitro* criteria were used to assess immunity. In those experiments, spleen cells from donors immunized 3 wk before cells were transferred, were inconsistent in conveying adoptive immunity (Twohy, unbuplished). This experiment was of a preliminary nature, and did not discern whether the advantage of "T-boosted" cells was a result of the cells having previous contact with the parasite antigens, or whether the enhanced immunity was a result of the enrichment of immunocompetent cells via a selective migration of immunocompetent cells to lymph nodes and spleens. The passive transfer of the anamestic response may more readily be demonstrated in this system by methods designed to detect an earlier onset of immunity rather than by comparing levels of infection later in the course of infection.

Synergy between central and peripheral T cell populations has been previously reported by Cantor and Asofsky (6) and Wagner (27) using the graft-versus-host system and the *in vitro* cytotoxic allograft response, respectively. Cantor and Asofsky found combinations of peripheral blood lymphocytes and thymocytes to be 2-3 times more potent than predicted from an additive effect while Wagner found a 20 fold increase in reactivity. In contrast, we found about a 10 fold increase in the level of

protection given by combination of thymocytes and LNC. Like the results of the previous workers, our results show that peripheral T cells were approximately 20-25 times more immunocompetent than thymocytes. The differences in the degree of synergy, as reported by the above investigators, may have resulted because the levels of immunocompetence present in the limiting or constant cell population were different.

Alternatively, each of the experimental systems may be different with respect to its sensitivity or its lymphocyte interactions and effector mechanisms. Nevertheless, evidence that T-T cell synergy may play a major role in immune responses against histocompatibility antigens and intracellular parasites unifies the concept of cellular immunity and may more clearly distinguish it from other cell-mediated immune phenomena, i.e., T-T cell synergy may be a criterion of cellular immunity.

It is evident that T cells are required for protection of mice against *L. donovani*. Anti-theta serum and Az have been shown to effectively abrogate T cell function (1,5,20,24). The differences observed between the inhibitory effects of Az and aBAθ serum are consistent with the concept that T2 cells recirculate more than T1 cells and are consequently more susceptible to the inhibitory effects of both agents. Treatment of thymocytes with aBAθ serum *in vitro* killed 100% of the cells while *in vivo* treatments 18-22 hrs after reconstitution only moderately inhibited the reconstitutive effects of thymocytes.

The results of this investigation provide convincing evidence that acquired resistance to *L. donovani* in mice relies principally on T cell mediated cellular immunity. Furthermore, the suppression-reconstitution model described provides a useful system for studying the cellular components which are responsible for protective immunity against infectious

intracellular parasites. In this regard the data suggest that the mechanisms of cellular immunity to intracellular parasites are at least fundamentally analogous to those described for the GVH system (6) and the *in vitro* cytotoxic allograft response (27).

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TABLE I

Inhibition of protection conferred by transplanted LNC or thymocytes by *in vivo* treatment of recipients with Az or aBAθ serum.

Cells used for reconstitution	Treatment *in vivo*	Log LDs/liver mean ± sd	Significance untreated[a]	Significance deprived[b]
None*	None	9.22 ± 0.172	P<0.001	
LNC (10^5)	Untreated	8.05 ± 0.370		P<0.001
LNC (10^5)	Az	9.11 ± 0.183	P<0.001	ns
LNC (10^5)	aBAθ	8.92 ± 0.392	P<0.02	ns
Thymocytes (10^6)	Untreated	7.63 ± 0.386		P<0.001
Thymocytes (10^6)	Az	8.64 ± 0.394	P<0.02	P<0.05
Thymocytes (10^6)	aBAθ	8.14 ± 0.154	P<0.05	P<0.001

* Designates deprived group.

[a] Treated group compared to untreated group; [b] group compared to deprived group; ns, not significant.

Figure 1. Experimental design. Bone marrow cells from thymectomized syngeneic donors were treated with aBAθ serum plus complement and used to restore the hemopoietic stem cell population in thymectomized, splenectomized, and irradiated (deprived) recipients. Following the infection of deprived recipients, they were treated with Az after about 20 hr, and reconstituted 5 days later with various numbers of thymocytes and/or LNC. The level of protection conferred by reconstitution was assessed 40 to 50 days post infection from the number of parasites counted in liver impression smears.

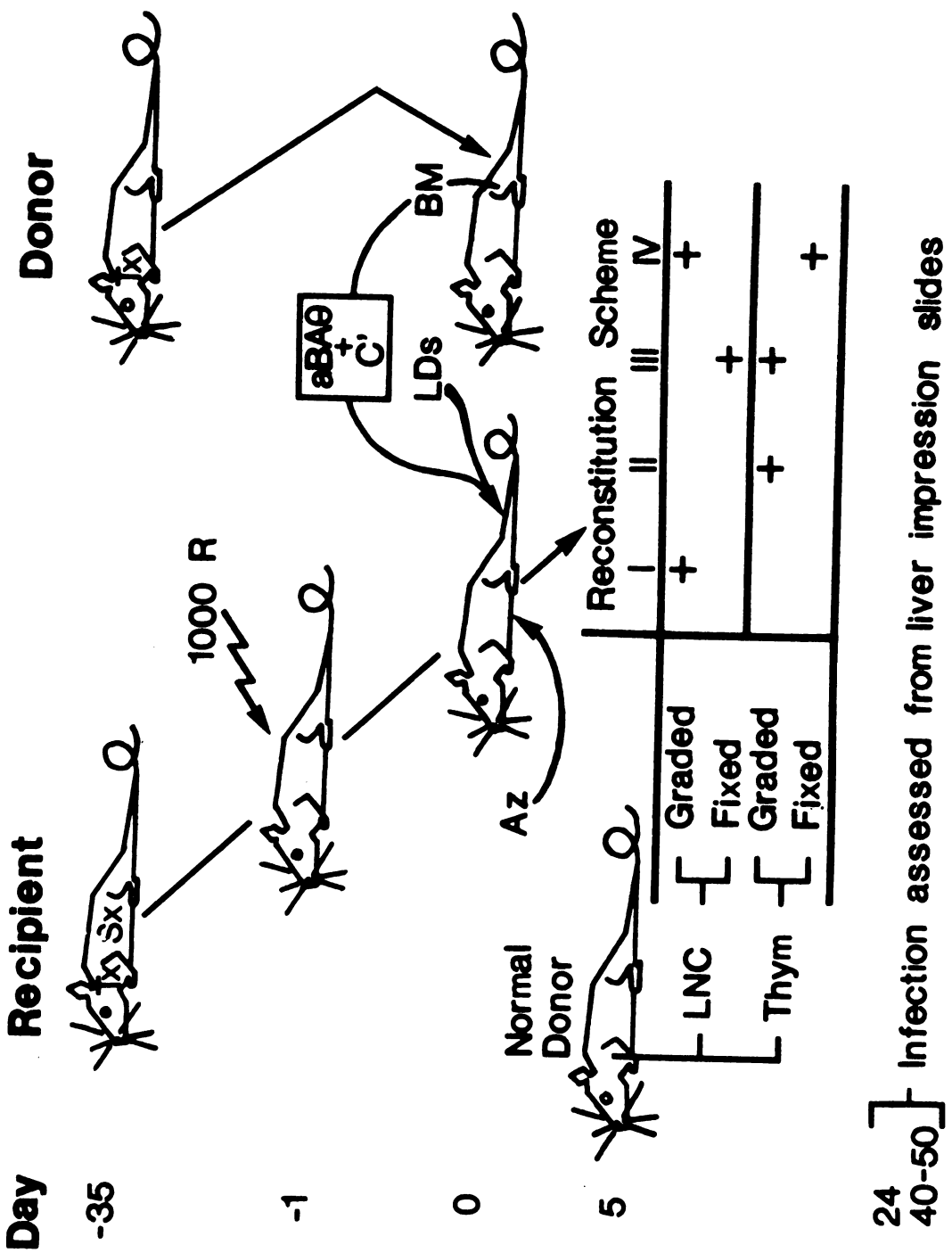
Day
Recipient
Donor
-35
Sx
1000 R
-1
aBAθ
+C'
LDs
BM
0
Az
5
Normal Donor
LNC
Thym
Graded
Fixed
Graded
Fixed
Reconstitution Scheme
I
II
III
IV
+
+
+
+
+
+
24
40-50
Infection assessed from liver impression slides

Figure 1

Figure 2. Relative immunocompetence of thymocytes and LNC. The log number of parasites/liver 41 days post infection were plotted against the number of thymocytes (▲) and LNC (●) that were injected for reconstitution. Each point represents the mean number of parasites/liver from 6 to 9 mice. Slopes of regression lines were calculated by the method of least squares. The range between the mean numbers of parasites in livers from control mice 24 and 41 days post infection are depicted by the vertical bar while the horizontal bar represents the mean parasite count in deprived mice on day 41.

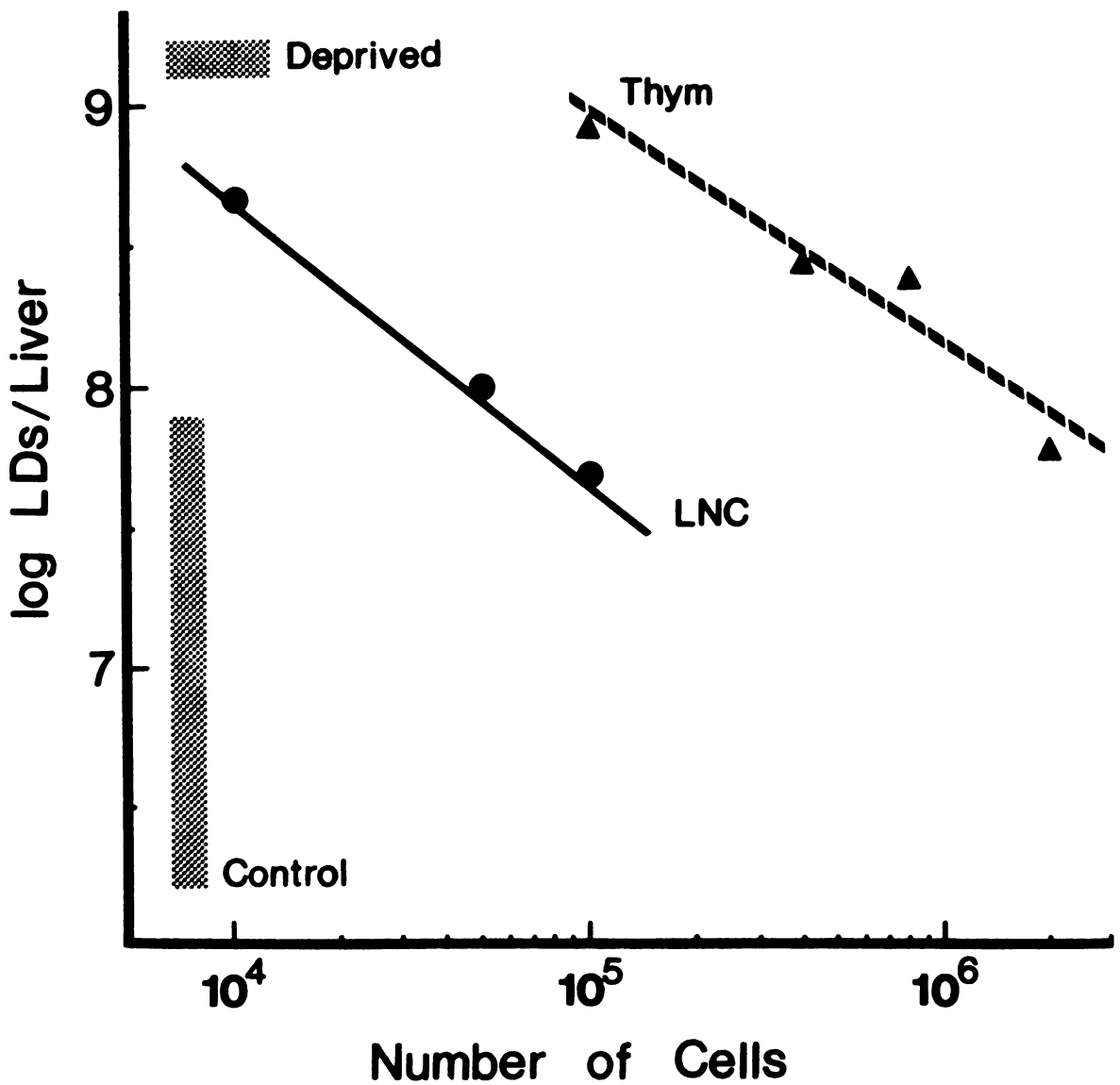
Deprived
Thym
LNC
Control
log LDs/Liver
9
8
7
10^4
10^5
10^6
Number of Cells

Figure 2

Figure 3. Synergistic effect of reconstitution with graded numbers of thymocytes mixed with 10^4 LNC. Each point represents the mean number of liver-parasites from 4 to 8 mice at 44 days post infection. Regression lines were calculated by the least squares method and represent the relative immunocompetence of thymocytes and LNC combined (○) and thymocytes alone (●). Control and deprived groups are represented by vertical and horizontal bars (see Fig. 2).

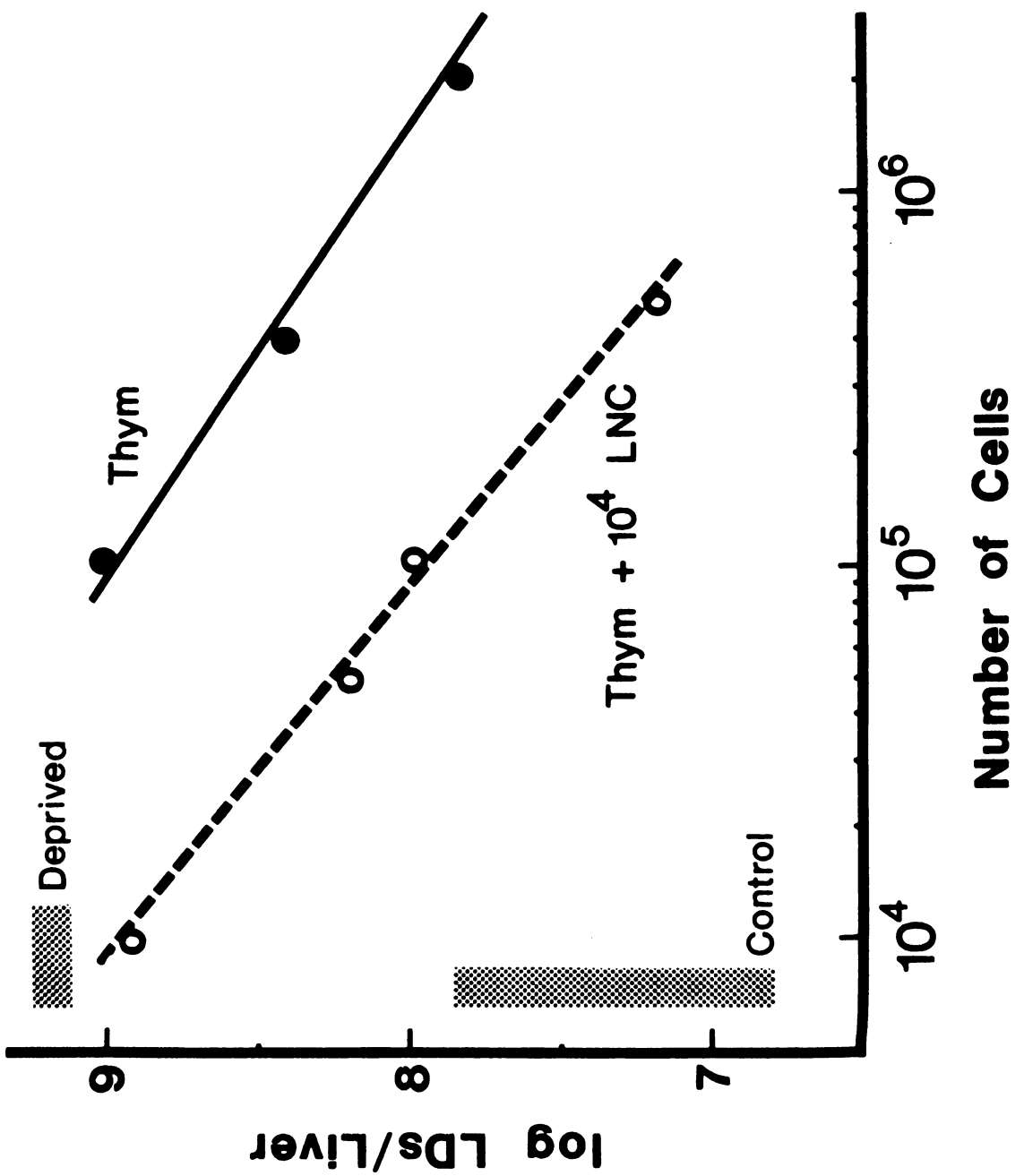
log LDs/Liver
9
8
7
Deprived
Thym
Thym + 10^4 LNC
Control
10^4
10^5
10^6
Number of Cells

Figure 3

Figure 4. Synergistic effect of reconstitution with graded numbers of LNC mixed with 10^5 thymocytes. Each point represents the mean number of liver-parasites in 5 to 7 mice at 47 days post infection. Regression lines (calculated by the least squares method) represent the relative immunocompetence restored by graded numbers of LNC conbined with 10^5 thymocytes (●) and LNC alone (○). Control and deprived groups are represented by vertical and horizontal bars (see Fig. 2).

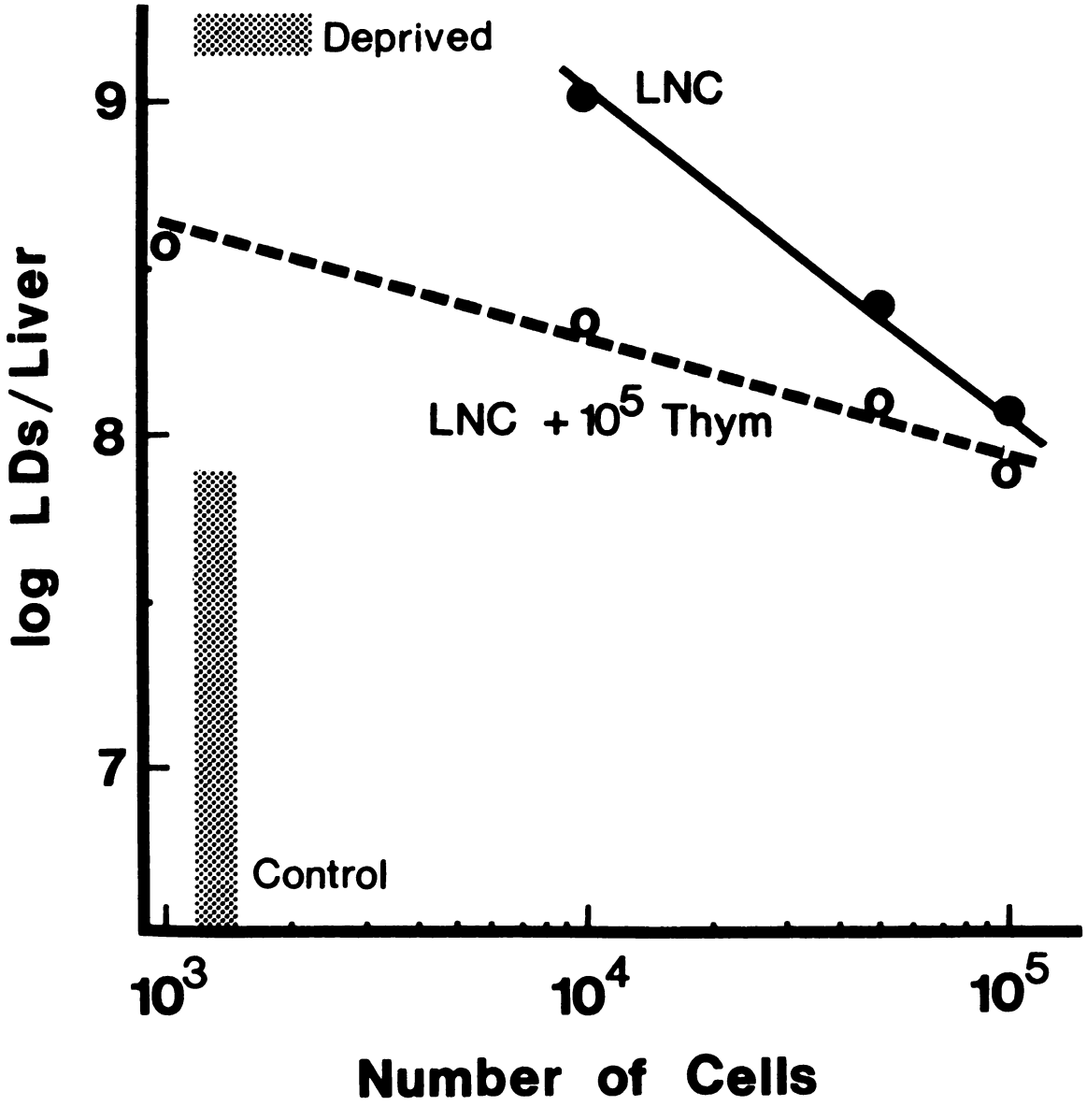
Deprived
LNC
LNC + 10^5 Thym
Control
log LDs/Liver
9
8
7
10^3
10^4
10^5
Number of Cells

Figure 4

Figure 5. Survival of parasites in peritoneal macrophages from control, reconstituted, and deprived mice 44 days after infection and harvested 2 days after i.p. injection of 5×10^6 LD bodies. Each point represents the mean number of cells in duplicate cultures from 6 mice.

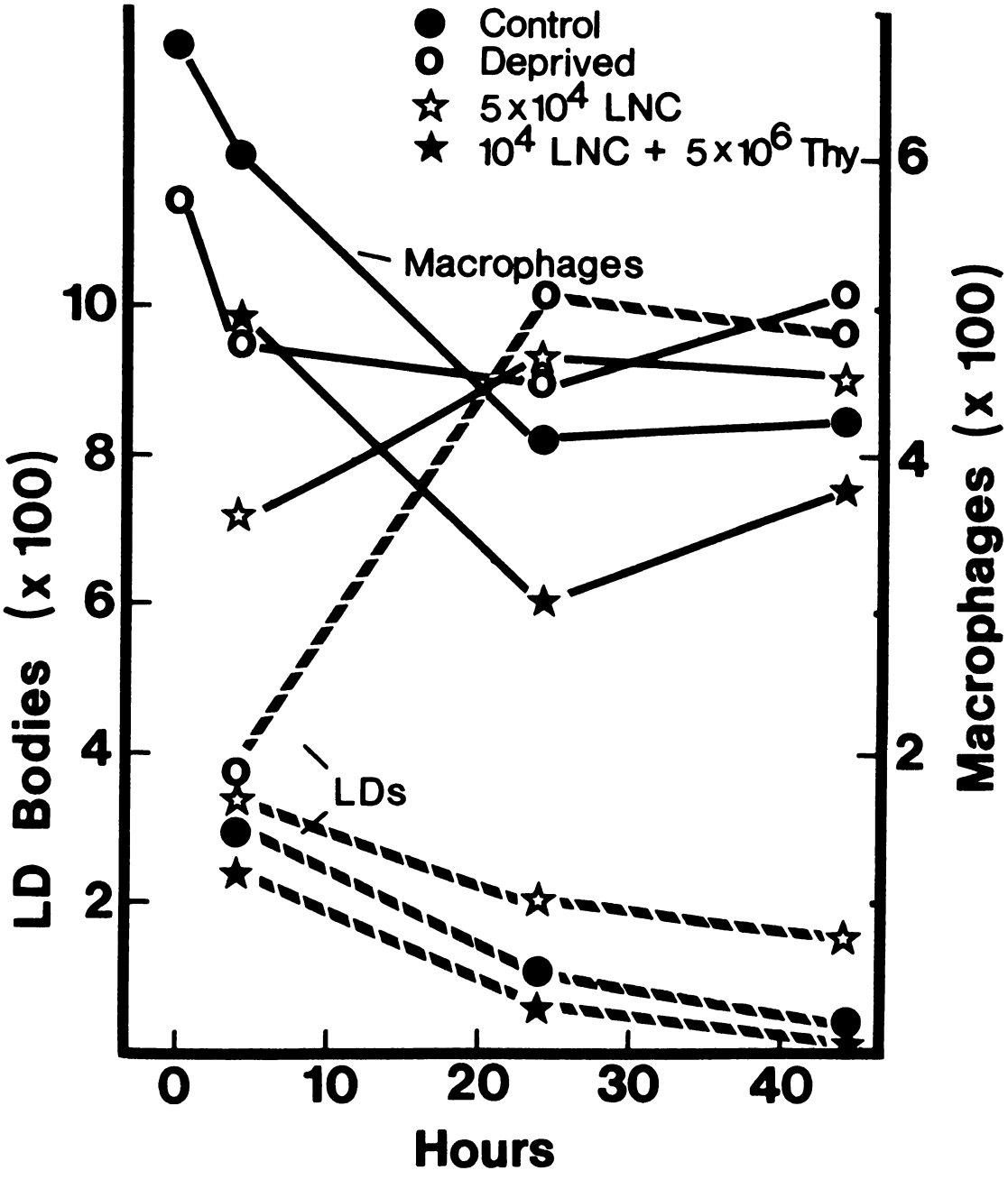
Control
Deprived
5x10^4 LNC
10^4 LNC + 5x10^6 Thy
Macrophages
LDs
LD Bodies (x 100)
Macrophages (x 100)
Hours
0
10
20
30
40
2
4
6
8
10
2
4
6

Figure 5

Figure 6. Protection conferred by cells from non-immune and immune donors. Each point represents 4 to 6 mice at 50 days post infection. Solid regression lines (calculated by the least squares method) represent standards for protection by gradients of normal thymocytes (○) or LNC (●). Relative levels of protection were compared for group-A, 10^5 spleen cells from 4 wk immune donors (✪); group-B, 10^5 normal thymocytes mixed with 10^4 normal LNC (◉); group-C, 10^5 spleen cells from 3 wk immune, "T-boosted" donors (★); and group-D, 10^5 normal thymocytes mixed with 10^4 immune LNC from "T-boosted" donors (☆). Control and deprived groups are represented by vertical and horizontal bars (see Fig. 2).

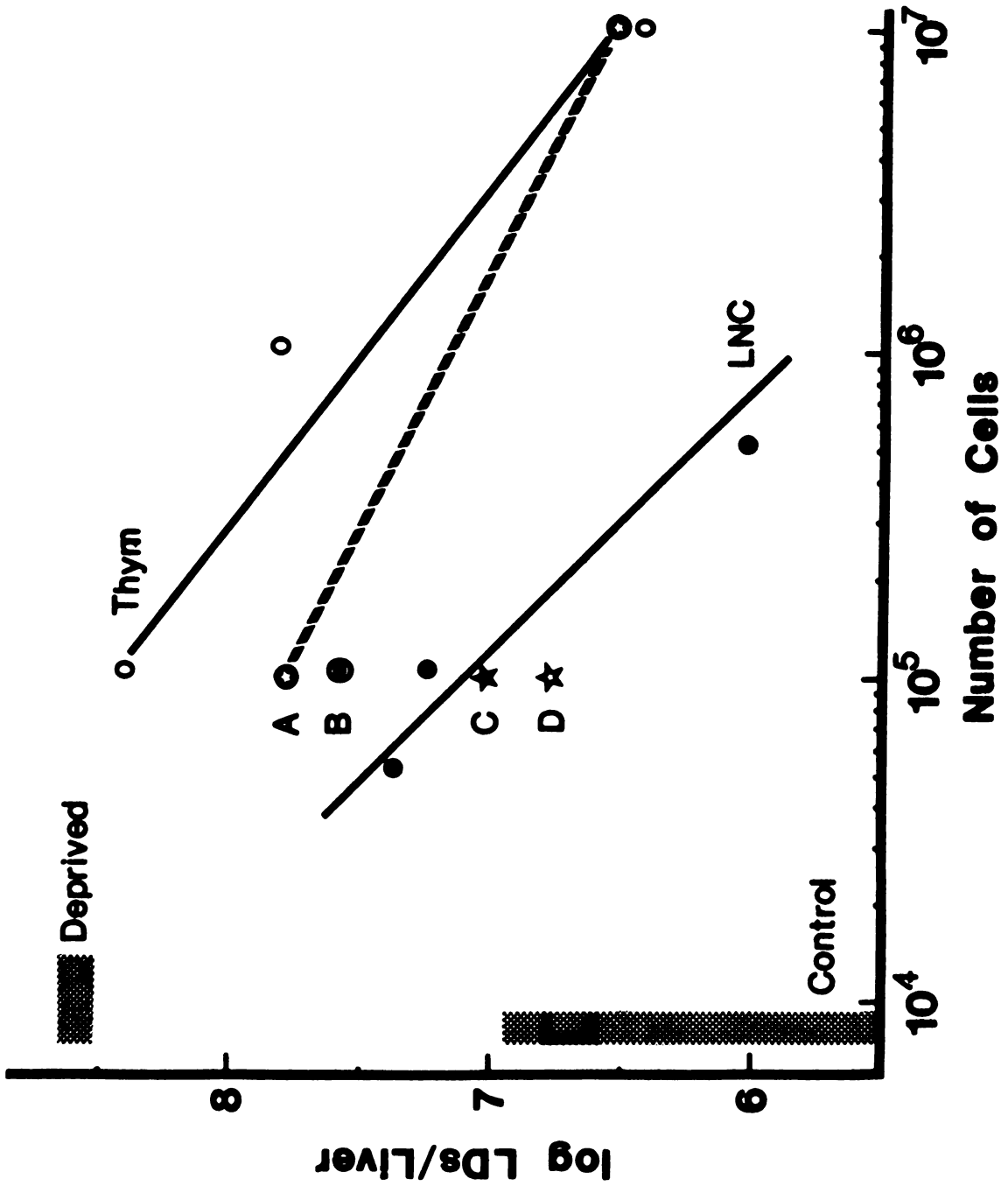
log LDs/Liver
8
7
6
Deprived
Control
10^4
10^5
10^6
10^7
Number of Cells
Thym
LNC
A
B
C
D

Figure 6

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