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LYMPHOCYTE BLASTOGENIC RESPONSES IN EXPERIMENTAL BLASTOMYCOSIS

*The University of Oklahoma* PH.D. 1982

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

# LYMPHOCYTE BLASTOGENIC RESPONSES IN EXPERIMENTAL BLASTOMYCOSIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

RICHARD PEARCE MORRISON

Norman, Oklahoma

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LYMPHOCYTE BLASTOGENIC RESPONSES IN EXPERIMENTAL BLASTOMYCOSIS

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I hear and I forget
I see and I remember
I do and I understand
-Confuscius

September 1982 Richard P. Morrison

DEDICATION

To Mom, Gram, Jan, and Sandra

With
Love

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# LYMPHOCYTE BLASTOGENIC RESPONSES IN EXPERIMENTAL BLASTOMYCOSIS

## CHAPTER I

## INTRODUCTION

Blastomycosis, first described by Gilchrist in 1894, is caused by the dimorphic fungus Blastomyces dermatitidis. Since its discovery, our understanding of this frequently serious infection has progressed slowly. Early contributions, such as, the portal entry of the organism (38), treatment with amphotericin B (18), and description of subclinical and acute self-limiting forms of the disease (35), have aided in our understanding of this disease. Gaps still remain, however, in our knowledge concerning the natural habitat of the organism, the clinical aspects and therapy of the disease, and the immunological responses of the host.

Research concerning the immune response to fungi such as Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum, and Coccidioides immitis, is progressing much more rapidly than that of B. dermatitidis. The low incidence of blastomycosis and the lack of a reliable antigen preparation may offer explanations for this. Nevertheless, the disease is often serious and the lack of understanding of the hosts interaction with this fungus warrants further investigation.

Delayed-type hypersensitivity (DTH) reactions can be thought of as in vivo models used to help define the cellular basis of cell-mediated immune (CMI) responses. DTH is considered to be a cell-mediated hypersensitivity in which the effector cell is a T lymphocyte capable of reacting specifically on contact with antigen. It is apparent from many studies that CMI is important in host defense against systemic fungal infections (13,17,36). B. dermatitidis often elicits a DTH response and to some extent a humoral response (36). DTH develops in a host as the result of previous contact or infection with the fungus. Patients with blastomycosis often develop strongly positive skin test reactions when challenged intradermally with blastomycin. This is not a consistant finding though, and may be due to altered immune functions or the lack of a suitable skin test antigen.

The importance of DTH in patients with blastomycosis was first observed by Smith in 1947 (43). He believed that the prognosis of these patients was dependent on a functional immune system. The relationship of DTH to immunity remains unknown. However, Spencer and Cozad present evidence that DTH indicates protection (46). They demonstrate that mice rendered hypersensitive to B. dermatitidis, either by an injection of merthiolate killed yeasts and Freunds incomplete adjuvant, or by the subcutaneous injection of viable B. dermatitidis yeast, were more resistant to a lethal challenge of B. dermatitidis than unsensitized mice. The idea that DTH indicates protection, however, does not hold for all fungal infections, since in an animal model of experimental candidiasis, DTH did not correlate with protection (19). Other studies have demonstrated that DTH elicited to B. dermatitidis can be transferred from immune mice to non-immune mice by spleen cells but not by serum (39). The transfer of DTH was measured by the foot pad swelling test and it is not known whether protection could also be

transferred. Recently, Cozad and Chang reported that protection parallels the development of DTH to B. dermatitidis (11). They measured protection by mortality studies and culture recovery of B. dermatitidis from organs of infected animals. Protection was found to be greatest during the period of highest DTH response.

There is evidence that CMI is not the sole defense mechanism against B. dermatitidis infection. For example, not all patients with blastomycosis have evidence of defective cell-mediated immune function (32,36). In fact, normal lymphocyte responses were observed in two patients with multiple relapses of pulmonary blastomycosis, which indicates that some other factor(s) is (are) responsible for the relapses (45). If the pathology of blastomycosis is examined, one not only finds the typical granulomata, but neutrophilic infiltrates are often observed.

Efforts to define the role of neutrophils in the immune response to B. dermatitidis have recently been initiated. Sixby et al. reported that culture filtrates of B. dermatitidis have chemotactic activity for neutrophils (41). In addition, they found that these neutrophils ingested B. dermatitidis yeast, but killed less than 30% of the inoculum. Inhibition of neutrophil chemotaxis by a factor found in the serum of patients with active blastomycosis, also implicates the neutrophil as a possible effector cell (31).

Results from other investigators do not support those of Sixby et al. Brummer and Stevens showed opposite effects of human macrophages and polymorphonuclear neutrophils (PMN) on the replication of B. dermatitidis (8). They found that whereas macrophages inhibited the in vitro replication of B. dermatitidis, PMN stimulated its replication. These authors were unable to

confirm any killing by PMN. They suggest that clumping of fungal cells could account for the decline in cell number, which was interpreted as killing by Sixby et al.

There is evidence suggesting that the macrophage is an important effector cell in the immune response to B. dermatitidis. Brummer et al. used an in vitro culture system to study the interaction of macrophages with yeast phase B. dermatitidis (6). Macrophages obtained from mice which had recovered from an infection with B. dermatitidis, significantly inhibited the growth of the fungus, more than macrophages from normal mice. Con A stimulated macrophages, likewise inhibited yeast growth. Evidence for an extracellular macrophage product, which inhibits yeast phase B. dermatitidis replication, is also presented in their report. Brummer et al. investigated the ability of normal and stimulated macrophages to inhibit the replication of several isolates of B. dermatitidis (7). Their results indicate that the virulence of B. dermatitidis isolates may be due, in part, to the organisms ability to escape inhibition by the macrophage.

Susceptibility of inbred strains of mice to pulmonary blastomycosis also implicates the macrophage as an important effector cell in the killing of B. dermatitidis (26). Several strains of mice with various H-2 backgrounds were tested for their resistance to B. dermatitidis. The C3H/HeJ strain was found to be highly susceptible and DBA/1J mice were resistant. Absence of complement component C5 or difference in H-2 type did not appear to be the explanation for susceptibility to blastomycosis. Difference between susceptible and resistant strains in lymphocyte proliferation and delayed-type hypersensitivity were not demonstrated. Moreover, antibody response to B. dermatitidis was higher in the susceptible strain. C3H/HeJ mice have a macrophage cytotoxicity defect, and

the authors believe that this may account for the susceptibility of this strain to blastomycosis. The A/HeJ strain, which also has a macrophage cytotoxicity defect, was found to be the second most susceptible strain.

It is evident that CMI and phagocytic cells are important effector mechanisms in host resistance to B. dermatitidis. Although it has not been demonstrated that one or the other can prevent the disease from fulminating, it is clear that both defense mechanisms are functioning. In vitro findings do not necessarily determine the in vivo role of cells in the killing and erradication of B. dermatitidis. Several elements of the immune system, e.g. specific antibodies, lymphokines, complement, lymphocytes, and macrophages, may be necessary adjuncts in killing in vivo.

CMI is mediated by specifically sensitized T lymphocytes, which can have a variety of functions (21). T cells have the ability to help B cells produce antibody. Some T cells may also act synergistically with other T cells. However, besides these amplifying actions, it is known that a certain subpopulation of T cells has an inhibitory action on B and T cell functions. The interaction of the various subpopulations of T cells with other cells of the immune system suggests a fine regulatory function of this class of lymphocytes.

The interaction of cell populations in regulating the immune response to B. dermatitidis is poorly understood. CMI and phagocytic cell functions are important in the hosts response to B. dermatitidis, but little has been done to characterize the cellular interactions in these responses. Immunological unresponsiveness, which is mediated through one type of cellular interaction, poses a serious threat to patients with fungal infections. It is clear that defective or compromised CMI functions facilitate dissemination of the organism, although

little is known about the immunoregulatory effects that can be induced by the infection in normal, immunocompetent individuals. Skin test anergy and changes in lymphocyte blastogenic responses to mitogens and antigens during disseminated disease suggests altered immunoregulation (2,15,29,36).

Suppression of the immune response during bacterial (10), viral (3), fungal (33), and protozoan (14,24) infections has been reported. Suppressor activity is not limited to a single population of cells, but has been attributed to T cells (21), adherent cells (1) and serum factors (25).

Rogers and Balish reported that lymphocyte proliferative responses to PHA and ConA were suppressed in mice systemically challenged with C. albicans (33). Suppression was evident at 7 but not at 14 days after challenged. Suppression also was demonstrated in mice immunized with BCG several weeks prior to systemic challenge with C. albicans. The PPD-specific lymphocyte blastogenic response was suppressed 95%, when BCG immunized mice were challenged with C. albicans. This suppression could not be attributed to an increase in the number of C. albicans - specific lymphocytes or release of toxic products from C. albicans. Depressed lymphocyte blastogenic responses during experimental endocarditis caused by C. albicans have been reported (42). The suppressed response to Candida antigen was observed primarily in rabbits that received a large C. albicans inoculum.

The effect of C. albicans on another limb of the immune response has been assessed by using the hemolytic plaque-forming-cell technique. Vardinon and Segal (50) reported that infection with C. albicans suppresses the plaque forming cell response to a T-dependent antigen (sheep red blood cells) but not to a T-independent antigen (LPS).

Recently, Murphy and Moorhead (28) described a murine model used to study the effects of cryptococcal polysaccharide antigen on the immune response. By injecting cryptococcal polysaccharide intravenously into mice, they established a level of antigenemia that is reported to occur naturally during human cryptococcosis. They characterized a suppressor cell that is found in the lymph node cell population. This cell suppresses the DTH response to cryptococcal antigen and also the development of T cells responsible for inhibiting the growth of C. neoformans in vitro. The suppressor cell is described as being a T cell that is antigen specific. It was shown to exert its effect on the afferent limb of the CMI response.

Disseminated histoplasmosis has been shown to induce complex disturbances in the immunoregulatory control mechanisms of the host. Artz and Bullock (4), using a murine model, demonstrated altered cellular immune responses. They produced a disseminated infection in mice that resolved over an eight week period. Immunosuppressive activity of spleen cells was observed from 1 to 3 weeks after inoculation. During this time, they observed depressed DTH responses to SRBC and histoplasmin, and impaired blastogenic responses to Con A and histoplasmin. Splenocytes from infected mice suppressed the T cell dependent antibody responses to SRBC by normal spleen cells in vitro as well as the T cell mediated cytotoxicity response of normal spleen cells. As animals recovered from the infection by week 8, there was a shift from suppressor activity to a helper mode. DTH responses became more vigorous, and cytotoxic activity and PFC responses became equal to or greater than normal control values.

Nickerson et al. (30), using the murine model of histoplasmosis described above, demonstrated that spleen cells from infected mice not only suppressed the

primary antibody response of normal spleen cells to a T dependent antigen (SRBC), but also suppressed the response to a T independent antigen (TNP-LPS). Suppressor activity could be demonstrated with lymph node cells as well. They described two populations of spleen cells that express suppressor function. One population was identified as T cells. The other was an adherent, macrophage-like, cell population.

Altered blastogenic responses of lymphocytes from mice infected subcutaneously with H. capsulatum has been reported (49). Lymphocyte proliferative responses to Con A and PHA were suppressed up to 21 days post-immunization. The response to LPS was suppressed to a lesser degree.

Some clinical observations support the findings of experimentally induced infections. For instance, there is a high prevalence of skin test anergy and altered lymphocyte proliferative responses among patients with systemic histoplasmosis (22,29,44). Stobo, et al. (48) reported that depressed T lymphocyte responses to antigens and mitogens in some patients with fungal infections may be due in part to a soluble product released from macrophages acting on a subset of regulatory T cells. However, patients with fungal infections are by no means a homogenous group immunologically. In some patients, immune responses are found to be altered, while in others they appear to be normal (2,9,47).

The mycoses are of considerable medical importance not only as secondary invaders of debilitated patients, but in some instances as true primary pathogens. Little is known as to how these organisms evade the hosts defense mechanisms or how they alter the immune response once the infectious process has begun. The elucidation of cellular interactions controlling the immune response to an infectious agent is one of the principle objectives of studying the immunobiology of infectious disease.

In this thesis, we make use of an in vitro method, the LT assay, which has been found to be particularly useful for investigating the effects of a disease process on the immune response. Antigen-induced blast transformation is a valuable model for studying antigenic recognition by sensitized lymphocytes. Specificities of B and T cell responses to mitogens has been evaluated by studying pure B and T cell populations (21). PHA and Con A have been shown to be T cell mitogens, whereas LPS stimulates blast transformation of B lymphocytes (21). Non-specific mitogen induced transformation represents a useful marker for evaluating various lymphocyte subpopulations and resembles, in many respects, antigen-induced stimulation. We describe altered lymphocyte proliferative responses to mitogens and antigen of spleen cells from mice either lethally infected or immunized with B. dermatitidis. The cell population responsible for the proliferative response to B. dermatitidis antigen is identified.

Relapses of blastomycosis in patients with "normal" CMI function, and identification of suppressor cell activity in some forms of fungal diseases, indicates the complexity of the immune response to fungal infections. By further identifying the cellular interactions during blastomycosis, we will hopefully gain a better understanding of the numerous clinical forms of the disease, and cellular functions during them.

# CHAPTER II

## MATERIALS AND METHODS

Animals. Male, C57BL/6 mice, 8 to 14 weeks old, were used throughout this study. This strain was originally obtained from Jackson Laboratories (Bar Harbor, ME), and bred and maintained in The Oklahoma University Department of Botany and Microbiology animal facilities. Mice were housed 8/cage and received food and water ad libitum.

Fungi and growth conditions. Blastomyces dermatitidis strain SCB-2 (ATCC 26199) and B. dermatitidis strain 242 (OU culture collection) were used throughout this study. Both strains had been isolated from cases of human blastomycosis. Stock cultures of yeast phase organisms were grown on brain heart infusion (BHI) agar (Difco) at 37°C for 4 days, then stored at 4°C. Cultures were transferred to fresh media monthly. Broth cultures were prepared by inoculating 50 ml of BHI broth with a loopful of yeasts from a 4 day old agar culture. Cultures were rotated at 180 rpm (Psycrotherm, New Brunswick Sci. Co., NJ) for 48 to 72 hours at 37°C. Yeast cells were harvested by centrifugation (200xg for 10 minutes) and washed three times in 10 ml of sterile saline. Fungal particles were counted using a hemocytometer, and diluted in saline to the appropriate concentration. Yeast cells from broth cultures tended to aggregate; aggregates of cells were counted as one. Viable colony forming units were determined by

plating triplicate 0.5 ml portions of an appropriate dilution of washed yeast cells onto plates of BHI agar containing 5% sheep blood (The Brown Laboratory, Topeka, KS). Plates were incubated at room temperature (22°C) for 2 weeks before the colonies were counted. Direct counts obtained using the hemocytometer were greater than plate counts in all experiments. The average viability of all inocula was 87% (range 74 to 96%). B. dermatitidis strain SCB-2 was used in all experiments unless otherwise stated.

Serum. Human plasma containing the anti-coagulant citrate-phosphate-dextrose was obtained from Oklahoma Blood Institute (Oklahoma City, OK). Plasma was clotted by the addition of purified bovine thrombin (Miles Laboratories, Inc., Elkhart, IN). Eighty units of purified thrombin were added to 40 ml of plasma, mixed and allowed to clot for 5 minutes at room temperature. The serum was collected by centrifugation and the procedure was repeated to remove any fibrin that remained after the first clotting. The resulting serum was heat inactivated at 56°C for 30 minutes and stored at -20°C. All sera were tested for nonspecific stimulation of mouse spleen cells in the lymphocyte transformation (LT) assay. Sera that were nonstimulatory were used.

Mitogens. Concanavalin A (Con A, type IV) (Sigma Chemical Co., St. Louis, MO), lipopolysaccharide (LPS) from E. coli (serotype 055:B5) (Sigma Chemical Co., St. Louis, MO) and phytohemagglutinin-P (PHA) (Difco Laboratories, Detroit, MI) were obtained in lyophilized form. Con A and LPS were reconstituted in RPMI 1640 (Gibco, Grand Island, NY) to a concentration of 2 mg/ml. PHA was reconstituted with 5 ml of distilled water. Reconstituted mitogens were stored at -20°C for no longer than one month. Appropriate dilutions of mitogens were prepared from these stocks for use in the lymphocyte transformation assay. Once thawed the unused portion was discarded.

Antigen preparation. Alkali-soluble-water-soluble Blastomyces dermatitidis antigen (B-ASWS) was prepared as described by Deighton et al. (12). Briefly, formalin killed yeast cells were harvested by centrifugation, washed extensively with distilled water and subjected to breakage in a Bronwill MSK homogenizer (Bronwill Scientific Inc., Rochester, NY). The resulting suspension of disrupted yeasts was centrifuged and the pelleted cell walls were washed repeatedly with distilled water. The cell walls were treated with trypsin and extracted with NaOH. The alkali supernatant fluid was filtered, then dialyzed against 3 daily changes of distilled water. The water insoluble glucan was removed by centrifugation, and the non-dialyzable B-ASWS was collected onto a PM10 ultrafiltration membrane (Amicon, Lexington, MA). The residue was eluted from the PM10 membrane, lyophilized and stored at -20°C.

Antigen used in the LT assay was reconstituted in RPMI 1640 to 2 mg/ml and stored at -20°C for no longer than one month. Appropriate dilutions of B-ASWS were prepared from this stock. Once thawed the unused antigen was discarded.

Immunization protocols. Three sensitization protocols were tested for their ability to elicit a protective immune response to B. dermatitidis. Immune protection was assessed by monitoring the resistance of sensitized mice to a lethal challenge of B. dermatitidis. Also, lymphocyte proliferative responses of sensitized mice were monitored using the LT assay. A nonlethal, subcutaneous (sc) infection with B. dermatitidis strain SCB-2 was produced in one group of animals (26). Yeast cells were harvested from broth cultures by centrifugation, washed 3 times with sterile saline, counted in a hemocytometer, and adjusted to a concentration of $2 \times 10^5$ yeast particles/ml in saline. The sc infection was

established by injecting 0.1 ml of the yeast cell suspension into mice at two sites over the front shoulders (0.05 ml/site). Inoculation sites were culture negative 5 weeks post-infection.

A sc inoculation of Merthiolate-killed B. dermatitidis was used to immunize a second group of animals (46). Equal volumes of an antigen suspension, containing 40 mg/ml dry weight equivalent of Merthiolate killed yeast cells (strain 242) and Freunds incomplete adjuvant (Difco Laboratories, Detroit, MI) were emulsified. Mice were inoculated subcutaneously in the inguinal area with 0.1 ml of the antigen-adjuvant emulsion. Each animal received two injections of 0.1 ml 7 days apart (days 0 and 7).

A third group of mice was immunized with formalin-killed B. dermatitidis strain SCB-2. B. dermatitidis yeast cells were treated with formalin (12), washed five times with distilled water, and adjusted to a concentration of 5 mg/ml dry weight equivalent. Each animal was injected intraperitoneally with 0.5 ml of the suspension.

Protection tests. To determine if any of the immunizations elicited a protective immune response, mice from each group were challenged with a lethal dose of B. dermatitidis yeast cells. At weekly intervals, beginning one week after the last immunization injection, 10 mice from each group were challenged intravenously (iv) with 500 yeast cells. Unimmunized control mice were also challenged. Numbers of dead mice were recorded daily for 90 days after challenge. Challenge experiments were repeated at least twice.

Infection protocol. We were interested in investigating the lymphocyte blastogenic response of spleen cells from lethally infected mice. Mice were infected iv with 500 B. dermatitidis yeast cells. Mice infected in this manner

survived for an average of 16 days. Spleens were harvested 4, 8 and 12 days post-infection and the cells were prepared for use in the LT assay. Spleen cells harvested from mice given 0.1 ml of saline iv, served as controls.

Preparation of spleen cells. Spleens from normal, infected or immune mice were removed aseptically, pressed through an 80 mesh stainless steel screen (Belco Glass, Inc., NJ) and collected in Hanks Balanced Salts Solution (HBSS) $Ca^{+2}$ and $Mg^{+2}$ free. The cell suspension was passed through a very thin layer of glass wool, to remove cell clumps. The cells suspension was centrifuged at 200 x g for 10 minutes, washed 3 times with HBSS and resuspended in RPMI 1640 supplemented with 5% heat inactivated human serum and 1% antibiotic-antimycotic (Gibco, Grand Island, NY). This supplemented RPMI 1640 is referred to as complete RPMI. The cells were counted in a hemocytometer and resuspended to 5 x $10^6$ cells/ml in complete RPMI. Viability was determined by trypan blue exclusion and cell types were determined by microscopic examination of Giemsa stained smears. In all experiments, viability exceeded 95% and the relative ratios of the different cell types were unaffected by passage over glass wool.

Spleen cells from infected and immunized mice were cultured for B. dermatitidis. After the last wash with HBSS, enough spleen cells were removed for the LT assay and the remaining cells were either plated directly onto BHI agar + 5% sheep blood, or diluted 1/10 or 1/100 and then plated. B. dermatitidis was never recovered from the spleens of any infected or immunized mice.

Separation of spleen cells by adherence. Passage of spleen cells over a nylon wool column as described by Julius et al. (20), was used as a means of obtaining a cell population enriched with T cells. Two grams of nylon wool, 3 denier, type 200, (Fenwal Labs, Deerfield, IL), was boiled for 10 minutes in double

distilled water. The water was decanted and the washing procedure was repeated 4 times, then the nylon wool was dryed overnight at 37°C. The dried nylon wool was teased apart, packed into a 30 ml syringe to the 16 ml mark, wrapped and autoclaved at 110°C for 15 minutes.

Prior to cell separation, the nylon wool was thoroughly rinsed with 50 ml of prewarmed (37°C) RPMI 1640 + 5% fetal calf serum and incubated for one hour at 37°C. Before adding the spleen cell suspension, the nylon wool was rinsed with 10 ml of media and allowed to run dry. Six ml of $5 \times 10^7$ cells/ml was added and allowed to penetrate the column. One half ml of media was added to the top of the column. The column was maintained at 37°C in a humidified atmosphere of 5% $CO_2$ for 45 minutes. Nonadherent cells were eluted by washing the column with 60 ml of prewarmed RPMI 1640 + 5% FCS. Adherent cells were removed by adding cold saline (0.75% NaCl w/v) and forcibly pushing the fluid out with a plunger. Cells recovered from the column were washed three times in HBSS, resuspended in complete RPMI and used in the LT assay.

The recovery of nonadherent (T cell enriched) cells ranged from 20 to 40% of the total cells applied to the column and consisted of 80 to 90% T lymphocytes as determined by trypan blue dye exclusion after treatment with anti-Thy 1.2 (Miles Laboratories, Elkhart, IN) and complement. The remainder of the nonadherent cell population consisted of 5-15% B lymphocytes, as determined by trypan blue exclusion after treatment with anti-mouse immunoglobulin (Bionetics, Kensington, MD) and complement, and less than 2% macrophages based on nonspecific esterase staining. Adherent cells accounted for 50% of the total cells added to the column. They consisted of 70 to 85% immunoglobulin positive cells and 10 to 30% T lymphocytes, as determined by the above methods.

Viability of all cell populations exceeded 95% as determined by trypan blue dye exclusion. All antisera were used at the working concentrations specified by the manufacturer in its technical information.

In some experiments peritoneal cells from normal mice were used to replace nylon wool adherent cells. The peritoneal cavities of 5 mice were lavaged with 10 ml of HBSS and the washings were centrifuged at 200 x g for 10 minutes. The cells were washed twice with HBSS, resuspended in complete RPMI, and mixed with nylon wool nonadherent cells in a ratio of 1:10 (peritoneal:nylon wool nonadherent).

Mass cytolysis. $5 \times 10^7$ spleen cells were suspended in 5 ml of RPMI 1640 containing the appropriate dilution of either monoclonal anti-mouse Thy 1.2 (Miles Laboratories, Elkhart, IN), monoclonal anti-mouse Lyt 1.2 (New England Nuclear, Boston, MA), monoclonal anti-mouse Lyt 2.2 (New England Nuclear, Boston, MA), or rabbit anti- mouse IgG and IgM (Bionetics, Kensington, MD). The cell suspension was incubated for 30 minutes at room temperature. Following one wash with 10 ml of HBSS (200 x g, 10 min) the cells were resuspended in 5 ml of RPMI 1640 containing an appropriate dilution of rabbit serum (complement source) and incubated at 37°C. Following 30 minutes of incubation, the cells were pelleted by centrifugation (200 x g, 10 min), washed twice with HBSS and resuspended in complete RPMI. The number of viable cells was determined by trypan blue exclusion. The concentration was adjusted to $5 \times 10^6$ viable cells/ml in complete RPMI. Their proliferative response to mitogens and B-ASWS was tested using the LT assay. Cells treated in a similar manner, but without the addition of antiserum during the first incubation, served as control cells.

Lymphocyte transformation (LT) assay. The LT assay was performed using the procedure of Fromtling et al. (16). Briefly, 100 ul of the spleen cell suspension (5 x $10^6$ cells/ml) was placed into each well of a 96 well round bottom microtiter plate (Cooke, Dynatech Laboratories Inc., Alexandria, VA). An equal volume of various concentrations of mitogens and B-ASWS was added to each well. All assays consisted of 4 replicates of each experimental condition. Cultures were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 3 days for mitogen responses and for 5 days for responses to B-ASWS. Eighteen hours before termination of the culture, 0.5 uCi of [$^3$H] thymidine, specific activity of 6.7 Ci/mmol, (New England Nuclear, Boston, MA) in 10 ul of RPMI 1640 was added to each well. Cells were harvested onto glass fiber filters (M.A. Bioproducts, Walkersville, MD), with a MASH II cell harvestor (M.A. Bioproducts, Walkersville, MD) and counted in a Beckman LS-100C liquid scintillation counter. The scintillation cocktail consisted of spectrafluor PPO-POPOP (42 ml/l) (Amersham, Arlington Heights, IL) in toluene. Results are expressed as counts per minute (cpm) of experimental cultures minus cpm of background $\pm$ standard error of the mean.

All LT assays were run on at least two different occasions and spleen cell suspensions were prepared from at least 5 spleens.

Statistical methods. The Student's t-test for unpaired data was used to determine the level of significance between means of samples. p values of 0.01 or less were considered significantly different.

# CHAPTER III

## RESULTS

Preliminary experiments were conducted to determine the experimental parameters required for optimal lymphocyte blastogenesis to various mitogens. The optimal concentration of PHA was a 1:4000 dilution of the stock solution (Difco, Detroit, MI) per culture. Optimal concentrations for Con A and LPS were 0.625 ug and 5.0 ug, respectively, per culture. Cultures harvested after 3 days of in vitro incubation demonstrated the best blastogenic response for all mitogens (Table 1).

Spleen cells were routinely cultured with 2 to 3 different concentrations of each mitogen, near that found to be optimum, because of the possibility that the optimal concentration (for blastogenesis) of mitogen might change after microbial challenge.

Dose response and time course for blastogenesis of spleen cells to B-ASWS. Spleen cells from mice inoculated sc with $2 \times 10^4$ viable B. dermatitidis yeast cells three weeks prior to testing, gave a maximum blastogenic response after 5 days of in vitro culture with 5 ug of B-ASWS per culture (Table 2). Splenocytes from normal mice responded poorly to all concentrations of antigen tested. Immune cells consistently produced higher background counts than cells from control mice, as expected due to the active immunization. In all subsequent

TABLE 1. Mitogenic responses of spleen cells from normal mice at various times of in vitro incubation.[a]

| Mitogen (ug/well) | [$^3$H] thymidine incorporation[b] Days of culture | | |
|---|---|---|---|
| | 3 | 4 | 5 |
| PHA 1:1000[c] | 32,190 (2,823) | 21,987 (1,938) | 1,063 (303) |
| 1:2000 | 42,876 (744) | 35,207 (1,694) | 15,297 (1,342) |
| 1:4000 | 55,091 (2,241) | 48,244 (3,125) | 18,398 (1,296) |
| Con A (5.0) | 2,189 (153) | 1,134 (232) | 997 (87) |
| (2.5) | 8,911 (382) | 2,399 (249) | 1,921 (104) |
| (1.25) | 96,775 (3,024) | 89,254 (2,879) | 36,490 (956) |
| (0.625) | 115,548 (3,825) | 98,799 (2,879) | 32,678 (2,431) |
| LPS (5.0) | 26,126 (1,232) | 18,921 (629) | 10,357 (401) |
| (2.5) | 22,533 (578) | 15,643 (538) | 9,491 (185) |
| (1.25) | 15,381 (783) | 9,691 (681) | 6,174 (238) |

[a]Pooled spleen cells from 5 mice were used. Values are the results of a typical experiment, which was repeated at least twice.

[b]Each value, expressed as counts per minute, represents the mean (of quadruplicate determinations) of the experimental minus background counts. Numbers in parentheses are standard deviations. In all our experiments, the background counts with medium alone, for cells from normal mice, ranged from $1.5 \times 10^2$ to $3 \times 10^3$ cpm.

[c]Final dilutions in well of stock PHA (Difco).

TABLE 2. Blastogenic response of spleen cells from control and immunized mice to B-ASWS.[a]

| Type of cells | Days of culture | [$^3$H] thymidine incorporation[b] B-ASWS (ug/culture) | | | |
|---|---|---|---|---|---|
| | | 5.0 | 2.5 | 1.25 | 0.625 |
| Control | 3 | 787 (209) | 513 (136) | 440 (130) | 1,296 (322) |
| | 4 | 1,559 (86) | 1,082 (193) | 581 (72) | 2,390 (325) |
| | 5 | 1,830 (411) | 1,234 (646) | 618 (223) | 6,855 (1,481) |
| | 6 | 3,534 (79) | 1,701 (722) | 3,366 (114) | 6,663 (816) |
| Immune[c] | 3 | 14,247 (2,178) | 15,787 (3,157) | 12,663 (1,376) | 9,028 (641) |
| | 4 | 17,421 (3,277) | 18,434 (2,211) | 13,417 (3,026) | 11,797 (247) |
| | 5 | 29,233 (2,582) | 23,723 (7,716) | 23,773 (6,530) | 16,103 (1,314) |
| | 6 | 15,640 (1,717) | 10,186 (3,296) | 9,326 (2,293) | 10,957 (3,461) |

[a]Pooled spleen cells from 5 normal or 5 immunized mice were used for the assay. Values are the results of a typical experiment, that was repeated at least twice.

[b]Each value, expressed as counts per minute, represents the mean (of quadruplicate determinations) of the experimental minus background counts. Numbers in parentheses are standard deviations. Background counts with medium alone ranged from $4.25 \times 10^2$ to $2.8 \times 10^3$ cpm for control cells and from $4.1 \times 10^3$ to $5.7 \times 10^3$ cpm.

[c]Mice were immunized subcutaneously with $2 \times 10^4$ viable B. dermatitidis yeast cells and blastogenic responses were determined 3 weeks later.

experiments, antigen cultures were incubated for 5 days, and 2 to 3 concentrations of B-ASWS near the optimum were used.

Effect of various immunization protocols: Protection against challenge with viable B. dermatitidis. Since spleen cells from mice infected sc with viable B. dermatitidis proliferated in the presence of B-ASWS, we attempted to determine if these animals were actually "immune" or just "sensitized" to the organism. Two additional immunization schedules were also tested for their ability to elicit a protective immune response as well as a lymphoproliferative response to B. dermatitidis antigen.

At weekly intervals after immunization, mice were challenged with approximately 500 colony-forming units (CFU) of B. dermatitidis. Prolonged survival after the iv challenge was equated with protection.

Mice, immunized sc with viable B. dermatitidis, demonstrated some protection by 1 week postimmunization (Figure 1). The first deaths of control animals occurred 14 days after iv challenge, and 100% of the control mice were dead by day 18. The first deaths of immunized mice occurred 24 days after iv challenge and 100% of these animals were dead by day 26. By the second week after immunization, protection was evident. All control mice were dead by day 14, whereas only 10% of the immunized mice died by day 50; 60% were dead by day 73. When the experiment was terminated 90 days after iv challenge, 40% of the immunized mice remained alive (Figure 2). With a three week delay after immunization, only 50% of the mice died during the course of the 90 day experiment, whereas all of the control mice died by day 14 (Figure 3). Immunized mice challenged at weeks 4, 5, and 6 (Figures 4,5,6) post-immunization, all survived the iv challenge. No deaths occurred during the 90 day experiment. One

Figure 1. Effect of prior sensitization on survival after iv challenge with B. dermatitidis: 1 week post-sensitization. (●) mice inoculated sc with viable B. dermatitidis, ( ■ ) mice inoculated ip with formalin killed B. dermatitidis, (○) normal mice, not sensitized to B. dermatitidis.

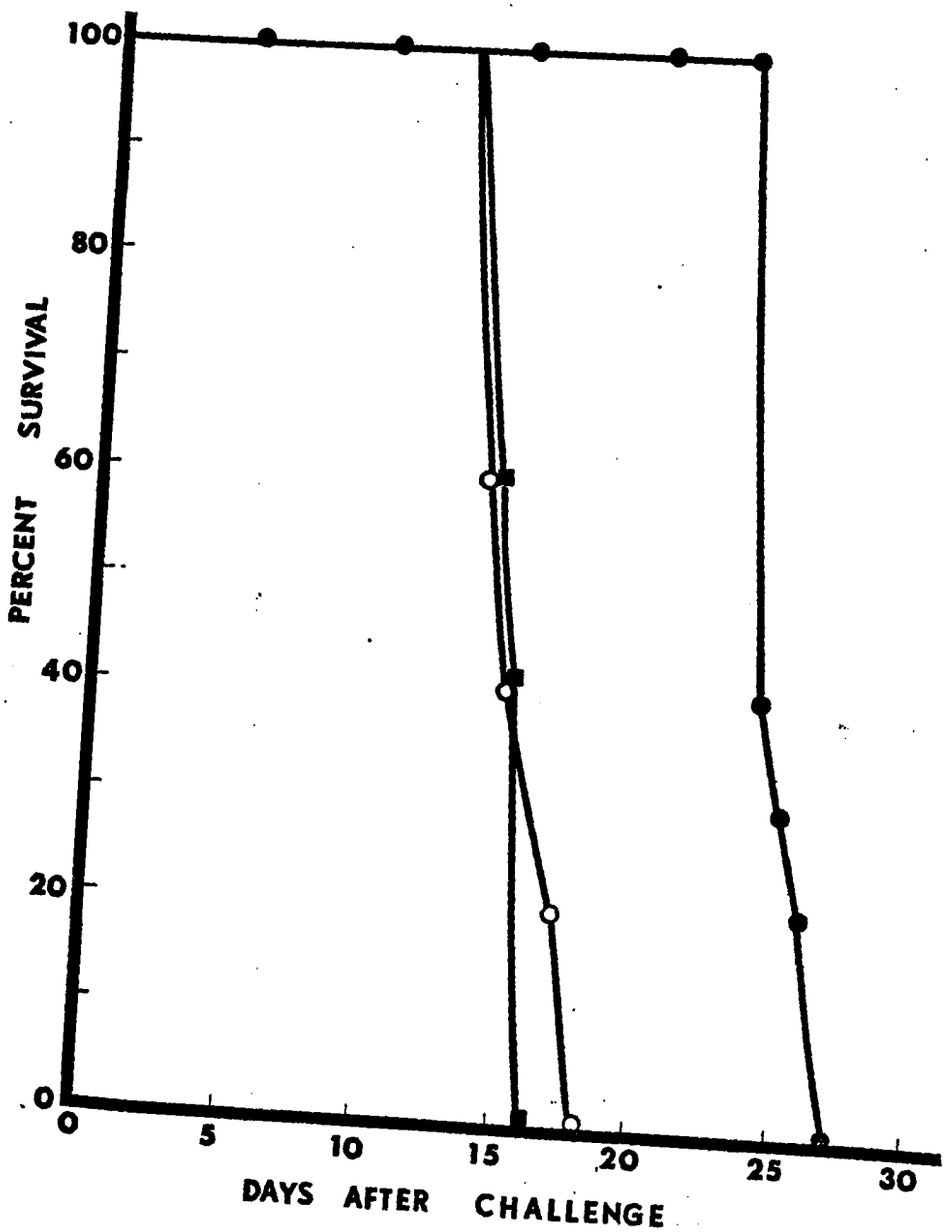

100
80
60
40
20
0
PERCENT SURVIVAL
0
5
10
15
20
25
30
DAYS AFTER CHALLENGE

Figure 2. Effect of prior sensitization on survival after iv challenge with B. dermatitidis: 2 weeks post-sensitization. ( □ ) mice inoculated sc with merthiolate killed B. dermatitidis. See Figure 1 for legend description.

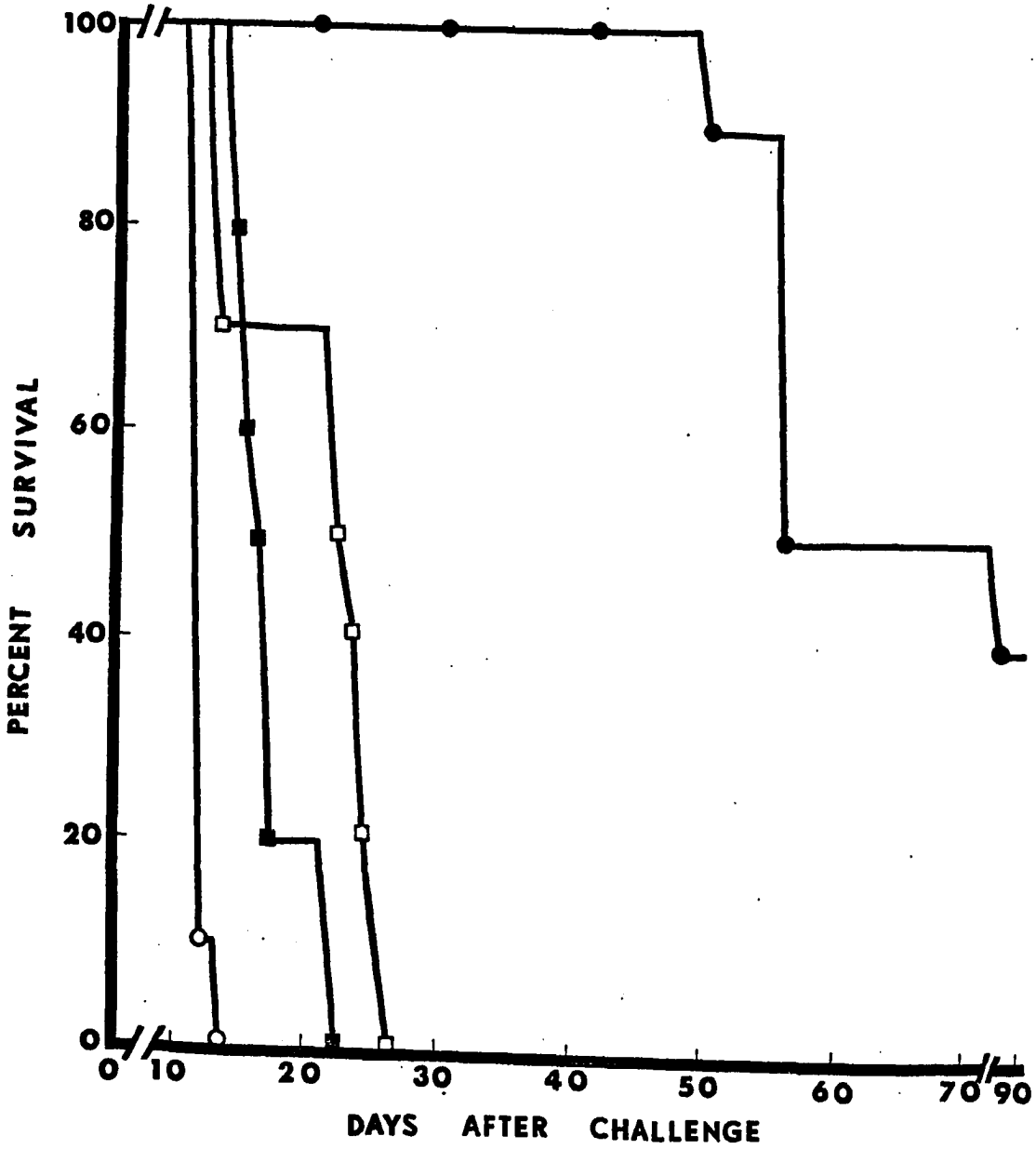

100
80
60
40
20
0
PERCENT SURVIVAL
0
10
20
30
40
50
60
70
90
DAYS AFTER CHALLENGE

**Figure 3. Effect of prior sensitization on survival after iv challenge with B. dermatitidis: 3 weeks post-sensitization. See Figures 1 and 2 for legend.**

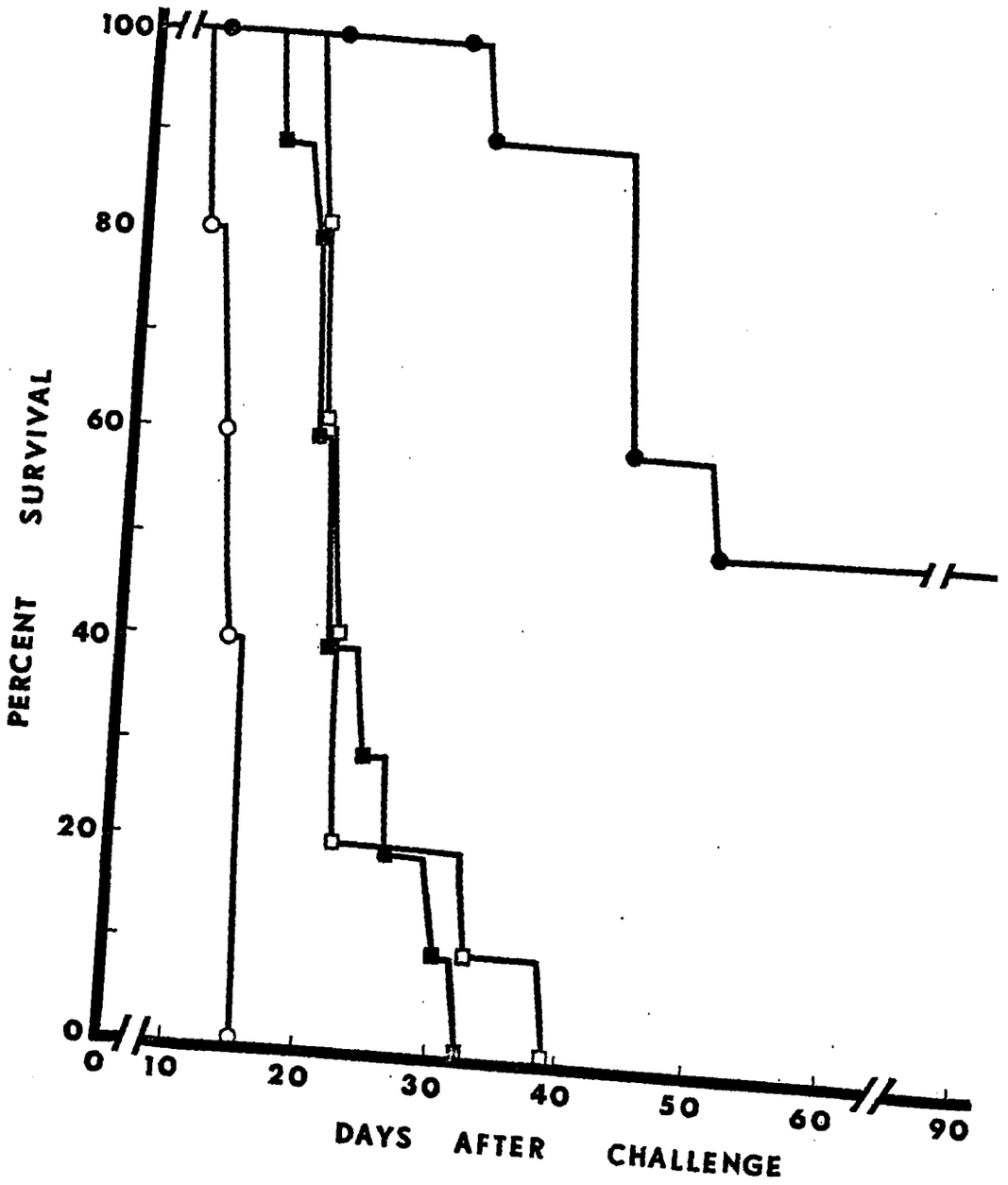

100
80
60
40
20
0
PERCENT SURVIVAL
0
10
20
30
40
50
60
90
DAYS AFTER CHALLENGE

Figure 4. Effect of prior sensitization on survival after iv challenge with B. dermatitidis: 4 weeks postsensitization. See Figure 1 for legend.

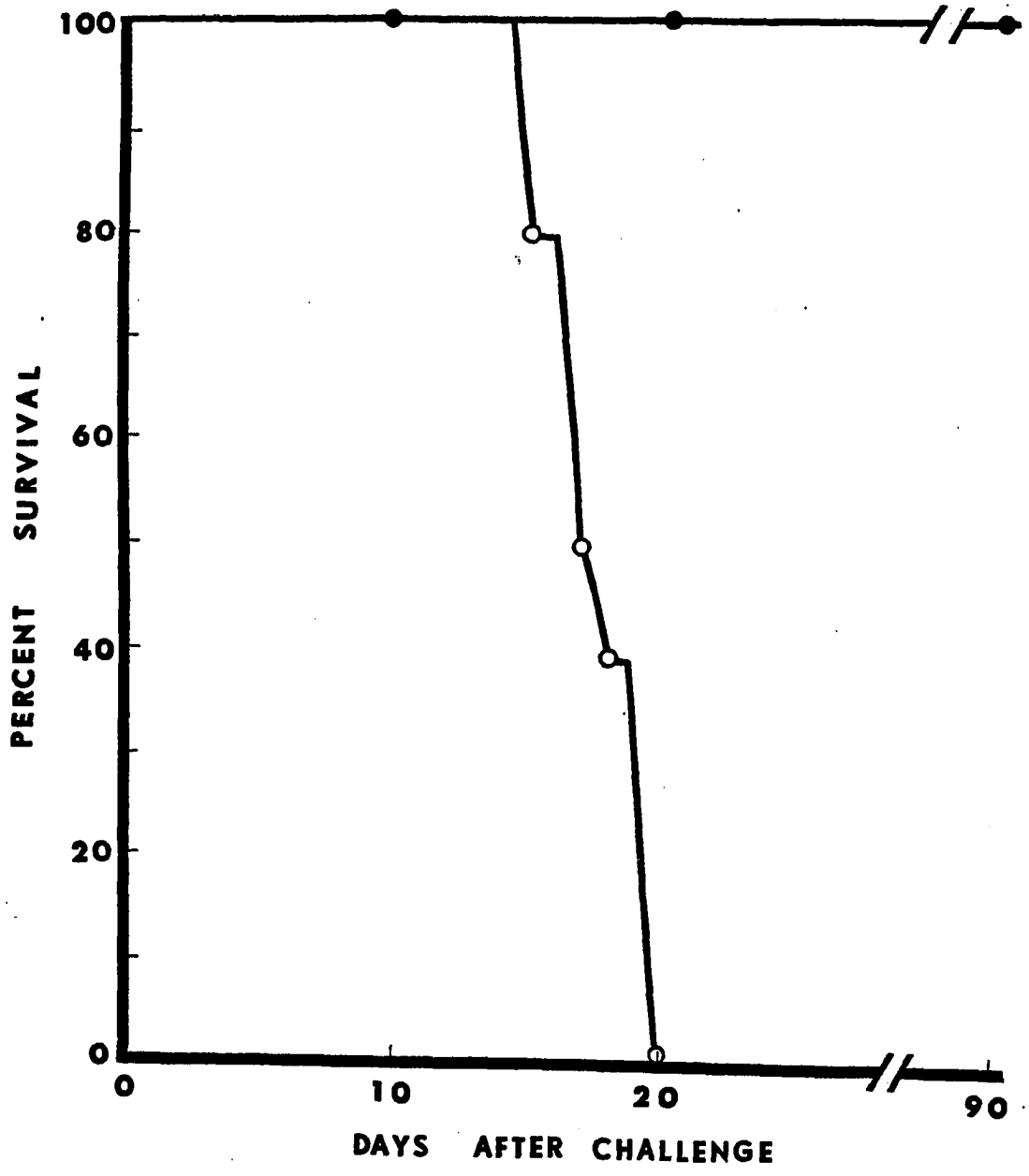

100
80
60
40
20
0
PERCENT SURVIVAL
0
10
20
90
DAYS AFTER CHALLENGE

Figure 5. Effect of prior sensitization on survival after iv challenge with B. dermatitidis: 5 weeks post-sensitization. See Figure 1 for legend.

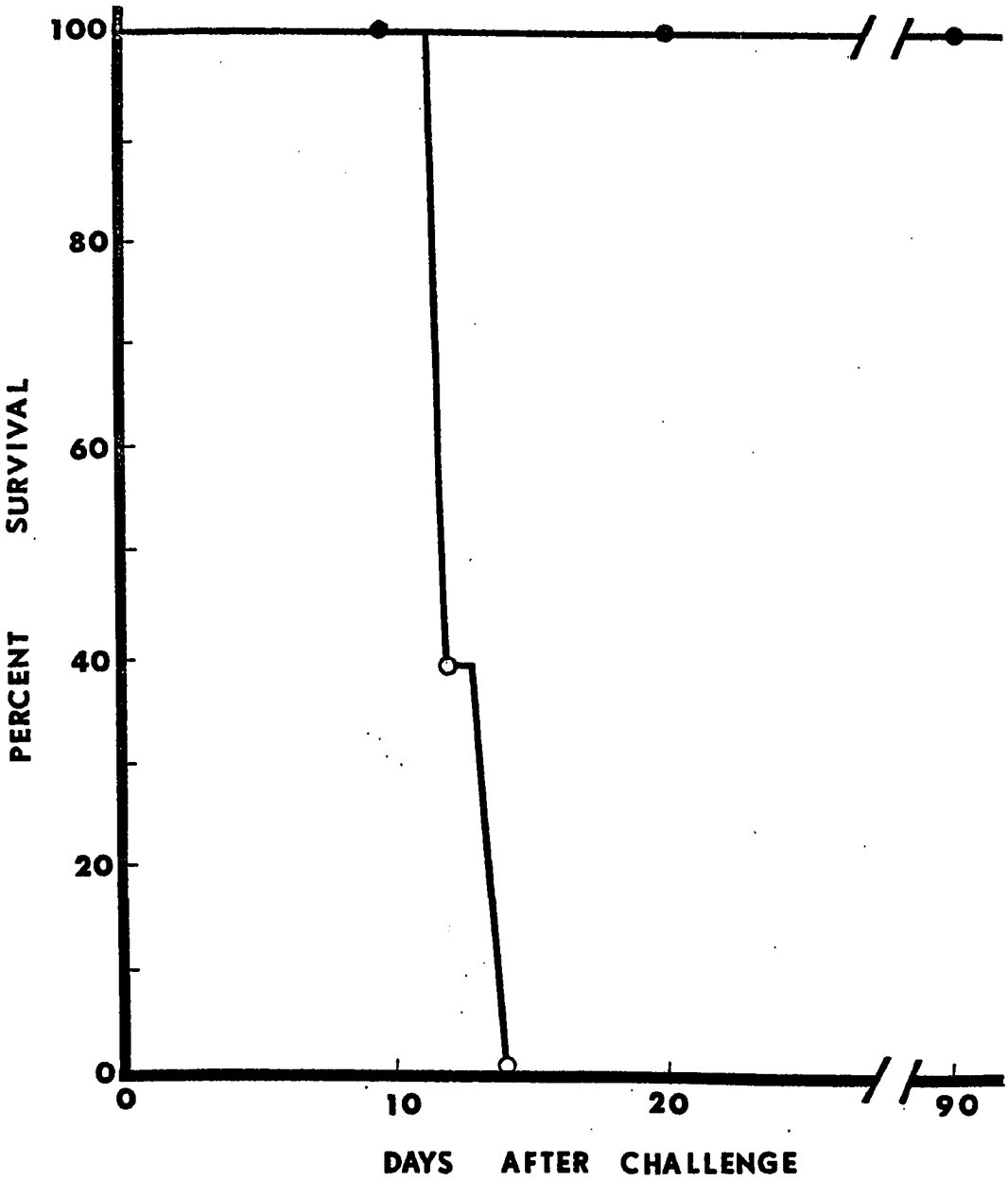
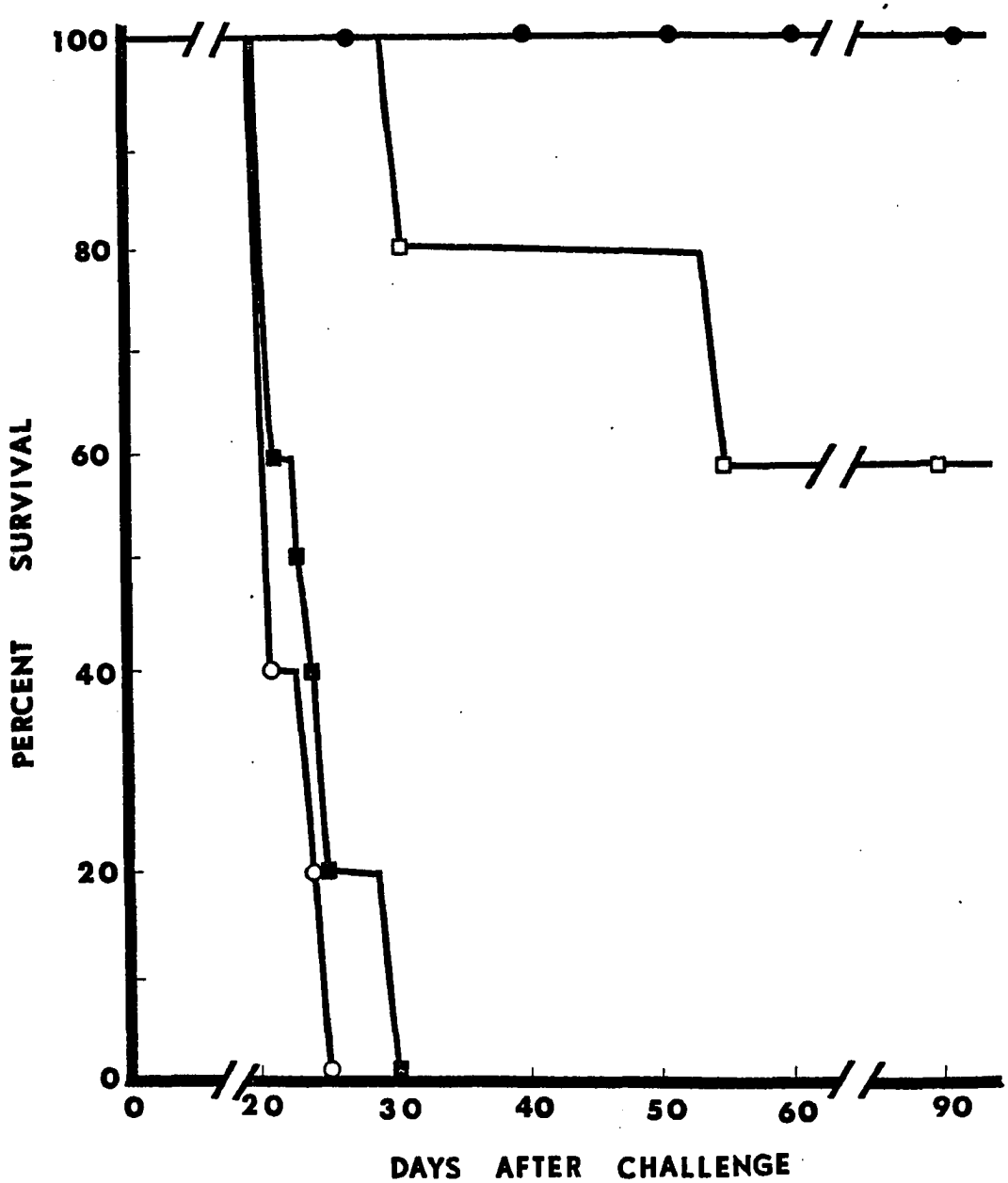

100
80
60
40
20
0
PERCENT SURVIVAL
0
10
20
90
DAYS AFTER CHALLENGE

Figure 6. Effect of prior sensitization on survival after iv challenge with B. dermatitidis: 6 weeks post-sensitization. See Figures 1 and 2 for legends.


100
80
60
40
20
0
PERCENT SURVIVAL
0
20
30
40
50
60
90
DAYS AFTER CHALLENGE

hundred percent of the control mice were dead by days 20, 13 and 25, respectively.

Mice that had been immunized sc with merthiolate killed B. dermatitidis showed some protection at weeks 2 and 3 after their first immunization (Figures 2,3). Although some immunized animals died on approximately the same day as controls, many survived longer. However, by 6 weeks post-immunization, only 20% of the immunized mice had died by day 30, and 60% remained alive at the termination of the 90 day experiment (Figure 6).

Mice immunized ip with formalin-killed B. dermatitidis showed no protection by week one (Figure 1), slight protection by weeks 2 and 3 (Figures 2,3) and were not protected at all 6 weeks after immunization (Figure 6).

Table 3 is a summary of the results presented in Figures 1-6. The mean survival time for each group of animals was calculated. The development of resistance of each group of sensitized mice to a lethal iv challenge of B. dermatitidis can be determined. Also, the persistance of the protective immune response elicited by the immunization protocols can be seen.

Effect of various immunization protocols: lymphocyte blastogenic response to B-ASWS. Spleen cells from control mice and from the various groups of immunized mice were tested for their ability to proliferate in the presence of B-ASWS (Figure 7). Spleen cells from all groups of sensitized mice demonstrated significant ($p < .001$) proliferation to B-ASWS compared to normal cells by one week post-immunization. The lymphoproliferative response of spleen cells from mice infected sc with viable B. dermatitidis remained high throughout the 6 week study; whereas the blastogenic response of spleen cells from mice immunized with either merthiolate or formalin killed B. dermatitidis waned by week 6.

TABLE 3. Mean survival time (days) of immunized mice after an iv challenge with *B. dermatitidis* yeast cells.[a]

| Type of Immunization | Weeks Post-immunization[b] | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| none[c] | 15.6 | 12.1 | 13.6 | 15.9 | 12.8 | 23.0 |
| sc live | 24.9 | 70.1 | 66.0 | > 90 | > 90 | > 90 |
| sc merthiolate killed | ND[d] | 21.4 | 21.6 | ND | ND | 70.8 |
| ip formalin killed | 14.8 | 17.9 | 22.8 | ND | ND | 24.1 |

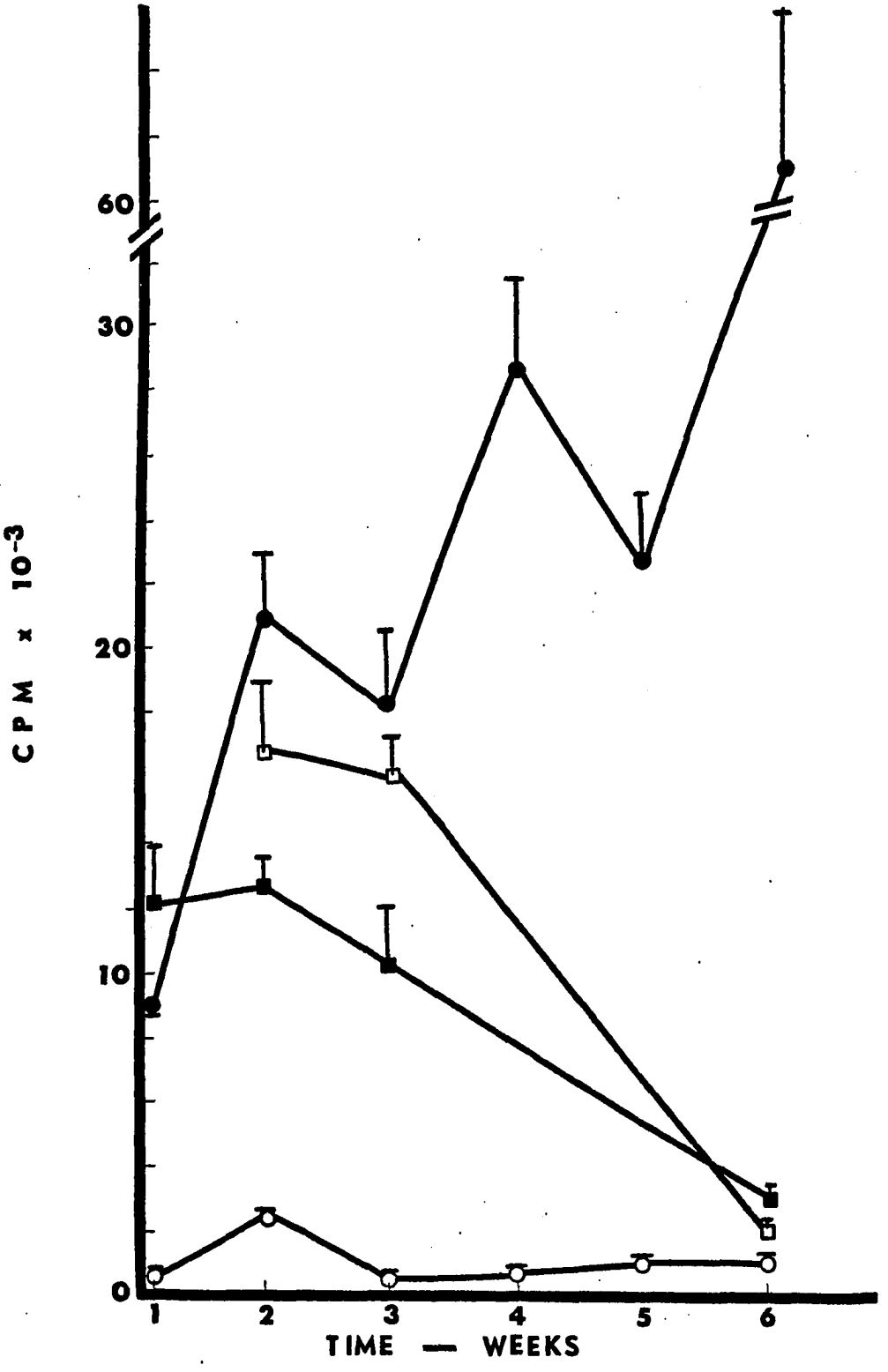
[a]This is a summary table of Figures 1-6. The mean survival time was calculated by determining the total number of days survived by the 10 mice in each group, and dividing the total by 10.

[b]Ten mice per group were challenged iv with approximately 500 *B. dermatitidis* yeast cells at weekly intervals after immunization.

[c]Control mice, not sensitized in any way.

[d]Not done.

Figure 7. Blastogenic response of spleen cells from normal and sensitized mice to B-ASWS. Pooled spleen cells from 5 mice for each time interval and sensitization protocol were used. Each value represents the mean of quadruplicate determinations of the experimental minus the background counts. Results are from a typical experiment repeated at least twice. ( ● ) mice inoculated sc with viable B. dermatitidis, ( □ ) mice inoculated sc with merthiolate killed B. dermatitidis, (■) mice inoculated ip with formalin killed B. dermatitidis, (O) normal mice, not sensitized to B. dermatitidis.


CPM x 10⁻³
60
30
20
10
0
1
2
3
4
5
6
TIME — WEEKS

Blastogenic response of spleen cells from normal and immunized mice to PHA, Con A and LPS at various times after immunization. One week after immunization, the mean blastogenic responses to PHA, Con A and LPS by spleen cells from immunized mice were significantly depressed (26%, $p<.01$; 20%, $p<001$; 36%, $p<.002$; % decrease, respectively), when compared to the response of normal mice (Figure 8). Two weeks after immunization, the blastogenic response to PHA was no longer depressed. However, the proliferative response of spleen cells from immunized mice to Con A remained depressed at weeks 2 and 3 post-immunization (34%, $p<.001$; 36%, $p<.001$, respectively), but by week 4 it was not significantly different from that of controls. The response of spleen cells from immune mice to stimulation by LPS was also depressed. The counts remained significantly lower than those of controls at weeks 2, 3, and 4 post-immunization (48%, $p<.001$; 60%, $p<.001$; 47%, $p<.002$, respectively), but returned to normal levels by week 5. Five weeks after immunization, the proliferative responses of spleen cells from immunized mice to all mitogens were similar to control values. Thus, subcutaneous infection did result in nonspecific temporary suppression of T and B cell responsiveness.

Blastogenic responses of spleen cells from lethally infected mice to PHA, Con A and LPS. Mice were inoculated iv with a dose of B. dermatitidis yeast cells that induced a lethal infection by days 15 to 20. Proliferation of spleen cells from these mice to mitogens was depressed, when compared to that of spleen cells from normal mice. The blastogenic response of cells from infected mice to PHA was significantly lower than control values at days 4, 8 and 12 postinfection (36%, $p<.002$; 39%, $p<.001$; 36%, $p<.004$; respectively) (Figure 9). Likewise, the blastogenic response to Con A at 4, 8 and 12 days after infection

Figure 8. Mitogenic responses of spleen cells from normal mice and mice infected sc with 2 x $10^4$ viable B. dermatitidis yeast cells, to PHA (1:4000/well), Con A (0.625ug/well) and LPS (2.5ug/well). Pooled spleen cells from 5 normal and 5 sensitized mice were tested at weekly intervals following sensitization. Each value represents the mean of quadruplicate determinations of the experimental minus the background counts. Results are from a typical experiment repeated at least twice. (●——●) PHA-normal, (● • • ●) PHA-sensitized, (■——■) Con A-normal, (■ • • • ■) Con A-sensitized, (○——○) LPS-normal, (○ • • • ○) LPS-sensitized.

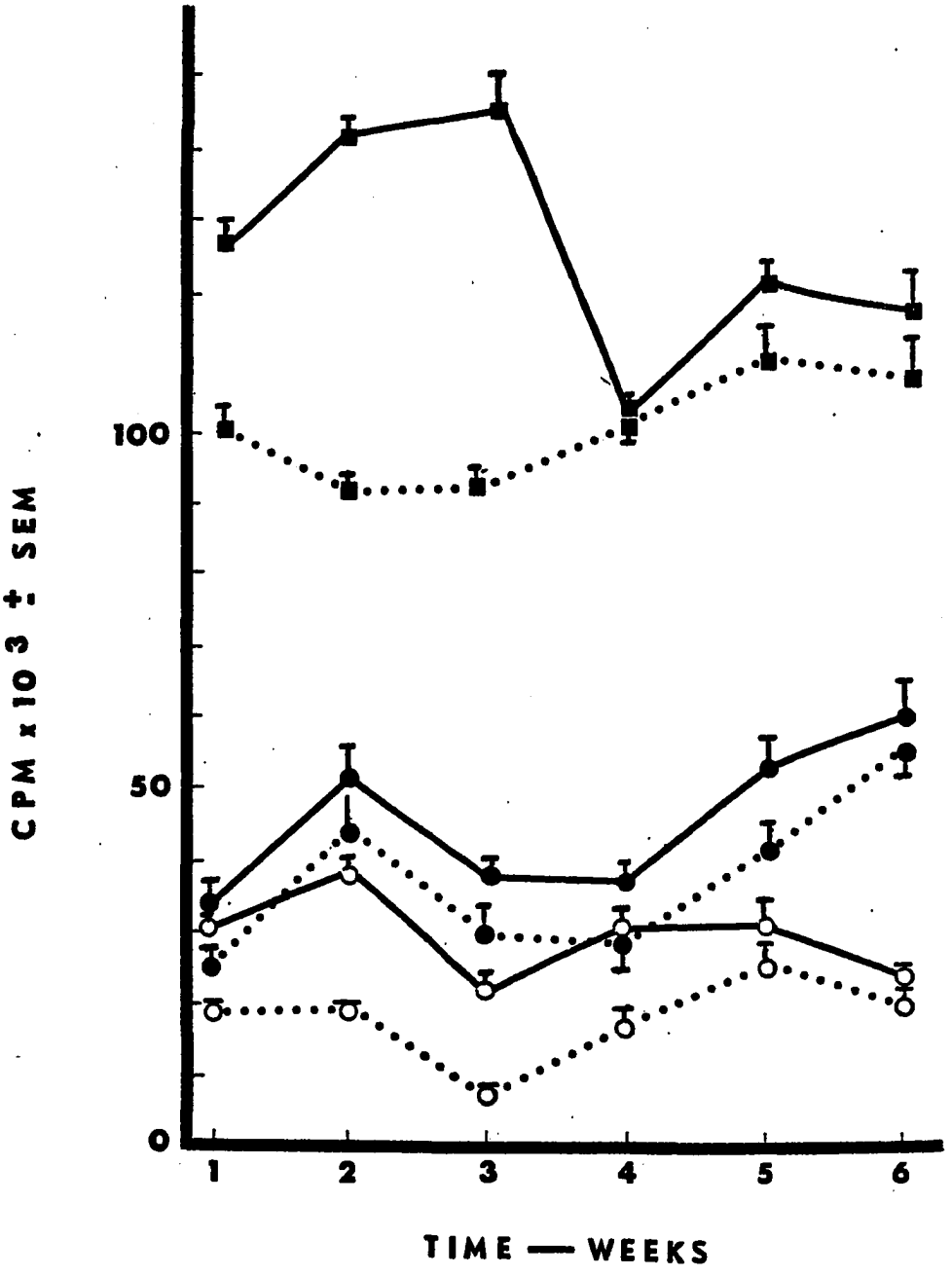

CPM x 10 3 ± SEM
100
50
0
1
2
3
4
5
6
TIME — WEEKS

Figure 9. Mitogenic response of spleen cells to PHA (1:4000/well) at various times following an iv inoculation. Mice were inoculated iv. with approximately 500 CFU of B. dermatitidis. Spleen cells were harvested at 4, 8 and 12 days after infection. Results are expressed as cpm of experimental cultures minus background counts.

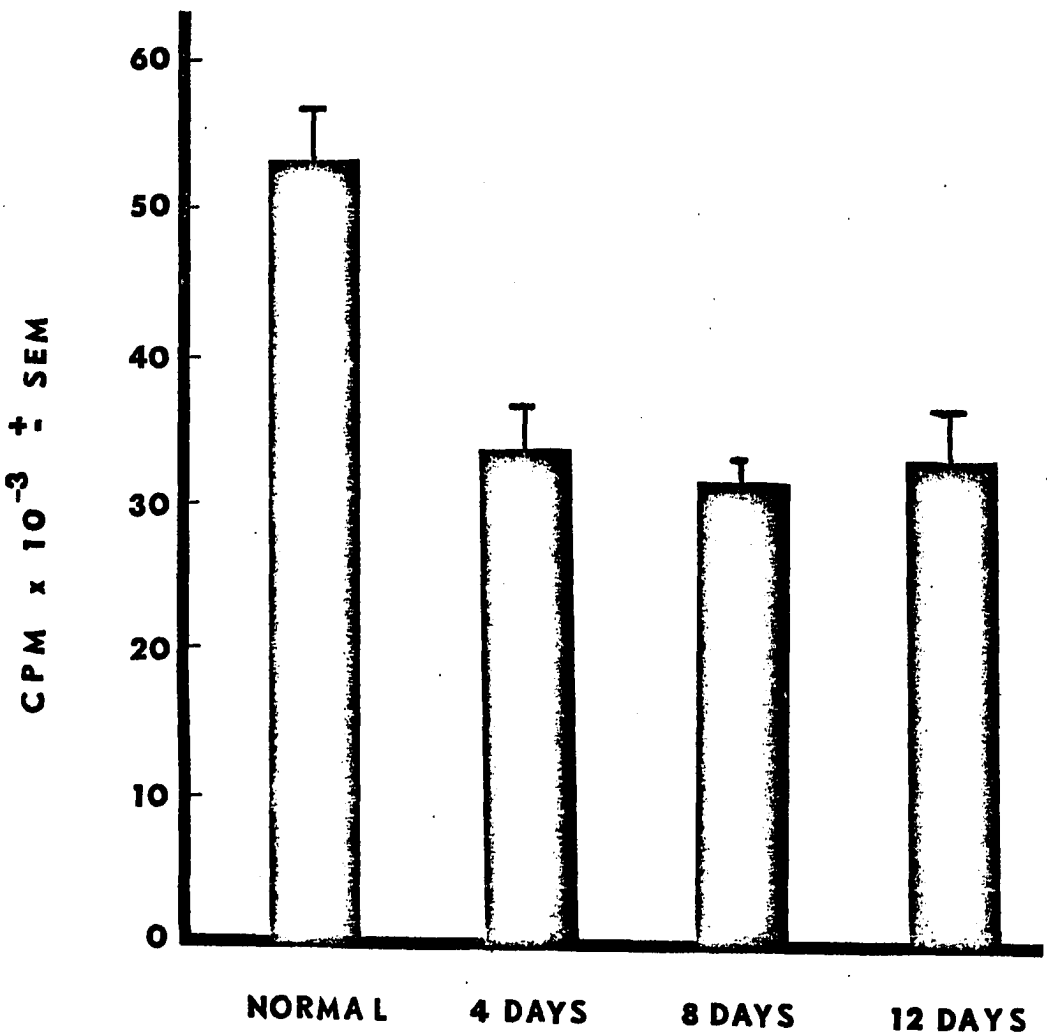

60
50
40
30
20
10
0
CPM x 10$^{-3}$ ± SEM
NORMAL
4 DAYS
8 DAYS
12 DAYS

Figure 10. Mitogenic response of spleen cells to Con A (0.625ug/well) at various times following an iv inoculation. Mice were inoculated iv with approximately 500 CFU of B. dermatitidis. Spleen cells were harveted at 4, 8 and 12 days after infection. Results are expressed as cpm of experimental cultures minus background counts.

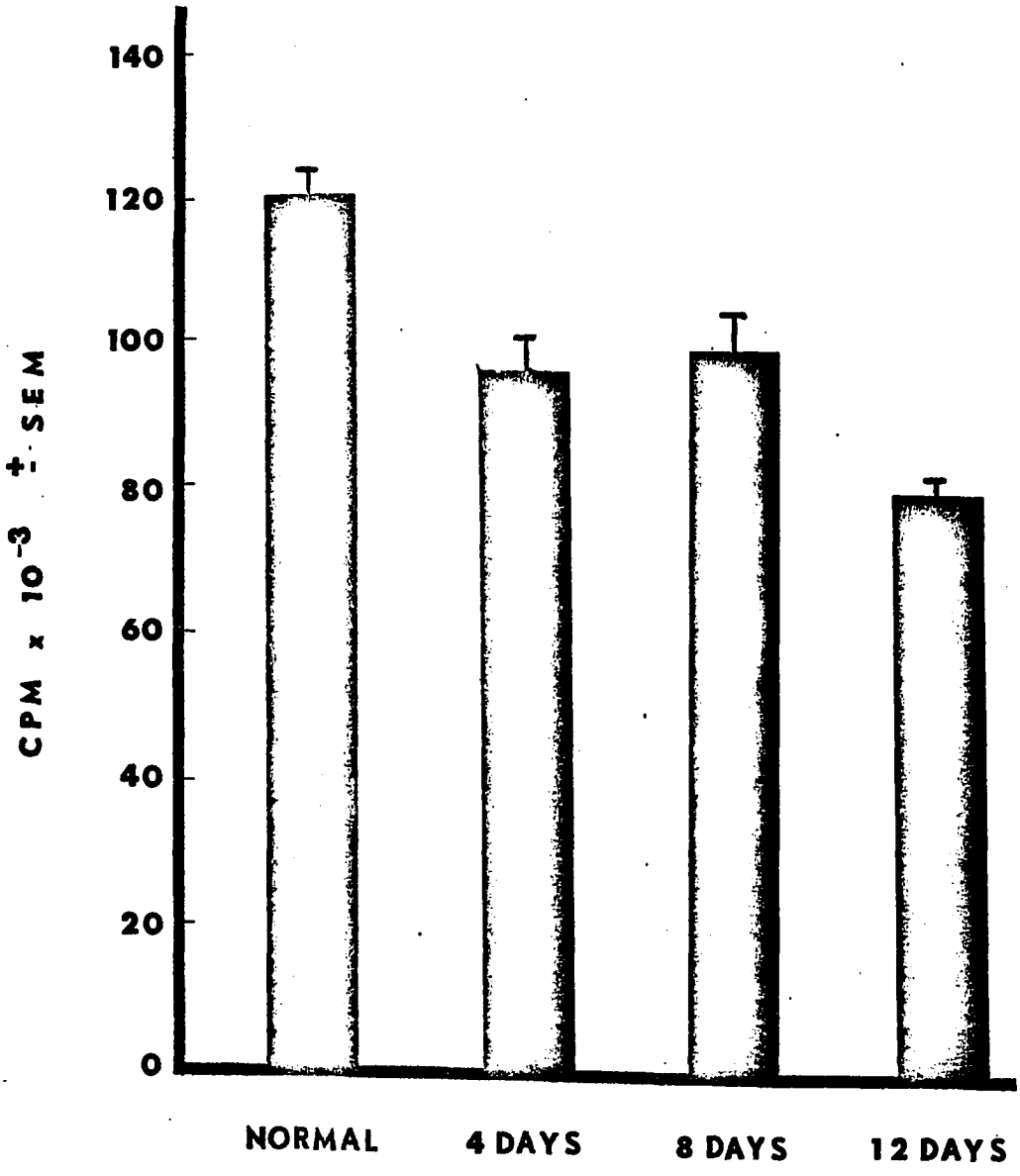

140
120
100
80
60
40
20
0
CPM x $10^{-3}$ ± SEM
NORMAL
4 DAYS
8 DAYS
12 DAYS

Figure 11. Mitogenic response of spleen cells to LPS (2.5ug/well) at various times following an iv inoculation. Mice were inoculated iv with approximately 500 CFU of B. dermatitidis. Spleen cells were harvested at 4, 8 and 12 days after infection. Results are expressed as cpm of experimental cultures minus background counts.

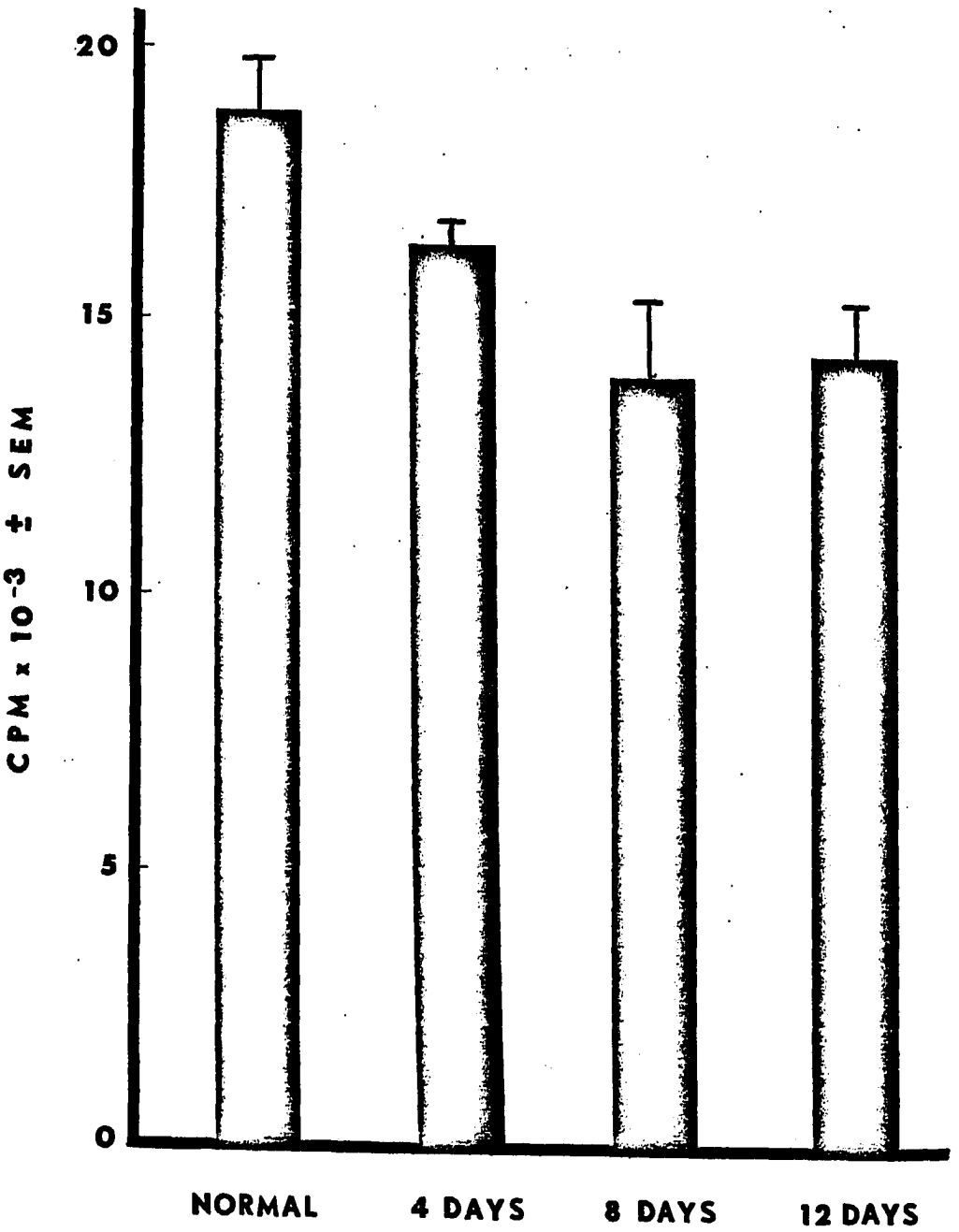

20
15
10
5
0
CPM x 10-3 ± SEM
NORMAL
4 DAYS
8 DAYS
12 DAYS

Figure 12. Mitogenic response to Con A (0.625ug/well) of mixtures of spleen cells from normal and iv inoculated mice. Spleen cells were harvested at 4, 8 and 12 days after infection. Normal cells were mixed 1:1 with cells from infected mice (i.e. $5 \times 10^5$ normal cells and $5 \times 10^5$ cells from infected mice/well). Results are expressed as cpm of experimental cultures minus background counts.

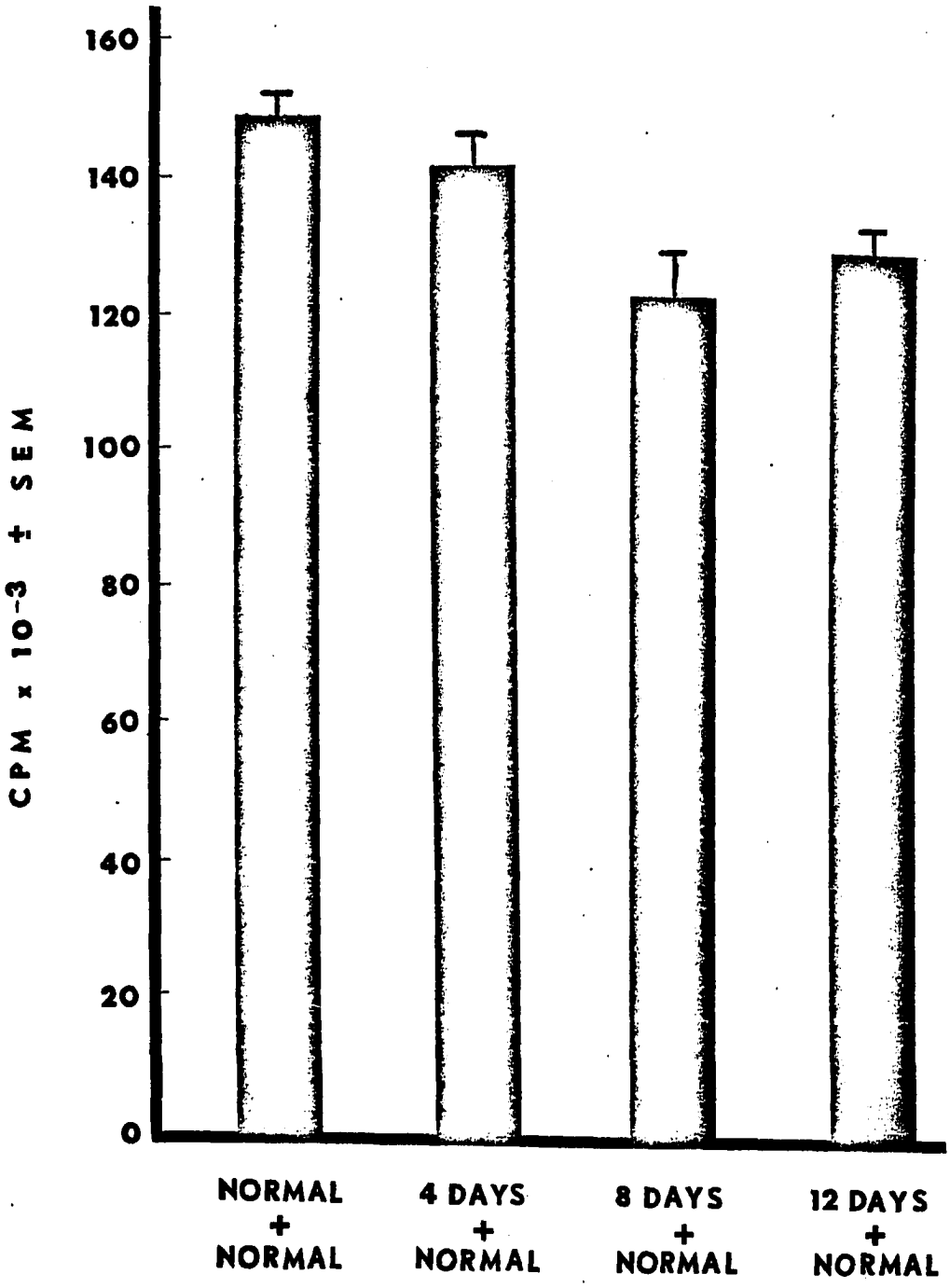
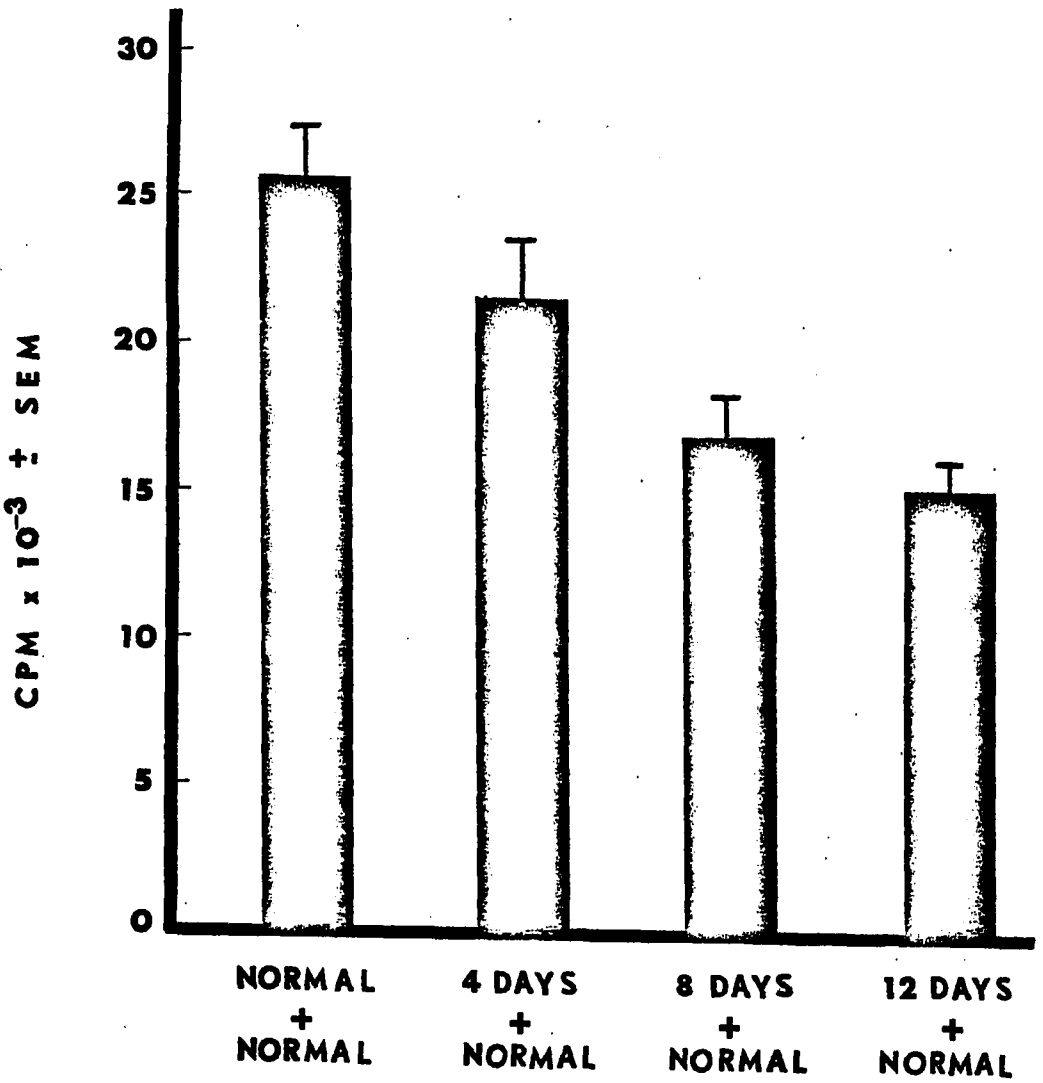

CPM x 10-3 ± SEM
160
140
120
100
80
60
40
20
0
NORMAL + NORMAL
4 DAYS + NORMAL
8 DAYS + NORMAL
12 DAYS + NORMAL

Figure 13. Mitogenic response to LPS (2.5ug/well) of mixtures of spleen cells from normal and iv inoculated mice. Spleen cells were harvested at 4, 8 and 12 days after infection. Normal cells were mixed 1:1 with cells from infected mice. Results are expressed as cpm of experimental cultures minus background counts.


30
25
20
15
10
5
0
CPM x $10^{-3}$ ± SEM
NORMAL + NORMAL
4 DAYS + NORMAL
8 DAYS + NORMAL
12 DAYS + NORMAL

was significantly less than control values (20%, $p<.001$; 19%, $p<.003$; 33%, $p<.001$, respectively) (Figure 10). Four days after infection, the blastogenic response of spleen cells to LPS was not significantly altered, however by 8 days postinfection, the response was lower than normal values (26%, $p<.006$) and remained depressed at 12 days postinfection (23%, $p < .004$) (Figure 11). Mitogen responses and response patterns at the different time intervals, were similar with both concentrations of mitogens tested.

Effect of splenocytes from infected mice on the lymphocyte blastogenic responses of cells from normal mice. Mixed populations of cells were assayed to determine if a population(s) of cells was present in the spleens of lethally infected mice that could suppress the proliferative responses of normal spleen cells to mitogens. The lymphoproliferative response to mitogens of normal spleen cells mixed with spleen cells taken from infected mice at days 4, 8 and 12 of the disease, was compared to that of normal cells. The proliferative response to PHA was not significantly affected by mixing the two cell populations (Data not shown). There was a significant decrease in the response to Con A, when cells from 8 or 12 day infected mice were mixed with normal spleen cells (17%, $p< .002$; 13%, $p < .003$, respectively), but no significant alteration was observed with mixtures of cells from 4 day infected mice (Figure 12). Since the response of mixed cells to Con A is not as depressed as that of unmixed cells from infected mice (Figure 10), it is unlikely that the depressed response is due to a population(s) of suppressor cells. However, it cannot be ruled out that a suppressor cell population might be involved. The lymphoproliferative response to LPS was significantly altered, when spleen cells from normal and infected mice were mixed (Figure 13). The suppression was not significant if spleen cells from 4

day infected mice were mixed with normal cells. However, the mixing of cells from either 8 or 12 day infected mice with normal cells significantly decreased the blastogenic response to LPS (34%, $p<.001$; 40%, $p<.001$, respectively). This response is comparable to that observed with cells from infected mice alone (Figure 11).

Development of antigen induced lymphoproliferative response of spleen cells from lethally infected mice. Mice lethally infected with B. dermatitidis developed lymphoproliferative responsiveness to B-ASWS, even though they eventually died from the disease (approximately 15 to 20 days post-infection). Responsiveness of spleen cells to B-ASWS was evident 4 days after infection, and was significant ($p< .001$) at 8 and 12 days postinfection (Figure 14). Responsiveness of spleen cells from lethally infected mice to B-ASWS was comparable to that of spleen cells from mice infected sc with viable B. dermatitidis. Thus, even though iv infected mice eventually died, they were able to respond specifically to B. dermatitidis antigen up to 3 to 5 days before death.

Characterization of the spleen cell popoulation responsible for the lymphoproliferative responsiveness to B-ASWS. To characterize the cell type responsible for the lymphoproliferative response to B-ASWS, spleen cells from normal mice and mice infected iv with B. dermatitidis, were fractionated on nylon columns or lysed with specific antisera and complement.

Cells from nylon separated fractions, nylon-passed and nylon-adherent, were tested for their proliferative responsiveness to B-ASWS (Figure 15). The nylon-passed cell fraction, enriched for T lymphocytes, proliferated in the presence of B-ASWS, but not as well as the unfractionated cells. Some proliferation to B-ASWS was also observed with the nylon adherent cells. Nylon

Figure 14. Blastogenic response of spleen cells to B-ASWS at various times following and iv inoculation. Mice were infected iv with approximately 500 CFU of B. dermatitidis. Spleen cells were harvested at 4, 8 and 12 days after infection. Results are expressed as cpm of experimental cultures minus background counts.

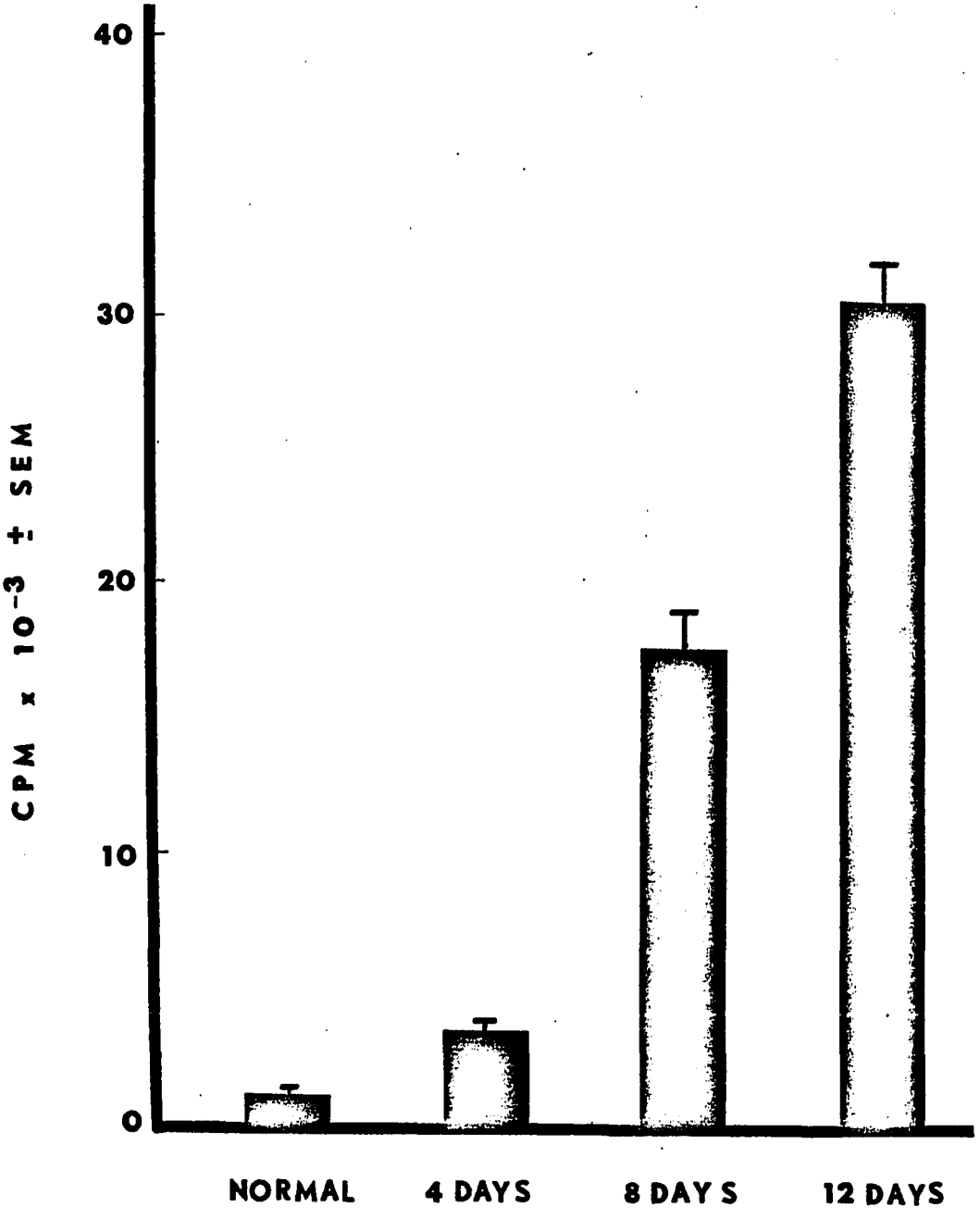
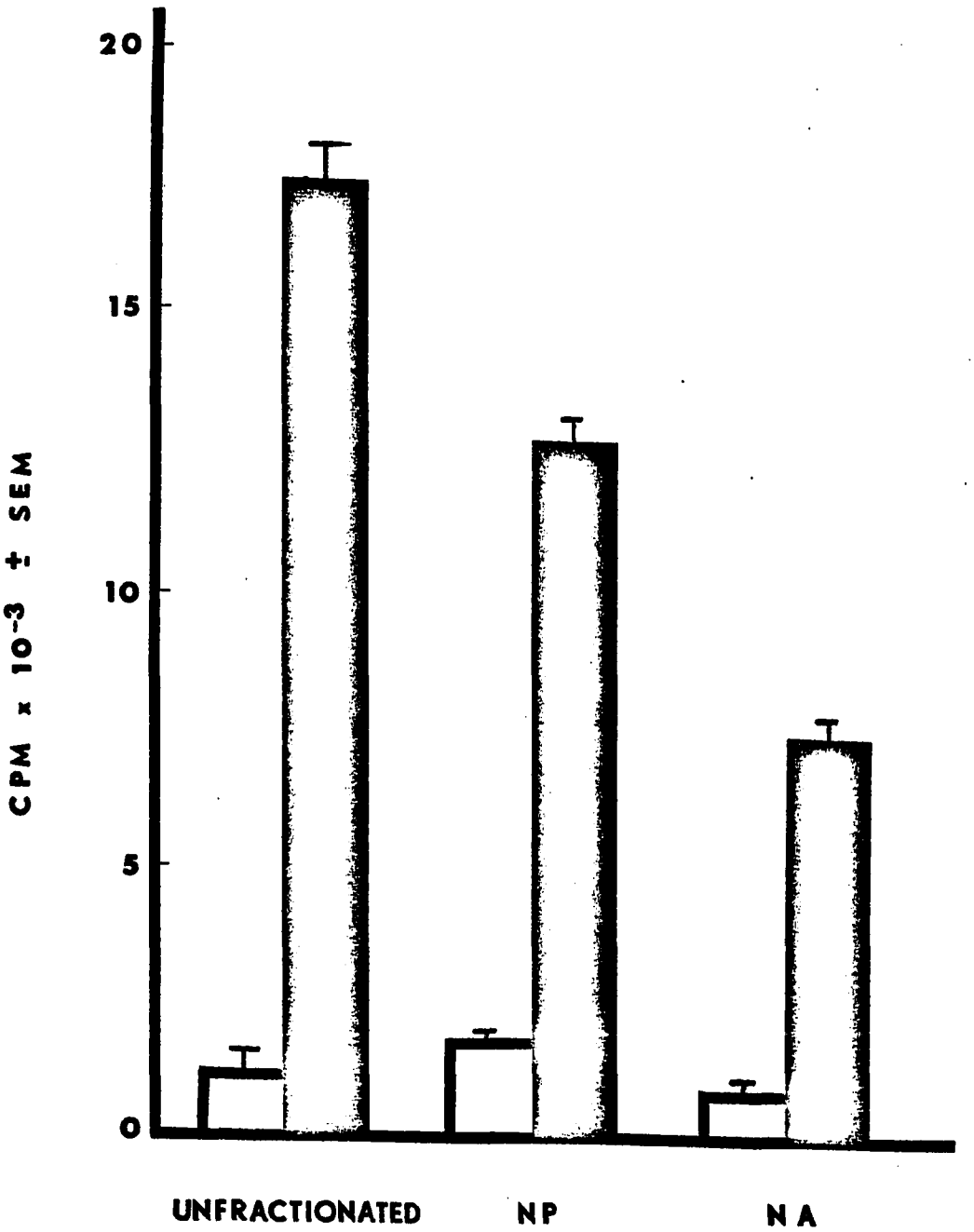

40
30
20
10
0
CPM x 10⁻³ ± SEM
NORMAL
4 DAYS
8 DAYS
12 DAYS

Figure 15. Blastogenic response of spleen cells from normal ( □ ) and 8 day iv inoculated mice ( ■ ), to B-ASWS, following passage over a nylon wool column. NP (nylon passed cell population), NA (nylon adherent cell population). Results are expressed as cpm of experimental cultures minus background counts. Results are expressed as cpm of experimental cultures minus background counts.


20
15
10
5
0
CPM x 10-3 ± SEM
UNFRACTIONATED
NP
NA

fractionated spleen cells from normal mice did not proliferate in the presence of B-ASWS.

To verify the efficacy of the separation procedure, the fractionated cell populations were tested for their mitogenic responsiveness to a T cell mitogen (PHA) and a B cell mitogen (LPS) (Table 4). Mitogenic responses of fractionated cells indicate that the T cell enriched fraction (nylon-passed) was contaminated with LPS responding cells. Likewise, the B cell enriched fraction (nylon adherent) was contaminated with PHA responding cells. Cross contamination of the fractions was also shown by specific antisera and complement lysis (see Materials and Methods).

In an attempt to restore the proliferative response of nylon-passed cells to that of unfractionated cells, peritoneal cells from normal mice were added to the nylon-passed fraction at a ratio of 1:10 (peritoneal cells:nylon-passed cells). Background counts (counts from cultures without B-ASWS) resulting from this mixture were often greater than experimental counts, thus the data were not interpretable (data not shown).

To characterize the responding cell population on the basis of membrane characteristics, certain populations of spleen cells from either 8 day infected or normal mice were removed by treatment with specific antisera and complement. The remaining cells were tested for their responsiveness to mitogens and B-ASWS. Mitogen responses were used to determine the effectiveness of the cytolysis procedure (Table 5). Spleen cells treated with anti-immunoglobulin proliferated poorly in the presence of LPS, but were more reactive to PHA than untreated cells. Treatment with anti-Thy 1.2 eliminated spleen cell responsiveness to PHA, while somewhat enhancing the response to LPS.

TABLE 4. Mitogenic responses of spleen cell subpopulations separated with nylon columns.[a]

| Cell fraction | Cell source | [$^3$H] thymidine incorporation[b] PHA[c] | LPS[d] |
|---|---|---|---|
| unfractionated | normal | 48,703 (2,451) | 23,227 (1,158) |
| | infected[e] | 35,921 (1,957) | 15,431 (938) |
| nylon passed | normal | 64,841 (3,662) | 7,787 (309) |
| | infected | 58,392 (2,921) | 8,543 (592) |
| nylon adherent | normal | 28,794 (2,252) | 36,441 (1,051) |
| | infected | 21,958 (1,833) | 31,157 (1,276) |

[a]Values are the results of a typical experiment, that was repeated at least twice.

[b]Each value, expressed as count per minute, represents the mean (of quadruplicate determinations) of the experiment minus background counts. Number in parantheses are standard deviations.

[c]1:4000/well.

[d]2.5ug/well.

[e]Mice were infected iv with approximately 500 CFU of B. dermatitidis yeast cells. Spleen cells were harvested 8 days after infection.

TABLE 5. Mitogenic responses of spleen cell populations separated by mass cytolysis.[a]

| Cell treatment | [$^3$H]thymidine incorporation[b] | |
|---|---|---|
| | PHA[b] | LPS[b] |
| none | 46,962 (2,841) | 16,491 (1,054) |
| anti-immunoglobulin[c] | 58,877 (3,364) | 5,372 (373) |
| anti-Thy 1.2 | 2,783 (837) | 21,567 (635) |
| complement control[d] | 42,423 (2,488) | 14,876 (956) |

[a]Pooled spleen cells from 10 mice were used. Spleen cells were harvested from mice that had been infected iv with approximately 500 CFU of B. dermatitidis yeast cells, 8 days prior to experimentation. Similar results were obtained with spleen cells from normal mice.

[b]See table 3.

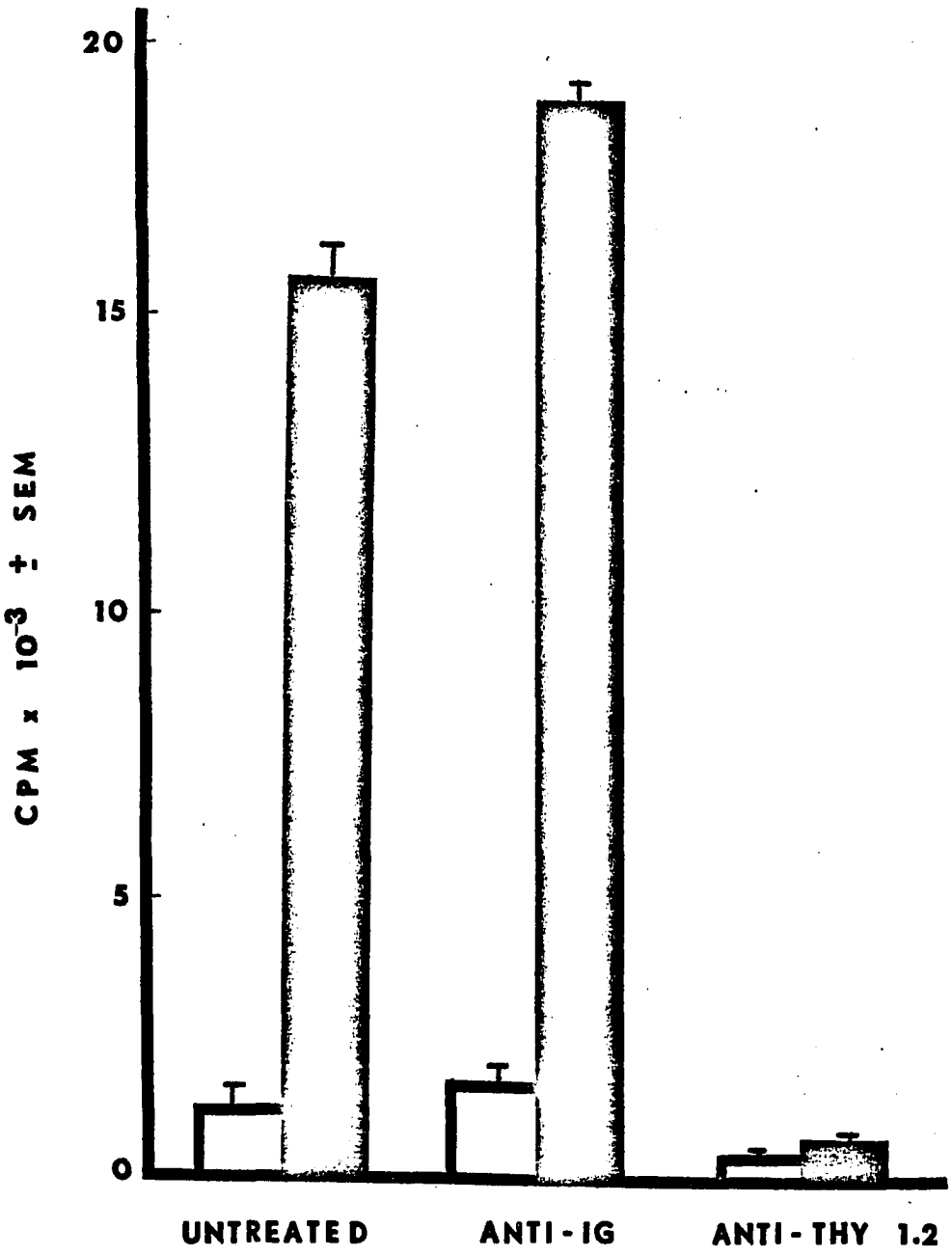
[c]Anti-mouse IgG and anti-mouse IgM were mixed before treating spleen cells.

[d]These cells were incubated in the presence of rabbit serum (C') only.

Treatment of spleen cells from infected mice with anti-Thy 1.2 completely eliminated the lymphoproliferative response to B-ASWS. Cells treated with anti-immunoglobulin were somewhat more reactive (Figure 16).

Attempts to characterize further the responding cell population with the use of antibodies to Lyt determinants failed. In all experiments, treatment of cells with either anti-Lyt 1.2 or anti-Lyt 2.2, resulted in background counts of 20,000 to 30,000 cpm with experimental values falling somewhere between (data not shown).

Figure 16. Blastogenic response of spleen cells from normal ( □ ) and 8 day iv inoculated mice ( ■ ) to B-ASWS following treatment with either anti-mouse immunoglobulin or anti-Thy 1.2. Results are expressed as cpm of experimental cultures minus background counts.


20
15
10
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CPM x $10^{-3}$ ± SEM
UNTREATED
ANTI-IG
ANTI-THY 1.2

# CHAPTER IV

## DISCUSSION

Results presented in this paper confirm that after, and even during, the resolution of a sc B. dermatitidis infection, highly significant resistance to a lethal iv challenge of this organism is established (Figure 1). Resistance to this pathogen was evident as early as 1 week after sc infection (Figure 1). Protection reached a maximum 4 weeks after infection (Figure 4) and remained high at 6 weeks post-infection (Figure 6). Mice immunized subcutaneously with viable B. dermatitidis demonstrate increased resistance to intranasal and ip inoculation, also. Mice develop resistance to a 70% lethal intranasal challenge dose about 1 week after sc infection and become completely resistant by 2 weeks (27). Protection against an ip inoculation of B. dermatitidis is evident by 9 days after sc infection (46). Our results (Figures 2,3,6), as well as those of Cozad and Chang (11), demonstrate that sc immunization with merthiolate killed B. dermatitidis elicits a protective immune response. Resistance, however, takes longer to develop, and the level of protection reached at 6 weeks after immunization is less than that induced by a nonlethal sc infection (Figure 6). Inoculation of mice ip with formalin-killed yeast induced some resistance at 3 weeks post-immunization, but by 6 weeks it had completely waned (Figure 6). Others have reported that injection of killed yeast ip could protect against iv challenge (23). This

discrepancy could be due to the use of different immunization schedules or to the virulence of the challenge organism. The B. dermatitidis strain used in these studies, and by Morozumi et al. (27), is the most virulent strain reported in the literature.

All groups of sensitized mice demonstrated a proliferative response to B-ASWS 1 to 3 weeks after sensitization (Figure 7). Lymphocyte proliferation to B-ASWS seemed to parallel protection in animals infected sc with viable yeast and in animals inoculated ip with formalin-killed yeast (Figure 7). The lymphoproliferative response to B-ASWS decreased as did protection in the latter group, whereas both properties remained high in the sc infected group. Proliferation of spleen cells from mice immunized sc with merthiolate killed B. dermatitidis to B-ASWS was not significantly greater than that of normal mice at 6 weeks after immunization. Yet, these animals demonstrated some resistance to an iv challenge. Since nonviable organisms would eventually be cleared from the host, thus eliminating the antigenic stimulus, one would expect these results. Upon challenge, however, the inoculum would stimulate memory cells to initiate protective effector functions in the host. The blastogenic response to B-ASWS remains high in sc infected mice. Viable yeast cells are present for 4 to 5 weeks after infection, and this constant supply of antigen may be responsible for the continued proliferative response. Although mice are culture negative for B. dermatitidis 4 to 5 weeks after infection, we can not be absolutely certain that the host has cleared the pathogen. It might remain latent in some organ, thus continually providing a potent antigenic stimulus.

Suppression and recovery of the proliferative responses to mitogens were observed in mice infected sc with viable B. dermatitidis yeast cells (Figure 8).

Suppressed responses to PHA, Con A and LPS were observed at 1 week post-infection. The PHA response returned to normal by 2 weeks. The Con A response remained suppressed for 3 weeks, and the LPS response did not return to normal until 5 weeks after inoculation. Blastogenic responsiveness to B-ASWS coincided with the suppression of mitogen responses. Responsiveness to B-ASWS was first observed at week 1 and increased, reaching a maximum at 6 weeks post-infection. Nonspecific suppression of mitogen responses was a transient event however, and responses to all mitogens were at normal levels 5 weeks after sc infection.

Subcutaneous infection of mice with B. dermatitidis provides a reproducible model for studying immunity to experimental blastomycosis (26,27). Morozumi et al. (27) report that mice are resistant to an intranasal challenge of B. dermatitidis yeast cells as early as 2 weeks after sc infection. They assessed several parameters of immunological responsiveness of sc infected mice. They found that: 1) These mice have positive DTH and humoral responses to B. dermatitidis antigen. DTH, as measured by skin testing, appears at 1 week after infection and remains strongly positive for 4 weeks. Antibody to B. dermatitidis first appears in sera 1 week after infection. The titer increases until 4 weeks and remains elevated for 6 weeks. 2) Peritoneal macrophages inhibit replication of B. dermatitidis as early as 1 week after infection. 3) Lymphoproliferative responses to B-ASWS appear in spleen cells by week 1 and in lymph node cells by 3 weeks after infection. We too found that spleen cells become responsive to B-ASWS by 1 week after infection. However, they report that the responsiveness of spleen cells from sc infected mice to Con A does not differ from cells of uninfected mice at any time during the 6 week course of infection. We show significantly suppressed mitogenic responses to PHA, Con A and LPS during the course of sc

infection. The same strain of B. dermatitidis and infection protocol were used in both studies. However, the mouse strains used differed, in that we used C57Bl/6J mice, while they used BALB/cByJ mice. These strains differ with respect to their H-2 haplotype. Therefore, the haplotype of their Ir genes would differ and this could very well account for the discrepancy, even though both strains are of average susceptibility to B. dermatitidis (26).

This study and those of Morozumi et al. (26,27) show that lymphoproliferative responses of spleen cells from sc infected mice to B-ASWS correlate with a high degree of resistance to a lethal challenge of B. dermatitidis. Our data indicate that during the course of the self-limiting infection, there seems to be an inverse relationship between depression of mitogen responses of spleen cells and the hosts capability to develop a protective immune response to B. dermatitidis. Proliferative responses to B-ASWS and protection from a lethal challenge were demonstrated during the period of depressed mitogenic responsiveness.

A similar phenomenon occurs in mice infected sc with Histoplasma capsulatum (49). Spleen cells from these mice become responsive to histoplasmin by day 4 and reach maximum responsiveness on day 42. Suppressed blastogenesis to Con A is detected 4 days after infection and remains depressed for 14 days. Blastogenic responses to PHA and LPS were suppressed for 21 days.

Similar situations have been described in which immune responses to an infectious agent are accompanied by nonspecific or specific suppression of other immunological parameters. T cells and adherent cells from spleens of Trypanosoma brucei infected mice suppress the in vitro antibody response to SRBC (14). Depressed lymphoproliferative responses occur during infection with Nippostrongylus brasiliensis (51), and Trepomema pallidum (37), and after vaccination with live rubella vaccine (3).

The depressed mitogen responses of spleen cells during the early stages of sc infection with B. dermatitidis could be explained by: 1) induction of nonspecific suppressor T cells, 2) activation of a macrophage population that suppresses lymphocyte function nonspecifically, 3) production of a suppressor factor that modifies lymphocytes, rendering them unresponsive to mitogens, or 4) by dilution of mitogen reactive lymphocytes in the spleen with cells unreactive to mitogens.

Subcutaneously infected mice developed a slight splenomegaly during the course of infection. During this time we observed the development of antigen-specific responsiveness to B. dermatitidis, as well as, an increase in spontaneous uptake of [$^3$H] thymidine by unstimulated cells, thus indicating activated cells. Therefore, a dilution effect of mitogen-reactive spleen cells with antigen-reactive, mitogen-unreactive cells, provides at least a partial explanation for the depressed mitogenic reactivity. Suppression is most evident during the acute phase of sc infection. Products released by the fungus could inhibit mitogenic responsiveness of certain populations of cells or prevent other cells from participating in the proliferative response. As infection clears and the antigenic load decreases, lymphocytes may recover their ability to respond. The continued blastogenic response to B-ASWS could be attributed to the production of memory cells during early infection or continued stimulation of the immune system by viable yeast cells that go undetected.

Lymphocyte proliferation was also examined during the course of a lethal B. dermatitidis infection, as opposed to a nonlethal sc infection. Mitogenic responses became depressed (Figures 9,10,11), and a lymphoproliferative response to B-ASWS developed (Figure 14), having reactivity equal to that developed during

a self-limiting sc infection. Mitogenic responses were depressed at 4 days and decreased throughout the infection. Spleen cells from lethally infected mice responded well to B-ASWS, even though the animals eventually died. In this case, responsiveness to B-ASWS does not indicate protection.

Antigen specific suppression occurs during the course of many infections. Antigen specific suppressor cells, that inhibit DTH to Trypanosoma cruzi antigen, but not to an unrelated antigen, are found in T. cruzi-infected mice (40). Splenocytes from rats infected iv with Candida albicans manifest depressed C. albicans specific blastogenesis 3 to 6 days after challenge (34). Immune suppression occurs during the early phases of infection with H. capsulatum (4). A disseminated model of histoplasmosis was induced by inoculating mice iv with H. capsulatum yeast cells (4). One to three weeks after iv inoculation, potent immunosuppressor activity was generated. DTH to histoplasmin and SRBC, as well as blastogenic responses to mitogens and H. capsulatum were depressed. Nickerson et al. (30) using the model of disseminated histoplasmosis described above, identified two populations of cells capable of suppressor function. One population was identified as T cells and the other had macrophage-like properties. Several investigators have shown that inoculation of mice iv with fungi generates antigen specific suppression (4,30,34). Our results indicate that inoculation of mice iv with yeast phase B. dermatitidis did not elicit this type of response.

Spleen cells from 8 day iv infected mice were mixed with normal spleen cells, in an attempt to determine if the depressed mitogen responses were due to a population of suppressor cells. The PHA response was not significantly suppressed (data not shown). The blastogenic response to Con A was less depressed than that of unmixed cultures. A suppressor cell functioning here would

be unlikely, because one would expect to see similar suppression in both instances. Spleen cells from 8 and 12 day iv inoculated mice significantly suppressed the proliferative response of normal splenocytes to LPS. The response was depressed as much as or greater than the LPS response of infected cells alone. Therefore, one cannot rule out the possibility of a suppressor cell population, or the production of a suppressive factor.

In an attempt to identify the population of cells responsible for the in vitro proliferative response to B-ASWS, the spleen cells from lethally infected mice were separated on the basis of their ability to adhere to nylon wool, or their membrane characteristics. Separation using nylon wool was unsuccessful (Table 3). Pure populations of cells were not obtained, but most of the responsive cells were found in the nylon passed fraction (Figure 15). Treatment of spleen cells with anti-Thy 1.2 and complement greatly reduced the proliferative response, whereas treatment with anti-immunoglobulin had little effect (Figure 16). T cells appear to be responsible for the lymphoproliferative response to B-ASWS in infected mice, but it could not be determined if an accessory cell (adherent or nonadherent) was necessary. Attempts to further identify the responding cells with anti-Lyt antisera failed. Even though 30 to 40% of the spleen cells were lysed following treatment with anti-Lyt 1.2 or anti-Lyt 2.2 and complement, tremendous background counts resulted (20,000 to 30,000 cpm) making it impossible to draw any conclusions.

In summary, a potent protective immune response and lymphocyte proliferation to a B. dermatitidis antigen was generated during a nonlethal sc infection with B. dermatitidis. Mitogenic responsiveness of spleen cells was depressed during early infection, but returned to normal by 5 weeks post-

infection. Inoculation of mice iv with viable B. dermatitidis yeast cells produced depressed responsiveness to mitogens, but stimulated cells to respond in vitro to an antigen extract of B. dermatitidis. Cells responsible for at least part of the proliferative response to B-ASWS were identified as T cells.

One should be cautious when interpreting lymphocyte transformation data. There are situations where DTH develops with in vitro production of mediators, but without in vitro antigen-induced transformation. Conversely, resistance to a pathogen may not develop, even though antigen-induced proliferation occurs in vitro.

Several parameters of lymphocyte blastogenesis have been examined and correlated with the development of protective immunity. A study of the relationship between the nonspecific suppression of mitogenic response and the development of protective immunity to blastomycosis warrants further characterization of the lymphocyte subpopulations at different time intervals after infection.

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