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IMMUNOMODULATION BY BLASTOMYCES DERMATITIDIS: FUNCTIONAL ACTIVITY OF MURINE PERITONEAL MACROPHAGES

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THE UNIVERSITY OF OKLAHOMA

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IMMUNOMODULATION BY *BLASTOMYCES DERMATITIDIS*: FUNCTIONAL ACTIVITY OF MURINE PERITONEAL MACROPHAGES

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LARRY SCOTT MCDANIEL

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FUNCTIONAL ACTIVITY OF MURINE PERITONEAL MACROPHAGES

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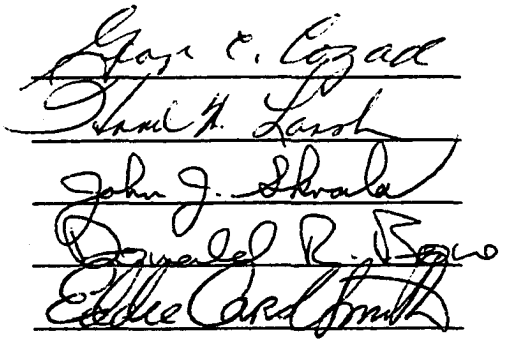

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July, 1981 L. Scott McDaniel RAH!

IMMUNOMODULATION BY *BLASTOMYCES DERMATITIDIS*: FUNCTIONAL ACTIVITY OF MURINE PERITONEAL MACROPHAGES

CHAPTER 1

BLASTOMYCOSIS, CELL-MEDIATED IMMUNITY, AND THE MACROPHAGE

The causative agent of blastomycosis is the dimorphic fungus *Blastomyces dermatitidis*. The disease was first described by Gilchrist at a meeting of the American Dermatological Association in 1894 (20). The description was based on microscopic observations of skin biopsy from a patient's hand. The disease was thought to be of protozoan origin, and the patient was described as having granulomatous lesions of the lungs, skin, and skeleton. In 1896, Gilchrist identified the agent as fungal (21), and Gilchrist and Stokes gave the organism its current name in 1898 (22).

Initially, the portals of entry were thought to dictate the pathogenesis of the cutaneous and systemic forms of the disease, i.e., the skin and lungs. However, in 1951, Schwartz and Baum (51) showed that the fungus is first inhaled and spread by hematogenous and lymphatic dissemenation.

Very little is known of the natural habitat of *B. dermatitidis*. It was thought to be geographically limited to the United States and Canada. According to Furcolow's 1970 study (19), the greatest number

of cases occurred in the Mississippi, Missouri, and Ohio River basin states. The disease has now been shown to be endemic in 15 of the 50 African countries (6, 13, 33, 56) as well as in Israel (32). There has also been a reported case in a life long resident of Poland (31).

The fungus has been isolated in Kentucky (11) and Georgia (10) in the mycelial form from soil. These findings were complicated by the fact that reisolation from the original samples or collection sites was not possible. Little was actually learned about the organism's environmental niche by these studies.

Blastomycosis has been diagnosed in lower animals. Among the species infected are the cat (2), dog (39), horse (5), and a captive sea lion (59). In animals the disease has been most frequently seen in the dog. The apparent absence of the infection among wild rodents and small mammals in the endemic areas has been noted (1). Much remains to be discovered about the environmental cycle of this fungus.

The definitive diagnosis of blastomycosis must be made by culture of the organism from clinical specimens. The currently available serological and skin tests are not conclusive (7, 14). Reviews of the disease and its history are available (14, 34, 47).

In studying the immunological processes active in experimentally induced murine blastomycosis, Spencer and Cozad (54) demonstrated that cell-mediated immunity provided protection to mice challenged with a lethal dose of the yeast. Further investigation in this laboratory have supported the involvement of cell-mediated immunity in blastomycosis. Such parameters as lymphocyte transformation (4), lymphokine production (23), and the passive transfer of hypersensitivity by cells but

not serum (52) have been examined.

As cell-mediated immunity has been shown to play such a dominant role in the immune response of mice to B. dermatitidis, it appeared worthwhile to look at aspects of cellular immunity as expressed by the macrophages of this system.

Macrophages are phagocytic cells distributed throughout the body. They differ in form depending on their anatomical site, but all possess the primary trait of phagocytosis, i.e., the ability to ingest and digest particulate matter.

The macrophage is a monocytic cell that apparently has its genesis in the bone marrow (55). A pleuripotential stem cell of the bone marrow is the precursor cell which differentiates into a promonocyte. The promonocyte matures to a monocyte. The monocyte is found in the peripheral blood at a normal level of 3 to 5% of the total circulating white blood cells. These circulating monocytes are blood-borne to the organs and tissues. The monocyte becomes a histiocyte or macrophage depending upon the area in which it comes to rest. The total lifespan of these mononuclear cells is measured in months (55).

Macrophages have been shown to be involved in a number of immunologic processes. Metchnikoff first described the ability of macrophages to ingest and destroy foreign bodies (40). The secretion of factors collectively called monokines (i.e., neutral proteases, complement cleavage products, interferons, prostaglandins, etc.) directly involves macrophages in the development of inflammation and immune amplification (3). These cells augment natural killer (NK) activity (12), and macrophages function in the presentation of antigen or mito-

gen signals to responder lymphocytes (53, 18). In addition, macrophages by themselves or cooperating with T-lymphocytes are involved in the activation of B-lymphocytes by thymus-independent antigens (8). Furthermore, though they are endowed with considerable destructive power, their good behavior and subservient nature are displayed by their willingness to obey T-and B-lymphocyte signals.

Phagocytosis itself can be divided into two very general stages. The first is attachment of the particle to the phagocytic cell, and this is followed by the second stage which is interiorization of the particle. The attachment phase may be immunologically specific (i.e., antibody or complement mediated) or non-immunological depending upon requirements for serum-recognition factors. Interiorization is accomplished by endocytosis of the attached particle. When the cell is ingesting large numbers of particles, as much as 50 to 60% of the plasma membrane may be interiorized in the form of phagosomes. It appears that phagocytosis of an initial particle results in a cooperative effect that enhances the uptake of subsequent particles (9). After ingestion of a large number of particles, the cell can begin to run short of membrane leading to the presence of particles in vacuoles that are not completely closed.

There has been considerable emphasis placed on the importance of the role of the macrophage in immunosurveillance (28, 36), and the capacity of macrophages to directly destroy tumor cells in vitro is well documented (26, 50). The mechanism of macrophage-mediated antitumor activity is yet undefined. Though phagocytosis has been implicated, recent microscopic observations have shown that it is not essen-

tial for tumoricidal activity (24, 43).

Our knowledge of the mechanisms involved in tumor cell destruction by macrophages has come from a variety of in vitro assays. These assays have looked at inhibition of target cell growth (15, 27), cleared zones of tumor cell monolayers (58), release of radioactive labels (45) cinemicrographic analysis (24), and sequential scanning and transmission electron microscopy (16).

Macrophages in vitro can either promote or impair the viability of target cells. Conditions which determine the outcome of this interaction are poorly understood. Parameters such as the functional activity of the macrophage, actual ratio of effectors to targets, and the susceptibility of the target cell are important. Fidler and colleagues have pointed out that a lack of standardization of these parameters and testing methods has lead to conflicting reports regarding macrophage-tumor cell interaction in vitro (46).

Macrophages resting within tissue are termed resident macrophages. When inflammatory stimuli increase, macrophages can become activated to a cytotoxic state which distinguishes them functionally and cytochemically from normal tissue macrophages. There is a growing body of evidence which indicates that activation of macrophages is accomplished primarily by the action of mediators on the membrane of the cell. Macrophages cultured with stimulated lymphocytes can show a maximal activation within one hour (42). This has been interpreted as resulting from an external rather than an internal action. In addition, effective adjuvants tend to be surface active molecules.

Mackaness coined the term macrophage activation to describe mor-

phological changes in mononuclear phagocytes obtained from animals immune to Listeria monocytogenes. The cells rapidly adhered to substratum and showed considerable ruffled membrane activity. Since their introduction, the terms "activation" and "stimulation" have been nondiscriminately and extensively used to describe diverse morphologic, biochemical, and functional phenomena. Mononuclear phagocytes respond differently to various material. The stimulatory or activation capacity of bacteria, immune complexes, activated lymphocytes, polyanions, interferon, and complement components varies greatly. Therefore, activation is a general term that does not define specific biochemical and cytological changes that can result from exposure to certain agents. It can be considered that macrophages are pluripotent cells capable of expressing a defined functinal state associated with inherent effector functions.

Activation of macrophages appears to be a complex process which may be brought about by a variety of agents acting through diverse biochemical routes. Other cell types and/or their products have a role in the regulation of macrophage effector capacities. In one study, homology at the I region between macrophages and T-cells as well as the presence of Ia molecules on the macrophages were necessary for the generation of cytocidal macrophages (17). The consistent observation that macrophages taken from nude mice manifest higher spontaneous cytotoxic activity than those from their normal congeners indicates that agents other than T-cell derived lymphokines can effectively trigger the activation process (44). However, agents such as BCG or C. parvum which are effective activators in vivo do not consistently cause activation

in vitro. This further supports the role of other cell types in activation. Nevertheless, agents such as endotoxin and double stranded RNA alone have been shown to be potent in vitro activators (58, 50).

A conceptual scheme has been put forth that outlines the sequences of reactions to develop activated macrophages (38). Given the presence of effective activation signals and competent mononuclear phagocytes, the following are three postulated phases which result in macrophage activation: 1) The first involves the formation of inflammatory macrophages by recruitment and differentiation of blood-derived mononuclear phagocytes. Therefore, the immediate precursor cells for activated macrophages are the blood-derived cells which accumulate nonspecifically at sites of inflammation. 2) Factors released from antigen stimulated lymphocytes (lymphokines) have a very profound effect on inflammatory macrophages. The effect of lymphokine activation signals on inflammatory macrophages constitutes the second phase of activation. These interactions generate noncytotoxic intermediate cells which have been termed primed macrophages. 3) The final phase of macrophage activation results from the response of lymphokine primed macrophages to other activation signals. These stimuli can be derived from invading parasites or tumor cells. These final activation signals are active on primed macrophages, but they cannot directly activate inflammatory macrophages.

The development of macrophage tumoricidal capacity following infection with BCG, Toxoplasma, Listeria, or interaction with lymphokines is influenced by genetic factors. For example, C3H/HeJ mice have at least two genetic defects. The first is unresponsiveness to

lipopolysaccharides (LPS), and the second abnormality is hyporesponsiveness of their macrophages to activation by a variety of agents (38). They possess a normal inflammatory response but require strong stimuli both in vivo and in vitro to develop tumoricidal capacity. The gene for control of one or more essential reactions in the development of nonspecific macrophage cytotoxic activity is either closely linked or identical to the LPS gene. Macrophages for at least six other mice strains with defective LPS genes resist development of normal tumoricidal activity (49). This defect is also associated with a failure to kill Rickettsia and Leishmania.

The identification of mouse strains with genetic defects in the development of macrophage cytotoxicity are a useful source for the characterization of macrophage activation. Abnormalities of phagocyte function occur in Chediak-Higashi syndrome. This genetic disease has been identified in C57BL/6J mice (41). There is a resultant pigment dilution, and the syndrome has been designated as the beige mutation in C57BL/6J. This mutation has become important in the study of host resistance with the discovery that these mice have a decreased resistance to some transplantable leukemias. The macrophages of the beige mouse possess lysosomal abnormalities and have giant cytoplasmic granules. However, the macrophage surface receptors, phagocytosis, and percent peroxidase positive cells are normal. Studies with the beige mouse may help elucidate the early steps in macrophage cytotoxic expression. A recent report also demonstrated that the beige mouse is deficient in NK cell funciton (48). The report suggests that this may provide the ideal system for distinguishing macrophage and NK mediated

defenses.

It has been shown that as macrophages become activated and acquire cytotoxic capabilities there is an associated expression of a distinctive macrophage surface antigen (25). The expression of Fc receptors on the surface of functionally active macrophages also increases (30). These findings are in line with the concept that natural cytotoxicity of macrophages may require distinctive surface structures on both the effector and target cell. Mononuclear phagocytes can not only discriminate between self and non-self but also between self and alterations to self. This is exemplified by the destruction of effete or damaged tissue components by macrophages. It has been suggested that anything which contacts the macrophage membrane is phagocytized unless the contacting material displays a signal that inhibits phagocytosis (57). This interaction is thought to occur between special membrane receptors (self receptors) and self determinants on the contacting material. Such a discriminatory capacity could have evolved from a simple expression in primitive species with refinement in phylogeny.

Studies involving fetal tissues has given insight into the structures recognized as non-self by mononuclear phagocytes. In some systems it has been shown that the growth of malignant tumors in experimental animals can be accompanied by the renewed formation and expression of fetal structures (57). Cytotoxicity of activated macrophages against various targets can be blocked in a dose dependent manner by irradiated syngeneic fetal liver cells but not by liver cells from adult donors (29). This competitive property falls off rapidly

after birth.

This discussion has centered on the generation, recruitment, activation, and cytotoxicity of macrophages as critically important components of host resistance. In this respect, the purpose of this study was to examine the functional activity of peritoneal macrophage populations from B. dermatitidis sensitized mice and evaluate the resultant resistance to tumor cell growth.

There were three parameters chosen to assess the functional activity of peritoneal macrophages with regard to the yeast phase of B. dermatitidis. These parameters were phagocytosis, metabolic activation, and intracellular killing of the fungus. Qualitative morphologic differences and quantitative functional differences between sensitized and non-sensitized macrophage populations were examined.

The second objective of this study was to determine the protective and therapeutic effects of B. dermatitidis induced resistance in mice using the syngeneic lymphoma EL4. The protective effects were evaluated by: 1) pre-immunization of mice with yeast cells and challenging with tumor cells at various times, 2) mixing yeast with tumor cells prior to injecting animals, and 3) therapy with yeast cells after the tumor load had been established. As a companion study to protection in vivo, in vitro assays to determine the interaction of activated macrophages and tumor cells were carried out.

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CHAPTER 2

ACTIVATION OF MURINE PERITONEAL MACROPHAGES BY *BLASTOMYCES DERMATITIDIS*

ABSTRACT

Cell-mediated immunity (CMI) plays the dominant role in the immune response of mice to *Blastomyces dermatitidis* infections. Since macrophages play an important role in CMI, the interactions between sensitized murine peritoneal macrophages and the yeast phase of *B. dermatitidis* were investigated. Scanning electron microscopy (SEM) showed that the sensitized macrophages were more efficient in phagocytizing *B. dermatitidis* than non-sensitized cells. In addition, there appeared to be activation of metabolic pathways within the sensitized macrophages as indicated by increased chemiluminescence activity during phagocytosis. There was a significant difference in the ability of sensitized macrophages to control intracellular proliferation of the yeast when compared to non-sensitized cells. This was determined by disruption of macrophages and plating for viable yeast. Scanning electron microscope observations offered further substantiation. Experiments with *Candida albicans* indicated that *B. dermatitidis* nonspecifically activated macrophages. At 2 hours post-phagocytosis, 30% fewer *C. albicans* in activated macrophages were able to form germ tubes. SEM indicated that a number of activated macrophages may interact with a single yeast

aggregate in vivo. A new mechanism of macrophage activation is suggested. These studies demonstrated the multipoteintial of activated macrophages with regard to their functional activity.

INTRODUCTION

The study of host resistance against systemic fungal infections has begun to receive considerable attention in the past decade. Up until that time, most immunological investigations of these organisms were concerned with the isolation and characterization of antigens for use in vaccines, diagnosis, and epidemiological studies. The lack of information as to the role of local defenses, antibody production, and cell-mediated reactions has prompted investigations into host response to various fungi. Recent studies have pointed to the importance of cell-mediated immunity (CMI) in fungal infections (6, 7, 14). Such studies have also pointed to the complexities involved in these host-parasite relationships.

Of the systemic mycoses, blastomycosis has been the most lacking in terms of the host response. Therefore, in our laboratory we have developed a mouse model system to study the role of cell-mediated mechanisms in resistance to Blastomyces dermatitidis infections. Previous studies in this laboratory have established a delayed hypersensitivity pattern which can be passively transferred with cells but not serum (26, 25). We have also correlated the delayed hypersensitivity pattern with other parameters of CMI responses such as lymphocyte transformation (1), lymphokine production (8), and cell-mediated immunoprotection (6). All of these studies emphasized the dominant role of CMI against B.

dermatitidis. It therefore appeared worthwhile to investigate the aspects of cellular immunity as expressed by the macrophages of this system. The macrophage is possibly a key cell being both processor of antigen and the destroyer of parasites through phagocytosis.

In a preliminary report (G. C. Cozad and L. M. Kronholm, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979. F51, P. 371), we demonstrated an increase in phagocytosis of latex particles by macrophages from immune mice. The prupose of this study was to further evaluate the functional activitiy of these macrophages with regard to yeast phase cells of B. dermatitidis. This was done by examining phagocytosis, metabolic activation of these macrophages, and intracellular killing of the fungus.

MATERIALS AND METHODS

Cultures. The killed whole yeast cell antigen was prepared by the method of Restrepo-Moreno and Schneidau (23) as modified by Spencer and Cozad (26). The yeast phase cultures of B. dermatitidis 242 was originally isolated from a fatal human case of blastomycosis. The culture was maintained on brain heart infusion (BHI) agar slants at 37°C. A 72 hour slant was harvested, and the yeast cells were washed with sterile 0.1% cysteine in physiological saline solution (PSS). After diluting with 0.04% trypan blue, the cells were examined in a hemacytometer to determine the concentration and viability. The cells were then centrifuged and resuspended at the desired concentration in RPMI 1640 (Gibco, Grand Island, NY.) supplemented with 1% heat-inactivated fetal calf serum (FCS) (MA Bioproducts, Walkersville, MD.), penicillin (100 units/ml), and streptomycin (100 ug/ml). These suspensions were used for in vitro infection of macrophage cultures. A sample was taken from the suspension and plated on BHI to determine the colony forming units (CFU).

Candida albicans D-76 was maintained by weekly transfers on modified Sabouraud dextrose agar. The yeast cells were grown on slants for 24 to 36 hours at 37°C, harvested, and washed with sterile PSS. The concentration and viability were assessed by hemacytometer counts using trypan blue. The desired concentration of yeast cells was suspended

in RPMI 1640 with 1% FCS.

Test animals. Two to three month old inbred C57BL/6J mice of both sexes were used in this study. The strain was originally obtained from Jax Laboratories, Bar Harbor, Maine, and bred in our animal facilities. The mice were separated by sex and given water and mouse chow (Ralston Purina) ad libitum.

Emulsion of yeast cell antigen with Freund incomplete adjuvant. Two methods were tested in the preparation of the antigen-emulsion (Ag-emulsion). The first procedure was that of Cozad and Chang (6). The Ag-emulsion was prepared by continuous grinding in a motar while a suspension containing 40 mg/ml (dry-weight equivalent) of the Merthiolate-killed yeast was added dropwise to an equal volume of Freund incomplete adjuvant (Difco Laboratories, Detroit, MI.). After the yeast cells were thoroughly ground, further emulsification was carried out by passing the mixture through an 18-gauge needle until a discrete droplet was formed on the surface of cold water. The control suspension was identically prepared except that a volume of PSS equal to the yeast cell suspension was used in place of the latter.

In the second procedure, the Ag-emulsion was prepared by forcing equal volumes of the yeast suspension and Freund incomplete adjuvant (Difco) back and forth between two 10 cc glass syringes connected by an 18-gauge emulsifying needle (Popper and Sons, Inc., New Hyde Park, NY.). This was continued until the emulsion formed a discrete droplet on the surface of cold water. In the control emulsion, PSS was substituted for the yeast cell suspension.

Sensitization and delayed hypersensitivity of mice. The mice were sensitized according to the method of Cozad and Chang (6). Briefly, the animals were divided into a test and control group. On days 0 and 7 the test animals were inoculated subcutaneously in the inguinal area with 0.1 ml of the Ag-emulsion (containing 2 mg dry weight equivalent of killed B. dermatitidis yeast cells). The control group was inoculated in the same manner with the PSS-emulsion.

To determine the delayed hypersensitivity (DH) pattern, three mice from each group were footpad tested on days 3, 15, and 30 after the initial injection. The footpad tests were done by the procedure of Youmans and Youmans (29). The tests were carried out by injecting 45 ug of dry-weight equivalent of Merthiolet-killed whole yeast cells contained in 0.03 ml of PSS into the right hind footpads and 0.03 ml PSS into the left hind footpads. The footpads were measured with dial gauge calipers immediately before and 48 hours after challenge. The mean differences in thicknesses between the right and left hind footpads served as the measure of DH.

Collection of peritoneal cells. Peritoneal cells were collected without the use of an exudate-inducing agent by a modification of the method described by Tolnai (28). At 15 to 18 days post-primary injection, three mice were selected from each group and killed by cervical dislocation. The abdominal skin was dissected away from the peritoneum. Then 5.0 ml of cold RPMI 1640 with 10 units/ml sodium heparin (Fellows Medical Manufacturing Co., Anaheim, CA.) and 1% heat-inactivated FCS was injected into the peritoneal cavity by a syringe with a 20-gauge needle. After a short massage, the fluid was withdrawn into

the same syringe by inserting the needle successively on both sides of the abdomen.

The cells from three animals were pooled, and their viability was assessed by trypan blue exclusion. After centrifugation the suspensions were adjusted to 1 x 10^6 cells/ml in cold RPMI 1640 with 1% FCS, penicillin (100 units/ml), and streptomycin (100 ug/ml). Five milliliters of each suspension were added to separate petri dishes containing 3 coverslips each. The cells wer allowed to attach for 60 minutes at 37° C in 5% CO_2. All non-adhering cells were washed away with warm Dulbecco's phosphate buffered saline (PBS). Fresh warm medium containing 10% FCS was added, and the cultures were incubated for 18 to 24 hours at 37° C in 5% CO_2.

Enumeration of macrophages. After the 24 hour incubation period, some coverslips were selected to enumerate the macrophages present. A modification of the nonspecific esterase stain by Koski, Poplack, and Blaese (13) was used. The coverslips were fixed for 30 seconds in a solution of 20 mg Na_2HPO_4 and 100 mg KH_2PO_4 in 30 ml of distilled water, 45 ml acetone, and 25 ml of 30% formaldehyde. The coverslips were rinsed with distilled water and air dried for 30 minutes. Then 1.0 ml of a pararosaniline solution (1.0 gm of pararosaniline hydrochloride in 25 ml of 2N HCl) was filtered and mixed with an equal volume of freshly prepared 4% sodium nitrite. The stain was prepared by mixing in sequence 44.5 ml of M/15 Sorenson's phosphate buffer (2.128 gm of Na_2HPO_4 and 6.984 gm of KH_2PO_4 in 1000 ml of distilled water at pH 6.3), 0.25 ml of the prepared pararosaniline solution, and 3.0 ml of an alpha-naphthyl solution (1.0 gm of alpha-naphthyl butyrate in 50 ml of dimeth-

yl formamide). The mixture was immediately filtered, and the coverslips were stained for 45 minutes in a 37°C water bath. The coverslips were then rinsed with distilled water, drained, and counterstained with 0.5% methyl green for 15 seconds. A final rinse with distilled water was followed by 30 minutes of air drying before mounting the coverslips. The entire procedure must be carried out in glass.

Challenge of macrophage monolayers. After the 24 hour incubation of the macrophage monolayers, in some experiments the medium was replaced with fresh RPMI 1640 containing 1% FCS and 1 x 10^6 B. dermatitidis yeast cells/ml. Routinely, 0.3 to 0.5 ml of fresh mouse serum from sensitized animals were added to the medium. The monolayers were again incubated at 37°C in 5% CO_2. At various time intervals, coverslips were removed and washed free of non-phagocytized yeast cells. These coverslips were either fixed in absolute methanol and stained with Wright stain for light microscopy or fixed in PBS containing 0.5% glutaraldehyde for scanning electron microscopy (SEM).

For SEM, the coverslips were allowed to fix for 18-24 hours at room temperature after which they were washed four times in PBS. The coverslips were then dehydrated through a series of ethyl alcohol baths ending in absolute ethanol. The coverslips were then placed in a solution of ethanol amyl acetate for critical point drying in a No. 99 Model H Pelco Critical Point Dryer (Ted Pella Company, Tustin, CA.) using liquid CO_2 with a critical pressure of 1072 psi. The coverslips were then gold coated and examined in an International Scientific Instruments Super II scanning electron microscope at the noted magnifications with an accelerating voltage of 25 KV.

Other experiments were carried out to assess the intracellular killing of phagocytized B. dermatitidis yeast cells. A procedure similar to the one described by Howard (11) was used. Briefly, macrophage cultures from immune and non-immune animals were allowed to phagocytize B. dermatitidis yeast cells as described above. After 3 hours, the extracellular yeast cells were removed by washing the cultures three times with PBS. Fresh medium was then added. A coverslip was removed as a zero time sample. Other coverslips were removed at 24 hour intervals. The coverslips were placed in sterile distilled water, and their surfaces were scraped to disrupt the macrophages. The suspension were diluted, plated on BHI agar, and counted to determine the colony forming units (CFU) after 14 days at room temperature. Total numbers of yeast cells were determined by direct microscopic counts before plating.

C. albicans was substituted for B. dermatitidis in the replacement medium for some experiments. After allowing the C. albicans to be phagocytized for 30 minutes, the extracellular yeast cells were removed by washing four times with warm PBS. At this point, some coverslips were removed and fixed in absolute methanol. These coverslips were stained with Wright stain. The percent of macrophages phagocytizing and the number of yeast cells per 100 phagocytic cells were determined. Other coverslips were reincubated with fresh medium and removed at one hour intervals to determine the percentage of phagocytized yeast cells forming germ tubes.

Chemiluminescence. The interaction of macrophages with yeast cells during phagocytosis was examined by previously described procedure for chemiluminescence activity (9). Peritoneal cells were harvested

from immune and non-immune mice. The cells were counted, centrifuged, and resuspended at 1 x 10^6 cells/ml in RPMI 1640 with 1% FCS. Then 4.0 ml of the suspensions were added to dark adapted Beckman Poly Q vials. The suspensions were counted at 1 minute intervals for 10 minutes to obtain backgroud counts. The counts were made in a Beckman LS 100C liquid scintillation counter at ambient light and temperature through the tritium window in the out-of-coincidence mode with the front photomultiplier disconnected. Then 1.0 ml of 4 x 10^6 B. dermatitidis in RPMI 1640 with 1% FCS and 0.1 ml of fresh serum from sensitized mice was added to each vial. Control samples of peritoneal cells received 1.0 ml of the medium without the fungus. After mixing, the vials were counted at 1 minute intervals until 30 minutes of counts were obtained for each sample. The average counts per minute (CPM) over five minute periods were determined and plotted.

In vivo phagocytosis. Three mice of each group, immune and non-immune, were selected. The abdominal area was washed with ethanol, and concentrations ranging from 1 x 10^4 to 1 x 10^7 B. dermatitidis yeast cells in Hank's Balanced Salt Solution (HBSS) were injected intraperitoneally (IP). At various time intervals after the IP injection, the mice were sacrificed, and their peritoneal cells were harvested as described early.

The cells were counted, centrifuged, and resuspended in RPMI 1640 with 1% FCS at 1 x 10^6 cells/ml. Then 5.0 ml of the suspensions were plated in petri dishes containing 3 coverslips. The cells were allowed to settle for 60 minutes at 37°C in 5% CO_2. At the end of this time, the cell cultures were washed four times with PBS and prepared for light microscopy or SEM as earlier outlined.

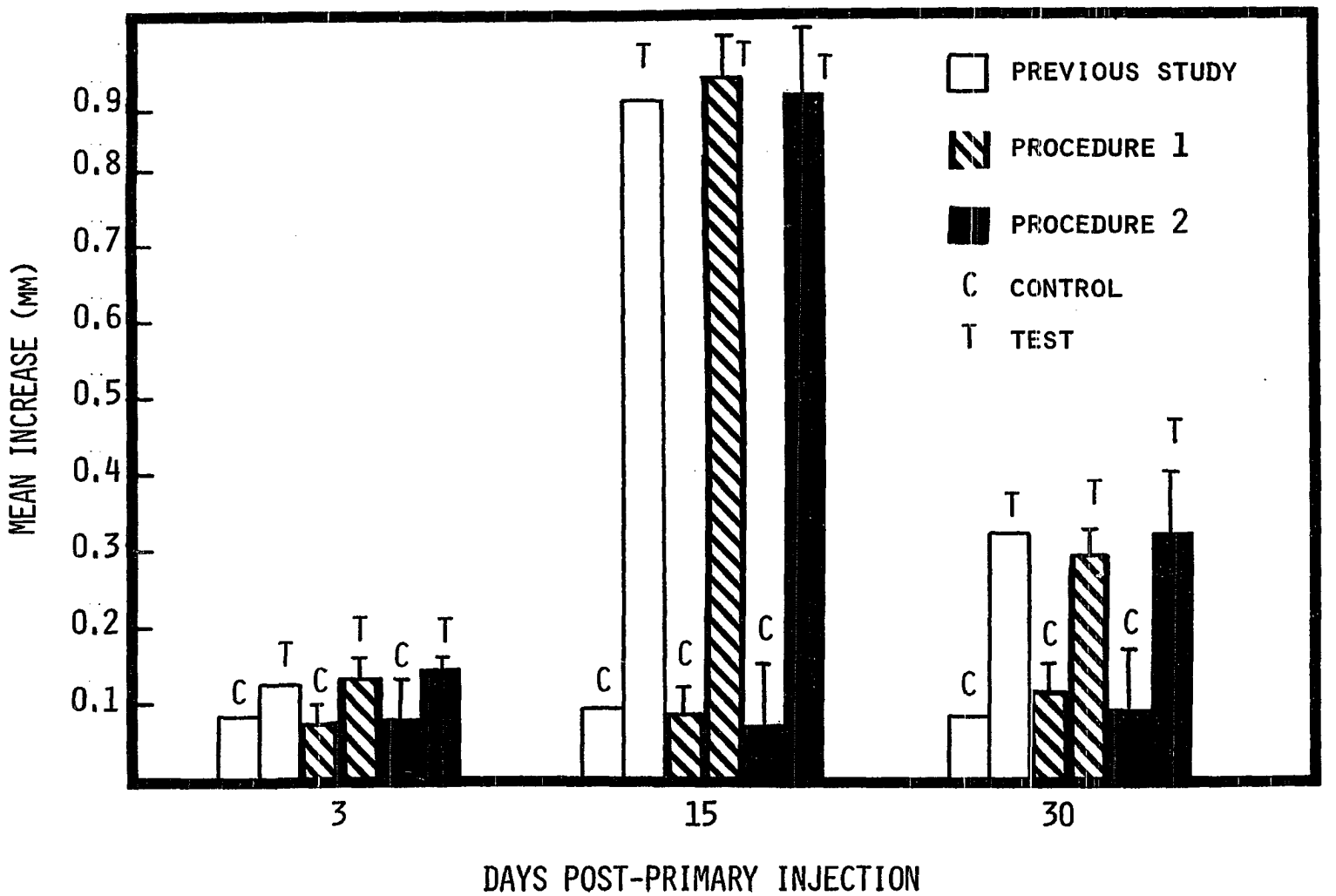
RESULTS

Establishment of delayed hypersensitivity. Previous studies in this laboratory have established a consistent delayed hypersensitivity pattern to B. dermatitidis in C57BL/6J mice. For this study, the pattern of delayed hypersensitivity was determined and compared to the results of a previous study (Figure 1). It can be seen that the established pattern was followed with a peak reaction on day 15 post-primary injection with a return to near normal values by day 30. Mice in a range of 15 to 18 days post-primary injection were considered to be sensitized to B. dermatitidis. It can also be noted that there was no difference in the hypersensitivity response when comparing the two methods of antigen emulsification.

Enumeration of macrophages. The composition and density of the peritoneal cell monolayers were determined by staining for nonspecific esterase. Nonspecific esterase has been demonstrated in monocytes (13). In the procedure used, esterase positive cells stained red while the esterase negative cells took up the green counterstain.

Coverslips stained immediately after the 60 minute settling period contained 80 to 85% mononuclear adherent cells. The remaining cells were lymphocytes and some polymorphonuclear cells. Coverslips stained after the 24 hour incubation period showed greater than 98% mononuclear cells (results not shown). The concentration of cells used

Figure 1. Delayed hypersensitivity pattern in C57BL/6J mice to killed B. dermatitidis yeast cells. Mean (± standard deviation) increase in footpad thickness of mice injected on days 0 and 7 with Ag-emulsion or PSS-emulsion.


MEAN INCREASE (MM)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
PREVIOUS STUDY
PROCEDURE 1
PROCEDURE 2
C CONTROL
T TEST
3
15
30
DAYS POST-PRIMARY INJECTION

was sufficient to produce an almost continuous monolayer of macrphages.

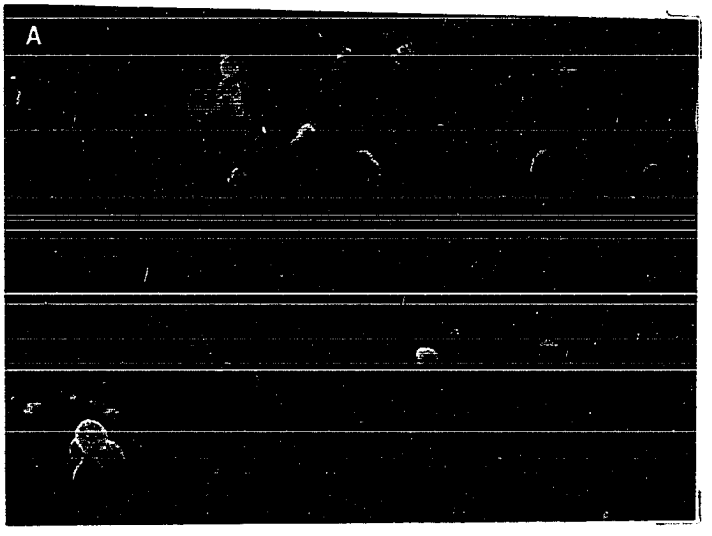
In vitro phagocytosis of B. dermatitidis. An initial effort was made to arrive at a phagocytic index with the light microscope. This could then be used to correlate the activity of macrophages with the delayed hypersensitivity pattern. However, due to the size and staining characteristics of the *B. dermatitidis* yeast cells this proved difficult. Therefore, the SEM was used to qualitatively assess the phagocytic activity of macrophages from immune mice.

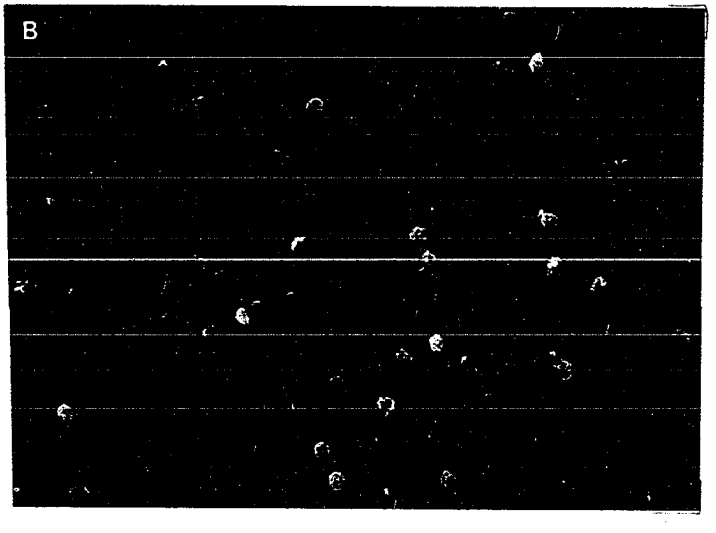
At about 5 to 10 minutes after exposure of *B. dermatitidis*, Figure 2 is representative of what one sees. In the sensitized macrophage culture (Figure 2A) there are several yeast cells attached or being phagocytized. Note also the morphology of these macrophages. They are rounded and highly ruffled. This is consistent with observations made of activated macrophages (18). In the non-sensitized macrophage culture (Figure 2B) at the same time interval, there are no yeast cells attached. Also these macrophages are flattened and lack the roughness seen in the sensitized macrophage culture.

Observations made during the first 30 minutes of phagocytosis showed that the sensitized macrophages more readily took up the *B. dermatitidis* yeast cells. Once a cell had been phagocytized, it was difficult to determine the actual number of yeast cells within the phagocyte. However, it was estimated that there were 2.5 ± 0.3 yeast cells per phagocytizing macrophage in the sensitized cultures after 30 minutes of interaction. This was compared to 1.4 ± 0.4 yeast cells per phagocytic cell in the non-sensitized cell cultures.

Figure 2A. Representative field from sensitized macrophage culture. Sample was fixed 5 to 10 minutes after the addition of B. dermatitidis. Note the presence of several attached yeast cells (X400).

Figure 2B. Comparable field from non-sensitized macrophage culture. Sample was fixed 5 to 10 minutes after the addition of B. dermatitidis. No yeast cells are attached to the macrophages (X400).


A


B

It was found that the normal phagocytic process occurred (Figure 3). There was attachment of the yeast cells to the macrophage via filopodia. Next the macrophage began to engulf the yeast cell, and finally the yeast cell or cells were completely phagocytized. This process was seen in the sensitized and non-sensitized cultures. However, it was observed with reduced efficiency in the non-sensitized macrophage cultures.

Chemiluminescence. There was a rapid increase in emitted light from sensitized peritoneal cells as compared to non-sensitized cells during interaction with B. dermatitidis yeast cells (Figure 4). The peak response occurred at about 15 minutes after addition of the yeast cells to the sensitized peritoneal cells. The non-sensitized cells showed only a gradual increase in emitted light over the 30 minute period of interaction. Controls containing peritoneal cells without the fungus remained constant throughout the counting time. Heat produced by the scintillation counter began to significantly effect the results after 30 minutes of counting.

Intracellular proliferation of B. dermatitidis. The results obtained for plating B. dermatitidis from lysed macrophage cultures are shown in Figure 5. The non-sensitized macrophages failed to prevent intracellular proliferation of the yeast cells. The phagocytized yeast cells proliferated at an almost logarithmic rate in the non-sensitized culture. While there was not dramatic killing as detectable by this system, the sensitized macrophages did inhibit the intracellular growth of B. dermatitidis yeast cells.

The cultures were followed with the SEM. Between 24 to 48 hours,

Figure 3. Phagocytosis of B. dermatitidis by sensitized peritoneal macrophages. A. Attachment of yeast cell to macrophage via macrophage philapodia (X4000). B. A macrophage in the process of engulfing a yeast cell (X5000). C. A macrophage that has completely phagocytized more than one yeast cell (X3000).

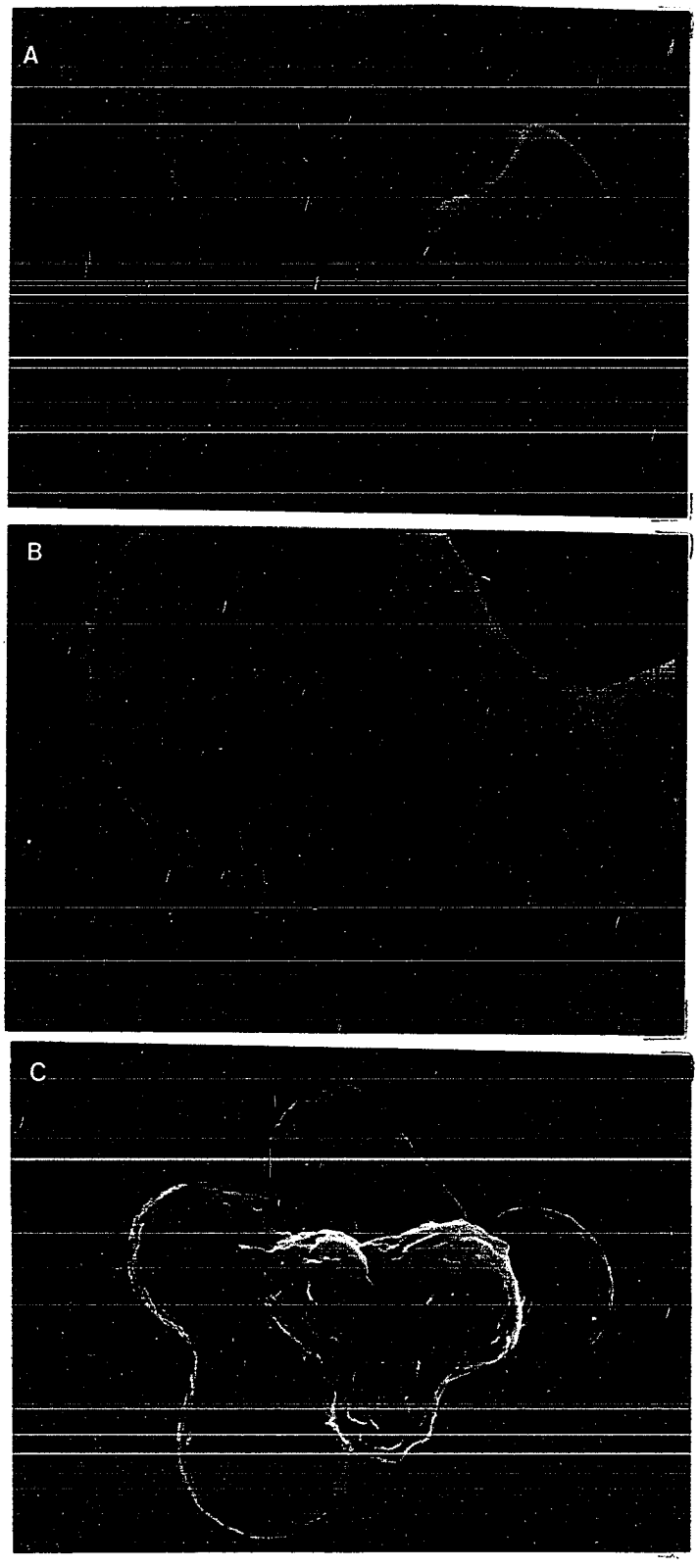

A
B
C

Figure 4. Chemiluminescence activity of macrophages during phagocytosis of B. dermatitidis. A 1:1 peritoneal to yeast cell ratio gave optimal results. Each point is the mean (± standard deviation) counts per minute for a five minute interval.

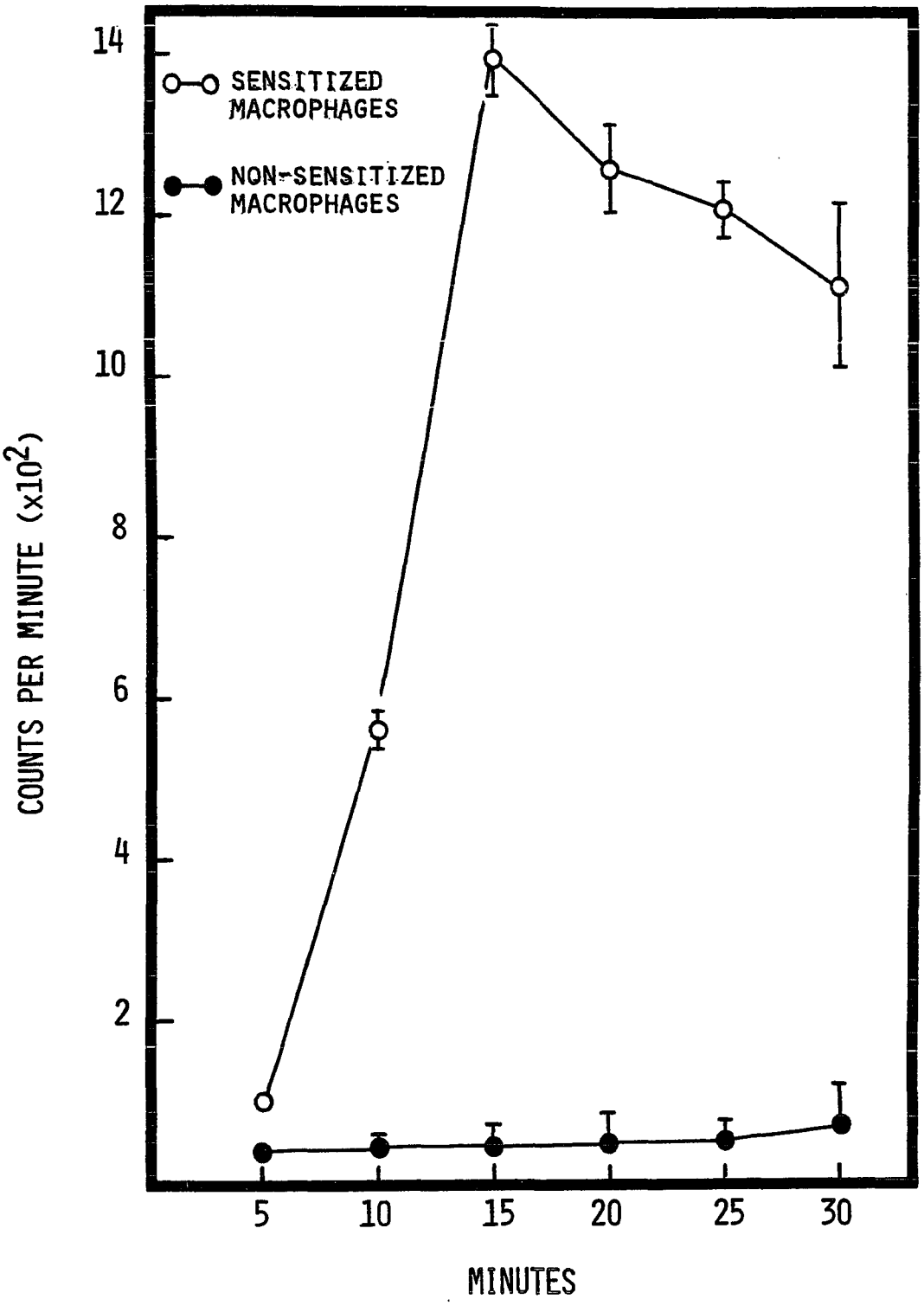
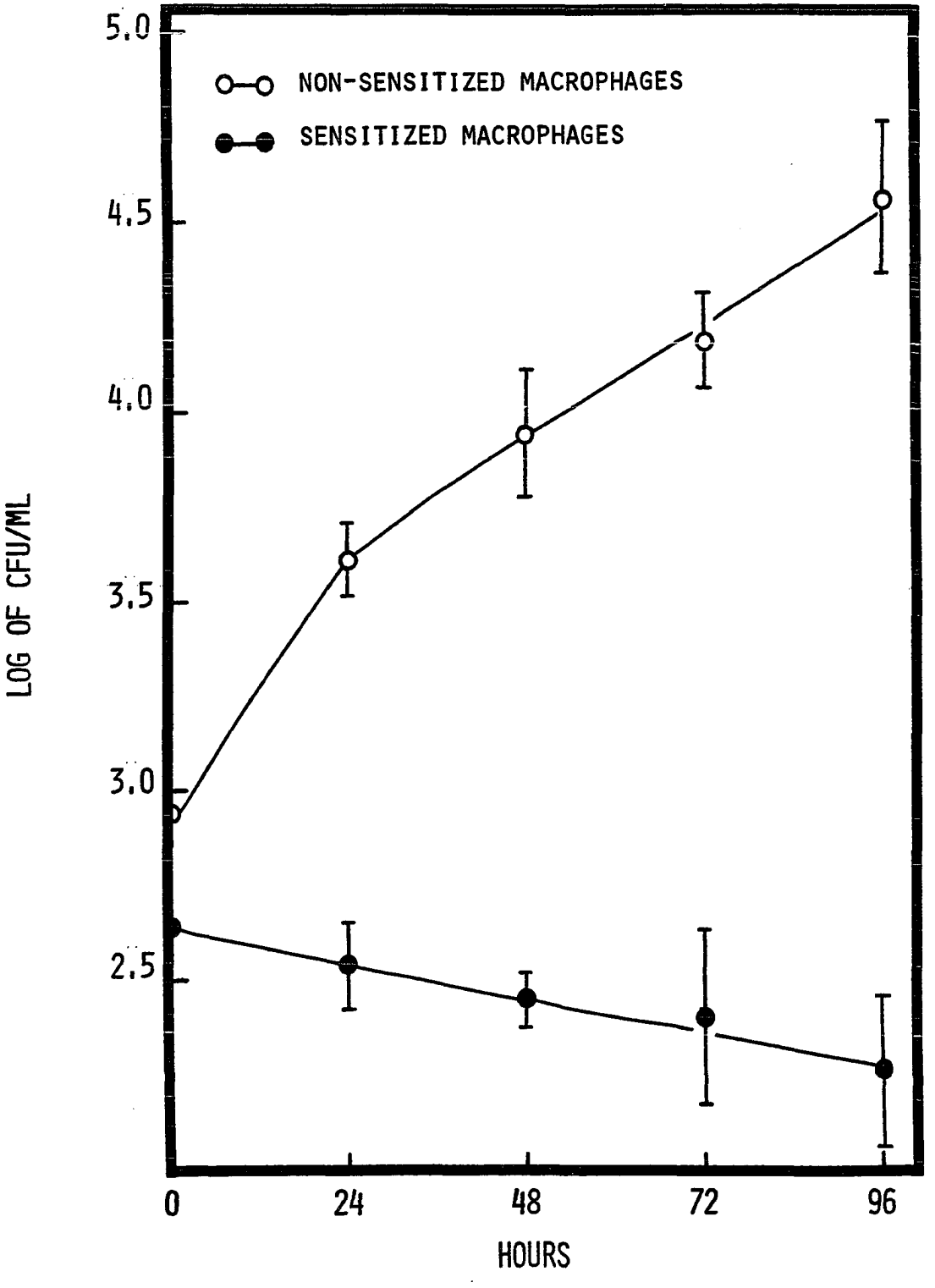

SENSITIZED MACROPHAGES
NON-SENSITIZED MACROPHAGES
COUNTS PER MINUTE (x10²)
14
12
10
8
6
4
2
5
10
15
20
25
30
MINUTES

Figure 5. Intracellular proliferation of B. dermatitidis. Macrophage: yeast cell ratio was 1:1 with results expressed as means (± standard deviation).


NON-SENSITIZED MACROPHAGES
SENSITIZED MACROPHAGES
5.0
4.5
4.0
3.5
3.0
2.5
LOG OF CFU/ML
0
24
48
72
96
HOURS

there were a significant number (greater than 60%) of non-sensitized macrophages with yeast cells protruding out. Figure 6 shows a representative non-sensitized macrophage. The yeast cell appeared to bud out of the macrophage. At about 90 hours, Figure 7A is typical of what was seen in the sensitized cell culture. The yeast cells appeared to be contained with a minimal amount of intracellular proliferation. In contrast, Figure 7B shows a compairable field from the non-sensitized macrophage culture at 90 hours. The yeast cells exhibited extensive proliferation. The non-sensitized macrophages had little or no effect over the intracellular proliferation of B. dermatitidis.

Figure 8 was obtained from a non-sensitized culture at 12 hours after exposure to B. dermatitidis. The yeast cell appeared to be located on the remenants of a macrophage that had attempted to phagocytize it. Approximately 3 to 5% of the macrophages in the non-sensitized cultures appeared to be lysed before the yeast cells budded out.

In vitro phagocytosis of C. albicans. When B. dermatitidis activated and control macrophages were infected with C. albicans, 69 to 74% of the former and 41 to 53% of the latter ingested yeast cells (Table 1). Furthermore, the activated macrophages significantly suppressed germ tube formation by the phagocytized C. albicans (Figure 9). At the end of 2 hours, 30% fewer yeast cells in the activated macrophages had germ tubes as opposed to those phagocytized by control macrophages. At the end of three hours, there was only about a 10% difference in germ tube formation by the phagocytized yeast cells. Beyond 3 hours the germ tubes were so numerous that it was not possible to make accurate observations.

Figure 6. Yeast cell budding out of a non-sensitized macrophage. Macrophages which had phagocytized yeast cells were incubated for 48 hours. It appears that the yeast cells are growing out of this macrophage (X2000).

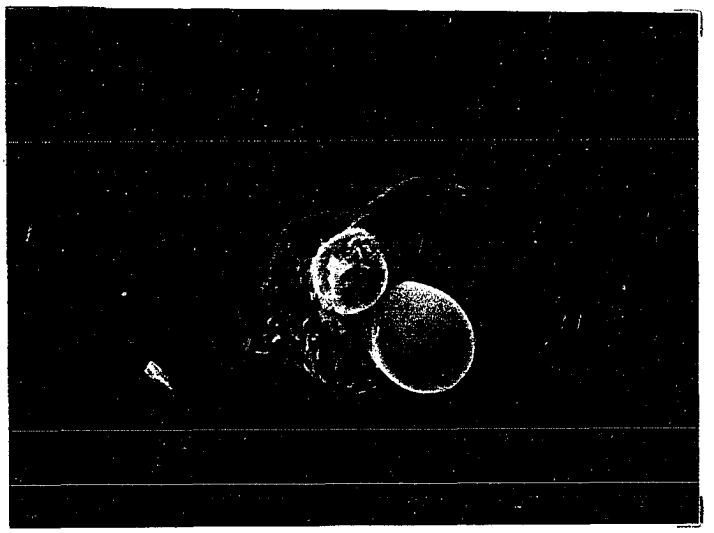
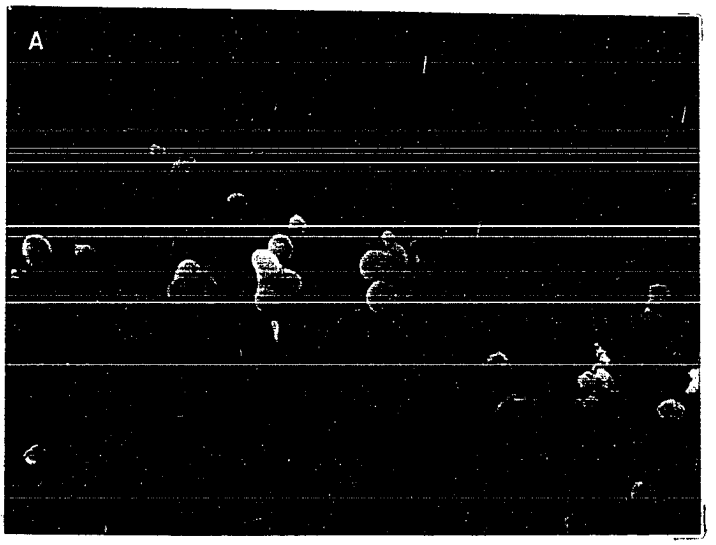


Figure 7A. Representative field from sensitized macrophage culture. Sample was fixed 90 hours after removal of non-phagocytized yeast cells. The B. dermatitidis appears to be well contained showing only a minimal amount of intracellular proliferation (X400).

Figure 7B. A comparable field from non-sensitized macrophage culture. Sample was fixed 90 hours after removal of non-phagocytized B. dermatitidis. The yeast cells show extensive proliferation and have escaped from the phagocytes. The non-sensitized macrophages have little or no effect on the growth of the yeast cells (X400).


A

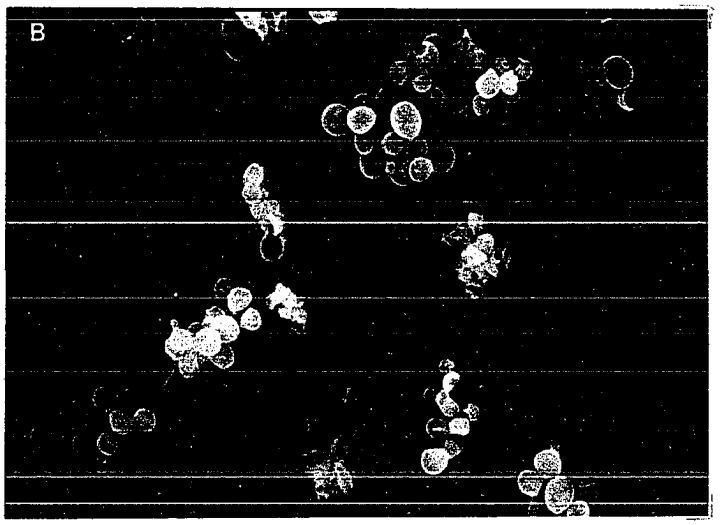

B

Figure 8. Macrophage lysed by B. dermatitidis yeast cell. Sample was fixed 12 hours after non-phagocytized yeast cells were removed. It appears that the macrophage has been destroyed without extensive proliferation of the yeast cell (X2000).

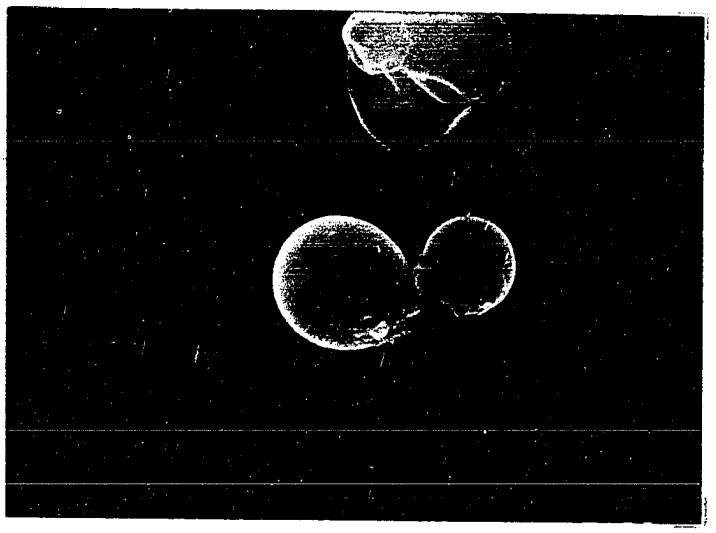
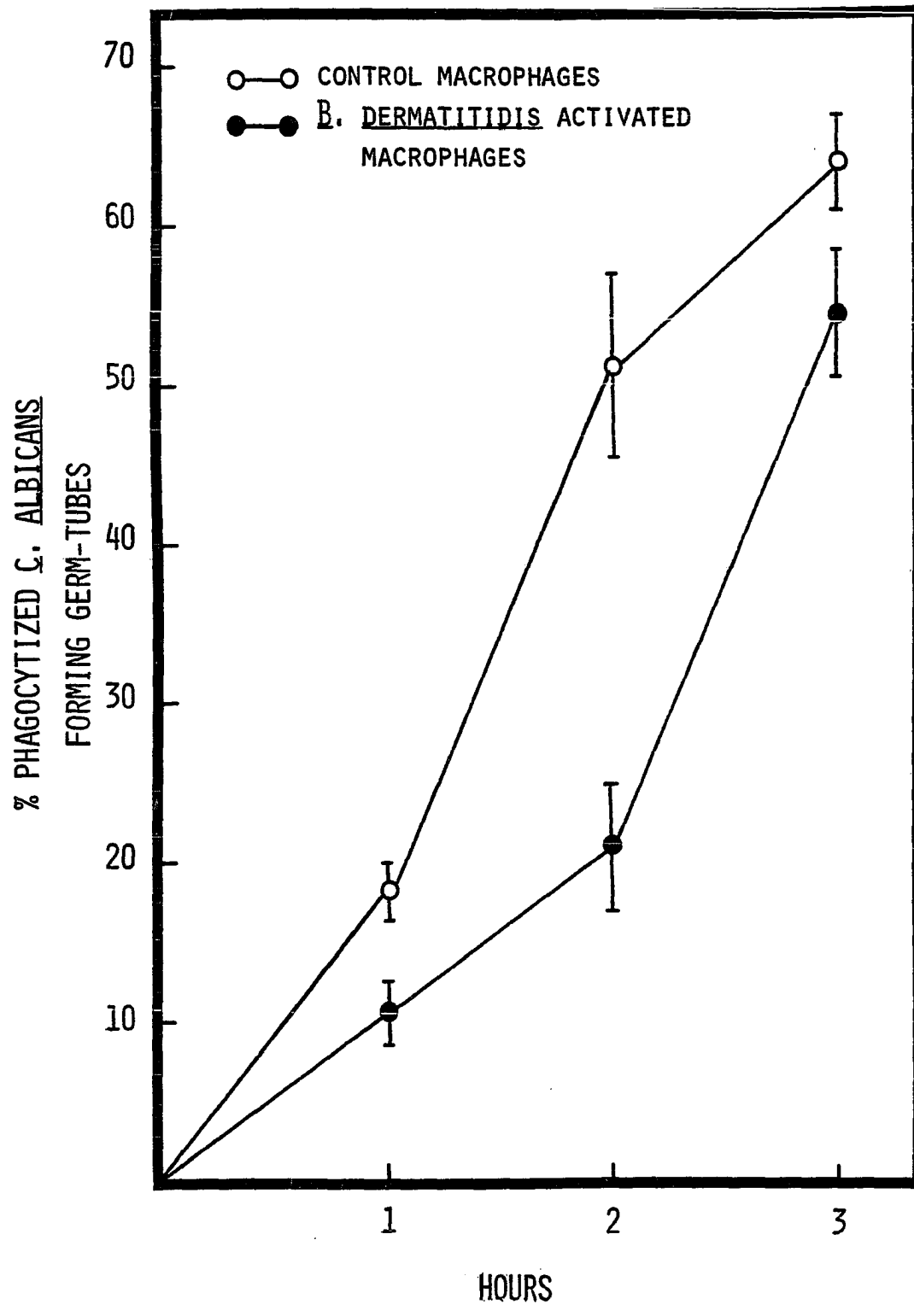


TABLE 1. FATE OF C. ALBICANS IN B. DERMATITIDIS ACTIVATED PERITONEAL MACROPHAGES

EXPT. NO.	MACROPHAGE	% MACROPHAGE PHAGOCYTIZING	NO. OF C. ALBICANS PER 100 PHAGOCYTIZING CELLS	P-VALUE*
1	A	72	288	<0.03
	C	49	219	
2	A	69	270	<0.01
	C	53	175	
3	A	74	262	<0.05
	C	41	202	

A. Activated macrophages
C. Resident macrophages
* Student t-test

Figure 9. Germ tube formation by C. albicans phagocytized by control macrophages and macrophages activated by B. dermatitidis. Results are expressed as means (± standard deviation).

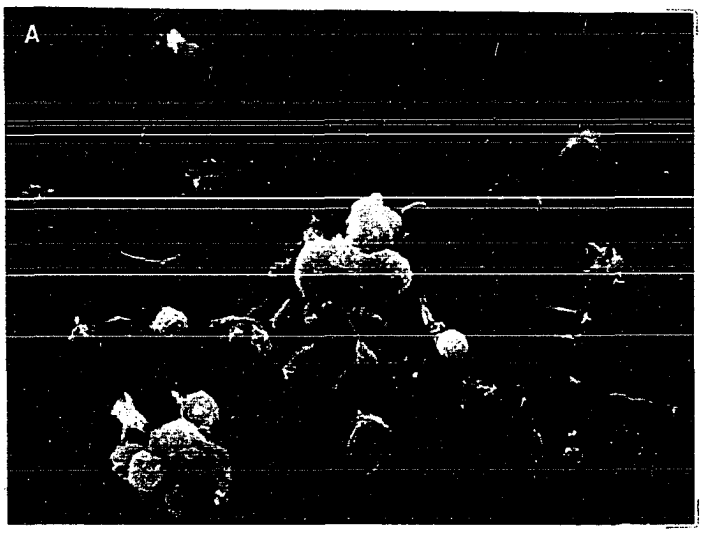

CONTROL MACROPHAGES
B. DERMATITIDIS ACTIVATED MACROPHAGES
% PHAGOCYTIZED C. ALBICANS FORMING GERM-TUBES
70
60
50
40
30
20
10
1
2
3
HOURS

In vivo phagocytosis of B. dermatitidis. The SEM was used to examine macrophages from sensitized and non-sensitized mice that had been exposed to B. dermatitidis yeast cells in vivo. It was not possible to quantitate the amount of phagocytosis. There was no way to accurately determine with the SEM if a macrophage contained one or more yeast cells once the cell was completely phagocytized. Qualitatively, the sensitized macrophages appeared more efficient at phagocytizing the yeast cells. Greater than 50% of the adherent cells obtained at 30 minutes after IP injection of B. dermatitidis contained yeast cells. This is in contrast to less than 30% of the adherent cells from control mice at the same time interval. However, adherent peritoneal cells collected at 2 and 3 hours after exposure showed very little evidence of phagocytized yeast cells.

Figure 10 shows the formation of a ring of macrophages around a yeast cell after harvesting the peritoneal contents. The macrophages from sensitized mice showed a cooperative effect during in vivo phagocytosis. The cells appeared to close in and engulf the yeast cells within the center of the ring.

Occasionally, cells taken from sensitized mice challenged in vivo with B. dermatitidis showed a high degree of clumping (Figure 11A). Observations of a mesh like film were also made (Figure 11B). The nature of this film was not determined. However, a recent report (10) demonstrated a network of cross-linked fibrin on the surface of elicited peritoneal macrophages which may explain these observations.

Figure 10. In vivo interaction of B. dermatitidis with sensitized macrophages. The contents of the peritoneum were harvested 30 minutes after injection of B. dermatitidis. A number of macrophages appear to be actively involved in phagocytizing the yeast cell. The yeast cell is centerally located (A = X800; B X3000).


A

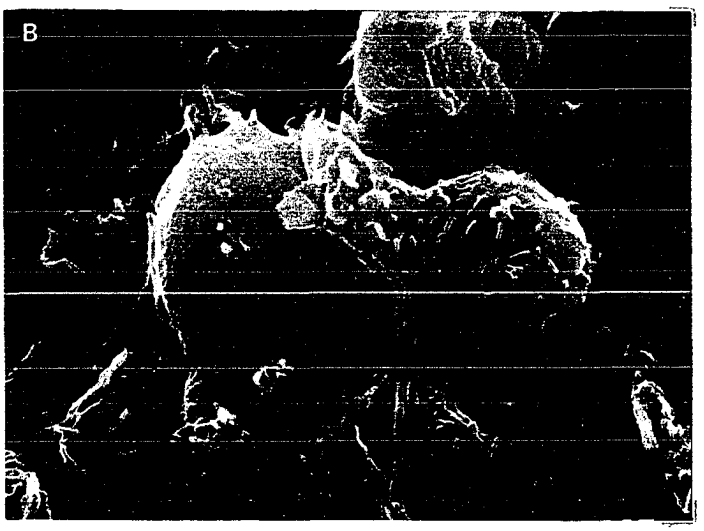
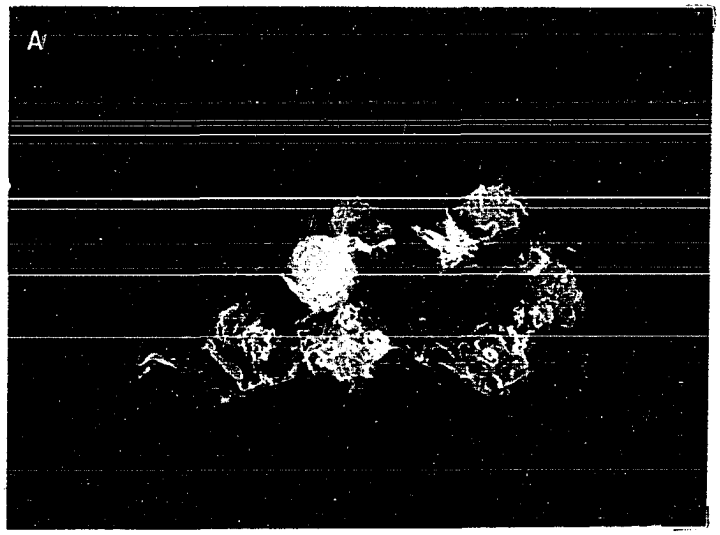
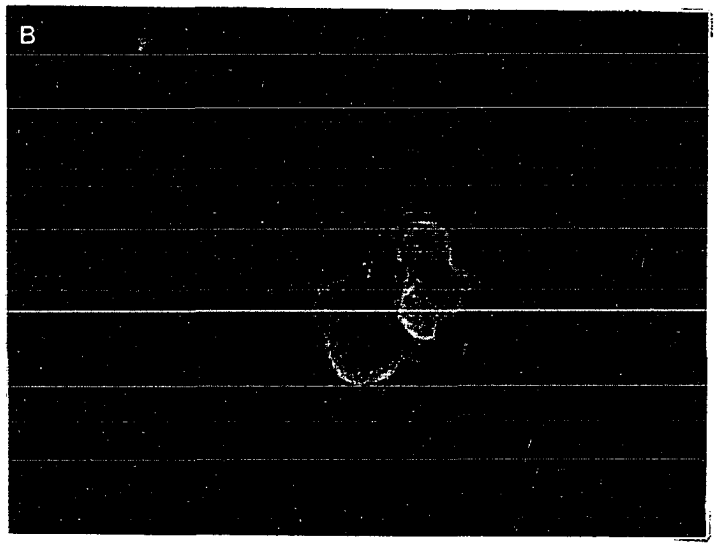

B

Figure 11. Formation of macrophage aggregates by sensitized macrophages. The contents of the peritoneum were harvested 30 minutes after injection of B. dermatitidis. A. In some cases there was extensive involvement of an apparent fibrin network (X1600). B. This micrograph shows a possible fibrin film associated with a macrophage aggregate (X1000).


A


B

DISCUSSION

It is well established that cell-mediated immunity plays a part in the mechanism of resistance to infectious diseases. The macrophage is a key cell in this phenomenon being both processor of antigen and destroyer of parasites through phagocytosis.

The data presented here show that peritoneal macrophages from sensitized C57BL/6J mice exhibit a definite increase in responsiveness to B. dermatitidis yeast phase cells. The peak in phagocytic activity of peritoneal macrophages appears to coincide with the peak in delayed hypersensitivity as determined by footpad testing. Macrophages collected from mice at 15 to 18 days post-primary injection are more efficient at phagocytizing B. dermatitidis yeast cells than those from non-sensitized mice.

The SEM proved to be an invaluable tool in assessing the phagocytic activity of mcrophages against B. dermatitidis yeast cells. The size and staining character of this organism precluded attempts to follow the phagocytic process with the light microscope. Phase contrast microscopy afforded some insight into the interaction of the yeast cells with macrophages, but it was still not possible to determine in all cases if the yeast cells were completely phagocytized by a macrophage or macrophages. The SEM left no questions. Because of their size, the yeast cells were readily discernable as phagocytized particles within

the macrophage. However, it was not possible to quantitate the total numbers of yeast cells per phagocytic cell with the SEM.

It has been reported that the composition of effector cell monolayers can significantly influence the outcome of in vitro cytotoxic assays (22). Such assays lack standardization. There is no standard assay for examining in vitro interaction of macrophages with target cells. Variations in experimental conditions can produce macrophage monolayers highly contaminated with granulocytes and even lymphocytes. Such contamination was significantly eliminated from our monolayers by culturing for 24 hours prior to challenge.

We examined the interaction of macrophages with yeast cells during phagocytosis by the technique of chemiluminescence. The phagocytic event produced a rapid increase in the amount of emitted light from the sensitized cell cultures. This phenomenon has been associated with the activation of certain metabolic pathways (19). It may indicate the formation of microbiocidal substances within the macrophages.

Non-sensitized macrophages phagocytized B. dermatitidis yeast cells as did sensitized macrophages but with reduced efficiency. Therefore, and obvious question is: What is the fate of the phagocytized yeast cells? In order to gain insight into this question, the intracellular killing of yeast cells was investigated. While there was not a dramatic decrease in the viability of yeast cells in the sensitized macrophage cultures, these macrophages did inhibit the intracellular proliferation of the yeast. The results obtained may reflect the limitations of the assay.

Macrophages are capable of killing many different microorgan-

isms rapidly following phagocytosis (27). However, a number of parasites are able to survive phagocytosis and proliferate within macrophages. Our data indicate that B. dermatitidis yeast cells possess at least two means of escaping phagocytosis. One is related to the ability of the organism to proliferate inside the phagocyte. By an as yet undetermined mechanism, phagocytized B. dermatitidis yeast cells continue to proliferate and bud out of the macrophage. The SEM revealed that greater than 90% of the phagocytized yeast cells were capable of physically growing out of non-sensitized macrophages in vitro by 90 hours. A second escape mechanism appears to involve yeast cell-mediated macrophage destruction. It has been reported that B. dermatitidis yeast cells contain cell wall associated toxin (5). The ability of these yeast cells to destroy non-sensitized macrophages in vitro without evidence of extensive proliferation may result from such yeast cell produced substances.

A recent report by Brummer et al. (3) indicated that virulent, avirulent, and attenuated strains of B. dermatitidis yeast cells can replicate in vitro in the presence of macrophages. There are wide differences in our assay procedures so that direct comparisons are not possible. Nevertheless, we consider our strain to be virulent for mice (21-day LD_{50} dose of 3.90×10^2 yeast cells intravenously). We did not observe the inhibition of replication which they reported by resident peritoneal macrophages at 24 hours. They failed to indicate if the "free growing" yeast they observed had ever been phagocytized. There was no effort to remove non-phagocytized yeast cells from their system. In addition, we question the cell composition of their monolayers. Our

staining procedures indicated a high potential for contamination of macrophage monolayers by granulocytes and lymphocytes. It has been reported that extended attachment periods (greater than 60 minutes) in the presence of 10% heat-inactivated fetal calf serum can result in very heterogeneous monolayer populations (22).

B. dermatitidis activated macrophage monolayers were infected with C. albicans in order to assess the ability of these macrophages to inhibit the growth of other fungi. The size of this organism made quantitation with the light microscope practical. This gave quantitative results as to the number and activity of phagocytic cells in the peritoneum. Also, it has been reported that lack of germ tube formation by phagocytized C. albicans is indicative of candidacidal activity by macrophages (21). The B. dermatitidis activated macrophages significantly suppressed formation of germ tubes by phagocytized C. albicans. The results indicate that B. dermatitidis can nonspecifically activate peritoneal macrophages.

Results obtained from IP challenge of B. dermatitidis were in agreement with the in vitro data. A significant number of macrophages collected from challenged sensitized mice showed evidence of phagocytized yeast cells. There appeared to be greater cooperation between macrophages in vivo in phagocytizing yeast cells. The formation of rings of macrophages around yeast cells may be a truer representation of macrophage yeast cell interaction in vivo. This tends to fit the histological picture of blastomycosis in which the pathogen may be extracellular or contained within giant cells (24).

The inability to recover large numbers of phagocytized B.

dermatitidis yeast cells beyond one hour after IP injection may relate to the macrophage disappearance phenomenon as described by Nelson and North (20). The surface of the macrophages may become "sticky" mediating attachment of yeast cells to membranes via macrophages. Alternatively, the cells may directly bind to organs within the peritoneum. A recent study indicated that the deposition of fibrin on the surface of immune macrophages may mediate their aggregation (10). It would be interesting to see what effect heparin would have on the formation of rings or aggregates of macrophages by IP challenged mice.

The activation of macrophages could be of major importance in preventing the development of systemic blastomycosis during infection. Cozad and Chang (6) showed a close parallel between delayed hypersensitivity and resistance to infection by B. dermatitidis. We present here evidence of correlation between macrophage activity and delayed hypersensitivity in mice. Mackaness (15) first demonstrated a relationship between macrophage in vitro activity and in vivo protection against infection with L. monocytogenes. Since macrophage populations are not uniform (12), it is possible that the failure of some macrophages to become activated and kill B. dermatitidis results in spread of the disease via the lymphatic system.

Additional evidence for the importance of macrophage activation came from a recent study by Morozumi et al. (17). They reported that C3H/HeJ mice are highly unresponsive to lipopolysaccharides, and their macrophages resist activation by a variety of agents (16). They concluded that defects in macrophage cytotoxicity may contribute to susceptibility in blastomycosis.

Finally, we feel that our system may represent a novel mechanism of macrophage activation. The question arises as to why cells in peritoneal cavity should exhibit activation from subcutaneous immunization with a killed organism. The mechanism for this is not clear. It is known that activation can be sustained over a period of time in chronic rather than acute inflammatory situations (4). As the activation of macrophages has not been accomplished by serum transfer, it would appear that a circulating activating factor does not account for the activity of the peritoneal cells. It has been reported that BCG infected mice challenged with Listeria produce systemic effects on macrophages during periods of intense antigenic stimulation (2). While the BCG data was with reference to active infections, it does postulate an undefined systemic activation at the height of response. The actual mechanism of systemic macrophage activation by B. dermatitidis should prove interesting.

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CHAPTER 3

IMMUNOADJUVANT EFFECTS OF *BLASTOMYCES DERMATITIDIS* AGAINST THE EL4 LYMPHOMA IN C57BL/6J MICE

ABSTRACT

We have previously shown that Merthiolate-killed *Blastomyces dermatitidis* yeast cells greatly enhance the cell-mediated immune response in C57BL/6J mice. Therefore, the use of this fungus as an immunopotentiator against EL4 lymphoma was investigated. Preimmunization resulted in a doubling of the mean survival time of mice at an initial tumor challenge of 10^2 to 10^4 EL4 cells. In some experiments, mice were given a range of 10^2 to 10^6 EL4 cells intraperitoneally or 10^2 to 10^6 EL4 mixed with killed *B. dermatitidis*. None of the animals receiving tumor cells alone survived. Mice treated with yeast cells were protected from as many as 10^4 tumor cells. Complete suppression of tumor growth was observed in treated animals at 10^2 and 10^3 tumor cells. The mice were not immune to further EL4 challenge. The lack of tumor-specific immunity indicated nonspecific suppression by macrophages. At 10 days after treatment, the peritoneal macrophages from mice showing complete suppression were tested for their ability to prevent in vitro tumor cells proliferation. These macrophages demonstrated 90% inhibition of ^{3}H-thymidine incorporation by EL4 at a 100:1 effector to target

ratio. Macrophages from treated animals at 10 and 15 days exhibited a two-fold increase in specific lysis of EL4 as compared to resident macrophages. Spleen and lymph node cells from protected animals showed no cytotoxic activity against EL4 in a ^{51}Cr-release assay. Treatment of tumor bearing mice with a single dose of B. dermatitidis was effective only if administered within 24 hours of tumor establishment.

INTRODUCTION

It is generally accepted that macrophages play a major role in resistance against growth of tumor cells within host tissue. The in vitro cytolytic capacity of activated macrophages is well substantiated (9, 20). Also, as the result of bacterial or protozoal infection macrophages become activated and are able to inhibit the growth of tumor cells in vivo (8, 11). The mechanisms whereby activated macrophages can destroy tumor cells but not normal cells are currently under investigation.

Microorganisms such as Bacillus Calmette-Guerin (BCG) (20), Toxoplasma gondii (8), or Corynebacterium parvum (11) are capable of inducing macrophages which have the capacity to distinguish tumor cells from normal cells. There is a great interest in agents which enhance the ability of macrophages to nonspecifically kill tumor cells. Various agents are also able to activate cultured resident macrophages to a tumoricidal state. These include lymphokines (20), lipopolysaccharide (LPS) (1), and poly I:C (28). Evidence continues to mount for the importance of macrophages as the primary target of such immunomodulating agents.

Past studies in our laboratory with yeast cells of the dimorphic fungus Blastomyces dermatitidis indicated that this organism greatly stimulates the cell-mediated immune system, and suggests its use as a

potent immunostimulant for suppression of tumor cell growth. We report here results from experiments aimed at determining the protective and therapeutic effects of B. dermatitidis induced resistance in C57BL/6J mice using the syngeneic lymphoma EL4.

MATERIALS AND METHODS

Culture. The Merthiolate-killed whole yeast cell antigen was prepared by the method of Restrepo-Moreno and Schneidau (21) as modified by Spencer and Cozad (26). The yeast phase culture of B. dermatitidis 242 was originally isolated from a fatal human case of blastomycosis. The culture was maintained on brain heart infusion (BHI) agar slants at $37^{o}C$.

Test animals. Two to three month old inbred C57BL/6J mice of both sexes were used in this study. The strain was originally obtained from Jax Laboratories, Bar Harbor, Maine, and bred in our animal facilities. The mice were separated by sex and given water and mouse chow (Ralston Purina) ad libitum.

Cell line. The thymus-derived benzopyrene-induced lymphoma EL4 of C57BL mice was used in this study. The cells were maintained as ascites tumors in 8 to 12 week old C57BL/6J mice. Transfers were made at 10 to 12 days by intraperitoneal (IP) injection of 10^{6} cells in 0.2 ml of Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY.). In vitro cultures of EL4 were maintained as suspensions in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS) (MA Bioproducts, Walkersville, MD.). Further supplements included 2 mM L-glutamine, 100 units/ml of penicillin, and 100 ug/ml of streptomycin (Gibco). Cells were carried in logarithmic growth (12 to

14 hour doubling time) within a cell density of 1 x 10^5 to 2 x 10^6 cells/ml. EL4 viability under these growth conditions was between 97 and 100% as determined by trypan blue exclusion.

Preparation of immunizing inoculum and assessment of delayed hypersensitivity. An antigen-emulsion (Ag-emulsion) of Merthiolate-killed B. dermatitidis yeast cells was prepared by forcing equal volumes of the yeast suspension and Freund incomplete adjuvant (Difco Laboratories, Detroit, MI.) through an 18-gauge emulsifying needle (Popper and Sons, Inc., New Hyde Park, NY.). This was continued until the emulsion formed a discrete droplet on the surface of cold water. A control suspension was prepared by substituting a physiological saline solution (PSS) for the yeast suspension.

The mice were sensitized according to the method of Cozad and Chang (4). On days 0 and 7 mice were inoculated subcutaneously in the inguinal area with 0.1 ml of the Ag-emulsion (containing 2 mg dry weight equivalent of killed B. dermatitidis yeast cells). A control group was inoculated in the same manner with the PSS-emulsion.

To determine the delayed hypersensitivity pattern, three mice from each group were footpad tested on days 3, 15, and 30 after the initial injection. The footpad tests were done by the procedure of Youmans and Youmans (30) as described by Cozad and Chang (4).

Tumor inoculations. Mice were divided into groups of 10 animals each. All experiments were carried out at least two times. Initial experiments were conducted to establish the median survival time of mice receiving from 10^2 to 10^7 tumor cells with no treatment.

Experiments were designed to test the effect of preimmunizing

mice with the Ag-emulsion. Mice were challenged with a range of 10^2 to 10^6 tumor cells IP on days 0, 3, and 15 after the initial Ag-emulsion injection. Some animals were given 0.1 or 1.0 mg of killed B. dermatitidis in 0.2 ml of Dulbecco's phosphate buffered saline (PBS) IP five days before tumor challenge. In all experiments the B. dermatitidis was washed 3 times in 10 volumes of sterile PSS to remove Merthiolate.

The protective effects were also evaluated by mixing the yeast dells with tumor cells prior to injecting into mice. The mice were given 0.2 ml IP injections of 10^2 to 10^6 EL4 cells mixed with 0.1 or 1.0 mg of killed B. dermatitidis yeast cells. The survival of the animals was checked daily for 2 months.

To test the therapeutic potential of B. dermatitidis, mice were inoculated IP with 10^2 to 10^6 EL4 cells and treated with 1.0 mg of B. dermatitidis in 0.2 ml of PBS IP at various times after tumor challenge. A control group of tumor bearing mice received 0.2 ml of PBS alone IP. The times of treatment were 2 hours, 1, 2, 5, 8, and 11 days.

Collection of peritoneal cells. Peritoneal cells were collected without the use of an exudate-inducing agent by a modification of the method described by Tolnai (29). The abdominal skin was dissected away from the peritoneum. Then 5.0 ml of cold RPMI 1640 containing 10 units/ml sodium heparin (Fellows Medical Manufacturing Co., Anaheim, CA.) and 1% FCS was injected into the peritoneal cavity by a syringe with a 20-gauge needle. After a short massage, the fluid was withdrawn into the same syringe by inserting the needle successively on both sides of the abdomen.

The cells from three animals were pooled, and their viability was assessed by trypan blue exclusion. After centrifugation the suspensions were resuspended to 5 x 10^6 cells/ml in cold RPMI 1640 with 15 FCS. Then 0.1 ml of the suspension was placed into wells of Falcon Microtest II plate style 3040 (Falcon Plastics, Oxnard, CA.). After adherence for 60 minutes at 37°C in 5% CO_2, nonadherent cells were removed by washing two times with warm PBS and suction through a Pasteur pipette, Then 0.1 ml of fresh warm medium containing 10% FCS was added to the chambers, and the plates were incubated for 18 to 24 hours at 37°C in 5% CO_2.

Cytostasis assay. A modification of the procedure described by Goldman and Bar-Shavit (6) was used. After the 24 hours incubation, 0.1 ml of RPMI 1640 with 10% FCS containing the desired concentration of EL4 cells was added to the wells. Controls of 0.2 ml RPMI 1640 with tumor cells alone were set up. The plates were reincubated for 7 hours. Then 0.5 uCi of ^{3}H-thymidine (20 Ci/mmole; New England Nuclear, Boston, MA.) was added. Controls containing macrophages alone with ^{3}H-thymidine were included. Then plates were again reincubated at 37°C and 5% CO_2 for an additional 24 hours. The samples were harvested with a MASH unit (MA Bioproducts). The filter strips were dried, and the discs were placed in toluene scintillation fluid and counted in a Beckman LS 100C liquid scintillation counter.

Cytotoxic assay. EL4 cells were labeled by incubating 5 x 10^6 cells for 3 hours in 1.0 ml RPMI 1640 with 10% FCS containing 10 uCi of ^{125}I-iododeoxyuridine (^{125}IUdR) (5 Ci/mg; Amersham, Arlington Heights, IL.) (14). After incubation the cells were washed three times with

medium and resuspended at 5 x 10^6 cells/ml.

The desired concentration of labeled EL4 cells in 0.1 ml of medium was added to wells containing 5 x 10^5 macrophages that had been incubated for 24 hours. Wells were set up for spontaneous and maximun release for each tumor cell concentration used. All samples were ran in triplicate. The plates were incubated for an additional 27 to 30 hours at 37°C and 5% CO_2. After incubation 0.1 ml of a 2N HCl solution was added to the maximum release wells to obtain total counts. The plates were centrifuged for 15 minutes at 200 x g. Then 0.1 ml samples were removed and counted in a Packard Tri-Carb gamma counter. The percentage of specific lysis was obtained using the following formula:

$$\% \text{ Specific lysis} = \frac{\text{cpm test sample -cpm spontaneous release}}{\text{maximum cpm of target cells}} \times 100$$

Scanning electron microscopy (SEM) of EL4 and macrophages.

Peritoneal cells were hrvested as described above. A 5.0 ml suspension of 1 x 10^6 cells/ml was plated in petri dishes containing three glass coverslips. After 60 minutes at 37°C and 5% CO_2, all non-adhering cells were washed away with warm PBS. Fresh warm medium containing 10% FCS was added, and the culture was incubated for 18 to 24 hours at 37°C in 5% CO_2.

After 24 hours, the medium was replaced with fresh medium containing 5 x 10^3 EL4 cells/ml. This was incubated for various time intervals up to 48 hours at 37°C and 5% CO_2. The medium was then aspirated, and the coverslips were fixed with 0.5% glutaraldehyde in PBS at room temperature for 18 to 24 hours.

The samples were further prepared for SEM by dehydration through a series of ethyl alcohol. The coverslips were critical point dried in a No. 99 Model H Pelco Critical Point Dryer (Ted Pella Company, Tustin, CA.) using liquid CO_2 with a critical pressure of 1072 psi. The samples were then gold coated in a Technics Hummer sputter coater and examined in an International Scientific Instruments Super II scanning electron microscope at the noted magnifications with an accelerating voltage of 25 KV.

Chromium release assay. The cytotoxic activity of lymphocytes was evaluated using an established procedure (25). EL4 cells, 1×10^7 in 1.0 ml, were incubated in serum free RPMI 1640 containing 100 uCi of ^{51}Cr-sodium chromate (500 mCi/mg; Amersham, Arlington Heights, IL.) for 1 hour at 37°C and 5% CO_2. After incubation the cells were washed twice and adjusted to 2×10^5 cells/ml (target cells) in RPMI 1640 with 10% FCS. Spleen or lymph node cells (effector cells) from mice showing tumor suppression at 10 or 15 days were adjusted to 1×10^7 cells/ml. Then 0.1 ml of target cells were placed in round bottomed microtiter plates (Linbro Scientific Co., Hadmen, CT.). The plate was incubated at 37°C in 5% CO_2 for 4 hours. At the end of this time, the plate was centrifuged at 200 x g for 15-20 minutes. Then 0.1 ml of the supernatant was carefully removed, transferred to a 12 x 75 mm disposable glass tube, and counted for 10 minutes in a Packard Tri-Carb gamma counter. The percent cytotoxicity was obtained by the following formula:

$$\% \text{ Specific lysis} = \frac{\text{cpm test sample} - \text{cpm spontaneous release}}{\text{maximum cpm of target cells}} \times 100$$

RESULTS

Hypersensitivity. Footpad tests demonstrated a delayed hypersensitivity pattern that is consistent for B. dermatitidis. For results see Chapter 2.

Survival time of normal mice. EL4 lymphoma cells were grown in the pweitoneal cavity of C57BL/6J mice. It was necessary to establish the survival time of untreated mice inoculated with various concentrations of EL4 cells. The median survival time is shown as a function of the initial tumor inoculum in Figure 1. The median survival time was directly proportional to the initial tumor inoculum.

These results were compared to a previous study (13) for reference purposes. The data are shown in Figure 2. As can be seen, the survival time of the mice in this study followed that of the previous study.

Preimmunization effects on mice survival. Mice were preimmunized with B. dermatitidis in Freund incomplete adjuvant. They were then challenged with various tumor inocula at different times post-primary immunizing dose. The results are presented in Figure 3. As can be seen, mice that were challenged on the same day or 15 days after the initial B. dermatitidis injection had mean survival times very similar to that of the controls. However, mice challenged 3 days after the initial B. dermatitidis injection showed a significant increase in mean survival

Figure 1. Median survival time of C57BL/6J mice as a function of the initial number of EL4 cells injected into the peritoneal cavity.

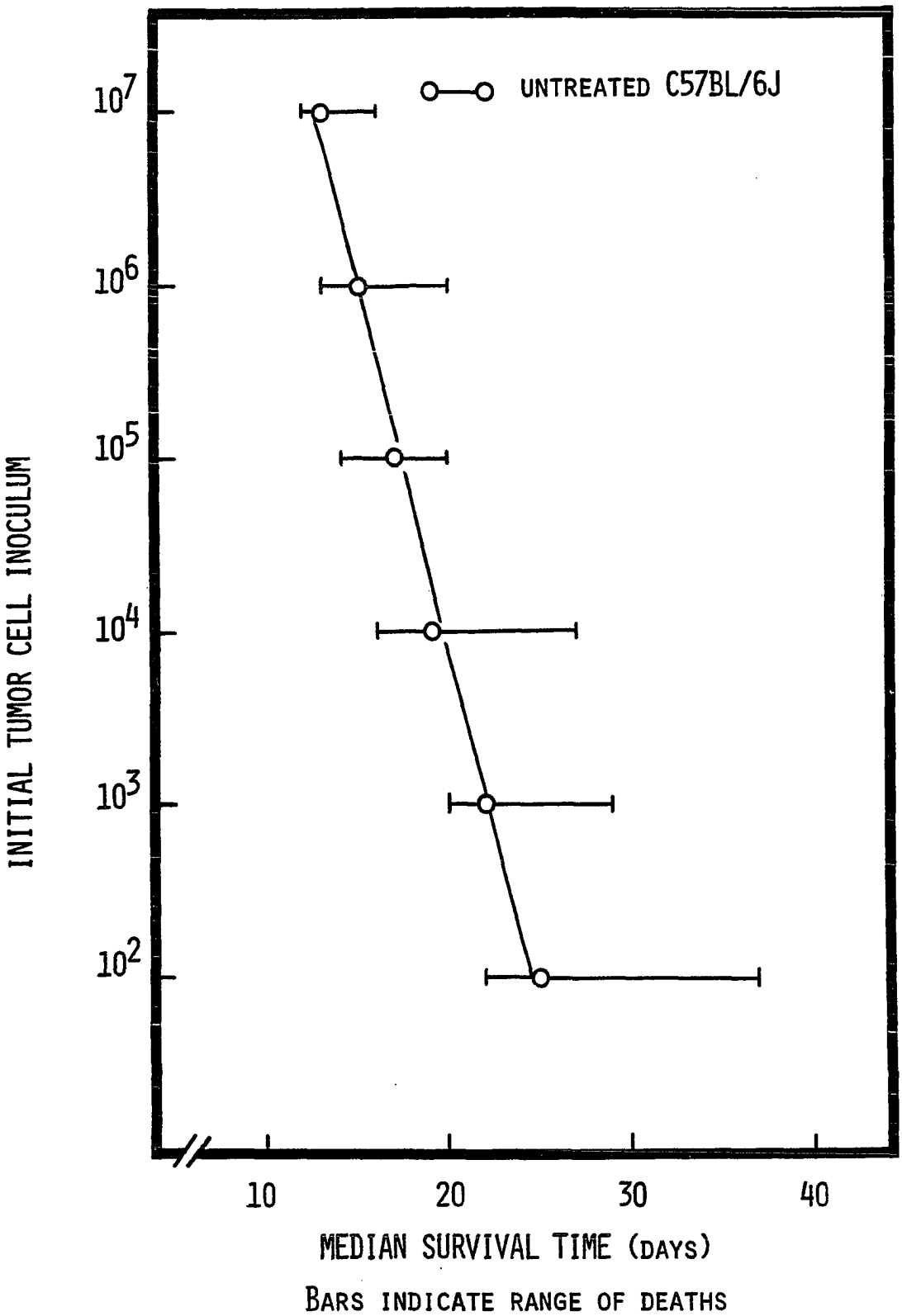
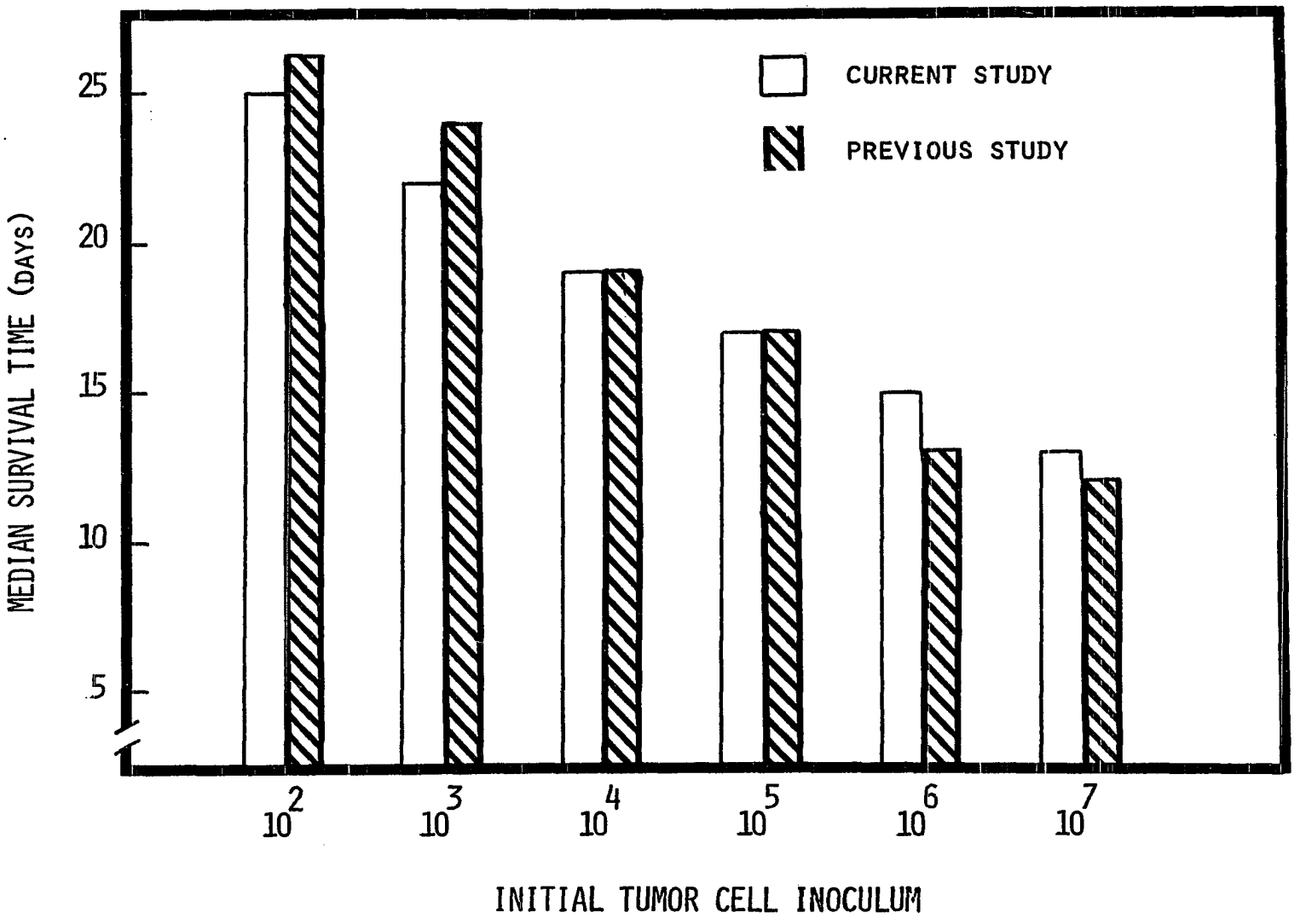
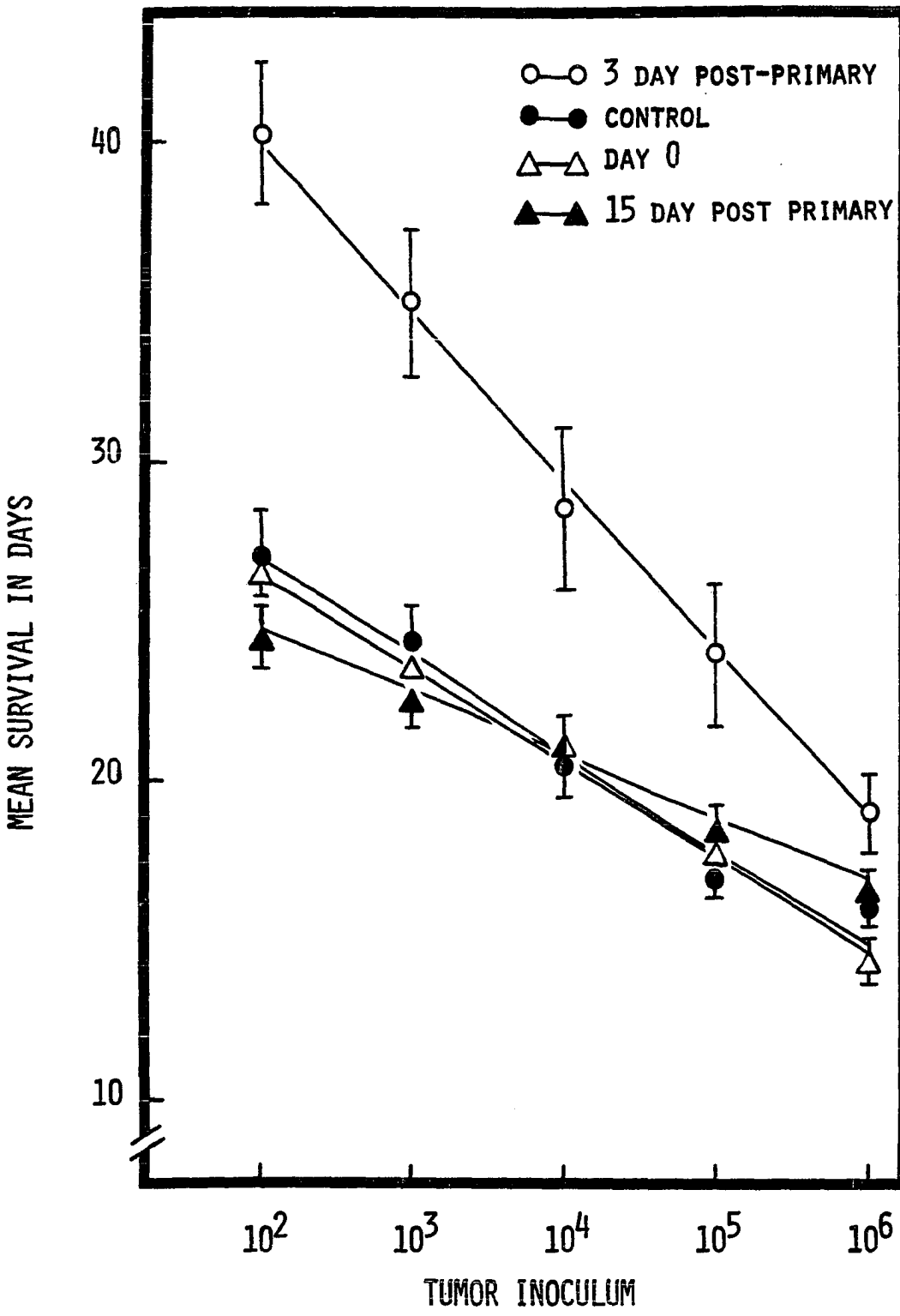

UNTREATED C57BL/6J
10^7
10^6
10^5
10^4
10^3
10^2
INITIAL TUMOR CELL INOCULUM
10
20
30
40
MEDIAN SURVIVAL TIME (DAYS)
BARS INDICATE RANGE OF DEATHS

Figure 2. Comparison of median survival time of C57BL/6J mice receiving EL4 to a previous study for reference purposes.


CURRENT STUDY
PREVIOUS STUDY
MEDIAN SURVIVAL TIME (DAYS)
25
20
15
10
5
10^2
10^3
10^4
10^5
10^6
10^7
INITIAL TUMOR CELL INOCULUM

Fugure 3. Preimmunization of C57BL/6J mice with B. dermatitidis in Freund incomplete adjuvant. Results are expressed as mean (± standard error) survival time.


3 DAY POST-PRIMARY
CONTROL
DAY 0
15 DAY POST PRIMARY
MEAN SURVIVAL IN DAYS
40
30
20
10
10^2
10^3
10^4
10^5
10^6
TUMOR INOCULUM

time. The lower the initial tumor inoculum, the more significant was the increase in mean survival time as compared to controls.

In some experiments, mice were given killed B. dermatitidis IP in PBS 5 days prior to tumor challenge. Figure 4 shows the increase in mean time of survival of mice treated with 0.1 or 1.0 mg of yeast cells. There was no significant difference in the survival of mice treated with 0.1 or 1.0 mg of yeast cells. The increase in survival was very similar to that seen in mice immunized 3 days before tumor challenge with B. dermatitidis in Freund incomplete adjuvant (Figure 3).

Mixing of EL4 with B. dermatitidis. A dry equivalent weight of 0.1 or 1.0 mg of killed B. dermatitidis was mixed with 10^2 to 10^6 EL4 cells and immediately injected IP to determine if B. dermatitidis could induce a host response capable of suppressing the growth of the tumor. The EL4 in these mixtures showed no significant decrease in viability even after 4 hours as determined by trypan blue exclusion. Within several days, all mice that had received the mixture developed an inflammatory response at site of injection. Mice that received EL4 alone showed no such response. At 30 days, the inflammation had receded in a majority of the animals that received B. dermatitidis. By 60 days, all mice in the control groups were dead as the result of tumor growth, but a significant number of animals that received the mixture were alive and free of tumors (Table 1). At 65 days, the living mice were rechallenged with 1×10^4 EL4 cells IP. Although the survival of the mice increased, they all died as result of tumor development.

Treatment of EL4 with B. dermatitidis. To determine if B. dermatitidis could cause regression of established tumors, mice inoculated

Figure 4. Preimmunization of C57BL/6J mice with B. dermatitidis by IP injection five days prior to challenge with EL4. Results are expressed as mean (± standard error) survival time.

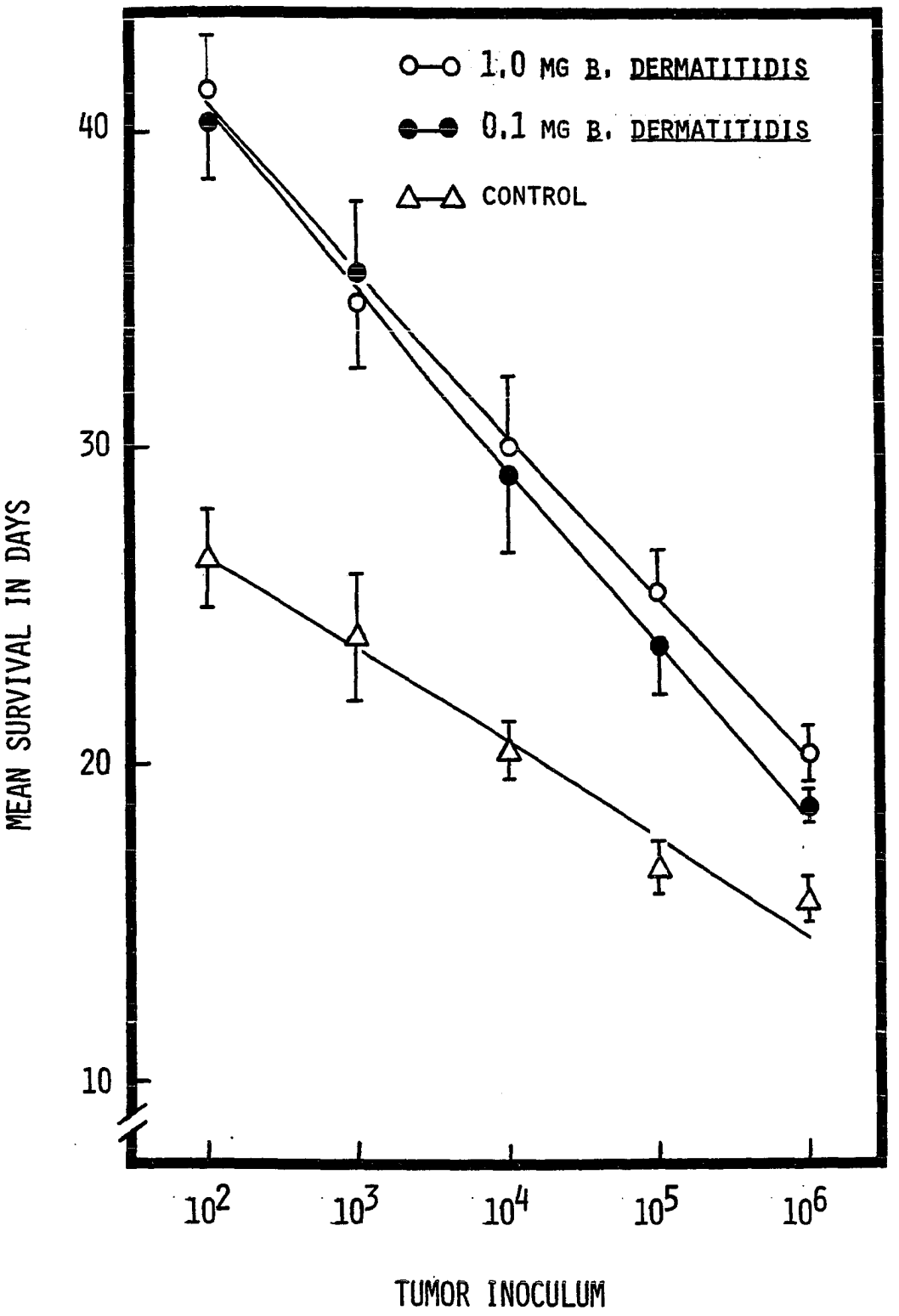

1.0 MG B. DERMATITIDIS
0.1 MG B. DERMATITIDIS
CONTROL
40
30
20
10
MEAN SURVIVAL IN DAYS
10^2
10^3
10^4
10^5
10^6
TUMOR INOCULUM

TABLE 1. SUPPRESSION OF TUMOR GROWTH BY B. DERMATITIDIS

CONC. EL4	RESULTS 60 DAYS POST TREATMENT					
	CONTROL		0.1 MG B. DERMATITIDIS		1.0 MG B. DERMATITIDIS	
	A	B	A	B	A	B
10^6	0/10*	0/10	0/10	0/10	0/10	0/10
10^5	0/10	0/10	1/10	0/10	2/10	1/10
10^4	0/10	0/10	10/10	6/10	10/10	8/10
10^3	0/10	0/10	10/10	10/10	10/10	10/10
10^2	0/10	0/10	10/10	10/10	10/10	10/10

* No. of animals tumor-free/no. given injections of tumor
A Experiment #1
B Experiment #2

with EL4 cells were treated at various times with 1.0 mg of B. dermatitidis (Table 2). Animals that received 10^5 or 10^6 EL4 cells showed no regression of tumor growth. There was no significant increase in the mean survival of these animals as compared with the controls. Mice given 10^2, 10^3, or 10^4 EL4 cells showed a significant percent survival if treated within 2 hours. The amount of survival decreased at least three-fold if the treatment was given at 1 day. No animals treated between 3 and 11 days with a single dose of B. dermatitidis showed significant increases in survival.

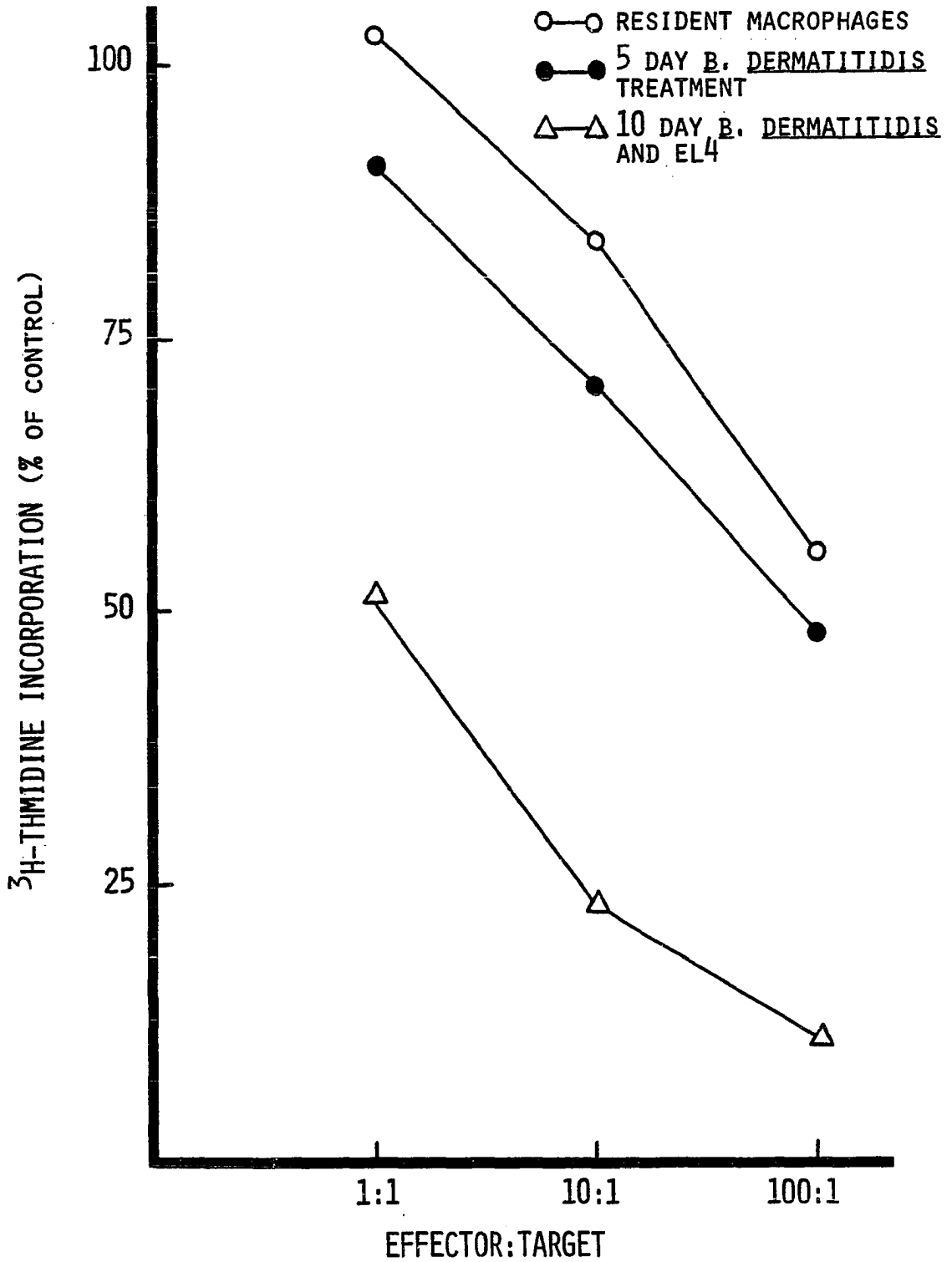
Effect of macrophages on tumor proliferation. To determine if peritoneal macrophages could halt the growth of EL4 cells in vitro, ^{3}H-thymidine incorporation by EL4 in the presence of various macrophage preparations were examined (Figure 5). At a 100:1 effector to target ratio, resident macrophages reduced ^{3}H-thymidine incorporation into EL4 by 44%. A significant difference in the amount of incorporation was seen in the presence of macrophages from mice given 0.1 or 1.0 mg of B. dermatitidis 5 days before harvesting. The results obtained using macrophages derived from animals given B. dermatitidis subcutaneously 3 days before harvesting were almost identical to those seen with 5 day prior IP injected animals (data not shown). Macrophages from mice that received a mixture of 10^4 EL4 and B. dermatitidis 10 days before harvesting produced 90% inhibition of incorporation at a 100:1 effector to target ratio. This was greater than a 45% difference when compared to resident macrophages at the same concentration. The difference between these two groups was even greater at 1:1 and 10:1 effector to target ratioes.

TABLE 2. REGRESSION OF ESTABLISHED TUMORS BY *B. DERMATITIDSI*

CONC. EL4	TREATMENT TIME	RESULTS 60 DAYS POST TREATMENT		P-VALUE[@]
		1.0 MG *B. DERMATITIDIS*	PBS	
10^4	2 HR	6/10[*]	0/10	<0.05
	1 DAY	2/10	0/10	<0.05
	2 DAY	0/10	0/10	
10^3	2 HR	9/10	0/10	<0.001
	1 DAY	2/10	0/10	NS
	2 DAY	0/10	0/10	
10^2	2 HR	10/10	0/10	<0.001
	1 DAY	6/10	0/10	NS
	2 DAY	4/10	0/10	NS

@ Mann-Whitney Rank-Sum test
* No. of animals tumor-free/no. given injections of tumor
NS Not significant

Figure 5. Macrophage-mediated cytostasis of EL4 assessed by inhibition of ^{3}H-thymidine incorporation. Macrophages were collected from normal mice (resident macrophages), from mice that had received 1.0 mg of B. dermatitidis 5 days earlier, and from mice showing complete suppression of tumor growth at 10 days which had received 10^{4} EL4 + 1.0 mg B. dermatitidis.


RESIDENT MACROPHAGES
5 DAY B. DERMATITIDIS TREATMENT
10 DAY B. DERMATITIDIS AND EL4
100
75
50
25
^{3}H-THMIDINE INCORPORATION (% OF CONTROL)
1:1
10:1
100:1
EFFECTOR:TARGET

In vitro cytotoxicity of peritoneal macrophages. Peritoneal macrophages taken from mice 10 or 15 days after receiving 0.1 or 1.0 mg of B. dermatitidis mixed with 10^4 EL4 cells were tested for their ability to release ^{125}IUdR prelabeled EL4 cells. The data in Fugure 6 show that there was at least a two-fold difference in the amount of lysis caused by macrophages from treated mice as compared to normal resident macrophages. There was a consistent drop in the amount of lysis when the effector to target ratio was increased from 10:1 to 100:1.

In initial experiments, an incubation period of 48 hours was used to the cytotoxic effects. However, under these conditions the spontaneous release was equal to the maximum release in the controls. If the incubation time was cut to 30 hours, the counts obtained showed greater than a five-fold difference for the spontaneous versus the maximum release of ^{125}IUdR.

Interaction between activated macrophages and target cells. The EL4 lymphoma cells were readily identifiable in the SEM. They were spherical and had numerous microvilli on their surfaces. When EL4 cells were seen in the presence of resident macrophages, there was no evidence of macrophage aggression toward the lymphoma cells (Figure 7A). Some tumor cells appeared to move across the surface of resident macrophages.

Macrophages collected from mice 10 days after receiving 1.0 mg of B. dermatitidis mixed with 10^4 EL4 cells (test macrophages) were examined for there ability to interact in vitro with EL4. Figure 7B shows a macrophage surround by 8 EL4 cells. Two of the cells have been lysed and several others show significant surface alterations. This micrograph also shows the apparent sequence that lead to the ultimate lysis

of tumor cells. The EL4 cells lost many of their surface microvilli eventually becoming smooth. These smooth surface cells would develop numerous perforations indicating lysis had occurred. Figure 7C shows a single EL4 cell that has been lysed by a test macrophage. There are several EL4 cells in close proximity to the macrophage that have not been lysed.

Figure 8A shows a test macrophage with 4 EL4 cells attached to it after 4 hours of interaction. Philapodia from the macrophage are actively engaged with the tumor cells (Figure 8B). Even at this early time, test macrophages showed strong evidence of interaction with tumor cells. However, there are no apparent alterations in the EL4 cells at this point.

Lymphocyte cytotoxicity. Cytotoxic lymphocytes were tested for in the spleen or lymph nodes of mice which had received 0.1 or 1.0 mg of B. dermatitidis mixed with 10^4 cells IP. The tests were carried out at 10 and 15 days after injection of the mixture. In one experiment, lymph node and spleen cells were taken at 10 days from mice which had received the above mixture on day 0, 3, and 5. Table 3 shows results from a representative experiment for lymphocytes collected 10 days after a single injection. No cytotoxic lymphocytes were detected in spleen and lymph node preparations from mice showing suppression of tumor growth.

Figure 6. Release of ^{125}I from EL4 target cells after 30 hours in culture. The target cells were cultured with macrophages (effector) collected from normal mice (resident macrophages) or from mice showing complete suppression of tumor growth at 10 or 15 days after receiving 10^4 EL4 + 1.0 mg B. dermatitidis. The data shown are means (± standard error).

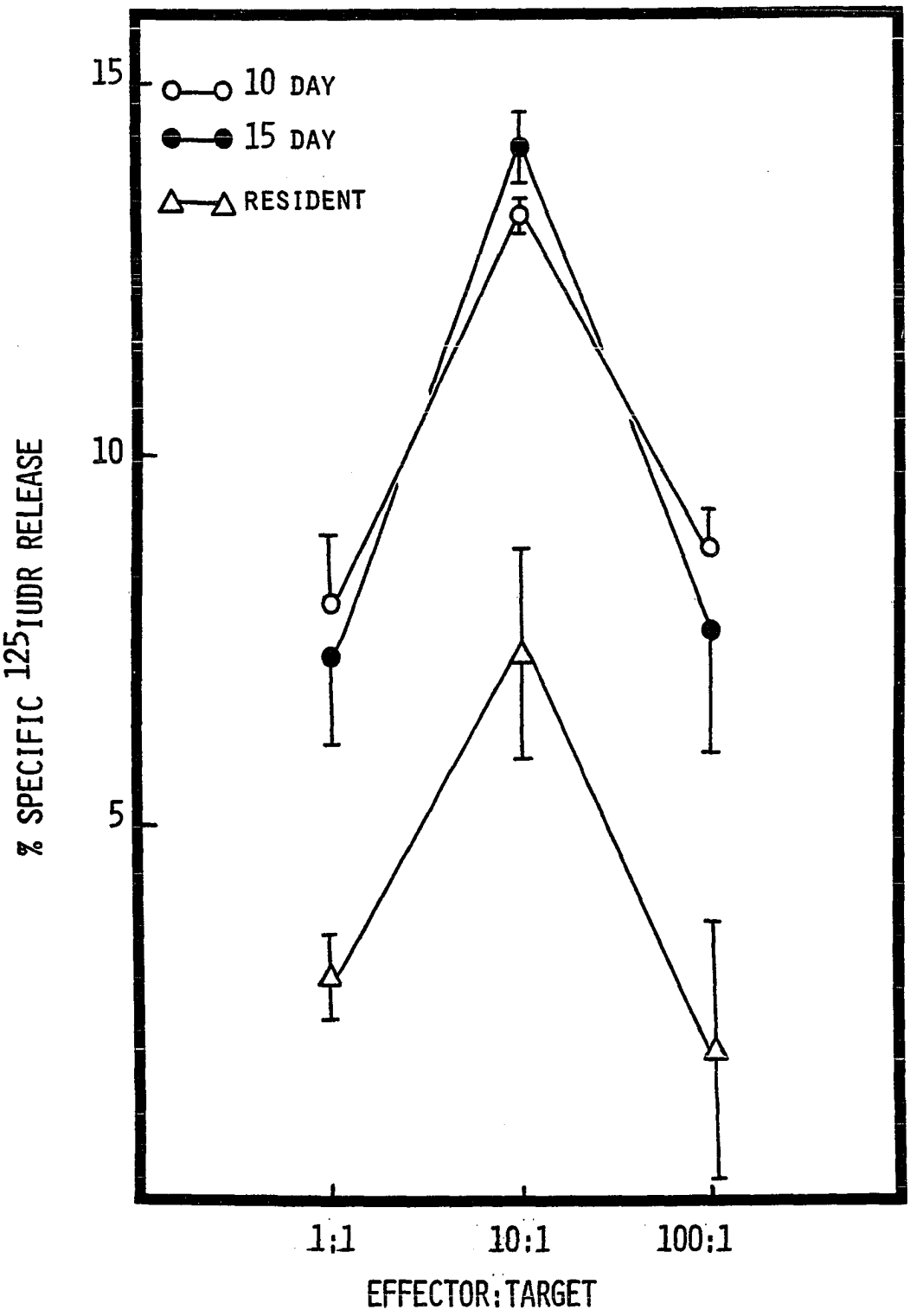

15
10
5
10 DAY
15 DAY
RESIDENT
% SPECIFIC 125IUDR RELEASE
1:1
10:1
100:1
EFFECTOR:TARGET

Figure 7A. Scanning electron micrograph of a resident peritoneal macrophage with seven tumor cells. Sample was fixed after 48 hours of macrophage:EL4 interaction in vitro. Note the presence of EL4 motility structures (X2000).

Figure 7B. Scanning electron micrograph of an activated peritoneal macrophage with eight tumor cells. Macrophages were collected from mice showing suppression of tumor growth at 10 days after receiving 10^4 EL4 + 1.0 mg of B. dermatitidis. Sample was fixed after 48 hours of macrophage:EL4 interaction in vitro. Two EL4 cells have been lysed and several show significant surface alterations (X2000).

Figure 7C. Scanning electron micrograph of an activated peritoneal macrophage in intimate contact with a single EL4 cell which has been lysed. EL4 cells in close proximity to the macrophage show no evidence of surface alteration. The sample was fixed after 30 hours of macrophage:EL4 interaction in vitro (X2000).

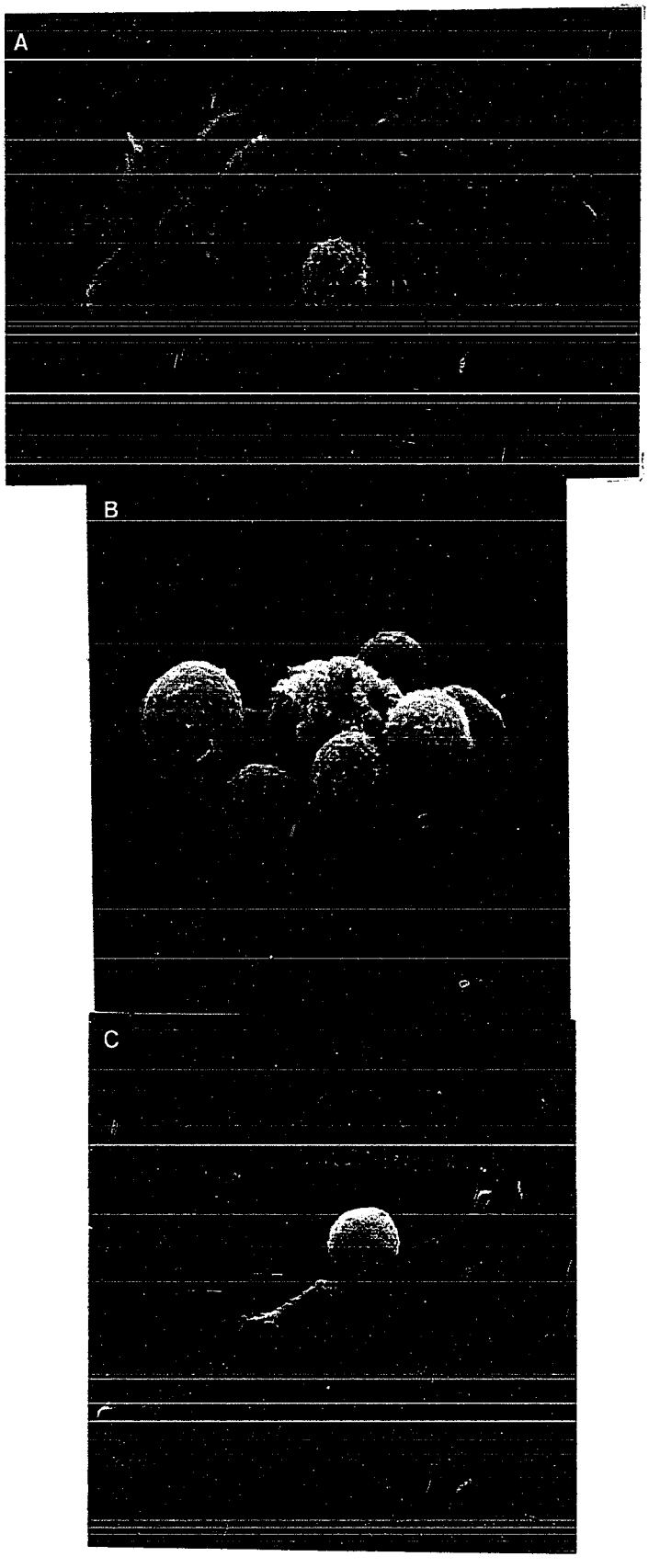
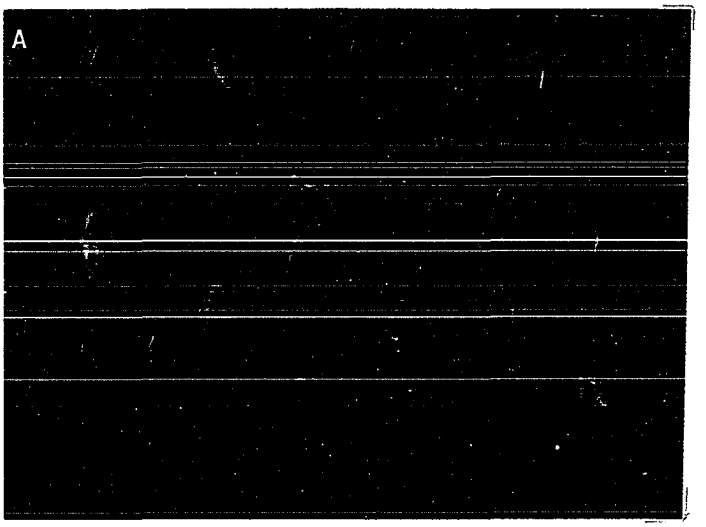

A
B
C

Figure 8. Scanning electron micrograph of an activated macrophage with four tumor cells. Sample was fixed after 4 hours of macrophage:EL4 interaction. There is already an indication of strong attachment of the EL4 cells to the macrophage via macrophage philapodia (A = X2000; B = X15000).


A

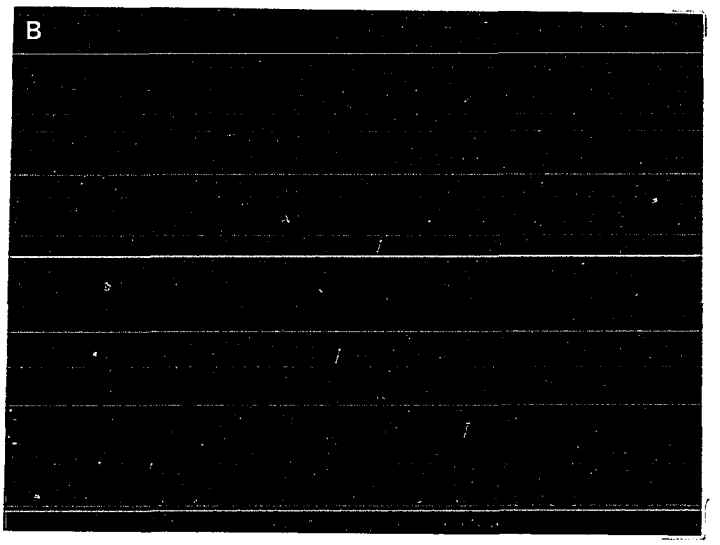

B

TABLE3. ^{51}CR-RELEASE ASSAY

EFFECTOR CELLS	% SPECIFIC LYSIS EFFECTOR:TARGET (50:1)
C57BL/6J, NORMAL SPLEEN	-2
C57BL/6J, SENSITIZED SPLEEN	5.1
C57BL/6J, NORMAL LYMPH NODES	2.2
C57BL/6J, SENSITIZED LYMPH NODES	3.0

DISCUSSION

The results presented here demonstrate that Merthiolate-killed B. dermatitidis yeast cells can cause suppression of EL4 lymphoma in C57BL/6J mice. Other studies on the mechanisms of tumor regression by immunopotentiating agents have shown that macrophages are important effector cells (8, 9). Previous studies in this laboratory have shown that B. dermatitidis can activate macrophages to become fungicidal. It is possible that this organism can activate macrophages to a tumoricidal state. Other mechanisms could be involved in the tumor suppression and cannot be ruled out. However, these experiments show that peritoneal macrophages from mice treated with non-viable B. dermatitidis yeast cells plus EL4 have an enhanced potential for in vitro tumor cell stasis and killing.

O'Neill and Stebbing (19) recently reported that preimmunization of mice with C. parvum failed to inhibit the growth of EL4 in C57BL/6J mice. In contrast to this, preimmunization with B. dermatitidis significantly increased the mean survival time of mice challenged with EL4. This protective effect was seen at 3 to 6 days after subcutaneous or IP injection of B. dermatitidis. It is curious that the route of injection had little on the time sequence of protective effects.

It was originally thought that the highest degree of enhanced survival would coincide with the peak in phagocytic activity of peri-

toneal macrophages (i.e., 15 to 18 days post-primary B. dermatitidis injection). However, this was not the case. The protective effects dropped off, and the mean survival time returned to that of the controls by day 15. This suggests that the cells involved in protecting preimmunized mice are not highly phagocytic. Similar results were observed by Rice and Fishman (22), and they reported that weakly phagocytic cells were more active in the inhibition of tumor growth than highly phagocytic macrophages.

B. dermatitidis produces a granulomatous reaction in tissue (24). Such reactions have been reported for other agents currently used in immunotherapy (7). The components of the yeast cells of B. dermatitidis which elicit tissue reaction are not completely defined. The yeast phase cell walls are mainly composed of glucose with small amounts of galactose, mannose, protein, and lipid (10, 2, 3). It has been reported that the lipid component may be responsible for induction of the granulomatous response (3). This is in contrast to the reported role of glucan from C. albicans being responsible for tissue reaction (17). In addition, glucan derived feom Saccharomyces cerevisiae has been shown to stimulate macrophages and produce resistance to bacterial infection and neoplasia (12, 23). The nature of the component responsible for the protective effects seen in this study is not known.

Bacterial endotoxin or LPS is capable of activating macrophages in vitro to a tumoricidal state (1). B. dermatitidis yeast cells have been reported to contain cell wall associated toxin (3). While the B. dermatitidis endotoxin has not been extensively characterized, it is doubtful that this toxin is identical to bacterial LPS. As evidence,

it has been reported that C. albicans and endotoxin which shares some of the properties of bacterial LPS but is not exactly comparable to it (5). Nevertheless, such a substance could be active in the protective effects we observed.

The possibility of direct interaction between non-viable B. dermatitidis and EL4 has been considered. Our data suggest that there may be some interaction since the highest degree of protection was obtained by mixing yeast cells with tumor cells prior to injection. However, we do not feel that the protective effects result solely from an adverse action of yeast cells against tumor cells. First of all, significant protection was obtained by preimmunizing mice with B. dermatitidis alone. There were no adverse effects on EL4 observed by the light microscope. Also, at a 10:1 ratio of non-viable yeast cells to tumor cells in vitro, the EL4 cells continued to incorporate ^{3}H-thymidine at near normal levels (data not shown). In addition, the protective effects were not limited to mixing yeast cells with tumor cells in vitro. The same results could be obtained by injecting yeast cells and tumor cells separately on opposite sides of the peritoneum.

The fact that the highest degree of protection resulted from mixing yeast cells with tumor cells may be clinically significant. It has been reported that a BCG-tumor cell mixture can enhance the survival of some cancer patients (27). It is possible that a non-viable B. dermatitidis-tumor cell mixture could be useful in the development of active immunity in cancer patients.

The preliminary results we obtained on the therapeutic potential of B. dermatitidis indicate that the yeast cells were beneficial

only if administered within 24 hours after tumor transplantation. It has been reported that immunoadjubants can generate cells which are capable of suppressing cell-mediated immune responses (15). It is possible that B. dermatitidis could suppress cellular immunity in tumor bearing mice thereby enhancing tumor growth. However, until further data has been gathered on the effects of B. dermatitidis on tumor bearing mice, no conclusions can be made. It is likely that a regimen involving several doses of B. dermatitidis over a period of time would be more therapeutically effective than a single dose.

The SEM revealed that there was strong interaction between macrophages from mice showing complete suppression of tumor growth and EL4 cells in vitro. The lack of such interaction between EL4 and resident macrophages was also noted. EL4 cells could be easily removed from resident macrophage cultures by washing. Such treatment failed to remove most EL4 cells interacting with macrophages from tuomr suppressed mice. Also, it has been reported that intimate contact between target and macrophages is required for tumor cell lysis in vitro (16, 18). Our observations of EL4 with the SEM agree with these findings.

We were unable to detect cytotoxic lymphocytes in the spleen or lymph nodes of mice showing suppression of tumor cell growth. It is possible that local immunity could have generated cytotoxic lymphocytes within the peritoneum. Such cells were not tested for. However, the fact that mice showing tumor suppression were not resistant to further EL4 challenge indicates a lack of specific immunity. This supports the idea of nonspecific suppression of tumor growth by macrophages.

These experiments indicate that non-viable B. dermatitidis

yeast cells can significantly effect the outcome of tumor growth in this system. Since the organism is not viable, the possibility of infection is eliminated. It will be interesting to see what component of B. dermatitidis yeast cells is involved in stimulating the immune system to suppress tumor growth. Studies in our laboratory are aimed at further evaluation of B. dermatitidis induced resistance to neoplasia.

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