A DITHIOTHREOTOL-DEPENDENT ANTIBACTERIAL FACTOR FROM LYSATE AND LYSATE FRACTIONS OF PERITONEAL CELLS FROM MICE INFECTED WITH MYCOBACTERIUM BOVIS, BCG

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from Mice Infected with Mycobacterium bovis, BCG

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ABSTRACT

A DITHIOTHREOTOL-DEPENDENT ANTIBACTERIAL FACTOR
FROM LYSATE AND LYSATE FRACTIONS OF PERITONEAL CELLS
FROM MICE INFECTED WITH MYCOBACTERIUM BOVIS, BCG

By

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Antilisterial activity was detected in lysates of mouse peritoneal mononuclear cells. Retention of the activity after collection was dependent on a reducing agent, dithiothreotol (DTT), but could be regenerated in stored lysates with DTT.

Lysates of peritoneal cells from mice immunized and prestimulated with Mycobacterium bovis, BCG, had slightly more activity than lysates from control mice. Peritoneal cells from BCG-immunized and prestimulated mice had a greater reduction potential than controls as measured by the ability to reduce nitro blue tetrazolium in vitro.

Activity was found in the pellet fraction containing lysosomes from homogenized peritoneal cells fractionated by differential centrifugation and correlated with acid phosphatase, a lysosomal enzyme marker.

Lysates of both glass adherent and nonadherent peritoneal cells from Swiss Webster mice had antilisterial activity; the former had more. Lysates of purified populations of peritoneal macrophages and spleen lymphocytes from CBA/J mice possessed low levels of antilisterial activity. Lysates of normal peritoneal macrophages which had been incubated

with culture fluid of BCG-sensitive spleen lymphocytes possessed antilisterial activity at much higher titers in the presence of DTT. Both lymphocyte culture supernatant fluid and lysates had slight activity.

Antilisterial activity in crude lysates was stable at 100 C for 10 minutes, but was partially lost after lyophilization or aeration. The material was acid labile in the absence of DTT.

Inactivation of stationary phase listeria over time in the presence of crude lysates was biphasic. A rapid decrease in the number of colony forming units occurred initially within 1½ hours and was followed by a slower rate of inactivation. There was no detectable effect on listeria in log phase.

Two fractions with antilisterial activity were detected in eluates from DEAE-cellulose chromatography of peritoneal cell lysates of BCG-immunized and prestimulated mice. The first fraction (DEAE-I) was found in eluates from cell lysates of control mice. The second (DEAE-II) was detected only in lysates from BCG-immunized mice. The fraction had optimal activity near neutral pH.

DEAE-II was less stable than the unfractionated lysate to heating and differed in listerial inactivation kinetics. Combining the two active DEAE fractions did not restore the original kinetic pattern. Although DEAE fraction II does not completely account for the activity found in unfractionated whole lysates, it does appear to be unique to lysates of peritoneal cells from BCG-immunized and prestimulated mice and may partially account for the increased antimicrobial properties of activated macrophages in antibacterial cell-mediated immunity.

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INTRODUCTION[1]

Antibacterial cell-mediated immunity is a host response to infection with a facultative, intracellular parasite. Animals which have mounted a cellular response against agents such as mycobacteria possess "activated" macrophages. These cells are nonspecifically more active against not only the same microorganisms, but also against many others. Whereas the macrophage is the cell which directly provides this protection and by which the response is observed, an immunologically specific sensitized lymphocyte population provides the stimulus for activating these cells. The lymphocytes are sensitized during the initial contact and the specificity of antibacterial cell-mediated immunity is the subsequent reaction between the same antigen and sensitized lymphocytes. The lymphocytes then elaborate "lymphokines" which activate macrophages. The greater antibacterial efficiency of these cells is measured by increased survival times of the host and decreased numbers of viable bacterial units. The bactericidal or bacteristatic mechanisms of activated macrophages are unknown.

The purpose of this study was to examine a phenomenon observed in previous research in which antistaphylococcal activity was detected in lysates of peritoneal cells from mice immunized and

[1]The material presented in this Introduction is discussed more completely and referenced in the following Literature Review.

prestimulated with Mycobacterium bovis, BCG, but not from control mice. This observation held promise for a possible correlation with antibacterial cellular immunity and study at a subcellular level.

This thesis is composed of four sections. The first is a literature review describing antibacterial systems of cells and tissues of normal and immune animals. The other three sections consist of manuscripts prepared for publication. The first paper describes the activity present in murine peritoneal cell lysates, the second the effect of BCG-sensitive lymphocytes on activity of normal peritoneal macrophages and the third the purification and characterization of active fractions.

LITERATURE REVIEW

I. Humoral antimicrobial systems.

A. "Normal" or nonspecific humoral factors.

The large number of antimicrobial substances and systems that have been identified in normal cells and sera are summarized in Table 1.

The early reports, dating back to the late 1800's, are often confusing and ill-defined. One of the earliest humoral substances reported was beta-lysin which is bactericidal, primarily for gram-positive microorganisms (166). Beta-lysin is a system composed of at least two components, released during blood coagulation, and probably is only activated in sites where tissue damage occurs and fibrin is formed (200).

Lysozyme is an enzyme, muramidase, found in the granule fraction of macrophages and polymorphonuclear leucocytes and in body secretions. It is bactericidal to some organisms (31, 147). Its range of activity can be increased by pretreatment of the organisms with specific antibody and complement (200), by an acidic environment or by lipid solvents (153).

Histone, protamines and other basic proteins and polypeptides have antimicrobial properties, primarily against gram-positive organisms (136). Histones are extracted from nuclei as complexes with nucleic acids (136). Protamines may be obtained from a variety of sources (135, 224). The mode of action appears similar to the cationic

TABLE I. Antimicrobial factors in normal cells and serum.

	Biological activity	Mode of action	Biochemical nature	Molecular weight	Heat stability	pH optimum	Other
Factors from serum and some body fluids:							
β-lysins (166)	G+ microorganisms, some G- microorganisms		protein	≥10,000	stable 60°, 30 min	unstable at alkaline pH (126)	at least 2 components necessary for activity (200)
Lysozyme (31,147)			basic protein (190)		stable 50°, 30 min		
Properdin (169)	certain G- microorganisms, viruses		euglobulins		in pure form, stable 56°, 30 min(220)		"natural" antibody; requires complement
Complement (155)	bactericidal to G- microorganisms, opsonic	Lytic			inactive 56°C, 30 min		sequential antigen-activation requires participation of antibody antibody;multicomponent system; requires participation of antigen and antibody; sequential activation
Factors from neutrophils:							
Histones (136)	G+ microorganisms	forms electrostatic bands w/ bacteria interfering w/ cell functions(200)	basic proteins, peptides		stable 56°, 30 min	alkaline pH	extracted from nuclei
Protamines (135,224)	G+ microorganisms	"	basic proteins, peptides		stable 56°, 30 min		
Leukins (73)	G+ microorganisms (199)	"	basic proteins (199)		stable 56°, 30 min (200)		
Phagocytin (77,78)	G+ & G- microorganisms	"	basic proteins (240)				
Lactoferrin (128)	bacteriostatic for microorganisms requiring Fe	interferes w/ Fe metabolism (112)	protein(128)	80-90,000			

Factors from macrophages:							
? (162)	antimycobacterial				heat labile		extracted from nuclei; higher levels in BCG-immunized guinea pigs
Free fatty acids (88,97,98)	antimycobacterial		fatty acids				higher levels in lysates from BCG-immune mice
Monocytin (60)	cidal for Sal. typhi, Sal. paratyphi, E. coli, Shig. dysenteriae, Staph. aureus		basic protein		stable, 100°, 1 hr		
Lysozyme (110)							
Mycosuppressin (234,235)	antimycobacterial	inhibits respiration	lipid (?)	≥10,000	stable, 98° C,	pH 6-7	protective only if incubated w/ mycobacteria prior to infection
Factors from other cells:							
Plakin (70)	G+ microorganisms	forms electrostatic bands w/ bacteria interfering w/ cell functions			inactive 80°, 30 min (4)	acid-stable alkaline-labile	extracted from blood platelets
Hematin, mesohematin (87,226)	G+ microorganisms	competes w/ essential porphyrins (47)	iron porphyrins		stable, autoclaving	stable - alkaline pH	extracted from red blood cells

proteins of polymorphonuclear leukocytes which is discussed in a later section (II.A.1).

Complement is perhaps the best studied humoral component, first described by Nuttall in 1888 (155). With antibody, complement is bactericidal to gram-negative organisms. Complement, with antibody may be lethal to some viruses (81), protozoa (198), and spirochetes (109) and may enhance phagocytosis (201) and immune aggregation (105).

The exact mechanism of the complement bactericidal reaction is unknown. Muschel (145) postulated the bacterial cell membrane was damaged. He theorized the complement activating antigen-antibody reaction took place at the cell wall and the activated complement components were transferred through the wall to the bacterial membrane.

The resistance of gram-positive microorganisms to complement has been explained by a thicker cell wall and a lower phospholipid content. Protoplasts of gram-positive Bacillus subtilus are affected by antibody and complement (146). The action of complement on the cell membrane may be an intermediate rather than the final reaction leading to bacteriolysis. Escherichia coli B can be made nonviable through the complement-antibody reactions but the cells do not lyse unless lysozyme is added (3).

Properdin, a protein present in normal serum, is bactericidal in the presence of complement against gram-negative organisms, and also neutralizes some viruses (169). The properdin system is now generally regarded as normal or natural antibody that is of low specificity. Properdin antibactericidal activity is not as effective when excessive

amounts are present and is inhibited by specific antibody to Shigella dysenteriae (220).

Leukins (73), plakin (70), hematin and mesohematin (87), and lactenin have been reported but not well-characterized. A comprehensive review written by Skarnes and Watson (200) describe these factors.

Other antimicrobial agents such as fatty acids, high CO_2 tension and lactic acid accumulation in inflammatory sites can also contribute to host resistance by acting singly or in concert with another antibacterial factors (47). Ionic environment and pH can also affect the outcome of an infection (51).

B. Specific induced antibodies.

Humoral immunity is important in most, if not all, types of infections. Sera of artificially immunized or convalescent animals contain antibodies, immunoglobulins which may confer protection when exposed to the same or similar cross-reacting microorganisms.

Immunoglobulins, or antibodies, provide protection through several mechanisms: 1) the neutralization of toxins secreted or associated with microorganisms, 2) neutralization of viruses, 3) with complement, bactericidal and lytic activity of gram-negative bacteria, and 4) opsonization of microorganisms for enhanced phagocytosis and removal from the host's system.

Potentially pathogenic agents such as Diplococcus pneumoniae and Haemophilus influenzae are generally disposed of by the humoral response. Antibodies are formed against facultative or obligate intracellular parasites, but apparently are ineffective in resolving these infections (122).

II. Cellular antibacterial systems

A. Normal cellular systems

1. Neutrophils

White cells (leucocytes) phagocytose, inactivate, kill and degrade many types of microorganisms. Polymorphonuclear leucocytes (PMN's) are present in large numbers in the tissues and circulation and are of prime importance in preventing infection. Phagocytosis by PMN's begins by ingestion of the microorganism, through the invagination of the cell membrane and formation of a vacuole (phagosome). Adjacent granules (primary lysosomes) fuse with the phagosome (now a secondary lysosome) and release their contents. This is generally followed by the death and degradation (digestion) of the ingested organism (94).

There are two or more separate populations of primary lysosomes in PMN's, each containing a somewhat different content of enzymes or factors. The populations differ as to size, density, enzyme content, and time and mode of origin (225).

Increased metabolic activity is associated with phagocytosis. Oxygen and glucose consumption and lactic acid production are increased as is increased glucose 1-C oxidation, a measure of hexose monophosphate shunt activity (192). The increased lactic acid production lowers the intravacuolar pH (127) which may contribute to antibacterial action on acid sensitive microorganisms such as the pneumococci.

The increased respiration is associated with H_2O_2 formation (10). The H_2O_2 formed by leucocytes may be bactericidal independently or as part of the myeloperoxidase system which is very effective against many bacteria, fungi and viruses (14, 93, 114, 209). The system

requires myeloperoxidase enzyme, H_2O_2, and iodide. Other halide cofactors such as bromide or chloride may substitute for iodide. Optimal activity occurs at pH 4.5 to 5.0 and the mode of action was originally postulated to be a halogenation of the infectious agent (93). More recently, it has been suggested that aldehydes are produced by amino acid decarboxylation and deamination. The myeloperoxidase system may catalyze formation of HOCl from Cl^- and H_2O_2 in an acid medium. The HOCl may then react with amino acids producing chloramines which spontaneously decompose to NH_3, CO_2, Cl^- and the bactericidal aldehyde (193, 241).

A number of cationic proteins with antimicrobial activities have been extracted from PMN granules. A crude preparation, termed phagocytin by Hirsch (77, 78) has an acid pH optimum and purified material is heat stable (100 C, 90 min.). The cationic proteins can be separated electrophoretically into a number of fractions with different antimicrobial specificities, including both gram-positive and gram-negative organisms (240). Unfractionated preparations also inactivate actinomyces (39). The effect of the material is dependent on the number of bacteria (77). There is an initial, rapid decrease in the numbers of viable bacteria followed by no additional killing. The proteins reportedly are bound by strong electrostatic forces, causing bacterial clumping, inhibition of oxygen consumption and damage to the cell membrane (239).

An iron-binding protein, lactoferrin, in leucocyte granules is bacteriostatic (128). Lysozyme, also present in leucocyte granules may lyse certain bacteria. Its antibacterial properties may be

enhanced by pretreatment of bacteria with specific antibody and complement (139), although its major contribution may be the degradation of microorganisms after they are killed by other mechanisms.

2. Eosinophils

Eosinophils may phagocytose and kill bacteria by mechanisms similar to those of neutrophils. These systems operate more slowly and less efficiently than do those of neutrophils and probably do not offer a major contribution to antiinfectious systems (26, 37). Morton et al. (143) presented evidence for an antibacterial system at the cell membrane which does not require phagocytosis.

3. Lymphocytes

If lymphocytes have phagocytic properties is a point of debate. Reports that lymphocytes phagocytose microorganisms (176, 242) are countered with those claiming they do not (22). Strauss et al. (210) reported spleen cells (95% lymphocytes) have a marked hexose monophosphate shunt activity when incubated with phagocytosable particles and suggested bactericidal activity may also be occurring at the cell surface. They did not observe phagocytosis, but by light microscopy, bacteria were seen in close association with the spleen cells. They proposed that lymphocytic antimicrobial systems are similar to PMN's, with differences being chiefly quantitative.

4. Monocytes and macrophages

It has been amply demonstrated that cells of the monocyte-macrophage series phagocytose, kill and degrade many infectious agents (28, 211).

The systems and mechanisms will be discussed later and compared with the antibacterial mechanisms of "activated" macrophages of antimicrobial cell-mediated immunity (AM-CMI).

B. Cell-mediated antibacterial immunity

1. Immunological aspects of cell-mediate immunity

Antimicrobial cellular immunity refers to the increased capacity of macrophages to kill or inhibit microorganisms after a host has been infected. The macrophages from immune animals are better able to inactivate and/or destroy microorganisms than macrophages from normal animals.

The criteria of the response categorize it as an immunologic reaction because 1) the host requires a finite period of time to respond, 2) immune animals slowly lose their resistance but respond more quickly to the second contact with the same organism, 3) resistance may be increased by additional injections with the same organism (120), and 4) the response requires a particular lymphocyte population (124).

The formal separation of cell-mediated immunity from humoral immunity is based on the capability of the latter system to passively transfer immunity with serum from immune animals. Cellular immunity may be passively transferred to normal recipients with lymphocytes from immune animals (113).

Antibody, or humoral immunity which can be passively transferred with serum, is more effective against acute bacterial infections whereas cell-mediated immunity is associated with chronic bacterial infections (121). Classical circulating antibody has no apparent

effect on the resolution of infections with most facultative or obligate intracellular parasites (172). In addition, antimicrobial cell-mediated immunity appears to be more of a local or centralized response rather than the systemic response observed in humoral or antibody-mediated immunity. For example, the activated macrophages in cellular immunity achieve the greatest activation at local tuberculous lesions (41, 222).

Although the cellular response requires a specific recall with the original infecting microorganism, once elicited the enhanced resistance is effective against other microorganisms. Immunization with Mycobacterium tuberculosis produces cell-mediated immunity which protects not only against M. tuberculosis but also against listeria, brucellae and staphylococci (35, 48, 113, 119, 123) and phylogenetically diverse organisms. Resistance to Toxoplasma gondii produces protection against listeria, salmonellae, mengovirus and besnoitia (187).

2. Cell-mediated immunity in microbial diseases

Cellular immunity is of primary importance in many bacterial (33, 83, 91, 124, 227), fungal (81), viral (141), rickettsial (221), protozoan (137, 208), metazoan (45) and mycoplasma (46) diseases. A prime requirement for the elicitation of cell-mediated immunity appears to be infection with viable, facultative intracellular parasites. Mycobacterium may be an exception in that killed cells induce some resistance although less effectively than a comparable number of viable cells (35, 237). The organisms must persist for some time in the tissues for acquired resistance to develop (34).

Although viable organisms are required to elicit the response, the anamnestic response can be recalled with killed organisms or specific antigenic fractions (71).

The prototype of cell-mediated immunity is anti-tuberculous immunity, but studies have been made with many types of facultative intracellular bacteria. Studies can be complicated by the activity of multiple systems. The resolution of salmonella infections (15, 33, 119) can include cell-mediated immunity and the concomitant humoral response that arises (32). Some investigators have shown a bactericidal effect of serum from animals infected with whole cell vaccines of S. typhimurium in vivo and in vitro (92). Others report humoral and cellular systems are important in the immune elimination of salmonella (215, 216). These latter reports show that serum from mice immunized with attenuated S. typhimurium, heat-killed bacterial suspensions or ribosomal preparations from S. typhimurium passively transferred protection to normal mice (215). The same antigenic preparations were capable of eliciting a cell-mediated immunity (216). Although this appears to be in direct contradiction to original observations for the requirement of viable intracellular parasites, there are an increasing number of reports which indicate that antibacterial cell-mediated immunity can be elicited with antigenic preparations from microorganisms (104), especially ribosomal fractions (36, 236, 238).

Development of AB-CMI in vivo is dependent on the size of the inoculum and the infecting organism. Listeria monocytogenes, which multiples more rapidly than M. tuberculosis, produces an immunity in mice four days after injection (118). Mice inoculated with large

numbers (10^7 viable units) of BCG develop a high level of resistance at 12-15 days (121). Inoculation of mice with small numbers (10^4 viable units) of BCG induces delayed-type hypersensitivity (DTH), but little protective response. The immunity persists only as long as the original antigenic stimulus persists, suggesting that specific activation of macrophages requires frequent or continuous stimulation (120).

3. Cellular mechanisms

a. Lymphocytes in cell-mediated immunity

Acquired resistance to infection with intracellular parasites depends on the interaction of at least two cell types: specifically committed lymphocytes and cells of the monocyte-macrophage series (122, 131).

There are two major types of lymphocytes involved in the body's immunological system, each with distinctive features and different functions. Bone-marrow derived lymphocytes (B-lymphocytes or B-cells) are thymus-independent cells and represent the cellular branch of the immune response responsible for antibody production and secretion. Thymus-dependent lymphocytes (T-lymphocytes or T-cells) arise from stem cells in the bone marrow and mature under the influence of the thymus, circulate through the body and respond to specific antigenic stimuli but do not secrete antibody. T-cells play a role in rejection of tumors and allografts (164), delayed-hypersensitivity reactions (117, 323), activating macrophages to resist infection (16, 79, 106) and cooperation with B-cells in some antibody responses (13). Table II lists characteristic properties of T-cells, B-cells and macrophages.

Table II. Distinguishing characteristics of T lymphocytes, B lymphocytes and macrophages. (Taken from reference 230).

Membrane markers	T lympho-cytes	B lympho-cytes	Macro-phages
IgG	-	+	-
Receptor for C 3 (erythrocyte-antibody-complement (EAC) rosettes)	-	+	+
Receptor for Ig or Ab-Ag complexes (Fc)	-	+	+
Thymus-specific antigens (θ, mouse thymocyte leukaemia antigen, etc.)	+	-	-
Receptors for sheep red blood cells (erythrocyte (E) rosettes)	+	-	-
In vitro stimulation of DNA synthesis by mitogens[a]			
Phytohaemagglutinin (PHA)	+	-[b]	-
Concanavalin A (Con A)	+	-	-
Lipopolysaccharide (bacterial endotoxin)[c]	-	+	-
Anti-Ig	-	+	-
Specific binding to antigen-coated beads	-	+	-
Mixed lymphocyte culture reactivity	+	-	-
Graft-versus-host reaction inducing capacity	+	-	-
Adherence to surfaces (glass, plastic)	-[d]	-[e]	+
Phagocytic	-	-	+

[a] These data derive mainly from experiments in mice, and their extrapolation to man is questionable.

[b] Some B lymphocytes may be recruited to divide secondarily by factors elaborated by activated T lymphocytes. B cells may also be stimulated when the mitogen is attached to a solid support.

[c] In mice.

[d] Except for blast cells.

[e] Except for mature plasma cells or when immune complexes are attached to B cells.

This is an active area of research. Recent reviews (90, 212) are available describing these cells and their interactions in the immune response.

Although antibacterial cell-mediated immunity is expressed by the macrophages, immunologically committed lymphocytes are the mediators responsible for the specificity of AM-CMI. Cellular immunity can be passively transferred by viable lymphocytes from immune donors (118, 142) but not with lysed cell preparations (58, 125). Animals at the peak of a cellular immune response, either during a primary infection to BCG or after a secondary challenge with BCG, have activated macrophages which are much more resistant to L. monocytogenes, B. abortus and S. typhimurium. When lymphoid cells from BCG-immunized donors are transferred to normal recipients, they confer no measurable protection to L. monocytogenes unless BCG is also injected (18, 113).

Immune lymphocytes have no direct effect against bacteria (121), however, recent reports in anti-tumor cell-mediated immunity indicate there is direct cytotoxic activity by immune lymphocytes against target cells operating without phagocytosis at the membrane level (164). A minor role for direct lymphocytic antibacterial activity may remain as a possible mechanism in AM-CMI.

Rats infected with L. monocytogenes produce a population of newly formed lymphocytes (100). In the afferent limb of the response, uncommitted lymphocytes transform, divide and differentiate to committed cells upon contact with the infecting organism, a similar sequence found in humoral mechanisms (125). This population of newly formed lymphocytes arises primarily from the spleen or regional lymph nodes

in animals injected intravenously or subcutaneously, respectively, with listeria or mycobacteria. Approximately 48 hours after injection, large numbers of pyroninophilic blast cells arise in the paracortical regions of the responding lymph nodes, these areas containing thymus-derived lymphocytes (63, 218). The blast cells continue to divide for 10-12 days, migrate to the lymph node medulla and then pass into the efferent lymph, developing into pyroninophilic small lymphocytes (124).

The newly formed large lymphocytes have a propensity to be drawn into inflamed tissues and accumulate in an induced exudate in the peritoneal cavity (100). Rats injected with vinblastine, an antimitotic agent, at the peak of their response against L. monocytogenes do not possess the population of specifically sensitized effector cells in their thoracic ducts nor are they found in inflammatory exudates induced in the peritoneal cavity (133). The drug must be injected within 5 days of the primary infection to interfere with the host response. After this time, the protective lymphocytes are vinblastine resistant and are presumably transformed to immunologically committed small lymphocytes. The numbers of large lymphocytes in the thoracic duct lymph of untreated animals have decreased to "normal" values at this time (133). The cells which transfer resistance have a short circulating life span. After intravenous transfer into uninfected recipients, protective donor lymphocytes do not recirculate to the thoracic duct in sufficient numbers to confer protective immunity to a second set of normal recipients (132).

Protective cells with blast cell morphology are seen to accumulate in nonspecifically induced inflammatory foci. Similar protective cells

are absent or present in small numbers in unstimulated peritoneal cavities (101). It has been suggested, that these cells respond to an inflammatory stimulus and migrate to the area where they provide the stimuli to attract monocytes and macrophages into the area and affect enhanced macrophage microbicidal activity. The large lymphocytes may transform into small lymphocytes which provide immunological memory. Upon future contact with the antigen, the cells are again stimulated to divide and transform into the large effector cells which would preferentially localize in infected tissues (133).

Little is known about the life history of the progenitors of effector T-cells or requirements of cooperation among cell types in the induction of AM-CMI. On the basis of delayed hypersensitivity and graft-versus-host reactions in mice, it has been proposed that induction can require two different classes of T-cells (7, 170). The two types may belong to different cell types or may represent different stages of maturation within a single cell line. The first type of T-cell postulated (T_1) may be normally present in the thymus and spleen and decrease in number in secondary lymphoid tissue 2-6 weeks after adult thymectomy, i.e., it might be in an early stage of post-thymic maturation in these tissues. The second type postulated (T_2) may be scarce in the thymus and may not decrease in number in secondary lymphoid tissue after adult thymectomy, i.e., it might be in a late stage of post-thymic maturation. It is suggested that T_1 cells are the progenitors of effector cells and that T_2 cells act as amplifiers of the response. Investigations of cellular requirements

for cell-mediated immune reactions in other systems suggest cooperation between T_1 and T_2 cell types is not essential (17, 206).

By definition, cell-mediated immunity may be passively transferred to normal recipients with lymphocytes, not with serum (58). One argument for the involvement of AM-CMI in a particular infection is the abrogation of the protective response by antilymphocyte or antithymocyte sera. Mice treated with antithymocyte serum develop little or no protective response to ectromelia virus (16) or vaccinia virus (79). There is also clinical evidence of a participatory role of T-cell dependence in cell-mediated resistance. Patients with immunological deficiency diseases lacking a thymus or thymus function have progressive and often fatal diseases with vaccinia (59). Similarly, passive transfer of infectious immunity may be eliminated by treating immune spleen cells in vitro with anti-θ serum and complement (106). This is not conclusive evidence for cell-mediated immunity, however. There are thymus-dependent antibody responses (13) and abrogation of an immune response after treatment with antithymocyte serum may not be indicative of a cell-mediated immunity.

b. Cell-cell interactions and biological mediators

1. Specificity and relationship with delayed-type hypersensitivity

The effect of lymphocyte mediators on macrophages must be discussed with delayed-type hypersensitivity although any relationship between delayed-type hypersensitivity and antimicrobial cell-mediated immunity has not been resolved. There are two opposed viewpoints:

1) delayed-type hypersensitivity is related and may provide the specificity for AM-CMI and 2) there is no relationship.

Delayed-type hypersensitivity *in vivo* is defined in terms of morphological observations and time sequence of events. The Arthus and anaphylactic-type skin reactions in immediate hypersensitivity develop early and are mediated by various classes of immunoglobulins. Histologic examination of the skin shows the predominant cell types to be granulocytes.

In delayed-hypersensitivity reactions, such as a tuberculin skin test, the reaction does not reach a maximum until 24-48 hours, and is characterized by a mononuclear cell infiltration including both lymphocytes and monocytes and macrophages. When normal macrophages with antigen are injected into the skin of a sensitized animal, a delayed-type reaction develops sooner and more intensely than when antigen only is injected. This suggests a major factor in the delayed reaction is the time required for accumulation of macrophages (76). Skin tests are used to measure DTH *in vivo* in many species, including man. In the mouse, a variation of the skin test, the foot pad test, is used.

At the time an animal begins to mount a cell-mediated immunity, it also generally develops a delayed-type hypersensitivity to the same microorganism (195) and like AM-CMI, delayed-type hypersensitivity is cell-mediated. It may be passively transferred to normal recipients with lymphoid cells but not with immune serum. Antilymphocyte serum (217) or antithymocyte serum (213) inhibits DTH reactions and thoracic duct lymphocytes can transfer sensitivity (27).

Like AM-CMI, there appears to be a reaction with antigen and specific effector cells followed by recruitment of noncommitted passive cell types. In transfer studies with donor cells labeled with ^{3}H-thymidine, the majority of cells which accumulate at the reaction site are those of the recipient animal (72, 148). Using isotopically labeled leucocytes and cell transfer techniques, McClusky (129) demonstrated that the majority of cells infiltrating the site of a dermal cellular hypersensitivity reaction were not specifically sensitized. Labeled lymphocytes from sensitized donors were always present in small numbers in the infiltrates (72, 148). Inhibition of macrophage migration (MIF test), an *in vitro* test for DTH which correlates with the *in vivo* skin test, can be obtained when 97.5% of the peritoneal exudate cells were from the unsensitized donor and only 2.5% were lymphocytes from a sensitized animal (44).

There appears to be two separate populations of cells involved in DTH, a thymus-dependent antigen sensitive lymphocyte population and a thymus-independent bone marrow-derived population (117, 232). Although the latter is generally believed to be monocytes, there is some evidence it may be a T-cell population. Youdin et al. (233) eliminated passive transfer of DTH by treatment with anti-θ serum and complement.

Passive transfer of AM-CMI with lymphocytes also transfers the corresponding DTH (118). Delayed-type hypersensitivity can be transferred in man with transfer factor from cell-free extracts from lysed leucocytes (107). With the appearance of delayed sensitivity, there is often a coincident recovery from a systemic infection such

as in the case of patients with generalized vaccinia, moniliasis and leprosy (108).

While DTH generally accompanies AM-CMI (195) it is also possible to have one response and not the other (223). Animals may be desensitized to tuberculin hypersensitivity (180, 229) or made hypersensitive without causing an increase in resistance (171). Delayed hypersensitivity can be elicited to Staphylococcus aureus without eliciting the increased resistance of AM-CMI (115). Goihman-Yahr et al. (62) reported that guinea pigs with delayed-type hypersensitivities, reactions of infectious allergy, graft rejection or allergic dermatitis did not possess enhanced listericidal activity and only macrophages from animals sensitized to BCG had a significant capacity to inactivate L. monocytogenes.

Certain fractions from salmonellae (215) and the tubercle bacillus (40, 238) give a measurable degree of resistance without producing cutaneous hypersensitivity. Youman's group (150, 238) reported mice immunized with ribosomal fractions of mycobacteria developed a specific immunity to mycobacteria without the development of delayed-type hypersensitivity. They argued against a role for tuberculin hypersensitivity in cell-mediated immunity on this basis. Mice and guinea pigs injected with ribosomal fractions and tested for DTH by the footpad test (238) and the in vitro test for MIF (150) had only slightly greater reactions over controls. The same authors reported a specific immunity to tuberculosis was induced with these fractions (35, 36). In contrast, reports by other authors noted that ribosomal preparations of mycobacteria are active in provoking DTH reactions (11, 12, 156).

Although skin sensitivity may be suppressed by daily injections of tuberculin, acquired resistance persists and may even increase (229). Mackaness argues that the absence of skin sensitivity implies nothing in respect to immunologic reactivity at the cellular level and the process of desensitization might only cause the temporary withdrawal of the sensitized cells involved in DTH from the circulation (121).

Results of skin tests in humans may not be definitive. Lymphocytes from patients with diseases associated with depressed or absent delayed hypersensitivity, such as sarcoidosis, Hodgkin's disease and chronic mucocutaneous candidiasis, were tested for hypersensitivity by the in vitro methods of antigen-induced MIF production and ^{3}H-thymidine incorporation (24, 181, 182, 184). Of the 37 patients tested, 16 exhibited cutaneous hypersensitivity to one or more antigens and had positive MIF and proliferation assays. Six patients had no cutaneous hypersensitivity reactions and had negative in vitro tests, indicating the lack of sensitive lymphocytes. Four of the remaining 15 patients had consistently negative skin reactions and repeatedly positive MIF and proliferation assays, indicating that despite the lack of skin reactivity, sensitive lymphocytes were present (24, 182). It was suggested that the lack of an in vivo response was a result of some dysfunction other than a lack of sensitive lymphocytes, possibly an abnormal macrophage response. The other 11 patients had consistently negative skin and MIF tests, but did show normal antigen-induced thymidine incorporation, showing a dissociation between MIF production and cell proliferation. Obviously DTH is not a simple response and seems comparable to biochemical lesions, i.e., any single enzyme deletion or

alteration in a biosynthetic pathway may result in the loss of the end product. Skin test results should therefore be interpreted with caution.

2. Factors from lymphocytes affecting macrophages

Delayed-type hypersensitivity may provide the basis for the specificity for AM-CMI and the ensuing nonspecific aspects due to reactions of macrophages, effector cells, after stimulation by lymphokines (120). A number of factors or lymphokines with biological activity have been detected from sensitive lymphocytes of animals with DTH (49). A list of these mediators with their characteristic properties are listed in Table III. It is not known whether the same cell can produce all these factors or whether different cell populations are involved. Flanagan et al. (54) report an MIF-like material in supernatant fluids from monkey kidney cells infected with mumps or Newcastle disease virus and suggest these mediators may be produced by a number of cell types.

Lymphocytes or lymphokines may affect macrophages in several ways. In vivo events of DTH may be postulated to occur in the following manner. After stimulation, small numbers of specifically committed lymphocytes undergo blast formation and produce and secrete factors which cause an inflammatory response and effect the accumulation first, of a granulocytic cell population followed by a monocytic infiltrate (chemotactic factor(s)). Once the monocyte-macrophage population has entered the area, they may be prevented from leaving by migration-inhibition factor (MIF). The macrophages may also be stimulated to divide (mitogenic factor(s)) and, in AM-CMI, stimulated to enhanced antimicrobial capabilities (macrophage resistance factor(s)).

TABLE III. Factors from lymphocytes or associated with delayed hypersensitivity-cellular immunity.

	Biological property	Chemical nature	Molecular weight	Electrophoretic mobility	Enzyme sensitivities	Heat stability	Other
Factors affecting macrophages:							
Migration inhibition factor (MIF)(43,179)	inhibits the migration of normal macrophages	guinea pig MIF-glycoprotein (177) human MIF-protein (183)	35-65,000(174) 12-25,000(20,231) 50-67,000(183)	faster than albumin (179)	sensitive to: chymotrypsin(177) neuraminidase (guinea pig) (human MIF resistant to neuraminidase) resistant to RNase, DNase(42) urea, guanidine or sodium dodecyl sulfate (19)	stable, 60°C 30 min(179) destroyed at 80°C (20)	concanavalin A-induced MIF has same properties as antigen induced MIF(178)
Macrophage aggregation factor (MAF)(116) (same as MIF?)	causes clumping of macrophages in suspension						
Macrophage chemotactic factor (MCF)(2,219)	causes macrophages to migrate through micropore filter along gradient	protein	35-50,000 and 150,000 12,500	like albumin	neuraminidase resistant	stable, 60°C, 30 min	distinct from C'-3 and C'-5
Macrophage resistance factor (postulated) (113)	renders macrophages nonspecifically resistant to certain microorganisms						
Cytophilic antibodies (21)	confer on macrophages specific reactivity with antigen			guinea pig-IgG-2 globulins			
Macrophage mitogenic factor (154)	induces macrophage proliferation						

Specific macrophage arming factor (SMAF) (52)	induces macrophages to inhibit growth of tumor cells			
Factors affecting lymphocytes:				
Blastogenic or mitogenic factor (BF or MF) (64,89)	induces blast formation and tritiated thymidine incorporation in normal lymphocytes			secreted by lymphocytes Ag-dependent (205) Ag-independent(204)
Potentiating factor (PF) (86)	stimulates sensitized lymphocytes to incorporate thymidine			secreted by antigen-stimulated, sensitive lymphocytes
Conditioned medium reconstituting factor (CMRF)(1)	stimulates lymphocytes to respond to antigen			produced by normal glass adherent cells
Inhibitor of DNA synthesis (IDS)(202)	inhibits DNA synthesis, neutralizes EGO activity	10,000	inactive at 56°C	
Enhanced gene operator (EGO) (202)	mitogenic for lymphocytes		stable at 56°C	
Factors affecting granulocytes:				
Inhibition factor (23)	inhibits the migration of human buffy coat cells or peripheral blood leucocytes			
Chemotactic factor (230)	causes granulocytes to migrate through micropore filter			

TABLE III. Factors from lymphocytes or associated with delayed hypersensitivity-cellular immunity(continued).

	Biological property	Chemical nature	Molecular weight	Electrophoretic mobility	Enzyme sensitivities	Heat stability	Other
Factors affecting cultural cells:							
Lymphotoxin (LT) (67)	cytotoxic for some cell cultures (mouse L cells, HeLa cells)	protein	80-150,000	human LT, like β, γ globulins; murine LT, like albumin(65)		human LT, inactive at 80°C(67) murine LT stable at 100°C, 15 min(65)	
Proliferation or cloning inhibition factor (PIF or CLIF) CLIF (111) PIF (69)	inhibits proliferation of cultured cells w/o lysing them		<10,000		trypsin sensitive	inactive at 100°C	
Interferon (85)	protects some cells against some viral infection	glycoprotein (53)	20,000		trypsin sensitive		multiple factors; may be produced by cells other than lymphocytes
Factors producing in vivo effects:							
Skin reactive factor (SRF) (168)	in normal guinea pigs, produces early skin reaction histology similar to DTH	protein	70,000	faster than albumin	sensitive to proteases	56°C-50% destroyed; 100°C-100% destroyed	does not require Ag or C'3
Macrophage disappearance factor (151)	intraperitoneal injection causes macrophages to adhere to peritoneal wall						

Transfer factor(108)	transfers delayed type hypersensitivity	<10,000	resistant to DNase, RNase and proteolytic enzymes	
Lymph node permeability factor (LNPF) (228)	causes permeability changes in tissue with accumulation of neutrophils and monocytes	protein		multicomponent systems

In diseases such as tuberculosis in which the infectious agents are exceptionally resistant to inactivation and degradation, a granuloma may be formed in which large differentiated macrophages prevent dissemination by walling off the organisms.

A soluble factor(s) elaborated by antigen-stimulated lymphocytes enhances certain in vitro macrophage functions. Increased macrophage adherence, membrane ruffled border activity, phagocytosis and hexose monophosphate oxidation appears after 3 days of incubation in MIF rich fractions (83). Lymphocytes from M. tuberculosis-immunized mice, when cultured in vitro with the same organism, produce supernatants which inhibit the growth of M. tuberculosis in normal macrophages (95, 158). Godal et al. (61) found that supernatant from rabbit mixed leucocyte cultures suppressed the multiplication of mycobacteria in normal peripheral blood monocytes after 10 to 17 days. Krahenbuhl and Remington (103) reported that resistance to listeria was conferred to normal guinea pig macrophages by supernatant fluids from spleen cells of toxoplasma-infected animals incubated with toxoplasma antigen. They also reported MIF activity in these supernatant fluids. The supernatant fluids had no direct effect on bacteria and did not inhibit the extracellular multiplication (55, 95). Pearsall et al. (161) reported some direct activity against certain yeasts by lymphokine preparations.

Supernatant fluids from splenic lymphocytes of mice immunized with L. monocytogenes cultured in vitro with listeria cause normal macrophages to inhibit the intracellular multiplication of virulent mycobacterium (96). However, if listeria is added to lymphocytes from

nonimmunized mice or to lymphocytes from mice immunized with H37Ra M. tuberculosis the supernatant fluids are still able to inhibit the intracellular growth of virulent tubercle bacilli to some degree. These in vitro studies mimic in vivo studies (122) in which lymphocytes react specifically with the antigen and confer on macrophages the ability to nonspecifically inactivate microorganisms.

Partially purified peritoneal lymphocyte preparations from immunized mice mediated a suppression of intracellular growth of Histoplasma capsulatum in normal mouse macrophages in vitro (81). An outbred strain of mice (Swiss-Webster) was used, however, and the presence of mediators produced from a mixed lymphocyte reaction cannot be eliminated.

Lymphocytes from peritoneal exudates of guinea pigs with delayed hypersensitivity to bovine gamma globulin (BGG) cultured with and without BCG confer no enhanced listericidal activity to normal peritoneal macrophages (196). The supernatants do have migration inhibitory activity, however. It is interesting in these experiments in which viable facultative parasites were not used, DTH was elicited but not AM-CMI. These data reflect in vivo situations in which AM-CMI may involve a response over and above DTH.

Lymphokines can be generated from antigen-induced sensitive lymphocytes or from lymphocytes incubated with mitogens, such as Conconavalin A (Con-A) and phytohemagglutinin (PHA). Both PHA and Con-A are plant mitogens that induce an increase in DNA synthesis by lymphocytes (96). Both substances stimulate lymphocytes to produce MIF and lymphotoxin (38, 66, 167, 194). Macrophages incubated with

antigen-induced lymphokine preparations inactivate 95% of the listeria to which they are exposed. However, little or no inactivation of listeria occurs in macrophages treated with PHA-induced lymphokines (189). Macrophages incubated with MIF-rich fractions of supernatants from Con-A stimulated guinea pig lymphocytes have 4-6 times fewer listeria than controls (55). The authors reported a bacteristatic rather than bactericidal medhanism. Klun and Youmans (96) found both mitogens cause the uptake of ^{3}H-thymidine by lymphocytes, but only Con-A stimulated lymphocytes to secrete a product which inhibited the intracellular growth of virulent tubercle bacilli within normal macrophages. Perlmann et al. (165) noted PHA, but not Con-A, was capable of inducing normal human lymphocytes to become cytotoxic to chicken erythrocytes. Stobo et al. (207) reported Con-A stimulated spleen cells from thymectomized mice but PHA did not. There were approximately 10% θ-positive cells present in spleens from thymectomized animals and if this same cell population was again incubated with anti-θ serum, all responsiveness to Con-A was abolished. Thus, it appears that Con-A may stimulate a separate population of T-cells than PHA, and it is the former population that is responsible for inhibition of facultative intracellular parasites.

3. Factors from macrophages affecting lymphocytes

Digested sheep erythrocytes recovered after various periods of time from guinea pig peritoneal macrophages incubated _in vitro_ no longer induce antibody formation and preferentially induce delayed-type hypersensitivity responses _in vivo_ (163).

A glass-adherent helper cell population is required for activation of lymphocytes in some cell-mediated responses. Removal of macrophages and monocytes from peripheral blood samples by glass bead columns prevents lymphocyte transformation by PPD, streptokinase-streptodornase and streptolysin O (9, 75). A glass-adherent cell population is required in mixed lymphocyte reactions, but the requirement for these cells can be replaced by supernatants of normal, unstimulated glass-adherent cells (9). Whether the requirement in these responses is for macrophage antigen processing, antigen presentation or production of nutrients is unknown.

4. Cytophilic antibody

Boyden and Sorkin (21) reported immunized animals possess an immunoglobulin, cytophilic antibody, most often described as IgG (distinguished from IgE) which may play a role in cell-mediated immunity (21) and delayed-type hypersensitivity (152). Cytophilic antibodies to PPD may be present in animals immunized with BCG (152, 203) and Amos et al. (5) reported cytophilic antibodies to tuberculoprotein prevented the migration of peritoneal exudate cells in the presence of the antigen.

No observations have been reported of morphological or biochemical changes occurring in macrophages as a result of binding of cytophilic antibody. It is argued that the nonspecificity of antimicrobial cell-mediated immunity would not be accomplished through a cytophilic antibody specific for a particular antigen (214), however it is possible for changes in macrophage functions to occur as a result of a reaction

at the macrophage membrane. In any case, the relationship, if any, of macrophage-cytophilic antibody and antimicrobial cell-mediated immunity remains to be clarified.

c. Macrophages in antimicrobial cell-mediated immunity

Macrophages ingest and degrade a variety of soluble (29, 50) and particulate (28, 211) materials and have been called the scavengers of the body. Whereas PMN's play a major role in pyogenic infections, monocytes and macrophages are more important in the control of intracellular parasites (144). Mononuclear phagocytes are distinguished by their nuclei, their phagocytic capacity, the nature and content of their lysosomes (which differ from polymorphonuclear leucocytes), their adherence to glass and other surfaces, and the presence on their plasma membrane of receptors for certain immunoglobulins or immune complexes (IgG1, IgG3, and IgM) (6) and complement (C'3) (84) (Table III). In addition, macrophages possess considerably more synthetic potential than PMN's and can be stimulated to form large amounts of lysosomal and other enzymes.

Macrophages can be "activated" both specifically and nonspecifically. Nonspecifically activated macrophages can be obtained from peritoneal exudates of animals stimulated with intraperitoneal injections of glycogen, casein hydrolysate, etc. These agents produce a general inflammatory response in the peritoneal cavity. Two to three days after prestimulation, a large population of macrophages arises with altered morphological and biochemical characteristics such as changes in cell size, motility, "stickiness", pinocytotic rate, phagocytic

capacity, and the number, size and content of lysosomes (31, 134). Nonspecifically activated macrophages do not possess the heightened microbicidal capacities that specific, immunologically activated macrophages possess, although they may appear similar morphologically (191).

Using time-lapse phase-contrast cinematography, peritoneal macrophages from normal or tuberculin sensitive guinea pigs in the absence of antigen (PPD) have numerous pseudopodia and migrate freely. Macrophages from normal or sensitized animals in the presence of sensitive lymph node or thymus cells have fewer pseudopodia and are much less motile. Nonsensitive macrophages aggregate and become associated with lymphocytes in the presence of PPD and sensitive lymphocytes. The migration of sensitive lymphocytes is not affected in the presence of PPD unless they are aggregated with macrophages (191).

The macrophages of the body are the cells in which antimicrobial immunity is expressed. These cells develop a greatly enhanced microbicidal capacity not only for the original infecting organism, but for a wide variety of microorganisms (121). This is reported in _in vivo_ and _in vitro_ studies usually with peritoneal macrophages but also with fixed phagocytes of the spleen, liver and lung (118).

Surprisingly, mice injected with anti-macrophage sera (AMS) during infection with _L. monocytogenes_ do not die sooner nor does the mortality rate increase. When treated with antithymocyte sera mice infected with listeria have decreased survival time and increased mortality (174).

Howard et al. (81) reported freshly harvested mononuclear phagocytes from immune Swiss albino mice inhibited the intracellular growth

of Histoplasma capsulatum, whereas cells maintained in culture for 48 hours did not. Hirt and Bonventre (80) noted freshly harvested peritoneal macrophages obtained from BCG-immunized mice phagocytosed staphylococci poorly but to a greater extent than control cells. The phagocytic and bactericidal activities of peritoneal macrophages cultured for 2 days was much greater than freshly harvested macrophages. After 2 days in culture, killing of staphylococcus by macrophages from control or BCG-immunized Swiss albino mice was the same.

McGhee and Freeman (130) observed that within 6 hours after infection of immune macrophages in vitro, 50% of surviving intracellular brucellae become sensitive to osmotic conditions and required media containing 0.2 M sucrose to survive. Osmotically sensitive brucella were not produced in normal macrophages.

It is suggested that the increased microbicidal properties of macrophages in AM-CMI is due to the increased lysosomal enzyme activity of activated macrophages (8, 102), assuming the killing and degradation of microorganisms takes place within these organelles (30). The activities of some lysosomal enzymes increase in animals infected with BCG (31), 57, 188) or Cornyebacterium ovis (74), although Franson and White (57) reported a decrease in activities of phospholipases A_1 and A_2 in BCG-infected rabbits. Lung macrophages possess higher activities of lysozyme than peritoneal macrophages but the latter are more efficient in killing bacteria (160). Results from experiments performed by Brown et al. (23) suggested that increased lysosomal activity in chemically activated macrophages is associated with vigorous bacterial

multiplication rather than bacterial inhibition. They postulated that lysosomal enzymes are not detrimental but actually may be beneficial to the survival of facultative intracellular parasites by providing growth stimulating low-molecular weight nutrients as products of hydrolysis.

Myeloperoxidase activity, a primary antibacterial system in PMN's has been found in mouse peritoneal macrophages (159), however the levels reported are barely above the lower limit of detectability. Furthermore, phagocytosing peritoneal macrophages have less activity than resting cells, which is the reverse of what is expected based upon results for PMN's. Simmons and Karnovsky (197) reported almost undetectable levels in peritoneal macrophages even though the cells were very competent in their killing ability.

Phagocytosis of listeria by human blood monocyte derived macrophages is inhibited by glycolytic poisons such as NaF but is unaffected by cyanide or 2,4-dinitrophenol (DNP), inhibitors of respiration (25). Macrophages from mice infected with listeria or BCG have enhanced phagocytic properties and glucose metabolism (175). Cohn (28) found macrophages from immune hosts did not differ from normal cells in their ability to degrade labeled bacteria. He noted the presence of immune serum had an inhibitory action on the rate of degradation. Iodoactate, arsenite and cyanide, inhibitors of glycolysis and respiration, had no influence on the degradation of labeled bacteria, suggesting the process is not dependent on energy requiring mechanisms (25, 28). Cline (25) reported maximal killing of listeria by normal human macrophages required oxygen but was unaffected by cyanide or DNP.

Miller (140) also found killing by normal mouse peritoneal macrophages was inhibited by anaerobiosis but unaffected by cyanide, antimycin A or amytal. Intracellular inactivation of bacteria was unaffected by uncouplers of oxidative phosphorylation (DNP, oligomycin or arsenite), by glycolytic (NaF and iodoacetate), Krebs cycle (sodium malate), or phosphogluconate (quinacrin and phenylbutazone) pathway inhibitors. Compounds with redox potentials of +0.22 volts or less were inhibitory to killing of Pseudomonas aeruginosa. Material of +0.36 volts and higher were not. He suggested the electron transport chain is intimately involved in the mechanism of bactericidal activity of normal mouse macrophages.

In metabolic studies with Con-A on polymorphonuclear leucocytes and normal alveolar macrophages there is a rapid enhancement in the rate of cell respiration, even in the presence of potassium cyanide (185). There is a KCN-insensitive increase in the rate of 1-^{14}C-glucose oxidation to $^{14}CO_2$ indicating a specific activation of the hexose monophosphate shunt. Immune macrophages do not undergo substantial changes in their oxidative metabolism as compared to normal macrophages even though fluorescent studies reveal a binding of Con-A.

It has been reported that macrophage lysates (186) and lysosomal extracts of Kuppfer cells (98) and peritoneal macrophages (157) possess no direct antibacterial activity. Outteridge et al. (157) noted lysates of pulmonary alveolar macrophages from 5 out of 15 sheep had some inhibitory, but no killing activity against listeria in lysosomal fractions.

Ramseier and Suter (173) found antimycobacterial activity in peritoneal mononuclear whole cell lysates. Activity in BCG-immunized guinea pigs was four times that of control animals and was apparently specific. There was no inhibition of Brucella abortus, Salmonella typhimurium, Staphylococcus aureus, or Escherichia coli. The material was heat labile and associated with the nucleus (174).

Some antimycobacterial activity by macrophage fractions is reported due to toxic free fatty acids produced as a result of hydrolsis of lipoproteins or phospholipids by lipases (88, 97, 98) with slightly higher levels in lysates from BCG-immune mice than in controls.

Gershon and Olitzki (60) reported a factor from mouse monocyte lysates, monocytin, which was bactericidal for Salmonella typhi, S. paratyphi, E. coli, S. dysenteriae and S. aureus. It was effective in protecting mice against S. typhi if injected 4 hours before the bacterial inoculum. The material was a basic protein, labile to acid treatment and trypsin and stable to heating at 100 C for 1 hour.

The exact nature and mode of microbial inactivation or killing in normal macrophages or those involved in AM-CMI are unknown.

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AN ANTILISTERIAL FACTOR FROM MOUSE PERITONEAL CELLS

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ABSTRACT

Lysates of mouse peritoneal cells contained antilisterial activity which was detectable in the presence of a reducing agent, dithiothreotol. Lysates from mice immunized and prestimulated with viable BCG had greater activity than lysates from mice immunized and prestimulated with heat-killed-BCG or control mice. Activity was associated with both glass adherent and nonadherent cells, but to a greater extent with the former. Peritoneal cells of mice immunized and prestimulated with heat-killed or viable BCG were able to reduce nitro blue tetrazolium (NBT) to a much greater extent than cells of control mice. After mechanical disruption of cells and differential centrifugation, antilisterial activity was found in the pellet fraction containing lysosomes.

INTRODUCTION

Phagocytes of the body provide a primary defense mechanism against infection. Antibacterial systems and mechanisms of action have been identified from polymorphonuclear leucocytes, including lysosomal cationic proteins (1) and a myeloperoxidase system (2). Macrophages phagocytize and kill many types of bacteria (3) and activated macrophages of cell mediated immunity have an increased antibacterial capacity (4, 5). With the exception of lysozyme, the mechanism(s) by which macrophages inhibit or kill microorganisms is unknown. This paper reports an oxygen sensitive factor from mouse peritoneal mononuclear cells which is bactericidal (or bacteriostatic) for Listeria monocytogenes.

MATERIALS AND METHODS

Microorganisms. Listeria monocytogenes was maintained on Brain Heart Infusion Agar (Difco Laboratories, Detroit, Mich.). Cultures used for measuring antilisterial activity were grown in Brain Heart Infusion Broth (Difco) at 37 C overnight with shaking. Attenuated Mycobacterium bovis (BCG) was grown in Dubos Broth base without enrichment or Tween 80 (Difco) supplemented with 0.5% dextrose at 37 C for two weeks.

Mice, immunization and prestimulation. Female Swiss albino mice (20-22g) (Carworth Farms, Portage, Mich.) were divided into four groups. The mice in the first group were inoculated ip with 0.2 ml of viable BCG suspension homogenized by mortar and pestle at a concentration equivalent to a #7 McFarland tube ($\sim 4 \times 10^8$ cells/mouse). Three to four weeks later, the mice were prestimulated by inoculating ip 0.2 ml of homogenized viable BCG at a concentration equivalent to a #2 McFarland tube ($\sim 1.2 \times 10^8$ cells/mouse). The mice in the second group were immunized and prestimulated with heat-killed BCG by the same method. The mice in the third group were not immunized but were prestimulated with 1.5 ml of 0.1% glycogen (Matheson, Coleman and Bell, Cincinnati, O.). The mice in the fourth group were not immunized or prestimulated.

Cell collection. Peritoneal cell exudates were collected three days after prestimulation of Groups 1, 2 and 3 by injecting 3-4 ml of Hank's balanced salt solution (HBSS) (Microbiological Associates, Bethesda, Md.) with 10 units/ml ammonium heparin (Scientific Products,

Evanston, Ill.). The abdomens were massaged and the exudates withdrawn with a syringe and a 22 gauge stainless steel needle with additional holes drilled in the shaft. Cells of mice in each group were pooled in siliconized centrifuge tubes on ice. The cell suspensions were centrifuged at 200 x g for 10 min in a refrigerated centrifuge (IEC, Needham, Mass.) and resuspended in HBSS. Measured aliquots of the cell suspension were added to 0.1 ml of a solution of 2.1% citric acid-0.1% crystal violet and counted in a Neubauer hemocytometer (Clay Adams, New York, NY). Peritoneal cells from control mice were collected and treated in the same manner. All exudates contained 50-90% macrophages, 10-40% lymphocytes and less than 2% granulocytes.

Cell lysis and fractionation. Unfractionated lysates were obtained from cells washed 2X and resuspended in citrate-phosphate buffer (6), pH 7.0 (PCB) and lysed with 20 HD_{50} units[1]/ml of staphylococcal delta hemolysin[2] by incubating for 20 min at ambient temperature. Cell lysis in all experiments was greater than 98%.

Cells from which lysates were to be fractionated were washed 2X in cold 0.25 M sucrose in 0.05 M Tris-acetate buffer at pH 7.4 and resuspended in 16 ml of the same solution. Four 4-ml aliquots were ground with a teflon-coated tissue homogenizer (TRI-R Instrument Inc., Rockville Center, NY) on ice at 600 rpm for 2, 4, 6, or 8 min. After homogenation, two 0.5 ml buffer rinses of the tissue grinder were added to the lysed cell suspension for a final volume of 5.0 ml. From 70 to 95% of the cells were disrupted within 2 to 8 min time periods. The homogenized lysates were fractionated by differential centrifugation by the method of Cohn and Wiener (7).

Antilisterial assay. The assay system used to measure antilisterial activity was a modification of that by Hirsch (8). All lysates and fractions were dialyzed against 1:10 PCB before testing. Serial 3-fold dilutions of the material to be tested were made in PCB containing 0.01% bovine serum albumin (Difco). After preliminary experiments indicated that the factor was oxygen-labile, the reducing agent, dithiothreotol (DTT) (Calbiochem, San Diego, Calif.) was included in the buffer system at a concentration of 10 mM. The final volume of each dilution tube was 1.0 ml. The control was a tube containing 1.0 ml of the diluent only. An 18 hour broth culture of Listeria monocytogenes was centrifuged, resuspended in 0.85% saline and diluted to ca. 2×10^5 cfu/ml. One-tenth milliliter was added to each dilution and the control tube. Each tube was flushed with argon and incubated at 37 C for 2 hrs. The suspensions were diluted and aliquots of each dilution added to 20 ml of warm (50 C), melted Brain Heart Infusion agar. After mixing, the contents were poured into petri dishes, allowed to solidify and incubated overnight at 37 C. The number of colonies were counted and expressed as the per cent kill calculated from the control.

Separation of glass-adherent and nonadherent cells. Peritoneal cells from mice immunized and prestimulated with viable BCG were suspended in 5 ml Eagles MEM (Microbiological Associates) with 20% fetal calf serum, supplemented with 1% each vitamins, amino acids, and glutamic acid, 100 units/ml of penicillin and 100 ug/ml streptomycin. The cell suspension was placed in plastic petri dishes and incubated for 2 hrs at 37 C in 95% air and 5% CO_2. After incubation, the nonadherent cells were removed. The cells were gently washed 2X with

HBSS and removed from the plastic dishes with a plastic policeman and salt solution of 0.8% NaCl, 0.02% KH_2PO_4, 0.02% KCl, 0.115% Na_2HPO_4 and 0.02% NaEDTA. Both groups of cells were centrifuged, washed 2X and resuspended in HBSS. Cell counts were made (65% macrophages) and the cells lysed with delta hemolysin. The nonadherent cells were centrifuged, washed 2X with HBSS, counted (99% lymphocytes) and lysed with delta hemolysin. The lysates were assayed for antilisterial activity.

Enzyme and protein assays. Acid phosphatase activity was measured using p-nitrophenyl phosphate (Sigma Chemical Co.) as substrate according to the method of Dipietro and Zengerle (9). Protein was measured by the method of Lowry et al. (10).

Nitro blue tetrazolium reduction by peritoneal cells. Peritoneal cells were collected from unstimulated or prestimulated mice immediately after sacrifice by cervical dislocation. The abdominal skin was reflected, and cells were withdrawn after each of two 5 ml injections of cold HBSS. The cells from each mouse were treated separately and bloody suspensions were discarded. The cells were centrifuged at 250 x g for 10 min at 4 C and resuspended in 0.5 to 1.0 ml of phosphate buffered saline (without magnesium or calcium) with 2 mg/ml dextrose and 5 units/ml heparin. The cells were counted and adjusted to 4×10^7 macrophages/ml. One-tenth milliliter of nitro blue tetrazolium (NBT) (Sigma, catalog #N 51293)[3] and 0.05 ml of 0.81 micron latex particles (Difco) were added to a siliconized test tube and prewarmed in a 37 C water bath for 2 min. One-tenth milliliter of the cell preparation was added, mixed, covered and incubated for 15 min at 37 C. One-half

milliliter of 0.5 N HCl was then added and the cells were centrifuged at 250 x g for 10 min at 4 C. The supernatant was removed by aspiration. One milliliter of pyridine (Reagent Grade, Mallinckrodt) was added to the cell pellet, mixed and extracted in a 95 C water bath, in a hood, for 10 min. The extracted cells were centrifuged at 500 x g for 10 min and the supernatant was measured spectrophotometrically at 515 nm.

RESULTS

Antilisterial activity of peritoneal cell lysates. The antilisterial activity of lysates was lost if stored overnight either at 2 C or -56 C. The addition of DTT restored the activity (figure 1). The initial concentration of lysate before dilution was 10^8 peritoneal cells/ml and had a protein concentratration of 6.7 mg/ml after dialysis. There was some inhibition of activity in the higher lysate concentrations and all graphs plotting activity versus lysate dilution were U-shaped when the higher concentrations were tested.

Activity from glass-adherent and nonadherent cells. More antilisterial activity was associated with lysates from glass-adherent cells than from nonadherent cells (figure 2). Cell concentrations for adherent and nonadherent cells after separation were 3.1×10^7 cells/ml (65% macrophages) and 7.6×10^7 cells/ml (99% lymphocytes), respectively.

Nitro blue tetrazolium reduction by peritoneal cells. Optical density readings for extracted, reduced NBT are plotted for individual mice in figure 3. Very little reduction of NBT was found in control, unstimulated mice or mice prestimulated with glycogen. Mice injected and prestimulated with heat-killed BCG had O.D. readings 3-9 times greater than control values. Mice injected and prestimulated with viable BCG had even higher values of 8-14 times greater than controls.

Comparison of antilisterial activity and acid phosphatase in lysate fractions. From hemolysin disrupted cells, 90% of the total acid phosphatase activity was in the high speed supernatant fluid after differential centrifugation. This was interpreted to mean that delta

hemolysin disrupted lysosomes. For this experiment then, cells were disrupted mechanically to obtain lysates for fractionation. Antilisterial activity in the supernatant fluid left after high speed centrifugation correlated with acid phosphatase activity at all dilutions tested (figure 4). In the low speed (nuclear) and high speed lysosomal) pellet fractions of homogenized cell suspensions, antilisterial activity did not parallel enzyme activity until the fractions had been diluted (figures 5 and 6). This effect corresponded to the inhibition observed in the higher concentrations of unfractionated lysate (figure 1).

Acid phosphatase and antilisterial activity of immunized and control mice. Total acid phosphatase activity per 10^6 macrophages/ml was greater from mice prestimulated with glycogen than from unstimulated control mice (table 1). Enzyme activity was highest from mice which were immunized and prestimulated with viable BCG. An intermediate level of activity was found for mice receiving heat-killed BCG. When high speed supernatant fractions from homogenized cell suspensions of each of the four groups of mice were adjusted to equivalent acid phosphatase enzyme activities (1×10^{-4} uM substrate hydrolyzed/hr) comparable numbers of listeria were inactivated (table 2).

DISCUSSION

Macrophages from animals exhibiting cell-mediated immunity are known to possess greater antibacterial capacities than macrophages from normal animals. Activated macrophages from animals which have mounted a specific cellular immune response against such facultative parasites as mycobacteria, listeria and salmonella characteristically exhibit a nonspecific heightened capacity to inactivate and degrade a wide range of apparently unrelated organisms (4, 5, 11, 12). The nature of the biochemical agent(s) responsible for this activity is, at present, unknown.

Intracellular degradation of radio-actively labeled bacteria takes place in the lysosomes of macrophages (3). Heat-killed bacteria are degraded more readily than viable bacteria and organisms such as Bacillus subtilus and Micrococcus lysodiekticus are degraded more rapidly than Staphylococcus aureus.

Greater numbers of lysosomes and higher activities of lysosomal enzymes were found in macrophages of animals with cellular immunity than in normal animals (7, 13). It was suggested that this, in part, may be responsible for the heightened antibacterial response in cell-mediated immunity. Contrary evidence suggested that lysosomal enzymes may be beneficial to facultative intracellular parasites by providing growth-stimulating low molecular weight compounds, as products of the hydrolytic enzymes (14). In addition, alveolar macrophages have higher lysosomal enzyme activities than peritoneal macrophages (7, 15), yet the latter are more efficient in killing bacteria (16).

The removal of bacteria from a system by a phagocyte is probably dependent on a three step process: 1) phagocytosis and fusion of lysosomes with phagosomes, 2) inactivation or killing, and 3) degradation by lysosomal enzymes (3, 18). Little is known of the biochemical events responsible for step two. We are reporting an antibacterial material which may contribute to elucidation of this step.

The activated macrophage in cell-mediated immunity is associated with an increased metabolic activity and a higher rate of phagocytosis (12). Miller (17) found killing required an intact respiratory electron transport chain in normal unstimulated mouse peritoneal macrophages, although an uncoupler of oxidative phosphorylation, 2, 4-dinitrophenol, had little or no effect on bactericidal activity (17). Cyanide, antimycin A, or amytal, inhibitors of respiration, interfered with bactericidal activity to some extent. Cohn (18) found no interference in bacterial degradation by cyanide. Inhibitors of glycolysis, the citric acid cycle, and the phosphogluconate oxidative pathway did not interfere significantly. The latter pathway is important for the generation of H_2O_2 in the bactericidal myeloperoxidase system in PMNs. Although Paul et al. (2) reported low levels of activity in macrophages from various sources, Miller (17) and Cline (19) found no evidence for a significant myeloperoxidase system in macrophages. Anaerobiosis has no apparent effect on phagocytosis by mononuclear cells and, although some antibacterial activity is expressed, maximal activity requires the presence of oxygen in human peripheral (19) and mouse peritoneal mononuclear phagocytes (17).

Cell lysates from all groups of mice had antilisterial activity when tested in the presence of DTT. Higher activities were found in lysates from prestimulated than nonstimulated control mice and the highest level of activity was in lysates from mice injected and prestimulated with viable BCG.

The antilisterial factor is probably bactericidal rather than bacteriostatic in nature. Continued incubation of petri plates caused no increase in the number of colonies of listeria.

Antilisterial activity was associated with lysates from both glass-adherent (65% macrophages) cells and with nonadherent cells (99% lymphocytes). More than 99% of the cells in the nonadherent cell population were morphologically lymphocytes but it is possible this activity may have been derived from the small percentage of contaminating monocytes and macrophages.

The increase in reduction potential in macrophages from mice receiving BCG was striking. Macrophages from mice in these groups reduced NBT to a much greater extent than controls. NBT reduction has been shown to occur in the electron transport system via an interaction with ubiquinone at $E_o' = +1.00$ volt (20). Miller (17) found various dyes and electron acceptors with redox potentials of less than $E_o' = 0.22$ volt interfered with bactericidal activity of normal macrophages and suggested the electron transport system was involved in macrophage antibacterial mechanisms. In our system, DTT, a reducing agent or electron donor with a redox potential of $E_o' = -33$ volt, activates an antilisterial factor. The almost minimal levels of reduction potential in control mice may mean the factor may be present, but all or most of

it perhaps in the inactive, oxidized form. The increased metabolism of the macrophages in mice with cell-mediated immunity may be generating the elevated reduction potentials required to bring the factor to the active reduced form. In previous experiments (21), only peritoneal cell lysates from BCG-immunized mice had antibacterial activity when assayed without DTT immediately after cell collection and lysis.

The requirements for a reducing compound, dithiothreotol, for antibacterial activity to be retained after release from the cell may also account for the lack of its detection in other studies. Enzymes have been isolated which have this same requirement. Some of these, such as arylamidases (22) and cathepsins (23), are present in lysosomes of rat liver and kidney.

All fractions from homogenized peritoneal cell lysates obtained by differential centrifugation had antilisterial activity. The relative activity found in the pellet obtained by centrifugation at low speed steadily decreased as homogenation time was increased and more cells were broken. Antilisterial activity paralleled phosphatase activity in the high speed supernatant fractions at each dilution tested. Enzymes such as phosphoprotein phosphatase can interfere with the assay for acid phosphatase (24) and the factor(s) may correlate with the cytoplasmic enzyme. However, antilisterial activity was found in the pellet fraction obtained by high speed centrifugation which may suggest a lysosomal origin for the factor(s). Noncorrelation of antilisterial and enzyme activities in the pellet fraction at the higher concentrations suggests the presence of an inhibitor, perhaps a membrane or membrane component.

Studies to isolate and characterize this factor(s) are in progress.

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FOOTNOTES

[1] HD_{50} unit/ml is the concentration which lyses 50% of the cells.

[2] Generously donated by Dr. Frank A. Kapral, Ohio State University.

[3] Prepared as suggested by manufacturer. A request for the exact concentration of NBT was refused by Sigma Chemical Co.

Figure 1. Restoration by 10 mM dithiothreotol (DTT) of antilisterial activity of mouse peritoneal cell lysate lost by overnight storage at 2 C (lysate dilutions with DTT ● , lysate dilutions without DTT ■). Undiluted lysate equivalent to 1 x 10^7 cells/ml (6.5 x 10^6 macrophages and 3.5 x 10^6 lymphocytes per ml).

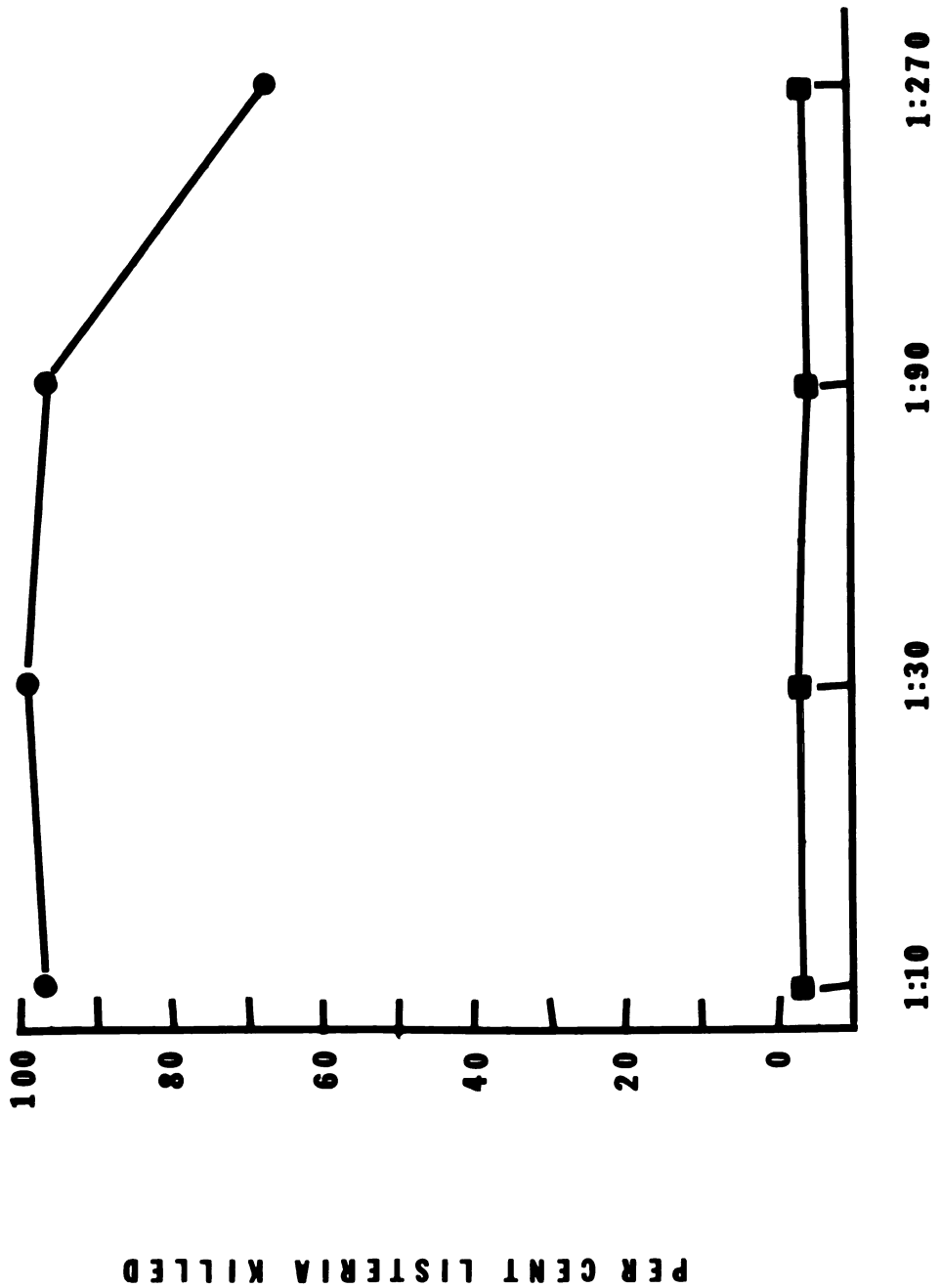
PER CENT LISTERIA KILLED
100
80
60
40
20
0
1:10
1:30
1:90
1:270
LYSATE DILUTION

Figure.1.

Figure 2. Antilisterial activity of lysates of subpopulations of mouse peritoneal cells, glass adherent cells O, and nonadherent cells Δ.

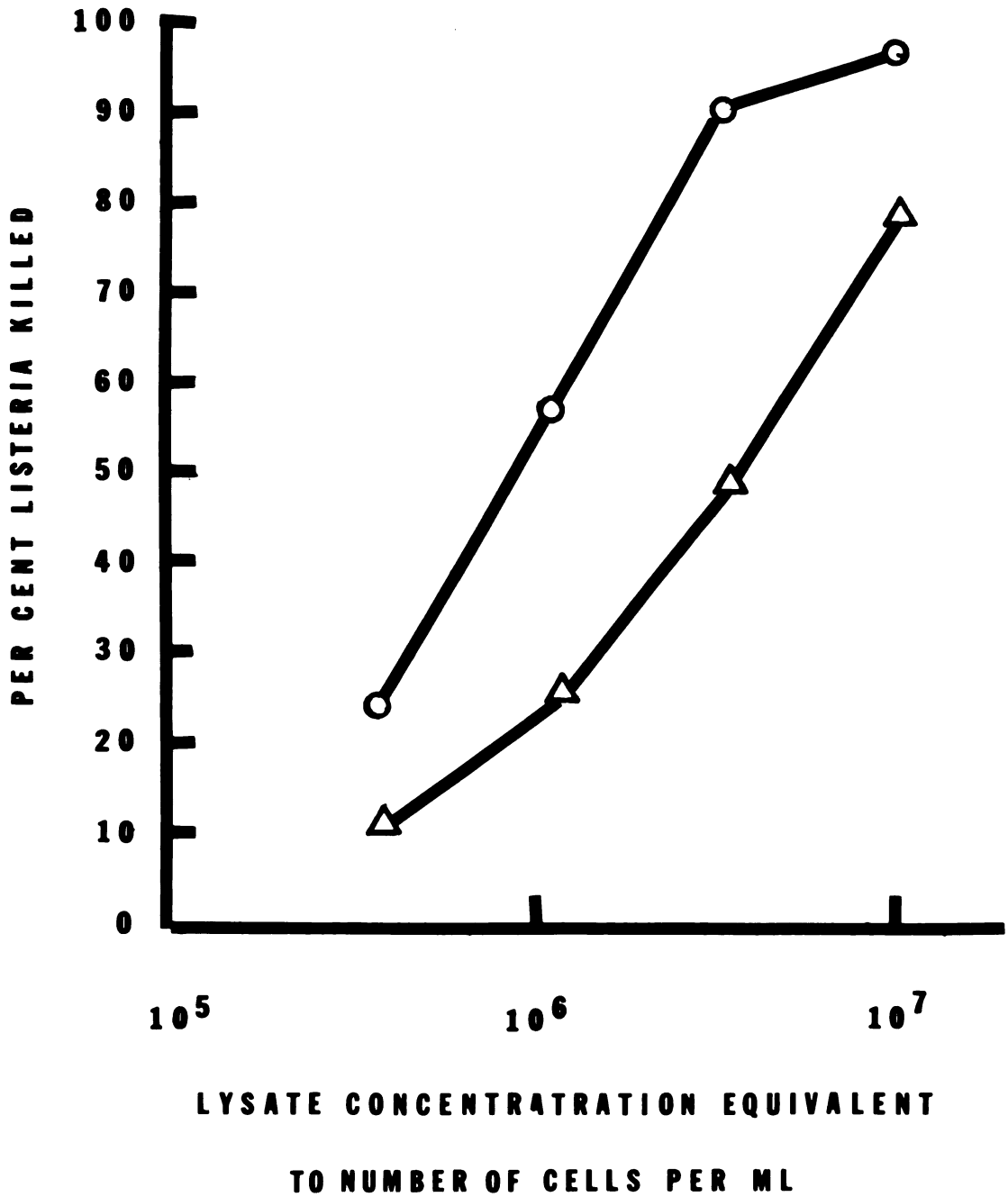
100
90
80
70
60
50
40
30
20
10
0
PER CENT LISTERIA KILLED
10^5
10^6
10^7
LYSATE CONCENTRATRATION EQUIVALENT
TO NUMBER OF CELLS PER ML

Figure 2.

Figure 3. Nitro blue tetrazolium reduction by mouse peritoneal macrophages. Optical density values of extracts of reduced NBT from 4×10^6 macrophages.

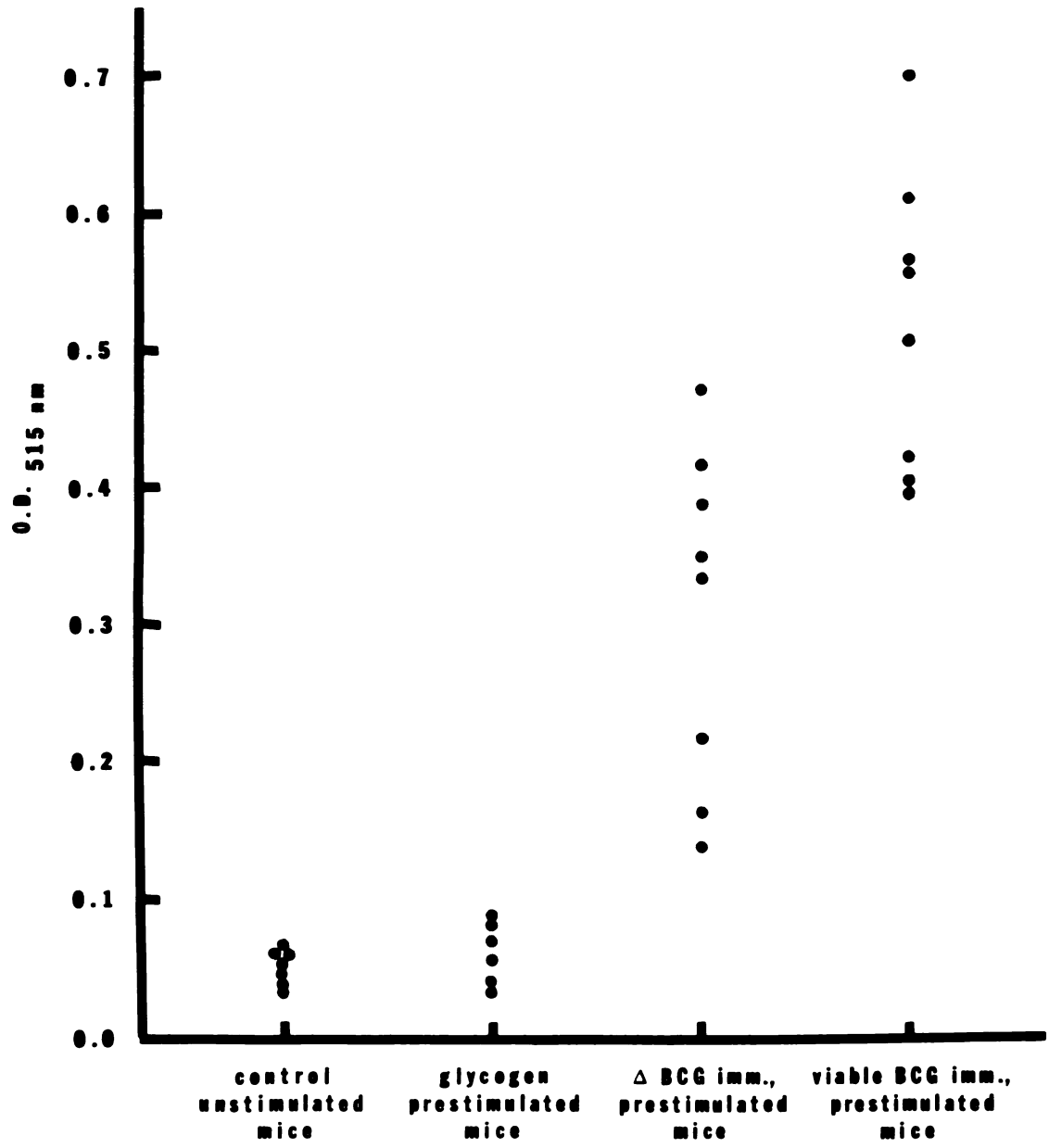
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
O.D. 515 nm
control unstimulated mice
glycogen prestimulated mice
Δ BCG imm., prestimulated mice
viable BCG imm., prestimulated mice

Figure 3.

Figure 4. Acid phosphatase and antilisterial activities of high speed supernatant fractions of mouse peritoneal cells homogenized 2, 4, 6, and 8 minutes. Antilisterial activities of fractions diluted 1:10, O ; 1:30, ◇ ; 1:90, △ ; and 1:270, □ . Undiluted acid phosphatase activities, ● .

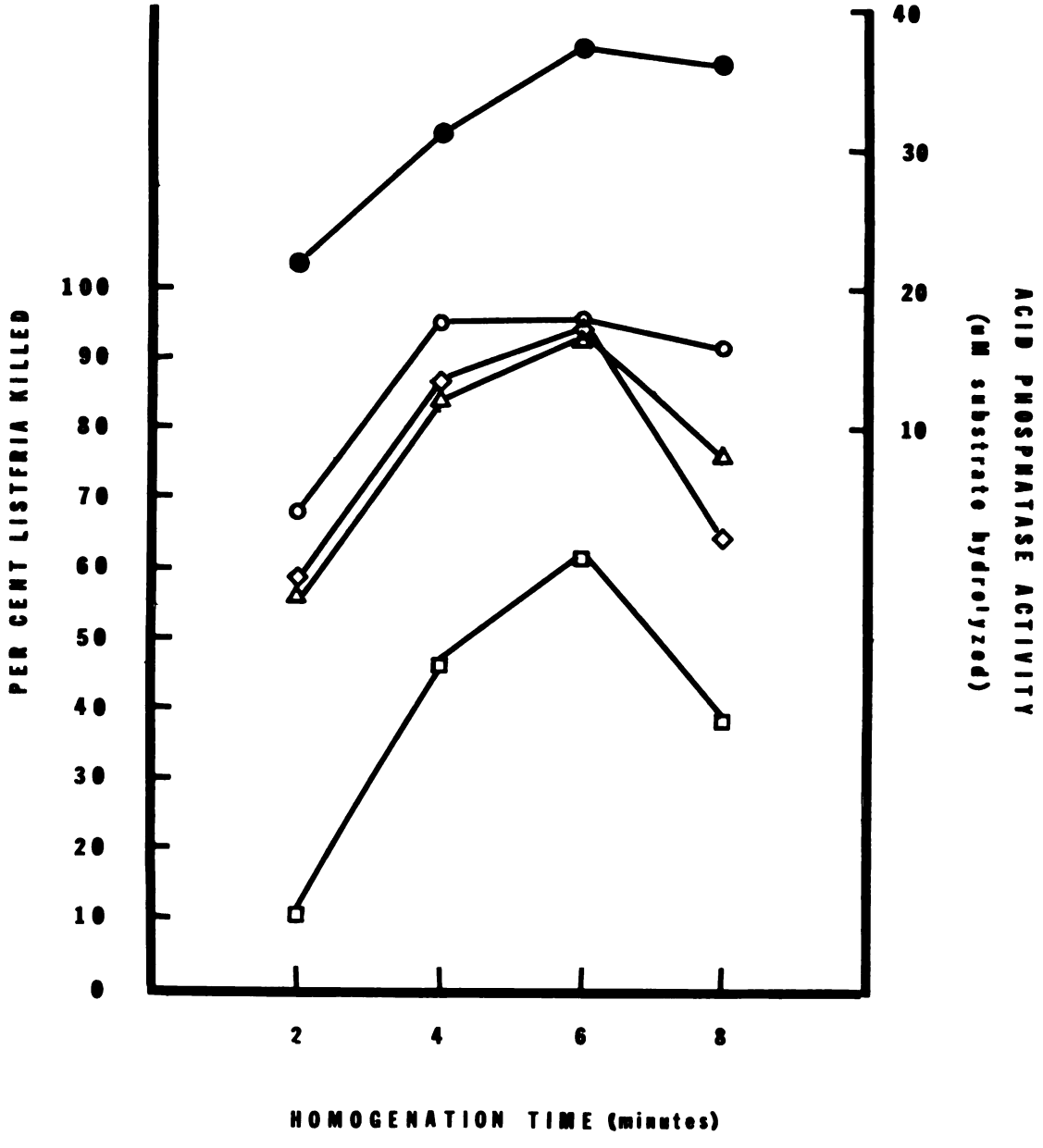
PER CENT LISTERIA KILLED
100
90
80
70
60
50
40
30
20
10
0
40
30
20
10
ACID PHOSPHATASE ACTIVITY
(nM substrate hydrolyzed)
2
4
6
8
HOMOGENATION TIME (minutes)

Figure 4.

Figure 5. Acid phosphatase and antilisterial activities of low speed pellet fractions of mouse peritoneal cells homogenized 2, 4, 6, and 8 minutes. Antilisterial activities of fractions diluted 1:10, O ; 1:30, ◇ ; 1:90, △ ; and 1:270, □ . Undiluted acid phosphatase activities, ● .

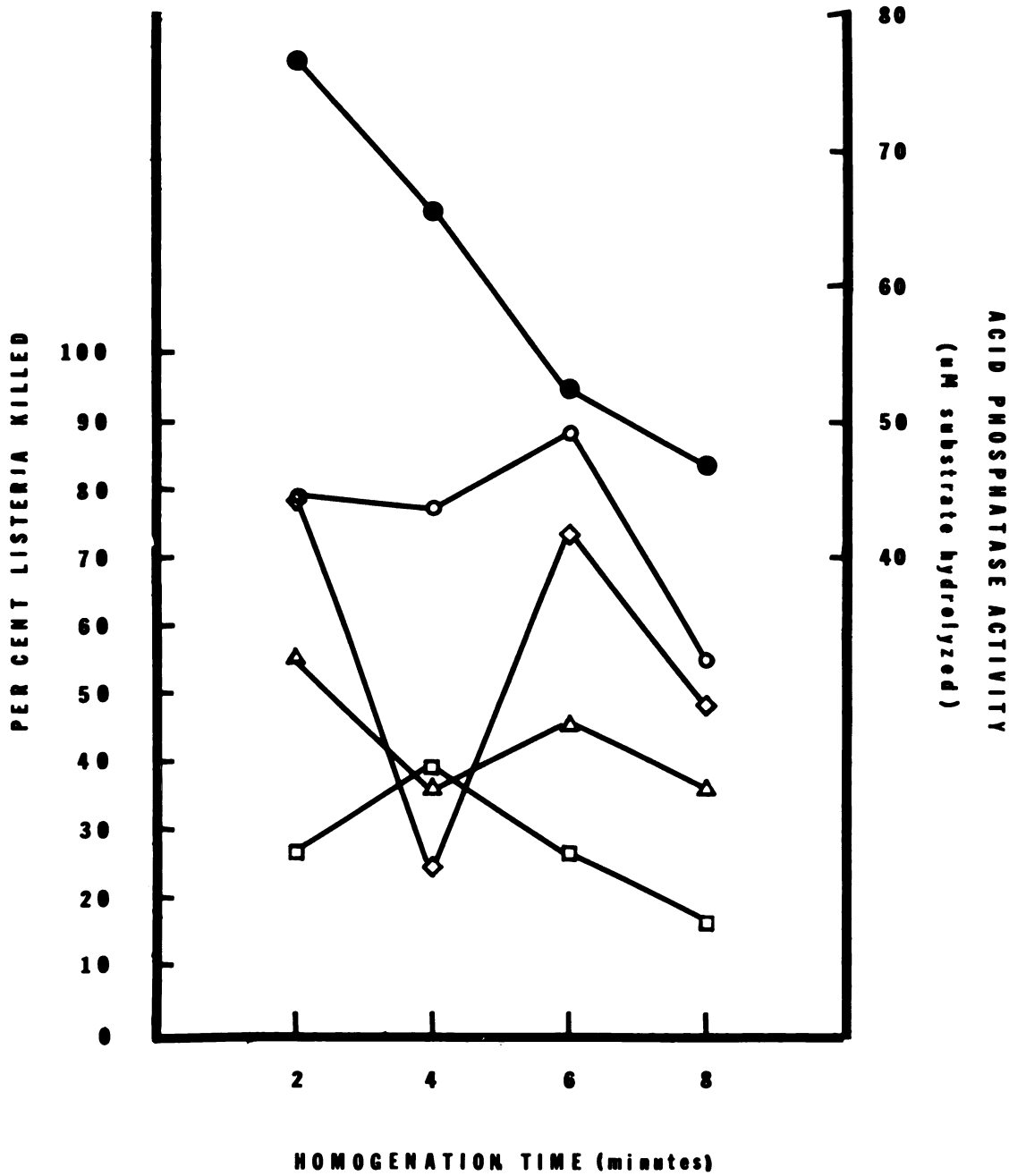
PER CENT LISTERIA KILLED
100
90
80
70
60
50
40
30
20
10
0
80
70
60
50
40
ACID PHOSPHATASE ACTIVITY
(uM substrate hydrolyzed)
2
4
6
8
HOMOGENATION TIME (minutes)

Figure 5.

Figure 6. Acid phosphatase and antilisterial activities of high speed pellet fraction of mouse peritoneal cells homogenized 2, 4, 6, and 8 minutes. Antilisterial activities of fractions diluted 1:10, ○ ; 1:30, ◇ ; 1:90, △ ; and 1:270, □ . Acid phosphatase activities of undiluted fractions, ● .

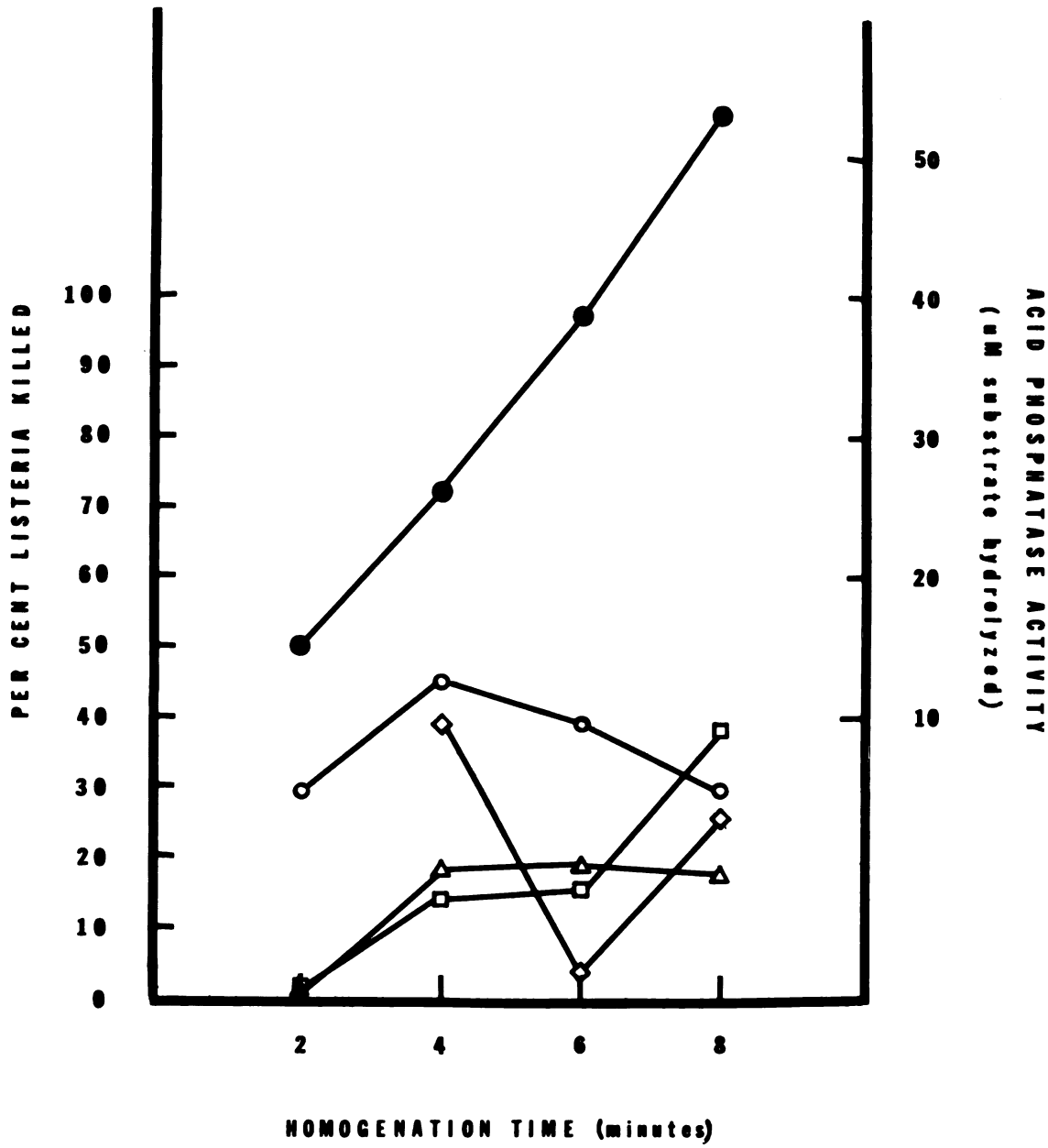
PER CENT LISTERIA KILLED
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
HOMOGENATION TIME (minutes)
50
40
30
20
10
ACID PHOSPHATASE ACTIVITY
(nM substrate hydrolyzed)

Figure 6.

Table 1. Acid phosphatase activities of high speed supernatant fractions from lysates of 10^6 macrophages per ml.

	Pretreatment of mice			
	Control, unstimulated	Control, glycogen prestimulated	Heat-killed BCG immunized & prestimulated	Viable BCG immunized & prestimulated
Acid phosphatase activity[1]	17.2	21.5	28.0	34.6

[1] uM of substrate hydrolyzed per hour

Table 2. Antilisterial activities of high speed supernatant fractions from peritoneal cell lysates from BCG-immunized and control mice diluted to equivalent acid phosphatase activity[1].

	Pretreatment of mice			
	Control, unstimulated	Control, glycogen prestimulated	Heat-killed BCG immunized & prestimulated	Viable BCG immunized & prestimulated
% Listeria killed[2]	73	58	68	79

[1]Fractions diluted to acid phosphatase activity hydrolyzing 8×10^{-4} uM substrate per hour

[2]Buffer control = 1.68×10^{4} cfu/ml

DITHIOTHREOTOL-DEPENDENT ANTILISTERIAL ACTIVITY OF LYSATES FROM NORMAL MACROPHAGES EXPOSED IN VITRO TO CULTURE FLUIDS FROM SPLEEN CELLS OF BCG-IMMUNIZED MICE

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ABSTRACT

The effect of culture fluids from mouse lymphocytes on the antilisterial activity of normal macrophages *in vitro* was determined. Titers of lysates of macrophages incubated with or without culture fluids from lymphocytes from control mice had titers of 10-270 with or without a reducing agent, dithiothreotol (DTT). Lysates of macrophages after incubation with culture fluids of spleen lymphocytes from BCG-immunized mice had antilisterial titers with and without DTT of 197,000 and 810 respectively. This may indicate a DTT-dependent antilisterial system inherent in activated macrophates of cell-mediated immunity. Lysates of incubated spleen cells and their culture fluids also had slight antilisterial activity.

INTRODUCTION

Culture supernatant fluids from lymphocytes of immunized animals incubated *in vitro* with specific antigen reportedly affects the activity of macrophages *in vitro*. Nathan et al. (7) reported increased macrophage adherence, phagocytosis and hexose monophosphate oxidation in macrophages from normal guinea pigs incubated with supernatants of lymphocytes from o-chlorobenzoyl bovine γ-globulin immunized animals after the lymphocytes were incubated with the specific antigen. Patterson and Youmans (8) found a significant decrease in the number of virulent *Mycobacterium tuberculosis* H37Rv in normal macrophages when splenic lymphocytes from mice immunized with avirulent *M. tuberculosis* H37Ra were added. They also observed intracellular inhibition when culture fluids from immune spleen cells incubated *in vitro* with specific antigen were added to normal macrophages. Howard et al. (3) reported similar observations when lymphocytes from *Histoplasma capsulatum* immunized mice were added to normal macrophages.

The increased macrophage antimicrobial activity is not specific for the immunizing organism. Culture supernatant fluids of lymphocytes from listeria-immunized mice incubated *in vitro* with listeria confer the ability to inhibit virulent mycobacteria on normal macrophages (5), and culture filtrates of BCG-immune lymphocytes incubated with BCG activate normal macrophages to greater killing of listeria (10).

We detected antilisterial activity in mouse peritoneal cell lysates which depends on the presence of a reducing agent, dithiothreotol (DTT) (6). This is a report of the detection of this activity in lysates of normal macrophages and an increased activity in lysates of macrophages incubated with culture fluids from BCG-immune lymphocytes.

MATERIALS AND METHODS

Mice

CBA/J female mice approximately nine weeks old were obtained from Jackson Laboratories, Bar Harbor, Maine. They were housed 5 per cage with water and food pellets, ad libitum.

Bacterial preparations

Mycobacterium bovis, BCG was grown at 37 C for 2 weeks in Dubos Broth base without enrichment or Tween 80 (Difco Laboratories, Detroit, Mich.) with 0.5% dextrose, centrifuged and homogenized with mortar and pestle. Cells were suspended in tissue culture media, the concentration estimated by centrifugation in a Hopkin's tube, and adjusted to approximately 1×10^8 organisms/ml.

Listeria monocytogenes was maintained on Brain Heart Infusion agar (Difco). Cultures used for measuring antilisterial activity were grown for 18 hrs in Brain Heart Infusion broth (Difco at 37 C with shaking.

Immunizations

Mice were inoculated once intraperitoneally with approximately 2 mg (wet weight) of BCG in physiological saline in a total volume of 0.2 ml.

Lymphocyte cultures

Spleens from control mice and mice immunized 4 weeks previously with BCG were removed aseptically and placed in cold Hank's basal salt solution (HBSS) (Microbiological Associates, Bethesda, Md). Both groups were treated in the same manner. The spleens were punctured at various sites, injected with cold HBSS and the cells expressed with sterile forceps.

The cells were dispersed by forcing them first through a 20-gauge and then through a 27-gauge syringe needle. The cells were centrifuged at 5 C for 10 min at 250 x g and resuspended in HBSS with 10% fetal calf serum and supplemented with 1% each glutamic acid, sodium pyruvate, glutamine, vitamin, essential and nonessential amino acid pools, and 100 ug/ml streptomycin and 100 units/ml penicillin.

Cell numbers were determined by hemocytometer. Differential cell counts were made in a solution of 2.1% citric acid-0.1% crystal violet. Viability counts were made by the trypan blue exclusion technique.

Spleen cell suspensions were adjusted to 1×10^7 viable lymphocytes per ml and 15 ml were placed in 100 x 15 mm plastic tissue culture dishes. BCG was added to one-third of the spleen cell cultures for a final concentration of 10^6 organisms per ml. The cell cultures were incubated for 2 hrs at 37 C in an atmosphere of 95% air and 5% CO_2. Nonadherent cells in the culture medium were transferred to new tissue culture dishes, incubated 3 days and centrifuged. The culture fluids were decanted and set aside. The lymphocytes were washed 1x and resuspended to 1×10^7 cells/ml in 0.1 M phosphate buffer, pH. 7. Cell suspensions after incubation were 93-99% lymphocytes.

Macrophage collection and culture

A modification of the method of Klun and Youmans (4) was used. Peritoneal cells of normal, unstimulated mice were collected as described previously (6). The cells were pooled, centrifuged 10 min at 5 C at 250 x g and resuspended in the above medium with 10% horse serum added. Differential cell counts and viability were determined as above and cell numbers adjusted to 1 x 10^7 macrophages/ml. Fifteen ml were placed in plastic tissue culture dishes and incubated at 37 C in 95% air and 5% CO_2. After two days, the medium and unattached cells were removed, fresh medium was added and the macrophages incubated for an additional 24 hrs.

After a total of 3 days incubation, the medium was removed and replaced with 10 ml fresh medium and 5 ml of lymphocyte culture fluid. The cell cultures were incubated 24 hrs. The media was decanted and the adherent cells rinsed quickly and gently with a 1:1 trypsin-EDTA at room temperature. The cells were covered with cold trypsin-EDTA and removed with a rubber policeman. The macrophages were washed, counted and resuspended to 2 x 10^6 macrophages/ml in 0.1 M phosphate buffer, pH 7.0. The cell suspensions were 99% macrophages.

Preparation of cell lysates and lymphocyte culture fluids for assay

Macrophage and lymphocyte suspensions were lysed with staphylococcal delta-hemolysin as described previously (6). When microscopic examination determined greater than 95% lysis had occurred, aliquots were removed and assayed immediately for antilisterial activity.

Supernatant fluids from cultured lymphocytes were dialyzed in distilled water which had been degassed and flushed with argon. The dialyzed culture fluids were assayed immediately.

Aliquots of cell lysates and dialyzed lymphocyte culture fluids were stored at 4 C and reassayed after 3 weeks.

Antilisterial assay

Antilisterial activity was measured using a modification of the assay described in a previous paper (6). Serial 3-fold dilutions of the material to be tested were made in two diluents, 0.1 M phosphate buffer pH 7 containing 0.01% bovine serum albumin with and without DTT at a concentration of 10 mM. The controls were tubes containing 1.0 ml of the diluent. An 18-hour broth culture of L. monocytogenes was centrifuged, resuspended in 0.85% saline and diluted to ca. 2×10^6 colony forming units/ml. One-tenth ml was added to each dilution and control tubes. The tubes were incubated for 2 hrs at 37 C. The suspensions were diluted and aliquots of each dilution added to 15 ml of warm, melted Brain Heart Infusion agar. After mixing, the contents were poured into petri dishes, allowed to solidify and incubated over night at 37 C. The number of colonies were counted and the titer taken as the highest dilution which inactivated 50% of listeria when compared to the buffer control.

RESULTS

Antilisterial activity of lymphocyte culture fluids

In the absence of DTT, no detectable antilisterial activity was found in fresh lymphocyte culture fluids except in those from BCG stimulated lymphocytes of BCG-immunized mice (Table 1). Without DTT, no antilisterial activity in culture fluids was detected after 3 weeks storage. Slight activity was detected in the presence of DTT culture fluids from control lymphocytes which had been incubated with BCG, and from BCG-immune lymphocytes incubated with or without BCG.

Antilisterial activity of lymphocyte lysates

There was antilisterial activity in lysates from lymphocytes from all groups, with and without DTT (Table 2). Titers of stored lysates were similar to initial titers.

Antilisterial activity of macrophage lysates

Endpoints were not reached in several of the initial titrations (Table 3). When reassayed, the greatest activity was found in lysates of macrophages incubated with culture fluids from BCG-immune lymphocytes incubated with BCG. In the presence of DTT, lysates in this group had titers of 197,430. Without DTT, the titer was 810. Macrophage lysates from cells incubated with culture fluids of BCG-immune lymphocytes, unstimulated *in vitro*, had similar titers. Endpoints reached with macrophage lysates in other groups were not greater than 270.

DISCUSSION

The lymphocyte culture fluids and lysates of spleen cells inactivated listeria to some extent. Whether the activity is due to a material produced and secreted by lymphocytes or whether this baseline activity is a result of the small number of macrophages present could not be determined.

Low levels of antilisterial activity were detected in lysates of macrophages with or without culture fluids from unsensitized lymphocytes. This was interpreted to represent the normal antibacterial factors of macrophages, although an effect by pinocytosed antibiotics cannot be eliminated. Titers with or without DTT did not differ markedly.

In the presence of DTT, lysates of macrophages incubated with culture fluids of BCG-stimulated lymphocytes from BCG-immunized mice had greater antilisterial activity than the above controls. Unpublished data indicates this is a qualitative difference, not quantitative. Culture fluid from BCG-immune spleen cells cultured *in vitro* without BCG were also able to stimulate macrophages and high titers were obtained with lysates in this group when DTT was included in the assay system. Spleen cells had been collected from mice one month after injection with BCG and, therefore, the cells may still have been near the peak of their immune response. Blanden et al. (1) found significant resistance to *L. monocytogenes* in mice infected with BCG four weeks earlier.

The nature of the stimulus affecting macrophages is unknown. The macrophages may be pinocytosing and concentrating an active lymphocyte product or receiving a signal to produce and/or activate a macrophage antilisterial product(s). The latter is more probable, assuming complete uptake of an active factor(s) from culture fluid and the action additive to normal macrophage antibacterial systems. There was not sufficient total activity in the volume of culture fluid added to macrophages to account for the increased activity, even using least favorable data. It is possible that a DTT-dependent antibacterial factor produced by lymphocytes is taken up and is acting synergistically with macrophage systems. Lysates of macrophages incubated with culture fluids of BCG-sensitized lymphocytes, incubated in vitro with or without BCG, had titers of 197,430 and 65,610, respectively. In this same test group, titers were 270 with or without DTT when BCG was added to culture fluid at the time of transfer to macrophages. This suggests the removal of an active factor(s) by the BCG.

We have reported a DTT-requiring antilisterial factor(s) from mouse peritoneal cell lysates. Slightly greater activities were detected in cell lysates from BCG-immunized and prestimulated mice (6). The dependency of the antilisterial factor on a reducing agent for activity may explain why it has not been detected by previous investigators. The elevated antilisterial titer, with DTT, of lysates of macrophages exposed to culture fluid of BCG-immune lymphocytes suggests the DTT-dependent factor(s) may be important in antibacterial cell-mediated immunity.

Other authors have reported an inhibitory influence of culture supernatant fluids from immune, stimulated lymphocytes on intracellular parasites in normal, infected macrophages (2, 3, 4, 5, 8, 9, 10). Pearsall et al. (9) reported a direct effect on yeast cells by lymphokine-containing lymphocyte supernatants. Klun and Youmans (4) and Fowles et al. (2) found no direct effect on extracellular mycobacteria or listeria by lymphocyte culture fluids but suggested these results may not be definitive. Fowles et al. (2) found the addition of sensitive lymphocytes had a greater effect on macrophage bacteriostasis than did supernatant fluids and suggested the mediators produced by lymphocytes may be labile. In their experiments, the effect of lymphocytes on macrophages was detected by determining the bacterial inactivation in intact macrophages. Our system offers the advantage of measuring bacterial inactivation in a cell free system and examining the process at a subcellular level.

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TABLE 1

ANTILISTERIAL ACTIVITY WITH OR WITHOUT DTT OF CULTURE FLUIDS FROM MOUSE SPLEEN LYMPHOCYTES, INCUBATED *IN VITRO* WITH OR WITHOUT BCG, FROM NORMAL AND BCG-IMMUNIZED MICE

Source of lymphocytes[a]	Added to lymphocytes for incubation *in vitro*	Included in assayed solutions	Antilisterial titer[d] when assayed immediately	Antilisterial titer of supernatants after storage
Unimmunized	0	0	< 10	< 10
		DTT[c]	< 10	< 10
	BCG[b]	0	< 10	< 10
		DTT	< 10	270
BCG-immunized	0	0	< 10	< 10
		DTT	> 270	810
	BCG	0	> 270	< 10
		DTT	90	270

[a] Lymphocyte concentration in *in vitro* culture = 1×10^7 cells/ml

[b] BCG concentration in *in vitro* culture = 1×10^6 cells/ml

[c] DTT concentration in assay solutions = 10 mM

[d] Titer = highest dilution which inactivated 50% of listeria

TABLE 2

ANTILISTERIAL ACTIVITY WITH OR WITHOUT DTT OF LYSATES OF MOUSE SPLEEN LYMPHOCYTES, INCUBATED *IN VITRO* WITH OR WITHOUT BCG, FROM NORMAL AND BCG-IMMUNIZED MICE

Source of lymphocytes[a]	Added to lymphocytes for incubation *in vitro*	Included in assayed solutions	Antilisterial titer[d] when assayed immediately	Antilisterial titer of supernatants after storage
Unimmunized	0	0	30	30
		DTT[c]	90	270
	BCG[b]	0	30	30
		DTT	90	90
BCG-immunized	0	0	> 270	270
		DTT	30	10
	BCG	0	> 270	90
		DTT	30	10

[a]Lymphocyte concentration in *in vitro* culture = 1×10^7 cells/ml

[b]BCG concentration in *in vitro* culture = 1×10^6 cells/ml

[c]DTT concentration in assay solutions = 10 mM

[d]Titer = highest dilution which inactivated 50% of listeria

TABLE 3

ANTILISTERIAL ACTIVITIES WITH OR WITHOUT DTT OF LYSATES OF NORMAL MOUSE PERITONEAL MACROPHAGES AFTER INCUBATION *IN VITRO* WITH CULTURE FLUIDS FROM SPLEEN LYMPHOCYTES, CULTURED WITH OR WITHOUT BCG, FROM NORMAL OR BCG-IMMUNIZED MICE

Macrophages[a] incubated with	Added to lymphocytes for incubation *in vitro*	Added to culture fluid when transferred to macrophages	Included in assayed solutions	Antilisterial titer[e] when assayed immediately	Antilisterial titer after storage
No lymphocyte supernatant fluid added	0	0	0	30	30
			DTT[d]	90	10
Supernatant fluid from lymphocytes[b] of control mice	0	0	0	<10	<10
			DTT	180	270
		BCG[c]	0	270	90
			DTT	>270	270
	BCG	0	0	>270	90
			DTT	270	270
Supernatant fluid from lymphocytes of BCG-immunized mice	0	0	0	>270	270
			DTT	>270	65,610
		BCG	0	270	270
			DTT	270	270
	BCG	0	0	>7290	810
			DTT	>7290	197,430

[a] Macrophages incubated with 5 ml lymphocyte culture fluid + 10 ml fresh medium

[b] Lymphocyte concentration in *in vitro* culture = 1×10^7 cells/ml

[c] BCG concentration in *in vitro* culture = 1×10^6 cells/ml

[d] DTT concentration in assay solutions = 10 mM

[e] Titer = highest lysate dilution which inactivated 50% of listeria

ISOLATION AND CHARACTERIZATION OF AN ANTIBACTERIAL FACTOR FROM PERITONEAL CELL LYSATES OF MICE IMMUNIZED WITH VIABLE MYCOBACTERIUM BOVIS, BCG

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ABSTRACT

Crude homogenates of peritoneal cells from BCG-infected mice contained antilisterial activity when a reducing compound, dithiothreotol (DTT), was included in the assay system. The material was oxygen sensitive, heat stable and active against Listeria monocytogenes, Staphylococcus aureus and Mycobacterium fortuitum but not Pseudomonas aeruginosa. Inactivation of L. monocytogenes in stationary phase was biphasic and continuous over time. There was no detectable inactivation of log phase L. monocytogenes.

Lysates from peritoneal cells of control mice had antilisterial activity in only one DEAE-cellulose fraction (DEAE-I), lysates from BCG-infected mice had an additional fraction with antilisterial activity (DEAE-II). The additional fraction had the same antibacterial spectrum as crude lysate. DEAE-fraction II was most active at a pH near neutrality. Inactivation rate kinetics were not the same as those of crude lysates, i.e., a short period of listerial inactivation was followed by no further decrease in viable units over time instead of steady continued inactivation. No additive effects were observed when fractions I and II were combined.

It is concluded that the active fraction unique for lysates of BCG-immunized mice only partially accounts for the antimicrobial properties in macrophage lysates of BCG-immunized and prestimulated mice.

INTRODUCTION

Lysates of mouse mononuclear cells were capable of inactivating Listeria monocytogenes (5). Only peritoneal cell lysates from mice immunized and prestimulated intraperitoneally with BCG had antibacterial activity when assayed immediately after cell collection and homogenation (4). When a reducing agent, dithiothreotol (DTT), was included in the assay system, lysates from all groups of mice immunized or control, had some antilisterial activity (5). Peritoneal macrophages from mice immunized and prestimulated with BCG had high reduction potentials when assayed in vitro, but cells from control mice or mice prestimulated with glycogen had little or none. This suggested that if the antilisterial factor(s) is present in normal cells, it may only be present in an inactive, oxidized form.

We have recently reported DTT-requiring antilisterial activity in lysates of normal macrophages which had been incubated in vitro with supernatant fluids from lymphocytes of BCG-immunized mice (6). DTT-dependent activity was present at much higher titers in these lysates than controls.

This is a report of partial separation attempts and characterization of the active factor(s).

MATERIALS AND METHODS

Animals. Female Swiss-Webster albino mice (Carworth Farms, Portage, Michigan) were housed 5 per cage and fed mouse pellets and water ad libitum. Immunization with viable BCG, peritoneal cell collection and homogenation procedures were as described previously (5). All fractionation and testing, unless indicated otherwise, was with cell lysates from mice immunized and prestimulated with viable BCG.

Microorganisms. Listeria monocytogenes, Staphylococcus aureus, and Pseudomonas aerugenosa were maintained on Brain Heart Infusion agar (Difco). Mycobacterium fortuitum and Mycobacterium bovis, BCG were maintained on Dubos oleic agar (Difco).

Antibacterial assay system. The assay system was as described previously (5) except phosphate buffer (PB), pH 7 replaced phosphate-citrate buffer, pH 7. The assay system used to measure antilisterial activity was a modification of that by Hirsch (1). All lysates and fractions were dialyzed against 1:10 PB before testing. Serial 3-fold dilutions of the materials to be tested were made in PB containing 0.01% bovine serum albumin (Difco). After preliminary experiments indicated that the factor was oxygen-labile, the reducing agent, dithiothreotol (DTT) (Calbiochem, San Diego, Calif.), was included in the buffer system at a concentration of 10 mM. The final volume of each dilution tube was 1.0 ml. The control was a tube containing 1.0 ml of the diluent. An 18 hour broth culture of Listeria monocytogenes was centrifuged, resuspended in 0.85% saline and diluted to

ca. 2 x 10^6 CFU/ml. One-tenth milliliter was added to each dilution and the control tube. Each tube was flushed with argon and incubated at 37 C for 2 hrs. The suspensions were diluted and aliquots of each dilution added to 20 ml of warm (50 C), melted Brain Heart Infusion Agar. After mixing, the contents were poured into petri dishes, allowed to solidify and incubated overnight at 37 C. The number of colonies were counted and expressed as the percent kill calculated from the control.

Ammonium sulfate precipitation. A saturated solution of ammonium sulfate was adjusted to pH 7 with ammonium hydroxide. Homogenized cell fractions were centrifuged at 20,000 x g for 20 minutes and dialyzed against phosphate buffer, pH 7. Fractionation procedures were performed at 0-4 C. Appropriate volumes of saturated $(NH_4)_2SO_4$ were added with constant stirring to obtain the desired percent saturated solutions. After stirring on ice for 1 hour the mixture was centrifuged at 20,000 x g for 20 min. The precipitate obtained between 50-80% was used in further fractionation. This fraction was redissolved and dialyzed against PB.

DEAE-cellulose chromatography. Diethylaminoethyl (DEAE)-cellulose (Reeve Angel, Clifton, NJ)was equilibrated with 0.05 M tris (hydroxymethyl)-amino methane (Tris)-hydrochloric acid buffer, pH 7.8 that contained 5 mM dithiothreotol (DTT) (Calbiochem, San Diego, CA). A column 1.7 by 27 cm was prepared. The sample was applied and eluted by pumping at a flow rate of 1 ml/min with 0.05 M Tris-HCl, pH 7.8 with 5 mM DTT followed by a linear gradient of 0.05 M Tris HCl-5mM DTT, pH 7.8 and 0.05 M Tris HCl-5mM DTT and 0.5 M NaCl, pH 7.8. The eluate

(3 ml/tube) was monitored at 280 nm using an Isco UV monitor. Individual fractions with activity were pooled, precipitated in 80% $(NH_4)SO_4$ and reconstituted and dialyzed against PB with 1mM DTT.

Gel filtration chromatography. Sephadex G-200 (Pharmacia) was swollen and equilibrated in 0.05 M PB pH 7. A column 2.8 x 39 cm was prepared and 1 ml of whole cell lysate applied and eluted at a constant head pressure of 10 cm. Fractions (3.0 ml) were collected and measured for absorbance at 280 nm, sterilized by millipore filtrations and tested for antilisterial activity.

Polyacrylamide gel electrophoresis. A modification of the method of Maizel (3) was used to monitor purification of the lysates. Samples and electrode buffers were prepared with 1 mM DTT. The gel was 7.5% in acrylamide; no stacking gel was used. Migration was to the anode in Tris-glycine buffer, pH 8.3 at a constant current of 3 ma per gel. Bromphenol blue was used as the tracking dye. The gels were stained with 0.25% coomassie blue (Schwarz/Mann, Orangeburg, NY) in 7.5% acetic acid and destained in 7.5% acetic acid. Gels were scanned at 580 nm with a modified Gilford spectrophotometer.

Heat stability studies. Dilutions of supernatant fluid from lysates after high speed centrifugation and DEAE fraction II were assayed for activity. Appropriate dilutions were prepared in phosphate-citrate buffer, pH 7 with 10 mM DTT. Aliquots were heated at 37 C for 30 min, 45 C for 30 min, 56 C for 30 min and 100 C for 10 min. The fractions were cooled on ice and tested for antilisterial activity.

Inactivation rate kinetics. Cultures of L. monocytogenes were inoculated into Brain Heart Infusion (BHI) broth (Difco) and grown to

stationary phase (18 hr culture) and mid-log phase (2.5 hrs or an O.D. of 0.22 at 620 nm). The cultures were centrifuged and resuspended in saline. Aliquots of the resuspended cultures were added to solutions to be tested in 0.07 M phosphate-citrate buffer at pH 7 with 0.1% bovine serum albumin and 10 mM DTT and also to controls without lysate. The suspensions were incubated at 37 C. Aliquots were removed at appropriate times and serially diluted. Aliquots of the dilutions were added to 20 ml of warm (50 C), melted BHI agar, mixed and poured into petri dishes. After solidifying, the plates were incubated overnight at 37 C. The number of colonies were counted and the difference between control and test values was used to calculate percent inactivation.

Relative activity against heterologous organisms. Eighteen hour cultures of L. monocytogenes, S. aureus, and P. aerugenosa grown in BHI broth and a 48 hr culture of M. fortuitum grown in Dubos broth with Tween 80 (Difco) were used. Bacterial cultures were centrifuged and resuspended in saline to ca 10^6 CFU/ml. Fractions were assayed as above with dilutions which inactivated 60% of listeria.

pH optima. After determining the optimal dilution for activity at pH 7 in 0.1 M phosphate buffer with 10 mM DTT, concentrated material purified by DEAE-cellulose chromatography was diluted in 0.1 M acetate buffer (pH 4 & 5), 0.1 M phosphate buffer (pH 6, 7, 8), and 0.05 M glycine-NaOH buffer (pH 9 & 10) and tested for activity. Each buffer system contained 10 mM DTT.

Protein determination. Protein was measured by the method of Lowry et al. (2).

RESULTS

Stability of active factor in crude lysates. Antilisterial activity was measured in lysate treated or stored under different conditions (Table 1). Some activity was lost in storage without DTT. There was substantial loss of activity in lyophilized or aerated samples. All activity was lost in samples stored in buffer at pH 5 without DTT. In all succeeding experiments, lysates and fractions were stored at 4 C or -56 C with 10 mM DTT.

Fractionation. A schematic diagram of the fractionation procedure is shown in Figure 1. Peritoneal cells from groups of 50 BCG-immunized and BCG-prestimulated mice were washed and resuspended in 30 ml of 0.25 M sucrose in 0.05 M Tris-acetate buffer, pH 7.4. The homogenized cell preparation was clarified by centrifugation, brought to 50% saturation with ammonium sulfate and stirred on ice for 1 hr. The precipitate left after centrifugation was discarded and the remaining supernatant fluid brought to 80% saturation with ammonium sulfate. The resulting precipitate was redissolved, dialyzed and applied to a DEAE-cellulose column. The elution pattern is shown in Figure 2.

Activity was detected in samples under peaks I and II. A similar elution pattern was observed with lysates from control mice, but antilisterial activity was detected only in the samples comparable to peak I. In several experiments with lysates from BCG-immunized mice, the second peak did not register but assays indicated the presence of

antilisterial activity in the region of peak II. Fractions under peak II were pooled and used in further testing.

The absorbance scan patterns of stained acrylamide gels from crude high speed supernatant fluid and DEAE-cellulose fractions I and II are given in Figure 3. DEAE-cellulose fraction I appears to be reduced to a two component system. Fraction II contained very little protein and attempts to resolve the sample by acrylamide gel chromatography were generally unsuccessful.

Table 2 lists protein concentration, activity and recovery values at each purification step. After DEAE-cellulose chromatography, approximately 0.2% of the original total protein was recovered in fraction II.

Figure 4 represents the elution pattern of whole, unfractionated lysate on Sephadex G-200. Antilisterial activity was detected only in the shoulder preceding the second major peak.

Heat stability. Material in crude lysate is heat stable (Table 3). There was less than a 10% loss in activity of lysates placed in a boiling water bath for 10 min. DEAE fraction II was less stable. There was a 40% loss in activity in samples heated at 100 C for 10 min.

Bacterial inactivation. The relative antibacterial activities of lysate and DEAE fraction II are listed in Table 4. There appeared to be some reduction in the numbers of S. aureus and M. fortuitum in the presence of both crude and purified material. L. monocytogenes was most sensitive. There was little or no decrease of P. aeruginosa with either fraction.

Inactivation of listeria in stationary phase by crude material was biphasic and continuous over time but there was no measurable effect on

listeria in log phase (Figure 6). Inactivation kinetics of stationary phase listeria by DEAE fraction II did not correspond with those of the crude material, in that listeria were inactivated for a short period of time followed by no further decrease in colony forming units instead of a continuous decrease. Combining DEAE-cellulose fractions I and II did not reestablish the original kinetic pattern. There was no clumping or decrease in numbers of listeria in preparations examined microscopically.

pH optima. A biphasic curve was observed with DEAE-cellulose fraction II in inactivation studies of L. monocytogenes at different pH values (Figure 6). There was a slight depression of activity at pH 7 with greatest activity at pH 6 and 8.

Except for Table 1, all experiments were run 2-3 times.

DISCUSSION

Antilisterial activity in murine peritoneal cyll lysates required the presence of a reducing agent, DTT, for activity (5). As detected in this study, the activity in crude lysates is oxygen-sensitive and acid-labile in the absence of DTT and is heat stable. By sephadex chromatography, activity was found in fractions eluting before a protein marker of 67,000 molecular weight. Listeria in stationary phase were inactivated, but not listeria in log-phase. There was a continuous biphasic reduction of listerial colony forming units over time.

A fraction with antilisterial activity was found in DEAE-cellulose eluates of cell lysates from BCG-immunized and prestimulated mice. No detectable activity was found at this position from lysates of control, unimmunized mice. Because of the very low concentration of protein in the pooled sample, it was not possible to make any conclusions on the homogeneity of the sample by acrylamide gel disc electrophoresis.

Like the crude material, the fraction was heat stable. It was most active at pH 6 and 8. Whether the fraction is affected directly or whether the bacteria are more stable at neutral pH is not known.

Although the numbers of S. aureus and M. fortuitum were reduced slightly by both crude lysate and DEAE fraction II at the concentration inactivating 60% of L. monocytogenes, these values were not statistically different from controls. The concentrations optimal

for the inactivation of listeria may not be the most effective for other bacteria. Additional studies will be required to determine if microorganisms other than listeria are affected.

Like the crude material, DEAE fraction II was effective against listeria, but the kinetics of inactivation were different. Whereas the crude material continued to inactivate more listeria, fraction II obtained through DEAE-cellulose chromatography reduced the number of listeria colony forming units for only a brief time after which there was no further inactivation.

A second fraction with antilisterial activity, DEAE-cellulose fraction I, was found in DEAE-chromatography eluates of both control and BCG-immunized mice. This was found to contain at least two components by acrylamide gel disc electrophoresis. Combining fraction I and fraction II produced no change in the kinetic pattern and the effect on listeria was less than additive.

Fraction II obtained by DEAE-cellulose chromatography does not completely account for the activity detected in crude lysate. It does appear to be unique for peritoneal cell lysates of BCG-immunized and BCG-prestimulated mice.

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FIG. 1. Fractionation diagram for purification of antilisterial factor.

FIG. 1.

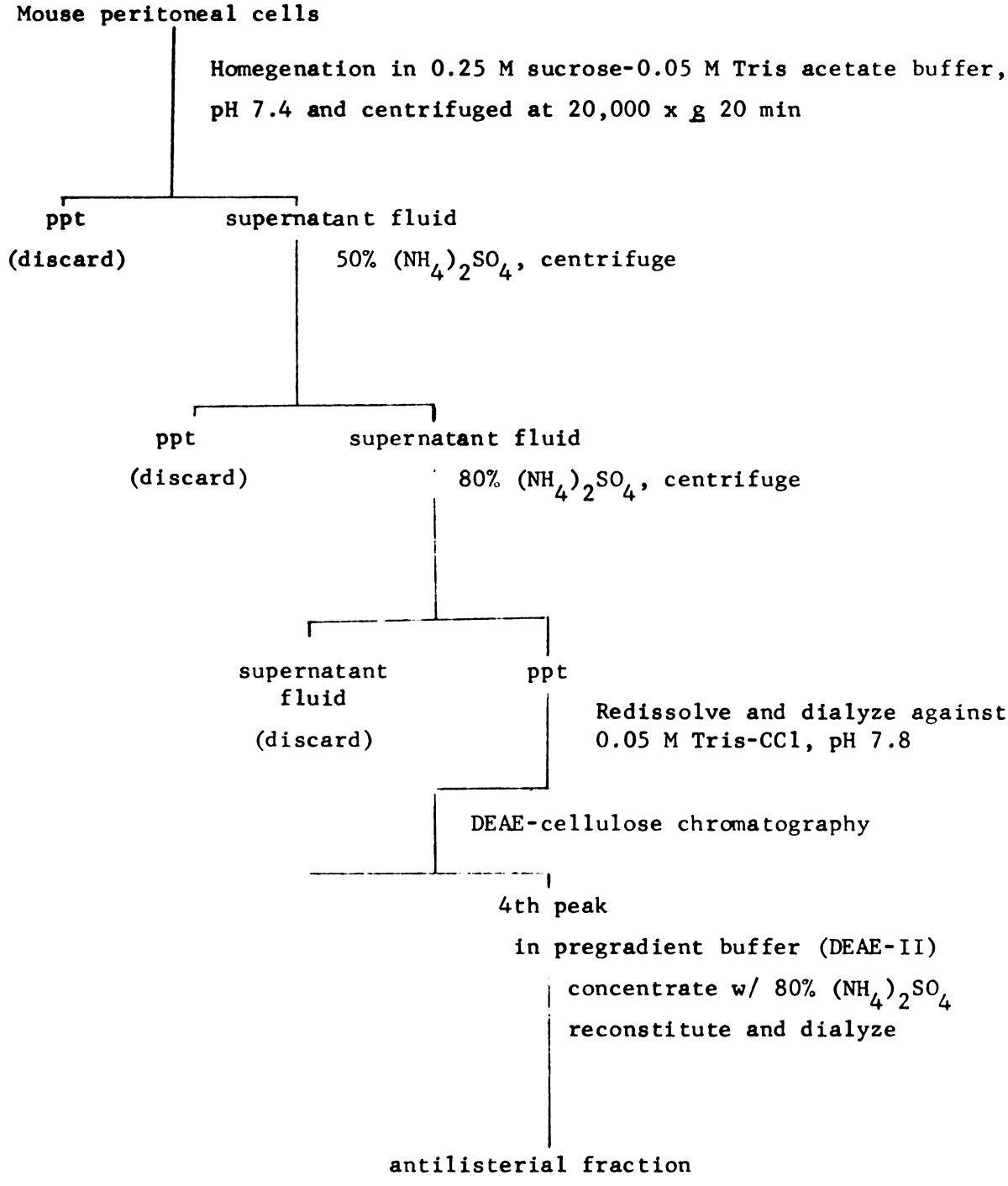
Mouse peritoneal cells
Homegenation in 0.25 M sucrose-0.05 M Tris acetate buffer, pH 7.4 and centrifuged at 20,000 x g 20 min
ppt
(discard)
supernatant fluid
50% $(NH_4)_2SO_4$, centrifuge
ppt
(discard)
supernatant fluid
80% $(NH_4)_2SO_4$, centrifuge
supernatant fluid
(discard)
ppt
Redissolve and dialyze against 0.05 M Tris-CCl, pH 7.8
DEAE-cellulose chromatography
4th peak
in pregradient buffer (DEAE-II)
concentrate w/ 80% $(NH_4)_2SO_4$
reconstitute and dialyze
antilisterial fraction

FIG. 2. DEAE-cellulose chromatography fractionation of high speed supernatant fluid in 0.05 M Tris-HCl, pH 7.8 with 5 mM DTT.

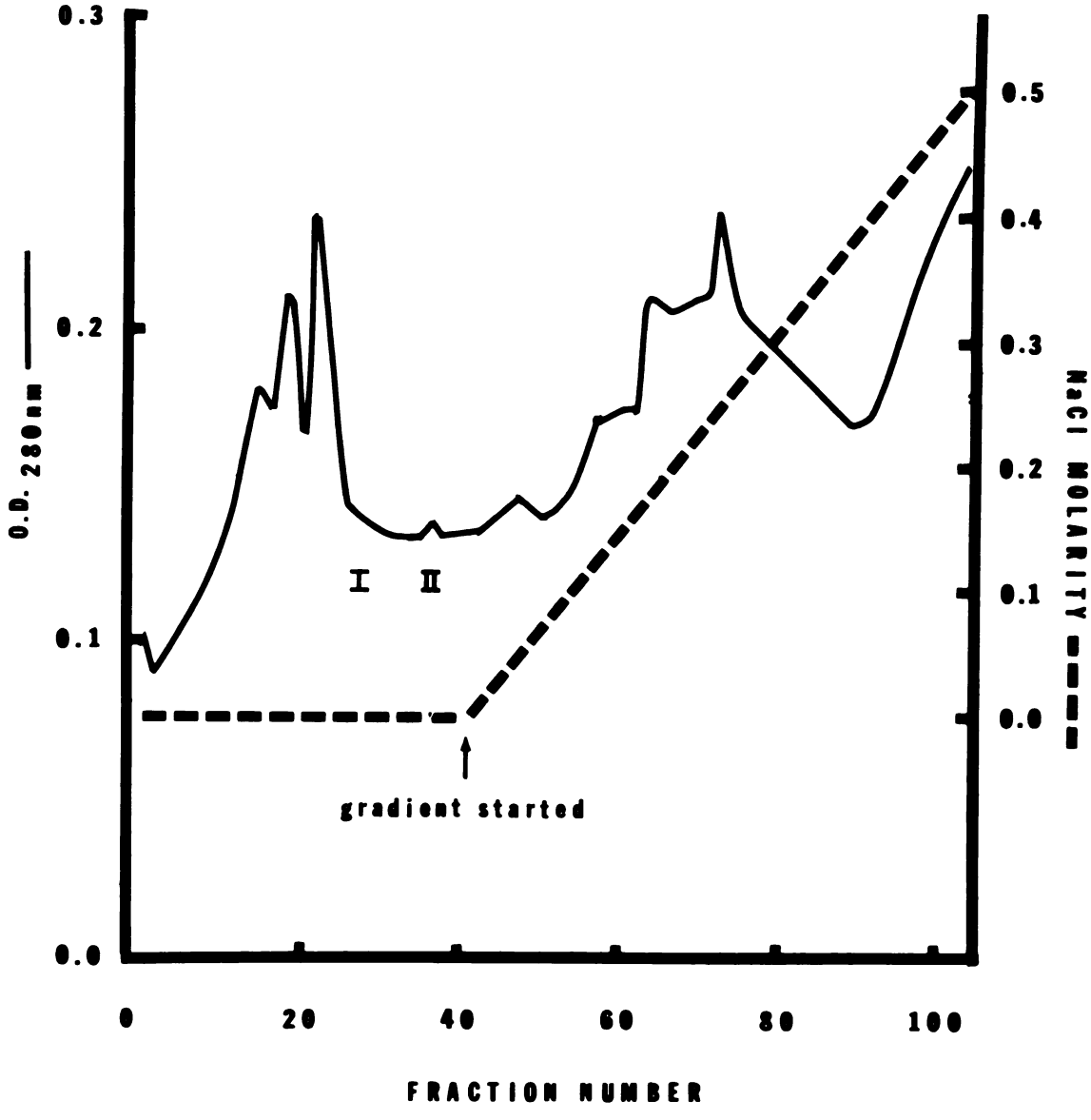
0.3
0.2
0.1
0.0
O.D. 280nm
0.5
0.4
0.3
0.2
0.1
0.0
NaCl MOLARITY
I
II
gradient started
0
20
40
60
80
100
FRACTION NUMBER

Figure 2.

FIG. 3. Relative absorbance patterns of stained acrylamide gels of unfractionated high speed supernatant fluid (HSS) and DEAE-cellulose fractions I and II separated electrophoretically.

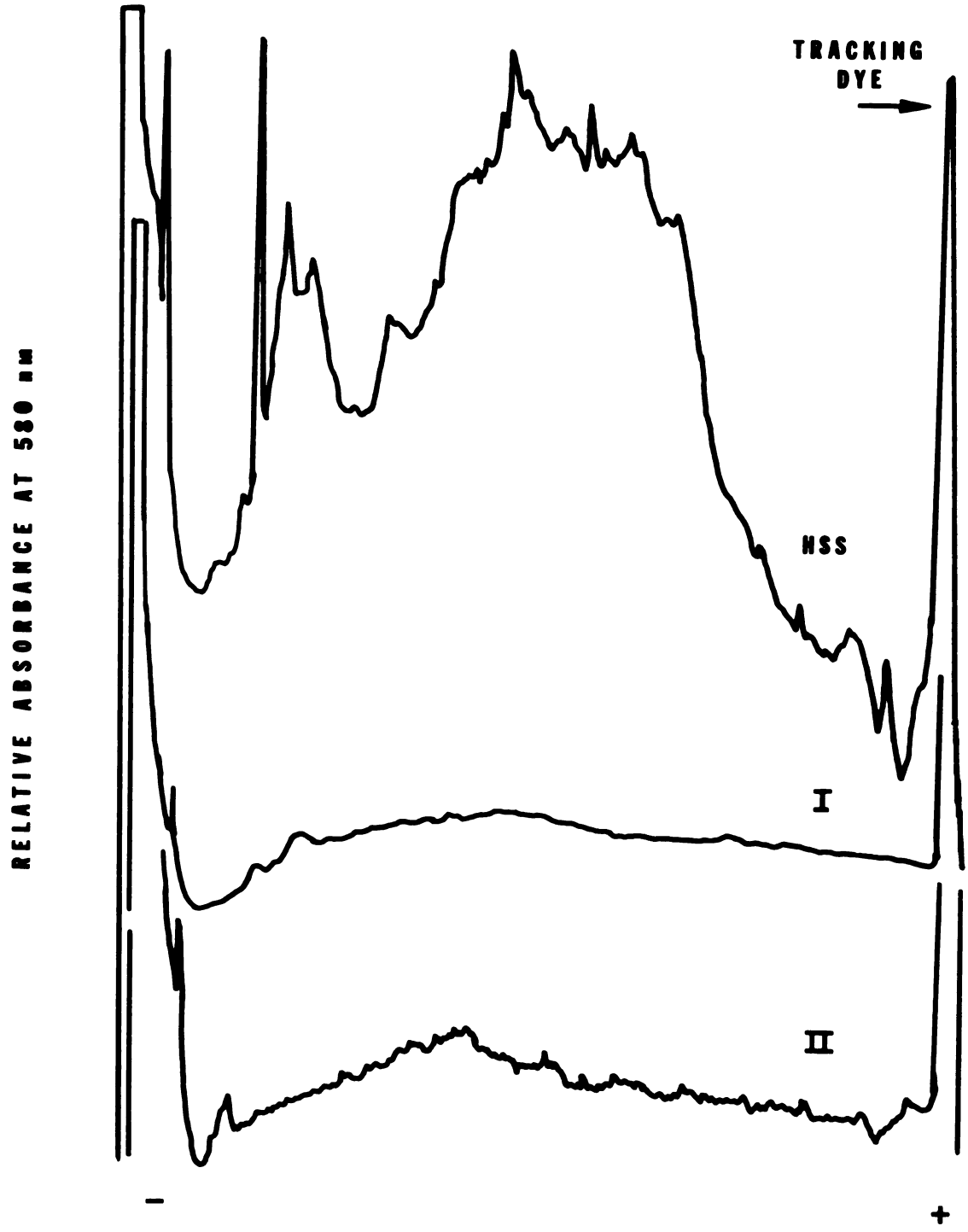

Figure 3.

FIG. 4. Sephadex fractionation of high speed supernatant fluid.

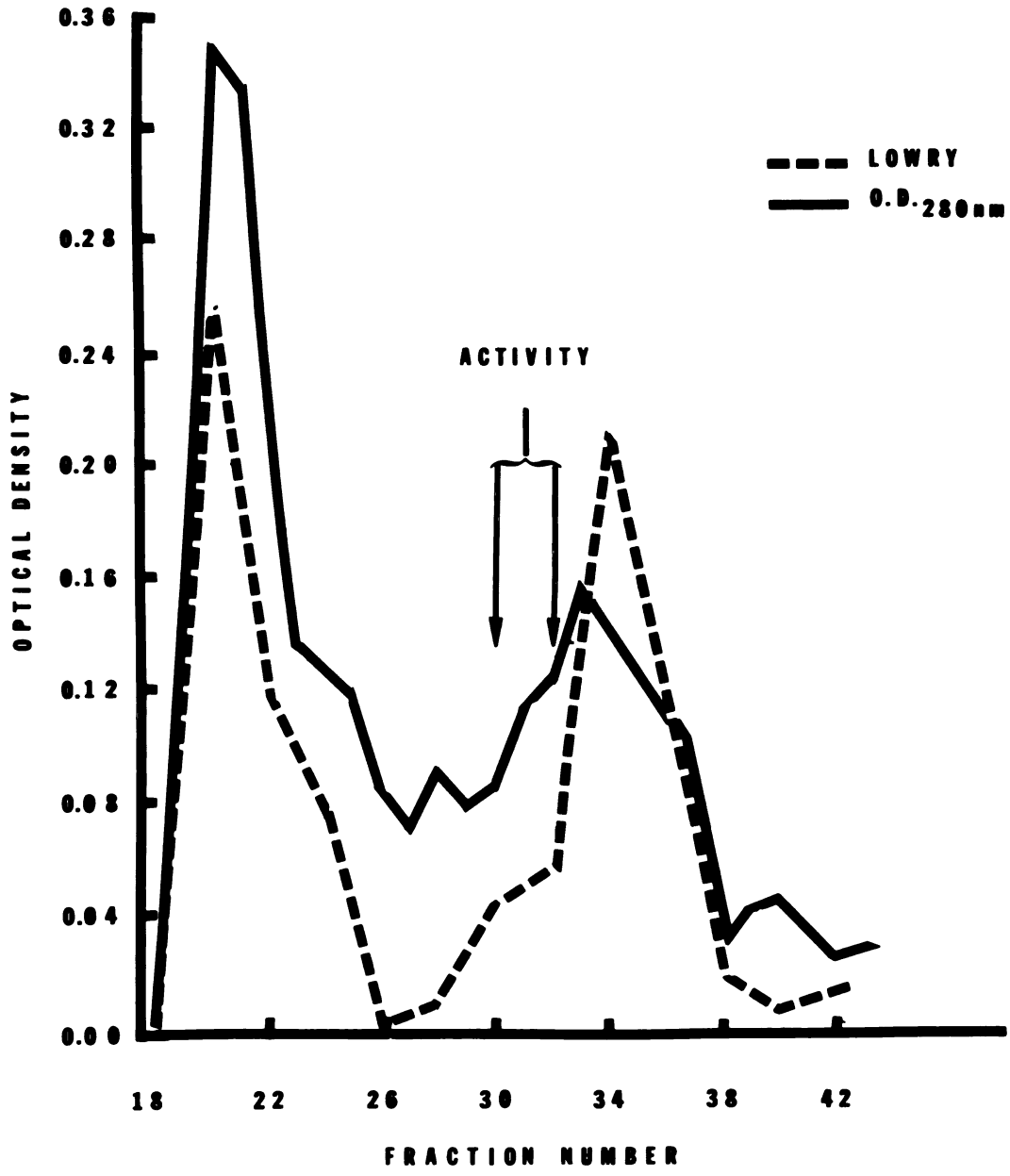
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
OPTICAL DENSITY
LOWRY
O.D.280nm
ACTIVITY
18
22
26
30
34
38
42
FRACTION NUMBER

Figure 4.

FIG. 5. Antilisterial activity of DEAE-cellulose fraction II at different pH.

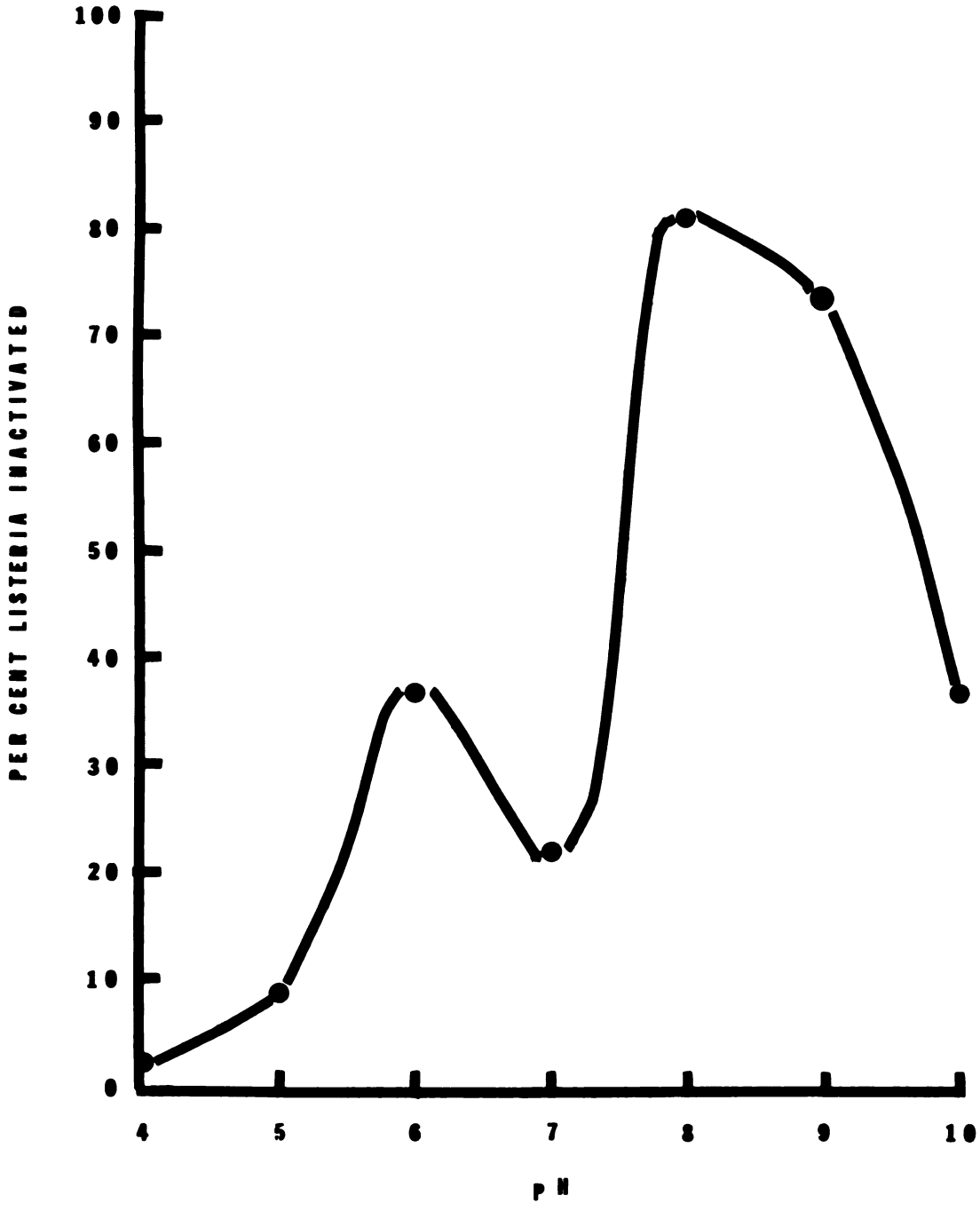
PER CENT LISTERIA INACTIVATED
100
90
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

Figure 5.

FIG. 6. Rate of listerial inactivation by unfractionate lysate, and fractions I and II from DEAE-cellulose chromatography. Inactivation of log-phase listeria by unfractionated lysate, □ ; inactivation of stationary-phase listeria by unfractionated lysate, ■ ; inactivation of stationary-phase listeria by DEAE-cellulose fraction II, ○ ; inactivation of stationary-phase listeria by DEAE-cellulose fraction I and II, ● .

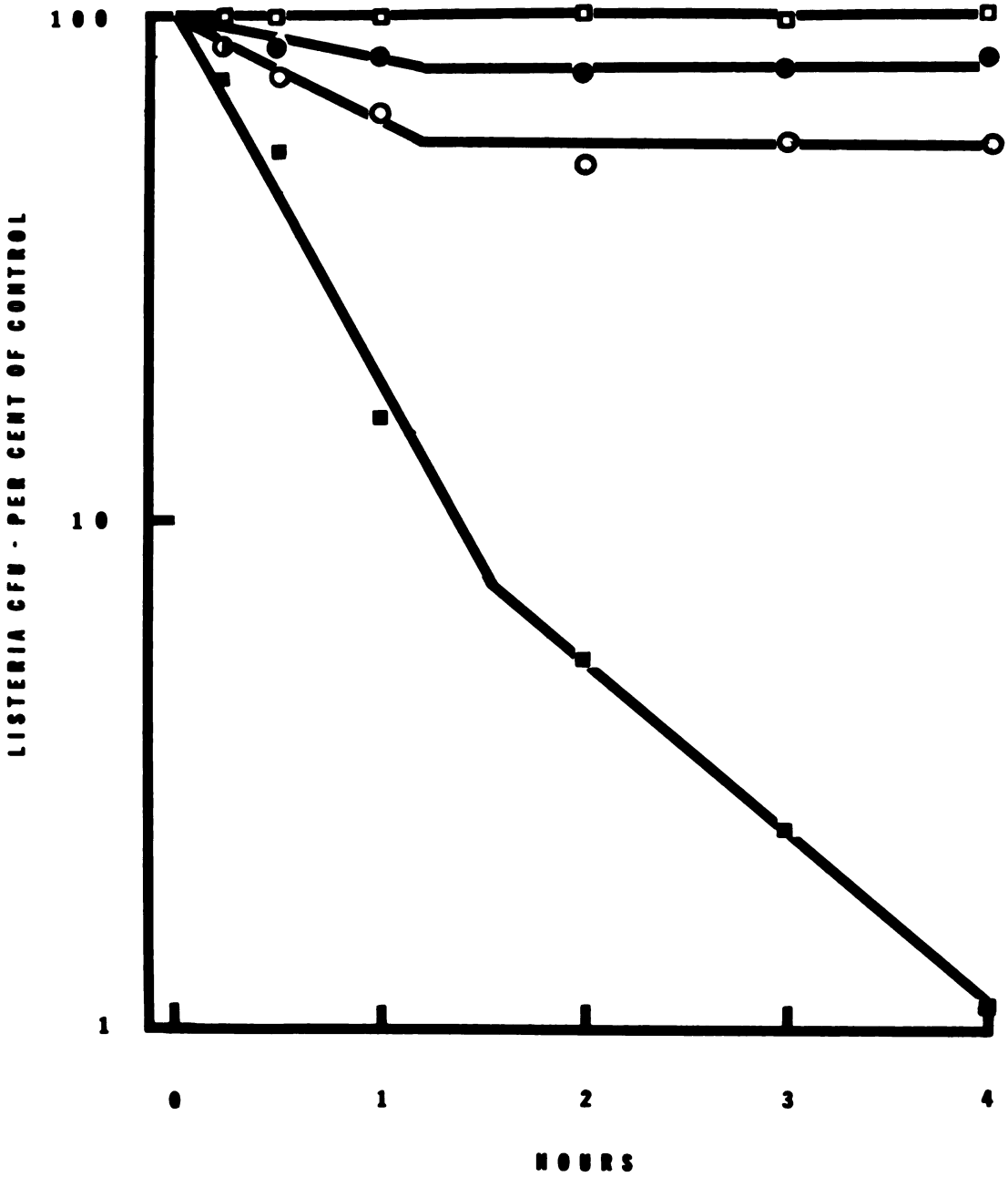

Figure 6.

TABLE 1. Stability of antilisterial factor in crude lysates.

Treatment	Percent activity remaining	
	with DTT[1]	without DTT[2]
24 hrs at 4 C		
in PCB, pH 7	100	75
24 hrs at -56 C		
in PCB, pH 7	100	89
Lyophilized		
in PCB, pH 7	40	38
Aerated, 15 min		
in PCB, pH 7	67	67
24 hrs at 4 C		
in PCB, pH 5[3]	94	0

[1]Lysate contained 10 mM DTT during treatment

[2]Lysate treated without DTT; 10 mM DTT added to lysate for assay

[3]Dialyzed against phosphate citrate buffer (PCB), pH 7 for assay

TABLE 2. Amount of antilisterial activity recovered in fractions from peritoneal cell lysates.

	280/260	Protein ug/ml	Total volume (ml)	Total protein (ug)	Specific[1] activity	Total activity	% recovery
Supernatant fluid[2]	0.618	1550	44.5	68,900	1.26×10^3	8.7×10^7	100
50-80% $(NH_4)_2SO_4$ ppt.	0.791	12,600	2.5	31,500	1.26×10^3	4.0×10^7	45
DEAE Fraction II	1.65	53	3.1	160	1.17×10^5	1.9×10^7	21

[1]Number of listeria inactivated per microgram protein

[2]After high speed centrifugation of lysate from 50 mice

TABLE 3. Antilisterial activity of crude lysates and DEAE-cellulose fraction II after heating.

Fraction	Control untreated	Percent activity remaining			
		37 C-30 min	45 C-30 min	56 C-30 min	100 C-10 min
HSS		100	99	95	92
DEAE-II		100	114	90	62

TABLE 4. Antibacterial activities of unfractionated lysate and DEAE-cellulose fraction II.

	Percent of bacteria inactivated			
	Pseudomonas aeruginosa	Staphylococcus aureus	Mycobacterium fortuitum	Listeria monocytogenes
Whole lysate	10%	25%	20%	95%
DEAE-cellulose fraction II	0%	22%	15%	45%

Standard deviation = 13%