STUDIES ON A POSSIBLE CELLULAR RESPONSE IN MICE IMMUNIZED WITH STAPHYLOCOCCUS AUREUS, SMITH STRAIN DIFFUSE

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By

Donna Y. Muirhead

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ABSTRACT

STUDIES ON A POSSIBLE CELLULAR RESPONSE IN MICE IMMUNIZED WITH STAPHYLOCOCCUS AUREUS, SMITH STRAIN DIFFUSE

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Intracellular inactivation of Staphylococcus aureus, Smith strain diffuse, by macrophages of unimmunized mice and mice immunized with dead, or live staphylococci, or BCG was impossible to determine without the addition of some antibiotic. Washing the parasitized macrophages did not reduce the extracellular population of staphylococci sufficiently to follow rates of intracellular killing; the extracellular population multiplied to such an extent, no differences could be detected. The addition of a small (3 ug/ml) amount of dihydrostreptomycin allowed differences to be determined. Macrophages from BCG immunized mice inactivated staphylococci to a measureable extent compared to macrophages from control, dead or live staphylococci immunized mice. No significant differences were observed among these latter groups.

At pH 5 bactericidal activity of macrophage lysates was detected for all test groups against both opsonized and unopsonized staphylococci. Inactivation of unopsonized staphylococci was observed

at higher lysate dilutions than opsonized staphylococci. Lysosomal acid hydrolases were implicated because of their acid pH optima. No bactericidal activity was seen for any of the four experimental groups at pH 5 or 7 in the presence of 5% homologous mouse serum. In the absence of mouse serum, staphylococcal inactivation at pH 7 was observed only with lysates from BCG immunized mice. A rough fractionation of lysate from this positive control group indicated a lysosomal origin for this activity. No tests were made to determine the cellular origin of this factor(s).

An increased percentage of mice immunized with live staphylococci survived an intraperitoneal challenge dose of 100 LD_{50} staphylococci. On the other hand, mice immunized with BCG or dead staphylococci were protected to some extent, but none of the control mice survived challenge.

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INTRODUCTION

Cellular immunity, an immunological state associated with an increased bactericidal activity of macrophages, is generally induced only with viable absolute or facultative intracellular parasites. The organisms associated with the induction of this phenomenon are phylogenetically diverse: viruses, bacteria and protozoa all appear as suitable stimuli (Ruskin et al., 1969). Although the response appears to be specifically elicited by intracellular parasites, the effect is nonspecific in nature, i.e., immunization with Mycobacterium tuberculosis induces a resistance against other infectious agents (Mackaness, 1964; Coppel and Youmans, 1969A, 1969B; Dubos and Schaedler, 1957; Youmans and Youmans, 1969).

Staphylococcus aureus is generally considered an extracellular parasite: however small numbers persist within macrophages and are inactivated only after an extended period of time (Kapral and Shayegani, 1959; Mackaness, 1964). A possible cellular involvement has been suggested for staphylococci on the basis of this slow degradation (Shayegani, 1968; Shayegani and Mudd, 1966). Staphylococcal infections are often associated with delayed-type hypersensitivity, an immunological state associated with cellular immunity (Mackaness, 1967; Taubler, 1968; Taubler and Mudd, 1968).

Most studies of cellular immunity are made by measuring

rates of bactericidal activity against homologous and heterologous organisms. In vivo testing most often involves counts of infectious units over time in specific body tissues such as spleen, liver and blood (Mackaness, 1964: Blanden et al., 1969). In vitro testing involves measuring the rate of intracellular killing within macrophages in monolayers (Ruskin et al., 1969: Miller and Twohy, 1969) or in suspension (Connel and Youmans, 1969A, 1969B). In vitro studies invariably incorporate the use of antibiotics to remove the extracellular population. Bonventre and Imhoff (1970) find dihydrostreptomycin has a bactericidal effect on intracellular mycobacteria.

On the basis of these observations we undertook a study to test for a possible cellular response in mice to injections of staphylococci. Our experiments were designed: (i) to determine the rates of intracellular inactivation by mouse peritoneal macrophages with and without the addition of dihydrostreptomycin, and (ii) to measure the bactericidal activities against staphylococci by macrophage lysates from mice immunized with staphylococci as compared with positive and negative control groups.

LITERATURE REVIEW

Cellular immunity

Immunological characteristics.--The ability to ingest and destroy invading microorganisms is a primary defense mechanism in animal systems. Occasionally, bacteria invade a host and are phagocytosed by macrophages but manage to resist intracellular destruction and even reproduce themselves intracellularly. The animal host eventually counters this challenge by producing "resistant macrophages" (Melly et al., 1960), highly efficient cells which are able to ingest and destroy the established organism. At this point, the animal is said to have developed a "cellular immunity" (Mackaness, 1964).

It has been shown (Mackaness, 1964; Coppel and Youmans, 1969A, 1969B; Dubos and Schaedler, 1957; Youmans and Youmans, 1969) that immunization with facultative intracellular parasites induces an immunity not only against the homologous immunizing organism, but also against heterologous facultative intracellular organisms and organisms normally considered to be extracellular, i.e., immunization with Mycobacterium tuberculosis produces an immunity which not only protects against mycobacterium but also against listeria, brucella and staphylococcus. This resistance

may extend to phylogenetically diverse organisms (Ruskin et al., 1969). Resistance to Toxoplasma gondii produces a cross protection against listeria, salmonella, mengo virus and besnoitia.

The phenomenon is not completely nonspecific, however, for it has been shown that the recall of the response is elicited only by the original immunizing organism. Although the resistance is nonspecific in its action, the state of cellular resistance is definitely related to the immune mechanisms because (i) the cells require a certain period of time to respond, (ii) immune animals slowly decrease their resistance but regain it quickly upon rechallenge with the immunizing organism, and (iii) resistance can be enhanced by additional injections with the same organism (Mackaness, 1967). An animal host which has developed a cellular immunity may eliminate both homologous and heterologous challenge organisms; however, the immunity appears much more efficient against the homologous infection (Ruskin et al., 1969).

Macrophages can be "activated" by many different types of irritants and toxins such as tuberculin, endotoxins, zymosan, colloids and hormones (Nelson, 1969). Injection of animals with these substances produces changes in morphological properties and activities of macrophages similar to those seen in immune cells. Responses due to nonspecific irritation and those of a purely immunological nature are difficult to separate although attempts were made by Coppel and Youmans (1969B, 1969C) and Youmans and Youmans (1969). These investigators observed that immunization with viable M. tuberculosis was at least several hundred times

more effective than killed cells in challenge against tuberculous infections. This superiority was not seen in mice challenged with Listeria monocytogenes or Klebsiella pneumoniae. They found that living and heat killed mycobacterial cells induced protection equally well against the heterologous organisms and suggested there may be at least two factors in mycobacterial cells important in immunization. They proposed that one might be a fairly stable cell wall component which acts similar to the manner of E. coli endotoxin and the other a thermolabile material which induced immunological protection only against M. tuberculosis. Mackaness (1967) suggested that the defense mechanisms against a homologous challenge were related closely with the development of delayed hypersensitivity, an immunologic phenomenon with a high degree of specificity.

Cellular immunity is associated with a form of resistance which cannot be transferred with immune serum, but only with cells of the reticuloendothelial system (Mackaness, 1964: Jenkin and Rowley, 1963). Transfer of an immunity to an intracellular parasite may be accomplished by lymphoid cells (mediators of delayed hypersensitivity) derived only from mice infected with that particular organism (Mackaness, 1968). Although the donor mice show resistance to heterologous bacterial challenge, mice which had received immune lymphoid cells manifested a resistance only against the homologous organism. Similar results were shown for facultative intracellular protozoans such as toxoplasma and besnoitia (Frenkel, 1967). Normal mice receiving lymphoid cells from toxoplasma immune animals were resistant only to toxoplasma and normal mice receiving lymphoid cells from besnoitia immune

animals were resistant only to besnoitia.

Most of the in vitro studies made on the intracellular degradation of staphylococci involved the use of antibiotic (usually dihydrostreptomycin and/or penicillin) in the test medium, a questionable practice in light of recent reports showing uptake of dihydrostreptomycin (Bonventre and Imhoff, 1970) and a bactericidal effect on intracellular mycobacterium (Patterson and Youmans, 1969). Patterson and Youmans observed no difference between normal and "immune" macrophages in their ability to inactivate intracellular mycobacterium in the absence of antibiotic, but saw only an increased survival rate of "immune" macrophages. They did observe increased bactericidal activity within "immune" macrophages in the presence of antibiotic and suggested this effect was caused by a greater permeability or uptake of dihydrostreptomycin by these cells as compared with normal cells. The rate of inactivation was proportional to the amount of dihydrostreptomycin added to the medium and they observed an effect with as little as 1.25 ug/ml. Bonventre and Imhoff (1970) observed that macrophages actually accumulated tritiated dihydrostreptomycin intracellularly to higher concentrations than were found in the extracellular milieu, although the rate of uptake was extremely slow in comparison to other substances which were transported across membranes. They reported concentrations only in terms of radioactive activity and did not relate this on a weight basis, therefore no direct comparisons could be made with other results. In in vitro tests using Mycobacterium tuberculosis sixteen times the concentration of antibiotic was required to inhibit the growth of intracellular bacilli than was needed to inhibit growth in culture medium (Mackaness, 1952). Despite this evidence which perhaps argued against a cellular mechanism, not all

work was done with *in vitro* systems using antibiotics. *In vivo* experiments prove this response was real and was not an artifact (Mackaness, 1964; Blanden et al., 1966, 1969; Dubos and Schaedler, 1957).

Metabolic characteristics.--Macrophages from immune animals with increased ability to inhibit the growth of tubercle bacilli ingested carbon, tubercle bacilli and staphylococci more readily than did macrophages from normal animals (Lurie, 1939) and had an accelerated mitotic activity (Khoo and Mackaness, 1964; Mackaness, 1962). Myrvik et al. (1962) and Evans and Myrvik (1967) noted enhanced metabolic activities by immune alveolar macrophages such as increases in oxygen uptake and a heightened response in the hexose monophosphate shunt. Increases in glycolytic activity of peritoneal macrophages as well as increases in acid hydrolases were associated with the cellular immune state (Saito and Suter, 1965). Cohn and Wiener (1963) found increased levels of acid phosphatase, lipase and lysozyme in BCG-induced alveolar macrophages as compared with control cells. Mouse peritoneal macrophages immune to *Corynebacterium ovis* had a total cell protein 1.85 times that of normal phagocytes (Hard, 1970). Substantially higher activities of acid phosphatase, β-glucuronidase, cathepsin D, lysozyme, N-benzoyl-DL-phenylalanine-1-naptholesterase, β-napthylacetate esterase and aryl sulfatase were observed in corynebacterium-immune macrophages as compared to control cells. Dannenberg and Bennett (1963), however, found no increase in levels of proteinase, esterase or lipase of immune peritoneal mononuclear cells from mycobacterium infected animals.

In spite of the mass of data on this subject the exact nature and the mechanistic details of this phenomenon remain to be clarified. Similarly, its relationship to other immunological responses such as humoral antibody production and delayed hypersensitivity is unclear.

Staphylococcal pathology and immunology

Survival within host tissues.--Staphylococcus aureus is generally considered an extracellular parasite although Melly et al. (1960) and Hunt and Moses (1958) reported not only intracellular survival, but actual multiplication within human and mouse leucocytes. Kapral and Shayegani (1959) noted virulent staphylococci survived but did not multiply in leucocytes of normal rabbits and humans. Mackaness (1964) found staphylococci more rapidly inactivated within granulocytes than within macrophages, a view in accord with Metchnikoff's morphological studies (Hirsch, 1959). Mackaness reported less than 0.1% staphylococci surviving within granulocytes after an incubation period of three hours. This survival rate contrasted with 3 to 10% in the case of macrophages. Long term studies of this small surviving population of staphylococci have not been made, primarily because of the difficulties involved in eliminating the extracellular microbial population.

The virulence of pathogenic staphylococci has been attributed to many factors. Breakdown in natural defenses and genetic defects within the host as well as environmental stress have been implicated in infectious events (Florman, 1968; Morse, 1968; Zeya and Spitznagel, 1968). Tauraso and White (1963) cited a variety of toxins produced by virulent staphylococci such as alpha hemolysin, dermonecrotoxin and leukocidin. The presence of antiphagocytic agents was also found to be contributory to the disease state. Blair (1965) observed staphylococcal leucocidin had a unique cyto-

toxic effect on white blood cells. Virulent staphylococci were generally associated with coagulase and other plasma coagulating factors that were secreted or retained at the cell surface to produce aggregates of infecting cells that were phagocytosed with difficulty (Smith, 1963).

The encapsulated Smith strain of Staphylococcus aureus was isolated from a patient with osteomyelitis in 1930 and has shown a consistent pathogenicity for mice. The diffuse and compact variants were distinguished by their colonial morphology in serum-soft agar (Yoshida and Ekstedt, 1968). The extreme virulence of the Smith diffuse variant has been attributed to some component of its antiphagocytic capsule. The Smith compact variant was avirulent and was rapidly ingested by peritoneal phagocytes. Both variants elaborated free coagulase and alpha hemolysin (Koenig, 1962; Koenig et al., 1962 A, 1962 B; Parker et al., 1965; Hunt and Moses, 1958). Rogers (1962) suggested these strains might be indicators of characteristics acquired by other pathogenic staphylococci in in vivo multiplication which were lost in vitro. Three lines of evidence were proposed which might support such a thesis: (i) virtually all adult human sera had detectable opsonizing antibody against the Smith diffuse variant suggesting the antigen to be fairly common; (ii) there was evidence of antigenic relationships between the Smith diffuse strain and pathogenic staphylococci from human infections as noted when immunization with these strains induced production of a heat-stable antibody which could opsonize the Smith strain; and (iii) there were bacterial precedents to support in vivo changes in bacterial characteristics. Certain strains of Pasturella pestis encapsulate and acquire resistance to phagocytosis in vivo.

No one factor has been consistently associated with virulence. Morse (1968) suggested all are important and the more virulence factors a strain possesses the greater are its chances for establishing infection.

Immunology.--Increased resistance to challenging doses of S. aureus measured by survival tests was observed after immunization with specific antigens and both live and dead vaccines (Ekstedt, 1965). Hyperimmune rabbit antisera against heat-killed vaccines of the Smith diffuse strain of S. aureus protected mice against challenge with the homologous organisms (Yoshida and Ekstedt, 1968). Absorption of this sera with purified teichoic acid removed the protective antibody. Immunization with specific antigens such as coagulase, alpha hemolysin, cell wall teichoic acids and protein A protected animals to some extent, but, in general, vaccination against staphylococci has met with little success (Rogers and Melly, 1965; Koenig and Melly, 1965; Lominski et al., 1962; Harrison, 1964; Spencer et al., 1964; Ekstedt and Yoshida, 1969).

Johnson et al. (1960) suggested staphylococci closely resembled the tubercle bacillus in its ability to maintain a long term residence within host tissues and survive within phagocytic cells. Rogers and Melly (1965) admitted intracellular residence might be an important attribute of virulent strains, but maintained extracellular multiplication was also of prime significance.

Goshi et al. (1961) noted repeated intradermal infection of rabbits could result in an increase in susceptibility to infection rather than an increase in resistance. This was associated with the development of delayed hypersensitivity to the staphylococci.

Malata et al. (1969) found 16% of healthy persons showed immediate and 18% showed delayed hypersensitivity to an antigenic complex of ten different staphylococcal strains. Eighty-eight percent of patients with furunculosis produced a positive immediate reaction to this complex, while 66% showed positive reactions of the delayed type. They proposed that an effective therapy would probably not be based on antibody production. Greenberg (1968) and Spencer et al. (1964) observed persons with persistent and recurring infections had relatively high titers of both alpha antitoxin and antileukocidin and reasoned there was little value in an attempt to increase these titers through immunization. Taubler (1968) and Taubler and Mudd (1968) have demonstrated delayed-type hypersensitibity to a nonencapsulated strain of Staphylococcus aureus by in vivo (footpad) and in vitro (migration inhibition) tests.

Although delayed-type hypersensitivity and cellular immunity develop almost simultaneously with mycobacterium infected animals, different components of the tubercle bacillus might cause the different responses. The protein (tuberculin) might be responsible for the delayed hypersensitivity and the lipids or polysaccharides or both may be responsible for the immunity (Dannenberg, 1968). Mackaness (1967) conceded that it is possible to have delayed hypersensitivity without cellular immunity and vice versa. A cellular immunity without delayed hypersensitivity may be achieved by desensitization with antigen. However, he does suggest a strong correlation between the two responses.

The surface antigen of the encapsulated Smith diffuse strain of S. aureus has been isolated, purified and characterized by Morse (1962). The antigen contained 70% carbohydrate. Rabbits immunized

with heat-killed cells of the Smith strain exhibited cutaneous hypersensitivity when small quantities of Smith surface antigen (SSA) were inoculated intradermally. Intracutaneous inoculation of 100 ug of SSA into normal rabbits produced no local reaction, whereas as little as 1 ug injected into immunized animals produced an erythema and swelling 1 to 2 centimeters in diameter. These reactions were maximal at 8 to 12 hours (immediate-type hypersensitivity) and gradually receded over the next 48 hours.

Shayegani (1968) and Shayegani and Mudd (1966) suggested a possible cellular involvement for staphylococci by virtue of the slow degradation process. They proposed that "these facultatively intracellular parasites" might outlive the monocytes in which they reside and by multiplying extracellularly produce constant, recurring infections.

MATERIALS AND METHODS

Immunization

Animals. Female Swiss Albino mice (Spartan Research Animals, Haslett, Michigan) weighing 24 to 26 grams were used in all experiments. They were housed 4 to 5 per cage. Food (Purina mouse chow, Ralston Purina Co., St. Louis, Mo.) and water were given ad libitum.

Bacterial strains. Staphylococcus aureus, Smith diffuse strain was donated by Dr. Joseph J. Kowalski, Dept of Microbiology and Public Health, Michigan State University. Mycobacterium tuberculosis, Bacille Calmette-Guerin was obtained from Dr. Virginia Mallman, Dept. of Microbiology and Public Health, Michigan State University.

Bacterial media. Modified Staphylococcal 110 medium was prepared by the method of Yoshida and Ekstedt (1968). Dubos broth (Difco Laboratories, Inc., Detroit, Mich.) supplemented with 0.5% dextrose was used to grow mycobacterium.

Test media for bactericidal assays. Measurements of intracellular populations of staphylococci in the presence of mouse peritoneal macrophages were made using 90% Hank's Basal Salt Solution (HBSS)(Microbiological Associates, Inc., Bethesda, Md.), 10% mouse serum and 0.0056 M glucose.

Measurements of staphylococcal growth in different concentrations of dihydrostreptomycin with and without the presence of mouse

peritoneal macrophages were made using 90% HBSS, 10% Fetal Bovine Serum (FBS)(Microbiological Associates, Inc., Bethesda, Md.) and 0.0056 M glucose to conserve mouse serum.

Vaccines and immunization schedules. Four groups of test mice were used: (i) control mice, (ii) mice immunized with dead staphylococci, (iii) mice immunized with live staphylococci, and (iv) mice immunized with live mycobacteria.

Control mice were not immunized. A killed staphylococcal vaccine was prepared using an 18 hour culture of S. aureus incubated at 37 C. The cells were centrifuged and resuspended in culture supernatant to 10^8 CFU/ml as determined by optical density readings at 620 nm. Ten milliliter aliquots of the suspension were transferred to sterile vaccine bottles and benzalkonium chloride was added to a concentration of 1:1600 (v/v). The suspension was shaken vigorously and placed in a 37 C water bath for 30 minutes. The vaccine was subsequently tested for sterility by inoculating into tubes containing Brain Heart Infusion Broth (Difco Laboratories, Detroit, Mich.). These tubes were then incubated at 37 C for 3 days and observed for growth. Mice were immunized on a biweekly basis for 1 month by injecting 0.1 ml of this vaccine (10^7 killed organisms) intraperitoneally. Mice immunized with live staphylococci were treated in a manner similar to the dead staphylococci-treated mice. On the day of immunization an 18 hour culture was centrifuged and resuspended in sterile 0.85% saline to 10^8 CFU/ml as calculated from previous measurements of viable staphylococci versus optical density readings (Bausch and Lomb Spectronic 20) at 620 nm. In addition to biweekly intraperitoneal injections of 10^7 CFU, an open, pustular lesion was induced 2 weeks prior to testing by injecting 0.1 ml of the live vaccine subcutaneously on the abdomen. (These healed in 3 to 8 days depending on the severity

of the lesion.) Mice immunized with mycobacteria were injected once intraperitoneally 4 weeks prior to testing with 0.2 ml of a saline suspension of a 10 day culture incubated at 37 C. The cells were suspended to an optical density reading of 0.15 at 620 nm which represented 10^8 cells/ml. The mice were therefroe injected with approximately 2 x 10^7 viable cells.

Bleeding procedure. Two days following a challenge immunization, blood samples were obtained via the retro-orbital plexus. Approximately 0.5 to 0.75 ml of whole blood was taken per mouse and about 4 ml of serum was obtained per 20 mice. Blood was taken from the mice not more often than once a month. Pooled serum from each group was frozen at -20 C until used.

Opsonization of staphylococci. A pellet of cells obtained by centrifuging 1 ml of an 18 hour culture (approximately 10^9 CFU) was suspended in 0.3 ml of the appropriate mouse serum. The cell-serum mixture was incubated at room temperature for 20 minutes. The staphylococci were then centrifuged and the serum removed. The cell pellet was resuspended and diluted in saline to a calculated 2 x 10^3 CFU/ml and used immediately in the bactericidal assay with macrophage lysates.

Prestimulation and collection of peritoneal macrophage exudates. All work was performed with macrophages of peritoneal origin. Two days prior to testing, mice were injected intraperitoneally with an immunizing dose of the appropriate vaccine and 1 ml of sterile 0.1% glycogen (Nutritional Biochemicals Corporation, Cleveland, Ohio). Control mice received glycogen only. On the day of testing, 2.5 ml of sterile HBSS containing 10 units/ml sodium heparin (Wolins Pharmaceutical, Inc., Farmingdale, N. Y.) was injected into the peritoneal cavity. The abdomen was then massaged by gentle kneading for a few

minutes. The peritoneal exudate was collected with a hypodermic syringe equipped with a special 22-guage stainless steel needle perforated at the needle tip with 3 to 4 small holes.

Determination of LD_{50} for Staphylococcus aureus. An overnight culture of staphylococci was centrifuged and resuspended in sterile 0.85% saline. Five-fold dilutions of the cell suspension were made in saline and 1.0 ml volumes were injected intraperitoneally. Mortality rates were based on the number of mice dead by 20 hours.

Bactericidal assays

Macrophage lysis by delta-hemolysin. Delta-hemolysin (supplied by Dr. Frank A. Kapral, Ohio State University) is an exotoxin produced by staphylococcus which lyses erythrocytes and leucocytes. A concentration of 25 to 50 HD_{50} units/ml was suggested for lysing mouse peritoneal macrophages (Dr. Frank Kapral, personal communication). Preliminary testing indicated that 95% or better of 10^6 large mononuclear cells/ml were lysed after 15 minutes incubation at ambient temperature with 50 HD_{50} units. This concentration was used in all experiments requiring lysis of mouse peritoneal cells.

Rate of intracellular killing. Mice were prestimulated 2 days prior to testing with an immunizing dose of antigen and 1 ml of sterile 0.1% glycogen as described above. On the day of the test macrophages were collected, washed in HBSS and resuspended in media consisting of 90% HBSS, 10% mouse serum and 0.0056 M glucose at pH 6.8. The cells were counted with a haemocytometer (American Standard Haemocytometer, Arthur H. Thomas Co., Philadelphia, Pa.) and suspended in test medium to 10^6 cells/ml. An 18 hour culture of S. aureus was centrifuged and resuspended in saline to a calculated concentration of 10^9 CFU/ml.

One-tenth milliliter of the staphylococcal suspension was added to 10 ml of the macrophage suspension for a final staphylococcus:macrophage ratio of 10:1. The cell suspensions were incubated at 37 C on a gyrotatory water bath (New Brunswick Scientific Co., In., New Brunswick, N. J.). One milliliter samples were taken at appropriate times over a 4 hour period and transferred to conical centrifuge tubes. The suspensions were centrifuged at 500 x g for 5 minutes (International Centrifuge, Model SBV, International Equipment Co., Boston, Mass.). The supernatant fluid was carefully removed with a sterile Pasteur pipette and transferred for serial dilution and plating on nutrient agar to provide the extracellular count. Additional 1 ml samples of the 10:1 suspension were removed and mixed with 0.1 ml of 500 HD_{50} units/ml of delta-hemolysin. After incubating for 15 minutes at room temperature the macrophage lysate was briskly pipetted up and down for one minute before dilution and plating on nutrient agar. This latter procedure provided the total staphylococcal population, i.e., both intracellular and extracellular counts.

In subsequent experiments to determine the rate of intracellular inactivation, macrophages were collected, washed with HBSS and 2×10^7 cells were resuspended in 1.0 ml of test medium consisting of 90% HBSS, 10% mouse serum and 0.0056 M glucose. An 18 hour culture of S. aureus was centrifuged and resuspended in saline to 2×10^9 CFU/ml. One-tenth milliliter of the staphylococcal suspension was added to the macrophage suspension to obtain a staphylococcus:macrophage ratio of 10:1. The suspension was incubated at 37 C for 15 minutes on a gyrotatory water bath, after which the cell suspension was centrifuged and washed twice with 30 ml saline. All centrifugation was done at low gravity forces

(500 x g for 5 minutes) to sediment the macrophages but leave the staphylococci in the supernatant fluid. The macrophage pellet was then resuspended in 1.05 ml of culture medium. One-half milliliter was transferred to an additional 9.5 ml of culture medium containing 3 ug/ml of dihydrostreptomycin (Pfizer Laboratories, New York, N. Y.) and 0.5 ml transferred to 9.5 ml of culture medium without dihydrostreptomycin. One milliliter of test suspension was removed from each system at appropriate time intervals over a 4 hour period. The macrophages were centrifuged, resuspended in 1 ml of 50 HD_{50} units/ml of delta hemolysin and incubated at ambient temperature for 15 minutes. The macrophage lysate was then serially diluted and plated on nutrient agar to obtain the concentration of intracellular staphylococci.

Effect of washing macrophages on extracellular growth of staphylococci. Peritoneal macrophages were collected, washed, counted and resuspended in 1.0 ml of test medium consisting of 90% HBSS, 10% mouse serum and 0.0056 M glucose for a final concentration of 10^7 large mononuclear cells/ml. One-tenth milliliter of an 18 hour culture of S. aureus, centrifuged and resuspended in saline to 10^9 CFU/ml, was added to the macrophage suspension for a staphylococci: large mononuclear cell ratio of 10:1. The cell suspension was incubated for 15 minutes at 37 C before centrifugation and washed twice with 30 ml of saline. After the last wash the macrophages were resuspended in a small volume of test medium and transferred to a sterile flask. Additional test medium was added for a final concentration of 10^6 large mononuclear cells/ml. One milliliter was immediately removed for a 0-time reading, transferred to a conical centrifuge tube and centrifuged. The supernatant fluid was carefully removed from the cell pellet with a sterile Pasteur pipette and the cell

pellet was resuspended in 1 ml of 50 HD_{50} units/ml of delta hemolysin. After incubation in delta hemolysin for 15 minutes at room temperature the cell lysate was briskly pipetted for a minute before serial dilution and plating.

Staphylococcal growth curves in the presence of dihydrostreptomycin. An 18 hour culture of staphylococci was centrifuged and resuspended in saline to a concentration of 10^8 CFU/ml. One-tenth milliliter of the bacterial suspension was added to flasks containing 10 ml of culture medium of 90% HBSS, 10% FBS and 0.0056 M glucose. Dihydrostreptomycin was added to produce a series of graduated concentrations and the flasks were incubated at 37 C in a gyrorotatory water bath. Samples were taken at appropriate times and serially diluted and plated on nutrient agar.

In tests involving the addition of macrophages, peritoneal exudate was collected as described, the cells were washed twice with sterile HBSS, resuspended in culture medium of 90% HBSS, 10% FBS and 0.0056 M glucose and counted. The test suspensions were prepared as above with the addition of enough large mononuclear cells for a final concentration of 10^6 cells/ml. The final staphylococci:macrophage ratio was 1:1. Flasks were incubated on a gyrorotatory water bath at 37 C. One milliliter samples were removed from each test flask at appropriate times over an 8 hour period and transferred to conical centrifuge tubes. The contents were centrifuged and 0.1 ml of the supernatant fluid was removed for serial dilution and plating on nutrient agar to determine the extracellular staphylococcal population.

Assay for bactericidal activity of macrophage lysates. This assay was a modification of that used by Hirsch (1958). Two days prior to testing, the mice were injected with an immunizing dose of

antigen and 1.0 ml of sterile 0.1% glycogen. On the day of the test, macrophages were collected, washed twice with HBSS, counted and resuspended in 4 ml of sterile 0.25 M sucrose. Delta-hemolysin was added to a concentration of 50 HD_{50} units/ml. The suspension was incubated in an ice bath for 1 hour with a magnetic stirrer. After incubation a small aliquot was removed and tested for completeness of lysis with an aqueous stain of 2.1% crystal violet and 0.1% citric acid.

Solutions of lysate were diluted in 0.07 M citrate-phosphate buffer with 0.01% Bovine Serum Albumin (BSA)(Sigma Chemical Co., St. Louis, Mo.) to represent a lysate from 10^6 large mononuclear cells per milliliter and serial 3-fold dilutions were then made from this solution with final test volumes of 1.0 ml. All lysate solutions were kept on ice until the time of incubation.

An 18 hour culture of Staphylococcus aureus was centrifuged and resuspended in saline to a calculated 2 x 10^3 CFU/ml. One-tenth milliliter of the staphylococcal suspension was carefully transferred to the lysate dilutions. The test suspensions were then incubated for 2 hours at 37 C on a gyrorotatory water bath. At the end of the incubation period, 20 ml of warm (48 C), melted nutrient agar was added to each test tube and the contents were mixed in a vortex blender. The agar suspension was poured into petri dishes and, after solidifying, incubated for 24 hours at 37 C before counting.

Fractionation of macrophage lysate for localization of bactericidal activity. Two days prior to testing mice were injected with an immunizing dose of antigen and 1 ml of sterile 0.1% glycogen. On the day of the test, peritoneal cells were collected, washed twice with HBSS, once with cold 0.25 M sucrose and resuspended in 5 ml of 0.25 M sucrose (Cohn and Wiener, 1963). Delta-hemolysin was added to a

concentration of 50 HD_{50} units/ml and the suspension was incubated in an ice bath with stirring for 1 hour. The cell suspension was checked for lysis as above. One milliliter of the lysate was removed for testing as the "total homogenate" fraction. The remaining lysate was subjected to an initial low speed centrifugation (500 x g for 12 minutes) at 0 C to obtain the "nuclear pellet". The "nuclear pellet" was resuspended in 4 ml of citrate-phosphate buffer and 0.1% BSA at pH 7.0 and the supernatant fluid was centrifuged at 15,000 x g for 12 minutes at 0 C to obtain the "lysosomal pellet". The supernatant fluid was removed and labeled "high speed supernatant" and the "lysosomal pellet" was resuspended in 4 ml of 0.07 M citrate-phosphate buffer and 0.1% BSA. The suspensions were allowed to sit overnight at 0 C after which the fractions were serially diluted in buffer and the bactericidal assay repeated.

RESULTS

Extracellular growth of staphylococci

Determination of LD_{50} of Staphylococcus aureus in mice via peritoneal route. The immunizing doses were estimated from an initial LD_{50} test. The dose required to kill 50% of the test group was 1.3×10^7 CFU/ml under the given test conditions (Table 1).

Staphylococcal growth in suspension with peritoneal macrophages. Staphylococci and macrophages were suspended in a test medium of 90% HBSS, 10% FBS and 0.0056 M glucose in a ratio of 10:1. No attempt was made to keep the extracellular staphylococcal population from multiplying. The increases in total and extracellular staphylococcal colony forming units with time are shown in Figures 1 and 2. No significant differences were seen and it was obvious that the very rapid rate of extracellular growth completely masked any intracellular killing which might be taking place. An attempt was then made to control the extracellular population of staphylococci by washing and/or the addition of antibiotic.

Extracellular growth of staphylococci after repeated washing of parasitized peritoneal macrophages. In additional experiments repeated washings of macrophages were made in an attempt to measure the rate of intracellular killing without the addition of antibiotic. The results of the experiment are shown in Figure 3. In spite of the washings, the extracellular staphylococcal population was not reduced sufficiently to prevent interference with the measurement of

Table 1. The LD_{50} for Staphylococcus aureus with female Swiss albino mice via the peritoneal route.

Calculated[a] staphylococcal population	Actual[b] staphylococcal population	Mortality[c] rate
CFU/ml	CFU/ml	
5.8×10^9	6.0×10^9	6/6
1.2×10^9	1.0×10^9	6/6
2.5×10^8	1.0×10^8	6/6
5.0×10^7	1.3×10^7	3/6
1.0×10^7	2.5×10^6	2/6

[a]Populations of staphylococci were estimated on the basis of optical density versus number of colony forming units per milliliter measurements at 620 nm.

[b]Actual populations were determined by making serial dilutions of the suspensions of staphylococci and plating out on nutrient agar. Plates were read at 18 hours after incubation at 37 C.

[c]Mortality rates were based on number of animals found dead at 20 hours compared to the total number tested.

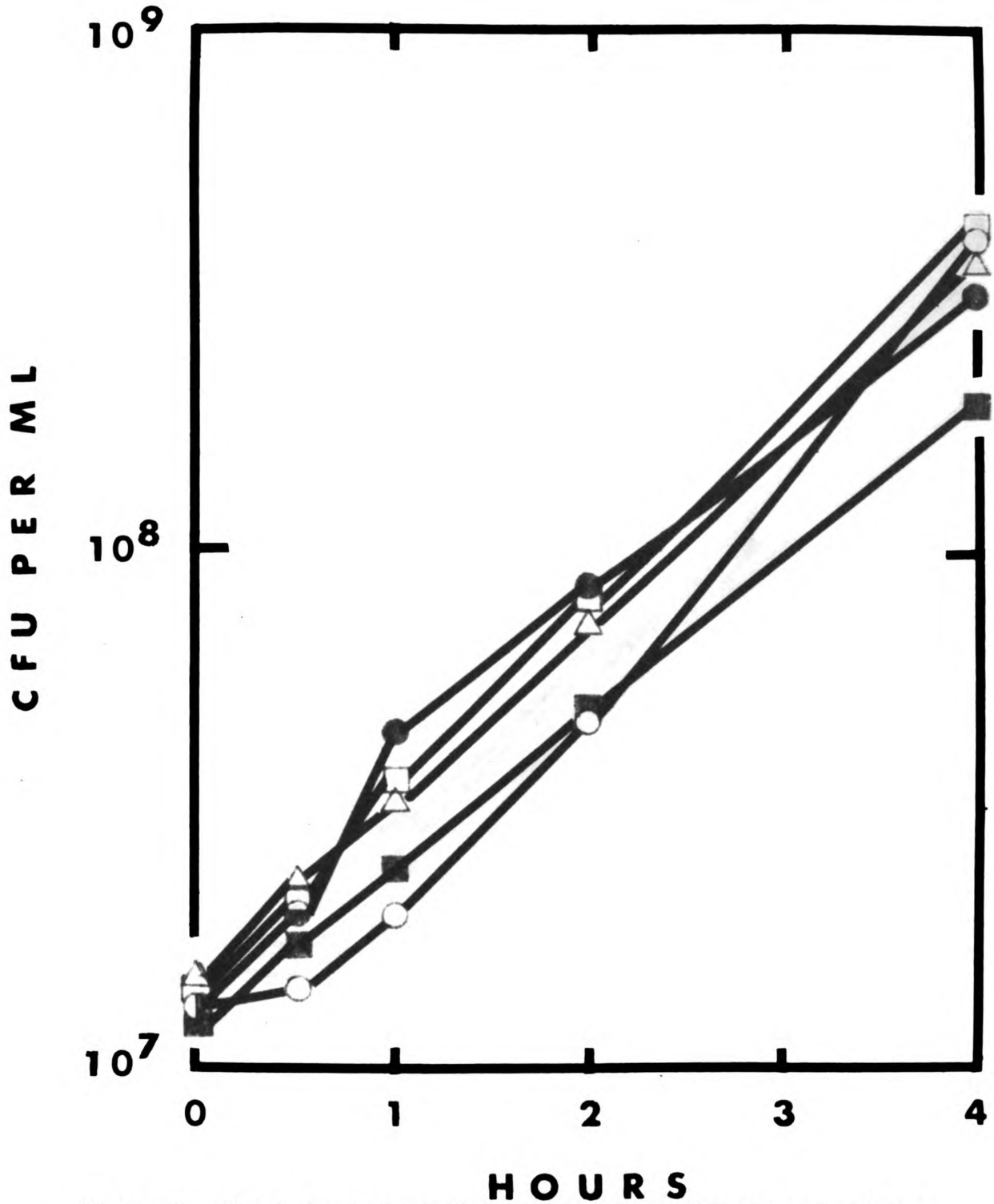


Figure 1. Total staphylococci, both extracellular and macrophage-associated, vs. time in the presence of 10^6 peritoneal large mononuclear cells/ml and without streptomycin. Control mice (○); mice immunized with live staphylococcus (▲); mice immunized with dead staphylococcus (●); mice immunized with BCG (□); staphylococcus growth control (no macrophages) (■).

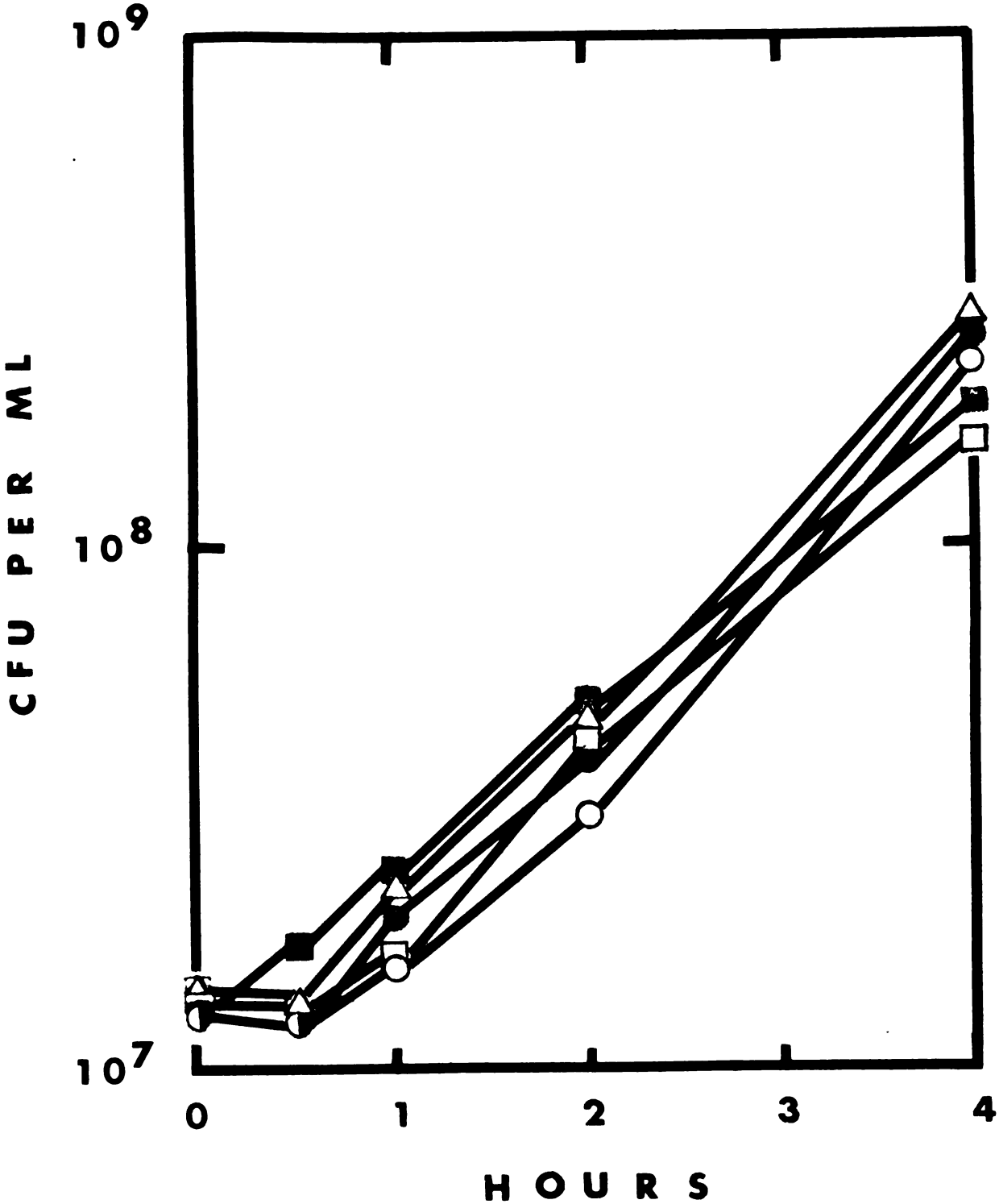
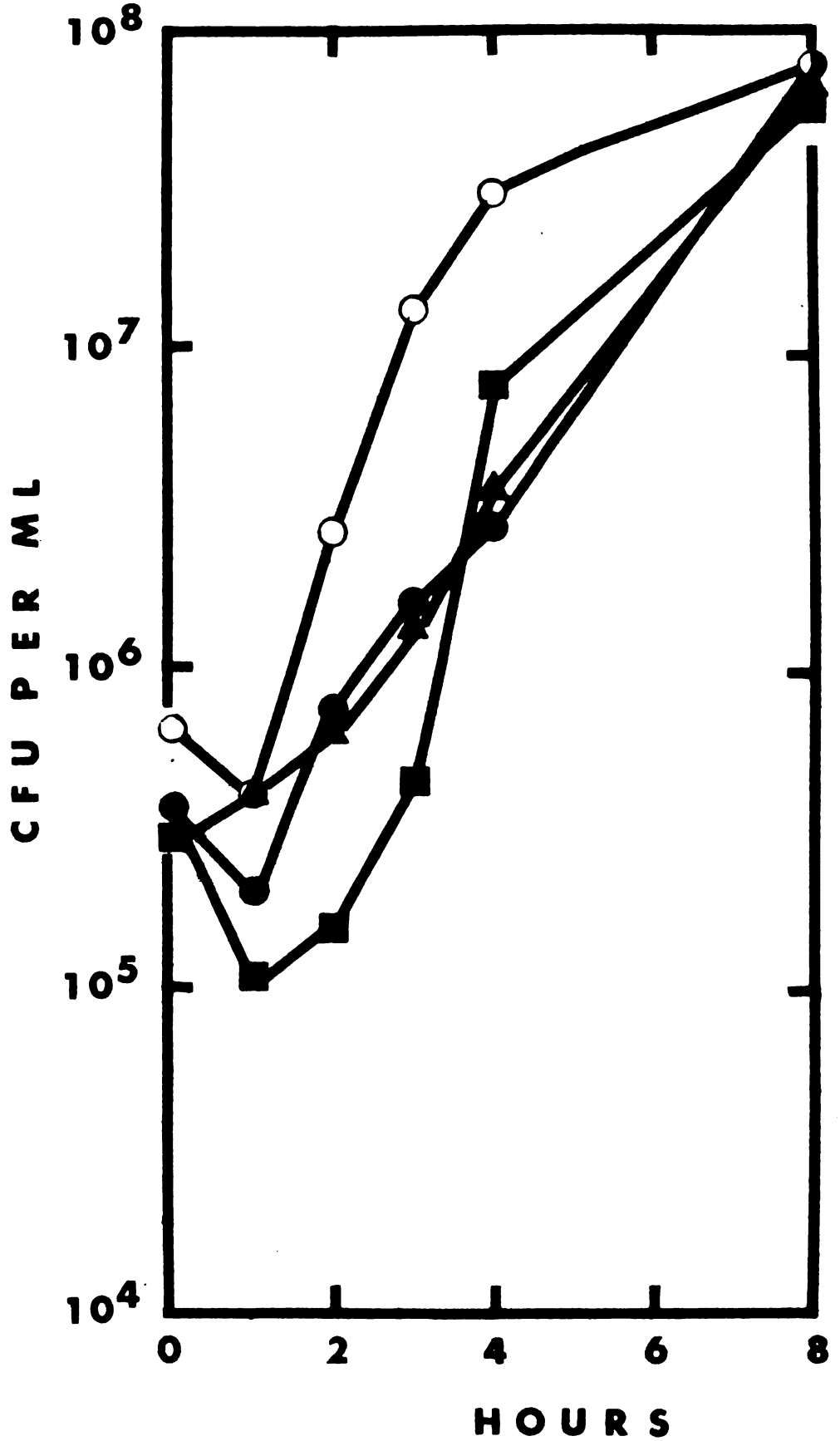


Figure 2. Extracellular population of staphylococci vs. time in the presence of 10^6 large mononuclear cells/ml and without streptomycin. Control mice (○); mice immunized with live staphylococci (△); mice immunized with dead staphylococci (●); mice immunized with BCG (□); staphylococcus growth control (no macrophages) (■).

Figure 3. Rate of killing of staphylococci by peritoneal mouse macrophages following repeated washings of macrophages after incubation with staphylococci for 15 minutes at 37 C and without streptomycin. Control mice (○); mice immunized with dead staphylococcus (▲); mice immunized with live staphylococcus (●); mice immunized with BCG (■).


10^8
10^7
10^6
10^5
10^4
CFU PER ML
0
2
4
6
8
HOURS

intracellular and cell associated staphylococci.

Staphylococcal growth in presence of different amounts of dihydrostreptomycin. The effect of different concentrations of dihydrostreptomycin on the viability and growth of staphylococci is shown in Figure 4. No peritoneal cells were added in this test. There was a very small difference in the concentrations of antibiotic which would allow growth or would kill staphylococci. While 0.1 ug/ml had no apparent effect, 0.5 ug/ml caused rapid killing. The effects of different concentrations of streptomycin on staphylococci in the presence of peritoneal macrophages is presented in Figure 5. The growth curves represent staphylococcal concentrations in the extracellular phase only. With macrophages present, 5 ug/ml of dihydrostreptomycin were required to prevent the extracellular growth of staphylococci. This was a ten-fold increase over the concentration required in the absence of macrophages and appeared in agreement with the work of Bonventre and Imhoff (1970) who reported the uptake and concentration of tritiated dihydrostreptomycin within macrophages.

Bactericidal activity of peritoneal macrophages and peritoneal macrophage lysates

Intracellular inactivation of staphylococci by washed mouse peritoneal macrophages in suspension with 3 ug/ml dihydrostreptomycin. Differences in the four test groups of mice were detected with the addition of 3 ug/ml dihydrostreptomycin to a parasitized and washed suspension. This concentration of antibiotic was chosen based on the above results. Using unwashed parasitized macrophages, 5.0 ug/ml dihydrostreptomycin checked staphylococcal growth while 2.5 ug/ml did not. In this test macrophages were washed after parasitization and a

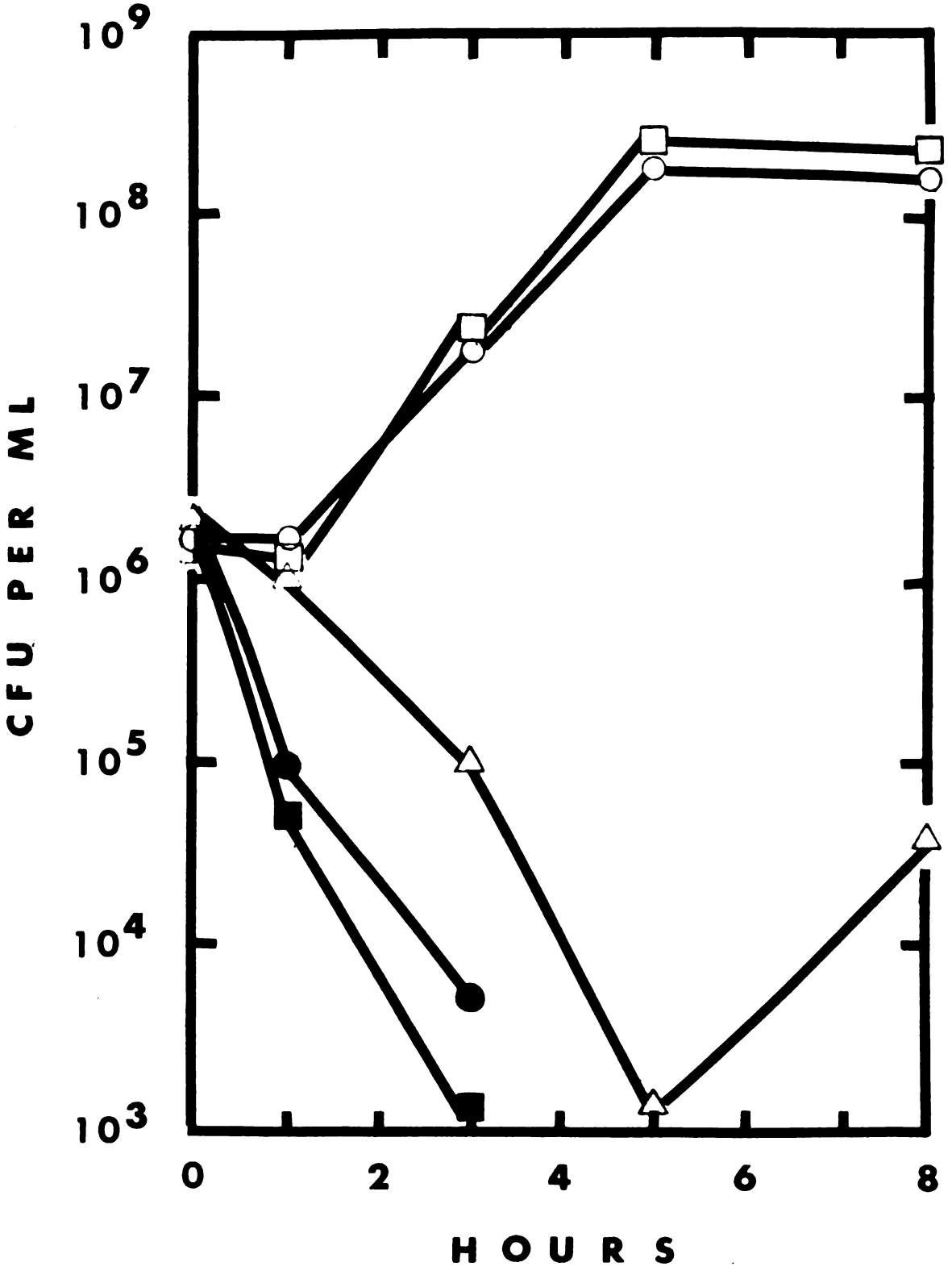


Figure 4. Staphylococcal growth curve in the presence of different amounts of streptomycin. Streptomycin concentrations: 0.05 ug/ml (○); 0.10 ug/ml (□); 0.5 ug/ml (△); 1.0 ug/ml (●); 5.0 ug/ml (■).

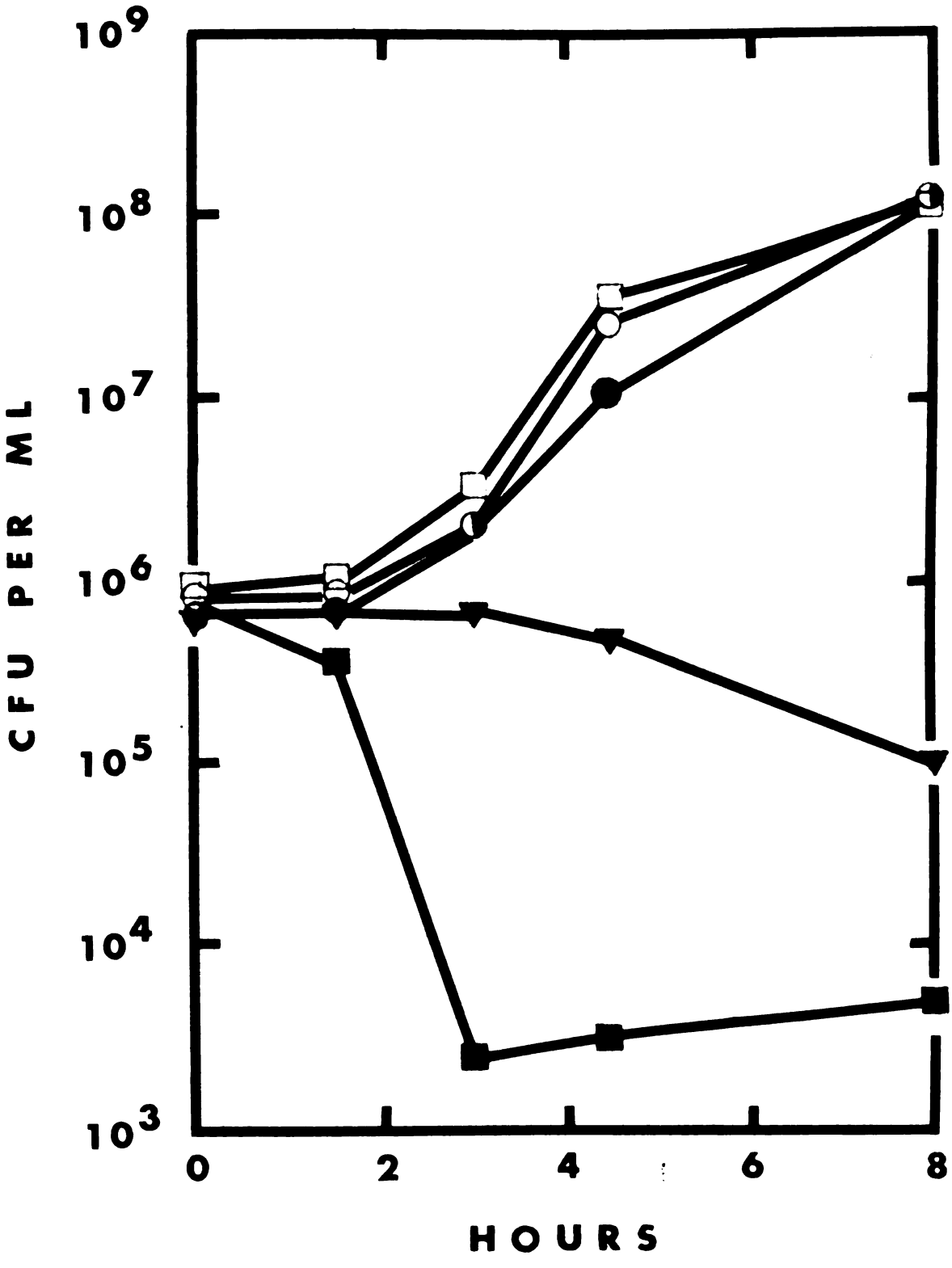


Figure 5.6 Extracellular staphylococcal growth curve in the presence of 10^6 peritoneal mouse macrophages/ml and different amounts of streptomycin. Streptomycin concentrations: 0.5 ug/ml (□): 1.0 ug/ml (○): 2.5 ug/ml (●): 5.0 ug/ml (▼): 10.0 ug/ml (■).

compromising value of 3 ug/ml dihydrostreptomycin was used in an attempt to use as little antibiotic as possible. Viability of macrophages was checked at each time period and was 95% or better throughout the test. No significant differences in intracellular inactivation were observed between the control mice or mice injected with either live or dead staphylococci. There was a slightly greater rate of bactericidal activity within macrophages of BCG immunized mice (Figure 6).

Bactericidal activity of diluted macrophage lysates against staphylococci. The effect of macrophage lysates on the viability of a standard population of staphylococci under various conditions are presented in Tables 2 through 7. The dilutions marked with arrows in these tables represent the ED_{50} for that lysate preparation, i.e., the dilution which was effective in killing 50% of the original staphylococcal population. No bactericidal activity was detected in macrophage lysates from any of the four test groups in the presence of 5% mouse serum at either pH 5 or 7 (Tables 2 and 3). It was possible that serum proteins coated the bacterial cells and prevented their inactivation. When 5% mouse serum was omitted, there was a definite bactericidal effect at pH 5 against both opsonized and unopsonized staphylococci with macrophage lysates from all test groups (Tables 4 and 6). The lysates from mice immunized with live staphylococci and BCG had a slightly higher titer although the differences are slight. A slightly higher titer was observed with unopsonized staphylococci (Table 6) as compared to opsonized staphylococci (Table 4) for all test groups. The bactericidal activity was most likely due to lysosomal acid hydrolases which have acid pH optima.

No bactericidal activity was detected at pH 7 against opsonized staphylococci in lysates from control, dead, or live staphylococcal

Figure 6. Rate of intracellular killing of staphylococci by peritoneal mouse macrophages in the presence of 3 ug/ml streptomycin following repeated washings of macrophages after incubation with staphylococci for 15 minutes at 37 C. Control mice (○): mice immunized with dead staphylococci (▲): mice immunized with live staphylococci (●): mice immunized with BCG (■).

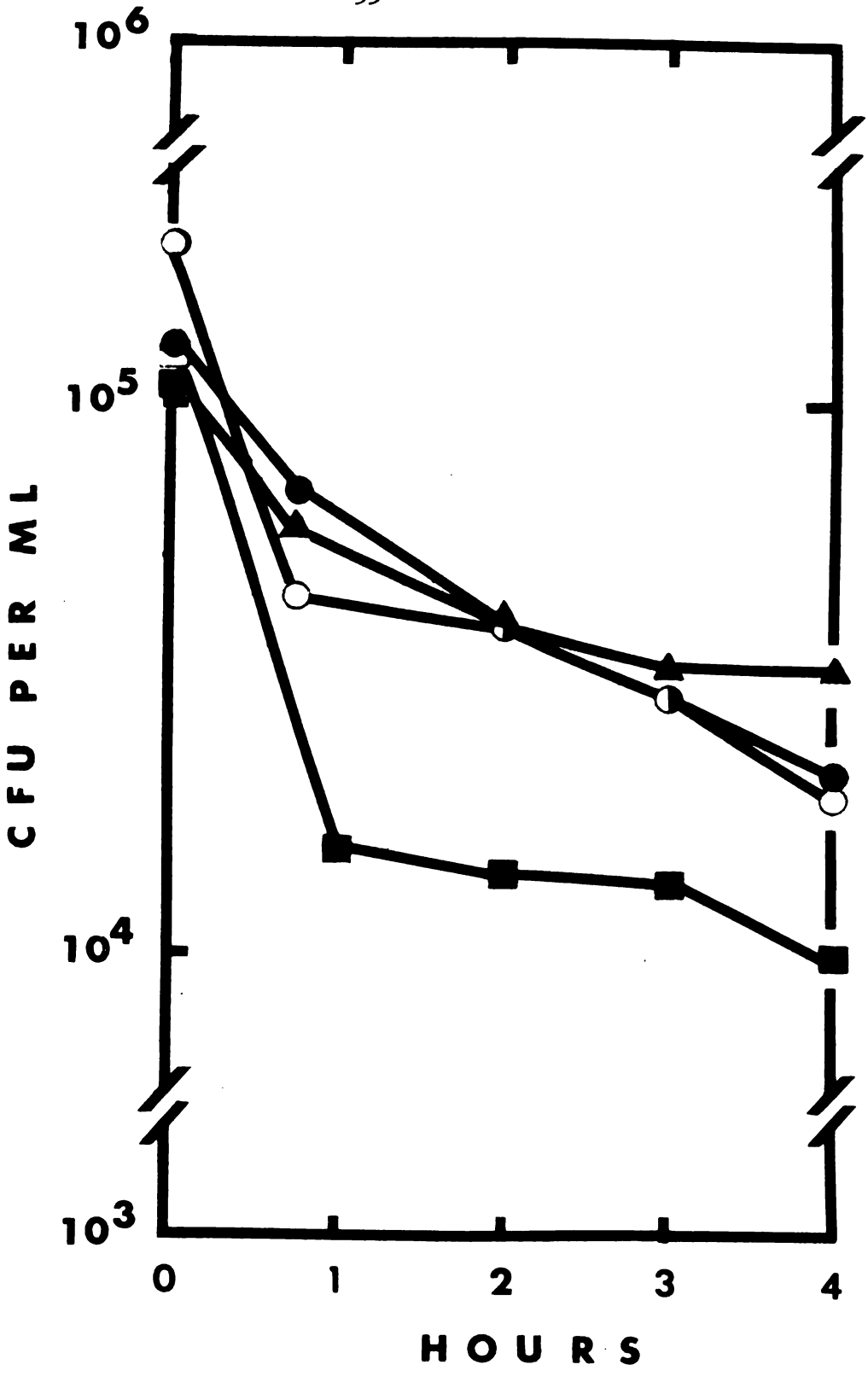

10^6
10^5
10^4
10^3
CFU PER ML
0
1
2
3
4
HOURS

Table 2. Effect of serial 3-fold dilutions of macrophage lysate on staphylococcal viability. Test medium: 0.07 M citrate-phosphate buffer, 0.01% BSA, 5% mouse serum[a] at pH 5.

Lysate dilution	Control mice	Dead staphylococci immunized mice	Live staphylococci immunized mice	BCG immunized mice
	Staphylococcal CFU[b]			
1:3	92	170	603	404
1:9	102	146	368	536
1:27	92	161	306	532
1:81	108	158	300	580
1:243	102	152	284	608
1:729	96	154	287	590
1:2,187	103	164	294	634
1:6,561	104	146	286	572
1:19,683	101	142	256	564
1:59,049	104	152	304	580
Buffer[c] control	102	154	320	552

[a]Serum used in each test group was obtained from mice immunized in a similar manner.

[b]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions.

[c]Number of staphylococcal CFU remaining at end of 2 hours incubation in buffer only.

Table 3. Effect of serial 3-fold dilutions of macrophage lysate on staphylococcal viability. Test medium: 0.07 M citrate-phosphate buffer,- 0.01% BSA,- 5% mouse serum[a] at pH 7.

Lysate dilution	Control mice	Dead staphylococci immunized mice	Live staphylococci immunized mice	BCG immunized mice
	Staphylococcal CFU[b]			
1:3	220	173	521	736
1:9	173	173	430	780
1:27	186	161	324	764
1:81	210	162	290	752
1:243	228	160	255	956
1:729	190	164	291	768
1:2,187	219	168	262	776
1:6,561	211	162	257	684
1:19,683	203	172	254	800
1:59,049	226	174	257	748
Buffer[c] control	180	168	261	766

[a]Serum used in each test group was obtained from mice immunized in a similar manner.

[b]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions.

[c]Number of staphylococcal CFU remaining at end of 2 hours incubation in buffer only.

Table 4. Effect of serial 3-fold dilutions of macrophage lysate on viability of opsonized staphylococci.[a] Test medium: 0.07 M citrate-phosphate buffer and 0.01% BSA at pH 5.

Lysate dilution	Control mice	Dead staphylococci immunized mice	Live staphylococci immunized mice	BCG immunized mice
	Staphylococcal CFU[b]			
[d] > 1:3	69	220	83	104
1:9	100	233	146	27
1:27	83	106	75	3
> 1:81	101	187	37	78
1:243	124	245	34	> 318
1:729	116	260	> 200	434
1:2,187	115	258	152	404
1:6,561	122	252	200	412
1:19,683	125	275	254	433
1:59,049	113	263	198	364
Buffer[c] control	117	256	217	409

[a]Staphylococci were opsonized with mouse serum from a corresponding test group.

[b]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions.

[c]Number of staphylococcal CFU remaining at end of 2 hours incubation in buffer only.

[d]ED_{50}, lysate dilution which kills 50% of added staphylococci.

Table 5. Effect of serial 3-fold dilutions of macrophage lysate on viability of opsonized staphylococci.[b] Test medium: 0.07 M citrate-phosphage buffer and 0.01% BSA at pH 7.

Lysate dilution	Control mice	Dead staphylococci immunized mice	Live staphylococci immunized mice	BCG immunized mice
	Staphylococcal CFU[b]			
1:3	139	346	553	361
1:9	112	261	301	149
1:27	105	227	264	131
1:81	100	272	284	168
1:243	110	258	296	263
1:729	115	240	280	322
1:2,187	110	240	296	339
1:6,561	105	242	318	390
1:19,683	114	261	314	368
1:59,049	130	205	286	334
Buffer[c] control	108	263	326	335

[a]Staphylococci were opsonized with mouse serum from a corresponding test group.

[b]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions.

[c]Number of staphylococcal CFU remaining at end of 2 hours incubation in buffer only.

[d]ED_{50}, lysate dilution which kills 50% of added staphylococci in 2 hours.

Table 6. Effect of serial 3-fold dilutions of macrophage lysate on viability of staphylococci. Test medium: 0.07 M citrate-phosphate buffer and 0.01% BSA at pH.5.

Lysate dilution	Control mice	Dead staphylococci immunized mice	Live staphylococci immunized mice	BCG immunized mice
	Staphylococcal CFU[a]			
1:3	27	158	0	1
1:9	7	175	49	0
1:27	27	91	79	0
1:81	39	89	15	2
1:243	105	144	11	134
1:729	130	224	63	284
1:2,187	138	259	112	477
1:6,561	134	237	166	510
1:19,683	129	205	140	378
1:59,049	132	258	137	448
Buffer[b] control	124	237	145	454

[c] > 1:81 (Control mice); > 1:243 (Dead staphylococci immunized mice); > 1:729 (Live staphylococci immunized mice); > 1:243 (BCG immunized mice)

[a]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions.

[b]Number of staphylococcal CFU remaining at end of 2 hours incubation in buffer only.

[c]ED_{50}, lysate dilution which kills 50% of added staphylococci. in 2 hours.

Table 7. Effect of serial 3-fold dilutions of macrophage lysate on staphylococcal viability. Test medium: 0.07 M citrate-phosphate buffer and 0.01% BSA at pH 7.

Lysate dilution	Control mice	Dead staphylococci immunized mice	Live staphylococci immunized mice	BCG immunized mice
	Staphylococcal CFU[a]			
1:3	172	186	366	495
1:9	129	182	304	369
1:27	119	211	222	0
1:81	123	156	274	6[c]
1:243	121	200	232	355
1:729	118	132	272	343
1:2,187	122	124	246	392
1:6,561	119	206	230	416
1:19,683	88	192	174	409
1:59,049	129	189	252	425
Buffer[b] control	115	179	255	411

[a]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions.

[b]Number of staphylococcal CFU remaining at end of 2 hours incubation in buffer only.

[c]ED_{50}, lysate dilution which kills 50% of added staphylococci in 2 hours.

immunized mice although there was a slight effect with macrophage lysates from BCG immunized mice (Table 5). There was definite bactericidal activity against opsonized staphylococci at pH 7 by lysates from BCG immunized macrophages, the positive control group (Table 7).

All tests in this series were made in triplicate and the figures represent averages of these values. Buffer controls were made with each test series and represent the number of staphylococci remaining viable in buffer alone. Although staphylococci were added to a calculated 200 CFU/ml in each test system, plate counts indicated some deviation. Lysate controls were also run with each series. One milliliter of lysate was added to the appropriate buffer and treated in the same manner as the test systems. Except for macrophages from mice injected with live staphylococci, no contamination was found associated with the cell lysate. Mice from this test group had been prestimulated with 10^7 live staphylococci two days prior to testing and some viable staphylococci were invariably found in the lysate contents. The values found in the tables representing this test group have been normalized by subtracting a calculated correction factor, i.e., one-third of the lysate control staphylococcal plate count was subtracted from the plate count obtained for the 1:3 dilution, one-ninth of the lysate control staphylococcal plate count was subtracted from the plate count obtained for the 1:9 dilution, etc.

Localization of bactericidal activity in macrophage lysates from BCG immunized mice. The presence of an active factor(s) at pH 7 from lysates of BCG immunized mice prompted an experiment to determine a possible association with lysosomes. Lysate from this

test group was separated in 0.25 M sucrose by differential centrifugation into three fractions: (i) a "nuclear" fraction, (ii) a "lysosomal" fraction, and (iii) the remaining "high speed supernatant" (Cohn and Wiener, 1963). Serial 3-fold dilutions were made with these fractions and part of the total homogenate and their bactericidal activities tested against a standard concentration of staphylococci. It appeared the major portion of the bactericidal activity was in the lysosomal fraction although a significant killing effect was also found in the supernatant fluid left from the high speed centrifugation (Table 8). It was possible the lysosomal membranes ruptured during treatment and released their contents into the nonsedimentable fraction.

Effect of staphylococcal challenge on survival of immunized mice. The four groups of mice were challenged with 2×10^9 CFU (100 LD_{50}) of staphylococci via the peritoneal route three days after immunization. The test group immunized with live staphylococci survived challenge best: 33% were dead by 20 hours (Table 9). The BCG and dead staphylococci immunized group had high death rates, 93% and 83% respectively. Control mice had a 100% mortality rate.

Table 8. Effect on staphylococcal viability of serial 3-fold dilutions of fractions from macrophage lysate obtained by differential centrifugation. Source of macrophages: BCG immunized mice. Medium: 0.07 M citrate-phosphate buffer and 0.01% BSA at pH 7.

Lysate dilution	Fraction			
	Total homogenate	Nuclear pellet	Lysosomal pellet	High speed supernatant
	Staphylococcal CFU[a]			
1:3	329	167	44	211
1:9	183	127	4	110
1:27	158	6	24	4
1:81	69	0	71	1
1:243	29	57	0	26
1:729	0	180	0	30
1:2,187	3	126	0	27
1:6,561	11	154	0	17
1:19,683	46	150	0	0
1:59,049	12	130	57	3

[a]Number of staphylococcal CFU remaining at end of 2 hours incubation in lysate dilutions. The number of staphylococci remaining at the end of 2 hours incubation in buffer only was 210 CFU.

Table 9. Effect of a challenge dose of staphylococci on the survival of test mice.

Mice test groups	No. of mice dead/ No. of mice tested [a]	Survival rate
Control	18/18	0%
Dead staphylococci immunized	13/14	7%
Live staphylococci immunized	6/18	67%
BCG immunized	15/18	17%

[a] Mortality rates are on the basis of number of animals dead at 20 hours.

DISCUSSION

Cellular immunity and the problem of antibiotics. A cellular immunity has been demonstrated for several kinds of microorganisms including mycobacterium, salmonella, brucella, listeria, toxoplasma, and leishmania (Mackaness, 1964; Dubos and Schaedler, 1957; Coppel and Youmans, 1969A, 1969B; Youmans and Youmans, 1969; Jenkin and Rowley, 1963; Miller and Twohy, 1969). In all cases the agent inducing a cellular immunity was an absolute or facultative intracellular parasite and with the exception of mycobacterium immunity was induced only with living organisms.

Instances of survival or degradation only over an extended period of time was demonstrated for staphylococci within monocytes (Shayegani and Kapral, 1962) and intracellular survival and multiplication was observed for a relatively nonvirulent strain of Serratia marcescens (Miller and Buckler, 1968) although a cellular response has not been demonstrated for either organism.

Our findings (Fig. 3) concurred with those of Kapral and Shayegani (1959) in which it was demonstrated that washing the host cells after parasitization was rather ineffective in controlling the extracellular growth of staphylococci for the purpose of studying intracellular inactivation of staphylococci. These high concentrations of viable extracellular Staphylococcus aureus

prevented any determination of differences in intracellular killing between normal and immunized animals. When 3 ug/ml of antibiotic were incorporated into a test system measuring intracellular degradation of washed macrophages, an increased rate of inactivation was observed in BCG immunized mice (Figure 6). Little difference in staphylococcal inactivation was observed between normal mice or mice immunized with dead or live staphylococci. Approximately a 33% decrease in colony forming units was observed after zero time between the positive controls (BCG immunized mice) and the other test groups. After an initial one hour period of intracellular degradation approximately 1.5×10^4 CFU of staphylococci were associated with 10^6 large mononuclear cells from BCG immunized mice, while about 5×10^4 CFU were found with the same number of cells in the other test groups. Possibly the macrophages from BCG immunized mice took up dihydrostreptomycin to a greater degree than macrophages from other groups and this additional amount might account for the differences observed in this test; however, the possibility of a real effect must also be considered.

Bactericidal activity of immune versus normal macrophages. Much work was been published about the rate of intracellular survival or inactivation of invading organisms by cells of the reticuloendothelial system (Lurie, 1939, Li et al., 1963; Mackaness, 1964; Rowley, 1958) and the hydrolytic action of lysosomal enzymes has been well documented (Cohn, 1963 A, 1963 B; Friedberg and Shilo, 1970; Friedberg et al., 1970). Degradation of ingested proteins, macromolecular carbohydrates and bacteria by lysosomal enzymes has been established (Aronson and DeDuve, 1968; Coffey and DeDuve, 1968; Ehrenreich and Cohn, 1969). Shayegani (1968) noted a bactericidal action of the

lysosomal contents from polymorphonuclear leucocytes on staphylococci.

Animals which have been immunized with BCG have elevated levels of lysosomal acid hydrolases (Cohn and Wiener, 1963; Saito and Suter, 1965) which might explain their elevated bactericidal activities in cellular immunity. Other investigators (Dannenberg and Bennett, 1963) have found no increase in levels of enzymes.

The experiments performed with serial dilutions of macrophage lysates showed bactericidal activity against both untreated and opsonized staphylococci in all test groups at pH 5 (Tables 4 and 6). The ED_{50} was at a lower titer in the case of opsonized staphylococci. This was in disagreement with the findings of Donaldson et al. (1956) and Rowley (1958) in which immune serum enhanced the killing of Escherichia coli within phagocytic cells. Cohn (1963 B) found, however, that specific antibody inhibited the degradation of E. coli. In our experiments there appeared to be inactivation of both opsonized and unopsonized staphylococci at greater lysate dilutions with macrophages from live staphylococci immunized mice than with the other test groups, but the difference between groups was not great (Tables 4 and 6). The fact that killing was observed at pH 5 and not at pH 7 would seem to implicate the lysosomal acid hydrolases which have their pH optima in this region (Aronson and DeDuve, 1968; Coffey and DeDuve, 1968). In experiments with alveolar macrophages, Cohn and Wiener (1963) found similar increases in lysosomal enzyme concentrations. In normal alveolar macrophages unit activity per 10^6 macrophages for acid phosphatase was 20.7 ug phosphorus/hr at 38 C, for lysozyme it was 3.2 ug egg white lysozyme equivalents at 22 C, and for lipase it was 0.31 umoles napthol/hr at 38 C. In BCG induced alveolar macrophages the corresponding activities were 37.0 for acid phosphatase, 9.2 for

lysozyme and 0.83 for lipase.

A bactericidal factor(s), presumably lysosomal, obtained from peritoneal cells of BCG immunized mice was active against staphylococci at pH 7 (Table 7). This factor(s) may or may not have been produced by the macrophages. Patterson and Youmans (1970) have observed the presence of a factor(s) apparently produced by immune lymphocytes and taken up by macrophages. The macrophages then exhibited an increased bactericidal activity. In our tests, (Table 7) no bactericidal activity was observed at pH 7 in any of the other test groups. In fractionation of the macrophage lysate, most of the bactericidal activity was located in the "lysosomal pellet" although some activity was observed in the "high speed supernatant". This latter activity might have been due to the inadvertant rupturing of lysosomal membranes during handling and treatment with the resultant "spilling" of the lysosomal contents. Further tests should be made to characterize this substance(s).

This bactericidal effect at pH 7 might account for the difference in intracellular killing within intact macrophages of BCG immunized mice as compared with the other test groups.

SUMMARY

A slightly greater intracellular killing was observed in macrophages from BCG immunized mice (positive controls) than in macrophages from control and dead and live staphylococcus immunized mice. A small amount of dihydrostreptomycin was incorporated into the test medium, however, and the observed differences may be due to a greater uptake of antibiotic by the immune macrophages from the positive control group.

Killing of opsonized and unopsonized staphylococci in the presence of macrophage lysates was observed in all test groups at pH 5 with a slightly higher ED_{50} obtained for animals immunized with live staphylococcus or BCG. Mice immunized with BCG produced (or exhibited) a factor(s) which was probably lysosomal in origin and active at pH 7.in bactericidal activity against staphylococci. This bactericidal effect was not seen in control mice or mice immunized with either live or dead staphylococci at this pH.

ADDENDUM

1. It was decided after all experiments were finished that an extra test group might well have been included here. A group of test mice which received one staphylococcal injection prior to testing, similar to the immunization schedule of the mice immunized with BCG, might have been added.

2. Although Swiss albino female mice were specified for use as test animals, the original mice with which the LD_{50} dose of staphylococci was determined and the actual test mice were supplied by different breeders. It was subsequently discovered that mice obtained from Carworth Farms (those with which the LD_{50} was determined) provided a more uniform and predictable response than those obtained from Spartan Research (those with which the body of this research was performed).

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