COMPARATIVE STUDIES OF CELLULAR RESISTANCE TO *CANDIDA ALBICANS* INFECTIONS IN MICE

by

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ABSTRACT

The quantitative extent of resistance induced by specific and nonspecific immunization to systemically induced C. albicans infection was investigated in mice. Animals were specifically immunized by i.p. injections of formalinized suspensions of C. albicans. Nonspecific resistance was induced by temporally spaced subcutaneous injections of endotoxin. The mice were challenged by i.v. injections of known numbers of C. albicans. Animals were sacrificed at 24 hour intervals; cultures of spleen, liver, and kidney for viable C. albicans were done on antibiotic blood agar.

In vitro studies were evaluated by comparing kinetic curves of inactivation of C. albicans by macrophages from various systems, that is, from animals that were immunized, endotoxin treated or treated with saline. Lymphocytes plus macrophages from the various systems were also challenged to determine if the lymphocyte was functionally involved with the destruction of C. albicans.

The most significant in vivo result was that fewer yeasts were found in the kidneys of immunized animals. The kidneys of the endotoxin treated animals and the saline treated animals were heavily infected. No significant differences were observed in the livers and spleens of the different groups throughout the experiment.

In vitro results indicate that macrophages from immunized animals have slightly but statistically significant enhanced cytopeptic capacities when compared to macrophages from endotoxin treated and saline treated animals. Lymphocytes appeared to enhance the cytopeptic response after a temporal delay which was not observed in cultures containing only macrophages.

The data suggest that macrophages from immunized animals, endotoxin treated animals or saline treated animals yielded a steeper inactivation curve, reflecting death of the challenge organism, than the systems containing lymphocytes from the various systems acting in conjunction with macrophages from a homologous or heterologous system. Lymphocytes from immunized animals and lymphocytes from endotoxin treated animals did enhance the cytopeptic capacities of macrophages from saline treated animals when comparisons to the lymphocyte-macrophage saline control system were made. This enhanced cytopepsis that occurred in the presence of the lymphocyte was not as pronounced in systems containing lymphocytes and macrophages from immunized animals and/or endotoxin treated animals.

The fact that endotoxin treated lymphocytes stimulate the cytopeptic capacities of saline treated macrophages indicates a mechanism for the induction of nonspecific resistance. This implies that the mechanism of nonspecific resistance includes cellular components and that both the lymphocyte and macrophage participate.

INTRODUCTION

There are few reports concerning the comparative efficacy of specific and nonspecific resistance in a single experiment. In this thesis I report, in terms of resistance to challenge with Candida albicans, data of quantitative nature concerning the relationship of normal, Boivin antigen, (endotoxin) treated and immunized animals. Endotoxin was used as a classical agent to induce nonspecific resistance.

It was planned to study the role of cellular resistance in fungal infections by in vivo and in vitro techniques. Previously, several investigators have reported the role of antibody in resistance to Candidiasis as being of minimal value. A significant role for cellular mechanisms in resistance to fungal infections has been reported. Recently the archival literature has reported that combinations of lymphocytes and macrophages from immunized animals have an enhanced resistance to challenge organisms when compared to lymphocyte-macrophage combinations from saline treated animals. These reports have been mainly concerned with a soluble product termed "macrophage inhibition factor" or MIF.

The studies now reported have been with the cytopeptic capacities of peritoneal exudate macrophages from the immunized, endotoxin treated, and saline treated control animals. The cytopeptic competence of the differently treated macrophages has been compared employing macrophages freed from lymphocytes before exposure to the infectious agents as well as macrophages exposed to defined lymphocyte populations at the same time

as exposure to the infectious agents. The experimental designs were chosen to further out knowledge concerning the role of macrophages, of lymphocytes, and of macrophages plus lymphocytes acting on the infectious fungal agent, Candida albicans.

REVIEW OF THE LITERATURE

It has been shown that acquired immunity to certain infectious agents is not dependent on the mechanism in which specific antibody interferes with the pathogenicity of the agent. These agents are found among the protozoa, fungi, bacteria, and viruses. The majority of the organisms are intracellular parasites and this may be a partial explanation for the ineffectiveness of the specific antibody. However, there are several pathogenic bacteria (37), fungi (25), and protozoan (47) parasites that are unaffected by antibody in an extracellular environment.

Metchnikoff (45) proposed the mechanisms of phagocytosis and subsequent intracellular killing and destruction (cytopepsis) by which these "special" parasites as well as any other invading body could be disposed of. In spite of his discovery that the macrophage had a role in host resistance, the subject was not investigated extensively for several years. This may have been due to the difficulties involved in working with cell cultures as compared to the ease of working with humors. Nevertheless, many investigators have recently studied cellular resistance in detail and many new concepts are evolving as a result of these investigations.

Origins of Lymphocytes and Macrophages

In order to understand the possible mechanisms of the cellular response to an invading agent, we should first have a general understanding of the cells involved.

The matrix from which "blood" cells are derived is the embryonic mesoderm. Most of the early studies and conclusions concerning the origin of cells in a developed fetus were based on morphological criteria and functional information was obtained with vital dyes and carbon particles. With newer techniques and radioactive labeling, it has become possible to draw more definite conclusions from investigations concerning the origin of mononuclear cells.

Experiments, using H^3-thymidine, bone marrow mononuclear phagocytes, peripheral blood monocytes and tissue macrophages, have indicated that these cells are proliferating cells or are capable of proliferation. Additional experiments differentiated mononuclear phagocytes into two cell types. One type has been referred to as promonocytes or immature proliferating phagocytes and the other type was referred to as a mature monocyte or macrophages (76). Labeling experiments have shown that the macrophages has the ability to multiply in the tissue.

North (54, 55) using the bacterium Listeria monocytogenes and mycobacterium Bacillus Calmette-Guerin (BCG) and H^3-thymidine, was able to demonstrate a pattern of cellular responses. The responses consist of intense proliferation of lymphoid cells in the spleen, a coincident proliferation of resident macrophages in the peritoneal cavity and subsequent emergence of a population of macrophages with a greatly increased ability to phagocytize inert particles and to spread on a foreign surface. North feels that the temporal relationship that exists between the cellular responses suggests a relationship between the lymphocyte and macrophage cell division and that division of both types of cells is necessary for the changes which occur in macrophages.

Glick (19) reported the abnormal development of immune mechanisms, cellular as well as antibody, after removal of the bursa of Fabricius shortly after birth of chickens. Later this worker implicated antibody formation as the major defect following ablation of the bursa.

Cooper (10) demonstrated a separation of immunologic function in the chickens. In the chicken, the bursa of Fabricius contains the larger lymphocytes (B-cells) and plasma cells and these cells are responsible for antibody production.

The thymus in the chicken represents the site of differentiation of a population of lymphocytes that are associated with cellular immunity. The thymus-dependent, or T-cell, system may also form antibody to selected antigens.

This divided immunologic system has been extended to other vertebrates. Only the chicken has a bursa of Fabricius but investigators have proposed other possible locations of a similar B-cell population development in higher vertebrates (1, 9, 20, 21).

A number of kinins have been reported to be present in active or precursor form and to be secreted by activated T-cell lymphocytes. Among these kinins are transfer factor, lymphocyte transforming factor, migration inhibition factor, lymphotoxin, chemotactic factors, growth inhibitory factors, mitogenic or blastogenic factors and cytotoxic factors. It should be noted that some of the kinins or factors may have more than one title at the present time. Some of these factors do not affect macrophages to our knowledge whereas others seem to have a specific effect on macrophages (11, 14, 15).

The reticuloendothelial system is composed in part of a network of reticular, i.e., supporting or structural cells of the spleen, thymus, and

other lymphoid tissues, together with cells lining the sinuses of the spleen, bone marrow, lymph nodes, the capillary endothelium of the liver, Kupffer cells, and the adrenal and pituitary glands.

The spleen possesses a combination of phagocytic, cytopoietic and antibody forming activities which are of great importance in providing immunity to organisms or antigens which get into the blood. These functions are shared with the other filtering organs, chiefly the liver and bone marrow, which contain macrophages strategically placed for contact with substances in the blood. The macrophages are the most active individually, but because of the size of the liver, it is the most important from the viewpoint of total phagocytic activity. In the cytopoiesis of phagocytes, the spleen is the most important in producing macrophages and the bone marrow in producing granulocytes.

The fixed and free macrophages of the connective tissue share the function of phagocytosis. Lymphocytes have not been shown to phagocytize particles.

In general phagocytosis is the process by which a cell extends pseudopodia around the foreign particulate matter which is then taken into the cytoplasma in vacuoles. Phagocytic vacuoles then merge with a lysosome and the lysosome contents are brought in contact with the ingested particles. Subsequent digestion of the particle results (6, 44, 49, 79).

Specific Cellular Immunity

The acquisition of antibody forming capacity, commonly referred to as immunologic competence, by the vertebrates has not lowered the importance of phagocytosis as a mechanism of resistance to infectious disease. Macrophages are able to ingest, kill, and digest many types of organisms that

gain entrance to the body. The role and quantitative extent of immunological assistance from the lymphoid system under such circumstances remains moot; macrophages, however, play an important role in immune responses of both cellular and humoral types.

Cellular immunity must be considered as resistance to foreign particles manifest by the phagocytic capabilities of macrophages acting in conjunction with cytotoxic secretions of lymphocytes.

Humoral antibodies may have only limited efficacy against those pathogens that are capable of intracellular survival and proliferation such as most viral, fungal, protozoal and several bacterial diseases. In such infections, cellular hypersensitivity mechanisms are the major participants in host defense (79).

Specific cellular immunity was first proposed by Metchnikoff. He hypothesized that phagocytic cells of immunized animals may be endowed with enhanced phagocytic and digestive activities independent of humoral antibody. Much of the research involving cellular immunity has corroborated Metchnikoff's hypothesis and has shown that cellular immunity can be specifically stimulated.

A. Cytophilic Antibody Theory

At the present time there are three principal theories to explain the mechanism of stimulation of specific cellular immunity. The first theory proposes the existence of cytophilic antibodies, e.g., that group of antibodies which show a predilection for macrophage surface antigen. This binding of antigen to macrophages may be the only function of the antibody and any subsequent cellular activity may be unrelated to the presence of the specific antibody. Alternatively, the combination of antigen with

cell-bound antibody may directly affect macrophage enzyme systems (46, 74, 75). The following chart summarizes schematically the first two theories.

POSSIBLE MECHANISMS OF ACTION OF CYTOPHILIC ANTIBODY ASSOCIATED WITH MACROPHAGES

(1) Antibody + macrophages → cell bound antibody + antigen → fixation of antigen → fixed antigen + immune macrophage → cytotoxic effect.

(2) Antibody + macrophages → cell bound antibody + antigen → activation of macrophage enzyme systems → subsequent change in macrophage activity.

(Adapted from Tizard, I.R., Macrophage-cytophilic antibodies and the functions of macrophage-bound immunoglobulins. Bacteriological Reviews 35:375, 1972.)

B. Soluble Chemical Mediator Theory

The second theory employs the concept of chemical mediators. The theory states that sensitized lymphocytes (probably thymus derived lymphocytes) secrete a variety of chemical mediators such as chemotactic factor, blastogenic factor, macrophage inhibitory factor, macrophage activating factor, and other factors. The factors affect macrophages in an inflammatory response in various ways.

This second theory depicts cellular immunity as a process involving two cell types, e.g., the lymphocyte and the macrophage. The lymphocytes that are involved in protection against infection are short lived. They are derived from the long lived immunocompetent "T" cells that circulate through lymphoid tissues to the blood and back again (40).

Mackaness has confirmed that a specific cellular response does occur in the presence of specific antigen. He also demonstrated a relationship

between the lymphocyte and macrophage to obtain specific response to the antigen (39, 40, 41). Further evidence to support the bicellular relationship for specific resistance has been furnished by Simon and Sheagren (70). They compared the bactericidal activity of normal macrophages after overnight incubation with either normal or bovine gamma globulin sensitized lymphocytes in the presence or absence of the antigen. They found that immune lymphocytes plus antigen were more effective than immune lymphocytes minus antigen which were similar to the control lymphocytes with and without antigen.

Salvin, Sell and Nishio (64) have studied the lymphocyte-macrophage relationship in relation to delayed hypersensitivity. They observed moderate inhibition of motility and activity of normal macrophages in the presence of sensitized lymphocytes and antigen. The effect was more dramatic with macrophages from sensitized animals in the presence of antigen. They attributed these effects to MIF and felt that this mechanism provided the greatest quantity of cells in delayed hypersensitivity reactions.

Salvin and Cheng (62) have shown a relationship of lymphoid cells and macrophages in guinea pigs that have been sensitized to Candida albicans. Howard, Otto, and Gupta (28) have shown that lymphocytes were the mediators of the suppression of intracellular growth of Histoplasma capsulatum. Simon and Sheagren (70) have shown by two techniques that peritoneal exudate macrophages from immune animals had a greatly enhanced bactericidal capacity when the exudate had been cultured with the antigen before challenge.

Characteristic of many of the above observations and indeed the system for determining the specificity of the two components was nonspecific

activation of macrophages. It should be noted that the nonspecific activation of the macrophages is a result of specific activation of the lymphocytes. For example, if spleen cells from a tuberculous donor were given to a normal recipient, there would be no overt effects on the functional state of the recipient's macrophages unless tubercle bacilli were injected simultaneously with the reactive spleen cells. When this was done, activated macrophages appeared in the peritoneal cavity within 24 hours, and the animals became resistant to Listeria challenge (33, 41). Thus, it would appear that sensitized lymphocytes can influence macrophage activity only in the presence of the specific antigen used to sensitize the host.

C. Specific Sensitization of Macrophages

The third theory is that the macrophage acquires a specific capacity for a particular antigen after the primary exposure. In many instances there is a direct relationship between the animal's ability to inactivate a second inoculum of bacteria in vivo and the ability of the macrophages to ingest and destroy the same bacteria in an in vitro system. Antibody has been reported to be of little or no value in many of the systems studied (18, 26, 48, 51, 73). This is not to say that antibody is not important. Under the proper set of circumstances, antibody may be equally as important as the cellular response. Antibodies are effective in protecting a host from most organisms that ordinarily cannot grow and divide in phagocytic cells (68).

Thorpe and Marcus (73) have presented data showing that phagocytes from specifically immunized animals had significantly enhanced cytopeptic abilities when compared to phagocytes from normal animals that had been challenged with Pasteurella tularensis. It was also shown that passively transferred specific antibodies did not alter the mortality rates in normal

mice, but decreases in mortality were observed when the animals were treated with passively transferred phagocytes from immunized animals.

Radioisotope studies carried out by Hill and Marcus (25, 26) demonstrated that macrophages from immune animals were able to limit the growth of the H. capsulatum. They were also able to show that these macrophages had an enhanced capacity for cytopepsis in the absence of lymphocytes.

Hackett and Marcus (22) reported that animal specifically immunized to Klebsiella pneumoniae had 1000 times the protection induced by treatment of animals with endotoxin when challenged with K. pneumoniae.

Maxwell and Marcus (34, 43) have shown that animals immunized with BCG, presumably in the absence of lymphocytes, were more active in ingesting and inactivating tubercle bacilli than were animals that had been treated with saline.

Mitsuhashi et al. (50) were able to demonstrate that cells could suppress intracellular proliferation of phagocytized, highly virulent Salmonelleae and finally digest them without addition of exogenous immune sera. This expression of cellular immunity could be established only after contact with live bacilli.

Sato et al. (64) isolated phagocytes from the liver and subcutaneous tissue of adult mice and demonstrated inhibition of intracellular multiplication of Salmonella enteritidis regardless of the presence of antibody in the cell cultures from immunized animals. Cell cultures from normal animals did not retard the growth of the organism either with or without antibody. Sato et al. (64) subsequently reported that mononuclear phagocytes from a normal mouse acquired cellular immunity against infection with Salmonella enteritidis in tissue culture if the cells were treated with

live vaccine of the same organism, whereas the cells treated with dead vaccine of S. enteritidis did not.

Osawa (56) and co-workers in a series of elaborate experiments concluded that contact of live organisms with the host cell is necessary for conferring post-infective immunity in salmonellosis.

Venneman and Berry (77, 78) have shown that resistance to Salmonella typhimurium can be induced with live organisms or extracts of ribosomal RNA. Heat-killed suspensions did not confer resistance to the recipients. Furthermore, they were able to demonstrate a resistance to Salmonella with serum from immunized mice. This immunity was short lived when compared to the immunity provided by the cellular response.

The effect of neonatal thymectomy on immunity of mice against Salmonella infection was studied by Saito et al. (61). They found a lowered peripheral lymphocyte count, reduced serum antibody response and partially reduced protection against infection with virulent Salmonella enteritidis after immunization with a live vaccine in mice thymectomized at birth. However, in vitro cellular resistance of peritoneal macrophages from immunized animals was not found to be reduced by neonatal thymectomy.

Perkins and Marcus (57, 58) studied the effects of x-irradiation on resistance to infectious organisms. They observed that antibody was an effective agent in protection of mice which had been subjected to sublethal doses of x-irradiation. However, antibody protection failed when animals were exposed to higher levels of x-irradiation. These investigators concluded that for antibody to be effective in protection, cellular activity cannot be impaired.

Mackaness (37) investigated the response of macrophages from normal and immune mice to Listeria monocytogenes. The response of normal and immune

cells from mice to infection with Listeria was tested using plaque formation in macrophage monolayers. The results indicated that macrophages from immunized animals were capable of destroying the parasite, whereas cells from normal animals were unable to control the Listeria challenge.

Mackaness (37) has reported a possible mechanism for specific cellular responses. He felt that an accelerated response was dependent upon the ability to generate a new population of resistant cells from a residuum of specifically sensitized macrophages or macrophage precursors still surviving in the tissues as a result of the immunological activation which occurred during the primary infection.

Cline (5) has reported on the bactericidal activity of human macrophages using a Listeria model. He reported that "differentiated" macrophages ingested and killed more organisms per cell than newly isolated macrophages.

Holland and Pickett (27) used guinea pig, rat or mouse macrophages and Brucella abortus, Brucella melitensis or Brucella suis and found that while all three strains of Brucella grew abundantly within normal macrophages, immune macrophages greatly restricted the intracellular growth of smooth and non-smooth Brucella. Although Brucella species are sensitive to antibodies, the growth of smooth Brucella species within either normal or immune cells was not influenced by the addition of specific antiserum to the medium. These studies suggest that macrophage immunized animals have an enhanced capacity to phagocytize and digest the specific challenge organism. These observations imply that macrophages can be specifically sensitized to an antigen and may have a memory capacity similar to that observed in antibody production.

Nonspecific Cellular Immunity

The normal body possesses a remarkable nonspecific resistance to infections. The first line of defense consists of the physical and chemical barriers presented by the epithelial tissues. The relative impenetrability of the skin, the stickiness of mucous membranes, ciliation of the upper respiratory tract, the flushing action of various fluids, the acidity or alkalinity of various parts of the digestive system, all are effective in protecting the host against numerous pathogens and potential pathogens.

Serum components of normal animals contain bactericidal substances which are nonspecific in their action. These substances have been referred to as natural antibody or opsonins. In 1904, Neufeld and Rimpau (53) drew attention to the thermostable opsonins of heated sera. He and his colleagues gave these heat stable opsonins the title "bacterotropins." The bacterotropins, however, are specific in their phagocytosis promoting action, whereas the original opsonins were nonspecific in their phagocytosis promoting action.

The complement molecule is also considered part of the nonspecific resistance system. Although the complement precursors are only assembled in the presence of an antigen-antibody complex, it should be noted that nearly any antigen-antibody complex will induce the molecules' assembly. This is evidenced by its multiplicity of actions; e.g., it may be involved in opsonisation, anaphylotoxin release, histamine release; it may result in in immune adherence, chemotaxis of neutrophils; it is an effector in hemolytic and bacteriolytic mechanisms and recently has been reported to be an integral portion of the properdin system (60).

Properdin was isolated and reported by Landy and Pillmer (34). They found the protein in normal human serum. It required the precursor of complement and magnesium ions to exhibit bactericidal action against gram negative bacteria and certain viruses.

There are several other antibacterial substances that have been isolated from animal tissues or fluids. Table 1 summarizes many of the substances which I shall not discuss.

Another mechanism of defense is nonspecific cellular immunity. Bacterial endotoxins and BCG constituents are most commonly encountered examples of nonspecific stimulants. When animals are inoculated with either BCG or lipopolysaccharide, they develop an enhanced resistance to challenge by a variety of bacteria.

In 1936 Boivin and Mesrobeanu isolated antigenic substances from a gram-negative bacillus, Salmonella typhi. It was soon realized that the latter substance not only represented the antigenic moiety of the bacterium but possessed at the same time a number of nonspecific and seemingly unrelated biological effects. Among these effects, the most important qualities were described as the ability to induce fever, leukopenia followed by leukocytosis, vascular reactions, hyperglycemia, depletion of liver glycogen, thrombocytopenia, fibrinolysis, to raise the properdin level, stimulate the R.E. system, to endow the organism with the ability to resist heterologous infection, and, in high doses, bring about prostration and death.

Data indicate that endotoxins migrate in an electric field as anions due to the presence of phosphoric acid esters. Although still under investigation, a current dogma holds that all known gram-negative endotoxins have

Table 1. Antibacterial Substances from Animals Tissue or Fluid

Compound	Source	Chemical Nature	Antibacterial Selectivity	Heat Stability
Complement	Serum	Euglobulin-carbohydrate	Gram negative	Labile
Properdin	Serum	Euglobulin	Gram negative	Labile to relatively stable
Natural Ab	Serum	Euglobulin	Gram negative	Labile to relatively stable
Phagocytin	Leukocytes	Globulin	Gram negative	Relatively stable
β Lysin	Serum	Protein	Gram positive	Relatively stable
α Lysin	Serum	Protein		
Leukins	Leukocytes	Basic peptides	Gram positive	Stable
Plakins	Blood platelets	Peptide (?)	Gram positive	Relatively stable
Lysozyme	Cells (?)	Small basic protein	Gram positive (Chiefly)	Stable
Histones	Lymphatics	Small basic protein	Gram positive	Stable
Protamine	Sperm	Small basic protein	Gram positive	Stable
Hematin (Mesohematin)	RBC	Iron porphyrins	Gram positive	Stable
Spermine (Spermidine)	Pancreas Prostate	Basic polyamines	Gram positive	Stable
Tissue Polypeptides	Lymphatics	Linear basic peptides	Gram positive	Stable

Labile.....Inactivated 56°C, 1/2 hr.
Relatively stable....Resists 56°C, 1/2 hr., but destroyed below 80°C, 1/2 hr.
Stable.....Resists 80-100°C, 1/2 hr. or more.

similar structure. They consist of lipopolysaccharide-protein-lipid complexes which can be split into the relative components. It has been found that the lipid portion of the complex is responsible both for toxicity and the manifold biological effects of endotoxin.

It has long been known that gram-negative bacterial lipopolysaccharides (LPS) enhance the resistance of experimental animals (3, 71). Studies clearly demonstrate that many apparently unrelated biological systems, both humoral and cellular, are affected by injection of LPS (4, 7, 71).

Investigators have purified endotoxin and have demonstrated that administration of purified endotoxin greatly alters the activity of cellular defense mechanisms. The phagocytic activity of the reticuloendothelial system and of mobile phagocytes has been shown to be stimulated (71).

Rowley (59) reported in 1956 that injection of gram-negative bacterial cell walls into animals resulted in, first, an increase in susceptibility to challenge followed by an enhanced state of resistance.

Bohme (3) has drawn the following conclusions concerning endotoxin:

1. Endotoxin preparations increase the resistance of mice to heterologous infections.
2. The amounts of endotoxin necessary are in the order of magnitude of hundreds of micrograms and less; the route of administration has no clear bearing on the results. Other reporters have also reported this (4, 22).
3. The protective effect becomes manifest 5-24 hours after administration depending on the dose.

4. The effect afforded by endotoxin is nonspecific in nature.

Bohme does not discuss, nor is there significant literature available to define the mechanism of resistance induced by endotoxin.

Macrophages or mononuclear leukocytes react to initiating stimuli such as LPS or BCG by becoming activated and proliferating in the vicinity of the irritation. The activated macrophage is characterized by increased "stickiness", morphology, increased acid phosphatase, increased oxygen uptake, increased glycolysis and lipid turnover, production of hydrogen peroxide, faster movement and more efficient ingestion (13, 35, 49).

The lysosomes and mitochondrial activity are increased nonspecifically for the stimulating agent involved (12). However, the destructive properties of the activated macrophage are nonspecific even though the origin of these properties was from an immunologic event that was specific.

MATERIALS AND METHODS

Organisms

Cultures of *Candida albicans* were obtained from the Department of Microbiology (University of Utah) pure culture collection. The organism was maintained on Sabouraud-dextrose agar slants. The organism was transferred every three months and was stored at room temperature. The organism was checked periodically for contamination by growth on chlamydospore agar and germ tube formation in human sera.

Cultures of *Klebsiella pneumoniae* (199-A) were also obtained from the Department of Microbiology pure culture collection (University of Utah). The organism was maintained on nutrient agar slants. The organism was also lyophilized for long-term storage. Biochemical analysis and Klebsiella antisera (Difco) identified the organism as *Klebsiella pneumoniae* type 1.

Endotoxins

The endotoxin was obtained from Difco Laboratories (Bacto Lipopolysaccharide B, *E. coli* 055:B5). This material was diluted in sterile saline to a concentration of 100 ug per ml and was stored at 4 C.

Vaccine

Candida albicans was grown on antibiotic blood agar at 37 C for 24 hours. The organisms were harvested in gel-saline (1% gelatin in 0.85% saline) from the blood agar plate. The harvested suspension was filtered

through sterile glass wool. The filtered suspension was observed microscopically for clumping. The suspension was adjusted to MacFarland tube #3 or MacFarland tube #6. The organisms were killed by addition of 0.5% formalin. The vaccine was checked for viability of the organisms at 48 hours. If viable organisms were present, the vaccine was stored and rechecked again at 72 hours.

MacFarland tubes prepared according to a standard method (69) were used to estimate the number of organisms for Candida vaccines and Klebsiella organisms to be used in experiments.

Media

Antibiotic blood agar (ABA) was used for all experimental cultures. The organisms were grown for 24 hours at 37 C. Antibiotic blood agar was also used for all cultures from organs sampled during the *in vivo* experiments. Antibiotic blood agar is prepared by the following recipe (42):

Tryptose phosphate broth	29.5 g	14.75 g
Agar	20.0 g	10.0 g
Human blood	60 ml	30.0 ml
Distilled water	1000 ml	500 ml
Penicillin	50 units/ml final concentration	
Streptomycin	50 mg/ml final concentration	

Phosphate buffered saline (PBS), pH 7.2, was used to wash tissue culture cells. PBS was also used to make appropriate dilutions of *in vitro* samples prior to plating the organism. The phosphate buffered saline was prepared by the following recipe:

Sodium phosphate	5.2 g
Potassium chloride	0.9 g
Potassium phosphate	0.9 g
Sodium chloride	36.0 g
Distilled water	4500 ml

Fluid thioglycolate medium (BBL) was reconstituted and sterilized. Thioglycollate was used to induce peritoneal exudates in the animals.

Chlamydospore agar (Difco Laboratories) was used to grow Candida albicans in the mycelial phase. The spores produced during the mycelial phase of growth aided in identification of the organism.

Sabouraud-dextrose agar (Difco Laboratories) was used to maintain the cultures of Candida albicans. The organism was transferred every three months. Two stock cultures on Sabouraud-dextrose agar were overlayed with light mineral oil for long-term storage.

Tissue Culture Fluid

Auto-Pow Eagles Minimum Essential Medium (EMEM) (Flow Laboratories, Rockville, Md; Cat. no. 1A-020) was used as a stock tissue culture medium. After sterilization, 150 mg of glutamine per 500 ml of EMEM, 100 units per ml of penicillin, and 100 mg/ml of streptomycin were added to the sterile media. The pH was adjusted to 7.4 by addition of sterile sodium carbonate. Fifty ml of fetal calf serum (Grand Island Biological Co., Berkeley, California; Cat. no. 614) was added immediately prior to use. The media was stored at 4 C. Prior to use, the media was warmed to 37 C.

Human serum was used to observe the short filament (germ) tubes formed by Candida albicans.

Dyes and Stains

Trypan blue dye was reconstituted in distilled water to yield a 0.2% Trypan blue solution. The dye was used to determine the number of viable cells per ml of exudate (23). Trypan blue was substituted for eosin y.

Wright's stain (Allied Chemical) was used to stain peritoneal exudate smears.

Antibiotics

Tetracycline hydrochloride (500 mg) suitable for intravenous (i.v.) injections was purchased from Lederle Laboratories (Pearl River, N.Y.). The sterile powder was weighed out in mg amounts and reconstituted in sterile saline.

Penicillin (100 units/ml) was mixed with streptomycin (100 mg/ml) and distributed in one ml portions to sterile disposable tubes. The penicillin-streptomycin was frozen until needed.

Mycostatin sterile powder (E.R. Squibb and Sons, N.Y.) was used in the tissue culture media. The powder was suspended in EMEM in a concentration of 2500 units/ml. One-tenth ml was added to 2 ml final volume in the culture tubes. The final concentration in the culture tubes was 125 units/ml. For use in tissue cultures, the suggested minimum concentration for mycostatin sterile powder is 100-300 units/ml of nutrient medium (Mycostatin insert).

Candidin

Candidin was prepared in this laboratory. A departmental culture collection organism was grown in Sauton's protein-free media for 2 weeks at 37 C. Formalin (0.5%) was added to the culture to kill the organisms. The broth was checked for viable organisms by culture. The sterile

suspension was filtered and stored at 4 C. The supernatant fluid contained 5.4 mg/100 ml total protein.

Equipment

Red tip heparinized capillary tubes were obtained from Sherwood Medical Industries (St. Louis, Missouri). The capillary had an internal diameter of 1.1 mm ± 0.1 mm and a length of 75 mm. Calculation indicated that 52 mm length of fluid was equivalent to 50 lambda. The capillary tubes were used during the clearance study.

Leighton tubes (16 x 150) were obtained from Bellco Glass, Inc. (Vineland, New Jersey).

Glass beads were sterilized and used to lyse the tissue culture cells.

A Deluxe Mixer (58220) from Scientific Products was used to aid in lysis of the tissue culture cells. The mixer was also used to mix dilutions prior to sampling.

A bright line hemocytometer (American Optical) was used to count peritoneal exudate cells and diluted suspensions of *Candida albicans*.

Animals

Adult albino mice (*Mus musculus*) obtained from local sources were used in these experiments. They were fed Purina chow and had free access to water.

Injection Schedule

The experiments involved both *in vivo* and *in vitro* systems. The systems will be discussed separately beginning with the *in vivo* system.

Mice were separated into groups consisting of 20 animals. The groups were assigned a treatment regimen by random drawing.

The animals that were specifically immunized were given an intraperitoneal injection of 0.25 ml of the prepared formalin vaccine three times a week for two weeks. The mice were then challenged following one week of rest.

The second group of animals was treated with the endotoxin. The animals received 10 ug of endotoxin subcutaneously in the nuchal region 24 hours prior to challenge. The total volume of solution injected per mouse was 0.1 ml.

The third group of animals was treated with saline. The animals received 0.1 ml of saline subcutaneously (s.c.) in the nuchal region 24 hours prior to challenge.

Preparation of C. albicans for Challenge

Stock cultures of Candida albicans were grown on antibiotic blood agar. A 24 hour culture was harvested in 1% gel-saline and mixed thoroughly. The suspension was filtered through glass wool and wet preparations of the suspension were observed microscopically. It was observed that less than 10% of the yeasts were budding. The organisms were diluted in PBS and counted in a hemocytometer. The organisms were then adjusted to the appropriate concentration for challenge. Plate counts of the challenge suspension were also done to determine the exact number of viable organisms. For in vitro experiments, the Candida suspension was adjusted to a concentration of 2×10^6 organisms/ml in gel-saline. The suspension was centrifuged at 3000 RPM for five minutes. The resulting pellet was resuspended in Eagles Minimum Essential Medium. The macrophages were challenged with 10^6 Candida albicans organisms (0.5 ml).

Challenge

Systemic moniliasis was induced by i.v. challenge. All mice were challenged sequentially over a one hour period with the same suspension of organisms. The suspension of organisms contained approximately 1×10^7 organisms/ml. The challenge dose was 0.1 ml which contained approximately 1×10^6 organisms/ml.

Five animals from each group, e.g., the saline treated group, endotoxin treated group and specifically immunized animal group, were sacrificed at 24 hours intervals post-challenge. The skin surrounding the peritoneal area was treated with 95% ethanol. The skin surrounding the peritoneal cavity was reflected without entering the abdominal cavity.

With a sterile set of instruments the abdominal cavity was entered and the liver, spleen, and kidney were excised. The organs were bisected and equivalent cross sections streaked onto antibiotic blood agar plates. The plates were then incubated 48-96 hours, the colonies were counted, and results recorded.

In vitro Experimental Design

The mice used for the *in vitro* experiments had the same immunization schedule as the specifically immunized mice used in the *in vivo* experiments. If the immunized mice were not used within a month of their last immunization injection, a 0.1 ml booster injection was administered 7 days prior to collection of the macrophages.

The endotoxin treated animals received 0.2 ml injections containing 20 ug of endotoxin. The injections were administered subcutaneously in the nuchal region. The first injection of endotoxin was administered 24 hours prior to stimulatin of the peritoneal exudate. The second injection

of endotoxin was administered 48 hours later, 24 hours after the injection of thioglycollate. The animals received injections of endotoxin daily for the following 3 days. The last injection of endotoxin was administered 24 hours prior to harvesting the macrophages.

The third group of animals received 0.1 ml saline for 5 consecutive days prior to harvesting the macrophages.

The peritoneal exudate was induced by injecting the animals with 5.0 ml of thioglycollate intraperitoneally. Five days later the animals were sacrificed by ether anesthesia. Immediately after the animal had expired, it was removed from the ether jar and injected i.p. with 5 ml of sterile PBS. The animals were pinned to a dissection board and 95% ethanol was applied to the abdominal region to moisten the fur of the abdominal surface. Sterile forceps and scissors were used to reflect the skin from the abdominal wall. A second pair of sterile tissue forceps and scissors were used to make a small opening in the abdominal wall. A sterile Pasteur pipette was used to aspirate the peritoneal exudate from the abdominal cavity. The exudate was placed in a sterile, silicon coated centrifuge tube. The cells from three or four animals in each group were pooled. The monocytes were centrifuged at 2000 RPM for five minutes in a table top centrifuge. The cells were washed twice in PBS and resuspended in 5 ml of tissue culture fluid. The monocytes were counted by the trypan blue dye exclusion technique. The cells were adjusted to a concentration of 4×10^6 monocytes/ml and dispensed in 0.5 ml quantities, 2×10^6 cells, to sterile Leighton tubes. The respective groups, e.g., cells from specifically immunized animals, cells from endotoxin treated animals and cells from saline treated animals were aligned one behind the other with the immune cells in the

front row, the endotoxin treated cells in the second row, and the saline treated cells in the back row. Tissue culture fluid, 1.5 ml, was added to each tube brining the total volume to 2 ml. The phagocytes were incubated for 24 hours at 37 C in a CO_2 incubator. After overnight incubation, the tissue culture fluid was aspirated and the cells were washed twice with sterile PBS. Tissue culture fluid, 1.5 ml, was added to each tube. These cells were then ready to be challenged.

Lymphocytes were collected from the mesenteric lymph node and the inguinal lymph nodes. The lymph nodes were removed aseptically and placed in 100 x 15 mm Falcon tissue culture petri dishes. The lymph nodes were suspended in 10 ml of tissue culture fluid until the nodes from all the animals in the respective group had been collected. The lymph nodes were then placed in a sterile strainer that contained a sterile one square inch section of 100 mesh stainless steel wire screen. The lymph nodes were drawn across the screen until only fatty tissue and connective tissue remained. Five ml of the tissue culture fluid in the dish was pipetted through the screen to wash the lymphocytes into the petri dish. The lymphocyte suspension was incubated at 37 C for 30 minutes. After incubation, the lymphocytes were filtered through sterile glass wool into a centrifuge tube. The cells were centrifuged at low speed (2000 RPM) for 5 minutes. The supernatant fluid was poured off and the cells were resuspended in 5 ml of tissue culture medium. Appropriate dilutions of the cell suspension were made and the cells were counted by the trypan blue dye exclusion technique. Approximately 90% of the lymphocytes were viable at this time. If the lymphocytes were to be incubated with the Candidin, 1.0 ml of the antigen (54 ug) was added at this point and the

lymphocyte plus antigen suspension was incubated together for 24 hours. At the end of the incubation the lymphocytes were added to the macrophages before the Candida suspension was added.

The cultured phagocytes (2×10^6) plus 1×10^6 lymphocytes per tube plus 1×10^6 Candida albicans per tube were incubated together for 30 minutes at 37 C in a CO_2 incubator. At the end of the incubation period, the supernatant fluid was aspirated and the phagocytes were washed twice with PBS. Tissue culture fluid containing 125 units of Nystatin was added to each tube. As soon as all of the tubes had received the Nystatin-tissue culture fluid, the first tube from the group was labeled and sampled. The supernatant fluid was aspirated from the sample tubes and 2 ml of cold PBS was added to each Leighton tube. Eight to 12 sterile glass beads were added to each tube. The cells were lysed by holding the Leighton tube on a Vortex mixer. This resulted in a rapid rotation of the beads within the tube. Each tube was held on the mixer for 30 seconds or longer if necessary for lysis of all the cells. The cell lysate was diluted in cold PBS and plate counts of the dilutions were done. The plate counts were done on antibiotic blood agar in duplicate. The plates were incubated for 48 hours at 37 C and colony counts were then made.

Experimental Design for Circulatory Clearance of C. albicans

In vivo clearance studies were done to determine if there were any differences in the clearance rate of immune, normal and tetracycline treated animals. Several animals in each group were tested to get an average at each time interval.

Mice were anesthetized with ether. Subsequently the animals were injected with 2×10^6 organisms intravenously. Blood samples were collected

by orbital puncture. The blood samples were collected in heparinized capillary tubes and diluted in cold PBS. Samples were collected prior to the injection of the organisms, immediately after the injection of the organisms and at 30 seconds, 1 minute, 2, 5, and 10 minutes. Plate counts were done in duplicate on each sample.

EXPERIMENTAL RESULTS

Experiments were carried out in albino white mice (20-27 grams). *In vivo* and *in vitro* experiments were designed to compare cellular resistance of white mice to *Candida albicans*.

Efficacy of Nonspecific Immunization

A. Dose Variation

Experiments were designed to determine the quantity of endotoxin necessary to induce nonspecific resistance to an infectious agent. White mice, in groups of 10, were treated with saline, 1 ug, 10 ug or 100 ug of endotoxin respectively. Twenty-four hours later the mice were challenged with *Klebsiella pneumoniae* (10 LD_{50}). The results, Table 2, indicate that 10 ug of endotoxin was as effective as 100 ug of endotoxin in affording protection, whereas 1 ug of endotoxin gave no significant protection to challenge with *Klebsiella pneumoniae*.

B. Schedule Variation

The extent of the nonspecific resistance induced with 10 ug of endotoxin was determined next. The mice were challenged with *Klebsiella pneumoniae* in quantities of 10 LD_{50}, 100 LD_{50}, or 1000 LD_{50}. The results, Table 3, show that 10 ug of endotoxin injected subcutaneously render the mice resistant to 100 LD_{50} challenge.

To determine if daily doses of endotoxin, 10 ug, resulted in enhanced, diminished, or equal protection, 24 mice were divided into four groups. The

Table 2. Induced Nonspecific Resistance to *Klebsiella pneumoniae* in Mice

	Amount of endotoxin injected			
Challenge	Control (gel-saline s.c.)	1 ug	10 ug	100 ug
10 LD_{50}	10/10*	7/10	1/10	1/10

*Number dead/total five days after challenge

Table 3. Nonspecific Resistance to Klebsiella pneumoniae Induced by Subcutaneous Injections of Endotoxin

	LD_{50} challenge doses				
Endotoxin injected	Saline	1 LD_{50}	10 LD_{50}	100 LD_{50}	1000 LD_{50}
10 ug 24 hr prior to challenge	0/10*	0/10	0/10	2/10	8/10
10 ug/day for 7 days prior to challenge	0/10	0/10	2/10	3/10	9/10

*Number dead/total five days after challenge

animals were treated with saline or 10 ug of endotoxin 24 hours prior to challenge, or 10 ug of endotoxin for 7 days prior to challenge the animals were challenged with K. pneumoniae, 10 LD_{50} or 100 LD_{50}, depending on the group. The results in Table 3 show that treating the animals for 7 days prior to challenge with 10 ug of endotoxin does not significantly differ from treating the animals with 10 ug of endotoxin 24 hours prior to challenge.

Circulatory Clearance of C. albicans in Mice

A clearance experiment was performed to determine how rapidly C. albicans was cleared from the circulatory system. Systemic monoliasis was induced by i.v. injections of 2×10^6 Candida albicans. Blood samples were obtained by intra-arterial bleedings. Samples were obtained at the time of injection, 60 seconds, 120 seconds, 300 seconds, and 600 seconds. Mice that had been immunized with C. albicans vaccine, injected with saline, and injected with lug tetracycline daily for 3 days prior to challenge were used for the clearance study. The results presented in Table 4 show that each group of animals cleared the Candida albicans to an asymptote level in 600 seconds. The tetracycline groups showed no significant differences from the saline group or the immunized group of animals.

In Vivo Experimental Results

In an effort to determine the extent of resistance in white mice to Candida albicans, animals were treated with saline, endotoxin, Candida albicans vaccine or tetracycline (67) (1 mg/mouse). Comparisons of induced resistance were made by doing plate counts on the liver, spleen

Table 4. Clearance of *Candida albicans* from the Circulatory System

	Challenge dose = 2×10^6 *C. albicans*		
Time in seconds	Immune	Tetracycline	Saline
Before injection	0	0	0
0	7.4×10^5	7.3×10^5	7×10^5
30	5.8×10^4	6.0×10^4	6×10^4
60	9.2×10^3	1.1×10^4	1×10^4
120	1×10^3	2.0×10^3	2×10^3
300	1×10^2	1.4×10^2	1.5×10^2
600	5×10^1	5.5×10^1	7×10^1

and kidney at designated time intervals. The results of the in vivo experiments are presented in Figure 1. It was observed that the kidneys were the most affected organs in all groups. By visual inspection, the kidneys from the immunized animals appeared more normal than the endotoxin, tetracycline treated or saline treated animals' kidneys. These latter kidneys appeared swollen, pale in color and had multiple petechial hemorrhages at the surface. The livers and spleens of all the animals, regardless of the group, appeared normal.

The average colony count per group with five mice per group for each of 4 days is given in Figure 1. If the number of colonies was greater than 200 or too numerous to count, the sample was scored as 200 on the graph.

At 24 hours post challenge, there were few differences. There were no significant differences in the colony counts from the liver samples. The spleen samples showed a small difference between the immune animals' spleens and the normal animals' spleens. There did not appear to be any significant differences between the immune or normal spleens when compared to the endotoxin and tetracycline spleen samples. The counts for the kidney samples from the tetracycline group and endotoxin treated group appeared to be greater than the counts for the immune kidney and normal kidney samples. There appeared to be no differences in counts between the immune kidney samples and the normal kidney samples. The liver samples tested on days 2, 3, and 4 showed few or no colonies. Thus, no significant differences in counts were observable in either liver or spleen samples tested on the second, third, or fourth days. The kidney samples on day 2 indicated that the saline treated animals' kidneys and

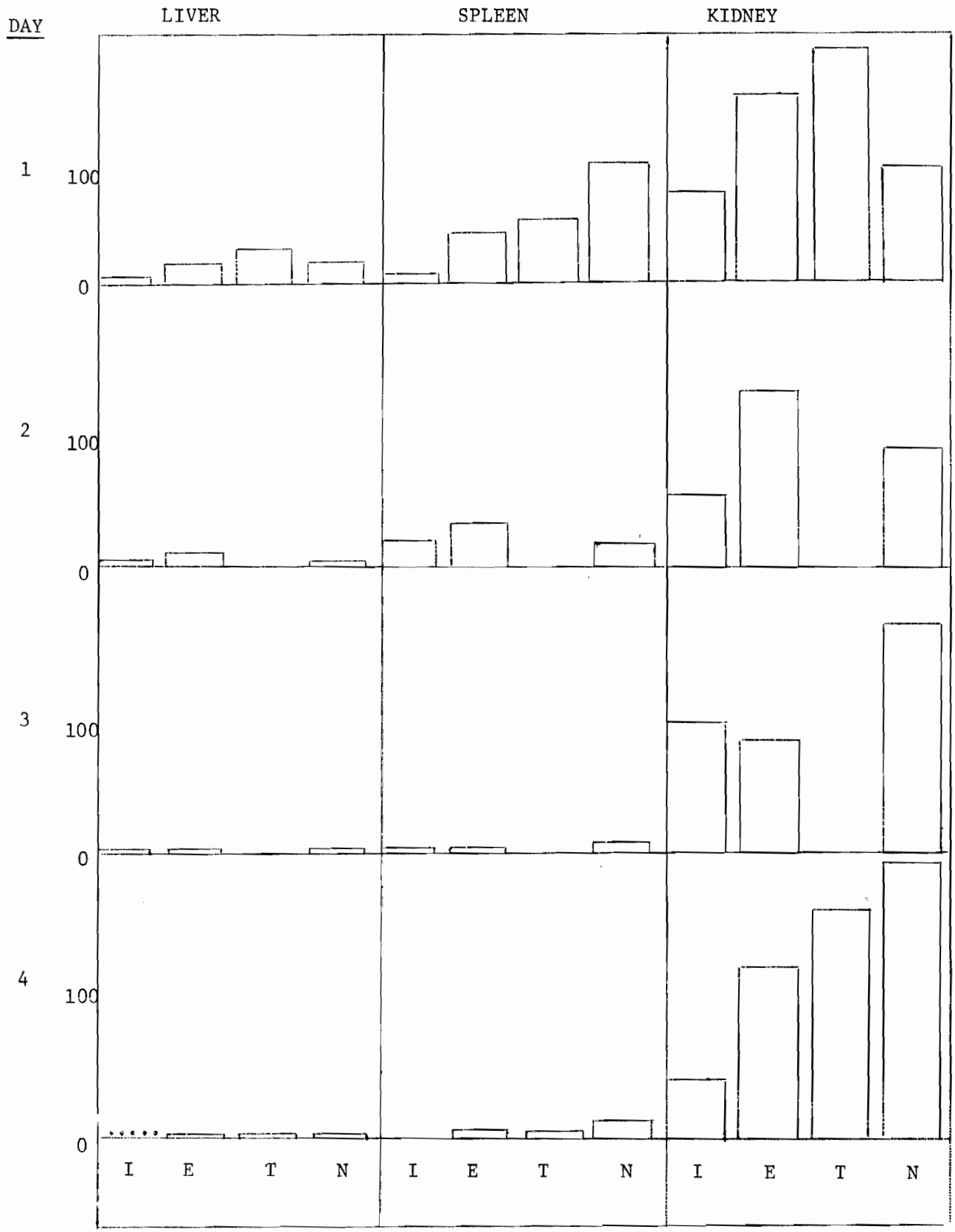

Figure 1. Candida albicans infection: post challenge plate counts from mouse organs. I=immunized, E=endotoxin treated, T=tetracycline treated, N=saline treated.

the immunized animals' kidneys were nearly equal in colony counts. The endotoxin treated animals' kidneys had a greater number of counts. However, it is unlikely that the difference is significant. By day three, the saline treated animals' kidneys had a greater number of colony counts than did the endotoxin treated or the immune animals' kidneys.

The samples collected on day four showed the saline treated animals' kidneys to contain too many organisms to be counted by the method used. The tetracycline treated animals' kidneys had high numbers of colonies but the colony counts were less than the colony counts in the group of saline treated animals. The endotoxin treated animals also had a high number of colony counts from the kidney samples; however, the colony counts were not as great as the tetracycline or the saline treated animals' kidney colony counts. The immunized animals' kidney samples had significantly fewer colony counts than the endotoxin, tetracycline and saline treated groups.

It would appear that by day four the immunized animals were starting to control the infection whereas the other groups were showing no significant change in the number of colony counts or an increase in colony counts from previously tested samples.

The apparent differences in colony counts in the kidney samples indicate some degree of enhanced resistance among the immunized animals when compared to the other groups. Reasons for these differences were sought at a cellular level.

Removal of C. albicans from Tissue Culture Monolayers by Washing with PBS

An experiment was designed to determine the success of removing extracellular organisms from a cell monolayer by washing the monolayer. One

million L-cells (Department of Microbiology, University of Utah), mouse fibroblasts that are poorly phagocytic and may not be phagocytic at all, were cultured in Leighton tubes. The cells were incubated for 48 hours at 37 C. After the incubation period the cells were challenged with 2×10^6 Candida albicans. The L-cells and Candida albicans were incubated for 30 minutes at 37 C. The cells were washed three times with PBS and tissue culture media was added after the final washing. A sample of the extracellular fluid was taken immediately after the tissue culture fluid had been added. Samples were also taken at 15 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 18 hours, and 24 hours. The results, presented in Table 5, indicate that no significant increase in extracellular organisms occurs during the initial 3 hours of incubation. At 18 hours of incubation the organism had multiplied to a significantly greater quantity of organisms per ml of tissue culture fluid.

These results also indicate that greater than 99.0% of the organisms were removed by washing the monolayer three times. Due to the small number of organisms that remained in the tubes despite the washings and the ability of the few residual organisms to multiply to a significant number of colonies, in a brief period, the decision was made to include 125 units of mycostatin in the media in further experiments (72).

In Vitro Cytopepsis: Macrophage Systems

Experiments were designed to determine the efficiency of macrophages from animals treated with saline, endotoxin or Candida albicans vaccine to phagocytize and destroy Candida albicans organisms.

Approximately equal numbers of macrophages (2×10^6) from the specifically treated animals were cultured in Leighton tubes. After

Table 5. Removal of Extracellular Candida albicans from L-cells by Washing

Sample Time	Number of Candida albicans recovered in 1 ml of extracellular fluid
0 minutes	210*
15 minutes	238
30 minutes	257
60 minutes	220
180 minutes	245
360 minutes	495
1080 minutes	TNC ($>10^5$)
1440 minutes	TNC

*Average colony count for 2 samples

overnight incubation, the cells were washed and challenged with approximately 1 x 10^6 organisms. In the experiments, the number of yeast cells initially phagocytized were nearly equal in all of the groups. However, the macrophages from the immunized animals appeared to be slightly more efficient in destroying the yeast cells when compared to the macrophages from the endotoxin treated and saline treated animals' macrophages. The macrophages from the endotoxin treated animals were intermediate in their cytopeptic capacities. By the sixth hour post challenge there appeared to be no differences in the cytopeptic activity in any of the groups, e.g., the number of viable yeasts were found to be nearly equal in each system. Results of the experiment are presented in Figure 2.

A modification of the above experimental design to include lymphocytes from one group of animals added to macrophages of all groups was proposed to determine if lymphocytes interact with macrophages to enhance cellular resistance.

in Vitro Cytopepsis: Macrophage Plus Lymphocyte Systems

In all of the following experiment the same basic experimental design has been employed as described below. The variable involved is the lymphocytes from the variously treated animals.

Experimental Design

1. Treat the animals with *Candida albicans* vaccine, endotoxin or saline.
2. Induce peritoneal exudate macrophages with thioglycollate.
3. Aseptically collect the macrophages from the peritoneal cavity.
4. Wash the macrophages with PBS.

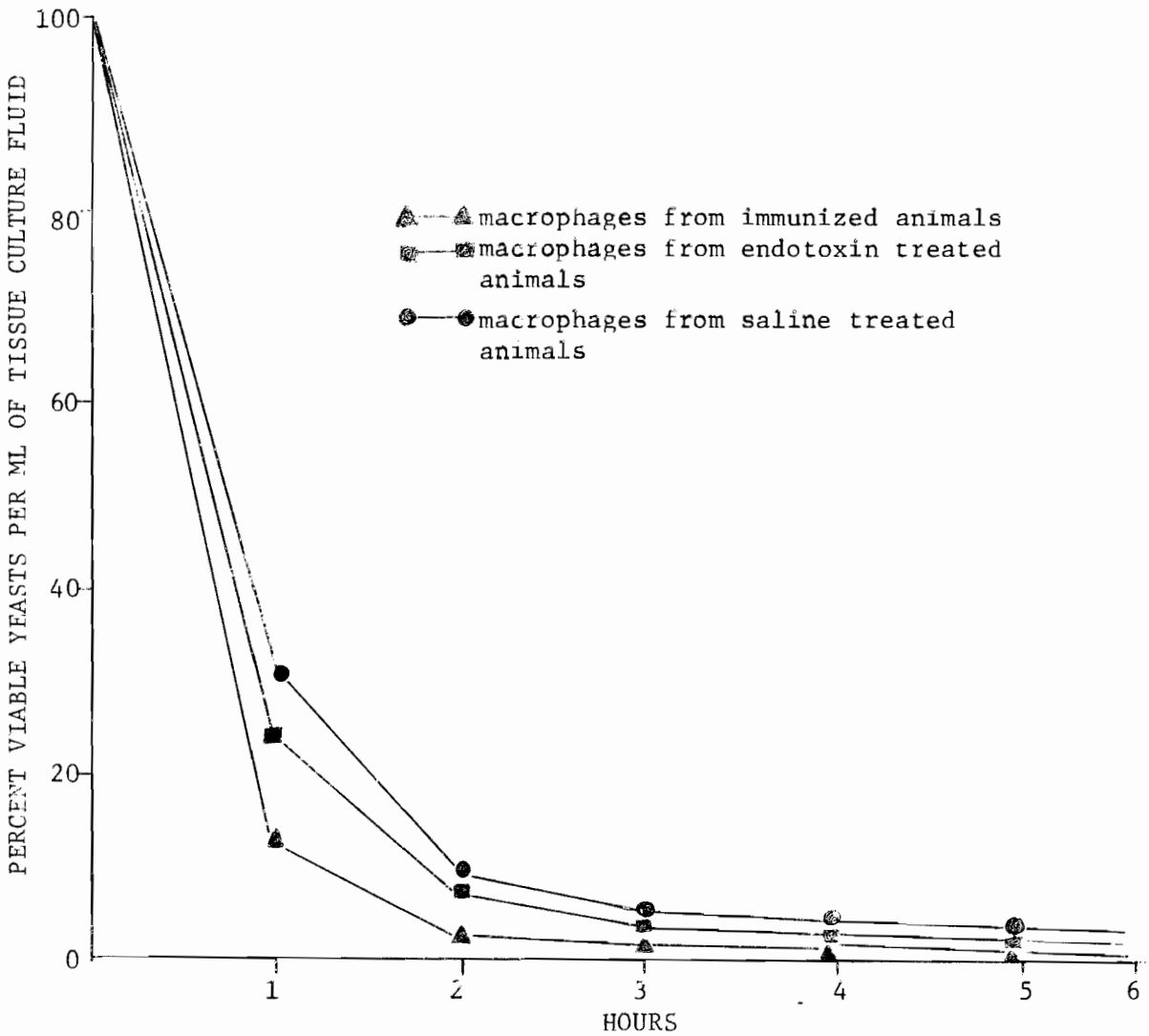

Figure 2. Comparison of the in vitro rate of destruction of Candida albicans: macrophages from immunized animals, endotoxin treated animals and macrophages from saline treated animals.

5. Aseptically collect the mesenteric and inguinal lymph nodes from the proper group of animals.

6. Count the macrophages and lymphocytes and adjust the cells to the proper concentration.

7. Incubate the exudate cells in the presence of Candidin overnight.

8. Wash the cells.

9. Challenge the cells with 1 x 10^6 Candida albicans.

10. Incubate the suspension for 30 minutes.

11. Wash the cells with PBS (3 times) and re-feed the cells.

12. Rupture the cells at specific time intervals.

13. Do plate counts for viable organisms in the resulting supernatant fluid.

Macrophages were cultured in duplicate. To one culture lymphocytes were added while the other culture was not exposed to lymphocytes. The media used to re-feed the cells after the challenge with C. albicans contained 125 units of mycostatin.

The following experimental results were obtained.

Figure 3 compares the cytopeptic activities of macrophages from immunized animals to macrophages from saline treated animals. The corresponding lymphocytes were added to each macrophage culture. The results are presented as the percent of surviving yeasts on the abcissa versus time, in hours, on the ordinate. The macrophages from the immunized animals destroyed most of the yeast cells during the initial two hours. The macrophages from saline treated animals were significantly less efficient at this time in destroying yeast cells.

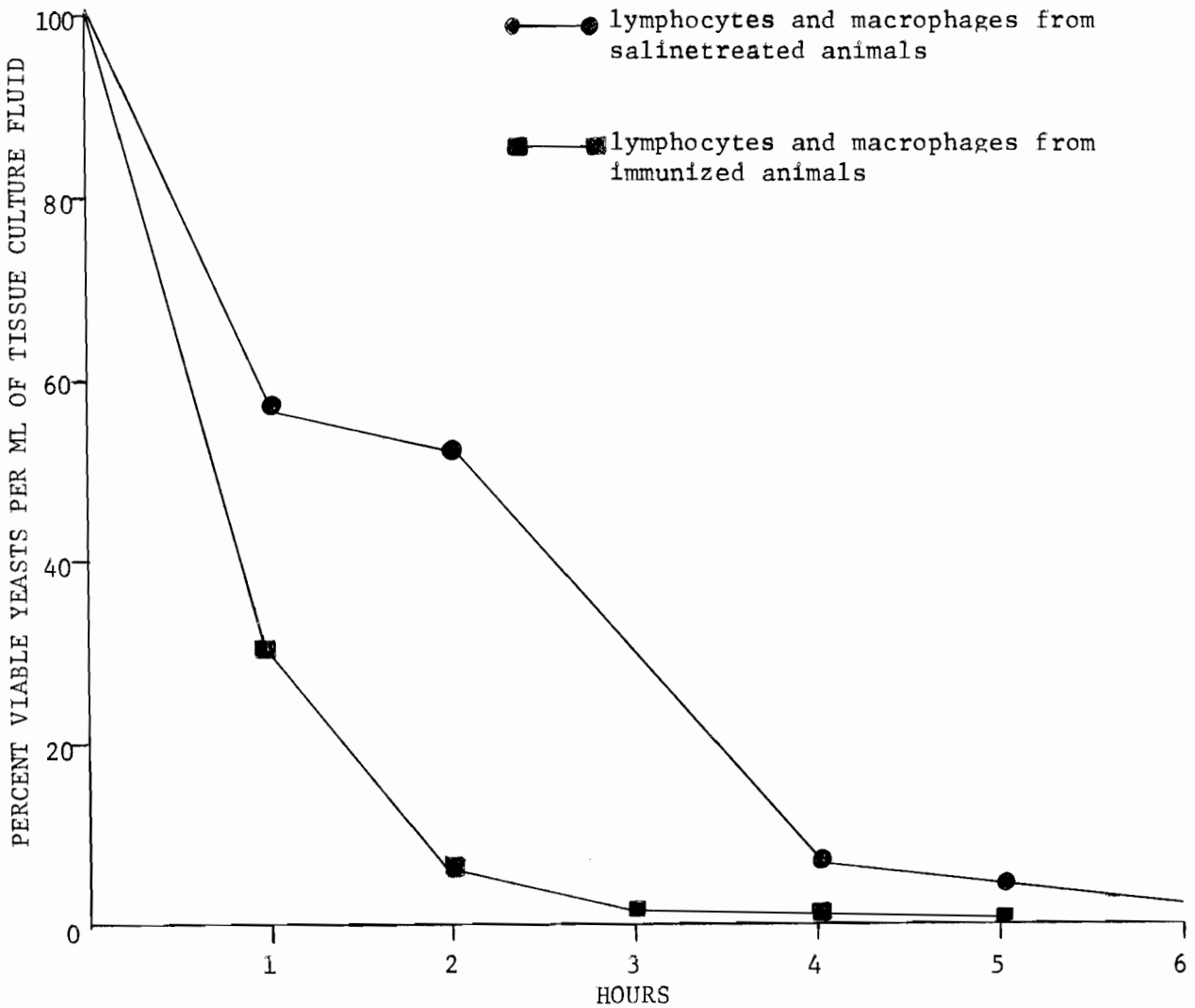

Figure 3. Comparison of the *in vitro* rates of destruction of *Candida albicans*: immune lymphocyte-macrophage system versus the saline lymphocyte-macrophage system.

By the end of the fourth hour the numbers of yeasts alive in normal and immune macrophages had diminished to insignificant differences. Only a few organisms were alive after six hours, that is, at the termination of the experiment. The most significant difference in the number of viable yeast cells was observed at two hours post challenge. The data in this figure was observed to be a first order rate reaction. Subsequent comparisons made between various combinations of lymphocytes and macrophages from immunized animals, endotoxin treated animals and saline treated animals, were also observed to be a first order rate reaction. Slope regression analysis (Figure 4) and estimation of 50% survival (ET_{50}) of the yeast cells indicate a significant difference at the 95% confidence level. The statistical analysis was done according to the method of Litchfield and Wilcoxin (37).

Figure 5 shows results obtained when macrophages from endotoxin treated animals plus lymphocytes from immune animals were compared to a lymphocyte-macrophage combination from saline treated animals. The figure shows that there is an enhanced capacity to destroy the C. albicans cells until the fourth hour. The immune cell system reaches its asymptote by the second hour; the "normal" cell system reaches a similar asymptote by the fourth hour. The slope regression analysis (Figure 6) shows the linear relationship of the two systems. The difference in ET_{50} and in slope regression was statistically significant at the 95% confidence levels.

Figure 7 compares cytopeptic capacities of the system containing lymphocytes from immunized animals plus macrophages from saline treated animals to lymphocytes and macrophages from saline treated animals.

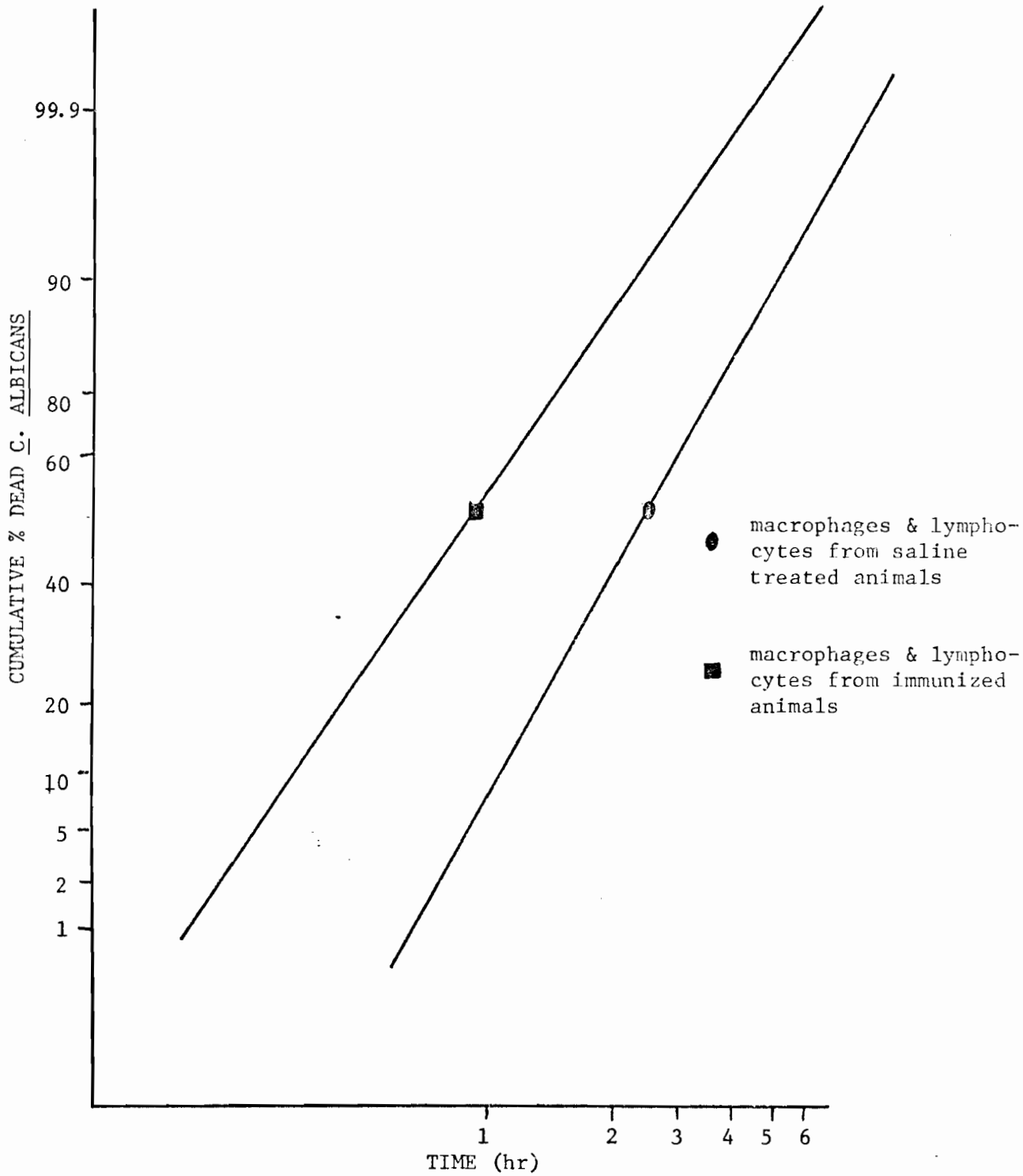

Figure 4. Slope regression analysis of the in vitro rate of destruction of Candida albicans in the systems containing lymphocytes and macrophages from immunized animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

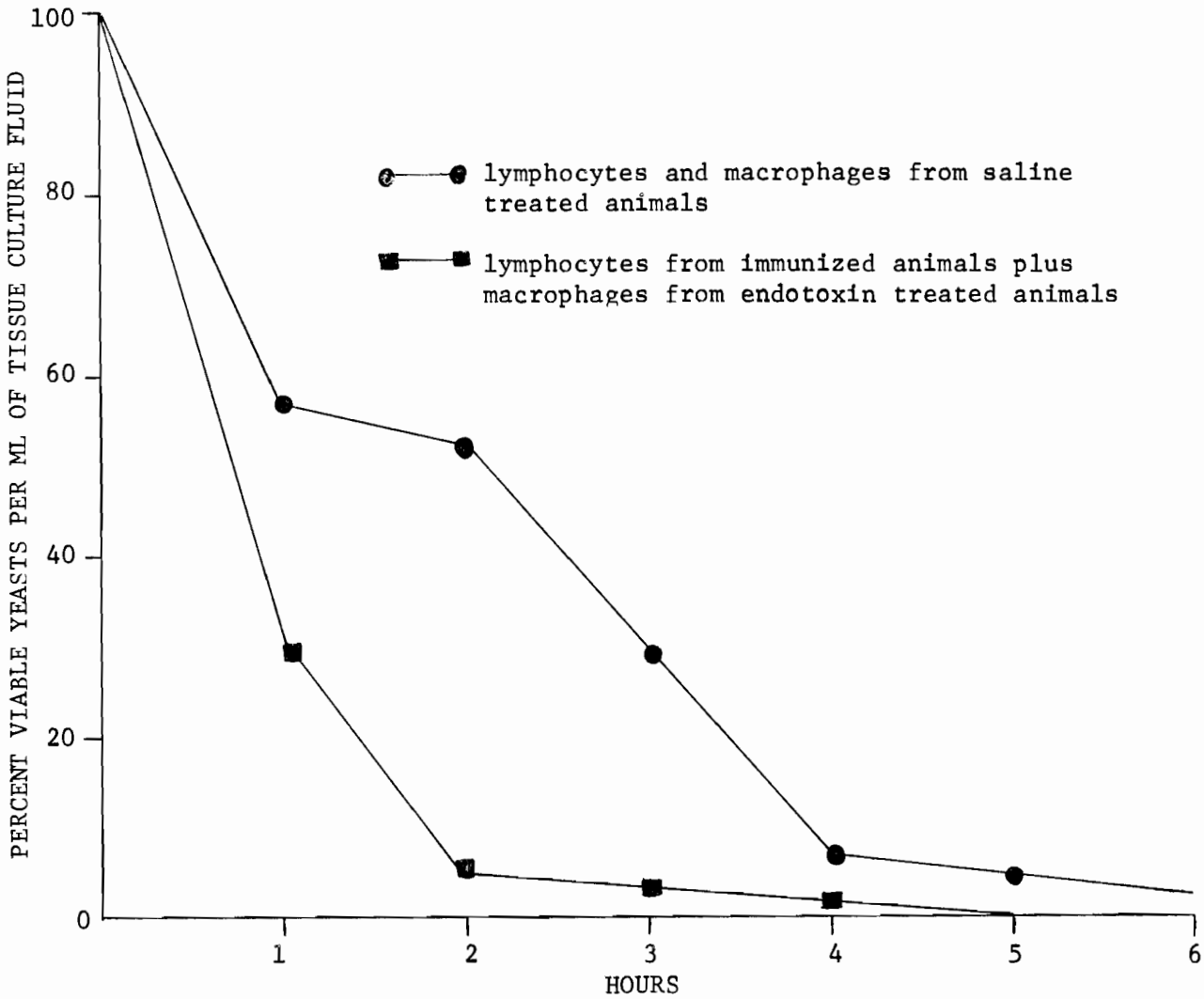

Figure 5. Comparison of the *in vitro* rates of destruction of *Candida albicans*: lymphocytes from immunized animals plus macrophages from endotoxin treated animals versus lymphocytes and macrophages from saline treated animals.

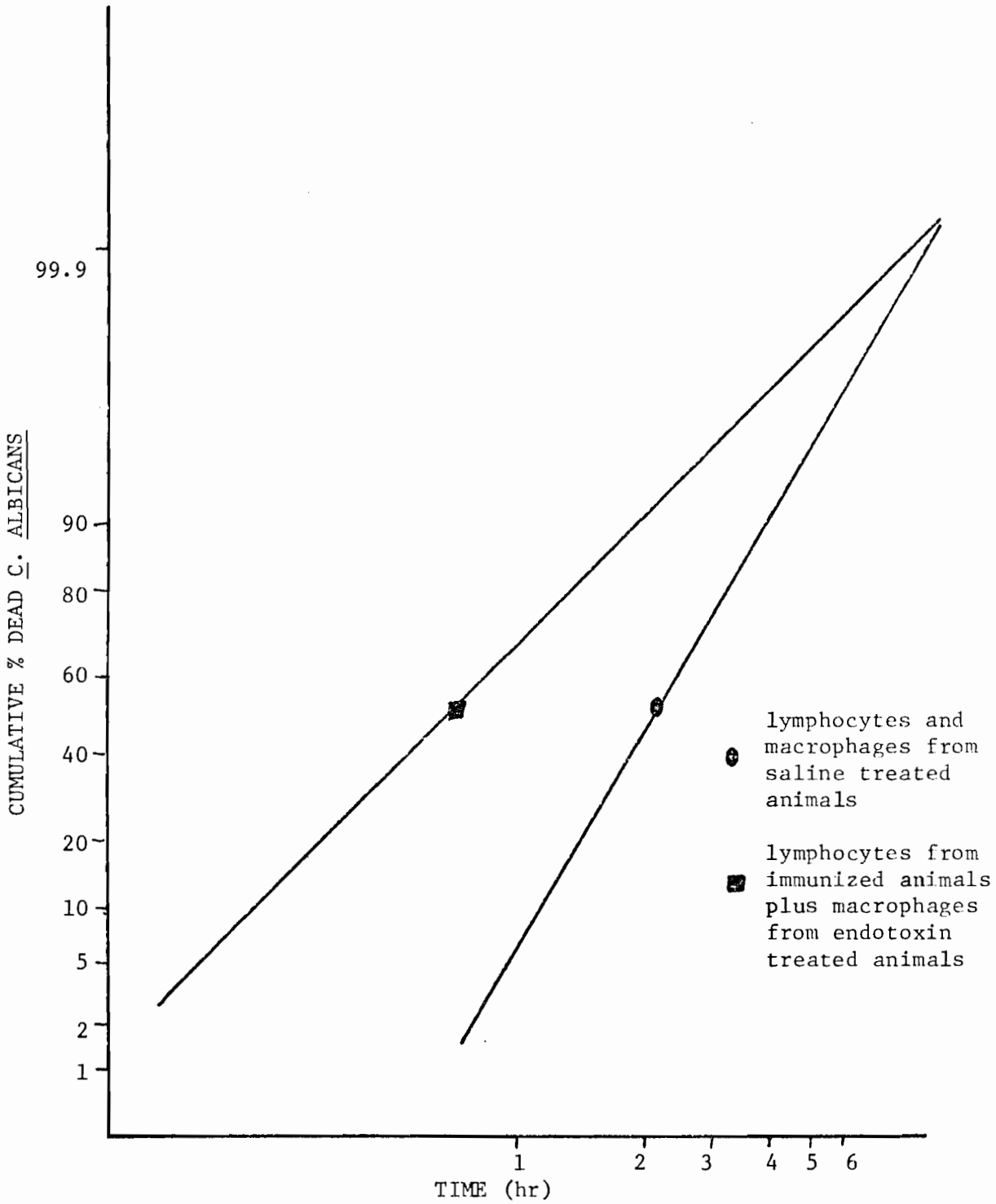

Figure 6. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes from immunized animals plus macrophages from endotoxin treated animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

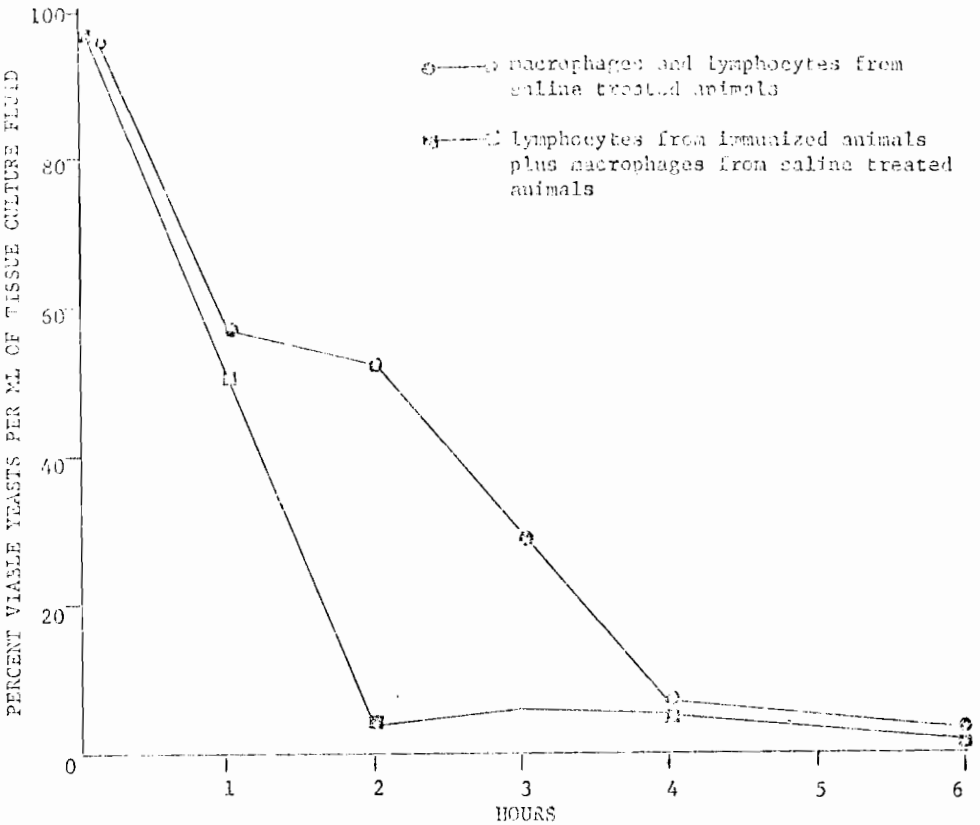

Figure 7. Comparison of the in vitro rates of destruction of Candida albicans: lymphocytes from immunized animals plus macrophages from saline treated animals versus lymphocytes and macrophages from saline treated animals.

During the initial hour the two systems seemed to be equally effective in destroying the yeast cells. However, by the end of the second hour the system composed of lymphocytes from immunized animals plus macrophages from the saline treated animals system. This increased efficiency diminished with time and by the fourth hour the cytopeptic capacities of the two systems had reached asymptomatic levels. Figure 8 presents the slope regression analysis for this system. The slope of the immune lymphocytes plus saline treated macrophage system is significantly different from the slope of the saline lymphocyte-macrophage system, as is the ET_{50} for the comparison of these two systems.

Figures 9 and 10 show the comparisons of the system containing lymphocytes from endotoxin treated animals plus macrophages from immunized animals to the system containing lymphocytes and macrophages from saline treated animals. The data show moderate differences developing after the initial hour post challenge. This system does not show the efficiency observed in the system containing macrophages and lymphocytes from immunized animals, or the system containing macrophages from endotoxin or saline treated animals. Slope regression analysis and the ET_{50} values are significantly different from the saline treated control system.

Figure 11 compares the lymphocyte-macrophage system from endotoxin treated animals to the lymphocyte-macrophage system from saline treated animals. The cells from the endotoxin treated animals were more efficient in post phagocytic killing of the yeast cells than the saline treated animal cells. This homologous system of endotoxin treated lymphocytes macrophages appears to be more active cytopeptically than the previously

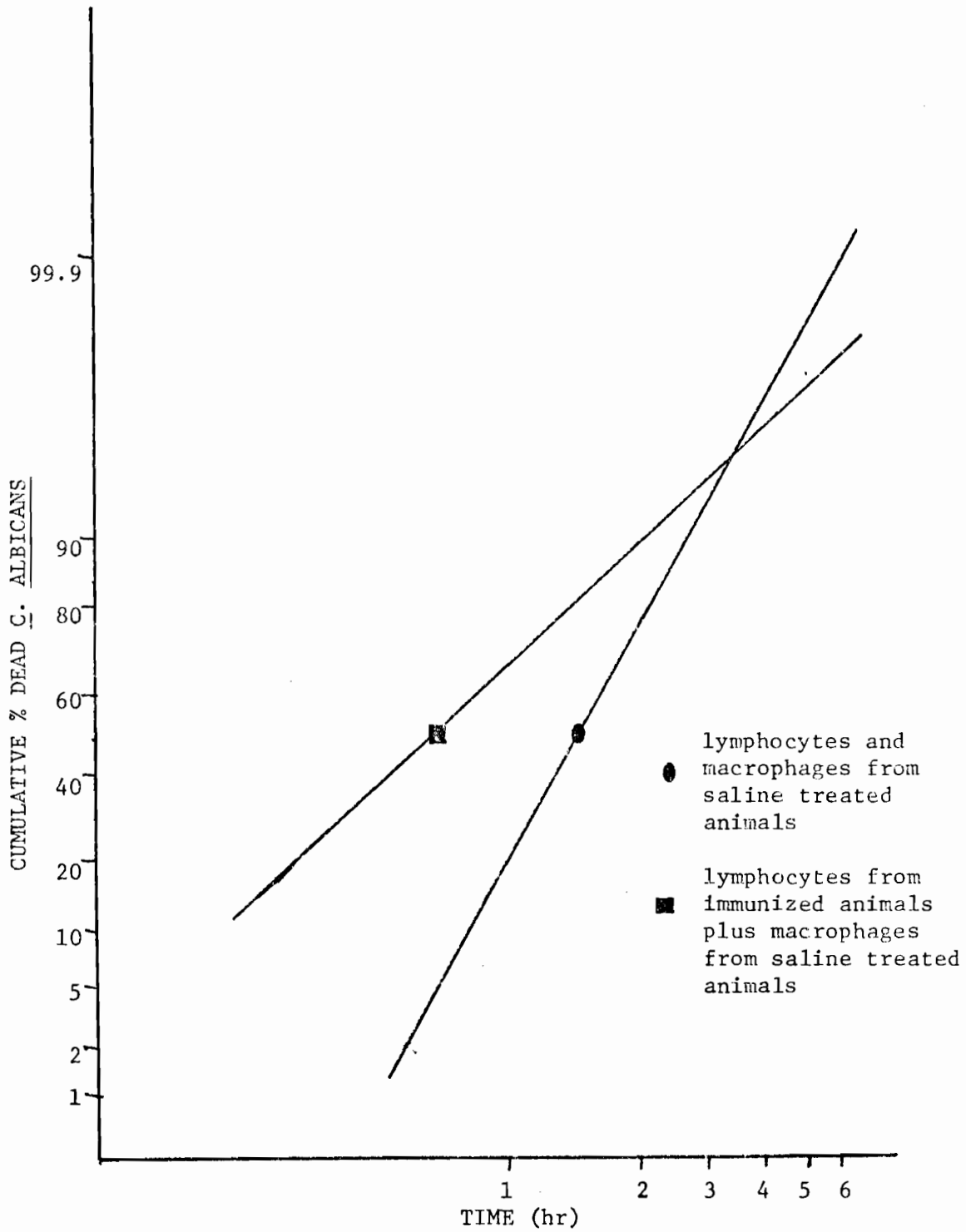

Figure 8. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes from immunized animals plus macrophages from saline treated animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

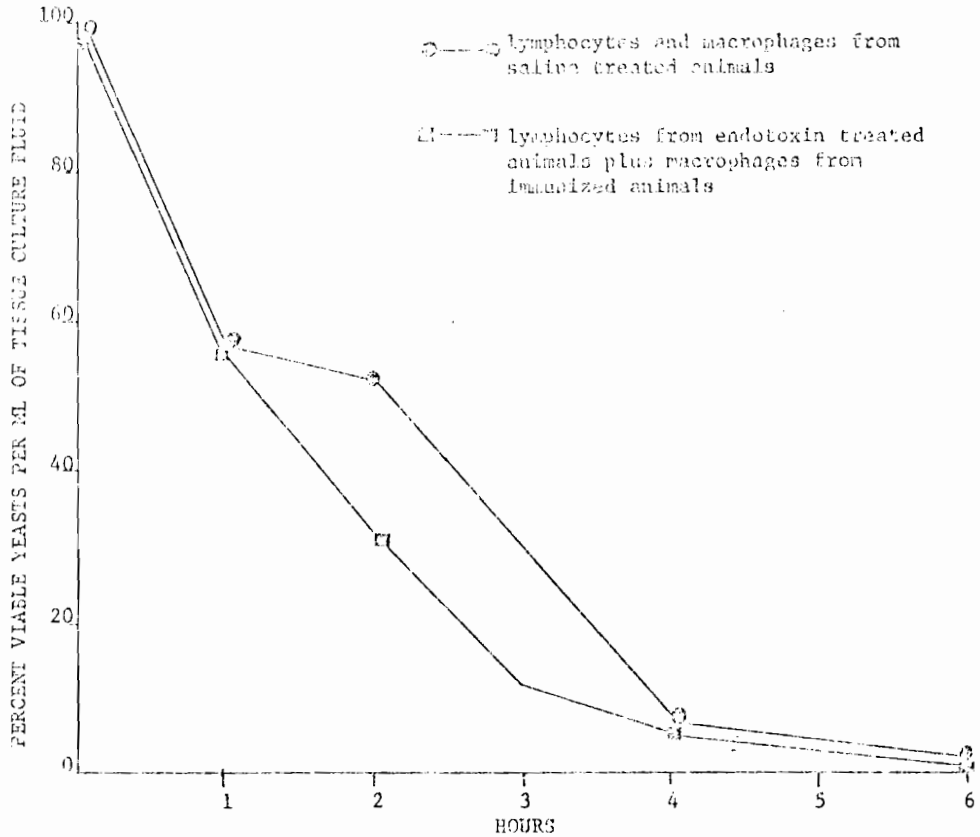

Figure 9. Comparison of the *in vitro* rates of destruction of *Candida albicans*: lymphocytes from endotoxin treated animals plus macrophages from immunized animals versus lymphocytes and macrophages from saline treated animals.

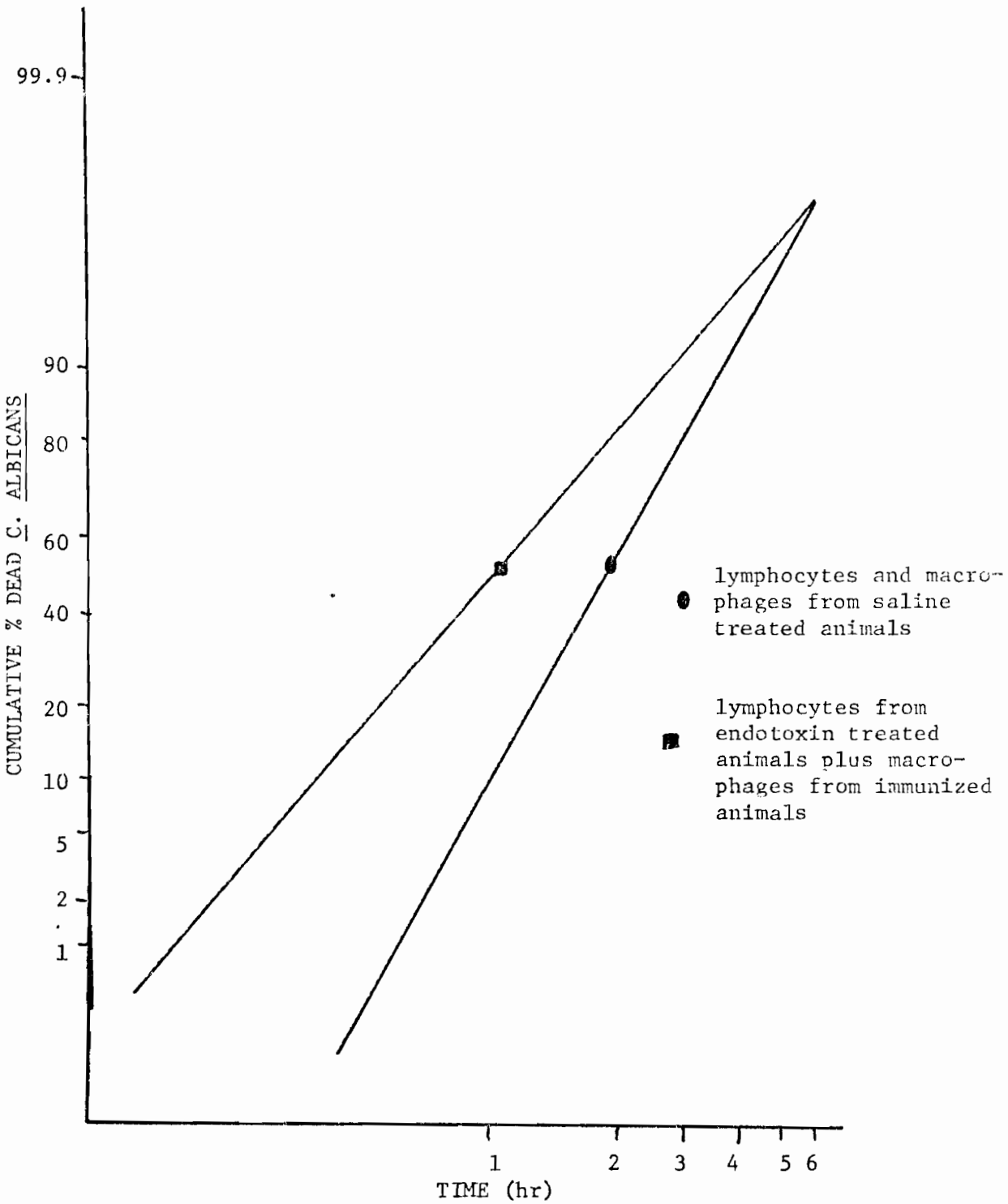

Figure 10. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes from endotoxin treated animals plus macrophages from immunized animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

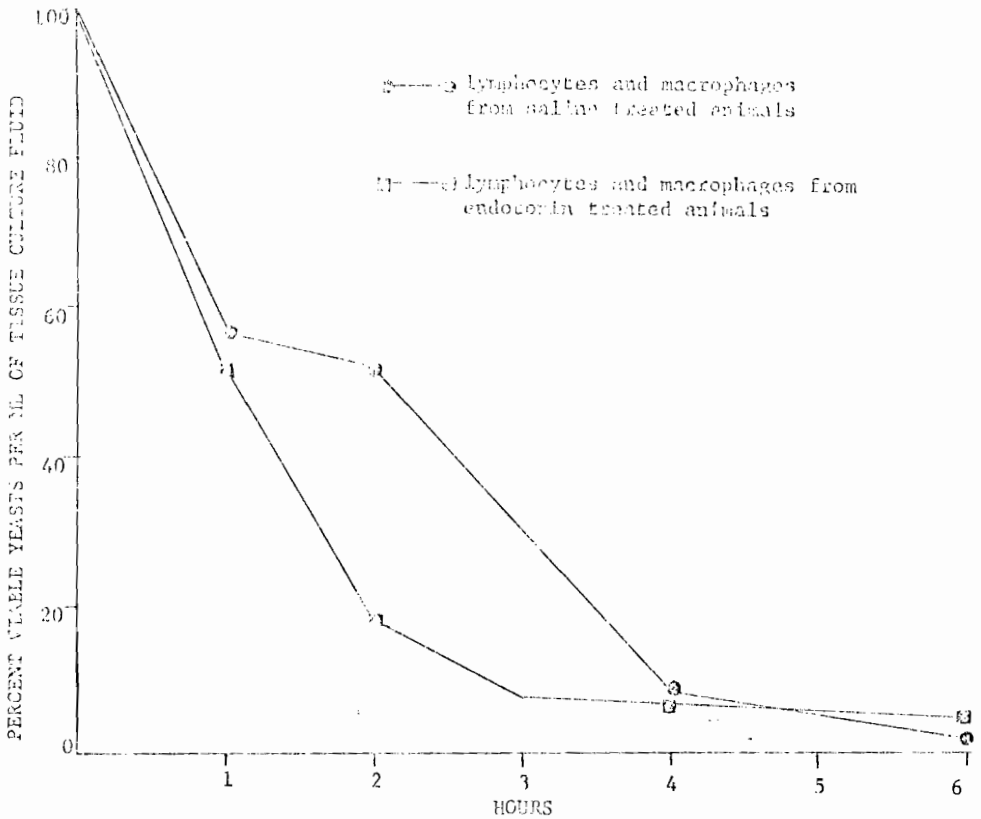

Figure 11. Comparison of the *in vitro* rates of destruction of *Candida albicans*: lymphocytes and macrophages from endotoxin treated animals versus lymphocytes and macrophages from saline treated animals.

examined heterologous system containing lymphocytes from endotoxin treated animals and macrophages from immunized animals. Statistical analysis of these data show that significant differences exist between the two systems in terms of ET_{50} and slope regression values (Figure 12).

Figure 13 shows a comparison of the system involving lymphocytes from endotoxin treated animals plus macrophages from saline treated animals to the system of lymphocytes and macrophages from saline treated animals. The data suggest moderate enhancement of the cytopeptic rate in the endotoxin involved system. As observed in previous systems, the differences diminished by the fourth hour of the experiment. The slope regression (Figure 14) and the ET_{50} values were significantly different at the 95% confidence level.

Figure 15 presents data from the system utilizing lymphocytes from saline treated animals plus macrophages from immunized animals. The data show no differences between this system and the saline control system during the initial hour of the experiment. The subsequent three hours show moderate differences which have diminished by the fourth hour of the experiment. Statistical analysis of the slope regression (Figure 16) shows that the slope of the test system is not parallel with the control system. Slope regression analysis and ET_{50} analysis indicate significant differences exist at the 95% level of confidence.

Figure 17 compares lymphocytes from saline treated animals plus macrophages from endotoxin treated animals to the system composed of lymphocytes and macrophages from saline treated animals. This figure shows little or no difference during the initial hour of the experiment. The second and third hour samples show marked differences from the

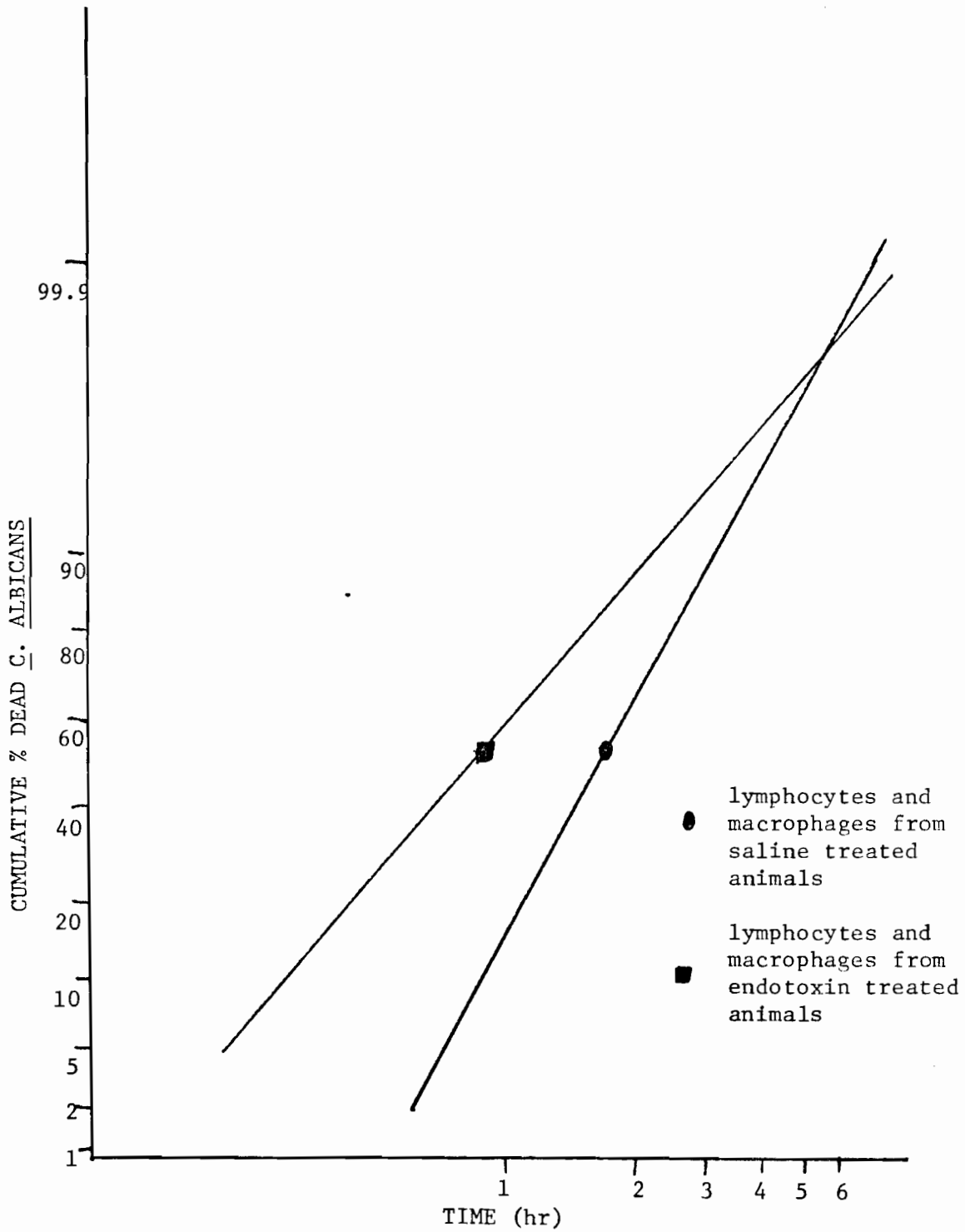

Figure 12. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes and macrophages from endotoxin treated animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

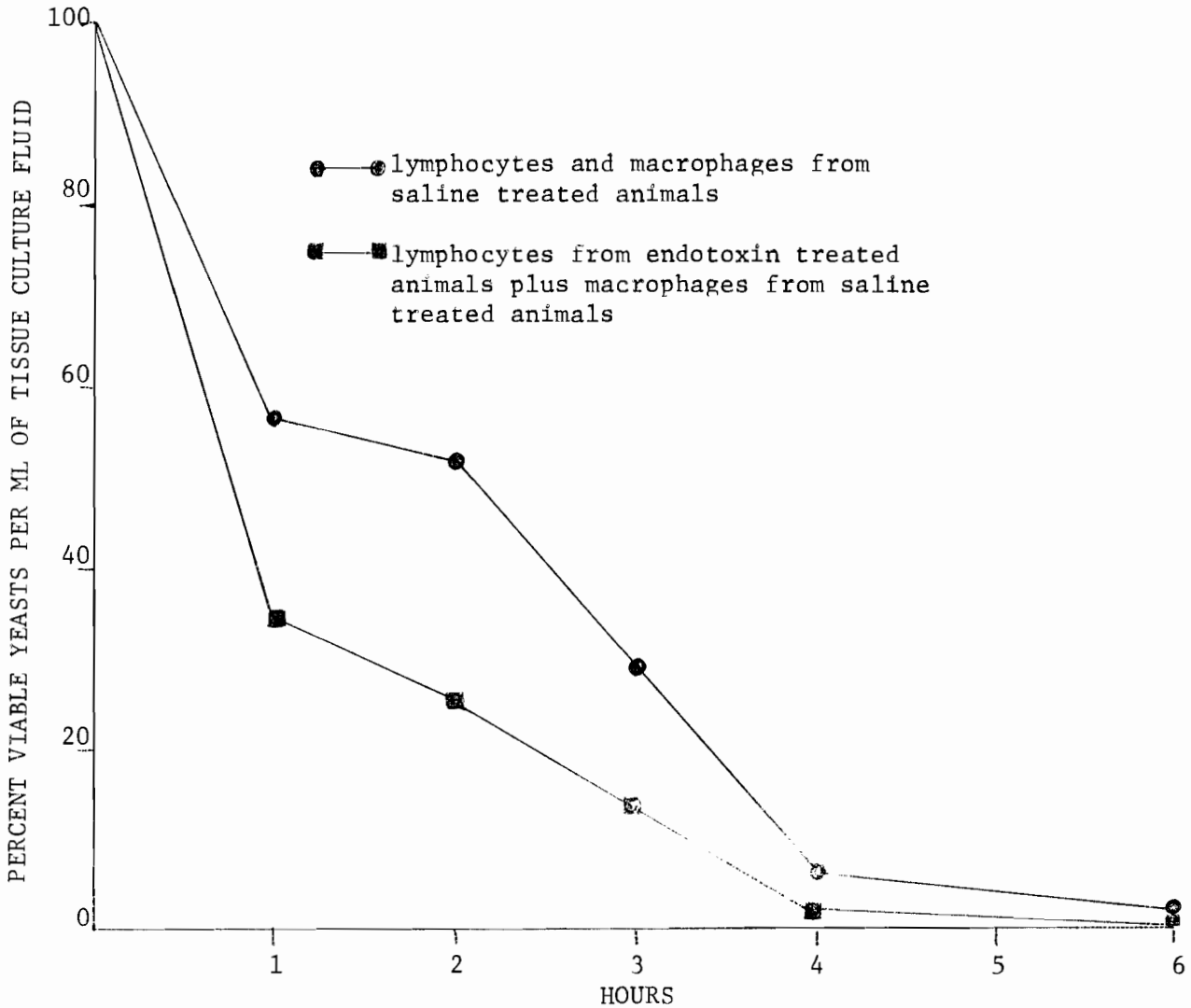

Figure 13. Comparison of the in vitro rates of destruction of Candida albicans: lymphocytes from endotoxin treated animals plus macrophages from saline treated animals versus lymphocytes and macrophages from saline treated animals.

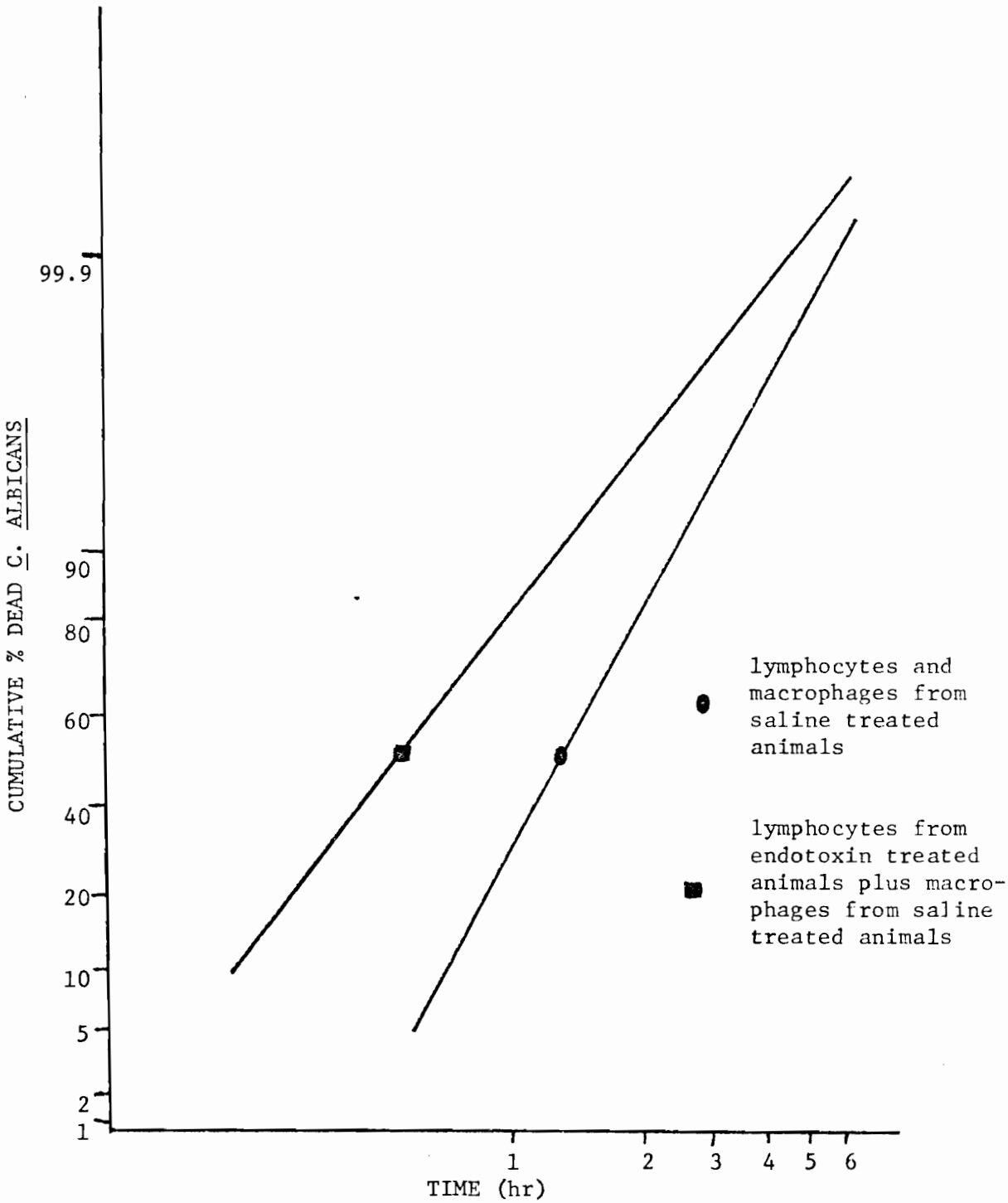

Figure 14. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes from endotoxin treated animals plus macrophages from saline treated animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

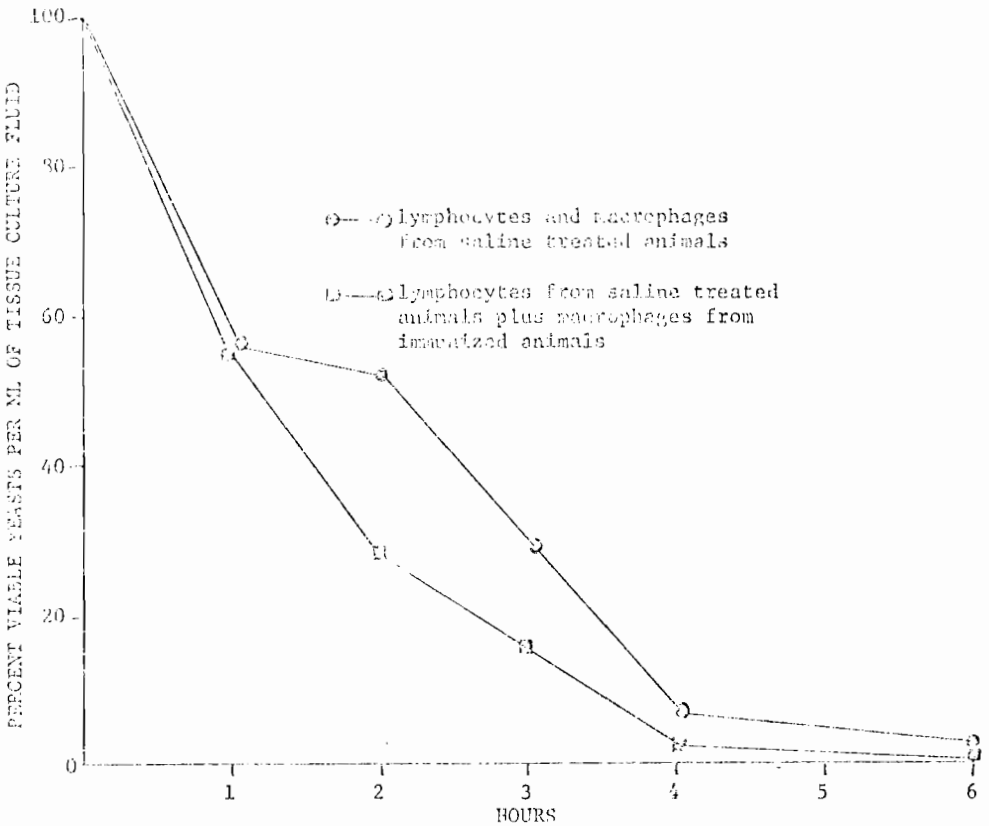

Figure 15. Comparison of the in vitro rates of destruction of Candida albicans: lymphocytes from saline treated animals plus macrophages from immunized animals versus lymphocytes and macrophages from saline treated animals.

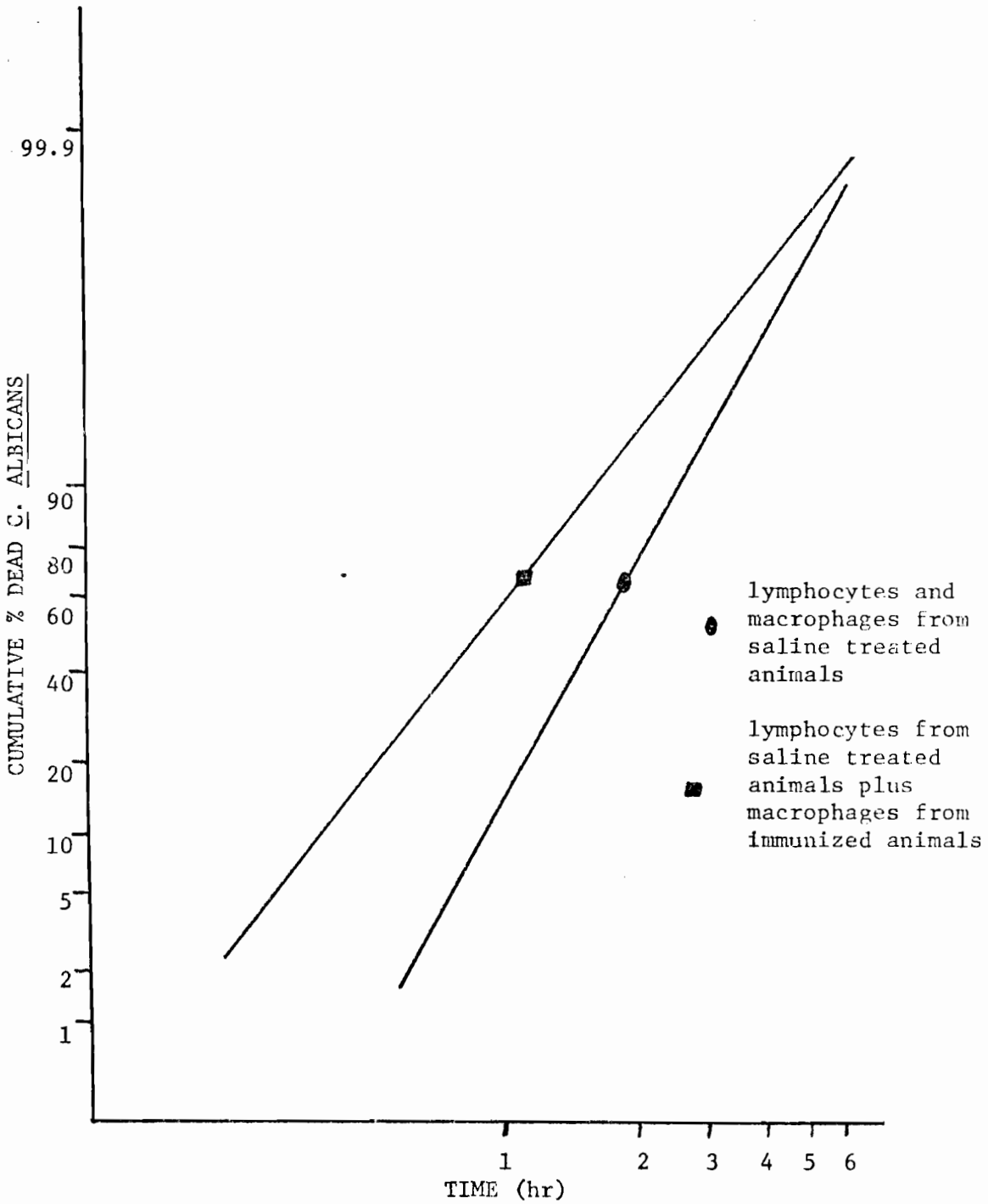

Figure 16. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes from saline treated animals plus macrophages from immunized animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

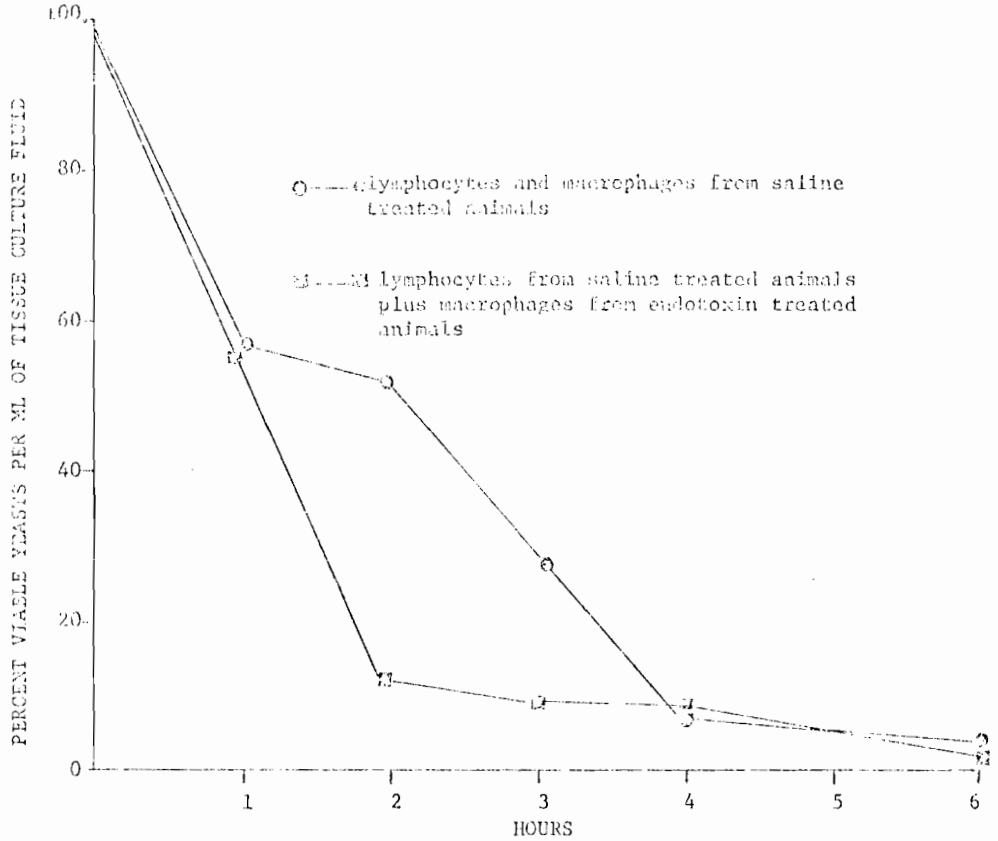

Figure 17. Comparison of the *in vitro* rates of destruction of *Candida albicans*: lymphocytes from saline treated animals plus macrophages from endotoxin treated animals versus lymphocytes and macrophages from saline treated animals.

saline control system. However, by the fourth hour the differences have diminished. Figure 18 is the slope regression comparison of the two systems and is significant at the 95% confidence level as are the ET_{50} values.

Macrophages from immunized mice, endotoxin treated mice and saline treated mice were cultured and challenged in the absence of lymphocytes. The macrophage systems were compared to a lymphocyte free macrophage system from saline treated animals in addition to the lymphocyte-macrophage system from saline treated animals.

When immune macrophages were compared to saline treated macrophages, slight but significant differences were observed. These data are shown in Figure 19. The macrophages from the immunized mice are represented by triangles and the macrophages from the saline treated animals are represented by circles.

When the macrophages from the immunized animals were compared to the lymphocyte-macrophage system from saline treated animals, the differences of greatest magnitude were observed. Figure 20 presents the comparison of the macrophages from immunized animals to the lymphocyte-macrophage system from saline treated animals.

Macrophages from endotoxin treated animals were compared to macrophages from saline treated animals. The slopes of the lines are nearly equal as shown in Figure 21. However, the magnitude of difference is great enough to be significant at the 95% level of confidence. The ET_{50} is statistically significantly different from the saline macrophage system. When the cytopeptic activity of macrophages from endotoxin treated animals was compared to the

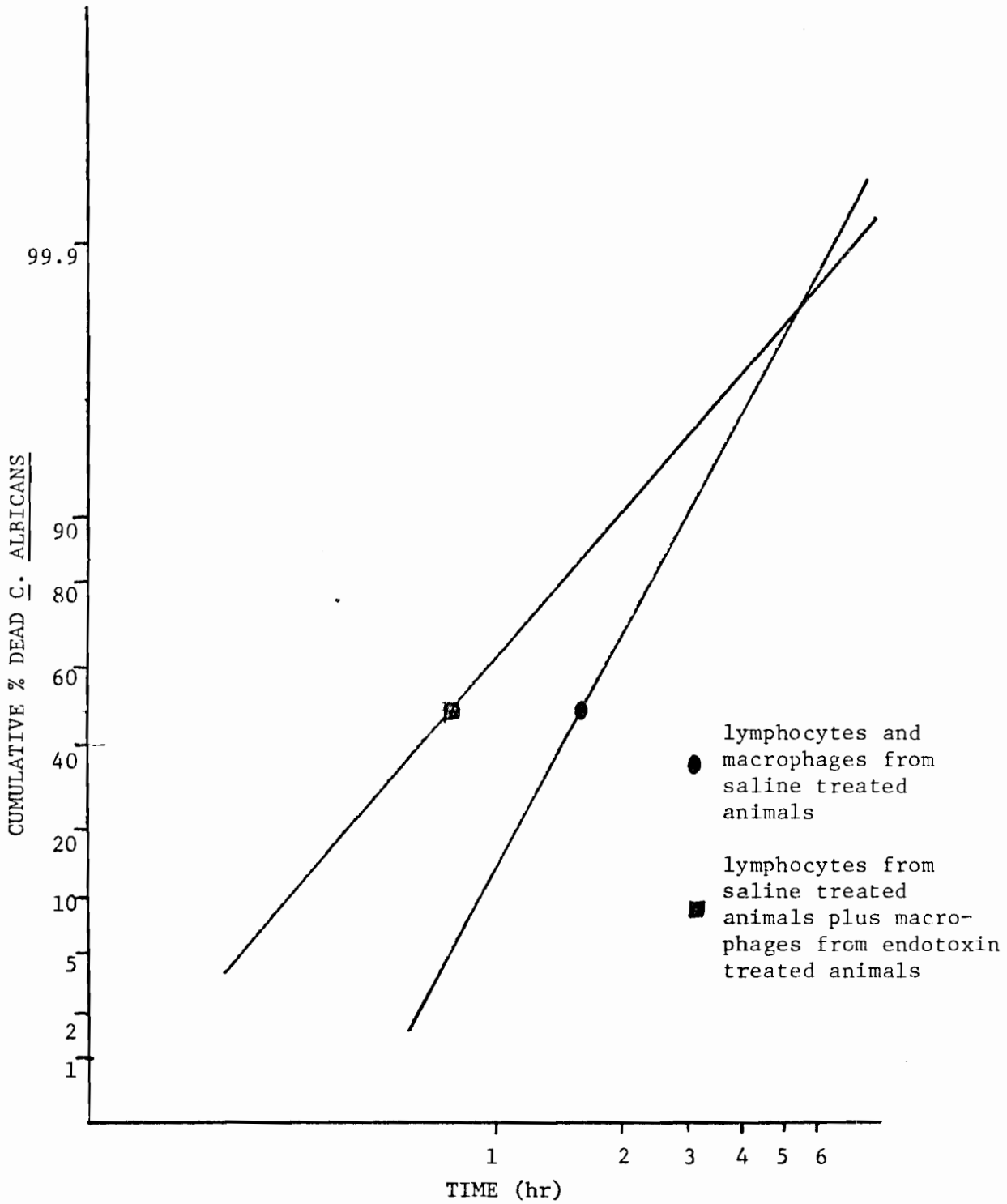

Figure 18. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing lymphocytes from saline treated animals plus macrophages from endotoxin treated animals versus lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

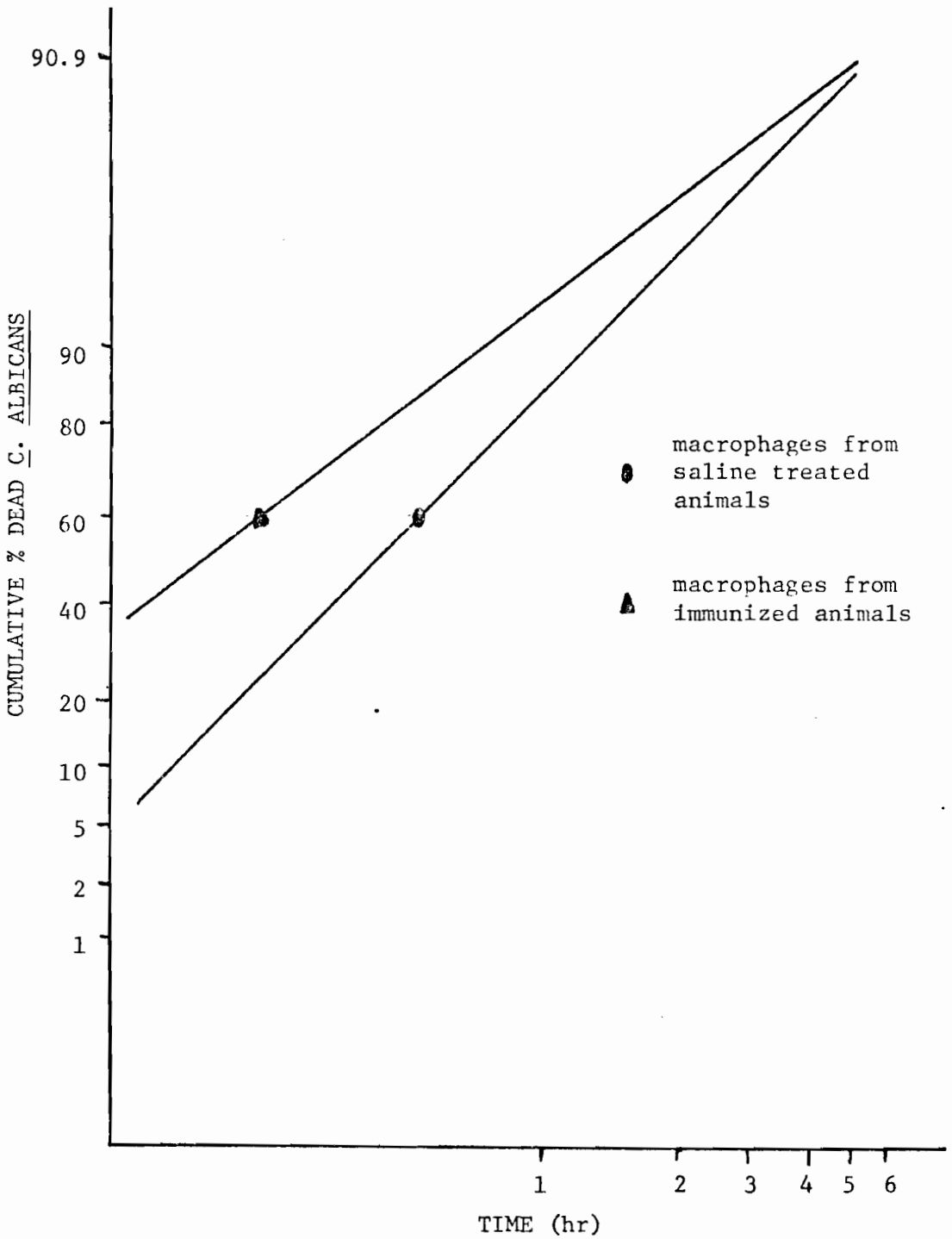

Figure 19. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the system containing macrophages from immunized animals vs. macrophages from saline treated animals. Symbols mark ET_{50} values.

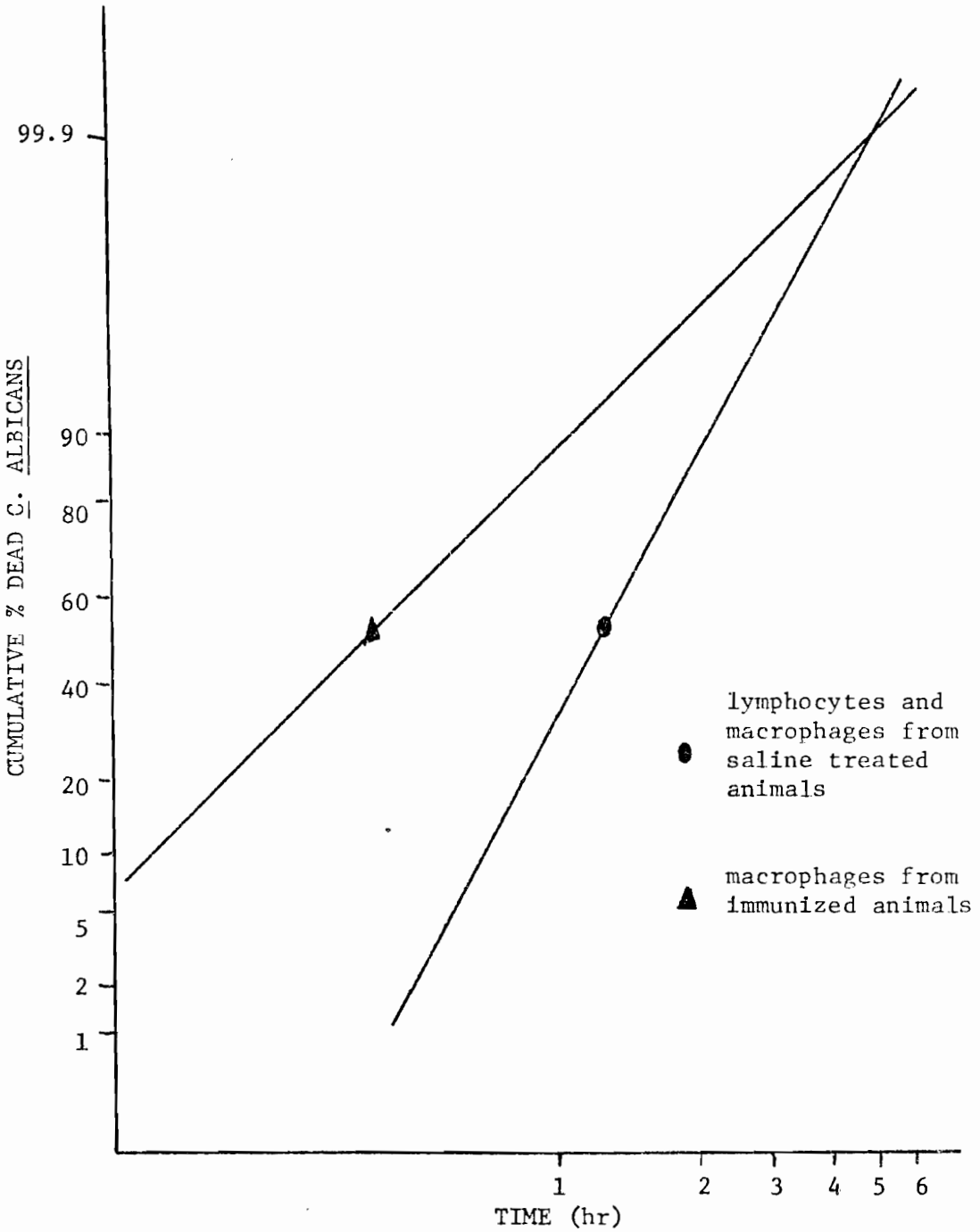

Figure 20. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing macrophages from immunized animals vs. lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

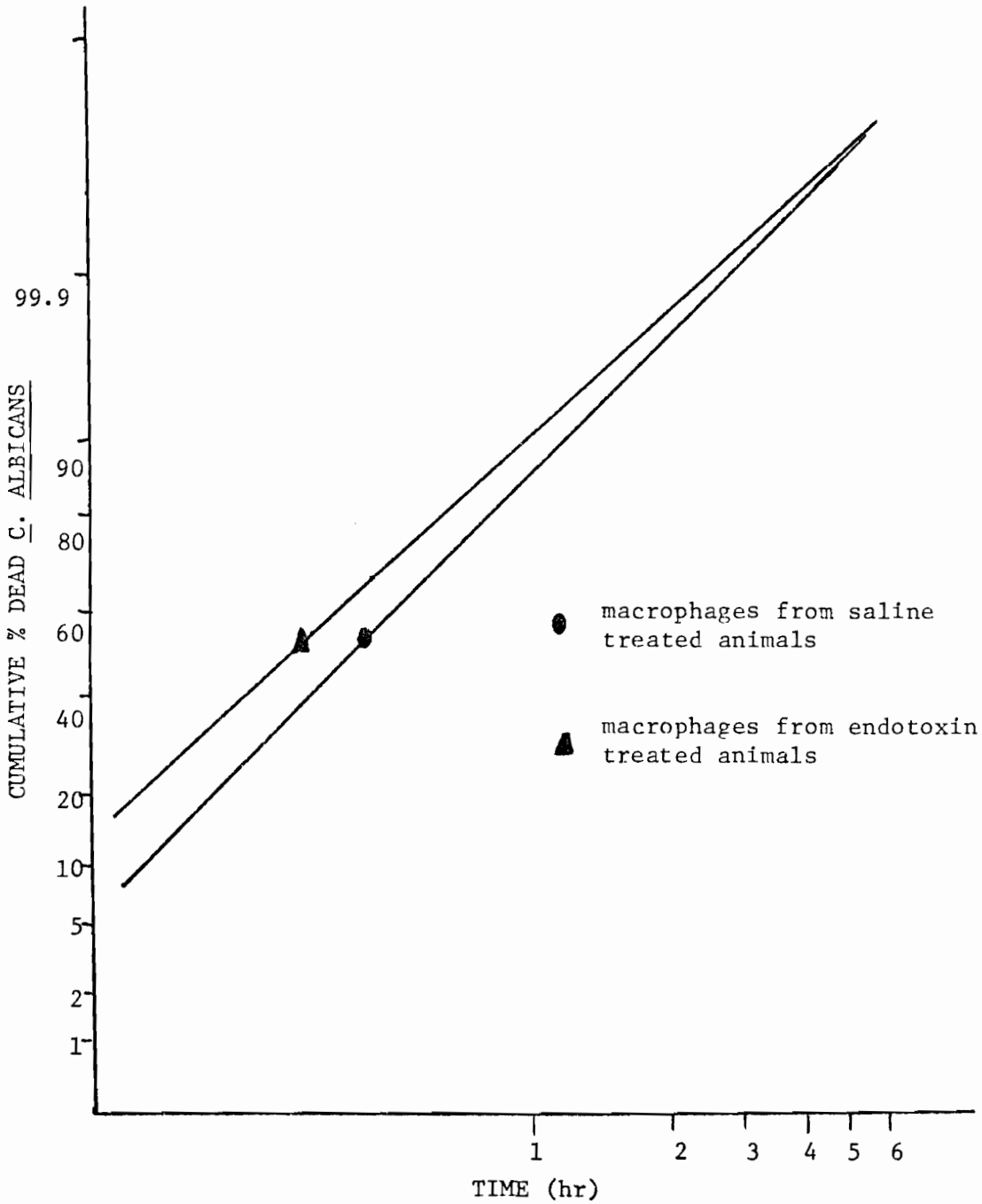

Figure 21. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing macrophages from endotoxin treated animals vs. macrophages from saline treated animals. Symbols mark ET_{50} values.

cytopeptic activity of the lymphocyte-macrophage system from saline treated animals, large differences were observed (Figure 22). The endotoxin treated macrophage system was significantly more cytopeptically active than the saline treated lymphocyte-macrophage system.

Surprisingly, when macrophages from the saline treated animal system were compared to the saline treated lymphocyte-macrophage system, significant differences were observed with the more rapid cytopepsis in favor of the macrophage alone. The lines representing the systems containing the lymphocytes plus macrophages from saline treated animals and the saline treated macrophages alone are shown in Figure 23. The ET_{50} of the saline treated system is also significantly different from the ET_{50} of the saline treated lymphocyte-macrophage system.

Figure 24 compares all of the lymphocyte-macrophage systems. The saline treated lymphocyte-macrophage system is represented by S-S. The saline treated lymphocyte-macrophage system is represented by S-E. The endotoxin treated lymphocyte-saline treated macrophage system is represented by E-S. The endotoxin treated lymphocyte-macrophage system is represented by E-E. The endotoxin treated lymphocyte-immune macrophage system is represented by E-I. The system containing lymphocytes from immunized animals plus macrophages from saline treated animals is represented by I-S. The system composed of the immunized animals' lymphocytes plus macrophages from endotoxin treated animals is represented by I-E. The system containing lymphocytes and macrophages from immunized animals is represented by I-I.

Figure 25 compares macrophages from immunized mice (represented by hexagons), macrophages from endotoxin treated animals (represented

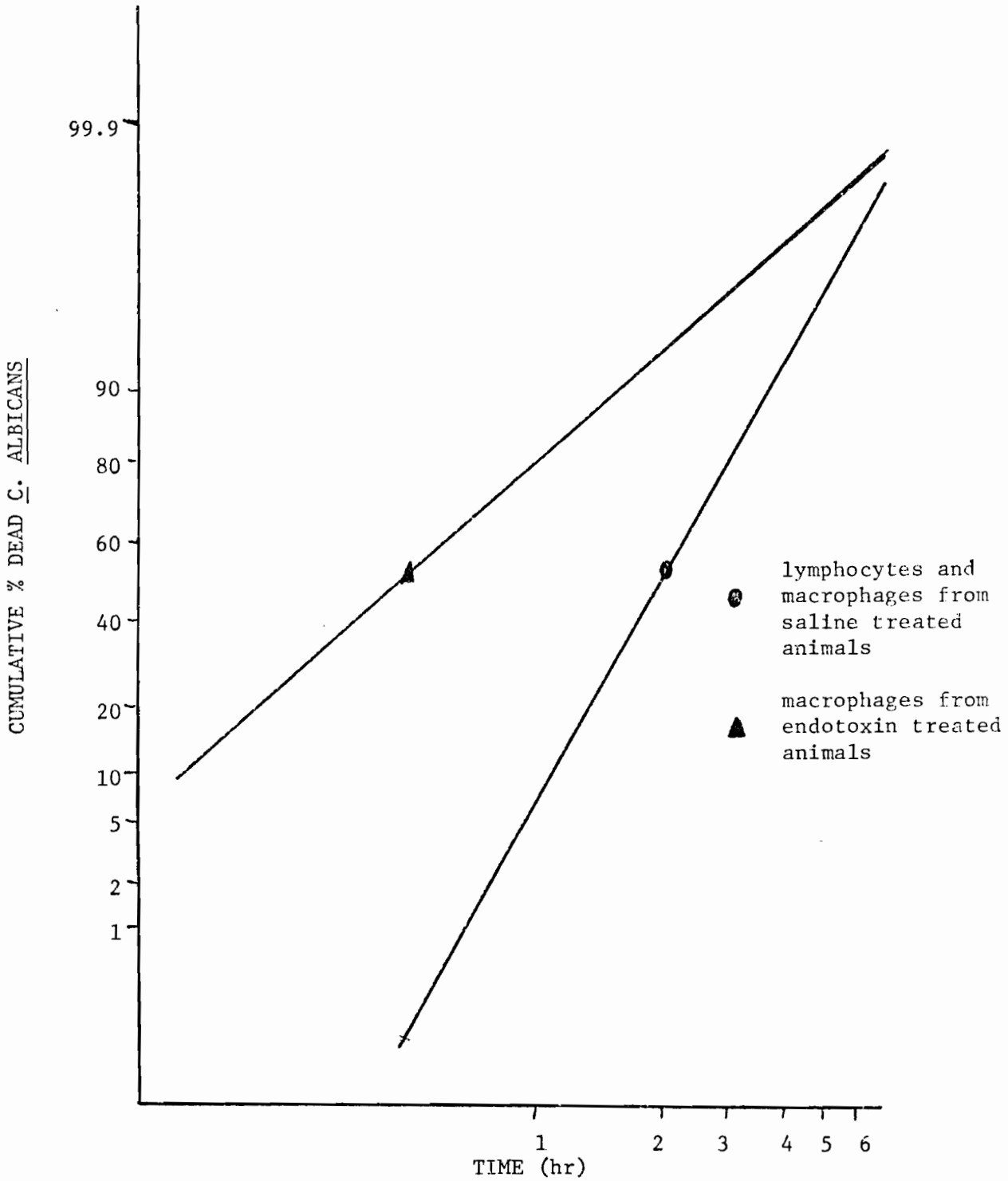

Figure 22. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing macrophages from endotoxin treated animals vs. lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

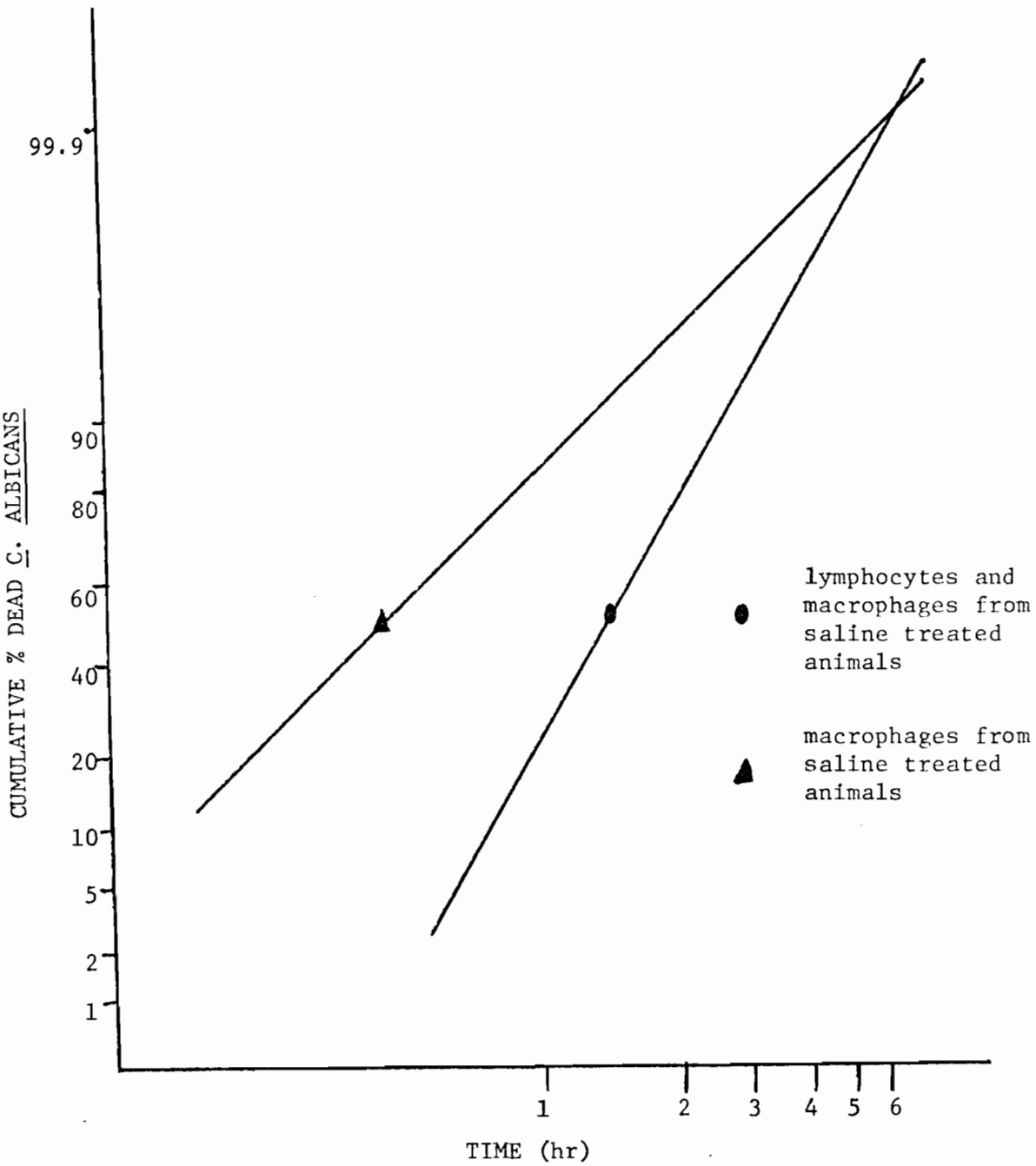

Figure 23. Slope regression analysis of the in vitro rates of destruction of Candida albicans in the systems containing macrophages from saline treated animals vs. lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

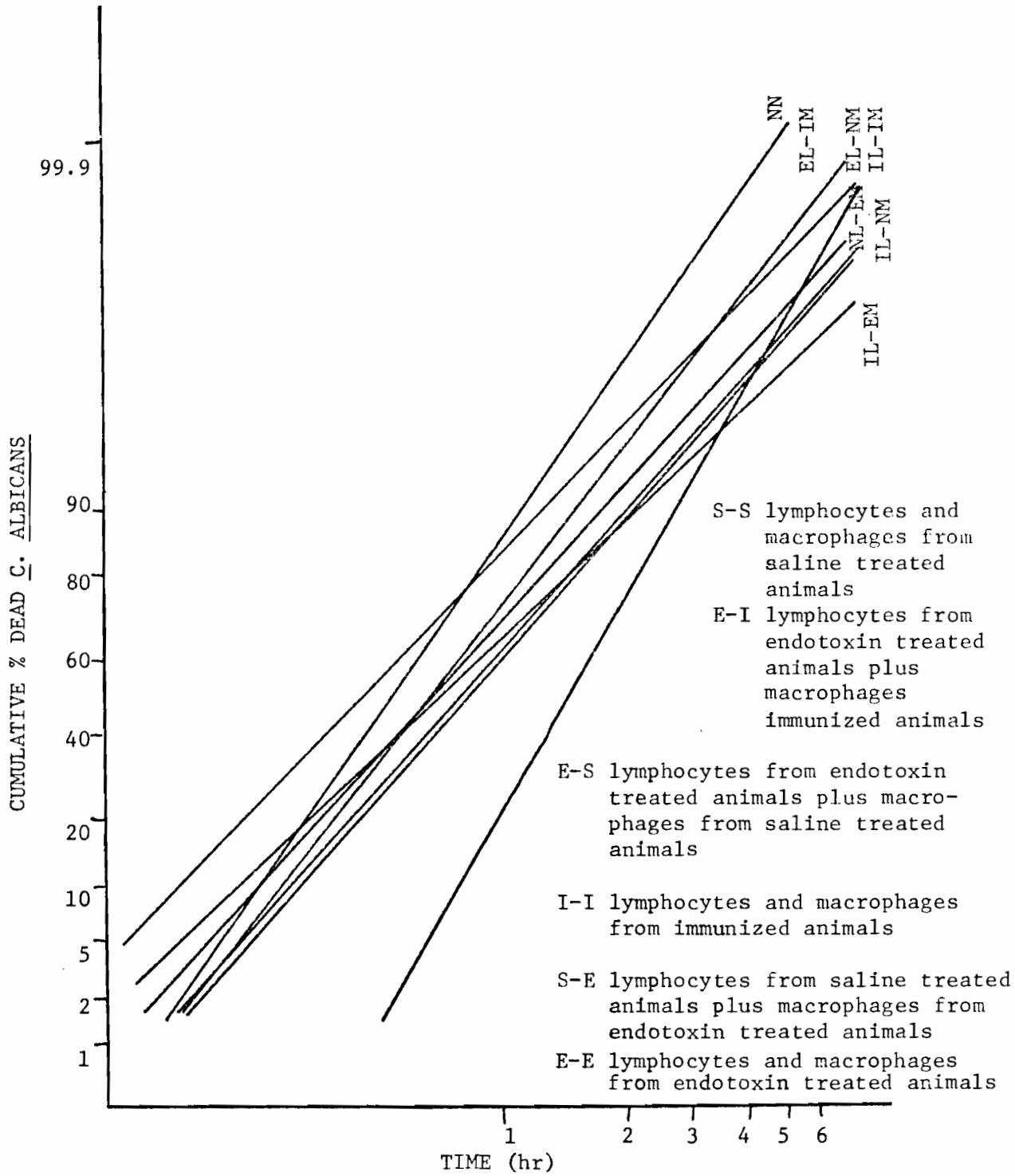

Figure 24. Comparison of the in vitro rates of destruction of Candida albicans: all of the lymphocyte-macrophage systems.

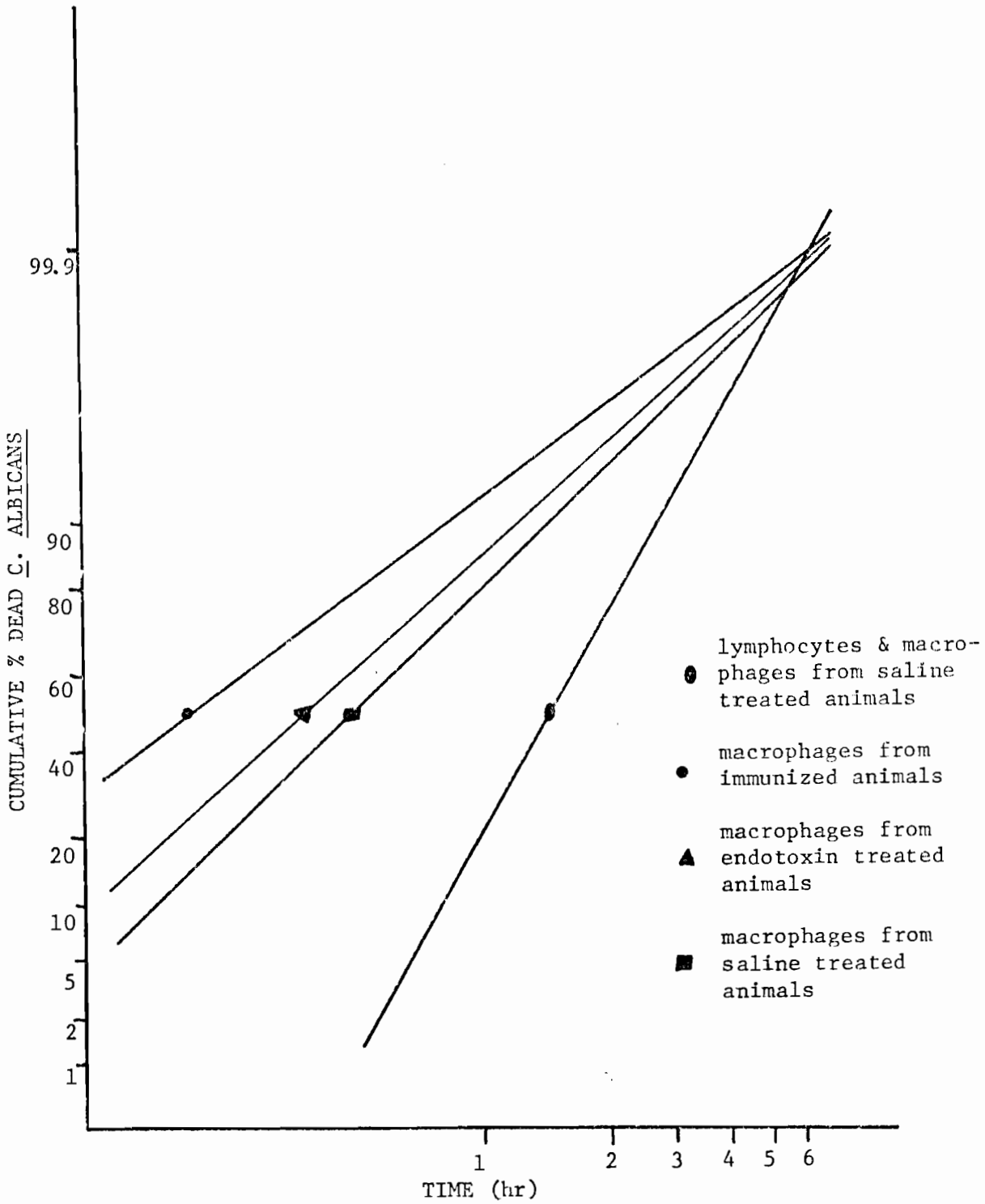

Figure 25. Comparison of the in vitro rates of destruction of Candida albicans: the systems containing only macrophages vs. lymphocytes and macrophages from saline treated animals. Symbols mark ET_{50} values.

by triangles), macrophages from saline treated animals (represented by squares) and lymphocytes plus macrophages from saline treated animals (represented by circles). The macrophages from the immunized animals are observed to have an enhanced cytopeptic capacity when compared to macrophages from the endotoxin treated animals or macrophages from saline treated animals.

Table 6 summarizes the data. The cell type and treatment are listed in columns one and two. Column three indicates the magnitude of the differences in percent dead yeasts between the experimental system and the control system. Column four lists the value and confidence limits (95%) for the slope of each system. The table indicates that each system is significantly different from the lymphocyte-macrophage system of cells from saline treated mice.

The data suggest with confidence that macrophages from immunized animals and macrophages from endotoxin treated animals, in the absence of lymphocytes, destroy the yeast cells more efficiently than the systems containing lymphocytes and macrophages. Correspondingly, the slopes of the lines which indicate rate of destruction for the systems containing only macrophages have greater values than the lymphocyte-macrophage systems. This finding implies that the rate of destruction of the C. albicans occurred at a more rapid rate in the systems containing only macrophages than in the systems containing lymphocytes and macrophages.

The most efficient lymphocyte-macrophage system consisted of lymphocytes from immunized animals plus macrophages from endotoxin

Table VI. Compilation of Experimental and Control Data

Cell type and treatment		Δ at 2 hours	Estimated time of	Slope of
Lymphocytes	Macrophages	in % dead yeasts	50% survival (ET_{50})	regression line
Saline	Saline	--	2.12 (2.105 - 2.131)	1.599 (1.591 - 1.607)
Immune	Immune	50	0.74 (0.7342-0.7460)*	1.7329(1.7226-1.7433)*
Immune	Endotoxin	48	0.65 (0.643 - 0.657)*	2.151 (2.134 - 2.168)*
Immune	Saline	42	1.00 (0.998 - 1.012)*	2.370 (2.3487-2.3914)*
Endotoxin	Immune	30	1.10 (1.089 - 1.110)*	1.994 (1.980 - 2.008)*
Endotoxin	Endotoxin	34	1.03 (1.02 - 1.04)*	2.068 (2.054 - 2.082)*
Endotoxin	Saline	45	0.96 (0.951 - 0.969)*	1.900 (1.887 - 1.913)*
Saline	Immune	34	1.15 (1.139 - 1.161)*	1.970 (1.956 - 1.984)*
Saline	Endotoxin	32	1.10 (1.089 - 1.111)*	2.107 (2.084 - 2.118)*
--	Immune	46	0.27 (0.266 - 0.274)*†	3.074 (3.041 - 3.108)*†
--	Endotoxin	47	0.51 (0.503 - 0.517)*†	2.530 (2.507 - 2.553)*†
--	Saline	39	0.68 (0.6719-0.6882)*	2.239 (2.219 - 2.259)*

*significantly different from the saline treated lymphocyte-macrophage control system

†significantly different from the saline treated macrophage system

treated animals (ET_{50} = 0.65). This system was slightly more efficient than the lymphocyte-macrophage system from immunized animals (ET_{50} = 0.74).

DISCUSSION

These studies have been concerned with the comparative efficacy of the two distinguishable cellular mechanisms of resistance in mice to Candida albicans. In vivo and in vitro comparisons were made on groups of animals and cells taken from such animals, that had been specifically immunized, treated with bacterial lipopolysaccharide or treated with saline. There are few reports in the literature that are concerned with this type of quantitative comparison.

Kemp and Solotorovsky (29) studied the pathogenesis of experimental candidiasis. They found rapid clearing of the organism from blood into the lungs, spleen, and liver. Also, cytopepsis of the Candida cells in these tissues was complete within a short time. In the kidney and heart a progressive infection was observed. Although we did not investigate the possibility of a progressive infection in the heart, our results with other organs agree with those of Kemp and Solotorovsky.

The protective effect of endotoxin administered 24 hours prior to challenge with Candida albicans has been demonstrated (16, 22, 33). Kimball et al. (30) have demonstrated a protective effect against other fungi by the same means. Several investigators (3, 4, 7, 16, 34, 59, 81) have reported on the protective effect afforded an animal treated with endotoxin. Furthermore, there are reports showing that animals specifically immunized against various fungi and bacteria have a greater degree

of resistance to challenge with the homologous organism than the degree of resistance achieved by injection of endotoxin. In these reports specific antibody did not appear to enhance either the phagocytic or the cytopeptic rate of the organism. Histologic studies on animals that had been immunized and then challenged showed fewer organisms in tissues than did the tissues of non-immunized animals (2, 25, 31, 62, 81).

Our studies with Candida albicans show that with time, immunized animals were able to control the growth of the organism in the liver and spleen and that the number of organisms in the kidney had decreased by the fifth day. Endotoxin and saline treated animals showed less resistance than the specifically immunized animals.

Experimental moniliasis has been studied by several investigators. In general, it has been reported that after intravenous injection of Candida, there is a marked leukocytosis and granulocytosis (32). There may be a marked lymphopenia however, and monocytes show a tendency to increase. The organisms are cleared rapidly from the blood (33) and seem to locate in all of the major organs (29). Chronic infections are the result of established foci in the kidney and/or the heart.

Clearance experiments were carried out to determine how rapidly the organisms were removed from the circulatory system. Ten minutes after i.v. injection of the organism, less than 70 organisms/ml (colony count) could be isolated from the blood. Comparisons of clearance rates were made among the immune, tetracycline, and saline treated animals. There were no differences in the rate of clearance in these groups. The results of the saline control group correlated with the data presented by Wright et al. (81).

Investigators have studied the effect of endotoxin treatment with Candida albicans infections. Reports on the efficacy of endotoxin are inconsistent. Wright et al. (80, 81) have reported protective effects from administration of endotoxin. They used 100-400 ug of endotoxin and found a serum factor that was active against Candida. Cellular responses were not recorded by these investigators. Hansenclever and Mitchell (24) were able to detect some protection to candidiasis in mice after treatment with 30 ug of endotoxin. Experiments reported by Dobias (16) showed a protective effect afforded by bacterial endotoxin when mice were challenged 5, 8, and 14 days after endotoxin treatment.

Dobias also reported a failure to confer specific immunity to Candida albicans in mice. His experiments employed multiple injections of dead Candida albicans followed by a two week rest before challenge. Larger doese of antigen caused extensive alopecia in the mice.

Many investigators have reported increased resistance to experimental fungal infections. Increased resistance to histoplasmosis and coccidiodomycosis has been reported by Anderson (2), Hill (25), Knight (31), Rowley (59), and others. These investigators reported enhanced resistance in animals after treatment with fungal spores, fungal extracts, dead cells, and live cells. As in several of the bacterial diseases, increased resistance appeared to be independent of humoral immunity.

Our results indicated enhanced resistance in the immunized group. The difference in protection was small but appeared consistently in each experiment.

Recently cellular immunity has been depicted as a process involving two cell types. Evidence presented by a number of investigators lends

credence to the hypothesis that cell mediated immunity involves collaboration between committed lymphocytes and phagocytic cells (8, 9, 39, 41, 71).

The mediator or effector cell is thought to be the committed lymphocyte which is derived from the long-lived small lymphocytes. The effector cell itself is produced specifically in response to an antigen and is short lived. In the presence of the specific antigen the effector lymphocytes have an influence on macrophages due to chemical mediators (14) or the elaboration of cytophilic antibody. The stimulating process results in activated macrophages and is effective in combating a variety of organisms (17, 39, 40, 41, 61, 62, 63, 70, 71).

There are reports in the literature that demonstrate restriction of intracellular growth of some fungi in mononuclear phagocytes from immunized mice (2, 24, 25, 26). Recently Howard et al. (28) have implied a lymphocyte mediated cellular immunity to histoplasmosis. Although they have been unable to detect any of the lymphoid mediators, experiments utilizing immune and nonimmune lymphocytes with the immune and nonimmune macrophages lend support to a lymphocyte mediated role in resistance.

Salvin and Cheng (63) have detected a lymphocyte mediator commonly referred to as macrophage inhibitory factor (MIF) (53) in guinea pig mononuclear cells. The cells were harvested from guinea pigs which had developed delayed hypersensitivity to Candida albicans. These investigators reported that such mononuclear cell mixtures phagocytized fewer numbers of Candida albicans in vitro than did monocytes from normal animals.

Our experiments were not designed to determine if any of the soluble mediators from lymphocytes were present. In vitro experiments utilizing

macrophages from normal and immune animals showed small but consistent differences in their cytopeptic capacities. We observed the greatest difference when lymphocytes from immune animals plus macrophages from endotoxin treated animals were compared to lymphocytes and macrophages from saline treated animals. The difference observed in this system was greater than the difference observed when only macrophages from immune animals were compared to macrophages from saline animals. Salvin and Cheng (62) reported a decrease in phagocytic and cytopeptic capacity in sensitized mononuclear cells in tissue culture when challenged with Candida albicans. We did not observe this. In our systems the macrophages, regardless of how they had been treated, phagocytized nearly equal numbers of yeast cells. The cells from saline treated animals were not as efficient in their cytopeptic capacities or at best they were equal to the cells from the immunized animals.

The effort to separate variables for study made necessary the in vitro system. Since lymphocytes and macrophages coexist in the in vivo situation, it cannot be presumed that an effect seen following therapy of the animal with any biological agent is the result of lymphocyte-macrophage interaction. In fact, this criticism applies to an unknown extent to the in vitro studies because the cells obtained from the mice could conceivably have been acted upon prior to setting up the in vitro experiment. This made necessary the variety of controls which have been used.

To determine the effect the lymphocyte had in resistance to Candida albicans numerous experiments were performed. These experiments consisted of comparing lymphocytes and macrophages from saline treated animals to

lymphocytes plus macrophages from immunized, endotoxin treated or saline treated animals. There were small differences between lymphocytes and macrophages from saline treated animals and lymphocytes from saline treated plus macrophages from immunized animals. It is possible that the macrophages from the immunized animals could have been coated *in vivo* with a specific cytophilic antibody. This would account for the small differences observed. It is also possible that the macrophages from the immunized animals were auto-activated in the presence of the antigen and the lymphocytes did not contribute to the resistance to *C. albicans*.

When lymphocytes and macrophages from saline treated animals were compared to lymphocytes from immunized animals and macrophages from saline treated animals, a modest difference in the cytopeptic abilities was observed from one to four hours post challenge. This leads one to consider the possibility of soluble lymphocyte kinins that activate macrophages. However, in other systems (28, 33, 38, 39, 40, 41). the enhanced activity of the macrophage was not reported to wane during the experiment. By the fourth hour there were no observable differences in the above system.

Lymphocytes and macrophages from saline treated animals were compared to lymphocytes from saline treated animals plus macrophages from endotoxin treated animals. After the initial hour post challenge, the macrophages from the endotoxin treated animals were more efficient at destroying the yeast cells. The differences observed were not as great or as early as the system which had lymphocytes from immunized animals plus macrophages from endotoxin treated animals. In the latter system it is possible that

a combination of specific and nonspecific factors enhanced the cytopeptic abilities of the macrophages. The former system, i.e., normal lymphocytes and endotoin treated macrophages, would not release the specific kinins according to the mediator theory. The results with the former system are very similar to the results obtained when the macrophages were challenged without previous exposure to lymphocytes or antigens.

Therefore, one could conclude that the nonspecific stimulation of the macrophages by the endotoxin was responsible for the enhanced capacity to destroy the yeast cells.

Further experiments were performed to determine if endotoxin treated lymphocytes would confer a state of enhanced resistance on macrophages prior to challenge with Candida albicans.

Lymphocytes from endotoxin treated animals were cultured with macrophages from immunized animals and challenged with C. albicans. When a comparison was made between the system containing lymphocytes from endotoxin treated animals plus macrophages from immunized animals with the same cells from the saline control system, no differences were observed during the initial hour.

A modest difference was observed during the subsequent four hours, but by the sixth hour no observable difference existed between the two systems. One might have expected to see greater differences in this system than were observed. This expectation seems logical considering data that others have obtained which suggests that macrophages from immunized animals become activated in the presence of specific antigen and that lymphocytes from endotoxin treated animals appear to have a slight activating effect on macrophages as suggested by our data.

One might expect this latter system to be very active cytopeptically. The data indicate that the amount of intracellular killing activity provided by the endotoxin-treated lymphocyte "immunized" macrophage system is about the same as the saline-treated lymphocyte "immunized" macrophage system. Thus, the differences observed in the endotoxin-treated lymphocyte "immunized" macrophage system may be due to the "immunized" macrophage activity alone with the lymphocyte contributing little or not at all to the overall killing effect in the system tested.

Lymphocytes and macrophages from endotoxin-treated mice were cultured and challenged with C. albicans. Once again, no differences were observed between the endotoxin-treated lymphocyte-macrophage system and the saline-treated lymphocyte-macrophage system during the initial hour post challenge. During the subsequent two hours, the endotoxin-treated lymphocyte-macrophage system was more efficient in cytopeptic capacities than the saline control system. However, by the fifth hour both systems showed equivalent killing capacities. If these results are compared to those obtained with the saline treated lymphocyte plus endotoxin treated macrophage system, one can see that the results are nearly equal. It appears that the degree of cytopeptic competency observed was due to the endotoxin treated macrophages and that the interaction of the lymphocytes with the macrophages provided little or no enhancement of killing capacity. If a degree of enhanced cytopeptic capacity is conferred on macrophages via the endotoxin treated lymphocyte, it should be observed when endotoxin treated lymphocytes are cultured with saline treated macrophages. When this system was challenged with C. albicans, there were modest differences observed between the endotoxin treated

lymphocyte plus saline treated macrophage system and the saline control system. The most marked difference was seen at the end of the first hour; this difference decreased steadily during the remainder of the experiment. These results seem to contradict some results presented previously. It is possible that the modest amount of cytopeptic capacity provided by the lymphocytes is not cumulative and therefore not evident in the presence of macrophages that have been activated by specific antigen or endotoxin. It is also possible that there is a cumulative effect. In this case, the activation provided by the lymphocyte may be so slight that activated macrophages do not evidence an enhanced capacity to kill the C. albicans when compared to activated macrophages and saline treated lymphocytes.

The results presented indicate a possible mechanism for the induction of nonspecific resistance. Saline treated macrophages, in the presence of endotoxin treated lymphocytes, show an enhanced capacity to kill C. albicans cells. How this enhanced cytopeptic capacity is transferred to the macrophage is not known at the present time. One could hypothesize that mediators similar to the mediators that are released from sensitized lymphocytes in the presence of specific antigen are released from the endotoxin treated lymphocytes but in smaller quantities. However, this does not explain the greatly enhanced activity of the endotoxin treated macrophage system that was not exposed to lymphocytes during the in vitro experiments. The activity of these macrophages may have been influenced in vivo by the homologous lymphocytes. If indeed this latter speculation were a factor, one would expect to see the same degree of lymphocyte influence on the system containing only

endotoxin treated macrophages and the system composed of saline treated lymphocytes and endotoxin treated macrophages. This was not observed in terms of the ET_{50} values obtained. The slope values were similar but significantly different.

From these data it appears that endotoxin has an effect on lymphocytes that results in moderate activation of "normal" macrophages. Macrophages from endotoxin treated animals are more active than the system containing lymphocytes and macrophages from endotoxin treated animals or the system containing lymphocytes from endotoxin treated animals plus saline treated macrophages from saline treated animals. This finding implies that the lymphocyte may actually retard the killing action of the macrophage under certain conditions.

SUMMARY

A model experimental system has been employed *in vivo* and *in vitro* which compares the efficacy of specific and nonspecific resistance. *Candida albicans* was used as the challenge organism in the experiments. Data of a quantitative nature was collected concerning the relationship of saline treated animals, Boivin antigen, i.e., endotoxin treated animals, specifically immunized animals and the respective lymphocytes and macrophage combinations from such animals have been discussed. *In vitro* experiments were performed using macrophages alone and lymphocyte-macrophage combinations. The results of the experiments suggest that *in vitro*, macrophages from immunized animals in the absence of lymphocytes are most active cytopeptically. Macrophages from endotoxin treated animals, in the absence of lymphocytes, are more active than any of the lymphocyte-macrophage systems. The data involving macrophages cultured in the absence of lymphocytes showed steeper slopes when compared to the saline treated lymphocyte-macrophage system data. The macrophage system appeared to destroy the yeast cells rapidly whereas the lymphocyte-macrophage systems were slightly delayed in their cytopeptic activities. In all systems equivalent numbers of yeast cells were phagocytized. The differences observed were a function of post phagocytic killing of the organisms.

Statistical analysis of the data show that each experimental system was significantly different from the lymphocyte-macrophage saline control

system. In addition, the systems containing only macrophages from immunized animals and macrophages from endotoxin treated animals were significantly different from the macrophages from saline treated animals.

Differences were observed in the lymphocyte-macrophage systems which implied a mechanism for induction of nonspecific resistance. The results suggest that lymphocytes from endotoxin treated animals are capable of enhancing cytopeptic capacities of macrophages from saline treated animals. The specific mechanisms or products involved have not been elucidated at the present time.

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