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EFFECTS OF ANTILYMPHOCTYE SERUM ON ANIMALS EXPERIMENTALLY INFECTED WITH *HISTOPLASMA CAPSULATUM* OR *CRYPTOCOCCUS NEOFORMANS*

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EFFECTS OF ANTILYMPHOCYTE SERUM ON ANIMALS EXPERIMENTALLY INFECTED WITH *HISTOPLASMA CAPSULATUM* OR *CRYPTOCOCCUS NEOFORMANS*

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EFFECTS OF ANTILYMPHOCTYE SERUM ON ANIMALS EXPERIMENTALLY INFECTED WITH *HISTOPLASMA CAPSULATUM* OR *CRYPTOCOCCUS NEOFORMANS*

CHAPTER I

INTRODUCTION

The fact that leucocytes of one species could be destroyed by antisera prepared in a second species was first described by Metchnikoff (21) in 1899. Pappenheimer (23) in 1917 prepared antisera against rat thymocytes in rabbits and noted that such sera had the ability to agglutinate donor lymphocytes and also to lyse them. Heating of the sera to 56 C for 30 minutes removed the lytic properties of the sera but failed to impair agglutination. Lymphopenia has been a consistent finding in animals treated with antilymphocyte serum (ALS) and has been well documented (4, 5, 26, 30). As the lymphocyte became steadily more inseparable from the immune process, the important potentialities of ALS became evident. In 1956, Inderbitzen (11) and Humphrey (10) in 1960 used heterologous ALS to destroy circulating lymphocytes as a technique for studying the participation of these cells in the delayed tuberculin reaction. In 1958, Wilhelm

(31) used the same technique to study contact sensitivity. The studies confirmed the growing evidence that the lymphocyte was a necessary element for delayed reactions, such as the tuberculin type, to occur.

Only within the last few years has the immunosuppressive activity of ALS become the subject of intense investigation. Antilymphocyte serum inhibited cutaneous delayed hypersensitive reactions (30), prolonged the survival of various tissue grafts (1, 27) and depressed primary immunologic responses (22). The strongly growing interest in ALS was due to its tremendous potential use as an immunosuppressant for human organ and tissue transplantation. Starzl et al. (27) demonstrated the usefulness of ALS in prolonging human renal homotransplants. He did, however, report a fatal pneumonitis in one patient and minor upper respiratory infections had developed in other patients being treated with ALS during transplantation therapy. Abaza et al. (1) reported that 4 of 8 dogs treated with ALS died from infection; two from distemper, one from hepatitus and one of pneumonia even though the dogs had been immunized against distemper and hepatitus.

The relative freedom from infection during ALS therapy to date has been due to stringent isolation of the patient and antibiotic therapy. Such precautions may be insufficient for viral or fungal infections due to the limited effectiveness of antibiotics in these infections.

Experimental infection studies combined with ALS treatment should be done in animals to fully access complications and hazards which may arise from infection during immunotherapy. These risks might be made more apparent by experimental infection used in combination with ALS in laboratory animals.

There has been no general agreement on the properties to which ALS owes its remarkable immunosuppressive powers. A number of points on which there has been general agreement is given below: 1. Antilymphocyte serum has the character of an antibody. Most of its activity is in the IgG (7S) serum fraction (12). 2. Antilymphocyte serum is species specific (9). Antilymphocyte serum prepared against the lymphoid cells of one strain of mouse will react against lymphoid cells of another strain but will not agglutinate or lyse lymphoid cells of rats, guinea pigs, etc. 3. Antilymphocyte serum is not directed against antigens peculiar to lymphoid cells (19). An antiserum prepared using mouse tail epidermal cells has the properties of ALS but may be less effective. 4. Lymphoid cells are the effective targets of ALS. Antilymphocyte serum which is first absorbed with lymphoid cells becomes impotent as an immunosuppressant (19). 5. Antilymphocyte serum does not act by causing lymphocytic depletion. Homograft survival, for example, persists long into the period of lymphoid recovery (1, 12, 17, 18, 33). 6. The immunosuppressive effects of ALS outlive its own metabolic lifetime. Grafts

live far longer than can be accounted for by the presence of ALS (22). 7. Antilymphocyte serum is particularly effective in cell-mediated immunities (less effective in suppressing humoral responses) (15). 8. Antilymphocyte serum affects peripheral lymphocytes (4, 5, 26, 30). For a discussion of these and other points see Levey et al. (19).

Experimental bacterial or mycotic infections in conjunction with ALS treatment have not been reported. This study was designed to assess the effect of ALS on the degree of susceptibility of laboratory animals to experimental infection by two fungal organisms: Histoplasma capsulatum and Cryptococcus neoformans.

Histoplasma capsulatum is a diphasic fungus which generally causes a mild, benign respiratory disease in humans. Rarely, it may disseminate to other organs causing a progressive, chronic, malignant disease which may result in death. In mice and guinea pigs, relatively large doses given intraperitoneally (ip) typically do not produce a fatal infection.

Cryptococcus neoformans is a budding yeast frequently isolated from nature. In humans and animals it may cause subacute or chronic infection which may involve the lungs, skin or other parts of the body but has a distinct predilection for the brain and meninges. In contrast to H. capsulatum, C. neoformans in relatively small amounts will cause death of mice within a short time. Therefore,

H. capsulatum provides an opportunity to study ALS treated animals with an infecting organism which generally does not kill the host while C. neoformans provides one which can very rapidly do so.

Some of the points which were considered in this study are given below: 1. What changes in susceptibility to experimental infection by the two fungal organisms mentioned occur in animals treated with ALS? Does ALS allow greater proliferation of an infecting organism in target organs such as liver, lungs and spleen? What effect on mortality from experimental infection results from ALS treatment? How does varying the infective dosage or the schedule of treatment with ALS affect mortality? Are ALS treated animals which are not experimentally infected more prone to acquire infections due to organisms in their environment? 2. What effect does ALS in the amounts given in this study have on humoral antibody formation? 3. Is delayed type hypersensitivity (DTH) which normally develops in guinea pigs infected with H. capsulatum affected by ALS and, if so, to what extent? 4. How does ALS affect the white blood cell and differential counts in guinea pigs and how are these counts modified by experimental infection with H. capsulatum?

In this study, the terms antilymphocyte serum (ALS) and antithymocyte serum (ATS) were used interchangeably. In studies of other workers both antisera have been produced

against lymphocytes, the only difference being that the source of lymphocytes for ALS was generally from lymph nodes while ATS was prepared only against thymic lymphocytes. The two sera seem to have identical properties and function in an identical manner as far as is known (4).

CHAPTER II

MATERIALS AND METHODS

Animals Used. Five to seven week old female, English Short Hair guinea pigs (Camm Research Institute Inc., Wayne, New Jersey) were used as thymocyte donors. Twelve week old guinea pigs were used for infection studies and treatment with ALS.

The mice used were Swiss white hybrids which are maintained in our laboratory. Both sexes were used.

New Zealand white rabbits weighing 2-3 kg were used for the production of all ALS used in this study.

Organisms Used. Histoplasma capsulatum, Scritchfield isolate, originally cultured from a human case of histoplasmosis, was used. The yeast phase of this organism was maintained on Brain Heart Infusion Blood Agar at 37 C. The mycelial phase of H. capsulatum was maintained on Sabouraud's Agar containing 2% dextrose.

For experiments that involved mice the yeast phase was used. Yeast cells from two day old cultures were removed from the surface of Brain Heart Infusion Blood Agar slants, washed in saline three times, counted, and diluted

to give suspensions containing 10^6 and 10^8 cells/ml. Mice were inoculated intravenously (iv) with 0.1 ml of the first or second suspension. Plating of suitable dilutions on Sabouraud's Agar (2% dextrose) showed that these two dosages represented 7×10^4 and 7×10^6 viable cells, respectively.

The mycelial phase of this organism was used for inoculation of guinea pigs. One month old cultures were harvested from Sabouraud's Agar slants into sterile saline, ground with a Teflon grinder and diluted to give 10^7 units/ml (a unit consisted of single or small clumps of conidia with or with out hyphal fragments). Guinea pigs were inoculated ip with one ml of the above suspension. Plating of appropriate dilutions on Sabouraud's Agar (2% dextrose) showed that this dosage represented 9×10^5 viable units.

Cryptococcus neoformans, strain 184, is a weakly encapsulated form of C. neoformans. This organism was maintained in our laboratory on Sabouraud's Agar containing 2% dextrose. For infection of mice, flasks of Neopeptone Dextrose Dialysate Broth (7) were inoculated and grown at 30 C with constant shaking for three days. The cells were then counted and diluted in saline. Mice received either 1 LD_{50} (2.2×10^5) or 0.1 LD_{50} (2.2×10^4) organisms iv contained in 0.1 ml. The viability was calculated to be 90% when cells of C. neoformans were diluted in saline, plated on Sabouraud's Agar with 2% dextrose, and grown at 37 C for 2 days.

Determination of LD_{50}. Preliminary studies using 120 mice were done to determine the 28 day LD_{50} for the strain of C. neoformans used. Calculations were performed according to the method of Reed and Muench (24).

Preparation of Antilymphocyte Serum. Two different preparations were used in this study but both were prepared in essentially the same manner.

a. Rabbit anti-guinea pig lymphocyte serum was prepared by removing the thymuses of 5-7 week old guinea pigs. The organs were then vein-stripped and carefully pressed through a fine stainless steel mesh into cold saline. The thymocytes were then washed three times in cold saline and resuspended to a concentration 5×10^8 cells/ml. Four New Zealand white rabbits were inoculated iv with two ml (10^9 cells) of the suspension. Two weeks later the procedure was repeated. The following week all rabbits were bled from the central ear artery using a 20 gauge needle. Fifty to sixty ml of blood were taken from each rabbit. One week later the rabbits received 5×10^8 thymocytes iv and were bled, as above, seven days later. Two days later, all rabbits were exsanguinated by cardiac puncture. All sera was stored at -20 C until the last batch was collected. At this time the sera were thawed, heated to 56 C for 30 min, filter sterilized (Millipore), cultured for sterility and stored in 10 ml amounts at -20 C until needed.

b. Rabbit anti-mouse lymphocyte serum was prepared as above except that mouse thymuses were used. The thymuses of five mice furnished approximately 10^9 cells. Mice four weeks old were used as donors; thymic involution occurs rapidly in mice older than this.

Thymic preparations were tested for viability by adding one drop of freshly made trypan blue, 0.2%, to a drop of thymocyte suspension, mixing and examining under 40X power. Viability was then determined by counting dead (stained) and living (unstained) cells. Viable cells should be used for preparing ALS (16). Preparations made using the technique above regularly produced thymocyte suspensions which were 95% viable.

Leucocyte Agglutination Tests. A modification of the method of Amos and Peacocke (3) was used. Thymocyte preparations were washed in 0.15 M saline three times and the cells then diluted to 2 x 10^7/ml in buffer prepared as follows: Mix solution A (1.3 g Na_2HPO_4, 3.0 g Na_4EDTA/500 ml 0.15 M saline) with solution B (1.3 g NaH_2PO_4/500 ml 0.15 M saline) to obtain pH 7. Equal volumes (0.1 ml) of cells and serial dilutions of serum were mixed in 9 x 75 mm tubes and read for macroscopic agglutination by gentle mixing after standing 2 hr at room temperature. The titer obtained was a measure of leucocyte agglutinating antibody of the pooled ALS. The procedure was the same for both anti-mouse and anti-guinea pig ALS except the source of the donor

lymphocytes. Spleen cells were tested in the same manner. Lymphoid cells in buffer only served as a control.

Cytotoxicity Testing. The method of Gray et al. (9) was used. Two drops of saline were placed in a ring formed by Vaseline petroleum jelly on a glass slide. Thymuses were cleanly cut and the end cut repeatedly touched to the saline. The cytotoxic activity of ALS was determined by adding an equal volume of serum and incubating 30 min at 37 C. Trypan blue (0.2%) was added and the viability determined. Normal rabbit serum was used as a control.

White Blood Cell (WBC) and Differential Counts.

a. Guinea pigs

All guinea pigs were bled by cardiac puncture prior to treatment. One portion of the blood was heparinated for WBC counts. Microhemtocrit determinations were also performed on this sample. Blood smears were also prepared at this time. A second portion of the blood was allowed to clot and the serum stored at -20 C until needed. This entire procedure was repeated weekly for five weeks after initiation of treatment.

b. Mice

Mice were subjected to total WBC counts and differentials before and after ALS treatment to determine the effectiveness of serum treatment. An ineffective ALS preparation would fail to produce lymphopenia.

Precipitin Testing. Serum collected from guinea pigs at weekly intervals was run against a series of histoplasmin antigens prepared as described earlier (2).

The above serum samples also were tested against normal rabbit serum. By so doing, it was possible to demonstrate humoral antibody production in ALS treated animals.

Hemolytic Plaque Assay Technique. Plaque assay tests were performed in a manner similar to that originally described by Jerne et al. (13). This procedure was done to measure the effect of ALS on plaque forming cells (PFC) and thus on the cellular immune response.

Thirty mice were used. Ten mice were inoculated with ip with 0.25 ml ALS the day before, the day of, and the day after receiving ip 1 ml of a 2% washed SRBC suspension. The second group of mice received normal rabbit serum (NRS) and SRBCs as above and a third group received only SRBCs. On the fourth day after immunization with SRBCs the spleens of all mice were removed, teased into cold Hank's balanced salt solution, and strained through a fine stainless steel mesh. One tenth ml of a 1 to 10 dilution of spleen cells was added to 1.9 ml of 0.75% agar solution in Hank's containing 2 mg of diethylaminoethyl dextran (Pharmacia, Uppsala, Sweden). This suspension was then decanted onto a layer of 1.5% agar in Hank's solution and spread evenly by swirling. After standing at room temperature for 15 min the

plates were incubated at 37 C for 1 hr. A 1:10 dilution of guinea pig complement, 1.5 ml/plate, was added and the plates were reincubated at 37 C for an additional 30 min. The plates were then placed at room temperature for two hours. The excess complement was then decanted and the plates stored at 4 C overnight. The plates were stained by adding 2 ml of the following mixture: a. 10 ml of 2% benzidine solution in glacial acetic acid with 0.5 ml of 30% hydrogen peroxide. b. 90 ml of distilled water. The plates were incubated until a blue color developed. The solution was then poured off and the plaques counted under a stereoscope (0.7X ocular).

Infection of Mice with Cryptococcus neoformans. One hundred and fifty mice, 8 weeks old and of both sexes were used. The mice were placed in 3 main divisions. The first division consisted of 80 mice, 10 in each group. All members of this division received 3 ip injections, 0.25 ml ALS/ injection. Four groups were treated with ALS the day before, the day of, and the day after infection (days -1, 0 and 1). These mice were infected with either 0.1 or 1 LD_{50}. Two other groups were treated with ALS in the same manner. One group received nonviable (2% formalin and heat killed) cells and the other group received no cells (ALS control). Two additional groups were given 1 LD_{50} on day 0. The first group received ALS on days 0, 1 and 2 and the second group received ALS on days 7, 8, and 9 post infection.

In the second division, the treatment of mice was similar except normal rabbit serum (NRS) was used instead of ALS.

In the third division only 2 groups (20 mice) were used. The first group was given 1 LD_{50}; the second group received 0.1 this dosage. These mice had no serum treatment.

The 1 LD_{50} given enabled direct comparisons of ALS, NRS, and non-serum treated animals infected with C. neoformans using a known reference point. The 0.1 LD_{50} was used primarily to determine if such a low dosage would kill the ALS treated mice. Complete details of this experiment are shown in Table 1.

Infection of Mice with Histoplasma capsulatum. One hundred and ten mice, 8 weeks old and of both sexes were used. The mice were placed in 3 main divisions. The first division consisted of 40 mice, 10 in each group. Each of these mice received 3 ip injections of ALS at days -1, 0, and 1 and were infected on day 0. Half of the mice in this division received 10^7 yeast cells of H. capsulatum iv. The second half received 10^5 yeast cells iv.

The second division also consisted of 40 mice. Half received 10^7 yeast cells iv while the second half received 10^5 cells iv. These mice were serum treated as above except that normal rabbit serum was used instead of ALS.

Table 1. Schedule for serum treatment and infection of mice with C. neoformans

Serum Treatment*	Days given**	Dosage in LD_{50}'s	Viability of cells***	Route given	No. mice/ group
ALS	-1,0,1	1	V	iv	10
ALS	-1,0,1	1	V	iv	10
ALS	-1,0,1	0.1	V	iv	10
ALS	-1,0,1	0.1	V	iv	10
ALS	-1,0,1	---	---	iv	10
ALS	0,1,2	1	V	iv	10
ALS	7,8,9	1	V	iv	10
ALS	-1,0,1	1	NV	iv	10
NRS	-1,0,1	1	V	iv	10
NRS	-1,0,1	0.1	V	iv	10
NRS	-1,0,1	1	NV	iv	10
NRS	-1,0,1	---	---	iv	10
NRS	7,8,9	1	V	iv	10
Saline	-1,0,1	0.1	V	iv	10
Saline	-1,0,1	1	V	iv	10

*ALS = Antilymphocyte Serum.

NRS = Normal Rabbit Serum.

**Days serum treatment administered relative to infection on day 0.

***V = viable cells. NV = non viable.

The third division consisted of 30 mice. These mice received no serum treatment. Ten mice were inoculated iv with 10^7 yeast cells; twenty received 10^5 cells iv, as in Table 2.

The high dosage (10^7) was used to determine if any differences could be observed in ALS and NRS treated animals, as compared with controls, when an overwhelming infection was introduced. The 10^5 dosage was employed primarily to create an infection but without rapid death (as in the high dosage). Thus, liver, lungs, and spleen, typically involved in histoplasmosis, were removed from some members of this group at 2 weeks and from others at 4 weeks post infection and quantitatively cultured. The extent of proliferation of H. capsulatum was compared in ALS, NRS, and untreated controls.

Skin Tests in Guinea Pigs Infected with H. capsulatum. Four groups of guinea pigs inoculated four weeks earlier with 9×10^5 viable mycelial particles of H. capsulatum were used. Group one received two injections of ALS prior to infection and weekly thereafter. Group two received NRS rather than ALS. Groups three and four were treated with saline on the same schedule as the other groups except group four received one additional injection, ALS, the night before skin testing. Table 3 shows details of this experiment.

Table 2. Schedule for serum treatment and infection of mice with H. capsulatum

Serum Treatment*	Days given**	No. cells given	Viability of cells***	Route given	No. mice/ group
ALS	-1,0,1	10^5	V	iv	10
ALS	-1,0,1	10^5	V	iv	10
ALS	-1,0,1	10^7	V	iv	10
ALS	-1,0,1	10^7	V	iv	10
NRS	-1,0,1	10^5	V	iv	10
NRS	-1,0,1	10^5	V	iv	10
NRS	-1,0,1	10^7	V	iv	10
NRS	-1,0,1	10^7	V	iv	10
Saline	-1,0,1	10^5	V	iv	10
Saline	-1,0,1	10^5	V	iv	10
Saline	-1,0,1	10^7	V	iv	10

*ALS = antilymphocyte serum.

NRS = normal rabbit serum.

**Days serum treatment administered relative to infection on day 0.

***V = viable cells given.

Table 3. Schedule for bleeding, serum treatment and inoculation of guinea pigs with H. capsulatum

Day**	Group 1	Group 2	Group 3	Group 4	Group 5
-7	Bled	Bled	Bled	Bled	Bled
-4	ALS	NRS	Saline	Saline	ALS
-2	ALS	NRS	Saline	Saline	ALS
-1	Bled	Bled	Bled	Bled	Bled
0	Infected	Infected	Infected	Infected	Saline
6	ALS	NRS	Saline	Saline	ALS
7	Bled	Bled	Bled	Bled	Bled
13	ALS	NRS	Saline	Saline	ALS
14	Bled	Bled	Bled	Bled	Bled
19	ALS	NRS	Saline	Saline	ALS
20	Bled	Bled	Bled	Bled	Bled
26	ALS	NRS	Saline	Saline	ALS
27	Bled	Bled	Bled	Bled	Bled
33	ALS	NRS	Saline	Saline	ALS
34	Bled	Bled	Bled	Bled	Bled
40	ALS	NRS	Saline	Saline	ALS
41	Bled	Bled	Bled	Bled	Bled
43				ALS	
44	ST*	ST	ST	ST	ST

*ST = skin tested, 0.1 ml histoplasmin id.

**Indicates day relative to day of infection (day 0).

Determination of Local Inhibition of Delayed Hypersensitivity. To determine if delayed type hypersensitivity could be inhibited locally by intradermal (id) injection of ALS, 10 guinea pigs with known positive skin tests were used. Both abdominal sides were shaved. On one side, histoplasmin (lot H-42, produced by United States Public Health Service), 1:25 dilution, was inoculated id. At distances of from 25 to 48 mm from this site, ALS was given id. A second injection of histoplasmin was given as a control at least 100 mm distant from the ALS injection site. Induration and erythema were measured at 24 and 48 hours. As an alternate study, ALS was given id and 24 hours later histoplasmin was injected near the first injection site. Normal rabbit serum was included as a control.

CHAPTER III

RESULTS

Leucoagglutination Tests.

a. Guinea pigs

The leucoagglutination titer of ALS prepared against guinea pigs was found to be 1:512 when tested against guinea pig thymocytes. When the same antiserum was tested against lymphocytes derived from splenic origin, the titer was 1:256. This serum was also run against spleen and thymic lymphocytes from donor mice. No agglutination was observed in tests involving heterologous cells. Guinea pig lymphoid cells, run as controls, in buffer alone or in buffer plus normal rabbit serum were not agglutinated. Lymphoid cell preparations for leucoagglutinin tests must be used within a few hours as autoagglutination occurred when cells were stored for longer periods. . .

b. Mice

Similar results were obtained when ALS prepared against mouse thymocytes was tested. The leucoagglutination titer against mouse thymocytes was 1:256 and against mouse spleen cells was 1:128. Fresh thymic or spleen cells did

not agglutinate in buffer alone or in buffer plus normal rabbit serum. Lymphoid cells from guinea pig donors failed to agglutinate in the presence of rabbit anti-mouse lymphocyte serum.

Cytotoxicity Tests. The results of these tests showed that ALS prepared against both guinea pig and mouse thymocytes had the ability to cause lysis and death (ascertained by the inability to exclude trypan blue dye) of lymphoid cells in the presence of complement. Fifty percent of the lymphocytes exposed to ALS were killed within 30 minutes as compared to 10% in controls which contained normal rabbit serum instead of ALS. Results of leucocytotoxicity tests of both guinea pig and mouse thymocytes are shown in Table 4.

Precipitin Tests. Four out of 35 guinea pigs infected with H. capsulatum had sera that reacted positively with crude histoplasmin. One animal which had a positive response had been treated weekly with ALS; the other three animals which responded positively had received saline.

All guinea pigs which had been treated with NRS weekly had precipitin bands against some components of NRS after two weeks of such treatment. All guinea pigs which had been treated with ALS weekly also showed precipitins against NRS. Some of these sera showed as many as five precipitin bands against NRS, two more than seen in tests with any serum of NRS treated guinea pigs.

Table 4. Cytotoxicity of ALS against thymocytes as assayed by trypan blue exclusion

Thymocytes from:	Percent viable cells after incubation with complement for 30 min in the presence of			
	RAMLS*	RAGPLS**	NRS***	SALINE
Guinea pig	90	50	94	92
Mouse	43	87	90	94

*Rabbit anti-mouse lymphocyte serum.

**Rabbit anti-guinea pig lymphocyte serum.

***Normal rabbit serum.

White Blood Cell Counts.

a. Guinea pigs

Normal white blood cell (WBC) counts for guinea pigs used were in a range of 3,500 to 13,000/mm^3, with most of the animals having a count of 7,000 ± 2,000/mm^3. The mean count in normal controls was 7,100 cells/mm^3. Animals treated with ALS exhibited a short-lived leucopenia and counts usually dropped 3,000 to 4,000 cells/mm^3 within a few hours of treatment. This was followed by a moderate leucocytosis within a day or two which peaked at 2 weeks after initial treatment. All infected animals regardless if they were ALS, NRS or saline treated showed the same general trend: a moderate leucocytosis developed within 1-2 weeks post infection and shortly thereafter began to return to normal values. Results are illustrated in Figure 1.

b. Mice

Normal mice were highly variable. Counts ranged from 6,200 to 19,600/mm^3. After 3 injections of ALS, 0.25 ml each, ip, given daily for 3 consecutive days the total white count average was only moderately increased. The mean pre-treatment white count was 9,100; shortly following completion of ALS treatment the mean count had increased to 19,700. White counts returned to normal in about 10 days.

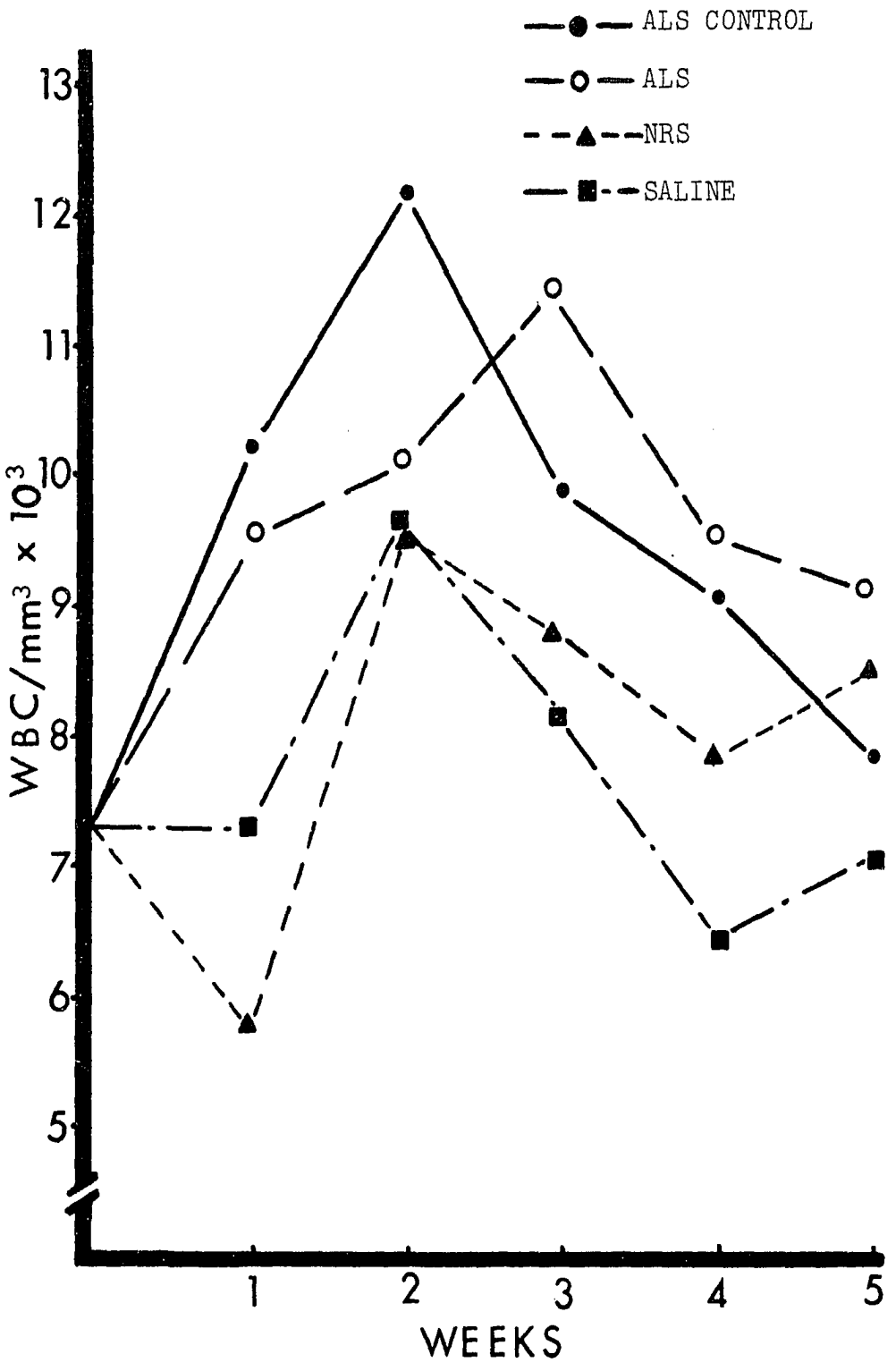

Figure 1. Effect of infection with H. capsulatum on white blood cell counts of guinea pigs treated with anti-lymphocyte serum (ALS), normal rabbit serum (NRS) or saline.

Differential Blood Counts

a. Guinea pigs

Guinea pigs treated with ALS showed a marked decrease in percentage of lymphocytes within 24 hours. Lymphopenia was pronounced for two weeks in noninfected controls with percent lymphocytes falling from 66 to 14. This trend failed to continue, however, regardless of weekly administration of ALS. Four weeks after commencement of ALS treatment the lymphocyte percentage had returned to within 14% of its normal base line average.

Animals which were ALS treated and also infected showed severe lymphopenia but the return toward normal was more gradual than in noninfected controls.

The groups of guinea pigs which were treated with NRS weekly or with saline showed only slight lymphopenia which could be attributed to infection with H. capsulatum. Figure 2 shows the relationships among the various groups.

Figure 3 shows that the severe lymphopenia in ALS controls was almost a mirror image reflexion on neutrophilic hyperplasia. Basophils and monocytes fluctuated only slightly and were not included in this figure. A notable eosinophilia was observed only in animals which had received serum treatment.

Figure 4 which represents an infected and ALS weekly treated group shows somewhat the same pattern as Figure 3.

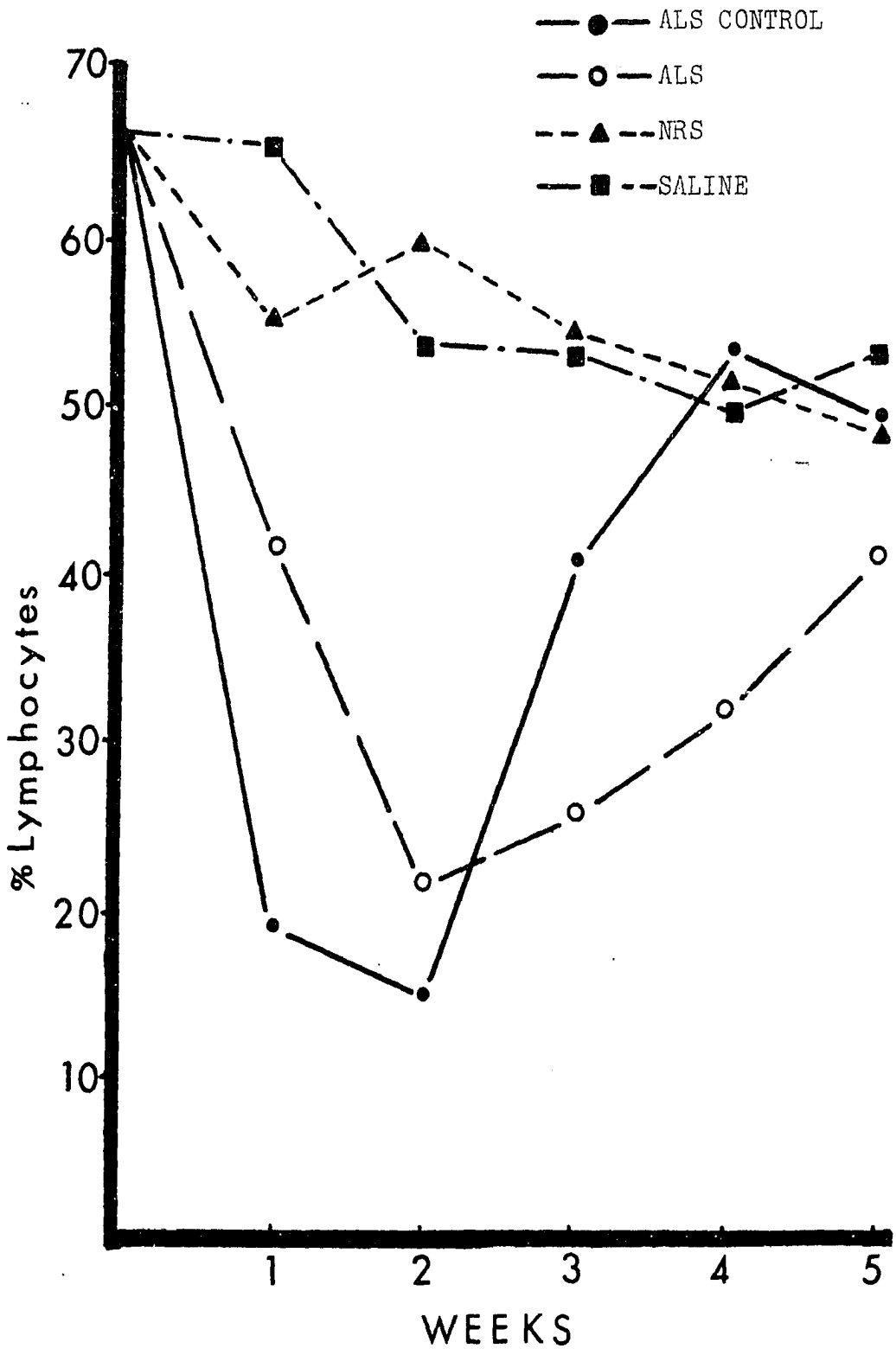

Figure 2. Effect of antilymphocyte serum (ALS), normal rabbit serum (NRS) or saline on peripheral lymphocyte counts of guinea pigs infected with H. capsulatum.

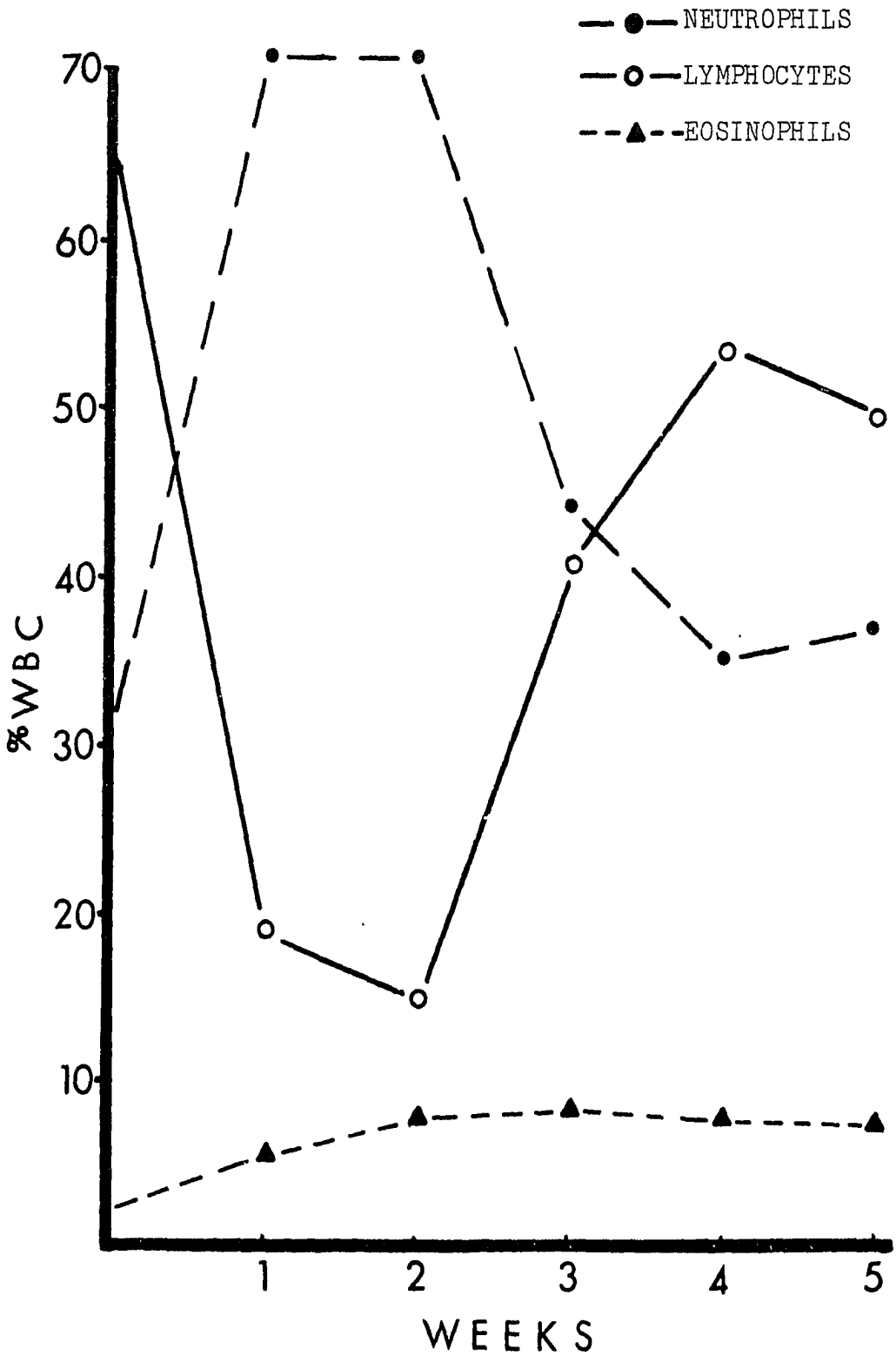

Figure 3. Effect of antilymphocyte serum on peripheral lymphocyte, neutrophil and eosinophil counts of normal guinea pigs.

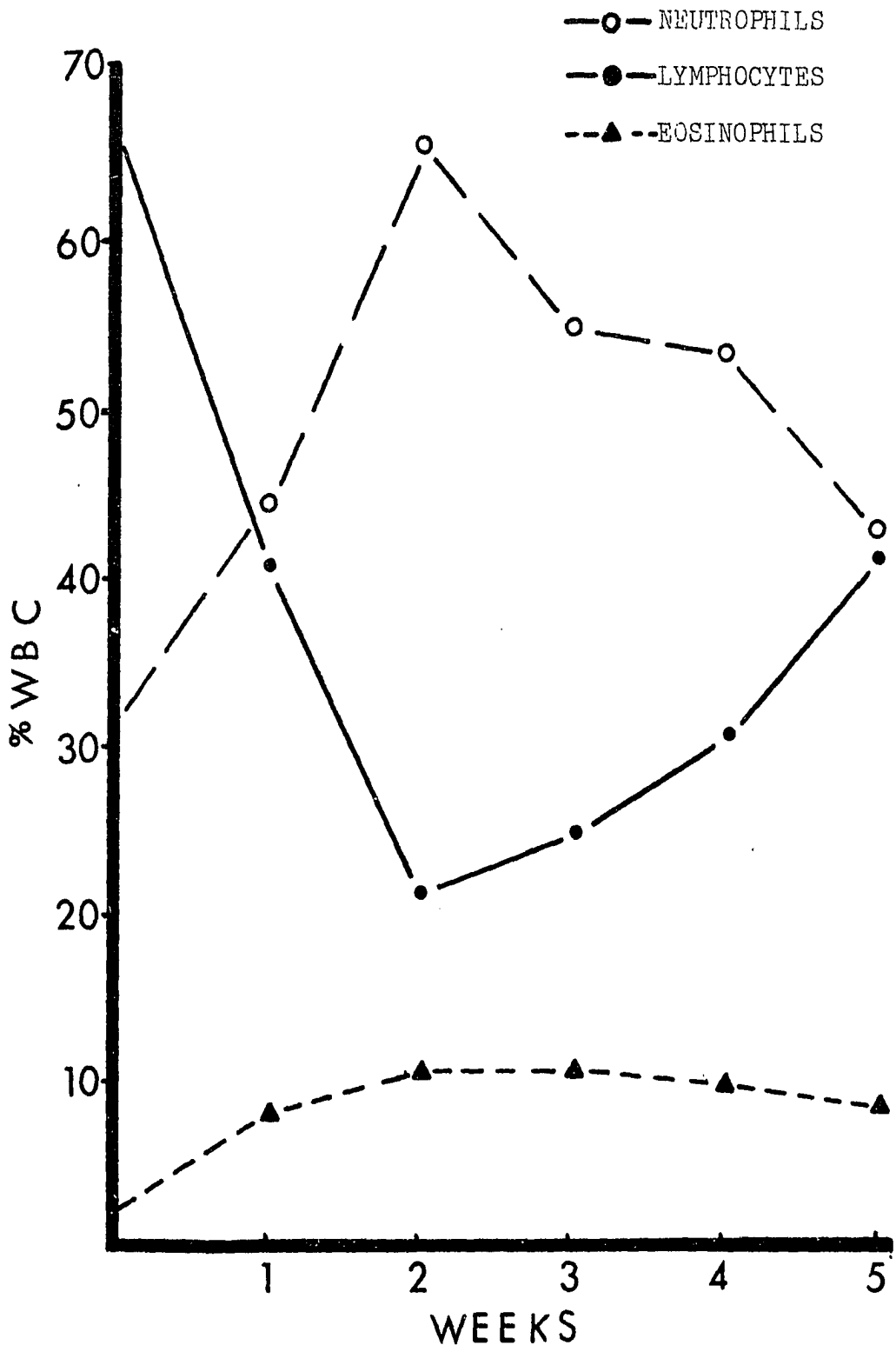

Figure 4. Effect of antilymphocyte serum (ALS) on peripheral lymphocyte, neutrophil and eosinophil counts of guinea pigs infected with H. capsulatum.

Lymphocyte percentage decreases rapidly and this decrease is compensated by an increase in neutrophils. After two weeks the two cell types have switched places with respect to their normal base line percentages. A gradual reversal then occurred as each cell type began to return toward its normal concentration.

Figures 5 and 6 representing animals infected and treated with NRS and animals infected and treated with saline respectively, show only gradual decreases in percent lymphocytes with compensating increases in neutrophils.

Microhematocrit determinations showed that the mean hematocrit dropped only three to four percent two or three weeks after infection. These values returned to their normal average (43%) within five weeks.

b. Mice

Differential counts performed on normal mice revealed the following values: neutrophils, 21%; lymphocytes, 74%; monocytes, 4%; eosinophils, 1%. After three consecutive daily doses, 0.25 ml ALS/dose given ip, the following values were obtained: neutrophils, 56%; lymphocytes, 38%; monocytes 3%; eosinophils, 3%. Marked anisocytosis and poikilocytosis were evident.

Effect of Intraperitoneal Inoculation of ALS on Delayed Type Hypersensitivity (DTH). Little effect was produced by weekly injections of guinea pigs with ALS. The mean induration of skin test readings measured in

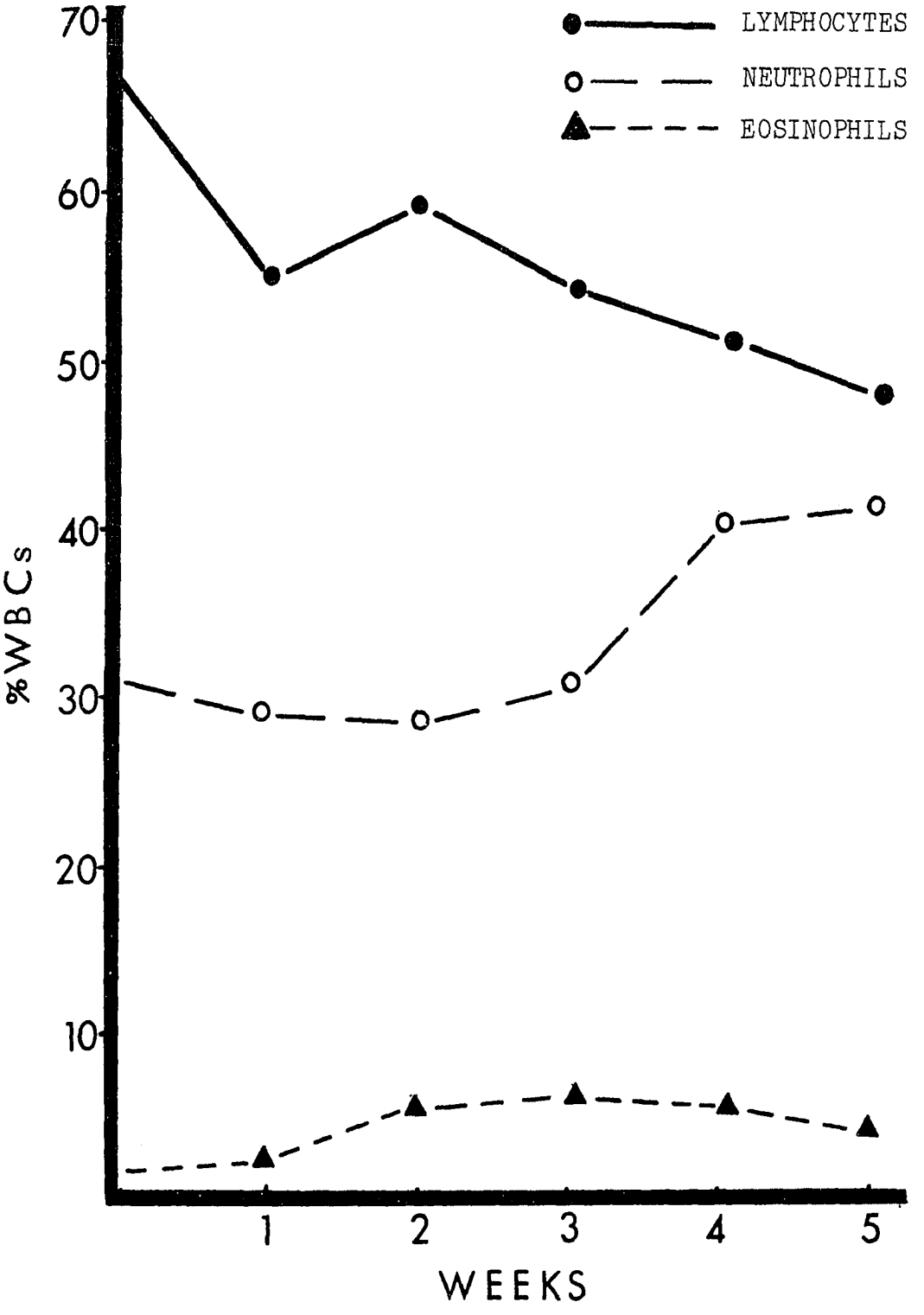

Figure 5. Effect of normal rabbit serum on peripheral lymphocyte, neutrophil and eosinophil counts in guinea pigs infected with H. capsulatum.

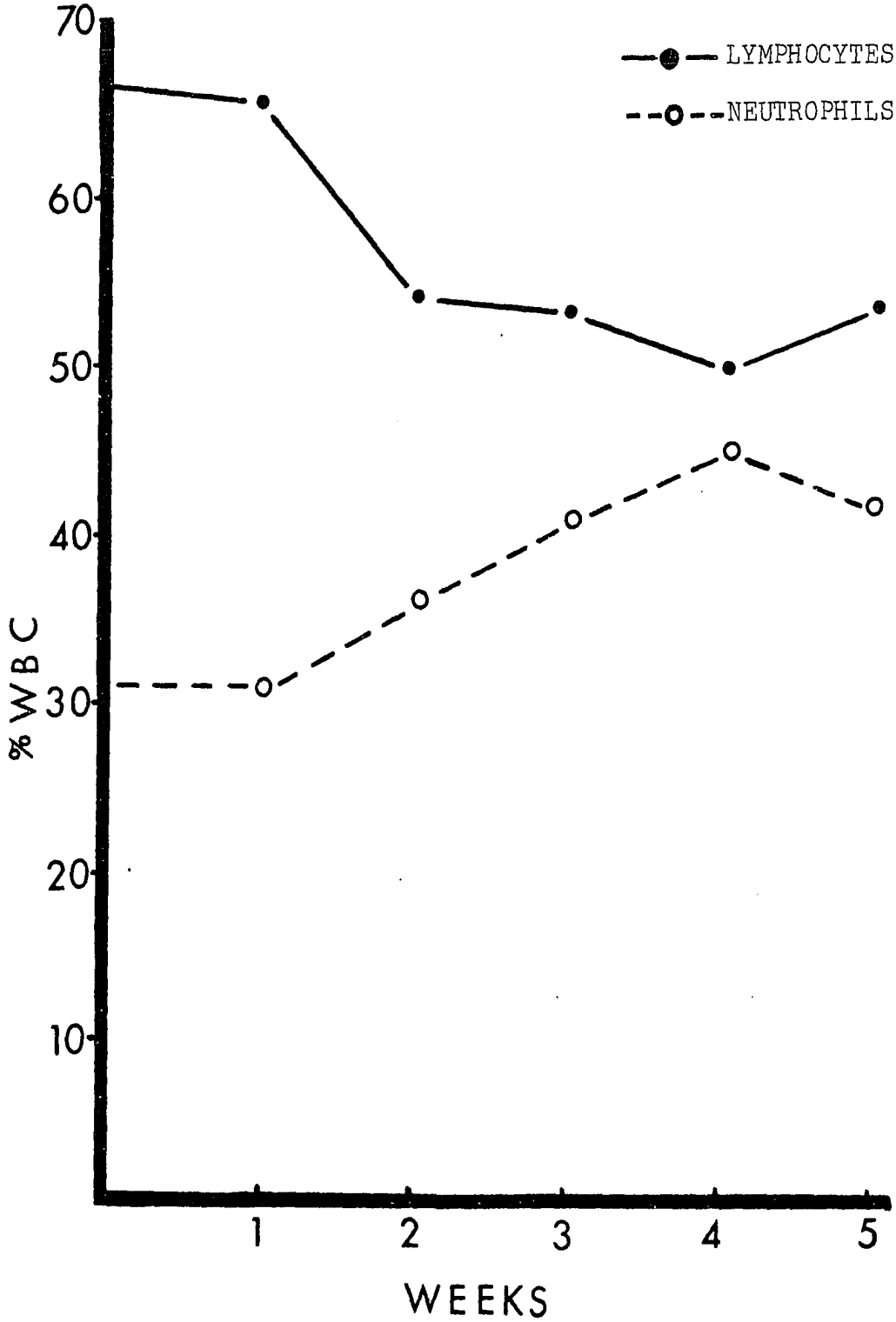

Figure 6. Effect of infection with H. capsulatum on peripheral lymphocyte and neutrophil counts of guinea pigs.

millimeters was 7.6 x 8.2. This was somewhat less than animals treated with NRS weekly (10.1 x 11.1) or saline (11.4 x 11.4). Marked suppression was exhibited, however, in the group which received only one injection of ALS on the night before skin testing. The mean 24 hour induration for this group was 1.7 x 1.8 mm. Results are shown in Table 5.

For purposes of comparison, the area of induration (IA) was calculated. The formula, $IA = \frac{\pi D1\ D2}{4}$, where D1 and D2 are two measurements of induration made perpendicular to one another, was used. The mean area of induration, or induration index (II) then would be expressed as: $II = \frac{\Sigma(IA)i}{n}$, where n equals the number of animals tested. The minimum positive value for II was defined as 19.75, choosing arbitrarily to let D1 and D2 both equal 5 (the minimum allowable measurement which was considered as positive). The 24 hour II for animals treated with ALS weekly was 52.9; for animals that received NRS weekly, 96.1; for saline treated animals, 139.3; for animals which received saline except for a single injection of ALS the night before skin testing; 3.5. Table 5 gives values for these readings and for 48 hour readings as well. The number of skin test positive animals involved in the four groups above are shown in Table 6.

Effect of ALS Given Intradermally on DTH. In guinea pigs infected with H. capsulatum, ALS given id markedly depressed the delayed skin reaction. As seen in Table 7, the

Table 5. Effect of antilymphocyte serum given intraperitoneally on delayed skin reactivity in guinea pigs infected with H. capsulatum

Treatment	24 hr Induration			48 hr Induration		
	D1*	D2	II**	D1	D2	II
Infected, NRS weekly	11.1	10.1	96.1	9.5	8.6	73.2
Infected, saline weekly	11.4	11.4	139.3	9.5	8.0	100.3
Infected, ALS weekly	8.2	7.6	52.9	5.6	5.6	28.9
Infected, ALS night before only	1.8	1.7	3.5	2.5	2.5	8.3

*Represents a measurement in mm of induration while D2 is a second measurement made perpendicular to the first. These figures are means of the groups indicated.

**Represents the mean area of induration.

ALS = antilymphocyte serum.

NRS = normal rabbit serum.

Table 6. Results of skin tests in guinea pigs infected with *H. capsulatum*

Treatment	Induration		Erythema	
	24 hr	48 hr	24 hr	48 hr
ALS weekly	4/5*	3/5	4/5	4/5
NRS weekly	10/10	8/10	10/10	8/10
Saline weekly	10/10	8/10	10/10	8/10
ALS, night before only	0/10	1/10	7/10	6/10

*Indicates the number of guinea pigs having positive skin test readings (at least 19.75 mm^2) over the total number tested.

effects of ALS are not limited to a small area around the site of injection. Skin reactivity to histoplasmin was inhibited, usually completely, within a radius of 40 mm. At distances greater than 40 mm little or no effect on skin test reactivity was apparent.

When histoplasmin was injected in the vicinity of the ALS injection site but 48 hours later, the delayed skin test reaction was still suppressed. This suppressive effect had usually disappeared within 72 hours.

Infection of Mice with C. neoformans. Mice were inoculated iv with a high dose (2.2×10^5 cells) or a low dose (2.2×10^4 cells) on day zero (except for controls which were not inoculated). These dosages represented 1 LD_{50} and 0.1 LD_{50}, respectively for 28 days. It was found that mice treated with ALS on days -1, 0, and 1, and infected with 1 LD_{50} died more rapidly than any other group. On day 17 (post infection) 50% of the mice in this group were dead. Mice infected with 1 LD_{50} and treated with ALS on days 7, 8, and 9 had 50% deaths by day 20. Mice infected with 0.1 LD_{50} and received ALS on days -1, 0, and 1 had 50% deaths on day 24. Only 1 mouse that had received ALS but no infecting dose (ALS control) died within 24 days. Table 8 shows these data.

Twenty percent of the mice treated with NRS and infected with either 0.1 or 1 LD_{50} had died between day 0 and day 28. The same was true for the group of mice which

Table 7. Effect of intradermal inoculation of ALS on histoplasmin skin tests in guinea pigs infected with H. capsulatum

Guinea pig No.	Distance* between sites, mm	24 hour**		Distance*** between sites, mm	24 hour	
		Ind	Ery		Ind	Ery
1	25	-	-	100	10 x 13	10 x 13
2	26	-	-	100	11 x 15	11 x 14
3	27	-	-	100	13 x 14	12 x 14
4	30	4 x 5	-	100	10 x 11	10 x 10
5	33	-	-	100	8 x 9	8 x 9
6	35	-	-	100	8 x 10	8 x 10
7	36	-	-	100	12 x 12	12 x 11
8	37	-	-	100	9 x 12	9 x 12
9	40	4 x 5	-	100	12 x 15	12 x 15
10	48	9 x 18	9 x 12	100	12 x 15	12 x 15

*Distance in mm between sites of injection of histoplasmin and ALS, both on same side.

**Ind = induration; Ery = erythema. (Both measured in mm.); - = negative.

***Distance in mm between sites of injection of histoplasmin and ALS injected on different sides.

Table 8. Number of mice dying after infection with C. neoformans

Treatment	Days given	LD_{50}	Inoc.*	Number dying at week 1	2	3	4	Total dead 4 weeks
ALS	-1,0,1	1	V	4	0	2	3	9
ALS	-1,0,1	1	V	4	0	4	1	9
ALS	-1,0,1	0.1	V	5	0	1	1	7
ALS	-1,0,1	0.1	V	0	0	2	2	4
ALS	0,1,2	1	V	1	1	3	3	8
ALS	7,8,9	1	V	0	3	4	2	9
ALS	-1,0,1	1	NV	1	0	2	0	3
ALS	-1,0,1	None	None	1	0	0	0	1
NRS	-1,0,1	1	V	0	0	0	5	5
NRS	-1,0,1	0.1	V	0	0	0	2	2
NRS	-1,0,1	1	NV	0	0	0	0	0
NRS	-1,0,1	None	None	0	0	0	0	0
NRS	7,8,9	1	V	0	0	4	1	5
Saline	-1,0,1	1	V	0	0	0	4	4
Saline	-1,0, 1	0.1	V	0	0	0	0	0

ALS = antilymphocyte serum, 0.25 ml/injection.

NRS = normal rabbit serum, 0.25 ml/injection.

*V = indicates viable yeast were given; NV = nonviable.

were infected with 1 LD_{50} but had no serum treatment. After 28 days a total of 45 mice (75%) which had been infected and treated with ALS had died out of a total of 60. During this same period, 12 out of 30 mice (40%) died which were infected and treated with NRS. Four of 20 (20%) died which were infected and treated with saline only.

In mice treated with ALS on days -1, 0, and 1, the first death occurred within two days post infection. The first death of a mouse treated with NRS on the same schedule occurred after 22 days. In mice treated with saline, the first death occurred 23 days post infection. Table 9 gives results of this experiment.

Infection of Mice with H. capsulatum. Mice infected with a large dosage of (10^7) yeast cells of H. capsulatum rapidly died irrespective of serum treatment. In a group of twenty mice treated with ALS, all mice were dead nine days post infection. The first death occurred on day three but 80% of the deaths were on days six and seven. In mice treated with NRS the first death was on day six and 50% were dead by day seven. Unlike mice treated with ALS, 15% were still living after four weeks.

The group of mice which received saline in place of serum also had 50% fatalities recorded on days six and seven. The first death in this group occurred on day six. Ten percent of the mice were alive after three weeks (see Table 10).

Table 9. Day of first death and 50% death of mice infected with C. neoformans

Treatment	Days given	LD_{50}	Inoc*	Day of 1st death	Day of 50% death	% dead, 6 wks
ALS	-1,0,1	1	V	2	17	100
ALS	-1,0,1	0.1	V	2	21	85
ALS	0,1,2	1	V	8	24	100
ALS	7,8,9	1	V	11	19	100
ALS	-1,0,1	1	NV	5	FO	30
ALS	-1,0,1	None	None	2	FO	10
NRS	-1,0,1	1	V	22	28	90
NRS	-1,0,1	0.1	V	22	FO	30
NRS	-1,0,1	None	None	FO**	FO	0
NRS	7,8,9	1	V	15	26	90
NRS	-1,0,1	1	NV	FO	FO	0
Saline	-1,0,1	1	V	23	31	60
Saline	-1,0,1	0.1	V	FO	FO	0

*V = cells given were viable; NV = cells not viable.

**FO= event failed to occur during the time allotted.

Table 10. Number of mice dying after infection with *H. capsulatum*

Treatment	Days given	Dosage, yeast cells	Number dying at week				Total dead
			1	2	3	4	
ALS	-1,0,1	10^5	0	0	0	2	2
ALS	-1,0,1	10^5	0	5*	0	5*	10
ALS	-1,0,1	10^7	9	1	0	0	10
ALS	-1,0,1	10^7	10	0	0	0	10
NRS	-1,0,1	10^5	0	0	0	0	0
NRS	-1,0,1	10^5	0	5*	0	5*	10
NRS	-1,0,1	10^7	8	2	0	0	10
NRS	-1,0,1	10^7	3	4	0	0	7
Saline	-1,0,1	10^5	0	0	0	1	1
Saline	-1,0,1	10^7	5	3	0	0	8

*These mice were sacrificed for autopsy at these times.

ALS = antilymphocyte serum, 0.25 ml/injection.

NRS = normal rabbit serum, 0.25 ml/injection.

In mice which received a low number of cells (10^5), no deaths had occurred by the end of three weeks in any group (ALS, NRS, or saline).

Autopsies were performed at two and four weeks post infection on a duplicate set of mice given 10^5 yeast cells iv. Mice treated with ALS had a number of characteristics not seen in mice treated with NRS or saline. Their fur was ruffled and they exhibited varying degrees of lethargy. Hepatosplenomegaly was common; the spleens were several times larger than those of the other two groups. The livers often were pale orange and had a mottled appearance. Culturing of these organs showed more extensive invasion of H. capsulatum (see Table 11).

Striking differences were found in the three groups of mice involved. Although all mice had received the same number of organisms initially, ALS treated mice exhibited grossly higher numbers of organisms as determined by colony counts on serial dilutions of organ homogenates. In the spleens of ALS treated mice, over 150 times as many colonies were isolated as compared to spleens of mice that were treated with NRS, and 30 times as many as in spleens of mice treated with saline. In the liver, the mean number of organisms recovered was 20 times higher in the ALS group than in the NRS group and 11 times that of the saline group. In the lungs, 122 times as many organisms were recovered

Table 11. Number of organisms in the spleen, liver, and lungs of mice autopsied at 2 and 4 weeks after infection with H. capsulatum, iv

Treatment	Dosage	Number of colonies x 10^3 after autopsy at					
		2 weeks			4 weeks		
		spleen	liver	lung	spleen	liver	lung
ALS	10^5	1,560	3,200	612	8,274	11,320	2,914
NRS	10^5	10	164	5	130	200	4
Saline	10^5	52	290	3	850	2,068	498

ALS = antilymphocyte serum.

NRS = normal rabbit serum.

from the lungs of ALS treated animals as in the NRS group and 204 times that of the saline group.

It was noticed that on the culture plates on which lung tissue was plated, 100% of the ALS lung tissue contained common saprophytic "contaminants." Twenty percent of the lung plates from mice treated with saline showed this finding, but no saprophytes were isolated from the lungs of NRS treated mice.

Hemolytic Plaque Assays. Antilymphocyte serum effectively reduced the number of plaque forming cells (PFC). Only 20% (6 PFC/10^6 lymphoid cells) as many PFC were formed by ALS treated mice compared to saline controls (30 PFC/10^6 lymphoid cells). Normal rabbit serum treated mice also showed some depression (15 PFC/10^6 lymphoid cells). These findings were in agreement with Barth et al. (4) who found marked depression of PFC in ALS treated mice and moderate depression in NRS treated mice. About 30 times as many plaques were demonstrated by Barth et al. (4) since they used primed mice whereas non-primed mice were used in the present study.

CHAPTER IV

DISCUSSION

The antilymphocyte sera produced for this study were effective immunosuppressants. The following facts concerning anti-mouse and/or anti-guinea pig ALS point to such effectiveness: 1. Substantial leucoagglutination titers were demonstrated. 2. Cytotoxic activity was present. 3. Moderate inhibition of plaque forming, antibody producing cells was observed. 4. Suppression of delayed skin test reactivity both systemically and locally was demonstrated. 5. Marked lymphopenic producing ability was shown.

No serum has yet been found which had immunosuppressive properties unless that serum also possessed leucoagglutinating ability (25). There is no <u>in vitro</u> assay method for predetermining the effectiveness of a particular antiserum, but one or more of the five points listed above have been characteristically present in efficacious antisera prepared by various workers.

In this study it was shown that treatment with ALS had a marked effect on mortality produced by experimental infection. Fifty percent of the guinea pigs which were

treated weekly with ALS died during the study. Two of seven control animals which were treated only with ALS also died. Autopsies on these two animals indicated that death resulted from a septicemic condition caused by Pseudomonas species. These findings indicated that experimentally infected guinea pigs treated weekly with ALS were more prone to develop fatal infections. Weekly treatment of control guinea pigs with ALS also seemed to predispose them to infection from organisms in the environment.

Findings in mice were similar. Mice treated with ALS and given 1 LD_{50} of C. neoformans died much sooner than infected controls given NRS or saline. Mice given 0.1 LD_{50} and ALS had 80% deaths at the end of 28 days whereas mice given saline and the same number of cells had no deaths during the same period. Those mice which died exhibited the characteristic occipital bulging caused by increased intracerebral pressure produced in cryptococcosis. The brains of such mice were culturally positive for C. neoformans. Mice which were not experimentally infected but were treated with ALS were not more susceptible to observable infection from agents in the environment than were normal, untreated control mice.

The inference drawn from this predisposition to mortality was that ALS presumably inhibited or destroyed antibody forming cells which would have ordinarily become committed to the infecting organism. The hypothesis of

"sterile activation" (18) might explain such a loss of commitment. This hypothesis is based on the observation that some lymphoid cells when exposed to ALS transform into blast cells. Perhaps, then, certain lymphocytes were ready to recognize a foreign antigen. When these cells were exposed to ALS they transformed into blasts. This rendered them incapable of recognizing any foreign antigen.

An interesting, though perplexing, phenomonon follows: Nine of 40 mice (22.5%) infected with 0.1 or 1 LD_{50} of C. neoformans and treated with ALS on days -1, 0, and 1 died on the second post infection day. This did not occur in mice that received ALS and killed cells or in mice which were infected but got ALS on days 0, 1, and 2. Neither did it occur in mice that received ALS on days -1, 0, and 1 and were infected with H. capsulatum, even though 100% of the mice in the group which received 10^7 cells were dead within nine days post infection (much more quickly than with the high dose of C. neoformans). Neither ALS nor C. neoformans alone, in the amounts used here, caused death in two days, but the combination of the two did. There can be no doubt that ALS caused a good deal of physiological stress, as evidenced by extreme lethargy, ruffled fur, diarrhea, and weight loss which accompanied ALS treatment. Perhaps the stress so created, plus some unknown contribution from the viable yeast cells were sufficient cause for death. Five additional mice died during the following five

days but no deaths occurred for the next eight days. The next group of deaths occurred then, but that was the expected result of infection although it occurred one week earlier than infected control mice given NRS or saline.

The lymphopenia produced in guinea pigs was severe but transient despite weekly treatment with ALS. This could be explained by the fact that relatively small doses were given. Since ALS is immunogenic, small doses may have been insufficient to suppress the formation of antibody against itself. An anti-ALS would then result. This was in fact shown in this study using precipitation in agar gel. Thus, subsequent inoculations of ALS must have resulted in its own removal by the process of immune elimination. If large doses of ALS were given initially this might suppress antibody formation against itself and a durable lymphopenia might have resulted.

The peripheral lymphocyte count correlated roughly with the extent of suppression of delayed hypersensitive reactions. Waksman et al. (30) showed some correlation between the extent of induration and lymphocyte count in the delayed tuberculin reaction. Russell and Monaco (26) demonstrated a relationship between depressed lymphocyte counts and prolongation of graft survival. These workers also reported that tissue lymphopenic recovery was reflected by recovery from blood lymphopenia. Administration of ALS resulted in a remarkable reduction of peripheral and

central lymphocytes which, in turn, resulted in suppression of the delayed reaction whether it be abrogation of skin hypersensitivity or prolongation of graft survival.

The mechanism responsible for the delayed skin reaction is speculatory. According to McCluskey et al. (20) and Feldman et al. (8) two steps are involved in the delayed reaction. The initial step is immunologically specific and involves relatively few lymphoid cells, probably lymphocytes, which have been altered [committed] by a first exposure to antigen and which, upon re-exposure to the antigen, trigger an inflamatory response. The second step is a nonspecific accumulation of mononuclear cells such as blast cells, other lymphocytes and monocytes.

The inhibition of delayed skin test reactivity by RAGPLS in this study was likely due to the effects of this antiserum on committed lymphocytes involved in the initial phase of delayed hypersensitivity. These cells may be coated or "blindfolded" with the globulin portion of ALS (17), or perhaps lysed in the presence of complement. Regardless of the mechanism of inactivation of committed lymphocytes, inhibition of skin reactivity would result since an insufficient number of lymphocytes would be available to trigger the mechanism.

This situation was somewhat more easily seen when ALS was given id. One-tenth ml of ALS injected id had an effective radius of about 40 mm. When histoplasmin was

injected id into an area less than 40 mm distant from the ALS site, it may have been that lymphocytes which were committed to H. capsulatum (or its by-products such as histoplasmin) would be drawn to the area (uncommitted lymphocytes which wander into the area would also be affected) and were acted upon by ALS. Once these cells were inactivated they became incapable of triggering the first stage of the delayed reaction. This system bears further investigation to determine how ALS was able to inhibit delayed type reactions over such a vast area of tissue since one might assume that ALS would be bound to various cells sharing antigens in common with lymphoid cells. This would tend to restrict the diffusion of ALS to an area close to its site of injection. This might be explained by reasoning that ALS perhaps attached to migrating lymphoid cells (as has been shown to occur in vitro)(29, 33) which migrated for some distance before they became incapacitated through the action of ALS. These cells, through close association, may have become adherent to other cells in the area due to the globulin coating the former cell's surface.

The mode of action of ALS is also debatable. Peripheral circulating lymphocytes may be coated with ALS in vivo and these coated cells are probably then subjected to a shortened life span, presumably by phagocytosis or opsonization in the spleen and liver. Consequently, the peripheral circulating lymphocyte pool is dramatically reduced. This is

the pool which is concerned with cellular and allograft immunities and this is the reason that ALS works so well in these immunities (A. P. Monaco, personal communication).

Denman and Frenkel (6), using autoradiography and immunofluorescence showed only limited penetration of ALS into spleen, thymus, and lymph nodes. This might be interpreted to mean that ALS was most effective on peripheral lymphocytes rather than on those within lymphoid tissue. Even so, Barth et al. (4) reported diminution of lymphoid cells including plaque forming cells in splenic tissue following ALS treatment. Although this condition persisted in the spleen during a secondary response, no effect on secondary antibody response to sheep red blood cells could be achieved with ALS. Thus, although it has been claimed that lymphopenia may not be essential for successful immunosuppression (15) some workers have noted a close correlation between lymphopenia and homograft retention (14).

The changes which occurred in splenic tissue, as mentioned above, may play a role in the extreme proliferation of H. capsulatum which occurred in various organs of ALS treated mice. This tremendous increase in the extent of tissue invasion provides some evidence that the cellular immune response is an important defense mechanism in limiting histoplasmosis to its typically benign form. Such an infection in ALS treated mice may well be attributed to an altered ability of the host to respond in an immunologically

normal fashion. This impairment of the immune system does not stem from competition of antigens (i.e. ALS with H. capsulatum yeast cells) for if this were so, mice treated with NRS should have more--not fewer--organisms/organ than those mice treated with saline. In fact, NRS seemed to have a mild inhibitory effect in this instance which was in accordance with the finding that no saprophytic fungi were isolated from the lungs of mice treated with NRS. Obviously, various fungal spores commonly present in the environment were continually being inhaled by these mice but the results of autopsies indicated that these common saprophytes do not proliferate to any great extent after access to the lung. This situation did not seem to prevail in mice treated with ALS. Such opportunistic growth may have resulted from treatment with ALS and/or be due to the dibilitated nature of the host which stemmed from infection with H. capsulatum.

CHAPTER V

SUMMARY AND CONCLUSIONS

The anti-mouse and anti-guinea pig antithymocyte sera prepared for this study were shown to contain cytotoxic and leucoagglutinating antibodies, and were capable of producing severe lymphopenia in these animals. The anti-mouse ALS was also shown to cause a reduction in the number of plaque forming cells when the hemolysis-in-gel plaque assay system was used.

It was found that guinea pigs treated weekly with ALS were more susceptible to development of fatal infection when inoculated with H. capsulatum. Guinea pigs which were treated weekly with ALS but not experimentally infected were more susceptible to infection from organisms in the environment than normal control animals. No fatalities occurred in guinea pigs infected with equal doses of H. capsulatum but treated with NRS or saline rather than ALS.

The time necessary to reach 50% fatality in mice infected with C. neoformans was greatly reduced by pretreatment with ALS compared to infected controls treated with NRS or

saline. When low dosages were used (0.1 LD_{50}) a similar pattern emerged but the effect was even more striking.

Culturing of spleen homogenates from mice inoculated ip with H. capsulatum showed that more than 150 times as many organisms were present than in the spleens of control mice that received the same infective dose but were treated with normal rabbit serum. Similar results were obtained from culturing the lungs but to a lesser degree. More organisms were recovered from the liver than from the spleen regardless of the type of treatment (ALS, NRS, or saline). The lungs contained the fewest number of organisms.

Delayed hypersensitive skin reactions were radically decreased or abrogated in H. capsulatum infected guinea pigs inoculated intraperitoneally 12 hours before skin testing with histoplasmin. When ALS was given weekly the influence on skin reactivity was less notable. Given intradermally, ALS was shown to inhibit the delayed reaction to histoplasmin within a radius of 40 mm.

The principal conclusions from this study were: 1. Animals treated with ALS shortly before and after experimental infection were far more likely to develop fatal infections than control animals. 2. Fatality was greatly accelerated by treatment with ALS. 3. Relatively low doses of C. neoformans (innocuous to saline treated mice) rapidly killed mice treated with ALS. 4. No impairment of humoral antibody production was observed but cellular

immunity was affected. It appeared likely that this impairment of cellular defense resulted in much higher mortality rates in animals treated with ALS. 5. Delayed hypersensitive skin reactions can be depressed or abrogated by ip or id treatment with ALS. 6. A moderate leucocytosis resulted from weekly treatment with ALS and was similar to that of H. capsulatum infected guinea pigs treated with ALS. Infection alone resulted only in a slight and transient leucocytosis. 7. Antilymphocyte serum in guinea pigs caused a severe but transient lymphopenia. Infection by H. capsulatum in ALS treated animals slowed the return of lymphopenia toward normal values.

It is suggested that ALS offers a useful tool for immunological investigations in animals which are experimentally treated with various microbial agents. Such studies might include selective inhibition of cellular immune mechanisms mediated by peripheral circulating lymphocytes and the subsequent effect on virulence and survival of the test organism used. Antilymphocyte serum might also be of value as a means of increasing susceptibility of animals to various "opportunistic" microorganisms which possess limited capability to cause infection.

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