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IMMUNOLOGICAL RESPONSE BY GUINEA PIGS CHRONICALLY INFECTED WITH HISTOPLASMA CAPSULATUM

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IMMUNOLOGICAL RESPONSE BY GUINEA PIGS CHRONICALLY INFECTED WITH *HISTOPLASMA CAPSULATUM*

CHAPTER I

INTRODUCTION

Serological methods for the study of histoplasmosis have been used since 1947 when Salvin measured complement fixation titer in sera obtained from animals infected experimentally with *Histoplasma capsulatum* (39). Various tests utilize the serological principles of complement fixation, agglutination, and precipitation reactions (8, 18, 42). Most investigations made to date have been attempts to establish information about factors which are responsible for the wide variation and peculiar relationship between histoplasmosis and circulating antibody titers demonstrated. Variation occurs in both man and experimental animals who are infected in one way or another to the etiological agent, *H. capsulatum*. The difficulty of interpretation may reside in the fact that a variety of techniques and materials have been employed (40, 45). For instance, Saslaw and Campbell

used a yeast phase antigen for complement fixation (C.F.) while Tenenberg used histoplasmin (8, 49). Specific isolates, antigenic materials, incubation periods, and reaction temperature are other examples of this variation (22).

Animal infection studies suggest several factors are influential and contribute to the apparent variation of results frequently obtained. Growth phase of the organism, route of inoculation, size and viability of specific isolates are all important factors to consider when one attempts to characterize the sensitizing agents.

Reports by investigators have given rise to a number of different interpretations of the wide range of immunological responses to this infectious agent. Generally the production of precipiting antibody precedes the occurrence of complement fixing antibodies (7, 42). Production patterns of agglutinin titers closely resemble those of precipitin titers with the exception that the former are demonstrable for a longer period of time. Several different diseases which have been studied demonstrate that the first immunoglobulin produced usually is the type characterized as 19S (IgM) type. The sequential production of 7S (IgG), is considered responsible for the complement fixation activity demonstrated (46). Positive identification of any similar

condition existing in H. capsulatum has not been completed.

Attempts to utilize serological tests in diagnosis and prognosis have been made with questionable success (7, 25, 42). A recent conversion to a positive skin test is considered suggestive of a relatively recent infection (7, 25). Increase of C.F. titers accompanied by negative precipitin titer is considered by some to reflect a poor prognosis (15). Grayston obtained results which show no correlation between serological titer and severity of disease (16). Presence of a substantial level of C.F. antibody also has been associated with an increased resistance to re-infection (38, 41). A loss of skin test reactivity concomitant with a loss of demonstrable antibody in chronically infected individuals has been observed (5).

Increased resistance to reinfection has been studied in still other ways attempting to understand immune response. Experimental investigations using L.D. 50's as well as increased levels of antibody titers are used most frequently (19, 29, 40, 42). Conflicting data prompt proposal of several hypotheses with respect to this aspect of histoplasmosis. The majority of individuals who resist infection or challenge exhibit demonstrable circulating antibody (41). However, an appreciable number have no apparent circulating antibody as

evidenced by negative serological tests (16, 42). Thus it appears that circulating antibody is not involved as the only mechanism of defense in resistance. Sweany (48) and others (50) discuss the probability that phagocytosis or tissue fixed antibody may be responsible for increased resistance. Howard (20) has shown that immunized mice contain peritoneal exudate cells with increased phagocytic activity compared with non-immunized subjects.

The immunological activity or inactivity contributing to variance in apparent inconsistency which may be due to the techniques employed is still primarily an unanswered question. However, the fact that the majority of individuals within a population experience only a mild primary pulmonary type infection suggests a high percentage of a population show immunological competence with regard to histoplasmosis (22).

Many individuals who experience a chronic infection by H. capsulatum are known to exhibit low levels of C.F. antibody (Larsh, Unpublished). Recognition of this phenomenon causes speculation that H. capsulatum may impair the functioning ability of the mechanisms involved in immune response.

H. capsulatum is known to exhibit a predilection for

the tissue of the reticulo-endothelial system (RES). The fact that the RES is considered to be involved in production of antibody would suggest that a condition may arise during infection which impairs or incapacitates the functioning ability of the RES. Our experimental testing has shown that C.F. antibody is produced in relatively large quantities, primarily several weeks after the initial infection.

Most of the investigations discussed in the literature on this topic are primarily concerned with the response elicited in the majority of the test subjects. However, another aspect which is inherent to all biological systems revolves primarily around the idea of variation in populations. Expression of this variation usually involves only a minority of a population. Variation in natural resistance has been studied and Loosli (27) observed that age and sex are definite factors. Saslaw and Schaefer observed that young and old males are the most susceptible to H. capsulatum infections (44).

It appeared pertinent to investigate a system which basically follows a format of previous investigators, while at the same time looking for identities with respect to the minority of a population. This aspect is logically valid because intensive study of this disease stems not from the fact

that a relatively small percentage of persons do become systemically infected alone, but also because of its immunological implications.

This study was designed to elicite information pertinent to the question of varied immunological responses observed in experimentally infected animals. Relatively new techniques were employed to measure several aspects of immunological response.

A technique introduced by Jerne, et al. (21) was employed to measure quantitatively the number of spleen cells exhibiting antibody production. Auzins (2) and others have used plaque forming cells as a quantitative indicator of cellular involvement.

Recent investigations which include a wide variety of diseases have established disc electrophoresis as a technique useful in characterization of sera. Tuberculosis, pneumonia, cancers and nerve disease are examples of diseases which exhibit reproductible characteristically specific patterns which differ from those of normal sera (35).

A study involving chronic and nonchronically infected guinea pigs was initiated. This study involves an assay system which employs chronically and nonchronically infected guinea pigs. Two groups of guinea pigs were subjected to

chronic infections of H. capsulatum and Listeria monocytogenes. Both groups were challenged with secondary infections and assessment of their ability to respond immunologically was performed. Serum protein from all test groups was partially characterized by acrylamide gel electrophoresis. These two groups were compared and tested with respect to their capacity to produce demonstrable antibodies and cells associated with antibody production.

Theoretically one should be able to characterize the significant differences in serum electrophoretically. Assessment of such a subtle variation has been made for antibody molecules with respect to reaction affinities as related by the work of Eisen (12).

CHAPTER II

MATERIALS AND METHODS

Organisms employed for infecting guinea pigs.

a. Listeria monocytogenes:

L. monocytogenes (A4413) was employed throughout these studies. A virulent culture was obtained from Dr. C. P. Sword, University of Kansas, Lawrence. The culture was obtained from tryptose agar slants. Inocula for experimental investigations were prepared by subculturing the organism in tryptose broth at 37C.

Seventy two-hour tryptose broth cultures of L. monocytogenes were washed and diluted in physiological saline to yield about 5×10^7 organisms per ml.

b. Histoplasma capsulatum:

H. capsulatum (Scritchfield) was employed in this study. This is a culture presently maintained in our laboratory. Sabouraud dextrose agar slants were inoculated and cultured for several days.

c. Salmonella typhosa:

S. typhosa (ATCC 12840) obtained from the

Table 1. Inoculation of guinea pigs with *H. capsulatum*

H. capsulatum isolate	Inoculation route	Inoculation schedule (day)	Inoculum size	
			Total parts	Viable parts
Scritchfield	IP	1	9.4×10^6	1.4×10^4
"	"	5	1.4×10^7	3.7×10^5
"	"	11	2.2×10^7	5.5×10^5
"	"	42	6.8×10^6	6.5×10^4
"	"	46	1.7×10^7	1.1×10^4
"	"	50	1.6×10^7	1.0×10^5
"	"	119	1.4×10^5	3.4×10^6
"	"	185	1.6×10^5	2.2×10^4

University stock culture was used in this study. The present culture of S. typhosa has been maintained on medium #23, but was subcultured on nutrient agar slants for use throughout these experiments.

Experimental Infection of Animals.

A total of 300, Hartley strain, male guinea pigs were selected for this study. Forty-five comprised a group which had received 10 serial intraperitoneal injections of H. capsulatum (Scritchfield). Each injection was 5×10^4 to 3×10^5 viable mycelial units contained in 1 ml as determined by plate counting, and shown in Table 1. Injection schedule spanned a period of 7 months. A second group of animals were serially injected intrapertoneally in a 45 day period with L. monocytogenes using an inoculum size of 5×10^7 to 2.5×10^7 as related in Table 2. Noninfected animals made up the remainder of the animals in this study.

Distribution and Sampling.

The noninfected group of animals was subdivided into 4 smaller regiments, to be utilized later for establishment of primary infected subgroups and controls. Final groupings on animals were made so as to include a minimum of 12 animals per specific test. For example, chronically H. capsulatum infected animals, which were subsequently superinfected with

Table 2. Inoculation of guinea pigs with L. monocytogenes

Organism	Inoculation route	Inoculation schedule (day)	Inoculum size Viable counts
L. monocytogenes	IP	1	5×10^7
"	"	5	2.5×10^7
"	"	8	"
"	"	14	"
"	"	21	"
"	"	28	"

L. monocytogenes were housed in groups of 4 animals per cage with a minimum of 3 cages per test.

Animals receiving the original series of inoculations of H. capsulatum and L. monocytogenes were superinfected with H. capsulatum, L. monocytogenes and S. typhosa.

Superinfection was accomplished by administering two injections per animal 7 days apart. All injections were made intraperitoneally as related in Table 3.

A heavy suspension (1:10 vv) of L. monocytogenes cells in distilled water was subjected to three, 5 minute periods of sonication, in a Blackstone Model sonicator. Microscopic examination demonstrated a highly fractionated sample of bacterial cells. The suspension was next centrifuged at 10,000 g's for 60 n minutes in a refrigerated centrifuge (Sorval Model RC-2). The supernatant extract was then used as the complement fixing antigen. This method for production of C.F. antigen was introduced by Seeliger and Cherry (1).

Histoplasmin (1:25) which was generously supplied by Dr. C. E. Palmer, U. S. Public Health Service, was employed as C.F. antigen in the histoplasmosis assay.

Twice washed hemologous cells of S. typhosa were used in the agglutination testing.

Table 3. Inoculation of guinea pigs with primary infecting agents

Organism	Inoculation route	Inoculum schedule (day)	Inoculum size Viable counts
H. capsulatum (Scritchfield)	IP	1 8	1×10^6 $.5 \times 10^6$
L. monocytogenes (A 4413)	IP	1 8	5×10^7 1×10^7
S. typhosa (ATCC 12840)	IP	1 8	1×10^6 * $.5 \times 10^6$

* Heat killed whole organisms

Collection of Sera.

Random samples from noninfected animal groups were bled using the cardiac puncture technique. These initial bleedings served as the controls in subsequent immunological tests. All bleedings were performed using 5 ml syringes and 1 inch 20 gauge needles. Samples from animals which had received the serial injections were similarly bled prior to superinfection and served as reference controls. At 0, 8, 12, 16 and 20 days post injection, random sample animals were bled and sacrificed. Four animals from each group were bled for each test. The 5 ml collections were divided; one ml was collected in citrated containers, while four ml were used as the source of test sera.

Sera were decanted from tubes and stored at 4 C until used.

All sera were coded by number so that the specimen was examined for titer and characterized electrophoretically without knowledge of the source of the specimen.

Preparation of Spleen Cell Homogenates.

Four animals from each test group were sacrificed and autopsied. Whole spleens were removed and superficially washed in sterile Hanks Balanced Salt Solution. Spleens were subsequently diced and cell contents of each spleen were

teased out into a culture medium. Finally, homogenates of diced spleens were obtained using teflon grinders. Minimum Essential Medium (MEM) without serum was used in these experiments. Cell suspensions were washed by repeated centrifugations and finally resuspended in MEM. Aliquots were taken and the final total cell counts were determined. The spleen cells in a sample from this suspension were stained with Giemsa stain and counted under the microscope. No determination of cellular type was attempted; only the total number of counts representing nucleated lymphoid cells was made. This total was usually in the order of 5×10^8 per spleen. These steps were carried out around room temperature using chilled MEM. Upon completion of counting, the cell suspensions were placed under refrigeration until final plating. Preparation of assay plates necessitated making appropriate dilutions of washed cell suspensions to give from 1×10^4 to 2×10^7 cells per ml from stored homogenates. Plating involved the use of a technique introduced by Jerne et al. (21) with minor modification (9, 45). Plastic petri dishes 9 cm in diameter were used. This preparation included the introduction of 15 ml of 1.4% Difco agar in MEM into each plate. These plates were stored in a 37 C incubator for 24 hours to evaporate a small amount of water. This procedure increases

the ability of the plate to reabsorb moisture from the top layer. All agar used in the top layer is first dissolved in boiling water. Two grams to dry DEAE cellulose is added in the form of hydrochloride. The DEAE is removed by filtration after brief boiling, and the resulting agar solution is dispersed in 2 ml quantities and maintained in a 45 C water bath. The DEAE agar treatment reduces the anticomplement activity of agar (21). To each tube is added 0.2 ml suspension of sheer tanned sheep red blood cells (SRBC) previously absorbed with 1:25 Histoplasmin and 0.2 ml suspension of spleen cell homogenates containing approximately 5×10^4 to 1×10^5 spleen cells. The contents were mixed and poured over the bottom layer of MEM agar. The plates were then incubated for 2 hours at 37 C. Plaque counts were made under 10 X magnification using a binocular microscope. Plaques appear as lysed areas in the layer of SRBC's, having a diameter of about 1.0 mm.

Blood Cell Determinations.

Duplication blood smears previously made were immediately stained with Wright Stain. Differential cell counts were made from these preparations.

Citrated blood samples were used immediately for determination of total WBC counts. All counts were made from suspensions in "white" leucocyte pipettes with 1.0% acetic

acid being employed to lyse the RBC's.

Assay of Serological Titers.

All sera collected in this study were titrated for antibody concentrations. The complement fixation technique described by Lackman (23) was used for titering of H. capsulatum and L. monocytogenes infected animals. Tube agglutination titer determinations were performed on S. typhosa infected animal sera according to the method of Burrell and Mascoli (6).

Electrophoresis of Sera in Acrylamide Gels.

Stock solutions for anionic gel systems having a running pH 9.3 are given in Table 4.

Original stock solutions are usable for 6 months, with the exception of those containing ammonium persulfate, which must be prepared at weekly intervals. Preparation of acrylamide gels was accomplished in the following manner.

1. The sample gel solution and chemicals were completed and all preparations were stored in tinfoil covered bottles and maintained under refrigeration.

2. Preparation of gel tubes.

a. All tubing had an inner diameter of 5 millimeters and 7.5 cm in length. Tubes were cleaned with 7X cleaning solution, rinsed through distilled water,

Table 4. Stock solutions for anionic gel systems

ANIONIC GEL SYSTEM				
	VOLUME RATIOS	COMPONENTS/100 ML		pH (25 C)
LOWER GEL	1	Acrylamide Bisacrylamide Water to volume	30gm 0.8gm	
	1	Tris 1 N HCl Temed Water to volume	18.15gm 24ml 0.24ml	9.1
	2	Ammonium persulfate Water to volume	0.14gm	
UPPER GEL	1	Acrylamide Bisacrylamide Water to volume	10gm 0.8gm	
	1	Tris 1 M H_3PO_4 Temed Water to volume	2.3gm 12.8ml 0.1ml	6.7
	1	Riboflavin Water to volume	2mgm	
	1	Ammonium persulfate Water to volume	80mgm	
UPPER BUFFER		Tris Glycine Water to volume	5.16gm 3.48gm	8.91
LOWER BUFFER		Tris 1 N HCl Water to volume	14.5gm 60ml	8.07

and finally rinsed with a 1:200 solution of Kodak Photo-Flo 200 solution and allowed to dry at room temperature.

b. Tubes were next fitted with the hollow rubber cap (Canalco #3-1768) to seal one end and placed in GEL Polymerization Rack (Canalco #3-1762). Adjustment of tube includes leveling all the bottom ends as well as leveling the entire apparatus.

c. Using a 1 ml syringe fitted with a 20 gauge needle and 10 ml length of plastic tubing, each tube was filled with 0.75 ml of the lower gel solution. The surface of the gel solution was next layered with approximately 0.1 ml of distilled water to insure the formation of a flat upper surface. Allow lower gel solution to polymerize for 30 minutes. Next the layering water was decanted along with any unpolymerized gel solution. This was most easily accomplished using rolled absorbing tissue paper.

d. Repeat the layering technique using 0.2 ml of the upper gel solution. Again overlay upper gel solution with 0.1 ml distilled water. A fluorescent light assembly (Canalco #3-1764) was placed approximately three inches behind the apparatus. The upper gel

solution was photopolymerized 20 minutes. Again the layering water and any unpolymerized gel solution was decanted.

e. Rubber caps were removed from the lower ends of the tubes and the tubes were placed in tube holder assembly of the upper electrophoresis chamber, positioning the upper gel in the upper position. Upper buffer solution was added to the upper buffer chamber, sufficient to cover the upper electrode of the plastic cover when it was in place. A hanging drop of upper buffer was added to the lower end of the tube to prohibit air bubble formation. The lower buffer chamber was partially filled with lower buffer solution to a level approximately 1 cm above the lower end of gel tube.

f. Two microliters of sample serum were carefully layered on top of the upper gel. Twelve tubes were run concurrently including a standard normal serum in each series of runs to insure the fact that uniformity exists between runs. The serum protein front was marked by adding 1 ml of a Bromophenol blue dye (.001%) to the upper buffer compartment.

g. Adjustment for a uniform current, 3.3 ma per

column, to pass through gel tubes was made. The leading migrating front marked by the dye was followed visually. The run was terminated when this front reached a distance of about 1-2 mm from the bottom of the gel column. The time for the run was approximately 50 minutes.

h. At completion of electrophoresis, the gel columns were removed from the glass tubes by rimming with a 22 gauge needle, while forcing water through the needle to aid in gel removal. Upon removal, gels were immediately fixed and stained for 60 minutes in a solution of 0.5% Amide Schwartz dye in 7% acetic acid.

i. Destaining the gel column was done in the assembly holder. Each tube was positioned in the tube holder assembly, both chambers were filled with 7% acetic acid and adjustment for a current of 5 ma per gel column from the power supply was made. All background stain could be removed in approximately 2-3 hours. Gel columns were stored in vials containing 7% acetic acids.

Evaluation of the Differences between Patterns.

Three visual criteria were used in the preliminary

assessment of the acrylamide gels: (a) number of bands in the gels; (b) the position of the bands; (c) the density and width of the bands as an estimate of the relative amount of protein in each fraction. Quantitation of the gel columns is based on the densitometer tracings.

Gel columns were read and recordings of the tracings were performed using a Canalco Model 5 Microdensitometer and a Servoriter II Recorder respectively.

The disc-electrophoresic method achieves its separations by electrophoresis of material in a polyacrylamide gel matrix of defined pore size and shape. The 7% gel used here produced an average pore around 50 Å in diameter. Separations are sharpened by the use of thin starting zones where a discontinuous buffer system is combined with a large (2½%) gel for an initial period of electrophoresis. The buffers and pH are selected to conform to conditions permitting the application of the Kohlrausch regulating function (9, 32). The initial period of electrophoresis occurs in the large pore gel which is referred to as the stacking gel. The function of this gel is to serve as a preconcentrating step. The proteins in the sample upon entry into the separating gel are stacked in bands, one above the other, in order of decreasing mobility.

CHAPTER III

RESULTS

Evaluation of Serological Determination.

Inoculations used in establishing infection were performed as related in the schedules listed in Tables 1, 2, and 3. Criteria for active infections were positive skin test reaction and/or production of C.F. antibody.

Animals receiving serial injections of H. capsulatum responded in a variable manner. Positive skin tests were observed in approximately 80% (165/210) of test subjects. Skin test reactions varied with respect to size of induration and erythema. Positive skin tests were recorded for all animals exhibiting an induration of 5 mm diameters or larger. Approximately 20% (45/210) of the animals exhibited no skin test reactivity. These as well as samples from those reacting were bled and all exhibited positive C.F. titers, ranging from 1:8 to 1:256. Any sera becoming positive as a result of previous skin testing has not been identified.

This series of experiments, as described earlier used H. capsulatum (Scritchfield isolate) for inoculation and

sensitization of 6 month old male guinea pigs. The results of Salvin (35, 37) indicate serological methods offer a better means of detecting this disease in guinea pigs than cultural methods, especially after the first four to six weeks.

A second group of animals receiving serial injection of L. monocytogenes were not skin tested. However, samples of this group were bled. Recognition was made that animals repeatedly infected with this organism demonstrated a typical listeriosis infection. Illustration of this is made in Figure 1, which clearly shows an increase in total leucocytes and specifically monocytes. Such a typical response in listeriosis is described in the work of Armstrong and Sword (1). They identified the primary resistance in listeriosis to be cellular. They had earlier recognized that an inoculation with a dose size in the order of 5×10^7 or greater is necessary to establish infection in guinea pigs.

Animals making up the aforementioned groups, those receiving a series of inoculations prior to superinfection, are subsequently referred to as chronically infected animals. Only those guinea pigs receiving two inoculations, as described in Table 3, are referred to as primary infected animals.

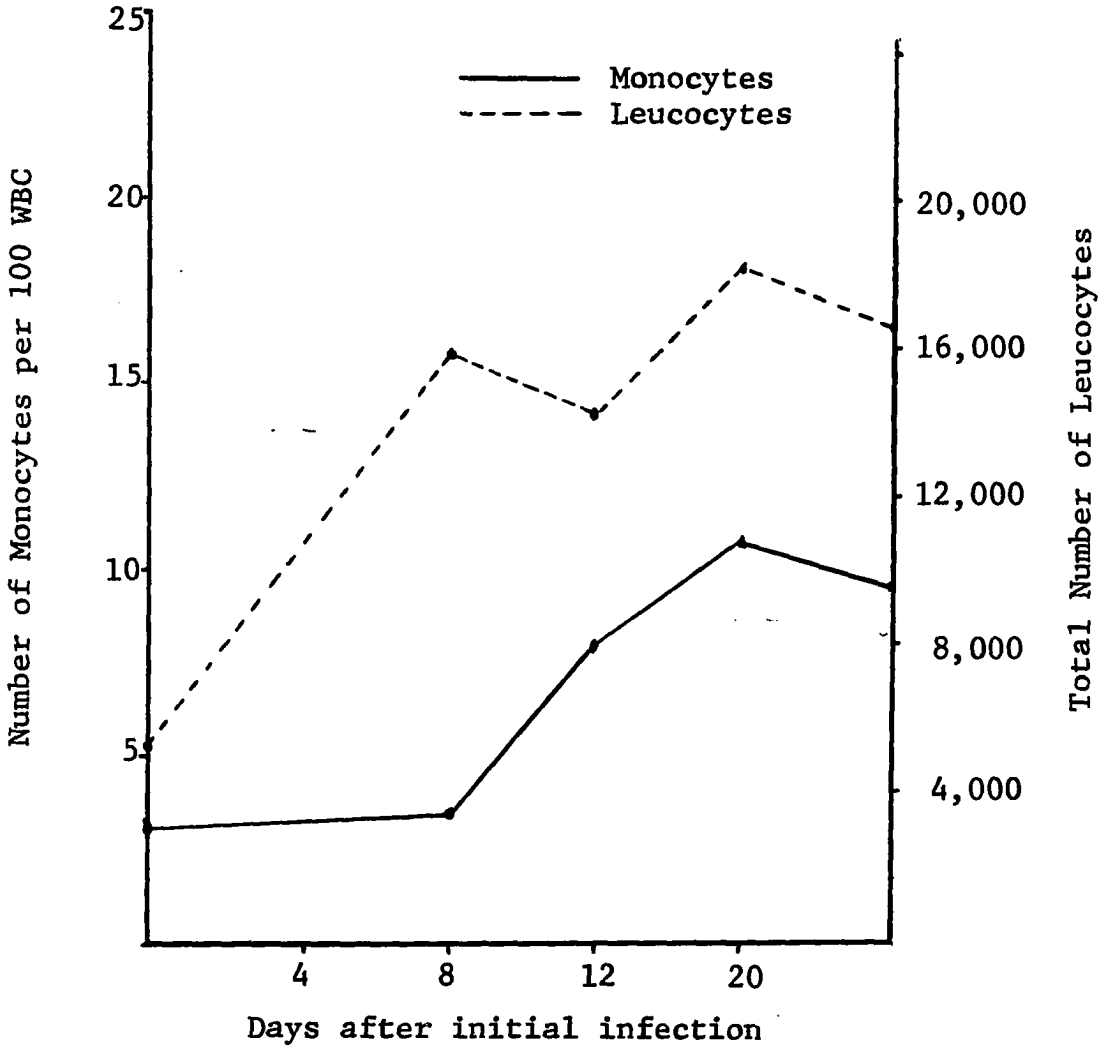


Figure 1. Variation in white blood cells in L. monocytogenes infection

Complement fixation titers of sera from the various test groups are listed in Figure 2. The mean titer of 12 test sera is used and expressed as the reciprocal of the dilution.

All titers in Figure 2 reflect the immunological response to two inoculations of the respective organism as related in the primary sensitization schedule listed in Table 1. Titers typifying those reported (8, 1, 42) earlier were demonstrated in the sera of the animals in this study. L. monocytogenes (1:32) sensitized animals appear to reflect a higher C.F. response than do the H. capsulatum (1:8) sensitized animals. However, there is no way in which one can actually compare these two separate systems.

Guinea pigs inoculated according to the schedule listed in Table 1 demonstrate a C.F. titer which is substantially higher (1:128) than the animals receiving only 2 inoculations. This is shown in Figure 3, and is in agreement with expected results (39). The range of titers for each group varied from 1:32 to 1:256. The foregoing is also true for the L. monocytogenes inoculated animals whose sera exhibited a mean value of 1:128 for the 12 sera tested.

It is of greater interest to note the immunological response of the animals serially inoculated with H. capsulatum

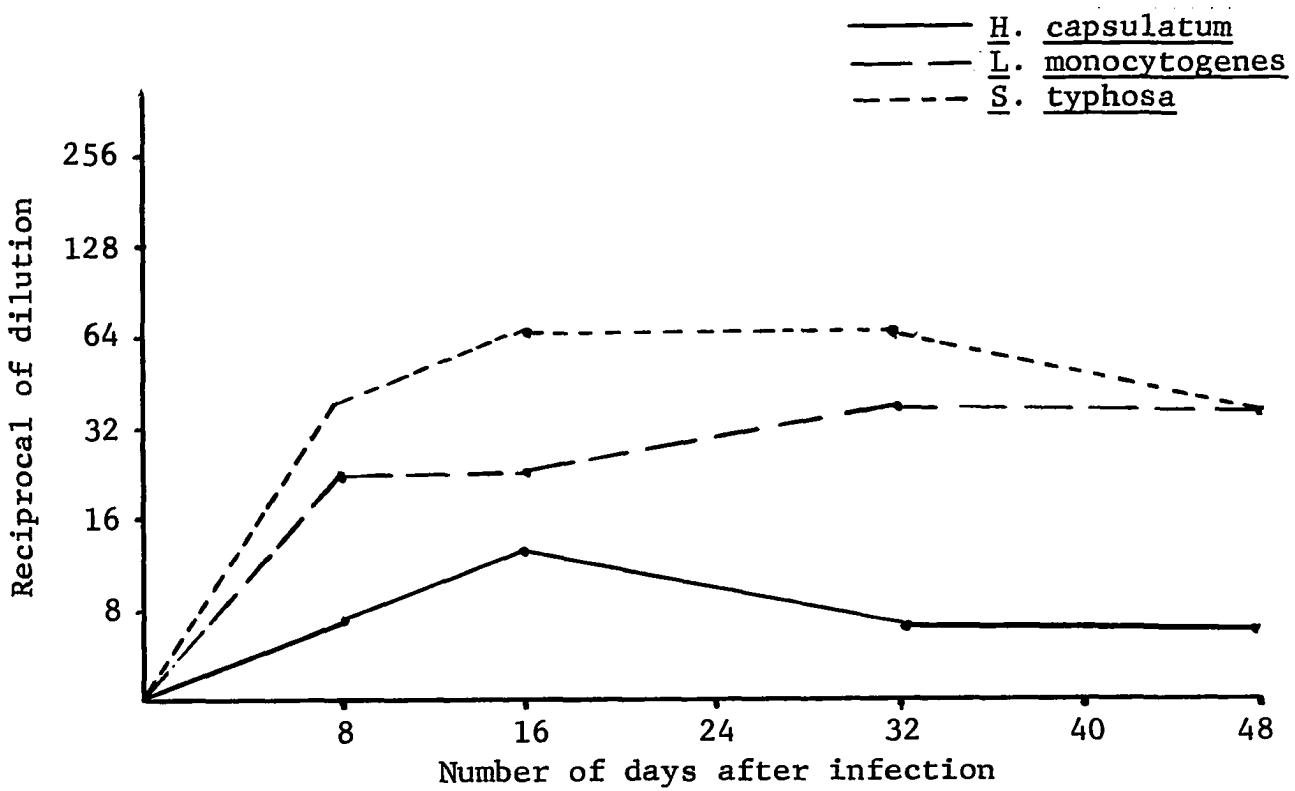


Figure 2. Complement fixation titers of sera from guinea pigs with primary infection of test organisms. H. capsulatum, L. monocytogenes and S. typhosa.

and L. monocytogenes which are subsequently challenged with a second infectious organism. It is from within this group that one can look for immunological incompetence.

It was observed that the C.F. titers of sera from H. capsulatum infected animals having an L. monocytogenes superinfection are similar (1:8) when compared with those of primary infected animals and is shown in Figure 3. The agglutination titers for S. typhosa infected animals were also in the same order of magnitude. A similar observation (Figure 4) was made with respect to C.F. titers (1:16) from sera of L. monocytogenes infected animals which were superinfected with H. capsulatum. The ability of both groups of chronically infected animals to respond immunologically to a second type infection is apparently the same.

Evaluation of the Spleenic Cellular Response

Results reflecting the cellular response aspect of this study are listed in Table 5. The number of cells capable of producing plaques as determined by the technique employed are related for each test group.

The number of plaques are directly proportional to the number of individual cells. "All cells capable of producing antimolecules (H. K. Eisen, 1962) are known to display

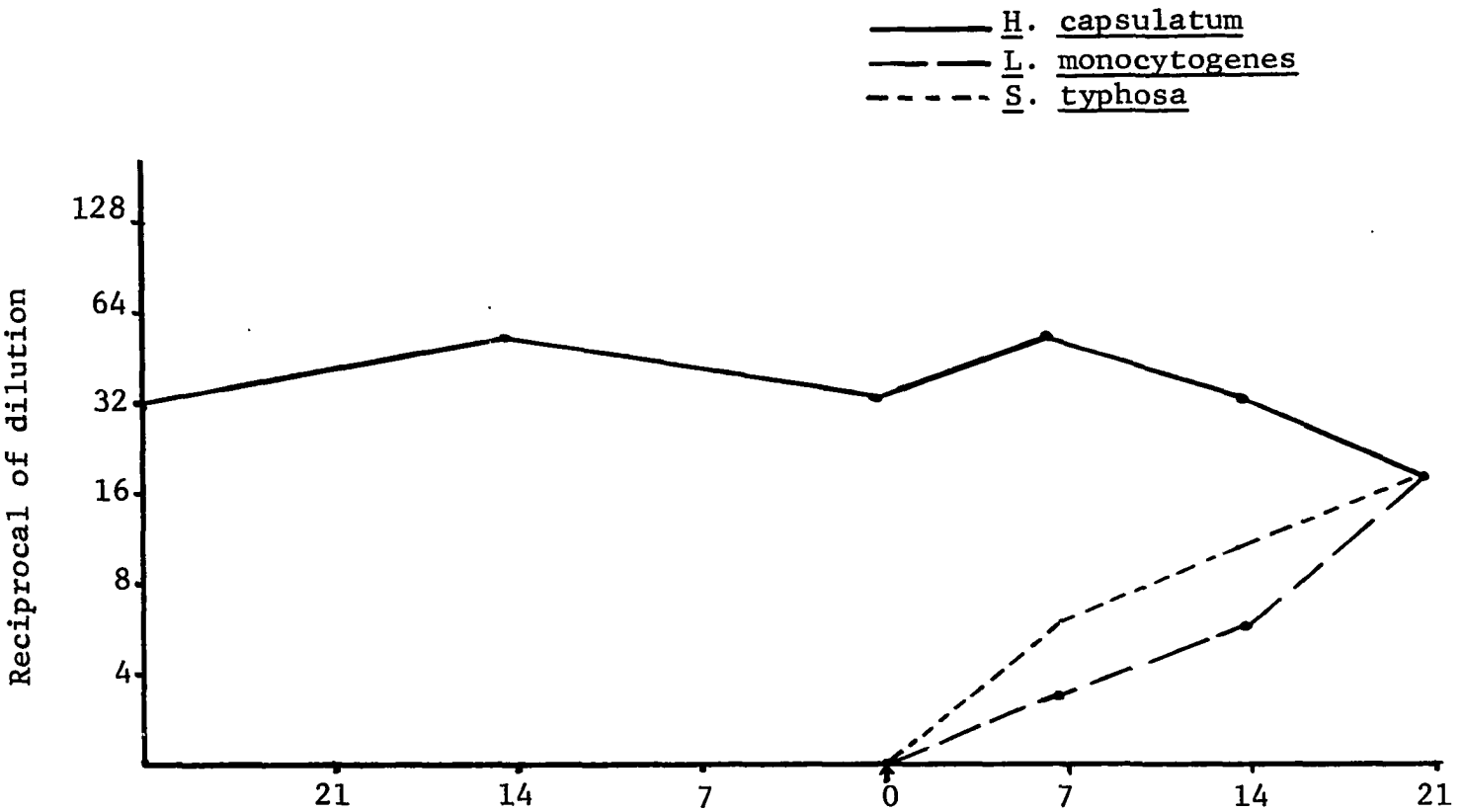


Figure 3. Complement fixation titers of sera from guinea pigs chronically infected with H. capsulatum. Superinfection titers shown at the right side of figure.

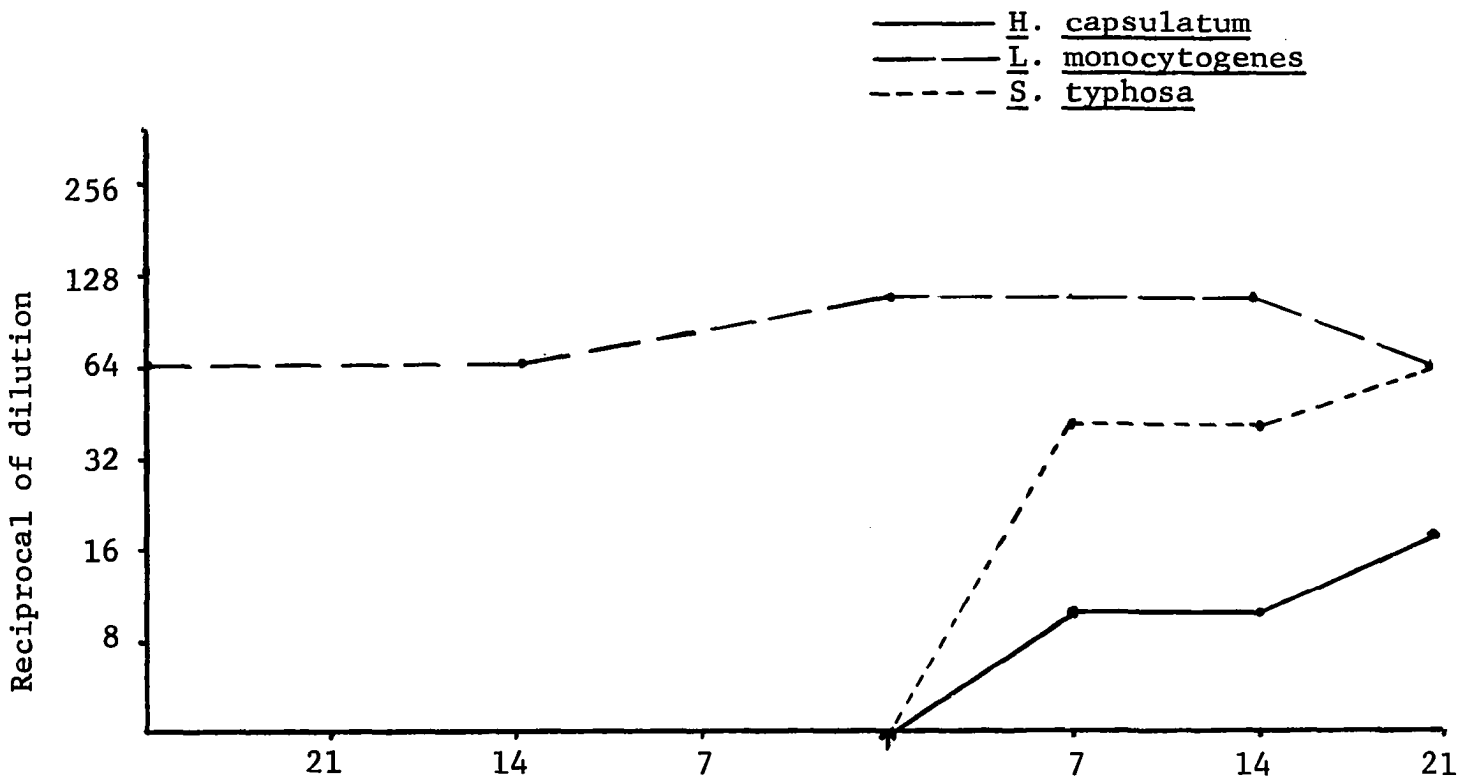


Figure 4. Complement fixation titers of sera from guinea pigs chronically infected with L. monocytogenes. Superinfection titers shown at the right side of figure.

Table 5. Plaque forming cells per spleen*

Infection classification and organism	Control	Primary infection	Homologous superinfection	Reciprocal superinfection
NON CHRONIC	10 - 30			
H. capsulatum		250 - 500		
L. monocytogenes		500 - 750		
Bovine serum		300 - 500		
CHRONIC	Prior to superinfection 30 - 50			
H. capsulatum		5000 - 7500	7500 - 10,000	250 - 300
L. monocytogenes		1500 - 2500	2000 - 2500	150 - 300
Bovine serum				

* All spleens were taken 16 days post infection.

some of these at their surface" (21). SRBCs previously adsorbed with specific antigen are mixed with spleen cell homogenates. Specific interaction between the cells containing antibody and adsorbed SRBCs results in lysis of the SRBCs.

Individual plaques reflect the number of cells producing the specific antibody. Spleens have exhibited the presence of antibody producing cells as well as other cell types. Lymph node cells also are known to be associated with antibody and have been studied in a similar manner (10). Table 5 illustrates the results obtained from the spleen cell homogenate studies. Four animals were sacrificed in each test group. Four replicates of each spleen homogenate were plated. All values listed represent the average of the four guinea pig spleens. More accurate measurement was made difficult because it was necessary to make dilution of whole spleen homogenates.

Similarity exists in the number of PFCs is parallel to the increase in 2-mercapto-ethanol sensitive antibody. Thus it is interpreted that the technique employed is primarily an in vitro detection of individual 19S (IgM), antibody producing cells. The relationship of PFCs to C.F. antibody titers is shown in Figure 5. A correlation between PFCs and titers is observed.

Chronically infected animals demonstrate an average number of plaques appreciably larger than animals receiving only 2 inoculations. It was observed that the chronically infected animals upon reinfectation of homologous organism showed an increase but not the same degree of increase as in the heterologous infections. By reviewing the response of the chronically infected animals to superinfection, a reduction in the number of PFCs as compared to non-chronically infected animals can be observed.

It was noted that the response patterns were similar for both types of chronically infected animals. It was recognized that control animals exhibited a reduced number of plaques, even though no prior history of specific infection was known. An explanation for this possibility resides in the non-specificity of some antibody present.

Evaluation of Acrylamide Disc Gel Electrophoresis.

Initial studies were performed to determine reproducibility for this method of electrophoresis. Aliquots of normal human serum and normal guinea pigs were electrophoresed on several separate occasions. Visual observations indicated good reproducibility between columns electrophoresed in this manner (Figure 6). Densitometer tracings of these columns were also in agreement with each other. It

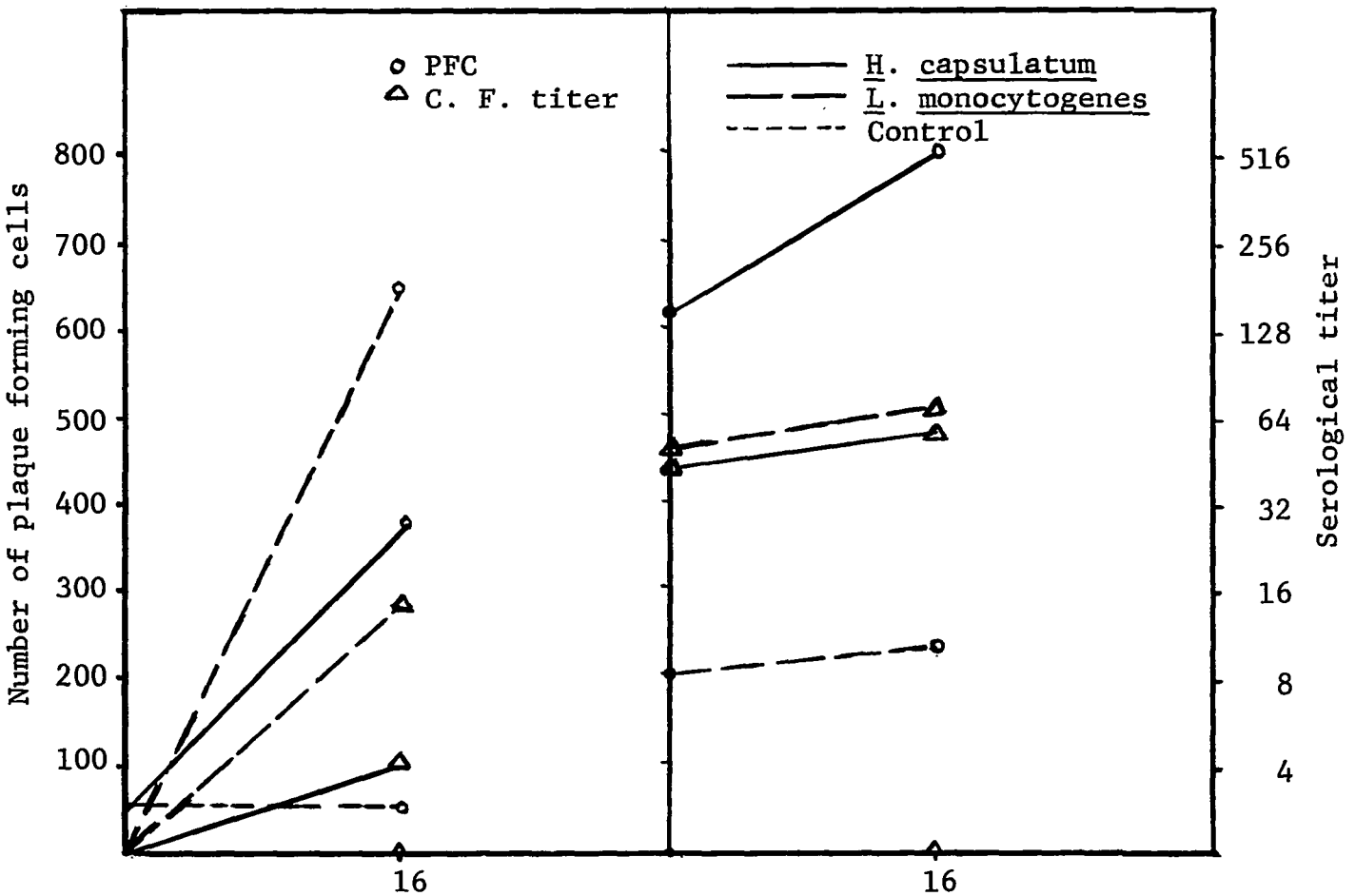
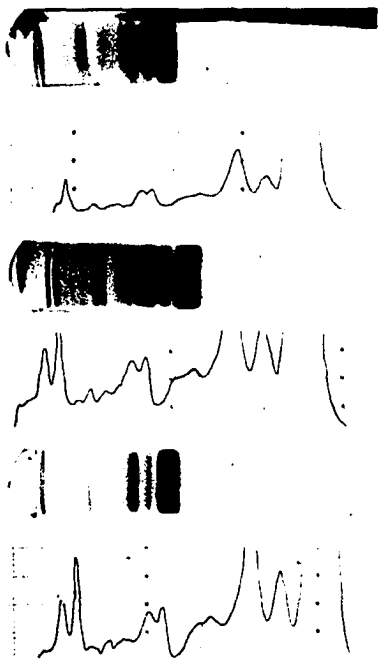
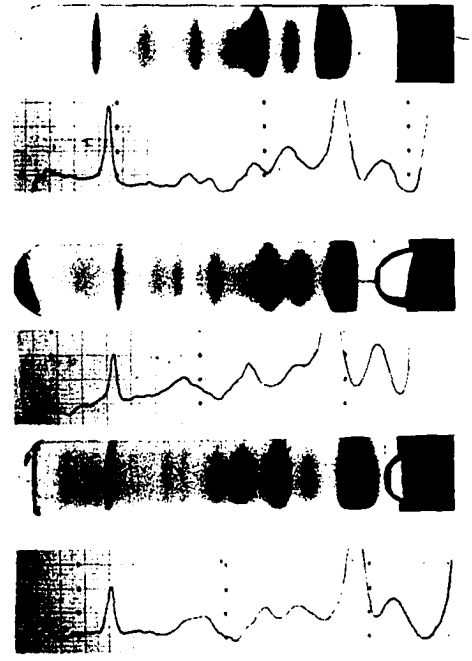


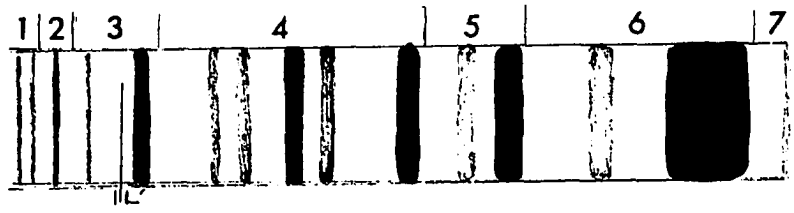
Figure 5. Relationship between PFC and C.F. titers from nonchronically infected guinea pigs.



NORMAL HUMAN SERUM



NORMAL GUINEA PIG SERUM



NORMAL GUINEA PIG SERUM

1. Gamma globulins
2. Gamma globulins (origin)
3. Gamma globulins, slow α-globulins
4. β-globulins
5. Transferrins, fast α-globulins haptoglobulins, albumins
6. Albumins
7. Prealbumins

Figure 6. Photographs, diagrams and densitometer tracing of normal human and normal guinea pig sera following electrophoresis.

was determined from preliminary studies that increasing the column volume (length) from 0.50 ml to 0.75 ml increased the resolution, especially of the trailing bands.

Partial characterization of guinea pig serum protein had been accomplished using acrylamide gel electrophoresis. Previous reports use several different methods to represent the distribution of protein bands in gel columns. The most commonly used is in the work of Ornstein (32) and is represented in Figure 6. One observes that the albumin fraction follows closely behind the front tracing dye. Investigation of animal sera has demonstrated that approximately 10% of the serum protein fails to migrate in the anionic system. A substantial amount of this stationary protein has been identified as immunoglobulin.

All samples of sera subjected to electrophoresis in this study were used in duplicate. Four different sera were tested from each test group. Therefore, a minimum of eight columns was examined for each test group. The greatest uniformity of separate bands is observed to occur in the region of RD values between 0.5 and 0.95. Variations occur most prominently in the lower RD values. This region is commonly referred to as γ 2.

Figure 7 represents a composite of several gel columns,

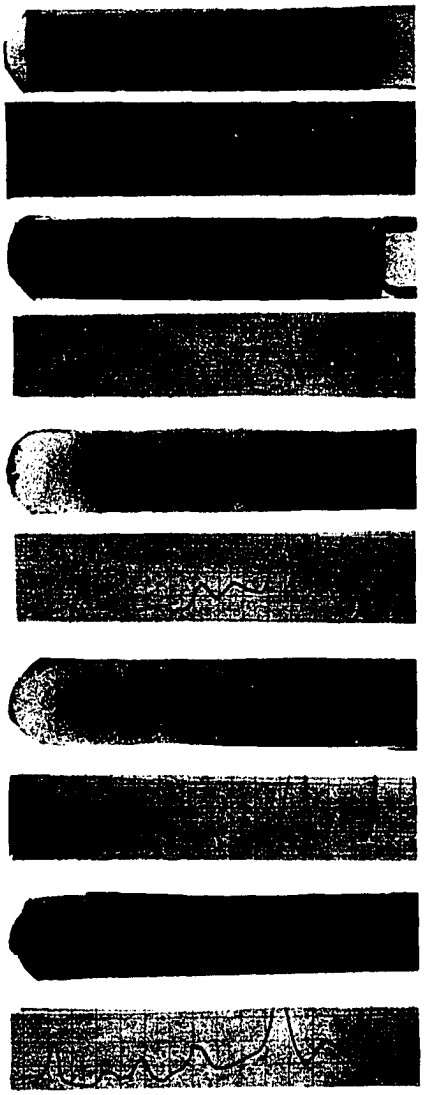


Figure 7. Electrophoretic patterns from guinea pigs infected with H. capsulatum. Various size skin test reactivity are grouped.

reflecting serum samples which had been collected from animals previously grouped according to skin test reactivity.

Included in this composite are sera from animals with the following classifications: non-reacting, induration sizes of 1-5, 6-12, and 13 mm in diameter or larger. Also included is a control serum. Several differences can be observed; however, the one difference of primary interest is labeled 1'. This band appears in all sera obtained from animals chronically infected with H. capsulatum with the exception of non-skin test reactive chronically infected animals. Shown in Figure 8 is the presence of band 1' in sera collected from animals receiving primary infection of H. capsulatum. These animals were bled at 8, 16, and 45 day intervals post injection Not all H. capsulatum infections are characterized by the presence of this band. A survey of all serum samples reviewed an appreciable percentage (2/24) of the animals receiving H. capsulatum inoculation failed to exhibit the presence of 1'. Normal guinea pigs are also characterized by the absence of this band. Results illustrated in Figure 9 exhibit the presence of band 1' in all sera obtained from animals chronically infected with L. monocytogenes. Animals receiving S. typhosa sensitization as well as primary infection with L. monocytogenes exhibit the presence of the same band (figure 10).

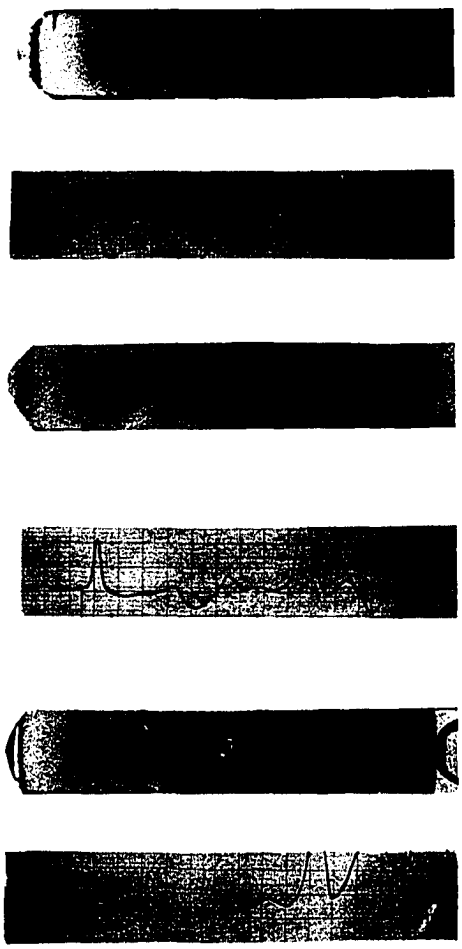


Figure 8. Electrophoretic patterns of sera from guinea pigs infected with H. capsulatum.

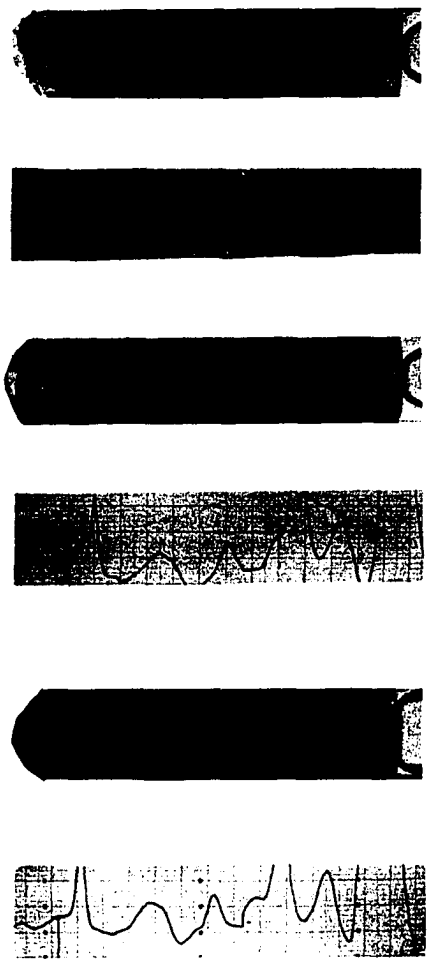


Figure 9. Electrophoretic patterns of sera from guinea pigs infected with L. monocytogenes.

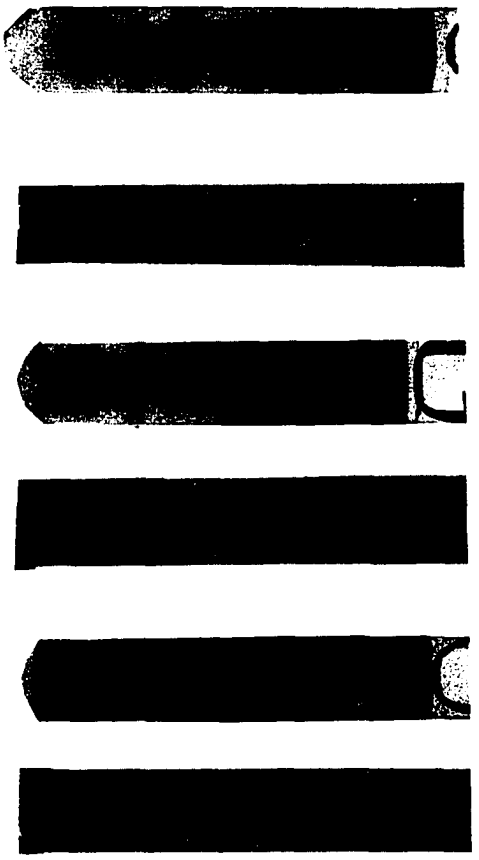


Figure 10. Electrophoretic patterns of sera from guinea pigs infected with S. typhosa.

Figures 11 and 12 illustrate the electrophoretic results of sera from animals chronically infected with H. capsulatum and superinfected with L. monocytogenes and S. typhosa respectively. No sera were obtained in sampling these animals which demonstrated the absence of band 1'. It should be pointed out that some animals, chronically infected with H. capsulatum, within this same experimental group had exhibited the absence of the 1' band. Animals chronically infected with L. monocytogenes and subsequently superinfected with H. capsulatum exhibited no serum samples without the 1' band (Figure 13).

The only characterization established with respect to this band was an RD of $0.12 \pm .03$. Relative distance (R.D.) is defined as the ratio of specific protein migration distance to the tracing dye migration distance.

Attempts to quantitate the amount of protein were unsuccessful due to the wide range of variability in densitometer tracings resulting from scanning the gel columns. Other differences of equal or greater importance are probably present in these gels. No attempt has been made to establish significant differences in these primarily because no distinct pattern was identified.

Results of differential white blood cell count

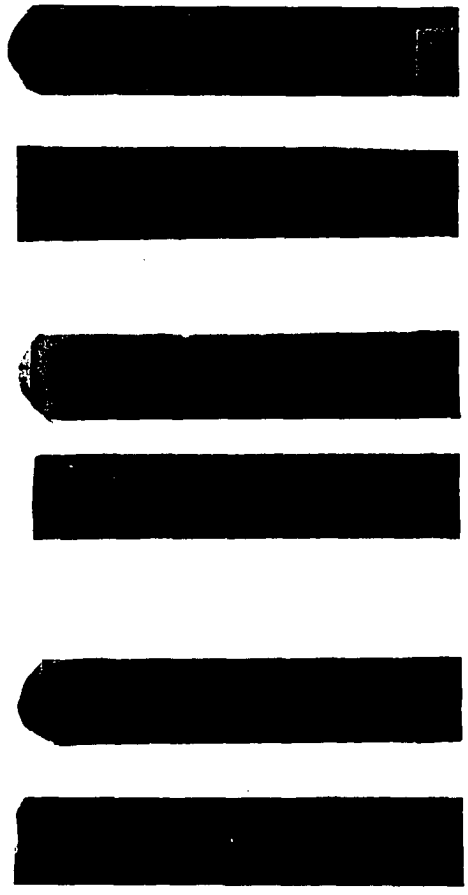


Figure 11. Electrophoretic patterns of sera from guinea pigs chronically infected with H. capsulatum and subsequently superinfected with L. monocytogenes.

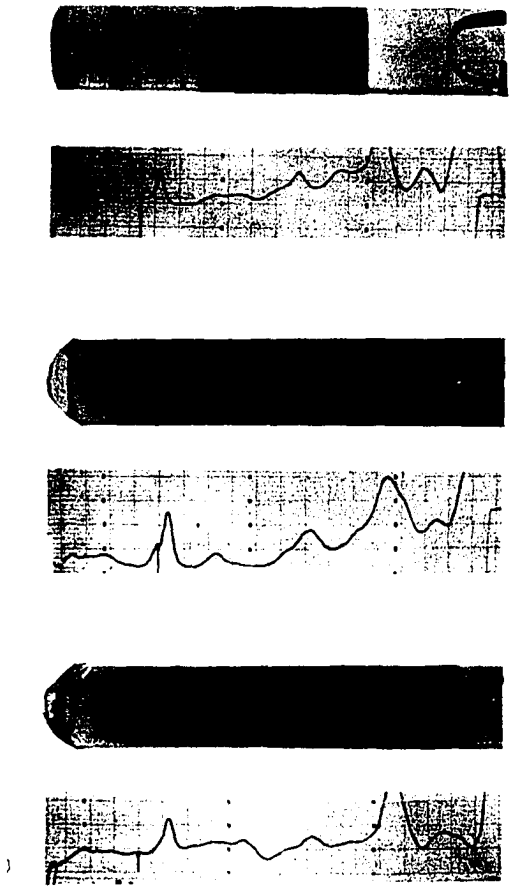


Figure 12. Electrophoretic patterns of sera from guinea pigs chronically infected with H. capsulatum and subsequently superinfected with S. typhosa.

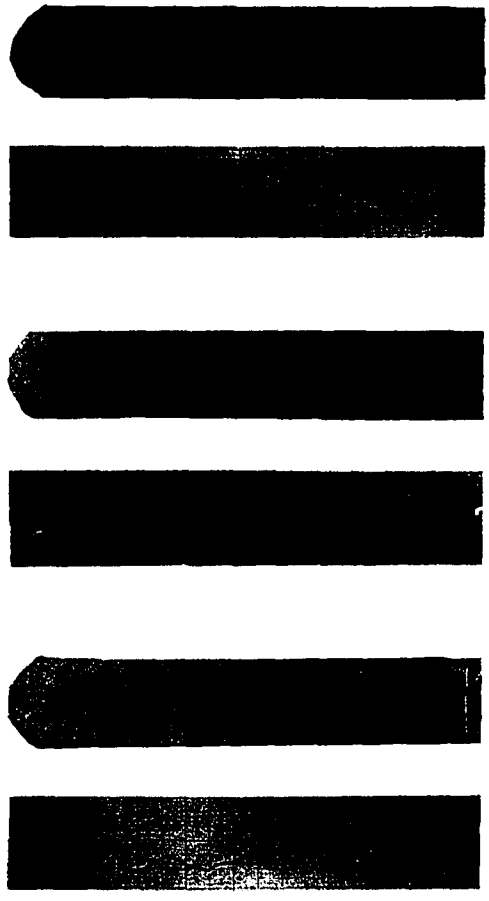


Figure 13. Electrophoretic patterns of sera from guinea pigs chronically infected with L. monocytogenes and subsequently superinfected with H. capsulatum.

determinations of blood taken 16 days after infection are listed in Table 6.

Segemented neutrophil, lymphocyte and monocyte plus total leucocyte counts are shown. The only instance in which a significant difference can be detected is in the case of L. monocytogenes infection.

No significant difference in cell types were recognized for any of the animals chronically infected with H. capsulatum. These animals exhibited an increased leucocytosis with increased skin test reactivity (13 mm and greater). A negative correlation was observed between skin test activity and C.F. antibody titers.

Table 6. Differential blood cell counts per 100 cells

Inoculating organism	Primary infection				Chronic infection H. capsulatum				Chronic infection L. monocytogenes			
	S*	L**	M***	WBC	S	L	M	WBC	S	L	M	WBC
H. capsulatum	44	46	4	8500					33	61	6	8500
L. monocytogenes	39	48	12	8250	30	55	15	6000				
S. typhosa	36	68	4	7500	20	75	5	7200	41	48	7	9500
Control	30	64	4	7000								

* Segmented neutrophiles
** Lymphocytes
*** Monocytes

CHAPTER IV

DISCUSSION

Interpretation of the immune responses by man and animals to infection arising both naturally and artificially is still problematic and at best difficult to make. Circulating and tissue fixed antibodies produced as a result of infection are widely used as a major criterion in diagnosis and prognosis of disease. However, antibody activities demonstrate a definite heterogeneity as characterized by their chemical and physical properties as well as their antigenic associations (13, 14, 30). Definite heterogeneity also exists with respect to molecular size, structure, and electrophoretic mobilities. Previous investigation has generally suggested a sequential appearance of antibody types, specifically 19S (IgM) and 7S (IgG), during the progression of a disease; however, more recent investigations reflect varied patterns with respect to this theory (33).

In a recent review, Pike discusses in some detail the fact that no strict pattern for this sequential production exists. Murray et al. (31) reports that the IgM type is

predominant in primary typhus infections, whereas the IgG type is more apparent in recrudescent disease. Reddin et al. (37), following studies of chronic brucellosis in man, have reported that sera of patients with bacteriologically proved chronic disease contain primarily 6S type agglutinins, in contrast to the sera of patients in whom the only evidence of chronic infection was a low titer of 19S agglutinins. The exact nature of the immunoglobulin type proteins produced in H. capsulatum infections has not been characterized completely. Thus the relationship between histoplasmosis type infections and the other previously described cannot be ascertained.

The absence of an absolute correlation between positive skin tests and positive C.F. sera may reflect the presence or absence of two separate varieties of immunoglobulins. Block et al.(3) observed that 7S γ2 yields complement fixation activity but fails to yield passive cutaneous anaphylaxis (hypersensitivity). Dyson et al. (11) have discussed the relationship of delayed hypersensitivity in experimental fungal infections.

Results from this investigation show that chronically and nonchronically infected animals yield demonstrable circulating antibody titers. Examination of Figure 3 suggests

that initial titers were first demonstrated in sera collected 8 days post infection or 16 days after initial inoculation. This is the apparent condition that exists for all types of inoculation in the study. Forty-five day titers were observed to be substantially higher than the 8 day titers.

Chronically infected animals showed antibody titers appreciably higher than those of animals with primary infections. No significant decrease in titers was observed in animals chronically infected with H. capsulatum. Animals with histoplasmosis produced antibody to a second type infection approximately the same as that of animals receiving primary infection. These data suggest that no appreciable change has occurred with regard to the ability of these test animals to produce specific antibody to a second or third type antigen. If the immunological response mechanism of animals chronically infected with H. capsulatum had been impaired or incapacitated one would expect a significant decrease in titers. However, one must keep in mind that the complement fixing and agglutinating titer determinations represent only partial evaluation of antibody production.

Results from the experiments involving spleen homogenates complement these obtained serologically. Jerne's technique provided an assay system for quantitation of

antibody producing cells.

An increase in number of PFC is a reflection of increase in number of antibody producing cells. Jerne's original work was done with mice spleen cell homogenates and yielded a strong correlation between the number of PFC's and hemolytic titers (21). Subsequent studies have employed lymph node cells as well as C.F. titers (10). The high correlation between the number of PFC's and antibody titers is indicative that this is an assay system which can be employed for fungal type studies.

Several aspects of the cellular phase in this study gave informative results. Control animals exhibited a low number of cells associated with antibody which suggests a nonspecific reaction may be occurring in this system. However, a significant difference in the number of cells producing antibody was easily recognized in chronic and nonchronic infections.

Superinfecting chronically infected animals with homologous organisms shows an increase in PFC's occurs; however, this increase is not in the same order of magnitude as that exhibited between chronic and nonchronic infections. Suggested explanation for this phenomenon is that a maximum level of specific cell differentiation may exist. Makinodin

has discussed cellular differentiation with respect to the type of antibody produced and has proposed one of several theoretical schemes found in the literature (28).

Chronically infected animals superinfected with a second organism were observed to yield PFC values similar to those for primary infections. Reduction in PFC is noted in the animals chronically infected with histoplasmosis. Reduction of this size is considered insignificant in view of the fact that similar reduction occurs in both types of infections. A more exacting evaluation of this point could be made if more types of different diseases had been included in this study.

Analysis of individual sera for protein components was accomplished using gel electrophoresis. Spacer gels, or upper gels, organize sera into bands of different proteins due to electrophoretic mobility. A more pronounced separation actually takes place in the lower gel, due to the combined influence of electrophoretic mobility and molecular sieving. Electrophoretic studies of samples from serial bleeding taken from all groups of animals suggest that distinctive patterns can be established for these diseases. Investigations of sera from several different animals as well as man show that good reproducibility of electrophoretic

patterns does exist for many different diseases (34, 35). These electrophoretic patterns are considered helpful in diagnosis of the disease.

The results depicted in Figures 10 and 11 demonstrate specific patterns are characteristic depending upon the previous history of the animals. After examination of several gels it was recognized that one distinct pattern of events was occurring. As related in the previous chapter, this pattern involves the 1' band.

It was noted that the band having a RD value of 0.12 $\pm$ .03 was present or absent depending on the source of the serum. Only a certain portion of animals demonstrated this variation as recognized by the absence of this band in electrophoresed sera. The absence of the band was first noted in non skin test reactive animals chronically infected with H. capsulatum. This prompted a study of sera from another group of animals which had not been previously skin tested. Also, animals chronically infected with listeriosis, which laterwere superinfected with H. capsulatum, failed to produce a portion of animals which showed the absence of this band even though the post inoculation time was increased. Finally, sera of all animals receiving 2 different antigenic stimuli exhibited the presence of the 1' band. Apparently only some

animals within this study group were unable to respond to the H. capsulatum infection.

Patterns of serum protein were recorded by densitometer readings. Quantitative evaluation was not made of any band. Several bands that can be seen visually were not clearly shown by the densitometer tracings. Improved techniques in reading gels or increased sensitivity of the instrumentation is deemed necessary to make quantitative assessments. A study of different H. capsulatum isolates should be performed before any definite conclusion can be made.

Chemical analysis as well as determination of the immunological activity of the 1' band is necessary for a better understanding of the significance of this band. However, the fact that only a percentage rather than all or none of the individuals in a population show this variation suggests a genetic character may be responsible.

These results contribute to the development of hypothetical or theoretical explanations for the observed responses.

Variation in genotypic and phenotypic expression is common in all biological systems. The basis for this phenomenon has been identified as genetic in nature. Certainly it seems plausible that within a population of animals one

could expect the establishment of several differences in potential with respect to immunological competence. Other aspects of immunological response observed in this study also suggest a variation in potential. Several animals from a group presently under investigation died after the second or third inoculation of the same organism. Death was accompanied by an inability to isolate viable organism from various tissues. Such animals correspond to those reported by Raffel (36) and are explained as having died from immunological shock. Variation in skin test sensitivity as well as the degree of sensitivity recorded in this study may be other expressions of potential differences existing in a population of animals.

The experimental design used did not include a method whereby one can assess whether the size of the inoculum, particular isolate used or whether a variation in expression of potential is responsible for the observed results.

The possibility exists that the distribution of antibody type molecules changes with progression of disease. Analysis of previously reported data suggests an increase in 7S molecules could actually occur, concomitant with a reduction in 19S antibody molecules. Generally the serological studies indicate that this is the sequence of events. The

inability of some individuals within a population to respond in this way could be the factor responsible for the observed results.

Another explanation for this phenomenon could be that the types of antibody molecules in a population do not change but rather a specific alteration in the molecular affinity, as expressed in reactivity, occurs. Eisen relates that a change in the number of molecules, within a population of molecules, occurs with respect to the affinities of the antibody types during the progression of a disease (10). Such an explanation could suggest that an inducible system is involved in the control of types of antibody molecules produced.

The significance of differential blood cell counts remains questionable. The only instance in which a significant difference can be determined is for L. monocytogenes.

In a comparative study of sera from animals exhibiting differences in skin test sensitivity another factor was observed. It was observed that the largest skin test reactions parallel the more advanced degree of leucocytosis. Equally apparent was the fact that the group with 1-5 mm induction size exhibited the highest C.F. titers (1:256). This is suggestive of a negative correlation existing between the increase of leucocytes and increased titers. The range of

values exhibited by the sample employed prohibited the detection of any significant evaluation of this factor.

CHAPTER V

SUMMARY AND CONCLUSIONS

A study was undertaken to gain further insight into the cause of the variability in the immunological responses often observed in experimental histoplasmosis. Variability in skin test reactivity, amount of demonstrable antibody, and control of the infecting agent has been observed in almost every test system. Specific aspects of this study were designed to measure the parameters which generally are considered indices of immune response.

Measurement of this immunological response involved an assay system comprised of chronically and nonchronically infected animals. Guinea pigs were chronically and nonchronically infected with H. capsulatum or L. monocytogenes. The ability of chronically infected animals to respond immunologically to superinfecting agents was determined. The immunological response of nonchronically infected animals was used as the reference for comparison. Assessment of this ability included serological titers, immunological competence of specific cells, and partial characterization of the serum

proteins. Through an evaluation of the results obtained, definite conclusions were established.

The results of this study reflect the fact that an infection caused by H. capsulatum does not necessarily impair the capacity of the RES in its role of humoral antibody formation.

Animals chronically infected with H. capsulatum or L. monocytogenes had approximately equal abilities to produce specific humoral antibody as shown by the testing techniques employed. Identification has been made of a spleenic cellular involvement which demonstrates a continual antibody synthesis by cells of these chronically and nonchronically infected animals. Sera from infected animals has been partially characterized using gel electrophoresis. It has been demonstrated also that sera from chronically and nonchronically infected animals differs with respect to the protein components of the sera. A specific difference occurs in the H. capsulatum infections. The absence of a qualitatively identified band in a significant number of sera has been shown.

Literature citations are included as evidence in support of these findings. These include present day information relevant to antibody heterogeneity and variation in antibody affinities.

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