This dissertation has been
microfilmed exactly as received

70-2294

ALY, Raza, 1935-

IMMUNOLOGICAL RESPONSE OF GUINEA PIGS CHRONICALLY INFECTED WITH REPEATED AND A SINGLE DOSE OF HISTOPLASMA DUBOISII.

The University of Oklahoma, Ph.D., 1969
Microbiology
University Microfilms, Inc., Ann Arbor, Michigan

Downloaded from SHAREOK. Persistent Handle URL: https://hdl.handle.net/11244/2634

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

IMMUNOLOGICAL RESPONSE OF GUINEA PIGS CHRONICALLY INFECTED WITH REPEATED AND A SINGLE DOSE OF *HISTOPLASMA DUBOISII*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

RAZA ALY

Norman, Oklahoma

1969

IMMUNOLOGICAL RESPONSE OF GUINEA PIGS CHRONICALLY INFECTED WITH REPEATED AND A SINGLE DOSE OF *HISTOPLASMA DUBOISII*

APPROVED BY

Howard H. Lamb

Leon J. Cierzko

Gear E. Cozad

Donald C. Cox

Eddie C. Smith

DISSERTATION COMMITTEE

ACKNOWLEDGEMENT

The author wishes to express his sincere thanks to Dr. Howard W. Larsh for his inspiration and continued guidance throughout this study.

A special note of thanks to Dr. G. C. Cozad, Dr. J. H. Lancaster, Dr. D. C. Cox, and Dr. L. S. Ciereszko for their assistance in the writing of this manuscript. Sincere appreciation to Dr. Peter A. Bartels for his assistance.

A special note of appreciation is also extended to Miss Janis Reeser, Miss Mary Jane Hook, and Mr. Don Wiggins for their technical assistance in this study. Also, a special note of appreciation to my wife Naheed for editing and typing the manuscript.

TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF ILLUSTRATIONS	vi
Chapter	
I. INTRODUCTION	1
II. MATERIALS AND METHODS	8
III. RESULTS	22
IV. DISCUSSION	42
V. SUMMARY AND CONCLUSIONS	48
BIBLIOGRAPHY	50

LIST OF TABLES

Table		Page
1.	Inoculation of guinea pigs with *H. duboisii*	10
2.	Inoculation of guinea pigs with *H. duboisii* for primary infection	11
3.	Inoculation of guinea pigs with super-infecting agents	13
4.	Stock solutions for cationic gel system	17
5.	Total and differential white blood cell count	32

LIST OF ILLUSTRATIONS

Figure		Page
1.	H. duboisii infected guinea pig with orchitis	23
2.	Complement fixation titers of guinea pigs infected with H. duboisii	26
3.	A comparison of complement fixation titers of guinea pigs superinfected with L. monocytogenes	27
4.	Hemolysin titers of guinea pigs against sheep red blood cells previously infected with H. duboisii	29
5.	Hemagglutination titer of guinea pig against sheep red blood cells previously infected with H. duboisii	30
6.	Number of rosette forming cells per spleen. Number of RF cells prior of superinfection on the left, number of RF cells with homologous superinfection on the right of figure	33
7.	Number of RF cells per spleen. Number of RF cells produced against sheep RBCs on the left, number of RF cells produced against L. monocytogenes on the right side of figure	34
8.	Rosette formation against sheep RBCs: (Top); rosette formation against H. duboisii: (Bottom)	36
9.	Electrophoretic patterns of sera from normal guinea pigs	38
10.	Electrophoretic patterns of sera from primary infected guinea pigs with H. duboisii	39

Figure Page

11. Electrophoretic patterns of sera from chronically infected guinea pigs with single inoculation of H. duboisii 40

12. Electrophoretic patterns of sera from guinea pigs infected with repeated inoculations of H. duboisii 41

IMMUNOLOGICAL RESPONSE OF GUINEA PIGS CHRONICALLY INFECTED WITH REPEATED AND A SINGLE DOSE OF *H. DUBOISII*

CHAPTER I

INTRODUCTION

During the past two decades, histoplasmosis has aroused more interest among medical mycologists than has any other fungus disease of man. The etiological agent of histoplasmosis was discovered in the Panama Canal Zone in 1906 by Samuel Darling. Histoplasmosis, once believed to be a rare and invariably fatal disease, is now considered to be an extremely common, benign infection in its endemic areas (17). It has been estimated that 30,000,000 people are infected in the United States (17).

The basis for this radical change lies in the use of the skin test antigen, histoplasmin, as an epidemiological tool, and better understanding of the serological methods applied. This also helps to determine the pattern of the disease, to delineate new cases, and to aid in the study of suspected cases (1, 18).

In addition to the classic type of histoplasmosis,

a different type of histoplasmosis occurs in Africa (1). Vanbreuseghn and Dubois, 1952, defined Histoplasma duboisii as the causative agent of African histoplasmosis. Although very similar in mycelial phase to Histoplasma capsulatum, H. duboisii nevertheless can be differentiated from it by the urease test (46). H. capsulatum hydrolyses urea very rapidly while H. duboisii remains inactive or acts very slowly. Rosenthal and Sokolsky (28) and Berliner (2) reported that the mycelial form of H. duboisii hydrolyzed gelatin whereas H. capsulatum does not.

It was also reported by Rosenthal and Sokolsky (28) that H. capsulatum elaborated tyrosin hydrolytic enzymes but H. duboisii did not. Recently Blumer and Kaufman (5) studied the hydrolytic activities of 10 strains of H. capsulatum and H. duboisii on gelatin, tyrosine and urea substrate. The data revealed that the physiological properties cannot be used entirely to differentiate H. capsulatum and H. duboisii and for definitive identification of H. duboisii, animal inoculation was suggested.

H. duboisii is characterized by a parasitic form, much larger than H. capsulatum, since it can attain 15 microns in diameter and is generally found in giant cells, while H. capsulatum is enclosed inside histiocytes (44, 38).

Though there are many similarities between African histoplasmosis and "classical" histoplasmosis, it seems

pertinent to distinguish these two. On the one hand, pulmonary affection, rare in the disease due to H. duboisii, are common in that caused by H. capsulatum. On the other hand, while cutaneous lesions are very frequent in the African histoplasmosis, they are rare in the American form (45). The fact that we do not as yet know of a benign or inapparent form of African histoplasmosis testifies more to our ignorance than to a real absence of these forms of the disease.

Okudaira and Schwarz concluded it was possible to differentiate H. duboisii from H. capsulatum by its size in the parasitic phase, its different pathogenicity and particular tissue response in hamsters and mice (24). Coudert and Coly reported differences in the colloiden agglutination test of both organisms (8, 34).

In studying animal infection, several factors are influential and contribute to the apparent variation of results frequently obtained. Growth phase of the organisms, age and sex of the animals, route of inoculation, and size and viability of specific isolates are all important factors and should be considered when one attempts to perform any immunological study.

Various serological tests have been employed in studying fungal infections. Tenenberg and Howell (43) employed histoplasmin as a complement fixing (CF) antigen, though Saslaw and Campbell employed whole yeast phase (36)

and ground yeast phase supernatant (37) as CF antigen. Hill and Campbell (13), in complement fixation tests with human sera and whole yeast and histoplasmin antigens, found that either antigen would have been diagnostic in many cases. Whole yeast antigen reacted in higher titer with sera from cases of "early primary histoplasmosis," whereas histoplasmin was more sensitive in chronic cases.

In the latter clinical group, whole yeast phase antigen frequently failed to reveal a significant titer. Tenenberg (42), in an extensive comparative study with different human sera, found whole yeast phase antigen to be more sensitive and more nonspecific than histoplasmin antigen in the CF test. It seems that a test, to be of a practical value, must possess a relatively high degree of sensitivity and specificity. Bear in mind that "increasing the sensitivity of a test jeopardises its specificity," so a compromise between sensitivity and specificity must be made if a test is to be practicable.

It has been shown that precipitin (32, 33, 31) and collodion agglutination tests (34, 35) tend to demonstrate their highest titers early in the disease and are transitory. These titers are weak to negative in the later stages of infection. Conversely, the complement fixation test tends to be positive in the later stages of infection (15, 16, 42). Several different diseases which have been studied demonstrate that the first immunoglobulin produced

is the type characterized as IgM type. The sequential production of IgG is considered responsible for the complement fixation activity (39). Any similar condition existing in H. capsulatum has not been fully demonstrated.

Four mechanisms of acquired resistance to infectious agents are understood in broad outline. They all depend upon humoral antibodies which serve to counteract or neutralize a toxic product of infecting organisms, to lyse them in the presence of complement, to promote their ingestion by phagocytic cells, or to prevent, in the case of viruses, the entry of the parasite into cells, where it is necessary for the parasite to replicate. So far we know these mechanisms operate against agents of acute infectious diseases. Most chronic infections are caused by microbial agents which can survive for long periods in the face of whatever resistance the host is capable of developing against them. Salmonella infections persist despite the development of an impressive level of immunity. The infected host can eliminate thousands of lethal doses of superinfecting inoculum but cannot get rid of the original infecting population. This shows that even a very efficient mechanism of immunity can be prevented by parasite from functioning effectively against organisms that establish themselves in large numbers at the original site of implantation. Another example is the parasite's innate capacity to survive in an intracellular environment

which has been observed in the case of tuberculosis and histoplasmosis.

Histoplasmosis is characteristically a chronic disease and has been shown to persist in the infected tissues for long periods of time. H. duboisii, like H. capsulatum, causes a generalized infection of the reticulo-endothelial system (RES). It has been demonstrated that these cells and tissues are responsible for antibody synthesis (29, 6, 22). This suggests that a condition may arise during infection which may impair or incapacitate the functioning ability of the RES.

This study was designed to obtain information about the immunological response of animals experimentally infected with H. duboisii. Guinea pigs were chronically infected with the mycelial phase of H. duboisii. Each injection was $1.6X10^3$ to $1.6X10^4$ of viable mycelial units as determined by the plate count. The injections were given intraperitoneally and their schedule spanned a period of seven months. Animals which received only two injections of H. duboisii over a period of 40 days were designated as the nonchronically infected group.

The ability of the chronically and nonchronically infected animals to respond immunologically to superinfecting agents was determined. The antibody production of infected animals was partially measured using serological methods and the rosette test (Immunocytoadherence

technique). The rosette test was employed to measure quantitatively the number of spleen cells exhibiting antibody production. The rosette test was developed by Nota et al, in 1964, to characterize the kinetics of spleen cell population during the immune response of mice to sheep red blood cells (RBCs). When a spleen suspension of animals, immune to foreign RBCs, are mixed with specific red cells, "rosettes" are formed that consist of isolated immune cells with red cells attached to their membranes. This technique is sensitive and simple to perform. It probably detects 19S and 7S antibodies (40).

The serum protein of infected and noninfected animals was partially characterized by using the disc electrophoresis. This technique is useful in analyzing proteins and other high molecular weight materials. A characteristic and reproducible pattern has been found in diseases such as rheumatic fever, sickle cell anemia, tuberculosis and cancer (27).

CHAPTER II

MATERIAL AND METHODS

The following agents were employed in this study:

1. Histoplasma duboisii

   H. duboisii (B 684), isolated from pulmonary histoplasmosis in Conakry, Guinea, in 1962 by Dr. E. Drouhet, was employed in this study. This organism was grown on Sabouraud's dextrose agar for twenty days at 25°C and the mycelia were scraped from agar slants into sterile physiological saline. The suspension was homogenized in a teflon grinder. The number of viable particles was determined by culturing respective serial dilutions on ten Sabouraud's dextrose agar plates.

2. Listeria monocytogenes

   Listeria monocytogenes (A 4413) was originally obtained from Dr. C. P. Sword, University of Kansas, Lawrence. The culture was grown in tryptose broth at 37°C for 72 hours, washed and diluted in physiological saline to a concentration of about $2X10^7$ organisms per ml.

3. Sheep Red Blood Cells

   Commercially available sheep red blood cells (Sheep

RBC's) were obtained from Brown Laboratories, Topeka, Kansas. The cells were washed three times with physiological saline and resuspended in same to the appropriate concentrations desired.

Animals Used

One hundred and seventy Hartley strain albino guinea pigs with an average weight of approximately 500gm were used. Sixty guinea pigs comprised a group (Group #3) which received 7 intraperitoneal injections of mycelial fragments homogenized in a teflon grinder. Each injection was $1.6X10^3$ to $1.6X10^4$ of viable particles contained in 1 ml as determined by viability plate count (Table 1). Fifty animals (Group #1) received only one intraperitoneal injection containing $6.1X10^4$ viable mycelial particles per ml. Injection schedules for these two groups spanned a period of 7 months.

Forty animals comprised a group (Group #2) which received only two intraperitoneal injections over a period of forty days, as shown in Table 2. This group was designated nonchronically infected animals, while the one with repeated doses and a single dose over a period of six months were designated chronically infected animals.

All the guinea pigs infected with H. duboisii were further divided into smaller subgroups, and superinfected with L. monocytogenes and H. duboisii. The third subgroup was inoculated intraperitoneally with 10% sheep

Table 1. Inoculation schedule of guinea pigs with H. duboisii

Organism	Culture age (days)	Inoculation route	Inoculation schedule (days)	Inoculum Size/ml Total Count	Inoculum Size/ml Viable Count
H. duboisii 2100 (B684)	20	IP	1	2.5×10^5	1.2×10^4
"	"	IP	6	1.6×10^6	1.6×10^3
"	"	IP	13	2.2×10^6	7.4×10^3
"	"	IP	31	1.8×10^7	1.6×10^4
"	"	IP	38	1.9×10^7	1.5×10^4
"	"	IP	60	2.1×10^7	1.2×10^4
"	"	IP	80	3.4×10^8	1.1×10^4

Table 2. Inoculation schedule of guinea pigs with H. duboisii for primary infection

Organism H. duboisii 2100	Culture age (days)	Inoculation route	Inoculation schedule	Inoculum Size Total Count	Viable Count
"	20	IP	1	2.4×10^7	2.6×10^4
"	20	IP	8	1.7×10^7	2×10^4

erythrocytes (9). These subgroups were arranged so that each test system included twelve animals. Superinfection was accomplished by inoculating with two injections seven days apart, except in the case of sheep RBC inoculations, where only one inoculation was performed as shown in Table 3.

Preparation of Antigen

A three month old culture filtrate of the mycelial phase of H. duboisii grown in liquid asparagine medium was employed as a complement-fixing (CF) antigen. Skin hypersensitivity was determined by injecting 0.1 ml of a five month culture filtrate in 1:10 dilution, intradermally. The millimeters of erythema and induration were measured after forty-eight hours.

A seventy-two hour old broth culture was washed three times with physiological saline. A heavy suspension (1:10 vv) of L. Monocytogenes cells in distilled water was sonicated for ten minutes, using a Blackstone Model sonicator. The degree of lysis was determined by microscopic examination. The suspension was centrifuged at 10,000 RPM for sixty minutes in a refrigerated centrifuge. The supernatant extract was employed as the complement-fixing antigen.

Bleeding of Animals

Random samples from noninfected animals were bled intracardially. These initial bleedings served as controls

Table 3. Inoculation schedule of guinea pigs with superinfecting agents

Inoculating agents	Inoculation route	Inoculum schedule	Inoculum size/ml Total Count	Inoculum size/ml Viable Count
	IP	1		$2x10^7$
L. monocytogenes (A4413)	IP	8		$1.4x10^7$
	IP	1		$6.1x10^4$
H. duboisii	IP	8		$3.9x10^4$
Sheep erythrocytes	IP	1		10 percent

in subsequent immunological tests. Samples from animals which had received repeated and a single dose were similarly bled prior to superinfection and served as reference controls. At 0, 7, 14, 21, and 28 days post-injection, four randomly selected animals from each group were bled. The 4 ml blood samples were allowed to clot and the sera were decanted from tubes and stored at -40°C until used for serology.

Determination of white blood cell (WBC) counts was made by using citrated blood. All WBC counts were made using "white" leucocytes pipettes with 1% acetic acid. Determination of differential counts was performed using Wright's stain.

Serological Studies

The sera collected from animals infected with H. duboisii and L. monocytogenes were titrated for C.F. antibody concentrations. The complement-fixing technique described by Lackman was used throughout the study (14).

Hemagglutination and hemolysin tests were used for the animals immunized with sheep RBCs. Hemagglutination test was performed in equal volumes of 0.5% suspension of sheep RBCs (0.5 ml) and serially diluted serum in saline; the tubes were incubated at 25°C for one hour.

The hemolysin test was performed in equal volumes of a 1% suspension of sheep RBCs (0.2 ml) and serially diluted serum vernol buffered saline, and the mixture was

incubated for 10 minutes. Then 0.2 ml of 1:25 dilution of complement was added to each tube and allowed to incubate for an additional 30 minutes at 37°C. Hemolysin was read by visual inspection and was scored as either 100% or 0.

Antigen Preparation for Rosette Test

Yeast phase culture of H. duboisii grown on brain-heart infusion agar at 37°C for three days was used as an antigen against the antibody forming spleen cells of animals infected with H. duboisii. Tanned sheep RBCs, sensitized with L. monocytogenes, was employed as the rosette antigen to detect the antibody producing spleen cells of animals infected with L. monocytogenes.

Removal of Spleen for Rosette Test

Four animals from each experimental group in addition to control animals were sacrificed, their spleens were removed and placed into Hanks Balanced Solution. The spleens were further diced and homogenized by teflon grinders. Each suspension was passed through a cheese cloth and washed three times in Hanks Balanced Salt Solution at 4°C in a refrigerated centrifuge. The viability of spleen cell was determined by using the trypan blue exclusion test. Aliquotes from the original suspension were taken and the final total cell counts were determined. No attempt to differentiate the types of cell was made.

The total number of nucleated cells was found to be approximately $2.2X10^8$ per spleen.

Yeast phase of H. duboisii and sheep RBC antigen were prepared by washing three times in phosphate buffered saline. In a clean Kahn tube 1 ml of $6X10^6$ spleen cells, 0.2 ml of $100X10^6$ cells of antigen in phosphate buffered saline and 0.05 ml of red blood cell-absorbed guinea pig serum were added. After 12 hours at 4°C, the capped tubes were repeatedly, but gently, inverted for 10 minutes to resuspend the settled cells without destroying the rosettes. The rosette count was made in a hemacytometer by examining nucleated spleen cells. The rosette count is expressed as the number of rosette-forming cells (RFC) per spleen.

Electrophoresis of Sera in Acrylamide Gels

Stock solutions for cationic gel systems having a running pH of 4.3 were used in this study (Table 4).

Original stock solutions were usable for six months, with the exception of those containing potassium 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 prepared and all preparations were stored in tinfoil covered bottles and maintained under refrigeration.
2. Preparation of gel tubes

Table 4. Stock Solutions for Cationic Gel Systems

CATIONIC GEL SYSTEM Running pH=4.3

	VOLUME RATIOS	COMPONENTS/100 ML		pH (25°C)
LOWER GEL	1	Acrylamide	30 gm	
	1	Bisacrylamide	0.8 gm	
	1	IN KOH	24 ml	
	1	Acetic Acid	8.6 ml	4.3
	1	Temed	0.24 ml	
	2	Potassium Persulfate	60 mgm	
	2	Riboflavin	2 mgm	
UPPER GEL	1	Acrylamide	10 gm	
	1	Bisacrylamide	0.8 gm	
	1	IN KOH	48 ml	
	1	Acetic Acid	2.87 ml	6.7
	1	Temed	0.1 ml	
	2	Potassium Persulfate	60 mgm	
	2	Riboflavin	2 mgm	
		COMPONENTS/LITER		
UPPER BUFFER		Acetic Acid	8 ml	4.5
		Beta Alanine	31.2 gm	
LOWER BUFFER		Acetic Acid	43 ml	4.3
		IN KOH	120 ml	

These buffers can be used at 1/10 strength

a. All tubing had an inner diameter of 5 millimeters and were 7.5 cm in length. Tubes were cleaned with 7X cleaning solution, rinsed through distilled water, 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 a hollow rubber cap (Canalco #3-1768) to seal one end and placed in a 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 5 inches 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. A flourescent light assembly (Canalco #3-1764) was placed approximately three inches behind the apparatus. The lower-gel solution was allowed to photopolymerize for 30 minutes. Next the layer of water was decanted along with any unpolymerized gel solution. This was most easily accomplished using rolled absorbent tissue paper.

d. The layering technique was repeated using 0.2 ml of the upper gel solution. Again the upper gel

solution was overlayed with 0.1 ml distilled water. A fluorescent light assembly 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 the 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 the gel tube.

f. Two microliters of sample serum were carefully layered on top of the upper gel. The front was marked by adding 1 ml of Methylgreen (.001%) to the upper buffer compartment.

g. Adjustment of 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 passed the bottom of the gel column. The time for the run was approximately 90 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 acid.

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 band; (c) the density and the width of the bands as an estimate of the relative amount of protein in each fraction. Quantitation of the gel columns is based on 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 approximately 50°A 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 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

All animals dying during the infection period were autopsied. The yeast phase of H. duboisii was cultured from liver and spleen in 70% of the autopsied animals. At autopsy, the animals had large livers and spleens; fibrinous exudate covered the peritoneum and the spleen. Some of the spleens developed a whitish-pink color, resembling total necrosis of the organ.

Animals infected chronically and nonchronically as indicated in tables 1, 2, and 3 were tested for infection. Criteria for establishing infections were positive skin test reaction and production of complement fixing antibody. In the animals inoculated with a single dose of H. duboisii, the precipitin test was used since, with the exception of two, these animals failed to develop detectable C.F. titer.

Female guinea pigs repeatedly inoculated with H. duboisii developed mastitis. Male guinea pigs incorporated later in the study developed severe orchitis (Fig. 1). Skin involvement with lesions was also noticed in some animals.



Figure 1. H. duboisii infected guinea pig with orchitis.

Animals inoculated with a single dose of H. duboisii did not show the presence of C.F. antibodies in the majority of cases; however, very weak precipitin antibodies were demonstrated using Ouchterlony techniques. The precipitin bands appeared after a period of seven days. Positive skin test reactions were observed in approximately 76% (23/30) of the animals. Positive skin tests were recorded for all animals exhibiting an induration of 5 mm or more. Less than 5 mm induration was considered to be negative. Animals with negative skin reaction were weakly positive for the presence of precipitin antibody.

When skin tested, 80% (40/50) of the guinea pigs that had been infected with repeated doses of H. duboisii gave positive reactions. Twenty per cent of the animals giving negative skin reactions were tested for the presence of C.F. antibodies. Only 40% (4/10) were C.F. positive, ranging from 1:8 to 1:64.

The second group which received two injections, as presented in Table 3, revealed the skin reaction as shown in animals with repeated injections. However, the C.F. titer was much lower in the former group.

Animals receiving a single and series of inoculations prior to superinfection were referred to as chronically infected animals, while those receiving two inoculations as shown in Table 2, were designated primary

infected animals.

Serological Study

All test sera were inactivated at 60°C for 30 minutes. The complement fixing titers of sera from different animals are listed in Figure 2. Mean C.F. titers of sera from 10 guinea pigs were plotted and expressed as the reciprocal of the dilution.

Figure 2 reflects the C.F. titer of all the guinea pigs which were inoculated with H. duboisii. Animals with repeated inoculations of this organism as indicated in Table 1 demonstrated a higher C.F. response than did the group inoculated with two injections. No C.F. response was observed in animals with a single inoculation. However, at the end of three months a titer of 1:16 was observed in 10% (2/20) of the animals. It seems that, in majority of the cases, a single inoculation is not enough to elicit C.F. antibody response in the guinea pig. The C.F. titer in animals with repeated doses varied from 1:16 to 1:256 and was in agreement with expected results (Sälvin, 30). The animals receiving only two inoculations did not develop high C.F. titers, and the titers varied from 1:8 to 1:32 (Figure 2).

The ability of these three groups of animals infected with H. duboisii to respond immunologically to a second type of injection was compared, as shown in Figure 3. It was observed that the C.F. titer of sera

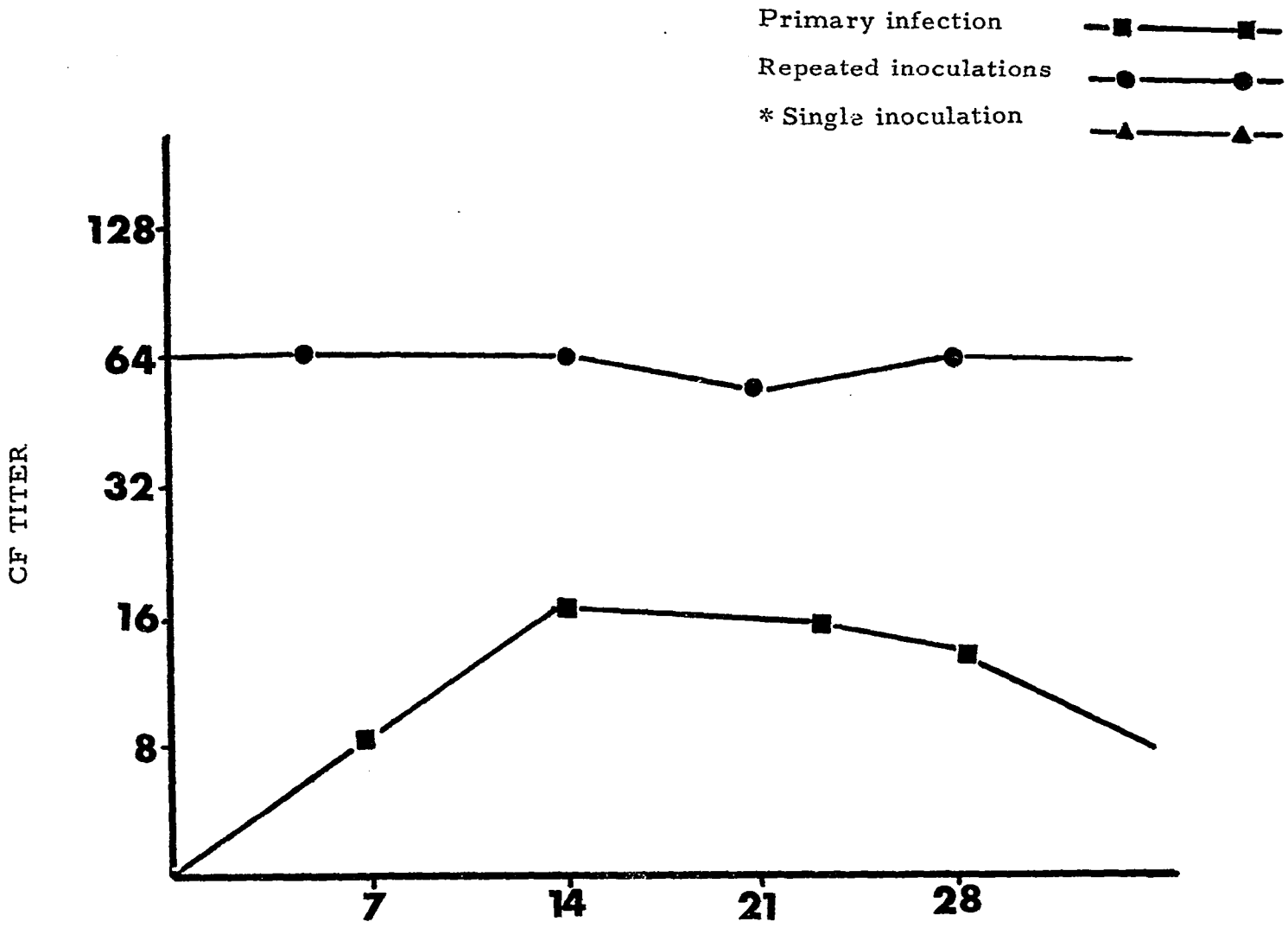

Figure 2. Complement fixation titers of guinea pigs infected with H. duboisii.

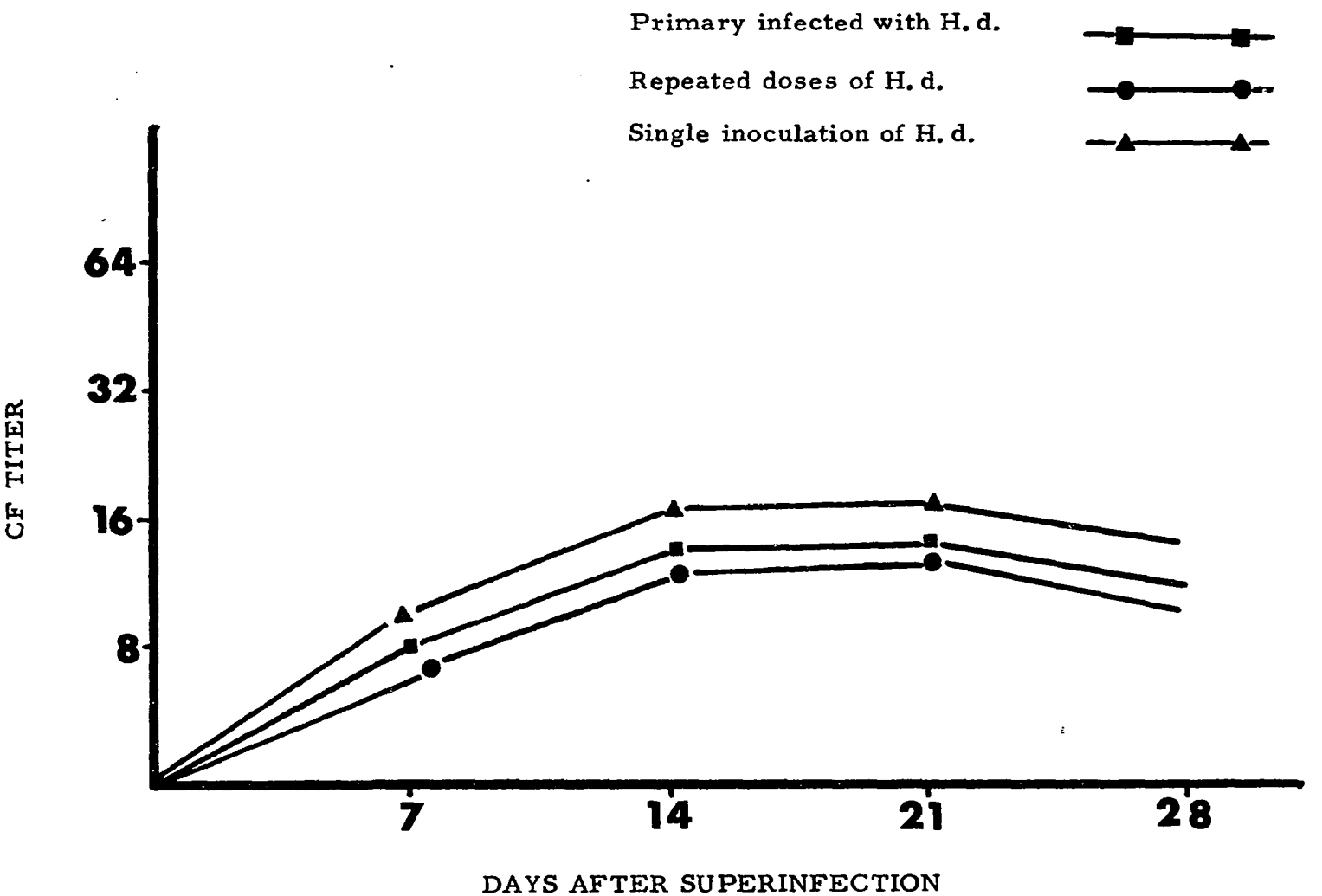

Figure 3. A comparison of complement fixation titer of guinea pigs superinfected with L. monocytogenes.

from H. duboisii infected animals having an L. monocytogenes superinfection were similar (1:16), including control animals. The ability of both groups of chronically infected animals to respond to a second type of injection is apparently the same.

The immunological response of guinea pigs infected with H. duboisii and immunized later with sheep red blood cells was also compared. The presence of hemolysin and agglutinin antibodies was demonstrated in all the animals. The hemolysin titer was much higher than the hemagglutination titer as indicated in Figure 4. It was observed that the hemolysin titers of sera from H. duboisii infected animals, immunized with sheep erythrocytes, were very close (ranging from 1:640 to 1280) when compared with each other. The range of differences observed was within one tube dilution.

The hemagglutination titer of sera from H. duboisii infected animals, having been immunized with SRBC was compared and indicated in Figure 5. The group of animals infected with one and two inoculations of H. duboisii have similar immunological responses (1:64) to the second type of immunization. However, a low H. A. titer (1:32) was observed in the group of animals infected with repeated doses of H. duboisii. Results of total and differential white blood count determinations of blood taken 16 days after the last inoculation are

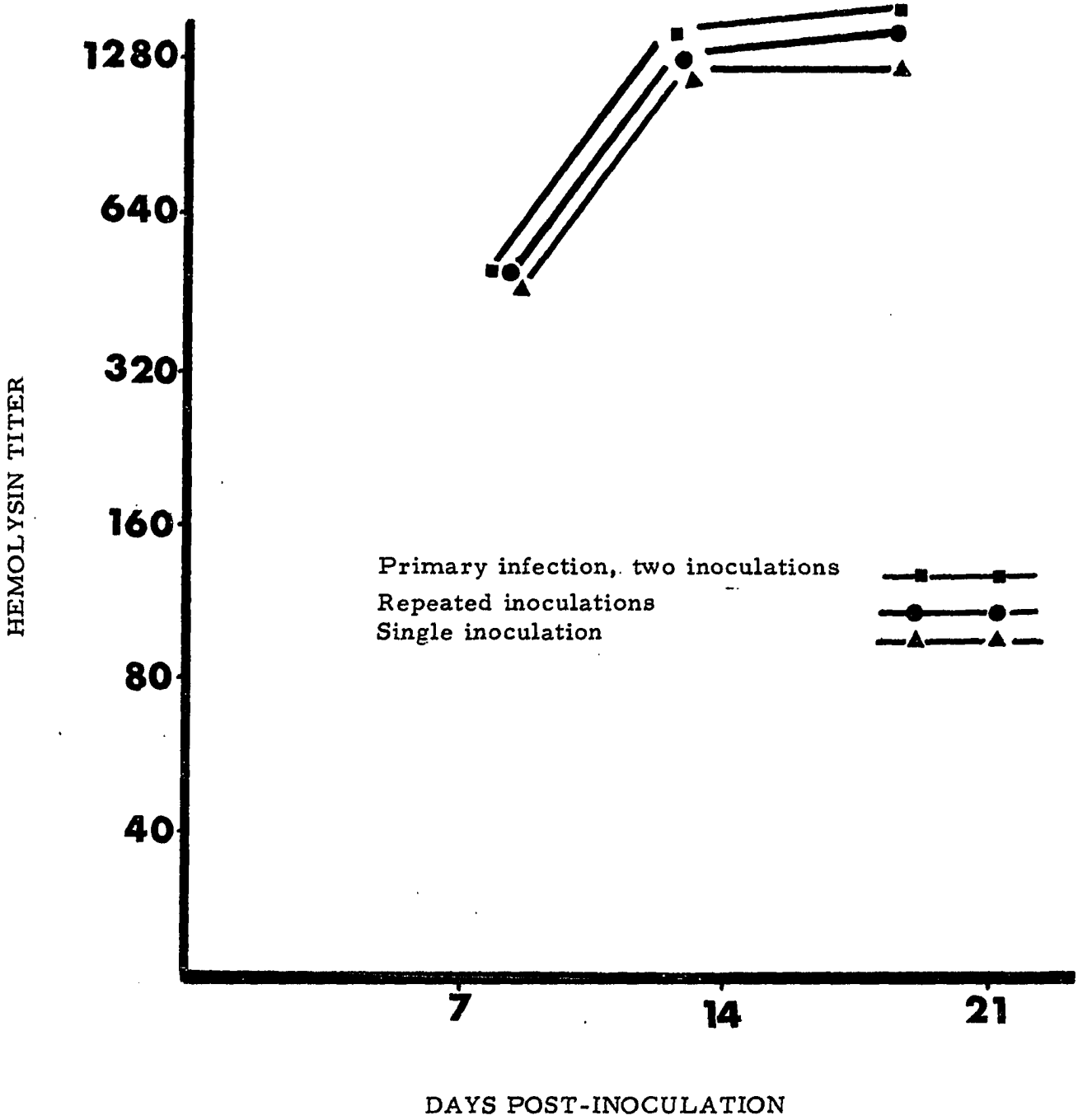

Figure 4. Hemolysin titer of guinea pigs against sheep RBCs previously infected with H. duboisii.

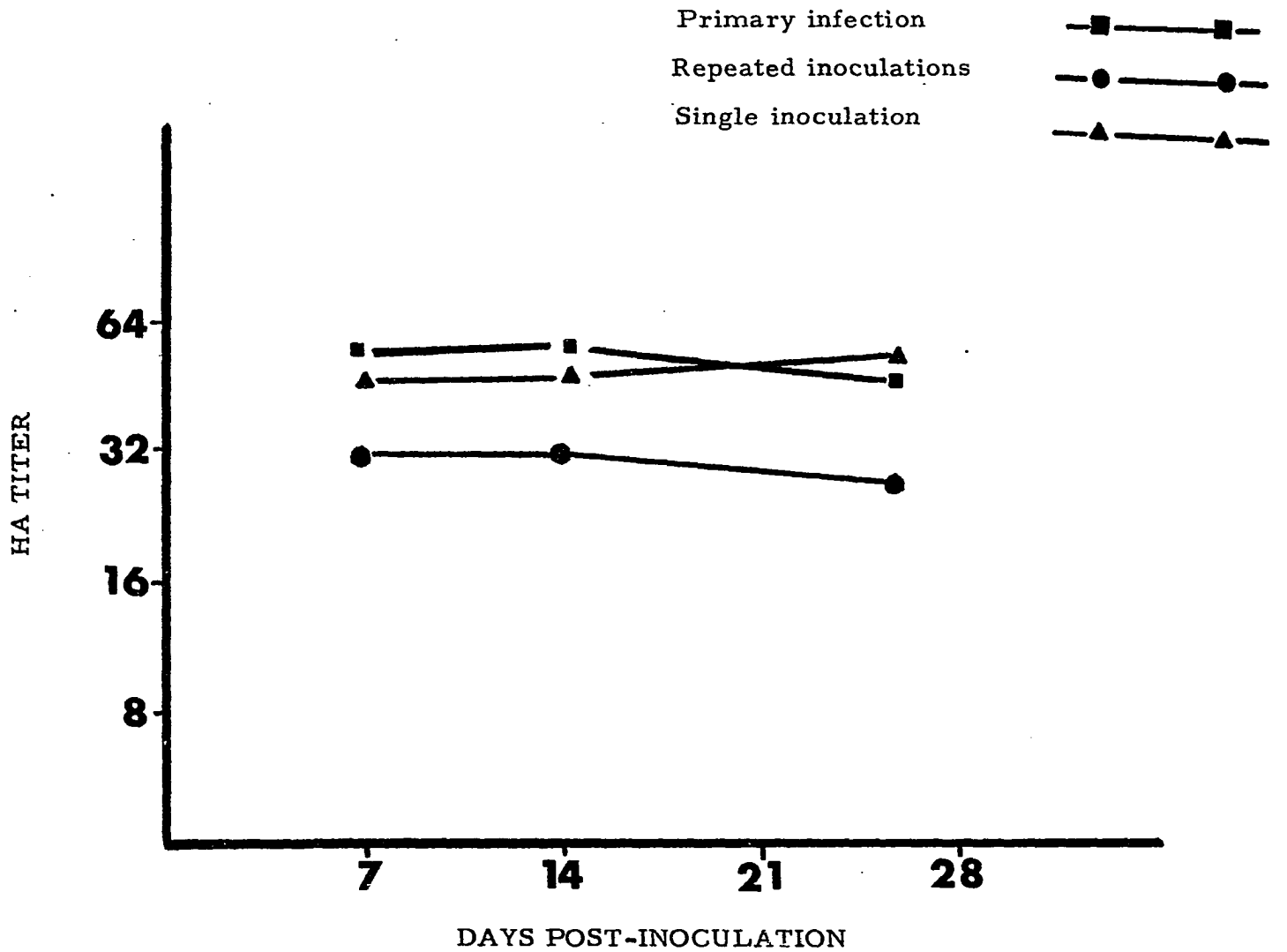

Figure 5. Hemagglutination titer of guinea pigs against sheep RBCs, previously infected with H. duboisii.

listed in Table 5. An increase in lymphocyte and total leucocyte count was observed in animals infected with repeated doses.

Evaluation of Cellular Involvement of Spleen

No attempts were made to estimate the number of nucleated cells which may have migrated into or out of the spleen following intraperitoneal injection of the antigen. Also no attempt was made to estimate the types of nucleated cell involved in the production of antibodies.

Figure 6 illustrates the number of RF cells per spleen. The number of Rosette-forming cells (RFC) are directly proportional to the number of individual cells which are responsible for producing antibodies. Lymph nodes are also associated with antibody producing cells and have been studied in a similar manner by Storb and Weiser in 1967, employing immunocytoadherence technique. The capacity to form rosettes was probably due to antibodies of more than one molecular species as suggested by the observation that incorporation of 0.1 M mercaptoethanol in the spleen cell and red blood cell (used as an antigen) mixture failed to completely suppress the formation of rosettes.

Results reflecting the response of the antibody forming spleen cells are listed in Figures 6 and 7. Four animals from each test group were sacrificed. Five samples from each spleen homogenate were diluted and used in

Table 5. Total and differential white blood cell counts

	PRIMARY INFECTION	SINGLE INOCULATION	REPEATED INOCULATIONS
Neutrophils	19	24	29
Lymphocytes	59	53	73
Monocytes	12	9	14
Total WBC Count	7,500	7,000	14,325

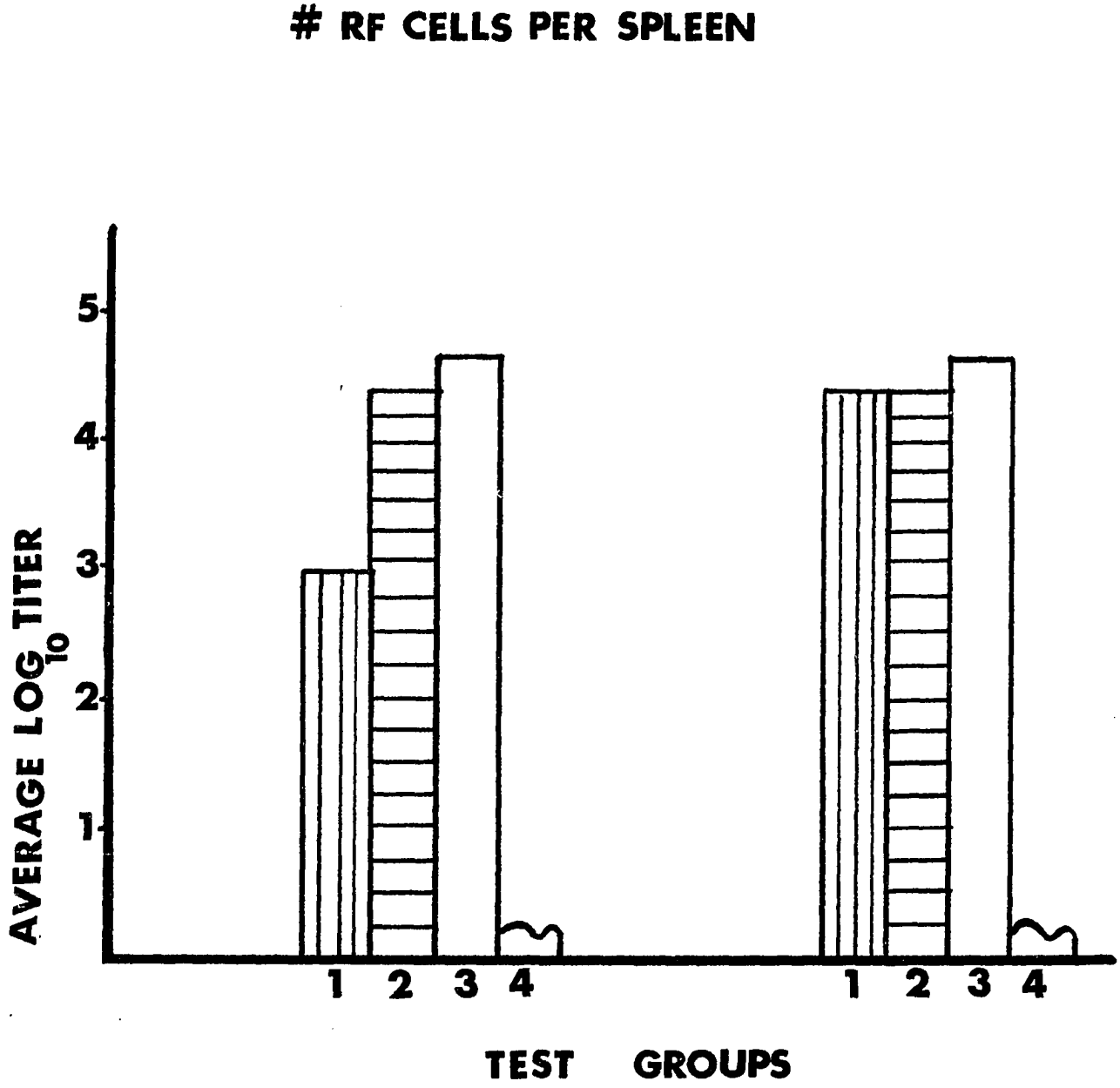

Figure 6. 1. Chronically infected group (one inoculation), 2. primary infected group, 3. chronically infected group (repeated inoculation), 4. control.
Left. Number of RF cells prior to superinfection; Right. Number of RF cells with homologous superinfection.

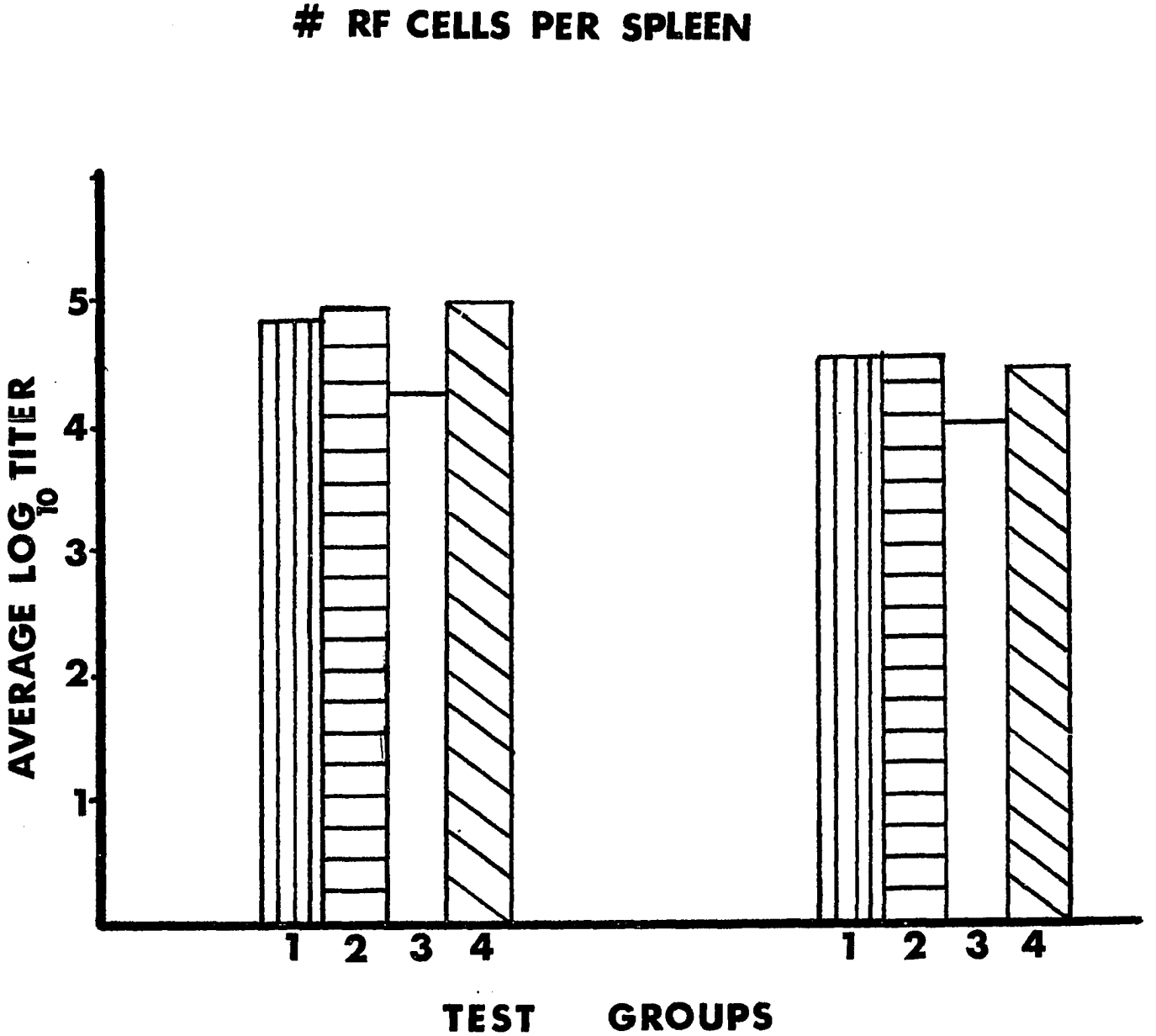

Figure 7. 1. Chronically infected group (one inoculation), 2. primary infected group, 3. chronically infected group (several inoculations), 4. control.
Left. Number RF cells produced against sheep RBCs;
Right. Number of RF cells produced against L. monocytogenes.
All spleens were harvested 16 days postinoculation.

this study. All values listed represent the average of the four guinea pig spleens.

Animals chronically infected with a single inoculation of H. duboisii (Group 1) demonstrated an average of $1X10^3$ RF cells/spleen, while primary infected animals (Group 2) and animals chronically infected with repeated inoculations (Group 3) demonstrated an average of $4.5X10^4$ and $6X10^4$ RF cells/spleen respectively. It was observed that Group 1, upon reinfection with homologous organisms showed an increase in the number of RF cells, while no significant increase was observed in Groups 2 and 3. Response of chronically infected animals with repeated inoculations to superinfection with L. monocytogenes demonstrated a reduction in the number of RFCs as compared to Groups 1 and 2. With the exception of a slight overall increase in the number of RF cells, a similar response was observed when the three groups were inoculated with sheep RBCs. This increase could be due to the application of a direct method as opposed to the indirect method utilized in the case of listeria superinfection. Figure 8 illustrates the formation of rosettes against sheep RBCs and H. duboisii.

Initial studies were performed using both anionic and cationic systems. Results obtained were reproducible and in agreement with the corresponding densitometer tracings. The cationic system was selected because it

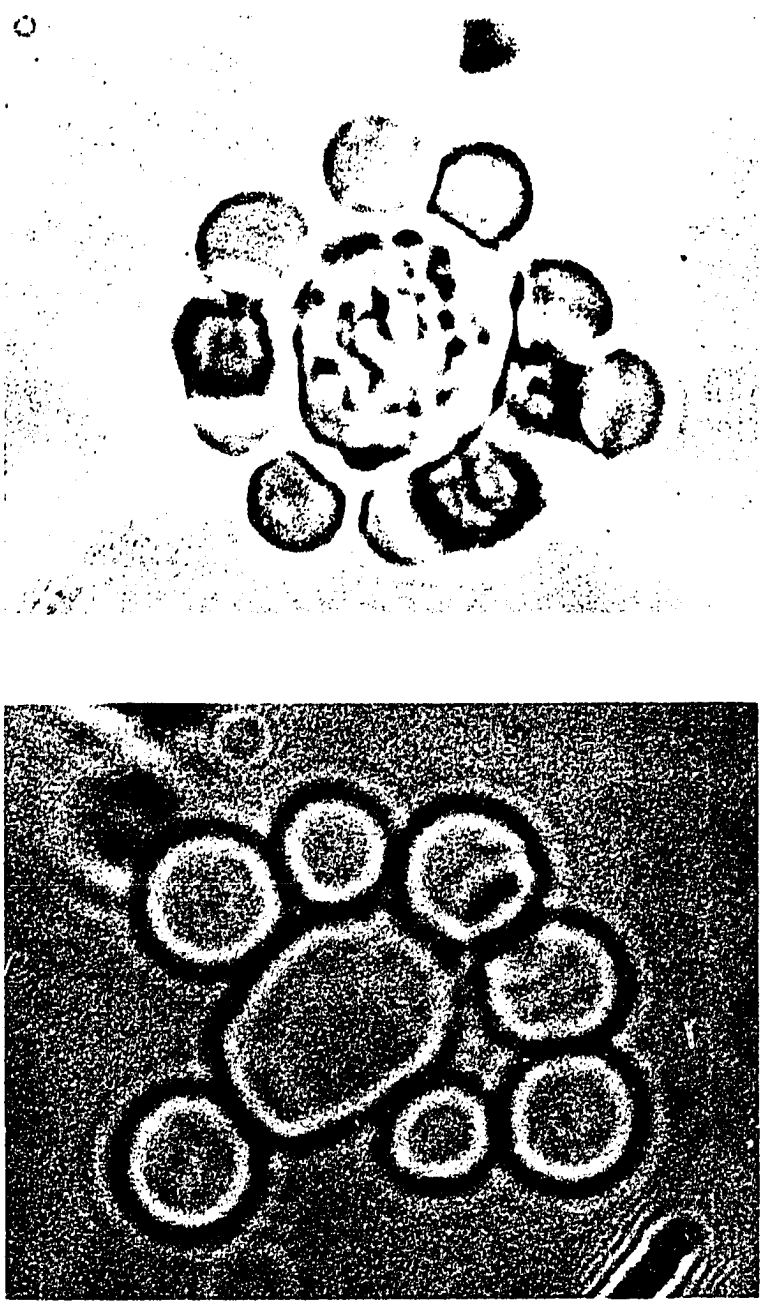

Figure 8. Top. Rosette formation against sheep RBCs.
Bottom. Rosette formation against H. duboisii.

yields better resolution in the gammaglobulin area.

All samples of sera subjected to electrophoresis in this study were used in duplicates. Twelve sera from each group were tested. Several differences in the number of bands were observed in the lower RD region of chronically infected and control animals (Figures 9, 10, 11 and 12). Of particular interest was an additional band which consistently (20/24) appeared in the sera of chronically infected animals (Figures 11 and 12). This band was absent in the sera of normal and primary infected animals. The presence of the band was marked with a small triangle as indicated in Figures 11 and 12.

The only characterization established with regard to this band was an RD of .02-.05. Relative distance (RD) is defined as the ratio of specific protein migration to the albumin migration distance.

A few inconsistent bands appeared within each group. It is difficult to assess any importance of these bands due to their irregular occurrence.

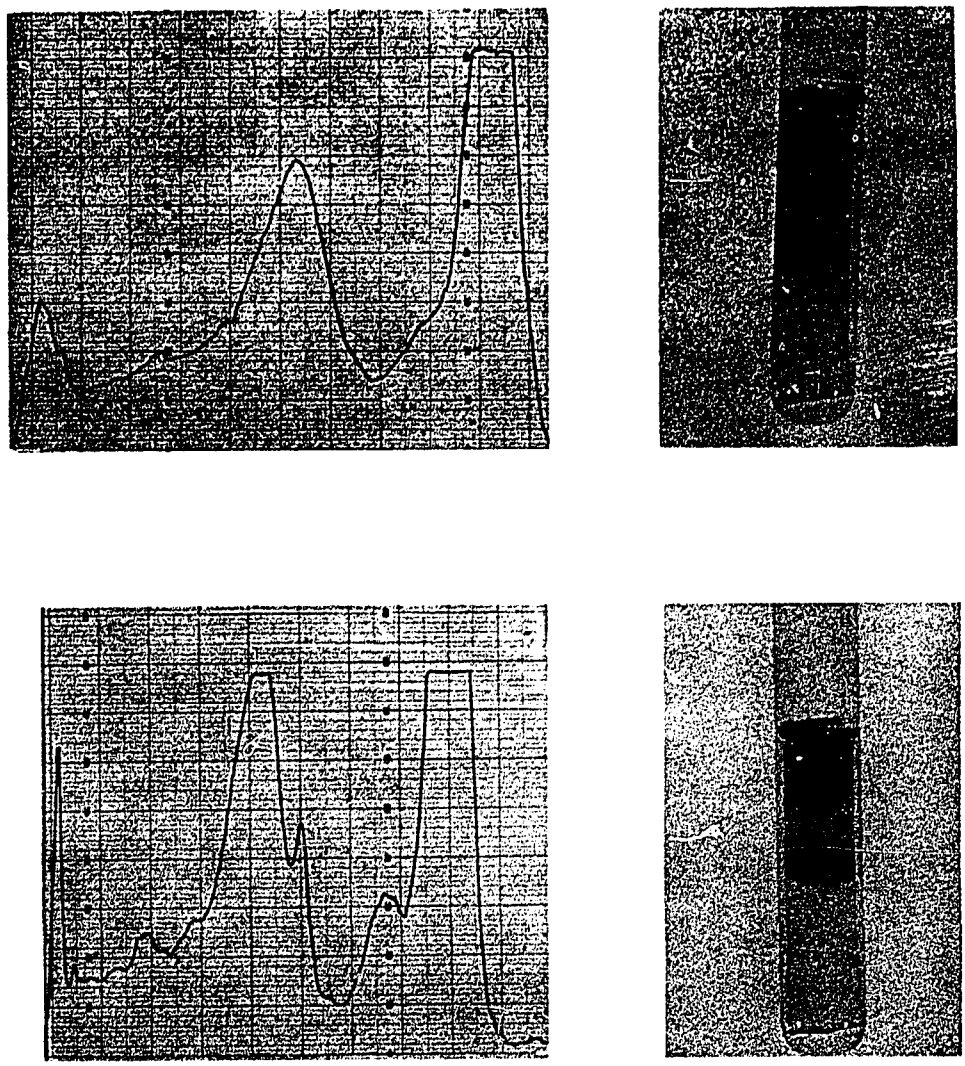

Figure 9. Electrophoretic patterns of sera from normal guinea pigs.

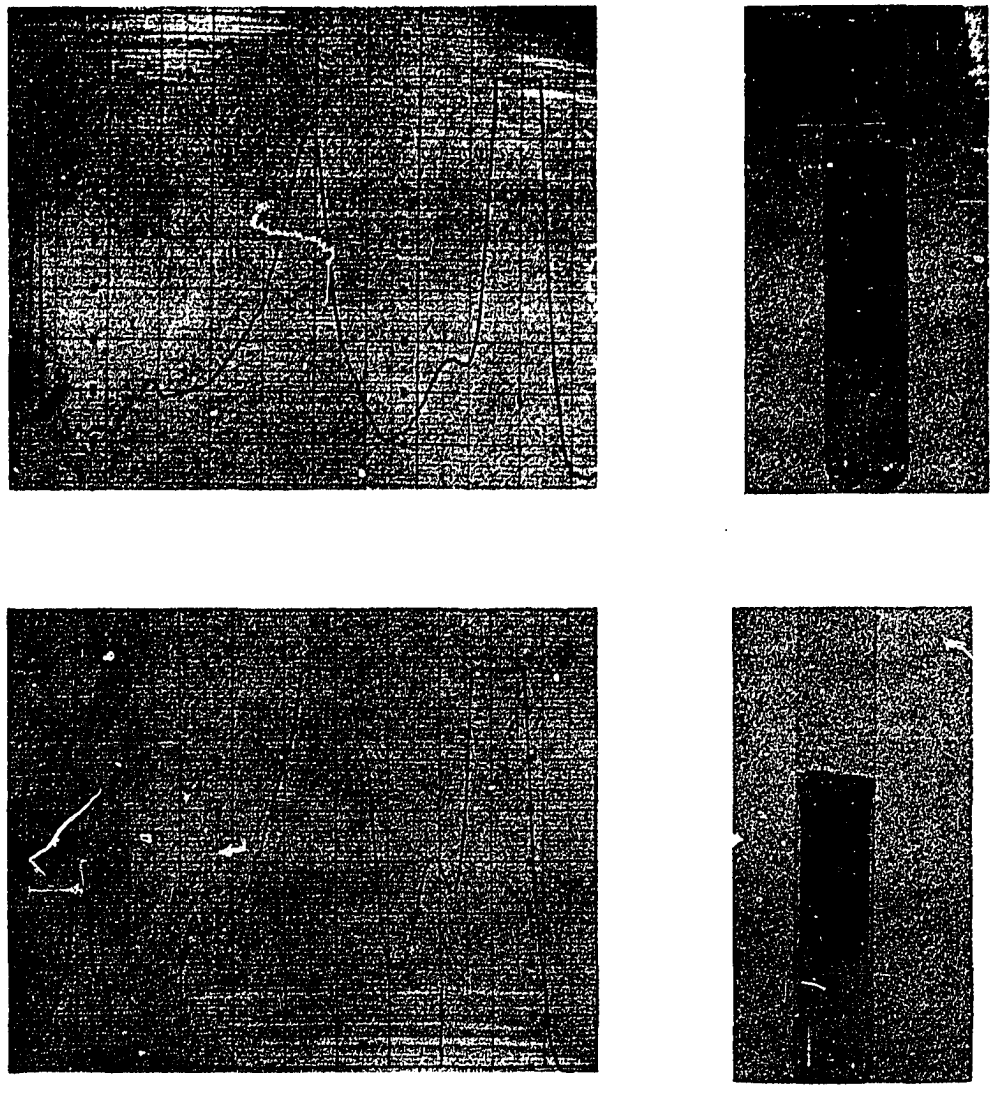

Figure 10. Electrophoretic patterns of sera from primary infected guinea pigs with H. duboisii.

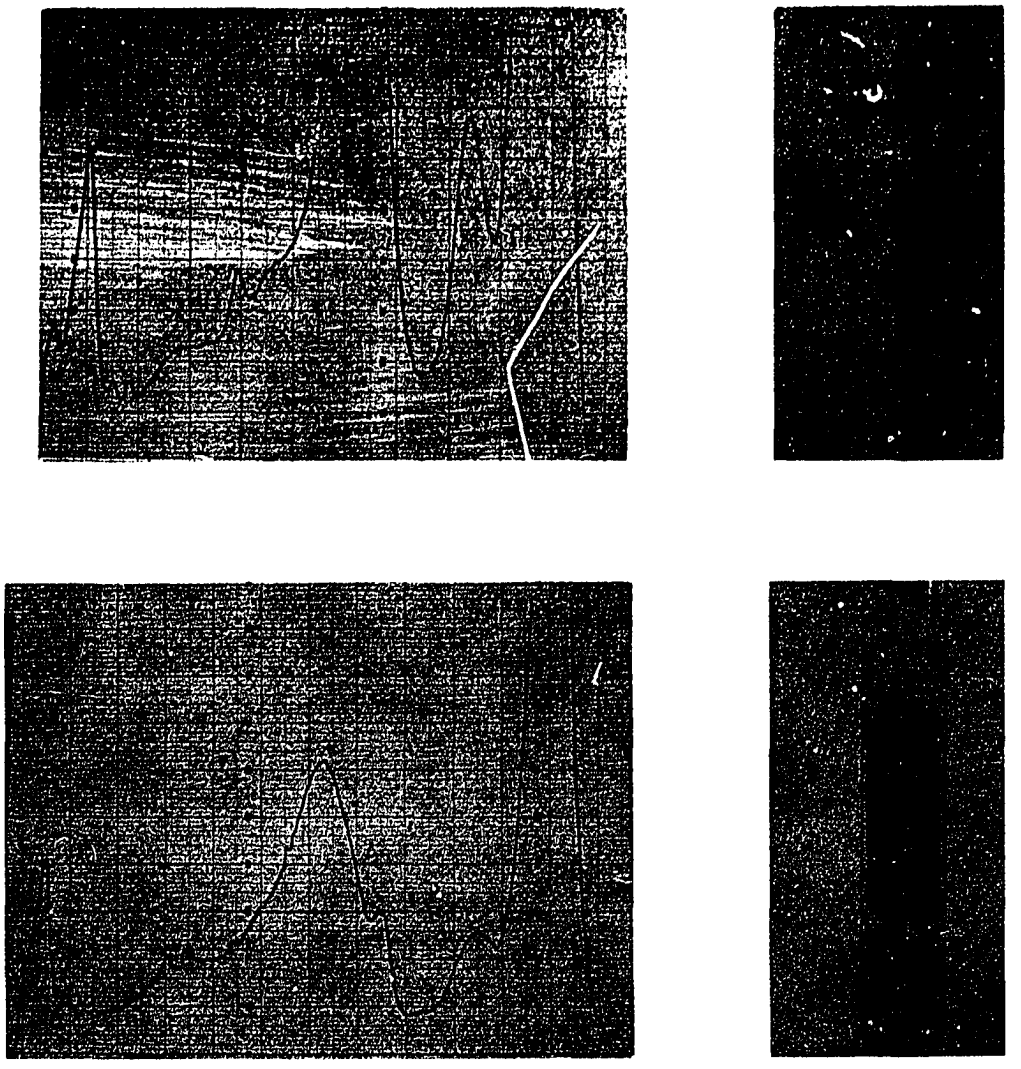

Figure 11. Electrophoretic patterns of sera from chronically infected guinea pigs with single inoculation of H. duboisii.

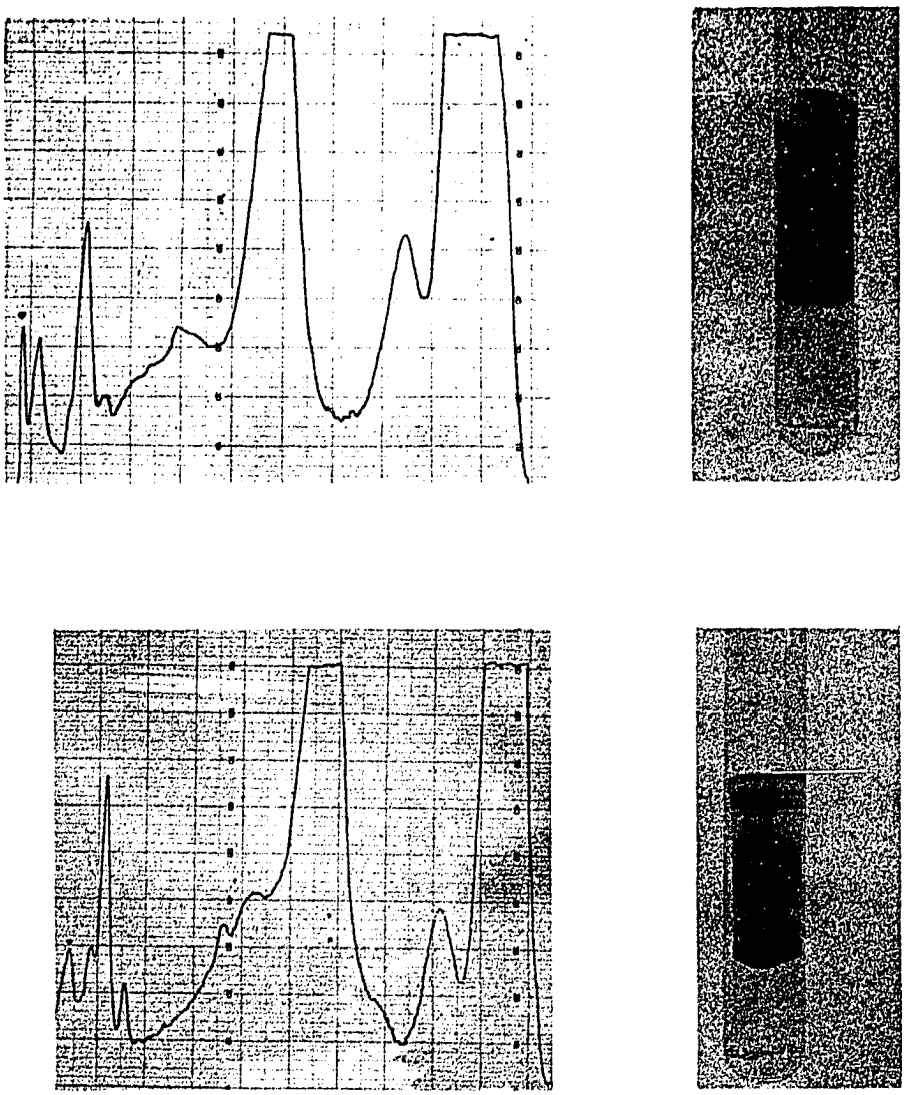

Figure 12. Electrophoretic patterns of sera from guinea pigs chronically infected with repeated inoculations of H. duboisii.

CHAPTER IV

DISCUSSION

The heterogeneity of antibodies has been a recurrent theme in immunology for many decades. There are a number of variations which have emerged from problems concerned with the stimulation and mode of production of antibody. This heterogeneity has also been observed with respect to structure, molecular weight, and electrophoretic mobilities. A sequential production of antibody types during the progression of a disease, particularly IgM and IgG, has been well demonstrated; an exception to this rule, however, has been noticed more recently in certain diseases (26).

Lately Wigzel (47) has demonstrated that actively produced as well as passively administered antibodies can inhibit antibody synthesis, thereby strengthening the concept that antibody is acting as a "feed back" factor during immunization (11). This prompted the inclusion of an additional group of animals in this study. This group was treated similarly to the chronically infected group, with the exception that only a single injection of H. duboisii was administered rather than several inoculations.

Examination of Figure 2 suggests that animals with a single dose of H. duboisii did not show the presence of C.F. antibodies in most of the cases; however, the presence of precipitin antibodies was demonstrated. Also positive skin test reactions were recorded in 76% of the animals. This indicates that one inoculation is not sufficient in eliciting C.F. antibodies, though presence of precipitin antibodies and positive skin reactions were demonstrated.

Animals which were chronically infected showed appreciably higher C.F. titer than the animals with non-chronic infection. Animals (Fig. 3) with histoplasmosis, when superinfected with L. monocytogenes, did not show any significant difference as far as their humoral response was concerned. A similar response was noticed when these infected test animals were inoculated with sheep RBCs. The data suggest that no appreciable change has occurred with regard to the ability of these test animals to produce specific antibody to homologous and heterologous antigens. If there was any impairment of the R.E. system, one would expect to find a significant reduction in the immunological response of these animals to superinfection.

Studies on the infection of mice and hamsters with H. duboisii have demonstrated a variation in the virulence of this organism. Schwarz et al. (38) reported that neither hamsters nor mice died from intraperitoneal

infection of large doses of one isolate of H. duboisii. However, the lack of virulence is not a definite characteristic for isolates from African Histoplasma. Recently, it was shown in this laboratory (41) that some isolates of H. duboisii were lethal to guinea pigs by intraperitoneal injection. In this study all male guinea pigs developed a severe orchitis and gradual involvement of the organs of the R.E. system. Also a mild mastitis as compared to severe orchitis was observed in some of the female guinea pigs. This suggests a relationship of sex hormones to the cultivation of the yeast phase of H. duboisii, particularly in male guinea pigs.

Quantitative detection of antibody producing cells in large populations of lymphoid cells isolated from animals immunized with certain antigens can be performed by the method of rosette formation. The rosette test has been applied to the cytodynamic study of the immune response in lymphnodes (4) and spleen (3). Recently, Zaalberg et al. (48) have compared the rosette test and the Jerne test for detection of antibody producing cells. He concluded that the rosette test is 100x more sensitive than the Jerne test. This technique is indeed sensitive, simple to perform and probably detects 19S as well as 7S antibodies.

Interpretation of the present results on antibody producing cells are based on the assumption that except

for macrophages and reticulum cells, all rosette-forming cells are antibody producing cells (40). Also the migration of antibody producing cells into and out of the spleen is not assessed. From the data obtained (12) and (31) on the passage of cells between tissues, lymph and blood, it can be assumed that in the present investigation the spleen both received and released at least some RF cells.

An increase in number of RF cells is a reflection of an increase in the number of antibody producing cells. It was observed that control animals did not demonstrate the presence of RF cells. However, this does not exclude the possibility of the presence of RF; perhaps there are some RF cells present in the spleen homogenate and are simply diluted out in the preparation for hemacytometer counting chamber.

It was observed that the chronically infected animals with a single inoculation, when challenged with homologous superinfecting agent, showed an increase in the number of RF cells. This suggests that the antibody forming capacity of these animals is not fully utilized by administering a single inoculation. However, the superinfection of chronically infected animals with homologous organism yielded no increase in RF cells. The suggested explanation for this phenomenon would be the existence of a maximum level of specific differentiation.

A similar suggestion has been made by Makinodin (21).

Chronically infected animals with repeated inoculations of H. duboisii displayed a slight reduction in RF cells upon superinfection with L. monocytogenes. A similar response was noticed in this group when inoculated with sheep RBCs, except that the RF cell count was higher. This may be due to the application of the indirect method in enumerating RF cells in the case of Listeria superinfection. Reduction of this type is not very significant in comparison with other groups similarly treated. One should bear in mind that spleen is not the only organ for producing antibody forming cells. The approach used to quantitate the antibody forming cells, however, was restricted to spleen cells only.

An important observation was made in the study: animals with histoplasmosis when superinfected with L. monocytogenes displayed a very low fatality rate. On the other hand, the noninfected animals when infected with Listeria demonstrated a 100 percent fatality rate.

Until recently it was common to dismiss this phase of cross resistance as an example of "nonspecific immunity" and any possible immunological significance of this phenomenon was ignored. Mackaness (20, 19) has demonstrated that animals infected with Listeria are, for a time, resistant to other facultative intracellular parasites. He further stated that this type of resistance

can be transferred with cells (macrophages), but not with serum. No definite explanation was given as to the mechanism of this nonspecific cross immunity.

An increase in lymphocytes and total white blood cell count was recognized in chronically infected animals. This increase parallels the increase in the number of RF cells and C.F. titer in chronically infected animals with repeated inoculations.

Electrophoretic patterns of sera for protein components showed that distinctive patterns can be detected in infected animals. Investigation of sera from different sources has shown that a good reproducibility of electrophoretic patterns does exist for many different diseases (27). These electrophoretic patterns are considered helpful in the diagnosis of disease.

After examination of different gels it was recognized that a band having a RD value of .02-.05 was present in chronically infected animals. The absence of this band was noted in normal and nonchronically infected animals. The fact that only a percentage rather than all of the individuals in a population display this variation suggests that a genetic character may be responsible. No attempt was made to chemically analyze the presence of this band.

CHAPTER V

SUMMARY AND CONCLUSIONS

A study to investigate the immunological response of guinea pigs was undertaken. Animals were infected chronically with H. duboisii and their ability to respond to superinfecting agents was determined. The immunological response of nonchronically infected animals was used as the reference for comparison. The chronically infected animals were given a single and repeated doses of H. duboisii.

The parameters chosen to assess the immunological ability of infected animals included serological titers, specific response of antibody forming cells and partial characterization of the serum proteins. Humoral response was assessed using hemolysin, complement fixation, and hemagglutination titers. Spleenic involvement was determined using the rosette test, which depicts both 19S and 7S antibodies.

In most of the cases, animals receiving a single dose of H. duboisii did not demonstrate the presence of complement fixing antibodies. However, positive skin and precipitin reactions were recorded. Chronically

infected animals displayed appreciably higher C.F. titer than those with nonchronical infections. No significant difference in serological titers was observed when different testing groups were superinfected. However, a slight reduction in the number of rosette forming cells of chronically infected animals with repeated doses against superinfection was noticed. It was demonstrated that sera from chronically and nonchronically infected animals differ with respect to protein components.

In conclusion, no noticeably significant impairment or damage to the reticuloendothelial system was observed in guinea pigs which were chronically infected with H. duboisii in regard to antibody production.

BIBLIOGRAPHY

1. Ajello, L., L. K. George, W. Kaplan, and L. Kaufman, 1963. Laboratory Manual for Medical Mycology. C.D.C.

2. Berliner, Martha D., 1967. Gelatin hydrolysis for identification of the filamentous phase of Histoplasma, Blastomyces and Chrysosporium species, Sabouraudia, 5 (4) 274-277.

3. Biozii, G., C. Stiffel and D. Mouton, 1966 b. A study of the antibody containing cells in the course of immunization. Inter. Symp. on Immunity Cancer and Chemotherapy, Buffalo 1966. (Edited by E. Minich). Academic Press, New York.

4. Biozii, G., C. Stiffel, D. Mouton, M. Liacopouloo-Briot, and Y. Bouthillier, 1966 a. Etude du phenomene de I'Immunocytoadherence au cours de I'immunisation. Ann. Inst. Pasteur. 110 (Suppl. 3), 7.

5. Blumer, S., and L. Kaufman, 1968. Variation in enzymatic activities among strains of Histoplasma capsulatum isolated from men, dogs, and soil. Mycopathologia, Haag. 17: 172-182.

6. Burnet, Sir McFarlane, 1954. How antibodies are made. Sci. Amer., 191: 74-78.

7. Burnet, Sir McFarlane, 1961. The mechanism of immunity. Sci. Amer., 204: 58-67.

8. Coudert, J., and M. Coly, 1956. Eassi d'application de la reaction d'agglutination des particles de collodion a quelques parasitoses. Ann. Parasitol. Humaine et Comparee. 31, 489.

9. Cozad, G. C. and G. M. Scalarone, 1969. Effect of histoplasmosis on antibody response to an erythrocyte antigen. J. Bact., 97: 576-580.

10. Darling, S. T., 1906. A protoscan general infection producing pseudotubercles in the lungs, and focal necrosis in the liver, spleen and lymph nodes. J. Amer. Med. Assoc., 46: 1283.

11. Finkelstein, M. S., and J. W. Uhr, 1964. Specific inhibition of antibody-formation by passively administered 19S and 7S antibody. Science, 146: 167.

12. Gowan, J. L., D. D. McGregor, D. M. Cowen, and C. E. Ford, 1962. Initiation of immune responses by small lymphocytes. Nature 214: 183-185.

13. Hill, G. B., and C. C. Campbell, 1956. A further evaluation of histoplasmin and yeast phase antigen of H. capsulatum in complement fixation test. J. Lab. and Clin. Med., 48: 255-263.

14. Lackman, D. B., 1951. National Institute of Health Circular No. 12. Revised.

15. Larsh, H. W., and M. L. Furcolow, 1952. Follow-up of the Camp Gruber epidemic. Proc. of the Conference on Histoplasmosis. Public Health Monogr. No. 39, 34-38.

16. Lehan, P. H., C. A. Brasher, H. W. Larsh and M. L. Furcolow, 1957. Evaluation of clinical aids to the diagnosis of chronic progressive cavitory histoplasmosis. Am. Rev. Tuberc. 75: 938-948.

17. Loosli, G. G., W. G. Beadenkoff, F. A. Rice, and L. J. Savage, 1951. Epidemiological aspect of histoplasmin, tuberculin, and coccidiodin sensitivity. Am. J. Hyg. 53: 33-57.

18. McDearman, Sara C., 1962. A modified histoplasmin in the hemagglutination test for histoplasmosis. Am. J. Med. Tech., 28: 146-160.

19. Mackaness, G. B., 1964. The immunological basis of acquired cellular resistance. J. Exp. Med., 120: 105-120.

20. Mackaness, G. B., R. V. Blanden, and F. M. Collin, 1966. Host parasite relation in mouse lymphoid. J. Exp. Med., 124: 573.

21. Mackinodin, T., and J. F. Albright, 1963. Cytokinetics of antibody response. Immunopathology, III Intl. Symp.

22. Nossal, G. J. V., 1964. How cells make antibodies. Sci. Amer. 211: 106-115.

23. Nota, N. R., M. Liacopoulos, C. Stiffel, and G. Biozii, 1964. L'immunocytoadherence: Une method simple et quantitative pour l'etude in vitro des cellules productrices d'anticorps. Compt. Rend. Acad. Sci. 259: 1277-1280.

24. Okudaira, M., and J. Schwartz, 1961. Infection with H. duboisii in experimental animals. Mycologia 53: 53-63.

25. Ornstein, L., 1964. Disc electrophoresis-backgroun and theory. Ann. New York Acad. Sci. 121: 321-344.

26. Pike, R. M., 1967. Antibody heterogeneity and serological reactions. Bact. Rev. 31: 157-174.

27. Price, W. H., H. Harrison, and J. Molends, 1966. Seromucoid patterns of normal and tuberculosis individuals as determined by disc electrophoresis technique. Amer. J. Epidem. 23: 152-175.

28. Rosenthal, S. A., and H. Sokolsky, 1965. Enzymatic studies with pathogenic fungi. Derm. Int., 72-79.

29. Sabin, Florence, 1939. Cellular reactions to a dye-protein with a concept of mechanism of antibody formation. J. Exper. Med., 70: 67-82.

30. Salvin, S. B., 1947. Complement fixation studies in experimental histoplasmosis. Proc. Soc. Exper. Biol. and Med. 66: 342-345.

31. Salvin, S. B. Precipitin and complement fixation tests with whole yeast phase antigen. Ibid. pp. 163-165.

32. Salvin, S. B., and M. L. Furcolow, 1954. Precipitin in human histoplasmosis. J. Lab. and Clin. Med., 43: 259-274.

33. Salvin, S. B., and M. L. Furcolow, 1952. The precipitin test in human histoplasmosis. Proc. of the Conference on Histoplasmosis. Publ. Health Monogr. No. 39, 1956. pp. 129-131.

34. Saslaw, S., and C. C. Campbell, 1949. A collodion agglutination test for histoplasmosis. Publ. Health Rep. 64: 424-429.

35. Saslaw, S. Collodion agglutination test and complement fixation test with ground yeast-phase antigen. Ibid., pp. 152-162.

36. Saslaw, S., and C. C. Campbell, 1948. The use of yeast-phase antigen in a complement fixation test for histoplasmosis. J. Lab. and Clin. Med., 33: 811-818.

37. Saslaw, S. and C. C. Campbell, 1948. The use of yeast-phase antigens in a complement fixation test for histoplasmosis. II. Results with ground antigens. Ibid. 33: 1207-1211.

38. Schwartz, J., and E. Drouhet, 1957. Morphologic features of an African strain of Histoplasma in hamster and mice. A.M.A. Archives of Pathology, 64: 409-413.

39. Smith, R. T., 1966. Human immunoglobulins. A guide to nomenclature and clinical application. Pediatrics 37 (5) Part I.

40. Storbe, U., and S. Weiser, 1967. Antibody carrying cells in the immune response. RES, J. Reticuloendothelial Soc. 4: 51-68.

41. Sunday, F. O., 1969. Ph.D. Thesis. Univ. of Oklahoma.

42. Teneberg, J. D., 1956. Histoplasmin and whole yeast phase H. capsulatum as antigen in complement fixation test on histoplasmosis. Proc. of the Conference on Histoplasmosis, 1952. Publ. Health Monogr. No. 39, pp. 150-152.

43. Teneberg, D. J., and Arden Howell, Jr., 1948. A complement fixation test for histoplasmosis. Public Health Rep. 63: 163-168.

44. Tsubura, E., M. Okudaira, G. Baum, J. Schwartz, and D. Artis, 1962. Comparative studies of Histoplasma capsulatum isolated from men, dogs and soil. Mycopathologia, Haag 17: 172-182.

45. Vanbreuseghem, R., 1956. Histoplasmin duboisii and large form of Histoplasma capsulatum. Mycologia 48: 264-269.

46. Vanbreuseghem, R., 1967. Recent advances of human and animal mycology. Proc. of the Intl. Dermatological Symp. 209-218.

47. Wigzel, H., 1967. Studies on the regulation of antibody synthesis. Cold Spring Harbor Symp. on Quant. Biol. 32: 507-515.

48. Zaalberg, O. B., V. A. Van Der Meual, and J. M. Van Twisk, 1966. Antibody production by single spleen cells: A comparative study of cluster and agar-plaque formation. Nature 210: 544.