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EXPERIMENTAL HISTOPLASMOSIS.

The University of Oklahoma, Ph.D., 1972
Microbiology

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THE UNIVERSITY OF OKLAHOMA
GRADUATE COLLEGE

A STUDY OF IMMUNOCYTOADHERENCE DURING
EXPERIMENTAL HISTOPLASMOSIS

A DISSERTATION
SUBMITTED TO THE GRADUATE FACULTY
in partial fulfillment of the requirements for the
degree of
DOCTOR OF PHILOSOPHY

BY
GLENN DALE ROBERTS

Norman, Oklahoma

1971

A STUDY OF IMMUNOCYTOADHERENCE DURING
EXPERIMENTAL HISTOPLASMOSIS

APPROVED BY

Samuel H. Land
Eddie Card Smith
George C. Cozad
Donald C. Cox
John H. Lancaster

DISSERTATION COMMITTEE

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ACKNOWLEDGMENTS

The author wishes to express his appreciation to his major professor, Dr. Howard W. Larsh, Research Professor and Chairman of the Department of Botany and Microbiology, for his patience and guidance throughout the length of my graduate education. Appreciation is also extended to Drs. George C. Cozad, Donald C. Cox, John H. Lancaster, and Eddie C. Smith for their helpful suggestions given during the course of the research and completion of this manuscript.

Much gratitude is expressed to the Schering Corporation for their interest in this project; for their generous financial support and Dr. George L. Evans, Director of Research of Schering Diagnostics who is primarily responsible for initiating this interest.

Special thanks are extended to the many people whose technical assistance made this study possible. One could find no better group with which to work.

A STUDY OF IMMUNOCYTOADHERENCE DURING EXPERIMENTAL HISTOPLASMOSIS

CHAPTER I

INTRODUCTION

Histoplasmosis is a mycotic disease which invades the lymphatic system, lungs, spleen, liver, kidneys, adrenals, and the reticuloendothelial system. Its protean nature makes it one of the most difficult infectious diseases to diagnose.

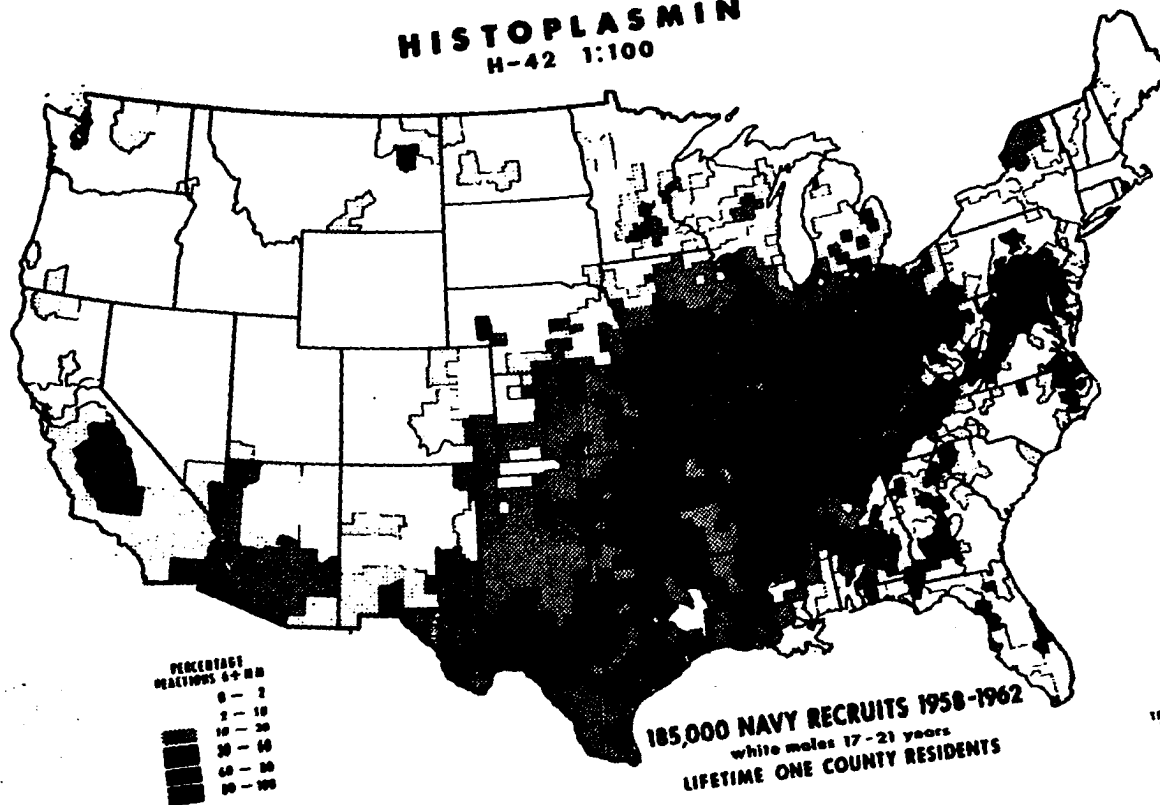
The etiology of the disease is attributed to a dimorphic fungus, Histoplasma capsulatum which grows as a parasite within the cells of the reticuloendothelial system of man and other animals. The parasitic form is described as a small, ovoid, budding yeast 2-4 microns in diameter. The fungus grows in soil and natural substrates as a mycelial phase characterized by small, smooth microaleuriospores and thick, smooth to spiny macroaleuriospores.

Histoplasmosis has been recognized in many parts of the world and is common in the central United States in the Missouri, Ohio, and Mississippi River Valleys. Figure 1 shows the areas which have the highest prevalence of positive reactors to the histoplasmin skin test. Although the incidence of infections is highest in the endemic areas, the relative ease of travel makes this disease of concern to practicing

Figure 1. Geographic distribution of histoplasmin skin test sensitivity.

HISTOPLASMIN

H-42 1:100



physicians in all parts of the world.

Loosli (22) has estimated that as many as 30 million people in the United States have experienced histoplasmosis. Furcolow (17) has extrapolated that approximately 500,000 new infections occur each year in this country. This estimate was based on skin-test surveys. These figures have created an awareness of the disease and there is now extensive literature dealing with research on H. capsulatum.

Except for their etiology, fungus infections differ little from bacterial infections. The close relationship between bacteria and fungi is apparent from the transitional forms connecting the two groups and from the similarity of the pathologic changes and clinical manifestations induced by them. The incidence of histoplasmosis is possibly as frequent as that of many bacterial infections, but the number of correctly diagnosed cases is markedly lower. This may be attributed to the variety of clinical symptoms exhibited by the disease and the lack of specific clinical diagnostic procedures.

A number of clinical classifications of histoplasmosis have been described, but most fall into one of three major groups: acute pulmonary histoplasmosis, chronic cavitary histoplasmosis, and disseminated histoplasmosis (35).

The most common form of the disease seen in the clinic is acute pulmonary histoplasmosis. This is usually a result of direct inhalation of some form of the organism and subsequent lung involvement. A large number of cases are asymptomatic and the infected individual may not realize he has had the disease. A still larger number of cases present only mild symptomatic features. These include an influenza-like illness with fever, chills, cough, slight chest

pain, and malaise. Symptoms usually last only a few days, but may persist for a few weeks. The radiological picture reveals a small nodular lesion or lesions resembling the type seen in tuberculosis. Hilar lymphadenopathy which may resemble sarcoid is frequently observed. The diagnosis of this type of disease is very difficult due to the short duration of clinical symptoms.

Chronic cavitary histoplasmosis is commonly found in adults of middle age or older. The symptoms resemble tuberculosis and some other mycotic diseases in many respects. They include: weight loss, fever, productive cough, and extreme fatigue. Radiological examination usually reveals large lesions in one or both lungs with frequent cavities. Chronic cavitary histoplasmosis is indistinguishable from pulmonary tuberculosis by radiological and clinical examinations. A confirmatory diagnosis is usually based on the isolation of the organism from the sputum or a biopsy of the lung.

Disseminated histoplasmosis is now recognized as a rather common form in patients suffering from debilitating diseases such as Hodgkin's disease, sarcoid, leukemia, or even tuberculosis (18). This results from the spread of the organism from the lungs via the blood to other areas of the body. Some forms of disseminated disease are classified as acute, and calcified lesions may be found in the spleens of otherwise healthy individuals. However, most cases of the disseminated type are chronic, and diagnosis is usually made at autopsy. This form of histoplasmosis may present lesions of the oral cavity and larynx (5) (27), cutaneous and prostatic lesions (28), cardiac lesions (37), peritoneal and small bowel lesions (26), and many other type clinical

symptoms.

Obviously, the disease presents no distinctive clinical picture, and an accurate diagnosis is extremely difficult. Confirmatory diagnosis of histoplasmosis is based almost entirely upon the recovery and identification of H. capsulatum from infected individuals. In many cases cultural proof is impossible and serology provides the presumptive diagnostic evidence. Such evidence is currently obtained by the complement fixation, immunodiffusion, latex agglutination, skin tests, and indirect immunofluorescent tests. A current serological review is presented by Kaufman (19) who describes these procedures. All of these tests can provide the physician with information of diagnostic and prognostic value. However, there are some disadvantages that must be considered: (a) the complement-fixation test is complex and costly, and must be performed by persons experienced in serology; (b) the skin test has limited value as a diagnostic tool because it cannot distinguish active from past infection; (c) titers obtained by most tests are very low and several consecutive serum samples are required to establish an increase or decrease in the production of antibodies over a given time period; (d) the requirement for analysis of consecutive serum samples further increases the time required to make a presumptive diagnosis which cannot be afforded by a patient awaiting treatment; (e) antibody titers detected by conventional tests are usually present for varying periods of time even after convalescence has occurred; (f) the interpretation of test results is often difficult because the commonly used antigens may react with sera taken from individuals having other systemic mycotic infections; (g) patients having disseminated

histoplasmosis may frequently be immunologically unresponsive and will exhibit no serologic evidence of active disease.

These disadvantages are minimized by the information gained from the immunoserology of the systemic mycoses; however, they were mentioned to emphasize the need for new and improved methods.

For several years immunologists have been investigating methods of detecting antibody production by single cells. Nota (25) and Biozzi (7) described the phenomenon of immunocytoadherence (ICA). They used this to study the kinetics of antibody production by cells of immunized animals. The test has been used as the basis for a simple and quantitative method of detecting and counting those cells among large populations of lymphoid cells which contain a certain amount of immunoglobulin on their surfaces. If a cell suspension is mixed with a particulate antigen, the antigen adheres firmly and specifically to the lymphoid cells involved in the immune response. The number of clusters or rosettes can be observed and counted in a hemacytometer.

Theoretically, antibodies belonging to all types of immunoglobulins should be detected by ICA, since rosette formation is dependent upon the binding of the antigen by the specific site of the antibody molecule (6). Zaalberg (38) has now shown that both 7S and 19S immunoglobulins can be detected by this method. Many other investigators have used ICA to study the cytodynamics of the immune response in animals immunized with sheep erythrocytes (34) (14) (13) (8) (7).

In 1969 I evaluated ICA as a method for detecting the immune response in animals having sporotrichosis (30). Results indicated that this method might be used as an index of active infection since the

relative number of rosettes correlated very well with the clinical picture exhibited by the animals.

A preliminary investigation of animals with histoplasmosis showed the same results which led to a study with the following objectives: (a) evaluation of immunocytoadherence as a serological tool and a comparison of its sensitivity to other serological tests; and (b) determination of the nature of the immunologic mechanism involved in immunocytoadherence.

Since this initial work, many medically important applications of the test have been described. Perrudet-Bodoux (4) investigated circulating human lymphocytes and sheep cells coated with thyroglobulin to detect lymphocytic antibodies in patients having immune thyroiditis. His results also indicated that lymphocytic antibodies correlated better with the clinical picture than did humoral antibodies. Bach (1) has done an in vitro evaluation of immunosuppressive drugs with the ICA test and has shown that it is a satisfactory method for evaluating the immunosuppressive potency of the drug in humans. Bach (3) also investigated ICA as an index of the immunosuppressive activity of antilymphocyte serum as applied to tissue transplantation. Bach (2) recently used ICA to detect rheumatoid arthritis in human patients. He has shown that the test is valuable in detecting active cases and is more accurate than other available methods.

The mechanism of immunocytoadherence is not yet well understood. Several possibilities exist: (a) the lymphoid cells may passively fix antibody to their surfaces; (b) the cells may be liberating a circulating antibody which has just been synthesized; (c) the rosette-forming

cell may be the vector of delayed hypersensitivity; and (d) the cells may be primed antigen-sensitive cells or memory cells.

Several workers have speculated on the mechanism of rosette formation. Coombs (11) states that there are IgM and IgG receptors on the surfaces of the cells. Moav (23) has preliminary data that suggest that the lymphoid cells are actively secreting immunoglobulins. In addition, Brain (9) has suggested that rosette formation is due to a substance, not primarily an antibody, elaborated by a large population of lymphocytes. Roberts (29) has been unable to demonstrate that rosette-forming cells are involved in mediating the delayed hypersensitive response but has shown that the cells have humoral antibody specificities on their surfaces.

Other applications of the ICA test justify its use as a serological tool in the study of histoplasmosis, and the controversy concerning the mechanism of the test makes this an intriguing problem.

Considering the simplicity and sensitivity of the test, it is hoped that it will lead to a new method which might be used to detect active histoplasmosis. The ICA test may supplement conventional serological tests and allow a more rapid and accurate diagnosis of histoplasmosis and other diseases.

CHAPTER II

MATERIALS AND METHODS

Preparation of Inoculum.

A culture of Histoplasma capsulatum, the Scritchfield isolate, was obtained from the University of Oklahoma stock culture collection. Yeast cultures were incubated in Brain Heart Infusion Broth for 3 days at 37 C with constant rotation in a Psychrotherm environmental shaker (New Brunswick Scientific). The cells were washed three times in sterile physiological saline solution (PSS) and resuspended to a concentration of 2.6×10^8 cells/ml.

Cell viability counts were made by plating the inoculum on Sabouraud's dextrose agar and counting the colonies after 10 days incubation at 25 C. Viability was recorded as 75%.

Animal Infection.

One hundred four normal female New Zealand rabbits, 12-14 weeks in age, were obtained for this study. Twenty-six animals were given intravenous (IV) injections of 2.6×10^8 cells/kg of body weight and 26 animals were given intratracheal (IT) injections of 1×10^8 cells contained in 0.2 ml PSS.

The remaining 52 rabbits were used as normal controls. Half were given IT injections of PSS and half were given IV injections of PSS. Another control group was made up of rabbits which were previously

infected with either Blastomyces dermatitidis, Listeria monocytogenes, or several other strains of H. capsulatum.

Skin Testing.

One half of the animals from each group were skin tested prior to infection and five weeks later. 0.1 ml of a 1:100 dilution of histoplasmin, lot H-42 (CDC, Atlanta, Georgia) was injected intradermally on the dorsal surface of each animal. Tests were read after 48 hours and recorded as positive if the induration was 5 mm or more in diameter.

Serological Studies.

Animals were placed in two groups and each was bled biweekly for eight weeks after the infecting dose had been given. Blood was withdrawn by cardiac puncture with Becton Dickinson vacutainers. Thirty ml were placed in heparinized tubes and 5 ml in a plain tube. The sera were collected after coagulation of the blood occurred and stored at -60 C for later serological studies.

Latex Agglutination Test.

The histoplasmin latex agglutination test antigen was obtained from Colab Laboratories. Two-fold dilutions of serum were made in PSS so that each tube contained 0.5 ml. Five tenths ml of a 1:10 dilution of latex antigen was added to each tube, mixed, and incubated for 2 hr at 37 C, followed by refrigeration overnight. Tubes were centrifuged at 2500 rpm for 3 min and observed macroscopically for the presence of aggregates.

Agar Gel Immunodiffusion.

The presence of precipitating antibodies in sera was determined by double diffusion in agar gel. Purified agar, 0.85 g, was added to

99 ml of borate saline prepared by adding 5 ml of borate buffer, pH 8.4-8.5 (boric acid 6.18 g; sodium tetraborate, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 9.53 g; NaCl, 4.38 g; distilled water to 1.0 liter) to 94 ml of saline. One ml of 1% of merthiolate was added and the solution was heated to boiling. Two and one half ml were added to an ordinary microscope slide and allowed to harden. Holes spaced about 4 mm apart were cut with a capillary pipette.

Histoplasmin (H-42) concentrated ten fold was used as the antigen and the sera were undiluted. The diffusion was allowed to proceed in a humidity chamber for 14 days at 25 C before the final evaluation was made.

Complement Fixation.

Complement fixation titers were determined by the method of Lackman (21). Block antigen titrations were performed on the yeast phase antigen, lot no. 5 (31) obtained from the Center for Disease Control in Kansas City, Kansas to determine the optimal amount used in the test.

Preparation of Immunocytoadherence (ICA) Test Antigen.

Yeast cultures of H. capsulatum were grown in Brain Heart Infusion Broth as previously described and killed by treatment with a 1:10,000 dilution of Merthiolate (Lily) at 4 C for 48 hr. The cells were washed three times in distilled water and finally suspended in phosphate-buffered saline (PBS) pH 7.0 so that each ml contained 2.6×10^7 yeast cells.

Lymphocyte Isolation.

One volume of a 3% gelatin in saline mixture was added to two

parts heparinized blood to facilitate erythrocyte sedimentation, and the suspension was incubated in a vertical position for 45 min at 37 C (12). The leukocyte-rich supernatant was collected and placed in a glass column 120 mm long, containing nylon wool fibers (Dow Type 200) for 1 hr at 25 C. The wool was washed with 25 ml minimal essential medium (MEM) pH 7.2. MEM, 100 ml Eagle's base, was supplemented with 200 mM glutamine, 1.0 ml; 50X minimal essential amino acids, 2.0 ml; 100X nonessential amino acids, 1.0 ml; 100X vitamins; 1.0 ml and 100 mM sodium pyruvate, 1.0 ml all obtained from Kam Laboratories. The eluate from the column was centrifuged for 10 min at 100 g and the cell button was washed three times in MEM. Before the last washing the contaminating erythrocytes were eliminated by hypotonic shock produced as follows: the sediment of the second washing was quickly mixed with 6 ml of distilled water for 25 seconds and finally 2 ml of 3.5% saline were added (16). Perfect isotonicity was regained after the third washing. The lymphocyte suspension was adjusted to 2.6×10^6 lymphocytes/ml.

Leukocyte and differential counts were made on whole blood to determine the purity of the lymphocyte suspension. Trypan blue was used to determine the viability of the cell suspension.

ICA Test.

A modification of Biozzi's method was used (7); the following ingredients were added to a clean Kahn tube: 0.5 ml of the lymphocyte suspension (1.3×10^6 total cells) and 0.5 ml of the ICA antigen (1.3×10^6 yeast cells). The mixture was centrifuged for 10 min at 200 g and then resuspended by gentle agitation for 3 min. The

microscopic reading was performed in a hemacytometer and rosette counts were expressed as the number per cm^3 . Lymphocytes having four or more yeast cells clustered around them were designated as rosettes (Figure 2). Only single lymphocyte rosettes were counted, clumps of cells having yeast attached were disregarded. Rosettes (Figures 3 and 4) were observed with the phase microscope to depict cell types involved.

Passive Fixation on Lymphocytes.

One ml of known positive serum was incubated for 90 min at 25 C with 2.6×10^6 lymphocytes from normal control animals. The lymphocytes were washed twice with MEM and mixed with the ICA antigen as previously described. Samples were assayed for the presence of rosettes.

Rosette Inhibition Test.

Heavy-chain goat antisera specific for rabbit IgG, IgA, and IgM immunoglobulins were custom prepared by Cappell Laboratories. Peripheral lymphocytes from infected animals were washed twice in PBS at 4 C and adjusted to a concentration of 2.6×10^6 cells/ml. To a volume (0.05 ml) of cell suspension was added an equal volume of either anti- α , anti- γ , or anti- μ serum, diluted 1:10. Control tubes were treated with a similar dilution of normal goat serum. The cells were then incubated for 1 hr at 4 C. All sera were heat inactivated for 30 min at 56 C. Without further washing, 0.5 ml of the ICA antigen was added to 0.5 ml of the cell suspension, and the ICA test was performed. The number of rosettes was counted and the percentages of inhibition were determined.

Figure 2. A simple Schema for the ICA test.

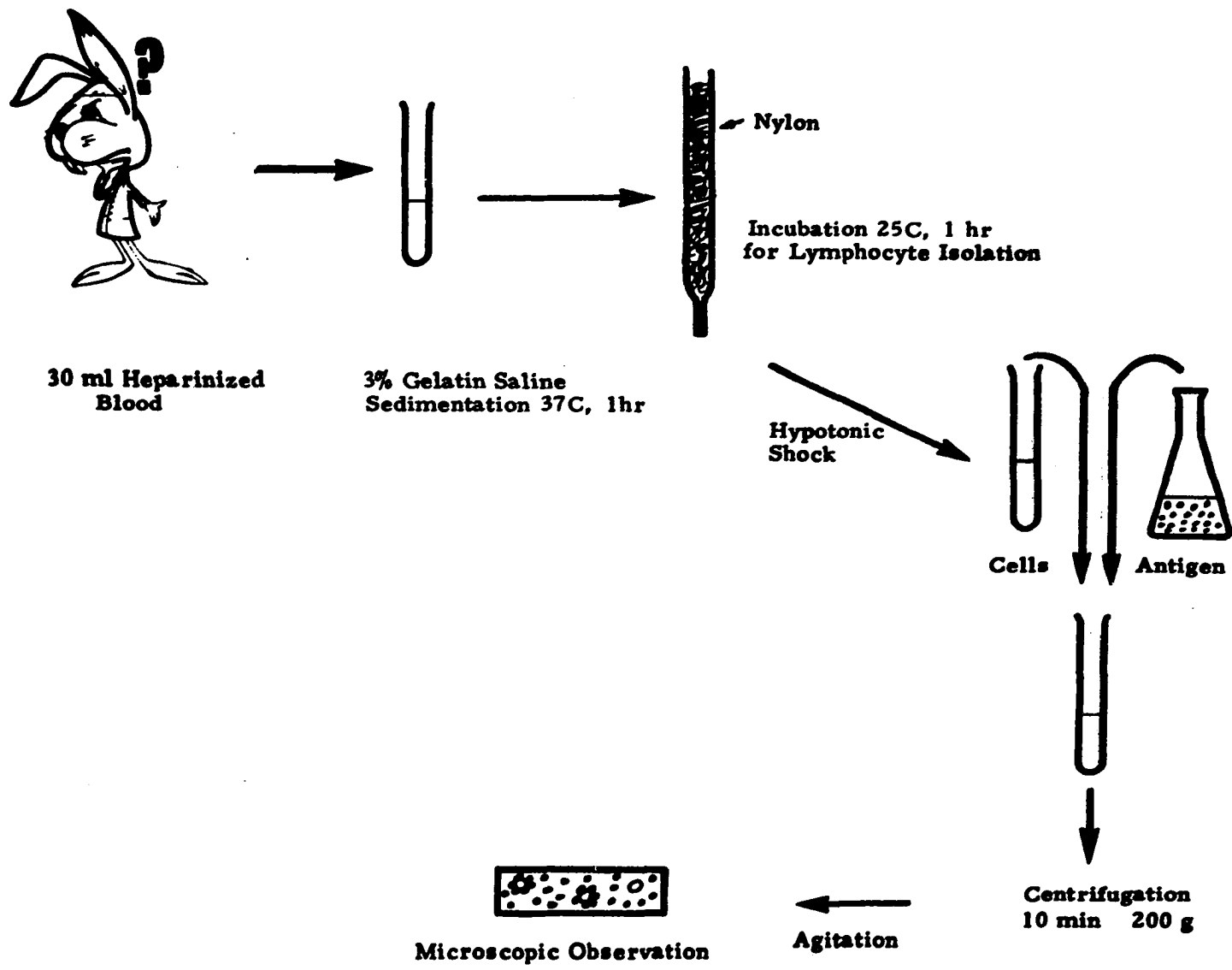
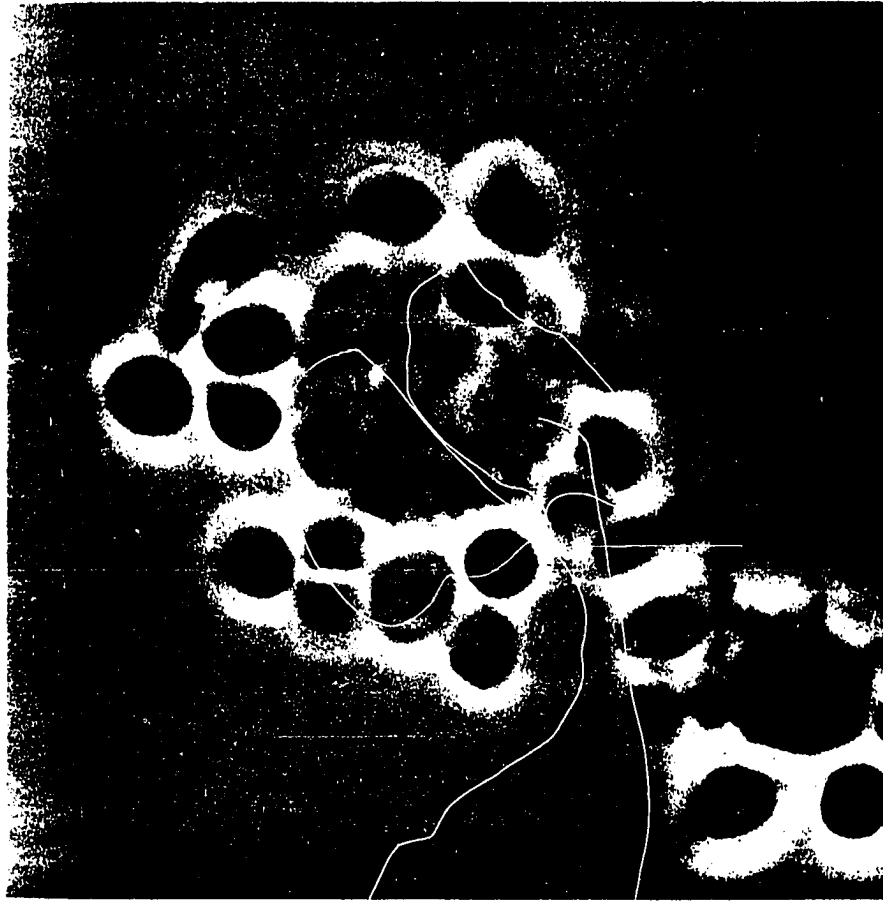


Figure 3. Typical rosette involving a small lymphocyte. A. Lymphocyte
B. Histoplasma yeast cells



Figure 4. Typical rosette involving a medium sized lymphocyte.

A. Lymphocyte B. Histoplasma yeast cells.



Detection of Immunoglobulins Produced by Peripheral Lymphocytes
as Demonstrated by Autoradiography.

Lymphocyte suspensions were prepared as previously described. The culture medium was prepared with 100 ml Hank's Balanced Salt Solution to which 2 ml of an amino acid stock solution (50X) (24), 0.5% (W/V) ovalbumin, glucose to a final concentration of 0.5% (W/V), 1% (V/V) MEM vitamin mixture (Kam Laboratories) and 75 µg/ml of Gentamicin (Schering Corporation) was added and the pH was adjusted to 7.4. The radioactive amino acids were then added: 1 microcurie (µc)/ml ^{14}C L Lysine (uniformly labeled 265 µc per millimole (mM) New England Nuclear) and 1 µc/ml ^{14}C L Isoleucine (uniformly labeled 273 µc/mM, New England Nuclear).

For each lymphocyte culture 1 ml of medium and 2 µc of the labeled amino acids were used. The cultures were gassed with CO_2 and incubated in 30 ml tissue culture flasks at 37 C with continuous rotation for 48 hr.

After incubation the culture fluid was centrifuged at 18,000 g for 10 min at 4 C. To remove the excess of radioactive amino acids the cell free supernatant solution was dialyzed against 0.015 M phosphate buffer pH 7.6 for 72 hr at 4 C. The culture fluid was concentrated by lyophilization and dissolved in 0.1 ml double-distilled water.

Immunoelectrophoresis.

Microscope slides were coated with a thin layer of colloidal adhesive (0.1 g purified agar, 0.05 ml glycerin, and 100 ml distilled water) and were allowed to dry for 15 min. The slides were then coated with 2.5 ml of electrophoretic medium containing 1.5 g purified agar,

75 ml distilled water, 25 ml High Resolution Buffer (Gelman, and 0.1 ml 1:100 solution of Merthiolate. Two circular wells and one rectangular trough were cut with an agar well cutter (National Instrument Laboratory, N. I. L.) and the circular plugs were removed by vacuum. Because the culture fluid often contains too little protein to provide a well-defined precipitin pattern, a carrier serum (normal calf) was added. The antigen well was first filled with normal rabbit serum and after it was absorbed into the agar the concentrated culture fluid was added three times.

The slides were placed in a N. I. L. Agafor Electrophoresis apparatus filled with Gelman High Resolution Buffer and the power supply was turned to 40 volts and allowed to continue for 45 min at room temperature. The center-trough agar plug was removed by vacuum and the trough was filled with histoplasmin H-42 as mentioned under immunodiffusion. The slides were placed in a humidity chamber and incubated at room temperature for 48 hr or until precipitin bands occurred.

The unprecipitated radioactive proteins on the slides were removed by washing PBS, for 72 hr with three changes. The slides were dried at room temperature for one day with a piece of filter paper placed over them.

Autoradiography.

After drying, the slides were sprayed with Omnispray (Isolab), warmed to 37 C and dipped into Kodak NTB3 Nuclear Track Emulsion (Eastman Kodak) which was heated to 40 C in a water bath. When the slides were withdrawn from the emulsion they were drained on a tissue

paper held against the lower edge of the group of the slides. The slides were allowed to dry for 30 min while standing on the moistened tissue paper. The coated slides were placed in plastic slide boxes which contained 15 or 25 g of indicating Drierite. The box was sealed with black tape and stored at -70 C on edge so that the slides were exposed in the horizontal position with the emulsion side up. Exposure times were determined by checking test slides at various intervals. They were processed with Dektol developer (Kodak) and acid fixed with distilled water rinses according to instructions provided by the manufacturer (20). The slides were examined for the presence of precipitin bands which indicated that radioactive globulin specific for a Histoplasma antigen was produced by the lymphocytes in culture.

Radial Immunodiffusion.

To determine the relative amounts of the immunoglobulin classes in the concentrated culture fluid, radial immunodiffusion was employed. The N. I. L. Saravis Radial Diffusion Quantitation Kit was used in conjunction with goat anti-rabbit-specific heavy chain sera prepared by Cappell Laboratories.

Seven-tenths ml of a 1:2 dilution of the antiserum was placed in the corner of a cellulose acetate strip (Millipore) and allowed to diffuse throughout its length. The saturated antiserum membrane was placed in the inner surface of the top template of the kit and a sheet of Parafilm was placed behind it. The base of the kit was positioned and bolted with a torque wrench. Excess antiserum was removed from the wells in the top template by suction.

Ten microliters of the concentrated culture fluid was placed

in each well with the Ziptrol applicator supplied in the N. I. L. kit. The template assembly was placed in a humidity chamber for 4 days at room temperature. The membrane was removed from the assembly and washed for 30 min through several changes of PSS. The washed membrane was placed in Ponceau-S dye for 15 min and then rinsed in several changes of 5% acetic acid. The stained membrane was placed between blotters and dried and the diameters of the resulting immunoprecipitin circles were measured by transmitted light and compared with a standard simultaneously tested in the same run. The standards were rabbit globulins with known amounts of antibody nitrogen; these were diluted and a standard curve established. The results of the experiment were graphed on semi-log paper and amounts of protein were determined accordingly.

Lymphocyte Transformation.

Lymphocyte suspensions were obtained from the peripheral blood of rabbits as described previously using 3% gelatin saline and nylon wool columns. The lymphocyte suspensions were washed in MEM containing 1% serum and re-suspended to give a concentration of 3×10^6 cells/1.6 ml. Of this suspension 1.6 ml was placed in 5 ml sterile screw cap tubes to which 0.025 ml of 10X concentrated histoplasmin H-42 was added. The screw caps were loosely replaced and the cultures were incubated at 37 C. After 24 hr $1\mu\text{Ci}$ of ^3H -methyl thymidine (16.1 curies/mM, New England Nuclear) was added. After 48 hr in culture, the lymphocytes were processed for determination of ^3H thymidine uptake (32). From the 1.6 ml suspension a button of cells was obtained by centrifugation and the pellet was washed once with PSS,

twice with 5% trichloroacetic acid, and once with methanol, using 1 ml volumes for each wash. Following the methanol wash and decantation of the methanol three drops of 1 N NaOH were added. The pellet was dissolved by heating at 56 C for 20 min. One ml of methanol was added and the mixture was transferred to a plastic counting vial. Ten ml of Aquasol (New England Nuclear) were added and radioactive counts were recorded by a Beckman scintillation counter.

CHAPTER III

RESULTS

Animal Infection.

Animals which received an intravenous injection of H. capsulatum began to exhibit symptoms of histoplasmosis on the eleventh day. They first appeared to have acute rhinitis with a nasal discharge and later during the second week developed conjunctivitis and dyspnea. Several animals became severely ill and expired during the second or third weeks. Most of the clinical symptoms disappeared during the third and fourth weeks. In one instance an animal showed a definite loss in weight after the fifth week and dyspnea became quite prominent. Blood was withdrawn during the ninth week and the animal later expired. Autopsy revealed chronic pulmonary histoplasmosis with cavitation. Left thoracic empyema and numerous lung lesions were observed; however, all other organs appeared normal. Cultures of the exudate produced colonies of H. capsulatum, and microscopic examination revealed numerous intracellular yeast.

Animals which received an intratracheal injection exhibited mild clinical symptoms during the progress of the infection. Slight dyspnea and coughing were observed during the second week; however, no animals appeared acutely ill at any time.

Serological Studies.

The animals were placed into two groups: one which was skin tested five weeks post injection, and the other which received no skin test. This was done to determine if a test following the preinfection one would have an effect on other serological results.

All initial skin tests were negative; 5 weeks later 70% of those animals receiving an intratracheal injection were reactive. Most of those animals had areas of induration 15 mm or greater in diameter. Fourteen percent of the animals which received an intravenous injection were reactive. These areas of induration were somewhat smaller; most were between 5 and 7 mm in diameter. All animals were tested during the tenth week to determine the number with positive skin reactions. Results are shown in Figure 5. Subsequent skin testing had no effect on any other serological results.

Agglutination titers are presented in Figure 6. The trend for both groups shows a rise in titer during the first week, followed by a maximum peak by the second week, and a gradual decline throughout the remaining weeks. The titers for those animals which were given intratracheal injections were markedly lower than those of the animals which received intravenous injections. Control groups presented no positive reactions to the agglutination test.

Agar Gel Immunodiffusion.

Agar gel immunodiffusion was used to detect the presence of precipitins in the sera of animals examined throughout the progression of the infection. The group which received intratracheal injections of H. capsulatum had a much smaller number of animals exhibiting

Figure 5. Correlation of skin hypersensitivity with lymphocyte transformation.

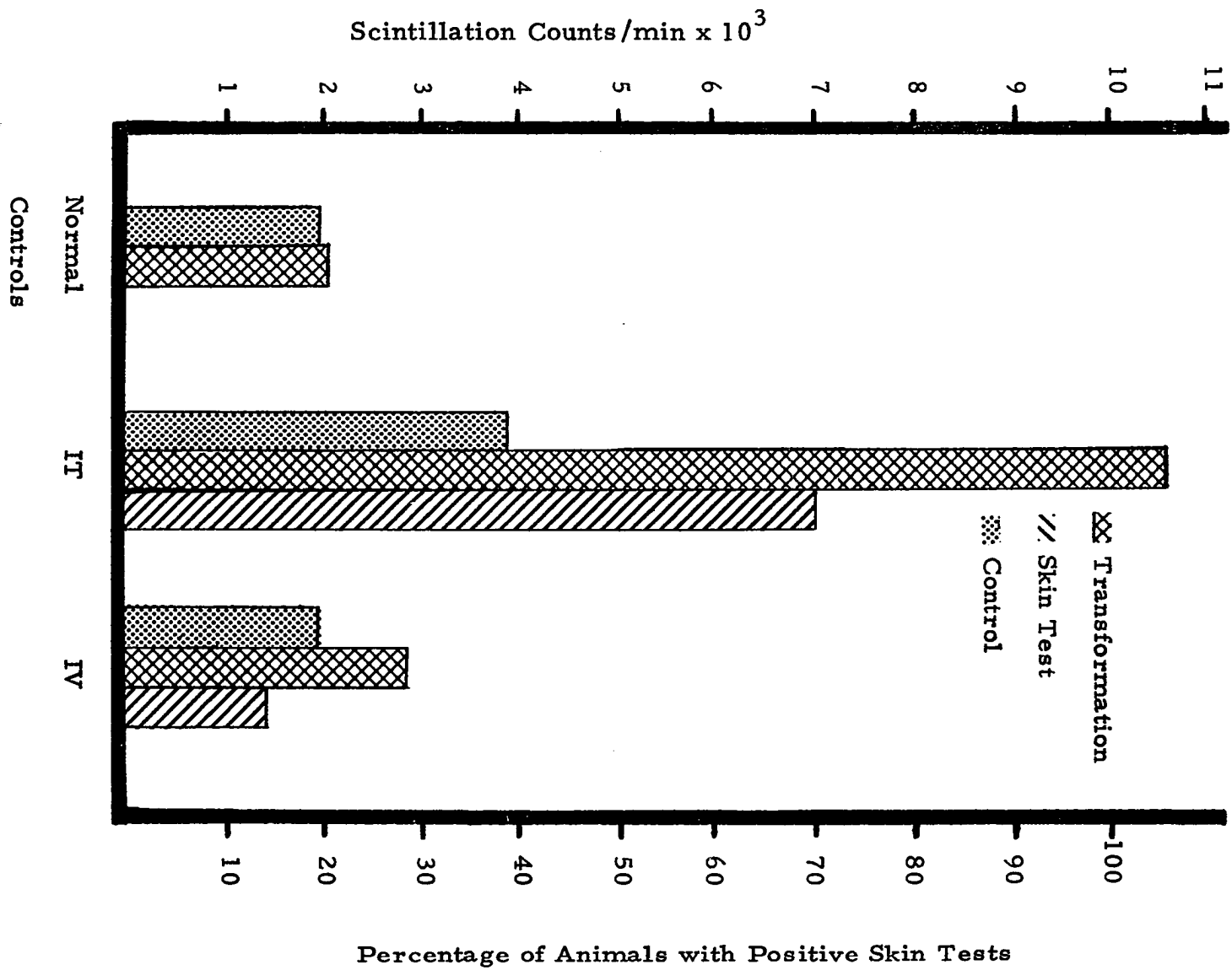
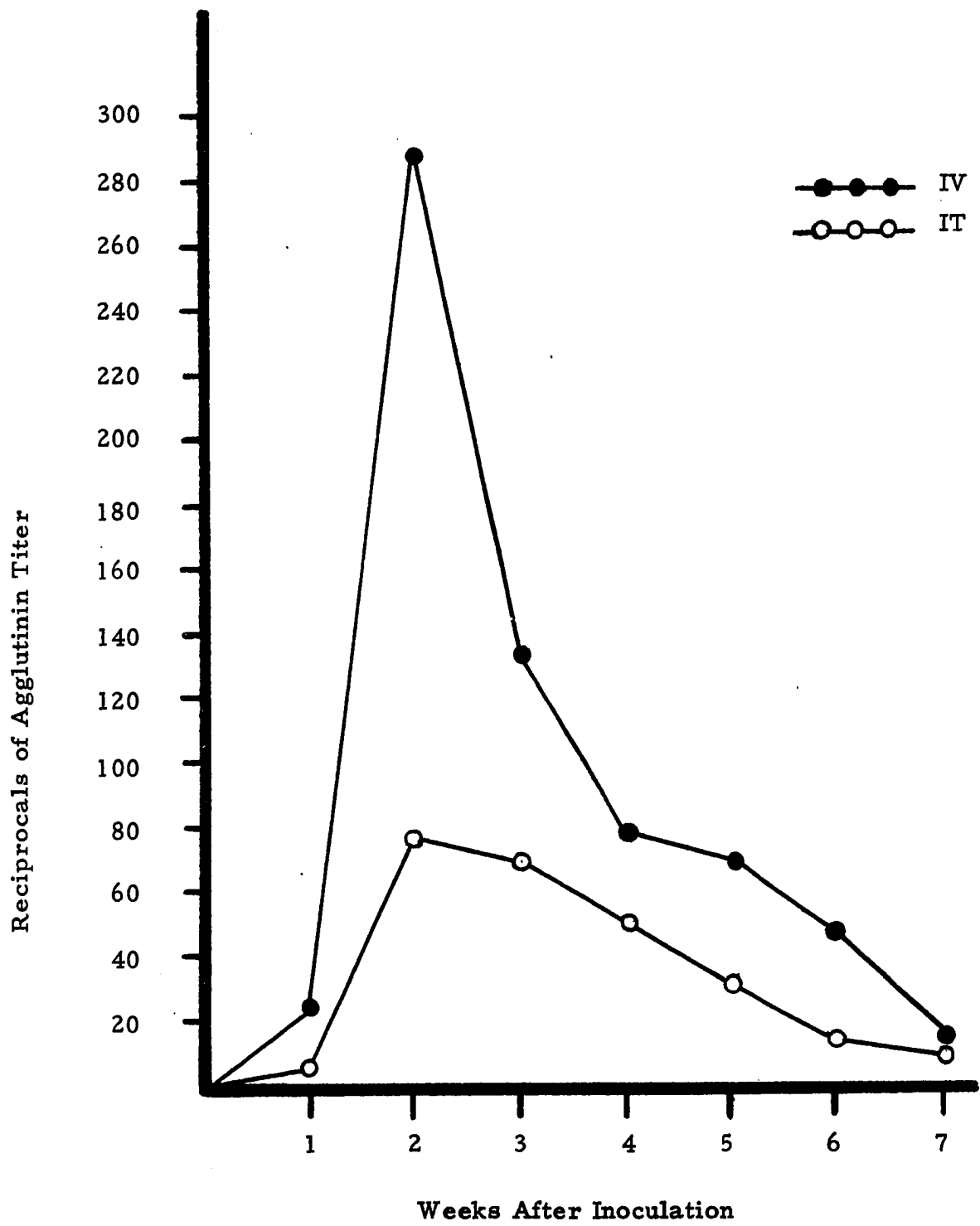


Figure 6. Results of Agglutination tests showing average titers.



precipitins than did the group which received intravenous injections. The percent of sera exhibiting precipitins is presented in Figure 7. Control animals showed no evidence of precipitin at any time during the study.

Complement Fixation.

The animals which received intravenous injections of H. capsulatum showed a rapid rise in titer with a maximum peak by the fourth week. Titers fell slightly by the seventh week but remained quite elevated.

Intratracheally injected animals exhibited a maximum titer by the third week and quickly declined to a low level by the seventh week. Complement fixation titers in this group were markedly lower, reached a maximum earlier, and declined more rapidly than the intravenously injected animals. Controls remained nonreactive throughout the study. Average titers are presented in Figure 8.

Lymphocyte Isolation.

The isolation procedure for relatively pure suspensions of peripheral lymphocytes was adequate for this study. The average number of cells obtained ranged from 1×10^6 to 1×10^7 cells per 25 ml blood. Trypan blue exclusion tests revealed a viability of 90-95%. Microscopic examination of the lymphocytes showed small, medium, and large lymphocytes; few neutrophils were seen.

Immunocytoadherence Test.

A rather striking relationship between the number of rosettes and the clinical picture was observed. It appears that as the symptoms of the infection became more severe, the number of rosettes increased.

Figure 7. Percentage of sera exhibiting precipitins.

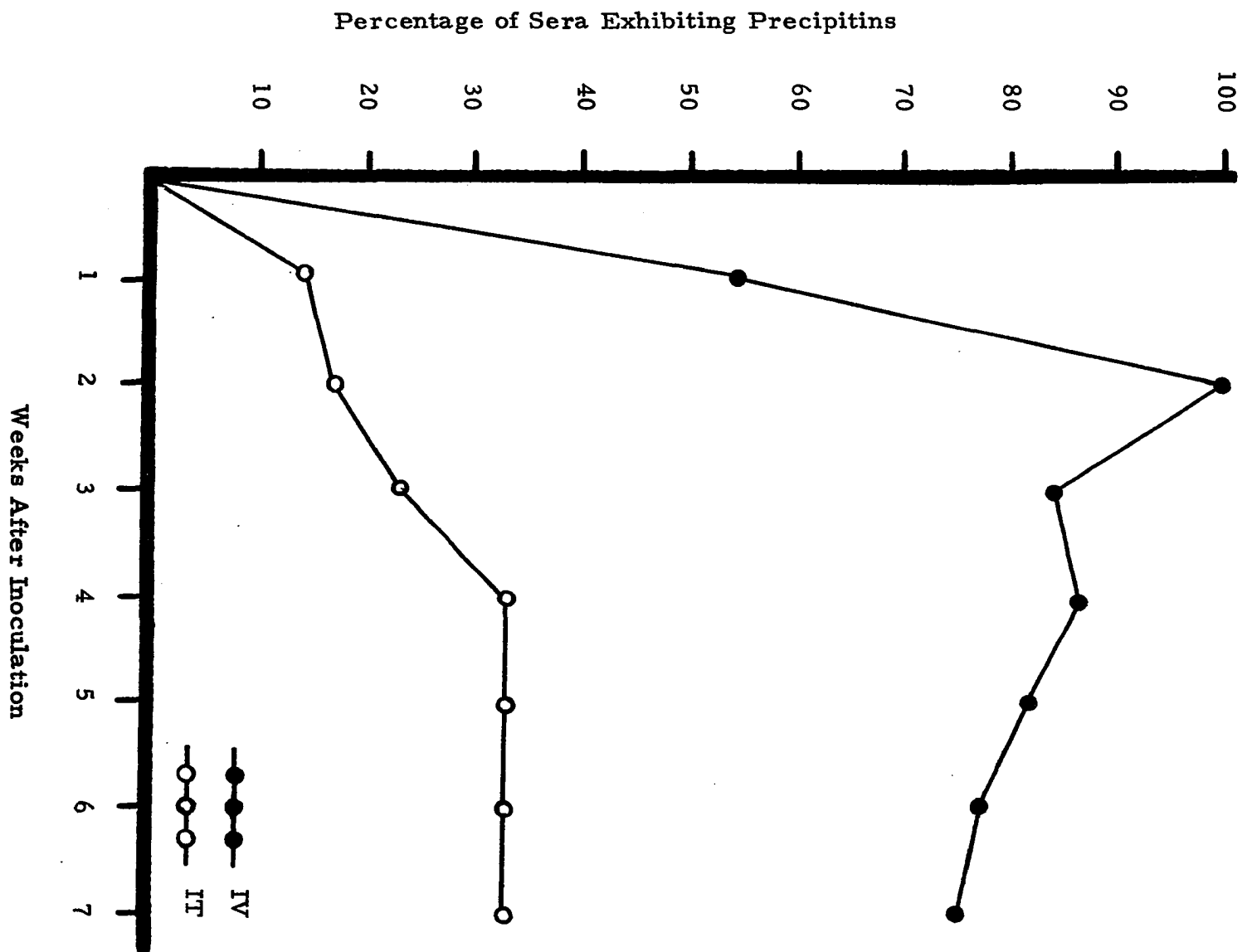
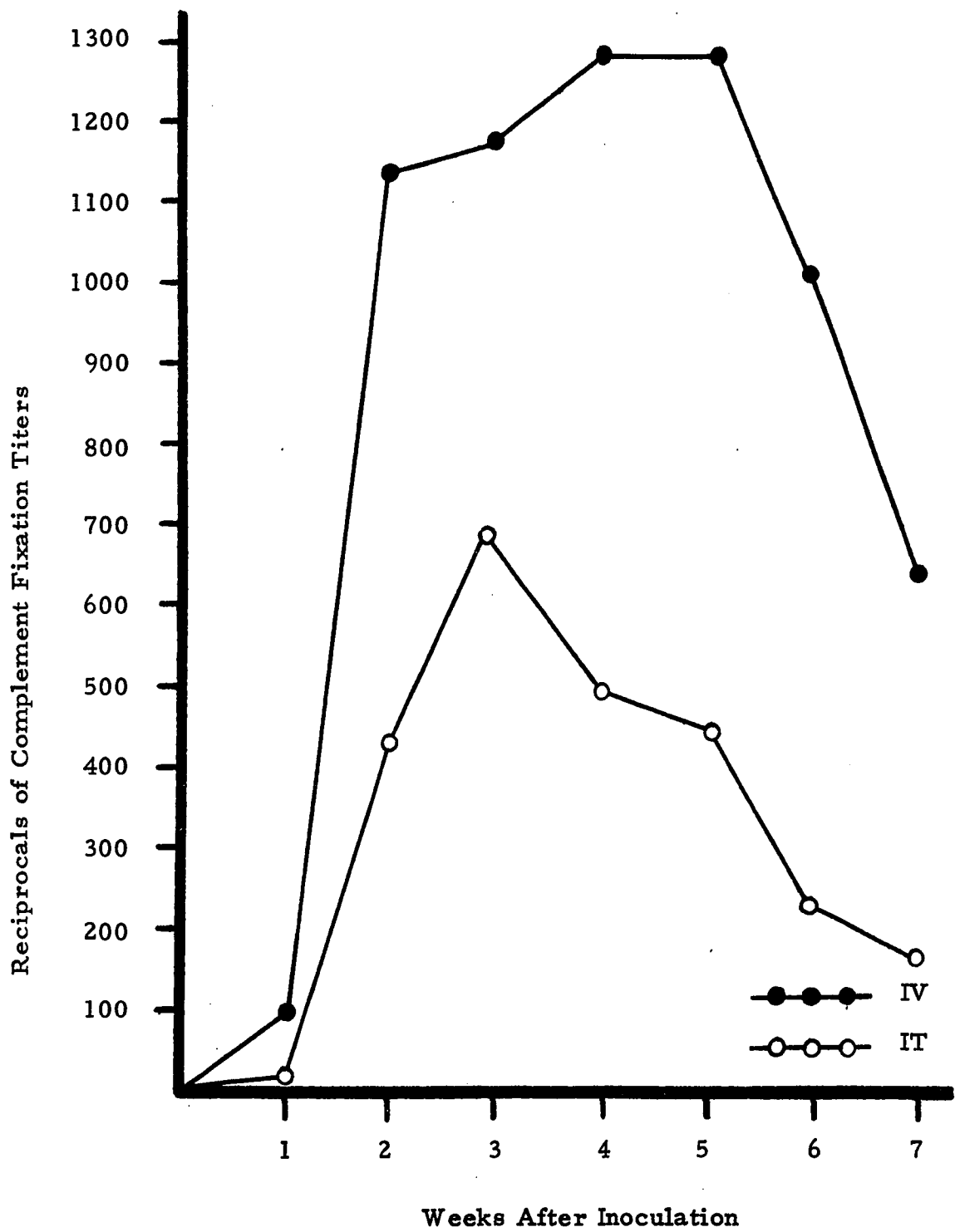


Figure 8. Results of complement fixation tests showing average titers.



In several instances animals became critically ill and rosette levels reached 8-15 per cubic centimeter (cm^3). Shortly after these levels were reached in the intravenously inoculated animals, death occurred. Animals in the IV group exhibited various degrees of symptoms and the number of rosettes varied with the severity of the infection. Tables 1-4 illustrate the correlation between the clinical picture and the number of rosettes.

The animals which received IT injections exhibited mild symptoms and low numbers of rosettes, which also showed a very good correlation with the clinical picture.

Those animals which received H. capsulatum yeast by the IV route showed a distinct trend. During the first week no rosettes were present; by the second week an average of 7.6 per cm^3 was reached, followed by a gradual decline which was almost beyond detection by the seventh week.

Animals receiving IT injections exhibited similar results except that there was a much lower number of rosettes with a maximum average of 3.1 per cm^3 which occurred during the fourth week. Figure 9 illustrates the average number of rosettes during the course of the infection for both IV and IT groups.

Rabbit number 13, which received an IV injection, became severely ill during the eighth week and exhibited a definite weight loss and dyspnea. The animal was bled and then later died. Twelve rosettes per cm^3 were noted. Autopsy revealed approximately 250 ml exudate, which contained many yeast cells. The lungs showed numerous lesions and cavitation. Again, the number of rosettes correlated directly with

TABLE 1

Correlation of ICA and Clinical Picture of IV Infected Animals

Group 1

Animal	Week 1		Week 3		Week 5		Week 7	
No.	ICA	CP	ICA	CP	ICA	CP	ICA	CP
56	0	as	12*	ss	3	ms	1	as
5	0	as	10	ss	-	d	-	-
2	0	as	9	ss	1	ms	0	as
1	0	as	8	ss	-	d	-	-
57	0	as	7	ms	2	as	1	as
6	0	as	5	ms	10	ss	-	d
58	0	as	5	ms	1	as	0	as
54	0	as	4	ms	6	ms	0	as
4	0	as	4	ms	0	as	0	as
49	0	as	3	ms	1	as	-	db
3	0	as	2	ms	4	ms	0	as
7	0	as	2	ms	0	as	0	as
53	0	as	1	ms	5	ss	-	db
	0	avg	5.5	avg	3.0	avg	0.25	avg
Controls	0.02	avg	0	avg	0	avg	0.02	avg

as = asymptomatic

d = death

ms = mild symptoms

db = death from bleeding

ss = severe symptoms

* = number of rosettes per
cubic millimeter

TABLE 2

Correlation of ICA and Clinical Picture of IV Infected Animals

Group 2

Animal No.	Week 2		Week 4		Week 6	
	ICA	CP	ICA	CP	ICA	CP
8	15*	ss	-n	d	-	-
60	15	ss	-	d	-	-
64	10	ss	3	ms	0	as
63	8	ss	5	ms	1	as
11	6	ss	5	ms	1	as
9	5	ms	4	ms	2	as
12	5	ms	3	ms	1	as
13	4	ms	3	ms	2	ms
61	0	ms	4	ms	1	as
62	-	d	-	-	-	-
55	-	d	-	-	-	-
	7.6	avg	3.9	avg	1.3	avg
Controls	0.01	avg	0	avg	0	avg

as = asymptomatic

* = number of rosettes per cubic
millimeter

ms = mild symptoms

d = death

ss = severe symptoms

TABLE 3
Correlation of ICA and Clinical Picture of IT Infected Animals
Group 1

Animal No.	Week 1		Week 3		Week 5		Week 7	
	ICA	CP	ICA	CP	ICA	CP	ICA	CP
85	0	as	8*	ms	2	as	1	as
29	0	as	4	as	1	as	0	as
83	0	as	3	ms	-	db	-	-
31	0	as	3	as	0	as	1	as
27	0	as	0	as	6	as	1	as
84	0	as	0	as	5	ms	1	as
32	0	as	0	as	4	as	1	as
79	0	as	2	ms	2	as	0	as
30	0	as	2	as	1	as	0	as
80	0	as	2	as	2	as	0	as
82	0	as	1	as	2	as	0	as
28	0	as	1	as	2	as	0	as
33	0	as	-	db	-	-	-	-
81	0	as	0	as	0	as	0	as
	0	avg	2	avg	2.2	avg	0.33	avg
Controls	0	avg	0.01	avg	0	avg	0.03	avg

as = asymptomatic

ms = mild symptoms

db = death from bleeding

* = number of rosettes per cubic millimeter

TABLE 4

Correlation of ICA and Clinical Picture of IT Infected Animals

Group 2

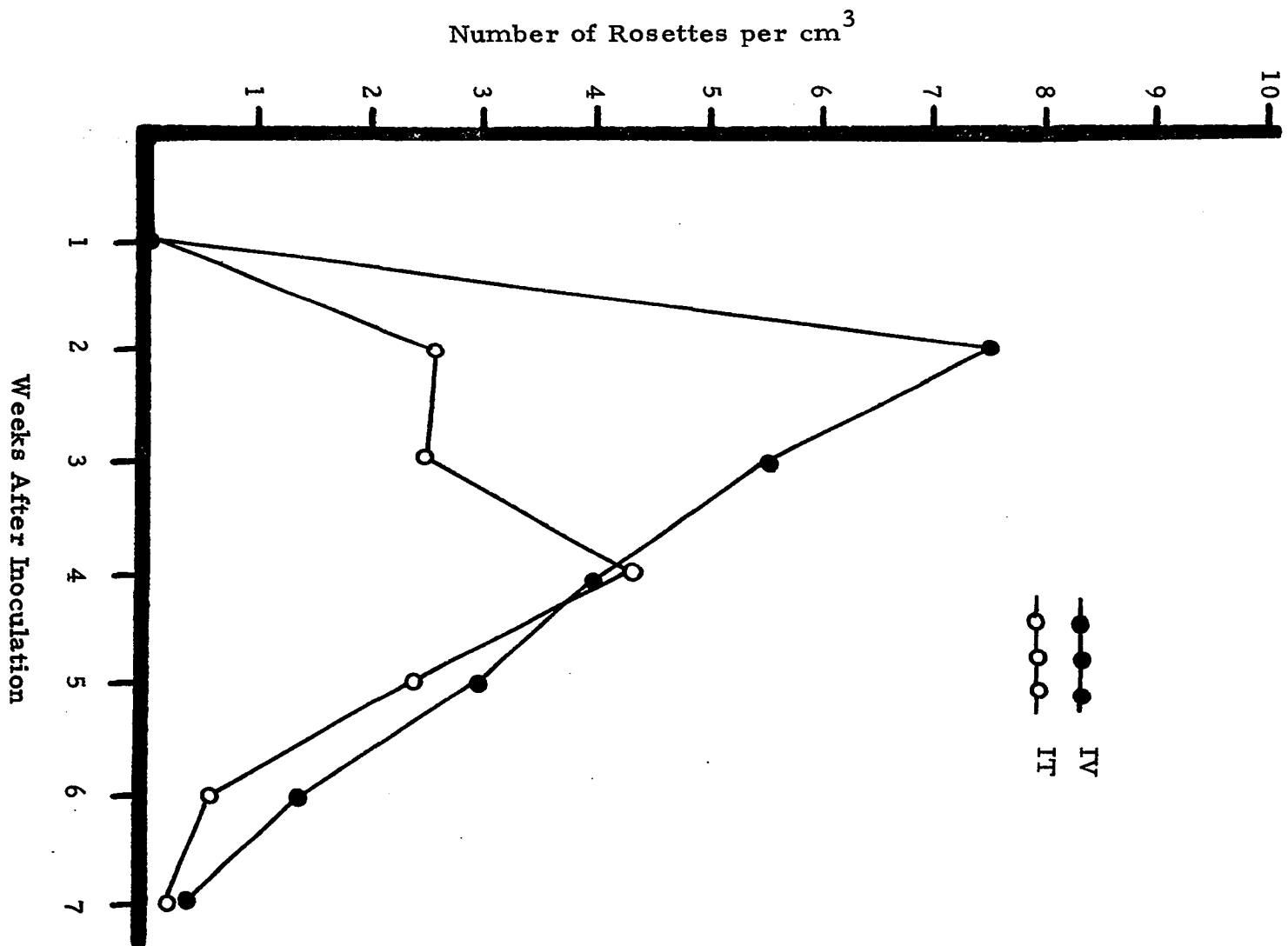
Animal No.	Week 2		Week 4		Week 6	
	ICA	CP	ICA	CP	ICA	CP
35	8*	ms	6	as	1	as
39	6	as	1	as	1	as
37	5	ms	4	as	0	as
38	5	as	2	as	0	as
36	2	as	6	ms	0	as
87	2	as	4	ms	0	as
86	1	as	4	as	2	as
34	1	as	2	as	0	as
88	0	as	4	as	2	as
89	0	as	2	ms	0	as
90	0	as	2	as	0	as
91	0	as	1	as	0	as
	2.5	avg	3.1	avg	0.5	avg
Controls	0.01	avg	0	avg	0.05	avg

as = asymptomatic

ms = mild symptoms

* = number of rosettes per cubic millimeter

Figure 9. Results of ICA test showing average numbers present.
Control values for weeks 1-7 are: .01, .01, 0, 0, 0,
.025, .025 respectively.



the clinical picture, in this instance chronic histoplasmosis.

Control animals showed an extremely low background level of rosettes with only an occasional one present. Animals having blastomycosis had an average of 5 per cm^3 during the height of infection; those injected with other strains of H. capsulatum exhibited a level comparable to the test group during the second week. Animals having listeriosis had no rosettes.

Both small and medium sized lymphocytes were involved in ICA, with an occasional large cell being seen. In many instances yeast attachment seemed to be polar, with binding occurring at one end. It appeared that those animals which were acutely ill had a larger number of yeast cells attached to the entire surface of small lymphocytes. The animal which exhibited chronic cavitary histoplasmosis showed only large lymphocytes involved in immunocytoadherence.

Passive Fixation on Lymphocytes.

In the experiment conducted to rule out the hypothesis of a passive sticking of antibodies on lymphocytes, the results showed that even after incubation with high titer anti-histoplasma sera, the lymphocytes from normal donors did not react in the immunocytoadherence assay.

Rosette Inhibition.

The reaction of peripheral lymphocytes in rosette formation appears to involve specific receptors situated on the cell membrane. To investigate the nature of these receptors and their relationship to immunoglobulins, attempts were made to block their reactivity for antigen by pretreatment of the cells with specific antisera.

The number of rosettes produced was not sufficient to give definitive evidence for the type receptor sites. The general trend showed that only slight inhibition of rosette formation was produced when cells were treated with anti- α sera. This was observed every week throughout the infection. Pretreatment with anti- μ and anti- γ showed a greater percentage inhibition during the first two weeks with anti- μ , and then later most inhibition was produced with treatment by anti- γ sera.

Autoradiographic Studies.

The concentrated supernatant fluid taken from lymphocyte cultures containing ^{14}C -labeled amino acids was allowed to react in agar gel with histoplasmin and also specific anti-globulins by using radio-immunodiffusion and radio-immunoelectrophoresis techniques.

No reaction was observed when histoplasmin was used as the antigen; however, only anti- γ serum reacted to form precipitin bands with samples from IV and IT injected animals, as demonstrated by both procedures. Only several samples from weeks one to three were reactive. When the slides containing precipitins were exposed to nuclear tracking gel and developed they showed that the bands were radioactive and were positioned the same as non-developed control slides.

One additional procedure was used to show that the concentrated culture fluid contained immunoglobulin specific for Histoplasma capsulatum. Indirect immunofluorescence was used and demonstrated that yeast cells treated with the fluid contained globulin on their surfaces. Rhodamine-conjugated anti-rabbit IgG was used as the indicator system

for this detection.

Radial Immunodiffusion.

Concentrated culture fluid samples were diluted and frozen after the autoradiography assays were completed. They were thawed for this study and it was noted that many of the samples contained coagulated material which could not be redissolved.

Radial immunodiffusion was performed with samples including those having insoluble material. Only a few gave results by this procedure; however, they showed the presence of IgM, IgA, and IgG immunoglobulins in samples from both IV and IT groups during the second week of infection. Zone sizes were measured, and it was noted that samples from the IV injected groups contained approximately twice the amount of immunoglobulin as did the IT groups.

Appropriate standards for the radial immunodiffusion quantitation were not available and the procedure was used semi-quantitatively to compare zone sizes and not concentrations of the immunoglobulins.

Since some of the samples contained insoluble material, no reaction occurred due to a lack of diffusion into the cellulose acetate membrane used in the assay.

Lymphocyte Transformation.

The skin reactivities and the degree of transformation in terms of counts per minute (cpm) of ^3H thymidine incorporated into the cells are shown in Figure 5. Those lymphocytes taken from the IT infected group exhibited a large degree of thymidine uptake, and the IV group showed only a small increase in cpm when compared to the controls.

It is also of interest to note that transformation was observed only in those samples taken from weeks five to seven.

CHAPTER IV

DISCUSSION

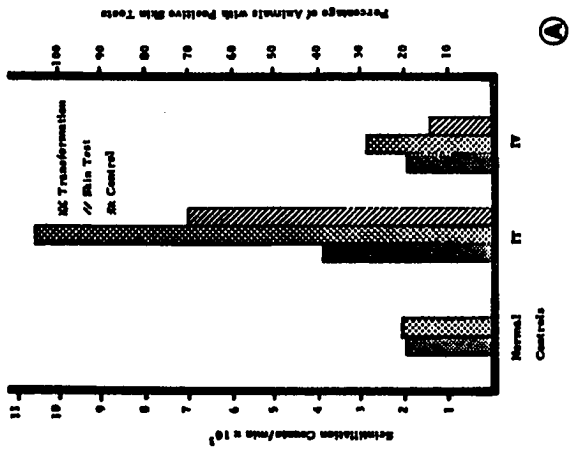
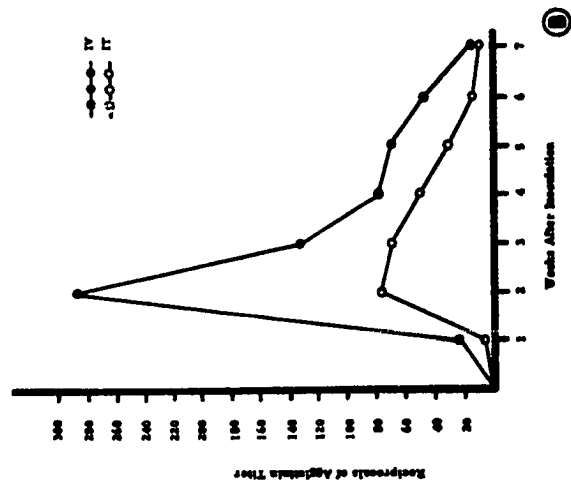
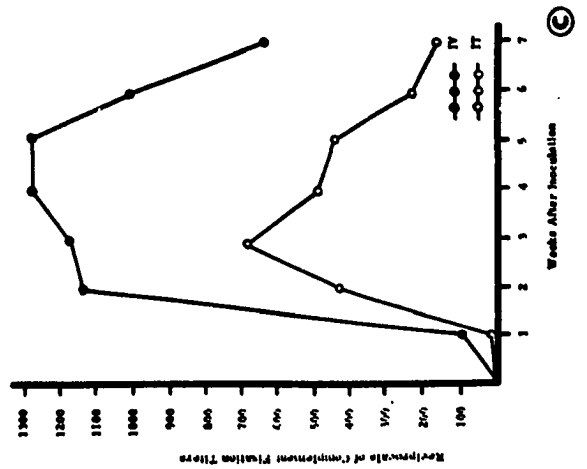
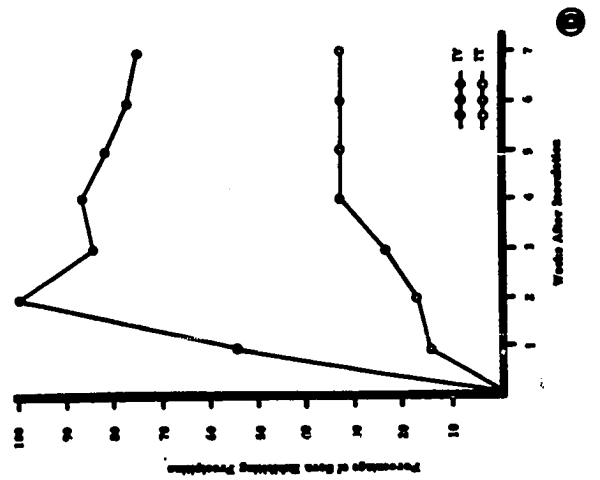
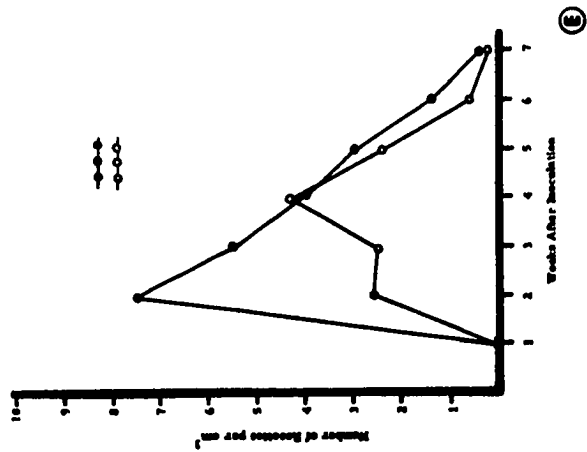
It appears that immunocytoadherence is a good method for detecting the immune response in animals having experimental histoplasmosis. One exceptional observation is the close correlation between the clinical picture and the relative number of rosettes present. The fact that these are found only when the disease is active suggests that ICA might be used as an index of active infection. This warrants evaluation in human cases of histoplasmosis, and if the results coincide with this study, immunocytoadherence may be a good immunologic tool for the diagnosis of histoplasmosis. The problem of specificity still exists since antigens from the yeast phase of B. dermatitidis give reactions similar to those of H. capsulatum; nevertheless, the test might indicate that a person has an active infection produced by one or both of these organisms.

Figure 10 shows a summary of all the serological tests and the relative number of rosettes present during the same time periods. It is obvious that rosettes reach a maximum at approximately the same time that the antibody titers peak. This is the case with both the IV and IT groups. Antibody levels are much higher in the IV groups, and the number of rosettes corresponds accordingly.

Intravenous injections of Histoplasma capsulatum stimulate

Figure 10. Summary of serologic results

- A. Correlation of skin test and lymphocyte transformation**
- B. Agglutination test**
- C. Complement fixation test**
- D. Percentage of sera exhibiting precipitins**
- E. ICA test**



antibodies to be produced by various organs, including the spleen, bone marrow, and lymph nodes; thus, a large number of antibody-producing cells are activated. In contrast, possibly the only sites for antibody production in pulmonary infections are the lungs and mediastinal lymph nodes; this might account for the smaller number of antibody-producing cells stimulated and for the lower level of antibody production seen in the group given IT injections.

The results suggest that rosettes are found in pulmonary and disseminated histoplasmosis. Although the infections established were of an acute nature the trend exhibited by the ICA test suggests that rosettes are present in acute and chronic cases. In one case of chronic infection, results show that immunocytoadherence may be used as a method for detecting the immune response in several types of histoplasmosis. Its sensitivity is indicated by the fact that rosettes are found only when active infection is present, in contrast to conventional methods which reveal antibodies even after convalescence has occurred.

The mechanism for immunocytoadherence has been investigated, and results lead to several conclusions. Experiments suggest that the receptors on peripheral lymphocytes possess a polypeptide structure in common with antibody molecules. From the preliminary studies on rosette inhibition it may be suggested that these receptors fall into three exclusive classes: those with IgA, IgM, and IgG receptors. However, the possibility that some cells possess more than one type of receptor was not investigated.

Passive transfer of high-titer anti-sera to the organism producing histoplasmosis never conferred immunocytoadherence properties

on normal lymphocytes. It is probable that the rosette-forming cell cannot be sensitized passively by antibodies.

The possibility that the lymphocytes involved in immunocytoadherence are mediators of delayed hypersensitivity was investigated. It was observed that the group of animals which had the greatest degree of delayed hypersensitivity, as exhibited by skin reactions and lymphocyte transformation, had the lowest number of rosettes. If lymphocytes from these animals were involved as mediators of delayed hypersensitivity, the antithesis of the results obtained would be expected. This suggests that ICA does not involve cells which are carriers of cellular immunity.

The hypothesis that the lymphocytes involved in immunocytoadherence are antibody-producing cells was investigated. The results show that cells maintained in culture produced ^{14}C -labeled antibody specific for *Histoplasma* yeast cells. The globulins were identified as IgA, IgG and IgM, which correlates well with the classes detected by the serologic tests employed earlier in this study. These immunoglobulins were produced at a maximum and at the same time that serum antibodies reached their highest levels. The number of rosettes observed in the IV group was much higher than the IT group; serum antibody levels appeared to be directly related. These results suggest that the most probable mechanism for immunocytoadherence is that antibody responsible for binding *Histoplasma* yeast cells to the surface of lymphocytes is being continuously released during incubation. In earlier studies it was shown that the number of rosettes formed with cells which have been incubated at 4 C is markedly lower than those

maintained at 25 C or 37 C. This suggests that antibody production is decreased or stopped at lower temperatures, further suggesting that antibody is produced by lymphocytes during incubation.

It is well known that 19S immunoglobulins are mainly produced at the beginning of histoplasmosis, while 7S antibodies dominate in the later phases (10). The 19S immunoglobulins have a shorter circulation life than 7S globulins (15). Therefore, a smaller number of antibody-producing cells will be required to sustain a high level of the long-lived antibody prevailing in the advanced phase of the disease. This might possibly explain why the number of rosettes decreases after the third and fourth weeks. However, after the fourth week only a few animals exhibited rosettes, and this may be due to the fact that the animals have recovered from the active infection, and antigen stimulation is no longer present. This demonstrates that the sensitivity of the ICA test is sufficient to make it useful as an immunologic tool for the detection of active histoplasmosis.

It is important to note that the results of the skin testing and lymphocyte transformation studies suggest that two types of immunity are produced in histoplasmosis. Of those animals which received IT injections, 70% exhibited skin hypersensitivity and a high degree of lymphocyte transformation. Clinical symptoms in these animals were negligible and humoral antibody titers were low. Of those animals which received IV injections, 14% exhibited skin hypersensitivity and a low level lymphocyte transformation; clinical symptoms were most severe and several animals died from the infection. These animals had very high humoral antibody titers. It might be speculated that cell-mediated

immunity plays an important role in protective immunity to histoplasmosis and that humoral antibodies play little or no role in protection. This is in agreement with the findings of Spencer (33) in a study designed to determine the role of cell-mediated immunity to protection from blastomycosis; it also agrees with other speculation concerning the role of delayed hypersensitivity in mycotic, viral, and bacterial infections (36).

CHAPTER V

SUMMARY AND CONCLUSIONS

Immunocytoadherence has proven to be a sensitive method for detecting an immunologic response in rabbits with experimental histoplasmosis. Results show that it may be used as an index of active infection since the number of rosettes correlates directly with the clinical symptoms. Immunocytoadherence warrants investigation in human cases of histoplasmosis because experimental studies indicate that it compares to current immunologic methods.

The mechanism of immunocytoadherence may be explained in that the lymphocytes obtained from peripheral blood are active in the synthesis of immunoglobulins specific for Histoplasma capsulatum. ICA detects those cells whose increase parallels that of humoral antibody levels. The number of cells involved in ICA drop to a negligible level as the disease process is arrested; however, humoral antibody titers remain elevated. This suggests that immunocytoadherence is a sensitive method for detecting active antibody synthesis in histoplasmosis.

BIBLIOGRAPHY

1. Bach, J. F., Dardenne, M. and Fournier, C. 1969. In vitro evaluation of immunosuppressive drugs. *Nature* 222: 998-999.
2. Bach, Jean-Francois, Delrieu, Francois and Delbarre, Florian. 1970. The rheumatoid rosette. *Amer. J. Med.* 49: 213-222.
3. Bach, J. F., Dormont, J., Dardenne, M. and Balner, H. 1969. In vitro rosette inhibition by antihuman antilymphocyte serum. *Transplantation* 8: 265-280.
4. Badoux-Perrudet, A. and Frei, P. C. 1969. On the mechanism of rosette formation in human and experimental thyroiditis. *Clin. Exp. Immunol.* 5: 117-127.
5. Bennett, D. E. 1967. Histoplasmosis of the oral cavity and larynx. *Arch. Int. Med.*: 417-427.
6. Biozzi, G., Binaghi, R. A., Stiffel, C. and Morton, D. 1969. Production of different classes of immunoglobulins by individual cells in the guinea pig. *Immunology* 16: 349-359.
7. Biozzi, G., Stiffel, C. and Morton, D. 1967. A study of antibody-containing cells in the course of immunization. pp. 103-109.
8. Biozzi, G., Stiffel, C., Morton, D., Bouthillier, Y. and Decreusefond, C. 1968. A kinetic study of antibody producing cells in the spleen of mice immunized intravenously with sheep erythrocytes. *Immunology* 14: 7-19.
9. Brain, P., Gordon, J. and Willetts, W. A. 1970. Rosette formation by peripheral lymphocytes. *Clin. Exp. Immunol.* 6: 681-688.
10. Chandler, J. W., Smith, T. K., Newberry, W. M., Chin, T. D. Y. and Kirkpatrick, C. H. 1969. Immunology of the mycoses: characterization of the immunoglobulin and antibody responses in histoplasmosis. *J. Inf. Dis.* 119: 247-254.
11. Coombs, R. R. A., McConnell, I., Munro, A. and Gurner, B. W. 1969. Studies on actively allergized cells. *Int. Arch. Allergy* 35: 209-227.

12. Coulson, A. S. and Chamber, D. G. 1964. Separation of viable lymphocytes from human blood. *Lancet*: 468-469.
13. Cunningham, A. J., Smith, J. B. and Mercer, E. H. 1966. Antibody formation by single cells from lymph nodes and efferent lymph of sheep. *Exp. Med.* 124: 701-715.
14. Duffus, W. P. H. and Allan, D. 1969. A study of the ontogeny of specific immune responsiveness amongst circulating leukocytes in the chicken. *Immunology* 16: 337-347.
15. Fahey, J. L. and Sell, S. 1965. The immunoglobulins of mice. V. The metabolic properties of five immunoglobulin classes. *J. Exp. Med.* 122: 41.
16. Fallon, H. J., Frei, H., Davidson, J. D., Trier, J. S. and Burk, D. 1962. Leukocyte preparations from human blood: evaluation of their morphologic and metabolic state. *J. Lab. Clin. Med.* 59: 779-791.
17. Furcolow, M. L. 1962. Opportunism in histoplasmosis. *Lab. Invest.* 11: 1134-1139.
18. Goodwin, R. A., Snell, J. D., Hubbard, W. W. and Terry, R. T. 1967. Relationships in combined pulmonary infections with Histoplasma capsulatum and Mycobacterium tuberculosis. *Amer. Rev. Resp. Dis.* 96: 990-997.
19. Kaufman, L. 1970. Serology: Its value in the diagnosis of coccidioidomycosis, cryptococcosis, and histoplasmosis. *Proceedings--International Symposium on Mycoses--Pan American Health Organization* 205: 96-100.
20. Kodak Data Release. 1971. Kodak materials for physics and autoradiography. Kodak Technical Pamphlet No. P-64.
21. Lackman, D. L. 1951. Rocky Mountain Laboratory Circular. No. 12.
22. Loosli, C. G. 1957. Histoplasmosis. *J. Chronic Dis.* 5: 473-485.
23. Moav, N. and Harris, T. N. 1970. Rosette formation in relation to active synthesis of antibody. *J. Immunol.* 105: 1501-1511.
24. Neuman, R. E. and McCoy, Th. A. 1958. Growth promoting properties of pyruvate, oxalate, and α -ketoglutarate for isolated Walker Carcinoma 256 cells. *Proc. Soc. Exp. Biol. Med.* 98: 303-306.

25. Nota, N. R., Liacopoulos-Briot, M., Stiffel, C. and Biozzi, G. 1964. 'L'immunocyto-adherence: une methode simple et quantitative pout l'e'tude "in vitro" des cellules productrices d'anticorps.' C. R. Acad. Sci. Paris. 259: 1277-1280.
26. Reddy, P. A., Brasher, C. A., Christianson, C. S. and Gorelick, D. F. 1970. Peritonitis due to histoplasmosis. Ann. Int. Med. 72: 79-81.
27. Reddy, P. A., Christianson, C. S., Sutaria, M. K. and Brasher, C. A. 1970. Oral lesions as presenting manifestation of disseminated histoplasmosis. Ann. Otology, Rhinology, and Laryngology 79: 389-397.
28. Reddy, P. A., Sutaria, M. K., Brasher, C. A. and Christianson, C. S. 1970. Disseminated histoplasmosis: cutaneous, visceral and prostatic histoplasmosis. South. Med. Journ. 63: 819-821.
29. Roberts, C. I., Brandriss, M. W. and Vaughan, J. H. 1971. Failure of immunocytoadherence to demonstrate delayed hypersensitivity. J. Immunol. 106: 1056-1064
30. Roberts, G. D. and Larsh, H. W. 1971. A study of the immunocytoadherence test in experimental sporotrichosis. J. Inf. Dis. In Press.
31. Schubert, J. H. and Ajello, L. 1957. Variation in complement fixation antigenicity of different yeast strains of Histoplasma capsulatum. J. Lab. Clin. Med. 50: 304-307.
32. Sell, S. and Gell, P. G. H. 1969. Studies on rabbit lymphocytes in vitro X. Immunology 16: 285-289.
33. Spencer, H. D. Personal communication. 1971.
34. Storb, U. and Weiser, R. 1967. Antibody-carrying cells in the immune response I. Identification of "rosette"-forming cells by light microscopy. J. Reticulo-Endothelial Soc. 4: 51.
35. Tosh, F. E. 1968. Histoplasmosis. Current Diagnosis 2. pp. 95-97. W. B. Saunders Company. Philadelphia.
36. Waksman, B. H. 1971. Delayed hypersensitivity: immunologic and clinical aspects. Immunobiology. pp. 28-35. Sinauer associates Inc., Stamford, Connecticut.
37. Weaver, D. K., Batsakis, J. G. and Nishiyama, R. H. 1968. Histoplasma endocarditis. Archives of Surgery 96: 158-162.

38. Zaalberg, O. B., Van Der Meul, V. A. and Van Twisk, M. J.
1968. Antibody Production by isolated spleen cells: a study
of the cluster and the plaque techniques. Immunology 100:
451-459.