LOMANITZ, Rachel, 1915–

CORRELATION OF CELLULAR COMPONENTS AND IMMUNE RESPONSE IN ANIMALS SENSITIZED TO CRYPTOCOCCUS NEOFORMANS.

The University of Oklahoma, Ph.D., 1964
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CORRELATION OF CELLULAR COMPONENTS AND IMMUNE RESPONSE IN ANIMALS SENSITIZED TO CRYPTOCOCCUS NEOFORMANS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

RACHEL LOMANITZ

Oklahoma City, Oklahoma

1964

CORRELATION OF CELLULAR COMPONENTS AND IMMUNE RESPONSE

IN ANIMALS SENSITIZED TO CRYPTOCOCCUS NEOFORMANS

APPROVED BY

George J. Prior

Edward L. Lasek

George C. Cozad

M R Shelton

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

I am deeply indebted to Dr. John M. Hale, Director of Laboratories, San Antonio State Tuberculosis Hospital, San Antonio, Texas, not only for the exceptional patience and forbearance with which he guided this research, but for his inspiration and encouragement, and even more so, for his kindness and understanding, which made working with him such a pleasure.

I also wish to express my heartfelt gratitude to Dr. George J. Friou, who so graciously accepted the difficult and thankless task of taking over the reins in mid-stream. His advice and suggestions with regard to the preparation of this manuscript have been most helpful.

My sincere thanks to the members of the Microbiology Faculty, both at the Medical Center and on the Norman Campus, who so willingly extended a helping hand.

This work was initiated with a grant-in-aid from the University of Oklahoma Chapter of the Society of Sigma Xi. It was supported in the main by an institutional research grant (G.R.S. 1-501-FR-05-111-01) from the National Institutes of Health.

TABLE OF CONTENTS

LIST OF TABLES

CORRELATION OF CELLULAR COMPONENTS AND IMMUNE RESPONSE IN ANIMALS SENSITIZED TO CRYPTOCOCCUS NEOFORMANS

CHAPTER I

INTRODUCTION AND HISTORY

The immunology of Cryptococcus neoformans (Torula histolytica, Cryptococcus hominis) has been, and still remains somewhat of an enigma. The organism, usually regarded as pathogenic, but probably more accurately termed opportunistic, was first isolated by Sanfelice, in 1894, from peach juice. Recognizing its yeast-like nature, Sanfelice called the organism Saccharomyces neoformans. At about the same time, Busse and Buschke described the first case of "Saccharomycosis hominis", reporting that they had recovered a yeast from a "sarcoma-like"lesion in the tibia of a woman. In 1896, Curtis cultured "Saccharomyces subcutaneous tumafaciens" from the hip of a patient. Five years later, Vuilleman, aware of the failure of the yeast in question to produce ascospores, removed it from the genus Saccharomyces, and renamed it Cryptococcus hominis. A yeast pathogenic for guinea pigs was isolated by Klein, from the "sediment of a sample of country milk", in 1901. During that same year, Weiss reported after careful investigation, that on the basis of morphological features, as well as numerous physiological characteristics, the two strains isolated by Sanfelice and Klein, from peaches and milk respectively,

were identical with those recovered by Sanfelice and Plimmer from human "cancers".

A case of "tuberculosis meningitis" was reported by von Hansenman (1905) in which gelatinous cysts containing yeast cells were found in the meninges. In 1915, Verse described correctly, ante mortem, the first case of human cryptococcosis with central nervous system involvement ("generalisierter blastomykose"). A short time later (1916) two cases of cryptococcosis were reported by Stoddard and Cutler, who, mistaking the capsular material for a product of histolysis, referred to the causitive agent as Torula histolytica.

According to Littman and Zimmerman (1956) and Littman (1958) Cryptococcus neoformans is unique among the pathogenic fungi in that it produces a capsule. This gelatinous envelope is composed primarily of two polysaccharides; one an iodine-staining amylose (1,4 glucosan) liberated into acid medium, and the other, a serologically active "pentosan", liberated into neutral medium. Acid hydrolyzed products of the latter were found to be: xylose, mannose, galactose and glucuronic acid. Chemical analysis indicated that the pentosan is negative for protein, amino acids, glycogen, glucosamine and starch.

By analogy with bacteria, it would be expected that C. neoformans should be highly antigenic. Surprisingly enough, however, early workers (Hoff, 1942; Kligman, 1947; and Stanley, 1949) failed to demonstrate either humoral antibodies or skin sensitivity. (Hoff did report an agglutinating titer of 1:180). These initial failures led to the impression that Cryptococcus neoformans is at best poorly antigenic, however,

in 1949, Evans reported the production of "high titer" antiserum (1:320) to three out of nine strains of C. neoformans. He also determined (1950) that the various strains of the organism can be divided into three serological types, and that specificity resides in the capsule. At about the same time, Salvin confirmed that there are qualitative differences in the immunogenecity and serological activities of Cryptococcus isolates. Neil and Kapros reported that closely spaced, large doses of antigen from weakly encapsulated strains of Cryptococcus are essential to the production of high titer antiserum. They also were able to demonstrate the presence of soluble antigen in the blood and tissues of mice by means of both precipitin tests and the Quellung reaction. Although the cryptococcus capsule is readily visualized in the presence of homologous antiserum, according to Evans and associates (1956) no capsular swelling occurs as in the typical Quellung reaction. Actually a slight shrinkage is detectable, as compared with capsule size in India ink smears. Evans suggested (1960) that visualization of the cryptococcus capsule may be due to the formation of a thin line of precipitation.

In their studies on the preparation of vaccines, Neil, Abrahams and Kapros (1950) found that those prepared from thinly encapsulated strains of Cryptococcus showed increased immunogenic capacity. Attacking the problem from a different angle, Neil, Sugg and McCauley, utilizing body fluids, from an infected patient, as antigen (rather than antibody) were able to demonstrate the presence of serologically active material in blood, urine and spinal fluid, by both precipitin and complement fixation reactions. The combined evidence of the above workers, indicates not only that some strains of Cryptococcus neoformans are

antigenic, but that the immunogenicity bears some type of inverse relationship to the amount of capsular material associated with the strain.

In 1959, Salvin suggested, on the basis of the information cited, that, contrary to earlier opinion, the capsular polysaccharide of Cryptococcus neoformans might well be producing such an enormous quantity of antigen that it actually floods the tissues, binding the antibody as rapidly as it is produced. The concept is supported by Gadebusch who reported, in 1958, that large doses of purified polysaccharide produced "immune paralysis" in mice, while small doses elicited minimal protection, which could be greatly enhanced by combining the polysaccharide with an ion exchange resin. Gadebusch demonstrated by tracer studies that the polysaccharide was eluted from the resin in minute quantities at a subcutaneous injection site.

Seelig, prompted no doubt, by the strong positive precipitin test he obtained when using a patient's spinal fluid as antigen (1960), also espoused the "immune paralysis" theory: "Les antigenes persistent longtemps dans le système réticulo-endothélial et se combinement immédiatement avec les anticorps qui sont formés continuellement". (The antigens persist for a prolonged period in the retuculo-endothelial system and combine immediately with antibody which is being produced continuously.) The "excess antigen" theory could of course explain the difficulty encountered in demonstrating antibody in the serum of both patients and experimental animals.

Recently methods have been devised for producing high-titered antiserum. Gadebusch reported, in 1960, that a highly specific enzyme, isolated from a soil microorganism, Alcaligenes spp., was capable of

depolymerizing the capsular material of one particular isolate of Cryptococcus neoformans, strain 3723. As the investigator himself suggests, it is highly probable that he is dealing not with one enzyme, but with several (precipitated with from 1 to 10% ammoniuum sulfate). In the hydrolysate of Fraction II several monosaccharides were identified: xylose, mannose, galactose and glucuronic acid.

According to Gadebusch, enzymatically decapsulated cells, injected intravenously produced antiserum with a considerable increased agglutinating titer (1:640 as compared with 1:160, when formalin-killed or Waring Blender-ground cells were used). Fluorescent studies showed that the cells were still antigenic, although no capsular material was discernible in India ink smears. Gadebusch suggests that the antigen resides in small bits of capsule tightly bound to the cell wall, (verification would undoubtedly require electron microscope studies) and that enzyme treatment unblocks potential antibody-inducing sites. He also points out that cells repeatedly subjected to enzyme treatment failed to fluoresce in the presence of labeled homologous antiserum, and were incapable of stimulating either protective or agglutinating antibodies.

In 1962, Kase and Metzger obtained a small-capsuled dry variant, ODH-DV, by treating the parent mucoid strain, ODH-127, with Wescodyne G. According to the investigators, the variant retained its stability in the face of repeated sub-culturing on artificial media, and even numerous animal passages. In the course of the present research, ODH-DV proved to be unaffected, as regards capsule production, by the shaking to which it was subjected in the preparation of antigen. Kase and Metzger reported that antiserum produced by the variant was quite high-titered (1:640 to

1:2560, as compared with 1:2 when the parent strain was used as the antigen).

Both of the methods just described indicate that cryptococcal cells are capable of stimulating the production of high-titer antiserum, provided that they are freed of the bulk of the normally associated capsular material. This can be interpreted as lending support to the "immune paralysis" theory, or it may simply mean that the antigen is located close to, or even in the cell wall, and the bulk of the capsular material must be removed before the antigen can become functional. In humans, encapsulated, but apparently non-viable, organisms have been found in the spinal fluid as long as a year following treatment with Amphotericin B, suggesting that the large capsule, so typical of the organism in vivo, is not readily metabolized. There is therefore evidence to support both of the theories advanced to account for the difficulty frequently encountered in demonstrating antibody in the serum of patients. It is also important to recognize that many cases of cryptococcosis, particularly in recent years, have occured in patients on drug therapy (e.g. steroids), or suffering from disease states frequently accompanied by severe depression of antibody production. However, this has not been true in all human infections. Finally, the possibility that present procedures are not sufficiently refined to detect antibodies, should not be dismissed.

The latter alternative undoubtedly stimulated the work leading to two recent papers. In 1962 a hemagglutination procedure was described by Pollock and Ward, in accordance with which they were able to demonstrate a titer of 1:320 in the serum of a patient. The same serum agglutinated to a maximum titer of 1:8 (depending on the variation of

the technique used). Precipitating antibodies were not detectable. A few months later, Abrahams, Gilleran and Weiss, resorting to an extremely sensitive technique, passive cutaneous anaphylaxis, found that seven out of ten guinea pigs reacted to as little as 0.04 micrograms of cryptococcal antibody.

While, in general, the immunology of Cryptococcus remains somewhat perplexing, the real paucity of information on the subject concerns the capacity of the organism to provoke a state of hypersensitivity. Cryptococcus neoformans has been isolated from the soil in various parts of the United States, Hawaii and Brazil (Emmons, 1951, 1954; Ajello, 1958; Silva, 1960; Hasenclever and Emmons, 1963; Muchmore and associates, 1963). It is frequently found in close association with pigeon excreta (Emmons, 1954, 1955, 1960; Kao and Schwartz, 1957; Littman and Schneirson, 1959; and others). The exact nature of this relationship is not known, however, since the pigeon is ubiquitous, it is clear that the excreta, thought to serve as an enrichment medium, is never wanting. It would therefore appear that exposure of humans to Cryptococcus neoformans is widespread. It follows that either the rate of susceptibility to infection is extremely low, or the number of sub-clinical cases, resulting in immunity, is quite high. If, as is suspected, hypersensitivity does develop on contact with the organism, a standardized, reproducible skin-test antigen would serve as an important epidemiological tool, and would in all probability reveal a goodly number of reactors.

Although the attempts of several of the earlier workers (Rappaport and Kaplan, 1926; Hoff, 1942; and Klingman, 1947) to demonstrate skin sensitivity met with failure, there are a few sporadic

reports in the literature of positive skin reactions to crude suspensions of yeast cells, in most cases made from the patient's own organisms.

In 1927, Berghausen prepared a boiled aqueous extract of "Torula" cultured from a patient, and described "a marked local reaction" when the material was injected subcutaneously. According to Carton, in 1930 Urbach and Zach reported "high-grade specific skin allergy" to intracutaneous injection of "blastomycin" (prepared from Cryptococcus neoformans). The same author states that in 1935, Bernhardt and associates also obtained a positive skin reaction to antigen prepared from Cryptococcus neoformans). Kessel and Holtzwart (1935) reported "swollen erythematous areas" on the forearm of a patient injected with his own (heat-killed) organisms, as well as with those cultured from two patients with CNS involvement. According to the authors, the latter antigens provoked a more marked reaction than did that from the patient's own organisms.

Three years later Dienst prepared a vaccine containing a billion organisms per ml. When 0.1 ml was injected into the patient's forearm, an erythematous area 2 cm. in diameter became visible within 24 hours and disappeared within 48 hours. Dienst also inoculated two rabbits intravenously, and two intratesticularly with a broth culture. He reported that when the animals were skin-tested thirty days later, erythematous areas 6 to 12 mm. in diameter developed within 48 hours and persisted for five to seven days. Four other rabbits, inoculated with living organisms, developed skin sensitivity, however their complement fixation titers remained negative. When the latter animals were skin-tested four months later, they reacted positively.

In 1952, a patient's own organisms, both capsulated and

HCl-decapsulated, were used by Carton as skin test antigens. Reactions to both were positive "as evidenced by appropriate wheals". No mention was made of the timing or appearance of the wheals. A patient suffering from cryptococcal osteomyelitis was skin-tested by Leopold (1953) who described a "wheal" 4.5 x 5 cm appearing at the end of 48 hours. This was thought to indicate "a marked degree of sensitivity, analagous to that found in tuberculosis, and was probably the explanation for rapid necrosis of the bone". Agglutinating antigen was not present in the patient's serum. Tuberculosis of the skin and bone are both known to produce a much greater degree of sensitivity than the pulmonary form of the disease. Arnason and Waksman attribute this to the route of entry of the organisms. Leopold's results suggest that the same situation exists in cryptococcosis.

The first systematic approach to the problem of developing a cryptococcal skin-test antigen was that of Salvin and Smith (1961). These investigators sensitized guinea pigs by means of two intraperitoneal injections of living cells. Three weeks to two months later, the animals reacted to skin-testing with antigen prepared by forcing live organisms twice through the tiny aperture of a pressure cell. According to the authors, the first passage removed most of the capsular material, as determined by electron microscopy, while the second passage caused "extensive disruption of cells". The sediment was described as appearing to consist largely of cell wall fragments. On the basis of the time required for appearance of the skin reaction, following intracutaneous injection of antigen, Salvin and Smith concluded that the reaction was of the delayed type. They reported further that antigen prepared from

ether-killed organisms, not pre-treated in the pressure cell, was higher in carbohydrate and lower in protein content, and failed to elicit a skin reaction.

In 1962 Pollock and Ward observed an immediate type skin reaction (which subsided in two hours) on injecting .001 mg of crude cryptococcal polysaccharide into the forearm of a patient.

The present study was undertaken in the hope of unfolding to some extent the mystery surrounding the antigenic composition and immunologic characteristics of Cryptococcus neoformans. More specifically, the objective was to determine whether experimental animals become sensitized to cryptococcal antigen, and if so, to establish what relationship, if any, exists between the development of skin sensitivity and the production of humoral antibodies - as detected by the hamagglutination technique.

CHAPTER II

MATERIALS AND METHODS

Source and Methods of Identification of Organisms

Source

A small-capsuled, Wescodyne-G produced variant, ODH-DV, was obtained through the courtesy of Col. Joseph F. Metzer, Armed Forces Institute of Pathology, Bethesda, Maryland. A large-capsuled isolate ("L") was cultured from the pulmonary lesion of a patient at the Veterans Administration Hospital in Oklahoma City. One strain, 45-466-59 ("Y") used for skin testing was supplied by the Communicable Disease Center, Chamblee, Georgia. The remainder, with the exception of one of unknown origin, were isolated from patients at various local hospitals.

Criteria for Identification of Cryptococcal Isolates

The genus Cryptococcus is characterized by capsule formation, light cream to tan pigmentation and rapid production of urease (Seeliger, 1956). These attributes could readily be demonstrated. All strains hydrolyzed urea, the majority within an hour, and the rest within 7 hours. The rate of urease production, as expressed by color conversion (to pink) of urea slants, is shown in Table 1.

Species identification was based on the following physiological behavior pattern: (a) growth at room temperature, as well as at 37C;

TABLE 1

PRODUCTION OF UREASE BY CRYPTOCOCCUS ISOLATES

Isolate	1 hr.	2 hrs.	5 hrs.	7 hrs.	24 hrs.
A	+	+	++	++	+++++
B	-	-	-	+	+++++
D	+	+	+	++	+++++
G	+	++	+++	+++	+++++
J	-	+	++	+++	+++++
L	-	+	+	++	+++++
N	+++	++++	+++++	+++++	+++++
Y	++	+++	+++	++++	+++++
UH_1	-	+	++	++	+++++
UH_2	+	++	+++	++++	+++++
UH_3	-	+	+	++	+++++
#2	+	++	++	+++	+++++
ODH-DV	-	+	+	++	++++

(b) ability to assimilate glucose and galactose but not lactose (Benham, 1955; Littman, 1958) (c) thiamine dependency (Rappaport and Kaplan, 1926; Littman, 1958) (d) inability to assimilate nitrates as the sole source of nitrogen (Littman and Zimmerman, 1956) and (e) virulence.

Materials for Assimilation Tests and Growth Requirements; Preparation of Media

Yeast carbon base, yeast nitrogen base, and vitamin-free yeast base were obtained from Difco Laboratories, Detroit, Michigan. These products were made up in 100 ml quantities, as directed by the manufacturer, at ten times the required concentration. Sugars (2.5 g), KNO_3 (0.78 g), or thiamine (1.0 mg) were added to 100 ml of the appropriate medium. The solutions were sterilized by Seitz filtration, diluted 1:10 with 1.7% autoclaved agar, and poured into plates. The nitrate assimilation, and thiamine requirement experiments were of such degree of sensitivity that it was necessary to use washed agar. Regular agar-agar was placed in a large Erlenmeyer flask and diluted to volume with distilled water. The water was changed every 48 to 72 hours for a period of ten days. Subsequently, most of the water was decanted; the balance was filtered through gauze, and the agar was washed well with distilled water; diluted to volume and autoclaved. Organisms were streaked lightly on the plates and checked for growth at 48 hours. Because of the sensitivity of the thiamine-dependency and nitrate-assimilation experiments, it was necessary to grow the organisms for five to six days to deplete them of stored material, and then transfer them.

Carbohydrate assimilation. All cultures were found to conform

to the pattern characteristic of the species neoformans.

Thiamine dependency. The isolates grew very poorly in the absence of thiamine (or under conditions as close to total absence as could be provided in the laboratory). Addition of a small amount of thiamine to the medium resulted in luxuriant growth.

Failure to assimilate nitrates. As is characteristic of the species neoformans, the isolates failed, with one exception, to assimilate nitrates as a sole source of nitrogen. In the latter case, assimilation was rather weak.

Virulence. Most of the strains were isolated from patients and are therefore known to be virulent for humans. Two isolates, "Y" and ODH-DV, have been proved virulent for mice. Isolate #2 is of unknown origin, and when checked in the laboratory, failed to demonstrate virulence for mice. All isolates grew equally well at room temperature and at 37C.

Storage of Stock Cultures and Cultivation Techniques

Cryptococcus cultures were stored at refrigerator temperature on mycophil slants. Mycophil agar, obtained from Baltimore Biological Laboratory, Inc., is particularly suited to growth and isolation of fungi. It consists of phytone (a plant peptone) dextrose and agar, and is high in thiamine content.

Preliminary Procedures

Selection of Strain

Since it was intended to decapsulate cells by agitation in a

sonic oscillator, it was desirable to select a well encapsulated strain which would lend itself readily to detection of capsules in India ink smears. These requirements were best fulfilled by "L".

Establishment of Optimal Growth Conditions

The criteria used for this purpose were degree of turbidity and percentage of encapsulated cells. The most favorable environment was found to be a temperature of 25 to 26C with agitation on a shaking machine.

Determination of Time Required to Heat-kill Organisms

Organisms were suspended in saline and heated in a water bath at 56C. At five minute intervals a small quantity was removed; one loopfull was streaked on mycophil agar; another was placed in a drop of .05M methylene blue and examined under high power for the presence of unstained cells. Under these conditions, living cells reduce methylene blue to the colorless form; dead ones are incapable of doing so, and remain blue.

Counting Methods

Counting of the organisms in antigen preparations, and of the white cells in peritoneal exudates, was done under high power in a Helber counting chamber.

Preparation of Antigens and Antigen Fractions

Encapsulated Cells

Organisms were transferred from mycophil slants to Erlenmeyer flasks containing mycophil broth and placed on a Burrell wrist-action shaker. They were allowed to vibrate at approximately 220 oscillations

per minute, for 24 to 48 hours, at 25 to 26C, to induce heavy growth and maximal encapsulation. A second series of flasks was inoculated generously (10 ml inoculum to 60 ml of broth) from the original flasks and permitted to remain on the shaker for 48 hours. By that time the cultures were extremely turbid due to the high concentration of yeast cells. The material was centrifuged for ten minutes at 1500 x g, and the supernatant was removed and discarded. (Decanting was possible when the small-capsuled variant was used, however, in the case of the mucilaginous, large-capsuled isolate, pipetting was necessary). The residue was then washed three times (0.05 M tris-hydroxymethylamino-methane -- buffer, pH 7.6, or sterile physiological saline were used interchangeably) and centrifuged following each washing. After the last washing, the cells were suspended in physiological saline and heat-killed by immersing in a water bath at 56C for 15 minutes.

Partially Decapsulated Cells

The saline suspension of heat-killed cells was introduced into a 10 KC sonic oscillator and agitated for three minutes (at which time some capsular material was still discernible in India ink smears). The suspension was then removed and centrifuged at 1500 x g, this time for five minutes, the supernatant was decanted; the residue was washed and centrifuged three times and taken up in sterile physiological saline. It was diluted to the desired concentration as needed.

Decapsulated Cells (Somatic Antigen)

The procedure was the same as for the preparation of partially decapsulated cells, except that sonic oscillation was continued for an

additional two to nine minutes (depending on the isolate used) until capsular material could no longer be detected with India ink. Following centrifugation the supernatant was decanted and set aside. The residue was washed and centrifuged five times to free the cells of any loose capsular material. It was then taken up in sterile physiological saline.

Capsular Antigen

The supernatant that had been set aside was shown with Bial's reagent, to contain a pentosan -- according to Littman (1958) the serologically active portion of the capsular polysaccharide. The pentosan content of the supernatant was standardized by the colorimetric method of Dische and Borenfreund (1957).

Antigens for Specificity Studies

Commercially prepared blastomycin, coccidioidin, histoplasmin, trichophytin and tuberculin were procured. Candida albicans antigen was prepared in the laboratory in the same manner as cryptococcal encapsulated cell antigen.

Crude Cell-Wall Material and Cell-Wall Fractions

Decapsulated cells of a Cryptococcus strain, UH_3, isolated from a patient, were subjected to agitation in the sonic oscillator for a period of an hour and forty-five minutes, at which time a count made under high power revealed approximately 95% breakage. (The number of whole cells per ml observed, was roughly five percent of the total originally introduced into the sonic oscillator). The suspension of cell-wall material (CW) was centrifuged in the cold for 20 minutes at 28,000 x g.

The supernatant, cell-free extract (CFX) was decanted. A portion of the residue, composed largely of cell-wall material, as ascertained by microscopic examination, was degraded by a procedure roughly in accordance with that used by Kessler and Nickerson (1959) in their work on Candida. No attempt was made to lyophilize the material prior to lipid extraction. Two fractions, probably glyco-protein complexes, according to what Kessler and Nickerson reported for Candida, were prepared from the cell-wall material; one consisted of the residue remaining after extraction with 1N KOH; the other was an ammonium sulfate precipitate. The latter was prepared by saturating the alkaline (KOH) supernatant and washings with ammonium sulfate. The flask containing the mixture was placed in the refrigerator over night to allow further precipitation. Subsequently the contents were dialized in the cold room for three days against circulating distilled water. The water was changed twice during the first 24 hours, and daily thereafter. Finally the material was centrifuged and redialized in the cold for 24 hours.

Polysaccharide Antigen for Hemagglutination Tests

Cultures of the large-capsuled variant, "L" were prepared as described under "Encapsulated cells". Following centrifugation for ten minutes, at 1500 x g, the supernatant was removed and the residue (live organisms) discarded. The polysaccharide antigen was precipitated from the supernatant by addition of two and a half to three volumes of absolute alcohol. To assure a maximum yield, the mixture was allowed to remain in the refrigerator for 24 hours (Pollock and Ward). Subsequently it was filtered through semi-crimped rapid filter paper; the residue was

washed three times with alcohol, once with a 1:1 mixture of alcohol and anhydrous ether, and finally dried with ether. The antigen, used for treating (sensitizing) the red blood cells, was made up when needed, as a 1% solution in M/30 phosphate buffered saline, pH 7.2. (Two vials of Parstain's phosphate buffer salts -- obtained from Hartman-Leddon Co., Philadelphia, Pennsylvania -- were dissolved in 400 ml distilled water containing 1.4 g of NaCl).

Immunization Procedures

Experimental Animals

Adult, white albino rabbits and white albino guinea pigs were used throughout the work, with the exception of the normal donors used for passive transfer experiments in guinea pigs. The latter were dark colored.

Sensitization of Rabbits

Unless otherwise specified, rabbits were sensitized by intravenous injections. Inocula consisted of 3×10^9 heat-killed organisms suspended in 0.5 ml of sterile physiological saline.

Sensitization of Guinea Pigs

Guinea pigs were injected intraperitoneally. Inocula consisted of 1.5×10^8 heat-killed organisms suspended in 0.1 ml sterile physiological saline.

Preparation of Peritoneal Exudates

Eight to ten days following the last dose of sensitizing antigen, all donor animals were injected intraperitoneally with sterile mineral oil. (Rabbits received 60 ml and guinea pigs 30 ml). Three to four days later the animals were exsanguinated. The peritoneal exudates were withdrawn by suction; the cavities and viscera washed several times with Ringer's balanced salt solution and the washings added to the peritoneal exudates. The exudates were then transferred to separatory funnels and allowed to stand for five to ten minutes -- until the oil emulsion layered on top. The exudates were drawn off from the bottom and centrifuged at 1500 x g for four to five minutes. The supernatant was decanted; the cells were taken up in a small volume of sterile physiological saline and counted.

Procedure for Passive Transfer With Peritoneal Exudates

Chase (1945) reported the first successful transfer of delayed hypersensitivity. He injected freshly harvested peritoneal exudate cells into normal guinea pigs intraperitoneally, and showed that after a latent period of 24 to 36 hours, skin sensitivity could be elicited with the proper antigen. In 1955 Metaxas and Metaxas-Buehler demonstrated that if suitable amounts of sensitized cells were mixed directly with an adequate dose of tuberculin and injected intracutaneously into normal recipients, reactions were observed with regularity, and they developed at "very much the same rate as skin responses of comparable severity in cell donors". Because of the simplicity and greater sensitivity of this method, a modification, described by George and Vaughn (1962) was adapted to

the present program. The maximum number of white cells obtainable (which varied from donor to donor) was always used.

Procedure for Passive Transfer With Serum

Normal animals were inoculated intracutaneously with 0.1 ml of each serum to be tested. Twenty-four hours later antigen was injected into the same sites.

Serologic and Skin-Test Procedures

Hemagglutination

The technique was a modification, as outlined by Pollock and Ward, of the method designed by Neter and associates for demonstrating antibodies to Enterobacteriaciae.

Human type O red cells were washed three times in phosphate buffered saline, prepared as described. Ten ml of a 1% solution of the antigen were added to 1 ml of packed red cells and the mixture was incubated in a water bath at 37C for 30 minutes. The treated red cells were then washed three times with phosphate buffered saline, and finally diluted to a 2.5% suspension by volume with the same solution. The serum was inactivated by heating in a water bath at 56C for 30 minutes.

Phosphate buffered saline (0.2 ml) was pipetted into each of a series of serological test-tubes. An equal volume of serum, diluted 1:5, was added to the first tube and appropriate serial dilutions were made. To each tube was added 0.2 ml of the red cell suspension. A control tube was included containing only red cells and buffered saline. As a further control, the entire procedure was duplicated, using untreated red cells, in order to detect non-specific hemagglutination. The number

of tubes showing hemagglutination with untreated red cells was subtracted from the number agglutinated with treated red cells. The difference was considered to represent the actual number of tubes of specific hemagglutination.

Slide Agglutination

A drop of the cell suspension was placed on a clean slide. A drop of the serum to be checked was added and the slide was agitated gently. Agglutination, if present, could usually be detected with the unaided eye. However, all results were confirmed microscopically. Two types of antigen (decapsulated and partially decapsulated cells) and a saline control were usually run on the same slide.

Skin-Testing

Skin-test material was injected intracutaneously, into marked areas on the flank of the animal. In the passive transfer experiments part of the testing was done on the abdomen. The animals were examined immediately following inoculation, and again one to two hours later for signs of immediate reaction.

Indurations (longest diameter) were measured at 24 and 48 hours after injection of antigen, and on some occasions at 16 and 40 hours as well.

Isolation of Candida spp. From Rabbit Feces

Fecal specimens were collected and placed in test tubes containing 10 ml of mycophil broth. The tubes were set aside for five to six hours to allow the bacteria to initiate growth. Penicillin, 0.2 ml

containing a total of 50,000 units, was added to each tube and the rack was placed on the shaker. It was agitated for 24 hours at 25 to 26C to stimulate growth of yeast. The cultures were then streaked lightly on mycophil agar. Occasionally heavy streaking on mycosel agar was of value when difficulty was encountered in isolating yeasts from mycophil plates. Representatives of all yeast colonies were streaked on corn meal-tween 80 agar and identified as belonging to the genus, Candida, on the basis of pseudomycelial information.

CHAPTER III

RESULTS

Preliminary

In the initial pilot study three rabbits were used. Two of the animals were given capsular polysaccharide (1 mg. and .01 mg.[1] of capsular material per injection respectively) while the third received decapsulated organisms -- 3×10^9 in each inoculum. After two series of injections on alternate days, each followed by a ten-day rest period, the rabbits were skin-tested with:

(a) 0.1 ml of 1:10 capsular polysaccharide

(b) 0.1 ml of somatic material containing 3.7×10^9 decapsulated organisms per ml

Neither rabbit reacted in any manner to the polysaccharide material. However, the animal immunized with somatic material manifested exquisite skin sensitivity (induration 21 mm, with central necrosis) to decapsulated cells. The induration reached maximum size and intensity within 48 hours, suggesting a delayed type of reaction.

---

[1]The rabbit receiving the smaller inoculum of capsular polysaccharide died (cause unknown) during the injection period.

Specific Nature of Skin Sensitivity to Cryptococcus Neoformans

Response to Decapsulated Cells of Various Strains

The sensitized rabbit reacted characteristically, when skin-tested with decapsulated cells of all twelve isolates of Cryptococcus neoformans. The reactions were erythematous and conspicuously elevated. Indurations measured at 24 hours varied from 9 to 15 mm.

Specificity of the Reactions in Rabbits

The same sensitized animal that reacted to the various strains of Cryptococcus neoformans, failed to respond to skin-testing with blastomycin, coccidioidin, histoplasmin, trichophytin or tuberculin. It did react to Candida albicans antigen. However, Candida spp. was isolated from its feces in large numbers, suggesting that the animal might be sensitized to its own normal flora. Subsequent experiments have confirmed this.

Six rabbits, not previously sensitized, were skin-tested with both Candida albicans and cryptococcal antigens. All of the animals were negative to cryptococcal antigen, but all responded in some measure (indurations varying from + to +++) to C. albicans antigen. Candida spp. was repeatedly isolated from the feces of five of these rabbits and an unidentified yeast from the sixth.

At a later date eleven rabbits were skin tested with both candida and cryptococcal antigens, prior to any known contact with Cryptococcus. Ten of the animals were positive to candida antigen; all were negative to cryptococcal antigen. (See Table 2). Rhodotorula spp.

TABLE 2

RESPONSE OF ELEVEN RABBITS TO SKIN TESTING WITH *CANDIDA ALBICANS* AND *CRYPTOCOCCUS NEOFORMANS* ANTIGEN. INDURATIONS MEASURED AT 48 HOURS.

Rabbit No.	*Candida albicans*	*Cryptococcus neoformans*
1	4 mm	-
2	6 mm	-
3	12 mm	-
4	9 mm	-
5	9 mm nodule	-
6	7 mm nodule	-
7	-[a]	-
8	small nodule	-
9	8 mm nodule	-
10	10 mm	-
11	8 mm	-

[a]Inoculum probably injected sub-cutaneously, resulting in false negative response.

was isolated from the feces of ten of the rabbits; the eleventh (No. 3) died (cause unknown) before a fecal specimen could be obtained. The rabbits were skin-tested approximately two weeks later with the previously used antigens plus Rhodotorula spp. isolated from rabbit No. 5. The results, recorded in Table 3, coupled with those in Table 2, (all animals were originally negative to Cryptococcus) clearly indicate that the skin response to Candida albicans is not due to cross reaction with either Cryptococcus neoformans or Rhodotorula spp. Following the second skin-testing, Candida spp. were isolated from the feces of all ten of the rabbits. An encapsulated yeast, probably Cryptococcus spp., but not neoformans (it failed to grow at 37C) was also isolated from No. 6.

Specificity of the Reaction in Guinea Pigs

Three sensitized guinea pigs were skin-tested with the same antigens used for specificity tests in rabbits. The concentration of the candida antigen was 5.14×10^9 organisms per ml., while that of the cryptococcal antigen was 1.5×10^9 organisms per ml. Two of the animals showed small indurations to candida antigen at 32 hours. These reactions had already diminished at 48 hours, while those to Cryptococcus had increased slightly. The weak responses were therefore regarded as being non-specific. The results, as summarized in Table 4, lend confirmation to the fact that skin sensitivity to cryptococcal antigen is highly specific.

Determination of Effective Skin-Test Dose for Rabbits

Four rabbits, two sensitized to encapsulated cells, and two to

TABLE 3

MAXIMUM RESPONSE OF RABBITS TO SECOND SKIN TEST WITH *CANDIDA ALBICANS* AND *CRYPTOCOCCUS NEOFORMANS* ANTIGENS, AND TO *RHODOTORULA* SPP. ISOLATED FROM RABBIT NO. 5. MOST INDURATIONS WERE MAXIMAL AT 40 HOURS. ALL SALINE CONTROLS WERE NEGATIVE.

Rabbit No.	Time Hours	Antigens - 3 x 10^9 orgs./ml		
		Rhodotorula	*C. albicans*	*C. neoformans*
1	24 hours	-	6 mm	-
2	40 hours	-	9.5 mm	4.5 mm
3	D I E D			
4	48 hours	5.5 mm	12 mm	4 mm
5	24 hours	5 mm	12 mm[a]	4 mm
6	40 hours	5 mm	15.5 mm[c]	4 mm
7	40 hours	-	13 mm	-
8	16 hours	-	7 mm	-
9	40 hours	4 mm[b]	10 mm	4.5 mm
10	24 hours	5 mm	12.5 mm	4.5 mm
11	40 hours	3 mm	13.5 mm	5 mm

[a]Induration at 16 hours

[b]Induration at 24 hours

[c]This is larger than either prior or subsequent indurations, and since at first some difficulty was experienced in controlling the disposable syringes, the possibility is suggested that the inoculum may have been slightly larger than 0.1 ml.

TABLE 4

RESULTS OF SPECIFICITY TEST IN GUINEA PIGS. NO ANIMALS REACTED TO BLASTOMYCIN, COCCIDIOIDIN, HISTOPLASMIN, TRICHOPHYTIN AND TUBERCULIN.

Guinea Pig No.		Induration in mm		
		Candida albicans	*Cryptococcus neoformans*	Saline
1	32 hours	-	9	-
	48 hours	-	10.5	-
2	32 hours	4.5	10.5	-
	48 hours	3.5	10.5	-
3	32 hours	5	9.0	-
	48 hours	4	10.0	-

NOTE: Concentration of *C. albicans* antigen = 5.14×10^9 orgs./ml
Concentration of *Cryptococcus* antigen = 1.5×10^9 orgs./ml

decapsulated cells, plus one normal animal, were skin-tested with three different concentrations of antigen. The sensitized animals were tested only with the specific material they had received previously, however, the control was tested with both encapsulated and decapsulated organisms. The dose originally selected, 3×10^9 organisms per ml, proved most satisfactory. The higher concentration of antigen produced a slight reaction in the control, while the lower one failed to produce a specific reaction in three of the four sensitized animals. See Table 5.

Sensitization of Rabbits to Various Types of Cryptococcal Antigens

Encapsulated and Decapsulated Organisms of a Small-Capsuled Variant

Two rabbits were sensitized to encapsulated, and two to decapsulated cells prepared from a small-capsuled variant, ODH-DV. Two rabbits were also inoculated with capsular polysaccharide. Each animal received a series of three injections followed by a ten-day rest period. Subsequently the rabbits were skin-tested with the same material they had been receiving. The animals sensitized with encapsulated and decapsulated cells, respectively, showed characteristic reactions, while those that had received polysaccharide failed to respond. Twenty-four hour (maximal) indurations are listed in Table 6.

Encapsulated Cells of a Large-Capsuled Variant

Encapsulated cells (3×10^9 organisms per ml) were prepared from strain "L", a large-capsuled variant isolated from a patient. Each

TABLE 5

RESULTS OF SKIN-TESTING WITH VARIOUS DOSAGES OF ANTIGEN

Rabbits	Indurations with various concentrations of antigen			
	2.1×10^{10} orgs./ml	3×10^{9} orgs./ml	4.4×10^{8} orgs./ml	Saline control
A_1				
24 hours	leaked out	7 mm	-	-
48 hours	leaked out	8 mm	-	-
A_2				
24 hours	28 mm	13 mm	7 mm	-
48 hours	26 mm	10 mm	5 mm	-
B_1				
24 hours	5 mm	-	-	-
48 hours	-	-	-	-
B_2				
24 hours	11 mm	7 mm	?	-
48 hours	8 mm	-	?	-
Control (non-sensitized)				
Decapsulated cells				
24 hours	?	-	-	-
48 hours	-	-	-	-
Encapsulated cells				
24 hours	-	-	-	-
48 hours	-	-	-	-

? = less than 5 mm; suspected of being non-specific reaction.
A - sensitized and skin-tested with encapsulated cells.
B - sensitized and skin-tested with decapsulated cells.
Control - skin-tested with both encapsulated and decapsulated cells.

TABLE 6

MAXIMAL SKIN RESPONSE TO SPECIFIC SENSITIZING MATERIAL
(TIME 24 HOURS; INOCULUM 3 x 10^8 ORGS.)

Rabbits	Induration (mm)
A_1	11
A_2	12
B_1	7
B_2	11
C_1	-
C_2	-

A sensitized with encapsulated cells

B sensitized with decapsulated cells

C inoculated with polysaccharide material

of two rabbits was given a series of three intracutaneous injections (0.1 ml) on alternate days. Following a three day rest period, the animals were skin-tested with the following antigens prepared from the sensitizing strain:

Encapsulated cells	Decapsulated cells
Heated encapsulated cells (80C - 5 min.)	Autoclaved encapsulated cells (115 lbs. - 5 min.)

They reacted not only to both encapsulated and decapsulated cells, but to encapsulated cells which had been heated or even autoclaved (See Table 7).

Passive Transfer Experiments

Transfer of Hypersensitivity with Peritoneal Exudates in Rabbits

The four animals previously sensitized to decapsulated and encapsulated cells of ODH-DV, plus two non-sensitized controls were used as donors. White cell suspensions were prepared from peritoneal exudates as described, mixed directly with antigen, the latter in three concentrations, and injected the same day, intracutaneously, into two normal recipients. Controls consisted of leucocyte suspensions (from non-sensitized donors) combined with the various concentrations of antigen, white cell suspensions (from all donors) without antigen, and antigen alone. The results are summarized (Table 8) and indurations compared with those obtained by skin-testing the actively sensitized donor animals. It was observed that the more sensitive the donor, and the higher the concentration of white cells, within the limits used, the larger the induration in the recipient.

TABLE 7

RESPONSE TO SKIN TESTING WITH SENSITIZING STRAIN;
TIME 24 HOURS; INOCULUM 3 x 10^8 ORGANISMS

Rabbit No.	Encapsulated Cells	Decapsulated Cells	Heated Encapsulated Cells (80C - 5 min.)	Autoclaved Encapsulated Cells (115 lbs. - 5 min.)
1	7 mm	7 mm	11 mm	6 mm
2	14 mm	9 mm	15 mm	11 mm

All saline controls were negative.

TABLE 8

PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY WITH PERITONEAL EXUDATES. COMPARISON OF INDURATIONS EXHIBITED BY ACTIVELY AND PASSIVELY SENSITIZED RABBITS.

Donor Rabbits	[a]No. Leucocytes Injected	Induration			
		Passively Sensitized Recipients			Actively Sensitized Donors
		Antigens			
		4.5×10^9 orgs./ml	3×10^9 orgs./ml	1.5×10^9 orgs./ml	3×10^9 orgs./ml
A_1	1.75×10^6	5 mm	5 mm	5 mm	7 mm
A_2	10^7	11 mm	9 mm	8 mm	10 mm
B_1	10^6	4 mm	-	-	-
B_2	10^6	8 mm	7 mm	4 mm	7 mm
C_1	$.06 \times 10^6$	-	-	-	-
C_2	$.5 \times 10^7$	-	-	-	-

A Sensitized with encapsulated cells
B Sensitized with decapsulated cells
C Previously received polysaccharide material.

[a]Leucocyte yields were variable. In each case the maximum obtainable was used.

All controls (white cells without antigen and antigen alone, in all three concentrations) gave negative results.

One rabbit exhibited some degree of sensitivity to the leucocyte-antigen mixtures from each of the four sensitized donor animals. However, in no case did it react to any of the controls. The second recipient failed to react to any inoculum.

Transfer of Hypersensitivity with Peritoneal Exudates in Guinea Pigs

The donors consisted of two sensitized and two normal animals. White cell-antigen suspensions were prepared as in the experiment with rabbits. In addition to somatic antigen (decapsulated cells) in two concentrations, crude cell-wall material, previously found to be antigenic, was used in dilutions of 1:10 and 1:100.

One of the three recipients reacted in some measure to all inocula, controls included, and was therefore regarded as hypersensitive. The other two guinea pigs reacted to sensitized leucocyte suspensions mixed with both concentrations of somatic antigen, as well as both dilutions of crude cell-wall material. The results are summarized in Table 9.

Since the results were considered somewhat equivocal, the experiment was repeated, using only the decapsulated cells as antigen. Again, one of the three guinea pigs reacted to leucocyte-antigen suspensions, not only from the sensitized donors, but to those from the normal donors. In the latter case, however, the indurations were considerably smaller and smoother. Since this time antigen 1 alone, produced indurations of 4 to 5 mm, the results obtained when it was added to the white cell suspensions are not significant, and should probably be disregarded. They

TABLE 9

PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN GUINEA PIGS. EXPERIMENT 1.

Recipient No.	[a]No. Leucocytes Injected	Antigens: Decapsulated Cells 3 x 10^9 orgs./ml	Antigens: Decapsulated Cells 1.5 x 10^9 orgs./ml	Antigens: Cell Wall 1:10	Antigens: Cell Wall 1:100
1	3.6 x 10^5	++	+	++	+
	1.45 x 10^6	+	+	-	-
2	3.6 x 10^5	Eryth	-	+	+
	1.45 x 10^6	+[b]	++[b]	++[b]	++
3	Hypersensitive - Reacted in some measure to all inocula.				

[a]Leucocyte yields were variable. In each case the maximum obtainable was used.

[b]Necrosis

All controls gave negative results. These included leucocyte-antigen suspensions from normal animals; leucocyte suspensions without antigen, from sensitized donors, and all antigens alone.

are, however, included in the data (Table 10).

Failure to Transfer Skin Sensitivity with Serum

The rabbits and guinea pigs from which peritoneal exudates were withdrawn were also used as serum donors. Serum (0.1 ml) from both sensitized and normal animals was inoculated intracutaneously (rabbit sera into rabbits and guinea pig sera into guinea pigs). Twenty-four hours later, antigen (in the same strengths as used in the experiments involving passive transfer with peritoneal exudates) was injected into the original sites. Controls included each serum without antigen, each antigen by itself, and Ringer's solution (with which the antigens were diluted).

No reaction was observed in either recipient rabbit. In guinea pigs sensitivity was observed at sites where the higher concentration of antigen (twice the normal skin-test dose -- as established experimentally) was injected following inoculation with serum from donor animals. However, no significance was attached to this, since a similar induration was provoked by the antigen itself.

Cellular Site of the Sensitizing Agent

Preliminary steps have been taken to establish the site of the antigen responsible for development of skin sensitivity to Cryptococcus neoformans. Cell-free extract and cell-wall material were prepared as described. Following centrifugation, the cell-wall residue was suspended in a volume of physiological saline approximately equal to that of the decanted cell-free extract. Subsequently the two cellular fractions were tested in both rabbits and guinea pigs for capacity to provoke skin

TABLE 10

PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN GUINEA PIGS. EXPERIMENT 2.

Guinea Pig No.	No. Leucocytes Injected	Induration (mm) Antigen 1 3×10^9 orgs./ml	Antigen 2 1.5×10^9 orgs./ml	Antigen 2 72 hours - 9 days
1	S_1 5.4×10^6	5 mm	7 mm	-
	S_2 4.6×10^6	7 mm	8.5 mm	Eryth
	N_1 2.0×10^6	5[a] mm	4 mm	-
	N_2 1.4×10^7	-	-	Smooth nodule
2	S_1 5.4×10^6	-	7 mm	Nodule
	S_2 4.6×10^6	7 mm	6 mm	Nodule
	N_1 2.0×10^6	-	-	-
	N_2 1.4×10^7	4 mm	4.6 mm	Tiny nodule[b]
3	S_1 5.4×10^6	4.5 mm	9 mm	Nodule
	S_2 4.6×10^6	5 mm	8 mm	Nodule
	N_1 2.0×10^6	5 mm	5.5 mm	Tiny nodule
	N_2 1.4×10^7	5 mm	4 mm	Tiny nodule

S_1 and S_2 are sensitized donors. N_1 and N_2 are normal donors.

[a]Antigen 1 alone produced indurations of 4 to 5 mm.

[b]Less than 4 mm.

Indurations were maximal at 24 hours.

Nodules (72 hours) from sensitized leucocyte-antigen suspensions were larger and rougher than those produced by normal leucocyte-antigen mixtures.

sensitivity.

Comparison of Skin Responses to Cell-Wall Material and Cell-Free Extract

Rabbits. Four rabbits, two sensitized to the large-capsuled variant "L", and two used previously as recipients in the serum transfer experiment, were skin tested with equal volumes (0.1 ml) of both cell-free extract and cell-wall material. Both sensitized animals, as well as one serum transfer recipient (the latter apparently sensitized by the numerous injections of antigen received in the previous experiment) developed indurations of 10 to 14 mm in diameter to cell-wall material. One of the sensitized rabbits failed to react to the cell-free extract, while the other two showed erythema only. The fourth animal was unresponsive (See Table 11).

Guinea pigs. When the experiment was carried out with guinea pigs (two sensitized and two normal) somatic antigen was used in three concentrations, including the usual skin-test dosage. The cell-wall material was injected full strength, as well as in dilutions of 1:100 and 1:1000.

Skin-testing, following the first series of three injections, elicited only insignificant reactions. Immunization was therefore continued, and on second skin-testing striking responses were obtained to all of the antigens. These are shown in Table 12.

As in rabbits, reactions to cell-free extract showed only erythema, while those to cell-wall material, even at the greatest dilution, were well indurated. The two higher concentrations produced necrosis.

TABLE 11

COMPARISON OF SKIN REACTIONS ELICITED IN SENSITIZED RABBITS TO CELL-FREE EXTRACT (CFX) AND CRUDE CELL-WALL MATERIAL (CW)

Rabbit		CFX	CW	Control
S_1	24 hours	-	9 mm	-
	30 hours	-	10 mm	-
S_2	24 hours	9 mm	9 mm	-
	30 hours	10.5 mm	11.5 mm	-
T_1	24 hours	10 mm	10.5 mm	-
	30 hours	9 mm	14 mm	-
T_2	24 hours	-	±	-
	30 hours	-	-	-

S_1 and S_2 are sensitized animals.

T_1 and T_2 were used as recipients in a serum transfer experiment, as a result of which T_1 became sensitized.

All reactions to CFX showed erythema only, while those to CW were indurated.

TABLE 12

COMPARISON OF SKIN REACTIVITY IN GUINEA PIGS TO CELL-FREE EXTRACT (CFX) CRUDE CELL-WALL MATERIAL (CW) AND DECAPSULATED CELLS (SOMATIC ANTIGEN).

					Somatic Antigen		
Guinea Pig	CFX	CW	CW 1:100	CW 1:1000	7.4×10^9 orgs./ml	1.5×10^9 orgs./ml	3×10^8 orgs./ml
S_1	11 mm (Eryth)	15 mm	6 mm	6.5 mm	12 mm	11 mm	7 mm
S_2	Eryth	12 mm	7 mm	6.5 mm	11 mm	9.5 mm	8 mm
N_1	-	-	-	-	-	-	-
N_2	10 mm (Eryth)	9 mm	5 mm	3.5 mm	9 mm	8.5 mm	7 mm

S_1 and S_2 are sensitized animals. N_1 and N_2 are normal controls, but N_2 apparently converted as a result of previous skin-testing (See text).

Reactions of sensitized animals to the two higher concentrations of somatic antigen, as well as to CW and CW 1:100, showed necrosis. All response to CFX showed erythema only.

One of the controls also displayed skin reactivity, but to a noticeably lesser degree. Apparently it had become sensitized by the battery of antigens injected when skin-testing was attempted earlier. This is consistent with the situation in rabbits, already described.

Capacity of Cell-Wall Fractions to Elicit Skin Reactivity in Guinea Pigs

Two cell-wall components were prepared as described. One consisted of the residue remaining, following extraction with KOH; the other was a precipitate, formed by saturating the KOH supernatant and washings with ammonium sulfate.

Three sensitized guinea pigs were skin-tested with the above fractions, as well as with the cell-wall suspension diluted 1:10, decapsulated cells (normal skin-test strength) and cell-free extract. Since no attempt was made to standardize the various skin-test materials, there was no basis for comparing their relative effectiveness. It was established, however, that both cell-wall components were capable of producing induration in sensitized guinea pigs. It was observed, further, that while reactions elicited by both cell-wall fractions reached a peak within 24 hours, 32 hours was required for maximal response to crude cell-wall material. See Table 13.

Duration of Skin-Sensitivity

Skin sensitivity induced in guinea pigs has been shown to be relatively long lasting. Sensitized animals still reacted when retested following a six-month rest period.

TABLE 13

RESPONSES OF SENSITIZED GUINEA PIGS TO CELL-FREE EXTRACT, CRUDE CELL-WALL MATERIAL (1:10) DECAPSULATED CELLS (SOMATIC ANTIGEN) NORMAL SKIN-STRENGTH, AND TWO CELL-WALL FRACTIONS. REACTIONS WERE MEASURED AT 24 HOURS, UNLESS OTHERWISE INDICATED.

Guinea Pig No.	Cont.	CFX	CW (1:10)	Somatic Antigen	Fraction I	Fraction II
1	-	-	-	10 mm	8 mm	10 mm
2	-	6 mm	6 mm 10 mm[a]	8.5 mm	10 mm	11 mm
3	-	-	5.5 mm 9 mm[a]	6 mm	9 mm 10 mm[a]	6.5 mm

CFX = Cell-free extract

CW = Cell-wall material

[a]32 hours

Serology and Skin Sensitivity

Circulating Antibody Level vs. Degree of Skin Sensitivity in Rabbits

The ten rabbits previously skin-tested with candida and cryptococcal antigen, in connection with the specificity studies, also were used in this experiment.

The animals were divided into three groups; Group A (two rabbits) received Candida albicans antigen and served as the control. Groups B and C (four rabbits each) were inoculated with partially decapsulated and decapsulated cells respectively. All injections were given intravenously in the ear vein, and consisted of 1.5×10^9 organisms suspended in 0.5 ml of sterile physiological saline. The rabbits received three injections on alternate days during the first week, and one injection weekly thereafter. Blood, for hemagglutination studies, was drawn prior to each injection. The animals were skin-tested six days, and twenty-two days respectively, following the first injection. The course of the development of humoral antibodies is illustrated in Table 14, while the skin sensitivity studies are summarized in Table 15.

It would appear from this experiment that there is no relationship between circulating antibody level, and degree of skin sensitivity. The results of the two phenomena are itemized for comparison in Table 16. It will be noted that Rabbit No. 6, with the highest hemagglutinating titer (1:320) manifested the poorest skin sensitivity -- induration 8 mm in diameter as compared to 17 mm with central necrosis, in Rabbit No. 10. Serum from the latter hemagglutinated only to two tubes (titer 1:40).

TABLE 14

DEVELOPMENT OF HEMAGGLUTINATING TITERS IN RABBITS INOCULATED WITH PARTIALLY DECAPSULATED AND [a]DECAPSULATED, HEAT-KILLED ORGANISMS

Rabbits	12/4	12/6	12/8	12/20	12/26	1/2	1/9
Group A							
1	-	-	-	Died			
2	-	-	-	-	-	1:20	1:20
Group B							
4	-	1:20	1:20	1:160	1:80	1:320	1:320
5	-	-	-	1:160	1:160	1:320	1:320
6	-	-	-	1:320	1:320	1:640	1:640
7	-	-	-	1:20	1:40	1:160	1:80
Group C							
8	-	-	-	1:20	1:40	1:20	1:20
9	1:20	-	-	1:20	1:40	1:20	1:20
10	1:20	-	-	1:40	1:40	1:40	1:40
11	-	-	Died				

All injections were given I.V. in the ear vein and consisted of 1.5×10^9 orgs. suspended in 0.5 ml sterile physiological saline.

[a]No capsules discernible in India ink smears.

Group A received Candida albicans antigen.

Untreated RBC's were used as controls to detect possible hemagglutination not due specifically to Antigen-Antibody reaction.

Group B received partially decapsulated cells of Cryptococcus neoformans.

Group C received decapsulated cells of Cryptococcus neoformans.

TABLE 15

MAXIMAL REACTIONS (DIAMETER IN MM) OF RABBITS TO *CANDIDA ALBICANS* AND *CRYPTOCOCCUS NEOFORMANS* SKIN-TEST ANTIGENS

Rabbits	10/16[a]	10/28		12/10		12/26	
	C. albicans	*C. alb.*	*C. neof.*	*C. alb.*	*C. neof.*	*C. alb.*	*C. neof.*
Group A							
1	4 mm	6 mm	-	18 mm	5 mm	Died	
2	6 mm	9.5 mm	4.5 mm	11.5 mm	5 mm	11 mm	9 mm
Group B							
3	12 mm	Died					
4	9 mm	12 mm	4 mm	8.5 mm	11 mm	10 mm	14 mm[b]
5	9 mm	12 mm	4 mm	11 mm	10 mm	11 mm	10 mm
6	7 mm	15.5 mm	4 mm	11 mm	5.5 mm	10 mm	8 mm
7	-	13 mm	-	8 mm	-	9.5 mm	8.5 mm
Group C							
8	Tiny nodule	7 mm	-	10.5 mm	10.5 mm	10 mm	9.5 mm
9	8 mm	10 mm	4.5 mm	11 mm	8.5 mm	13.5 mm	11.5 mm
10	10 mm	12.5 mm	4.5 mm	13.5 mm	15.5 mm	15.5 mm	17 mm[b]
11	8 mm	13.5 mm	5 mm	Died			

[a]None of the animals reacted to *Cryptococcus neoformans* on preliminary testing.

[b]Central necrosis.

TABLE 16

COMPARISON OF HEMAGGLUTINATING TITER WITH DEGREE OF SKIN SENSITIVITY

Rabbits	Hemag. titer 12/26	Skin reaction
Group A		
1	Died	Died
2	-	9 mm
Group B		
4	1:80	14 mm[a]
5	1:160	10 mm
6	1:320	8 mm
7	1:40	8.5 mm
Group C		
8	1:40	9.5 mm
9	1:40	11.5 mm
10	1:40	17 mm[a]

[a]Central necrosis

Group A Received *Candida albicans* antigen.

Group B Received partially decapsulated cells of *Cryptococcus neoformans*.

Group C Received decapsulated cells of *Cryptococcus neoformans*. (No capsular material discernible in India ink smears).

The results also indicate, that although decapsulated cells are almost incapable of stimulating the production of hemagglutinating antibodies, they are just as capable, as partially decapsulated cells, of inducing skin sensitivity.

Slide Agglutination

Serum from the last blood samples drawn for hemagglutination studies was used for slide agglutination tests. Partially decapsulated as well as decapsulated cells, were used as antigens. Serum from Rabbits Nos. 6 and 10 was also checked against fully encapsulated cells. In the latter case the results were negative. Serum from Rabbits Nos. 4, 5, and 6 (recipients of partially decapsulated cells) and of Rabbit No. 10 (inoculated with decapsulated cells) agglutinated both antigens. In the case of the decapsulated cells, while clusters were usually smaller, the agglutination was much tighter than when partially decapsulated cells were used (See Table 17).

TABLE 17

RESULTS OF SLIDE AGGLUTINATION TESTS; RABBITS WERE THE SAME AS IN PREVIOUS EXPERIMENTS. ANTIGENS AS INDICATED - PARTIALLY DECAPSULATED, DECAPSULATED, OR FULLY ENCAPSULATED CELLS OF ISOLATE "L" (USED FOR SENSITIZING).

		Antigens		
Rabbits	Hemagglutinating titer	Partially decapsulated cells	Decapsulated cells	Fully encapsulated cells
Group A				
2	1:20	-	-	
Group B				
4	1:320	+	+	
5	1:320	+	+ (weak)	
6	1:640	+	++[a]	-[b]
7	1:80	-	-	
Group C				
8	1:20	-	-	
9	1:20	-	-	
10	1:40	+	+++[a]	-

[a] Decapsulated cells were more tightly agglutinated than partially decapsulated cells.

[b] Only serum from Rabbits No. 6 and 10 was tested against fully encapsulated cells.

Group A Received Candida albicans antigen.

Group B Received partially decapsulated cells of Cryptococcus neoformans.

Group C Received decapsulated cells of Cryptococcus neoformans. (no capsular material discernible in India ink smears).

CHAPTER IV

DISCUSSION

It has been shown that both rabbits and guinea pigs can be sensitized readily to Cryptococcus neoformans. Fully encapsulated cells, partially decapsulated, and decapsulated cells have all been used, with seemingly equal effectiveness as sensitizing material.

There is reasonably convincing evidence that the skin response, elicited by subsequent testing, was of the cellular or tuberculin type. Hypersensitivity can be of two types -- immediate, mediated by interaction between antigen and antibody, in which the response is apparent (and frequently maximal) within two hours, but subsides quickly, and delayed, or cellular, in which reactivity usually reaches maximum extent and severity within 24 to 48 hours, and persists for several days. In the cellular type of hypersensitivity, classical antibodies do not appear to be involved. This phenomenon was originally thought to be synonymous with "bacterial hypersensitivity". Subsequently, however, it was shown that many types of antigens, ranging from simple chemicals to fungi, and even viruses, are capable of inducing delayed hypersensitivity. The classical studies of this phenomenon have been done with Mycobacterium tuberculosis, and the response is frequently referred to as "tuberculin-type" sensitivity.

Perhaps the most important difference in the two types of sensitivity is the medium by means of which passive transfer can be effected. Since immediate hypersensitivity is associated with circulating antibody, it can be transferred readily with serum from sensitized donors. However, numerous attempts to transfer tuberculin sensitivity in this manner have met with failure. Delayed hypersensitivity to other antigens, e.g. dinitrochlorobenzene (Hexthausen) and Streptococcal products (Lawrence) has also defied serum-transfer procedures.

The first successful transfer of tuberculin sensitivity was reported by Chase in 1945. It was accomplished with sensitized, living cells from peritoneal exudates, rather than serum. In 1951, Hexthausen passively transferred sensitivity to dinitrochlorobenzene in guinea pigs, by means of white cells from pooled, heparinized blood. A year later, Lawrence succeeded in transferring, to streptococcal negative individuals, delayed reactivity induced by heat-killed beta-hemolytic streptococci and streptococcal products. He used sensitized leucocytes prepared from heparinized venous blood. In 1960, Rapaport and associates transferred delayed sensitivity to coccidioidin, in man, with leucocyte extracts prepared from sensitive donors.

In the original passive transfer experiments, Chase injected leucocyte suspensions intravenously into normal guinea pigs, and found that after a latent period of 24 to 36 hours, tuberculin sensitivity could be elicited by intracutaneous injection of the proper antigen. Subsequently, Metaxas and Metaxas Bühler (1955) showed that if the tuberculin was added directly to the sensitized cells and the mixture was injected intradermally, the latent period could be eliminated and

sensitivity increased. This procedure, as described by George and Vaughan (1962) was followed in the present research.

Another characteristic feature of delayed reactions is the lack of correlation between circulating antibody level and degree of skin sensitivity. Under certain experimental conditions, circulating antibody may be present without delayed skin sensitivity, and under different circumstances, delayed hypersensitivity can exist in the absence of circulating antibodies. The latter was demonstrated by Uhr, Salvin and Pappenheimer in (1957). These investigators prepared antigen-antibody complexes by precipitating them in the region of antibody excess. They found that minute amounts of such complexes (0.3 micrograms of either diphtheria toxoid, or ovalbumin) incorporated in oil-water emulsion, could induce delayed hypersensitivity in guinea pigs at least two to three weeks before antibodies were demonstrable in the serum. Three years later, Salvin and Smith found that guinea pigs injected in the foot pad with 0.5 micrograms of egg albumin developed delayed hypersensitivity within 7 to 14 days. However, serum antibodies were not demonstrable, either by the Arthus reaction, or by hemagglutination, for at least 40 days following injection.

In all of the respects described, skin sensitivity to Cryptococcus neoformans appears to conform to the criteria established for delayed hypersensitivity.

Timing

Reactions reached maximum extent and severity within 24 to 48 hours following intradermal injection of antigen. No immediate reaction

was ever observed, although animals were examined promptly following skin-testing, and again an hour or two hours later.

Passive Transfer

The skin sensitivity was transferred in both rabbits and guinea pigs (rabbit to rabbit, and guinea pig to guinea pig) with peritoneal exudates from sensitized animals. Furthermore it was observed in rabbits that the more sensitive the donor, and the higher the concentration of leucocytes, the greater the degree of sensitivity in the recipient. A similar relationship has been reported to exist with regard to streptococcal products (Lawrence) and tuberculin (Metaxas and Metaxas Bühler and others).

Attempts to transfer the sensitivity with serum obtained from the donors of peritoneal exudates, were unsuccessful. As mentioned previously, this is characteristic of delayed hypersensitity to other antigens. Summarizing the situation, Arnason and Waksman stated in their review article: "Passive transfer with living cells, coupled with failure of passive transfer with serum, has come to be regarded as the key experiment in proving the delayed nature of a given immunologic response".

Lack of Correlation Between Circulating Antibody Level and Degree of Skin Sensitivity

A group of rabbits, inoculated intravenously with decapsulated and partially decapsulated cells of *Cryptococcus neoformans*, showed no relationship between circulating antibody level and degree of skin sensitivity. The rabbit with the highest hemagglutinating titer (1:320) showed

minimal skin reactivity, while the most highly sensitized animal (induration 17 mm with central necrosis) had a hemagglutinating titer of only 1:40. A second rabbit showing a high degree of skin sensitivity (14 mm with severe necrosis) had a hemagglutinating titer of 1:160, and a control animal, immunized intravenously with Candida albicans and intradermally with Cryptococcus neoformans, developed sensitivity to Cryptococcus (induration 9 mm) in the absence of demonstrable hemagglutinating antibodies. It should be stressed that this seemingly chaotic situation is in complete conformity with the picture established for delayed reactions.

This characteristic lack of relationship between circulating antibody level and degree of skin sensitivity is also consistent with the possibility that the two immune responses are directed against different antigenic components. This is frequently the case with both mycotic and bacterial agents, and there is evidence that it may also apply to Cryptococcus neoformans. Gadebusch (1960) concluded, on the basis of his work with enzymatically decapsulated cells, substantiated by fluorescent studies, that the antigen responsible for the production of humoral (protective and agglutinating antibodies) resides "in bits of capsular material tightly bound to the cell wall". The hemagglutinating studies, carried out in the course of the present research, would appear to corroborate this finding. Rabbits immunized with partially decapsulated cells developed relatively high titers, compared with a maximum of 1:40 in animals receiving decapsulated cells. The low titers in the latter group probably mean that the material injected contained a small number of intact antigenic sites. The sharp difference in antibody levels exhibited by the two groups of rabbits, clearly emphasizes that removal of

the capsular material, beyond a certain point, results in decreased antigenicity of the organisms. In contrast, the degree of skin sensitivity is quite comparable in the two groups. The fact that additional sonic agitation markedly reduced the capacity of the cells to stimulate the production of circulating antibody, but had no effect on their ability to induce skin sensitivity, strongly suggests that separate antigens are responsible for the two immunologic responses.

On the basis of studies carried out with crude cell-wall material and cell-wall fractions, as opposed to cell-free extract, it appears that the agent which elicits skin sensitivity resides in the cell wall. This could explain why it was unaffected by sonic agitation.

The picture appears to be somewhat complicated by the results of the slide agglutination tests. It is possible, however, that the slide agglutination and hemagglutinating antigens may be two separate entities. Of particular interest is the agglutination of decapsulated cells. This phenomenon might be explained in several ways: (a) decapsulation was not complete, and antigenic sites are still present; (b) agglutinating antigen may be present in both the capsule and the cell wall; and (c) agglutinating antigen may reside only in the cell wall. Should this be the case, some of the antigenic sites in partially decapsulated cells probably remain blocked. The fact that decapsulated cells were much more tightly agglutinated than partially decapsulated ones would tend to support the latter contention. Fully encapsulated cells were not agglutinated, suggesting that the abundance of capsular material may be preventing contact between antigen and antibody. This appears to corroborate further

Gadebusch's findings -- that the seat of the antigen responsible for the production of agglutinating antibodies is very close to the cell wall.

It has long been known that route of inoculation is an important factor in the production of delayed hypersensitivity. Baker (1935) reported that the intracutaneous route is most effective. This has been attributed (Yoffey and Courtice, 1956) to the rich supply of lymphatic plexi available for transporting antigen to the lymph nodes.

The ease with which intradermal injection induces delayed sensitivity undoubtedly accounts, in part, for the sensitizing effect of skin-test antigens. In 1959, Sills and associates reported that several originally negative patients converted to positive as a result of skin-testing with histoplasmin. Two years later Bauer and Stone found that repeated skin-testing with PPD could sensitize guinea pigs.

It is not surprising, therefore, that Rabbit T_1 (Table 10) previously a recipient in a serum transfer experiment, behaved as a sensitized animal when skin-tested subsequently with cell-free extract and cell-wall material.

Similarly, Guinea pig N_2 (Table 11) apparently developed sensitivity as a result of earlier skin-testing.

Another unanticipated skin response was manifested in the course of the present research: A rabbit sensitized to Cryptococcus neoformans failed to react to other skin-test antigens, with one exception--Candida albicans. This was therefore first suspected of being a cross-reaction. However, isolation of Candida spp. from the animal's feces suggested that the rabbit might have become sensitized to a constituent of its own

normal flora. Vogel and associates (1962) reported a similar phenomenon in guinea pigs, attributing the spontaneous hypersensitivity to the presence of Candida albicans in the intestinal tract.

In two subsequent experiments, rabbits (six in the first group, and eleven in the second) were skin-tested prior to any known contact with Cryptococcus. All reacted to Candida albicans antigen; none responded to C. neoformans. Thus, cross reactivity was ruled out.

Isolation of Candida spp. from the feces of five rabbits out of the group of six, and from ten out of the eleven (one died before a fecal specimen was obtained) adequately substantiated the theory that the animals were sensitized to their own normal flora. In no case was Candida albicans isolated, but this is hardly unexpected, since rabbits are highly susceptible to systemic infection with that species of Candida. A high percentage of humans (95% of adults) also react to Candida antigen, thus negating the diagnostic value of skin-testing.

The fact that rabbits, ordinarily considered poor developers of delayed hypersensitivity, were readily sensitized with fully encapsulated cells of a large-capsuled strain of Cryptococcus neoformans, appears to be significant. It demonstrates that the large capsule, highly characteristic of the organism in vivo, does not prevent the specific antigen, which seemingly resides in the cell wall, from inducing a state of hypersensitivity, at least in rabbits. The next step, similating much more closely the circumstances thought to surround human infection, would be to inoculate animals intranasally with living organisms. If sensitivity to Cryptococcus neoformans develops under such conditions, it is reasonable to suspect that humans, too, become sensitized on contact with the

organism, and would therefore respond to subsequent skin-testing with the proper antigen.

It would seem that many chapters on the immunologic characteristics of Cryptococcus neoformans still remain unwritten. Since the antigenic components of the organism have not as yet been isolated in sufficiently pure form to permit the preparation of highly specific antiserum, such potentially valuable procedures as fluorescent and electron microscopy cannot be exploited at the present time. Marshall and associates (1961) showed that fluorescent staining is not specific for the organism, but for the capsular polysaccharide - even free in the medium. It therefore has diagnostic value, but can contribute little to immunologic studies. The cell-wall is known to contain polysaccharide, which, if antigenic, would also fluoresce, probably making it impossible to determine the site or sites of fluorescence. In addition, it has never been demonstrated that polysaccharide antigens induce skin-sensitivity. Therefore, present techniques would offer no help in pin-pointing the location of the skin sensitizing antigen.

Basically the limitations on fluorescent microscopy would also apply to electron microscopy. Electron micropraphs of Candida albicans (Bakerspigel, 1964) show that the cell wall and the cell membrane of that yeast are clearly delineated. Presumably the same would apply to Cryptococcus neoformans and would make it possible for an investigator to determine whether he is working with pure cell-wall material. Beyond this, no contribution can be made to the immunology of the organism until highly specific antisera are available. Ferritin tagging of such sera

would undoubtedly be a valuable means of searching for antigens. The paramount problem remains; isolation of antigenic components in relatively pure form.

CHAPTER V

SUMMARY

It has been shown that Cryptococcus neoformans can readily induce a state of hypersensitivity in rabbits and guinea pigs. Heat-killed, fully encapsulated cells, from both large and small-capsuled variants, as well as partially decapsulated and decapsulated cells, served equally well as sensitizing agents.

There is strong evidence indicating that the skin reactivity is of the tuberculin, or cellular type. Timing was always typical for delayed hypersensitivity. Reactions reached maximal severity and extent within 24 to 48 hours. No immediate reaction was ever observed. What is more important, the hypersensitivity has been passively transferred with peritoneal exudates, while attempts to transfer it with serum from sensitized animals have failed completely.

The total lack of correlation between circulating antibody level (as demonstrated by hemagglutination) and degree of skin sensitivity, is also in conformity with the established picture for delayed hypersensitivity to tuberculin and other substances.

Relatively high hemagglutinating titers (1:320 to 1:640) were produced in three out of the four rabbits inoculated intravenously with partially decapsulated cells. Of the three animals receiving decapsulated cells none developed a hemagglutinating titer above 1:40. These

low values could be non-specific. On the other hand, it is highly possible that some few antigenic sites remained intact even in decapsulated cells. In contrast, the degree of skin sensitivity exhibited in the two groups was quite comparable.

Slide agglutination experiments indicate that the seat of the agglutinating antigen is either in the capsule, close to the cell wall, or possibly in the cell wall itself. There may be two antigens involved.

The specificity of the skin-sensitizing antigen was confirmed in both guinea pigs and rabbits. The latter reacted to Candida albicans skin test antigen. However, later studies ruled out the possibility of cross-reactivity, and showed that the response to Candida was due to sensitization of the rabbits resulting from the presence of Candida spp. in their own intestinal tract.

The sensitizing agent appears to reside in the cell wall. Skin reactivity has been elicited with crude cell-wall material, even considerably diluted, as well as with each of two cell-wall fractions (one, the residue remaining following extraction with potassium hydroxide, and the other, the precipitate formed by saturating the KOH supernatant and washings with ammonium sulfate). In contrast, cell-free extract produced erythema only.

REFERENCES

Abrahams, I., T. G. Gilleran, and C. B. Weiss. 1962. Quantitative studies on reverse cutaneous anaphylaxis in mice with progressive cryptococcosis. J. Immunol. 89: 684-690.

Ajello, L. 1958. Occurrence of Cryptococcus neoformans in soil. Am. J. Hyg. 67: 72-77.

Arnason, B. G. and B. H. Waksman. 1964. Tuberculin sensitivity - immunologic considerations. Adv. in Tuberculosis Res. 13: 1-97.

Baker, A. B. 1935. Complement fixation as related to resistance and allergy in experimental tuberculosis. Am. Rev. Tuberculosis 31: 54-61.

Bakerspigel, A. 1964. Some observations on the cytology of Candida albicans. J. Bacteriol. 87: 228-230.

Bauer, J. A. and S. H. Stone. 1961. Isologous and homologous lymphoid transplants. I. The transfer of tuberculin hypersensitivity in inbred guinea pigs. J. Immunol. 86: 177-189.

Benham, R. W. 1955. The genus Cryptococcus; the present status and criteria for identification of species. Trans. N. Y. Acad. Sci. 17: 418-429.

Berghausen, O. 1927. Torula infection in man. Ann. Int. Med. 1: 235-240.

Buschke, A. 1895. Ueber eine durch coccidien hervorgerufene Krankheit des menchen. Deutsche Med. Wchschr. 21: 14, cited in Cryptococcosis , Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Busse, O. 1895. Ueber saccharomycosis hominis. Virchows Arch. f. Path. Anat. 140: 23-46, cited in Cryptococcosis, Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Carton, C. A. 1952. Treatment of central nervous system cryptococcosis: A review and a report of four cases treated with actidione. Ann. Int. Med. 37: 123-154.

Chase, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exptl. Biol. Med. 59: 134-135.

Curtis, F. 1896. Contribution à l'étude de la saccharomycose humaine. Ann. Inst. Past. 10: 449-468, cited in Cryptococcosis, Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Dienst, P. B. 1938. Cryptococcus histolyticus isolated from a subcutaneous tumor. Arch. Dermatol. Syphilol. 37: 461-464.

Dische, Z. and E. Borenfreund. 1957. A new color reaction for the determination of aldopentose in the presence of other saccharides. Biochim. Biophys. Acta 25: 639-642.

Emmons, C. W. 1951. Isolation of Cryptococcus neoformans from soil. J. Bacteriol. 62: 685-690.

Emmons, C. W. 1954. The significance of saphrophytism in the epidemiology of the mycoses. Trans. N. Y. Acad. Sci. Ser. II 17: 157-166.

Emmons, C. W. 1955. Saprophytic sources of Cryptococcus neoformans associated with the pigeon. (Columbia livia). Am. J. Hyg. 62: 227-232.

Emmons, C. W. 1960. Prevalence of Cryptococcus neoformans in pigeon habitats. Pub. Health Repts. 75: 362-364.

Evans, E. E. 1949. An immunologic comparison of twelve strains of Cryptococcus neoformans (Torula histolytica). Proc. Soc. Exptl. Biol. Med. 71: 644-646.

Evans, E. E. 1950. The antigenic composition of Cryptococcus neoformans. I. A serologic classification by means of the capsular agglutination reaction. J. Immunol. 64: 423-430.

Evans, E. E. 1960. Capsular reactions of Cryptococcus neoformans. Ann. N. Y. Acad. Sci. 89: 184-192.

Evans, E. E., H. P. R. Seeliger, L. Kornfeld, and C. Garcia. 1956. Failure to demonstrate capsular swelling in Cryptococcus neoformans. Proc. Soc. Exptl. Biol. 93: 257-260.

Gadebusch, H. H. 1958. Active immunization against Cryptococcus neoformans. J. Infect. Dis. 102: 219-226.

Gadebusch, H. H. 1960. Specific degradation of Cryptococcus neoformans 3723 capsular polysaccharide by a microbial enzyme. III. Antibody stimulation by partially decapsulated cells. J. Infect. Dis. 107: 406-409.

George, M. and John H. Vaughan. 1962. In vitro cell migration as a model for delayed hypersensitivity. Proc. Soc. Exptl. Biol. Med. 111: 514-521.

von Hansemann, D. 1905. Ueber eine bisher nicht beobachtete gehirnerkrankung durch hefen. Verhandl. d deutsch. path. Gesellsch. 9: 21-24, cited in Cryptococcosis, Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Haxthausen, H. 1951. Passive transmission of dinitrochlorobenzene allergy with white blood cells from sensitized guinea pigs. Acta Dermatol. Venerol. 31: 659-665.

Hasenclever, H. F. and C. W. Emmons. 1963. The prevalence and mouse virulence of Cryptococcus neoformans strains isolated from urban areas. Am. J. Hyg. 78: 227-231.

Hoff, C. L. 1942. Immunity studies of Cryptococcus hominis (Torula histolytica) in mice. J. Lab. Clin. Med. 27: 751-754.

Kao, C. J. and J. Schwartz. 1957. Isolation of Cryptococcus neoformans from pigeon nests, with remarks on the identification of virulent cryptococci. Am. J. Clin. Path. 27: 652-663.

Kase, A. and Col. Jos. F. Metzger. 1962. Isolation of a dry variant from a mucoid strain of Cryptococcus neoformans and preliminary comparative studies. J. Bacteriol. 83: 926-928.

Kessel, J. F. and F. Holtzwart. 1935. Experimental studies with Torula from a knee infection in man. Am. J. Trop. Med. 15: 467-483.

Kessler, G. and W. J. Nickerson. 1959. Glucomannan-protein complexes from cell walls of yeasts. J. Biol. Chem. 234: 2281-2285.

Klein, E. 1901. Pathogenic microbes in milk. J. Hyg. 1: 78-95.

Kligman, A. M. 1947. Studies of the capsular substance of Torula histolytica and the immunologic properties of Torula cells. J. Immunol. 57: 395-401.

Lawrence, H. S. 1952. Cellular transfer in humans of delayed cutaneous hypersensitivity to hemolytic streptococci. J. Immunol. 68: 159-177.

Leopold, S. S. 1953. Pulmonary moniliasis and cryptococcal osteomyelitis in the same patient. Med. Clin. North Am. 37: 1737-1746.

Littman, M. L. 1958. Capsule synthesis by Cryptococcus neoformans. Trans. N. Y. Acad. Sci. 20: 623-648.

Littman, M. L. 1959. Cryptococcosis (Torulosis) current concepts and therapy. Amer. J. Med. 27: 976-998.

Littman, M. L. and S. S. Schneirson. 1959. Cryptococcus neoformans in pigeon excreta in New York City. Am. J. Hyg. 69: 49-59.

Littman, M. L. and L. E. Zimmerman. 1956. Cryptococcosis - Torulosis or European Blastomycosis. Grune Stratton and Co., New York.

Marshall, J. S., L. Sverson, W. C. Eveland, and A. Kase. 1961. Application and limitation of fluorescent antibody stain in specific diagnosis of cryptococcosis. Lab. Invest. 10: 719-728.

Metaxas, M. N. and M. Metaxas - Bühler. 1955. Studies on the cellular transfer of tuberculin sensitivity in the guinea pig. J. Immunol. 75: 333-347.

Muchmore, H. G., E. Rhoades, F. G. Felton and G. Nix. 1963. Occurrence of Cryptococcus neoformans in the environment of three geographically associated cases of cryptococcal meningitis. New Eng. J. Med. 268: 1112-1114.

Neill, J. M., I. Abrahams, and C. E. Kapros. 1950. A comparison of the immunogenicity of weakly encapsulated and strongly encapsulated strains of Cryptococcus neoformans. J. Bacteriol. 59: 263-275.

Neill, J. M., and C. E. Kapros. 1950. Serological tests on soluble antigens from mice infected with Cryptococcus neoformans and Sporotrichum schenckii. Proc. Soc. Exptl. Biol. Med. 73: 557-559.

Neill, J. M., J. Y. Sugg, and D. W. McCauley. 1951. Serologically reactive material in spinal fluid, blood and urine from a human case of cryptococcosis (torulosis). Proc. Soc. Exptl. Biol. Med. 77: 775-778.

Pollock, A. and L. M. Ward. 1962. A hemagglutination test for Cryptococcosis. Am. J. Med. 32: 6-15.

Rapaport, F. T., Lawrence, H. S., Miller, J. W., Pappagianis, D. and Smith, C. E. 1960. Transfer of delayed hypersensitivity to Coccidioidin in man. J. Immunol. 84: 358-367.

Rappaport, B. Z. and B. Kaplan. 1926. Generalized Torula mycosis. Arch. Path. 1: 720-741.

Salvin, S. B. 1950. Quantitative studies in the serologic relationship of fungi. J. Immunol. 65: 617-626.

Salvin, S. B. 1959. Current concept of diagnostic serology and skin hypersensitivity in mycoses. Am. J. Med. 27: 97-114.

Salvin, S. B. and R. F. Smith. 1960. Delayed hypersensitivity and the anamnestic response. J. Immunol. 84: 449-457.

Salvin, S. B. and R. F. Smith. 1961. An antigen for detection of hypersensitivity to Cryptococcus neoformans. Proc. Soc. Exptl. Biol. Med. 108: 498-501.

Sanfelice, F. 1894. Contributo alla morfologia e biologia dei blastomiceti che si sviluppano nei succhi di alcuni frutti. Ann. d'ig 4: 463-495, cited in Cryptococcosis, Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Seeliger, H. P. R. 1956. Use of urease test for screening and identification of cryptococci. J. Bacteriol. 72: 127-131.

Seeliger, H. P. R. 1960. Les phénomènes d'immunité dans les Cryptococcoses. Path. et Biol. 8: 297-306.

Sills, R. D., J. Rom, and J. E. Berk. 1959. Altered skin reactivity induced by repeated histoplasmin skin tests. J. Allergy 30: 541-544.

Silva, M. E. 1960. Occurrence of Cryptococcus neoformans and Microsporum gypseum in soils of Bahia Brazil. Bol. funacao concalo. 17: 7-14.

Stanley, N. F. 1949. Polysaccharide haptens from Torula histolytica. Austral. J. Exptl. Biol. Med. Sci. 27: 409-415.

Stoddard, J. L. and E. C. Cuttler. 1916. Torula infection in man. Monographs of the Rockefeller Inst. for Med. Res. No. 6: 1-98, cited in Cryptococcosis, Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Uhr, J. W., S. B. Salvin, and A. M. Pappenheimer, Jr. 1957. Delayed hypersensitivity. II. Induction of hypersensitivity in guinea pigs by means of antigen - antibody complexes. J. Exptl. Med. 105: 11-24.

Versé, M. 1914. Ueber einen fall von generalisierter blastomykose beim menschen. Verhandl. d. deutsch. path. Gesselsch. 17: 275-278, cited in Cryptococcosis, Littman, M. L. and L. E. Zimmerman. (New York: Grune Stratton and Co., 1956).

Vogel, R. A., M. Koger, and M. Johnson. 1962. Tuberculin hypersensitivity associated with immunization of guinea pigs with Candida albicans and the presence of this organism in normal guinea pigs. Mycopathol. Mycol. Appl. 16: 117-124.

Weiss, J. D. 1902. Four pathogenic Torulae (blastomycetes). J. Med. Res. 2: 280-311.

Yoffey, J. M. and F. C. Courtice. 1956. Lymphatics, Lymph and Lymphoid Tissue. Harvard Univ. Press. Cambridge, Mass.


6
97