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COMPARATIVE STUDIES OF HISTOPLASMA CAPSULATUM AND
A TRICHOSPORON-LIKE ORGANISM:
MORPHOLOGY, PATHOGENICITY, IMMUNOLOGY, AND
CROSS-PROTECTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Ram Pratap Tewari


* * * * * * *

The Ohio State University
1966

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PLEASE NOTE:
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INTRODUCTION

An increasing number of mycotic infections of man and animals has been recognized in recent years. No doubt this is due in part to the greater awareness on the part of both physicians and mycologists of the possibility of such infections. On the other hand there has also been a real increase in the number of infections due to an ever-increasing use of antibiotics and corticosteroids in the treatment of other ailments. This is more true for the infections recognized due to the "opportunist fungi," such as Mucor, Candida, Aspergillus, Rhizopus and possibly others, especially in chronically hospitalized patients. At least 50 of the 50,000 to 200,000 known species of fungi have been recognized as human pathogens (Emmons, Binford and Utz, 1963). One indication of the prevalence of systemic mycotic infections can be derived from recent statistics for histoplasmosis. According to Campbell (1965a) at least 30 million human infections have been recognized, as of the present (even though the disease is not necessarily active), and this number is increasing by at least 100,000 cases annually.

Members of the form genus Trichosporon, besides being present as common saprophytes, have been found repeatedly in association with different disease processes of man and animals, but have always been considered of questionable etiologic significance. Their presence in the sputum of hospitalized patients is
alarming and points to the possibility of some more significant role, rather than the usual one as a contaminant. However, pathogenicity to experimental animals (rabbits) of such an isolate from sputum has been well documented on only one occasion (Gilbert and Fetter, 1962).

Serological tests have acquired a very significant place in the study and diagnosis of systemic mycotic infections, viz., histoplasmosis, coccidioidomycosis, and blastomycosis. The use of these technics has led to the detection of numerous otherwise unrecognized cases of such infections. However, countless difficulties have been encountered in the interpretation of serological tests in establishing a definite clinical diagnosis, because of alleged cross-reactions between these three, and possibly other, fungal antigens. In fact serological cross-reactions in systemic mycotic infections have been accepted as the rule rather than the exception.

Skin tests using mycotic "-ins," such as histoplasmin, blastomycin, and coccidioidin, have been used quite extensively in determining the geographic distribution and prevalence of coccidioidomycosis, histoplasmosis and to a lesser extent blastomycosis. Like tuberculin, while of tremendous value, they have always been associated with false positive reactions. According to Campbell (1965a), "It was recognized from the outset that the skin test antigens were "crude" and contained components that were cross-reactive in these infections and possibly other infections of mycotic origin for which skin test antigens have never been available."
There is undoubtedly an urgent need for a safe and effective vaccine against histoplasmosis especially for laboratory personnel and for certain high-risk populations who are more liable to infection. Toward this goal, numerous attempts have been made during the last one and one-half decades, by different investigators. Although resistance to infection with *Histoplasma capsulatum* in experimental animals has been repeatedly induced by sublethal infection or by immunization with killed cells or fractions thereof, none has been found of any practical significance (Salvin, 1960). Similarly, even the cross-protection in mice against histoplasmosis by sublethal infection with *Blastomyces dermatitidis* is of considerable academic significance but has no real practical importance (Sethi et al., 1964).

From the above, it is obvious that the immunological relationship between *H. capsulatum* and other fungi is very complicated and rather poorly understood. Certain species of *Trichosporon*, particularly the one *Trichosporon*-like organism which is the subject of the present investigation, and the genus *Geotrichum*, occupy an interesting intermediate position morphologically between the true dimorphic fungi and yeasts. Four strains of this *Trichosporon*-like organism were isolated from the sputum of a human case of vague respiratory ailment and from skin lesions of a dog and two horses.

The present study was undertaken to explore the extent of the relationship between *H. capsulatum* and a *Trichosporon*-like
organism. Specifically the points to be investigated were:-

(1) How close is the morphological resemblance, and does it suggest a taxonomic relationship?

(2) Does this Trichosporon-like organism have a significance as a pathogen for experimental animals and by implication for the human?

(3) What effect, if any, do cortisone and streptomycin have on the susceptibility of experimental animals to H. capsulatum and this Trichosporon-like organism?

(4) Is there a possibility of Trichosporon species being responsible for false positive skin test and serological reactions in histoplasmosis?

(5) Could a vaccine against histoplasmosis be obtained by using this Trichosporon-like organism?
REVIEW OF LITERATURE

I. Pathogenicity of Histoplasma capsulatum in Mice: Effect of Cortisone and Streptomycin

In his famous studies on histoplasmosis in 1931, DeMonbreun not only described the phenomenon of dimorphism in *H. capsulatum*, but also, for the first time, demonstrated its pathogenicity to experimental animals (monkey and dog). Since then, extensive experimental work by different investigators has established well its pathogenicity in mice by various routes of inoculation (Negroni, 1965).

An increasing number of human infections with the "opportunist fungi," such as *Candida*, *Mucor* and *Aspergillus*, have been recognized in chronically ill patients, particularly in those who have been treated with cortisone or ACTH and with broad spectrum antibiotics during some stage of their ailments (Sidransky and Friedman, 1959 and Baker, 1962).

Conflicting reports have appeared in the literature regarding the effect of cortisone on experimental mycotic infections. Enhancement of infection has been demonstrated with *Achorion quinckenum* and *Trichophyton mentagrophytes* in guinea pigs (Riess and Caroline, 1952 and Kligman et al., 1951), *Blastomyces dermatitidis* and *Candida albicans* in mice (Syverton et al., 1952), *Candida albicans* in rabbit (DeMitr et al., 1963), *Coccidioides immitis* in rats (Cavallero and Sala, 1951, and
Redaelli et al., 1951) and Cryptococcus neoformans in rats and
guinea pigs (Gadebusch and Gikas, 1965).

A great deal of skepticism has existed regarding the role
of cortisone in experimental infections with H. capsulatum.
Enhancement of infection following cortisone treatment has been
observed in dogs by Farrell, Cole, Prior and Saslaw (1953), in
rats by Konigsbaur (1953), in guinea pigs by Vogel et al. (1955)
and in mice by Mankowski (1955) and Grunberg and Titworth (1963).
However, Baum et al. (1954) and Louria et al. (1960) were unable
to demonstrate any augmentation of H. capsulatum infection in mice
following cortisone treatment.

Similarly, in human infection with H. capsulatum both
beneficial and harmful effects of cortisone have been reported.
Packard et al. (1957), Tegeris (1958) and Saliba and Beatty (1962),
have reported successful treatment of two, one, and 19 cases of
disseminated histoplasmosis with cortisone, respectively. On
the contrary, Murray and Sladden (1965) have recently reported a
case of disseminated, fatal histoplasmosis, in a sixty-six-year-
old woman following long-term steroid therapy for reticulosarcoma.
The patient had lived for many years in India, returning home in
1952, and was considered to have acquired the infection in India,
where histoplasmosis is known to exist.

Enhancement of growth of certain fungi, viz. Sporotrichum
schenckii, Coccidiodes immitis, Phialophora verrucosa, Trichophyton
mentagrophytes and H. capsulatum (Campbell and Saslaw, 1949a) and
that of Cryptococcus neoformans (Foley and Farber, 1948), in vitro, have been observed by incorporating varying concentrations of streptomycin in the laboratory media. However, in a subsequent study, Campbell and Saslaw (1951) failed to demonstrate the enhancement of the infectivity of yeast cells of H. capsulatum in mice simultaneously treated with streptomycin. There is a general consensus, on the basis of clinical experience, that streptomycin has a deleterious effect on the course of human histoplasmosis, but there is no experimental work supporting this observation.

The present experiment on the effect of cortisone and streptomycin alone or in combination on the pathogenicity of H. capsulatum in mice has been designed in an attempt to obtain further information which may be applicable to human beings regarding the role of these two rather important chemotherapeutic agents in laboratory animals.
II. Pathogenicity of Trichosporon

The genus *Trichosporon* was first described by Behrend in 1890. Eight species and one subspecies of this form genus were described by Diddens and Lodder (1942) and confirmed by Lodder and Kreger-Van Rij (1950), on the basis of morphological, cultural, and biochemical properties. Recently three more new species, viz. *T. figureirae*, isolated from faeces of two human patients suffering from diarrhea (Batista and Silveira, 1960), *T. loboi* from skin lesions of a man (Batista et al., 1962) and *T. penicillatum* from different sources (Do Carmo-Sousa, 1965), have been described in this genus.

*T. cutaneum* (*T. beigelii*) is the only species in which the etiologic association with white piedra of hair in man (Conant et al., 1955) and in animals (Kaplan, 1959) has been well documented. Other members of the genus *Trichosporon*, besides being present as common saprophytes, have also been found in association with different disease processes of man and animals, but have always been considered of questionable etiologic significance. On the other hand, pathogenicity for laboratory animals is much more convincing.

Gilbert and Fetter (1962) first showed that *T. capitatum*, originally isolated from a human sputum, was pathogenic for rabbits by intravenous inoculation of saline suspension of spores and hyphal fragments. Nine of the 15 rabbits died after
inoculation and the organism was recovered from all at autopsy. Mycotic abscesses were present in the kidneys, which ruptured through into the tubules. The organism also invaded the brain and heart, where it evoked a mixed reaction.

Michail and Styliona (1964) isolated a strain of T. cutaneum from the sputum of a man suffering from bronchial asthma and it was pathogenic for a rabbit after intravenous inoculation of a heavy suspension of the organism.

The present experiments were designed to show the pathogenicity of a Trichosporon-like organism for normal mice, and for mice treated with cortisone and streptomycin, alone or in combination.
III. Immunology

A. Skin Test Cross-Reactivity between H. capsulatum and Other Fungi

The skin hypersensitivity reaction to histoplasmin in man and animals infected with H. capsulatum was almost simultaneously demonstrated in the early forties by Negroni (1940), VanFerais et al. (1941), and Zarafonetis and Lindberg (1941). Zarafonetis and Lindberg defined histoplasmin as the antigenic substance, or substances, derived from the mycelial growth of H. capsulatum in broth. However, the present classical histoplasmin was first prepared by Emmons et al. in 1945 in a synthetic medium. They prepared histoplasmin, blastomycin, coccidioidin and haplosporangin and demonstrated cross-reactivity in skin tests in animals experimentally infected with fungi from which these antigens were derived. Almost complete cross-reaction occurred between histoplasmin and blastomycin and to a lesser extent with coccidioidin and haplosporangin.

Skin test antigens used, viz. histoplasmin, blastomycin, coccidioidin, and haplosporangin, were prepared from H. capsulatum, Blastomyces dermatitidis, Coccidioides immitis and Haplosporangium parvum, respectively. Haplosporangium parvum is a saprophyte present in soil and infects wild rodents, producing cystic elements resembling those of C. immitis.

Howell (1947) infected guinea pigs with yeast cells of H. capsulatum and B. dermatitidis intraperitoneally and
demonstrated skin test cross-reactions both with mycelial phase antigens (histoplasmin and blastomycin) and heat-killed saline suspension of the yeast cells. Cutaneous responses to histoplasmin and blastomycin reached their height at 24 hours, and occasionally disappeared within 48 hours, but with those of yeast-phase antigens usually persisted 48 hours or longer. Degree and intensity of cross-reactions depended on the strength of antigens employed and could be eliminated by dilution.

Subsequent investigations by many workers have well established the occurrence of cross-reactions in skin tests in man and experimental animals using both crude and partially purified mycelial and yeast-phase mycotic "-ins," viz. histoplasmin, blastomycin and to a lesser extent with coccidioidin (Smith et al., 1949, Gluskar et al., 1950, Dyson and Evans, 1955, Edwards and Palmer, 1957, Knight et al., 1959a, Edwards et al., 1961, Salvin, 1959 and 1965, and Drozdos, 1964).

More recently Asgari and Conant (1964) have demonstrated cross-reactions of skin test sensitivity between histoplasmin and chrysosporin prepared from the growth of H. capsulatum and Chrysosporium keratinophilum respectively, in guinea pigs. C. keratinophilum but not H. capsulatum was isolated from the soil of a village in Iran, where histoplasmin-positive individuals without any clinical evidence of histoplasmosis were present. The authors postulated that C. keratinophilum present in soil might have been responsible for the positive histoplasmin
skin test amongst the village people.

Members of the genus Trichosporon have been repeatedly isolated from the sputum of the hospitalized patients by routine diagnostic laboratory procedures and have invariably been considered as sputum contaminants (Tewari, 1961, Gilbert and Fetter, 1962 and Michail and Styliona, 1964). It has been long suspected that fungi other than H. capsulatum, B. dermatitidis, and C. immitis might be responsible for positive skin test reactions with mycotic "-ins," in routine surveys of human populations (Emmons et al., 1945, Palmer et al., 1957 and Campbell, 1965a).

A working hypothesis was postulated that species of Trichosporon by virtue of their presence in human sputum might play some role in the sensitization of the individuals against some of the classical mycotic "-ins." The present experiment for skin test cross-reactivity between histoplasmin and trichosporin (?) in guinea pigs infected with yeast cells of H. capsulatum and a Trichosporon-like organism was designed to test this hypothesis with respect to infection with H. capsulatum and this Trichosporon-like organism.
B. Serological Cross-Reactions between H. capsulatum and Other Fungi

Several serological tests have been developed and used extensively during the last one and one-half decades in the study and diagnosis of systemic mycotic infections (histoplasmosis, blastomycosis, and coccidioidomycosis). The earlier claims, regarding the specificity of serological reactions of fungus antigens (Furcolow et al., 1948, Campbell and Saslaw, 1949b, Smith, 1949, Smith et al., 1950, and Salvin, 1952) have been proved to be erroneous. On the contrary, the occurrence of serological cross-reactions in systemic mycotic infections has been accepted as a rule rather than exception (Smith et al., 1949, Salvin, 1949, Campbell and Binkley, 1953, Labzofsky et al., 1957, Campbell, 1960 and 1965). Histoplasma antigens cross-react with the sera from cases of blastomycosis and coccidioidomycosis and Blastomyces antigens cross-react with the sera of histoplasmosis and coccidioidomycosis. Even coccidioidin, the most specific of the three antigens, occasionally also cross-reacts with the sera from proven cases of histoplasmosis.

Besides these, major antigenic sharing, less recognized minor cross-reactions between the antigens of H. capsulatum and C. neoformans and Candida albicans, have been demonstrated (Salvin, 1949, 1950). Also, C. neoformans and A. fumigatus, have been isolated sporadically from patients who gave positive serologic reactions with H. capsulatum and B. dermatitidis antigens (Heller et al., 1957 and Campbell, 1965a).
Kaufman and Brandt (1964), in their fluorescent antibody studies of the mycelial form of *H. capsulatum* and morphologically similar fungi, have shown cross-reactions between *H. capsulatum*, *S. amphotericum* and *C. cinerea*. *H. capsulatum* was also found to share antigen(s) with *B. dermatitidis*, *H. farcinosum*, and *Emmonsia parva*.

Seeliger and Schroter (1963), in an extensive serologic study on the antigenic relationships of the form genus *Trichosporon*, on the basis of agglutination, precipitation, agar-gel diffusion and complement fixation tests with the whole cell antigens and crude polysaccharide extracts, have shown that eight species of the genus *Trichosporon* can be subdivided in three, or possibly four, serological groups. "Strains belonging to serogroup-1 (T. fermentans, T. capitatum, T. cericeum) give numerous unilateral or reciprocal cross-reactions with *Geotrichum candidum*, and *Nadsonia elongata*. Strains of serogroup-2 (T. cutaneum, T. infestans, T. margaritiferum) show common partial antigens with *Cryptococcus* species, *Candida curvata*, and *G. humicola*. T. pullulans holds an intermediate position between serogroup 1 and 2. T. behrendii is serologically distinct from the remaining *Trichosporon* species."

Torheim (1963) in an immunochemical investigation in *Geotrichum* and certain related fungi, classified them into three serological groups. Group-1 includes *Geotrichum candidum*, *G. steroides*, *G. javanense*, *G. matalense*, *G. pulmonium*, *G. versiforme*, and *G. pullulans*. Group-2 includes *T. pullulans* and *T. capitatum*. Group-3 includes *T. pullulans* and *T. fermentans*.
Endomyces lactis and E. magnusii. Group 2 includes G. amyelicum, G. hirtum and T. cutaneum. Group 3 comprises only Endomycopsis selenospora. The above findings suggest that serologic relationships are extensive and probably very complex.

The possibility of antigenic sharing between H. capsulatum and other still unrecognized mycotic agents causing systemic infections has been pointed out repeatedly (Emmons et al., 1945, Palmer et al., 1957, Campbell, 1960 and 1965a and others), but no published records are available to support this contention. Certain species of Trichosporon, and the genus Geotrichum, occupy an intermediate position morphologically between the true dimorphic fungi and yeasts. It may be that Trichosporon, by virtue of its interesting position, has antigens in common with the true dimorphic fungi like H. capsulatum.

The present serological experiment was planned to show possible antigenic sharing between a Trichosporon-like organism and H. capsulatum by using collodion agglutination and immunodiffusion tests.
IV. Cross-Protection between *H. capsulatum* and Other Fungi

The first successful attempt to immunize mice against histoplasmosis by using formalin-killed acetone-dried yeast cells, mycelial broth filtrate, and yeast-phase broth filtrate as immunizing agents was made by Salvin in 1953. Significant protection to intracerebral challenge with 300-400 LD-50's of yeast cells of *H. capsulatum* was observed six days after intraperitoneal immunization with either of the three vaccines. Out of several immunization routes, viz. intraperitoneal, intravenous, subcutaneous, intracerebral, oral and intrapodial, the first two were found to be most effective. Cross-protection between *H. capsulatum* and *B. dermatitidis* was not demonstrated, when acetone-dried yeast cells were used as immunizing agents.

Farrell *et al.* (1953) have reported that although dogs inoculated intra-tracheally with mycelial phase organism showed no clinical symptoms, they were resistant to subsequent challenge with mycelial phase organisms plus cortisone. This combination produced severe disease in control dogs.

Schaefer and Saslaw (1954) have shown significant protection in mice immunized with either heat killed cells or sublethal living cells to subsequent intraperitoneal challenge with yeast cells of *H. capsulatum* six weeks later.

Marcus and Rambo (1955) have reported successful immunization of mice by i.p. inoculation of formalin killed yeast cells to
Salvin (1955a) has shown that sublethal infection of mice with yeast cells of *H. capsulatum* gives significant protection against subsequent lethal intracerebral challenge. Neither the size nor the route of administration of the sublethal infection was of great importance, since mice injected i.p., i.v., i.c., or s.c. all showed resistance to lethal intracerebral challenge. No cross-protection after sublethal infection with either *H. capsulatum*, *C. albicans* or *B. dermatitidis* was demonstrated. In marked contrast to the lack of cross-reaction between these fungi, resistance to *R. typhi* and *R. tsutsugamushi* has been demonstrated in mice previously injected with sublethal doses of yeast cells of *H. capsulatum* (Salvin and Bell, 1955).

Salvin (1955b) while further studying the immunization of mice against *H. capsulatum* infection, reported that mice immunized with acetone-dried yeast cells of *H. capsulatum* had fewer organisms than non-immunized mice. Immunization was relative in that it tended merely to lower the number of cells in various tissues, depending on the route of challenge.

Grayston and Salvin (1956) have reported a comparative histopathological study of experimental *H. capsulatum* infection in mice immunized with formalinized yeast cell vaccine and prior sublethal infection. Immunization by either method ameliorated the course of the disease following intracerebral challenge.

Rowley and Huber (1956) have shown significant protection
to subsequent intravenous challenge in mice infected intraperitoneally with sublethal doses of yeast cells of *H. capsulatum*. Immunization of mice with heat-killed, formalin-killed or asphyxiated vaccines did not protect them against i.v. challenge with yeast cells of *H. capsulatum*.

Saslav and Schaefer (1956) reported that *H. capsulatum* was recovered frequently from reticuloendothelial tissues as late as 45 weeks after intraperitoneal infection with yeast cells of *H. capsulatum*. Mice surviving previous sublethal challenge or those previously immunized with heat killed vaccine showed little variation as to the presence of organisms in tissues, when challenged, from those receiving primary challenge.

Hill (1959) reported that mice immunized with killed whole yeast-phase cells or protein polysaccharide fractions of *H. capsulatum* showed significantly higher protection to intravenous challenge. A significant increase in resistance was also encountered in animals with induced chronic sublethal infection. Slight but significant increases in numbers surviving challenge with *B. dermatitidis* occurred after injection with a less virulent strain.

Hill and Marcus (1959) have shown induced resistance to subsequent intravenous challenge with yeast cells of *H. capsulatum* in mice immunized with formalinized yeast cell vaccine by intraperitoneal, intramuscular, or subcutaneous methods but not by the oral route. Similar protection was observed after sublethal
intraperitoneal infection with yeast cells of *H. capsulatum*. No significant difference was found between the extent of resistance induced by infection and that induced by injection of killed vaccine.

Successful immunization of mice against intravenous homologous challenge was obtained with polysaccharides derived from yeast phase cells of *H. capsulatum* (Knight et al., 1959). However, formalinized yeast cell vaccine gave greater protection than extracted polysaccharide vaccine.

Cross-protection between *H. capsulatum* and *B. dermatitidis* has been demonstrated by using sublethal doses of yeast cells as immunizing agents (Salfelder and Schwarz, 1964 and Sethi, Salfelder and Schwarz, 1964). The protection was tested by lethal intravenous challenge of the immunized mice with the heterologous organisms. Significant homologous protection was also observed in mice immunized with live yeast cells of *H. capsulatum*. Surprisingly, no homologous protection could be demonstrated when *B. dermatitidis* sensitized animals were challenged with *B. dermatitidis*.

This experiment has been designed to obtain information on the extent of cross-protection against lethal challenge by *H. capsulatum* provided by the immunization of mice with "vaccines" prepared from a *Trichosporon*-like organism. It is hoped that the information obtained may ultimately contribute to the development of an effective vaccine for human use.
MATERIALS AND METHODS

Organisms, sources and maintenance

The strains of *Histoplasma capsulatum* used in this study were provided by the courtesy of Professor Charlotte C. Campbell, Harvard University School of Public Health, Boston, Massachusetts, and Miss Virginia Torbet, of The Ohio State University Hospital.

The available information regarding the sources and dates of isolation of the nine strains of *H. capsulatum* studied have been summarized in Table 1. Hereafter each strain will be referred to merely by the corresponding number from this table. The strain Y-4, a Trichosporon-like organism used in this study, was isolated in India in 1961.

**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Place and Date of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH-1</td>
<td>Soil</td>
<td>Weymouth, Massachusetts, May, 1963</td>
</tr>
<tr>
<td>G-2</td>
<td>Human</td>
<td>Received from Dr. N. F. Conant (#715), January, 1947</td>
</tr>
<tr>
<td>G-13</td>
<td>Dog</td>
<td>Walter Reed Army Hospital, Washington, D.C.</td>
</tr>
<tr>
<td>G-46</td>
<td>Human</td>
<td>Thoracentesis fluid or blood - Walter Reed Army Hospital, Washington, D.C., February, 1956</td>
</tr>
<tr>
<td>G-76</td>
<td>Human</td>
<td>Infant spleen, Walter Reed Army Hospital, Washington, D.C., March, 1961</td>
</tr>
<tr>
<td>G-89</td>
<td>Bat</td>
<td>Bat spleen - Panama, C.Z., October, 1961</td>
</tr>
<tr>
<td>G-91</td>
<td>Human</td>
<td>Minneapolis, Minnesota, Date Unknown</td>
</tr>
</tbody>
</table>
The yeast-phase stock cultures were maintained on a modified Brain Heart Infusion Agar (Difco) (BHIA) slant having the following composition:

- Infusion from calf brain: 200 gm
- Infusion from beef heart: 250 gm
- Proteose Peptone, Difco: 10 gm
- Bacto-Dextrose: 2 gm
- Sodium chloride: 5 gm
- Disodium phosphate: 2.5 gm
- Agar: 15 gm
- Distilled water: 1000 ml

This medium was supplemented with 1 per cent dextrose and 0.001 per cent cystine hydrochloride and sterilized by autoclaving for 15 minutes at 121°C. The cotton gauze plug of each slant was sealed with vaspar (three parts paraffin 55°C plus one part liquid petroleum). Each strain was subcultured at 3-4 weeks intervals to fresh BHIA slants, incubated at 37°C for 48 hours then regrigerated at 4°C.

The mycelial-phase stock cultures were maintained on Emmons' modification of Sabouraud Dextrose Agar (ESDA) slants having the following composition:
Neopeptone Difco 10 gm
Bacto-Dextrose 20 gm
Bacto Agar 15 gm
Distilled 1000 ml

The medium was sterilized by autoclaving for 15 minutes at 121°. Each strain was subcultured at 2-3 months' intervals and kept at room temperature in a rack with two open test tubes of tap water, the whole rack being covered by a plastic sack.

**Standardization of inoculum**

The yeast cell suspensions of G-91 for animal inoculation were standardized turbidometrically with a Bausch and Lomb "Spectronic 20" spectrophotometer at 550 lambda. Cell suspensions were prepared from yeast cultures of G-91 grown on BHIA at 37° for 72 hours. Growth was harvested with 0.5 per cent formol saline and refrigerated overnight at 4°. The suspension was washed twice with 0.1 per cent formol saline and centrifuged at 500 rpm for two minutes in a Sorvall refrigerated centrifuge to remove large cell aggregates. The supernate was serially diluted with 0.1 per cent formol saline to yield eight suspensions of from 20 per cent to 90 per cent light transmittance, at 10 per cent intervals. The cell counts were made from each of the eight suspensions in a hemocytometer chamber. Buds larger than one-third of parent cells were counted as separate cells. The dilution and counts were repeated until reproduceable results were obtained. The cell counts were plotted against the corresponding light transmittance to get a standard curve. All subsequent suspensions for animal
inoculation were made on the basis of this curve (Graph I).

The standard curve for Y-4 was made in essentially the same way as described for G-91 except that 24-hour cultures were used and centrifugation was for three minutes at 500 rpm (Graph I).

Laboratory animals

Mice: White, Swiss, male mice (ICR strain), randomly bred, weighing 14-16 gm, used in this study were obtained from Harlan Industries, Inc., Cumberland, Indiana. The animals were distributed in groups of five each in individual disposable cages by a random procedure and were fed Purina laboratory chow and water ad libitum.

Rabbits: White, male, New Zealand rabbits weighing 4-6 lb., obtained from a local dealer, were used for the preparation of antisera. Animals were housed in separate cages and were fed rabbit chow and water ad libitum.

Guinea pigs: White, male guinea pigs, weighing 300-350 gm obtained from a local dealer were used for the demonstration of skin test cross-reactivity. Two guinea pigs were housed in each cage, and fed laboratory chow and water ad libitum.
GRAPH - I  STANDARD CURVE OF G-91 AND Y-4
I. Morphology

The morphological characteristics of Y-4 were studied both in yeast and mycelial phases and compared with the corresponding phases of G-91.

A. Yeast-phase
1. Gross morphology - was studied by growing the organism on BHIA and blood agar (BA) media at 37° for seven days. Preparation of BHIA has been described earlier. The blood agar (BA) plates were prepared from Difco blood agar base, supplemented with 5 per cent fresh citrated human blood.
2. Microscopic morphology - was studied by making lactophenol cotton blue mounts of the yeast-like colonies on BHIA medium.

B. Mycelial phase
1. Gross morphology - of the organisms was studied over a period of time by developing a single colony on ESDA medium at room temperature.
2. Microscopic morphology - was studied by making slide cultures of the organisms on the ESDA and BA media according to the procedure described by Ajello et al. (1963).
II. Pathogenicity

A. Pathogenicity of *H. capsulatum* in mice

1. Preliminary pathogenicity trial of nine strains of *H. capsulatum*: Nine strains of *H. capsulatum* (GH-1, G-2, G-13, G-46, G-76, G-89, G-91, G-132, G-X) were screened in a preliminary trial for mouse pathogenicity in order to select a suitable strain for LD-50 determination.

**Inocula:** Inocula were prepared from yeast cultures of *H. capsulatum* grown on BHIA at 37° for 72 hours. Growth was harvested with cold 0.1 per cent cysteine saline (CSS) as recommended by Rowley and Huber (1955). The yeast cell suspension was washed twice with CSS and centrifuged at 500 rpm for two minutes in a Sorvall refrigerated centrifuge to remove large aggregates. The supernate was diluted with CSS and adjusted to yield a suspension with 50 per cent light transmittance at 550 lambda on a Bausch and Lomb "Spectronic 20" spectrophotometer. This suspension containing approximately 64 million cells per ml, when buds larger than one-third the size of the parent cell were counted as separate cells, was taken as the first inoculum. A portion of this suspension was diluted with CSS to yield the second suspension containing eight million cells per ml. All the cell suspensions from the time of harvesting to mouse inoculation were maintained in an ice bath as described by Campbell et al. (1954).
Mouse inoculation: Ten mice were used for each of the nine strains of *H. capsulatum* and were distributed randomly into two groups (a and b) of five each in separate cages (Table 2). Prior to intravenous injections, the mice were exposed for 15 minutes to a 100 watt electric bulb to produce peripheral vasodilatation. Each mouse of group-a was inoculated intravenously with 32 million cells and that of group-b with 8 million cells suspended in 0.5 ml of CSS respectively. Five mice were kept as controls and inoculated intravenously with 0.5 ml of CSS (Table 3).

All inoculated mice were examined every morning for a period of 30 days, deaths recorded, and at least one mouse of each group was autopsied and a portion of liver and spleen was cultured on a BHIA plate. The plates were incubated at room temperature for a period of four weeks before being discarded as negative.

2. LD-50 determination of G-91

On the basis of preliminary pathogenicity trial, strain G-91 of *H. capsulatum* was selected for LD-50 determination. A total of 80 white, male, Swiss mice weighing 14-16 gm were randomly distributed into eight groups of 10 mice (five per disposable cage). Another group of five, similar mice was kept as control.

The procedures for the preparation of yeast-phase cell suspension of G-91 and mouse inoculation were exactly the same as described earlier under preliminary pathogenicity trial. The
suspension containing approximately 64 million cells per ml was
diluted serially in twofold dilutions to yield eight different
suspensions containing 32, 16, 8, 4, 2, 1, 0.5 and 0.25 million
cells in 0.5 ml of CSS. Each suspension was transferred into a
separate sterile, 20 ml vaccine bottle and kept in an ice bath.
Each group of mice was inoculated intravenously with 0.5 ml of
one of the suspensions. Animals of the control group were inocu­
lated with 0.5 ml of CSS (Table 4).

All inoculated mice were counted every morning for a
period of 30 days, deaths recorded and at least two mice of
each group were autopsied and pieces of spleen and liver were
cultured for H. capsulatum. All plates were incubated at room
temperature for four weeks.

3. Effect of cortisone and streptomycin

The suspension containing approximately 64 million cells
per ml was diluted with CSS to obtain the final inocula contain­
ing one and four million cells per 0.5 ml respectively. All the
cell suspensions from the time of harvesting to the mouse inocu­
lations were maintained in an ice bath.

Experimental procedure: A total of 110 white, male, Swiss
mice were randomly distributed into 11 groups each consisting of
10 mice (five per disposable cage). The animals of groups I, II,
III, VI, VII and X were inoculated subcutaneously with five mgm of
cortisone (Cortone Acetate, Merck Sharp and Dohme) two days before
and were given two supplementary doses of 2.5 mgm two and five days
after intravenous infection with \textit{H. capsulatum} (Sidransky and Friedman, 1959). The animals of groups I, II, IV, VI, VIII and XI each were given daily intraperitoneal injection of 5 mg streptomycin sulfate (Squibb) suspended in 0.5 ml saline, starting from two days before to 30 days after the intravenous injection with \textit{H. capsulatum} or until death. The animals not receiving cortisone were inoculated subcutaneously with 0.5 ml saline at the time when the other mice were given cortisone (Table 5).

Infection: Prior to intravenous injection the mice were exposed for 15 minutes to a 100 watt electric bulb attached to a table lamp to produce peripheral vasodilatation. Injections of the standardized yeast suspension of G-91 in 0.5 ml CSS were made in a lateral caudal vein using a 0.5 ml tuberculin syringe with a \( \frac{1}{2} '' \), 25 gauge needle. The mice of groups II to V were each infected intravenously with one million yeast cells while those of groups VI to IX were infected with four million yeast cells of \textit{H. capsulatum} two days after starting cortisone and streptomycin treatment. The mice of groups I, X and XI were kept as non-infected controls for cortisone and streptomycin in combination, cortisone alone and streptomycin alone, respectively, and were inoculated intravenously with 0.5 ml of CSS.

All animals were weighed two days before and 10, 20, and 30 days after the inoculation. The mice were examined every morning, deaths recorded, and autopsies performed. Portions of spleen and liver were cultured on a BHIA and on a Brain Heart Infusion Agar with chloramphenicol and cyclohexamide (Difco),
(BHCCA). BHIA plates were prepared according to the formula described earlier for the maintenance of yeast-phase cultures. The BHCCA (Difco) has essentially the same composition as BHIA (Difco) except the former has been supplemented with 0.005 per cent of chloramphenicol and 0.05 per cent of cycloheximide (Actidione-Upjohn) to make it more selective for the isolation of most pathogenic fungi. The inoculated plates were incubated at room temperature for four weeks before being discarded as negative.

All surviving animals were sacrificed on the 30th day after inoculation, but the autopsy and culture work were done only on _H. capsulatum_ infected mice.

B. Pathogenicity of Y-4 in mice

1. Preliminary pathogenicity trial

   Mice: Thirty white, male, Swiss mice weighing 14-16 gm were distributed in four groups (five per disposable cage) by a random procedure; groups A and B consisting of 10 mice each while groups C and D each were comprised of five mice.

   Inoculum and mouse inoculation: The inoculum containing approximately 70 million yeast cells per ml was prepared according to the standard procedure described earlier, from a 24-hour growth at 37° on BHIA medium. Mice of group A were inoculated intravenously with 0.5 ml of the suspension (35 million cells) and those of group B were inoculated intraperitoneally with 1 ml of the suspension (70 million cells). Mice of groups C and D were kept as
controls and were inoculated intravenously with 0.5 ml CSS and intraperitoneally with 1 ml CSS respectively (Table 7).

All mice were examined every morning, deaths recorded the day they occurred, autopsies performed, and a piece of spleen, kidney, liver, lung and brain were cultured separately on a BHCC and ESDA plates. The plates were incubated at room temperature for 30 days before being discarded as negative. Two of the surviving mice of group A were sacrificed on the 30th day and the remaining two were sacrificed on the 60th day. Five of the 10 mice of group B were sacrificed on the 30th day and the remaining four surviving were sacrificed on the 60th day. All control mice of groups C and D were sacrificed on the 60th day. Autopsies were performed on all sacrificed mice and culture was done as described above with mice dying spontaneously.

**Histopathological examination:** A portion of spleen, kidney, liver, lung and brain from autopsied mice was preserved in 10 percent formol saline in a separate container for each. The sections were cut after paraffin embedding, were stained by hematoxylin and eosin (H & E) and also by Gomori's Methanamine-Silver nitrate technic (G.M.S.) (Grocott, 1955).

2. Demonstration of "toxin" production by Y-4

This experiment was designed to arrive at some possible explanation of a neurologic syndrome shown by mice, inoculated intravenously with Y-4, in terms of some "toxin" production by the organism.
Preparation of "toxins": Three different "toxic" preparations were prepared from the yeast cells of Y-4, according to the procedure described below:

**A-Filtrate from broken cells:** Forty-eight-hour growth of Y-4 at 37° was harvested with a small amount of cold sterile saline to make a thick suspension. The cells were broken with help of 0.2 mm glass beads by grinding for 30 minutes in a Virtis homogenizer No. 32, while keeping the suspension in an ice bath. The broken cell suspension was diluted with 20 ml of cold saline and sterilized by filtration through a cold Selas' filter (0-2 porosity) and was kept frozen in a deep freeze at -15°.

**B-Filtrate from growth in Salvin's medium:** Yeast cells of Y-4 were grown at 37° for seven days in a 250 ml Erlenmyer flask containing 100 ml of Salvin's liquid medium (Salvin, 1950) on a rotatory shaker. The composition and preparation of Salvin's medium will be described in a later section.

Growth in liquid medium was filtered through Selas' filter (0-2 porosity) and filtrate was stored frozen at -15°.

**C-Heat-killed cell suspension:** Forty-eight-hour growth of Y-4 at 37° on BHIA medium was suspended in cold sterile saline. The suspension was washed three times with cold saline and adjusted turbidometrically to yield a suspension containing approximately 34 million cells per ml. The suspension was kept in a 60° water bath for one hour and was then kept frozen at -15°.

All three "toxic" preparations (A, B and C) were tested for sterility by subculture on blood agar plates and in thioglycollate
broth tubes incubated both at 37° and room temperature for seven days. Frozen suspensions were thawed immediately before inoculation.

**Experimental procedure:** Forty male, white, Swiss mice weighing 14-16 gm were distributed in eight groups of five each, by a random procedure. Mice of groups I, III, V, VII and VIII were inoculated subcutaneously with 5 mg of cortisone (Cortone Acetate, Merck, Sharp and Dohme) two days before and 2.5 mg 2, 5 and 7 days after intravenous injection with the different "toxic" preparations. Mice of groups II, IV and VI were inoculated subcutaneously with 0.5 ml of saline at the time when others were given cortisone treatment. Mice of groups I and II were injected with filtrate from broken cells (A); groups III and IV with Salvin's medium culture filtrate (B); groups V and VI with heat killed cell suspension; group VII with uninoculated Salvin's medium and group VIII with saline. The first injection of the different "toxic" preparation was given intravenously in 0.5 ml quantities two days after the start of cortisone treatment. The second dose was given intraperitoneally in 1 ml quantities seven days later, the day when fourth injection of cortisone was given (Table 10).

All animals were weighed two days before and 10, 20, and 30 days after injections with different suspensions. Mice were examined every morning, deaths recorded the day they occurred, autopsies performed and a portion of spleen and liver was cultured on a BHCCA and BHIA plates. The inoculated plates were incubated at room temperature for four weeks.
All surviving mice at the end of a 30 day period were sacrificed and no autopsy or culture work was done on these animals.

3. Effect of cortisone and streptomycin

Mice: A total of 45 white, male, Swiss mice weighing 14-16 gm were randomly distributed into five groups; A, B, C, and D consisting of 10 mice each, while group E (control) contained five mice. Mice were housed five per disposable cage.

Inoculum and mouse inoculation: A yeast cell suspension of Y-4 containing approximately 3^4 million cells per ml was prepared from a 24-hour culture according to the standard procedure described earlier. Mice of group A and C were inoculated subcutaneously with 5 mg of cortisone (Cortone Acetate, Merck, Sharp and Dohme) two days before, and 2.5 mg two and five days after intravenous infection with Y-4. Mice of groups C and D were given daily intraperitoneal injections of 5 mg streptomycin sulfate (Squibb) in 0.5 ml saline, starting from two days before to 30 days after, intravenous injection with Y-4 (or until death). Mice not receiving cortisone were inoculated subcutaneously with 0.5 ml saline at the time when others were given cortisone. Mice of groups A, B, C, and D were infected intravenously with 17 million cells of Y-4 in 0.5 ml CSS and those of group E were injected intravenously with 0.5 ml CSS only (Table II).

All mice were examined every morning, deaths recorded the day they occurred, autopsies performed and a piece of spleen,
kidney, liver, lung and brain was cultured on a BHCC and ESDA plates. The plates were incubated at room temperature for four weeks before being discarded as negative.

Histopathological examination: Portions of spleen, kidney, liver, lung and brain were preserved in 10 per cent formol saline soon after death. Sections were cut by paraffin embedding and were stained by H and E and G.I.S. technics.
III. Immunology

A. Skin test cross-reactions between *H. capsulatum* (G-76 and G-91) and Y-4

Preparation of antigen: Antigens were prepared from both yeast and mycelial phases of two strains of *H. capsulatum* (G-76 and G-91) and one strain of Y-4.

Mycelial phase: Mycelial phase antigen was prepared according to the procedure described by Emmons et al. (1945) in a synthetic asparagine medium having the following composition:

1-Asparagine........................... 14.00 gm
Dipotassium phosphate
c.p. (K₂HPO₄)......................... 1.31 gm
Sodium citrate c.p. .................... 0.90 gm
Magnesium sulphate (U.S.P.)......... 1.50 gm
Ferric citrate (U.S.P.) Scales........ 0.30 gm
Dextrose (Difco)....................... 10.00 gm
Glycerine c.p. (U.S.P.)................. 25.00 gm
Demineralized double distilled water to make.................. 1,000.00 ml

The medium was dispensed in one liter Erlenmeyer flasks, 250 ml per flask, and sterilized by autoclaving at 115° for 25 minutes. Bits of dry mycelium from ESDA slant culture were floated on the surface of the medium and the flasks were incubated in the dark at room temperature with frequent shaking for 2-3 months. The culture was then filtered through Whatman filter paper No. 1 and sterilized by filtration through Selas' filter (0.2 porosity). Sterility of the filtrate was tested by subculturating on a B.A. plate and in thioglycollate broth for two weeks at 37° and at room temperature. Merthiolate was added to the filtrate to give a final concentration
of 1:10,000. The stock solution was stored at 4°. The stock solution was diluted 1:10 for use as a skin test antigen.

**Yeast-phase antigen:** Yeast phase antigen was prepared by growing the organism in Salvin's medium (Salvin, 1950b), having the following composition:

- Casamino acids: 10 gm
- Dextrose (Difco): 3 gm
- Sodium chloride: 2.5 gm
- Potassium chloride: 2.5 gm
- Dipotassium phosphate: 2.5 gm
- Biotin: 20 microgram
- Penicillin: 20,000 units
- Streptomycin: 40,000 units
- Demineralized double distilled water - to 1000 ml

All ingredients except penicillin, streptomycin, and biotin were dissolved in distilled water, adjusted to pH 7.2, dispensed in 100 ml quantities, in Erlenmeyer flasks and sterilized by autoclaving at 121° for 15 minutes. Sterile solutions of biotin, penicillin and streptomycin were added to each flask separately after autoclaving. The flasks were inoculated each with 1 ml of yeast cell suspension adjusted to 20 per cent light transmittance at 550 lambda on a Bausch and Lomb "Spectronic 20" spectrophotometer and were grown on a rotatory shaker at 37° for three weeks. The culture medium was filtered through a sintered glass filter (grade F), lyophilized and stored at -15°. A 1:100 aqueous solution of this lyophilized material, after filtration through Selas' filter (0-2 porosity) and sterility testing was used as skin test antigen.
Sensitization of guinea pigs: Eight white, male guinea pigs weighing 300-350 gm each were distributed into four groups of two per cage by a random procedure. Guinea pigs of groups A and B were skin tested with yeast-phase antigen of G-91 and those of group C were tested with Y-4 antigen. Guinea pigs of group D were skin tested both with G-91 and Y-4 antigens and were kept as non-infected controls (Tables 12 and 13).

The yeast cell suspensions of *H. capsulatum* and Y-4 for guinea pig inoculation were prepared according to the standard procedure described earlier. Guinea pigs of groups A (Pigs \#1 and \#2) and B (Pigs \#3 and \#4) were infected with approximately six million cells (two mouse LD-50 of G-91) of G-76 and G-91 respectively. Guinea pigs of group C (Pigs \#5 and \#6) were infected with approximately 34 million cells of Y-4 (corresponds to 10 mouse LD-50 of G-91) and those of control group D (Pigs \#7 and \#8) were injected with 1 ml CSS. All injections were made intraperitoneally with cells suspended in 1 ml CSS.

The guinea pigs were skin tested by intradermal inoculation of 0.1 ml of both yeast and mycelial phase antigens at 6 and 12 weeks' intervals after infection, according to the plan presented in Tables 12 and 13. Uninoculated sterile Salvin and asparagine media were used simultaneously for skin testing to eliminate the possibility of their cytotoxicity. Skin reactions were read at 24, 48 and 72 hour intervals. Only reactions showing an area of induration 5 mm or more were considered as positive.
B. Serological cross-reactions between G-91 and Y-4

Preparation of Antisera: The hyperimmune sera, prepared in rabbits, by different methods, against six strains of \textit{H. capsulatum} (GH-1, G-2, G-46, G-76, G-89 and G-91) were provided by Professor Campbell. Antisera against Y-4 were prepared in rabbits by using yeast cell vaccine.

Vaccine: Formalinized yeast cell vaccine of Y-4 containing approximately 34 million cells per ml was prepared according to the procedure to be described later under cross-protection studies.

Animal: Three white male New Zealand rabbits weighing 4-6 pounds were used for the preparation of antisera. Prior to immunization baseline bleeding was done on each rabbit by cardiac puncture.

Immunization: Immunization of rabbits was performed according to the procedure recommended by Campbell (1965b). Immunization was done by intravenous inoculation in the marginal ear vein of 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1, 2 and 2 ml of vaccine on days 1, 2, 3, 4, 7, 8, 9, 10, and 11 respectively. Trial bleeding was done on the 18th day and at weekly intervals from then on, following a booster dose of 0.5 ml of vaccine. Blood after clotting was stored overnight at \(4^\circ\), serum separated and stored at \(-19^\circ\) after the addition of merthiolate to the final concentration of 1:10,000.
Antigens: Preparation of soluble yeast phase antigens used in serological procedures has been described earlier (under skin cross-reactivity in guinea pigs). Lyophilized yeast phase antigens of *H. capsulatum* (G-91) and (Y-4) were dissolved in distilled water (100 mg per ml) and stored at -15°.

All sera were tested simultaneously by collodion agglutination and immunodiffusion tests using yeast phase lyophilized antigens of G-91 and Y-4.

1. Collodion agglutination test

This test was performed according to the procedure described by Saslaw and Campbell (1948).

Collodion particles: Collodion particles, using liquid collodion (Merck), were prepared according to the method of Cavelti (1947) modified by Saslaw and Campbell (1948). The final particle suspension of uniform size (4-5 micron) was obtained by collecting the supernate after centrifugation for three minutes at 2500 rpm in a No. 2 International centrifuge. The suspension was then diluted with demineralized double distilled water and adjusted to five per cent light transmittance at 500 lambda on a Bausch and Lomb "Spectronic 20," spectrophotometer. The particle suspension was stored in the dark in a large rubber stoppered flask at room temperature.

Titration of antigen: The optimum dilution of yeast phase antigens of G-91 and Y-4 for use in the collodion agglutination test was determined by titration against homologous sera as shown in Tables 14 and 15.
Procedure: Collodion particle suspension was sensitized by adding an equal volume of 1:200 dilution of lypholized yeast phase antigen (5 gm per ml). The mixture was shaken thoroughly and kept at room temperature for one hour. All sera were inactivated at 60° for 30 minutes and serial dilutions were made in normal saline so that each tube contained 0.5 ml. To each tube 0.5 ml sensitized particles was added. The control tube contained 0.5 ml of the lowest dilution of the serum (1:5) plus 0.5 ml of the unsensitized particles diluted with an equal quantity of saline. The tubes were shaken vigorously, incubated at room temperature for two hours, centrifuged for two minutes at 1400 rpm in a No. 2 International centrifuge. The results were read by gently flicking the tubes in front of a table lamp using a black screen as the background. Agglutinations were considered four plus, when the diameter of the flakes was about 1 to 1 and ½ mm or over, with the decreasing gradually of three plus, two plus and one plus.

2. Immunodiffusion test

Immunodiffusion test was carried out according to the technic described by Huppert and Baily (1963) with slight modification. Immunodiffusion reactions were performed in 0.75 per cent Ionagar (Oxoid) No. 2, buffered at pH 7.0 with M/15 phosphate buffer which was prepared every week. The suspension was dissolved, sterilized at 15 pounds pressure for 15 minutes, cooled to 50°, aqueous merthiolate solution was added to a final concentration of
1:10,000. This medium was poured in 15 ml quantities into siliconized 100 ml plastic Petri dishes and stored overnight at 4°, before use. Five and seven well patterns of 6 mm diameter, consisting of a central well surrounded by 4 and 6 concentric wells respectively with wall to wall distance of 10 mm were cut with a cork borer. The sera were put into the wells two hours before the addition of antigen and incubated in a moist chamber at room temperature. Readings were made daily for five days.
IV. Cross-Protection

Preparation of vaccines: Both live and formalinized vaccines prepared from yeast cells of *H. capsulatum* (G-91) and *Y-4*, grown on BHIA medium in Kolle flasks at 37°, were used for the immunization of mice.

Formalinized vaccine: Seventy-two-hour growth of yeast cells of G-91 was harvested with 10 ml of 0.5 per cent formol saline and refrigerated overnight at 4°. The cell suspension was washed and diluted as previously described to yield approximately eight million cells per ml. The vaccine was transferred aseptically to a 20 ml sterile bottle and tested for sterility by subculturing on a blood agar plate and in a thioglycollate broth, incubated at 37° and at room temperature for 14 days. Similarly two other vaccines were prepared at weekly intervals, each containing approximately 16 million cells per ml, and were used to provide first, second and third immunizing doses.

Three *Y-4* formalinized vaccines containing 8, 16 and 16 million cells per ml were prepared as described above for the G-91 vaccine, except that a 48-hour growth was used and centrifugation was at 500 rpm for three minutes.

Live vaccines

G-91 vaccine. A yeast cell suspension containing 0.5 million cells per ml was prepared from a 72-hour culture at 37° on a BHIA medium according to the standard procedure described earlier.
**Y-4 vaccine.** A Y-4 live vaccine containing 0.5 million yeast cells per ml was prepared from a 24-hour growth in the same way.

**Mice:** A total of 100 white, male, Swiss mice were distributed in five groups, viz. A, B, C, D, and E, each consisting of 20 mice (five per disposable cage), by a random procedure.

**Immunization:** Mice of group A were immunized by intraperitoneal inoculation of 0.5 million live cells of G-91, contained in 1 ml of CSS while those of group B were immunized by intraperitoneal inoculation with 0.5 million cells of Y-4 suspended in 1 ml of CSS. Immunization with formalinized vaccine was carried out by three intraperitoneal injections of 1 ml of formalin-killed vaccine. The first, second and third doses contained approximately 8, 16 and 16 million cells per ml respectively. Mice of group C were immunized with formalinized vaccine of G-91 and those of group D were immunized with formalinized vaccine of Y-4. Mice of group E were kept as non-immunized control and were given three intraperitoneal injections of 1 ml of 0.5 per cent formal saline at the time when mice of other groups were given formalinized vaccines (Table 18).

**Challenge:** An inoculum containing approximately 64 million yeast cells of *H. capsulatum* (G-91) per ml used for challenging the mice was prepared by the standard procedure described earlier. Both immunized and non-immunized mice were challenged intravenously on the 21st day after the first immunizing dose with
approximately 32 millions (10 X LD-50) of G-91 suspended in 0.5 ml CSS. All mice were examined every morning for 30 days and deaths recorded on the day they occurred. At least two mice of each group were autopsied and a piece of spleen and liver was cultured on a BHIA plate. The plates were incubated at room temperature for four weeks.
RESULTS

I. Morphology

A comparative account of the morphological characteristics of Y-4 and G-91 is presented in Table 2.

TABLE 2

<table>
<thead>
<tr>
<th>MORPHOLOGICAL CHARACTERISTICS OF Y-4 AND G-91</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Y-4</strong></td>
</tr>
<tr>
<td>A. Yeast-phase</td>
</tr>
<tr>
<td>1. Gross morphology</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>EHIA slant at 37°C: The fungus grew rapidly and developed into a cream-colored, yeast-like culture in 24 hours; at 48 hours wrinkles began to appear on the surface and spread over the growth within 7 days (Fig. 1).</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>BA plate at 37°C after 96 hours: Round, elevated, non-hemolytic colonies with entire edge formed. At 72 hours a hazy zone of partial hemolysis developed around the colonies; this became more pronounced at 96 hours but did not develop into a frank hemolysis (Fig. 2).</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>EHIA plate at 37°C after 96 hours: Round elevated cream-colored colonies with entire edge were formed (Fig. 3).</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>G-91</strong></td>
</tr>
<tr>
<td>Similar to Y-4 but growth rate was slower, 48-hour growth being comparable to that of Y-4 after 24 hours. Wrinkling of the surface was minimal (Fig. 1).</td>
</tr>
<tr>
<td>Similar to Y-4 except that growth rate was slower and the colonies were non-hemolytic.</td>
</tr>
<tr>
<td>Similar to Y-4 except that growth rate was slower.</td>
</tr>
<tr>
<td>TABLE 2--Continued</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
</tbody>
</table>

**ESDA plate at room temperature after 7 days:**
Hyphae began to appear at the margin of the colonies and almost completely converted to the filamentous form after 4 weeks (Fig. 4).

**2. Microscopic morphology:**
Lactophenol mount from 24-hour growth on BHIA showed mainly oval and elongated budding cells (2.5-3.5 u in diameter). On aging it showed a mixture of blastospores and arthrospores. (Fig. 5).

**B. Mycelial phase**

1. **Gross morphology:**
ESDA at room temperature for four weeks: white, granular, filamentous colonial growth formed with a yellowish-brown slightly diffusible pigment (Fig. 7).

2. **Microscopic morphology:**
Slide culture preparation on BA at room temperature for four weeks showed branching septate hyphae forming oval to pyriform one or two-celled microconidia (2-4 u in diameter), branching arthrospores (1.5-2.5 by 2.5-3.5 u), occasional round to oval smooth-walled chlamydospores, and a few typical budding yeast cells (blastospores). (Figs. 9, 11 and 13.)

Similar to Y-4 except that growth rate was slower.

Lactophenol mount from 48-hour growth showed round to oval budding cells (2-4 u in diameter). There were no arthrospores but a few hyphal fragments formed on aging (Fig. 6).

White, fluffy filamentous growth with a buff-tan non-diffusible pigment (Fig. 8).

A similar slide culture preparation showed branching septate hyphae with oval to pyriform microconidia (2-4 u in diameter) and tuberculate macroconidia (chlamydo-spores) 7-12 u in diameter (Figs. 10 and 12).
Fig. 1. Yeast phase growth of Y-4 and G-91 on HEIA at 37°C after 72 hours.

Fig. 2. Yeast phase hemolytic colonies of Y-4 on BA at 37°C after 96 hours.
Fig. 3. Yeast phase colonies of Y-1 on EHIA at 37° after 96 hours.

Fig. 4. Yeast phase colonies on BSDA at room temperature. Conversion to filamentous form.
Fig. 5. Lactophenol mount of Y-4 in yeast phase from a 24-hour growth on BHIA. (X 1000)

Fig. 6. Lactophenol mount of G-91 in yeast phase from a 48-hour growth on BHIA. (X 1000)
Fig. 7. A mycelial colony of Y-4 on ESDA at room temperature after four weeks.

Fig. 8. A mycelial colony of G-91 on ESDA at room temperature after four weeks.
Fig. 9. Slide culture of Y-4 (mycelial) at room temperature after four weeks. Hyphae with microconidia, arthrospores and small chlamydospores. (X 640)

Fig. 10. Slide culture of G-91 (mycelial) at room temperature after four weeks. Hyphae with microconidia and tuberculate macroconidia (chlamydospores). (X 640)
Fig. 11. Slide culture of Y-4 (mycelial) at room temperature after four weeks. Arthrospores, macroconidia and chlamydospires. Higher magnification (X 1000).

Fig. 12. Slide culture of G-91 (mycelial) at room temperature after four weeks. Tuberculate macroconidia and microconidia. Higher magnification (X 1000).
Fig. 13. Slide culture of Y-4 (mycelial) at room temperature after four weeks. Branching chains of arthrospores. (X 1000)
II. Pathogenicity

A. Pathogenicity of H. capsulatum in mice

1. Preliminary pathogenicity trial of nine strains of H. capsulatum

The results of screening nine strains of H. capsulatum for comparative pathogenicity to mice, after intravenous inoculation are presented in Table 3. There was a great variation in the pathogenicity to mice of the nine strains. With 32 million infecting cells mortality ranged from 0 to 100 per cent. Strains G-2, G-76, G-91, G-132 and G-X killed all the infected mice while the strain GH-1 caused no deaths. Strains G-13, G-89, and G-46 gave 80, 40 and 20 per cent mortalities respectively. Similarly, with lower infecting dose of eight million cells, strains G-2 and G-X gave 100 per cent mortalities while the strains GH-1, G-46, and G-89 showed no deaths. Strains G-91, G-132, G-76 and G-13 gave 80, 80, 40 and 20 per cent mortalities, respectively.

H. capsulatum was consistently isolated from the spleen and liver of all autopsied mice of the infected groups. None of the control mice died nor gave positive cultures.

2. LD-50 determination of G-91

The results obtained in terms of 30-day mortality in mice according to the Reed-Muench method (1938) for the calculation of LD-50, are presented in Table 4.
## TABLE 3

RESULTS OF SCREENING OF NINE STRAINS OF H. CAPSULATUM
FOR MOUSE PATHOGENICITY

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Strain of H. capsulatum</th>
<th>Estimated No. of yeast cells in millions</th>
<th>30-day mortality No. died</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-a</td>
<td>GH-1</td>
<td>32</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>1-b</td>
<td>GH-1</td>
<td>8</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>2-a</td>
<td>G-2</td>
<td>32</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>2-b</td>
<td>G-2</td>
<td>8</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>3-a</td>
<td>G-13</td>
<td>32</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>3-b</td>
<td>G-13</td>
<td>8</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>4-a</td>
<td>G-46</td>
<td>32</td>
<td>1/5</td>
<td>20</td>
</tr>
<tr>
<td>4-b</td>
<td>G-46</td>
<td>8</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>5-a</td>
<td>G-76</td>
<td>32</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>5-b</td>
<td>G-76</td>
<td>8</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>6-a</td>
<td>G-89</td>
<td>32</td>
<td>2/5</td>
<td>40</td>
</tr>
<tr>
<td>6-b</td>
<td>G-89</td>
<td>8</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>7-a</td>
<td>G-91</td>
<td>32</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>7-b</td>
<td>G-91</td>
<td>8</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>8-a</td>
<td>G-132</td>
<td>32</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>8-b</td>
<td>G-132</td>
<td>8</td>
<td>4/5</td>
<td>80</td>
</tr>
<tr>
<td>9-a</td>
<td>G-X</td>
<td>32</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>9-b</td>
<td>G-X</td>
<td>8</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>10 control</td>
<td>bCSS alone</td>
<td>0</td>
<td>0/5</td>
<td>0</td>
</tr>
</tbody>
</table>

*aNumerator - number of deaths. Denominator - total number in group bC.S.S. - Cysteine Saline diluent
TABLE 4

ARRANGEMENT OF DATA USED IN COMPUTATION OF LD-50 TITER BY REED-MUENCH FORMULA

<table>
<thead>
<tr>
<th>No. of cells X 10^P</th>
<th>Dilution</th>
<th>Mortality ratio</th>
<th>Died</th>
<th>Survived</th>
<th>Accumulated Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Died</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Survived</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mortality Ratio</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Percent</td>
</tr>
<tr>
<td>32</td>
<td>1:1 (10^-0)</td>
<td>10/10</td>
<td>10</td>
<td>0</td>
<td>39/39</td>
</tr>
<tr>
<td>16</td>
<td>1:2 (10^-0.3)</td>
<td>10/10</td>
<td>10</td>
<td>0</td>
<td>29/29</td>
</tr>
<tr>
<td>8</td>
<td>1:4 (10^-0.6)</td>
<td>7/10</td>
<td>7</td>
<td>3</td>
<td>19/22</td>
</tr>
<tr>
<td>4</td>
<td>1:8 (10^-0.9)</td>
<td>7/10</td>
<td>7</td>
<td>3</td>
<td>12/18</td>
</tr>
<tr>
<td>2</td>
<td>1:16 (10^-1.2)</td>
<td>4/10</td>
<td>4</td>
<td>6</td>
<td>5/17</td>
</tr>
<tr>
<td>1</td>
<td>1:32 (10^-1.5)</td>
<td>0/10</td>
<td>0</td>
<td>10</td>
<td>1/23</td>
</tr>
<tr>
<td>0.5</td>
<td>1:64 (10^-1.8)</td>
<td>1/10</td>
<td>1</td>
<td>9</td>
<td>1/32</td>
</tr>
<tr>
<td>0.25</td>
<td>1:128 (10^-2.1)</td>
<td>0/10</td>
<td>0</td>
<td>10</td>
<td>0/41</td>
</tr>
</tbody>
</table>

* Numerator - number of animals died
Denominator - total number in the group

Calculations

The mortality at 1:8 dilution was higher than 50 per cent; that in the next lower dilution of 1:16, was considerably lower.

The necessary proportionate distance between these dilutions was obtained as follows:

Proportionate distance

\[
\text{Proportionate distance} = \frac{(\text{Percent mortality above 50%} - (50\%))}{(\text{Percent mortality above 50%}) - (\text{Percent mortality below 50%})}
\]

\[
= \frac{66.7 - 50}{66.7 - 29.4}
\]

\[
= \frac{16.7}{37.3}
\]

\[
= 0.448
\]
The proportionate distance was corrected by multiplying by 0.3 (logarithm of 2, the dilution step employed)

\[ 0.448 \times 0.3 \]

\[ = 0.133 \]

**Negative log of LD-50 end point titer**

= Negative log of the dilution above 50% mortality plus Corrected proportionate distance

\[ (-0.9) \text{ plus } (-0.133) \]

\[ = -1.033 \]

log LD-50 titer = \[ 1.08 \]

LD-50 titer = \[ 10^{-1.08} \]

The LD-50 in terms of number of cells was obtained by dividing 32 millions by 10.8 (dilution).

\[ \therefore \text{LD-50} = \frac{32}{10.8} = 2.96 \text{ million cells} \]

The value of LD-50 was also computed by Karber's method (1931) which came to 3.1 million cells. These two values were fairly close and thus for this study a round figure of three million yeast cells of G-91 was considered as LD-50.

3. Effect of cortisone and streptomycin

The results are presented in Table 5, and the per cent survival with regard to time in days is presented in graphs II and III. No mice died after intravenous infection with approximately one and four million yeast cells of *H. capsulatum* or after daily treatment with streptomycin alone during the 30 day observation
**TABLE 5**

MORTALITY IN MICE AFTER CORTISONE AND STREPTOMYCIN TREATMENT AND INFECTION WITH H. *CAPSULATUM*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Infection with Y-cells of H. <em>capsulatum</em></th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortisone</td>
<td>Streptomycin 5 mg/day</td>
<td>Ratio</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>+</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>-</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>+</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
<td>1 x 10^6</td>
</tr>
<tr>
<td>VI</td>
<td>+</td>
<td>+</td>
<td>4 x 10^6</td>
</tr>
<tr>
<td>VII</td>
<td>+</td>
<td>-</td>
<td>4 x 10^6</td>
</tr>
<tr>
<td>VIII</td>
<td>-</td>
<td>+</td>
<td>4 x 10^6</td>
</tr>
<tr>
<td>IX</td>
<td>-</td>
<td>-</td>
<td>4 x 10^6</td>
</tr>
<tr>
<td>X</td>
<td>+</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>XI</td>
<td>-</td>
<td>+</td>
<td>None</td>
</tr>
</tbody>
</table>

*a* 5 mg cortisone two days before and 2.5 mg cortisone on second and fifth days after intravenous infection with *H. capsulatum*.

*b* Numerator = number of animals died
Denominator = total number in the group
GRAPH-II Comparative survival of mice treated with Cortisone, Streptomycin and Cortisone and Streptomycin alone, following infection with *H. capsulatum* (G-91, 1 million cells).

GRAPH-III  Comparative Survival of mice treated with Cortisone, Streptomycin, and Cortisone-Streptomycin, following infection with H. capsulatum (G-91 - 4 million cells).

period. The mice treated with cortisone and streptomycin (groups II and VI) or cortisone alone (groups III and VII) following intravenous infection with one or four million yeast cells of H. capsulatum all died within 11 days. Those receiving four million cells of H. capsulatum died in a slightly shorter time (within nine days) than those receiving one million cells. The groups IV and VIII receiving streptomycin alone, following intravenous infection with one and four million cells of H. capsulatum, showed 20 and 70 per cent mortality respectively. The deaths in these groups occurred 11 to 21 days after infection as against 2 to 11 days in the cortisone-treated mice. The control mice receiving cortisone and streptomycin (group I) had 10 per cent mortality while those receiving cortisone alone had a 40 per cent mortality.

The weights of the surviving animals are presented in Table 6 and in graph IV. The mice receiving streptomycin alone (group XI) were apparently normal and showed rather exponential increase in mean body weight up to 20 days after which the gain was less marked, reaching 32.8 gm on the 30th day. This group can be considered as normal in terms of weight-gain for the purpose of comparison in this experiment. The mean body weight of mice receiving cortisone alone (group X) or cortisone and streptomycin (group I) was significantly lower on day 10 (17.3 and 16.3 gm respectively) than those receiving streptomycin alone (group II, 24.1 gm). The mice of groups V and IX infected respectively with
TABLE 6

EFFECT OF CORTISONE AND STREPTOMYCIN TREATMENT AND INFECTION WITH *H. capsulatum* ON BODY WEIGHT OF MICE

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>Treatment Streptomycin</th>
<th>Infection with Yeast cells of G-91</th>
<th>Average Body Weight in Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mg/day</td>
<td></td>
<td>-2 day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>None</td>
<td>15±1</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>1 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>1 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>1 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>1 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>VI</td>
<td>+</td>
<td>4 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>VII</td>
<td>+</td>
<td>4 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>VIII</td>
<td>-</td>
<td>4 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>IX</td>
<td>-</td>
<td>1 x 10⁶</td>
<td>15±1</td>
</tr>
<tr>
<td>X</td>
<td>+</td>
<td>None</td>
<td>15±1</td>
</tr>
<tr>
<td>XI</td>
<td>-</td>
<td>None</td>
<td>15±1</td>
</tr>
</tbody>
</table>

*5 mg cortisone two days before 2.5 mg cortisone on the second and fifth days after intravenous infection with yeast cells of *H. capsulatum* (G-91).*

*The number in parentheses indicates the number of animals weighed.*
GRAPH-IV  Body weight curve of surviving mice after Cortisone and Streptomycin treatment, and following infection with N. capsulata (G-91).

Group XI: Streptomycin alone; I: Streptomycin + Cortisone; III: Cortisone alone; IV: Streptomycin + G-91; V: G-91 alone (1 million); VIII: Streptomycin + G-91 (4 million); IX: G-91 alone (4 million).
one and four million yeast cells of *H. capsulatum* without any
other treatment had mean body weights of 23.6 and 20.4 gm as com-
pared with 24.1 for the controls on the 10th day, 28.1 and 25.2 gm
as compared to 30.9 gm on day 20 and 32.1 and 32.2 gm as compared
to 32.8 gm of the 30th day. The mice of groups IV and VIII re-
ceiving streptomycin alone, infected respectively with one and
four million cells of *H. capsulatum*, had average body weights of
21.2 and 17.0 gm on day 10 (control 24.1 gm); 24.5 and 23.3 on
day 20 (control 30.9 gm); and 29.4 and 26.3 on day 30 (control
32.8 gm).

The animals receiving cortisone and streptomycin (group I)
or cortisone alone (group X) were very sick, with ruffled coats,
during first 10 days of treatment; thereafter they began to im-
prove in physical appearance. The improvement was accompanied by
an increase in body weight after the cortisone was discontinued.
All other animals were apparently normal except those in groups
IV and VIII, which received streptomycin and one and four millions
cells of *H. capsulatum*. They were sick, with ruffled coats, but
the symptoms started after the 10th day and became more pronounced
during the latter part of the observation period. The mice of
group VIII, with the higher dosage, were sicker than those of
group IV.

All animals infected with *H. capsulatum* (groups II to IX)
yielded positive cultures of *H. capsulatum* on autopsy after death
or after being sacrificed on the 30th day. One mouse of group I
(cortisone and streptomycin) and four of group X (cortisone alone) died. They yielded a culture of several species of bacteria, predominantly Gram negative organisms, but no *H. capsulatum* at autopsy.

B. Pathogenicity of Y-4 in mice

1. Preliminary pathogenicity trial

The results, in terms of mortality obtained, after intravenous and intraperitoneal inoculation of mice are presented in Table 7.

**TABLE 7**

MORTALITY IN MICE AFTER INTRAVENOUS AND INTRAPERITONEAL INOCULATION WITH Y-4

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Inoculation with Y-4 cells</th>
<th>60-day mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. died</td>
</tr>
<tr>
<td>A</td>
<td>35 millions - i.v.</td>
<td>6/10</td>
</tr>
<tr>
<td>B</td>
<td>70 millions - i.p.</td>
<td>1/10</td>
</tr>
<tr>
<td>C</td>
<td>C.S.S. alone - i.v.</td>
<td>0/5</td>
</tr>
<tr>
<td>D</td>
<td>C.S.S. alone - i.p.</td>
<td>0/5</td>
</tr>
</tbody>
</table>

^a Numerator = number of deaths. Denominator = total number in the group
^b C.S.S. = cysteine saline

A marked difference was observed between the 60 day mortality in intravenous (60%) and intraperitoneally (10%) inoculated mice although the intravenous dose was only half (35 millions) of the intraperitoneal dose (70 millions). In group A five mice died within 19 days and one died on day 41, after intravenous
infection. In group B the only death was on the 49th day after intraperitoneal infection.

Mice after intravenous infection showed a peculiar neurologic syndrome, manifested by hyperirritability, jumping, circling, ataxia, and staggering gait. These symptoms appeared 4-5 days after infection, reaching the peak around 7-10 days followed by death within three weeks or apparent recovery. Although mice inoculated intraperitoneally showed a similar neurologic syndrome, it was less severe and followed by apparent recovery in most cases. None of the control mice died or showed any neurologic syndrome.

The results of cultures from spleen, kidney, liver, lung and brain of the mice dying spontaneously or sacrificed up to 60 days, after intravenous and intraperitoneal inoculation are summarized in Tables 8 and 9, respectively. Table 8 shows that kidney and brain were positive in only five out of nine cases. The organism persisted in mouse tissues at least up to 60 days after intravenous infection.

Table 9 shows that after intraperitoneal inoculation of approximately 70 million cells of Y-4 the highest proportion of positive cultures was obtained from spleen (9/10), followed by kidney (8/10), liver (6/10), lung (3/10), and brain (3/10). Generally speaking the number of positive cultures after intravenous inoculation were significantly higher than after intraperitoneal inoculation. The difference is most noticeable in the brain.
### Table 8

**Survival of Y-k in Mouse Tissues After Intravenous Inoculation of 35 Million Cells**

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Duration of infection in days</th>
<th>Cultures (Spleen, Kidney, Liver, Lung, Brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1</td>
<td>6</td>
<td>+, ND, +, +, ND</td>
</tr>
<tr>
<td>M-2</td>
<td>18</td>
<td>+, ND, +, ND, ND</td>
</tr>
<tr>
<td>M-3</td>
<td>18</td>
<td>+, ND, +, - ND</td>
</tr>
<tr>
<td>M-4</td>
<td>18</td>
<td>+, ND, +, +, ND</td>
</tr>
<tr>
<td>M-5</td>
<td>19</td>
<td>+, +, +, -, +</td>
</tr>
<tr>
<td>M-6</td>
<td>30</td>
<td>+, +, +, +, +</td>
</tr>
<tr>
<td>M-7</td>
<td>30</td>
<td>+, +, -, -, +</td>
</tr>
<tr>
<td>M-8</td>
<td>41</td>
<td>+, +, +, -, +</td>
</tr>
<tr>
<td>M-9</td>
<td>60</td>
<td>+, +, +, +, +</td>
</tr>
<tr>
<td>H-10</td>
<td>60</td>
<td>-, +, +, +, +</td>
</tr>
</tbody>
</table>

ND = not done; mice no. M-5 and M-8 died spontaneously and the remaining were sacrificed.
TABLE 9
SURVIVAL OF Y-4 IN MOUSE TISSUES AFTER INTRAPERITONEAL INOCULATION OF 70 MILLION CELLS

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Duration of infection in days</th>
<th>Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>M-11</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>M-12</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>M-13</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>M-14</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>M-15</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>*M-16</td>
<td>49</td>
<td>+</td>
</tr>
<tr>
<td>M-17</td>
<td>60</td>
<td>+</td>
</tr>
<tr>
<td>M-18</td>
<td>60</td>
<td>+</td>
</tr>
<tr>
<td>M-19</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>M-20</td>
<td>60</td>
<td>+</td>
</tr>
</tbody>
</table>

*M-16 died spontaneously, others sacrificed.
**Histopathology**: Five of the 10 mice of groups A and B (Table 7) inoculated intravenously and intraperitoneally with Y-4 respectively, were examined histopathologically. In group A only one of the six mice that died spontaneously could be examined histopathologically. No significant inflammatory reaction nor fungal elements were demonstrated in the brain, lungs, or liver of any of the animals examined. Including this animal and the four that were sacrificed, only three showed lesions. Two had macroscopically necrotic foci in the renal medulla. All three showed inflammatory infiltrates (polymorphs and macrophages) with necrosis of tissue histologically. Fungal elements (arthrospores and blastospores) could be seen in all three. The other organs were essentially normal, except for splenomegaly in all five animals, but organisms could not be demonstrated.

In group B organs of the two of the five mice examined showed inflammatory response of a similar nature. Mouse 15 (M-15) had a large abscess with organisms in the renal pelvis and moderate infiltration of polymorphs and mononuclear cells in the cortex. Mouse 16 (M-16) showed a small abscess with organisms in the pelvis with marked infiltration of polymorphs and mononuclear cells in the medulla and perinephric tissues (Figs. 14, 15 and 16).

2. Demonstration of "toxin" production by Y-4

The results of comparative mortality of cortisone-treated and non-treated mice after inoculation with different "toxic" preparations (along with the average body weight of the surviving
Fig. 14. Section of kidney (M-15).
Abscess in renal medulla.
H. & E. (X 80).

Fig. 15. Section of kidney (M-15).
Polymorph. and mononuclear cell infiltration. H. & E. (X 450).
Fig. 16. Section of kidney (M-15). Showing G.M.S. positive arthrosopes and blastospores. (X 450)
animals taken at 10 day intervals) are presented in Table 1. None of the non-cortisone treated mice (groups II, IV, and VI) died after inoculation with "toxic" preparations. However, in three of the cortisone-treated groups, one mouse died, either after inoculation with "toxic" preparation from liquid medium (group III) or killed cells (group V) or saline alone (group VIII). In the other two groups none of the cortisone-treated mice died following inoculation with either broken cell filtrate (group I) or uninoculated medium (group VII).

The average weights of the surviving animals, recorded at 10 day intervals, after inoculation with different "toxic" preparations are presented in Graph VI. The mice (groups II, IV, VI) without cortisone treatment were apparently normal and showed rapid increase in body weight for 20 days, after which the gain was less marked. Generally speaking the weights of these mice were higher than those of cortisone-treated mice irrespective of subsequent inoculation with "toxic" preparations (groups I, III and V), uninoculated liquid medium (group VII) or saline alone (group VIII). The difference was more marked on day 10, while mice were still under the effect of cortisone treatment. The weights of non-cortisone treated mice were sick, with dry and ruffled coats. After this these animals began to improve in the physical appearance. On day 20 the differences in the weights of cortisone and non-cortisone mice was less marked, being 31.0 to 33.2 gm and 29.5 to 29.0 gm respectively. On day 30 there was no significant difference
TABLE 10
EFFECT OF DIFFERENT Y-4 "TOXIC" PREPARATIONS ON CORTISONE-TREATED AND UNTREATED MICE

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>aCortisone Treatment</th>
<th>Preparation Inoculated</th>
<th>Mortality</th>
<th>Average Body Weight in Grams</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2-day</td>
<td>10-day</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean Range</td>
<td>Mean Range</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>aFilt. A</td>
<td>0/5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>Filt. A</td>
<td>0/5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>Filt. B</td>
<td>1/5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>Filt. B</td>
<td>0.5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>Killed cells</td>
<td>1/5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>Killed cells</td>
<td>0/5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>VII</td>
<td>+</td>
<td>Liquid medium alone</td>
<td>0/5</td>
<td>15±1 (5)</td>
</tr>
<tr>
<td>VIII</td>
<td>+</td>
<td>Saline only</td>
<td>1/5</td>
<td>15±1 (5)</td>
</tr>
</tbody>
</table>

aCortisone treatment - 5 mg two days before and three supplementary injections of 2.5 mg each on 2, 5, and 7 days after first dose of "toxic" preparations.

bFiltrate A - filtrate from broken cells; filtrate B - filtrate from culture in liquid medium and preparation C = heat killed cells, non-filtered.
First dose of 0.5 ml "toxic" preparation was given intravenously on day zero and second dose of 1 ml suspension was given intra-peritoneally seven days later.

cThe number in parentheses indicates the number of animals weighed.
Graph-V Body weights of surviving mice after Cortisone and Streptomycin treatment, and following inoculation with "Toxic" preparations (Y-4).

Group I: Broken cells + Cortisone; II: Broken cells alone;
III: Culture filtrate + Cortisone; IV: Culture filtrate alone;
V: Heat killed cells + Cortisone; VI: Heat killed cells alone;
VII: Liquid medium alone + Cortisone; VIII: Saline + Cortisone.
in the weights of non-cortisone and cortisone treated mice with the possible exception of group III (29.0 gm). Similarly, there did not appear to be any significant difference in the mean weights of the mice inoculated with different "toxic" preparations.

3. Effect of cortisone and streptomycin

The results in terms of 30 day mortality of control, cortisone, cortisone and streptomycin treated mice following intravenous infection with 17 million cells of Y-4 are presented in Table II. There was a marked difference in the mortalities of cortisone and non-cortisone treated mice. Cortisone-treated mice (groups A and C) showed 100 per cent mortality as against 10 per cent in non-treated mice (groups B and D). There was no difference in the mortalities of the streptomycin-treated (group D - 10 per cent) and non-treated (group B - 10 per cent) mice. Streptomycin in combination with cortisone (group C) had the same mortality (100 per cent) as that of cortisone alone (group A).

Comparative survival with regard to time in days of different groups of mice is presented in graph VI. All the mice of group A (cortisone treated) and group C (cortisone and streptomycin treated) died within 6 and 11 days respectively, after infection with Y-4. Of the 10 mice of each group B (non-treated) and D (streptomycin treated) only one died in each group, 8 and 11 days after inoculation.

All Y-4 infected mice showed a peculiar neurologic syndrome, as described earlier in first pathogenicity trial, but it varied
in intensity and duration in different groups. In cortisone alone or combined cortisone and streptomycin-treated mice (groups A and C) neurologic symptoms appeared 24 hours after infection and continued until all animals died, within 6 and 11 days respectively. No significant difference was observed in the neurologic syndrome of these mice. In group B (non-treated) symptoms were less severe, appeared 4-7 days after infection, reaching the peak around 7-10 days followed by death or apparent recovery within three weeks. In streptomycin treated mice (group D) the symptoms were more severe, both in intensity and duration, than that of non-treated mice (group B) and continued until death or the 30th day when the experiment was terminated. None of the control mice died or showed any neurologic syndrome.

In summary, all mice inoculated with Y-4 showed neurologic symptoms. These were more severe in the streptomycin and/or cortisone treated groups. The symptoms were more marked and did not regress. Streptomycin did not increase the mortality, but did slightly extend the life span of animals treated with cortisone, even though the neurologic symptoms did not improve.

Y-4 was consistently recovered, at least from one organ, of all the mice autopsied and cultured. The spleen, kidney and brain from all the mice dying spontaneously or sacrificed, were positive for Y-4. Positive cultures also were obtained from liver (10/10), lung (8/8), of group A; liver and lung (5/9 each) of group B; liver (3/3) and lung (2/3) of group C; and liver (9/9) and lung (7/9) of group D.
GRAPH-VI  Effect of Cortisone and Streptomycin, alone or in combination, on survival of mice following intravenous infection with 17 million cells of Y-4.

Group A: Cortisone + Y-4; B: Y-4 alone; C: Cortisone - Streptomycin + Y-4; D: Streptomycin + Y-4; and E: Control (saline).
TABLE 11

COMPARATIVE MORTALITY OF CONTROL, CORTISONE- AND STREPTOMYCIN-TREATED MICE, FOLLOWING INTRAVENOUS INFECTION WITH 17 MILLION CELLS OF Y-4

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>Treatment</th>
<th>i.v. infection with 17 million cells of Y-4</th>
<th>30-day mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortisone</td>
<td>Streptomycin 5 mg/day</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>b10/10 100</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>1/10 10</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>10/10 100</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+</td>
<td>1/10 10</td>
</tr>
<tr>
<td>E</td>
<td>Saline</td>
<td>only</td>
<td>0/5 0</td>
</tr>
</tbody>
</table>

\[a\] For dosage see text.

\[b\] Numerator = number died; denominator = number of mice in the group.

**Histopathology**

**Group A** (cortisone treated): Macroscopically, no significant pathological lesions were noticed, except congestion of internal organs, in the mice treated with cortisone alone, following infection with 17 million cells of Y-4. Microscopically kidney and brain showed the most significant tissue reaction. In the kidney the lesions were located predominantly in the cortical region and were characterized by the presence of multiple small areas of necrosis with polymorph and mononuclear cell infiltration, mostly in the glomerular region. There was moderate congestion of blood vessels with slight degenerative changes in the tubular
epithelium. In one mouse (M-42), in addition to the above lesions, multiple suppurative foci were seen in the tubules of the medulla. G.M.S. positive organisms (arthrospores and blastospores) were present in eight out of the ten kidneys examined (Figs. 17, 18, 19 and 20).

In the brain tissue reaction was characterized by marked hyperaemia of the cortical and meningeal blood vessels accompanied by focal areas of polymorph and mononuclear cell infiltration. G.M.S. positive organisms were present in seven out of the ten brain tissues examined. In some, e.g. M-49, reaction was very severe, with focal necrosis associated with extensive proliferation of the fungus (Figs. 23 and 24).

The liver in general showed marked congestion of blood vessels, but in mice (M-43, M-44 and M-50) the reaction was also accompanied with the areas of infarction. G.M.S. positive budding yeast cells but not arthrospores were present in three out of the ten sections examined.

In the lung the general reaction was that of marked hyperaemia and pulmonary haemorrhage. G.M.S. positive budding yeast cells were present in and around small blood vessels in one out of ten sections examined.

The spleen showed increased infiltration of polymorphs, suggestive of acute splenitis. G.M.S. positive organisms were found in one out of ten sections examined.
Fig. 17. (M-42; cortisone treated.)
Section of kidney. Infiltration of polymorphs and mononuclear cells and tubular degeneration and necrosis. H. & E. (X 640).

Fig. 18. (M-49; cortisone treated.)
Fig. 19. (M-49; cortisone treated.)
Section of kidney. Infiltration of polymorphs and mononuclear cells in and around a glomerulus. H. & E. (X 640).

Fig. 20. (M-41; cortisone treated.)
Fig. 21. (M-65; cortisone and streptomycin treated.) Section of kidney.
Arthrosapores and blastospores of Y-4.
G.M.S. (X 640).
Pig, 22, (M-1*9; cortisone treated.)
Section of brain. Infiltration of polymorphs and mononuclear cells and necrosis. H.& E. (X 640).

Fig. 22. (M-49; cortisone treated.)
Section of brain. Infiltration of polymorphs and mononuclear cells and necrosis. H.& E. (X 640).

Pig, 23* (M-49; cortisone treated.)

Fig. 23. (M-49; cortisone treated.)
Fig. 24. (M-65; cortisone and streptomycin treated.) Section of brain. Arthrospores and blastospores of Y-4. G.M.S. (X 640).
Fig. 25. (M-65; cortisone and streptomycin treated.) Section of lung. Slight infiltration of polymorphs and mononuclears. H. & E. (X 640).

Fig. 26. (M-42; cortisone treated.) Section of lung. Fungal elements of Y-4 in and around a blood vessel. G.M.S. (X 640).
Fig. 27. (M-64; cortisone and streptomycin treated.) Section of liver.
Blastospores in sinusoids. G.M.S. (X 640).

Fig. 28. (M-66; cortisone and streptomycin treated.) Section of spleen.
Blastospores. G.M.S. (X 640).
Group B (untreated): Only four of the ten mice infected intravenously with 17 million cells of Y-4, without any treatment, sacrificed after 30 days, were examined histopathologically. Macroscopically, no significant lesions other than the slight enlargement of the spleen were seen. Microscopically, the most striking lesions were found in the kidney and brain. In the kidney tissue reactions were predominantly located in the medullary region. In the cortical region there was moderate hyperaemia associated with infiltration of polymorphs and mononuclear cells around blood vessels and glomeruli. In the medullary region there was an increased infiltration of polymorphs and mononuclear cells. The renal pelvis showed inflammatory exudation with polymorph infiltration. G.M.S. positive organisms were present in three out of four sections examined.

In the brain the tissue reaction was characterized by focal areas of congestion and minimal polymorph infiltration. G.M.S. positive fungal elements were present in the basal part of the brain of one out of four sections examined (M-54) (Figs. 29 and 30).

The liver showed slight congestion without the presence of any organism.

The lung showed slight congestion. G.M.S. positive budding yeast cells were in the blood vessels of one out of four sections examined (M-52).

The spleen showed increased infiltration of polymorphs and mononuclear cells with formation of giant cells. No organisms were seen.
Fig. 29. (M-54; untreated.) Section of brain. Slight cellular infiltration. H. & E. (X 450).

Fig. 30. (M-54; untreated.) Section of brain. Fungal elements of Y-4. G.M.S. (X 640).
**Group C (cortisone and streptomycin treated):**

Histopathological examination was performed on the tissues of three of the ten mice which died after cortisone and streptomycin treatment followed by infection with Y-4. In general, the tissue reaction was of similar nature to that of group A described earlier. However, there was more extensive proliferation of the organism throughout the tissue of the organ involved. G.M.S. positive fungal elements were present in the kidney, brain, and liver of all three mice (Figs. 23 and 24). In the liver, however, only budding yeast cells were present (Fig. 27). Budding yeast cells were also found in the spleen of one mouse (M-66) (Fig. 28).

**Group D (streptomycin treated):** Histopathological examination was performed on the organs of nine out of ten mice treated with streptomycin followed by infection with Y-4 and sacrificed after 30 days. Macroscopically the spleen was moderately enlarged in all the animals, and abscesses were present in at least one kidney of five of the nine mice examined. Other organs were apparently normal.

Microscopically, most significant inflammatory reactions were found in the kidney. The pathological lesions were intermediate in intensity between cortisone treated mice (group A) and non-cortisone treated mice (group B). In the kidneys the tissue reactions were predominantly located in the medulla and renal pelvis, with abscess formation. G.M.S. positive organisms were present in four out of nine sections examined.
The brain - only two sections (M-73 and M-74) showed slight inflammatory response characterized by congestion and increased infiltration of polymorph and mononuclear cell. G.M.S. positive organisms were present in one section (M-74).

The liver showed slight congestion.

The lung showed congestion and hemorrhage.

The spleen showed increased infiltration of polymorphs and mononuclear cells with occasional giant cells. No organisms were seen in the liver, lung, or spleen.
III. Immunology

A. Skin test cross-reactions between *H. capsulatum* (G-76 and G-91) and Y-4

The results of skin tests of the guinea pigs at 6 and 12 weeks after infection with *H. capsulatum* and Y-4, and of the control animals, are presented in Tables 12 and 13. Infected guinea pigs developed skin reactivity to homologous antigen at six weeks after infection. None of the *Histoplasma* antigens gave positive reaction at six weeks when tested on guinea pigs infected with Y-4. However, three out of four guinea pigs infected with *H. capsulatum* gave weak positive reactions (but still more than 5 mm diameter) when tested with yeast phase antigen of Y-4. Only one of the three reacted weakly with the mycelial phase antigen (Table 12). The fourth animal was negative with both antigens.

At 12 weeks all the infected guinea pigs developed clear-cut skin reactivity of equal degree to homologous and heterologous antigens (Table 13). All reacted with both yeast and mycelial phase antigens. The reactions at 12 weeks were generally more pronounced than at six weeks. The skin reactions to mycelial antigen reached their height at 24 hours and were greatly reduced in intensity at 48 hours. The reactions to yeast phase antigens were generally more pronounced and persisted 48 hours or longer.

None of the antigens tested in non-infected guinea pigs gave a positive reaction. Similarly, uninoculated Salvin and asparagine media did not show any skin reactivity in either the infected or the non-infected animals.
### TABLE 12

RESULTS OF SKIN TESTS IN GUINEA PIGS SIX WEEKS AFTER INFECTION WITH H. CAPSULATUM AND Y-4

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>Skin reactions in mm in guinea pigs infected with:</th>
<th>Control (Non-infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-76</td>
<td>G-91</td>
</tr>
<tr>
<td></td>
<td>Pig-1</td>
<td>Pig-2</td>
</tr>
<tr>
<td>G-76-yeast</td>
<td>10 mm</td>
<td>12 mm</td>
</tr>
<tr>
<td>G-76-mycelial</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>G-91-yeast</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>G-91-mycelial</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Y-4-yeast</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Y-4-mycelial</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Salvin's medium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(uninoculated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine medium</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(uninoculated)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Control animals were not infected, but were skin-tested with yeast phase of both G-91 and Y-4 a week prior to the infection of other animals.*
TABLE 13
RESULTS OF SKIN TESTS IN GUINEA PIGS 12 WEEKS AFTER INFECTION WITH H. CAPSULATUM AND Y-4

<table>
<thead>
<tr>
<th>Test Antigen</th>
<th>Skin reactions in mm in guinea pigs infected with:</th>
<th>Control (Non-infected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-76</td>
<td>G-91</td>
</tr>
<tr>
<td>G-76-yeast</td>
<td>10 mm</td>
<td>ND</td>
</tr>
<tr>
<td>G-76-mycelial</td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td>G-91-yeast</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td>G-91-mycelial</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>Y-4-yeast</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>Y-4-mycelial</td>
<td>7</td>
<td>ND</td>
</tr>
<tr>
<td>Salvin's medium</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>(uninoculated)</td>
<td>Asparagine medium</td>
<td></td>
</tr>
<tr>
<td>(uninoculated)</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

^a Control animals were not infected, but were skin-tested with yeast phase of both G-91 and Y-4 a week prior to the infection of other animals.

^b Animal died before second skin test.

^c ND = not done.
B. Serological cross-reactions between G-91 and Y-4

The titration results of yeast phase antigens of G-91 and Y-4 with homologous antisera are presented in Tables 14 and 15. A 1:200 dilution of each antigen containing 5 mg per ml of the lyophilized material was found optimum for collodion agglutination.

The results of collodion agglutination and immunodiffusion tests using homologous G-91 as well as heterologous Y-4 antigens against different anti-\textit{H. capsulatum} rabbit sera are presented in Table 16. All sera gave positive reaction with G-91 antigen in both tests. Significant cross-reactions between Y-4 antigen and anti-\textit{H. capsulatum} sera were observed both in collodion agglutination and immunodiffusion tests. All except two anti-\textit{H. capsulatum} sera (G-2 and G-76) reacted with Y-4 antigen in both tests. Anti-G-2 sera gave a heterologous 1:8 collodion agglutination titer but failed to show any reaction in immunodiffusion. Anti-G-76 was immunodiffusion positive and collodion agglutination negative with Y-4 antigen. All sera except anti-G-89 gave stronger reactions with G-91 antigen than with Y-4. Anti-G-89 gave 1:256 collodion agglutination titer with Y-4 antigen and only 1:64 with G-91 antigen, although the immunodiffusion reactions were almost the same with both antigens.

It will be noted that G-91 antigen reacts more weakly with its homologous serum than the other antigen. Earlier bleeding showed a higher collodion agglutination titer but was negative in immunodiffusion tests and did not cross-react.
### TABLE 14

TITRATION OF YEAST PHASE ANTIGEN OF G-91 FOR COLLODION AGGLUTINATION USING HOMOLOGOUS RABBIT ANTISERUM

<table>
<thead>
<tr>
<th>Antigen used 0.5 ml</th>
<th>Positive serum dilutions</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>Amount in mg/ml</td>
<td>1:5 1:10 1:20 1:40 1:80 1:160 1:320</td>
</tr>
<tr>
<td>1:10</td>
<td>100</td>
<td>+     -     -     -     -     -     -</td>
</tr>
<tr>
<td>1:50</td>
<td>20</td>
<td>4+    4+    3+    1+    -     -     -</td>
</tr>
<tr>
<td>1:100</td>
<td>10</td>
<td>4+    4+    4+    3+    +     -     -</td>
</tr>
<tr>
<td>b 1:200</td>
<td>5</td>
<td>4+    4+    4+    4+    3+    -     -</td>
</tr>
<tr>
<td>1:400</td>
<td>2.5</td>
<td>4+    4+    3+    +     -     -     -</td>
</tr>
<tr>
<td>1:800</td>
<td>1.25</td>
<td>3+    1+    1+    +     -     -     -</td>
</tr>
<tr>
<td>1:1000</td>
<td>1.0</td>
<td>1+    1+    +     -     -     -     -</td>
</tr>
</tbody>
</table>

a In antigen control sensitized particles were mixed with 0.5 ml of saline. Serum control = positive serum + unsensitized collodion.

b 1:200 dilution selected as optimum on the basis of comparative titer.

### TABLE 15

TITRATION OF YEAST PHASE ANTIGEN OF Y-4 FOR COLLODION AGGLUTINATION USING HOMOLOGOUS RABBIT ANTISERUM

<table>
<thead>
<tr>
<th>Antigen used 0.5 ml</th>
<th>Positive serum dilutions</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>Amount in mg/ml</td>
<td>1:5 1:10 1:20 1:40 1:80 1:160 1:320</td>
</tr>
<tr>
<td>1:10</td>
<td>100</td>
<td>+     -     -     -     -     -     -</td>
</tr>
<tr>
<td>1:50</td>
<td>20</td>
<td>4+    3+    -     -     -     -     -</td>
</tr>
<tr>
<td>1:100</td>
<td>10</td>
<td>4+    4+    +     -     -     -     -</td>
</tr>
<tr>
<td>b 1:200</td>
<td>5</td>
<td>4+    4+    4+    4+    3+    1+    -</td>
</tr>
<tr>
<td>1:400</td>
<td>2.5</td>
<td>4+    4+    3+    2+    1+    1+    -</td>
</tr>
<tr>
<td>1:800</td>
<td>1.25</td>
<td>3+    1+    1+    1+    +     -     -</td>
</tr>
<tr>
<td>1:1000</td>
<td>1.0</td>
<td>2+    1+    1+    1+    -     -     -</td>
</tr>
</tbody>
</table>

a In antigen control sensitized particles were mixed with 0.5 ml of saline. Serum control = positive serum + unsensitized collodion.

b 1:200 dilution selected as optimum on the basis of comparative titer.
### TABLE 16

COMPARATIVE RESULTS OF COLLODION AGGLUTINATION AND IMMUNODIFFUSION TESTS WITH ANTI-**H. CAPSULATUM** RABBIT SERA USING G-91 (HOMOLOGOUS) AND Y-4 (HETEROLOGOUS) ANTIGENS

<table>
<thead>
<tr>
<th>Sera</th>
<th>H. capsulatum (G-91) Antigen</th>
<th>Y-4 Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collodion aggl. 1:8 or higher</td>
<td>Immuno-diffusion</td>
</tr>
<tr>
<td>Anti-GH-1</td>
<td>1:256</td>
<td>+, +s, +v</td>
</tr>
<tr>
<td>Anti-G-2</td>
<td>1:256</td>
<td>-</td>
</tr>
<tr>
<td>Anti-G-46</td>
<td>1:128</td>
<td>+, +s, +</td>
</tr>
<tr>
<td>Anti-G-76</td>
<td>1:64</td>
<td>+s, +</td>
</tr>
<tr>
<td>Anti-G-89</td>
<td>1:64</td>
<td>-</td>
</tr>
<tr>
<td>Anti-G-91</td>
<td>1:8</td>
<td>+, +s, +</td>
</tr>
</tbody>
</table>

Each + sign indicates one definite precipitin line; s and v denote very strong or very weak reactions.

### TABLE 17

COMPARATIVE RESULTS OF COLLODION AGGLUTINATION AND IMMUNODIFFUSION TESTS WITH ANTI-Y-4 RABBIT SERA USING Y-4 (HOMOLOGOUS) AND G-91 (HETEROLOGOUS) ANTIGENS

<table>
<thead>
<tr>
<th>Anti-Y-4 sera</th>
<th>Y-4 Antigen</th>
<th>H. capsulatum (G-91) Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collodion aggl. 1:5 or higher</td>
<td>Immuno-diffusion</td>
</tr>
<tr>
<td>1</td>
<td>1:80</td>
<td>* +s, +</td>
</tr>
<tr>
<td>2</td>
<td>1:80</td>
<td>+s, +</td>
</tr>
<tr>
<td>3</td>
<td>1:160</td>
<td>+s, +</td>
</tr>
</tbody>
</table>

* Each + sign indicates one definite precipitin line; s and w denote very strong or very weak reactions.*
Table 17 shows the results of collodion agglutination and immunodiffusion tests with Y-4 and G-91 antigens against three anti-Y-4 rabbit sera. All sera gave positive reactions in both tests with homologous Y-4 antigen, but no cross reactions between G-91 antigen and anti-Y-4 sera were observed.

Photographs of the plates showing immunodiffusion reactions are presented in Figures 31 to 34. Figure 31 shows immunodiffusion reactions with anti-\textit{H. capsulatum} sera and G-91 and Y-4 antigens. All sera except six and eight gave at least one precipitin line with either antigen. Serum six did not give any reaction with either antigen while serum eight gave a weak precipitin line with G-91 antigen only.

Figure 32 shows immunodiffusion reactions of reverse type from Figure 31. The \textit{H. capsulatum} antisera (A through D) are in the central wells, and peripherally we have the antigens Y-4 (Y), G-91 (g) and G-76 (G). Serum B did not react with any antigen. Sera A and D reacted with Y-4 and G-91, but not with G-76. Serum C reacted with all the antigens, and shows at least one "pattern of identity" between Y-4 and G-91, and also between Y-4 and G-76. Serum A, on the other hand, shows at least one "pattern of non-identity" between Y-4 and G-91, but no such line is visible between Y-4 and G-76.

Figure 33 shows immunodiffusion reactions with Y-4 antigen and homologous antisera. All sera except non-immunized control
6 gave two precipitin lines, the inner being much stronger and
more distinct than the outer line.

Figure 34 shows immunodiffusion reactions of G-91 antigen
with anti-Y-4 and anti-G-91 sera. G-91 gave two precipitin lines
with homologous sera but did not show any precipitin lines with
either heterologous serum (anti-Y-4) or with non-immunized
controls.
Fig. 31. Immunodiffusion test with anti-H. capsulatum sera and G-91 and Y-4 antigens. Central wells contained antigens Y-4 (Y) and G-91 (G), outer wells different antisera (1 to 8).

Fig. 32. Immunodiffusion test with anti-H. capsulatum sera and H. capsulatum and Y-4 antigens. Central wells contained antisera (A, B, C & D), outer well Y-4 (Y) and G-91 (G) and G-76 (G) antigens.
Fig. 33. Immunodiffusion test with Y-4 antigen and homologous antisera. Central well contained Y-4 antigen; outer wells 1 to 5 antisera and 6 normal serum.

Fig. 34. Immunodiffusion test with G-91 antigen and anti-Y-4 and G-91 sera. Central well contained G-91 antigen; outer wells 2 and 3 anti-Y-4 sera; wells 4 and 5 anti-G-91 sera, wells 1 and 6 normal sera.
IV. Cross-Protection

Table 18 shows the mortality of non-immunized mice after intravenous challenge with $10 \times \text{LD}_{50}$ of G-91 yeast cells.

<table>
<thead>
<tr>
<th>i.p. immunization</th>
<th>Challenge (10 x LD$_{50}$ of G-91)</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 21</td>
<td>No.</td>
</tr>
<tr>
<td>A</td>
<td>0.5 m. living G-91 cells</td>
<td>2/20</td>
</tr>
<tr>
<td>B</td>
<td>0.5 m. living Y-4 cells</td>
<td>20/20</td>
</tr>
<tr>
<td>C</td>
<td>3 doses of killed G-91 cells</td>
<td>13/20</td>
</tr>
<tr>
<td>D</td>
<td>3 doses of killed Y-4 cells</td>
<td>9/20</td>
</tr>
<tr>
<td>E</td>
<td>Formol saline only</td>
<td>17/20</td>
</tr>
</tbody>
</table>

Numerator = number of animals died; denominator = total number of animals in group.

For details see under "Methods."

The mortality data were also analyzed statistically by normal approximation of the binomial distribution, according to the method described in "Introduction to Mathematical Statistics," p. 149, "3rd ed.," Paul G. Haul, John Wiley and Sons, Inc., New York, 1962. The highest protection was observed in the mice of group A, immunized with G-91 live vaccine (0.5 million cells), followed by groups D, C, and B in descending order. Group A showed 10 per cent mortality as compared to 85 per cent of the control group E, which is statistically highly significant ($p < 0.01$). Mice of group B immunized with Y-4 live vaccine
did not show any protection against intravenous challenge with G-91. As a matter of fact, group B had higher mortality (100%) than control group E (85%).

Mice of group C and D, immunized with killed vaccine of G-91 and Y-4, respectively, showed significant protection against intravenous challenge with G-91. Groups C and D respectively had 65% and 45% mortalities as compared to 85% of the control group, which is statistically highly significant (p < 0.01). Surprisingly, the protection provided by heterologous vaccine (Y-4) was greater than that of the homologous vaccine (G-91) but the difference was statistically significant at the 5 per cent confidence level, but not at 1 per cent.

Comparative survival of the immunized and non-immunized mice after intravenous challenge with yeast cells of G-91 with regard to time in days is presented graphically in Graph VII. All deaths in mice immunized with live vaccines (groups A and B) occurred between 8 and 12 days after challenge, while deaths in mice immunized with killed vaccines (groups C and D) were observed between 11 and 29 days. An intermediate situation was encountered in the control mice (group E). Thirteen of the 17 deaths occurred between 8 and 12 days while the other four died between 13 and 23 days.

Organs of all the mice autopsied (at least two from each group) yielded positive cultures of H. capsulatum. Positive cultures for Y-4 could not be obtained in group B.
Comparative survival of control mice and mice immunized with live and killed vaccines of G-91 and Y-4, following intravenous challenge with yeast cells of G-91 (10 x LD -50).

Group A: G-91 - live vaccine; B: live Y-4 vaccine; C: G-91 - killed vaccine; D: Y-4 - killed vaccine; and E: Control.
DISCUSSION

I. Morphology

The morphological features observed in this study indicate that there is a superficial resemblance between \textit{H. capsulatum} (G-91) and Y-4. However, the morphological differences between these two organisms are so striking that there is a very little chance of confusion in the differential diagnosis.

Dimorphism in \textit{H. capsulatum} and other systemic fungi is a well documented phenomenon. Like \textit{H. capsulatum} (G-91), Y-4 also appears to have two forms under appropriate conditions. At 37°, on EHIA, it formed a yeast-like growth indistinguishable from G-91, at least in the earlier phase of growth. However, the production of more wrinkling on the surface of Y-4 slants was found to be an important distinguishing point. On human BA at 37° Y-4 appeared hemolytic while G-91 produced non-hemolytic colonies. The absence of hemolysis in G-91 may simply be a reflection of strain variation rather than being a difference of any diagnostic significance, since production of hemolysins by \textit{H. capsulatum} strains, have been reported by Salvin (1951).

Microscopically the difference between these two organisms was more marked. Y-4 consisted of oval to elliptical budding cells while G-91 yeast cells were round to oval in shape, although elliptical and somewhat elongated yeast cells have been observed in rare strains of \textit{H. capsulatum} (Fine et al., 1964).
appearance of surface wrinkling was associated with arthrospore formation which was absent in G-91.

On ESDA at room temperature after four weeks, both Y-4 and G-91 showed almost identical filamentous colonial morphology except that the former was granular while the latter was more filamentous. The morphological differences were more marked in the filamentous form as compared with the yeast phase. G-91 produced only two types of spores; viz., microconidia and the diagnostic tuberculate macroconidia (chlamydospores).

Y-4 produced four types of spores, but there is nothing resembling the classical tuberculate macroconidia of H. capsulatum. These were: (1) microconidia, almost similar in appearance to that of G-91; (2) chlamydomspore-like structures, analogous but much smaller and with smooth walls (4-5 microns); (3) branching chains of arthrospores and (4) occasional yeast-like budding cells (blastospores) were additional features that were absent in G-91. The arthrospores in particular serve to exclude H. capsulatum since it does not produce anything resembling these structures. In conclusion, on the basis of morphological features observed in this study, Y-4 appears to have an interesting intermediate position between the true dimorphic fungi and yeasts.
II. Pathogenicity

A. Pathogenicity of H. capsulatum in mice

1. Preliminary pathogenicity trial and LD-50 determination

It is evident from the results presented herein that there was a great variation in the pathogenicity of the nine strains of H. capsulatum for mice, after intravenous inoculation of yeast cells. With 32 million cells as the infecting dose mortalities ranged from 0 to 100 per cent. Strains G-2, G-76, G-91, G-132, and G-X gave 100 per cent mortalities while the strains GH-1 caused no deaths. Strains G-13, G-89 and G-46 gave 80, 40 and 20 per cent mortalities respectively. Similarly with a lower infecting dose of eight million cells strains G-2, G-X gave 100 per cent mortality while strains GH-1, G-46, and G-89 showed no deaths. Strains G-91, G-132, G-76 and G-13 gave 80, 80, 40, and 20 per cent mortalities, respectively.

It has been well established that strains of Histoplasma vary in virulence, and that intravenous injection of $10^6$ to $10^7$ yeast cells are required to kill a mouse (Emmons et al., 1963). However, mice are highly susceptible to H. capsulatum infection even though death may not result. It is interesting to note that Ajello and Runyon (1953) were able to infect a mouse with a single macroconidium from the mycelial phase culture and Rowley and Huber (1955) found that 1 to 10 yeast cells of the fungus were infective.
The present findings regarding the variation in virulence of nine strains of *H. capsulatum* (*vide supra*) and the LD-50 of three million yeast cells (approximately) of G-91 for mice after intravenous inoculation as determined by this study, are consistent with those of earlier investigators.

2. Effect of cortisone and streptomycin

The findings of this experiment indicate that normal mice receiving one and four million yeast cells of *H. capsulatum*, although becoming infected as was evident by the clinical picture, presence of pathological lesions, and positive cultures on autopsy, did not die during the 30 day observation period. However, treatment with cortisone and streptomycin, alone or in combination, increased the susceptibility of mice to fatal histoplasmosis. It is also apparent from these results that cortisone was more effective than streptomycin in enhancing the susceptibility of mice to histoplasmosis.

At least two independent earlier observations have shown the enhancing effect of cortisone treatment on susceptibility of mice to infection with *H. capsulatum* (Mankowski, 1955 and Grunberg and Titsworth, 1963). Similarly enhancement of *H. capsulatum* infection following cortisone treatment has been observed in other experimental animals, viz. dogs (Farrel et al., 1953), rats (Konisbaur, 1953) and guinea pigs (Vogel et al., 1955). In the present experiment all cortisone-treated mice (groups III and VII), but none of the untreated mice (groups V and IX), died after
intravenous inoculation with either one or four million cells of *H. capsulatum*. The group receiving four million cells died a little earlier (nine days) than those receiving one million cells (11 days). This suggests a definite enhancement effect of cortisone on pathogenicity of *H. capsulatum* for mice and is consistent with the findings cited above. On the contrary, Baum et al. (1954) and Louria et al. (1960) were unable to demonstrate augmentation of *H. capsulatum* infection in mice following cortisone treatment. This discrepancy may be due to the difference in the strain of mice or strain of *H. capsulatum* or route, doses and schedule of cortisone treatment, or a combination of all these factors. The different studies are not all comparable in these respects.

The mechanism of cortisone enhancement of *H. capsulatum* infection is not fully understood but as suggested by Kass (1960) for infection in general, is probably due to anacastic effect on certain host defenses particularly those of reticuloendothelial system. Cortisone is known to inhibit, in high dosage, the primary, but not the immune antibody response.

Streptomycin also increased the susceptibility of mice to *H. capsulatum* infection as is evident by the higher mortality of the streptomycin-treated mice in comparison with non-treated mice after infection with one and four million cells. Two of the ten (20%) streptomycin-treated mice died as compared to no deaths in non-treated mice after inoculation with one million cells. This
difference in mortality was more pronounced when the infecting dose was increased to four million cells. Streptomycin-treated mice showed 70 per cent mortality as against no deaths in the non-treated group. Campbell and Saslaw (1949) showed enhancement in growth of H. capsulatum, in vitro, by streptomycin, but were unable to show similar enhancement of infectivity in mice (Campbell and Saslaw, 1950). Again, the difference between their findings and the present study is probably due to the difference in strain of mice or route of inoculation, or both. On the basis of earlier findings of Campbell and Saslaw (1949) it is suggested that the increased susceptibility of streptomycin-treated mice to H. capsulatum is due to the direct enhancing effect of streptomycin on the organism rather than any deleterious effect on the host, since mice treated with streptomycin alone were perfectly normal.

The enhancing effect of streptomycin following infection with H. capsulatum was also indicated by the severity of the disease syndrome as manifested by general appearance and loss in body weight of streptomycin-treated as compared to non-treated mice. The difference was again more pronounced when mice inoculated with four million cells were compared with non-treated mice.

Mice treated with cortisone and streptomycin in combination following infection with one and four million cells showed increased susceptibility to H. capsulatum infection similar to that observed with cortisone alone.
It is obvious from the body weight records of the surviving mice presented in Table 2 and Graphs II and III that cortisone in the doses used was toxic to mice, manifested by poor physical appearance, slower gain in body weight and 40 per cent mortality from cortisone alone (group X). The toxic effect was more prominent during the first ten days and the mice began to improve in general condition after the withdrawal of cortisone treatment. This probably is due to the anti-anabolic effect of cortisone on protein metabolism and has been observed by earlier investigators (Hart and Rees, 1950, Louria et al., 1960, Godebusch and Gikas, 1965 and Ferriman, 1965). However, Sidransky and Friedman (1959) reported no toxic effect of cortisone in mice. Their recommended dosage was used in this study with the striking toxic manifestations reported. This again probably may be a reflection of differences in the strains of mice, since they do not state the strain, or the age of the mice, and both males and females were used in their study.

It is interesting to note that mice treated with cortisone alone (group X) and cortisone and streptomycin in combination (group I) without infection showed 40 and 10 per cent mortality. On autopsy, the mice yielded a mixed growth of bacteria, predominantly of gram-negative organisms. It appears that streptomycin may have a protective effect by inhibiting the bacterial growth in cortisone-treated mice. This is in conformity with the thesis of Kass and Finland (1958) that a sufficient dose of a specific
antibiotic usually overcomes the adverse effect of a given amount of corticosteroid.

The implication for the human would be to emphasize the importance of differentiating tuberculosis and histoplasmosis. Cortisone and streptomycin both have a role in the treatment of tuberculosis, but both are potentially harmful in histoplasmosis. The usual dosage in the human is much smaller than that used in these mice, but at the same time, cortisone-induced dissemination of infection in the human, involving a variety of bacteria, viruses, protozoa, helminthes and fungi, is well documented.

B. Pathogenicity of Y-

1. Preliminary pathogenicity trial

It is obvious from the clinical picture, particularly the "peculiar neurologic syndrome," 60 per cent mortality, the presence of lesions in the kidneys, and isolation of organisms at autopsy, that the infection of Y-1 in mice after intravenous injection was established.

The first and only well documented report of the pathogenicity of a Trichosporon species (T. capitatum) in rabbits was reported by Gilbert and Fetter in 1962. The organism was disseminated but significant proliferation was found only in kidneys, brain, and heart with predominance of lesions in kidneys in the form of mycotic abscesses. The present findings regarding pathogenicity of Y-1 in mice are comparable but not identical to those
described in the rabbit. The peculiar neurologic syndrome in mice manifested by hyperirritability, jumping, circling, ataxia, and staggering gait observed in the present study was not described by the previous workers in rabbits. Nothing can be said comparing the myocardial lesions since the mouse hearts were not studied histologically in the present investigation. The report of Gilbert and Fetter (1962) was found only after this phase of the investigation had been completed.

The intravenous route was more effective than the intraperitoneal route for the establishment of Y-4 and its subsequent pathogenicity to mice as is evident by the higher mortality and more severe neurologic syndrome. Once the organisms were introduced into mice, they were able to survive in the tissue at least for 60 days when the surviving animals were sacrificed.

The main histological lesions took the form of necrotic foci accompanied by inflammatory infiltrates (polymorphs and macrophages) and fungal elements (arthrospores and blastosporues). Occasionally abscess formation was found, always in the renal medulla. This is in contrast with findings of previous authors who found mycotic abscesses mainly in the renal cortex of the infected rabbit. It should be noted that organisms could be recovered from spleen, liver, lung, brain of the infected mice even though significant lesions could not be found.

Chang and Buerger (1964) reported a case of disseminated geotrichosis in a fifty-three-year-old white woman after
fluorouracil therapy for adenocarcinoma of the ascending colon. Histopathologically, inflammatory lesions associated with fungus elements were present in colon, lungs, heart, and spleen. On re-evaluation of the microscopic sections this case appears to be caused by a *Trichosporon* sp. rather than by *G. candidum*. The organism in tissues shows both blastospores and arthrospores which is characteristic for the genus *Trichosporon* while in *Geotrichum* only arthrospores are present (Lodder and Diddens, 1952). Moreover, the organisms seen in tissues are morphologically similar to those seen in mouse tissues in the present study and those in the rabbit described by Gilbert and Fetter (1962).

The neurologic syndrome observed in mice following infection with Y-4 can be explained either by direct invasion of the brain by the organism or indirectly by the production of some neurotoxin elsewhere in the body. Attempts to explore the pathogenesis of Y-4 to mice will be reported later.

2. Demonstration of "toxin" production by Y-4

It is evident from these data that the attempt to explain the peculiar neurologic syndrome of mice in terms of a toxin produced by Y-4 was unsuccessful.

Three so-called "toxic" preparations were tested in cortisone and non-cortisone treated mice. Preparation A, the filtrate from unwashed broken cells, could be expected to contain both "exo-" and "endotoxin(s);" preparation B, the filtrate from culture in liquid medium would have mainly exotoxin(s) and...
preparation C (the washed cell suspension) endotoxin(s) alone. No significant difference was observed in physical appearance, gain in body weight or mortality between the cortisone and non-cortisone treated mice when inoculated with different "toxic" preparations or with saline alone. This makes it very unlikely that toxin production by Y-4 is the cause of the neurologic syndrome in mice infected with this organism. This leaves the other alternative, of invasion of brain by Y-4 to explain the pathogenesis of the neurologic syndrome. This will be investigated more thoroughly in the next experiment.

Again, in this experiment there was definite evidence of cortisone toxicity.

3. Effect of cortisone and streptomycin

In this experiment normal mice, after intravenous inoculation with 17 million cells of Y-4, although they developed infection with the peculiar neurologic syndrome, pathological lesions and positive cultures on autopsy, showed only ten per cent mortality during 30 days' observation period. However, treatment with cortisone and streptomycin alone or in combination rendered the mice highly susceptible to infection with Y-4. It is also apparent from these findings that cortisone was more potent than streptomycin in reducing the resistance of mice to infection with Y-4. The fact that large doses of cortisone generally depress resistance of the host to infection with bacterial, viral, fungal,
protozoal, and even helminthic agents has been well documented (Kass and Finland, 1958).

In this experiment all the cortisone treated mice (group A) died within six days with severe neurologic symptoms as compared to only ten per cent mortality and mild neurologic symptoms in non-cortisone treated mice (group B) following infection with Y-4. This indicates a marked deleterious effect of cortisone on the course of Y-4 infection in mice. This conclusion is also supported by the post-mortem findings of the two groups. In cortisone treated mice (group A), although the organism was disseminated, the most significant lesions with proliferation of organisms (arthrospores and blastospores) were present in kidneys and brain. In the kidney, the lesions were predominantly in the glomerular zone of the cortex in the form of multiple small areas of necrosis with polymorph and mononuclear cell infiltration. There was moderate congestion of blood vessels with slight degenerative changes in the tubules. In the brain there was marked hyperemia of cortical and meningeal blood vessels accompanied by focal areas of polymorph and mononuclear cell infiltration. In some brain sections, although there was extensive proliferation of organisms, tissue reaction was minimal. This is analogous to the situation described by Gilbert and Fetter (1962) in the rabbit brain following infection with T. capitatum. In liver, lung, and spleen, although the organisms were seen as budding yeast cells, no arthrospores were present, and the tissue reaction was not very
marked. This suggests that the conditions prevailing in these organs are probably not suitable for the establishment and proliferation of Y-4.

In non-cortisone treated mice (group B), the tissue reactions were less severe but were still mainly confined to kidneys and brain. In the kidneys lesions were mostly confined to the medullary region and the renal pelvis. In brain the reaction was characterized by focal areas of congestion, minimal polymorph infiltration, and few fungal elements. By contrast, in the brains of cortisone-treated mice extensive fungal proliferation was seen. In lung and liver there was no significant tissue reaction. Budding yeast cells were seen in the blood vessels of only one of the four lung sections examined and in none of the livers. The spleen showed a more chronic type of reaction, with increased infiltration of polymorph and mononuclear cells and giant cell formation, but no organisms were seen.

In mice treated with cortisone and streptomycin (group C) all animals died within 11 days after infection with Y-4. Also in this group the neurological symptoms were most marked of any other group. Since in group A (cortisone-treated) all mice died within six days, there seems to be a slight beneficial effect of streptomycin in prolonging the life span of the treated mice, probably due to bacteriostatic effect against the normal bacterial flora. The tissue reaction was of similar nature to that of cortisone-treated mice (group A) but with a more extensive proliferation of
the fungus throughout the organs involved. This may simply be a reflection of the longer survival with more time for proliferation of the organism.

There was no difference in the mortality of streptomycin-treated mice (group D) and non-streptomycin-treated mice (group B) following infection with Y-4 (one mouse died in each group), but the neurologic symptoms were more severe in the former group but not nearly as severe as in group C. The tissue reactions in animals of the streptomycin-treated group were of intermediate intensity between non-treated mice (group B) and cortisone-treated mice (group A) but again lesions were mainly in kidney and brain. In the kidney tissue reaction in the form of suppurative foci was predominantly in the medullary region. In the brain, the tissue reaction was characterized by congestion and increased infiltration of polymorph and mononuclear cells with the presence of G.M.S. positive organisms. These findings point to the fact that treatment with streptomycin enhanced susceptibility of mice to infection with Y-4, but the effect was less marked than that of cortisone treatment.

On the basis of this experiment and the attempted "toxin" production (vide supra), it appears that the neurologic syndrome of mice infected with Y-4 is due to the direct invasion of brain tissue by Y-4 rather than to any toxin production.

In summary, the main difference noted is that *H. capsulatum* produces lesions in reticuloendothelial tissue and
organs (as is already well known) whereas the major lesions of Y-4 are in organs with little or no reticuloendothelial tissue (kidney and brain). Even where spleen and liver are involved by Y-4 the reticuloendothelial tissue does not seem preferentially affected.
III. Immunology

A. Skin test cross-reactivity between H. capsulatum (G-76 and G-91) and Y-4

It is evident from the results of this experiment that there is almost complete skin test cross-reactivity between the yeast and mycelial antigens of G-91 and G-76 (histoplasmin) on the one hand and Y-4 (trichosporin?) on the other. It is of interest that on first skin-testing six weeks post-infection only homologous reactions were well developed. Heterologous skin reactions were either absent or of borderline significance. On second test, after 12 weeks post-infection homologous and heterologous reactions were almost of equal intensity. Thus, development of skin test reactivity appears to be a sequential event beginning with homologous, followed by heterologous antigen reactivity.

No strictly quantitative comparison can be made but the skin test reactions with yeast phase antigens were more pronounced and usually persisted 48 hours or longer in contrast to a 24-hour duration with mycelial antigens. This is in conformity with the findings of Howell (1947) and supports the contention of Salvin (1965) and Tompkins (1965) regarding the superiority of yeast phase over mycelial phase antigens, for other purposes as well as skin testing.

Sensitization of guinea pigs by repeated skin testing with histoplasmin and blastomycin without infection has been reported
(Howell, 1948 and Smith et al., 1963). However, diluted antigens are less likely to cause this "false-positive" reaction. In the present study the control uninfected animals of group D (Figs 7 and 8) remained negative to both antigens throughout the study. They were tested three times in all, over a 13-week period, at the same times as the experimental animals.

Skin test cross-reactivity of mycotic "-ins," viz., histoplasmin, blastomycin, coccidioidin and haplosporangiin has been well documented (Emmons et al., 1945, Howell, 1947, Smith et al., 1949, Salvin, 1959 and others). Also, Asgari and Conant (1964), on the basis of their experiments with histoplasmin and chrysosporin in guinea pigs have suggested the possibility of heterologous sensitization of human beings with Chrysosporium keratinophilum causing false positive reactions with histoplasmin. This suggestion is supported by circumstantial evidence since C. keratinophilum (but not H. capsulatum) was isolated from the soil of a village in Iran, where histoplasmin-positive individuals, without any clinical evidence of histoplasmosis, were found. Further, they supported Palmer et al. (1957) who suggested that sensitization of 30 per cent of navy recruits to histoplasmin might be due to a fungus whose identity was not known at that time. They indicated that C. keratinophilum was a good possibility, since this organism has now been reported from the soil of Southern California (Carmichael, 1962).
Similarly on the basis of present findings of skin test cross-reactivity in guinea pigs between \textit{H. capsulatum} and Y-4 (a fungus isolated in India), the sensitization of human populations to histoplasmin by Y-4 in India and/or elsewhere is possible. It would be of interest to test people showing skin reactivity to several mycotic antigens with Y-4 antigen also.

B. \textbf{Serological cross-reactions between G-91 and Y-4}

Seeliger and Schroter (1963) have used direct agglutination, precipitation, immunodiffusion and complement-fixation tests with the whole yeast cell antigen and with crude polysaccharide extracts, in the classification of the genus \textit{Trichosporon} and have encountered nonspecific agglutination with whole yeast cells. The collodion agglutination test has been proved to be an important technic in the serology of histoplasmosis (Saslaw and Campbell, 1948 and Schubert and Wiggins, 1963). The results described herein with rabbit sera suggest that collodion agglutination and immunodiffusion tests can be employed successfully in serologic studies of Y-4.

The comparative results of collodion agglutination and immunodiffusion tests indicate the presence of some common antigen(s) between \textit{H. capsulatum} and Y-4. Serological cross-reactions between histoplasmosis, blastomycosis and coccidioidomycosis have been well documented (Smith \textit{et al}., 1949, Salvin, 1949, Campbell \textit{et al}., 1953, Labzoffsky \textit{et al}., 1957 and
Relatively less well known is the antigenic sharing between *H. capsulatum*, *C. albicans* and *C. neoformans* (Salvin, 1949 and 1950) and *H. capsulatum*, *Sepedonium*, *Chrysosporium*, *H. farcinosum* and *E. perva* (Kaufman and Brandt, 1964). On the basis of preliminary serological cross-reactions described herein, it is suggested that *Y-U* by virtue of its interesting position between dimorphic fungi and yeasts and its occasional isolation from sputum specimens, may be an agent of still unrecognized systemic mycotic infections responsible for the false positive serological reactions with *H. capsulatum* antigen, as was strongly suggested by Campbell (1965a). It is interesting to note the higher heterologous collodion agglutination titer (1:256) with *Y-U* antigen, as compared to G-91 antigen (1:64) with anti-G-89 (*H. capsulatum*) antiserum is analogous to the situation commonly observed with histoplasmosis patients who give higher titers with *Blastomyces* antigen. The failure to demonstrate cross-reaction between *H. capsulatum* antigen and *Y-U* antisera, is difficult to explain, but several possibilities may be suggested. (1) The immune sera prepared against *Y-U* may not have had enough time to develop the cross-reacting antibodies against G-91 antigen, as compared to hyperimmune anti-*H. capsulatum* where cross-reacting antibodies against *Y-U* antigen were demonstrated. (2) The anti-*H. capsulatum* sera which showed cross-reactions with *Y-U* antigen, were hyperimmune and thus were less specific than anti-*Y-U* sera.
(3) The cross-reacting antigens are a minor component of Y-4 and therefore less effective antigens. (4) The cross-reacting G-91 antigen is chemically different from Y-4 and thus was destroyed during preparation. (5) Too little cross-reacting antigen to show up in G-91 antigen.
IV. Cross-Protection

The data presented herein show that significant protection of mice against lethal challenge with \textit{H. capsulatum} can be achieved by suitable immunization. The greatest protection was observed in mice immunized with G-91 live vaccine and was definitely superior (at least as regards mortality) to that obtained by immunization with killed cells. This is not in agreement with the findings of Rowley and Huber (1956) who obtained protection with live vaccine, but no protection whatsoever with the killed vaccine. On the other hand, Schaefer and Saslaw (1954) and Hill and Marcus (1959) did not find any significant difference in the protection given by live and killed vaccines following lethal challenge with \textit{H. capsulatum}. It is quite possible that the discrepancies between present findings and those reported earlier are the result of differences in the strains of \textit{Histoplasma}, or in the methods of immunization and in the route of challenge, and in the challenge dose.

Significant cross-protection against \textit{H. capsulatum} in mice immunized with Y-4 killed, but not with Y-4 live vaccine was observed. This difference is difficult to explain but may either be due to insufficient number of living Y-4 cells inoculated or to differences in the pattern of infection of Y-4 and \textit{H. capsulatum} as was seen in pathogenicity studies (\textit{vide supra}).
The protective effect of killed Y-4 vaccine is presumably mediated by antibody. It can be assumed that the live Y-4 cells will also have some effect in this direction. On the other hand, the live Y-4 cells are also producing an infective process which may increase susceptibility to other types of infection (in this case, G-91). The end result of immunization by Y-4 live cells would then depend on which process was the more significant. The results in Table 18 suggest a protective effect of killed Y-4 which is masked in the living vaccine by the increased susceptibility suggested above.

All deaths in mice immunized with live vaccines occurred between 8 and 12 days after challenge while deaths in mice immunized with killed vaccine were observed between 11 and 29 days. An intermediate situation was encountered in the control mice. Thirteen of the 17 deaths occurred between 8 and 12 days while the other four died between 13 and 23 days. These findings are comparable with those of Schaefer and Saslaw (1954), who observed all deaths within the first four days after challenge in the mice immunized with live organisms as compared to deaths occurring between 7 and 22 days in the control group.

Cross-protection between H. capsulatum and B. dermatitidis has been demonstrated earlier by Salfelder and Schwarz (1964) and Sethi et al. (1964). On the contrary, Salvin (1953, 1955a) was unable to show any cross-protection between H. capsulatum, B. dermatitidis and C. albicans. However, resistance to R. typhi and
R. tsutsugamushi has been demonstrated in mice previously injected with sublethal doses of yeast cells of H. capsulatum (Salvin, 1955b). Hedgecock (1961) reported increased resistance in mice against tuberculosis by immunization with a killed vaccine prepared from mycelial phase but not from yeast phase of H. capsulatum. This resistance was acquired in seven days and maintained for about 29 days. Resistance was also induced 14 days after the injection of preparation from Brucella abortus, Cryptococcus neoformans, and both phases of Sporotrichum schenckii and Blastomyces dermatitidis. The resistance of all animals except those injected with yeast phase of B. dermatitidis decreased after 29 days.

All of these findings indicate that cross-protection mechanisms are very complex and that we do not have enough data and understanding to offer an hypothesis to explain why protection does or does not occur.
SUMMARY

The importance of systemic mycotic infections such as histoplasmosis, in this modern era of increasing antibiotic and corticosteroid therapy, the problems associated with its diagnosis and treatment due to antigenic-sharing with other fungi and lack of an effective treatment, and an urgent need for a vaccine have been well recognized. The interesting taxonomic position of a Trichosporon-like organism, and its apparent morphological resemblance with *Histoplasma capsulatum* led to the study reported herein which was designed to define the extent of relationship between these two organisms, using morphologic criteria and studies of pathogenicity, immunology and cross-protection.

A superficial morphological resemblance between *H. capsulatum* (G-91) and a Trichosporon-like organism (Y-4) was demonstrated. Like G-91, Y-4 appears to have two growth phases (yeast and mycelial) under appropriate conditions. However, the differences between these two organisms are so striking that there is a very little chance of confusion in the differential diagnosis.

A great variation in the virulence of nine strains of *H. capsulatum* for mice was observed, after intravenous inoculation of yeast cells. The LD-50 of G-91 was found to be approximately three million yeast cells. Treatment with cortisone and streptomycin, alone or in combination, increased the susceptibility of mice to fatal histoplasmoses. In this respect cortisone was more
deleterious than streptomycin. Cortisone alone in the doses used was toxic to mice. The importance of differentiating tuberculosis and histoplasmosis is emphasized, since cortisone and streptomycin both have a role in the treatment of tuberculosis, but both are potentially harmful in histoplasmosis.

A heretofore unrecognized infection in mice with $Y^{-4}$ was established. The infected mice showed a peculiar neurologic syndrome manifested by hyperirritability, jumping, circling, ataxia, and staggering gait, before death or recovery. Attempts to demonstrate the production of a neurotoxin by $Y^{-4}$ were unsuccessful. Here, again, treatment with cortisone and streptomycin alone or in combination greatly increased the susceptibility of mice to infection with $Y^{-4}$. Histopathological examination revealed that the kidneys and brain were the main sites for the invasion and proliferation of $Y^{-4}$.

Guinea pigs were infected with yeast cells of $H. capsulatum$ and of $Y^{-4}$ and skin-tested 6 and 12 weeks post-infection both with homologous and heterologous antigens. There was almost complete skin test cross-reactivity between histoplasmin on the one hand and $Y^{-4}$ antigen (trichosporin?) on the other. These findings in laboratory animals suggest the possibility of sensitization of human populations to histoplasmin by apparent or inapparent infection with $Y^{-4}$.

The serological cross-reactions between $H. capsulatum$ and $Y^{-4}$ yeast-phase antigens were demonstrated by collodion
agglutination and immunodiffusion tests. There was a strong cross-reaction between anti-,_H. capsulatum_ hyperimmune sera and Y-4 antigen. However, cross-reaction between anti-Y-4 sera and _H. capsulatum_ antigen could not be demonstrated. It is suggested that Y-4 may be an agent of still unrecognized systemic mycotic infections responsible for the false positive serological cross-reactions with _H. capsulatum_ antigen.

The cross-protection studies were carried out by immunizing mice intraperitoneally with live and formalin-killed yeast cells of G-91 and Y-4. Immunized and non-immunized mice were challenged intravenously 21 days later with the yeast cells of G-91 (10 x LD-50). The greatest protection was observed in mice immunized with G-91 live cells and was definitely superior to that obtained with G-91 killed vaccine. Significant protection against challenge with _H. capsulatum_ in mice immunized with Y-4 killed but not with Y-4 live cells was observed. The mechanisms of cross-protection are very complex and we do not have enough data and understanding to offer an hypothesis to explain why protection does or does not occur. The possible significance of these findings is discussed.
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