A COMPARISON OF THE IMMUNOGENICITY OF WEAKLY ENCAPSULATED AND OF STRONGLY ENCAPSULATED STRAINS OF CRYPTOCOCCUS NEOFORMANS (TORULA HISTOLYTICA)[1]

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This paper deals with a comparison of the antibody-evoking capacities (in rabbits) of weakly encapsulated and of strongly encapsulated cells of *Cryptococcus neoformans* and with the influence of the number of the fungus cells (amount of vaccine) upon the strength and the promptness of the antibody response. The data on these two points contribute toward a better understanding of the immunogenic properties of this fungus and, in addition, present an instance in which antibodies reactive with the large capsules of strongly encapsulated forms of a species were produced more effectively by immunization with weakly encapsulated forms than by immunization with the strongly encapsulated forms of the species.

The prevailing impression that *Cryptococcus* antiserums are difficult or inconvenient to produce has come from the fact that a number of workers failed with all strains tried, others failed with some strains although succeeding with others, and all who have reported the production of reasonably potent antiserums have employed prolonged series of injections over periods ranging from 6½ to 12 weeks. The literature consists of the papers of Stoddard and Cutler (1916), Sheppe (1924), Rappaport and Kaplan (1926), Benham (1935), Cox and Tolhurst (1946), Kligman (1947), Drake (1948), and Evans (1949). Most of the authors give no information either on the degree of encapsulation or on the density of the *Cryptococcus* suspensions employed for the immunizations. Cox and Tolhurst (1946), and also Evans (1949), have reported differences in the immunogenic effectiveness of different strains but did not mention any differences in encapsulation. Kligman (1947) did describe differences in encapsulation of the test strains, but his results (with immunizing procedures much different than the one we will describe) were uniformly negative with a weakly, an intermediate, and a strongly encapsulated strain.

Textbooks and literature reviews commonly state that a high degree of encapsulation is responsible for the reportedly poor immunogenicity of this fungus. However, there has been little, if any, experimental evidence. The opinions expressed are always based (when any supporting reference is given) upon the results Benham (1935) obtained by immunization with *Cryptococcus* cells that had been allowed to stand (presumably at room temperature) for 1¼ hours in the presence of 0.05 N HCl. Benham (1935) does not actually state what effect this

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mild acid treatment had upon the capsules; and in our own trials, and also in Kligman's (1947), similar treatment certainly did not destroy *Cryptococcus* capsules and, indeed, seemed to have no appreciable effect upon either their size or morphology.

STRAINS OF CRYPTOCOCCUS

A collection of 15 strains, when examined in India ink preparations (Weidman and Freeman, 1924), were found to fall into three groups on the basis of their encapsulation on routine agar slants. The first group comprised 4 strains that consisted entirely, or almost entirely, of cells with only narrow and poorly defined capsules. The second comprised 3 strains that consisted predominantly of cells with relatively wide and prominent capsules. The third group comprised 8 strains that consisted of mixtures of weakly and strongly encapsulated cells with neither form in predominance.

The 4 weakly encapsulated and the 3 strongly encapsulated strains were selected

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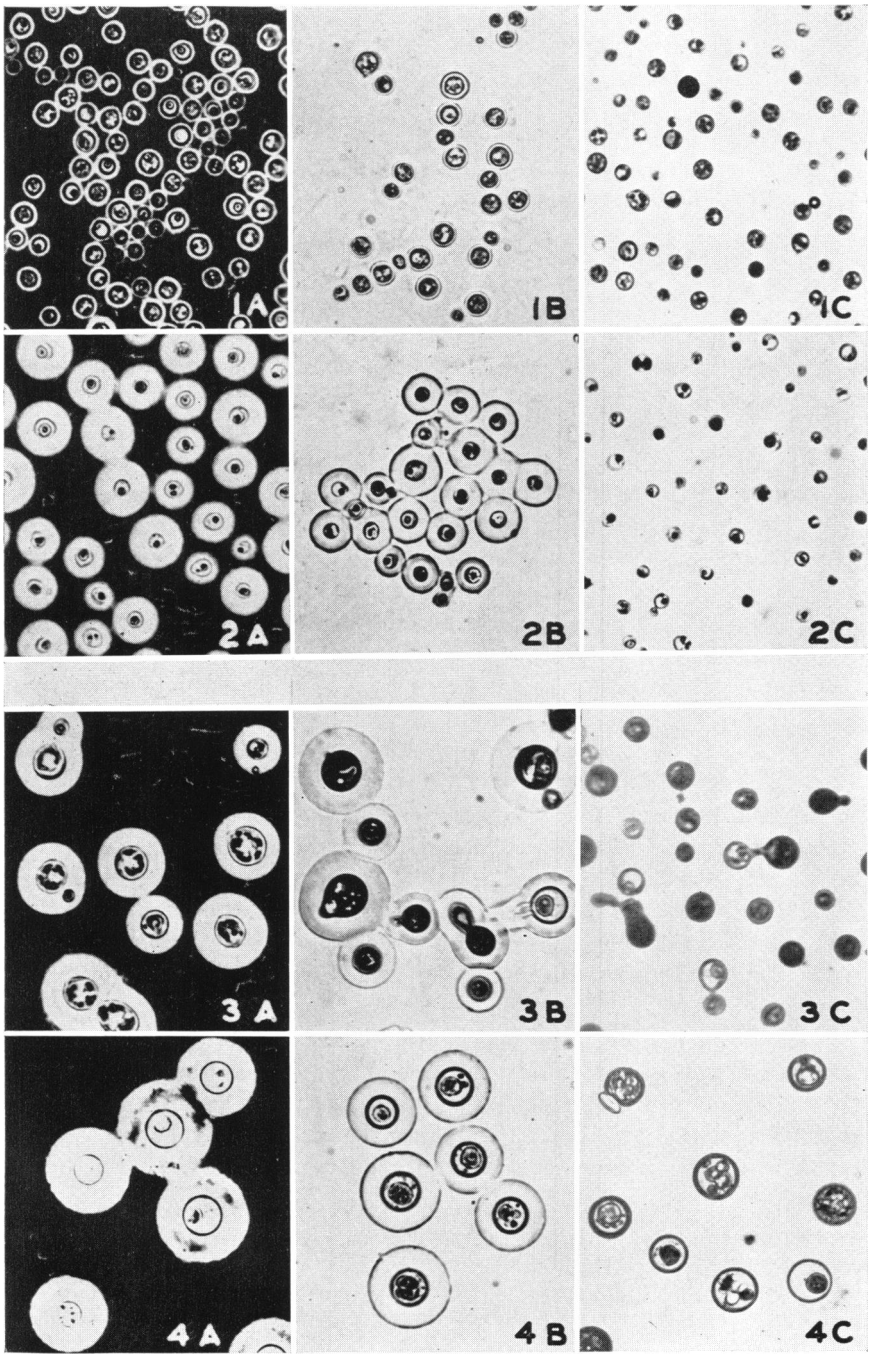
Figure 1. Cells from an agar culture of a weakly encapsulated strain (no. 1) of *Cryptococcus neoformans*. 1*A*: *Cryptococcus* cells plus India ink (without serum). 1*B*: *Cryptococcus* cells plus *Cryptococcus* antiserum. 1*C*: *Cryptococcus* cells plus normal rabbit serum, as a control. Note that the capsules give a definite quellung reaction in 1*B*, although of course it is not so prominent as the quellung reaction with the large-capsuled cells in figure 2. (The capsules of the predominating cells of strain nos. 2, 3, and 4 were slightly narrower than those shown for strain no. 1.)

The magnification in all the figures in this paper is × 560.

Figure 2. Cells from an agar culture of a strongly encapsulated strain (no. 5) of *Cryptococcus neoformans*. 2*A*: *Cryptococcus* cells plus India ink (without serum). 2*B*: *Cryptococcus* cells plus *Cryptococcus* antiserum. 2*C*: *Cryptococcus* cells plus normal rabbit serum, as a control. (The capsules of the predominating cells of strain nos. 6 and 7 were the same as shown for no. 5. All of this group contained a small proportion of cells with narrower capsules than are in the illustration.) Note that the size of the capsules appears about the same in the India ink preparations without serum (figure 2*A*) as in the quellung test preparation with the reactive antiserum (figure 2*B*); this phenomenon, which is apparent also in figures 3 and 4, is commented upon in footnote 4 to the text.

Figure 3. *Cryptococcus* cells from mice injected with the same strain (no. 1) whose agar-grown cells are illustrated in figure 1. 3*A*: *Cryptococcus* cells plus India ink (without serum). 3*B*: *Cryptococcus* cells plus *Cryptococcus* antiserum. 3*C*: *Cryptococcus* cells plus normal rabbit serum, as a control. All the strains that were only weakly encapsulated in agar cultures showed much larger capsules when obtained from infected mice; with one exception (strain no. 2), the capsules were as large as those illustrated here for strain no. 1. Note by comparison with figure 1 that the "endocellular" or noncapsular proportion also is larger in the *Cryptococcus* obtained from mice than is the case with the *Cryptococcus* grown on our routine agar medium.

Figure 4. *Cryptococcus* cells from mice infected with the same strain (no. 5) whose agar-grown cells are illustrated in figure 2. 4*A*: *Cryptococcus* cells plus India ink (without serum). 4*B*: *Cryptococcus* cells plus *Cryptococcus* antiserum. 4*C*: *Cryptococcus* cells plus normal rabbit serum, as a control. Note by comparison with figure 2, that the capsules of this strain are somewhat larger when obtained from mice than when obtained from agar cultures, but the difference is insignificant in comparison to the difference shown for the strain illustrated in figures 1 and 3. Note also that the increase in size of the endocellular or noncapsular portion when the *Cryptococcus* are from mice was as great with this strain as with the other strain.



Figures 1–4

for the study. All but one strain (which was from a nasal tumor of a horse) were from spinal fluid, lung tissue, or skin lesions of human cases of cryptococcosis (torulosis). The dates of original isolation ranged from 1912 to 1945, but there was no relationship between the degree of encapsulation and the distance from the time of isolation. All the strains killed mice when injected intraperitoneally; relatively large doses were required for fatal infections with any of them, but there was no consistent difference between the weakly encapsulated and the strongly encapsulated strains in respect either to minimal killing dose or survival time.

The difference in degree of encapsulation of the cells from agar cultures of the two groups of strains has been a prominent and consistent property throughout the period of our study. However, all the strains formed large capsules when they grew in mice; in fact, under those conditions, the capsules of the strains that produced only small capsules on agar were (with one exception) apparently as large as the capsules of the other group of strains.[2] The encapsulation of the cells obtained from agar cultures of a strain of each group is illustrated in figures 1 and 2; the encapsulation of the *Cryptococcus* cells from mice that had been infected with agar cultures of the same strains is illustrated in figures 3 and 4. Although only agar-grown cells were used as immunizing material, it is important to recognize that the strains that formed only small capsules on routine agar had a *potential* capacity to produce large capsules even though they could not manifest the capacity when grown under the nutritional and environmental conditions presented on our routine agar medium.

METHODS

Immunizing material. The vaccines were made from cultures grown on our routine agar medium (20 g agar, 10 g Difco peptone, 10 g glucose, 2 g Difco yeast extract, and 5 g NaCl, per liter). The *Cryptococcus* cells were suspended in salt solution containing 2.0 per cent formalin, stored 18 hours at 22 C to 24 C, washed once, heated 30 minutes at 56 C, washed a second time, and then stored in the icebox in the presence of 0.5 per cent formalin. Each day's supply of antigen was given a third washing immediately before being used for the injection of the rabbits. The object of the repeated washings was to reduce to a minimum the amount of free soluble antigen in the immunizing material. The suspensions of all the strains were adjusted to the same turbidity in a Klett-Summerson colorimeter with a green filter. The number of *Cryptococcus* cells in the adjusted suspensions ranged in hemocytometer count from 700 to 950 million per ml,

[2] It is significant that the highly encapsulated *Cryptococcus* recovered from the mice infected with the weakly encapsulated agar cultures failed to produce large capsules when transferred back to agar. That is, although the heart's blood and peritoneal washings from these mice contained (as far as could be told by microscopic examination) only highly encapsulated forms, the colonies that developed on plates inoculated with those materials consisted of weakly encapsulated forms not appreciably different in appearance from the cells in ordinary agar cultures of the same strains before the animal passage. (The possible influence of several successive mouse passages, before transfer to the agar medium, was not tried.)

without any regular difference between the suspensions of the strongly and the weakly encapsulated cells. (Apparently the capsules did not contribute to the optical density of the suspensions.)

Differential counts of India ink mounts showed that the suspensions of all the weakly encapsulated strains were entirely free of cells with wide capsules comparable to the capsules of the predominating cells of the other group of strains, and contained less than 1 per cent of cells with intermediate-sized capsules. The suspensions of the strongly encapsulated strains were less homogeneous; many cells with only moderate-sized capsules and also appreciable numbers (8 to 12 per cent) of cells with small capsules were present. The proportion of small-capsuled cells in the suspensions of the strongly encapsulated strains is important because it is a factor to take into account in the interpretation of the antibody-evoking capacities of the suspensions.

The three different doses of vaccine used in the experiments (referred to in the text as $100x$, $10x$ and $1x$) represented 3-ml amounts of the described stock suspensions; undiluted, diluted 1:10, and diluted 1:100, respectively. Although the stock suspensions had been adjusted on the basis of turbidity, the largest dose can be considered to be approximately 2.5 billion *Cryptococcus* cells per injection; the smallest dose, 25 million per injection.

Tests of the antiserums. The antigens used in the agglutination tests were prepared by diluting the previously described stock suspensions to a density of about 15 million cells per ml; the diluted suspensions were given an additional washing before their use. The precipitation test antigens were solutions of polysaccharide (supplied by Dr. E. J. Hehre and Mr. A. S. Carlson) from a culture of a weakly encapsulated strain and a solution of unpurified-alcohol-precipitable material from a culture of a strongly encapsulated strain (Neill, Castillo, Smith, and Kapros, 1949). The test mixtures, comprised of equal volumes of antigen and of serum or serum dilution, were incubated for 2 hours at 37 C and then stored in the icebox, for 3 days in the cases of the precipitation tests on both the antigens and of the agglutination tests on the weakly encapsulated strains, and for 5 days in the case of the agglutination tests on the strongly encapsulated strains. The icebox storage caused a considerable increase in the titers above that evident after 2 hours at 37 C.

The quellung test antigens were prepared from infected mice by the procedure described previously (Neill, Castillo, Smith, and Kapros, 1949). Since the titers were influenced both by the concentration and by the thoroughness of the washing of the *Cryptococcus* cells, all the suspensions were adjusted to comparable cell counts (about 12 per low-power field) and washed four or five times immediately before each experiment.

EXPERIMENTAL RESULTS

Immunogenicity of the strains. The antibody-evoking capacities of the four weakly encapsulated strains (nos. 1, 2, 3, and 4) and of the three strongly encapsulated strains (nos. 5, 6, and 7) were compared by immunizing pairs of rabbits with each test dose of each *Cryptococcus* strain. The first course consisted

of 13 daily injections, and bleedings were taken 7, 14, and 21 days after the last injection. Two to four additional courses were given; the amount of vaccine and number of injections were the same as in the first course in the case of the $1x$ series of rabbits, but they were considerably reduced for the others in order to avoid the occurrence of anaphylactic reactions; only 7-day bleedings were taken in the later courses.

As a control on possible qualitative serological differences among the strains, samples of antiserums of strain nos. 1 to 6 that had relatively high potency against the homologous strains (no. 7 did not yield a reactive antiserum) were tested against all the strains. Each of these antiserums gave both agglutination and quellung reactions with the *Cryptococcus* cells of each of the seven strains; and, although the titers were not identical, the differences observed were insufficient to indicate qualitative serological differences among the strains.[3]

Following the foregoing preliminary experiment, the entire collection of antiserums were tested for agglutination against the three strongly encapsulated and two or more weakly encapsulated strains; quellung tests were made with *Cryptococcus* cells from mice infected with strain no. 1 and with strain no. 6; and precipitation tests were made with the solutions of the previously described soluble antigens obtained from broth cultures of strain nos. 1 and 5.

The influences of the degree of encapsulation and of the amount of vaccine can be illustrated by the data from the tests on the first bleeding following the first course of injections. In the summary of these data (table 1) the agglutination titers are presented only for the homologous strain and for one weakly encapsulated (no. 2) and one strongly encapsulated strain (no. 6). The titers for these strains are fairly representative of the titers for the other weakly and the other strongly encapsulated strains that were tested, and afford a convenient basis for the comparison of the serums. The titers for the homologous strain are included to show that the conclusions drawn from the results of the tests against antigens of heterologous strains were not complicated by any minor qualitative serological ("type") differences (see footnote 3) that might exist among our strains.

The superior immunogenic capacity of the weakly encapsulated *Cryptococcus* strains is apparent throughout the data (table 1), whether the comparison is based upon the agglutination of the homologous (see footnote 3) or the heterologous strains, upon the precipitation of soluble antigens, or upon the quellung[4]

[3] In a paper that appeared after the completion of our experiments, Evans (1949) has reported the occurrence of qualitative serological ("type") differences among his collection of *Cyptococcus* strains. Although the capacities of the antiserums of our strain nos. 1 to 6 to agglutinate and to give quellung reactions with the cells of strain nos. 1 to 7 show that all our seven strains are closely related, some differences of the sort found by Evans (1949) might be revealed by tests with absorbed serums. However, the data presented for the reactions against the homologous strains seem an adequate control on the validity of the conclusion drawn from the data on the antigens from the other strains included in this paper.

[4] The term "quellung" is used in the present paper for convenience and also because of the similarity in immunological principle between the reactions of the capsules of *Crypto-*

reactions. The data from all the tests contribute to the completeness of the evidence. But it is particularly significant that the superior immunogenic capacity of the weakly encapsulated *Cryptococcus* applied to antibodies that caused agglutination and quellung reactions with the strongly encapsulated cells.

TABLE 1

Influence of the size of the capsules and of the amount of vaccine upon the antibody response of rabbits to cells of Cryptococcus neoformans

IMMUNIZATION MATERIAL			TITERS* OF THE ANTISERUMS AFTER ONE COURSE OF 13 DAILY INJECTIONS				
			Agglutination of cells from agar cultures				
Size of the capsules	Amount of vaccine injected†	Strain from which the vaccine was made	Homologous strain	Strain no. 2 (small capsules)	Strain no. 6 (large capsules)	Quellung of *Cryptococcus* from mice	Precipitation of soluble antigens
Small	1*x*‡	No. 1	3, 40	3, 40	U, 10	0, U	0, 3
		No. 2	10, 10	10, 10	3, 3	0, 0	0, 0
	10*x*	No. 1	160, 320	160, 320	40, 40	10, 10	10, 10
		No. 2	80, 160	80, 160	20, 40	10, 10	10, 10
		No. 3	80, 320	80, 160	20, 80	3, 10	3, 20
		No. 4	160, 640	160, 640	40, 160	10, 40	10, 40
	100*x*	No. 1	160, 320	160, 320	80, 80	10, 10	10, 20
		No. 2	80, 80	80, 80	20, 40	3, 10	3, 10
Large	1*x*‡	No. 5	0, 0	0, 0	0, 0	0, 0	0, 0
		No. 6	0, 0	0, 0	0, 0	0, 0	0, 0
	10*x*	No. 5	U, U	3, 3	U, U	0, U	0, 0
		No. 6	0, 0	0, 0	0, 0	0, 0	0, 0
		No. 7	0, 0	0, 0	0, 0	0, 0	0, 0
	100*x*	No. 5	3, 10	20, 20	3, 3	3, 3	3, 3
		No. 6	20, 20	40, 80	20, 20	3, 10	3, 10

* The titers presented are the maximum dilution of serum that gave positive reactions when mixed with an equal volume of the test antigen. The titers for the 2 rabbits immunized with the same material are separated by commas.

† The 1*x* dose contained about 25 million *Cryptococcus* cells per injection; the 10*x* and 100*x* doses, 10 and 100 times that amount.

‡ Additional courses of the 1*x* doses caused an increase in the titers in the case of strain nos. 1 and 2, but not in the case of strain nos. 5 and 6.

This phenomenon presents an instance in which antibodies reactive with antigens on the capsules of highly encapsulated forms of a species were produced much more effectively by immunization with weakly encapsulated forms than by immunization with strongly encapsulated forms of the species.

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coccus and the so-called quellung reactions of the capsules of pneumococci and other bacteria. However, as pointed out previously (Neill, Castillo, Smith, and Kapros, 1949), the major part of the visible phenomenon in the case of *Cryptococcus* seems to be due to a change that makes the capsule more readily seen rather than to any great increase in the size of the capsule. This point, not referred to in the text, can be seen by comparison of the India ink and quellung test preparations in figures 2, 3, and 4.

That the agglutination titers of *all* the antiserums were higher in the tests against the weakly encapsulated *Cryptococcus* cells than in the tests against the strongly encapsulated *Cryptococcus* cells can, we believe, be regarded as being due merely to a quantitative difference in the "sensitivity" of the test suspensions. A relatively low degree of "agglutinability" of highly encapsulated microbial cells is a common observation with Friedlander's bacilli and other bacteria.

The data on the strains (nos. 1, 2, 5, and 6) that were tried in three different doses show that the amount of vaccine influenced the responses to the suspensions of all the strains and that, even with the weakly encapsulated strains, relatively large doses had to be employed in order to obtain potent *Cryptococcus* antiserums within the short period of 20 days (13 days for injections plus a 7-day rest).

However, none of the data answer the fundamental question of the intrinsic immunogenicity of large-capsuled *Cryptococcus* cells because a considerable part (perhaps all) of the antibody response to the relatively large doses ($10x$ and $100x$) of the strongly encapsulated vaccines might have been evoked by the small but nevertheless significant proportion (about 10 per cent) of poorly encapsulated cells that were present.[5] Other factors increase the difficulties of interpretation, among which is the possibility that the predominating large-capsuled cells might have some inhibitory influence upon the potential immunogenic capacity of the accompanying small-capsuled cells.

Weakly and strongly encapsulated forms isolated from cultures. A weakly encapsulated and a strongly encapsulated form were isolated from strain no. 5, which was one of the strongly encapsulated cultures employed in the preceding experiment. The weakly encapsulated form was obtained from one of the relatively small, opaque, and faintly pigmented colonies that occurred in small numbers on plates inoculated with the parent culture. The strongly encapsulated form was from one of the relatively large, translucent, mucoid, and nonpigmented colonies that predominated on the plates. As shown in figures 5*A* and 5*B*, the capsular difference between these two forms was as great as the capsular difference between the two groups of strains that were used in the preceding experiment. The strongly encapsulated form seemed to have a greater average capsular size than the original culture (no. 5), but the proportion of cells with narrow capsules was not much less (3 per cent in comparison to 8 per cent in the original culture). However, the weakly encapsulated form was quite homogeneous, since no cells having more than very narrow capsules were found in thorough examinations of many India ink preparations.

The antibody-evoking capacities of the two forms were compared by giving rabbits one course of 13 daily injections of $10x$ doses of the vaccines. The antiserums were tested against the same antigens used in the preceding experiment

[5] In the event that the antibody response to the large doses of the strongly encapsulated strains did come from the accompanying small-capsuled forms, one would have to consider the theoretical possibility that even the $1x$ dose used in our experiment represented an excess of antigen for the strongly encapsulated cells. Although we believe that possibility is remote, we are now immunizing rabbits with smaller doses in order to get some data on that question.

and also for agglutination and quellung reactions against the cells from agar cultures of the newly isolated strongly encapsulated form. For comparison, the

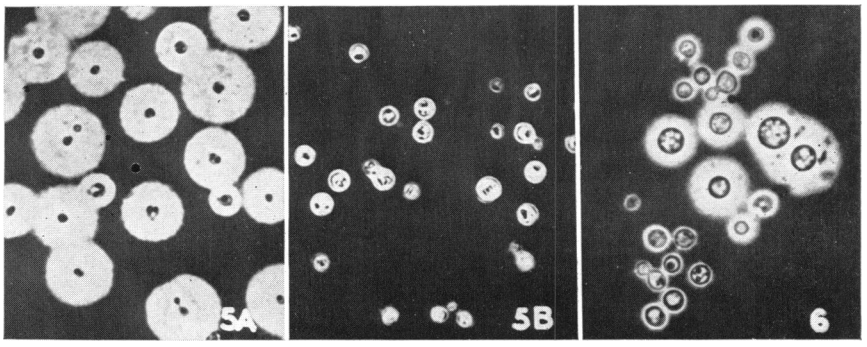

Figure 5. India ink preparations (without serum) of cells from agar cultures of the two forms that were isolated from a highly encapsulated strain (no. 5, which is illustrated in figure 2). *5A*: The strongly encapsulated isolated form. *5B*: The weakly encapsulated isolated form.

Figure 6. India ink preparation (without serum) of cells from an agar culture of a strain (no. 8) that consisted of a mixture of weakly and strongly encapsulated cells with neither form in noticeable predominance. The cells of the weakly encapsulated and of the strongly encapsulated forms that were isolated from this strain (no. 8) showed a difference in capsular size comparable to that illustrated in figures *5A* and *5B* for the corresponding forms isolated from strain no. 5.

TABLE 2

Comparison of antiserum produced by immunization with the weakly encapsulated form and with the strongly encapsulated form isolated from a culture (strain no. 5) that consisted predominantly of strongly encapsulated forms

STRAIN USED TO PRODUCE THE ANTISERUM	AMOUNT OF VACCINE INJECTED	TITERS* versus ANTIGENS PREVIOUSLY USED IN THE EXPERIMENT PRESENTED IN TABLE 1				TITERS versus THE NEWLY ISOLATED STRONGLY ENCAPSULATED FORM	
		Agglutination of cells from agar cultures		Quellung of *Cryptococcus* cells from mice	Precipitation of soluble antigens	Agglutination of cells from agar cultures	Quellung of cells from agar cultures
		Strain no. 2	Strain no. 6				
Weakly encapsulated form	10*x*	160, 320	80, 160	10, 20	10, 20	80, 160	10, 10
Strongly encapsulated form	10*x*	U, 3	0, U	0, 0	0, 0	0, 0	0, 0
Stock culture of no. 5	10*x*	3, 3	U, U	0, U	0, 0	0, U	0, 0
Stock culture of no. 5	100*x*	20, 20	3, 3	3, 3	3, 3	3, 3	U, U

* The titers presented are the maximum dilution of serum that gave positive reactions when mixed with an equal volume of the test antigen. The titers for the 2 rabbits immunized with the same material are separated by commas.

antiserums obtained by immunization with 10*x* and 100*x* doses of cells from the original culture (strain no. 5) were included in all of the tests. The data are presented in table 2.

The data (table 2) show that the weakly encapsulated form isolated from strain no. 5 was a much more effective immunizing agent than either the newly isolated strongly encapsulated form or the original culture of strain no. 5. The greater effectiveness is apparent in all the tests, including the agglutination and quellung tests made against the strongly encapsulated form. Furthermore, as can be seen by comparison with table 1, this weakly encapsulated form, isolated from a culture that was only poorly immunogenic, had an immunogenic capacity comparable to that of any of the weakly encapsulated strains (nos. 1, 2, 3, and 4) used in the preceding experiment.

A weakly encapsulated form and a strongly encapsulated form were isolated also from a strain (no. 8) whose routine agar cultures consisted of about equal proportions of the two forms (figure 6). Antiserums made by immunization with vaccines prepared from these isolated forms showed the same superior immunogenic capacity on the part of the weakly encapsulated cells as that shown in table 2 for the forms isolated from strain no. 5.

The results of these experiments show that cultures of *Cryptococcus* contain forms of markedly different immunogenic capacity that can be separated from one another by plating, and indicate also that one might predict in advance the difference in the immunogenicity of the isolated forms by simple microscopic examination for degree of encapsulation. These facts have obvious implications for the general subject of microbial variation. From the standpoint of difference in capsular size on agar medium, the two *Cryptococcus* forms are comparable to the "mucoid" and the "smooth" forms of *Torulopsis rotundata*, which were described by Mager and Aschner (1947) but not studied in regard to immunogenicity. However, the investigation reported here was not planned as a study on microbial variation, and until our experiments designed for that purpose are further advanced, it would be unprofitable to discuss that aspect of *Cryptococcus neoformans*.[6]

DISCUSSION

The preceding analysis of the experimental results brought out two new points of information on *Cryptococcus neoformans:* first, the superior immunogenicity of weakly encapsulated in comparison to strongly encapsulated cells of that species, and, second, the influence of the number of the fungus cells (amount of vaccine) upon the strength and the promptness of the antibody responses. That the su-

[6] Although description of the cultural and detailed morphological properties is beyond the scope of this paper, we do not want to leave the impression that either the weakly or the strongly encapsulated forms isolated from one strain were identical to the corresponding forms isolated from the other strain in properties other than degree of encapsulation. The original cultures from which they came differed in various cultural properties, and both of the forms isolated carried with them some of these strain differences. The analogous point should be noted for the strains that were employed in the experiment of table 1. The division into groups of weakly and strongly encapsulated strains was purely on the basis of capsular size, and the strains of each group differed from one another in various cultural and detailed morphological properties. There did seem, however, to be some association between the degree of encapsulation and the degree of pigmentation, the weakly encapsulated cultures tending to be the more pigmented (tan or faint pink).

periority was prominent with each of three different amounts of vaccine that comprised a 100-fold range of dosage made the comparison much more valid than if only one amount of vaccine had been included in the experiments. However, our chief interest in the influence of the amount of vaccine was that, with adequate dosage, *Cryptococcus* antiserums of reasonably high potency were consistently produced within a period of 20 days (13 days for injections plus 7 days' rest) in the case of each of the weakly encapsulated strains we tried.

The regularity and the promptness with which these antiserums were produced are in marked contrast to the reports in the existing literature. A number of the previous workers have failed with all strains tried, others have succeeded with some strains and failed with others, and all who have reported reasonably potent antiserums have employed relatively long periods of immunization. Rappaport and Kaplan's (1926) injections covered 9½ weeks; Benham's (1935) 10 to 12 weeks; Cox and Tolhurst's (1946) 45 days or longer; Evans' (1949) 12 weeks; and Drake (1948), although giving no details, states that "antibodies appear slowly in the circulating blood and usually several series of injections are necessary."

A way of producing potent *Cryptococcus* antiserums with regularity and without prolonged periods of immunization should aid in making antiserums more generally available for use in the various practical and academic studies that are suggested by our demonstration (Neill, Castillo, Smith, and Kapros, 1949) of the serological reactions of the capsules and of a polysaccharide of this pathogenic fungus. Although we got antiserums of somewhat higher titer by additional courses of injections, we would prefer, for practical purposes, to give one course of 13 daily injections of at least 250 million (but not so many as 2.5 billions) of weakly encapsulated *Cryptococcus* cells and to take a large bleeding after 5 to 7 days as a safeguard against losses of animals from anaphylactic reactions, which may occur during later courses of immunization. That all strains that are weakly encapsulated would be effective immunizing agents is of course not known, but that most would be is suggested by the fact that antiserums fully equal in potency to these obtained with the four weakly encapsulated strains we tried (table 1) were produced by immunization with the weakly encapsulated form (table 2) isolated from a highly encapsulated culture that itself had only weak immunogenicity. Adequate dosage and close spacing of the injections, as well as the use of a weakly encapsulated strain, are apparently essential. For example, Kligman (1947) got negative results with the one poorly encapsulated strain he tried, but the number of cells injected is not stated, and the injections, although continued for 16 weeks, were spaced a week apart.

The new information presented in this paper for *Cryptococcus* emphasizes the general principle that strain differences and the influence of the method of immunization must be taken into account in the appraisement of the immunogenic capacity of any species. The strongly encapsulated forms of *Cryptococcus* would, from our data, certainly be regarded as only poorly immunogenic in rabbits. The weakly encapsulated forms might also perhaps be considered to have a somewhat lower immunogenic capacity than that of most bacteria on the

basis of the amount of microbial material that has to be injected. But, on the basis of the promptness and the regularity with which one can produce antiserums that have reasonably potent capacities to precipitate the soluble antigens and to agglutinate and cause quellung reactions with the highly encapsulated microorganism, the weakly encapsulated forms of *Cryptococcus neoformans* appear to possess an immunogenic capacity quite comparable to that possessed by most species of bacteria.

SUMMARY

Agar slant cultures of 15 strains of *Cryptococcus neoformans* were separated into three groups: predominantly weakly encapsulated, predominantly strongly encapsulated, and mixtures in which neither form predominated. Vaccines made from the weakly encapsulated group had much greater immunogenic capacity than did vaccines made from the strains that were strongly encapsulated. The amount injected influenced the antibody response to all the vaccines. Reasonably potent antiserums were always obtained within a total period of 20 days by employing 13 daily injections of about 250 million cells of the weakly encapsulated strains per injection.

Weakly and strongly encapsulated forms, separated from cultures by plating, showed the same difference in immunogenic capacity as that found between the cultures of the predominantly weakly and the predominantly strongly encapsulated strains.

The comparison of the antibody responses included not only agglutination of the cells but also precipitation of a *Cryptococcus* polysaccharide and quellung reactions on strongly encapsulated *Cryptococcus* cells. The results of the quellung tests illustrated the principle that in some instances antibodies reactive with antigens on the capsules of a highly encapsulated form of a species are produced more readily by immunization with a weakly encapsulated form than by immunization with a highly encapsulated form.

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