Failure to Demonstrate Capsular Swelling in *Cryptococcus neoformans.** (22725)

E. Edward Evans, H. P. R. Seeliger,† Lottie Kornfeld,‡ and Corina Garcia§

Department of Microbiology, University of Alabama Medical Center, Birmingham, and Dept. of Bacteriology, University of Michigan, Ann Arbor

The capsule which surrounds certain microorganisms is ordinarily water-clear and difficult to see without appropriate staining procedures. If, however, specific antibody is added, a reaction occurs which renders the capsule clearly visible. This antigen-antibody reaction which was first demonstrated with the pneumococcus(1) has been of interest not only for identifying and classifying microorganisms, but also because it affords direct cytological evidence for the combination of antibody with a surface antigen. Although this combination is frequently referred to as the "Quellung" or "capsular swelling" reaction, several investigators(2-5) have recently suggested that the capsule does not actually increase in size in bacteria like pneumococcus, the *Klebsiella* group and the *Bacillus* group. Tomcsik in discussing the morphological arrangement of immunopolysaccharides in the bacterial cell(5) suggested that the essential feature of the reaction is precipitation and that swelling might be a secondary phenomenon due probably to capsular hydration. Johnson and Dennison(6) presented experimental data which indicated that a volume increase actually occurs when the pneumococcus capsule reacts with antibody. This is in conflict with the results of the previously mentioned authors as well as with work on the capsular reaction of *Cryptococcus neoformans* by Neill, *et al.*(7) and by members of our group(8).

The purpose of the present study is to describe further experiments on this interesting reaction. *Cryptococcus neoformans* has been used as the test organism since its relatively large size and spherical shape facilitate direct microscopic measurements.

Methods. Two strains of *C. neoformans* were selected because of their ability to produce relatively large capsules. Both strain DU (Type A) and strain L2 (Type B) were isolated from patients at the Los Angeles County General Hospital. Cultures were maintained on neopeptone-glucose medium (8). In certain experiments, cells with larger capsules were obtained by injecting mice intraperitoneally with 4 x 10^9 *C. neoformans* and washing the cells from the peritoneal cavity with saline solution on the sixth day of infection. Phenol, 0.5%, was added to cell suspensions that were to be stored for a period of time. All cells were centrifuged and washed 4 times with saline solution immediately before use to remove dissolved polysaccharide. Antiserum was prepared in rabbits as described in an earlier report(9). Antiserum No. 12 was polyvalent with respect to Types A, B, and C. Serum taken from rabbits before immunization was used for control experiments. Among the methods used to study possible change in capsular size during the antigen-antibody reaction was the measurement of packed volumes of centrifuged cells with and without added antibody. For these experiments, strain DU was grown in neopeptone-glucose broth for 4 days at 25-27°C with continual agitation, and then killed by the addition of phenol to a final concentration of 1%. Suspensions of strain L2 from infected mice were also used. Cells were removed by centrifugation, washed four times with saline solution and resuspended in saline or phosphate buffer solutions at pH 7.0 ($\mu = 0.1$). The cell concentration was adjusted to 85% transmittance in a Klett Summerson

* Supported in part by research grants E-436 and E-1066 from N. I. H., Public Health Service.

† Permanent address: Hygiene-Institut, Universitat Bonn, Germany. Temporarily assigned to Department of Microbiology, University of Alabama Medical Center during tenure of a W. H. O. Fellowship.

‡ Present address: Department of Medicine, University of Chicago.

§ Present address: Avenida Vilaflor, Quinta Puerto Rico, Sabana Grande, Caracas, Venezuela.

TABLE I. Volumes of Packed Cells following Two Hours Centrifugation at 2500 × Gravity.

Diluent	Cell strain	Serum dilution	Anticryptococcal serum #12 (mm^3)	Normal rabbit serum (mm^3)
Phosphate buffer pH 7 ($\mu = 0.1$)	DU	1: 4	12	13
		1: 16	12	13
Saline solution	"	1: 4	13	14
		1: 16	13	13
Saline solution	L2	1: 8	30	37
		1:160	30	36

DU strain was harvested from cultures and the L2 strains from infected mice. Capsules of the latter strain were extremely large (see Table III for mean diameters).

Colorimeter using a No. 54 filter. Both normal rabbit serum and anticryptococcal serum for use in the experiment were subjected to preliminary centrifugation at 2500 x g for 30 minutes although both had been clarified by filtration. To Hopkins centrifuge tubes were added 4 ml of cell suspension and 4 ml of a given serum dilution. Following a 5 minute incubation at room temperature, a sample was taken for microscopic examination to verify the presence or absence of a capsular reaction. The tubes were then centrifuged at 2500 x g for 30 minutes and the cell volume noted. The tubes were centrifuged at the same force for additional 30 minute periods until a total time of 2 hours centrifugation was reached. Similar experiments were conducted at 400 x g and 1700 x g and with saline or cultural supernate replacing the phosphate buffer as diluent. Although other investigators have found forces above 2500 x g necessary for demonstrating volume changes in pneumococcus(6), increasing the force above this level did not alter the relative volume in experiments with cryptococcus. For direct microscopic measurement of capsular diameter, suspensions containing 10^7 cells per ml of saline solution were used. In one procedure, individual cells were located on a slide containing no serum and just enough India ink to permit the capsular outline to be seen. The diameters of the capsule and of the somatic portion of the cell were then measured with a Carl Zeiss filar micrometer. Following this, diluted antiserum was allowed to flow under the cover glass while the cell was held in the microscopic field. The same cell was measured again after it had produced a capsular reaction. In other experiments, 0.2 ml portions of cell suspension were placed in 10 x 75 mm test tubes. To some tubes were added 0.04 ml of antibody solution, to others, 0.04 ml of dilute India ink. After mixing, a drop of each was added to cover glasses for the preparation of hanging drop or flat slides. In other experiments, a counting chamber of uniform (0.1 mm) depth was used. In some instances, India ink was added to slides containing cells plus anticryptococcal serum and to control slides with cells and normal serum. After an incubation period of 5 to 15 minutes at room temperature, the diameters of 30 cells were measured with an ocular micrometer. The averages of these values were then calculated.

Results. The results of centrifuging Cryptococcus (DU) cells for 2 hours at 2500 x g are presented in Table I. There was no significant difference in packed volumes of aliquots of cells which had been mixed with specific antiserum and with normal rabbit serum. The substitution of saline for phosphate buffer had no effect on the results. Decreasing the force to 1700 or 400 x g failed to disclose any volume increase in the cells mixed with antiserum. In some experiments, the volume was slightly greater in normal serum which could have been due to shrinkage or to firmer packing in the antigen-antibody aggregate. When the experiment was repeated with cells possessing extremely large capsules (diameter 28 to 50 μ), there was evidence of capsular shrinkage in the presence of antibody. Dilution of the antibody to the point where the capsular reaction was relatively weak (one twofold dilution be-

TABLE II. Effect of Dissolved Polysaccharide in Cultural Supernate on Apparent Packed Cell Volume.

Diluent added to washed cells	Serum (1:8) Anticryptococcal #12 (mm^3)	Serum (1:8) Normal rabbit serum (mm^3)
Saline	10	10
Cultural supernate		
1: 4	>50*	11
1:20	39	10

* The packed cell-precipitate mass greatly exceeded the graduations in the tube. The vol was estimated to lie between 300-400 mm^3.

Tubes were centrifuged at 2500 × gravity for 2 hr.

Strain DU was used in this exp.

low the extinction point) did not change the relative difference between tubes with antiserum and those with normal serum.

The effect of extracapsular polysaccharide on the apparent volume of packed cells is shown in Table II. It may be seen that when washed cells were resuspended in the culture fluid in which they had been grown and then mixed with antiserum, a great increase in volume resulted as compared to values in normal serum. This represents precipitation of dissolved polysaccharide and possibly other antigens present in the supernatant cultural fluid.

Direct microscopic measurements on single cells before and after the addition of antibody demonstrated that there was no swelling and indeed, the capsules appeared to shrink when antibody was allowed to flow under the cover glass. Control slides in which additional India ink was substituted for antibody also showed some degree of shrinkage, indicating that at least part of the observed decrease in diameter was due to mechanical lifting of the cover glass, permitting the flattened capsule to assume a spherical shape. Because of the technical shortcomings of this method, another procedure was adopted. Two hanging drop slides were prepared, one with cell suspension plus antiserum, the other with cell suspension plus India ink. The diameters of 30 cells were measured on each slide, and the mean diameter calculated. Similar experiments were set up using a counting chamber of uniform depth and a flat microscope slide with a cover glass. In no case was capsular swelling observed (Table III); moreover, mean capsular diameters of the cells in antiserum were significantly lower than those in India ink.

Discussion. In studying the mechanism of this antigen-antibody reaction, it is important to know whether swelling of the capsular polysaccharide-antibody complex is an essential feature of the reaction. The data presented here indicate that it is not. The difference in our results and those of others(6) cannot be adequately explained without further work. A different organism, the pneumococcus, was used in the earlier study and it is possible that the capsular reaction differs among different species or types depending on the physicochemical nature of the capsular antigen.

The pneumococcus is known to have an extra-capsular slime layer(2,10) and this may be responsible for the apparent swelling in the pneumococcal capsule. It may be significant that previous investigators(6) did not wash their cells to free them of extra-capsular polysaccharide. In their centrifugation experiments, the apparent increase in volume may have been due to precipitate. In our experiments with cryptococcus, a 1:4 dilution of cultural supernate added to washed crypto-

TABLE III. Comparison of Means of Capsular Diameters in India Ink and in Antiserum.

Exp. No.	Treatment	Mean diameter (μ)	S.D.	S.E.	P value
1	India ink	36.72	2.18	.40	<0.001
	Antiserum	34.62	2.00	.37	
2	India ink	38.43	3.22	.59	"
	Antiserum	34.90	2.66	.49	
3	India ink	38.00	3.43	.63	"
	Antiserum	34.76	2.49	.46	
4	India ink	43.86	2.28	.42	"
	Antiserum	41.16	2.21	.40	

Means were calculated from measurements on 30 cells each. Exp. 1 was done with a hanging drop slide; Exp. 2 and 3 in a counting chamber; Exp. 4 on a flat slide with a cover glass.

Abbreviations: S.D., stand. dev.; S.E., stand. dev. of the mean; P, probability calculated by Student's "t" test.

Strain L2 harvested from mice was used for these experiments.

coccal cells yielded so much precipitate that the total mass remained far above the graduated level in Hopkins tubes. The difference between this and the normal serum control was indeed striking, and could have been interpreted as capsular swelling had not the experiment with washed cells been conducted.

Although there is no capsular swelling, the data presented here suggest that there is some degree of capsular shrinkage. This is particularly apparent in cells harvested from mice because these cells have extremely large capsules. Further experimentation will be necessary to determine whether this apparent shrinkage has biological significance although from a statistical standpoint the differences in mean diameters presented in Table III are significant. It is possible that the apparent shrinkage seen under the microscope does not represent any volume change in the capsule but merely the change from an ellipsoid to a sphere during the antigen-antibody reaction. A shortcoming of the direct microscopic measurement is the fact that capsular boundaries in India ink are not as sharp as in antiserum, particularly in the counting chamber where the fluid depth is 0.1 mm. This accounts for the somewhat larger standard deviations obtained in counting chamber experiments. This criticism cannot be directed at centrifugation experiments; however, it is possible that the shrinkage observed here represents better packing in the presence of antibody.

Summary. 1. In studying the mechanism of the "capsular reaction" one of the first questions to be answered is whether or not the capsule swells when antibody is added. Data presented here indicate that it does not, using *C. neoformans* as a test organism. Exposure of washed, encapsulated *C. neoformans* to antibody results in a prominent clarification of capsular outline, but no increase in capsular size as determined by packed cell volume after centrifugation and by direct microscopic measurement. When cells with large capsules are employed, there is an apparent *decrease* in capsular size during the reaction with antibody. 2. If capsulated cells are not washed free of soluble capsular polysaccharide present in the cultural supernate, the soluble antigen coprecipitates when antibody is added. This may lead to the erroneous conclusion that there is an increase in capsular volume.

1. Neufeld, F., *Z. Hyg.*, 1902, v40, 54; Neufeld, F., and Etinger-Tulczynska, *ibid.*, 1931, v112, 492.
2. Klienberger, Nobel E., *J. Hyg.*, 1948, v46, 345.
3. Duguid, J. P., *J. Path. and Bact.*, 1951, v63, 673.
4. Edwards, P. R., and Fife, M. A., *J. Inf. Dis.*, 1952, v91, 92.
5. Tomcsik, J., *Ann. Rev. Biochem.*, 1953, v22, 351.
6. Johnson, F. H., and Dennison, W. L., *J. Immunol.*, 1944, v48, 317.
7. Neill, J. M., Castillo, C. G., Smith, R. H., and Kapros, C. E., *J. Exp. Med.*, 1949, v89, 93.
8. Evans, E. D., *J. Immunol.*, 1950, v64, 423; Banish, G., and Evans, E. E., unpublished data.
9. Evans, E. E., Sorensen, L. J., and Walls, K. W., *J. Bact.*, 1953, v66, 287.
10. Wood, W. B., and Smith, M. R., *J. Exp. Med.*, 1949, v90, 85; ———, *ibid.*, 1950, v92, 85.

Received August 30, 1956. P.S.E.B.M., 1956, v93.

Effect of Biotin Deficiency on Follicular Melanocytes of Mice. (22726)

WALTER C. QUEVEDO, JR.* (Introduced by H. M. Patt)
Biology Department, Brown University, Providence, R. I.

It has been known for some time that pigmented mice and rats produce grey hairs when made biotin deficient(1,2,3). Unlike greying induced by X-rays, which is permanent(4), greying resulting from biotin deficiency is reversible(3). It would appear that melanocytes persist within the hair follicles of biotin deficient mice whereas they are destroyed in X-irradiated mice. Thus far,

* Predoctoral Fellow of the U. S. Public Health Service. Present address: Argonne National Laboratory, Lemont, Ill.