Passive Immunization Against *Cryptococcus neoformans.** (24123)

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A number of investigators(1) have been successful in producing relatively potent antiserums in rabbits against *C. neoformans* by various immunization regimens, but none have employed these sera for *in vivo* studies. The present investigation was undertaken in an effort to extend our knowledge in this field.

Materials and methods. (1) *Antiserum Production.* Antisera were prepared against *C. neoformans,* isolates 3723, DU, LE, 1523 and RE by growing the organisms on asparagine medium(2) with 2% agar added. After 48 hours' incubation at 25°C, the cells were harvested, washed twice with saline (0.85% sodium chloride solution) suspended in 1% formalin and stored at 37°C for 12 hours. Finally, the cells were washed in saline, standardized by hemocytometer counts, and frozen at –27°C in 14 separate vials for each organism. Vaccination was performed by injecting 300 million cells on the first and second day intravenously (IV) into rabbits. Subsequent vaccinations were accomplished by injecting 250 million cells daily from the third through the fourteenth day. Each day one vial of each organism was thawed and used for that day's injection. The animals were bled 2 weeks following the last immunizing dose and the sera absorbed as follows:

(a) anti-RE absorbed with CN 1523 and 3723 cells
(b) anti-LE absorbed with CN 1523
(c) anti-1523 absorbed with CN RE
(d) anti-3723 absorbed with CN DU and LE cells
(e) anti-DU serum was not absorbed, since it had been determined that this serum was type specific(3).

Polyvalent antisera were prepared by mixing equal quantities of monovalent, absorbed sera. Agglutinin titers for each immunological component were reduced by approximately one-third. Finally, the mixed antisera were sterilized by filtration. (2) *Determination of agglutinin titer. Cryptococcus* cells were grown on asparagine medium for 48 hours and suspended in 0.5% formalin after 3 saline washes. The suspensions were standardized to McFarland No. 3 standard and mixed with serial dilutions of antiserum. Incubation was carried out in a water-bath for 2 hours at 37° C followed by storage at 5°C for 3 days. Titers thus obtained were as follows: anti-RE 1:320; anti-1523 1:320; anti-DU 1:320; anti-3723 1:160; and anti-LE 1:160. (3) *Virulence determination.* Virulence of the strains, CN DU and 3723, was titrated by intraperitoneal (IP) injections as previously reported(2) except that organisms were suspended in 5% hog gastric mucin. (4) *Antiserum administration.* Antisera were injected IP into white, male, Swiss (Rockland) mice in 0.5 ml doses daily for a total of 14 days following IP challenge with 10^6 cells of *C. neoformans* DU or $10^{3.8}$ of strain 3723. The antiserum injection on the first day followed the challenge dose by some 2 hours. The animals were observed until all had expired. (5) *Antiserum on phagocytosis* in vitro *and* in vivo. *In vitro* studies were initiated by isolation of mouse polymorphonuclear leucocytes (PMN)(4). Suspensions of organisms in silicone-coated vials were prepared to contain 10,000 cells of *C. neoformans* 3723 or DU and the following: (a) 0.4 ml PMN suspension and 0.4 ml normal rabbit serum (NRS). (b) 0.4 ml PMN suspension and 0.4 ml *C. neoformans* 3723 antiserum. Two 0.2 ml aliquots were transferred to separate vials, rotated at 55 rpm (37°C) for 40 minutes and plunged into ice water. Blood films were prepared from each vial, dried and stained by Wright's method. Recordings of degree of phagocytosis achieved were made by examination of smears to determine percent of

* Statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of Veterans Admin.

TABLE I. Effect of Passive Immunization on Mice Challenged with Selected Dose* of *C. neoformans* 3723.

Antibody†	No. of mice	No. of mice surviving at various intervals (Days) 5	10	20	30	40	50	70	90	110	130	150
RE-1523-LE	10	10	7	6	4	2	0					
DU-1523-LE	10	10	7	6	3	2	0					
DU-3723-LE	10	10	10	9	9	8	7	6	4	2	0	
DU-3723	10	10	10	9	9	9	7	7	5	2	0	
DU	10	10	8	6	2	1	0					
3723	10	10	10	10	10	9	8	8	6	3	2	0
Normal rabbit serum†	10	9	7	5	3	1	0					

* The selected dose was approximately one LD_{50} as determined in a prior experiment.
† Animals received daily inj. of antiserum or normal serum in control group for 14 days following challenge.

PMN containing ingested organisms. In view of the fact that *C. neoformans* RE could be separated into small and large capsule variants by plating(3); the above procedure was repeated employing both variants separately and mixed in 50/50 proportion. *In vivo* experiments were limited to studies in which pure preparations of small and large capsule variants of *C. neoformans* RE in gastric mucin were injected IP into separate groups of mice. Two hours later NRS or anti-RE serum was injected IP. After 24 hours, 2 mice from each group were injected IP with 0.5 ml saline, sacrificed 30 minutes later, and stained slides prepared from the peritoneal washing. The remainder of the animals received a dose of NRS or anti-RE serum. In this manner animals were sacrificed and additional inoculations were given for a period of 6 days. A number of mice were observed for 4 days after stopping passive immunization. Slide examinations of peritoneal washings and estimates of phagocytosis using the Wright procedure were made on all animals.

Results. Tables I and II quite clearly demonstrate that mice were protected against a lethal challenge dose of *C. neoformans* during the period of passive immunization by the action of antiserum. This protection is apparently type-specific. As soon as the antiserum is withdrawn, however, the animals develop the disease, although there appears to be a considerable delay in onset of the disease process.

To elucidate the mechanism of antiserum protection, ability of mouse PMN to phagocytize *Cryptococcus* cells was determined. *In vitro* experiments (Table III) show that a definite distinction must be made between small and large capsule variants of a given *Cryptococcus* culture. It is quite evident that the small capsule variants (SCV), whether

TABLE II. Effect of Passive Immunization on Mice Challenged with Selected Dose* of *C. neoformans* DU.

Antibody†	No. of mice	No. of mice surviving at various intervals (Days) 5	10	20	30	40	50	70	90	110	130	150	170	190
DU-1523-LE	10	10	10	10	9	8	8	6	5	4	2	2	0	
DU-3723-LE	10	10	10	10	10	9	8	7	6	5	3	2	0	
RE-3723-LE	10	10	10	10	9	8	7	6	5	4	2	2	0	
RE-1523-LE	10	10	10	9	8	7	6	5	4	2	2	1	0	
DU-3723	10	10	10	10	10	9	8	7	7	6	4	2	0	
RE-3723	10	10	10	10	10	9	8	8	7	6	5	3	0	
DU	10	10	10	10	10	9	9	8	8	6	5	3	1	0
3723	10	9	7	5	4	3	2	0						
Normal rabbit serum†	10	9	7	5	4	2	1	0						

* The selected dose was approximately one LD_{50} as determined in a prior experiment.
† Animals received daily inj. of antiserum or normal serum in control group for 14 days following challenge.

tested in a mixed culture, containing both large and small capsule varieties, or in pure culture (*C. neoformans* RE), are capable of being phagocytized in significant numbers. On the other hand, LCV are almost completely resistant to phagocytosis.

When phagocytosis of pure cultures of LCV and SCV of the RE strain were studied *in vivo* (Table IV), intraperitoneal phagocytosis of SCV decreased sharply in the presence of normal rabbit serum while phagocytosis of the LCV occurred rarely under any conditions. In the presence of homologous antiserum, however, there was no appreciable change in the phagocytic response to SCV during the immunization period, but a decrease in response was noted thereafter.

SCV and LCV strains of *C. neoformans* RE have been found to be stable variants on successive transfers *in vivo*. When SCV cultures were injected into animals, the size of their capsules increased. On subsequent transfer to solid or liquid media, SCV organisms were found exclusively. This observation has also been made(1) with other strains of cryptococci.

Discussion. Passive immunization has been demonstrated against a number of strains of *C. neoformans* by the use of mono and polyvalent, mixed antisera. The protection achieved in mice by homologous monovalent serum was always greater than that observed with divalent and trivalent sera. This difference is attributed to the lesser amount of homologous monovalent serum contained in the polyvalent types.

TABLE III. Ability of Mouse PMN's to Phagocytize *C. neoformans* Cells *In Vitro*.*

Organism	Capsule type ratio (small to large) Originals†	Final‡	Phagocytosis of Small capsule type % §	Large capsule type % §
CN 3723	20/80	5/157	86.2	1
CN DU	5/80	3/163	90.0	1
CN RE	50/50	7/190	94.0	1
	100/0	12/0	92.8	
	0/100	0/382		1.3

* Results are avg of 2 exp.
† Indicates ratio in original culture.
‡ Indicates ratio of No. of uningested to ingested cells observed.
§ % calculated on basis of pure culture of variant.

TABLE IV. Ability of Mouse PMN's to Phagocytize *C. neoformans* RE, Small and Large Capsule Variants *In Vivo*, on Successive Days.*

Normal rabbit serum† Phagocytosis of Small capsule type	Normal rabbit serum† Phagocytosis of Large capsule type	*C. neoformans* RE antiserum* Phagocytosis of Small capsule type	*C. neoformans* RE antiserum* Phagocytosis of Large capsule type
%		%	
56	3.0	60.5	3.5
49	3.5	59.5	4.0
38	4.5	60.0	6.0
21.5	6.5	62.5	7.0
9.5	5.5	65.0	6.5
4.5	6.5	66.5	6.5
2.5	6.0	66.0	5.5
2.5	6.0	58.5	6.0
2.0	6.5	39.0	6.0
2.0	7.0	26.5	5.0

* Results are avg of 2 exp.
† Serums were given daily for 2 days.

Evans(3) has classified the strains employed here by capsule type as follows: Type A—strains DU and RE; type B—strain 1523; type C—strain LE and untypable—strain 3723. On the basis of these immunization studies and the data of Evans it appears that the specificity of the protective response resides in the capsule.

It has been shown conclusively that isolated mouse PMN are capable of phagocytizing small, but rarely large capsule *Cryptococcus* variants (LCV). *In vivo* studies have confirmed this observation and, in addition, have demonstrated what appears to be a low grade opsonophagic response in one instance.

In spite of the apparent inability of mouse PMN to phagocytize a majority of LCV organisms, protection has been shown to cover the period of passive immunization. This observation suggests the possible presence of ablastins or other nonabsorbable humoral factors acquired as a result of immunization.

With these studies in mind it may be possible at the clinical level to employ type-specific antiserum to "contain" the disease, so that present-day antibiotics such as cycloheximide (Actidione†), even though toxic, can be employed in low doses or for short periods of administration.

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Summary. 1) Mice were capable of being protected against a number of isolates of *C. neoformans* by passive immunization with antiserum. This protection was limited to the immunization period and was type-specific. 2) *In vivo* and *in vitro* phagocytosis of *Cryptococcus* cells has been demonstrated. When large and small capsule variants were studied separately, the former were practically resistant and the latter relatively susceptible to ingestion by mouse PMN. 3) The probable mechanism for the protection achieved has been discussed and a possible use for antiserum has been proposed.

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Delayed Hypersensitivity *in vitro*. III. Effect of Cortisone on Cytotoxicity of Mumps Viral Antigen.* (24124)

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Development of delayed hypersensitivity is a characteristic response of the human host in a number of infectious diseases, including mumps and tuberculosis(1-7). Recent studies have demonstrated development of delayed hypersensitivity in experimental mumps virus infections in guinea pigs and its manifestation *in vitro* by a cytotoxic effect of mumps virus antigen on cells of tissue cultures prepared from spleens of infected animals similar to that seen in tuberculosis(8). Successful treatment of complications of mumps virus infections(9-15), particularly mumps orchitis, with corticosteroids, combined with evidence that corticosteroids will alter delayed hypersensitivity response to tuberculin *in vivo*(16-20) and suppress cytotoxic effect of tuberculin *in vitro*(21), suggest a possible relationship between hypersensitivity to mumps viral antigen and therapeutic action of corticosteroids in mumps virus infections. It was our purpose to study the effect of cortisone on *in vitro* cytotoxicity of mumps virus antigen for hypersensitive cells. A tissue culture system which permits observation and evaluation of the effect of cortisone on a cellular level independent of vascular components and nonspecific tissue responses was used.

Materials and methods. The egg-adapted strain of mumps virus used was obtained from Dr. John F. Enders. Preparation of stock virus and antigen has been described(8). Guinea pigs, weighing at least 250 g, were infected with mumps virus by 2 routes. Following ether anesthesia, the eye was immobilized and 0.03 ml of virus-containing fluid was injected into the anterior chamber with tuberculin syringe; 0.1 ml of virus fluid was also dropped into nasal passages of the animal. Three to 4 weeks after infection the guinea pigs were skin tested with mumps virus antigen prepared as previously described(8). The tissue culture method was the same as outlined by Gangarosa *et al.*(4). The experimental animal was sacrificed, the spleen removed under sterile conditions, and the tissue minced in a small test tube with Hanks' balanced salt solution (BSS)(22). Fragments about 1 mm^3 were placed on a rooster plasma coagulum in T-9 flasks (Konte Glass Co., Vineland, N. J.). Two ml of medium were added to each flask. The flasks were sealed with a silicone stopper and incubated at 37°C. The medium consisted of 60% BSS, 30%

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