# Detection of *Cryptococcus neoformans* Antigen in Body Fluids by Latex Particle Agglutination.* (28586)

NORMAN BLOOMFIELD†, MORRIS A. GORDON AND DUMONT F. ELMENDORF, JR.
(Introduced by Rachel Brown)
(With the technical assistance of Edward Lapa and Margit S. Rubin)
*Division of Laboratories and Research, New York State Department of Health, Albany*

We have demonstrated antigen of *Cryptococcus neoformans* in serum and cerebrospinal fluid (CSF) of patients with central nervous system (CNS) cryptococcosis by agglutination of antibody-coated latex particles, a technic analogous to that employed by Singer and his coworkers(1) to detect C-reactive protein in serum. Soluble antigens of *C. neoformans* have been demonstrated previously by the precipitin reaction(2) in peritoneal washings, blood, and tissue extracts of infected mice and by complement-fixation and precipitin tests(3) in serum, CSF, and urine from human patients.

*Materials and methods.* Globulin was precipitated at one-half saturation with $(NH_4)_2SO_4$ from serum of rabbits immunized intravenously by the method of Neill *et al.*(4) with Neill's small capsule strain of *C. neoformans*. The 4% globulin solution used throughout the study had a cryptococcus whole yeast cell agglutinin titer of 1:5120. Dow polystyrene latex, 0.81 $\mu$ diameter, was contributed by the Dow Chemical Co., Midland, Mich. The diluent for specimens of serum or CSF was glycine-buffered NaCl solution, pH 8.2(5) to which 0.1% bovine serum albumin (Armour) was added (GBS-BSA). In the absence of added BSA minimal nonspecific agglutination occurred with highly dilute serum or CSF. Buffered saline without albumin (GBS) was used to dilute rabbit anti-*C. neoformans* globulin.

The stock suspension of latex particles in distilled water was such that when diluted 1:100 in a round 12-mm cuvette its OD was 0.3 at 650 m$\mu$ in a Coleman model 6A Spectrophotometer. To stock latex was added an equal volume of a maximally reactive GBS dilution of rabbit anti-*C. neoformans* globulin (a 1:500 dilution of the 4% globulin), yielding a latex-globulin suspension stable upon storage at 3-6°C for at least 3 months. In the test, 0.04 ml of serum or CSF (diluted or not) was placed in a corner of a 1-inch square on a paraffin-ruled 2- × 3-inch glass slide; 0.02 ml of the latex-globulin suspension was placed in the opposite corner and the reagents were mixed and spread on the slide with the aid of an applicator stick. The slide was agitated for 2 minutes, either on a rotating apparatus at 180 rpm or by manual tilting, and the results recorded as negative to 4+. A 2+ or greater reading was considered reactive; reactivity below this level was disregarded because it was sometimes obtained with normal sera in low dilutions.

It is advisable in screening specimens to test diluted as well as undiluted serum, since occasional zone reactions were observed with weakly reactive specimens. We have found it convenient to prepare a 1:4 dilution directly on the slide, pipetting 0.01 ml of serum and 0.03 ml of diluent before adding the antibody-latex mixture. We have not thus far observed an appreciable zone reaction with CSF.

*Results.* Results of latex slide tests of serum and/or CSF from 9 patients with culturally-proven CNS cryptococcosis are illustrated in Table I. In 5 of 7 cases serum was reactive and in 6 of 8 antigen was demon-

TABLE I. Latex Slide Reactions of Serum and Cerebrospinal Fluid from Patients with CNS Cryptococcosis.

| | Titer* of first specimen | | | Titer* of first specimen | |
|---|---|---|---|---|---|
| Patient | CSF | Serum | Patient | CSF | Serum |
| EM | 512 | 16 | SM | Neg | Neg |
| FB | Neg | Neg | EP | 64 | 32 |
| VG | 1024 | | CG | 8 | 16 |
| RK | | 32 | NB | 2 | 16 |
| AA | 2048 | | | | |

* Reciprocal of greatest reactive (2+) dilution, *excluding* dilution by antibody-latex suspension.

* This work was supported in part by grant from Nat. Inst. Allergy and Inf. Dis., U.S.P.H.S.

† Deceased Sept. 1, 1963.

TABLE II. Examination of Serial Blood and CSF Specimens from Patients with CNS Cryptococcosis.

| Patient | Date collected | Antigen titer* (Latex) CSF | Antigen titer* (Latex) Blood | Antibody titer† (Agglutinin) CSF | Antibody titer† (Agglutinin) Blood | CSF culture |
|---|---|---|---|---|---|---|
| EM | 4/25/61 | 512 | | Neg | | Neg§ |
| Late severe CNS cryptococcosis. Treated | 5/ 1/61 | | 16 | | Neg | |
| with Amphotericin B before and dur- | 5/ 9/61 | | Neg | Pos‡ | 10 | |
| ing period when specimens were col- | 5/10/61 | <100 | | | | Neg |
| lected. | 5/22/61 | 2048 | Neg | 2 | 40 | " |
| EP | 12/26/62 | | 32 | | Neg | |
| Moderately late advanced CNS and pul- | 12/27/62 | 64 | | Neg | | Pos |
| monary cryptococcosis. Treated with | 1/ 6/63 | | 16 | | 2 | |
| Amphotericin B starting 12/28/62. | 1/24/63 | 16 | 8 | " | 5 | Neg |
| | 2/25/63 | Neg | 4 | " | Neg | " |
| | 4/13/63 | 8 | 16 | " | 2 | " |
| CG | 3/27/63 | 8 | 16 | " | ± | Pos |
| Late advanced CNS and pulmonary | 4/11/63 | 4 | Neg | " | Neg | " |
| cryptococcosis and Hodgkin's disease. | 5/16/63 | 4 | " | " | " | Neg |
| Treated with Amphotericin B follow- | | | | | | |
| ing 3/27/63 specimen. | | | | | | |
| NB | 5/11/63 | 4 | 8 | " | " | Pos |
| Moderately advanced CNS cryptococ- | 5/24/63 | Neg | 2 | " | " | " |
| cosis. Treated with Amphotericin B | 6/ 6/63 | 1 | 2 | " | " | Neg |
| beginning 5/30/63. | 6/13/63 | 4 | 8 | " | " | " |

* Reciprocal of greatest dilution (*excluding* that by antibody-latex suspension) in which agglutination occurred.

† Reciprocal of greatest dilution (*including* that by antigen suspension) in which agglutination occurred.

‡ Not tested for agglutinin. Antibody demonstrated by immunofluorescence.

§ Earlier CSF culture positive.

strable in CSF. Titers shown are those of the first specimen of blood and CSF examined in each case.

In case FB, antigen was not demonstrated in any of 16 samples of CSF and 2 of serum examined over a 7-month period, probably because agglutinating antibody was present in the initial specimen of the patient's serum (but not in his CSF) and was maintained and increased by therapeutic administration of rabbit anticryptococcus globulin. All 7 specimens of CSF and 4 of serum from patient SM also failed to react. These were taken early in the recrudescence and then during Amphotericin B therapy of an apparently chronic case in which very few colonies of *C. neoformans* appeared in culture, and these only from the first CSF specimen collected.

From each of 4 patients a series of blood and CSF samples was collected; results of tests with these are shown in Table II. Patient EM had been treated with Amphotericin B for several months preceding examination of the first specimen listed, and *C. neoformans* had been cultured from earlier CSF specimens. Treatment with Amphotericin alone proved ineffective; when rabbit anti-*C. neoformans* globulin was added to the therapeutic regimen (6), antigen titer in blood and CSF fell. In cases EP and CG response to treatment with Amphotericin B is reflected in reduction of antigen content of blood and CSF. Cases CG and NB are of particular interest because latex tests on the first specimens indicated the presence of cryptococcus antigen in both blood and CSF, despite the fact that no organisms were seen upon direct microscopic examination of the CSF. *C. neoformans* later appeared in cultures of the CSF from both these patients.

Negative results (—, ±, or 1+ readings) were obtained with 24 presumably normal sera (premaritals); 8 sera from patients with histoplasmosis; 27 CSF with elevated protein but no evidence of cryptococcosis; 10 apparently normal CSF; and 24 CSF (from 22 patients) examined for evidence of cryptococcosis because of suggestive history or the presence of

unidentified yeasts, but from which *C. neoformans* could not be cultured. Weak reactions, which exceeded neither the 1:2 dilution nor the 2+ level, were obtained with 2 of 18 sera that contained various amounts of rheumatoid factor as measured by the latex-fixation test for rheumatoid arthritis. A 2+ reaction was obtained with a 1:2 dilution of 1 of 2 CSF specimens, and with 1:2 and 1:4 dilutions of successive serum specimens from a patient with a CNS disorder clinically consistent with cryptococcosis, in whose CSF *C. neoformans* was unsuccessfully sought in repeated attempts.

Heating for 30 minutes at 56°C eliminated the slight agglutination in low dilutions observed with some unheated normal sera; however, it also reduced the agglutinating activity of some cryptococcosis sera and was therefore excluded from routine use.

To determine whether the reactivity of the antibody-coated latex particles might be serotype specific, tests were performed with antigens derived from cultures of *C. neoformans* serotypes A, B, and C(7), of the organisms isolated from CSF of patients FB and SM (whose serum and CSF failed to react in the latex test), and of a nonvirulent encapsulated cryptococcus known as *C. neoformans* var. innocuous. Roughly equal volumes of each strain of yeast (from cultures on Sabouraud's agar slants) were suspended in 2 ml of GBS-BSA, shaken for ½ hour at 37°C, centrifuged, and the supernatant fluids were subjected to the latex test. Reactions were obtained with all of the extracts, but that of serotype C was of markedly lower titer than the others. The serotype of the strain used to produce the antibody has not been determined.

*Discussion.* The latex slide agglutination technic affords a means for rapid diagnosis of cryptococcosis in many cases. Negative results may occur early in the course of the disease, in chronic low-grade infections, or during remission induced by Amphotericin B therapy. Diagnostic usefulness of the test was evident particularly in cases CG and NB, where cryptococcosis was indicated in spite of a negative direct microscopic examination of CSF; *C. neoformans* was later demonstrated in culture. The procedure appears to have adequate specificity; the very few apparently nonspecific reactions were of low degree. In cases where both serum and CSF were available, both reacted (prior to modification by treatment) or neither did. The test appears to have prognostic value, inasmuch as response to treatment was reflected in declining reactivity.

Rheumatoid factor in serum appears to be an occasional cause of false positive reactions of low degree, possibly by reacting with either the rabbit γ-globulin on the latex particles or the patient's own γ-globulin which may also have become adsorbed on the latex particles. Reactivity of an individual's rheumatoid factor with his own γ-globulin has been reported in tests of rheumatoid sera with untreated latex particles(5).

Evans(7) found it necessary to absorb 11 of 12 rabbit antisera against individual *C. neoformans* strains with cells of heterologous serotypes in order to achieve type specificity; 1 of 8 antisera of type A was type specific without absorption. Cross-reactivity among serotypes is of advantage, however, in detection of cryptococcus antigen. Poor reactivity with type C antigen may prove a problem in detection of cryptococcosis by latex particle agglutination but nonreactivity of specimens from FB and SM was not due to type specificity of the globulin, since extracts of organisms cultured from CSF of these 2 patients were amply reactive. The poor reactivity of the C antigen in the latex test parallels similar observations made in this laboratory of low reactivity of type C yeast cells in agglutination and fluorescent antibody tests with antisera of the type used for coating the latex particles.

*Summary.* Antigen of *C. neoformans* was detected in serum and/or CSF from 7 of 9 cases of CNS cryptococcosis by a rapid, sensitive slide test in which antibody-coated latex particles are agglutinated. The procedure yielded few nonspecific reactions, which were of low degree. Decline in reactivity following treatment suggests that the test has prognostic as well as diagnostic value. Lack of reactivity of blood and CSF from 2 patients was shown not to be attributable to serotype variation.

The authors wish to thank Dr. John K. Miller

for his cooperation in the collection of specimens from hospitalized patients and Dr. E. Edward Evans, Jr., for supplying strains of *C. neoformans* of known serotype.

1. Singer, J. M., Plotz, C. M., Pader, E., Elster, S. K., *Am. J. Clin. Path.*, 1957, v28, 611.
2. Neill, J. M., Kapros, C. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 557.
3. Neill, J. M., Sugg, J. Y., McCauley, D. W., *ibid.*, 1951, v77, 775.
4. Neill, J. M., Abrahams, I., Kapros, C. E., *J. Bact.*, 1950, v59, 263.
5. Singer, J. M., Plotz, C. M., *Arthritis and Rheumatism*, 1958, v1, 142.
6. Gordon, M. A., *Abst. 8th Internat. Congress for Microbiol.*, Montreal, 1962, 132.
7. Evans, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 644.

Received June 20, 1963. P.S.E.B.M., 1963, v114.

## Evidence of Inhibitive Role of Hippocampus in Neural Regulation of ACTH Release.* (28587)

KARL M. KNIGGE AND MYRON HAYS
*Department of Anatomy, University of Cincinnati, Cincinnati, Ohio*

Several studies(1-7) have examined the role of extrahypothalamic neural structures in regulation of adenohypophyseal secretion of ACTH. In rats stressed by physical immobilization(8), amygdaloid nuclei appeared to be necessary for the normal rapid release of ACTH; an inhibitive role of hippocampus was suggested by the observation that basal, non-stressed plasma corticoid levels were significantly higher after bilateral electrolytic lesions of this area. The present study was designed to investigate further the possible interrelationship between these excitatory and inhibitory areas.

*Materials and methods.* Bilateral electrolytic lesions (3 ma, 30 seconds) were placed by stereotaxic means in hippocampus, amygdala, habenula and midbrain reticular formation of adult Sprague-Dawley rats. The position and extent of the lesions in hippocampus and amygdala were as previously described (8). Anterior, vertical and lateral coordinates for lesions in midbrain reticular formation were: (+1.0, −2.0, ±0.6 mm) respectively accordingly to the atlas of deGroot(9); current parameters were 1.5 ma for 30 seconds. Two weeks after lesions in amygdala or reticular formation, adrenocortical response to acute ether anesthesia was tested. After 3-5 minutes of anesthesia, 1.5 ml blood was obtained by cardiac puncture. Plasma corticosterone levels, used as a measure of ACTH release and consequent adrenocortical response, were determined fluorometrically with 0.5 ml plasma aliquots(8). One-half of the lesioned animals that were unresponsive to this stressor subsequently received additional bilateral lesions in hippocampus or habenula (coordinates: +3.8, +0.7, ±0.7 mm; 1 ma for 15 seconds). The remaining were left as "controls" and their adrenocortical response to ether anesthesia tested a second time. Animals lesioned additionally in hippocampus or habenula were ether stressed again 2-4 weeks after lesioning. In one group of animals the order of lesioning was reversed; hippocampal lesions were made first, followed by lesions in reticular formations.

*Results.* Table I summarizes results of these experiments. Plasma corticosterone level in non-lesioned, non-stressed rats (Group I) was 13.4 μg/100 ml. Etherization and withdrawal of 1.5 ml blood by heart puncture led rapidly to a 4-fold increase in corticoid level (Group II). Bilateral lesions in amygdala or midbrain reticular formation were capable of preventing this acute response (Groups III, V). In animals with either of these lesions and unresponsive to etherization stress, placement of additional bilateral lesions in hippocampus resulted in a maximal output of adrenocorticoids when subjected to a second etherization test (Groups IV, VI). In ani-

* Supported by grants from U.S.P.H.S. and N.S.F.