Epidemiological and Immunological Studies of Cryptococcus neoformans

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ABSTRACT

WALTER, JINKS E. (University of Pittsburgh, Pittsburgh, Pa.), and ROBERT W. ATCHISON. Epidemiological and immunological studies of Cryptococcus neoformans. J. Bacteriol. 92:82–87. 1966.—The complement-fixation fluorescent-antibody test provided a means of differentiating between antibodies of Cryptococcus neoformans and Candida albicans. The test was applied to the sera of 134 pigeon fanciers for detection of antibodies to C. neoformans only. About 22% were positive as compared with 3% of a control group composed of 36 non-pigeon breeders. Positive reactions were observed only with C. neoformans types A and B cells. It was concluded that the pigeon fanciers had presumably been infected previously with C. neoformans type A or type B. Moreover, 48 of 49 isolates of C. neoformans cultured from the pigeon habitats of 72 fanciers studied were serotype A. These findings would seem to substantiate the hypothesis that pigeon habitats serve as reservoirs for human infections, and also that subclinical cryptococcosis is more prevalent than is realized.

Although cryptococcal meningitis has been seen with increasing frequency during the last decade (21), the source of infection and mode of transmission remain obscure. Since 1955, several investigators have reported that Cryptococcus neoformans abounds in pigeon habitats (1, 4, 11, 12, 20). More recently, there has been sporadic, but suggestive, epidemiological evidence that pigeon habitats may serve as reservoirs for Cryptococcus infections (19, 24, 37). These observations, together with the high case fatality of the disease, the ineffectiveness of most therapeutic agents, and the lack of dependable serological tests for diagnosis, have aroused concern. The large number of pigeons that inhabit residential and municipal buildings may pose a potential health problem of sizable magnitude.

Concurrently with the increase in cryptococcal meningitis, subpleural and pulmonary granulomas, suspected as being malignancies or diseases such as tuberculosis or histoplasmosis, have proven culturally, with increasing frequency, to be cryptococcosis (5, 10, 18, 23, 26, 36). The fact that many patients with cryptococcal granulomas remain asymptomatic, and are only recognized fortuitously, suggests that these infections are more common than is generally realized.

To investigate the relationship of cases of cryptococcosis to pigeon habitats, and also to evaluate the hazards of pigeon habitats as sources of human infections, a serological survey was undertaken to detect antibodies to C. neoformans in two selected populations. The frequent isolation of Cryptococcus from pigeon habitats prompted the selection of adult volunteers from a club of pigeon fanciers as the test population; business executives, assumed to be non-pigeon breeders, served as controls. Because conventional serological tests have been neither sensitive nor specific, the indirect fluorescent-antibody (FA) and the complement-fixation fluorescent-antibody (CF-FA) tests (8, 14) were investigated for possible use in the survey.

By use of the indirect FA assay, Vogel, Seller, and Woodward (35) found that 8% of normal human sera tested gave positive fluorescence to C. neoformans. However, many of these positive sera had agglutinin titers to Candida albicans, a
fungus that is far more ubiquitous than Cryptococcus (3, 22, 33). Since sorption of these sera with Candida failed to eliminate fluorescence to Cryptococcus, the authors implied that the test might be adopted for use in detecting subclinical cases of cryptoccocosis. Although there are no reports on the use of the CF-FA test for investigating cryptoccocosis, three reports on the use of complement-fixing tests have been published (28) and also with C. albicans by the agglutination test (19). Preliminary results revealed that the CF-FA test offered some advantages over the indirect FA test, and the CF-FA test was therefore adopted for the survey.

**Materials and Methods**

Organisms. Cultures of C. neoformans types A, B, and C were used throughout the study. These were obtained from M. Littman of the New York City Public Health Department, New York, N.Y. A culture of C. albicans was procured from N. Conant of Duke University Medical School, Durham, N.C. D. pneumoniae types 2 and 14 by the precipitin test (28) and also with C. albicans by the agglutination test (19). The CF-FA test was therefore adopted for the survey.

**Preparation of cellular antigens.** Cellular antigens of both Cryptococcus and Candida were prepared by the method of Neill, Abrahams, and Kapros (25). These were used in subsequent serological tests and also in the preparation of hyperimmune rabbit sera. Cultures of D. pneumoniae types 2 and 14 were grown on Brain Heart Infusion Agar (Difco). After incubation at 37 C for 30 hr, the cultures were killed with 1% phenol-treated saline. The cells were washed three times with saline, and the concentration was adjusted visually to a McFarland no. 3 standard; they were then used as antigens both for the serological tests and rabbit immunizations.

**Preparation of hyperimmune sera.** Rabbit antisera against the three serotypes of C. neoformans were prepared by a slight modification of the method of Neill, Abrahams, and Kapros (25). In addition to the 13 daily intravenous inoculations of 25 X 10⁶ cells used by these authors, a booster injection containing the same number of cells was given 7 days after the last daily inoculation. The rabbits were test-bled 1 week after the booster, and those with adequate tube agglutinin titers were exsanguinated. The method of Hasenclever and Mitchell (16) was adopted for preparing C. albicans antisera. Antisera against D. pneumoniae types 2 and 14 were obtained by inoculating two rabbits intravenously with each antigen. The initial inoculum was 0.1 ml and was doubled each time for five additional injections given on alternate days. The animals were bled-out 4 days after the last injection, and the titers were determined by the tube agglutination test and Quellung reaction. Before sorption, the agglutination titers (expressed as serum dilution) of the rabbit antisera against their homologous antigens were as follows: anti-type A, 1:32; anti-type B, 1:128; and anti-type C, 1:256. Anti-C. albicans sera had a 1:1024 titer against C. albicans cells. All antisera were stored at -20 C.

Antiserum against rabbit globulins was made in sheep according to the technique described by Proom (27). The antiserum against guinea pig serum was made in rabbits. Four weekly intravenous injections of 1.0 ml each were given, followed by a fifth 10.0-ml injection of fresh whole guinea pig serum. Animals were bled 10 days after the final injection of antigen. Only rabbit anti-guinea pig sera that gave positive standard ring test precipitin reactions with dilutions of guinea pig complement greater than 1:12,800 were used in this study.

**Preparation of FA conjugates.** Antibody globulin fractions of the sheep anti-rabbit serum and rabbit anti-guinea pig serum were made either by the ammonium sulfate technique (7) or the ethyl alcohol method (9). Conjugations of antibody globulins were carried out at 4 C for 16 hr with fluorescein isothiocyanate (FITC) dye. The ratio of FITC to protein was 1:50. Conjugate samples were lyophilized and stored at -20 C. Prior to use, free FITC was removed from the labeled antibody preparation by means of a Sephadex G25 column (29). The conjugates were then twice sorbed with mouse liver powder (6) and were used within 10 days.

**FA staining.** FA staining was done on no. 1 cover slips (10 by 20 mm) on which the fungal and bacterial cellular antigens were fixed by air-drying. In the indirect FA test, control and unlabeled test sera were diluted 1:2 with 0.85% saline, and the sheep anti-rabbit globulin conjugate was used undiluted. In the CF-FA test, control and unlabeled test sera were also diluted 1:2 and inactivated at 56 C for 30 min. Guinea pig complement, stored at -65 C, was thawed just prior to use, diluted 1:10, and mixed with the unlabeled test serum already applied to the cover slip containing the antigen. The rabbit anti-guinea pig globulin conjugate was used at a dilution of 1:2. In the CF-FA test, a diluent of 2.5% aqueous bovine serum albumin (BSA) solution was preferred for diluting complement and conjugate. All necessary controls were routinely carried out for each FA test. In both types of FA tests, cover slips with the unlabeled test or control sera and conjugates were each incubated at 37 C for 30 min. After each incubation period, the cover slips were washed three times with 0.01 M Sorenson's phosphate-buffered saline (pH 7.2). The fluid of Rodriguez and Deinhardt (30) was used to mount the cover slips to slides for observation with American Optical fluorescent equipment. A Schott
BG-12 exciter filter and Wratten 15G and Leitz Euphos barrier filters were used with the Osram HBO-200 light source.

**Assay methods of test materials.** Sera from 134 pigeon fanciers and 36 business executives were assayed for antibodies to *C. neoformans* by the CF-FA test. Dried pigeon droppings were aseptically collected from the coops of 72 fanciers from whom blood was taken. These were studied for the presence of *C. neoformans.* p-Dichlorobenzene (Fisher Scientific Co., Pittsburgh, Pa.) was used according to Ajello et al. (2) to destroy arachnids, which are capable of causing cross-contamination of tubes containing the fecal samples. The dilutions (1:25) of feces were made with an aqueous 0.1% glycine solution (pH 7.0). Preliminary studies showed that *C. neoformans* was more stable in this diluent than in either deionized distilled water or 0.07 M phosphate-buffered saline (pH 6.9). A 0.1-ml amount of each fecal suspension was inculcated on plates of Sabouraud Dextrose Agar and Littman Medium (BBL) containing 20 μg of chloramphenicol per ml of medium. The inocula were dispersed by the spread plate technique with a bent-glass rod and a turntable. The plates were incubated at 37 C and examined at 48, 72, and 96 hr for the yeastslike colonies. The criteria for the identification of *C. neoformans* were those described by Ajello et al. (2). The tests used included growth at 37 C, colonial and cellular morphology, urease production, carbon and nitrogen assimilation tests, and mouse pathogenicity.

Serological typing of *C. neoformans* isolated from pigeon droppings. Serotype-specific sera for *C. neoformans* types A, B, and C were prepared by sorption of each rabbit antiserum twice with its heterologous cellular antigens. The final dilution of each specific serum was 1:2 in a slide agglutination test used to type each pigeon isolate. Cellular antigens of each pigeon isolate of *C. neoformans* were prepared as described previously for stock cultures of *C. neoformans*. Each was adjusted to a turbidity of 0.3 McFarland tube. The agglutination test was performed by placing a drop of each pigeon isolate on three microscope slides. A drop of each type-specific *Cryptococcus* antiserum was mixed with the antigens. After 30 min of incubation at 37 C, agglutination was observed microscopically at a magnification of 100 times.

**RESULTS**

**FA tests.** The feasibility of using either the indirect FA or CF-FA test in the survey was determined by utilizing rabbit hyperimmune sera to ascertain the cross-reactions among *C. neoformans*, *C. albicans*, and *D. pneumoniae* types 2 and 14. When *D. pneumoniae* types 2 and 14 antisera were tested against *C. neoformans* in the indirect FA test, equivocal fluorescence (±) was observed. *Candida* antiserum produced strong (3+) capsular fluorescence with the three serotypes of *C. neoformans*. The indirect FA test was therefore considered inadequate as a tool for specifically detecting antibodies to *C. neoformans.*** Of great importance, however, was the finding that *Candida* antiserum failed to fluoresce any of the three types of *C. neoformans* in the CF-FA test. Also, *C. neoformans* type C antiserum consistently gave less fluorescence (3+) with its homologous cellular antigen than the 4+ reactions obtained with type A and B cells. Type A antiserum failed to fluoresce type C cells, but intensely stained (4+) type A and B cells. The hyperimmune sera against all three types of *Cryptococcus* gave weak fluorescence (1+) when directed against the cells of *C. albicans*. Controls consisting of sera from animals prior to immunization, 2.5% BSA solution, and inactivated guinea pig complement were negative. These results (Table 1) show the advantage of the CF-FA test over the indirect FA test. Therefore, the CF-FA test was used in the subsequent epidemiological survey with human sera.

**Assay of human sera.** Positive human sera revealed an intensity gradient from 1+ to 4+ fluorescence of type A and B cryptococcal cells, but none with type C cells. In both the indirect and the CF-FA tests, the number of cryptococcal cells reacting with human sera and rabbit antiserum used as a positive control was not uniform. Fluorescence varied from approximately 10 to 60% of the total cells and was confined to the capsular

| TABLE 1. Summary of the extent of cross-reactions observed among *Cryptococcus neoformans* types A, B, and C, *Candida albicans*, and *Diplococcus pneumoniae* types 2 and 14 by the complement-fixation fluorescent-antibody test |
|----------------------------------|-------------------|----------------|
| Rabbit hyperimmune serum used   | Antigen used (whole cells) | Result |
| Anti-C. neoformans type A and B sera | *C. neoformans* type A | 4+ |
|                                  | *C. neoformans* type B | 4+ |
|                                  | *C. neoformans* type C | 1+ |
|                                  | *C. albicans*       | 1+ |
| Anti-*C. albicans* serum         | *C. neoformans* type A | — |
|                                  | *C. neoformans* type B | — |
|                                  | *C. neoformans* type C | — |
|                                  | *C. albicans*       | 1+ |
| Anti-*D. pneumoniae* type 2 and 14 sera | *C. neoformans* type A | ± |
|                                  | *C. neoformans* type B | ± |
|                                  | *C. neoformans* type C | ± |
|                                  | *C. albicans*       | 3+ |
|                                  | *D. pneumoniae* type 2 | 4+ |
|                                  | *D. pneumoniae* type 14 | 4+ |
| Prebleed sera, BSA*, and heat-inactivated complement controls | All antigens used | — |

* Bovine serum albumin, fraction V.
poly saccharide of the organism. Examples of positive fluorescence are illustrated in Fig. 1–3.

The results of the serological survey showed that 29 individuals (21.6%) in the pigeon breeder population had detectable antibodies to *C. neoformans*. Only 1 of 36 (2.8%) of the assumed non-pigeon breeders had a positive reaction by the test.

Isolation of *C. neoformans* from the pigeon coops. Of the 72 pigeon coops studied, 49 (68.1%)

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**Fig. 1.** Complement-fixation fluorescent-antibody (CF-FA) staining of Cryptococcus neoformans type A cells by a pigeon breeder's serum giving a 4+ reaction. × 550.

**Fig. 2.** CF-FA staining of Cryptococcus neoformans type A cells by a pigeon breeder's serum giving a 2+ reaction. Area shows some cells which did not stain. × 550.

**Fig. 3.** CF-FA staining of Cryptococcus neoformans type B cells by hyperimmune rabbit antiserum, made against homologous fungal cells, giving a 4+ reaction. × 550.
yielded positive cultures of *C. neoformans* which, with a single, doubtful exception, were identified as serotype A by the slide agglutination test. The results of the serum CF-FA tests of each fancier were related to the presence or absence of *C. neoformans* in the droppings obtained from his pigeon coop. Of the 72 pigeon fanciers from whose coops droppings were collected, 19 (26.4%) gave positive serological reactions. *C. neoformans* was recovered from the habitats of all the positive reactors with two exceptions. There were, however, 32 serologically negative persons (44.5%) whose pigeon coops also yielded *C. neoformans*. These results are summarized in Table 2.

**TABLE 2.** Relationship between pigeon fecal isolates of *C. neoformans* and serological reactions of the sera of pigeon fanciers with the complement-fixation fluorescent-antibody test

<table>
<thead>
<tr>
<th>Isolation of <em>C. neoformans</em></th>
<th>Antigens to <em>C. neoformans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
</tr>
<tr>
<td>No. positive</td>
<td>17</td>
</tr>
<tr>
<td>No. negative</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
</tr>
</tbody>
</table>

The significantly larger number of positive reactors among the pigeon fanciers implies that people who associate with pigeon-ridden environments have a greater risk of acquiring cryptococcal infections than those with less exposure. In addition, the serological reactions produced by the sera of positive fanciers are compatible with a *C. neoformans* type A or B infection. This finding agrees with the results of the isolation study in which all of the *C. neoformans* isolates were serotype A with a single possible exception.

Meaningful interpretation of the 32 serologically negative fanciers from whose coops *C. neoformans* was isolated requires some knowledge of the duration of immunity conferred by infections with *Cryptococcus*. Neither the duration of raising pigeons nor the age of the fanciers appeared to have any relationship to their serological positive reactions. The relatively large size of the fungus, which varies from 4 to 20 μ in wet mounts (13), might be a limiting factor in infections with *Cryptococcus*. The probability of particles larger than 5 μ being deposited in the pulmonary alveoli is rather low.

The preponderance of *C. neoformans* type A in pigeon habitats in this area raises the question of whether or not type A is associated with pigeon sources elsewhere. Confirmation of our findings would support the usefulness of typing isolates from patients and associated environmental sources, leading to a better understanding of the epidemiology of this disease.

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**LITERATURE CITED**

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