Some Observations on the Serology of Pneumocystis carinii Infections in the United States

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An indirect immunofluorescent (IF) test with antigen of partially cleaned Pneumocystis carinii cysts was evaluated for sensitivity and specificity. The test was reactive at the 1:8 and above level for 44% of the persons who had suspected and confirmed infections, for 7.3% of the healthy contacts, and for 1.4% of the general population. Results of tests with sera from people with measurable antibodies to other diseases and those on immunosuppressant therapy suggest that only titers of 1:20 and above are specific. At this level, tests with sera of 32% of the cases, 4% of the contacts, 0% of the healthy controls, and from 0% to 37% (average 5%) of individuals on long-term immunosuppressive therapy or with antibodies to other diseases were positive. The high percentage of positive reactions in cytomegalovirus and in fungal infections could represent double infections. The complement fixation test was less sensitive than the IF test. Antibodies measured by the IF test were immunoglobulin G. No differences in antigenicity were demonstrated by the IF test among the cysts from eight human infections or from induced rat infections.

In epidemics of interstitial plasma cell pneumonia in Europe, both the complement fixation (CF) (1, 2, 26, 27) and the indirect immunofluorescent (IF) (4) tests have been found to be very efficient diagnostic techniques, revealing antibodies in over 75% of the suspected cases. In our laboratory in the United States, on the other hand, we have not shown comparable reactivity in sera from Pneumocystis carinii pneumonia cases (21), and the occasional references in the North American literature indicate that serological testing has not been generally successful (6, 9, 19, 22). The cause of the low level of reactivity has usually been attributed to poor antibody response in patients who are, almost without exception, on immunosuppressant drugs or are otherwise immunodeficient. However, the roles played by other factors, such as antigenic differences and strain or species differences in the organisms (10, 12) causing the disease, should be investigated. Since the supply of heavily infected human lungs necessary for preparing antigens and serum from confirmed infections is very limited, exclusive studies cannot be performed in a single laboratory. Answers to the many problems associated with the serological diagnosis of pneumocystosis must, therefore, come from compilation of data from many sources. As a contribution to this data, we are presenting the results of an evaluation of an IF test (21) which utilizes as antigen dried mounts of cysts partially separated from host tissue cells. Sera from Americans with and without P. carinii or other infections have been titrated. In addition, some aspects of the antigenicity of cysts as well as the labeled antiglobulins used in the IF test have been investigated. Results of a limited comparison of the CF and IF tests are included.

MATERIALS AND METHODS

Antigens. Both particulate (cyst) and soluble antigens were prepared from naturally infected human lungs and from the lungs of rats in which pneumocystis infections were induced with cortisone acetate (8). Many small lots of cyst antigen were made from eight human lungs, but most of the testing was done with the preparations from four lungs. The soluble antigen was prepared from two of the infected human lungs and from pools of the infected rat lungs. A few slides of Candida albicans were prepared for controls.

Preparation of the IF antigens has been described in detail (21). In brief, it consists of processing pieces of infected lung as follows: (i) brief homogenization in Hanks balanced salt solution in Waring blender and
filtration through wet gauze to free the parasitic material, both the extracellular stages and the cysts, from the clumps of connective tissue; (ii) fractional centrifugation at low speeds (usually 400 x g to 500 x g), followed by passage of those fractions richest in parasites through a discontinuous sucrose gradient to separate most of the host tissue from the parasite cells; (iii) sonic treatment for periods varying from 1 to 2 min to disintegrate the host cells; and (iv) washing of the final suspension of cysts and preparation of air-dried mounts of slides. All steps were monitored by staining the smears with toluidine blue (5) and Giemsa. Slides were stored at -70 C until used and showed no loss of antigenicity for at least 6 months.

The CF antigen, a saline extract of delipidized and dehydrated infected lung, was prepared by the modification of Vivell (25) of Casals' virus antigen. The antigen was stored either frozen at -20 C or lyophilized. Antigens for controls were also made from normal human lung and both infected and normal rat lungs.

**Antisera.** Sera (1,100) were tested. For evaluation of the IF test, the sera were selected to include samples from 58 persons with confirmed P. carinii infection, from 135 persons suspected of infection on the basis of clinical symptoms, and from 183 healthy American adults, some of whom had been in contact with patients. In addition, there were 661 sera from 437 persons with other diseases. The groups included many on immunosuppressant therapy. For comparisons of antigenicity of the cysts from rat and human lungs, 104 sera from infected humans were tested.

**Other reagents.** The fluorescein-labeled antihuman globulins included antihuman globulin Wellcome. F/P ratio [E495nm:E280nm] = 0.82, molar ratio 3.7. Optimally diluted for use in the test 1/100 to 1/200 antihuman IgG CDC (Center for Disease Control) molar F/P ratio 7.5. Optimal dilution for test 1:15 to 1:20; anti-human immunoglobulin (lg) M Mann. Optical density ratio [280/495] = 1.5. Diluted 1:5 for use.

**Tests.** The standard IF test procedure was followed with incubation periods for both the serum and conjugated antiglobulins of 45 min at 37 C. The sera were all treated with Wellcome conjugated antihuman globulin as the standard reagent, and selected groups were also tested with anti-IgG (CDC) and anti-IgM (Mann). Optimal dilutions of all lots of conjugate were determined as the highest concentration that, in the presence of a 1:500 or 1:1,000 dilution of Evans blue, showed no fluorescence of organisms with normal control serum and no more than plus one or minus one dilution variance from the known titers of the positive control sera. The sera were initially screened at dilutions of 1:8 and 1:40, and all reactive sera were titrated by serial dilutions starting at 1:5. The organisms typically appeared as rings or clumps of rings but were at times more uniformly fluoresced. Although some host cell detritus occasionally showed fluorescence, only typical cyst reactions were considered positive. Fluorescence was read with a Leitz Ortholux scope equipped with an HBO-200 mercury lamp and BG-12 and Leitz 510 filters.

The CDC Laboratory Branch Complement Fixation (LBCF) microtiter test was followed without modification. Optimal dilutions of lots of the Vivell antigen varied from 1:8 to 1:25. Serum dilutions started at 1:2, and reactions at 1:4 and above were considered positive. Occasional sera were positive at the 1:2 dilution when tested with control antigen of normal lung tissue.

**RESULTS**

**IF test.** The IF test was easily read under low-power magnifications. Fluorescence was usually brilliant. Some variability in the reading and determination of the IF titers was observed, but such fluctuations were kept at a minimum by using a single lot of antigen smears and comparing the degree of fluorescence with that of several standard control sera each day. As a result, the titers of sera, even those frozen and thawed several times during the 3-year study, were very reproducible.

The reactivity of sera from 183 healthy American adults and from 191 pneumocystis cases is shown in Table 1. One of 74 (1.4%) healthy controls and eight of 109 (7.3%) people in contact with infected persons had sera that were reactive at or above the 1:8 level. Of the 191 P. carinii cases, 58 were confirmed by tissue examination, and the remainder were suspected by clinical findings. Sera from 26 (45%) of the confirmed cases and 59 (44%) of the suspected cases were reactive. None of the normal controls, 3.7% of the contacts, and 32.5% of the cases showed titers of 1:20 and above. The distribution of reactors from negative titers to titers of 1:320 (Table 1) is bimodal in character and suggests that IF test titers below 1:20 could include nonspecific reactions. Titers of 1:20 and above are probably specific. The results of testing 661 sera from sick people on long-term immunosuppressant therapy and from people with antibodies to other diseases (Table 2) supports the conclusion that a titer must be 1:20 or higher to be diagnostic. Twenty percent (134 sera) were reactive at the 1:8 level, but only 5% (32) had titers of 1:20 or over. With the sera from patients on immunosuppressants, 20% to 34% were reactive, whereas only 4% to 10% were positive. In general, sera with antibodies to other diseases showed a lack of high titers to pneumocystis. The exceptions, the sera from cytomegalovirus infection and various fungal diseases, are particularly important because of the strong possibility of double infections in these cases (27). It is not possible from these data to determine whether the reactive sera from patients with cytomegalovirus (57%) and to fungal disease (27%) should be attributed to...
cross-reactions or to mixed infections, and amounts of serum were inadequate for adsorption studies. However, since 10% of the virus cases without specific antibodies showed above 1:20 pneumocystis titers, it seems possible that the positive pneumocystis reactions were specific.

In addition to testing the sera from patients with malignancies who were also on immuno-suppressants by IF for pneumocystis, 242 sera were also tested by IF for Toxoplasma gondii antibodies by the CDC Serology Section. Forty-eight percent were positive with titers of 1:16 to 1:1,024 for toxoplasma and 21% were reactive for pneumocystis at the 1:8 level, with 5% at the 1:20 to 1:40 level. The distribution of the reactions is shown in Table 3. There is no evidence of cross-reactivity between the two antibodies.

Although we have tested 120 serial specimens from 53 of the pneumocystis cases and 197 sera from 72 of those with malignancies, there is not enough data to show the pattern of appearance of antibodies during disease or after treatment. Such data must be cautiously interpreted, since the appearance of measurable amounts of antibody may be associated with changes in dosage of cortisone (8, 16).

**Antigens for the IF test.** Lots of antigen prepared both with and without sonic treatment (which ruptured the extracystic bodies and host cells) from eight infected human lungs, showed no evidence that the organisms were antigenically different. Extracystic bodies also were antigenic, but they were difficult to recognize in the smears. The yield of clean cysts, ease of separation of cysts from lung tissue, and brilliance of fluorescence varied among the lots prepared from the same lung as well as among the lots from different lungs. Only one of the infected lungs contained cysts that fluoresced brilliantly without the addition of antibody. After treating a suspension of these cysts with citrate buffer to remove possible antibody globulin from the organisms (3), an antigen of these cysts was usable. The fluorescence of host cells, which occurred with some sera, was a consistent
serum reaction and was not associated with a particular lung.

To further elucidate the role of host material and to detect possible antigenic differences between cysts from man and rat, 104 human sera were titrated with the two kinds of antigen. Results are presented in Table 4. Fluorescence with both kinds of cysts was brilliant, and the optimal dilutions of conjugate were the same for both. There was no fluorescence of cell detritus on the slides of antigen from rats which made them easier to read. No marked difference in the antigenicity of the two kinds of cysts was demonstrated. Forty-five sera were negative and 31 sera were positive with both. The three sera positive with only human cyst antigen and the three positive with only rat cysts were within one dilution of the significant titer. The appearance of the tests with 14 sera reactive with human cysts and negative with rat suggests that at least part of the fluorescence seen with human cyst antigen is due to reaction with host components.

Although many human sera contain antibodies to Candida albicans as evidenced by the fluorescence of these organisms on the control slides, there was no evidence of cross-reactivity with pneumocystis antibodies. Therefore, C. albicans controls were not deemed necessary for routine testing. However, since occasional yeast cells are found in all cyst antigen smears, the fluorescence of these rare organisms must not be confused with a positive pneumocystis reaction.

Conjugated antiglobulins. When optimal dilutions of the conjugated antihuman and anti-IgG globulins used in this study were limited to those concentrations that, with 1:500 or 1:1,000 dilution of Evans blue, gave no fluorescence with negative control serum and brilliant fluorescence to titer with the positive control sera, both conjugated antihuman globulins and conjugated anti-IgG produced similar titers with the sera tested. Sera (150) were tested with the two conjugates simultaneously using the same lots of cyst antigen. Of the 76 sera from possible pneumocystis cases, 27 were positive with the antiglobulin and 28 with the anti-IgG. Of the remaining sera, from healthy people and those on immunosuppressants, six were positive with the antiglobulin and only two with the anti-IgG. In addition, sera from 137 possible or confirmed infections were tested with a 1:5 dilution of labeled anti-IgM (Mann). The sera, of which 75 were positive with the anti-IgG conjugate, were tested in serial dilutions from 1:2 through 1:32. Five sera reacted: two with titers of 1:2, two with titers of 1:4, and one with a titer of 1:8. All five were also positive with anti-IgG. The antibodies measured by this IF test appear to be IgG.

CF test. Sera from 155 persons with possible pneumocystis infections, from 40 healthy contacts and from 25 healthy adults, were tested by both IF and LBCF tests. IF titers of 1:20 and above and CF titers of 1:4 and above were called "positive." Comparison of the results showed that 44 (20%) were positive by IF, 22 (10%) were positive by CF, and 31 (14%) were anticomplementary (AC). None of the sera from healthy adults were positive by CF, while 4 (6%) were positive by IF. Of the sera from possible pneumocystis cases, 40 were positive by IF and 22 positive by CF. Fifteen sera were positive by both tests. Fourteen, positive by IF, were negative by CF, and an additional 11 were AC. Seven sera were positive by CF only. Thus, although the two tests may be measuring some of the same antibodies, the IF appears a little more sensitive.

**DISCUSSION**

*P. carinii* has been found in some human lungs wherever autopsy tissue has been examined specifically for its presence. In European reports, incidence of positive lungs ranged from 1% to 10% (20, 24, 28). In Texas (17), 4% of the lungs of the lungs of children with leukemias were found to harbor cysts, and Esterly (6, 7) reported 4% of 200 of the routine autopsies of adults were positive in central United States. The presence of the organisms in animal hosts has also been noted in both Europe and North America (8, 14, 15, 23). It is realistic to assume that this opportunistic parasite is omnipresent. Nevertheless, the rates of serological reactivity measured by the IF test performed at CDC—31.5% specifically positive of possible cases, 7.3% positive of contacts, and 14% of the healthy population—were disappointingly low and considerably less than those reported during European epidemics. Although we have tested a limited number of European sera, an extensive exchange of sera is necessary before the results of serological testing can be finally interpreted.
Antigenic differences among the organisms present in different lungs is a moot question. We could detect no qualitative differences among the cysts from 8 human lungs tested by the IF test. Meuwissen and Leenwenberg (18), in the Netherlands, found that CF antigen prepared from American lung samples was comparable to their antigens prepared from local infected tissue. We have noted a difference in the degree of fluorescence of tissue section antigen prepared from lightly and heavily infected lungs, those showing typically plugged alveoli being more reactive. However, when cysts were separated from the conglomerates and lung cells, there were no consistent differences seen in their antigenicity by IF. Although the work of Kim et al. (12) suggests possible differences between cysts from rat and human lung, our results are more compatible with the findings of Kucera and Kramar (14), Lim et al. (16), and Gentry et al. (9) and suggest that species differences, if they exist, are not detectable by the CDC-IF test.

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LITERATURE CITED