Use of the Immunodiffusion Test in the Serodiagnosis of Aspergillosis

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The diagnostic value of an immunodiffusion (ID) test with standardized precipitogens derived from five Aspergillus species was determined with sera from 60 proven and 12 suspected cases of aspergillosis. The data demonstrated that the greatest number of aspergillosis cases were detected by the concurrent use of A. fumigatus and A. niger precipitogens. With these precipitogens, the ID test permitted the serodiagnosis of aspergillosis in 82% of the 60 proven cases and in 83% of the 12 suspected cases. The presence of one or more precipitins was indicative of aspergilloma, of allergic bronchopulmonary aspergillosis, or of invasive aspergillosis. Precipitins were detected in 93% of the sera from patients with aspergilloma, in 50% of the sera from patients with allergic bronchopulmonary aspergillosis, and in 88% of the sera from patients with invasive aspergillosis. Although the presence of one or two precipitin bands could indicate any form of aspergillosis, the presence of three or four was strong evidence of either aspergilloma or invasive aspergillosis. The ID test was found to be 100% specific in an evaluation of its effectiveness with 65 sera from individuals with other systemic mycotic infections, bacterial or neoplastic diseases, and from apparently normal humans. In diagnosed cases of aspergillosis, the examination of serial serum specimens provided information about the clinical course of the disease. A reduction in the number of precipitin bands and significant titer changes were noted as the patients responded to therapy.

The nonspecific clinical and radiological pulmonary manifestations of aspergillosis create diagnostic problems. A combination of cultural and histological evidence provides the only basis for an unequivocal diagnosis. Such evidence, however, cannot always be obtained. In such situations, serological methods may be useful adjuncts in establishing a diagnosis of aspergillosis.

The need for a reliable serological test for aspergillosis has long been recognized. Various investigators have studied different serological methods, such as complement fixation (1, 9, 15), immunodiffusion (2-4, 11, 14), latex agglutination (6), and electrophoretic tests (1, 7, 8, 15). Of these procedures, immunodiffusion is the simplest and most widely performed test for Aspergillus antibodies.

In response to an increasing number of requests for aspergillosis antibody tests, this study was undertaken to evaluate the diagnostic adequacy of the immunodiffusion test and its prognostic value.

MATERIALS AND METHODS

Serum specimens. Sera used in this study were obtained from patients with proven aspergillosis and other systemic mycotic infections, from patients with pulmonary disease of unconfirmed etiology, patients with asthma, bacterial, and neoplastic diseases, and from apparently normal humans. In each case, the clinical diagnosis and the cultural data were obtained from the attending physician. In 17 of the aspergillosis cases, only the generic identification of the etiologic agent was provided. The aspergillosis cases were classified into three categories: (i) aspergilloma with evidence of fungus ball(s), (ii) allergic bronchopulmonary aspergillosis with no tissue invasion but with wheezing, mucous plugs with hyphae, eosinophilia, or transitory pulmonary infiltrates, and (iii) invasive aspergillosis in which the fungus had invaded tissue. All sera were preserved with Merthiolate (0.01%).

ID tests. Immunodiffusion (ID) tests were performed in 1% Noble agar and 0.25% phenol in 25 ml of pH 8.6 Veronal buffer (LKB) in 75 ml of distilled water. Glass slides (25 by 75 mm) were cleaned with alcohol, placed in slide frames, and coated with a 0.1% solution of the buffered agar and 0.05% glycerine. After the slides had dried, 10 ml of the 1% agar was added (10 ml per three slides). The slides were incubated at 37 C for 1 hr in a moist chamber before wells were cut.

Antigens were 8X-concentrated, acetone-precipitated culture filtrates from 5-week-old Sabouraud
RESULTS

Sera from 60 patients with culturally, histologically, or radiologically proven aspergillosis were studied (Table 1). *A. fumigatus* was reported to have been isolated from 23 of these patients, *A. niger* from four, *A. flavus*, *A. terreus*, and *A. versicolor* from one each, and unidentified aspergilli from 17. In 10 of the aspergilloma cases and three of the allergic bronchopulmonary cases, aspergilli were not isolated. In these cases, diagnosis was based solely upon clinical and histopathological data. The absence of positive cultures from patients with aspergilloma and allergic bronchopulmonary aspergillosis has been reported previously. Campbell and Clayton (3) noted that the mycelium in a fungus ball is of low viability, and Pepys (10) found that sputum cultures from patients with allergic bronchopulmonary aspergillosis are frequently negative during episodes of pulmonary infiltration and that sputum cultures from patients with aspergilloma may be negative.

The data in Table 1 show that 7 of the 60 patients appeared to have primary cases of

| Table 1. Clinical and cultural data on 60 proven cases of aspergillosis |
|---------------------------------------------------------------|
| **Clinical type** | **Culture isolated** | **Preexisting disease** |
|-------------------|----------------------|------------------------|
| Aspergilloma (30)* | *Aspergillus fumigatus* (9) | Tuberculosis (16) |
| | *A. niger* (2) | Sarcoïdosis (5) |
| | *A. versicolor* (1) | COPD* (3) |
| | *Aspergillus* sp. (8) | Tuberculosis + COPD (1) |
| | | Emphysema (1) |
| | | Bronchiectasis (1) |
| | | Rheumatoid arthritis (1) |
| Allergic bronchopulmonary (14) | *A. fumigatus* (6) | Tuberculosis (4) |
| | *A. niger* (2) | Carcinoma (1) |
| | *A. flavus* (1) | COPD (1) |
| | *Aspergillus* sp. (2) | Diabetes (1) |
| | | Pneumonia (1) |
| | | Asthma (4) |
| | | None (2) |
| Invasive (16) | *A. fumigatus* (8) | Tuberculosis (3) |
| | *A. terreus* (1) | Sarcoïdosis (2) |
| | *Aspergillus* sp. (7) | Sporotrichosis (1) |
| | | Lupus erythematosus (1) |
| | | Chronic lung disease (1) |
| | | Leukemia (1) |
| | | Emphysema + pyelonephritis (1) |
| | | Emphysema + bronchiectasis (1) |
| | | Bronchiectasis (1) |
| | | None (4) |

* Values in parentheses indicate number of cases.

* Chronic obstructive pulmonary disease.
aspergillosis. The other 53 patients had a variety of underlying diseases. Tuberculosis, the most frequently occurring, was found in 23 of the 53 patients.

Sera from 12 patients with pulmonary disease of unknown etiology were also studied. In these cases, aspergillosis was strongly suspected, but a diagnosis could not be confirmed. *A. fumigatus* was isolated from two of these patients, *A. niger* from one, and *Aspergillus* sp. from four others. No aspergilli were isolated from the other five. These patients had a variety of underlying diseases; the most prominent was tuberculosis.

The precipitin reactivity of sera from patients with aspergillosis, patients with other pulmonary diseases, and from apparently normal subjects is shown in Table 2. The immunodiffusion test was positive in a total of 49 of the 60 (82%) aspergillosis cases studied: 28 of 30 (93%) of the aspergilloma cases, 7 of 14 (50%) of the allergic bronchopulmonary cases, and 14 of 16 (88%) of the invasive aspergillosis cases. Ten of the 12 (83%) sera from patients with pulmonary disease of unknown etiology were precipitin-positive as were 2 of the 17 (12%) from asthma patients. Fifty-five sera from proven cases of other systemic mycotic infection, bacterial diseases, or neoplastic diseases and 10 sera from apparently normal humans were all negative for precipitins to the five *Aspergillus* sp. tested.

Of the 95 sera from aspergillosis cases studied, 75 were reactive in the immunodiffusion test (Table 3). Seventy-four of the 75 reactive sera demonstrated precipitins to *A. fumigatus* antigens; 52 of the 75 sera contained only precipitins for *A. fumigatus*, whereas 22 contained, in addition, precipitins to other *Aspergillus* sp. The one serum which did not react with *A. fumigatus* reacted only with *A. niger*.

Seven of the 16 sera from patients with pulmonary diseases of unknown etiology showed precipitin activity only with *A. fumigatus* antigens. Two reacted only with the *A. niger* antigen. The other three precipitin-positive sera in this group reacted with precipitogens to *A. fumigatus* and other *Aspergillus* sp. The two precipitin-positive sera from patients with asthma reacted only with *A. fumigatus* antigens. None of the sera in this study had precipitins to *A. flavus, A. nidulans, or A. terreus* in the absence of demonstrable precipitins to *A. fumigatus*.

The number of precipitins noted in aspergillosis case sera varied from one to four. Sera reacting with *A. fumigatus* or *A. niger* antigens produced as many as four precipitin bands. In contrast, sera reacting with antigens to the other species of *Aspergillus* showed only one

| Clinical category | No. of subjects | *Aspergillus* precipitin test | Per cent positive |
|-------------------|-----------------|-----------------------------|------------------|
|                   |                 | Positive | Negative |                   |
| Aspergillosis     | 60              | 49       | 11       | 82                |
| Aspergilloma      | 30              | 28       | 2        | 93                |
| Allergic bronchopulmonary | 14 | 7       | 7        | 50                |
| Invasive          | 16              | 14       | 2        | 88                |
| Pulmonary disease, unknown etiology | 12 | 10       | 2        | 83                |
| Asthma            | 17              | 2        | 15       | 12                |
| Other mycotic diseases |               |           |          |                   |
| Blastomycosis     | 4               | 0        | 4        | 0                 |
| Candidiasis       | 1               | 0        | 1        | 0                 |
| Coccidioidomycosis| 3               | 0        | 3        | 0                 |
| Cryptococcosis    | 3               | 0        | 3        | 0                 |
| Histoplasmosis    | 2               | 0        | 2        | 0                 |
| Paracoccidioidomycosis | 1 | 0        | 1        | 0                 |
| Sporotrichosis    | 2               | 0        | 2        | 0                 |
| Bacterial diseases |               |           |          |                   |
| Nocardiosis       | 4               | 0        | 4        | 0                 |
| Tuberculosis      | 28              | 0        | 28       | 0                 |
| Neoplastic diseases |               |           |          |                   |
| Carcinoma         | 2               | 0        | 2        | 0                 |
| Hodgkin's disease | 3               | 0        | 3        | 0                 |
| Leukemia          | 2               | 0        | 2        | 0                 |
| Normal subjects   | 10              | 0        | 10       | 0                 |

**Table 2. Aspergillus precipitin test reactivity of sera from normal subjects and patients with aspergillosis and other pulmonary diseases**
precipitin. The data in Table 4 show the number of precipitin bands produced after reaction of sera from patients with different clinical forms of aspergillosis with *A. fumigatus* antigens. Sero-positive aspergillosis cases, regardless of clinical type, usually demonstrated one to two precipitins. Only 2 of the 43 positive sera from the aspergilloma cases produced three precipitin bands, and three sera produced four bands. None of the nine positive sera from the allergic bronchopulmonary cases produced more than two precipitin bands. Four of the 22 positive sera from invasive cases produced three precipitin bands, and two produced four bands. All 22 produced at least one band of identity with the reference sera.

Table 1 shows reactions obtained with sera from aspergilloma cases and sera from pulmonary invasive cases with the band produced by the human reference serum against *A. fumigatus* antigen B1172. Figure 2 illustrates reactions of *A. fumigatus* antigen B1172 and aspergilloma case serum, invasive case serum, and allergic bronchopulmonary case serum in reference to proven human case serum and rabbit reference serum. The three precipitin bands produced against *A. niger* precipitinogen by a serum from a patient with an *A. niger* aspergilloma are shown in Fig. 3. This serum also reacted with *A. fumigatus* precipitinogens, producing a band which shows partial identity with the reference *A. fumigatus* human serum.

Examination of the sera from the patients with suspected aspergillosis revealed one or two precipitin bands in 9 of the 12 positive sera and three bands in only one serum. The two sera in this group that reacted only with the *A. niger* antigen produced only one precipitin band. Precipitin-positive sera from the two patients with asthma produced only one band with *A. fumigatus* precipitinogens.

Seventy-two positive sera were titrated with the *A. fumigatus* antigens (Table 5). Of 42 positive sera from cases of aspergilloma, 34

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**Table 3. Spectrum of precipitin reactions of sera from proven or suspected cases of aspergillosis, pulmonary disease with unknown etiology, and asthma with antigens from five Aspergillus sp.**

| Antigen | Aspergilloma (49)* | Allergic broncho-pulmonary (20) | Invasive (25) | Pulmonary disease, unknown etiology (16) | Asthma (19) |
|---------|--------------------|---------------------------------|--------------|------------------------------------------|-------------|
| A. *fumigatus* | 33 | 7 | 12 | 7 | 2 |
| A. *niger* | 1 | 0 | 0 | 2 | 0 |
| A. *fumigatus* | - A. *niger* | 1 | 0 | 1 | 0 | 0 |
| + *A. flavus* | 2 | 0 | 0 | 1 | 0 | 0 |
| + *A. terreus* | 0 | 0 | 2 | 1 | 0 | 0 |
| A. *fumigatus, A. flavus* | + *A. terreus* | 1 | 0 | 1 | 0 | 0 |
| + *A. niger* | 1 | 0 | 1 | 0 | 0 | 0 |
| A. *fumigatus, A. terreus, and A. flavus* | + *A. niger* | 0 | 0 | 1 | 0 | 0 |
| + *A. nidulans* | 0 | 0 | 1 | 0 | 0 | 0 |
| All Aspergillus sp. | 0 | 0 | 1 | 0 | 0 | 0 |

* Values in parentheses indicate total number of sera.

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**Table 4. Number of Aspergillus *fumigatus* precipitin bands by clinical type of aspergillosis**

| Clinical type of aspergillosis | Total no. of sera | No. of sera with no. of precipitin bands |
|-------------------------------|------------------|------------------------------------------|
| Aspergilloma                  | 49               | 0 1 2 3 4                               |
| Allergic broncho-pulmonary    | 20               | 11 6 3 0 0                              |
| Invasive                      | 26               | 4 11 5 4 2                              |
| Total no. of sera             | 95               | 21 35 28 6 5                            |

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Fig. 1. Immunodiffusion test with A. fumigatus strain B1172 antigen (F) in center well against human reference serum (R.), sera from aspergilloma cases (S.), and sera from pulmonary invasive cases (S) in the peripheral wells.

Fig. 2. Immunodiffusion reactions obtained with A. fumigatus strain B1172 antigen (F) against sera of patients with different clinical forms of aspergillosis: aspergilloma (S.), invasive (S), allergic bronchopulmonary (S), and rabbit A. fumigatus antiserum (R.).

Fig. 3. Immunodiffusion reactions of serum (S.) from a patient with an A. niger aspergilloma against A. fumigatus strain B1172 antigen (F) and A. niger strain 107 antigen (N) compared to precipitin bands resulting from reaction of human reference serum (R,) and A. fumigatus antigen (F). 1:64 produced either three or four precipitin bands.

With the exception of one serum, the titers of the positive sera from the suspected aspergillosis group of patients varied from undilute to 1:16. The exceptional serum, from a patient with chronic pulmonary disease, showed a titer of 1:512 and produced only two precipitin bands. The two precipitin-positive sera from asthma patients demonstrated titers of 1:1 and 1:2, respectively.

Serial sera from four patients with proven aspergillosis were studied to determine the prognostic value of the ID test. The pertinent clinical, laboratory, and serological data from these patients are given in Table 6. It shows that the antibody response of patient ER reflected his clinical state. The number of precipitin lines dropped from two to zero, and the titer dropped from 1:4 to zero. In the case of BC, the number of precipitin lines escalated from two to three during treatment and then declined to one, whereas the corresponding titers changed from 1:8 to 1:16 to 1:4. When the third specimen was taken, the physician considered the patient clinically well. JE is the patient with an A. niger aspergilloma whose serum reactions were referred to in Fig. 3. The second serum from patient JE was the only positive one from the proven aspergillosis cases that did not react with A. fumigatus antigens. Case DR represents an inadequately
TABLE 5. Aspergillus fumigatus precipitin titers by clinical type of aspergillosis

| Clinical type of aspergillosis | Total no. of positive sera | No. of sera with titer of |
|-------------------------------|-----------------------------|---------------------------|
|                               | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 |
| Aspergilloma                  | 42  | 9   | 13  | 7   | 5    | 2    | 1    |
| Allergic bronchopulmonary     | 8   | 1   | 3   | 3   | 1    | 0    | 0    |
| Invasive                      | 22  | 4   | 3   | 7   | 3    | 1    | 3    |

TABLE 6. Immunodiffusion test results with serial serum specimens from four patients with proven aspergillosis

| Case | Clinical form | Specimens received | Date | Laboratory or clinical data | Immunodiffusion results |
|------|---------------|--------------------|------|-----------------------------|-------------------------|
|      |               |                    |      |                             | No. of precipitin bands | Antigen | Titer |
| ER   | Aspergilloma  | 1                  | 10/69| Numerous fungus balls; A. fumigatus isolated | 2                        | A. fumigatus | 1:4   |
|      |               | 2                  | 1/70 | Clinically well             | 2                        | A. fumigatus | 1:2   |
|      |               | 3                  | 6/70 | X rays cleared              | Zero                     | 0      |
| BC   | Pulmonary     | 1                  | 3/1/70| Cavitary aspergillosis diagnosed; A. fumigatus isolated | 2                        | A. fumigatus | 1:6   |
|      | invasive      | 2                  | 4/23/70| Amphotericin B administered (3/70 to 5/70) | 3                        | A. fumigatus | 1:16  |
|      |               | 3                  | 6/12/70| Clinically well             | 1                        | A. fumigatus | 1:4   |
|      |               | 1                  | 1967 | Some amphotericin given in 1967, tolerated poorly; A. niger repeatedly isolated | 1                        | A. niger | 1:16  |
| JE   | Aspergilloma  | 2                  | 1969 | Unchanged fungus balls, 1969 | 1                        | A. niger | q.n.s.|
|      |               | 1                  | 9/11/69| Old tuberculosis; A. terreus and S. schenckii isolated from leg; Aspergillus sp. and Penicillium sp. isolated from sputum | 3                        | A. fumigatus | 1:32  |
|      |               | 2                  | 10/28/69|                              | 4                        | A. fumigatus | 1:32  |
|      |               | 3                  | 12/4/69| Patient refused treatment and subsequently died (11/70) | 3                        | A. fumigatus | 1:32  |

DISCUSSION

Our studies indicate that the ID test for aspergillosis is an excellent aid in the laboratory diagnosis of aspergillosis. The presence of precipitating antibodies, regardless of the number of bands or titer, indicates infection, colonization, or allergy due to an Aspergillus species. Use of the ID test permitted the diagnosis of 28 of 30 (93%) cases of aspergillosa, 14 of 16 (88%) cases of invasive aspergillosis, and 7 of 14 (50%) patients with allergic bronchopulmonary aspergillosis. These results are in agreement with the findings of other investigators. Longbottom and Pepys (7) found that 98% of the sera from 57 patients with aspergillosa had demonstrable precipitins. Similarly, Campbell and Clayton (3) found 91% of 23 aspergillosa patients to be precipitin-positive and 70% of 87 patients with allergic aspergillosis to be positive. These authors suggested that the presence of serum precipitins bears no direct relationship to allergic bronchopulmonary aspergillosis but probably signified active or recent infection. Our ID test data with sera from allergic bronchopulmonary cases support this contention.

Our experiences with the ID test indicate that it is specific. Aspergillus sp. precipitins were not detected in any of the 55 sera from patients with other systemic mycotic infections or bacterial or neoplastic diseases or in 10 sera from apparently normal humans. These results conflict with the view of Stallybrass (14) that precipitins may be formed from chance contact with Aspergillus sp. antigens and may...
persist in otherwise healthy individuals for many years. We did find precipitins in two patients with asthma and in 10 of 12 patients with pulmonary disease of unknown etiology; however, aspergillosis was strongly suspected in all of these patients. We do not regard the reactions noted in the sera from the asthma patients and the patients in the "strongly suspected" group as merely cross-reactions. We feel that a positive precipitin reaction is good evidence of aspergillosis or hypersensitivity to an *Aspergillus* sp. Longbottom and Pepys (7) reported positive precipitin reactions in 63% of 93 patients with asthma and pulmonary eosinophilia. The same report showed that 8% of 185 patients with different pulmonary conditions had precipitins in their sera and that 14% had positive skin test reactions. Campbell and Clayton (3) included in their study eight precipitin-positive sera from patients with a clinical diagnosis of asthma. They also found sera from patients from whom *A. fumigatus* was repeatedly isolated and who had a long history of bronchitis and productive cough to be precipitin-positive. In these cases, as in our strongly suspected group, a diagnosis of aspergillosis was not confirmed.

In 1967, English and Henderson (4) reported on their study of 21 patients with various lung conditions. In determining the diagnostic significance of the ID test, these investigators stressed the importance of "reactivity," which they defined as the number of precipitin lines produced, and of the "range," or number of antigenic extracts with which the serum reacted. Our results do not support this approach. They do agree, however, with the view of Walter and Jones (15) that the presence of precipitating antibodies, regardless of the number of precipitin bands or titer, indicates infection with, or development of, an allergy to an *Aspergillus* sp.

Antigen preparation was based on the recommendations of Longbottom and Pepys (7) who found that 3- to 5-week-old surface cultures on Sabouraud medium yielded the most suitable antigens free of C-substance glycopeptide. We found that standardized and reproducible precipitinogens can be prepared by acetone precipitation of 5-week-old Sabouraud dextrose broth cultures. We did not investigate the use of other antigenic extracts. We used precipitinogens to four *A. fumigatus* strains; however, a serum specimen that does not react against all four of the *A. fumigatus* antigens is rare.

Our results indicate that precipitinogens to *A. fumigatus* and *A. niger* may be used for the maximal detection of aspergillus precipitins. Use of these two antigens permitted a presumptive diagnosis of aspergillosis in 49 of 60 (82%) patients whose sera were examined. We found three cases of proven or suspected aspergillosis in which a serum specimen failed to produce precipitin lines with *A. fumigatus*. All three sera reacted only with *A. niger* precipitinogens. Several investigators (3, 7, 8) have reported cases of aspergillosis due to species other than *A. fumigatus* in which serum specimens failed to react with *A. fumigatus* antigens. These infections were due to *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*. On the basis of these studies, we used a battery of precipitinogens prepared from *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, and *A. terreus*.

We observed an association between the number of precipitin bands and the clinical type of aspergillosis (Table 4). One or two precipitin bands occurred in sera from patients with each form of aspergillosis. None of the sera from the allergic bronchopulmonary cases produced more than two bands. Our data suggest that three or four precipitin bands may be indicative of an aspergilloma or of pulmonary or disseminating invasive aspergillosis.

Although a titer of 1:16 may be useful for differentiating aspergilloma and invasive aspergillosis from the allergic bronchopulmonary form of this disease, our data showed that sera with titers of 1:16 or greater from patients with proven aspergillosis also produced three or four precipitin bands. Consequently, the titration of positive sera with *A. fumigatus* precipitinogens does not appear to be useful in determining the clinical form of aspergillosis. Titration might prove useful in following the clinical course of the disease. However, our studies (Table 6) indicate that, with significant titer changes, a corresponding drop in the number of precipitin lines occurs. This observation is in accord with reports of other investigators (5, 12, 14, 15) that precipitating antibodies diminish or disappear with treatment. Apparently, therefore, titration is not needed to determine the progress of an infection and the patient’s response to therapy.

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