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Serologic Immunodiagnosis of Invasive Aspergillosis

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Two sensitive methods, counterimmunoelectrophoresis (CIE) and enzyme-linked immunosorbent assay (ELISA), for detecting antibodies to *Aspergillus* were used to study serial specimens from patients with histologically proven invasive aspergillosis for measurement of conversion from negative to positive immunoprecipitin reactions and changes in ELISA titers during immunosuppression. Sera from 12 granulocytopenic patients without invasive aspergillosis served as controls. Positive CIE reactions were demonstrated in 70% of patients with clinically suspected aspergillosis. In addition to a greater sensitivity (80%) of ELISA, the serial determination of antibody response by ELISA allowed for separation of seropositive patients into two groups. A serial rise in ELISA titer appeared to correlate with histologically documented recovery from infection, whereas those with declining or persistently intermediate titers were found to have disseminated aspergillosis at autopsy. Thus, serial antibody determination by ELISA was valuable as both a diagnostic and prognostic tool.

Invasive aspergillus infections in febrile granulocytopenic patients with hematologic malignancies are difficult to diagnose, and such diagnoses are frequently made at necropsy [1–3]. Earlier diagnosis with resultant earlier appropriate antifungal therapy has been found to lead to greater therapeutic success [4]. Unfortunately, techniques for definitive histologic diagnosis, such as open-lung biopsy, have risks. Because of this it is clear that a pressing need exists for noninvasive methods of establishing the presence of disseminated mycoses, including aspergillosis. Several serodiagnostic techniques have previously been evaluated for the early diagnosis of invasive aspergillosis, but the results have been conflicting [5–12].

In this study the sensitive enzyme-linked immunosorbent assay (ELISA) [13, 14] and the more refined serologic technique for precipitin detection, counterimmunoelectrophoresis (immunoelectroosmophoresis) (CIE) [15–17], have been used to detect an antibody response to *Aspergillus* in serial serum samples from febrile granulocytopenic patients with acute leukemia and aplastic anemia complicated by invasive aspergillosis. All of our patients have had culture and/or histologic evidence of aspergillosis and were compared with a control group similar in underlying diseases. The results of these tests are analyzed retrospectively for determination of the clinical reliability of these procedures. We report here our experience that either of these methods, with use of a well-characterized protein-glycoprotein antigen from *Aspergillus*, is useful in detecting infection in the great majority of cases and that ELISA antibody measurements may have prognostic value.

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Material and Methods

Patients. Serial serum samples from two groups of patients were used in this study. The first group consisted of 10 patients with acute leukemia, aplastic anemia or immunodeficiency disease, and clinically suspected invasive aspergillus infection. The clinical diagnosis of aspergillosis was made antemortem when clinical evidence of infection was found at the suspected site in association with appropriate systemic signs and symptoms. Diagnosis of disseminated aspergillosis was made upon examination of histologic sections.

The retrospective examination of the records of the patients included determination of age, sex,

underlying debilitating disease; duration of courses of chemotherapy, radiotherapy, and steroid therapy for treatment of hematologic malignancies or immunodeficiency disease; bone-marrow transplantation; concentrations of IgG, IgA, and IgM in the serum; absolute granulocyte count; and cultural evidence of *Aspergillus* from suspected sites. The intervals between the day of onset of clinical signs of suspected invasive aspergillosis, the day of death, and serologic determinations were recorded. A summary of the characteristics of the patients in this group appears in table 1.

The second group of compromised hosts consisted of 12 granulocytopenic patients without histologic evidence of invasive aspergillosis. Ten had acute leukemia, one had aplastic anemia, and one had carcinoma of the lung. Nine of this group (including the patient with lung carcinoma) received a marrow transplant. Serum specimens from these patients were used as controls.

All patients died within a few days to two months after their high-risk episode. Autopsy reports of all patients were available.

Sera. Specimens of serum from the two groups of patients were submitted to the Medical Mycology Section, National Bacteriological Laboratory (SBL), Stockholm, Sweden, from the Division of Infectious Diseases, UCLA School of Medicine, Los Angeles, Calif., for tests for antibody response to species of *Aspergillus*. Serial serum samples were available from both groups of patients during high-risk episodes that were clinically compatible with invasive aspergillosis. Serologic tests were done on serum samples taken within three weeks before and two weeks after the time of onset of clinical signs of a high-risk episode. All sera were tested at the same time.

Antigens. One reference strain of *Aspergillus fumigatus* (CBS 13361) and one strain of *A. fumigatus* isolated from a patient with aspergillosis at the Mycology Section, SBL, were grown in 100 ml of Czapek-Dox broth with an initial pH of 5.0 in one-liter Erlenmeyer flasks at room temperature (about 24 C) for six weeks. The culture filtrate was separated from mycelia by passage through Whatman no. 42 paper. Filtrates from five flasks of each strain of *A. fumigatus* were pooled and concentrated (1:10) by dialysis against polyethylene glycol (mol wt cutoff, 20,000) using Visking cellulose membrane (diameter, 1 cm).

The concentrated culture filtrate was then centrifuged (10,000 *g* at 4 C for 30 min), and the deposit of particulate material was discarded. A solution of 75% saturated ammonium sulfate was added slowly with stirring. The precipitates were removed by centrifugation (10,000 *g* for 15 min at 4 C), dissolved in small volumes of phosphate-buffered saline (pH 7.2), dialysed free from ammonium sulfate at 4 C, and freeze-dried. The freeze-dried material was reconstituted in phosphate-

Table 1. Characteristics of patients with acute leukemia or aplastic anemia and clinically suspected invasive aspergillosis.

Patient no.	Neoplastic disease	Immunosuppression			Organ involved with *Aspergillus* histologically	Organism cultured
		Cortico-steroids	Cytotoxic	Radiation therapy		
1	AML, BMT		+	+	Lung*	−*
2	ALL		+	+	Spleen, hepar	ND
3	AML, BMT		+	+	Lung	ND
4	AML	+			Lung	*Aspergillus fumigatus*
5	ALL, BMT		+	+	Lung	*A. fumigatus*
6	ALL, BMT		+	+	Lung	*A. fumigatus*
7	Systemic vaculitis	+			Lung, kidney	*A. fumigatus*
8	ALL, BMT		+	+	Lung, brain	*A. fumigatus*
9	T-cell suppressor immuno-deficiency disease					*A. fumigatus*
10	Aplastic anemia, BMT		+	+	Lung*	−*

NOTE. Abbreviations: ALL = acute lymphocyte leukemia; AML = acute myelogenous leukemia; BMT = bone-marrow transplantation; ND = no culture performed; + = present; − = negative culture.

* Diagnosis of pulmonary aspergillosis was made by lung biopsy while the patient was receiving amphotericin.

buffered 0.85% NaCl, pH 7.2, with various bases (wt/vol), depending on the purpose of the immunologic study.

This protein-glycoprotein antigen preparation of *A. fumigatus* (SBL 04/76) was used as the primary standard for the CIE and ELISA tests throughout this study. In addition, two standard antigen preparations of *Aspergillus flavus* (ATCC 11500) and *Aspergillus niger* (CBS 12228) were produced by the same method as above and used with each serum in all CIE tests.

All preparations were examined by crossed immunoelectrophoresis according to Weeke [18] and by isoelectric focusing in thin-layer polyacrylamide gels according to Vesterberg [19] and were further characterized by crossed immunoelectrofocusing as described previously [20]. The protein content of all antigen preparations was determined by the method of Lowry et al. [21]. Carbohydrate content was determined by a phenol-sulfuric acid method [22]. Chemical and immunoelectrophoretic analyses of the standard reference *A. fumigatus* antigen preparation are summarized in table 2 and figure 1.

Table 2. Composition of the standard preparation of polyvalent *Aspergillus fumigatus* antigen used in this study.

Composition	Value
Protein/carbohydrate ratio	2.94
No. of stained bands on polyacrylamide gel electrophoresis	
Coomassie blue	35
Periodic acid–Schiff reagent	7
No. of detectable antigens	
Counterimmunoelectrophoresis	18
Crossed immunoelectrophoresis	40
Crossed immunoisoelectrofocusing	
pH 3.5–9.0 gradient	33
pH 4.0–5.0 gradient	19

Antiserum. Rabbits (weight, 3–4 kg) were im-

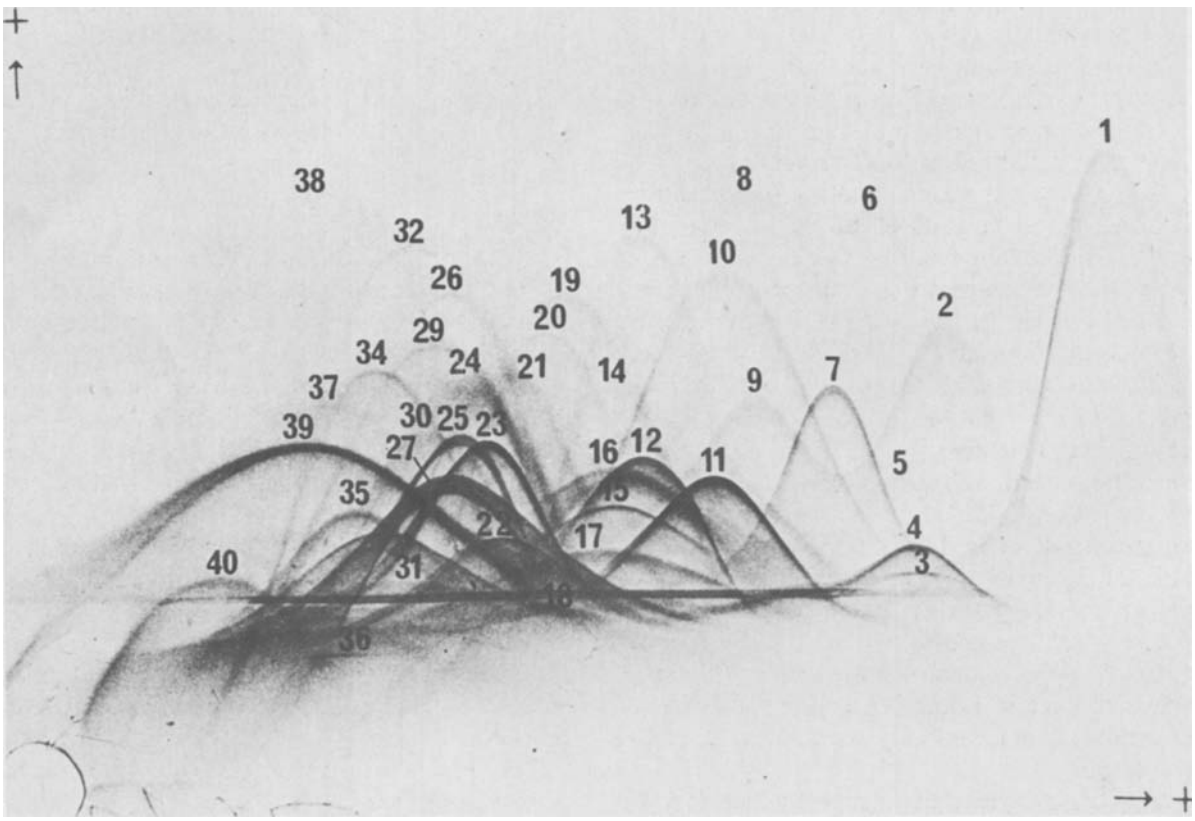

Figure 1. Crossed immunoelectrophoresis of 5 μl of a standard polyvalent antigen preparation of *Aspergillus fumigatus* (SBL 04/76). The second-dimension gel contained 5 μl of a reference rabbit antiserum to *A. fumigatus* (SBL 2D/76)/cm². The second-dimension electrophoresis was done at 2 V/cm overnight.

munized with 1 ml of each antigen preparation; 0.5 ml of antigen in Freund's incomplete adjuvant was given sc, and at the same time 0.5 ml of the antigen was given iv. Rabbits were immunized for at least three months. A reference antiserum for each species of *Aspergillus* was prepared from pooled serum samples from at least nine to 10 rabbits for which blood from repeated bleedings had given precipitin reactions to the immunogens. The antibody fraction of the pooled antiserum was purified by salt precipitation and ion-exchange chromatography on DEAE-cellulose at pH 5.0. The γ-globulin fraction was concentrated to ~25 mg of antibody protein/ml and stored frozen.

CIE. CIE was performed in 1.0% (wt/vol) Noble agar (Difco, Detroit, Mich.) cast as a layer 1-mm thick on 20- × 10-cm glass slides. The buffer in the gels and the electrode vessels was 0.075 M barbital buffer, pH 8.6. Wells 4 mm in diameter were cut in parallel rows 1 cm apart. Four rows of 24 pairs of wells were cut on each plate. The antigens were placed in the wells nearest to the cathode and the grouping sera in the others. Antigens and antisera (10 μl) were assayed in twofold dilutions. Electrophoresis was carried out in an apparatus constructed for quantitative (rocket) immunoelectrophoresis according to Laurell [23]. After electrophoresis at 10 V/cm for 15–30 min at 4 C, the plate was washed in 0.5% sodium citrate in saline for 24 hr at 20 C and then overnight in 0.9% NaCl before staining with Coomassie brilliant blue and examination of precipitin lines.

ELISA. The ELISA was performed essentially according to Engvall and Perlmann [24] as modified by Carlsson et al. [25] using 1 M deethanolamine • HCl, pH 9.8, as substrate buffer. Disposable polystyrene tubes (11 × 55 mm; Heger Plastics, Stallarholmen, Sweden) were coated by incubation (18 hr at 25 C) with 1 ml of *A. fumigatus* antigen dissolved in 0.05 M carbonate buffer, pH 9.6. A polyspecific sheep antiserum to human immunoglobulin, purified by affinity chromatography, that was conjugated with alkaline phosphatase (calf intestinal mucosa, type VII; Sigma Chemical Co., St. Louis, Mo.) was used for determination of total antibody response to *A. fumigatus.*

The enzyme reaction was performed for 100 min or until the absorbance at 400 nm reached ~0.8. The reaction was stopped by adding 0.1 ml of 5 M NaOH. The absorbance at 400 nm was then determined in a spectrophotometer with a 1-cm light path. The assays were always run in duplicate, and the mean absorbance value × 100 was taken as the result. The duplicate rarely deviated by >5% from the mean absorbance.

In primary assays to develop the model system of ELISA for aspergillus serology in routine diagnostic work, the optimal coating concentration of the standard antigen preparation of *A. fumigatus* was determined to 10 μg of antigen/ml; the binding capacity of the antibody to the antigen and the optimal dilution of human sera that most effectively discriminated between positive and negative sera was a dilution of 1:1,000.

To determine reference values for the ELISA test for *A. fumigatus*, different groups of sera from healthy individuals, noncompromised hosts with allergic aspergillosis and aspergilloma, and febrile granulocytopenic patients with proven deep-seated infections with virus (cytomegalovirus, herpes simplex virus), protozoa (*Pneumocystis carinii*), or bacteria (*Klebsiella, Escherichia coli, Pseudomonas*) were assayed [26] (figure 2). In the healthy controls and the febrile controls, an ELISA titer of <1.0 was consistently registered when expressed as the absorbance value at 400 nm per 100 min with a serum dilution of 1:1,000.

Results

All patients had radiologic evidence of pneumonitis and documented pulmonary aspergillosis by lung biopsy (patients no. 1 and 10), by brain biopsy (patient no. 9), or in autopsy tissue (table 1). Noteworthy was patient no. 9, who had a poorly understood adult immunodeficiency disease characterized by the presence of circulating T-lymphocyte suppressor cells. This patient had diffuse pulmonary infiltrates, and an open-lung biopsy specimen revealed pneumonitis but no causative organisms by special stains and cultures. He was then treated empirically with high-dose corticosteroids and developed a right frontal lobe mass lesion six months later. An open-brain biopsy specimen yielded *A. fumigatus*, and the patient subsequently died.

The serologic tests for antibody response to *Aspergillus* in the infected group and the control group revealed that in seven (70%) of 10 episodes in 10 patients, a positive precipitin reaction against *A. fumigatus* could be demonstrated by

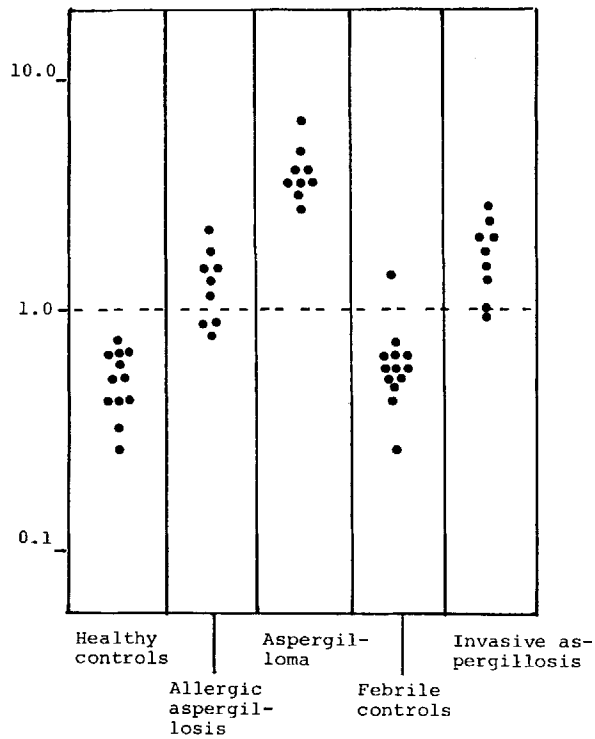

Figure 2. Results of enzyme-linked immunosorbent assay, expressed as absorbance at 400 nm per 100 min, for determination of antibody response to *Aspergillus fumigatus* in sera from 12 healthy blood donors, nine patients with hypersensitive pneumonitis, nine patients with aspergilloma due to *A. fumigatus*, 13 granulocytopenic patients with deep-seated bacterial, viral, and protozoal infections, and nine patients with invasive aspergillosis due to *A. fumigatus* (reproduced from [26]).

CIE in available serum samples taken within 14 days before the onset of clinical signs of suspected invasive aspergillosis. No precipitin reactions against *A. niger* and *A. flavus* antigens were detected. An ELISA titer of >1.0, scored as positive for antibodies to *A. fumigatus*, was demonstrated in the serum samples from eight (80%) of the 10 episodes.

None of the 12 febrile control patients with underlying diseases similar to the *Aspergillus*-infected group had precipitins detected by CIE against antigen preparations from *A. fumigatus*, *A. niger*, and *A. flavus*. One of the control patients had a slightly elevated ELISA titer (1.26) for antibodies to *A. fumigatus*; the remaining were <1.0 and were scored as negative.

All precipitin-positive episodes were also associated with an elevated ELISA titer (>1.0), except for one episode (patient no. 9) (table 3). In this episode the serial CIE was consistently positive while the serial ELISA titers were primarily elevated although declining.

Table 4 lists the serial ELISA titers for antibody response to *A. fumigatus* and the precipitin reactions against the same antigen preparation in CIE tests in available sera from the 10 episodes by the date and in relation to the number of days before and after the onset of clinical signs of aspergillosis and to days before death.

A conversion from a negative to a positive precipitin reaction was registered in serum samples taken from two patients within two weeks before the onset of clinical signs compatible with invasive aspergillosis. No positive precipitin reaction of serial tests reverted to negative. In three episodes the serial CIE tests for precipitins were consistently negative. In serial ELISA titers a rise of >0.15 absorption units (significant) could be demonstrated in five episodes. Two of these patients survived their high-risk episodes. They subsequently died 13 and 49 days later, respectively, but aspergillosis was not the primary cause of death. Necropsy revealed aspergillus lung infection that was apparently resolving by the time of death.

In one episode, the positive serial ELISA titers were persistently intermediate, and in four episodes the serial ELISA titers were declining. Three of these four latter patients died, with evidence of aspergillosis as the major factor contributing to death (patient no. 9, whose serum samples were retrieved only after death, was in this group). They had an elevated titer (1.95) at the time of the nondiagnostic open-lung biopsy, which declined to 0.27 just before death from intracerebral aspergillosis. In one patient, no. 10, a positive ELISA became in serial determinations negative by the time he developed cavitary pulmonary aspergillosis after a bone-marrow transplant. A thoracot-

Table 3. Comparison between antibody responses to *Aspergillus fumigatus* as determined with enzyme-linked immunosorbent assay (ELISA) and counterimmunoelectrophoresis (CIE) in *Aspergillus*-infected patients.

	ELISA titer		
CIE	<1.0	>1.0	Total
Positive	1	6	7
Negative	1	2	3
Total	2	8	10

Table 4. Results of serologic tests for antibody response to *Aspergillus* as correlated by the days of onset from clinical signs of aspergillosis and days from death.

Patient no.	Date of serum	Days before (+) and after (−) onset of clinical signs	Days before death	ELISA titer (OD × 100)*	Precipitin reaction†
1	6/78/76	+7	8	1.40	+
	6/25/76	0	1	1.67	+
2	6/23/76	+12	25	1.34	+
	7/2/76	+3	16	2.62	+
3	5/5/76	+15	65	1.44	−
	5/21/76	−1	48	2.55	−
4	1/13/77	+1	9	0.99	+
	1/19/77	−5	3	1.37	+
5	4/4/75	+17	19	1.25	−
	4/13/75	+8	10	1.34	+
6	10/16/75	+18	19	2.02	−
	10/28/75	+6	7	2.75	+
7	3/4/76	+12	24	1.85	+
	3/25/76	−9	3	1.62	+
8	12/7/76	+8	9	0.98	−
	12/10/76	+5	6	0.25	−
9‡	7/2/78	+21	26	1.95	+
	7/22/78	+1	6	0.62	+
	7/27/78	−4	1	0.30	+
10§	4/2/77	+19	61	2.00	−
	4/13/77	+8	50	0.75	−
	4/24/77	−3	39	0.55	−
	5/10/77		23	0.52	−
	5/31/77		2	0.56	−

* ELISA = enzyme-linked immunosorbent assay.
† By counterimmunoelectrophoresis (immunoelectrosmophoresis).
‡ Rapidly developing intracranial abscess, 7/23/78.
§ Thoractomy with lobectomy for removal of aspergilloma, 5/8/77.

omy and excision of infarcted lung were required to stop hemoptysis (thereby documenting aspergillosis in the lung). Subsequent serial determinations remained consistently negative for antibody response to *Aspergillus* both by CIE and ELISA. He subsequently died of graft-vs.-host disease and cytomegaloviral pneumonia, but aspergillosis was not found in the lung.

There was no relationship between levels of serum immunoglobulins and detectable antibodies to *Aspergillus*. Three of four patients with increasing granulocyte levels demonstrated a rise in ELISA titers.

Discussion

The clinical diagnosis of invasive aspergillosis is difficult since the clinical features and their roentgenologic manifestations are variable and are not diagnostic [3, 4]. The criteria for a definite diagnosis of invasive aspergillosis are based on demonstration of characteristic hyphae in tissue sections [3]. This criterion is required since sputum examination is not diagnostic and both false-negative and false-positive results occur [27, 28]. Instead, more aggressive diagnostic methods, such as transtracheal aspiration or percutaneous or open-lung biopsy, are warranted to establish the diagnosis in the immunocompromised host. However, aggressive diagnostic procedures are often contraindicated on these severely ill patients.

In this situation serologic tests for the diagnosis of invasive aspergillosis could be a valuable adjunct in the noninvasive, early diagnosis of inva-

sive aspergillosis and could lead to improved clinical management. Numerous researchers have demonstrated that the immunodiffusion (ID) test is a useful adjunct in establishing a diagnosis of aspergillosis in noncompromised patients [5–9, 11, 12], particularly in those with aspergilloma and allergic aspergillosis. The detection of precipitins in patient serum is presumptive or even confirmatory evidence where radiologic, clinical, and mycologic evidence of aspergilloma or allergic aspergillosis exists [29]. In recent studies the use of CIE (immunoelectroosmophoresis) for detection of antibody to *Aspergillus* in sera from patients has emerged as a rapid and reproducible serodiagnostic procedure [30]. CIE has been found to detect antibody in comparison to ID in twice as many sera from noncompromised patients with a diagnosis of allergic aspergillosis and aspergilloma even after concentration of the serum.

The reports of Murray [31] and Henderson et al. [32] that an ID test could be helpful also in diagnosing systemic invasive aspergillosis in immunocompromised hosts were supported by the studies of Coleman and Kaufman [10] but are contrary to the report of Young and Bennett [9]. The latter report involved 16 patients with fatal neoplastic diseases, none of whom had a detectable antibody response, as measured by passive ID. In contrast, Schaefer et al. [12] repeated ID tests on concentrated sera routinely at two-week intervals and found this to be a specific, though not always a sensitive (60%), test for invasive aspergillosis in adult leukemic patients. It seems likely that the differences between some of these earlier reports and our results are due to differences in the timing of sample collection and of antigen preparation and our use of more sensitive antibody detection techniques.

CIE rarely has been used in any published report for serodiagnosis of invasive aspergillosis in severely immunosuppressed patients. It is clear the CIE has two exploitable advantages when compared with ID, namely, greater speed and sensitivity. The greater sensitivity of CIE appeared to be an advantage in tests for aspergillus precipitins in immunosuppressed patients with invasive aspergillosis, since they were not only uncommon in febrile controls of granulocytopenic patients with leukemia complicated with invasive diseases of other microorganisms but can also be implicated in active disease process [30].

The aim of this study was to observe whether serial serologic CIE tests would be helpful in the diagnosis of invasive aspergillosis during episodes clinically compatible with invasive disease. The patients studied in this series are typical of those immunosuppressed states where disseminated aspergillosis has been a serious clinical management problem [27], such as acute leukemia and bone-marrow transplantation. The precise mode of death of patients was clearly documented by complete histologic examination at autopsy. The results showed that in the 10 episodes of histologically proven invasive disease the CIE was valid in seven cases (70%). A conversion from negative precipitin reaction in serial determination occurred in two of these episodes and was deemed mandatory to evaluate invasive disease. False-negative results occurred in only three episodes. Thus, serial immunoelectroosmophoresis tests for aspergillus precipitins during high-risk episodes appeared to be a highly suggestive indicator of invasive aspergillosis. It further appears that positive and clinically useful results can be obtained irrespective of levels of circulating granulocytes and serum immunoglobulins.

Since enzyme immunoassays have given promising results in quantitative assays in viral, bacterial, and parasitic infections as well as in the detection of antibodies to *Candida* and *Aspergillus* in serum from noncompromised patients, its potential in aspergillus serology for early diagnosis of invasive aspergillosis in severely immunosuppressed patients appeared to be worth investigating [14]. Little information about the antibody response detectable by ELISA against *Aspergillus* in invasive aspergillosis has been published previously. Preliminary data suggest that the ELISA might well be helpful in diagnosing invasive forms of the disease [33]. The results from this study indicate that an antibody response to *Aspergillus*, which in the ELISA used resulted in an elevated ELISA titer (>1.0) scored as positive, accompanied invasive aspergillosis in eight of the 10 episodes. A significant rise in ELISA titers developed in seropositive patients within a two-week interval before the onset of clinical signs of suspected aspergillosis. In two patients, this was associated with histologically proven recovery from active infection.

These results reflect the high sensitivity of the established ELISA for serology of *A. fumigatus*. The amount of detectable antibody protein is in

nanogram quantities. The enzyme immunoassays have also the ability to measure the primary interaction between antigen and antibody and do not require a secondary phenomenon such as formation of precipitin or agglutinin for detection of the reaction. Furthermore, the ELISA measures antibody response of all classes of immunoglobulins of approximately equal efficiency, not mainly that of a particular class of antibodies [13]. Another factor contributing to the great sensitivity of the ELISA for *Aspergillus* is related to the antigen preparation [34, 35]. As demonstrated by our results, the antibody-binding capacity of the antigen preparation in ELISA-positive human sera is great. This suggests the presence in the system of a large number of antigenic protein components against which the patients' sera contain antibodies following invasive aspergillosis. Further work is needed to determine which antigens are most consistently associated with the immune response or which antibodies might correlate with invasion and/or protection.

The demonstration and quantitation of circulating humoral antibody response to *Aspergillus* by CIE and ELISA may improve the antemortem diagnosis of invasive aspergillosis in immunosuppressed patients. Additional prospective studies are now clearly warranted to confirm this hypothesis and, perhaps most important, to evaluate the value of serologic information in the clinical decision to initiate systemic antifungal therapy.

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