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Invasive Aspergillosis: Antiserum for Circulating Antigen Produced after Immunization with Serum from Infected Rabbits

PAUL F. LEHMANN AND ERROL REISS*
Mycology Division, Center for Disease Control, Atlanta, Georgia 30333

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We describe a method for producing an antibody capable of detecting an antigen of *Aspergillus fumigatus* in serum and urine from infected rabbits and humans.

Various groups of patients, particularly those receiving chemotherapy for maintaining an organ transplant or for leukemia, are susceptible to invasive aspergillosis, a fungal disease caused by species of *Aspergillus,* most commonly *A. fumigatus* Fres. (3). The absence of anti-*Aspergillus* antibodies is typical in this infection, which is commonly only recognized postmortem (2, 7). The antibiotic amphotericin B is available, but early diagnosis seems to be essential for successful treatment (3). We have found a carbohydrate antigen in the serum of infected rabbits and humans that can be detected by an antiserum raised in rabbits by a novel means. Serum taken from an infected rabbit was the source of immunizing antigen used to produce this antiserum.

Detection of circulating antigens from microorganisms poses the problem of determining which of the materials present in the organism are likely to circulate. Many metabolites present in the whole organism or in crude extracts may be antigenic; however, they do not circulate in detectable quantities, either because they are not released in vivo or because they are rapidly metabolized. In addition, owing to antigenic competition (6) or to degradation into nonimmunogenic forms during chemical extraction, there may be a negligible antibody response in immunized animals to antigens that do circulate. Figure 1 shows that antiserum raised in rabbits against an alkaline extract of *A. fumigatus* mycelium reacted with many materials present in this and other extracts. A similar pattern was found with antisera raised against extracts of *A. fumigatus* mycelium prepared by extraction in hot neutral citrate buffer (4) or in aqueous pyridine (1). However, the antiserum X12 (Fig. 1), raised by using serum from an immunosuppressed and infected rabbit as source of antigen, detected predominantly a single antigenic moiety.

These antisera and the reference immunodiffusion antiserum (2) used in screening for *A. fumigatus* antibodies by the Center for Disease Control (this is raised against acetone-precipitated material from culture filtrates) were tested by counter-immunoelectrophoresis against urine and serum from immunosuppressed rabbits and serum from a leukemic child in whom invasive aspergillosis was diagnosed postmortem by histology. Only the antiserum X12 detected antigen.

Eight rabbits received 30 mg of cortisone acetate 1 day before intravenous injection of 10^5 or 10^6 conidia; antigen was detected in both their sera and urine 60 to 120 h after infection. All animals died within 8 days, showing evidence of invasive aspergillosis. Figure 2 shows that antigen was present in the child's serum taken postmortem but had not been present 4 months earlier. Unfortunately, serum samples were not obtained from this child in the days just before death. Figure 2 also shows that the reactivity of the antiserum X12 was removed after it was absorbed with the alkaline extract of *A. fumigatus* mycelium (CA; see legend to Fig. 1). Its activity was not removed by adsorption with glutaraldehyde-insolubilized human or rabbit serum, and thus the antigen detected appeared to be of fungal origin. We have not found this antigen in uninfected animals, in animals infected with *Candida albicans,* or in several normal human sera.

We have partly characterized the alkali-extracted antigen detected by X12. Figure 3a shows that the antigen was destroyed by treatment with sodium metaperiodate but not by an agarose-bound protease, which indicates that it is either wholly or partly carbohydrate. Figure 3b shows that both the chemically extracted antigen and the antigen found in the serum of an infected rabbit have a cathodic nature at pH 7.4. To increase the stock of specific antiserum,

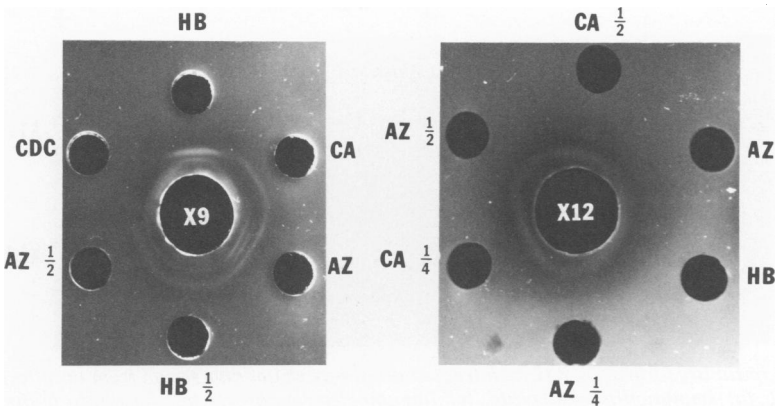

FIG. 1. *Precipitin arc formed by sera X9 and X12 with extracts of A. fumigatus (2085, London School of Hygiene and Tropical Medicine) mycelium. Mycelium was grown in 15 liters of Czapek Dox broth (54 h, 22°C, 18-liter carboy, with aeration and stirring) after seeding with a 48-h starter culture prepared in 600 ml of broth. Mycelium was killed with 1.5 g of thimerosal, collected over unbleached cotton muslin, and washed with saline and water before extraction. Soluble extracts were dialyzed against water and lyophilized. They were used at 1 mg/ml or at dilutions shown in the figure. HB, Mycelium extracted with hot citrate buffer, pH 7 (4). CA, Mycelium extracted from residue from hot buffer extraction by 0.4 N NaOH and 0.1 N $NaBH_4$ (ice cold, 20 h). AZ, Mycelium extracted with ether and then pyridine (1). CDC, Acetone-precipitated culture filtrate from a different A. fumigatus isolate (2). Full details of the preparation of these extracts will be published later. Antisera X9 and X12 were prepared in rabbits. X9, Antiserum response to 1 mg of CA in complete Freund adjuvant injected intramuscularly, followed after 5 weeks with a second injection intradermally distributed in 10 sites. X12, Antiserum response to 0.5 ml of infected rabbit serum emulsified in complete Freund adjuvant and injected in the same manner as for X9. The infected rabbit serum was collected 6 days postinfection from a rabbit that was immunosuppressed (200 mg of cyclophosphamide and 30 mg of cortisone acetate, subcutaneously) 24 h before intravenous injection of 10^6 A. fumigatus conidia.*

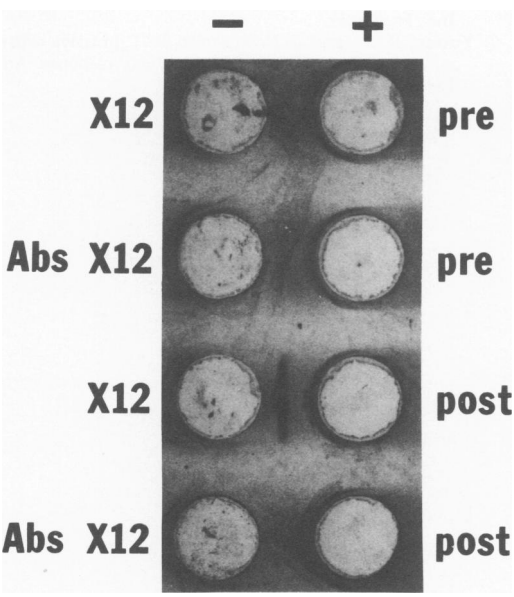

FIG. 2. *Antigen in a child's serum shown by counter-immunoelectrophoresis in agarose gel (Seakem agarose (Marine Colloids, Rockford, Me.), 1% [wt/vol], electroendosmosis $-m_r = 0.19$; barbital buffer (pH 8.6), ionic strength 0.075; gel thickness, 1.25 mm; 17 V/cm, 90 min). Abs X12, X12 (see legend to Fig. 1) absorbed with CA (3.2 ml of X12:1 mg of CA). X12 and Abs X12 were used at 80% initial concentration. Pre, Child's serum taken 4 months before death. Post, Child's serum taken post-mortem. Sections of liver, kidney, and pulmonary artery taken after death showed hyphae which reacted positively with sera specific for Aspergillus species.*

further batches of antiserum have been prepared by using precipitin lines as immunogen (5). We have found that it is necessary to use serum from an infected rabbit as a source of antigen since precipitins containing the antigen in CA do not induce an antibody response.

This appears to be the first reported detection of antigen from a filamentous fungus in sera and urine of animals and humans. We are developing a more sensitive assay system with the X12 antiserum to be evaluated for antemortem diagnosis of invasive aspergillosis. The method of raising the antiserum is applicable to other diseases where microbial antigens may be present. By immunizing animals with test materials such as urine, cerebrospinal fluid, and serum from an infected animal, it should be possible to induce an antibody response that is restricted to those antigens present in the fluids.

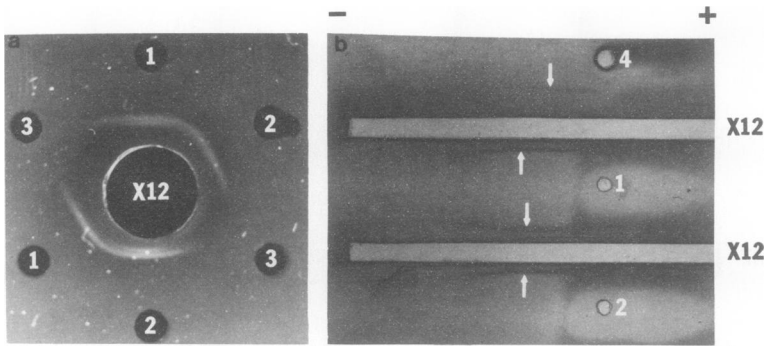

FIG. 3. *Precipitin arc formed by X12 with treated alkaline extract and serum from an infected immunosuppressed rabbit. (a) Immunodiffusion plate. (b) Immunoelectrophoresis in phosphate buffer (pH 7.4; ionic strength, 0.075). (1) CA (see legend to Fig. 1) at 0.5 mg/ml. (2) Protease-treated CA. A 3-mg portion of CA and a 2.5-mg portion of Pronase (Enzite-agarose-protease, Miles Laboratories, Elkhart, Ind.) were mixed (4 h, 37°C, in 1.5 ml of barbital buffer, pH 8). After centrifugation, 1 ml of supernatant was diluted to 4 ml in water before testing. (3) Periodate-treated CA. 1 mg of CA was dissolved in 0.5 ml of 0.05 M $NaIO_4$ (16 h, 4°C). 1.5-ml portion of water was added before testing. (4) Serum from an immunosuppressed and infected rabbit (see legend to Fig. 1).*

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