Galactomannan Antigenemia and Antigenuria in Aspergillosis: Studies in Patients and Experimentally Infected Rabbits

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Purified galactomannan (GM) from *Aspergillus fumigatus* was used in both a radioimmunoassay and an enzyme-linked immunoassay for antigen detection. Results of the two tests seemed interchangeable. By one or both assays, GM was detected in serum from four of 12 rabbits lethally infected with *A. fumigatus* in concentrations ranging from 108 to 356 ng/ml. Serum antigen was detected in only two of 12 patients with invasive aspergillosis. Results of assay for GM in urine were far more encouraging. Urinary GM was detectable throughout the course of lethal aspergillosis in all 16 rabbits, in concentrations of 24–1,900 ng/ml. Urine from seven of 13 patients with invasive aspergillosis had GM concentrations of 1–83 ng/ml. Antigen excretion roughly paralleled extent of disease.

Detection of fungal antigen in serum [1–3] and bronchoalveolar lavage fluid [4] of rabbits infected with *Aspergillus fumigatus* has been reported, as has detection of antigen in serum [5, 6] and in bronchoalveolar lavage fluid [7] of some patients with aspergillosis.

Excretion of microbial antigen into urine of the infected host has been observed during infection in humans with *Streptococcus pneumoniae* [8–10], *Haemophilus influenzae* [9–12], *Streptococcus agalactiae* [13–15], *Neisseria meningitidis* [9, 10], *Klebsiella pneumoniae* [16], *Cryptococcus neoformans* [17], *Escherichia coli* [18], and *Legionella pneumophila* [19]. Urinary antigen has not been reported in humans infected with *Aspergillus* spp.

The current study used RIA and ELISA techniques specific for a well-characterized neutral polysaccharide present in *A. fumigatus*. This galactomannan (GM) antigen was first described by one of us [20] and later described in greater detail [21]. Both serum and urine from rabbits infected with *A. fumigatus* as well as from patients with aspergillosis were tested for the presence of GM.

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Materials and Methods

Experimental infection. New Zealand white rabbits were injected iv with *Aspergillus* conidia. Animals weighed 2–3 kg except where stated otherwise. Three rabbits were injected iv with 10^6 viable conidia once a month for three doses and then infected with 1–2.1 × 10^7 conidia. These infected rabbits are referred to as previously immunized animals. Inocula were grown on Sabouraud's dextrose agar for 48–72 hr at 34 C, harvested in Tween-saline (0.15 *M* NaCl containing 0.025% Tween 20 [Fisher Scientific, Pittsburgh]), filtered through sterile gauze, and counted in a hemacytometer. Conidia maintained a viability of 80%–90% when stored in Tween-saline at 4 C for up to three months. Viability of each inoculum was assessed by plating of dilutions in Tween-saline on the surface of Sabouraud's agar. Rabbits were bled from the central ear artery. Urine was collected on a plastic sheet under the cage floor. A screen was interposed between the floor and sheet to prevent stool contamination. Urine accumulation was checked several times daily, but sick animals sometimes did not void for two days. Urine samples were centrifuged at 4,000 *g* for 10 min, and supernatants were stored at −40 C. Colony counts of *A. fumigatus* in organs of rabbits were assessed at intervals after infection. Rabbits were killed by CO_2 narcosis, organs were weighed, and representative portions were ground in PBS (150 m*M* NaCl and 7 m*M* phosphate, pH 7.4) with a Tenbroeck tissue grinder. Tenfold dilutions in PBS were plated in duplicate on Sabouraud's agar and incubated at 37 C.

Galactomannan. Preparation and structural analysis of the lot of GM used in this study have been reported previously [21]. *A. fumigatus* strain 5233 was grown in a shaken, chemically defined broth medium for four days at 37 C. The homogenate of washed mycelia was centrifuged at 10,000 *g* for 30 min, and the concentrated supernatant was chromatographed sequentially on columns of DEAE cellulose (DE52; Whatman, Clifton, NJ) and concanavalin A-Sepharose® (Pharmacia Fine Chemicals, Piscataway, NJ). The α-methylmannoside eluate from the latter was dialyzed against water and lyophilized. The material contained 0.7% nitrogen, 0.17% phosphate, and only galactose and mannose on monosaccharide analysis.

Clinical specimens. Invasive aspergillosis was diagnosed by culture and histological demonstration of hyphae in an area of inflammation. Specimens from and information about three patients (nos. 4, 21, and 22, respectively) were supplied by Drs. Scott Hammer (New England Deaconess Hospital, Boston), Daniel E. Keim (Fairfax, Va), and Robert Clark (University of Iowa, Iowa City). Except for those outside specimens, which were sent on ice, serum and clean-voided urine specimens were collected fresh and stored at −10 C.

Specimen processing. Serum was prepared for antigen detection by fourfold dilution in PBS, heating at 100 C for 10 min, and centrifugation at 12,800 *g* for 10 min. This procedure resulted in essentially complete recovery of GM added to normal human serum, as measured by RIA. Urine samples were tested unconcentrated and, for human urine specimens, after 10-fold concentration in a Minicon® B15 filtration device (Amicon Corp., Lexington, Mass). Because urine inhibited the antigen assays, both unconcentrated and concentrated urine specimens were dialyzed overnight against PBS. Elution through 9-ml disposable Sephadex® G25 columns (PD-10; Pharmacia Fine Chemicals, Uppsala, Sweden) also freed urinary antigen from interfering substances but was cumbersome and seemed to give more variable results than dialysis.

ELISA. Antiserum was raised in 2-kg New Zealand rabbits by iv injections of 10^6 live conidia of *A. fumigatus* about once a week for several months. For antigen detection, rabbit antiserum was diluted 1:100 in PBS that contained 0.05% Tween 20 (PBS-Tween) mixed with specimens or standards in a 1:10 ratio (usually 25 µl of antiserum and 250 µl of sample) and preincubated for 2 hr in a water

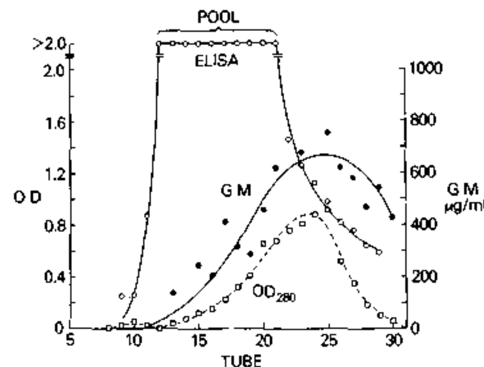

Figure 1. Preparation of coating antigen for ELISA. Unconjugated GM and BSA were partially separated from the GM-BSA conjugate by chromatography on Sephadex G200. The column effluent was monitored for BSA by measurement of OD_{280} and for GM by phenol-sulfuric acid. The GM-BSA conjugate was detected by adherence to plastic followed by an ELISA for GM. Fractions pooled for use as coating antigen are labeled "Pool".

bath at 37 C. Then 0.1-ml aliquots were placed in duplicate antigen-coated wells for 2 hr. The procedure for antigen coating of the ELISA plates is described below. After incubation at 37 C for 2 hr, wells containing the samples were washed three times in PBS-Tween. Rabbit antibody was detected by incubation for 1 hr at 37 C of peroxidase-conjugated goat antibody to rabbit IgG (Cappel Laboratories, Cochranville, Pa) diluted 1:1,000 in PBS-Tween, followed by substrate containing 0.1 *M o*-phenylenediamine (Aldrich Chemical, Milwaukee, Wis). After 1 hr, 20 µl of 8 *N* H_2SO_4 was added. Color was quantified in an ELISA plate reader (model MR 600; Dynatech Instruments, Torrance, Calif).

All antigens were bound to ELISA plates in 0.044 *M* carbonate buffer, pH 9.6, overnight at 4 C. Initial studies used MicroTiter® polyvinyl chloride plates (Dynatech Laboratories, Alexandria, Va). These round-bottomed wells bound GM but also bound conjugated antibody later in the assay, a problem only partially relieved by coating the wells with 3% bovine serum albumin (BSA; Pentex, Elkhart, Ind) after antigen coating. So that the optically superior Immulon® I plate (Dynatech) could be used and the binding of PBS-Tween–diluted conjugate would be decreased, GM was conjugated to BSA with use of CNBr. The same conditions were used as previously published for conjugation of GM with tyramine [21]. The conjugate, containing 22 mg of GM and 42 mg of BSA, was chromatographed on a column of Sephadex G200 (Pharmacia) with borate-buffered

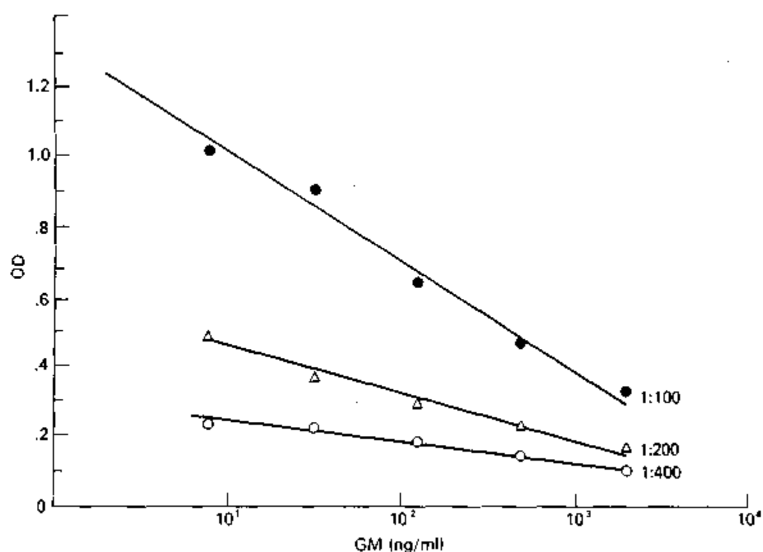

Figure 2. ELISA for GM with three different dilutions of rabbit antibody to GM.

saline (150 m*M* NaCl and 40 m*M* borate, pH 8.0; BBS) as eluant (figure 1). BSA was quantitated by measurement of OD_{280}, and GM was quantitated by the phenol-sulfuric acid technique [22]. The GM-BSA conjugate was detected by adherence of GM to Immulon I plates with use of rabbit antibody to GM and peroxidase-labeled antibody to rabbit IgG. The high-molecular-weight fraction with maximal adherence was chosen for subsequent work and used at a GM concentration of 21 μg/ml for coating plates. Typical standard curves constructed with plates coated by GM-BSA are shown in figure 2. Standard curves prepared in dialyzed urine were identical to those prepared in PBS.

RIA. Tyraminyl GM was prepared and iodinated with ^{125}I as described previously [21]. The specific activity of the ^{125}I-labeled GM was 3.1–5.3 × 10^7 cpm/μg. ^{125}I-labeled GM was used in a Farr-type RIA for antibody [23]. About 5 × 10^3 cpm in 50 μl of BBS was mixed with 100 μl of sample and either 150 μl of BBS for antibody assay or 50 μl of rabbit antiserum plus 100 μl of BBS for antigen assay. The mixture was incubated at 4 C overnight, and then 100 μl of fetal calf serum (Flow Laboratories, McLean, Va) solution, 600 μl of BBS, and 1.0 ml of saturated ammonium sulfate were added. The precipitate was centrifuged at 2,500 g for 30 min at room temperature (~25 C), washed in half-saturated ammonium sulfate, and counted for radioactivity in a gamma spectrometer (model 1185; Nuclear Chicago, Chicago). Each time the assay was done a BBS blank and a 1:40 dilution of a standard antiserum were tested. Percent bound was calculated as 100 × ([sample cpm − BBS cpm]/[added cpm − BBS cpm]). The BBS blank, which contained radioactivity bound or trapped in the fetal calf serum precipitate, never exceeded 10% of added cpm. The standard serum precipitated 60%–70% of the radioactivity at a dilution of 1:40 but 90% when undiluted. This control was included in each performance of antibody assays. All serum antibody results reported here, except the antibody control, refer to undiluted serum, with positivity being defined as at least 20% binding.

Definition of positivity in ELISA and RIA. Specimens of urine and serum from both uninfected rabbits and normal volunteers were tested for antigen in the same manner as other samples, including

Table 1. Results of antigen assays of normal specimens.

Specimen, assay	No. of subjects	Mean (%)*	SE	Mean − 1.96 SD
Human urine				
ELISA	36	105	3.01	70
RIA	35	100	1.38	84
Human serum				
ELISA	11	93	0.78	88
RIA	11	104	1.39	94
Rabbit urine				
ELISA	32	101	2.29	75
Rabbit serum				
ELISA	11	93	2.75	75
RIA	11	90	2.15	76

* As a percentage of buffer control values.

10-fold concentrations of human urine. OD values from the ELISA and cpm values from the RIA were converted to percentages of the buffer control value of that test performance. Means and SEs of these percentages are given in table 1, together with the mean minus 1.96 SD. This limit was calculated to be beyond 97.5% of the normal values, if a normal distribution is assumed. Specimens being tested for antigen were considered positive when the OD or cpm was a lesser percentage of the assay performance's buffer control value than the limit given in table 1. The RIA of normal rabbit urine was not included in table 1 because urine from infected animals was tested only by ELISA.

Sensitivity of assays. All standards and specimens were tested in duplicate. A standard curve was included in each assay performance. The sensitivity of each performance was determined by the intercept of that day's standard curve with the appropriate cutoff value given in table 1. This sensitivity limit ranged from 10 to 40 mg/ml in both the RIA and ELISA. Results from assays that were less sensitive were discarded. Because human urine specimens were ultrafiltered to 1/10th their original volume, antigen concentrations were considered to have risen 10-fold. For this reason the lower limit of the GM content reported for concentrated urine ranged from 1 to 4 ng/ml.

Isolates of Aspergillus. Isolate 5233 of *A. fumigatus* was used for GM preparation and animal injection except where noted otherwise. This and the other isolates of *Aspergillus* were identified and provided by Dr. K. J. Kwon-Chung (Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases).

Results

Lethality and serum antigen in experimental infection. The dose-response curve as measured by lethality was extraordinarily steep, with all rabbits surviving inocula of $\leqslant 3 \times 10^6$ conidia and 20 of 22 rabbits dying after inocula of at least 10^7 conidia (figure 3). Animals died within eight days or survived at least 30 days. Colony counts in organs of rabbits given conidia iv are shown in table 2. Histopathologic sections showed widely disseminated abscesses in rabbits given lethal inocula. Serum antigen levels in rabbits are shown in figure 4. Only four rabbits had antigen demonstrated. Antigenemia occurred between the second and sixth day, with the day of

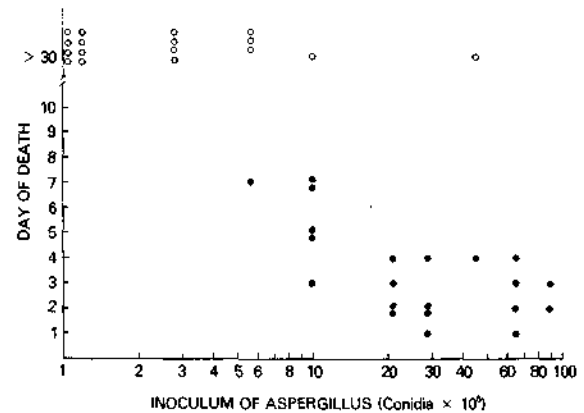

Figure 3. Day of death in rabbits injected iv with *A. fumigatus* conidia. *Open circles* denote surviving animals.

injection counted as zero. Antigen concentrations ranged between 108 and 356 ng/ml. These results suggested that antigenemia required at least 48 hr to occur and tended to occur in fatal infections shortly before death. There was a suggestion that antigenemia occurred earlier with higher inocula. All surviving rabbits became antibody positive. All but one seroconversion occurred by day 14. One antigenemic rabbit had his initial rise in antibody titer the day antigenemia was detected. Four rabbits had serum antibody before infection. One was given the lowest inoculum of 2×10^6 viable cells and simply remained antibody positive. The other three were

Table 2. *A. fumigatus* in organ homogenates after iv injection of conidia.

Experiment, tissue	Colony count/g of organ			
	24 hr	48 hr	72 hr	96 hr
Experiment 1				
Kidney	750	850	200	400
Spleen	17,000	4,000	550	2,500
Liver	14,000	9,000	1,550	8,500
Lung	13,000	450	170	35
Experiment 2				
Kidney	1,500	1,200	500	400
Spleen	10,100	15,500	3,500	600
Liver	8,500	4,100	3,600	2,500
Lung	5,100	300	700	600

NOTE. In experiment 1, 1.2×10^7 conidia were injected into 2-kg rabbits; in experiment 2, 1.9×10^7 conidia were injected into 4-kg rabbits. One rabbit was studied at each interval after inoculation.

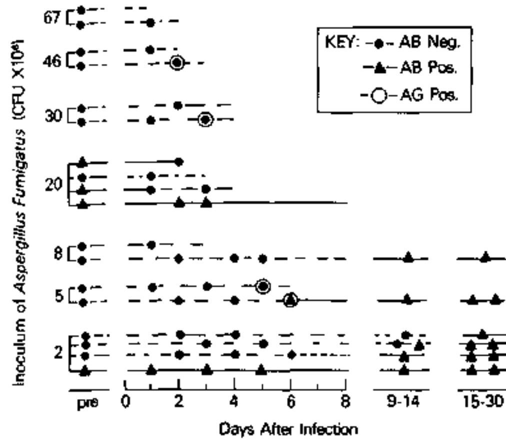

Figure 4. RIA for antigen in serum of rabbits injected iv with *A. fumigatus* on day 0: serum antigen (○), antibody positivity (▲), and antibody negativity (●). Each line represents results with one rabbit; the lines terminating before day 9 indicate the day of death.

given a high inoculum of 2 × 10^7 viable conidia. Two became antibody negative one and three days before death. The third was still antibody positive five days before death, but subsequent bleedings from the ear were unsuccessful. None of the four rabbits with preinfection antibody developed detectable antigenemia.

Four pairs of rabbits were inoculated iv with *Aspergillus flavus* isolate 5239 (8, 200, 200, and 400 × 10^6 conidia, respectively). Death occurred on days 2, 3, 4, and 10 in four animals receiving doses of 200–400 × 10^6 conidia. Neither these four nor the four surviving animals had antigen in serum obtained every 24–48 hr during the first eight days after infection.

Antigen in human serum. Antigen was detected in only two of 12 patients with invasive aspergillosis (table 3). The GM concentrations were near the limit of sensitivity of the assay, when the 1:4 dilution of serum is taken into consideration. The negative RIA with a positive ELISA on a serum, a finding that was repeatable, was attributed to slightly greater sensitivity of the ELISA at that time.

Urinary antigen in experimental infection. Aspergillus GM was found in urine of all 13 fatally infected, unimmunized rabbits but only in three of 16 surviving, unimmunized animals (table 4). There was virtually no delay in onset of antigenuria, in contrast to antigenemia. Whenever the first urine sample was obtained, antigen was in it. Antigen concentrations tended to rise as infection progressed but were not closely correlated with size of the original inoculum. Small amounts of naturally occurring antibody present in serum before infection did not seem to alter antigenuria. Of the three highly immunized rabbits that were infected, antigenuria occurred in two.

The possibility that urinary antigen reflected growth of *Aspergillus* in urine was examined by culture of 0.5 ml of uncentrifuged urine as well as the centrifuged sediment of 20 ml of urine. Ten urine samples from four rabbits given 55 × 10^7 conidia were tested. All contained antigen by ELISA. Cultures were placed on Czapek's agar with chloramphenicol (50 µg/ml) and incubated at 40 C for two weeks. One urine sample yielded one colony of *A. fumigatus:* no other urine sample contained this fungus. The possibility that growth of *Aspergillus* in the kidney accounted for all the urinary antigen appeared unlikely in view of the rising antigen con-

Table 3. Characteristics of *Aspergillus* antigenemia in patients.

Group, patient	Species or diagnosis	Days before death	Antigen concentration (ng/ml) ELISA	RIA
Group 1				
1	*A. fumigatus*	4	500	90
2	*A. fumigatus*	4	NT	0
3	*A. fumigatus*	6	NT	0
4	*A. fumigatus*	30	NT	0
Group 2				
5	Histology only	1	150	0
6	Histology only	4	0	0
7	Histology only	4	0	0
8	Histology only	4	NT	0
9	*A. terreus*	5	NT	0
10	*A. fumigatus*	12	NT	0
Group 3				
11	*A. fumigatus* (lung)	8*	0	0
12	*A. flavus* (palate)	7†	0	0
Group 4				
13	Invasive pseudallescheriasis		NT	0
14	Mucormycosis		NT	0
15–19	Cancer		0	0

NOTE. Group 1 comprised patients with fatal disseminated infection; group 2 comprised those with fatal pulmonary infection; group 3 comprised those with localized, nonlethal infection; and group 4 was the control group. 0 = not detectable; NT = not tested.

* Before lobectomy.

† After onset.

Table 4. Antigenuria in unimmunized and immunized rabbits infected with *A. fumigatus*.

Immunization, inoculum (conidia × 10^6)	Rabbit no.	Serum antibody preinfection (% bound)	Urinary antigen (ng/ml) at day after inoculation							
			1	2	3	4	5	6	7	8
Unimmunized										
65	1	8	300 (D)							
	2	5	100	370 (D)						
	3	10	*	*	440	* (D)				
	4	2	39	660	* (D)					
21.6	5	26	220	1,900 (D)						
	6	31	80	*	* (D)					
	7	28	60	290	*	* (D)				
	8	18	120	500 (D)						
10	9	31	42	125	*	700	* (D)			
	10	14	31	125	*	*	100	*	37 (D)	
	11	27	*	50	*	*	*	*	190 (D)	
	12	19	28	95	*	1,900	* (D)			
5.8	13	24	*	640	640	*	NT	NT (D)		
	14	17	*	*	*	270	NT	NT	0	0
	15	NT	*	43	24	*	NT	NT	*	0
	16	21	33	38	35	*	NT	NT	NT	0
2.8	17	19	0	0	0	NT	NT	0	NT	NT
	18	3	0	0	0	NT	NT	0	NT	NT
	19	3	0	0	*	NT	NT	0	NT	NT
	20	4	*	0	0	NT	NT	*	0	NT
1.2	21	15	*	*	0	NT	NT	NT	NT	NT
	22	12	0	0	0	NT	NT	NT	NT	NT
	23	13	*	0	0	NT	NT	NT	NT	NT
	24	10	0	*	0	NT	NT	NT	NT	NT
1	25	17	0	*	0	0	NT	NT	NT	NT
	26	12	0	0	0	NT	NT	NT	NT	NT
	27	42	*	0	0	NT	NT	NT	NT	NT
	28	54	*	0	0	0	NT	NT	NT	NT
Immunized										
10	29	76	0	0	0	0	NT	NT	NT	NT
10	30	81	*	76	31	0	NT	NT	NT	NT
21	31	74	58	250	155	0	NT	NT	NT (D)	

NOTE. NT = not tested. (D) = day of death.
* No urine voided.

centration in urine with time and of the stable colony count in the kidney (table 2).

Urinary antigen was also sought in four rabbits infected with *A. flavus* isolate 5239. Two receiving 3.90 × 10^8 conidia died on days 2 and 3. Both animals receiving 2 × 10^8 conidia survived the 30-day observation period. Urine samples were obtained daily for five days, except from the two animals that did not void for the first 24 hr after infection. The specimens were concentrated 10-fold on the Minicon B15 apparatus and dialyzed against PBS for 24 hr. All 13 urine samples contained GM by RIA, and 11 of 13 contained GM by ELISA. Concentrations of urinary GM, when the 10-fold ultrafiltration on the Minicon B15 device is taken into account, rose to a mean peak of 295 ng/ml by RIA and 422 ng/ml by ELISA. In the surviving pair of animals, GM concentrations in urine began to fall 72 hr after infection.

Molecular weight of GM in rabbit urine. Interest in the efficacy of concentrating urinary GM led to a study of its molecular weight. Dialyzed and undialyzed urine from one infected rabbit, both 1.5-ml volumes, was eluted through as 15- × 300-mm Sephadex G200 column with BBS. Fractions of 1.5 ml were collected and analyzed by ELISA for GM. Urinary antigen had a disperse molecular weight that, when compared with linear dextran standards (Pharmacia), centered around 18,000 (figure 5). Four urine samples from different rabbits with antigenuria were concentrated 10-fold in a Minicon B15 appara-

tus. ELISA of dialyzed fresh and dialyzed concentrated urine found an average increase in antigen concentration of 7.3-fold (range, 4.8- to 9.5-fold). Tenfold concentration of eight negative urine samples from rabbits infected with *A. fumigatus* did not yield detectable antigen.

Antigen in human urine. Fifty-one urine specimens were collected from 13 patients with biopsy- or autopsy-proven invasive aspergillosis. GM was detected in urine from seven of these patients (table 5). Five of these patients were also included in table 3. Patient 11 had urinary antigen each of the nine days immediately preceding surgical excision of the lung lesion. Serum antigen was negative on the second day of study. In the first 24 hr after excision, urinary antigen assays gave only borderline values. Daily urine specimens for the next 11 days were clearly negative. In parallel with this loss of antigenuria, the patient promptly improved postoperatively. This case may be relevant to the situation with patient 24, who had no antigenuria when she was first studied two days after complete excision of her lung lesion. She received no antifungal therapy but recovered with surgical excision alone. The negative result in patient 2 could be a result of the prolonged iv administration of amphotericin B that preceded urinary assay, although hyphae were clearly visible in the prostate at autopsy, and a culture of urine had contained *A. fumigatus* seven days before urinary antigen testing. Amphotericin B administration had been stopped temporarily but was reinstituted and given in the week before study.

Patient 26 was notable in that he had chronic granulomatous disease of childhood, a condition in which vascular invasion by hyphae does not occur and in which hyphae tend to be more sparse within lesions. Patient 12 had only a small palatal lesion that responded to amphotericin B as his leukemia improved. Patient 27 had hyper-IgE syndrome with recurrent episodes of bacterial pneumonia, prior right upper lobe resection, and a fungus ball of at least two years' duration in the superior segment of the right lower lobe. Hemoptysis and increased sputum production of one month's duration led to resection of the cavity and fungus ball. Despite absence of fever or leukocytosis, the resected lung contained not only a *A. terreus* fungus ball but also rare clumps of hyphae in alveoli surrounded by chronic inflammation. This patient and patients 11, 12, 24, and 26 were apparently cured of their aspergillosis. Of these five individuals, only patient 11 had urinary antigen,

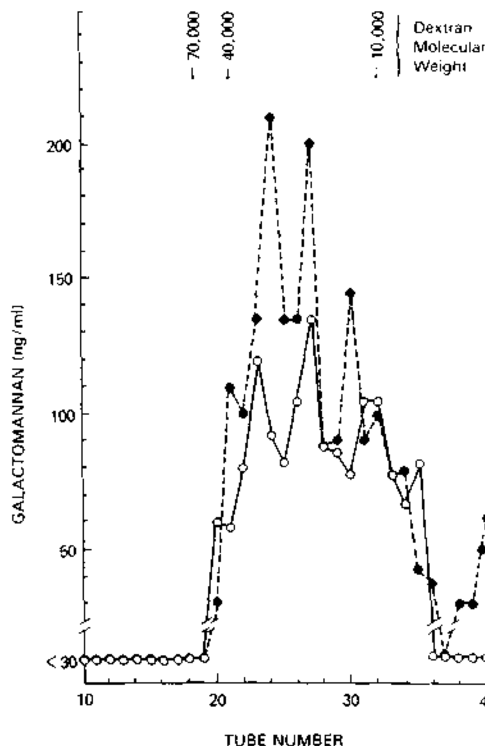

Figure 5. Chromatography of dialyzed (○) and undialyzed (●) urine from an infected rabbit on Sephadex G200 shows the GM concentration measured by ELISA. Elution peaks of linear dextran standards are indicated at the top.

whereas six of eight patients who died had antigenuria. The patient without antigenuria who died (patient 25) was studied three days after the pulmonary lesion was excised. Autopsy showed aspergillosis in the brain and diaphragm.

Eight patients were suspected of having invasive aspergillosis, but an alternative diagnosis was proven for all but patient 28, who remained undiagnosed despite lung biopsy. None of the eight had urinary GM (table 6). As stated in Materials and Methods, antigen-positive urine was defined by confidence limits around values from normal urine specimens. Because these RIA and ELISA values for normal urine had no outlying numbers, all normal urine specimens fell within the range called antigen negative.

Discussion

Published experience with immunoassay for *Aspergillus* antigen in serum of infected rabbits [1] and

Table 5. GM in urine of patients with proven invasive aspergillosis.

Patient	Age (years)	Sex	Underlying disease	Aspergillosis: Species	Aspergillosis: Site	Autopsy and/or surgery	Time of study	No. of specimens positive/ no. tested	Concentration range (ng/ml): ELISA	Concentration range (ng/ml): RIA
3	18	M	ALL	*A. fumigatus*	Lung, adrenal gland, stomach, bowel	Autopsy	Last 8 days of life	4/4	1-13	NT
4	62	M	Hemolytic anemia, corticosteroids	*A. fumigatus*	Heart valves, brain, bone, skin	Surgery, autopsy	1 month before death and after 3.5 g of iv amphotericin B	1/1	10	1
11	32	M	Hodgkin's disease, pneumocystis, CMV	*A. fumigatus*	Lung	Surgery	9 days before to 12 days after lobectomy	9/21	15–68	2–21
20	27	F	Breast cancer, corticosteroids	*A. fumigatus*	Lung, brain?	Surgery	Last 6 days of life	6/6	8–49	1–5
21	35	F	Behcet's disease, corticosteroids	*A. fumigatus*	Lung, heart, brain, kidney	Autopsy	Last 2 days of life	2/2	11–15	6–8
22	25	M	Aplastic anemia, bone marrow transplant, CMV	*A. fumigatus*, *Aspergillus glaucus*	Lung, skin, rib, brain	Surgery, autopsy	After 1 month of iv amphotericin B, 4 days before death	1/1	6	2
23	13	F	ALL, corticosteroids	*A. fumigatus*	Lung, thyroid, heart, kidney, spleen	Autopsy	After 17 days of illness, 3 days before death	1/1	83	11
2	70	F	Hemolytic anemia, corticosteroids, nocardiosis	*A. fumigatus*	Prostate, lung, brain, pericardium	Autopsy	2 and 7 days before death	0/2	ND	ND
12	47	M	AML	*A. flavus*	Palate	Surgery	Days 6-8 of illness	0/3	ND	ND
24	34	F	SLE, azathioprine, corticosteroids	*A. fumigatus*	Lung	Surgery	2–6 days after total excision	0/5	ND	ND
25	11	F	Aplastic anemia, corticosteroids	*A. flavus*	Lung, brain, diaphragm	Surgery, autopsy	3 days after lobectomy, 11 and 12 days before death	0/2	ND	ND
26	12	M	CGD	*A. fumigatus*	Skin, brain, lung	Skin aspirate	2 weeks after onset	0/2	ND	ND
27	25	F	Hyper-IgE syndrome	*Aspergillus terreus*	Lung	Surgery	3 weeks before excision	0/1	ND	ND

NOTE. ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia; CGD = chronic granulomatous disease; CMV = cytomegalovirus; ND = not detectable; NT = not tested; SLE = systemic lupus erythematosus.

Table 6. GM in urine of patients with suspected aspergillosis.

Patient	Age (years)	Sex	Underlying disease	History	Urinary antigen*	
					ELISA	RIA
28	24	M	CGD	*A. fumigatus* in sputum smear and culture and increased chest radiographic abnormalities	NT	0/1
29	15	M	CGD	*Acremonium strictum* pneumonia	0/6	0/5
30	7	F	ALL	*Pseudomonas aeruginosa* sepsis with nodular pulmonary infiltrates, no aspergillosis on autopsy	0/2	0/2
31	17	M	ALL	*Staphylococcus aureus* sepsis with bibasal pulmonary infiltrates	0/2	0/2
32	67	M	CML with blast crisis	Pneumocystis pneumonia, autopsy proven	0/2	0/2
33	19	F	CGD	Pneumonia, responding to antibacterial antibiotics	0/1	0/1
34	5	M	CGD	Diffuse lymphadenopathy, improving with antibacterial antibiotics	0/1	0/1
35	15	F	Aplastic anemia	*Candida tropicalis* infection of maxillary and ethmoid sinuses	0/1	0/1

NOTE. ALL = acute lymphocytic leukemia; CGD = chronic granulomatous disease; CML = chronic myelogenous leukemia; NT = not tested.

* No. positive/no. tested.

humans [5] has largely come from one laboratory. In a recent report from that laboratory, their RIA found antigen in serum from six of eight patients with documented invasive aspergillosis but in none of 45 controls [24]. A subsequent abstract stated that 14 (74%) of 19 individuals with invasive aspergillosis were positive by RIA compared with two of 31 controls [25]. Their results with rabbits were also extremely encouraging; they detected antigen in serum from 86% of lethally infected animals [1]. Two laboratories published preliminary confirmatory reports in 1979, with antigen being detected in serum of both the infected rabbits and patients with invasive aspergillosis [6, 26]. Clinical details were sparse [6] or absent [26] about the three and six patients, respectively, who were tested, but all patients and rabbits were antigenemic. In 1985 Sabetta et al. [27] used RIA to detect an undefined carbohydrate antigen in serum from 11 of 19 subjects with invasive aspergillosis, with all cases being proven by autopsy or biopsy. Our results with detection of serum antigen were less promising. We detected GM in serum from only four of 12 lethally infected rabbits and two of 12 patients. The reason for the discrepancy remains unclear. Only one of the previous reports used a method likely to be specific for GM, and that report included no data on sensitivity of their CIE for GM [26].

The test for serum antigen reported here used fourfold dilutions and heat to dissociate antigen-antibody complexes. The method was simple but decreased sensitivity by dilution of the specimen. Although dissociation techniques are not used for antigen detection in CSF or urine, they are often essential for serum samples [28] and were necessary to detect antigen in our serum specimens. Although the aforementioned CIE for *Aspergillus* did not use dissociation [3], all three reports of RIA included this step. Shaffer et al. [6] precipitated protein with trichloracetic acid and then dialyzed and lyophilized the supernatant, a process resulting in a 10-fold concentration of the serum. This cumbersome method was also used by Sabetta et al. [27]. Weiner et al. [5] used citric acid, heat, pepsin, and a 10-fold dilution to dissociate antigen. We did not pursue the problem of insensitivity of our serum assay or test additional control serum specimens because urine assay seemed satisfactory.

There is only one prior report to our knowledge that mentions detection of *Aspergillus* antigen in urine [3]. CIE was used to demonstrate antigen in urine of eight lethally infected rabbits between 60 and 120 hr after infection. Our experience in the experimentally infected rabbit confirms that GM appears in urine promptly after infection and increases in concentration until death. Nonlethal infections caused antigenuria transiently and only at maximal inocula. Survivors uniformly developed antibody to GM, an observation indicating that the antigen was present in tissue of the infected animal but was not

detectable in our urine assay. The molecular weight of GM in rabbit urine, which was ~18,000 by comparison with linear dextrans, was consistent with clearance by glomerular filtration. Renal excretion of dextrans was studied by Wallenius [29] in a variety of animals. Dextrans with molecular weights up to ~45,000 that had been injected iv were excreted into the urine of rabbits. In dogs, renal clearance was 20% of glomerular filtration rate when the dextran had a molecular weight of 22,000, a figure approximating the value for our GM. Our estimate of the GM molecular weight suggested the usefulness of concentration of the urine by use of a simple ultrafiltration unit. Concentration was not necessary for rabbit urine but improved sensitivity substantially for human urine specimens. Further concentration to 50-fold, rather than 10-fold, took more time and increased the coefficient of variation with normal human urine (data not shown).

ELISA and RIA gave comparable results in our hands, but each had particular advantages. ELISA required no isotopes and had a long reagent shelf-life. Variability in testing human urine was clearly greater with the ELISA than with the RIA. For example, the coefficient of variation with 10-fold concentrated human urine was 17% with ELISA and 8% with RIA. Variation was also manifested by occasional ELISA standard curves with sensitivity inadequate to detect 40 ng/ml. Results of such tests were discarded.

Detection of antigen in urine was potentially useful because most of the patients for whom this test was negative had had recent resection of their lung lesions (patients 24 and 25), focal disease in a small lesion (patient 12), or prior prolonged therapy with amphotericin B (patient 2). The most obvious failure occurred for a patient with chronic granulomatous disease and several abscesses in skin and brain (patient 26). Negative results for the patient with *A. terreus* could be explained by the unusual species or the rather rare areas in which the fungus invaded tissue (patient 27). Her major diagnosis was fungus ball of the lung, a saprophytic state that one might anticipate to cause chronic, low-grade antigen release.

Our study did not enroll enough patients infected with *A. flavus* for assessment of sensitivity of our assay during human infection with this species. We did detect antigen in concentrated urine of rabbits infected with *A. flavus* but not in serum. We did not attempt to purify GM from that species. Whether other species cross-react in our test is unknown. Further studies conducted prospectively will help in the definition of the clinical usefulness of this diagnostic procedure.

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