40-month period, from October 1990 to January 1994, 1,849 strains of 12 species or subspecies of *Campylobacter*, Helicobacter, and Arcobacter were isolated from 7,680 diarrheal stool samples. Two hundred and fifty-five (13.8%) of these isolates were dependent on H2-enhanced microaerophilic growth conditions, an essential requirement of *C. concisus*, *C. mucosalis*, *C. curvis*, and *C. rectus* (7).

All 255 clinical isolates plus isolate NCTC 12408 were positive for oxidase and nitrate reductase but were negative for catalase, indolyl acetate, hippurate, and urease. Every isolate produced abundant H2S, detectable in triple sugar iron agar and often completely blackening lead acetate strips. Isolates tolerate 1% glycine but not 3.5% NaCl. These phenotypic characteristics are shared by *C. concisus* and *C. mucosalis* (7).

*C. mucosalis* can be differentiated from *C. concisus* by its ability to grow at 25°C, its cephalothin sensitivity, and its dirty yellow colony color (6). The issue of growth of *C. mucosalis* at 25°C is controversial (6, 7). Almost all the clinical isolates, plus isolate NCTC 12408, did not grow at 25°C.

Fifteen clinical isolates that were cephalothin sensitive (inhibitory zone sizes of up to 40 mm) and that had a dirty yellow colony color were selected. In other words, as defined by phenotypic criteria, these isolates were *C. mucosalis*. All 15 clinical isolates plus isolate NCTC 12408 were positive for both arylsulfatase and pyrazinamidase, which is characteristic of *C. concisus* and not *C. mucosalis* (1). High-stringency DNA-DNA hybridization studies were performed with these probes: *C. mucosalis* NCTC 11000, *C. concisus* NCTC 11485, *C. curvis* NCTC 11649, and *C. rectus* NCTC 11489. Isolate NCTC 12408 and all 15 clinical isolates reacted strongly with the *C. concisus* probe and did not react with any other probe. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2) confirmed that the 16 isolates were *C. concisus*.

The phenotypic description of *C. mucosalis* isolates was based on only a few isolates (6). Isolate NCTC 12408 was characterized at NCTC by phenotypic, not molecular, methods as “*C. mucosalis*-like” (5a). As more and more clinical laboratories adopt filtration and H2-enhanced microaerophilic growth conditions, the problems of differentiating *C. mucosalis* and *C. concisus* and related species are becoming apparent. Molecular methods must be used for the precise identification of these underdetected pathogens.

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Pitfalls in Immunoblot Detection of *Aspergillus* Antigens Associated with Invasive Infection

Haynes et al. published an article about the immunoblot detection of *Aspergillus* antigens associated with invasive infection in humans (2). Since invasive aspergillosis is a life-threatening disease to immunocompromised patients, fast and
accurate diagnosis is of vital importance. Antigen detection is very important in this respect. Over the years several methods for the detection of *Aspergillus* antigens have been published (1, 3, 4).

Haynes' method uses concentrated urine specimens which are electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and blotted to polyvinylidifluoride paper. The blotted antigens are detected by a detector serum, rabbit serum directed against cell wall antigens from *Aspergillus fumigatus*. Haynes also published the molecular weights of antigens which he claims to be specific for aspergillosis. Considering that this method seemed very promising for the rapid and accurate detection of *Aspergillus* antigens and that it is able to detect defined antigens, we introduced it in our laboratory.

Experiments with urine specimens from patients with proven invasive aspergillosis obtained from Haynes showed that our detector serum was able to detect the same antigenic bands on the blots as did Haynes' detector serum. Our detector serum was immune serum directed against cell wall antigens from *A. fumigatus* from a conventionally raised New Zealand White rabbit (Haynes did not specify the type of rabbit to be used for immunization). Because we wanted to have negative controls in the test, we investigated 10 urine specimens from healthy individuals and 10 urine specimens from patients without aspergillosis but with urinary tract infections.

In the urine specimens from patients with urinary tract infections, we found strong reactions on the blots in places specific for *Aspergillus* antigens. From two patients, the causative organism could be isolated; both isolates were *Escherichia coli*. We preincubated the detector serum from the immunoblot with *E. coli* that had been treated for 1 h at 100°C. This treatment was intended to remove specific lipopolysaccharide epitopes on the *E. coli* surface and to improve the exposure of common gram-negative surface antigens (5). When this "absorbed" detector serum was used in the immunoblot test, the previously found reactions in the patients' urine specimens disappeared almost completely.

When investigating urine specimens from patients with urinary tract infections by using unabsorbed sera from conventionally raised rabbits, one should be aware of this pitfall. *E. coli* is not the only organism able to cause urinary tract infections, we investigated three *Aspergillus* detector serum specimens for the presence of antibodies against several other organisms. In all three we found, along with antibodies against *E. coli*, antibodies against (non-protein A-producing) *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Proteus mirabilis* in varying amounts.

Two of the *Aspergillus* detector serum specimens were absorbed with *E. coli*, *S. saprophyticus*, and *K. pneumoniae* organisms. For this purpose both merthiolate-killed and heat-treated (1 h, 100°C) organisms were used. The reactions of the "absorbed" detector sera with water-soluble antigen from *A. fumigatus* NCPF 2109 on the immunoblot were not affected. On the contrary, the intensity and the number of bands found on blots when "absorbed" detector sera were used in combination with urine specimens from patients with urinary tract infections were lower than when "unabsorbed" detector sera were used.

The use of *S. saprophyticus* as an absorbing organism had no effect in the reaction between the urine antigens and the treated sera.

After the problems we experienced, we recommend that one check the *Aspergillus* detector sera before using them in the immunoblot test for the presence of antibodies against common organisms causing urinary tract infections and, if necessary, one incubate the sera before using them with the organisms against which antibodies have been found.

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Utility of Quantitative Enzyme Immunoassay Reactivity for Predicting Human Immunodeficiency Virus Seropositivity in Low- and High-Prevalence Populations

Hou et al. (2) reported the practical value of quantitative enzyme immunoassay (EIA) reactivity in predicting human immunodeficiency virus type 1 (HIV-1) seropositivity. For HIV-1 EIA, the relationship between antibody titer and absorbance is not linear, and therefore this test was never intended to be quantitative (1). Because of the serious consequences of a positive diagnosis of HIV-1 infection, a report of a reactive HIV-1 EIA should never be made without supplementary, confirmatory testing.

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Detection of *Aspergillus* Antigens Associated with Invasive Infection

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Serial urine samples were collected from 33 neutropenic patients, 10 of whom developed invasive aspergillosis (IA) while undergoing bone marrow transplantation or remission induction therapy for leukemia. Concentrated urine samples from the infected patients were subjected to polyacrylamide gel electrophoresis, blotted, and then incubated with antiserum raised to a cell wall extract of *Aspergillus fumigatus* (anti-CW) or an immunoglobulin G monoclonal antibody to *A. fumigatus* galactomannan (EBA1). When IA patient urine blots were probed with anti-CW, major bands at 11 and 18 kodaltons (kDa); intermediate bands at 13, 14, and 29 kDa; and minor bands at 38 and 44 kDa were seen. In contrast, EBA1 showed diffuse staining at molecular masses larger than 45 kDa and a single weak band at 21 kDa. Urine samples from the 23 patients with no evidence of IA were unreactive with both anti-CW and EBA1. These antigen bands are likely to represent immunodominant antigens which are excreted during IA and should play a valuable role in the development of rapid diagnostic tests for aspergillosis.

The current high mortality that is associated with invasive aspergillosis (IA) in severely immunocompromised patients (22) is due, in large part, to difficulties in establishing a clinical and/or microbiological diagnosis in the course of infection (17, 33, 34). A rapid, sensitive, and specific diagnostic test should justify the early administration of antifungal drugs, thereby improving the poor response rates that have been reported (34). Furthermore, the need for early empirical therapy of IA has become increasingly apparent since centers which practice this approach have reported lower mortality rates (2, 5).

Techniques to detect specific anti-*Aspergillus* antibodies have demonstrated poor sensitivity (32), so that increasing attention has focused on antigen detection tests. This approach has been used successfully in other opportunistic mycoses, notably cryptococcosis (18). Previously, *Aspergillus* antigen has been demonstrated in serum, urine, and bronchoalveolar lavage fluid samples from both infected humans (9, 10, 23, 25, 26, 28, 31; K. A. Haynes and T. R. Rogers, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1146, 1989) and experimental animals (1, 8, 14, 15, 19, 21, 24, 27, 29). However, with the exception of galactomannan (GM), the antigens used in these assays, and as a consequence the antigens that are circulating or excreted during IA, have not been identified or characterized.

We examined serial urine samples from patients who were documented as having IA with the aim of identifying immunodominant *Aspergillus* antigens that could be used in a rapid diagnostic assay.

**MATERIALS AND METHODS**

**Patients and specimen collection.** We collected serial urine samples from 10 patients with histologically and/or microbiologically proven IA. In eight patients (U1 to U6, U8, and U9), the diagnosis of IA was confirmed by histological demonstration of septate branched hyphae in, and culture of *Aspergillus* spp. from, deep tissue specimens obtained postmortem. In the remaining two (U7 and U10), diagnosis was based on the following criteria: (i) fever (>38°C) for >5 days unresponsive to systemic antibacterial antibiotics, and (ii) repeated isolation of the same *Aspergillus* sp. from sputum and/or bronchoalveolar lavage fluids in the presence of pulmonary infiltrates.

The causative organism of IA in seven cases (U2 to U4 and U6 to U9) was *Aspergillus fumigatus*, and in one case each it was *A. flavus* (U1) and mixed A. *fumigatus-A. flavus* (U10); whereas in the last patient (U5) a fungus from the *A. glaucus* group was isolated.

Eight patients had undergone bone marrow transplantations for leukemia (five patients), congenital immunodeficiency (one), or inborn errors of metabolism (two); the remaining two patients received remission induction therapy for leukemia.

The mean age of the patients was 18.3 years (range, 1.6 to 63), and they were all neutropenic at the onset of IA.

In addition, urine samples from 23 neutropenic patients who showed no evidence of fungal infection were collected and pooled. Of these patients, 12 had hematological malignancies and 11 had inborn errors of metabolism. Seventeen underwent bone marrow transplantation, and the remaining six received remission induction therapy for their leukemia. At the time of collection, four patients had positive blood cultures containing coagulase-negative staphylococci.

Urine samples were filtered (0.5-μm-pore-size filter; Flow Laboratories, Rickmansworth, United Kingdom), dialyzed overnight at 4°C against distilled water, and concentrated 10-fold under a vacuum in a Rotavapor (Technik AG, Flawil, Switzerland). Concentrated urine samples were centrifuged at 13,500 × g for 20 min, and the supernatants were stored at −70°C until analyzed.

**Antigen preparations.** A water-soluble (WS) antigen fraction of *A. fumigatus* was prepared as described by Wilson and Hearn (30). Briefly, 10° conidia of *A. fumigatus* NCPC 2109 (Mycological Reference Laboratory, National Collection of Pathogenic Fungi, Colindale, London, United Kingdom) were inoculated into 300 ml of a 2% (wt/vol) glucose–1% (wt/vol) peptone liquid medium in a 2-liter Erlenmeyer flask and cultured for 3 days with constant aeration at 37°C. This starter culture was transferred to 3 liters of the same medium in a 5-liter Erlenmeyer flask and incubated for 4

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more days under identical conditions. The mycelial mat was separated from the culture filtrate (CF) by filtration through Whatman no. 1 filter paper (Whatman, Maidstone, United Kingdom) and washed with at least 3 liters of sterile distilled water.

The washed mycelial mat was homogenized in a kitchen blender in 0.4% (wt/vol) NH$_4$HCO$_3$ (pH 7.3). The resultant slurry was mixed 2:1 with 0.1-mm (diameter) glass beads in a Vi-4 cell disintegrator (Edmund Buhler AG, Tubingen, Federal Republic of Germany) and disrupted until more than 80% cell breakage had occurred. Mycelial breakage was checked by light microscopy.

The mycelial extract was separated from the glass beads on a sintered glass funnel and centrifuged at 12,000 rpm. This supernatant was filtered (0.1-μm-pore-size filter; Flow), dialyzed extensively against distilled water, and then lyophilized. This extract was designated the WS extract.

In addition, a CF antigen from a 2-day fermenter culture of *A. fumigatus* was prepared against a cell wall extract of *A. fumigatus* (NCPF 2109) (11) was a kind gift from V. M. Hearn (Myco-Charco, United Kingdom) and washed with at least 3 liters of sterile distilled water and then lyophilized. This extract was designated CF.

**Antisera and monoclonal antibody.** Rabbit antiserum (anti-CW) prepared against a cell wall extract of A. *fumigatus* NCPF 2109 (11) was a kind gift from V. M. Hearn (Myological Reference Laboratory).

Rat immunoglobulin G (IgG) monoclonal antibody (EB1A) to *A. fumigatus* GM was a kind gift from D. Stynen, Eco-Bio nV, Genk, Belgium. This antibody was specific for the GM component of all species of *Aspergillus* tested, including *A. fumigatus, A. flavus,* and *A. glaucus* (7).

**Affinity chromatography.** CNBr-Sepharose 4B (Pharmacia, Milton Keynes, United Kingdom) was swollen in 1 mM HCl, pH 4°C. The swollen gel was washed extensively with 1 mM HCl. A total of 50 mg of WS or CF antigen was dissolved in coupling buffer—0.1 M NaHCO$_3$ (pH 8.3)—0.5 M NaCl and incubated with the swollen gel overnight at 4°C. The mixture was turned end over end throughout. Nonbound ligand was removed by washing in coupling buffer, and free binding sites were blocked with 0.1 M Tris hydrochloride (pH 8.0) containing 0.1% (vol/vol) Tween 20 for 16 h at 4°C. The gel was then washed with 3 cycles of 0.1 M CH$_3$COONa (pH 4.0)–0.5 M NaCl followed by 0.1 M Tris hydrochloride (pH 8.0)–0.5 M NaCl.

Anti-CW was diluted 1:10 in phosphate-buffered saline (PBS) and incubated with the ligated gel overnight at 4°C. After completion of binding, the gel was placed into disposable column (Bio-Rad Laboratories, Watford, United Kingdom) and the nonbound fraction (nonbound anti-CW) was eluted with PBS. This was dialyzed extensively against distilled water and then concentrated back to its original volume.

**Electrophoresis and immunoblotting.** Concentrated urine samples, WS antigen, and molecular mass standards (phosphorylase b, 97.4 kilodaltons [kDa]; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa) were boiled separately in sample buffer containing 20% (vol/vol) 2-mercaptoethanol and electrophoresed in 15% polyacrylamide gels according to the discontinuous buffer system of Laemml (13). Electrophoresis was carried out in a minigel apparatus (Bio-Rad) at 175 V until the dye front reached the bottom of the gel (approximately 45 min). The gel contents were transferred onto nylon sheets (Immobilon PVDF; Millipore, Harrow, United Kingdom) by using a Trans-blot cell (Bio-Rad) run at 30 V overnight with 25 mM Tris–192 mM glycine (pH 8.3) containing 20% (vol/vol) methanol as the transfer buffer. The transfer efficiency was checked by staining with 0.3% (wt/vol) Ponceau-S in trichloroacetic acid. The blots were cut into strips and destained with PBS. Free binding sites were blocked by incubation for 60 min in 5% (wt/vol) nonfat milk (Sainsbury’s, London, United Kingdom) in PBS containing 0.05% (vol/vol) Tween 20 (NFMPBST). The strips were then incubated with one of four different primary antibodies: (i) preimmunization rabbit serum diluted 1:750 in 0.1% (wt/vol) NFMPBST, (ii) anti-CW diluted as for preimmunization serum, (iii) nonbound anti-CW diluted as for preimmunization serum, or (iv) EB1A diluted 1:250 in 0.1% (wt/vol) NFMPBST for 4 h at 25°C. The strips were washed and then incubated for 60 min at 25°C with an appropriate secondary antibody, which was either anti-rabbit IgG (Dako Ltd., High Wycombe, Buckinghamshire, United Kingdom) or anti-rat IgG (Sigma Chemical Co., Poole, United Kingdom). Both antibodies were conjugated to horseradish peroxidase and diluted 1:1,000 in 0.1% (wt/vol) NFMPBST. After two washings each with PBS-Tween 20, PBS, and 50 mM phosphate buffer (pH 7.4), bands were visualized by using 0.4% (wt/vol) dianinobenzidine in 50 mM phosphate buffer (pH 7.4) containing 0.01% (vol/vol) H$_2$O$_2$.

In some experiments, 125I-protein A and 125I-anti-rabbit IgG were used instead of horseradish peroxidase-conjugated antibodies and bands were revealed by exposure on X-ray film.

**RESULTS**

When the urine samples from IA patients (U1 to U10) were probed with anti-CW, seven antigens were detected with apparent molecular masses of 44, 38, 29, 18, 14, 13, and 11 kDa (Fig. 1, lanes 1b to 10b). The molecular masses of the different antigens detected were calculated by reference to the graph of log molecular mass versus relative mobility of each standard. Each urine sample showed a different antigen band pattern. However, two bands at 18 and 11 kDa were major antigens, since they were found in 9 and 10 of the IA patients, respectively. Three bands (29, 14, and 13 kDa) were intermediate antigens, occurring in up to six urine samples; and the remaining two bands at 44 and 38 kDa were minor antigens, found in only two and three urine samples, respectively. Diffuse bands with molecular masses greater than 60 kDa were also present in some urine samples (Fig. 1, lanes 5b to 10b). Similar antigen patterns were seen when 125I-protein A or 125I-anti-rabbit IgG was used instead of horseradish peroxidase-conjugated anti-rabbit IgG, thereby confirming the specificity of the binding. Prior absorption of anti-CW with the WS and CF antigen extracts of *A. fumigatus* led to a large reduction in binding to most of the antigen bands (Fig. 2, lanes 1b, 1c, 2b, and 2c). This suggests that homology exists between components of these two in vitro preparations and the antigens present in urine samples from patients. Furthermore, bands at 11, 18, and 29 kDa were clearly visible in the WS extract when it was stained with
anti-CW (Fig. 1, lane A; Fig. 2, lane 1a). In addition, as absorption of the anti-CW with the 48-h CF extract led to a reduction in binding, the urinary antigens were probably produced early in the growth of the fungus. None of the seven antigen bands could be detected with preimmunization serum, but weak nonspecific staining was seen at 100, 67, 59, and 23 kDa (Fig. 1, lanes 1a to 10a).

Sufficient urine was available from three patients (U5, U7, and U10) to allow probing with the EBA1 monoclonal antibody to *A. fumigatus* GM. A totally different band pattern was seen with this antibody when compared with anti-CW (Fig. 3, lanes 2 to 4). Staining was weaker, and bands were more diffuse. Most of the staining occurred at molecular masses greater than 45 kDa (Fig. 3, lanes 1 to 4). However, two urine samples (U5 and U10) showed in each case a weak band at approximately 21 kDa (Fig. 3, lanes 2 and 4). None of the antigens revealed with anti-CW could be demonstrated when the urine samples were probed with EBA1, confirming that they do not contain GM.

Pooled urine from the 23 neutropenic patients without evidence of aspergillosis showed the same staining patterns when incubated with either anti-CW or preimmunization serum. Diffuse, lightly stained bands were seen at approximately 50, 40, 25, and 16 kDa. EBA1 did not stain any component of these urine samples (Fig. 3, lane 5).

**DISCUSSION**

Most of the methods described to detect circulating *Aspergillus* antigens during IA have employed ill-defined reagents. For example, the concanavalin A-binding fractions often used in enzyme-linked immunosorbent assays (15, 31) have been shown to be complex mixtures comprising at least 14 components (30); a positive test result could therefore indicate the presence of any one of these molecules. Currently, the only defined antigen known to circulate during IA is GM (9, 14, 27; Haynes and Rogers, 29th ICAAC). GM has been characterized chemically (3, 4) after extraction from mycelium produced in vitro and was shown to have a
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molecular mass of between 25 and 50 kDa. However, the circulating GM-containing antigen detected by Reiss and Lehmann had a mass of greater than 125 kDa (14); it was suggested that this increased size was due to complexing of the polysaccharide to protein in vivo (21). We found that the majority of GM-containing antigens migrate with an apparent molecular mass of greater than 45 kDa (Fig. 3, lanes 1 to 4). The diffuse staining seen in these blots is characteristic of a highly glycosylated moiety.

By using an experimental animal model of IA infection, De Repentigny et al. (8) found circulating antigenic components that did not bind to concanavalin A but could inhibit binding of anti-Aspergillus antibody in their enzyme-linked immunosorbent inhibition assay; this suggests that non-GM antigens were present. Similarly, we used an inhibition enzyme-linked immunosorbent assay methodology, incorporating either a monoclonal antibody to A. fumigatus GM or a polyclonal high-titer human anti-Aspergillus serum, and found 11% of specimens from patients with IA in which antigen could only be detected in the polyclonal assay (Haynes and Rogers, 29th ICAAC). Our present study confirms these observations and reveals the non-GM antigens that are present during IA.

Phillips and Radigan (19) have also demonstrated the presence of several Aspergillus antigens in the sera of rabbits experimentally infected with A. fumigatus; the major protein had an apparent molecular mass of 80 kDa. We were unable to detect a band at this molecular mass in the urine samples of humans with IA. Our preliminary experiments with sera (data not shown) from IA and noninfected subjects were not successful, due to a high degree of nonspecific binding of the anti-CW rabbit antiserum to components in human sera.

The antigens described here were found in urine samples from patients with IA caused by species of Aspergillus other than A. fumigatus (U1, U5, and U10). This finding is not surprising, as much work has shown that different species share certain antigenic determinants (3, 4, 11, 30). In addition, the WS antigen fraction used here contains common antigens, as shown by both enzyme-linked immunosorbent assay and immunoblotting, when prepared from either A. fumigatus NCPF 2109 or A. flavus NCPF 2008 (unpublished results).

Our results show that both GM and non-GM antigens are present during IA. This pattern is repeated in other fungal infections in which both polysaccharide (6) and protein (20) antigens can be demonstrated in body fluids. For example, in systemic candidiasis it has been shown that both mannann-containing glycoprotein (12) and a 47- to 48-kDa protein (16) antigens are circulating.

In this study, we identified at least seven non-GM antigens that are present during IA. The purification and characterization of these non-GM antigens should lead to the development of more sensitive reagents which would allow the rapid detection of Aspergillus infection and which may be used to predict the development of invasive disease.

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LITERATURE CITED