Monoclonal Antibodies to Hyphal Exoantigens Derived from the Opportunistic Pathogen *Aspergillus terreus* ▼

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*Aspergillus terreus* has been difficult to identify in cases of aspergillosis, and clinical identification has been restricted to the broad identification of aspergillosis lesions in affected organs or the detection of fungal carbohydrates. As a result, there is a clinical need to identify species-specific biomarkers that can be used to detect invasive *A. terreus* disease. Monoclonal antibodies (MAbs) were developed to a partially purified preparation of cytolytic hyphal exoantigens (HEA) derived from *A. terreus* culture supernatant (CSN). Twenty-three IgG1 isotype murine MAbs were developed and tested for cross-reactivity against hyphal extracts of 54 fungal species. Sixteen MAbs were shown to be specific for *A. terreus*. HEA were detected in conidia, hyphae, and in CSN of *A. terreus*. HEA were expressed in high levels in the hyphae during early stages of *A. terreus* growth at 37°C, whereas at room temperature the expression of HEA peaked by days 4 to 5. Expression kinetics of HEA in CSN showed a lag, with peak levels at later time points at room temperature and 37°C than in hyphal extracts. Serum spiking experiments demonstrated that human serum components do not inhibit detection of the HEA epitopes by MAb enzyme-linked immunosorbent assay (ELISA). Immunoprecipitation and proteomic analysis demonstrated that MAbs 13E11 and 12C4 immunoprecipitated a putative uncharacterized leucine aminopeptidase (Q0CAZ7), while MAb 19B2 recognized a putative dipeptidyl-peptidase V (DPDP). Studies using confocal laser scanning microscopy showed that the uncharacterized leucine aminopeptidase mostly localized to extracellular matrix structures while dipeptidyl-peptidase V was mostly confined to the cytoplasm.

*Aspergillus terreus* is a filamentous fungus associated with organic detritus decay in the soil rhizosphere. Since the identification of *A. terreus* by Thom in 1918 (59), the species has been utilized in the biotechnology industry as a source of organic acids (11, 20), the serum cholesterol-lowering compound lovastatin (7, 62), and proteases and peptides that hydrolyze proteins (32). In contrast to its economic importance in biotechnology, *A. terreus* causes significant losses by spoiling food and agricultural products (2, 14, 31). Furthermore, *A. terreus* has been reported as a human pathogen and can cause superficial, cutaneous, and subcutaneous mycoses that affect the nail bed (28), outer ear canal (60), and skin (16). More recently, *A. terreus* has been associated with postoperative osteomyelitis (43), endophthalmitis (22), and peritonitis (63), and has been reported as a human pathogen and can cause osteomyelitis (43), endophthalmitis (22), and peritonitis (63), and has been reported as a putative virulence factor (10, 44). The species produces terrelysin, a hemolytic protein that has been recently reported as a putative virulence factor (10, 44). The species also produces secondary metabolites that may have toxic effects on host cells and may help facilitate invasive disease (19, 39, 41).

Species-specific diagnosis of *A. terreus* opportunistic infections is clinically important due to this pathogen’s resistance to the primary antifungal therapeutic amphotericin B (13, 56, 66). To date, the identification of *A. terreus* infections has challenged the most seasoned clinicians (29). Clinical diagnosis of *A. terreus* infection is subjective and has been restricted to macroscopic and microscopic characterization of tissue samples (72), computed tomography imaging (15), and detection of serum galactomannan or 1,3-β-D-glucan (30, 58). These diagnostic methods are limited and prevent the identification of the specific causative agent (30). Other more specific methodologies such as PCR have been recently developed but are limited by a number of other confounding factors (6, 30). Due to increasing *A. terreus* infections reported in the literature, resistance to amphotericin B, and the high mortality rate associated with infection, it is critical to develop sensitive and specific diagnostic tests (33). In this study, we describe the production and characterization of species-specific monoclonal antibodies (MAbs) to a partially purified cytolytic preparation of hyphal exoantigens (HEA) from *A. terreus*. Using the MAbs, we characterized the cross-reactivity profiles, kinetics of anti-
gen expression, and identity of several of the exoantigens. These data demonstrate that these MAbs may be useful for immunodiagnostic assays to detect invasive _A. terreus_ disease.

**MATERIALS AND METHODS**

**Preparation of _A. terreus_ hyphal exoantigens and rabbit polyclonal antibodies.** _A. terreus_ was purchased from the American Type Culture Collection (ATCC 1012; Manassas, VA). Conidia were inoculated in yeast extract broth (TSB) and grown in liquid culture for 7 days. HEA were partially purified from TSB using molecular sieves and gel filtration steps to isolate the cytoplolic fraction, as previously described (65).

Polyclonal antibodies to HEA were generated in rabbits and affinity purified by Bethyl Laboratories (Montgomery, TX) using HEA immobilized on activated Sepharose columns.

**Production of monoclonal antibodies to HEA.** Five to 14-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were housed under controlled environmental conditions in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood chip bedding. Mice were provided Teklad 7913 rodent chow (Harlan Laboratories, Madison, WI) and autoclaved tap water ad libitum. Sentinel mice housed in the animal quarters were free of viral pathogens, parasites, mycoplasma, and _Helicobacter_ spp. The animal protocol was approved by the University of California Occupational Safety and Health (NIOSH) Animal Care and Use Committee. The NIOSH animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Mice were immunized (six times) intraperitoneally at biweekly intervals with 25 μg of HEA emulsified in TiterMax adjuvant (TiterMax USA, Norcross, GA). Mice received a final boost (7th immunization) of 25 μg of HEA without adjuvant 3 days prior to hybridoma production. Pre- and postbleed mouse IgG-specific titers to HEA were tested using an indirect enzyme-linked immunosorbent assay (ELISA) method. Briefly, 96-well Nunc Immuno MaxiSorp microplates (Thermo Fisher Scientific, Rochester, NY) were coated with 1 μg/ml HEA in carbonate coating buffer (CBB: 60 mM sodium carbonate, 140 mM sodium bicarbonate, pH 9.6). Antibody binding from mouse serum was detected using biotin-SP-conjugated goat anti-mouse IgG Fc (Jackson ImmunoResearch Laboratories, Inc.). ELISA plates were developed with 4-nitrophenyl substrate (Sigma, St. Louis, MO) and read at 405 nm after 30 min as previously described (45, 51).

Three days following the final boost, the immunized mice were euthanized by CO2 asphyxiation, the spleens were removed, and spleenocytes were fused with SP2-O-Ag14 ATCC myeloma cells (ATCC CRL-1581). Hybridomas were selected by growing the cells in Dulbecco’s modified Eagle medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 50 μg/ml penicillin, 100 μg/ml streptomycin, 0.292 mg/ml lysozyme, 100 mM sodium hypoxanthine, 16 mM thymidine, 10% fetal calf serum (FCS) (HyClone, Logan, UT), and 100 μM interleukin-6 (IL-6; Boehringer, Mannheim, Germany). DMEM was additionally supplemented with 10% fetal calf serum to maintain the level of antigen during the 10 days of hybridoma growth and to select for bulk MAbs production. Hybridoma cell lines of individual clones were frozen in 10% dimethyl sulfoxide (DMSO) and 10% FCS, stored at −80°C for 2 weeks, and then transferred to a liquid nitrogen facility for long term storage.

**HeA capture ELISA.** In brief, 96-well Nunc Immuno MaxiSorp microplates (Thermo Fisher Scientific, Rochester, NY) were coated with 100 μl/well rabbit anti-HEA polyclonal antibody (1 μg/ml) in CBB and incubated overnight at room temperature (RT). Wells were washed three times by incubation with 200 μl/well phosphate-buffered saline (PBS, pH 7.4) for 5 min each, and were then blocked with 100 μl/well 5% nonfat dry milk (NFDM) in PBS for 30 min. Plates were then processed for the detection of IgG antibody by the addition of hybridoma culture supernatant (CSN), followed by goat anti-mouse IgG (45). Negative-control values were obtained by replacing hybridoma CSN with complete DMEM.

**Isotyping and quantification of IgG antibodies.** Isotyping of individual MAbs was determined by an indirect ELISA. Plates were coated with CSN from _A. terreus_ diluted in CCB (1 μg/ml) and incubated overnight. Plates were then blocked with PBSTM and incubated with MAb solutions from individual hybridomas. MAbs bound to HEA were detected using biotin-SP-conjugated AffiniPure goat anti-mouse IgG, IgG2a, IgG2b, and IgG3 secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) at a dilution of 1:5,000 in PBSTM. ELISA plates were developed using methods described previously.

For quantification, MAbs were serially diluted and captured on ELISA plates coated with AffiniPure goat anti-mouse IgG Fc fragment of subclass 1a, 2a, or 3 at a concentration of 1 μg/ml (Jackson ImmunoResearch Laboratories Inc.). IgG1, IgG2a, IgG2b, and IgG3 standards (Sigma) were used as a standard curve for quantification purposes. AP-conjugated goat anti-mouse secondary antibodies (1:5,000) diluted in PBSTM were used and developed using methods described previously.

**Preparation of _A. terreus_ extracts for characterization of MAbs.** (i) Conidial extracts. Conidia were collected from 10- to 14-day-old _A. terreus_ cultures grown on malt extract agar (MEA) by rolling approximately 1 g of 0.5-mm glass beads (BioSpec Products Inc., Bartlesville, OK) over the plate. Glass beads with conidia were collected into a 2-ml screw cap microcentrifuge tube and processed in a Mini Bead Beater (BioSpec Products Inc.). Mechanical bead beating was carried out for 1 min to disrupt the outer cell wall of the conidia. Conidium extracts were extracted in 50 mM ammonium bicarbonate buffer, pH 8.0, containing 0.5 M EDTA, 0.1 M phenylmethylsulfonyl fluoride, and Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). The suspension was centrifuged at 4,100 × g for 10 min, and the supernatant fluid was collected and lyophilized overnight. Lyophilized conidium protein extract was reconstituted in PBS, pH 7.4, and stored at −80°C.

(ii) Hyphal extracts and culture supernatants. _A. terreus_ conidia were inoculated in 50 ml of minimal medium consisting of 1% glucose, nitrates salts, and trace elements (27). Viability of conidia was determined by a Live/Dead BacLight viability kit (Invitrogen, Corp, Carlsbad, CA) as per the manufacturer’s instructions and as previously reported (17). Cultures were seeded with 2.5 × 10^9 viable conidia and grown at RT or 37°C with shaking (200 rpm) for various intervals of time. For MAb reactivity assays, cultures were grown for 6 days, and for the kinetic assays, cultures were grown for 12 days, with samples collected at 24-h intervals. Mycelial cultures were harvested by centrifugation at 4,100 × g for 5 min, and the hyphal (pellet) and CSN were collected and concentrated by lyophilization. The lyophilized CSN was reconstituted in 5 ml of PBS, pH 7.4, containing Complete Mini Protease Inhibitor Cocktail and stored at −80°C for further analysis. Lyophilized hyphal mycelial pellets were macerated in a mortar containing liquid N2 and suspended in cold PBS, pH 7.4, containing Complete Mini Protease Inhibitor Cocktail, and proteins were extracted overnight at 4°C on a rocker. The mycelial extract (ME) was centrifuged at 4,100 × g for 5 min, and the supernatant fluid was collected, aliquoted, and stored at −80°C for further analysis. Protein concentrations in all fungal extract preparations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE).

**Characterization of HEA antigen expression in fungi.** For cross-reactivity analyses, 46 fungal species (Table 1) were inoculated on MEA medium and grown for 7 to 10 days. The conidia (1.25 × 10^5) were collected and inoculated into 50 ml of TSB medium. Cultures were grown for 4 days at RT and centrifuged at 4,100 × g for 5 min to collect the mycelial pellet. The pellet was washed three times with cold PBS, pH 7.4, macerated, and then centrifuged at 4,100 × g for 5 min as previously described. The supernatant fluid was collected, protein concentration was determined by NanoDrop spectrophotometer, and antigen concentration was determined using the previously described indirect ELISA method. Positive reactivity was determined by an optical density (OD) of ≥0.2 (negative-control value plus 3 standard deviations). The ODs of the negative controls ranged from 0 to 0.18 for different fungal extracts. The kinetics of antigen expression during culture was also examined. Individual MAbs were tested against _A. terreus_ spore, mycelial, and CSN extracts, used at 100 μg/ml total protein to determine the level of antigen expression during different phases of _A. terreus_ growth. A polyclonal antibody (PAb)-based capture ELISA was used for this analysis, and all MAbs were normalized to 500 ng/ml in PBSTM. Plates were developed using secondary antibodies and reagents as previously described.

**HEA detection assay in spiked serum.** Detection of HEA by the MAbs in the presence of human serum was studied using the ELISA methods described earlier. CSN was collected from a 6-day culture of _A. terreus_ and was diluted to 100 μg/ml of total protein. Pool serum (CSN) was spiked with CSN and assayed in the capture ELISA at a final concentration of 50% total volume using 100 μl of sample. Additionally, samples were also incubated in PBS and...
TABLE 1. Fungal species tested for cross-reactivity.

| Fungal species          | Culture collection identification no.* | Reactive MAb(s) |
|-------------------------|-----------------------------------------|-----------------|
| Aspergillus species     |                                         |                 |
| A. terreus              | ATCC 1012                               | All             |
|                         | FGSC A1156                              | All             |
|                         | ATCC 16794                              | None            |
| A. niger                | NRYL 78                                 | None            |
|                        | SRC 2174                                | None            |
| A. flavus               | ATCC 24689                              | 67G7            |
| A. fumigatus            | FGSC A1100                              | None            |
| A. nidulans             | NRYL 15-22-48                           | None            |
| A. niger                | ATCC 24684                              | None            |
| A. parasiticus          | ATCC 26961                              | 67G7            |
| A. repens               | NRYL 13                                 | 2D7, 38B6, 40C6 |
|                         |                                        | 52G7, 64B3, 67G7 |
| A. sydowi              | ATCC 9507                               | 2D7, 67G7       |
| A. ustus                | NRYL 275                                | None            |
| A. versicolor           | ATCC 44408                              | None            |
| Other                   |                                         |                 |
| Acromonium strictum     | ATCC 46646                              | 22D9            |
| Alternaria alternata    | ATCC 11612                              | None            |
| Alternaria brassicicola | ATCC 96836                              | None            |
| Botrytis cinerea        | ATCC 11542                              | None            |
| Chaetomium globosum     | ATCC 6205                               | None            |
| Cladosporum herbarum    | ATCC 6506                               | None            |
| Cladosporum cladosporioides | ATCC 11288          | None            |
| Epicoccum nigrum        | ATCC 34929                              | None            |
| Exserohilum rostratum   | ATCC 26856                              | None            |
| Fusarium moniliforme    | PS M6311                                | None            |
| Geotrichum candidum     | UAMH 7863                               | None            |
| Memnoniella echinata    | NRRL 2373                               | None            |
| Memnoniella subsimplex  | ATCC 32888                              | None            |
| Myrothecium verrucaria  | NRYL 2003                               | None            |
| Penicillomyces variotii | ATCC 66705                              | None            |
| Penicillium aurantiogriseum | NRRL 971                         | None            |
| Penicillium expansum    | NRRL 973                                | None            |
| Penicillium fellutanum  | NRRL 7415                               | None            |
| Penicillium puiggarioum  | NRRL 1062                               | None            |
| Penicillium roqueforti  | NRRL 844                                | None            |
| Rhizopus stolonifer     | NIOSH 17-59-14                          | None            |
| Stachybotrys albus      | ATCC 18873                              | None            |
| Stachybotrys babyi      | ATCC 18825                              | None            |
| Stachybotrys chartarum  | IBT 7711                                | None            |
|                         | IBT 9201                                | None            |
|                         | IBT 9460                                | None            |
|                         | IBT 9466                                | None            |
|                         | IBT 9631                                | None            |
|                         | IBT 9633                                | None            |
|                         | IBT 14915                               | None            |
|                         | IBT 14916                               | None            |
| Stachybotrys chlorohalanata | ATCC 201863                      | None            |
| Stachybotrys cylindeoropa | ATCC 16276                       | None            |
| Stachybotrys kamptakens | ATCC 22705                              | None            |
| Stachybotrys neprophora | ATCC 18393                              | None            |
| Stachybotrys oenothae | CBS 252.76                              | None            |
| Stachybotrys parvispora | CBS 100155                              | None            |
| Sclerotioropsis breuittii | ATCC 16278                  | None            |
| Stemphylium botryosum   | ATCC 26851                              | None            |
| Trichoderma viride      | ATCC 16640                              | None            |
| Wallinia sebi           | NIOSH 26-41-01                          | None            |

* Sources for 46 species representing 20 different genera of fungi are as follows: ATCC, American Type Culture Collection; NRRL, Agricultural Research Service Culture Collection; NIOSH, National Institute for Occupational Safety and Health; FGSC, Fungal Genome Initiative Center; UAMH, University of Alberta Microfungus Collection and Herbarium, Canada; PS, Pennsylvania State University; IBT, Institutet for Bioteknologi, Denmark; CBS, Centraalbureau voor Schimmelcultures, Netherlands.

Individual MAb (500 ng/ml) for 1 h with shaking. The membrane was washed with PBST and incubated with AP-conjugated goat anti-mouse IgG (H+L) (Promega, Madison, WI) diluted 1:5000 in PBST for 1 h on a shaker. The membrane was then washed with PBST and developed using 1-step NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate) substrate (Promega). The substrate reaction was developed for 15 to 20 min and stopped by washing the membrane with distilled water.

**RESULTS**

Characterization of MAb reactivity to fungal extracts. Twenty-three murine IgG1 isotype hybridomas were developed against HEA (Table 1). Since the MAbs were developed against a partially purified cytolytic extract, reactivity of the MAbs to A. terreus culture extract antigen was tested. MAb reactivity was highest to mycelial extracts; however, weak reactivity to conidial extracts was also observed (Fig. 1a). As

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expected, the MAbs also showed significant reactivity to 6-day culture supernatant from cultures grown at room temperature and greater reactivity to CSN grown at 37°C (Fig. 1b). Collectively these results suggest that the MAbs react with hyphal antigens that are secreted into CSN.

Cross-reactivity of MAbs toward different fungi. Table 1 lists the 46 fungal species from 20 different genera that were tested for cross-reactivity in the capture ELISA. Positive reactivity was defined as an OD at 450 nm (OD$_{450}$) of ≥0.2. All MAbs showed reactivity to ME from each *A. terreus* strain tested in this study. Of the 23 MAbs that react with HEA, 16 (70%) did not cross-react with any of the fungal species tested (Table 1). Seven MAbs cross-reacted with other fungi. Most of the cross-reactivity observed in these seven MAbs was limited within the genus *Aspergillus*, except for MAb 22D9 that also cross-reacted with ME derived from *Acremonium strictum*. Five MAbs (22D9, 38B6, 40C6, 52G7, and 64B3) cross-reacted with ME from at least one other fungus, while 24D7 cross-reacted with two different fungi (Table 1). The MAb 67G7 exhibited the greatest cross-reactivity, reacting to hyphal extracts derived from four different *Aspergillus* species. Of the fungal species tested, *Aspergillus sydowii* and *Aspergillus repens* hyphal extracts showed highest cross-reactivity.

Western blot analysis of HEA. Figure 2 shows the Western blot analysis of *A. terreus* CSN using the 16 species-specific MAbs and the cross-reactive MAb 67G7. The results demonstrated differences within the MAb reactivities to antigens in *A. terreus* CSN. The MAbs 13E11, 19B2, 24C8, 29C9, and 61E5 showed similar patterns with strong reactivity to bands at ~18 kDa, 45 kDa, and ~70 kDa. The MAb 19B2 identified unique high-molecular-weight bands at ~100 kDa and ~150 kDa. It is possible that the immune reactivity to the 10-kDa and the 45-kDa antigens is due to binding of antibodies to shared epitopes or to carbohydrate antigens.

Kinetics of *A. terreus* HEA expression. Four MAbs (13E11, 15D3, 19B2, and 19B7) were chosen for further study based on
differences in patterns of reactivity in ELISA and Western blotting. Figure 3 illustrates the time course of HEA expression in *A. terreus* cultures. For MAbs 13E11, 15D3, and 19B7, reactivity to HEA preparation rapidly increased in ME during days 3 to 5 and peaked at day 6 (Fig. 3a). Reactivity to HEA in the CSN followed a similar pattern but appeared to lag behind that in ME by 24 h (Fig. 3b). At 37°C, HEA MAb reactivity was readily detectable by 24 h in both ME (Fig. 3c) and CSN (Fig. 3d) and peaked by days 3 and 4, respectively. Interestingly, HEA MAb reactivity could be detected in both CSN and ME beyond day 6 but appeared to decline in CSN after 10 days at 37°C. The MAb 19B2 antigen, while having a similar pattern as the other MAbs, appeared to lag in expression, suggesting that this MAb may recognize a different antigen than other MAbs tested in this study. Also, we observed a more rapid degradation of MAb 19B2 antigen after day 10 in CSN of *A. terreus* cultures grown at 37°C. This could probably be due to proteolytic degradation of the antigen.

**Human serum detection assay.** To determine the ability of these MAbs to be used in an immunodiagnostic assays, we tested 10 MAbs for reactivity to *A. terreus* antigens spiked in pooled human serum (Fig. 4). CSN grown at 37°C was mixed with human serum and assayed using a capture ELISA. Overall, there was a slight reduction in the detection of HEA when
HEA was spiked into human serum compared to PBS for all MAbs tested; however, there did not seem to be any significant binding to serum components for any of the antibodies.

**Immunoprecipitation and proteomic analysis.** Three MAbs (13E11, 19B2, and 12C4) were selected for further analysis based on differential patterns of reactivity to *A. terreus* extract (Fig. 2). The MAbs were incubated with *A. terreus* hyphal extract, and protein-antibody complexes bound to the protein G beads were eluted and separated using SDS-PAGE. Using MAb 13E11 to stain the immunoblot showed immunoprecipitation of an ~65-kDa band, slightly higher than the antibody heavy chain band. Interestingly, MAb 13E11 also showed this same band in the immunoprecipitate of MAb 12C4 but not that of MAb 19B2 (Fig. 5). This suggests that MAb 13E11 and MAb 12C4 recognized similar antigens. In contrast, MAb 19B2 demonstrated immunoreactivity to an antigen localized at ~100 kDa (middle panel). While MAb 12C4 precipitated a 66-kDa band that was recognized by 13E11 (left panel), this MAb does not recognize this band when it is used to stain the Western blot (right panel), indicating that it may recognize a conformational epitope.

Specific bands were excised from a parallel SDS-PAGE gel on which immunoprecipitated samples from each MAb were separated. Samples were subjected to UPLC tandem MS (MS/MS) analysis to determine the identity of the proteins. By comparing peptide masses of recovered peptides *in silico* to the generated database for *A. terreus*, we identified peptides for a putative uncharacterized protein (Q0CAZ7) in immunoprecipitates from both MAbs 13E11 and 12C4. The protein Q0CAZ7 has >60% sequence homology to a leucine aminopeptidase found in other *Aspergillus* species such as *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus oryzae*, and *Aspergillus flavus*. Peptides for a probable dipeptidyl peptidase V (Q0C8V9) were also identified following the UPLC MS/MS analysis of MAb 19B2 immunoprecipitates (Table 2). Both of these proteolytic enzymes contain putative N-glycosylation sites and are secreted after processing of a signal peptide.

**Immunolocalization of HEA.** The immunolocalizations of *A. terreus* leucine aminopeptidase (MAb 13E11) and dipeptidyl peptidase V (MAb 19B2) in the hyphae were determined using confocal laser scanning microscopy. Interestingly, the leucine aminopeptidase (13E11) was localized in extracellular matrix (ECM) structures (Fig. 6). Immunostaining was also observed within the *A. terreus* hyphae. MAb 19B2 immunostaining for a probable dipeptidyl peptidase V was diffuse but uniform over the entire hypha, suggesting that this protein might be present in the cytoplasm of *A. terreus* hyphae. No staining was observed in the extracellular matrix for MAb 19B2. The MAb 9B4 served as an isotype control for these studies and did not stain any structures.

**DISCUSSION**

Invasive aspergillosis develops in the lungs of immunocompromised subjects following the inhalation of viable *Aspergillus* conidia from the environment (33, 70, 73). Upon germination,
the conidia differentiate into a vegetative hyphal form that can cause damage to the host tissue and may allow the fungus to disseminate to other parts of the body (52). To date, the diagnostic methods have been limited to identifying macroscopic and microscopic characteristics in lung biopsy specimens (30, 53). Serological diagnostics have also been developed but are limited to the detection of galactomannan and β-D-glucan (30, 53). Although this methodology may confirm a fungal infection, it does not identify the specific pathogenic species involved. Previous studies have detected Aspergillus antigens in the sera and urine of patients diagnosed with invasive aspergillosis (18, 25, 26, 36). These results suggest that during infection, Aspergillus species secrete proteins that could be used to serologically detect the organism. Based on the limitations of available detection methodologies, it is critical to identify biomarkers that could be used to serologically identify individual pathogenic Aspergillus species.

Although A. fumigatus is the most widely known etiological agent of invasive aspergillosis, A. terreus has emerged as an opportunistic pathogen that has been attributed to a variety of infections including fatal disseminated aspergillosis (4). To our knowledge, no immunodiagnostics have been developed for the specific detection of A. terreus in clinical samples. In this study, 23 IgG1 MAbs were produced using a partially purified cytolytic HEA preparation that was isolated using a methodology previously used to purify stachylysin from Stachybotrys chartarum (65). The MAbs developed in the present study specifically detect antigens localized in A. terreus conidia and hyphae, but, more importantly, these hemolytic antigens were detected in the CSN fluid. These findings demonstrate that these antigens may be actively secreted during hyphal differentiation and growth and may be candidate biomarkers for immunodiagnostic assays. Detection of hemolytic antigen in higher concentrations in hyphae is consistent with our previous studies of stachylysin (50, 61, 65, 71). Sixteen MAbs were found to be species specific while seven cross-reacted with other species. The species-specific MAbs did not cross-react with mycelial extracts from other Aspergillus pathogenic species including A. fumigatus, A. flavus, Aspergillus niger, and A. nidulans. No cross-reactivity was observed with other fungal species belonging to the genera Penicillium and Fusarium. Most cross-reactivity was minimal with OD450 values of ≤0.5.

Previously, differences were reported in metabolic activities, growth rates, and virulence capabilities of different A. terreus strains depending on their environmental source (49). We were curious to see if any of these differences were reflected in altered expression of HEA. All MAbs reacted with the mycelial extracts from the four A. terreus strains; however, comprehensive testing with additional clinical strains of A. terreus will be critical prior to the development of diagnostic screening methods for use in the clinical setting. Moreover, other species that are closely related to A. terreus such as Aspergillus carneus, Aspergillus niveus, and the newly identified Aspergillus alabamensis, as well as other unrelated species including Scedosporium and Rhizopus stolonifer (5, 38), should be tested for cross-reactivity.

A. terreus growth is accompanied by conidial germination during favorable nutrient and environmental conditions. This process involves the swelling of conidia, initiation of primary metabolism, and hyphal extension and aggregation. In this study, we observed that the antigens were detected earlier in hyphal extracts than in CSN, suggesting an active secretion of these proteins. The concentration of these antigens appeared to correlate with the total biomass of the culture and protein concentration (data not shown) during HEA kinetic experiments. MAb reactivity to HEA was also observed to increase proportionally with increases in the mycelial pellet size. Most importantly, HEA were continuously detected in CSN at 37°C, emphasizing the relative stability of these antigens to proteolytic degradation for longer period of time.

Detection of the antigens in CSN may not fully reflect antigen production during invasive disease. Furthermore, secreted...
antigens may bind to serum proteins or other factors that alter the confirmation of the epitope and subsequently reduce the availability of the epitope for MAb detection. Certain fungal proteins are known to bind serum components in vitro (21). In tests with pooled human serum, there was only a slight reduction in the detection of epitopes using our MAbs. This suggests that the HEA epitopes do not interact with serum components, and this may have potential use for serodetection of invasive A. terreus disease.

Leucine aminopeptidase and dipeptidyl-peptidase V are both predicted to possess putative N-glycosylation sites as determined by N-Glycosite (74). These proteins are secreted with putative signal peptides as determined by SignalP, version 3.0, in silico analysis (9, 23, 46). This has been confirmed experimentally by us in this study and previously by others for A. terreus and other fungal species (24, 64). Homologues of dipeptidyl-peptidase V in other fungal species have been reported as a potential virulence factor or allergen and as important for tissue invasion and modulation of host immune responses (8, 34, 35, 54, 64, 67, 68).

Immunolocalization studies demonstrated that the putative leucine aminopeptidase identified by MAb 13E11 was localized to extracellular structures containing DNA. Similar structures containing extracellular DNA have been reported in vitro and in vivo and have been identified as the extracellular matrix of fungi (1, 37, 42). ECMs have only been recently identified, and there is little information on their role in the pathogenesis of fungal infections. MAb 13E11 may be a useful tool in studying ECMs, and, more importantly, the putative leucine aminopeptidase may function as a biomarker of invasive A. terreus disease. In contrast, MAb 19B2 recognized a probable dipeptidyl peptidase V, and immunostaining was primarily localized within the cytoplasm. These MAbs also have the potential to be used for the immunofluorescent detection of A. terreus in bronchoalveolar lavage samples.

In conclusion, we observed that HEA were released from vegetative hyphae and into CSN in a time-dependent manner. The MAbs developed in this study recognized these antigens, and this may have potential use for serodetection of invasive A. terreus disease.
