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Production of Specific Monoclonal Antibodies to *Aspergillus* Species and Their Use in Immunohistochemical Identification of Aspergillosis

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Two anti-*Aspergillus* murine monoclonal antibodies (MAbs), designated 164G and 611F, have been produced; both specifically recognize cytoplasmic antigens of *A. fumigatus*, *A. flavus*, and *A. niger* by enzyme-linked immunosorbent assay. The MAbs can identify *Aspergillus* spp. both in frozen sections by immunofluorescence and in paraffin-embedded clinical specimens by immunofluorescence and immunoperoxidase staining.

Invasive aspergillosis, usually caused by *Aspergillus fumigatus*, is second only to *Candida* species as a cause of fungal infections in the immunocompromised host (1, 10) and is a relatively frequent cause of morbidity and mortality in these patients. The diagnosis is usually clinical and is often difficult to confirm using current laboratory methods. The most successful laboratory methods described to date have been based on the detection of antigen (13, 14, 17). However, the sensitivity of these methods may be limited by the need to obtain sequential samples from each patient (9). PCR-based methods have also been developed (14, 16), although such approaches have not yet gained general acceptance. Thus, cultural and histopathological identifications remain the definitive methods of diagnosing invasive aspergillosis. However, histopathological identification may encounter difficulties, particularly in relation to the differentiation of *Aspergillus* species from other fungi. The implications of this are mainly therapeutic; the diversity of fungi causing disease in neutropenic patients is increasing, and some of these fungi, notably *Pseudallescheria boydii*, are resistant to amphotericin B, which would normally be used to treat infections caused by *Aspergillus* and many other species. Thus, it has become increasingly important to be able to differentiate *Aspergillus* species from other fungi where tissue is available for examination. Specific antibodies which may be used to label fungal hyphae in tissue sections would be very useful in these situations; however, polyclonal antibodies are frequently cross-reactive among fungal species, and even monoclonal antibodies (MAbs) may suffer the same limitations (3, 8, 12). In this report, we detail the production and partial characterization of two *Aspergillus*-specific MAbs which have been used to specifically identify *Aspergillus* hyphae in histological sections.

Initially, lyophilized isolates of *A. fumigatus* (NCPF no. 2010 and 2078), *Aspergillus flavus* (NCPF 2208 and 2617), *Aspergillus terreus* (NCPF 2026), *Aspergillus niger* (NCPF 2599), *Aspergillus nidulans* (NCPF 2232 and 2078), *Candida albicans* (NCPF 3343), *Candida tropicalis* (NCPF 3114), *Cryptococcus neoformans* (NCPF 3081 and 3168), *Penicillium piceum* (NCPF 2720), *Penicillium marneffei* (NCPF 4160), *P. boydii* (NCPF 2216), *Sporothrix schenckii* (NCPF 3181), *Trichosporon beigelii* (NCPF 4874), and *Histoplasma capsulatum* (NCPF 4100) were obtained from the National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Colindale, London, United Kingdom. Three species of zygomycetes (*Absidia corymbifera*, *Rhizomucor pusillus*, and *Rhizopus arrhizus*), were also obtained as clinical isolates from the Mycology Laboratory, St. John's Institute of Dermatology, St. Thomas's Hospital, London. Lyophilized isolates were reconstituted with 0.5 ml of sterile water and cultured for 48 h on Sabouraud agar slopes before subculture with continual shaking for 3 to 7 days in 2.5 liters of Sabouraud liquid culture medium at room temperature. Cultures were filtered through Whatman no. 2 filter paper to remove media and were then washed twice in phosphate-buffered saline (PBS) (0.01 M, pH 7.4). Cytoplasmic antigens (CAs) were then prepared as previously described (4). Some of the filtered mycelia and yeast were also frozen for use in the production of cryostat sections (see below). In the case of *A. fumigatus* NCPF 2078, the culture filtrate was retained and concentrated 50-fold by dialysis against polyethylene glycol 8000, divided into aliquots, and frozen at −70°C (*A. fumigatus* filtrate antigen [FA]).

For the production of specific MAbs, cyclophosphamide was used as an immunomodulator (2, 4). In this context, cyclophosphamide has its effect via the suppression of B-cell responses to an initial primary antigen (which may contain a large number of cross-reactive epitopes); subsequently, when a second antigen is used as an immunogen, only B cells specific to the latter will respond. On day 0, five BALB/c mice were inoculated intraperitoneally with *A. flavus* CA (NCPF 2208; 50 μg of protein per mouse) in Freund's complete adjuvant. Five control BALB/c mice received the same inoculation. Two days later, cyclophosphamide (Sigma, Poole, Dorset, United Kingdom) at a dose of 40 mg per kg of body weight in PBS was injected intraperitoneally into the first group of 5 mice; the control mice were not treated. On day 15, *A. fumigatus* CA (NCPF 2010; 50 μg of protein in Freund's incomplete adjuvant per mouse) was used to inoculate control and test mice. This protocol was repeated on day 21. Two days later, all mice were bled, and the serum was tested by enzyme-linked immunosorbent assay (ELISA) (see below) to ascertain which animal had the greatest differential response to *A. fumigatus* CA, compared with *A. flavus* CA. This mouse was given a further intravenous inoculation of *A. fumigatus* CA (50 μg of protein) in PBS, and its spleen was used in a fusion 3 days later.

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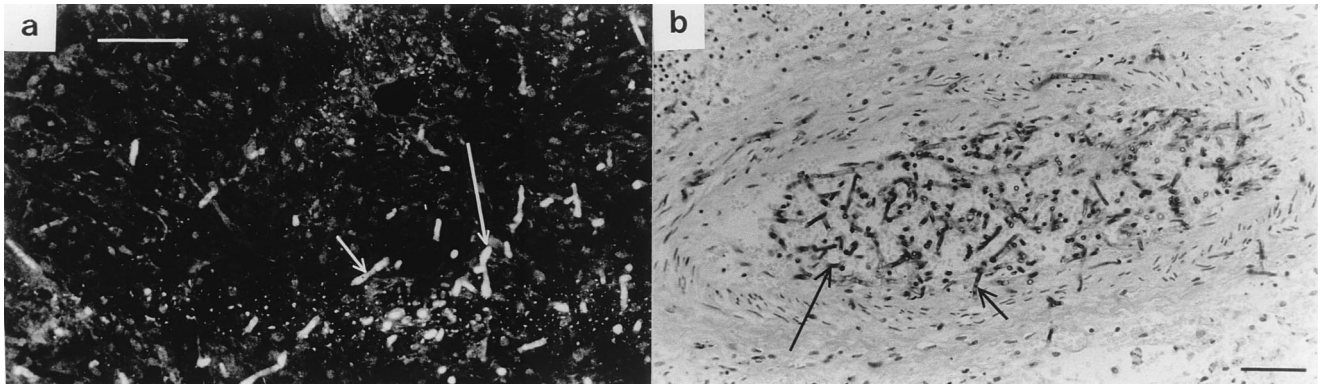

FIG. 1. Immunofluorescence and immunoperoxidase reactivities of MAb 611F in paraffin wax-embedded tissue. (a) Immunofluorescence staining of paraffin wax-embedded section of lung tissue from a patient with invasive aspergillosis caused by *A. fumigatus*. Representative hyphal elements are indicated by arrows. Bar represents 30 μm. (b) Immunoperoxidase staining of paraffin wax-embedded section of liver from a patient with invasive aspergillosis caused by *A. fumigatus*. Representative hyphal elements are indicated by arrows. Bar represents 25 μm.

MAbs were produced as previously described by using the myeloma line sp. 2/0 (2, 4). Hybridomas were screened for differential reactivity by ELISA (see below) against *A. fumigatus* and *A. flavus* CAs. Clones showing either species specificity or a markedly stronger reaction to *A. fumigatus* than *A. flavus* were subcloned twice, and those clones of interest were used for ascites formation in mice. MAbs were subsequently tested by ELISA for activity against all of the fungal CAs, together with the *A. fumigatus* FA detailed above, and were also subclassed as appropriate (5). An ELISA was performed as described previously (2, 4), with the following modifications. To determine the individual mouse with the greatest differential response to *A. fumigatus* CA, mouse sera at dilutions of 1:200, 1:400, 1:800, 1:1,600, and 1:3,200 in PBS–0.05% Tween 20 were used. Goat anti-mouse immunoglobulin G (IgG) peroxidase-linked conjugate (Jackson Immunochemicals, West Grove, Pa.) was used at a dilution of 1:1,000. To determine the reactivities of the MAbs to all the fungal CAs, ascitic fluid was used at dilutions of 1:100, 1:500, and 1:1,000 in PBS-Tween. Goat anti-mouse IgG peroxidase-linked conjugate was used at a dilution of 1:5,000 in PBS-Tween. MAb P4, produced against *Paracoccidioides brasiliensis* (2), was used as a negative control antibody (and was also used in this manner in the immunohistochemical studies described below). All assays were performed in duplicate.

For the production of cryostat sections, small aliquots of mycelium or yeast cultures from the various fungal pathogens (see above) were embedded in Cryo-M-Bed compound (Bright Instruments, Huntingdon, Cambridgeshire, United Kingdom), and 5-μm sections were cut, mounted, air dried, and fixed for 5 min in 100% acetone. In addition, paraffin-embedded tissue blocks from the following patient groups (all postmortem) were cut (5-μm sections), mounted on glass slides, and incubated at 37°C overnight: 11 culture-confirmed *A. fumigatus* infections (6 from lung, 2 from liver, 2 from paranasal sinuses, and 1 from brain tissue), 1 confirmed *A. flavus* infection (from lung), 5 confirmed *C. albicans* infections (2 from lung, 2 from liver, and 1 from skin), 4 confirmed *P. brasiliensis* infections (2 from lung, 1 from myocardium, and 1 from lymph node tissue), 3 confirmed *C. neoformans* infections (all brain), and 1 each of confirmed *Conidiobolus coronatus* infection (from peripheral nervous tissue) and *Chaetomium globosum* infection (from lung). Those sections destined for immunofluorescence staining were dewaxed in xylene, rehydrated in an ethanol series, washed in Tris-buffered saline (TBS) (0.1 M, pH 7.6), treated with 0.05 mg of protease V8 (Sigma)/ml in distilled water for 30 min at 37°C, and finally washed in TBS. For immunoperoxidase staining, sections were dewaxed, placed in absolute ethanol for 8 to 10 min, and then treated with a solution of 100 ml of methanol containing 2.8 ml of 3.6% H_2O_2 and 0.6 ml of 10 M HCl (10 min), followed by washes in distilled water and PBS.

For immunofluorescence staining, cryostat and paraffin sections were incubated with MAbs 611F and 164G and control MAb P4 as ascitic fluid diluted 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 in PBS for 1 h at 37°C in a humid chamber. After three washes in PBS, sections were incubated for 1 h at 37°C with rabbit anti-mouse IgG fluorescein conjugate (Ortho Diagnostics Systems, Raritan, N.J.) used at dilutions of 1:20 in PBS. After three washes in PBS, the sections were mounted and examined as previously described (4). Additional negative controls consisted of sections incubated with PBS instead of either the primary or secondary antibodies. For immunoperoxidase staining, paraffin sections were incubated for 10 min in normal swine serum (NSS) diluted 1:5 in TBS, which was then removed prior to incubation with MAbs 611F and 164G as ascitic fluid diluted 1:40 and 1:80 in TBS (1 h at 37°C) in a humid container. Negative controls were as described previously. After washing in TBS the sections were incubated with rabbit anti-mouse IgG peroxidase conjugate (Dakopatts, Glostrup, Denmark), diluted 1:50 in NSS, and then washed in TBS before incubation as described above with peroxidase anti-peroxidase (Dakopatts) diluted 1:100 in NSS. Following another TBS wash, 3,3′ diaminobenzidine (1 mg/ml in TBS) was added, and sections were incubated in the dark for 10 min at 37°C prior to a final TBS wash. Sections were counterstained with Mayer's haemalum, washed in tap water, and mounted.

Serum from one of the BALB/c mice immunized successively with *A. flavus* CA, cyclophosphamide, and *A. fumigatus* CA demonstrated a marked differential ELISA response to *A. fumigatus* CA, and the spleen from this individual was used in the fusion (data not shown). The five control mice showed no difference in reactivity to the two antigen preparations. Two MAbs, designated 164G and 611F, were obtained after screening by ELISA and subcloning. When these MAbs were tested by ELISA against CAs of all the fungal pathogens produced as described above, both reacted strongly to *A. fumigatus* CA (with optical densities at 492 nm of greater than 1.0). The MAbs were less reactive to the *A. flavus* and *A. niger* CAs, with no recognition of any of the other fungal antigens compared to

negative controls (data not shown). Both MAbs were also reactive against *A. fumigatus* FA by ELISA (data not shown). MAb 164G was found to be of the IgG1 subclass, while 611F was found to belong to the IgG3 subclass.

Both MAbs 164G and 611F demonstrated bright fluorescence staining of cultured *A. fumigatus* and *A. flavus* in cryostat sections with a limited reaction to *A. niger* (data not shown). Staining of both cytoplasm and cell wall was evident. There was no recognition of either *A. nidulans* or *A. terreus* or of any of the other fungal pathogens examined (data not shown). Both MAbs were able to stain *A. fumigatus* hyphae to a titer of 1:320, although optimal staining was seen at a titer of 1:80. MAb 611F clearly identified *Aspergillus* hyphae in all 12 paraffin-embedded clinical specimens from patients with culture confirmed invasive aspergillosis by both immunofluorescence (Fig. 1a) and immunoperoxidase staining (Fig. 1b). Both staining methods provided a clear contrast between the fungal elements and background tissue. Fungal elements were not recognized by MAb 611F in any of the 14 patients with other fungal infections (data not shown). MAb 164G stained *Aspergillus* hyphae in paraffin sections to a much lesser degree than 611F (data not shown), although the pattern of staining was broadly similar to that seen with 611F.

We have produced two MAbs which specifically recognize the three species of *Aspergillus* which most commonly cause disease in humans: *A. fumigatus*, *A. flavus*, and *A. niger*. There are now a substantial number of reports in the literature on the production of MAbs against *Aspergillus* species, although in most cases these antibodies have been found to be cross-reactive with other fungal pathogens to a greater or lesser extent (3, 8, 11, 12). Attempts to use MAbs to detect *Aspergillus* in histopathological sections have been more restricted and either have demonstrated cross-reactivity (15) or have been limited in scope, preventing a meaningful assessment of specificity (6). To date, the application of the *Aspergillus* MAb apparently specific to most species has been restricted to the identification of this pathogen in tissue from cattle (7).

While the immunization protocol described in this article was designed to maximize antibody production specifically to *A. fumigatus*, the MAbs produced specifically recognize, by ELISA, *A. fumigatus*, *A. flavus*, and *A. niger*. Both MAbs are also able to specifically recognize these three species of *Aspergillus* in frozen sections of fungal hyphae and are also reactive in paraffin-embedded tissue sections containing *A. fumigatus* and *A. flavus* hyphae. Neither MAb reacted with cryostat sections from a broad range of fungal pathogens, and paraffin sections from a more limited number of fungal infections were also negative. These observations confirmed the specificity of the two MAbs which was revealed by the ELISA data. MAb 611F was particularly useful in the specific identification of *Aspergillus* hyphae in pathological specimens.

The ability to immunolabel fungi in tissue sections is a considerable aid to histological identification. Discrimination between *Aspergillus* species and other fungi in situ, such as *P. boydii*, is notoriously difficult, and the increasing incidence of unusual filamentous fungal infections in neutropenic patients, some of which are poorly sensitive to amphotericin B, makes the correct identification of fungal species extremely important clinically. Using our MAbs, we have been able to differentiate infections caused by *Aspergillus* species from those caused by a large number of other fungi using standard immunohistochemical techniques which are easily applicable to routine histopathology. However, it is important to note that this study was retrospective and was performed using only postmortem material. As a result, we have no precise information yet as to how effective this method might be in improving clinical outcomes via the direct identification of *Aspergillus* infection in biopsy material from patients actually undergoing clinical evaluation. A large-scale study of this type is currently being planned; however, there is every reason to believe that these MAbs, particularly 611F, will be an extremely useful adjunct to standard histological techniques for the identification of conditions arising from infection with *Aspergillus*.

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