INFECTION AND IMMUNITY, Dec. 1991, p. 4478–4485
0019-9567/91/124478-08$02.00/0

Vol. 59, No. 12

Analysis of *Aspergillus nidulans* Conidial Antigens and Their Prevalence in Other *Aspergillus* Species

P. PUENTE, M. C. OVEJERO, N. FERNÁNDEZ, AND F. LEAL*

Departamento de Microbiología y Genética and Instituto de Microbiología Bioquímica, Universidad de Salamanca/Consejo Superior de Investigaciones Científicas, 37008 Salamanca, Spain

Received 29 March 1991/Accepted 20 September 1991

Aspergillus nidulans **is an ascomycetous fungus that reproduces asexually by forming multicellular conidiophores and uninucleate spores called conidia. These elements constitute the main vehicle for the transmission of this and other pathogenic *Aspergillus* species and are the starting point of the different forms of aspergillosis. In order to use *A. nidulans* as a potential source of useful antigens for the immunodiagnosis of these diseases, we have examined the total protein composition of conidial extracts of this fungus by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in gels of different percent T. Injection of SDS-extracted conidial proteins into rabbits allowed us to raise a battery of polyclonal antibodies which have defined some important immunogenic polypeptides. Several of these immunogens were both present in mycelial extracts and recognized by antimycelium antibodies. Four of them, designated cdA, cdB, cdC, and cdE, were also found in conidial extracts of other pathogenic *Aspergillus* species. Only cdE was undetectable in cell extracts of the nonrelated species *Fusarium culmorum* and *Phycomyces blakesleeanus*.**

Aspergillus spp. are considered to be the agents responsible for certain important lung diseases such as allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis. *Aspergillus fumigatus* is the main etiological agent of these diseases, but other species such as *A. nidulans*, *A. niger*, *A. flavus*, and *A. terreus* are occasionally involved (29).

The detection of circulating *Aspergillus*-specific antibodies has been tested as an aid to the diagnosis of the various forms of aspergillosis. Several fungal fractions (culture filtrate extracts and cell extracts from mycelia or conidia), mainly from *A. fumigatus*, have been used as detector antigens in routine serological procedures (17, 20), and despite variations in the selected strain, growth conditions, antigen preparation, and method of detection, all fractions have shown a high detection rate of anti-*Aspergillus* antibodies (for a complete review, see reference 11). Circulating antibodies to both mycelial and conidial antigenic components have been detected, with no significant differences in sera from patients suffering *Aspergillus*-related diseases; also, both kinds of antigens are useful in the diagnosis of aspergilloma, allergic bronchopulmonary aspergillosis, and, to a lesser extent, invasive aspergillosis (1). Since the route of transmission of these pathogenic fungi is through the conidia, we thought that these elements might play an important role in the triggering of the first stages of the immune response. Conidial antigens were first analyzed by Kauffman et al. (13) and, in a more complete study, by Piechura et al. (25), who followed the fate of *A. fumigatus* cytoplasmic antigens during the growth of conidia to mature mycelia and defined some important antigens. The objective of our work was to complete these previous studies by analyzing not only cytoplasmic but also membrane or cell wall conidial antigens from another pathogenic *Aspergillus* species with a very well characterized genetic system, *A. nidulans*. Additionally, we considered it to be of interest to check the degree of conservation of the detected antigens among the more commonly pathogenic aspergilli. The need for reproducible and standard serodiagnostic reagents has been emphasized on many occasions (5, 16, 19, 24); consequently, the antigens present in all species in different growth phases prove to be of particular interest from the point of view of the development of accurate diagnostic tests.

* Corresponding author.

MATERIALS AND METHODS

Fungal strains. The fungal strains used were *A. clavatus* (IMI 54399), *A. flavus* (NRRL 6540), *A. fumigatus* (ATCC 9197), *A. niger* (ATCC 9642), *A. terreus* var. *aureus* (ATCC 11877), and *Fusarium culmorum* (EAN 51 Botany School, Cambridge), all of them obtained from the Spanish Type Culture Collection (CECT), Valencia, Spain. *A. nidulans* G1059wt (*adF17 pabaA1 yA2*) was obtained from A. J. Clutterbuck, Glasgow, Scotland, and *A. nidulans* G1059fA3 (*adF17 pabaA1 yA2 fluF1*) was obtained from M. Tamame et al. (27), Salamanca, Spain. *Phycomyces blakesleeanus* (NRRL 1555) was kindly provided by A. Pérez Eslava, Salamanca, Spain.

Growth conditions. All *Aspergillus* strains were maintained on solid YED medium (1% D-glucose, 1% Difco yeast extract, 2% agar). *F. culmorum* was maintained on solid potato dextrose agar (PDA) (Difco). Plates were incubated at 28°C for 3 or 4 days and then stored at 4°C. *P. blakesleeanus* was maintained on rich medium (6) containing (per milliliter) 20 mg of D-glucose, 2 mg of asparagine-H_2O, 1 mg of Bacto-Casitone, 1 mg of yeast extract (Difco), 5 mg of KH_2PO_4, 500 μg of $MgSO_4 \cdot 7H_2O$, 28 μg of $CaCl_2$, 2 μg of thiamine · HCl, 2 μg of citric acid · H_2O, 1.5 μg of $Fe(NO_3)_3 \cdot 9H_2O$, 1 μg of $ZnSO_4 \cdot 7H_2O$, 300 ng of $MnSO_4 \cdot H_2O$, 50 ng of $CuSO_4 \cdot 5H_2O$, 50 ng of $NaMoO_4 \cdot 2H_2O$, and 2 mg of purified agar (Difco). The glucose was autoclaved separately. The final pH of the medium was 5.1.

To obtain high yields of conidia, all species of *Aspergillus* were grown on solid *Aspergillus* minimal medium (4) containing 0.1% glucose, 0.6% $NaNO_3$, 0.052% $MgSO_4$, 0.052% KCl, 0.15% KH_2PO_4, traces of $FeSO_4$ and $ZnSO_4$, and 1.5%

agar (pH 6.5). Both *A. nidulans* strains were supplemented (27) with 10 mg of *p*-aminobenzoic acid per liter and 200 mg of adenine per liter to give *Aspergillus* complete medium. Plates were incubated at 28°C for at least 4 days. *F. culmorum* gave the best yields after growing for several days on PDA solid medium at 28°C. *P. blakesleeanus* gave satisfactory results in maintenance medium.

For liquid growth cultures, all *Aspergillus* species and *F. culmorum* were grown by inoculation of 10^5 conidia per ml in 11 Erlenmeyer flasks containing 500 ml of liquid YED medium and by 25 h of incubation at 28°C in a Gallemkamp orbital shaker at 280 rpm. For *P. blakesleeanus*, the spores, previously activated at 48°C for 15 min, were inoculated in 500-ml Erlenmeyer flasks containing 250 ml of minimal medium (7), with the same composition as the above-mentioned rich medium (omitting the Bacto-Casitone and yeast extract), and incubated for 25 h at 22°C at 130 rpm.

Preparation of cell extracts. Mature conidia were harvested from solid medium plates by scraping the surface with a sterile spatula in 0.01% Tween 80. Conidia were pelleted from the suspension by centrifugation at 1,500 × *g* for 15 min and washed in sterile water. The conidial pellet was immediately frozen and kept at −70°C until used. Mycelial contamination was excluded by microscopic examination.

Conidia were thawed and mixed with lysing buffer (100 mM Tris-HCl [pH 7.5], 1 mM EDTA, 5 mM dithiothreitol, and 1 mM freshly added phenylmethylsulfonyl fluoride [PMSF; Sigma Chemical Co.] plus 5 μg of aprotinin and 5 μg of pepstatin A [both obtained from Boehringer Mannheim] per ml) to give a dense suspension. Then, an equivalent volume of Ballotini glass beads (1 mm) was added and samples were disrupted in a Braun MSK homogenizer with CO_2 refrigeration. Complete breakage was monitored by microscopic observation. After removing the glass beads, 2% sodium dodecyl sulfate (SDS; final concentration) was added to the lysed conidia and the lysate was incubated for 10 min at 100°C. Clarified extracts (12,000 × *g*, 15 min) were aliquoted and stored at −70°C.

Fractionation of cell extracts from mycelia was carried out by differential centrifugation. Briefly, mycelia broken as described for conidia were spun at 3,000 × *g* for 10 min to pellet cell walls; the resultant supernatant, after further ultracentrifugation at 150,000 × *g* for 1 h, was considered to be the cytosol; the pellet, after washing with the same buffer, was considered to be the total membrane fraction. Cell walls were washed twice each time with 10, 5, and 2% NaCl and 10 times with double-distilled water. The three fractions were extracted by boiling in 2% SDS, clarified, and stored as described above. Protein was quantitated by a modification of the Lowry method (23). Extracts containing less than 1 mg of protein per ml were concentrated by precipitation with 5 volumes of cold acetone at −70°C for at least 3 h. Precipitated protein was pelleted by spinning for 20 min at 12,000 × *g* at 4°C, dried in a vacuum evaporator (Savant Instruments), carefully resuspended in 2% SDS to the desired concentration, and clarified by centrifugation at 3,000 × *g* for 15 min.

Immunogens and immunization procedures. Conidia were disrupted and extracted as described above. Boiled, clarified extracts were acetone precipitated; the dried pellets were resuspended in PBS buffer (10 mM K_2HPO_4 [pH 7.5], 150 mM NaCl) and used as immunogens. Antibodies were raised in four New Zealand White rabbits by injection of 1 mg of conidial extract protein, emulsified with complete Freund's adjuvant, and intracutaneously distributed over 10 sites (100 μl per site). Booster injections of 0.5 mg of conidial extract protein emulsified with incomplete Freund's adjuvant were given subcutaneously on days 32, 57, 69, 78, 88, 90, and 94. Sample bleedings were taken on days −6 (preimmune serum), 50, 67, 77, 84, 124, 127, and 130. The animals were bled on day 148. Rabbits C and D were immunized with extracts from *A. nidulans* G1059wt, and rabbits E and F were immunized with extracts from *A. nidulans* G1059fA3.

IgG fraction purification. Sera were applied to a column of CM Affi-Gel Blue (Bio-Rad Laboratories) according to the manufacturer's instructions. The flowthrough, containing the immunoglobulin G (IgG) fraction, was concentrated by addition, while stirring, of the necessary amount of solid ammonium sulfate required to achieve a 50% saturated solution at room temperature and incubation at 4°C for 30 min. After 48,000 × *g* centrifugation for 10 min, the protein pellets were dissolved in a minimal amount of double-distilled water and then clarified and dialyzed for 24 h against three changes of double-distilled water at 4°C. The dialyzed solutions were centrifuged at 48,000 × *g* for 10 min to remove any insoluble material, aliquoted, and stored at −70°C.

SDS-PAGE. Polyacrylamide gel electrophoresis (PAGE) was carried out on a Protean II or Mini-Protean apparatus (Bio-Rad) on an isotropic 8, 10, 12, or 14% (wt/vol) acrylamide slab gel (16 by 18 by 0.1 cm or 8 by 6 by 0.1 cm) by using the discontinuous buffer system of Laemmli (18). A constant current of 25 mA per slab was applied until the bromophenol blue tracking dye front reached the separation gel, and then the current was switched to 30 mA per slab until the dye front reached the end of the slab. The molecular weight protein standards used were Bio-Rad low-molecular-weight standards, both normal and biotinylated, and Bethesda Research Laboratories high- and low-molecular-weight, prestained markers.

Proteins on gels were detected by a sensitive silver stain (22) or by staining for 30 min with 0.5% Coomassie brilliant blue R-250 in acetic acid-isopropanol-water (1:3:6) and destaining in acetic acid-methanol-water (10:5:85).

Electrophoretic blotting procedures and immunological detection of proteins. Proteins from extracts were first subjected to electrophoresis as described above and then transferred to nitrocellulose sheets (0.45 μm [pore size]; Schleicher & Schuell) in a Trans-Blot cell (Bio-Rad) by the method of Towbin et al. (28). The efficiency of the transfer was monitored by Coomassie brilliant blue or silver staining of the transferred gel or by colloidal gold staining (Bio-Rad) of the blot. The electrophoretic blots were soaked for 15 min in 10 mM Tris-HCl–150 mM NaCl (pH 7.5; TNa) and then in 3% fraction V bovine serum albumin (Miles) or 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5)–500 mM NaCl for 1 h at room temperature to saturate additional protein-binding sites. They were then incubated for 2 h with antiserum appropriately diluted in TNa, with concentrations and species as indicated in the legends to the figures. The sheets were washed in TNa (three times for 10 min each time) and incubated with horseradish peroxidase-conjugated goat IgG anti-rabbit IgG preparations (Bio-Rad) at 1/1,500 dilutions in TNa. The blots were incubated for 2 h at room temperature and washed in TNa (four times for 10 min each time). For the color reaction, the blots were soaked in a solution of 0.5 mg of 4-chloronaphthol (Sigma) per ml of 0.025% H_2O_2 in 50 mM Tris-HCl (pH 7.7)–150 mM NaCl. This was prepared fresh from stock solutions of 3 mg of 4-chloronaphthol per ml of methanol. After color development, usually for 5 to 10 min, the reaction was stopped by washing with water. The blots were dried between filter paper, photographed, and stored protected from light. When biotinylated molecular

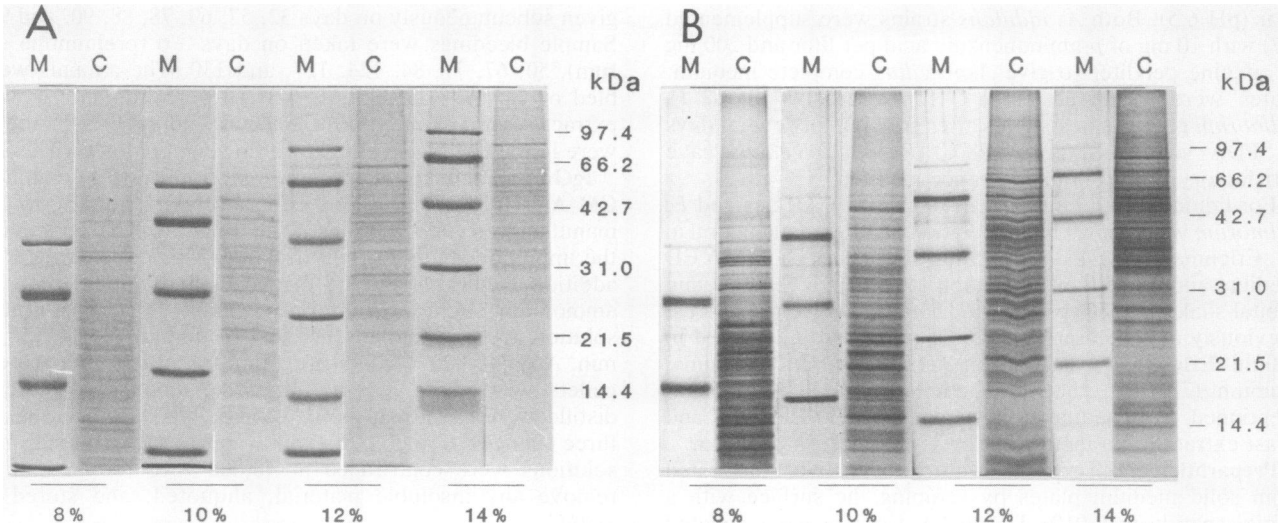

FIG. 1. Coomassie brilliant blue-stained (A; 20 μg of protein per lane) and silver-stained (B; 4 μg of protein per lane) SDS-PAGE profiles of *A. nidulans* G1059wt conidial extracts (lanes C) on gels of different percent T. Bio-Rad low-molecular-weight markers (lanes M) were used as standards.

weight markers were used, avidin-horseradish peroxidase conjugate (Bio-Rad) was included in the second antibody solution at a 1/3,000 dilution.

Photography. Stained gels were photographed with a 32 ASA, black-and-white Panatomic-X (Kodak) film by using an orange filter (Coomassie brilliant blue stain) or blue filter (silver stain). Immunoblots were photographed with an orange filter or without any filter.

RESULTS

SDS-PAGE of *Aspergillus* conidial proteins. *A. nidulans* G1059wt conidial proteins, prepared as described in Materials and Methods, were subjected to SDS-PAGE in 8, 10, 12, and 14% T gels and stained with Coomassie brilliant blue (20 μg per lane of protein load) or silver nitrate (4 μg per lane of protein load). Figure 1 shows that at least 52 polypeptides were detected with the first stain (A) and 66 with the second (B). Molecular masses ranged from 10 to 200 kDa, and the polypeptides were not randomly distributed throughout the entire apparent molecular mass range, being concentrated between 25 and 100 kDa. Polypeptides stained similarly with both reagents in the zone from 30 to 200 kDa, but Coomassie brilliant blue staining was clearly less sensitive for apparent molecular masses lower than 30 kDa.

A. nidulans conidial proteins were compared with those of other pathogenic *Aspergillus* species and of unrelated fungi such as *P. blakesleeanus* or *F. culmorum* by 14% T SDS–PAGE and silver staining (results not shown). The polypeptide profiles obtained were similar and confirmed that the amount of protein loaded in each lane was about the same; in addition, there was no proteolytic degradation in our conidial extracts. Most bands were concentrated between 25 and 100 kDa, and polypeptides of apparent molecular masses higher than 100 kDa were much less abundant in all the species. The number of proteins detected in each case was around 70, although the profiles did not coincide exactly. A zone of particular interest seems to be that between the 43- and 97.4-kDa molecular mass markers, showing many more polypeptide bands in *A. nidulans* and *F. culmorum* than in the other species. Both *A. nidulans* strains gave exactly the same results in this and all the other experiments.

Immunogenic potential of *A. nidulans* conidial proteins. Protein extracts obtained from mature conidia of two *A. nidulans* strains (G1059wt and G1059fA3) were used to immunize New Zealand White rabbits. The immune response was monitored qualitatively over time by Western blot (immunoblot) analysis. Proteins (60 μg per lane in normal gels and 10 μg per lane in minigels) from *A. nidulans* wild-type conidial extracts were separated by 14% T SDS–PAGE, transferred to nitrocellulose paper, and reacted with the antibodies present in the sera from successive bleedings of the immunized rabbits. Figure 2 shows the different

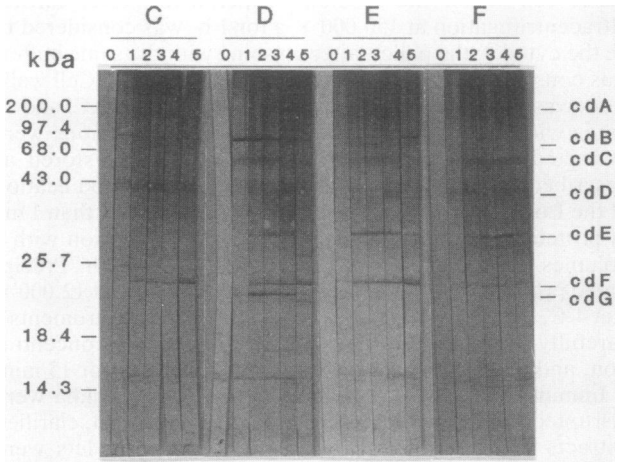

FIG. 2. Immunoblots of *A. nidulans* G1059wt conidial extracts (60 μg of protein per lane) developed by using total sera from consecutive bleedings of four different rabbits (C, D, E, and F) as the first antibody. Lanes: 0, preimmune serum; 1 to 5, 50, 67, 84, 124, and 148-day bleedings, respectively, all at a 1/800 dilution. Bethesda Research Laboratories high- and low-molecular-weight, prestained markers were used as standards.

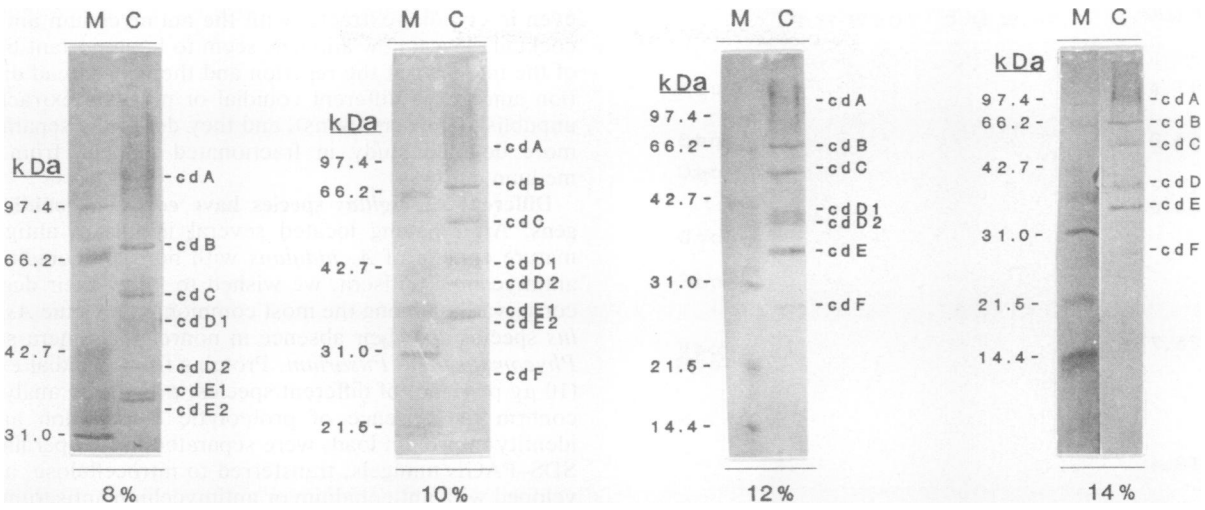

FIG. 3. Immunoblots of *A. nidulans* G1059wt conidial extracts (lanes C; 10 μg of protein per lane) separated on different percent T acrylamide gels and developed by using as the first antibody a cocktail of the IgG fractions purified from the best sera of Fig. 3 at a 1/800 dilution. Bio-Rad low-molecular-weight biotinylated markers (lanes M) were used as standards.

antigenic patterns obtained. For all the bleedings, the number and the intensity of the detected antigens increased with time, reaching a plateau and then occasionally decreasing in intensity later. Preimmune sera gave only a slight reaction in a narrow zone around 15 kDa.

Seven proteins, or rather polypeptide zones, proved to be immunogenic to a greater or lesser extent. We have designated them in order of decreasing apparent molecular weights: cdA, cdB, cdC, cdD, cdE, cdF, and cdG. Antigenic zones cdB, cdD, cdE, and cdF were clearly detected with sera from all the rabbits from the beginning of the immunization (cdB and cdD from the first bleeding and cdE and cdF from the second one). The cdC antigen was detected in three of four rabbits from the first bleeding. Finally, antigen cdA was barely detected in one of four cases, as was the case with cdG, but with a quite intense reaction from the second bleeding.

To characterize these antigens more exactly, they were separated in different percent T SDS-PAGE gels, transferred to nitrocellulose paper, and reacted with an IgG fraction, previously purified from a cocktail serum obtained after pooling the best bleedings (D2, D3, D4, D5, E2, E3, E4, E5, and F4) of the previous experiment. Figure 3 shows the antigenic patterns obtained. Ten percent and 8% T gels demonstrated that the polypeptides cdD and cdE were not single bands but rather consisted of two components each, giving rise to the new ones designated cdD1, cdD2, cdE1, and cdE2 antigens, making a total of eight antigenic polypeptides (because cdG is almost undetectable with the cocktail serum). With the data from these blots, a Ferguson plot was constructed to check the electrophoretic behavior of the antigens and markers. As can be seen in Fig. 4, cdA, more intensely so, and cdD1 displayed anomalous electrophoretic behavior, which together with their staining patterns suggests that they are glycoproteins. For this reason, their approximate apparent molecular weights were calculated from the asymptotic minimal molecular weight obtained after plotting the apparent molecular weight values, obtained by comparison with the markers in gels of different acrylamide concentrations, against the percent T. Retardation coefficients (K_rs) were calculated for standards of known molecular weights and for the remaining antigens. Then, a standard curve was constructed by plotting the K_rs of markers against their molecular weights and used to determine the apparent molecular weights of the antigens. The values thus calculated are as follows (in kilodaltons): cdA, 120; cdB, 71; cdC, 55.3; cdD1, 42; cdD2, 40.8; cdE1, 35.9; cdE2, 35.4; and cdF, 28.5. An apparent molecular mass of 26 kDa was calculated for cdG. Prestained markers have never been used for molecular weight determinations because of the broad zones they provide and the alterations in migration provoked by the attached dye (as an example, the

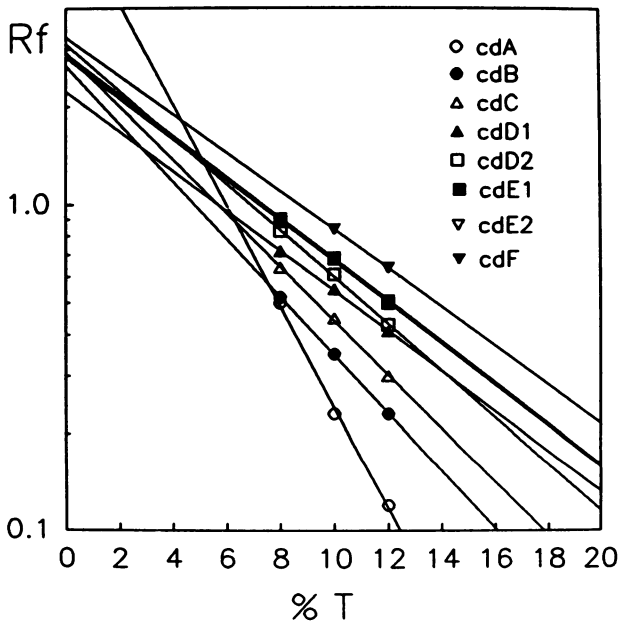

FIG. 4. Ferguson plot of detected conidial antigens calculated according to data from Fig. 3 (note the anomalous behavior of the cdA and cdD1 antigens).

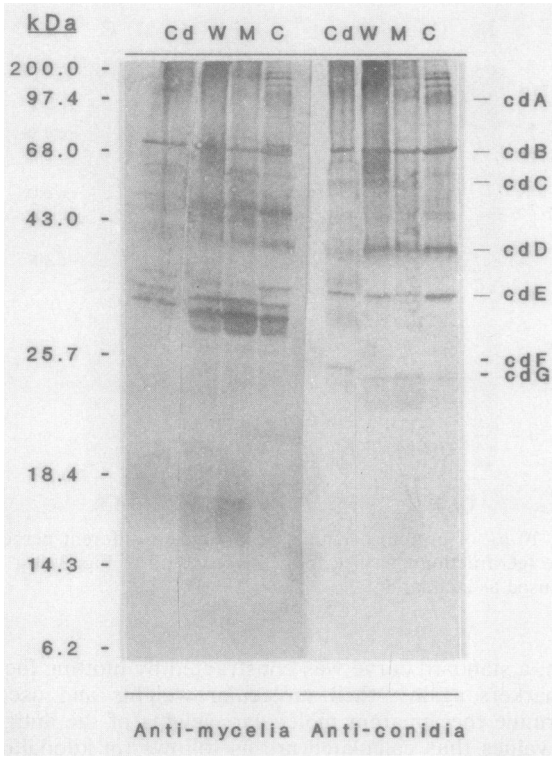

FIG. 5. Immunoblots of extracts from different growth phases of *A. nidulans* G1059wt (60 μg of protein per lane), developed by using, as the first antibody, cocktails of IgG fractions purified from sera raised against mycelial or conidial extracts at 1/800 dilutions. Bethesda Research Laboratories high- and low-molecular-weight prestained markers were used as standards. Lanes: Cd, conidial extracts; W, mycelial cell wall fraction; M, mycelial total membrane fraction; C, mycelial cytosolic fraction.

prestained 25.7-kDa marker runs almost the same as the 31-kDa unstained marker).

Conidial antigens are present throughout mycelial growth. Since the conidia of the pathogenic *Aspergillus* spp. enter the lung and germinate there, it was of great interest to ascertain whether the described conidial antigens were present throughout the vegetative growth of the fungus. To do this, we looked for the presence of the antigens in three different fractions, those of the cell wall, total membranes, and cytosol from 25-h mycelia of *A. nidulans* G1059wt grown in YED liquid medium, compared with conidial extracts. Figure 5 shows the results of a Western blot experiment in which the right panel was developed with the cocktail of anticonidium antisera used in previous experiments. In this case, the antigens designated cdA, cdB, cdD, cdE, and cdG were readily detected in all the vegetative fractions (the cdG reaction was almost undetectable in conidia, as in the previous experiment, probably due to a lower concentration than in mycelia); cdC appeared both in membranes and the cell wall but was barely detectable in the cytosolic fraction, and cdF was totally absent in the mycelial fractions. When a parallel blot was developed with another cocktail of antimycelium antisera (to be described), some of these antigens were again recognized, namely, cdA, cdB, cdD, and cdE. Some others, such as cdC, were almost undetectable and absent in the cytosolic fraction, and cdF and cdG were not reactive at all.

Some other new major antigenic bands were detected, even in conidial extracts, with the antimycelium antiserum cocktail. These new antigens seem to be important because of the intensity of the reaction and the widespread distribution among the different conidial or mycelial extracts (our unpublished observations), and they deserve a separate and more detailed study in fractionated mycelia from liquid medium cultures.

Different *Aspergillus* species have common conidial antigens. After having located several important antigens in mature conidia of *A. nidulans* with both anticonidium and antimycelium antisera, we wished to know their degree of conservation among the most common pathogenic *Aspergillus* species and their absence in nonrelated genera such as *Phycomyces* and *Fusarium*. Proteins from conidial extracts (10 μg per lane) of different species, previously analyzed to confirm the absence of proteolytic degradation and the identity in protein load, were separated in two parallel 14% SDS–PAGE minigels, transferred to nitrocellulose, and developed with anticonidium or antimycelium antiserum cocktail, respectively. Figure 6 shows that *F. culmorum* (lane H) gave no reaction at all with anticonidium antibodies and only a slight one with antimycelium antibodies. *P. blakesleeanus* (lane I) reacted with both antibodies but more markedly with the antimycelium ones in zones cdA, cdB, and cdC.

All of the *Aspergillus* species analyzed in our assays shared certain antigenic zones. With anticonidium antiserum, the following antigenic zones were recognized in the following species: cdA, cdB, and cdC in all *Aspergillus* species; cdE in *A. flavus* (Fig. 6, right panel, lane B), *A. fumigatus* (lane C), and *A. terreus* (lane D); cdF and cdG in *A. nidulans* (lane F) and *A. niger* (lane A); and cdD only in *A. nidulans*. With antimycelium antiserum, cdA, cdB, cdC, and cdE proved to be present in all the *Aspergillus* species; cdD was lightly detected in all the species except *A. flavus* (left panel, lane B) and *A. terreus* (lane D).

The previously detected new major antigens reactive only with antimycelium antiserum were present in all the *Aspergillus* spp. and absent in the other genera.

DISCUSSION

A considerable volume of information has been accumulated in recent years on the antigenic properties of different fractions of *A. fumigatus* cultures. New separation and detection methods have been applied to obtain higher sensitivity and accuracy in the detection of immunogenic molecules. As a result of these efforts, the serodiagnosis of aspergillosis is beginning to be a more reliable diagnostic method. However, some important problems still remain unsolved, mainly those concerning reproducibility in the preparation of antigenic extracts and the purification and characterization of defined antigens that can be produced in large amounts and used as routine reagents.

A. nidulans is a fungus with a very well characterized genetic system, and it has been used extensively to clone and overexpress different protein molecules, mainly enzymes. This suggested to us the idea of carefully analyzing the antigenic properties of this species and testing whether they were, at least in part, coincident with those of *A. fumigatus* and the other pathogenic aspergilli. If this were the case, in the future we would theoretically be able to clone the genes coding for the antigens of interest and overproduce them in a convenient system. We have started the analysis of conidial antigens assuming that, since these forms of resistance are the first fungal element to enter into contact with the lung, they are probably responsible for triggering the im-

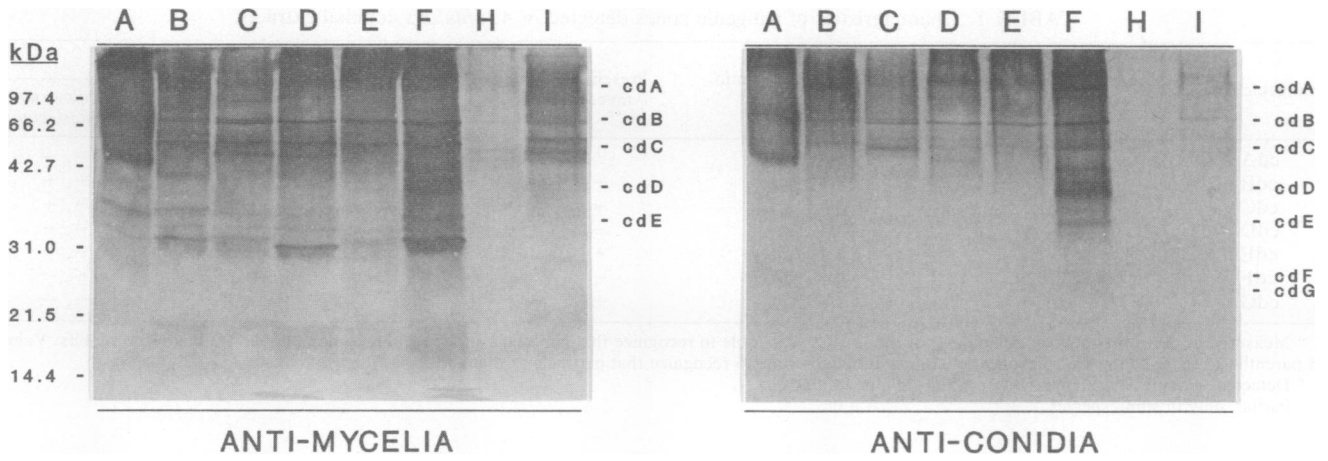

FIG. 6. Immunoblots of *Aspergillus* conidial extracts (10 μg of protein per lane), developed by using, as the first antibody, cocktails of purified antimycelium IgG and anticonidium IgG at 1/800 dilutions. Bio-Rad low-molecular-weight biotinylated markers were used as standards. Lanes: A, *A. niger*; B, *A. flavus*; C, *A. fumigatus*; D, *A. terreus*; E, *A. clavatus*; F, *A. nidulans* G1059wt; H, *F. culmorum*; I, *P. blakesleeanus*.

mune response. In addition, in spores, the presence of important structural antigenic proteins is not so dependent on the culture medium used. We describe here a comparative analysis of *Aspergillus* conidial proteins. Previous work has compared the protein composition of mycelial phases from different *A. fumigatus* strains (2, 21). The patterns shown in these works are quite similar to those presented here with respect to the number of bands detected and their molecular weight distribution. These results contrast with those described in several papers in which the number of bands is very low, even when two-dimensional electrophoresis and sensitive silver staining methods were used (15, 25). This is probably due to the differences in the ages of the cultures or the nature of the cellular fraction analyzed, since cytosolic fractions of old cultures tend to lose high numbers of proteins present in young cultures due to the action of their own internal proteolytic activities (26).

An analysis of the total protein composition by SDS-PAGE provided very little information about the degree of similarity between conidia from different *Aspergillus* species. However, it did demonstrate the lack of proteolytic degradation in our conidial preparations, and, as a consequence, the convenience of using these preparations to check their ability as immunogens. This is an important difference between this and other studies, in which only the ability of conidial proteins to react with antimycelium or anti-culture filtrate antibodies had been analyzed. We used an immunization protocol characterized by injecting low amounts of protein per shot and long intervals between shots, with the idea of raising antibodies only against the more highly immunogenic components of the extracts. Following the time course production of specific antibodies by Western blot, we were able to define those antigens which induced antibody formation from the beginning and throughout the whole immunization protocol. Additionally, we used several rabbits and two close *A. nidulans* strains to confirm the immunogenicity of the antigens detected in different individuals. Further, we prepared a cocktail of the purified IgG fraction of the best antisera to define the most important antigens more precisely. Since many of the previously described *Aspergillus* antigens are glycoproteins (8, 10, 12, 14) and since these molecules usually behave abnormally in SDS-PAGE, we decided to use different percent T acrylamide gels to obtain a better view of the proteins present in our extracts. The results showed two anomalously running antigens and also showed that using a single percent T gel is generally insufficient to discriminate all the antigens present in a particular extract.

This approach allowed us to add two new antigens to those initially described. Considering these observations, in this paper we refer to antigenic zones instead of antigenic proteins. Two-dimensional electrophoresis is currently helping us to unravel the actual number of polypeptides present in each antigenic zone. That the antibodies present in the cocktail serum were raised against antigens of conidial preparations does not mean that these are exclusive or specific to conidia. To ascertain whether this was the case for any of the antigens detected, we searched for cross-reactions with cell fractions obtained from exponentially growing mycelia of *A. nidulans*. We checked extremely carefully that no contamination was occurring between mycelial and conidial extracts. Mycelia were obtained after 25-h growth in liquid culture (in which *A. nidulans* is unable to conidiate), and, as confirmed by microscopic examination, nongerminated conidia were totally absent from the medium. Conidia were collected from plates with medium containing only 0.1% D-glucose, and, under these conditions, the amount of aerial hyphae produced was extremely low, most of the mat being constituted by conidiophores and conidia. Conidiophore and mycelial contaminants were easily washed away after several centrifugation steps. The purity of conidial preparations was confirmed by microscopic examination. With respect to the subcellular fractionation used, we never considered any of the separated fractions (i.e., those of the cell wall, membranes, or cytosol) as pure fractions but only as a more operative way to obtain a more sensitive detection and more-detailed information about the protein composition of *A. nidulans*. Consequently, the presence of an antigen in one of these fractions was never considered as a criterion for assigning an exclusive localization to that antigen.

Piechura et al. (25) have described the changes detected in cytoplasmic antigens during the growth of conidia to mature mycelia by using an antiserum raised against mature myce-

TABLE 1. Characteristics of antigenic zones detected in *A. nidulans* conidial extracts

Antigenic zone	Apparent molecular mass (kDa)	Immunogenicity ratio (bleeding)[a]	Presence in mycelia	Antimycelium reaction	Presence in:	
					Aspergillus spp.	*F. culmorum*, *P. blakesleeanus*[b]
cdA	120	1/4 (2nd)	−/+	+	General	+, +
cdB	71	4/4 (1st)	+	+	General	+, +
cdC	55.3	3/4 (1st)	+	+	General	−, +
cdD	40–42	4/4 (1st)	+	+	Partial[c]	−, −
cdE	35–36	4/4 (2nd)	+	+	General	−, −
cdF	28.5	4/4 (2nd)	−	−	Partial	−, −
cdG	26	1/4 (2nd)	+	−	Partial	−, −

[a] Measured as the ratio between the number of rabbits with sera able to recognize that particular antigen and the total number of immunized rabbits. Values in parentheses indicate the first bleeding containing antibodies able to recognize that particular antigen.
[b] Detected with either anticonidium or antimycelium antibodies.
[c] Partial, only in some species.

lial cytosol as a detector. In our case, we have noticed no significant variations, probably due to the fact that our serum was an anticonidium antiserum and above all because we used total conidial extracts, not only cytosol, to raise our antibodies. As mentioned above, the degree of conservation of antigenic proteins is higher in the structural fractions of different strains and growth phases. Only one antigen (cdF) seems to be of exclusive conidial localization and could be used in the future as a specific marker for this stage of differentiation.

There have been some descriptions in cross-reactivity studies among *Aspergillus* spp. limited to mycelial or culture medium antigens. The conclusion is that the presence of species-specific antigens is more frequent than the common ones (2, 3, 11, 16, 24). Our results suggest that, by using extracts in which structural proteins are well represented, it is easier to find antigens common to different species. The SDS extraction protocol we used is expected to provide a small amount of structural sugar components (such as glucans, chitin, galactomannan, etc.). For this reason, the observed cross-reactivity is probably due only to the polypeptide or glycoprotein components. Furthermore, the cross-reaction with members from unrelated genera could be abolished without the loss of specific reactions by further dilution (1/1,600 or more) of the detector sera.

In conclusion, as summarized in Table 1, the antigens designated cdB, cdC, cdD, and cdE seem to be important because they are immunogenic from the beginning of the immunization protocol; they are also detectable in mycelial fractions and can be recognized by antibodies raised against mycelial fractions. Zones cdB, cdC, and cdE have the additional interest of being common to all the *Aspergillus* species tested. Only cdE is totally absent in nonrelated genera such as *Phycomyces* or *Fusarium*. Work is in progress in our laboratory to check whether any of these antigens can be recognized by antisera from individuals suffering from *Aspergillus*-related diseases.

Recently, a very interesting 58-kDa *A. fumigatus* antigen has been reported (8, 9). It is a glycoprotein accounting for almost 50% of the Coomassie brilliant blue-stained protein from mycelial extracts. Two classes of monoclonal antibodies (IgG and IgM) to this antigen have been elicited and have been used in affinity purification protocols. The purified antigen showed reactivity with 90% of 38 human serum samples from invasive-aspergillosis-affected individuals. This fact confers potential value to both the antigen and monoclonal antibodies for the immunodiagnosis of aspergillosis. It is difficult to establish any correspondence between this and other previously detected antigens, considering that the strains used as the source of immunogens and sometimes even the methods of detection are different from those of other studies. In this paper, we describe polypeptides with a similar molecular weight detected in extracts from all the *Aspergillus* species tested (i.e., cdC). However, none of them was present in such high proportions as the 58-kDa antigen. Since our preparations probably contained a higher number of proteins (because of the SDS extraction), this might result in dilution of that particular antigen. Only comparative Western blot analysis after two-dimensional electrophoresis will provide useful information on the possible correspondence among antigens from different sources.

ACKNOWLEDGMENTS

We thank Carlos Belinchón for photographic services and Ramón Santamaría for critical reading of the manuscript. We are grateful to Nicholas Skinner for revising the English manuscript.

This work was supported by grant PB86-0206 from the DGICYT of the Ministerio de Educación y Ciencia (Spain).

REFERENCES

1. **Bardana, E. J., S. Craig, K. Strangfeld, and A. Montanaro.** 1988. Antigenic differences between exoantigens, mycelial and conidial antigens in *Aspergillus*, p. 345–354. *In* E. Drouhet, G. T. Cole, L. de Repentigny, J. P. Latge, and B. Dupont (ed.), Fungal antigens. Plenum Press, New York.
2. **Burnie, J. P., R. C. Matthews, I. Clark, and L. J. R. Milne.** 1989. Immunoblot fingerprinting *Aspergillus fumigatus*. J. Immunol. Methods **118**:179–186.
3. **Chaparas, S. D., L. Kaufman, S. J. A. M. Kim, and D. W. McLaughlin.** 1980. Characterization of antigens from *Aspergillus fumigatus*. V. Reactivity in immunodiffusion tests with serums from patients with aspergillosis caused by *Aspergillus flavus*, *A. niger* and *A. fumigatus*. Am. Rev. Respir. Dis. **122**:647–649.
4. **Clowes, R. C., and W. Hayes.** 1968. Experiments in microbial genetics, p. 159–190. Blackwell Scientific Publications, Ltd., Oxford.
5. **de Repentigny, L., and E. Reiss.** 1984. Current trends in immunodiagnosis of candidiasis and aspergillosis. Rev. Infect. Dis. **6**:301–312.
6. **Eslava, A. P., M. I. Alvarez, P. V. Burke, and M. Delbruck.** 1975. Genetic recombination in sexual crosses of *Phycomyces*. Genetics **80**:445–462.
7. **Eslava, A. P., M. I. Alvarez, and M. Delbruck.** 1975. Meiosis in *Phycomyces*. Proc. Natl. Acad. Sci. USA **72**:4076–4080.
8. **Fratamico, P. M., and H. R. Buckley.** 1991. Identification and characterization of an immunodominant 58-kilodalton antigen of *Aspergillus fumigatus* recognized by sera of patients with invasive aspergillosis. Infect. Immun. **59**:309–315.

9. **Fratamico, P. M., W. K. Long, and H. R. Buckley.** 1991. Production and characterization of monoclonal antibodies to a 58-kilodalton antigen of *Aspergillus fumigatus*. Infect. Immun. **59**:316–322.
10. **Harvey, C., and J. L. Longbottom.** 1986. Characterization of a major antigenic component of *Aspergillus fumigatus*. Clin. Exp. Immunol. **65**:206–214.
11. **Hearn, V. M.** 1988. Serodiagnosis of aspergillosis, p. 43–74. *In* H. Van den Bosche, D. W. R. Mackenzie, and G. Cauwenbergh (ed.), *Aspergillus* and aspergillosis. Plenum Press, New York.
12. **Hearn, V. M., E. V. Wilson, and D. W. R. Mackenzie.** 1988. Characterization of purified wall antigens obtained from *Aspergillus* species, p. 355–366. *In* E. Drouhet, G. T. Cole, L. de Repentigny, J. P. Latge, and B. Dupont (ed.), Fungal antigens. Plenum Press, New York.
13. **Kauffman, H. F., S. van der Heide, F. Beaumont, J. de Monchy, and K. de Vries.** 1984. The allergenic and antigenic properties of spore extracts of *Aspergillus fumigatus*: a comparative study of spore extracts with mycelium and culture filtrate extracts. J. Allergy Clin. Immunol. **73**:567–573.
14. **Kurup, V. P., K. V. John, A. Resnick, and J. N. Fink.** 1986. A partially purified glycoprotein antigen from *Aspergillus fumigatus*. Int. Arch. Allergy Appl. Immunol. **79**:263–269.
15. **Kurup, V. P., A. Resnick, G. H. Scribner, M. Gunasekaran, and J. N. Fink.** 1986. Enzyme profile and immunochemical characterization of *Aspergillus fumigatus* antigens. J. Allergy Clin. Immunol. **78**:1166–1173.
16. **Kurup, V. P., A. Resnick, G. H. Scribner, J. H. Kalbfleisch, and J. N. Fink.** 1984. Comparison of antigens and serological methods in *Aspergillus fumigatus* antibody detection. Mykosen **27**:43–49.
17. **Kurup, V. P., E. Y. Ting, and J. N. Fink.** 1983. Immunochemical characterization of *Aspergillus fumigatus* antigens. Infect. Immun. **41**:698–701.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Longbottom, J. L.** 1986. Applications of immunological methods in mycology. *In* D. M. Weir (ed.), Handbook of experimental immunology, 4th ed., vol. 4. Applications of immunological methods in biomedical sciences. Blackwell Scientific Publications, Ltd., Oxford.
20. **Longbottom, J. L., C. Harvey, M. L. Taylor, P. K. C. Austwick, P. Fitzharris, and C. A. Walker.** 1989. Characterization of immunologically important antigens and allergens of *Aspergillus fumigatus*. Int. Arch. Allergy Appl. Immunol. **88**:185–186.
21. **Mishra, S. K.** 1984. Antigenic profile of some typical and septate phialide strains of *Aspergillus fumigatus*. Sabouraudia **22**(2):91–100.
22. **Morrisey, J. H.** 1981. Silver stain for proteins in polyacrylamide gel: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
23. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**:346–356.
24. **Piechura, J. E., V. P. Kurup, J. N. Fink, and N. J. Calvanico.** 1985. Antigens of *Aspergillus fumigatus* III. Comparative immunochemical analyses of clinically relevant aspergilli and related fungal taxa. Clin. Exp. Immunol. **59**:716–724.
25. **Piechura, J. E., R. S. Riefel, and L. J. Daft.** 1987. Electrophoretic and serological analyses of cytoplasmic antigens from *Aspergillus fumigatus* during growth of conidia to mature mycelia. J. Med. Vet. Mycol. **25**:243–254.
26. **Puente, P., M. C. Ovejero, and F. Leal.** Unpublished data.
27. **Tamame, M., F. Antequera, J. R. Villanueva, and T. Santos.** 1983. High frequency conversion to a "fluffy" developmental phenotype in *Aspergillus* spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. Mol. Cell. Biol. **3**:2287–2297.
28. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
29. **Walsh, T. J., and P. A. Pizzo.** 1988. Nosocomial fungal infections: a classification for hospital-acquired fungal infections and mycoses arising from endogenous flora or reactivation. Annu. Rev. Microbiol. **42**:517–545.