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Variability of *Aspergillus nidulans* Antigens with Media and Time and Temperature of Growth

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The influence of culture medium and time and temperature of growth on the appearance of *Aspergillus nidulans* antigens was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by silver staining or Western blot (immunoblot), of the proteins present in total cellular extracts or culture supernatants. Samples in the exponential, deceleration, and stationary growth phases were selected by biochemical, morphological, and ultrastructural criteria. Protein and antigen patterns (detected with rabbit antibodies) from total extracts were very similar in all cases, and the major differences observed seemed to depend on the age of the cultures. Culture supernatant patterns were highly dependent on the type of medium (complex or defined) and the age of the culture. Temperature did not significantly influence these results. The reproducible reactivity of selected human sera from aspergilloma-affected individuals was strictly associated with the use of defined media, especially Czapek Dox-AOAC, in both total extracts and culture supernatants. Extended growth times were necessary in the case of metabolic antigens (those obtained from culture supernatants). Screening of a battery of 10 selected human serum samples from patients with aspergilloma or invasive aspergillosis demonstrated that two of the antigens (96 to 98 and 45 kDa) from stationary-phase culture supernatants in Czapek Dox-AOAC medium were consistently reactive. When considered together as one unit, both antigens reacted with more than 50% of the sera, and at least one or the other of the antigens reacted with more than 90% of the sera. Less consistent results were obtained for two somatic antigens (from total cell extracts) of 45 to 50 and 20 to 22 kDa.

In both humans and other animals, infection with different species belonging to the genus *Aspergillus*, mainly *Aspergillus fumigatus* and *A. flavus* but also *A. terreus*, *A. clavatus*, *A. niger*, and *A. nidulans*, elicits several pathologies of increasing severity depending on the immunological situation of the individual affected. From the more localized fungal ball or aspergilloma in tuberculosis patients in ancient times to the serious disseminated aspergillosis in situations of immunosuppression (neutropenic, leukemic patients, transplantation recipients, etc.) to lung invasion or local invasive aspergillosis or allergy reactions such as allergic brochopulmonary aspergillosis, a rapid and accurate diagnosis is needed for timely application of the appropriate therapy. At present, several methods are routinely used (for a review, see reference 10), but all of them suffer from the same problems and limitations: the lack of standardized antigens or antibodies and methods leads to wide variations in the results from different laboratories. Commercial preparations are not uniform, and consequently many hospital mycological diagnostic services produce their own reagents. In recent years, considerable effort has been devoted to the search for standard antigens. As a result, some major antigens of *A. fumigatus* of potential use in diagnosis have been characterized. In a series of papers, Kurup's group described the CS2 complex (2, 16) and two relevant glycoprotein antigens (9, 11); Fratamico et al. isolated a 58-kDa glycoprotein (4, 5), and Latgé et al. found an 18-kDa antigen which was also present in the urine of patients with invasive aspergillosis (13), identified with the mycotoxin restrictocin (1). Additionally, circulating galactomannan has been detected in both urine and serum specimens in disseminated cases (3). Recently, a monoclonal antibody which constitutes the base of a commercially available latex agglutination test has been raised (20). However, further work is needed to characterize more antigens from *A. fumigatus* and other *Aspergillus* species and to produce them in a quick, easy, and reproducible way. It was for these reasons that we undertook the task of identifying and characterizing the antigenic properties of *A. nidulans* with the idea of future use of this species as a source of antigens or as an expression system for *A. fumigatus* antigens. In two previous papers (17, 18), we had concentrated our efforts on the ability of *A. nidulans* proteins to elicit an antibody response in rabbits. Here, we demonstrate that those antibodies were raised against proteins constitutively expressed in several culture media and that they do not recognize the same set of somatic antigens as human sera from aspergilloma patients. However, they do react with some metabolic antigens reproducibly recognized by human sera. These results indicate that common epitopes are probably shared by somatic and metabolic antigens of the same or different *Aspergillus* species and validate the use of *A. nidulans* as a valuable source of antigens.

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MATERIALS AND METHODS

Organism and growth conditions. The fungal strain used was *A. nidulans* G1059wt (*adF17 pabaA1 yA2*) from A. J. Clutterbuck, Glasgow, Scotland.

The organism was maintained on solid YED medium (1% D-glucose, 1% Difco yeast extract, and 2% agar). To obtain high yields of conidia, the fungus was grown on solid *Aspergillus* complete minimal medium (AMM) 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 (18). Plates were incubated at 28°C for at least 4 days.

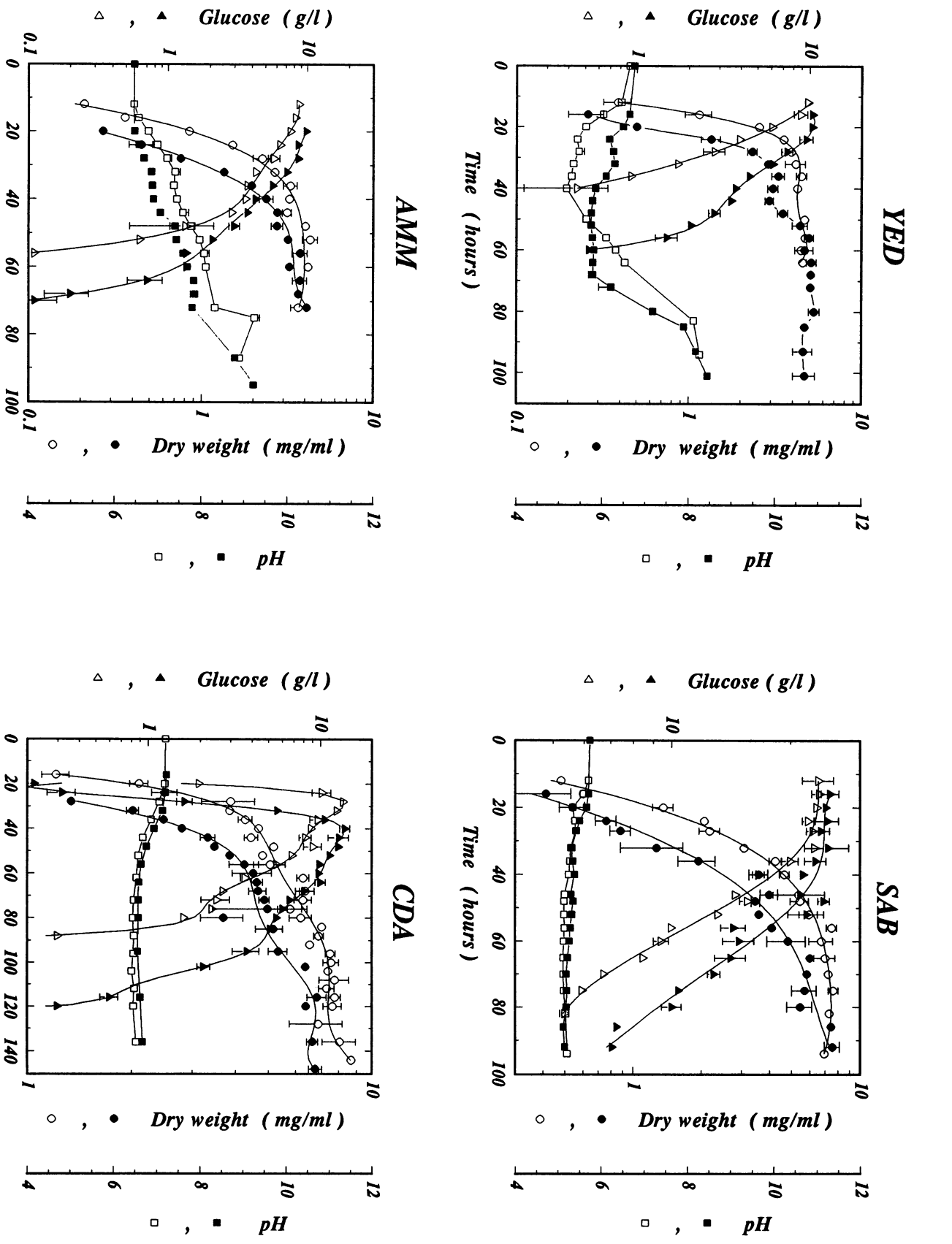

FIG. 1. Growth patterns, at 28°C (closed symbols) or 37°C (open symbols), of *A. nidulans* in four different media, characterized by changes in pH (squares) or glucose content (triangles) of the culture supernatant and dry weight yield of the mycelium (circles).

TABLE 1. Kinetic characteristics of *A. nidulans* growth in different media

Medium	Temp (°C)	Specific growth rate (α_{max},h^{-1})[a]	Doubling time (t_d, h)[a]	Duration of exponential phase (h)	Maximal dry wt (mg/ml)	Time taken to reach maximal dry wt (h)	Sampling times (h)[b]		
							First (E)	Second (D)	Third (S)
YED	28	0.188	3.68	28	5.07	93	23	44	120
	37	0.235	2.95	24	4.58	64	16	28	96
SAB	28	0.076	9.15	36	7.53	92	31	73	163
	37	0.129	5.37	24	7.60	75	24	55	144
AMM	28	0.125	5.54	36	4.10	72	28	53	116
	37	0.169	4.08	24	4.28	52	20	42	96
CDA	28	0.083	8.36	36	7.53	190	39	112	232
	37	0.091	7.60	28	8.05	136	24	73	192

[a] Calculated from the equation, $t_d = \ln 2/\alpha$.
[b] E, exponential; D, deceleration; S, stationary phase.

For liquid growth cultures, four different media were used: YED, AMM, Bacto Sabouraud dextrose broth (SAB), and Bacto Czapek Dox broth-Bacto synthetic broth AOAC (CDA). SAB and CDA media were obtained from Difco Laboratories (Detroit, Mich.). AMM, SAB, and CDA media, both solid and liquid, were supplemented with 10 mg of *p*-aminobenzoic acid and 200 mg of adenine per liter.

Aspergillus was grown by inoculation of 10^5 conidia per ml in 1-liter Erlenmeyer flasks containing 300 ml of the corresponding liquid medium and incubation at 28 or 37°C in an Adolph Kühner orbital shaker at 280 rpm.

Mycelia were harvested from liquid medium cultures by filtering through Whatman GF/C or filter paper and washed thoroughly with double-distilled H_2O. The wet cake was immediately frozen and kept at −70°C until used.

Preparation of cell culture supernatants. After growth, cell culture supernatants were immediately frozen at −70°C and freeze-dried in a Virtis bench top lyophilizer. The dry product was dissolved in a minimal amount of Milli-Q-grade water (10 to 15 ml) and exhaustively dialyzed at 4°C against the same type of water. After being spun at 12,000 × *g* for 15 min at 4°C to sediment insoluble melanin and denatured proteins, the clear supernatants were mixed by vortexing in Corex tubes with 7 volumes of acetone at −70°C and maintained overnight at the same temperature. Precipitated protein was recovered by spinning at 12,000 × *g* for 15 min at 4°C. Pellets were vacuum dried in a Speed-Vac system (Savant Instruments Co.), redissolved in 1 to 2 ml of Milli-Q water, and frozen at −70°C until use.

Preparation of cell extracts. Frozen mycelia were thawed and mixed with lysing buffer (100 mM Tris-HCl [pH 7.5] containing 1 mM EDTA, 5 mM dithiothreitol, 1 mM freshly added phenylmethylsulfonyl fluoride [Sigma Chemical Co.], 5 μg of aprotinin per ml, and 5 μg of pepstatin A per ml [both obtained from Boehringer Mannheim]) to give a dense suspension. Samples were then disrupted in a French press (SLM Aminco), using the 20,000-lb/in^2 cell, previously refrigerated at −20°C, at a pressure of 16,000 lb/in^2. Complete breakage was monitored by microscopic observation. Sodium dodecyl sulfate (SDS), 2% (final concentration), was added to the lysed mycelia, 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. Extracts containing less than 1 mg of protein per ml (see below for determination) were concentrated by precipitation with 7 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.

Analytical determinations. The glucose-oxidase method (kit 510-A; Sigma) was used, according to the manufacturer's instructions, to determine the amount of glucose present in cell culture supernatants. Protein was quantitated in total cell extracts or culture supernatants by a modification of the Lowry method (15).

SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoreses were carried out on a Protean II or Mini-Protean apparatus (Bio-Rad) on isotropic 14 or 16% (wt/vol) acrylamide slab gels (16 by 18 by 0.1 or 8 by 6 by 0.1 cm), using the discontinuous buffer system of Laemmli (12). Molecular weight protein standards were Bio-Rad low-molecular-weight standards and Bethesda Research Laboratories high- and low-molecular-weight or Sigma prestained markers.

Proteins in gels were detected by a sensitive silver stain (14) 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).

Rabbit and human sera. The rabbit immunoglobulins (immunoglobulin G [IgG]) used were those previously described (17, 18).

Human sera were provided by the microbiology laboratories of hospitals from various geographical locations in Spain. Nine of these serum samples were from aspergilloma cases, one was from a patient with invasive aspergillosis, and two were from healthy individuals.

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; Schleicher & Schuell) in a Trans-Blot Cell (Bio-Rad) according to the method of Towbin et al. (19). The 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 (for three 10-min periods) and incubated with horseradish peroxidase-conjugated goat IgG anti-rabbit or anti-human 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

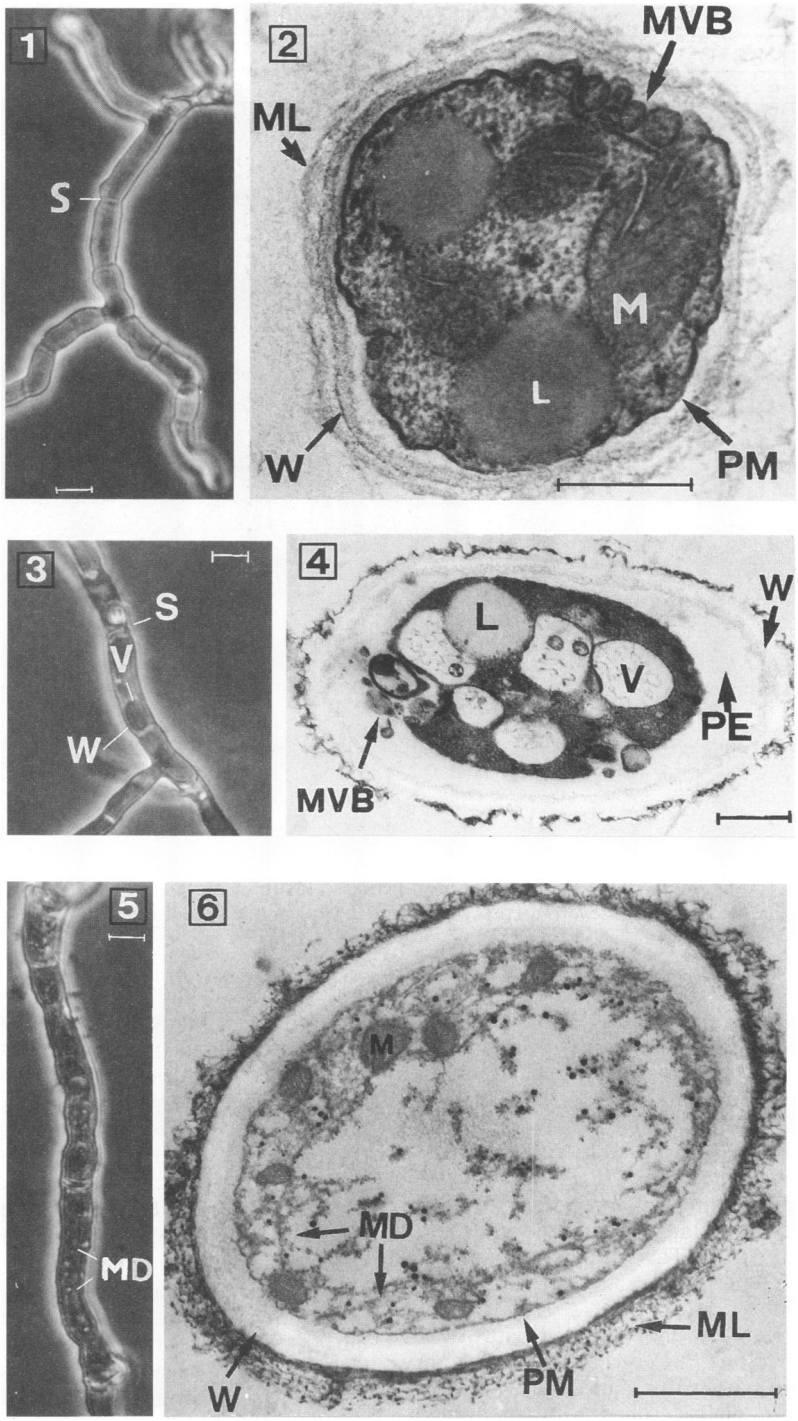

FIG. 2. Morphology of *A. nidulans* mycelium during different phases of growth in CDA (exponential phase, upper panels; deceleration phase, middle panels; and stationary phase, lower panels). Phase-contrast (panels 1, 3, and 5) or transmission electron (panels 2, 4, and 6) micrographs are shown. Bars: 5 μm (panels 1, 3, and 5); 1 μm (panels 2, 4, and 6). Details are given in the text, including definitions of abbreviations.

(four 10-min periods). 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 freshly from stock solutions of 3 mg of 4-chloronaphthol per ml in methanol. After color development, usually 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.

Light microscopy. Samples from liquid cultures were observed with a Nikon Optiphot phase-contrast microscope and photographed with an attached Nikon FX-35 camera, using 100-ASA black-and-white Panatomic X (Kodak) film.

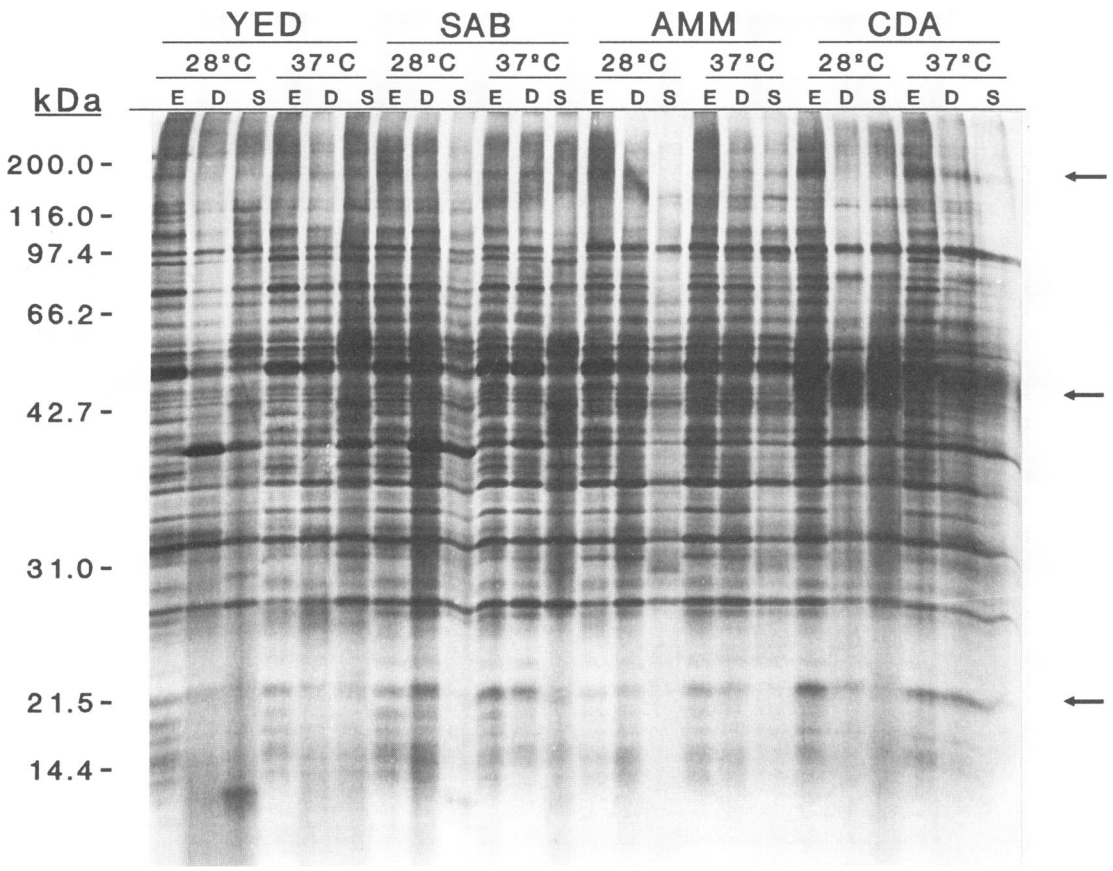

FIG. 3. Silver-stained SDS-PAGE profiles of *A. nidulans* mycelial extracts (15 μg of protein per lane) separated on 16% acrylamide gels. Bio-Rad high- and low-molecular-weight markers were used as standards. E, exponential phase; D, deceleration phase; S, stationary phase.

Electron microscopy. Hyphae were fixed with 1% (vol/vol) glutaraldehyde–1% formaldehyde in 0.05 M phosphate buffer (pH 7.2) for 30 min on ice (0 to 4°C). Samples were washed three times for 10 min each with the same phosphate buffer and postfixed with 1% (wt/vol) osmium tetroxide in phosphate buffer over 2 h in the dark at 4°C. Samples were dehydrated and embedded in Spurr resin. Sections were cut with glass knives on an ultramicrotome (Ultratome III; LKB Produkter AB, Bromma, Sweden), and staining was carried out with 2% uranyl acetate (pH 4 to 4.5) at room temperature for 20 min and then with alkaline lead citrate. The sections were viewed and photographed under a Phillips EM 300 electron microscope.

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

RESULTS

Characteristics of the growth of *A. nidulans* in complex or synthetic media. The growth of *A. nidulans* in different media was determined by measuring the dry weight, glucose consumption, and pH values at both 28 and 37°C. Data were plotted and analyzed (Fig. 1) with Sigmaplot 5.0 software (Jandel Scientific). Semilogarithmic plots were used (dry weight and glucose consumption) since they provided a more precise definition of the growth phases, and regressions of 4 or 5 orders were applied. In all four media, at both temperatures, the five classic phases were observed: lag phase (not shown), exponential phase (where growth rate is maximal), deceleration phase (when carbon source consumption is between 50 and 90%), stationary phase (when exogenous carbon source is absent from the medium), and lysis phase (when dry weight decreases; not shown). The duration of each phase as related to the others was dependent only on the medium and not on the temperature. YED medium and AMM (Fig. 1) showed longer exponential phases (Table 1), clearly delimited from the shorter deceleration and stationary phases. In SAB and CDA (Fig. 1), exponential growth was very brief compared with decelerative growth. In all cases, the increase in dry weight during the exponential and deceleration phases was a logical consequence of carbon source consumption and was followed by a fall in the original pH towards the acid zone (with the exception of AMM, which underwent a continuous increase throughout growth). When the sugar content declined to zero, the pH remained almost constant in SAB and CDA but increased to basic values in YED medium and AMM. Table 1 shows the values obtained with all media at each temperature for the following: the maximal specific growth rate (α_{max}; the slope of the first-order regression line which defines exponential growth and changes with culture conditions); the corresponding doubling time (t_d); the duration of the exponential phase; and the maximal dry weight yield and the time taken by the fungus to achieve it. For all media, α_{max} was lower at 28°C. The highest α_{max}, and consequently the lowest t_d, was obtained

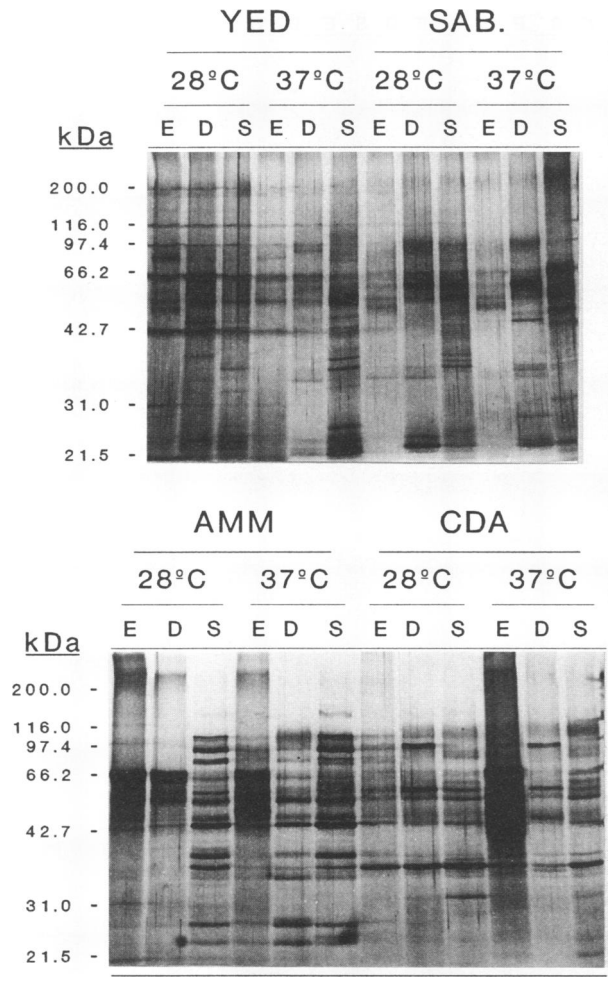

FIG. 4. Silver-stained SDS-PAGE profiles of *A. nidulans* culture supernatants (15 μg of protein per lane) separated on 16% acrylamide gels. Bio-Rad high- and low-molecular-weight markers were used as standards. E, exponential phase; D, deceleration phase; S, stationary phase.

in YED medium at both temperatures, followed by AMM, SAB, and CDA (at 28°C, CDA gave values slightly higher than, although very similar to those of, SAB). Maximal dry weight values were not influenced by temperature and were maximal and similar for SAB and CDA (2% glucose and 3% sucrose as carbon source, respectively) and approximately 1.6-fold lower for YED medium or AMM (both with 1% glucose). The growing time needed to achieve maximal dry weight was shorter for AMM than for YED medium and SAB and quite long for CDA.

Selection of sampling times. Three points of the curves were selected to study the morphology, ultrastructure, and polypeptide and antigenic composition of the fungus. Table 1 (last three columns) shows the chosen growth times for all media at both temperatures. Selection was made on the basis of the level of carbon source consumption for the exponential (around 80% remaining glucose), deceleration (40% remaining glucose), and late-stationary (0% remaining glucose) phases.

Morphology and ultrastructure of *A. nidulans* grown in complex or synthetic media. Hyphal morphology was similar in all media independently of temperature, although with slight variations. According to light microscopy, fungal hyphae growing in the exponential manner (Fig. 2-1) appeared quite refractive and highly branched, with evident septa (S). Transmission electron microscopy (Fig. 2-2) disclosed well-defined cell walls (W), plasma membranes (PM), large mitochondria (M), and vesicular bodies (MVB). Very thin melanin layers (ML) were observed.

Decelerative growth was characterized by a gradual increase in the vacuolization (V) and the appearance of some partially empty hyphae with prominent cell walls (Fig. 2-3 and -4). In synthetic media, a characteristic swelling in some of the hyphal middle cells or tips was formed, giving rise to globose structures of up to 6 to 8 μm in diameter, with thick cell walls and a large vacuole exactly in the middle (not shown). Transmission electron microscopy (Fig. 2-4) showed that the plasma membrane had shrunk, leaving an empty space towards the cell wall (PE). Lipid droplets (L) were very apparent, and multivesicular bodies were numerous.

The stationary phase was characterized by the abundance of almost empty nonrefringent cell walls (Fig. 2-5 and -6), with some membranous vesicle debris (MD).

In the three above-described growth phases, the hyphal population was not uniform and the reported observations corresponded to the majority of the population.

Polypeptide patterns of total extracts and culture supernatants of *A. nidulans* grown in complex or synthetic media. Mycelia from cultures in YED medium, SAB, AMM, and CDA, at either 28 or 37°C, were collected at the times indicated in Table 1, and total extracts were prepared as described above. Figure 3 shows silver-stained profiles of the polypeptides present in each sample after separation through SDS–16% PAGE. The patterns of all samples were similar, although differences in the amounts of protein in several particular bands were detected. The profiles of any selected time chosen (exponential, deceleration, or stationary phase) in any medium at 28°C were basically the same at 37°C. The profiles of any time of a particular medium, at either temperature, were almost totally coincident with those of the corresponding time of the other media. The number of polypeptides was slightly higher in complex than in synthetic media, and their apparent molecular weights ranged from 14,000 to 200,000, those from 25,000 to 97,000 being more abundant. The number of bands detected in each sample decreased with time for all media and temperatures, being more evident in samples from stationary phases (especially in CDA). Certain peculiarities were striking: (i) CDA profiles at both temperatures were easily distinguishable from the rest, mainly due to the presence of two broad bands around 45 to 55 kDa (Fig. 3, second arrow); (ii) a polypeptide of 20 to 22 kDa (Fig. 3, third arrow) was present in all samples from the exponential deceleration and phases but was absent in the stationary phase. The absence of this polypeptide could be used as a marker for the stationary phase.

Silver-stained polypeptides of culture supernatants, corresponding to the total extracts analyzed previously, separated through SDS–14% PAGE, are shown in Fig. 4. A few faint bands, ranging from 35 to 97 kDa, were detected in complex media (Fig. 4, top), especially in SAB. Synthetic media provided a more characteristic polypeptide pattern (Fig. 4, bottom), with a higher number of more intensely stained bands ranging in apparent molecular weight between 22,000 and 97,000. The number of bands increased with time for all media at either temperature.

Reactivity of polypeptides present in total extracts with rabbit or human sera. To study the immunoreactivity of the polypeptides present in total extracts of *A. nidulans*, prepared

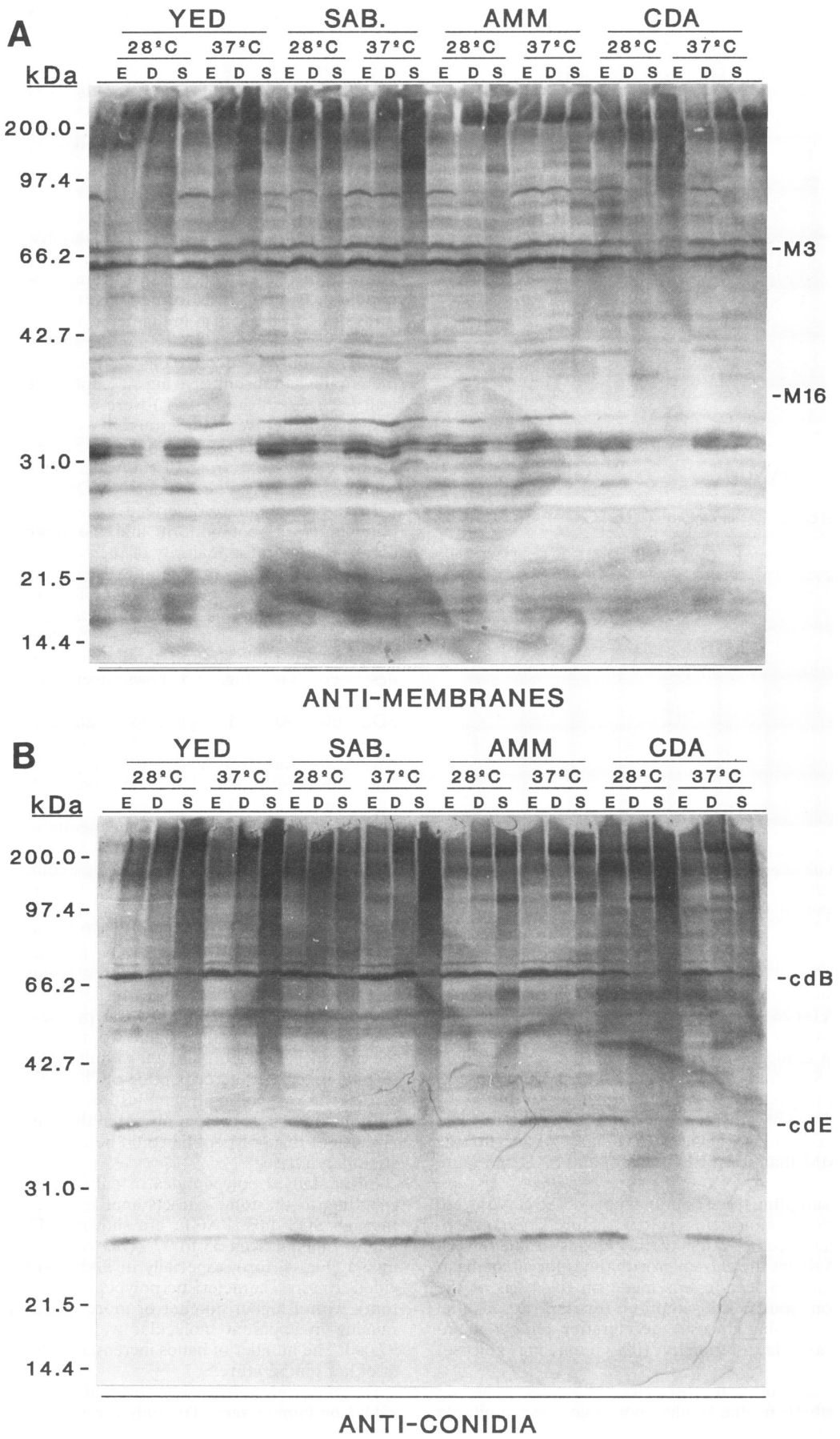
A
YED
SAB.
AMM
CDA
28°C
37°C
E D S
kDa
200.0
97.4
66.2
42.7
31.0
21.5
14.4
-M3
-M16
ANTI-MEMBRANES
B
-cdB
-cdE
ANTI-CONIDIA

FIG. 5. Immunoblot of *A. nidulans* mycelial extracts (60 μg of protein per lane) separated on 14% acrylamide gels and developed using as the first antibody a cocktail of the IgG fractions purified from antimembrane (A) or anticonidium (B) rabbit sera at a 1/400 dilution. Bio-Rad high- and low-molecular-weight prestained markers were used as standards. M3, M16, cdB, and cdE refer to representative antigens consistently detected in previous studies by anti-total membrane (17) or anticonidium (18) rabbit sera. E, exponential phase; D, deceleration phase; S, stationary phase.

as indicated above, four SDS-PAGE gels were transferred to nitrocellulose paper and challenged with antibodies previously raised in rabbits against subcellular fractions from mycelia or total conidial extracts. Figure 5 shows two of the blots, developed with antimembrane (5A) and anticonidium (5B) sera. Analogous results were obtained with the other two sera (not shown). For each serum sample, a different pattern, identical to that previously described (17, 18), was evident (for some representative bands, the published designation is included in Fig. 5 for reference). The pattern defined by each serum sample was quite constant for all media and temperatures. The only differences were the detection of a more intense nonspecific reaction, in the upper part of the blots, in the lanes containing extracts from synthetic media (especially with anticonidium and anti-cell wall sera) and the variability in the intensity of certain particular bands. Temperature did not significantly influence the number of bands detected, which gradually decreased in the deceleration- and stationary-phase samples for all media and temperatures, as did the intensity of most of the bands. All sera recognized antigens of 14 to 200 kDa. Antimembrane serum was the most reactive (as related to both number and intensity of bands), followed by anticytosol, anti-cell wall, and anticonidium sera.

When four selected human serum samples from aspergilloma patients were used to develop the blots, the results changed dramatically (Fig. 6 shows a representative blot). The patterns of complex media were different from those of synthetic media. CDA showed a differential and highly reactive pattern that was quite distinct from those shown by the rest of the media. Three zones, which reacted to varying extents with all sera, were consistently recognized and easily identified in silver-stained extracts (arrows in Fig. 3 and 6 indicate the

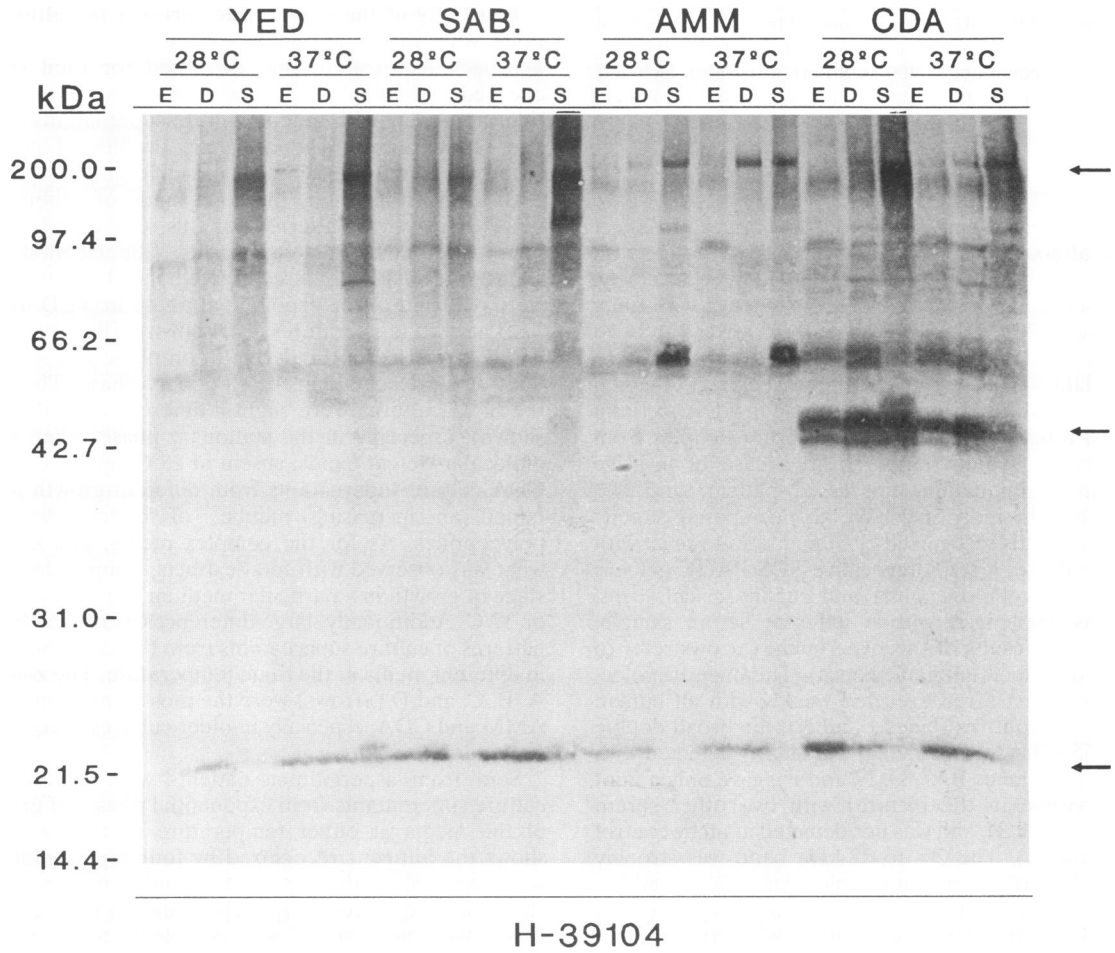

FIG. 6. Immunoblot of *A. nidulans* mycelial extracts (60 μg of protein per lane) separated on 14% acrylamide gels and developed using as the first antibody a selected human serum sample from an aspergilloma case at a 1/250 dilution. Bio-Rad high- and low-molecular-weight prestained markers were used as standards. E, exponential phase; D, deceleration phase; S, stationary phase.

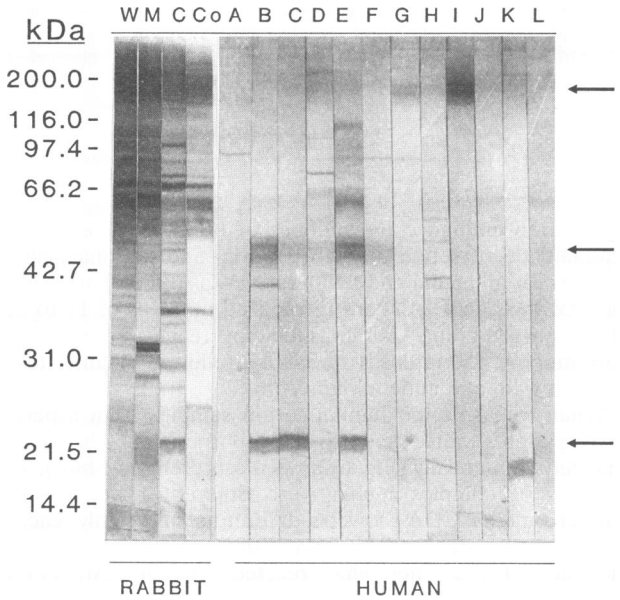

FIG. 7. Immunoblot of *A. nidulans* mycelial extracts (60 μg of protein per lane) separated on 14% acrylamide gels and developed using as the first antibody selected human sera from aspergilloma (lanes A, B, C, D, E, G, H, I, and L)- or invasive aspergillosis (lane F)-affected individuals (1/50 dilution). Control human sera (lanes J and K) or a cocktail of the IgG fractions purified from anti-subcellular fractions of rabbit sera (W, anti-cell wall; M, antimembrane; C, anticytosol; Co, anticonidium; 1/400 dilution) were also included. Bio-Rad low-molecular-weight prestained markers were used as standards.

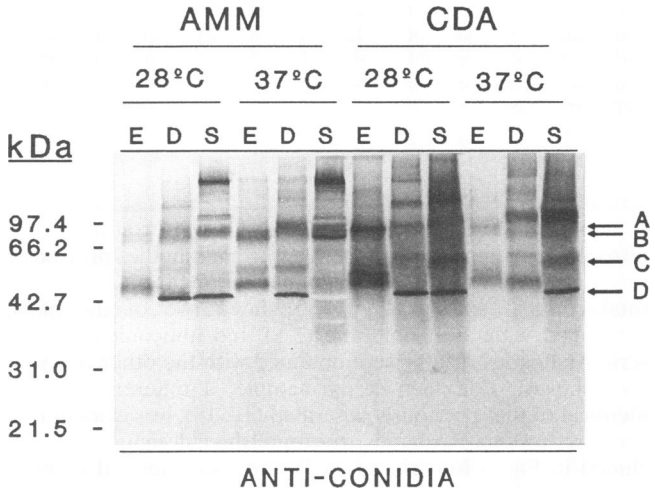

FIG. 8. Immunoblot of *A. nidulans* cell culture supernatants (20 μg of protein per lane) separated on 14% acrylamide gels and developed using as the first antibody a cocktail of the IgG fractions purified from anticonidial rabbit sera at a 1/400 dilution. Bio-Rad low-molecular-weight prestained markers were used as standards. E, exponential phase; D, deceleration phase; S, stationary phase.

corresponding zones). Temperature did not appear to affect the reactivity, although this reactivity increased with time with respect to the high-molecular-weight antigens. Conversely, polypeptides below 55 to 60 kDa were less reactive with time, especially those of 20 to 22 kDa (Fig. 6, third arrow) and two broad bands around 45 to 55 kDa (Fig. 6, second arrow). In view of these data, we decided to use the total extract obtained from *A. nidulans* grown to exponential phase in CDA medium at 28°C to test a battery of nine human serum samples from aspergilloma patients, one sample from a case of invasive aspergillosis in a nonimmunosuppressed patient, and two samples from healthy individuals. We also compared directly the ability of the bands recognized by human sera to react with rabbit sera. To do so, a semipreparative SDS-PAGE gel was transferred to nitrocellulose paper and cut into 1-cm strips. Each strip was challenged with a different serum sample. Figure 7 shows the results of this experiment (arrows refer to the previously described antigenic zones). The highest-molecular-weight zone (first arrow) reacted weakly with all human sera, including the controls (lanes J and K); the broad double band of 45 to 55 kDa (second arrow) was clearly detected with five serum samples (lanes B, C, D, E, and F), gave only a faint reaction (not visible in the picture) with two other serum samples (lanes A and G), and was not detected at all by control sera (lanes J and K). The 20- to 22-kDa band was strongly recognized by three of the serum samples (lanes B, C, and E), gave a weaker reaction with four other serum samples (lanes A, D, F, and L), and did not react at all with control sera. Anticytosol rabbit sera detected a band of identical mobility and aspect. The remaining visible bands reacted in a nonreproducible way or were also present in control sera.

Reactivity of the polypeptides present in culture supernatants with rabbit antibodies or human sera. Following an approach identical to that described for total extracts, we checked the ability of rabbit antibodies to recognize polypeptides appearing with time in culture supernatants of all media and temperatures. The antigenic profiles of complex and synthetic media were drastically different. Surprisingly, for each type of medium, the four kinds of rabbit antibodies detected identical patterns. For this reason, only blots developed with anticonidial antibodies (the cleanest ones) are shown in Fig. 8. Only three to five bands heavier than 65 kDa were detected at the exponential phase in YED medium and SAB at both temperatures (not shown). The intensity of these bands decreased in the deceleration phase and was extremely faint or undetectable in the stationary phase. The patterns of the 37°C cultures were slightly different in both media (not shown), especially in the stationary phase, when some high-molecular-weight bands, absent at 28°C, appeared. AMM and CDA culture supernatants from different growth phases contained an increasing number of better-defined reactive polypeptides. As for the complex media, and conversely to what was observed with total extracts, samples from the same stage of growth in a particular medium were not identical at 28 or 37°C. Additionally, large differences were seen between the patterns of culture supernatants from the same metabolic stage in different media at the same temperature. The zones labelled A, B, C, and D (arrows) were the most consistently reactive in AMM and CDA, especially in older cultures (deceleration and stationary phases).

Sera from aspergilloma patients were not reactive with culture supernatants from exponential phases of growth in any of the media at either temperature (not shown). Figure 9 shows the antigens recognized by four representative human serum samples in the deceleration and stationary phases. Two clearly different profiles (panels A and B) were evident. Type B sera were not reactive with complex medium polypeptides. Only two zones, one of 96 to 98 kDa (arrows A and B) and another of 45 to 50 kDa (arrow D), were recognized in synthetic media. Both zones were always present in type A

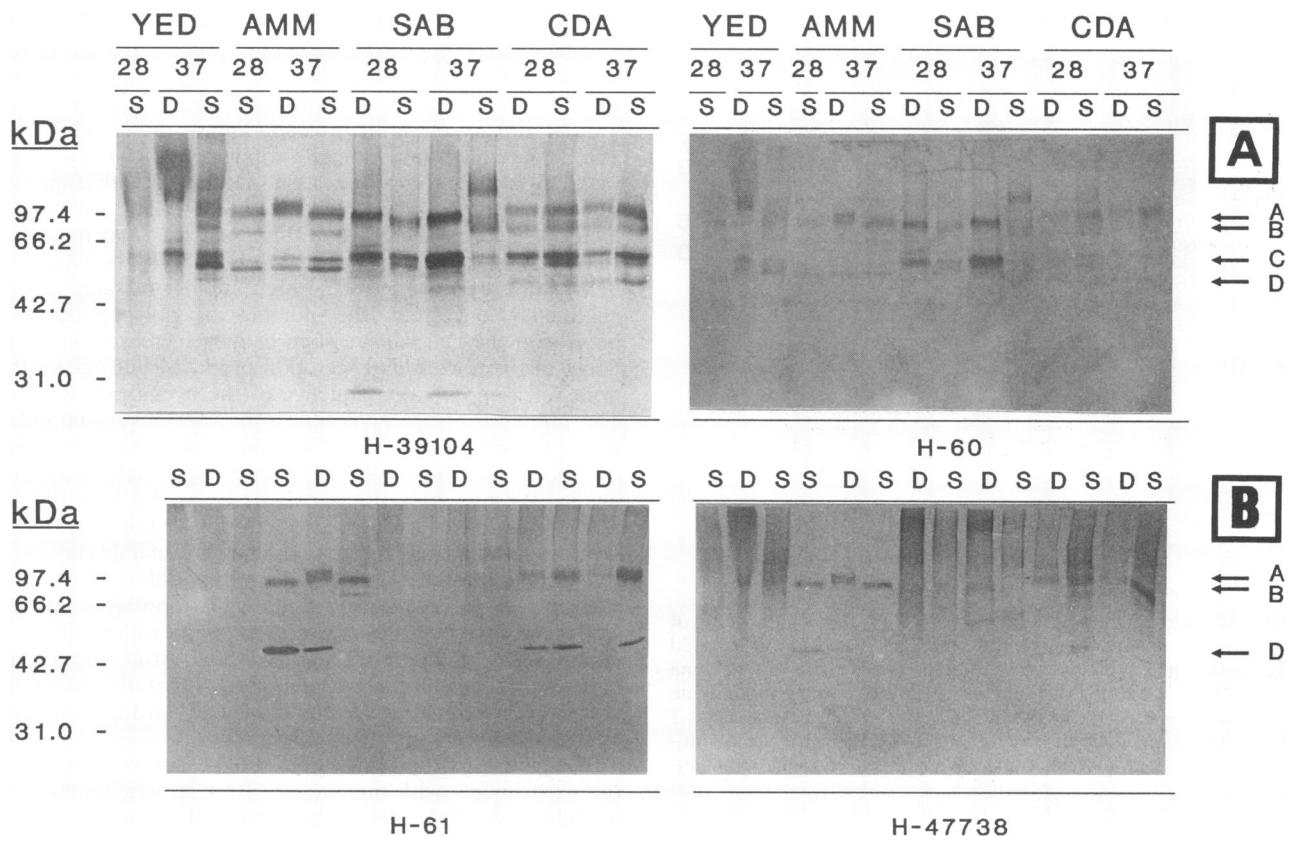

FIG. 9. Immunoblot of *A. nidulans* culture supernatants (20 μg of protein per lane) separated on 14% acrylamide gels and developed using as the first antibody four selected human serum samples (designated with our internal reference numbers: H-39104, H-60, H-61, and H-47738) from aspergilloma cases at 1/250 dilutions. Bio-Rad low-molecular-weight prestained markers were used as standards. S, stationary phase; D, deceleration phase.

profiles (panel A). In the latter profile type, a more reproducible pattern and a higher number of bands were observed. However, a marked degree of variability and lack of a strict correlation between a particular band and the time and temperature of growth in a single medium were noticeable.

We decided to discard complex media due to their lack of reactivity with some of the human sera. Stationary-phase culture supernatants from CDA at 37°C were selected to test the above-mentioned battery of human sera and to study the possible reactivity of the antigens thus recognized with rabbit antibodies. Figure 10 shows that, although each of the serum samples provided its own pattern, the response was much more uniform than in the case of total extracts. A zone of 116 to 125 kDa was detected by the four rabbit antibodies and six human serum samples (lanes B, C, D, E, F, and G). The strongest reaction was seen in a diffuse, probably double band of around 97 kDa (arrows A and B) detected with all sera, very intensely with seven of the serum samples (lanes B, C, D, E, F, H, and I), and weakly with another three samples (lanes A, G, and L). A zone of the same mobility reacted with the four rabbit antibodies. Finally, a thinner band of 45 to 50 kDa (arrow D) was developed by nine aspergilloma serum samples with different degrees of intensity (lanes A, B, C, D, E, F, G, I, and L) and by the four rabbit antibodies (especially those against cell walls and membranes). Three repeatedly reactive bands were also weakly present in the control sera (reaction of these bands could be abolished by further dilution of the sera or by diminishing the amount of antigen in the blot). The remaining bands were detected only occasionally and were not considered (the one designated by arrow C was the most frequently reactive).

DISCUSSION

Traditionally, *A. nidulans* has not been considered a useful source of antigens in the immunodiagnosis of the different forms of aspergillosis. Although, like other species of the genus, it is a potential pathogen, reports on its isolation from patients are occasional compared with those concerning *A. fumigatus*. However, in our opinion, advances in the molecular genetics of *A. nidulans* suggest that it would be worthwhile to attempt standardization of antigen production by this species. In two previous papers, we reported on the ability of total conidial extracts (18) or different subcellular mycelial fractions (17) from *A. nidulans* to elicit antibodies in rabbits. The IgG fractions thus raised cross-reacted with antigens from the well-documented pathogens *A. fumigatus* and *A. flavus* and also *A. terreus*, *A. clavatus*, and *A. niger*. Unfortunately, when we used the same antigenic mixtures to test their reactivity with human sera from aspergilloma patients, the response was very poor. Good results on the influence of culture conditions of *A. fumigatus* on the reactivity of antigenic preparations with human sera have been reported (7, 8, 21, 22). Since previously we had used only extracts from conidia or 25-h growth in YED medium at 28°C, we decided to undertake a detailed search of the culture conditions under which *A. nidulans* might be able to

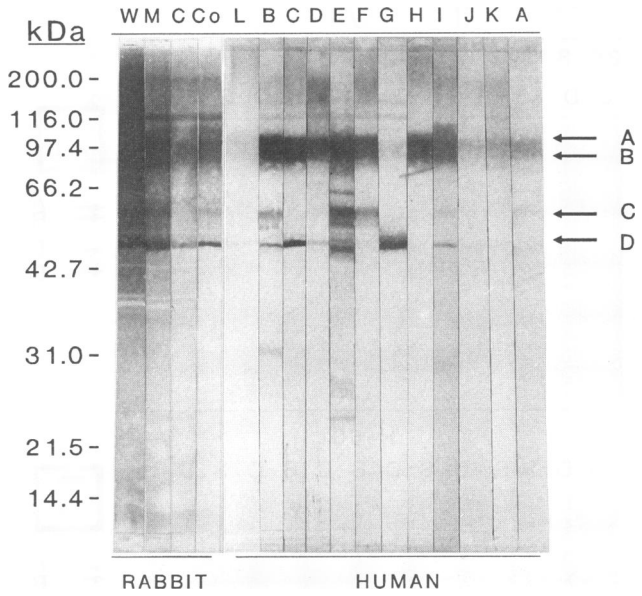

FIG. 10. Immunoblot of *A. nidulans* culture supernatants (60 μg of protein per lane) separated on 14% acrylamide gels and developed using as the first antibody selected human sera from aspergilloma (lanes A, B, C, D, E, G, H, I, and L)- or invasive aspergillosis (lane F)-affected individuals (1/50 dilutions). Control human sera (lanes J and K) and a cocktail of the IgG fractions purified from anti-subcellular fractions of rabbit sera (W, anti-cell wall; M, antimembrane; C, anticytosol; Co, anticonidium at 1/400 dilutions) were also included. Bio-Rad high- and low-molecular-weight prestained markers were used as standards.

demonstrate its potential as a source of good and reproducible antigens for immunodiagnosis of aspergillosis. Both complex and synthetic media have been used to grow *A. fumigatus* for the preparation of antigenic mixtures (for a review, see references 6 and 10). For this reason, we tested two complex media (YED and SAB) and two synthetic media (AMM and CDA). It seemed quite logical to grow the fungus at the human body temperature (37°C), although since this temperature differs considerably from those of natural conditions, we decided to use 28°C also. Efficient growth was obtained in all media at both temperatures from a strictly controlled inoculum of 10^5 conidia per ml. The final dry weight values (4 to 8 mg/ml) were comparable to those reported for *A. fumigatus* by Kim and Chaparas (7) and van der Heide et al. (21) and higher than those described by Kurup et al. (11). From unpublished results, we know that some *A. fumigatus* strains give very low dry weight yields. The pH profiles, however, were different from those obtained in the aforementioned papers, even when growth took place in similar medium (Czapek) at the same temperature; the inclusion of AOAC could be responsible for such differences. Only the pH profiles from SAB were similar to those of *A. fumigatus* growing in Czapek medium supplemented with yeast extract (21).

From the point of view of antigenic standardization (10), it is crucial to obtain the most precise definition of the metabolic stage of the fungus at the time of collecting material to elaborate antigenic mixtures. We used morphological, ultrastructural, and biochemical criteria to characterize three well-defined states of growth curves: exponential, deceleration, and late-stationary phases. Regardless of the temperature, the morphology and ultrastructure corresponded to those of healthy young hyphae with well-organized cellular architecture (exponential phase), mixed cultures with some partially empty or disorganized hyphae (deceleration phase), and old cultures with almost no organelles inside dark lysed hyphae containing membranous debris which was also present outside the cell wall (stationary phase). Corresponding to the metabolic situation, the number and intensity of polypeptide bands detected by SDS-PAGE and silver stain decreased with time, and smearing at the bottom of the gels strongly indicated an increase in proteolytic activities, which was maximal in the stationary phases. Total extracts (obtained by boiling in 2% SDS) were used owing to the high number of samples to be analyzed. Conversely, the number of polypeptide bands present in the cell culture supernatants increased with time. Some of them were real secretion products, but many others were released as a consequence of the hyphal age and lysis. The influence of age and culture media was very important, unlike the situation observed in total extracts, and determined the appearance of very distinct patterns, especially in synthetic media. Western blot (immunoblot) using the four different rabbit antibodies corroborated the results obtained with silver stain, suggesting that those antibodies are mainly specific for constitutive proteins expressed in very different metabolic situations. The fact that extracellular patterns were not coincident with those from total extracts indicates either the existence of hydrolytic activities which trim the original polypeptides, eliciting a different electrophoretic mobility, or the prevalence of some common and strongly antigenic epitopes in different polypeptides.

The most important findings of the present work came from the experiments with human sera from aspergilloma- or aspergillosis-affected individuals. Four well-documented serum samples helped us to choose CDA as being able to support the production of polypeptides reactive with human antibodies. This characteristic was independent of the temperature of growth but was affected, particularly for metabolic antigens, by the age of the cultures. The exponential phase was the best for total extracts, and the stationary phase was best for cell culture supernatants. Both samples were used as source antigens to screen a battery of 10 human serum samples from patients with aspergilloma or aspergillosis and two control serum samples. Rabbit antibodies were checked in parallel to ascertain whether they recognized the same antigens as those of human origin. In CDA culture supernatants, one group of antigenic zones was strongly and consistently recognized by most of the patients' sera and only very weakly by control sera. This faint response by the control sera has been reported several times (13) and could be due to the ubiquity of *Aspergillus* spp. Surprisingly, the four rabbit antibodies recognize an identical group of antigenic zones, which indicates that they are specific against some immunodominant epitopes quite resistant to proteolytic degradation.

In total extracts, a certain degree of reaction against the three previously described antigenic zones was observed, but this was not as consistent as in the case of metabolic antigens. Recognition of the same group of antigens by rabbit antibodies was unclear, and only anticytosol antibodies recognized the 20- to 22-kDa band.

The validity of these data and the conclusions drawn depend on this isolate of *A. nidulans* being representative of the whole species. In this respect, it is interesting to note that all problem sera tested were also reactive in our immunoblot assay with culture supernatants or cytosolic extracts from *A. fumigatus* (three isolates), and some of them were also reactive with analogous samples from *A. flavus* (one isolate). Furthermore, three different antisera raised against *A. fumigatus* cytosolic extracts were also reactive with the antigens described above in

A. nidulans (unpublished data). Although we have insufficient information concerning the species isolated from the aspergilloma or invasive aspergillosis cases tested, we do know that at least four of the serum samples were also positive in double-diffusion assays with commercial preparations of *A. nidulans*, *A. fumigatus*, and *A. flavus*. All of these data strongly suggest the existence of a high degree of conservation, in several *Aspergillus* species, among some of the epitopes responsible for the reaction with human sera.

At this time, it is not possible to establish any relationship between the described antigenic zones and some of the antigens already described for *A. fumigatus* (CS2-related glycoprotein antigens [2, 9, 11, 16], 58 kDa [4], 18 kDa [13], etc.). However, we believe that the identification of antigens that can be produced consistently by *A. nidulans* (we have reproduced both the metabolic and the somatic patterns in four different batches) under strictly controlled growth conditions demonstrates the usefulness of this species as an alternative or complementary source of reagents for the immunodiagnosis of some forms of aspergillosis. Work is being done in our laboratory to extend these results to a greater number of patients and control sera and to purify the three described metabolic antigens.

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