Surface Proteins of Two Aflatoxin-Producing Isolates of *Aspergillus flavus* and *Aspergillus parasiticus* Mycelia. 1. A Comparative Immunochemical Profile

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The surface antigens from *Aspergillus flavus* and *Aspergillus parasiticus* mycelia were characterized by immunochemical analysis on agarose gels. Results of immunoabsorption experiments showed that a least two antigens present in *A. flavus* were not detected in *A. parasiticus*. Semiquantitative and qualitative assays by line and crossed immunoelectrophoresis showed extreme heterogeneity in precipitin formation between the two fungi. A major antigen in *A. parasiticus* appeared in trace amounts in *A. flavus*; that antigen might be useful as a chemical marker in species identification studies. The avidity of antibody responses varied between the two isolates but the homology of the profiles support their close phylogenetic relationship as members of the same genus.

INTRODUCTION

The *Aspergillus* genus contains many species that have been categorized into several groups as a taxonomic treatment of the genus (Raper and Fennell, 1965). *Aspergillus flavus* and *Aspergillus parasiticus* are two species with economic impact because they produce toxic metabolites, aflatoxins, which are highly carcinogenic and mutagenic (Bennett and Papa, 1988; Boldblatt, 1969). Strains and isolates of *A. flavus* and *A. parasiticus* are not easily identified by chemical means. Klich and Pitt (1988) concluded that the texture of the conidial wall was a physical criterion for distinguishing the two species. Another report concluded that *A. flavus*, *A. parasiticus*, *A. sojae*, *A. oryzae*, and *A. tamarii* could be separated from one another by morphological and microscopic criteria (Klich and Pitt, 1985). Electrophoretic comparisons of mycelial enzymes from the two species showed no distinct differences in protein patterns (Schmidt et al., 1977). Earlier enzyme/protein electrophoretic studies (Nasumo, 1974; Nealson and Garber, 1967; Sorenson et al., 1971) of *A. flavus* failed to establish differences between the species; however, these reports suggested the merit of developing similarity indices that might complement or relate to physical characteristics of diverse mycelial surfaces. Several immunochemical studies relevant to aspergillosis showed very close relationships in the antigenic profiles of several isolates categorized in the *Aspergillus* group (Hearn and Mackenzie, 1980a,b; Tran Van Ky et al., 1971). A more recent observation in our laboratory indicated that the detection of a specific enzyme involved in the biosynthesis of aflatoxin might serve as a probe to identify the aflatoxigenic potential of *Aspergillus* species (Bhatnagar and Neucere, 1989).

This preliminary study deals with serological and chromatographic analyses of the surface mycelial antigens from two different aflatoxin-producing species of the *A. flavus* group as a basis for comparing other aflatoxin-producing isolates in the genus. The results describe qualitative and semiquantitative precipitin-in-gel profiles of the extracted antigens. A second parallel physicochemical study employed several aspects of HPLC including reverse phase, ion exchange, and gel permeation to analyze surface proteins.

MATERIALS AND METHODS

Fungal Isolates. *A. flavus* SRRC (1000A) and *A. parasiticus* SRRC (2004), both known to produce aflatoxins, were kindly provided by Dr. M. A. Klich from our laboratory.

Mycelial Growth and Extractive Procedures of Surface Antigens. Spores were maintained on potato dextrose agar as described earlier (Neucere and Zeringue, 1987). Fungal suspensions of 100×10^7 spores/mL were made in 0.001% Triton X-100 for both strains. Mycelia were prepared by inoculating 100 mL of 4% sucrose and 2% yeast extract (Scott Laboratories, Inc.) with 1.0 mL of each spore suspension in 500-mL Erlenmeyer flasks. After incubation for 6 days at 28 °C on a rotary shaker (150 rpm), mycelia were collected by filtration through cheese cloth and washed several time with cold deionized water. Surface proteins were extracted by stirring 100 mg of intact mycelia in 5 mL of 0.15 M phosphate-buffered saline (PBS), pH 7.2 (Hierholzer and Suggs, 1969) for 3 h at 25 °C. Supernatants were filtered through Whatman no. 2 paper and clarified by centrifugation at 12000*g* for 3 min at 10 °C.

Immunochemistry. *Preparation of Polyclonal Antibodies.* Antibodies to a mycelial PBS extract of *A. flavus* were raised in three rabbits under contract by Pel-Freez Inc. (Rogers, AR) as follows: Two milligrams of protein was added to 1.0 mL of complete Freund's adjuvant (Difco) and injected into the foot pads of each animal at biweekly intervals to total four injections. Blood was collected 12 days after the third injection and again 7 days following the last injection. Sera were separated from red blood cells by 1000*g* centrifugation and immediately frozen. Preimmune sera for control assays were provided from each animal. Whole sera (no purification steps) were used in all of the assays.

Immunoabsorption. The protein (antigen) extracts at 1.0 mg/mL from both isolates were dialyzed against veronal buffer for 3 h, and analyses were carried out as previously described (Daussant et al., 1969). For each extract, 0.5 mL was mixed with 0.5 mL of antiserum made against *A. flavus* and allowed to stand at 5 °C for 12 h. Precipitated antigen–antibody complexes were removed by centrifugation at 5000*g* for 30 min at 10 °C. The absorbed sera were used along with unabsorbed serum in a double diffusion format on microscope slides as shown in Figure 1A. All wells were filled with 50 μL of sample and allowed to diffuse for 24 h. Unreacted proteins were removed by washing with 1% saline. Slides were dried under filter paper and stained with 0.12% Coomassie Blue R250.

Crossed Immunoelectrophoresis. Crossed immunoelectrophoresis was performed according to Weeke (1973) using 1.5% agarose (Bio-Rad, 162-001) and 3% polyethylene glycol (Sigma,

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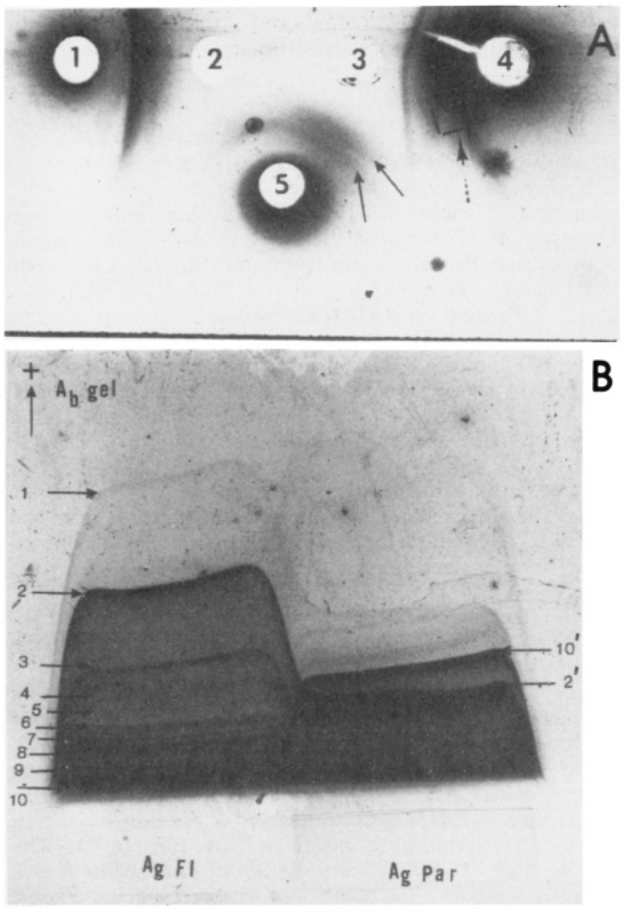

Figure 1. Qualitative analysis of antigens in *A. flavus* and *A. parasiticus* by immunoabsorption (A) and semiquantitation of antigenic components in the two strains (B). In (A), wells 1 and 4 both correspond to antiserum to *A. flavus*. Wells 2 and 3 denote antigen samples from *A. parasiticus* and *A. flavus*, respectively, and well 5 corresponds to antibodies to *A. flavus* absorbed with the antigen extract from *A. parasiticus*. Analysis by line immunoelectrophoresis of the two isolates is shown in (B). Ag Fl and Ag Par correspond to agar molds of antigen samples containing 250 μg of protein each from the two isolates. The antibody gel contained 6% antiserum to *A. flavus*. Arrows point to the major resolved precipitin lines. See text for details.

P-3640) in sodium barbital (Sigma) buffer, pH 8.2, ionic strength 0.05. Fifty milligrams of protein from each isolate was run in parallel in the first-dimension electrophoresis at 200 V (constant) for 1.5 h at 20 °C. Second-dimension gels contained 6% antiserum against *A. flavus* and were run at 80 V (constant) for 14 h at 20 °C. Plates were processed as described above.

Line Immunoelectrophoresis. This procedure was conducted according to Kroll (1973) on agarose gels. The rectangular sample gels of *A. flavus* and *A. parasiticus* each contained 250 μg of protein and the antibody-containing gel contained 6% antiserum against *A. flavus*. Electrophoresis was conducted in veronal buffer at 100 V (constant) for 18 h at 20 °C. Plates were processed as described above.

Chemicals. All chemicals were of reagent or analytical grade.

Protein Estimation. Protein concentrations for all of the analyses were determined either by the Lowry et al. (1951) method or the BCA protein assay (Pierce) with ovalbumin as standard.

RESULTS AND DISCUSSION

Qualitative (comparative) analysis of total proteins in the *A. flavus* and *A. parasiticus* extracts using absorbed antiserum is shown in Figure 1A. Wells 1 and 4 contained antibodies to *A. flavus* and wells 2 and 3 contained the antigens from *A. parasiticus* and *A. flavus* respectively. Antibodies against *A. flavus* absorbed with antigens from *A. parasiticus* was used in well 5. Note that the precipitin lines between wells 3 and 5 (solid arrows) are absent in the corresponding format of wells 2 and 5. In addition, several precipitin lines (dashed arrow) between wells 3 and 4 were not observed in the opposing format (wells 1 and 2) establishing distinguishable profiles. The results show that two or more antigens present in *A. flavus* are not present in *A. parasiticus*. Further analyses of the two extracts by line immunoelectrophoresis are shown in Figure 1B. In this procedure, the continuity of identical precipitin lines between adjoining patterns allows direct qualitative and semiquantitative comparison of the two samples. With equal quantities of protein in each molded sample gel, at least 10 precipitin lines can be differentiated. The dominant precipitin line in *A. flavus* (Figure 1B, arrow 2) is of much lesser quantity in *A. parasiticus* (arrow 2^1). The major component in *A. parasiticus* (arrow 10^1) represents a very small quantity in *A. flavus* (arrow 10). Another component (arrow 3) is not discerned in the *A. parasiticus* sample. These overall results show a major difference in the proportionality of individual antigens in the two extracts.

Representative analyses by crossed immunoelectrophoresis are shown in Figure 2. This procedure displays the individual antigens as precipitin peaks in a two-dimensional array. Over 25 antigens were apparent in each sample. The height and intensity of staining for each peak is a function of concentration. Considerable heterogeneity in both quantity of antigens and electrophoretic migration was observed for the two samples. One major difference is the occurrence of a dominant peak in *A. parasiticus* (arrow b in Figure 2B) which is not readily detected in *A. flavus* (Figure 2A). One other peak in *A. flavus* (a in Figure 2) was not detected in *A. parasiticus*. For better comparison of the two profiles, the gel plates were superimposed and photographed as in Figure 2C. Select antigenic peaks are shown as solid arrows for *A. flavus* and dashed arrows for *A. parasiticus*. That construction reveals more clearly the immense heterogeneity of content and electrophoretic properties of the antigens.

Extracts from *A. flavus* and *A. parasiticus* mycelia yield complex mixtures of antigenic substances detectable by several methods of immunochemistry in agarose. At the time of this research, antiserum against *A. parasiticus* was not available; therefore our results are based only on the antigenic proteins that react with polyvalent antiserum against *A. flavus*. Under optimum analytical conditions with the available antiserum, at least two antigens present in *A. flavus* were not detected in *A. parasiticus*. The analyses by line immunoelectrophoresis and crossed immunoelectrophoresis are both semiquantitative because immunoprecipitates are detectable only within a narrow range of antigen and antibody concentrations. Therefore, antigens not detectable as precipitins in gels can still induce antibody formation. This would explain the observation, for example, of a high-titre antibody response for antigen b in *A. parasiticus* compared to *A. flavus*. Other corresponding antigens present in both isolates reflect variations in the avidity of their antibody responses. Microheterogeneity of electrophoretic mobilities is not easily explained. Minor differences in exposure of residues containing basic or acidic amino acids and conformation changes can reflect both anodic and cathodic shifts.

In conclusion, this preliminary study showed that the similar antigenic reactions of the surface proteins from the two isolates support their close phylogenetic relationship as members of the same genus. Perhaps further investigations of several different isolates from both species and parallel studies with diverse antisera from each will yield a better view of this system. It is known for example that different isolates of *A. flavus* exist in the field (Cotty,

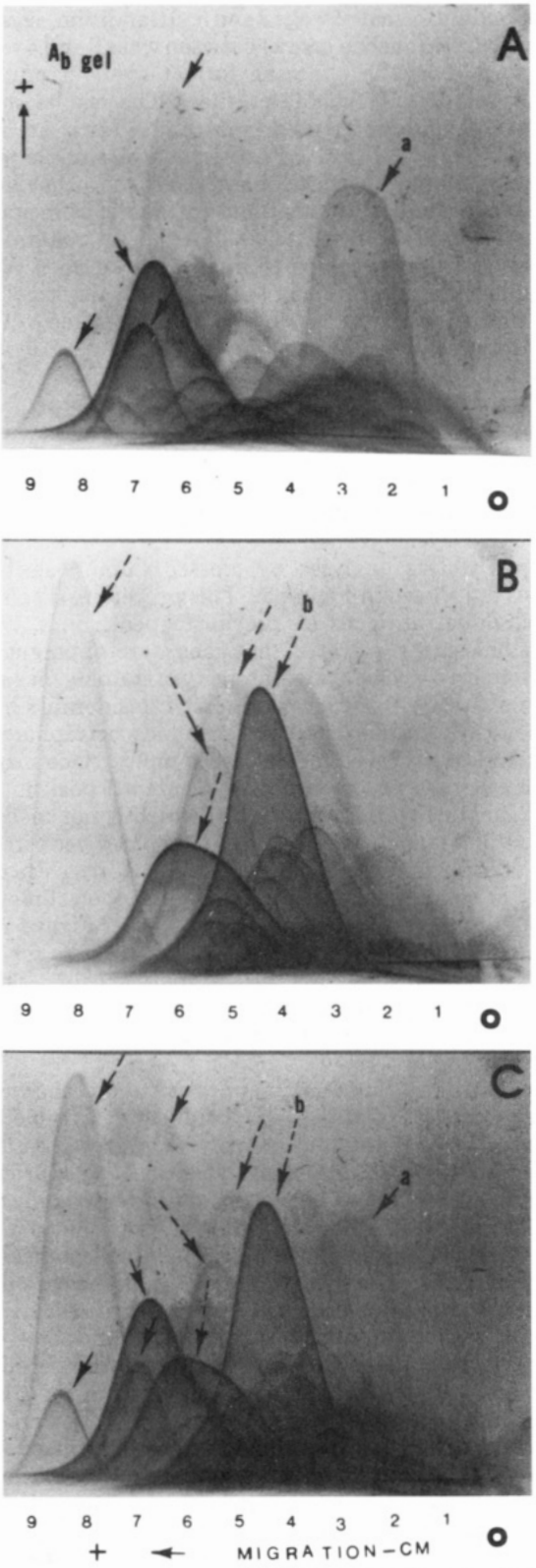

Figure 2. Two-dimensional immunoelectrophoresis of antigens from the two isolates of fungi. For the first dimension, the antigen wells (O) were loaded with 50 μg of protein from *A. flavus* (A) and *A. parasiticus* (B). Antiserum (6%) to *A. flavus* was employed in the antibody gel for the second dimension. (C) Superimposition of analyses A and B.

1989; Stoloff, 1977), but the homology of mycelial surface proteins from diverse isolates found in nature is unknown. A chemical marker such as antigen b in *A. parasiticus* could serve as a valuable tool or adjunct in either inter or intra species identification studies. Implementation of hybridoma technology for production of monoclonal antibodies could even further enhance both specificity and sensitivity of analytical strategies for identification of subtle isolate differences.

LITERATURE CITED

Bennett, J. W.; Papa, K. E. The aflatoxigenic *Aspergillus* spp. In *Advances in Plant Physiol.*; Ingram, D. S., Williams, D. H., Eds.; Academic Press: New York, 1988.

Bhatnagar, D.; Neucere, J. N.; Cleveland, T. E. Immunochemical Detection of Aflatoxigenic Potential of *Aspergillus* Species with Antisera Prepared Against Enzymes Specific to Aflatoxin Biosynthesis. *Food Agric. Immunol.* **1989**, *1*, 225–234.

Cotty, P. J. Prevention of aflatoxin contamination with strains of *Aspergillus flavus*. *Phytopathology* **1989**, *79*, 1153.

Daussant, J.; Neucere, J. N.; Yatsu, L. Immunochemical studies on aradis hypogaea proteins with particular reference to reverse proteins. I. Characterization distribution and properties of α-arachin and α-conarachin. *Plant Physiol.* **1969**, *44*, 471–479.

Goldblatt, L. A., Ed. *Aflatoxins*; Academic: New York, 1969.

Hearn, V. M.; Mackenzie, D. W. R. Mycelial antigens from two strains of *Aspergillus fumigatus*: an analysis by two dimensional immunoelectrophoresis. *Mykosen* **1980a**, *23*, 549–562.

Hearn, V. M.; Mackenzie, D. W. R. Antigenic activity of subcellular fractions of *Aspergillus fumigatus*. In *Medical Mycology, Zbl. Bakt. Suppl. 8*; Preusseur, H. G.; Ed.; Gustan Fischer Verlag; New York, 1980b.

Hierholzer, J. C.; Suggs, M. T. Standard viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions. *Appl. Microbiol.* **1969**, *18*, 816–823.

Klich, M. A.; Pitt, J. F. The Theory and Practice of Distinguishing Species of the *Aspergillus flavus* Group. In *Advances in Penicillium and Aspergillus Systematics*; Samson, R. A., Pitt, J. I., Eds.; Plenum Publishing: New York, 1985; pp 211–220.

Klich, M. A.; Pitt, J. F. Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Trans. Br. Mycol. Soc.* **1988**, *91*, 99–108.

Kroll, J. Crossed-line immunoelectrophoresis. *Scand. J. Immunol. Suppl. II* **1973**, *2*, 61–67.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Nasuno, S. Further evidence on differentiation of *Aspergillus Sojae*' from *Aspergillus oryzae* by electrophoretic patterns of cellular, pectin-lyase, and acid proteinase. *Can. J. Microbiol.* **1974**, *20*, 413–416.

Nealson, K. H.; Garber, E. D. An electrophoretic survey of esterases, phosphatase and leucine aminopeptidases in mycelial extracts of species of *Aspergillus*. *Mycologia* **1967**, *59*, 330–336.

Neucere, J. N.; Zeringue, H. J., Jr. Inhibition of *Aspergillus flavus* growth by fractions of salt-extracted proteins from maize kernel. *J. Agric. Food Chem.* **1987**, *35*, 806–808.

Raper, K. B.; Fennell, D. I. *The Genus Aspergillus*; Williams and Wilkins: Baltimore, 1965.

Schmidt, A. L.; Curtis, C. R.; Bean, G. A. Electrophoretic comparisons of mycelial enzymes from aflatoxin-producing and non-producing strains of *Aspergillus flavus* and *A. parasiticus*. *Can. J. Microbiol.* **1977**, *23*, 60–67.

Sorenson, W. G.; Larsh, H. W.; Hamp, S. Acrylamide gel electrophoresis of proteins from *Aspergillus species*. *Am. J. Bot.* **1971**, *58*, 588–593.

Stoloff, L. Aflatoxins—An Overview. In *Mycotoxins in Human and Animal Health*; Pathotox Publishers: Park Forest South, IL, 1977; pp 7–27.

Tran Van Ky, P.; Biquet, J.; Vaucelle, T.; Fruit, J. *Sabouraudia* **1971**, *9*, 210–220.

Ullah, A. H. J.; Neucere, J. N.; Cleveland, T. E. Surface proteins of *Aspergillus flavus* and *Aspergillus parasiticus* mycelia. 2. HPLC mapping by gel permeation, ion exchange, and reverse phase chromatography. *J. Agric. Food Chem.* **1992**, following paper in this issue.

Weeke, B. Crossed immunoelectrophoresis. *Scan. J. Immunol., Suppl. II* **1973**, *2*, 43–56.

Received for review October 3, 1991. Revised manuscript received March 26, 1992. Accepted June 8, 1992.