Surface Antigens of Intact *Aspergillus fumigatus* Mycelium: their Localization Using Radiolabelled Protein A as Marker

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(Received 14 September 1983; revised 10 November 1983)

Isotopically-labelled Protein A from *Staphylococcus aureus* was used as a marker to quantify binding of IgG molecules to surface antigens of *Aspergillus fumigatus* mycelium. The IgG class antibodies were obtained from rabbits inoculated with mycelial fractions and from patients suffering from aspergillosis and aspergillus-related diseases. The highest incorporation of label was obtained with an antiserum from rabbits inoculated with *A. fumigatus* wall material. Antibodies raised to other antigenic fractions of *A. fumigatus* and antibodies from patients infected with aspergillus gave lower levels of incorporation of Protein A. In competitive binding experiments, pre-incubation of antibodies with partially purified aspergillus antigens depressed subsequent binding to the mycelial surface by 20–30% of that of the control values. Low molecular weight disaccharides and oligosaccharides were without effect in this system. Pre-incubation of *A. fumigatus* with lectins having specificities for defined sugar residues did not reduce subsequent antigen/antibody interaction. Treatment of the mycelial surface with certain proteolytic or polysaccharolytic enzymes led to a decrease in antibody binding, while pre-treatment of *A. fumigatus* with the hydrolytic enzyme mixture of *Trichoderma harzianum* culture filtrate gave increased antibody binding. *Aspergillus* species showed different susceptibilities to enzyme action and their surface structures could be differentiated from *A. fumigatus* on this basis. These differences were not obvious in direct binding experiments where antibodies raised to *A. fumigatus* wall bound with equal facility to antigenic sites located on the walls of other *Aspergillus* species.

INTRODUCTION

Fungal polysaccharides located in the outer layer of the cell wall are usually covalently bound to form peptido-polysaccharide complexes (Lloyd, 1970; Ballou, 1976). These are the cell wall components primarily responsible for reactions with antibodies (Lloyd & Bitoon, 1971) and lectins such as concanavalin A (Travassos *et al.*, 1977). Lloyd *et al.* (1978) used immunofluorescence with specific rabbit antisera to determine the distribution of antigenic polysaccharides on the surface of the different cell types of *Sporothrix schenckii*. Other studies have been based on the action of lytic enzymes. Thus, Previato *et al.* (1979) found that treatment of isolated walls of *S. schenckii* with a (1→3)- β -exo,endo-glucanase resulted in a general disorganization of the skeletal, glucan-rich layer, and Sunayama & Suzuki (1970) destroyed the precipitating ability of mannan of *Saccharomyces cerevisiae* with an α -mannosidase from *Arthrobacter*. Sequential enzyme treatment has been used to obtain information concerning polymer distribution in the cell walls of *Neurospora*, *Schizophyllum* and *Phytophthora* (Hunsley & Burnett, 1970) and to release antigens from the cell walls of *Histoplasma* (Reiss, 1977). Evidence for a layered arrangement of hyphal cell walls was provided by the finding that treatment of *Neurospora crassa* with an exo-(1→3)- β -glucanase followed by chitinase yielded *N*-acetylglucosamine residues while chitinase alone released very little free hexosamine (Potgieter & Alexander,

Abbreviations: ConA, concanavalin A; SpA, staphylococcal Protein A; SEM, scanning electron microscopy.

1965). A similar pattern has been described for *Aspergillus oryzae* (Horikoshi & Arima, 1962) and for *A. nidulans* (Bull, 1970). With fluorescein-conjugated lectins as probes, Stoddart & Herbertson (1978) reported strong hyphal staining in tissue sections of *A. fumigatus* incubated with lectins specific for *N*-acetylgalactosamine and β -D-galactose, and moderate staining with one specific for α -D-glucose and α -D-mannose residues.

Galactomannan, glucan and glycoprotein structures with antigenic activity have been isolated from *A. fumigatus* mycelium (Azuma *et al.*, 1969). Little is known about the location in the organism of these and other antigenic residues and their accessibility to immunoglobulin molecules. In this study, antibodies raised to partially purified antigenic fractions of *A. fumigatus* were used in an attempt to obtain information concerning surface structures accessible for direct binding to specific IgG molecules. This has been measured by uptake of [125 I]Protein A (SpA), a technique which has been widely used for the detection of cell surface antigens (Dorval *et al.*, 1974; Biberfeld *et al.*, 1975; Zeltzer & Seeger, 1977). *Aspergillus* belongs to the chitin-glucan group of fungi (Bartnicki-Garcia, 1968) with galactomannan as a minor constituent. Therefore, saccharides with related structures and lectins with specificities for these sugars were used as probes in competitive-binding experiments to try and elucidate the nature of the binding sites. In addition, hydrolases, which included a variety of glucanases, mannosidases and chitinase, were tested for their ability to disrupt surface interactions.

A preliminary report on some of this work has appeared elsewhere (Hearn *et al.*, 1981).

METHODS

Preparation of mycelial pellets. The following species of *Aspergillus* were used (accession numbers are for the National Collection of Pathogenic Fungi): *A. fumigatus* NCPF 2109, *A. flavus* NCPF 2008, *A. terreus* NCPF 2026, *A. niger* NCPF 2022 and *A. nidulans* NCPF 2020. Neutral glucose/peptone medium (100 ml) supplemented with 0.2% (w/v) yeast extract and 0.5% (w/v) Righelato's salt solution (Righelato *et al.*, 1968) was inoculated with 2×10^6 spores of each *Aspergillus* species. The organisms were grown for approximately 40 h at 30 °C in a Gallenkamp orbital shaker incubator at 100 r.p.m. Mycelia were harvested in the form of compact spheres 2–2.5 mm diameter, the culture medium being removed by filtration through nylon membranes. The size of the pellets was such that they could be taken up on the tip of a Pasteur pipette while being too large to penetrate into it. Mycelial pellets were washed in 0.01 M-PBS at pH 7.4 containing 4 mM- Na_2EDTA , before distribution in disposable test tubes (type LP3 from Luckham, Burgess Hill, Sussex, UK) at 4 pellets per tube.

Preparation of antigens. A 3 d preparation of *A. fumigatus* mycelium grown in neutral glucose/peptone medium at 30 °C was harvested by filtration, and the resultant mycelial mat was washed with water until the washings were clear (Proctor, 1976). Rupture of the organisms was achieved using a Dymomill cell disintegrator (Glen Creston, London, UK) at 4 °C; after centrifugation, the insoluble pellet was put aside and used as a source of wall material. The supernatant was concentrated by counter-dialysis against a 10% (w/v) aqueous solution of PEG 6000; this constituted the total water-soluble material of the mycelium (Hearn & Mackenzie, 1979).

Antigenic fractions were obtained by salt precipitation in a two-step process whereby the water-soluble material was saturated with solid ammonium sulphate at room temperature to give a precipitate which was collected. The ammonium sulphate-soluble supernatant fraction produced at this stage was also retained. After redissolving the solid ammonium sulphate fraction in water, both fractions were exhaustively dialysed against water, and any insoluble residue was removed by centrifugation. The soluble supernates were concentrated to a suitable volume by counter-dialysis against PEG 6000 and stored at –20 °C.

Additional antigenic fractions were separated from water-soluble material of *A. fumigatus* on the basis of their attachment or non-attachment to concanavalin A (ConA). A column of ConA Sepharose (Pharmacia) was equilibrated with 1 mM-Tris/HCl buffer, pH 7.2, containing 1 mM- CaCl_2 , 1 mM- MnCl_2 and 0.25 M- NaCl . The water-soluble material in the same buffer was applied to the column surface. Elution was effected in the same buffer at 4 °C and resulted in the elution with the void volume of protein-rich material. Subsequent application of 0.2 M-methyl α -D-mannopyranoside in 1 mM-Tris/HCl buffer resulted in the elution of carbohydrate-rich material which had been bound to ConA (Wilson & Hearn, 1983). The relevant column fractions were pooled, dialysed and concentrated to a suitable volume against PEG 6000 and stored at –20 °C.

Cytoplasmic antigens, free of cell wall components, were prepared as protoplasts obtained by controlled enzymic lysis of an 18 h culture of *A. fumigatus* mycelium (Hearn *et al.*, 1980). The protoplasts were separated from the growth medium by filtration and washing and were stored at –20 °C.

The insoluble residue obtained during the preparation of the water-soluble material was washed repeatedly in hot water (50–60 °C) until the washings were clear. While some material, including labile components, is lost by this process, the requirement was for cell wall material free from cytoplasmic and nuclear contaminants (Hearn &

Mackenzie, 1979). The pellet was dried to constant weight over P_2O_5 and stored at $-20^\circ C$. This constituted crude, unextracted cell wall material.

Production of antisera. Antibodies were produced in New Zealand White rabbits. For predominantly carbohydrate antigens (cell wall and carbohydrate-rich fraction) the following protocol was used. Antigen (10 mg dry weight) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a buttock. Ten days later the same amount of antigen was mixed with an equal volume of Freund's incomplete adjuvant and injected into the other buttock. Ten days later the same level of antigen with Freund's incomplete adjuvant was injected into several subcutaneous sites. Ten days later 5 mg antigen was given intravenously without adjuvant. One week later the animal was test-bled. Sometimes another intravenous injection was required to obtain a serum of adequate titre.

The protocol used for protein-enriched antigens (cytoplasm and protein-rich fraction) was as follows. Antigen (0.5 mg dry weight) was mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a buttock. Ten days later a similar injection was made into the other buttock. The rabbits were left for five months and then inoculated subcutaneously in many sites with 0.5 mg antigen mixed with Freund's incomplete adjuvant. After ten days the animals were given 0.1 mg antigen intravenously without adjuvant. A test-bleed was taken ten days later. Sometimes an additional intravenous injection was required.

Antiserum to the total water-soluble material from ruptured mycelium was obtained as previously described (Hearn & Mackenzie, 1980).

An antiserum (SBS) raised to a mixture of mycelial and culture filtrate antigens of *A. fumigatus* was a generous gift from Dr K. Holmberg of the State Bacteriology Laboratory, Stockholm, Sweden.

An antiserum (X-9) raised to an alkali extract (Lehmann & Reiss, 1978) of *A. fumigatus* mycelium was a generous gift from Dr E. Reiss of the Center for Disease Control, Atlanta, Georgia, USA.

Serum was collected from rabbits prior to injection of antigens for use as a negative control.

Human sera. Sera were obtained from patients fulfilling the diagnostic criteria for aspergillosis. Some were specimens received by our laboratory, while the remainder were kindly donated by Dr J. Edwards of Sully Hospital, Glamorgan, UK. Three serum pools were prepared, numbered human pool 1, 2 and 3; all were highly reactive in serological tests. Serum from subjects negative for specific IgG by an enzyme-linked immunosorbent assay (Wilson & Hearn, 1983), was also collected for use as a negative control.

Preparation of an IgG fraction. The bulk of the plasma proteins were precipitated with caprylic acid (Steinbuch & Audran, 1969). The IgG component (and a portion of the IgA) which remained in the supernatant was concentrated five-fold. Azide (0.1%, w/v) was added as a preservative and the solutions were stored at $4^\circ C$. This procedure was carried out on all test sera.

Radio-iodination of SpA and ConA. The staphylococcal Protein A (SpA) used in these experiments was partially-purified material prepared from *Staphylococcus aureus* (Cowan strain) and obtained from Sigma (catalogue no. P-9267). Initial experiments were performed using SpA trace-labelled with the Bolton and Hunter reagent *N*-succinimidyl 3-(4-hydroxy 5-[^{125}I]iodo phenyl) propionate (Amersham), essentially as described by Bolton & Hunter (1973). SpA (0.05 mg) in 0.2 M-sodium borate buffer at pH 8.5 was added to 0.5 mCi (18.5 MBq) dried reagent and allowed to react overnight at $4^\circ C$. The [^{125}I]SpA was recovered from a commercially prepared G-25 Sephadex column (Pharmacia) when it eluted in the void volume. It was stored at $-70^\circ C$ in the presence of gelatin; new batches of [^{125}I]SpA were prepared every three weeks. ConA (A grade, from Calbiochem), was similarly labelled by the procedure of Bolton & Hunter (1973) and stored at $-70^\circ C$. The specific activity of [^{125}I]SpA and [^{125}I]ConA was 10^4 c.p.m. μg^{-1} in each case.

Polysaccharides and oligosaccharides for competitive binding assays. Polysaccharides used for binding to specific *A. fumigatus* antibodies were soluble starch, laminarin [(1 $\rightarrow$ 3)- β -D-glucan] which was kindly supplied by Dr F. Chattaway, formerly of Leeds University, and a (1 $\rightarrow$ 3)- α -D-glucan from *Polyporus betulinus* which was a welcome gift from Dr E. T. Reese (US Army Laboratories, Natick, Mass., USA.). The sugars used in this study were obtained as partial hydrolysis products of polysaccharides from *A. fumigatus* antigens by Dr P. A. J. Gorin, Prairie Regional Laboratory, Saskatoon, Canada. They included: β -D-Glcp-(1 $\rightarrow$ 3)-D-Glc; β -D-Glcp-(1 $\rightarrow$ 3)- β -D-Glcp-(1 $\rightarrow$ 3)-D-Glc (laminaribiose and laminaritriose, respectively); β -D-Galf-(1 $\rightarrow$ 5)-D-Gal; β -D-Galf-(1 $\rightarrow$ 5)- β -D-Galf-(1 $\rightarrow$ 5)- β -D-Galf-(1 $\rightarrow$ 5)-D-Gal; α -D-Manp-(1 $\rightarrow$ 2)-D-Man and α -D-Manp-(1 $\rightarrow$ 2)- α -D-Manp-(1 $\rightarrow$ 2)-D-Man. A synthetic trisaccharide with the structure:

was a gift from Dr T. Ogawa, Institute of Physical and Chemical Research, Saitama, Japan.

Exposure of mycelial surfaces to hydrolytic enzymes. α -Amylase (EC 3.2.1.1) ex *Bacillus subtilis*, β -amylase (EC 3.2.1.2) ex barley and pronase ex *Streptomyces griseus* were purchased from Koch-Light. β -Galactosidase (EC 3.2.1.23) ex bovine liver, *S. griseus* protease, papain (EC 3.4.22.2) from papaya latex, chitinase (EC 3.2.1.14)

ex *S. griseus* (with slight β -glucanase activity), α -mannosidase (EC 3.2.1.24) from jack bean, α -chymotrypsin (EC 3.4.21.1) from bovine pancreas and β -glucosidase (EC 3.2.1.21) from almonds were commercial preparations from Sigma. Trypsin (EC 3.4.21.4) and lytic enzyme L_1 from *Cytophaga* [contains several endo-(1 $\rightarrow$ 3)- β - and endo-(1 $\rightarrow$ 4)- β -glucanases] were both from BDH. An exo-(1 $\rightarrow$ 3)- β -glucanase (Basidiomycete QM806) free of cellulase and β -glucosidase was from Dr E. T. Reese. A mixture of exo- and endo- β -mannosidases (from the culture filtrate of a strain of *Aeromonas* species F-25) was kindly provided by Drs T. Araki and M. Ito, both of the Department of Fisheries, Fukuoka, Japan. Unfractionated culture filtrate from *Trichoderma harzianum* grown on *A. fumigatus*, which is capable of hydrolysing a wide range of polysaccharides in addition to proteins, was also tested.

Enzymes were used at a final concentration of 0.4% (w/v) except for crude lytic enzyme L_1 (1%, w/v). The culture filtrate of *T. harzianum* was diluted in acetate buffer at pH 5.0 to the extent where it caused a measurable effect on *A. fumigatus* mycelium without causing total degradation. α -Mannosidase solution was used at a 1:2 dilution in acetate buffer at pH 4.5. The exo-glucanase from Basidiomycete QM806, which has a broad pH optimum (Dr E. T. Reese, personal communication), was used at pH 5.0 and the mannosidase mixture from *Aeromonas* was used at pH 6.0, as recommended by Dr T. Araki. Commercial enzyme preparations were used at the pH specified by the supplier; the buffer concentration in each case was 0.05 M. Fungal pellets were routinely incubated for 2 h in a shaker bath at 37 °C. Chitinase was incubated with *A. fumigatus* for 3 h; a longer time period caused damage to the controls. Although the minimum hydrolysis time with chitinase is considered to be 12 h, this enzyme affected the surface of *A. fumigatus* germ tubes in 30 min at the same temperature (V. M. Hearn, unpublished results). It should also be noted that the temperature selected was suboptimal for pronase, which has optimal activity at approximately 50 °C.

Lectin-binding experiments. Lectins were used as possible competitive inhibitors of antibody binding to surface carbohydrate antigens of *A. fumigatus* mycelium. ConA, A grade, with specificity for α -D-mannopyranose and α -D-glucopyranose sugars and closely related structures, was from Calbiochem; *Triticum vulgaris* lectin (wheat germ agglutinin) and lectins from *Ricinus communis* (castor bean type I and type II) with specificities for *N*-acetylglucosamine, *N*-acetylgalactosamine and D-galactose respectively, were from Sigma. ConA was tested at a final concentration of 1.25% (w/v) in PBS; the other lectins were tested at a final concentration of 0.25% (w/v).

Immunoassay. The radioimmunoassay was carried out as follows. *Aspergillus fumigatus*, distributed at four mycelial pellets per tube, was washed three times with 0.01 M-PBS containing 4 mM-EDTA, which constituted the wash solution. After each wash, the supernatant was removed by suction, using a Pasteur pipette. The pellets were incubated for 1 h at room temperature with 200 μ l bovine serum albumin solution (5%, w/v). This mixture was replaced by 50 μ l IgG ($\approx$ 0.5 mg protein) in PBS and incubated for a further 18 h at 4 °C. The cells were washed twice, 85 μ g [125 I]SpA in bovine serum albumin (1%, w/v) was added and the mixture was incubated for 30 min at room temperature. After washing three times the radioactivity bound to pellets was measured in a counter (LKB, 1275 Minigamma). Each assay was done in triplicate. The assay was repeated if the coefficient of variation was >12%. The protocol is based on that of Dorval *et al.* (1975).

Competitive binding experiments involved a pre-incubation of IgG with the appropriate antigen or sugar (2 h at 25 °C) before exposing them to the fungal pellets and proceeding as above. The effect of lectins on IgG uptake by mycelium was determined by incubating the specified lectin with the fungal pellets in PBS for 2 h at room temperature. The pellets were washed three times before incubating with IgG and proceeding as above.

Scanning electron microscopy (SEM). Fungal pellets were fixed in 3% (w/v) glutaraldehyde (at pH 7.4 in cacodylate buffer) and osmium tetroxide, dehydrated through acetone and critical-point dried. They were mounted on aluminium stubs, sputter-coated with gold and examined in a Jeol JSM25 scanning electron microscope.

RESULTS

Experimental procedure

Preliminary experiments were required in order to obtain optimal reaction conditions together with minimal background readings. An IgG fraction gave less non-specific attachment to the mycelial pellets than did whole serum; this may have been due to surface binding of IgM and IgA, which can sometimes interact with SpA (Harboe & Fölling, 1974). A mild fixation step with paraformaldehyde after antibody binding, in order to prevent spontaneous dissociation of the antigen-antibody complexes (Biberfeld *et al.*, 1974), was omitted when it was found that the increase seen with aspergillus-positive sera was accompanied by a corresponding increase with negative sera. A proportion of [125 I]SpA bound directly to mycelial pellets (see Table 1) but it was preferred to labelled immunoglobulin which led to a twofold increase in non-specific attachment to surface structures (cf. Biberfeld *et al.*, 1975).

Fig. 1

Fig. 1. Proportional increase in [^{125}I]Protein A (SpA) uptake by *A. fumigatus* mycelial pellets relative to the concentration of specific IgG in the incubation mixture. An IgG fraction produced in rabbits to *Aspergillus* wall material was used.

Fig. 2

Fig. 2. Measurement of direct binding of [^{125}I]ConA to the mycelial surface of *A. fumigatus*. For conditions of incubation see Methods.

Table 1. Comparison of uptake by *A. fumigatus* mycelial pellets of IgG from rabbit and human sources

Rabbit		Human	
IgG* raised to specified antigens	Percentage binding†	IgG from patients with aspergillosis	Percentage binding†
Wall	100	Human pool 1	63.0 ± 21.8
Cytoplasm	29.0 ± 9.3	Human pool 2	48.0 ± 12.4
Water-soluble	45.0 ± 7.3	Human pool 3	94.3 ± 5.2
Protein-rich	37.7 ± 15.5	Human negative	20.6 ± 7.4
Carbohydrate-rich	34.5 ± 10.3		
Mycelial/filtrate (SBS)	45.5 ± 10.3		
Alkali extract (X-9)	50.5 ± 4.9		
Rabbit negative	19.4 ± 2.5		
—	10.6 ± 4.1		

* For details of IgG fractions see Methods.

† Binding of wall IgG to surface receptors is expressed as 100%.

The mean value for 12 experiments was 31019 c.p.m. ± 6359 c.p.m. (i.e. coefficient of variation = 20.5%). Other IgG values represent the mean of at least three experiments ± SD. Results with human sera are given relative to the rabbit wall fraction.

Initial experiments were done with SpA labelled with the Bolton & Hunter reagent. No detectable differences were apparent when the chloramine-T protocol for iodination was investigated (Hunter & Greenwood, 1962), and parallel experiments with SpA labelled by the two procedures were analysed. Either a 90 min incubation with antibody at 25 °C or an overnight incubation at 4 °C proved satisfactory while periods > 30 min did not increase uptake of SpA. SpA was added in excess of antibody in order to quantify binding of the latter to cellular sites. The supplier's instructions were followed in this case. The amount of [^{125}I]SpA bound to the mycelial pellets was directly proportional to the amount of antibody taken up by the cellular surface (Fig. 1). The uptake of specific IgG relative to the total IgG started to plateau at a concentration of 2 mg protein per 4 pellets.

A comparison was made of the ability of IgG from different sources to bind to receptor sites on the mycelial surface (Table 1). Highest uptake was seen with the IgG fraction prepared from antiserum raised to crude wall material while an antiserum raised to cytoplasmic components showed binding only 10% above that given by negative control sera. Sera raised to antigenic fractions gave intermediate values, as did antiserum raised to a water-soluble mycelial

Fig. 3. Inhibition of antibody binding to *A. fumigatus* by pre-incubation of the mycelium with antigenic fractions. For conditions of incubation see Methods. Fractions: ▨, water-soluble; ▩, protein-rich; ▤, carbohydrate-rich; ▦, protein-rich, ammonium sulphate-insoluble; □, carbohydrate-rich, ammonium sulphate-soluble; ■, control (no antigen). The bars represent SD.

preparation, and one raised to a mixture of mycelial and culture filtrate antigens. An antiserum raised to an alkali-soluble extract of *A. fumigatus* and considered to be directed mainly against galactomannan and (1→3)- α -D-glucan moieties (by analogy with extracts prepared from *A. nidulans* and *A. niger*: Bull, 1970; Bardalaye & Nordin, 1977) was also in this category. Human sera showed variable binding capacities to surface structures, indicating qualitative differences in their IgG fractions.

Effects of lectins on IgG uptake

Blocking of receptor sites by pre-treatment of the mycelium with lectins with primary specificities for D-hexose and hexosamine residues was attempted. All lectins were ineffective at the concentrations tested. The extent to which ConA bound to non-antigenic sites on the mycelial surface was investigated by the use of [125 I]ConA, following a 2 h incubation at room temperature (Fig. 2).

Competitive binding experiments with antigenic fractions

A number of soluble antigens were examined for their ability to block the binding of specific IgG to cell-bound antigens. Two of the antigens used effected > 20% inhibition and one > 30% inhibition of cellular binding at a concentration of 50 μ g dry wt per incubation. Increased inhibition was not seen with higher concentrations of these antigens. The predominantly carbohydrate antigens had to be used at 100 μ g (ammonium sulphate-soluble) and 200 μ g (carbohydrate-rich) per incubation to achieve this degree of inhibition to the system (Fig. 3). Pure polysaccharides, low molecular weight oligosaccharides and disaccharides, as specified in Methods, were without effect at 200 μ g dry wt per incubation. Higher concentrations of sugars were not examined.

Effect of enzymes on surface interactions

An enzyme effect was considered significant if uptake of [125 I]SpA varied by > 20% of the control values. On that basis, pronase, papain, trypsin and chymotrypsin were without effect

Table 2. Effect of polysaccharidases on antibody binding to *Aspergillus* mycelial surfaces

Results are expressed as percentage binding of antibody relative to control values. Hydrolytic enzyme control, β -mannosidase, gave a mean binding value of $93\% \pm 4.2$. NS, Not significant (set at an arbitrary value of $100 \pm 20\%$ with a permissible coefficient of variation among replicates of 10%); dis, mycelial disintegration.

Species	<i>T. harzianum</i> culture filtrate	<i>Cytophaga</i> lytic enzyme	Exo-(1 $\rightarrow$ 3) β -glucanase	α -Amylase	β -Amylase	α -Mannosidase	Chitinase
<i>A. fumigatus</i>	121	75	NS	65	NS	75	NS
<i>A. flavus</i>	NS	NS	134	67	NS	NS	NS
<i>A. terreus</i>	77	dis	NS	NS	55	57	124
<i>A. niger</i>	NS	dis	NS	64	NS	NS	76
<i>A. nidulans</i>	NS	66	NS	62	64	NS	NS

while with bacterial protease uptake of SpA was reduced to 77% of that of the control value. Of the polysaccharidases, only *T. harzianum* culture filtrate, α -amylase, α -mannosidase and *Cytophaga* lytic enzyme L₁ at 1% (w/v) had an effect (at 0.4%, w/v, results with the latter enzyme were not reproducible). A β -mannosidase was included as a negative control since β -linked mannose residues are unknown for *Aspergillus* (Azuma *et al.*, 1971; Bardalaye & Nordin, 1977). Other *Aspergillus* spp. were included at this stage for comparative purposes and quite considerable differences in enzyme action were detectable (Table 2). These differences were not apparent in direct binding experiments where an IgG fraction raised to *A. fumigatus* wall bound with equal facility to the surface of all species tested.

SEM studies

No differences were apparent in thin sections when *A. fumigatus* pellets treated with *Cytophaga* lytic enzyme were compared with control samples. An effect was noted when the mycelium was examined by SEM. This effect was much more severe in the cases of *A. terreus* and *A. niger* treated under the same conditions (Fig. 4).

DISCUSSION

The use of [¹²⁵I]SpA to quantify antibody-binding to *A. fumigatus* mycelium showed a clear distinction between non-immunized and hyperimmunized sera. Major differences in chemical composition between wall and cytoplasm were emphasized, this technique revealing few shared components. Human sera showed variable binding capacity but two of three pools tested had very strong reactivity.

When incubated with *A. fumigatus* soluble antigens ConA caused antigen precipitation and subsequent inhibition of antigen-antibody binding (Hearn & Mackenzie, 1979). The fact that binding of ConA to the mycelial surface occurred without causing a reduction in antibody uptake indicated that the sites of greatest affinity for the lectin are not the antigenic sites. Other lectins used here are capable of binding only to residues at the non-reducing terminals of saccharide chains. If a large part of the galactose residues in *A. fumigatus* is in the furanose form, this would preclude its reacting with castor bean lectin.

The effect of enzymes on mycelial surfaces was analysed. It has been shown that walls contain autolysing enzymes but the concentration of sugars released in 2 h at 37 °C was low (Polacheck & Rosenberger, 1975). Odds *et al.* (1974) found that, despite substantial release of sugars from the cell wall of *Histoplasma capsulatum* with a mixture of (1 $\rightarrow$ 3)- β -D-glucanase and chitinase, only a small and probably insignificant change in antigenic titre occurred, as measured by complement fixation tests. I have observed substantial changes in antibody uptake with relatively young cultures containing a low concentration of melanin, which can inhibit enzymic hydrolysis (Kuo & Alexander, 1967). San-Blas (1982) suggests that (1 $\rightarrow$ 3)- α -D-glucan protects young, non-melanized hyphae, presumably because of its ability to hinder hydrolytic enzymes from reaching their substrate. I was unable to inhibit antigen-antibody binding with a (1 $\rightarrow$ 3)- α -

Fig. 4. Scanning electron microscopy of *Aspergillus* mycelium after enzyme treatment. (a) *A. fumigatus* incubated with *Cytophaga* lytic enzyme (for details see Methods); (b) *A. fumigatus* incubated in the absence of enzyme; (c) *A. terreus* incubated with *Cytophaga* enzyme; (d) *A. terreus* incubated in the absence of enzyme; (e) *A. niger* incubated with *Cytophaga* enzyme; (f) *A. niger* incubated in the absence of enzyme. The bar markers represent 10 μm .

D-glucan preparation; however, an antiserum raised to a presumptive galactomannan and (1 $\rightarrow$ 3)- α -D-glucan-containing extract showed appreciable binding to fungal pellets.

Studies on *A. niger* and *A. nidulans* have shown a cell wall composed of glucan (40–60%) and chitin (10–20%); the glucans are predominantly alkali-soluble (1 $\rightarrow$ 3)- α -linked residues and alkali-resistant (1 $\rightarrow$ 3)- β - and (1 $\rightarrow$ 4)- α -linked polymers (Johnson, 1965; Bull, 1970; Zonneveld, 1971; Stagg & Feather, 1973). Cell wall galactomannan from *A. niger* contains (1 $\rightarrow$ 2)- α - and (1 $\rightarrow$ 6)- α -linked D-mannopyranosyl units with terminal galactofuranose units as non-reducing end-groups of D-galactopyranosyl side chains (Bardalaye & Nordin, 1977). This galactomannan corresponds closely with that isolated from *A. fumigatus* extracts by Azuma *et al.* (1971), who

also found a (1→4)- α -linked glucan with (1→6)- α -linked branch points. Enzymes with the ability to hydrolyse these linkages were tested for their effect on surface structures of *A. fumigatus*. A decrease in binding of IgG to the cell surface was caused by α -amylase, α -mannosidase and *Cytophaga* extract, which contains a mixture of relatively non-specific endoglucanases. On the other hand, β -amylase and exo-(1→3)- β -glucanase, which act on the non-reducing end-groups of polysaccharide chains, had no significant effect. It is difficult to evaluate the effect of *T. harzianum* culture filtrate, which constitutes a very complicated hydrolytic mixture (de Vries & Wessels, 1972). However, it is of interest that extra sites are exposed, rather than destroyed, by this treatment.

Whether this alteration in IgG binding is due to a direct enzyme effect on antigenic sites or to a disorganization of the cell surface has not been established. In some cases, e.g. in the action of *Cytophaga* extract on *A. niger* and *A. terreus*, it was apparent from SEM studies that the effects of hydrolysis were widespread and the total surface architecture was damaged. What is clear is that release of (1→4)- α -, (1→3)- β - and (1→4)- β -linked glucose residues, in addition to (1→2)- α - and/or (1→3)- α -linked mannose units from the mycelial surface, affected IgG binding to antigen sites.

Of the enzymes tested, α -amylase had the most widespread effect on surface architecture as measured by reduced antibody binding to the aspergilli. β -Amylase effected changes in only two species, namely *A. terreus* and *A. nidulans*, the former being the only one not affected by α -amylase. *Aspergillus flavus* appeared singularly unaffected by most enzyme treatments; however, (1→3)- β -glucanase caused increased binding and α -amylase caused decreased binding of IgG to surface structures. Despite the battery of enzymes present in the culture filtrate of *T. harzianum*, only *A. terreus* in addition to *A. fumigatus* was affected. While chitin is considered to constitute an underlying layer of hyphal wall material, chitinase was responsible for an increase in antibody binding in the case of *A. terreus* and a decrease in the case of *A. niger*. However, it cannot be ruled out that the low level of (1→3)- β -glucanase activity present in the commercial preparation made sites accessible for chitin hydrolysis.

The results obtained provide additional information on the nature of *A. fumigatus* surface structures. As purified and chemically defined moieties are isolated from *Aspergillus* they may be used to advantage in this system as possible inhibitors to antibody uptake and to produce monospecific antisera for binding assays.

I am indebted to Dr D. W. Dresser (Immunology Department, National Institute for Medical Research, Mill Hill, London) for the protocols used for raising antibodies. The electronmicrographs were kindly prepared by Mr M. Smith and Mrs E. Wilson. I am grateful to Mr G. Shennan and Miss J. John for excellent technical assistance. My thanks are due to Dr M. Kelemen (Microbiology Department, School of Pharmacy, Brunswick Square, London) for constant advice and encouragement.

This work was supported by a grant from the Medical Research Council.

REFERENCES

- AZUMA, I., KIMURA, H., HIRAO, F., TSUBURA, E. & YAMAMURA, Y. (1969). Biochemical and immunological studies on *Aspergillus*. II. Immunological properties of protein and polysaccharide fractions obtained from *Aspergillus fumigatus*. *Mycopathologia et mycologia applicata* **37**, 289–303.
- AZUMA, I., KIMURA, H., HIRAO, F., TSUBURA, E., YAMAMURA, Y. & MISAKI, A. (1971). Biochemical and immunological studies on *Aspergillus*. III. Chemical and immunological properties of glycopeptide obtained from *Aspergillus fumigatus*. *Japanese Journal of Microbiology* **15**, 237–246.
- BALLOU, C. (1976). Structure and biosynthesis of the mannan component of the yeast cell envelope. *Advances in Microbiology and Physiology* **14**, 93–158.
- BARDALAYE, P. C. & NORDIN, J. H. (1977). Chemical structure of the galactomannan from the cell wall of *Aspergillus niger*. *Journal of Biological Chemistry* **252**, 2584–2591.
- BARTNICKI-GARCIA, S. (1968). Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annual Review of Microbiology* **22**, 87–108.
- BIBERFELD, P., BIBERFELD, G., MOLNAR, Z. & FAGRAEUS, A. (1974). Fixation of cell-bound antibody in the membrane immunofluorescence test. *Journal of Immunological Methods* **4**, 135–148.
- BIBERFELD, P., GHETIE, V. & SJÖQUIST, J. (1975). Demonstration and assaying of IgG antibodies in tissues and on cells by labelled Staphylococcal Protein A. *Journal of Immunological Methods* **6**, 249–259.
- BOLTON, A. E. & HUNTER, W. M. (1973). The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent.

- Application to the radioimmunoassay. *Biochemical Journal* **133**, 529–539.
- BULL, A. T. (1970). Chemical composition of wild-type and mutant *Aspergillus nidulans* cell walls. The nature of polysaccharide and melanin constituents. *Journal of General Microbiology* **63**, 75–94.
- DORVAL, G., WELSH, K. I. & WIGZELL, H. (1974). Labelled Staphylococcal Protein A as an immunological probe in the analysis of cell surface markers. *Scandinavian Journal of Immunology* **3**, 405–411.
- DORVAL, G., WELSH, K. I. & WIGZELL, H. (1975). A radioimmunoassay of cellular surface antigens on living cells using iodinated soluble Protein A from *Staphylococcus aureus*. *Journal of Immunological Methods* **7**, 237–250.
- HARBOE, M. & FÖLLING, I. (1974). Recognition of two distinct groups of IgM and IgA based on differential binding to staphylococci. *Scandinavian Journal of Immunology* **3**, 471–482.
- HEARN, V. M. & MACKENZIE, D. W. R. (1979). The preparation and chemical composition of fractions from *Aspergillus fumigatus* wall and protoplasts possessing antigenic activity. *Journal of General Microbiology* **112**, 35–44.
- HEARN, V. M. & MACKENZIE, D. W. R. (1980). Mycelial antigens from two strains of *Aspergillus fumigatus*: an analysis by two-dimensional immunoelectrophoresis. *Mykosen* **23**, 549–562.
- HEARN, V. M., WILSON, E. V. & MACKENZIE, D. W. R. (1980). The preparation of protoplasts from *Aspergillus fumigatus* mycelium. *Sabouraudia* **18**, 75–77.
- HEARN, V. M., WILSON, E. V. & MACKENZIE, D. W. R. (1981). Antigen analysis of *Aspergillus fumigatus* mycelium. In *Proceedings of the VIth International Symposium on Glycoconjugates*, p. 381. Edited by T. Yamakama, T. Osawa & S. Handa. Tokyo: Japan Scientific Societies Press.
- HORIKOSHI, K. & ARIMA, K. (1962). X-ray diffraction patterns of the cell wall of *Aspergillus oryzae*. *Biochimica et biophysica acta* **57**, 392–394.
- HUNSLEY, D. & BURNETT, J. H. (1970). The ultrastructural architecture of the walls of some hyphal fungi. *Journal of General Microbiology* **62**, 203–218.
- HUNTER, W. M. & GREENWOOD, F. C. (1962). Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature, London* **194**, 495–496.
- JOHNSON, I. R. (1965). The composition of the cell wall of *Aspergillus niger*. *Biochemical Journal* **96**, 651–658.
- KUO, M.-J. & ALEXANDER, M. (1967). Inhibition of lysis of fungi by melanins. *Journal of Bacteriology* **94**, 624–629.
- LEHMANN, P. F. & REISS, E. (1978). Invasive aspergillosis: antiserum for circulating antigen produced after immunization with serum from infected rabbits. *Infection and Immunity* **20**, 570–572.
- LLOYD, K. O. (1970). Isolation, characterisation and partial structure of peptidogalactomannans from the yeast form of *Cladosporium werneckii*. *Biochemistry* **9**, 3446–3453.
- LLOYD, K. O. & BITOON, M. A. (1971). Isolation and purification of a peptido-rhamnomannan from the yeast form of *Sporothrix schenckii*. Structural and immunochemical studies. *Journal of Immunology* **107**, 663–671.
- LLOYD, K. O., MENDONÇA-PREVIATO, L. & TRAVASSOS, L. R. (1978). Distribution of antigenic polysaccharides in different cell types of *Sporothrix schenckii* as studied by immunofluorescent staining with rabbit antisera. *Experimental Mycology* **2**, 130–137.
- ODDS, F. C., KAUFMAN, L., McLAUGHLIN, D., CALLAWAY, C. & BLUMER, S. O. (1974). Effect of chitinase complex on the antigenicity and chemistry of yeast-form cell walls and other fractions of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. *Sabouraudia* **12**, 138–149.
- POLACHEK, Y. & ROSENBERGER, R. F. (1975). Autolytic enzymes in hyphae of *Aspergillus nidulans*: their action on old and newly formed walls. *Journal of Bacteriology* **121**, 332–337.
- POTGIETER, H. J. & ALEXANDER, M. (1965). Polysaccharide components of *Neurospora crassa* hyphal walls. *Canadian Journal of Microbiology* **11**, 122–125.
- PREVIATO, J. O., GORIN, P. A. J. & TRAVASSOS, L. R. (1979). Cell wall composition in different cell types of the dimorphic species *Sporothrix schenckii*. *Experimental Mycology* **3**, 83–91.
- PROCTOR, A. G. (1976). Mycological methods. In *Microbiological Methods*, 4th edn, pp. 181–193. London: Butterworths.
- REISS, E. (1977). Serial enzymatic hydrolysis of cell walls of two serotypes of yeast-form *Histoplasma capsulatum* with α -(1→3)-glucanase, β -(1→3)-glucanase, pronase and chitinase. *Infection and Immunity* **16**, 181–188.
- RIGHELATO, R. C., TRINCI, A. P. J., PIRT, S. J. & PEAT, A. (1968). The influence of maintenance energy and growth rate on the metabolic activity, morphology and conidiation of *Penicillium chrysogenum*. *Journal of General Microbiology* **50**, 399–412.
- SAN-BLAS, G. (1982). The cell wall of fungal human pathogens: its role in host-parasite relationships. *Mycopathologia* **79**, 159–184.
- STAGG, C. M. & FEATHER, M. S. (1973). The characterization of a chitin-associated D-glucan from the cell walls of *Aspergillus niger*. *Biochimica et biophysica acta* **320**, 64–72.
- STEINBUCH, M. & AUDRAN, R. (1969). The isolation of IgG from mammalian sera with the aid of caprylic acid. *Archives of Biochemistry and Biophysics* **134**, 279–284.
- STODDART, R. W. & HERBERTSON, B. M. (1978). The use of fluorescein-labelled lectins in the detection and identification of fungi pathogenic for man: a preliminary study. *Journal of Medical Microbiology* **11**, 315–324.
- SUNAYAMA, H. & SUZUKI, S. (1970). Studies on the antigenic activities of yeasts. V. Effect of α -mannosidase digestion on the immunochemical properties of the mannan from *Saccharomyces cerevisiae*. *Japanese Journal of Microbiology* **14**, 197–207.
- TRAVASSOS, L. R., DE SOUSA, W., MENDONÇA-PREVIATO, L. & LLOYD, K. O. (1977). Location and biochemical nature of surface components reacting with Concanavalin A in different cell types of *Sporothrix schenckii*. *Experimental Mycology* **1**, 293–305.
- DE VRIES, O. M. H. & WESSELS, J. G. H. (1972). Release of protoplasts from *Schizophyllum commune* by a lytic

- enzyme preparation from *Trichoderma viride*. *Journal of General Microbiology* **73**, 13–22.
- WILSON, E. V. & HEARN, V. M. (1983). Use of *Aspergillus fumigatus* mycelial antigens in enzyme-linked immunosorbent assay and counterimmuno-electrophoresis. *Journal of Medical Microbiology* **16**, 97–105.
- ZELTZER, P. M. & SEEGER, R. C. (1977). Microassay using radioiodinated Protein A from *Staphylococcus aureus* for antibodies bound to cell surface antigens of adherent tumour cells. *Journal of Immunological Methods* **17**, 163–175.
- ZONNEVELD, B. J. M. (1971). Biochemical analysis of the cell wall of *Aspergillus nidulans*. *Biochimica et biophysica acta* **249**, 506–514.