

Radioallergosorbent test (RAST) studies with insolubilized polysaccharides

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Summary

Polysaccharide preparations from *Aspergillus fumigatus* and *Candida albicans* when used with some human sera were found to fix complement and precipitate with specific antibodies present in the sera. Precipitins consisted predominantly of IgG with traces of IgM and IgA. Following insolubilization of the fungal polysaccharide preparations with the *N*-carboxyanhydride of L-leucine, reactivity with serum precipitins was retained and reaction with IgE antibodies was demonstrated using the radioallergosorbent test (RAST). Results showed that for *A. fumigatus* and *C. albicans* at least, polysaccharide-specific IgE antibodies probably occur in sera from some hypersensitive individuals. We suggest that chemical methods of the type described which induce polymerization or cross-linking will be useful for detecting IgE antibodies in the RAST. Such an approach could be used with a variety of polysaccharide preparations which cannot be coupled to polysaccharide solid supports by the usual methods.

Introduction

In recent years, the radioallergosorbent test (RAST) has become widely accepted as a method for detecting IgE antibodies with specificities for various allergens (Wide, Bennich & Johansson, 1967; Baldo & Turner, 1975). At present, the RAST is the most suitable method for providing at least a semi-quantitative assessment of the presence of these antibodies in man but, the method as it is normally used, has a major drawback. Chemical coupling procedures which can be employed to attach allergens to solid supports do not easily allow most polysaccharide allergens to be examined with the technique (Axén, Porath & Ernback, 1967; Weetall, 1973).

The RAST has been used to detect IgE antibodies which show specificity for fungal allergens (Arbesman *et al.*, 1974; Turner *et al.*, 1974; Baldo, Quinn & Turner, 1975) but *in vitro* attempts to assess the relative contributions of the protein and polysaccharide determinants to the total allergenicity of the extracts have not yet been made. If the RAST is to be successfully used in such an investigation, suitably convenient methods will have to be employed to immobilize soluble polysaccharide antigens.

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With this in mind, we attempted to convert some fungal carbohydrates into an insoluble form so that the antigenic determinants were still available for reaction and, at the same time, the need for an insoluble matrix was avoided. In this communication we present details of the insolubilization of *Candida albicans* and *Aspergillus fumigatus* polysaccharides and the results obtained when the latter preparation was used in RAST experiments. It is suggested that the procedures described might be generally employed to detect IgE antibodies to other polysaccharides which cannot be coupled to insoluble polysaccharide supports by the usually employed methods.

Materials and methods

Fungal preparations

Aspergillus fumigatus was grown at 37°C in Sabouraud's medium and the somatic polysaccharide prepared as described by Longbottom & Pepys (1964). The preparation contained 5.1% protein as measured by the Lowry method and hence will be referred to as *A. fumigatus* peptido-polysaccharide (PPS). Dried *A. fumigatus* spores and mycelia obtained from Greer Laboratories Inc., Lenoir, North Carolina, U.S.A., were extracted with dimethyl sulphoxide as described by Baldo, Quinn & Turner (1975). *Candida* culture filtrate was obtained from *C. albicans* strain A207 grown in Sabouraud's broth at 37°C as described by Faux (1968). *Candida albicans* mannan A was prepared using the method of Peat, Whelan & Edwards (1961). The purified preparation contained 0.29% N.

Preparation of polyleucyl fungal derivatives

Aspergillus fumigatus PPS (60 mg) and *C. albicans* mannan A (60 mg) were each copolymerized with an equal quantity of the *N*-carboxyanhydride of L-leucine (Miles-Yeda, Rehovoth, Israel, lot no. LE45) at 4°C in a vehicle of 0.07 M sodium bicarbonate (Tsuyuki, van Kley & Stahmann, 1956; Kaplan & Kabat, 1966). The insoluble product was sedimented at 15,000 rev/min for 1 hr and washed twice with distilled water. Yields of 92 and 94%, respectively, were obtained for the insoluble *Aspergillus* and *Candida* preparations. The preparations were air-dried and stored at -20°C.

RAST procedures

Aspergillus and *Candida* proteins were coupled to microcellulose (Merck) or to Whatman grade 44, 6 mm paper discs using the cyanogen bromide procedures described by Axén, Porath & Ernback (1967) and Ceska, Eriksson & Varga (1972). *Aspergillus* proteins extracted with dimethyl sulphoxide were used at a concentration of 7.5 mg per 100 mg of cellulose and 20 mg per 100 paper discs. For *C. albicans* culture filtrate the quantities used were 2.5 mg per 100 mg of cellulose and 7.5 mg for each 100 discs.

For use in RAST experiments, polyleucyl-carbohydrate derivatives were ground to a fine powder in a mortar and mixed with microcellulose in 0.1 M phosphate buffered saline pH 7.3 containing 0.5% Tween 20 and 0.2% bovine serum albumin. Suspensions were shaken vigorously with glass beads before dispensing into tubes containing the sera to be examined. Quantities of insoluble fungal polysaccharides were adjusted so that each 0.5 ml volume contained 10, 20, 50 or 100 µg of the fungal preparation and 0.5 mg of cellulose.

Discs, together with test, reference or cord sera, were incubated for 3 hr before washing and addition of Pharmacia immunoadsorbent-purified rabbit anti-human IgE

labelled with ^{125}I to a specific activity of approximately $6.2 \mu\text{Ci}/\mu\text{g}$. Tubes containing proteins coupled to microcellulose were treated as already described (Baldo, Quinn & Turner, 1975). After incubation with labelled anti-IgE, discs and cellulose were washed five times before counting.

Tubes containing insoluble polyleucyl derivatives, cellulose and serum were rotated overnight at room temperature and washed before addition of the labelled anti-IgE. Cord serum samples were included in the tests as 'IgE-free' serum controls. After five washings, the radioactivity remaining was measured by counting each tube for 5 min in a Packard Auto-Gamma Spectrometer.

To guard against the detection of polymerized IgE, each serum examined in the RAST experiments was also incubated with a paper disc (not activated) or 0.5 mg of cellulose only. This control is necessary since some sera, especially if stored for long periods and not centrifuged before RAST examination, contain material which reacts with ^{125}I -anti-IgE after the washing and spinning procedures involved in the RAST (Baldo, Wells & Pepys, 1974; Baldo, unpublished; Edge & Pepys, 1976).

Quantitative precipitin method

This was carried out on a micro scale as previously described (Baldo & Uhlenbruck, 1975a) using the method of Kabat (1961) and Schiffman, Kabat & Thompson (1964). Nitrogen in the washed precipitates and in fungal preparations was determined by the ninhydrin method (Schiffman *et al.*, 1964).

Complement fixation

This was carried out as described by Mayer (1961) using lyophilized guinea-pig serum (Wellcome Research Lab., Beckenham, England, lot no. K4935) as a source of complement and rabbit anti-sheep erythrocyte haemolytic serum (Wellcome, lot no. K3396B). The human sera examined were first heated at 56°C for 30 min.

Affinity chromatography

Aspergillus fumigatus PPS and *C. albicans* mannan A insolubilized with polyleucyl NCA were each mixed with Sepharose 4B (Pharmacia) in the ratio 2:1, suspended in PBS and allowed to stand for 24 hr. The mixtures were then poured into small columns and washed extensively with PBS. Human sera to be investigated for PPS- or mannan-specific antibodies, were added to the columns and eluted with PBS. Fractions were collected and examined for absorbance at 280 nm. Specific elution of bound antibodies was effected by adding Tris-glycine buffer pH 3 to the column. Fractions showing absorbance at 280 nm were pooled, dialysed, concentrated, lyophilized and examined on immunoplates and in quantitative precipitin experiments with the appropriate polysaccharide.

Results

Examination of the reactivities of A. fumigatus and C. albicans polysaccharides

(1) *Quantitative precipitin studies.* Polysaccharides from both species reacted readily with Con A (Fig. 1a) and with human sera from some asthmatic patients (Fig. 1b, c). Reactions with Con A presumably occur via the mannose units present in both of the polysaccharide preparations and demonstrate that this lectin could be useful for the isolation of these saccharide components using affinity chromatography techniques.

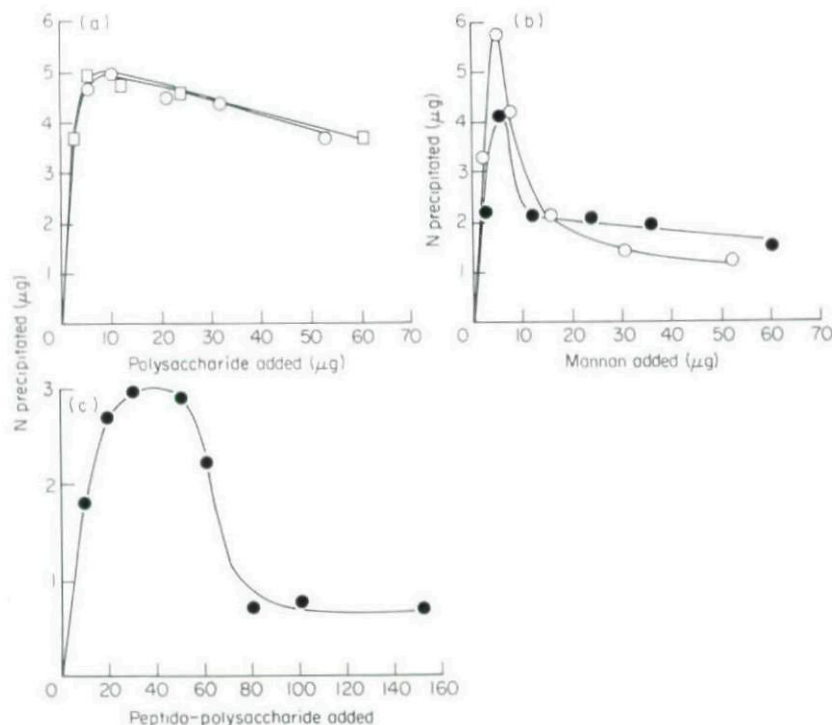


Fig. 1. Precipitation reactions of *Candida albicans* mannan A and *Aspergillus fumigatus* peptido-polysaccharide.

(a) Precipitation of the fungal preparations by Concanavalin A. Con A (29.1 μg, 4.7 μg N) was added to tubes containing *C. albicans* mannan A (□) and *A. fumigatus* peptido-polysaccharide (○). Total volume in each tube, 125 μl.

(b) Precipitation of *C. albicans* mannan A by human sera How (○) 100 μl and Bray (●) 50 μl. Total volume in each tube, 150 μl.

(c) Precipitation of *A. fumigatus* peptido-polysaccharide by human serum Ald, 100 μl. Total volume, 175 μl.

In precipitation studies using human sera and *C. albicans* mannan A, maximum precipitation occurred with small amounts of polysaccharide and sharp equivalence points were observed (Fig. 1b). Four sera obtained from adults with allergic aspergillosis, clearly precipitated with *A. fumigatus* PPS. With these sera, maximum precipitation occurred at higher antigen concentrations than was seen with the *C. albicans* mannan precipitation reactions and, in contrast to the latter reactions, no sharp, well-defined equivalence points were observed. Fig. 1c shows the results obtained with serum Ald, obtained from one of the allergic aspergillosis subjects.

(2) *Complement fixation.* The reactions of the fungal polysaccharides with human serum antibodies was further examined in complement fixation experiments. Both saccharide preparations fixed complement when used with the same sera used in quantitative precipitin studies. For example, with *C. albicans* mannan A and serum How diluted 1 in 80, complement fixation was demonstrated with as little as 0.0195 μg of mannan.

Isolation of A. fumigatus PPS- and C. albicans mannan-reactive antibodies from human sera via direct precipitation of soluble polysaccharides

Aspergillus fumigatus PPS (1 mg) and *C. albicans* mannan A (150 μg) were mixed with

human sera (serum Ald (3 ml) for the *Aspergillus* PPS and serum How (3 ml) for the *Candida* mannan), and allowed to stand for 5 days at 4°C. The proportions used were previously determined in quantitative precipitin studies (see Figs 1b and c). Precipitates which formed were washed five times with physiological saline and then extracted with two successive portions of 15% sodium chloride solution (Kabat, 1971). Each extraction was carried out with shaking first at 37°C for 1 hr and then at room temperature for 30 min. Pooled supernatants were concentrated by ultrafiltration against physiological saline until the volume had been reduced to 0.5 ml and then examined on 'Tri-Partigen' immunoplates for the presence of IgG, IgM and IgA. Results clearly showed that the polysaccharide preparations used reacted with antibodies in the human sera. For both fungal preparations, IgG was the predominant complementary immunoglobulin present in the human sera but small amounts of IgM and trace amounts of IgA were also present.

Demonstration of the retention of reactivity by the insolubilized fungal polysaccharide preparations

Addition of serum Ald and serum How to small columns (1 × 12 cm) of the *A. fumigatus*- and *C. albicans*-polyleucyl preparations, respectively, showed that the ability of these insoluble preparations to bind human antibodies was not lost following polymerization. Antibodies (predominately IgG) which were 83–90% precipitable by the complementary soluble polysaccharide preparation were eluted from the columns with acid buffers (Tris-glycine or citric acid-glycine, pH 3).

RAST examinations

Following the demonstration that reactivity with human IgG was retained after polymerization, the *Aspergillus* and *Candida* insoluble preparations were examined in RAST experiments with sera from allergic patients. Findings with the insoluble mannan and PPS were compared with results obtained when the fungal proteins were chemically coupled to paper discs or microcellulose. Of fifty-one sera from asthmatic patients, 39% showed positive RAST reactions to the *Candida* culture filtrate extract and 22% showed weak but definite positive reactions with the insoluble purified mannan A.

Sera from nine patients diagnosed as allergic aspergillosis, three cord sera and sera from eighteen apparently healthy human adults selected at random were used in RAST experiments with polyleucyl-*A. fumigatus* PPS. As the clinical history of each of these nine patients is known and the diagnosis well established, the RAST results obtained with each serum are presented in detail. When used with *A. fumigatus* proteins coupled to paper discs, marked uptake of label was observed. Six of the nine samples examined showed uptakes greater than 24% of the original counts added (Table 1). The three cord and eighteen normal serum samples showed percentage radioactive uptakes of less than 2%. Significant binding of serum IgE was also demonstrated when the insoluble PPS was examined although, with this preparation, the highest radioactive uptake observed was only 5.1%. Comparisons with cord and control sera, however, indicated that PPS-specific IgE antibodies were being detected in each of the nine sera examined. Table 2 shows the results obtained when 10 µg and 100 µg quantities of polyleucyl-PPS were examined. Radioactive uptake not dependent on the presence of allergen-specific IgE antibodies increased as the quantity of polyleucyl-PPS increased. This is demonstrated in Table 2 where it can be seen that the percentage uptake of

Table 1. Reaction of sera from allergic aspergillosis sufferers with *Aspergillus fumigatus* proteins coupled to paper discs

Serum	Radioactive uptake (%)	Radioactive uptake ratio test serum:cord serum
Cord	1.3	
A	9.3	7.1
E	27.8	21.4
H	3.8	2.9
K	32.7	25.1
M	32.7	25.1
Ald	6.4	4.9
Ben	30.2	23.2
Cov	24.4	18.8
Erk	32.0	24.6

Sera A, E, H and K were diluted 1:3 before examination.

Table 2. Reaction of sera from allergic aspergillosis sufferers with insoluble polyethyl derivative of *Aspergillus fumigatus* peptido-polysaccharide

Serum	Quantity of insoluble peptido-polysaccharide per tube			
	10 µg		100 µg	
	Radioactive uptake (%)	Test serum:cord	Radioactive uptake (%)	Test serum:cord
Cord	1.0		3.9	
A	3.5	3.5	14.6	3.7
E	2.4	2.4	9.9	2.5
H	2.2	2.2	7.7	2.0
K	4.5	4.5	16.0	4.1
M	5.1	5.1	18.7	4.8
Ald	3.3	3.3	10.9	2.8
Ben	2.5	2.5	11.5	2.9
Cov	2.8	2.8	9.5	2.4
Erk	1.9	1.9	8.1	2.1

Sera A, E, H and K were diluted 1:3 before examination.

label for both cord and test serum samples increased when 100 µg of polyethyl-PPS was used instead of 10 µg. Even so, the ratio of the radioactive uptake of test to cord samples remained almost the same for tubes containing 10 µg or 100 µg of the insoluble PPS. With 10 µg quantities of polyethyl-PPS, radioactive uptake by all three cord sera was 1% or less and only two of the eighteen normal sera bound more than 1.4 but less than twice the amount of label taken up by cord sera.

Discussion

If insolubilization of a substance suspected of being allergenic is to be an acceptable alternative to the proven methods of coupling allergens to solid supports such as paper

or microcellulose, then a number of requirements must be fulfilled. Firstly, the insoluble product must retain its reactivity with its complementary antibodies and, when used in the RAST, non-specific uptake of labelled iodine, with consequent high background counts, should not occur. Other requirements include a fine particle size suitable for easy distribution to large numbers of tubes and when in suspension, but, the product must still be of sufficient density to allow sedimentation after washing and before counting. The polyleucyl-fungal preparations studied here satisfied the above requirements although relatively high background counts were observed when larger quantities (100 μg) were used. The results showed, however, that it was unnecessary to use quantities of this order. Similar results relative to controls were obtained with 10 μg and 100 μg amounts of *A. fumigatus* polysaccharide.

Acid anhydrides such as the leucine derivative used here are susceptible to nucleophilic attack and hence are suitable for use with sugars containing free hydroxyl groups. The method has been used successfully to insolubilize a number of glycopeptides and polysaccharides for antibody and lectin purification and the polymers obtained have been found to retain their original antibody or lectin binding specificities (Kaplan & Kabat, 1966; Hammarström & Kabat, 1969; Moreno & Kabat, 1969; Lloyd, 1970; Galbraith & Goldstein, 1972; Baldo & Uhlenbruck, 1975b). Both of the polyleucyl-fungal preparations we studied were also found to have retained their reactivity for the complementary antibodies in patients' sera.

The observations that some glycopeptides and polysaccharides are capable of eliciting immediate skin reactions in infected laboratory animals and humans (Kabat, 1961; Barker *et al.*, 1962; Holborow & Loewi, 1962; Grappel, Bishop & Blank, 1974) has led to a desire to examine the nature and extent of the reagin response to many different carbohydrate allergenic determinants. A number of attempts have been made to separate and characterize antigens from different species of fungi and study their role in hypersensitivity reactions (Grappel, Bishop & Blank, 1974). In particular, carbohydrate-containing antigens from *Aspergillus*, *Candida* and dermatophyte species have been shown to produce immediate hypersensitivity reactions (Pepys *et al.*, 1968; Azuma *et al.*, 1969; Pepys, 1969; Kabe *et al.*, 1971; Grappel, Bishop & Blank, 1974).

The capacity of many so-called pure polysaccharides to induce immediate hypersensitivity responses has been discussed by Pepys & Longbottom (1973).

In our study, some sera were found to contain IgE antibodies which reacted with the insolubilized *Candida* and *Aspergillus* preparations. Only with the insoluble *C. albicans* mannan however could it be claimed that carbohydrate specific IgE antibodies were detected. This has since been confirmed in RAST tests using a suspension of finely ground and insoluble mannan, polymerized by treatment with epichlorohydrine (Sandula & Kuniak, 1974) and reported by Edge & Pepys (1976). Heat-stable, short-term homocytotropic antibodies to *C. albicans* mannan have also been demonstrated in human sera by PCA tests in the monkey (Longbottom *et al.*, 1976). Thus there are at least two antibody mechanisms for the production of immediate reactions to this polysaccharide antigen. Antibody reactions with both the *C. albicans* mannan and the *A. fumigatus* preparation can be accompanied by complement fixation and this too has to be taken into account in assessing the reactions they can elicit. However, carbohydrate preparations which elicit immediate reactions often contain peptide moieties of varying sizes and the peptide itself may be responsible for the observed immediate reactions (Pepys & Longbottom, 1973). The *A. fumigatus*

preparation we used contained 5.1% protein and the IgE antibody detected may have had specificity for the peptide portion of this peptido-polysaccharide. The experiments described here were not designed to investigate this point, but to examine the possibility of using material suspected of being allergenic in an insoluble form for RAST experiments in place of the normally used allergen-cellulose or allergen paper disc conjugates. This report and that of Edge & Pepys (1976) show that the use of reagents which can induce polymerization or cross-linking may provide a useful means for detecting IgE antibodies to natural and synthetic saccharide structures.

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