

The primary interaction of antibody to components of aspergilli

I. Immunologic and chemical characteristics of a nonprecipitating antigen

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*The experiments presented in this report describe the preparation of a radiolabeled antigen which can be used in the ammonium sulfate test to measure antibodies against aspergilli. The antigenic components were in the trichloroacetic acid (TCA)-soluble fraction from sonically disrupted aspergilli. The major radiolabeled ligand appeared to be an immunologically nonprecipitating anionic mucopolysaccharide which was insensitive to DNase or RNase, slightly sensitive to pepsin, and very sensitive to periodate oxidation and borohydride reduction. Results of quantitative studies indicate that the radiolabeled antigens had a relatively low affinity for antibody populations produced in hyperimmunized rabbits. Quantitative inhibition experiments as well as immunodiffusion studies demonstrated similarities and differences among species of the genus *Aspergillus* as well as other fungi. Commercial house dust extracts appear to contain significant amounts of the TCA-soluble aspergilli antigens.*

Antibodies to components of aspergilli have been demonstrated by several immunologic techniques including immunodiffusion in agar,¹⁻¹² immunoelectrophoresis (IEP),¹³⁻¹⁶ complement fixation,¹⁷⁻¹⁹ and latex agglutination.²⁰ Since these tests do not measure the primary interaction between antigen and antibody, a negative test does not necessarily indicate the absence of antibody. Some qualitative primary tests, e.g., indirect radioimmunodiffusion (RID),²¹ indirect radioimmunoelectrophoresis (RIEP),²² and the indirect fluorescent antibody test,^{23, 24} have also been employed to detect antibody to *Aspergillus fumigatus*. Although sensitive, the latter techniques do not permit quantitative determinations.

The present experiments describe the isolation and characterization of a radiolabeled antigen which can be used to distinguish between certain species of aspergilli and is suitable for use in the ammonium sulfate test.²⁵ In view of apparent rising incidence of infections by these usually saprophytic fungi,^{24, 26-28}

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such a test may have clinical applicability as will be discussed in further detail in an accompanying report.²⁹

MATERIALS AND METHODS

Microorganisms and growth conditions

Fungi. Unless otherwise noted, all fungi were obtained from the stock culture collection maintained at this institution. *Aspergillus fumigatus*, *A. nidulans*, and *A. flavus* were grown at 37° C. in Sabouraud's dextrose broth (Difco) and harvested after 3 weeks of growth. *Candida albicans*, *Trichophyton rubrum*, and *Nocardia asteroides* were similarly grown and harvested after 3 to 50 days of growth. Fungi were killed by inspissation at 80° C. for 60 minutes, collected after filtration through several layers of cheesecloth, and washed 3 times by repeated centrifugation at 12,000 × g for 30 minutes and resuspension in sterile pyrogen-free distilled water.

Mycobacteria. *Mycobacterium tuberculosis* strain H37Rv (from Trudeau Institute, Saranac Lake, N. Y.), *M. bovis* strain BCG (obtained from the United States-Japan Medical Science Program), *M. intracellulare*, and *M. kansasii* were grown on the surface of modified Wong's medium as previously described.³⁰ All mycobacteria were grown at 37° C. and harvested during the late log phase of growth, heat killed, and washed as described above.

Non-acid-fast bacteria. *Staphylococcus aureus*, Cowan strain No. 1, *S. epidermidis*, *Streptococcus pyogenes*, Lancefield group A, *Escherichia coli*, *Neisseria catarrhalis*, and *Listeria monocytogenes* were grown in Tryptose phosphate broth (Difco) at 37° C. overnight with vigorous shaking for aeration. *Thermoactinomyces vulgaris* was grown on brain-heart infusion broth (Difco) at 45° C. for 24 hours. The bacteria were heat killed and washed as described above.

Preparation of antigens (see Fig. 1)

Fungi.

SS ANTIGEN. Suspensions of killed, washed fungi were sonicated for 3-hours as previously described.³² Cellular debris was removed after centrifugation for 120 minutes at 22,500 × g. The supernatant was dialyzed against deionized water and designated as the sonicated supernatant or SS antigen.

TCA-S ANTIGEN. An equal volume of 1:10 normal rabbit serum was added as carrier to samples of SS antigen. Trichloroacetic acid (TCA) was added to a final concentration of 10 per cent and the samples kept at 4° C. for 30 minutes. The resulting precipitate was removed after centrifugation at 3,000 × g for 30 minutes. The supernatant was dialyzed against borate buffer at 4° C. and designated as TCA-S antigen. This preparation was found to be free from rabbit serum by immunodiffusion and radioimmunodiffusion as described below.

Mycobacteria.

SS PREPARATIONS. Suspensions of heat-killed, washed mycobacteria were sonicated for 60 minutes.³²

Non-acid-fast bacteria.

SS PREPARATIONS. Suspensions of killed, washed bacteria were sonicated 5 minutes and thereafter treated as above for mycobacteria.

PROTEIN A. Protein A was prepared from the cell wall of *S. aureus* as previously described.^{37, 38}

BACTO-C PROTEIN STANDARD (CRP). Bacto-C protein standard was obtained from Difco Laboratories, Detroit, Michigan.

Other antigens.

BSA. Bovine serum albumin, crystallized, was obtained from the Reheis Chemical Co., Chicago, Ill.

HOUSE DUST PREPARATIONS. House dust concentrate, 2.5 per cent (1:40) (Lot No. AN131A), was obtained from Endo Laboratories, Inc., Garden City, N. J., and house dust allergenic extract, 12,000 protein nitrogen units (PNU) per milliliter, (Lot No. D1408205),

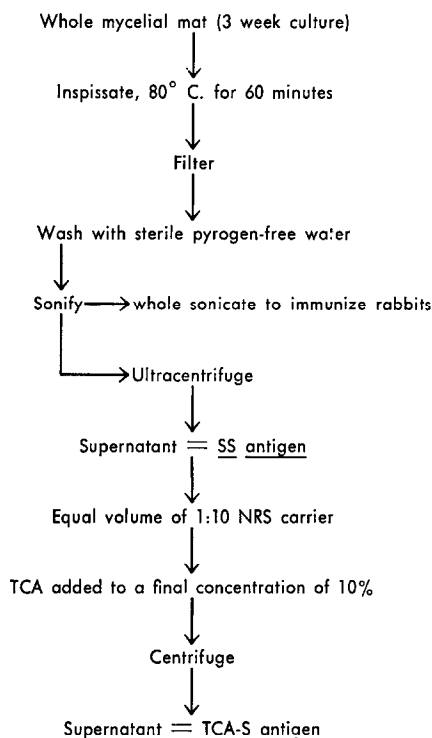


FIG. 1. Outline of procedures employed to prepare the test antigens used in this study.

was obtained from Hollister Stier Laboratories, Dallas, Texas. Both these preparations were concentrated by lyophilization, redissolved, and dialyzed in borate buffer for use in immunodiffusion and inhibition tests.

Radioisotope labeling

Test antigens were labeled with either ^{125}I or ^{131}I by a modification of the chloramine-T method.³⁵ The labeled SS and TCA-S antigens derived from *A. fumigatus* are referred to as ^{131}I -SS-Fum and ^{125}I -TCA-S-Fum, respectively, and components from *A. nidulans* as ^{131}I -SS-Nid and ^{125}I -TCA-S-Nid, respectively.

Analysis of antigens

Polyacrylamide electrophoresis.

UNLABELED SS AND TCA-S ANTIGENS. These antigens were analyzed by nonlinear gradient polyacrylamide electrophoresis with the use of four gel concentrations (3.75, 4.75, 7, and 12 per cent).³⁶ Gels were stained for protein with Coomassie brilliant blue, for polysaccharide and glycoprotein by the periodic acid-Schiff (PAS) reaction, for mucopolysaccharide by alcian blue, and for lipid by oil red O.³¹ In addition, after electrophoresis of some antigens, gels were embedded in a 2 mm. layer of 2 per cent Ionagar II (Oxoid) on 8.3×10.2 cm. glass slides. Agar was dissolved in 0.9 per cent saline containing 0.1 per cent sodium citrate. Troughs were cut on both sides of the gel and were filled with appropriate anti-*Aspergillus* rabbit antisera prepared as described below. The separated components within the gel were allowed to react with antisera for 48 to 72 hours.

RADIOLABELED SS AND TCA-S ANTIGENS. These antigens were also analyzed by nonlinear gradient polyacrylamide electrophoresis and the resulting gels exposed to x-ray film (AA54, Kodak Industrial). The radioactive areas on the film were then compared to duplicate stained gels.

Nitrogen and carbohydrate content. The nitrogen (N) content of the antigens was determined by a modification of the micro-Kjeldahl method with a Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.).³⁷ Carbohydrate (CHO) content was determined by a modification of the orcinol method³⁸ with the use of galactose standards.

Effect of enzyme treatment. ¹²⁵I-TCA-S-Fum and ¹²⁵I-TCA-S-Nid antigens at a concentration of 0.02 µg N per milliliter were exposed to a variety of enzymes, and the effect of this treatment on the capacity of antigens to react with hyperimmune sera was tested. The enzymes used were purified deoxyribonuclease-T₁ (DNase) from *A. oryzae*, calf thymus DNase-1, calf thymus ribonuclease-A (RNase-A) (all from Worthington Biochemical Corp., Freehold, N. J.), and pepsin (Nutritional Biochemicals Corp., Cleveland, Ohio).

Duplicate 5 µg samples of DNase-T₁ and DNase-I in 0.01M Tris (hydroxymethyl methylamine)-0.006M MgCl₂ buffer, pH 7.4, were mixed with an equal volume of radiolabeled antigen in 0.01M Tris, pH 7.4, and kept at 37° C. for 15 minutes.³⁹ Duplicate 5 µg samples of RNase-A in 0.5 ml. of 0.1M Tris-0.05M NaCl-0.001M ethylenediaminetetraacetic acid (EDTA), pH 7.4, were similarly incubated with an equal volume of radiolabeled antigen.⁴⁰ The reaction was stopped by chilling the tubes in an ice bath and adding 3 ml. of 5 per cent cold TCA. The mixture was then passed through a nitrocellulose filter (Type B-6, Schleicher and Schuell, Keene, N. H.). Denatured deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) adhere to the filter, but digested oligonucleotide segments will pass through.⁴¹ Similarly, intact SS and TCA-S antigens were found to adhere to the filter. By comparing the radioactivity remaining on the filter with the radioactivity of an untreated antigen, the per cent antigen digested can be calculated. Nuclease-treated and -untreated ³H-DNA and ¹²⁵I-RNA served as additional controls.

Binding activity was determined by incubating 0.5 ml. of antigens treated as above with 0.5 ml. of a 1:5 dilution of a hyperimmune rabbit serum prepared as described below for 18 hours at 4° C. Control experiments with untreated labeled antigen were also performed. The ammonium sulfate test was then carried out as described below.

Pepsin treatment of both ¹²⁵I-TCA-S antigens was carried out by a modification of the Nisonoff⁴² method. Labeled antigen preparations were dialyzed in 0.1M acetate buffer, pH 4.1. Pepsin was then added to a 3 ml. sample of both labeled test antigens at a ratio of 50:1 (antigen to enzyme) on a weight basis. The preparations were incubated for 20 hours at 37° C., then dialyzed at pH 7.0 and 4° C. ¹²⁵I-BSA was similarly treated as a control antigen.

Periodate oxidation and borohydride reduction of labeled antigen. These procedures have been used to preferentially degrade carbohydrate-polysaccharide moieties.⁴³ A 0.01M sodium metaperiodate solution, 1 ml., was added to 3 ml. of labeled antigen (0.02 µg N per milliliter) and incubated for 5 days at 25° C. The solution was dialyzed against borate buffer, and a portion was evaluated for binding activity with hyperimmunized rabbit sera with the use of the ammonium sulfate test. The remainder was subjected to borohydride reduction by the addition of 1 ml. of 0.1 per cent sodium borohydride solution and incubation for 5 hours at 25° C. Following the addition of 0.5 ml. of 6N acetic acid, the mixture was dialyzed against deionized water and then against borate buffer. Binding of treated antigen was determined with specific rabbit antisera with the use of the ammonium sulfate test. Labeled BSA was used as a control antigen.

Antisera

Rabbit. New Zealand white rabbits were immunized by the intramuscular route with portions of the *A. fumigatus* and *A. nidulans* whole sonicates in equal parts of incomplete Freund's adjuvant (Fig. 1).³² Control rabbit antisera to BSA were similarly prepared.

Goat. Antisera to rabbit IgG was produced as previously described.³²

Measurement of antibodies

Qualitative tests. Immunodiffusion, IEP, direct and indirect RID, and indirect RIEP were carried out on 1 inch × 3 inch glass slides coated with a 2 mm. layer of 1.5 per cent Ionagar II in 0.9 per cent saline and 0.1 per cent sodium citrate. Sodium citrate was used

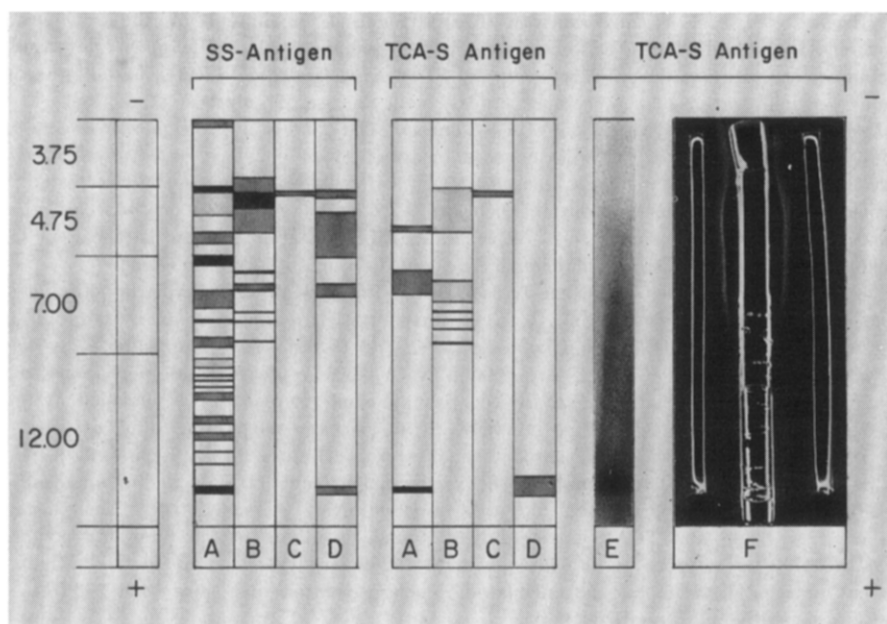


FIG. 2. Schematic representation of staining patterns of SS and TCA-S antigens from *A. fumigatus* following nonlinear gradient polyacrylamide electrophoresis. Figures at left represent concentrations of acrylamide gel. **Column A**, Proteins stained with Coomassie brilliant blue; **Column B**, polysaccharides and glycoproteins stained with periodic acid-Schiff; **Column C**, lipids stained with oil red O; **Column D**, mucopolysaccharides stained with alcian blue. **Column E** is the radiogram produced after electrophoresis of ^{125}I -TCA-S-Fum. In **Section F** the TCA-S antigen after electrophoresis in the acrylamide gel was reacted against anti-*A. fumigatus* serum. Precipitin bands developed only opposite the cathodal components of TCA-S.

to avoid interference with CRP.³ RID and RIEP were carried out as previously described^{32, 44} with the following exceptions: Slides were washed in one per cent potassium iodide (KI) containing 0.1 per cent sodium citrate for 24 hours prior to the addition of ^{131}I -SS-Fum or ^{131}I -SS-Nid. Reactions were then allowed to incubate 24 hours at 25° C. Each slide was washed in one per cent KI 5 times for one hour, dried, and exposed to x-ray film. Details of the procedures and control experiments to establish specificity of the reactions have been described.⁴⁴

Quantitative tests. The ammonium sulfate test was employed to quantitate binding of ^{125}I -TCA-S antigens by anti-*Aspergillus* rabbit sera, since the TCA-S antigen was soluble in a 50 per cent saturated ammonium sulfate solution (SAS/2). In this study the percentage of ^{125}I -TCA-S antigen in the SAS/2 precipitates was determined at increasing dilutions of antisera as previously described.^{25, 45} However, calculations were made on the basis of the per cent of ^{125}I -TCA-S antigen in the SAS/2 precipitates without correction for precipitation of antigen by normal rabbit serum and/or TCA-precipitable material. Antigen binding capacity (ABC)-33 values were also calculated as previously described⁴⁵ by finding the dilution of an antiserum that would precipitate 33 per cent of the labeled test antigen. As previously defined, an ABC-33 value represents the micrograms of N of an antigen that are bound by 1 ml. of undiluted serum.

Inhibition of the ^{125}I -antigen-antibody reactions by serial dilutions of unlabeled test antigens was also measured as previously described.³² The rabbit anti-*A. fumigatus* sera employed for this purpose had ABC-33 values of 0.6 to 1.1 μg N for ^{125}I -TCA-S-Fum and

TABLE I. Composition of SS antigens with respect to nitrogen and carbohydrate

Nitrogen and carbohydrate content	<i>A. fumigatus</i>	<i>A. nidulans</i>
Dry weight (DW) ($\mu\text{g/ml.}$)	307	319
Nitrogen ($\mu\text{g N/ml.}$)	13.9	10.8
% DW	4.5	3.4
Carbohydrate ($\mu\text{g CHO/ml.}$)	21.3	39.0
% DW	6.9	12.2

were diluted to bind 40 per cent of 0.01 $\mu\text{g N}$ of labeled test antigen. The rabbit anti-*A. nidulans* sera had ABC-33 values of 0.3 to 0.7 $\mu\text{g N}$ for ^{125}I -TCA-S-Nid antigen. The amount of unlabeled test inhibitors needed to reduce the binding in the standard systems by 50 per cent are referred to as the concentrations needed to reach the I-50 end point.

Nature of antigen binding. IgG in whole rabbit sera from immunized animals was digested with papain (Worthington Biochemical Corp.).⁴⁶ IEP was performed to confirm the results of digestion. Antisera specific for rabbit Fab and Fc were produced in this laboratory in guinea pigs by use of incomplete Freund's adjuvant. Binding by the ^{125}I -SS antigens from *A. fumigatus* and *A. nidulans* to the Fab and Fc precipitin bands was then evaluated by RIEP.

RESULTS

Analysis of antigens

Nonlinear gradient polyacrylamide electrophoresis. Staining patterns of the SS and TCA-S antigens from *A. fumigatus* are shown diagrammatically in Fig. 2. The SS antigen had about 18 protein-staining bands (*Column A*) distributed throughout the gel layers. The TCA-S antigen had 3 major protein bands: a diffuse and a narrow band within the 4.75 per cent and 7.0 per cent gels and a narrow band at the anodal end of the 12 per cent gel. Only minor differences in the polysaccharide and glycoprotein PAS-staining material (*Column B*) and no differences in lipid-staining material (*Column C*) were observed. The presence of an anodal mucopolysaccharide band with the TCA-S antigen was in contrast to the preponderance of cathodally staining material with the SS antigen (*Column D*). When the ^{125}I -TCA-S antigen was electrophoresed and applied to x-ray film and the radiogram (*Column E*) compared with the stained preparations (*Columns A to D*), the majority of radioactivity was at the anodal end of the gel which contained a narrow protein-staining band and a wider mucopolysaccharide-staining band. Unlabeled TCA-S antigen was electrophoresed and reacted against serum from a rabbit which had been immunized with whole sonicated aspergilli (*Column F*). Precipitin bands developed against the cathodal area containing protein, glycoprotein, or polysaccharide material which was not associated with significant radioactivity. Similar results were obtained with antigens derived from *A. nidulans*.

Nitrogen and carbohydrate. Nitrogen and carbohydrate content of the SS antigens in relation to dry weight are summarized in Table I. The nitrogen content relative to dry weight of *A. fumigatus* and *A. nidulans* was comparable, whereas the carbohydrate content of *A. nidulans* was 12.2 per cent or about twice that of *A. fumigatus* (6.9 per cent).

Effect of enzymes on ^{125}I -TCA-S antigens. Neither DNase T₁, DNase-I, or RNase-A degraded the labeled antigens, since approximately 95 per cent of

TABLE II. Effect of enzyme treatment and oxidation-reduction on the capacity of ^{125}I -TCA-S test antigens to bind specific antisera

Treatment	% Reduction of binding of test antigens at 0.02 μg N/ml. by 1:5 dilution of specific antisera		
	^{125}I -TCA-S-Fum	^{125}I -TCA-S-Nid	^{125}I -BSA
DNase- T_1	0	0	NT*
DNase-I	0	0	NT
RNase-A	0	0	NT
Pepsin	9	6	92
Periodate oxidation	30	22	5
Periodate oxidation and borohydride reduction	72	55	11

*Not tested.

radioactivity was recovered from the nitrocellulose filters. Labeled RNA and DNA used as controls were almost completely degraded, less than 5 per cent being recovered on the filters. The effects on binding activity of radiolabeled antigens by the enzymes are summarized in Table II. DNase- T_1 , DNase-I, and RNase-A had no effect on binding of labeled antigens. The effect on labeled BSA was not tested, but it has previously been shown not to be affected by these enzymes.³¹ When pepsin was employed to treat ^{125}I -TCA-S antigens, only small (6 to 9 per cent) reductions in activity were seen. Control ^{125}I -BSA was degraded by more than 90 per cent.

Periodate oxidation and borohydride reduction. This resulted in substantial reductions in the amount of ^{125}I -TCA-S antigens bound by a rabbit antiserum (Table II).

Measurement of antibodies

Qualitative tests. With the use of standard immunodiffusion techniques, a significant degree of cross-reactivity between precipitating antibodies and SS antigens in fungi and mycobacteria was observed, confirming many previous reports.^{1, 3-5, 15, 16, 19}

When indirect RID was performed with rabbit anti-*A. fumigatus* and ^{125}I -SS-Fum, sharp, single radioactive precipitin bands were demonstrated (Fig. 3). This reaction was inhibited by the prior addition of excess unlabeled SS-Fum. When direct RID tests were carried out with labeled *Aspergillus* antigens and anti-*Aspergillus* sera, radioactive bands were not produced. These results confirmed the previous observation (Fig. 2, Columns E and F) that the radioactive label in the test antigens employed was associated with a nonprecipitating component of these organisms. This is in contrast to standard immunodiffusion studies with SS antigens which demonstrated many precipitin bands.

Quantitative tests. Dilutions of sera from rabbits 92 days after immunization with whole sonicates of *A. fumigatus* or *A. nidulans* were tested for capacity to bind ^{125}I -TCA-S-Fum and ^{125}I -TCA-S-Nid. Data depicting the per cent of 0.01 μg N ^{125}I -TCA-S-Nid and ^{125}I -TCA-S-Fum bound by dilutions of homologous antisera are shown in Fig. 4. The maximum per cent of ^{125}I -antigen bound by any of the hyperimmune sera varied between 50 and 60 per cent at the lowest

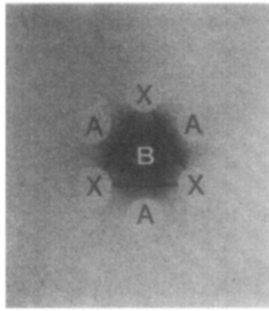


FIG. 3. Indirect RID with ^{131}I -SS-Fum antigen and anti-*A. fumigatus* (A). Radioactivity is seen over the precipitin bands that developed between anti-RGG (B) and anti-*A. fumigatus* (A). X represents unfilled wells.

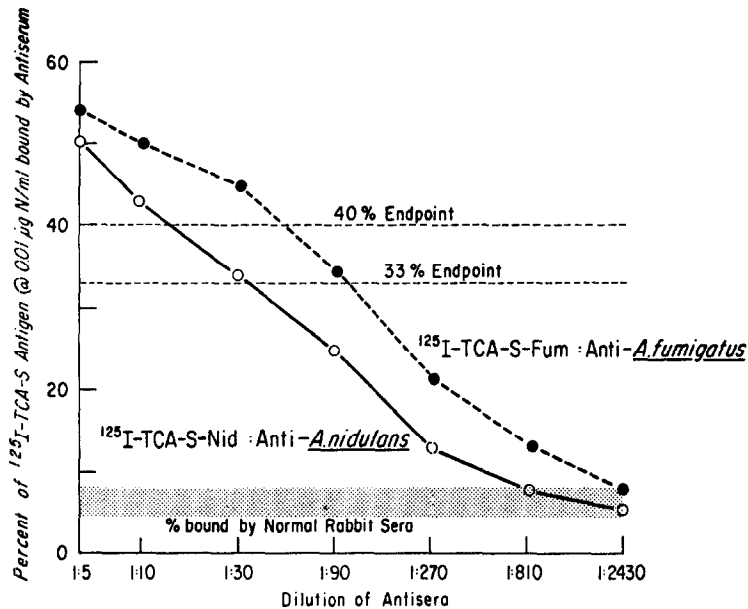


FIG. 4. Per cent of ^{125}I -TCA-S-Fum and ^{125}I -TCA-S-Nid precipitated by serial dilutions of antisera to *A. fumigatus* (broken line) and *A. nidulans* (solid line). The dilutions of antisera that precipitate 33 per cent of ^{125}I -TCA-S-Fum and ^{125}I -TCA-S-Nid test antigens are 1:105 and 1:35, respectively. The dilutions of antisera that precipitate 40 per cent of the antigens (40 per cent endpoint) are 1:50 and 1:14.

serum dilution tested. Sera from nonimmunized control animals precipitated 5 to 8 per cent of the labeled test antigens, and this value was not reduced if serum was incubated with unlabeled antigen prior to the addition of labeled antigen.

Experiments were carried out to evaluate the relative avidities of these reactions by repeating the studies described above using tenfold differences in

TABLE III. ABC-33 values using ^{125}I -TCA-S antigens at tenfold different concentrations

Rabbit No.	^{125}I -TCA-S-Fum (conc. $\mu\text{g N/ml.}$)		Avidity index* (%)	^{125}I -TCA-S-Nid (conc. $\mu\text{g N/ml.}$)		Avidity index* (%)
	0.01	0.001		0.01	0.001	
Anti- <i>A. nidulans</i> 2	0.26	0.044	17	0.17	0.027	16
Anti- <i>A. fumigatus</i> 1	0.52	0.05	10	0.35	0.05	14

*Avidity index = (ABC-33 at 0.001)/(ABC-33 at 0.01) $\times$ 100.

TABLE IV. Comparison of inhibitory capacities of unlabeled SS antigens from *A. fumigatus* and *A. nidulans*

Antisera diluted to bind 40 % of the homologous ^{125}I -TCA-S antigen at 0.02 $\mu\text{g N/ml.}$	Amount of unlabeled inhibitor ($\mu\text{g N}$) required to inhibit binding by 50 %	
	SS-Fum	SS-Nid
Anti- <i>A. fumigatus</i>	2.5	3.1
Anti- <i>A. nidulans</i>	2.1	3.0

antigen concentrations. Ratios of the ABC-33 values obtained using different antigen concentrations were calculated according to the following equation:

$$\frac{\text{ABC-33 at } 0.001 \mu\text{g N/ml.}}{\text{ABC-33 at } 0.01 \mu\text{g N/ml.}} \times 100.$$

Previous studies with a BSA-anti-BSA system demonstrated that the closer this value is to 100 per cent, the stronger the antigen is bound by the antibody populations present.⁴⁷ This ratio, also referred to as the avidity index, ranged from 10.0 to 17.0 per cent and was not significantly different with the various antisera tested (Table III). These low values demonstrate the relatively low avidity of these reactions between the antigens and antibodies tested. Also of interest in Table III is the observation that both anti-*A. nidulans* and anti-*A. fumigatus* bound more ^{125}I -TCA-S-Fum than ^{125}I -TCA-S-Nid when calculated on a nitrogen basis. Similar results were found when antisera produced by other animals were tested.

Inhibition studies. Inhibition studies summarized in Table IV showed only minor differences between the ability of unlabeled SS antigens derived from *A. fumigatus* and *A. nidulans* to inhibit the homologous test system. Anti-*A. nidulans* and anti-*A. fumigatus* were diluted so that they bound 40 per cent of the homologous labeled test antigen at 0.02 $\mu\text{g N}$ per milliliter. The inhibitory effects of progressive increments of unlabeled SS antigens (based on N content) on this binding were then compared. As expected, in an anti-*A. fumigatus*/ ^{125}I -TCA-S-Fum system, a lesser amount of the homologous SS antigen was required to inhibit the reaction than was required of the SS-Nid. In contrast, in an anti-*A. nidulans*/ ^{125}I -TCA-S-Nid system, (in keeping with the direct binding data), the I-50 end point was achieved with 2.1 $\mu\text{g N}$ of SS-Fum, whereas 3.0 $\mu\text{g N}$ of SS-Nid was needed to produce the same effect.

Experiments were carried out comparing the capacity of increasing amounts of SS-Fum and a variety of unlabeled SS preparations to inhibit the homologous

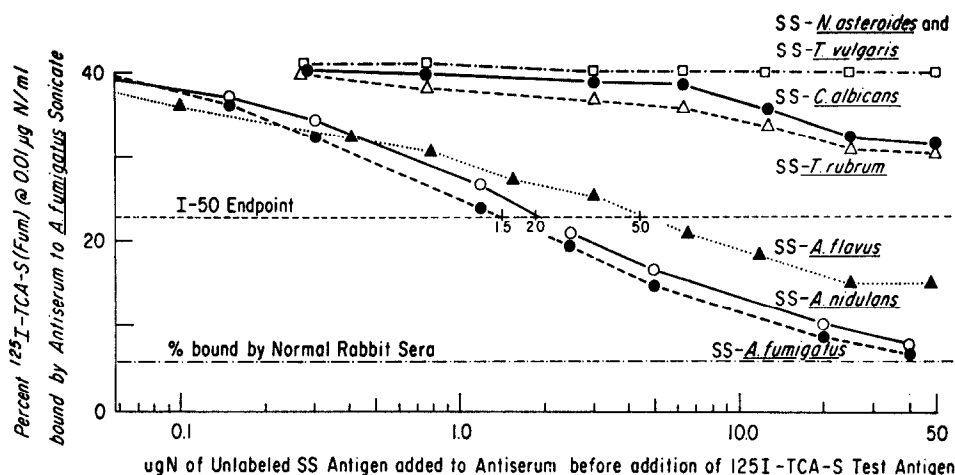


FIG. 5. Inhibition of ^{125}I -TCA-S-Fum/anti-*A. fumigatus* reaction by unlabeled sonicates of other species of *Aspergillus* and other fungi.

anti-*A. fumigatus*/ ^{125}I -TCA-S-Fum reaction (Fig. 5). More than 3 times as much SS-Flav (5 $\mu\text{g N}$) was required to achieve 50 per cent inhibition than by the homologous SS-Fum (1.5 $\mu\text{g N}$). Furthermore, complete inhibition by SS-Flav was not obtainable even with 50 $\mu\text{g N}$. The preparations derived from *C. albicans* and *T. rubrum* only partially inhibited the reaction, and those from *N. asteroides* and *T. vulgaris* had no inhibitory effects at the concentrations employed.

When the inhibitory effects of a variety of sonicates derived from other microorganisms were tested, only minimal inhibition was observed. For example, 5 to 50 $\mu\text{g N}$ of sonicates derived from *M. tuberculosis*, *M. bovis*, *M. kansasii*, *S. epidermidis*, *N. catarrhalis*, and *L. monocytogenes* resulted in minimal inhibition. No inhibition was observed by comparable amounts of *S. pyogenes*, *E. coli*, a crude preparation of protein A, by CRP. However, two commercial preparations of house dust demonstrated significant inhibition. Results with one of these (Endo house dust) are illustrated in Fig. 6. To reduce the binding by 50 per cent, 60 $\mu\text{g N}$ was required (or 40 times as much as the SS-Fum antigen). This suggests that 2.5 per cent of the house dust preparation consisted of nitrogenous material that was immunologically equivalent to the SS-Fum antigen. A second commercial house dust preparation (Hollister-Stier Labs.) similarly inhibited this binding system, but to a lesser extent.

Nature of antigen binding. Because of the observation that protein A from *S. aureus* binds to the Fc fragment of rabbit IgG in a nonimmunologic fashion,^{33, 34, 48} experiments were performed to evaluate whether binding observed by radiolabeled antigens in this study was in fact binding to the rabbit Fab fragment of IgG. Whole sera from animals immunized with either sonic preparations of *A. fumigatus* or *A. nidulans* were digested with papain. RIEP was carried out with the use of the papain-treated sera and ^{125}I -SS antigens of *A. fumigatus* and *A. nidulans*. Radioactivity was associated with Fab precipitin bands in both sera with both labeled antigens. The addition of excess amounts of

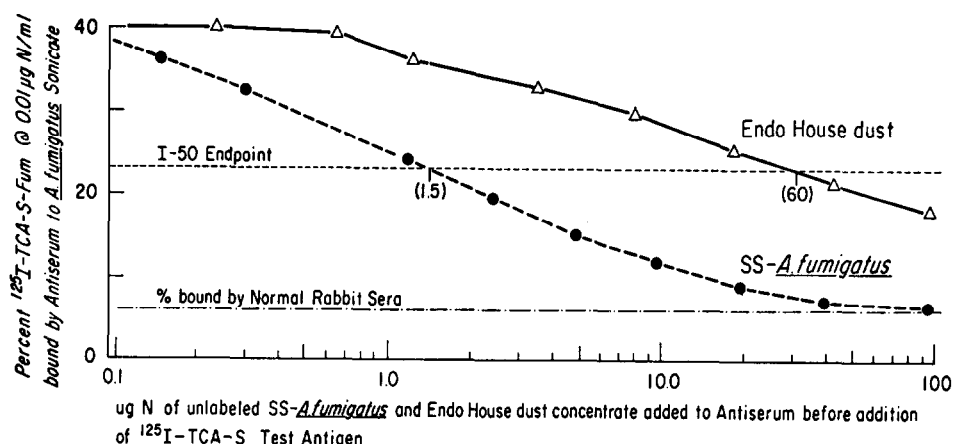


FIG. 6. Inhibition of ^{125}I -TCA-S-Fum/anti-*A. fumigatus* reaction by house dust concentrate.

unlabeled antigen prior to addition of labeled antigen inhibited the majority of the binding to Fab.

DISCUSSION

The present studies were undertaken to isolate a soluble antigen from aspergilli which could be employed in primary binding tests to detect antibodies to components of aspergilli. Since the cell walls of fungi are resistant to sonic disruption,⁴⁹ sonification was carried out for 3 hours. The soluble supernatant, consisting of a number of potentially antigenic substances, was shown to be a mixture of proteins, polysaccharides, lipids, and mucopolysaccharides of varying electrophoretic mobilities and molecular sizes.

When this crude material was further purified by eliminating TCA-precipitable material, an SAS/2-soluble fraction was obtained which bound antibodies in sera from immunized rabbits. Polyacrylamide electrophoresis of this component revealed that it also contains protein, polysaccharide, mucopolysaccharide, and lipid components. Unlabeled antigens which formed precipitin bands when reacted against a specific rabbit antiserum were associated with proteins, polysaccharides, and lipids near the cathode in the 3.75 and 7.0 per cent gels. On the other hand, most of the radioactivity was found in an anodal protein-mucopolysaccharide fraction. This radiolabeled fraction did not form precipitin bands with antibodies but was shown to bind to antibodies in immunized rabbit sera. Although the TCA-S labeled antigens did not precipitate with antibody, immunodiffusion studies both in this and other laboratories indicate that many other antigens in fungal and house dust extracts do have the capacity to precipitate and crossreact with one another.^{1, 3-5, 15, 16, 19}

Because not more than 50 to 60 per cent of the labeled antigens bound to the most concentrated antisera tested, it would appear that approximately 40 per cent of the radioactivity was not associated with antigenic components critical to the measurement of antibodies in these antisera. The relatively low avidity of the antisera from animals immunized with aspergilli (Table II)

was similar to previous data obtained using heterogeneous antigens obtained from tubercle bacilli³²; it was in contrast to the avid quality of antibody frequently obtained with a relatively pure immunogen, BSA.^{25, 47}

Labeled components from *A. fumigatus* and *A. nidulans* were further characterized by testing their susceptibilities to various enzymes. The labeled antigens used in this study did not require DNA or RNA as constituents for reactivity. Pepsin treatment did not appreciably affect their antigenicity, but there was considerable loss of binding following periodate oxidation and borohydride reduction. These data strongly suggest that the mucopolysaccharide and not the protein moiety plays a critical role in the binding properties of these labeled antigens.

Unlike staphylococcal protein A, which binds to the Fc and not the Fab fragment of IgG, the labeled SS antigens from both *A. fumigatus* and *A. nidulans* clearly bound to the Fab, or antigen-combining portion, of the immunoglobulin molecule. Furthermore, this reaction was inhibited by the prior addition of excess unlabeled homologous SS antigen.

Results of both binding and inhibition experiments suggested that more ¹²⁵I-TCA-S-Fum than ¹²⁵I-TCA-S-Nid was bound by both anti-*A. fumigatus* and anti-*A. nidulans* sera, and, similarly, unlabeled SS-Fum exhibited superior inhibitory properties when tested against ¹²⁵I-TCA-S-Nid as well as ¹²⁵I-TCA-S-Fum. The unlabeled SS antigen from *A. nidulans* was capable of binding all populations of anti-*A. fumigatus* which had the capacity to bind ¹²⁵I-TCA-S-Fum. The similarity of slopes of the inhibition curves obtained with both unlabeled SS-Fum and SS-Nid suggests close identity between these antigens (Fig. 4). Taken together, these data suggest that the *A. fumigatus* antigen has a higher specific binding activity per milligram of an antigen also present in *A. nidulans*.

The inhibition experiments also demonstrated some of the immunological differences known to exist among fungi. The flat slope of the *A. flavus* inhibition curve between 20 and 50 μ g N suggests a degree of cross-reactivity rather than identity between antigens in *A. flavus* and those in SS-Fum. SS antigens of both *C. albicans* and *T. rubrum*, though capable of some binding to antibodies, were completely lacking in at least some determinants present in ¹²⁵I-TCA-S-Fum because there was a plateau in their inhibition curves when more than 25 μ g N of the sonicates were added (Fig. 5).

The slope of the curve using house dust as an inhibitor implies the presence of antigens identical to *A. fumigatus*. On the basis of these data, approximately 2.5 per cent Aspergillus-blocking equivalents were present in a given volume of house dust when tested in relation to the ¹²⁵I-TCA-S-Fum/anti-*A. fumigatus* system. The presence of reactants in commercial house dust which share components with aspergillus species is in keeping with previous studies in which *A. fumigatus* was isolated from house dust.^{50, 51} It also warrants further study into the interpretation of immediate type I reactions in human beings with these apparently heterogeneous skin test materials.

These labeled antigens were also employed to study binding by antisera from human beings with a variety of diseases, including those caused by aspergilli, and this study is reported in the accompanying paper of this series.²⁹

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