Antigens from Moldy Hay Involved in Farmer's Lung.* (30804)

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The physiopathological features of farmer's lung disease have been described by Rankin *et al*(1,2) and Emanuel *et al*(3). The symptoms consist of shortness of breath, cough, chills, and fever which occur from 4 to 8 hours following exposure to moldy plant material. An acute granulomatous interstitial pneumonitis is often associated with the disease. Kobayashi *et al*(4) and Pepys *et al* (5) have detected specific precipitating antibodies against antigens from moldy hay in the sera of patients with farmer's lung. Recently, we prepared trichloroacetic acid (TCA-) soluble antigens from moldy hay and fractionally precipitated these antigens between the limits of 50 to 90% alcohol; this preparation was tested for pathogenicity by Barbee *et al*(6). Six patients with farmer's lung and 10 controls were given aerosol inhalations of this extract. The symptoms seen in the 6 farmer's lung patients were identical to those of acute farmer's lung disease. Controls showed only slight reactions. These results suggest that there is a causal relationship of the antigens present in moldy hay with the clinical disease. This paper describes the preliminary purification and properties of these TCA-soluble antigens from moldy hay.

Materials. The serum used in this study was obtained by Dr. J. Rankin from a patient in whom a firm diagnosis of farmer's lung had been established by appropriate clinical studies. This serum was typical of many other farmer's lung sera. The moldy hay used for this study was designated as H-1 moldy hay, and it has been described previously(4). The various antigen preparations were tested against serum by double diffusion in agar gels(7) and by immunoelectrophoresis in agar(8).

Methods and results. Eighteen kilograms of H-1 moldy hay were ground in a Model D Fitzmill Grinder equipped with a No. 2 screen. This material was extracted in a glass-lined fermentor with 150 l of 5% TCA for 3 days with continuous stirring. The slurry was centrifuged in a large basket centrifuge, and the supernate was dialyzed in 1⅞″ Visking dialysis tubing against running tap water for 60 hours. The non-dialyzable material was concentrated to 8 l in a Mojonnier evaporator at 35°C and reduced pressure. After standing overnight at 4°C, a precipitate formed which was removed by centrifugation; the supernate was then freeze-dried.

Twenty grams of this TCA-soluble material were dissolved in 300 ml of distilled water, and precipitates were obtained by slowly adding 95% ethanol to the vigorously stirred solution. Only the material which precipitated between the limits of 50 to 60, 60 to 70, and 70 to 90% alcohol gave precipitins with the serum used in this study. Fig. 1 shows the 3 precipitins obtained in immunoelectrophoresis when the TCA-soluble material from moldy hay was reacted with farmer's lung serum. These precipitin lines are indicated by A, B, and C. Antigen A was fractionated by alcohol into 3 components, and antigen B was split into 2 components. Antigen C was detected only in the material which precipitated between the limits of 70 to 90% ethanol.

Ten mg samples of each of the antigenic fractions precipitated by alcohol were dissolved in 1 ml of 0.5 N sulfuric acid and autoclaved for 8 hours at 6 psi in sealed ampules. The samples were neutralized with barium hydroxide and concentrated to 0.5 ml under an infrared lamp. About 25 μl of each sample was spotted on Whatman No. 1

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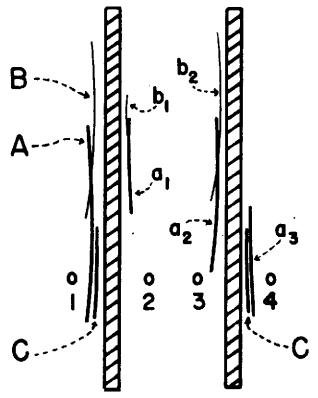

FIG. 1. Immunoelectrophoresis of the trichloroacetic acid (TCA-) soluble antigens from moldy hay. Five μl, containing 1 mg of TCA-soluble material or 0.5 mg of the various alcohol fractions, were placed in wells 1, 2, 3, or 4. After electrophoresis at 250 volts for 1 hour, the central trough was removed and filled with 0.1 ml of serum. In each case the positive electrode was toward the top of the gel. A TCA extract of moldy hay contained 3 antigens, indicated by precipitin lines A, B, and C. Antigen A was fractionated by alcohol into 3 components, indicated by lines a_1, a_2, and a_3, in fractions precipitated by 50-60%, 60-70%, and 70-90% ethanol, respectively. Antigen B was split into 2 components; b_1 was detected in alcohol fraction F 50-60, and b_2 was present in alcohol fraction F 60-70. Antigen C was found only in the material precipitated by 70 to 90% ethanol.

paper and chromatographed in 80% phenol (v/v). After drying, the papers were chromatographed in the second direction with either butanol:acetic acid:water (4:1:5) or with ethyl acetate:acetic acid:water (3:1:3). After drying, the papers were sprayed with recrystallized o-aminodiphenyl reagent(9) and dried at 95°C to produce brightly colored spots. Fig. 2 demonstrates the sugars identified by 2-directional co-chromatography. The three alcohol fractions each contained glucose, galactose, mannose, xylose, arabinose, and rhamnose. Glucuronic acid was also present in the material precipitated by 50 to 60% and 60 to 70% ethanol, but none was detected in the material precipitated by 70 to 90% alcohol.

The amino acid and amino sugar content of each of the antigenic fractions precipitated by alcohol was determined quantitatively on a Beckman/Spinco Model 120 amino acid analyzer using a 150 cm Dowex sulfonated styrene column operated at 50 C(10,11). The results in Table I show the various amino acids and amino sugars detected in acid hydrolysates of the 3 antigenic fractions. Tryptophan and cystine were not determined. The preparations contained large amounts of glutamic acid, aspartic acid, and hydroxyproline. It is interesting to note the similarity in the amino acid composition of the material precipitated by 60 to 70% alcohol and that precipitated by 70 to 90% alcohol.

The 3 antigenic fractions which were precipitated by ethanol were further purified on a 2.5 $\times$ 20 cm column of diethylaminoethyl (DEAE-) cellulose equilibrated with 0.05 M Tris (hydroxymethyl) aminomethane-HCL (Tris-HCl) buffer, pH 8. The samples (75 to 200 mg) were applied to the column in 5 ml of the same Tris-HCl buffer. The frontal peak was eluted with 0.05 M Tris-HCl buffer,

TABLE I. Amino Acid and Amino Sugar Composition of the Antigenic Material Fractionated by Alcohol Precipitation. Values are given as μg of each amino acid or amino sugar in 100 mg of each fraction precipitated by alcohol.

Amino acid or amino sugar	Material precipitated at ethanol concentration		
	50–60%	60–70%	70–90%
lysine	246	355	343
histidine	63	111	108
arginine	113	219	223
aspartic acid	679	1,244	1,297
glutamic acid	713	1,302	1,257
threonine	464	738	768
serine	546	851	851
hydroxyproline	655	1,160	1,553
proline	299	564	616
glycine	371	657	698
alanine	481	717	695
valine	421	767	901
methionine	7	15	15
allo-isoleucine	65	118	98
isoleucine	124	236	282
leucine	170	308	361
tyrosine	90	190	235
phenylalanine	82	157	223
glucosamine	260	385	528
galactosamine	340	761	579
Total	6,189	10,855	11,631

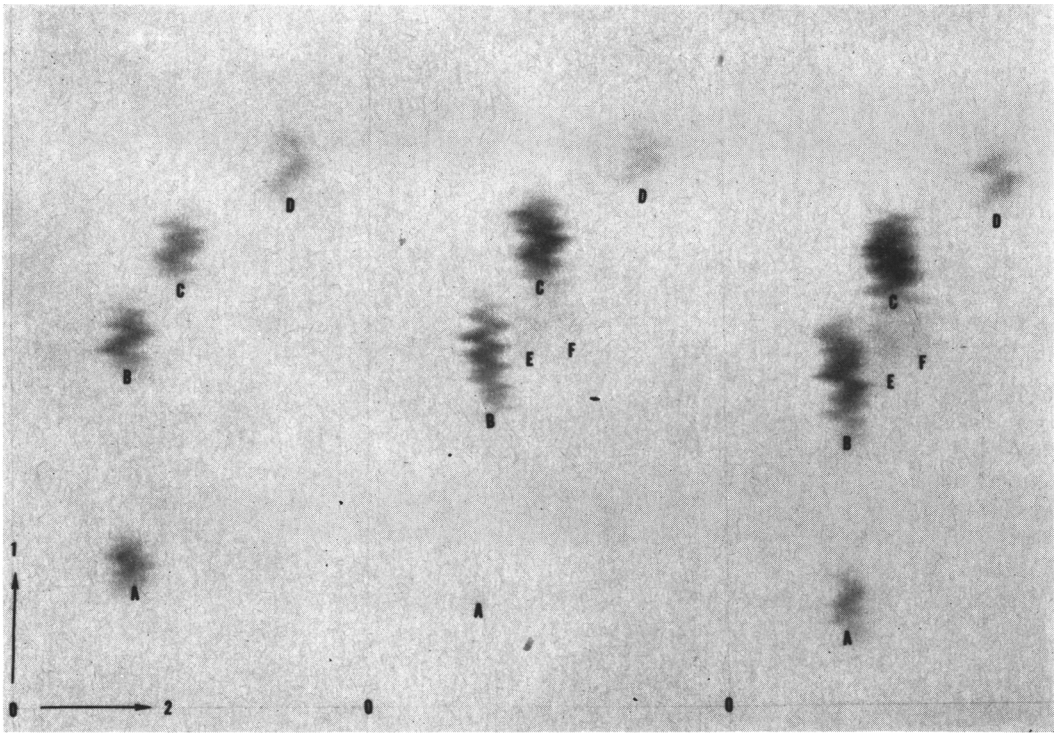

FIG. 2. Co-chromatography of an acid hydrolysate of the antigenic material precipitated by 50 to 60% ethanol with known sugars. In each case, the sample was applied at "0," and the paper was irrigated with 80% phenol in direction 1 followed by chromatography in direction 2 with butanol:acetic acid:water (4:1:5). The chromatogram on the left shows the location of 4 known sugars, and the chromatogram in the center contains a hydrolysate of the antigenic material precipitated by 50 to 60% ethanol. The chromatogram on the right shows the patterns obtained when this hydrolysate was co-chromatographed with the 4 known sugars. The known sugars are as follows: (A) glucuronic acid, (B) galactose, (C) arabinose, and (D) rhamnose. The unknown sugars in the hydrolysate were later identified as: (B) galactose and glucose, (E) mannose, and (F) xylose.

pH 8, before an exponential sodium chloride gradient was started. The gradient, as developed in this laboratory by W. Woodbury, was made with a small cylinder, containing 35 ml of 4 M NaCl in 0.05 M Tris-HCl buffer (pH 8), connected to a larger cylinder, containing 500 ml of 0.05 M Tris-HCl buffer (pH 8), from which the gradient was pumped onto the column. At the end of the gradient, the column was washed with 0.1 N HCl and then re-equilibrated with Tris-HCl buffer. The effluent from each column was collected in a fraction collector, and from 0.2 to 0.5 ml of each collected fraction was assayed for polysaccharide using 4 ml of anthrone reagent (12). The antigenic fractions produced a good color with anthrone, and this served as a useful tool for locating the antigens. The collected effluent of each alcohol fraction was then pooled according to its anthrone value, and each pooled fraction was dialyzed for 30 hours against distilled water and freeze-dried.

Fig. 3 shows the chromatogram obtained when the material precipitated by 60 to 70% alcohol was chromatographed on DEAE-cellulose. The chromatograms from each of the alcohol fractions were similar in that 2 large polysaccharide peaks were obtained; one peak was eluted by Tris-HCl buffer, and the other peak was eluted by NaCl. Antigens were present in the latter peak in all of the antigenic fractions precipitated by alcohol, but only the material precipitated by 70 to 90% alcohol produced a chromatogram with antigens in the polysaccharide peak eluted by Tris-HCl buffer.

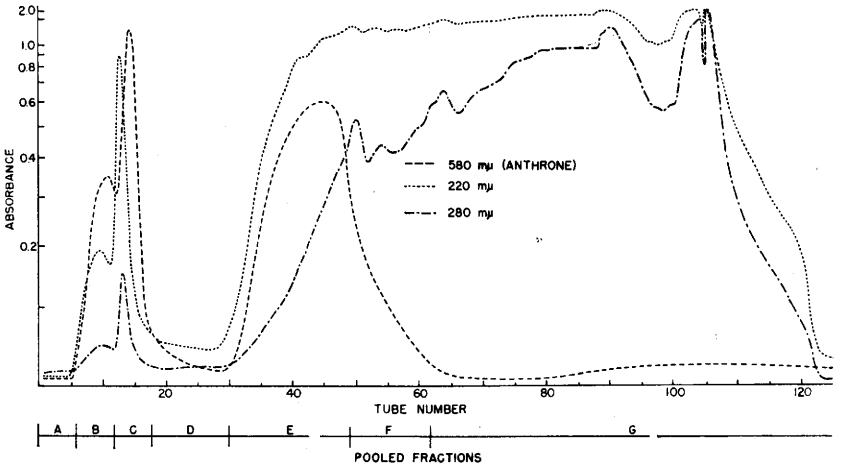

FIG. 3. DEAE-cellulose column chromatography of the antigenic material precipitated by 60 to 70% ethanol. The antigenic material (178 mg) was eluted using an exponential gradient from 0 to 4 M NaCl at a flow rate of 0.76 ml/min. The column effluent was monitored continuously at 280 and 220 mμ. Fractions were collected at 10.5 min intervals; 0.2 ml of each tube was tested with anthrone reagent. The frontal peak was eluted with Tris-HCl buffer; the gradient was started at tube 16; and the column was then washed with 0.1 N HCl at tube 90, followed by Tris-HCl buffer at tube 107. Fractions were pooled as indicated and tested for antigenicity; antigens were found only in Fractions E, F, and G.

Each of the pooled fractions from DEAE-celluose columns was tested for antigenicity by immunodiffusion using the method of antigen dilution end-point titer. Fig. 4 shows the dilution end-point titer of the pooled fractions from Fig. 3. Pooled fraction F produced a precipitin with 0.05 ml of farmer's lung serum when diluted to 3.9 μg. This represents a considerable increase in dilution end-point titer when compared to other fractions. For example, the TCA-soluble material from moldy hay only gave precipitins with an end-point titer which varied from 62.5 to 125 μg. After DEAE-cellulose column chromatography, the dilution end-point titer ranged from 3.9 to 31.2 μg.

Discussion. The choice of trichloroacetic acid (TCA) as a solvent to extract farmer's lung antigens was based on the work of Kobayashi *et al*(4) who found that TCA extracts from moldy hay gave precipitins with the sera of all symptomatic patients and with 17 of 29 asymptomatic farmers, some of whom had completely recovered from the disease, and gave no reaction with 38 controls. TCA also removed most of the protein from the samples. These TCA-soluble antigens were heat stable, stable to wide ranges of pH, and reacted with Schiff's reagent for carbohydrate (13) which suggested that the antigens contained much polysaccharide.

It was of interest, therefore, to find that after alcohol fractionation of the TCA-soluble material, the antigenic fractions still contained a peptide with 18 amino acids. These amino acids accounted for 5.6, 9.7, and 10.5% of the weight in the material precipitated by 50 to 60%, 60 to 70%, and 70 to 90% alcohol, respectively. Eight sugars and two amino sugars were also detected in these fractions.

The data suggested that these farmer's lung antigens were glycoproteins, and that they might be amenable to further purification by column chromatography on DEAE-cellulose. Also, some of the antigens were negatively charged as shown by their immunoelectrophoretic behavior.

Several antigenic fractions were found when the material precipitated by 70 to 90% alcohol was chromatographed on DEAE-cellulose. The frontal peak eluted by Tris-HCl buffer contained 2 antigens which would not adsorb to carboxymethyl cellulose at pH 7.2, suggesting that these antigens were not charged. Another antigenic fraction was eluted from DEAE-cellulose by low concentrations of NaCl, and it also contained 2 an-

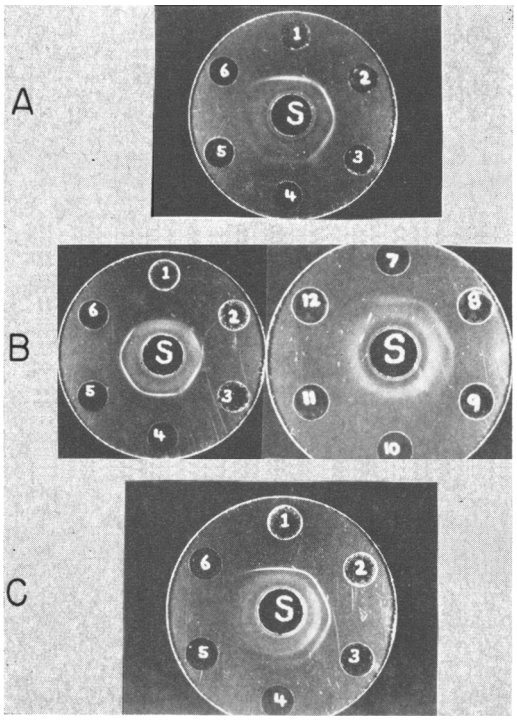

FIG. 4 The dilution end-point titer by immunodiffusion of antigenic fractions from DEAE-cellulose column chromatography of the material precipitated by 60 to 70% ethanol (Fig. 3): (A) Fraction E, (B) Fraction F, and (C) Fraction G. In each case, well S contained 0.05 ml of farmer's lung serum, and well 1 contained 0.5 mg of each fraction. Serial 2-fold dilutions were made counterclockwise from well 1.

tigens. These antigens had a relatively small negative charge when tested by immunoelectrophoresis.

The material precipitated by 50 to 60% and 60 to 70% ethanol each contained negatively charged antigens which could be eluted from DEAE-cellulose by higher concentrations of NaCl. No antigens were found in the frontal peak from these columns.

Analysis of the antigenic fractions obtained by DEAE-cellulose column chromatography showed that each of these fractions was composed primarily of polysaccharide, but that some amino acids were also present in each fraction. These results suggest that complex peptides or simple proteins are combined with a polysaccharide portion of glycoproteins in these farmer's lung antigens.

Summary. Trichloroacetic acid (TCA) extracts of moldy hay contained 3 antigens which produced precipitins with a typical farmer's lung serum. These antigens were fractionally precipitated with ethanol; antigens were present in the fractions precipitated between the limits of 50 to 60%, 60 to 70%, and 70 to 90% alcohol. Each of these alcohol fractions was further purified on diethylaminoethyl (DEAE-) cellulose and analyzed for sugars and amino acids. It is concluded that these TCA-soluble antigens involved in farmer's lung disease are composed of peptides and carbohydrates which were not separated by TCA extraction, alcohol fractionation, or by chromatography on DEAE-cellulose ion exchange columns.

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