Antigens from *Thermopolyspora polyspora* Involved in Farmer's Lung.* (30805)

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Farmer's lung is a disease of hypersensitivity which occurs among agricultural workers, and it is associated with inhalation of dust from moldy plant materials. The symptoms appear several hours after exposure to moldy hay or grain and increase in severity after repeated exposures(1-3). Farmer's lung patients usually possess circulating antibodies which form precipitins with antigens from moldy hay(4,5). We have shown(6) that trichloroacetic (TCA) extracts of H-1 moldy hay contain three antigens and that these antigens are composed of peptides and carbohydrates which were not separated by alcohol fractionation or by DEAE-cellulose column chromatography. Aerosol inhalations of these TCA-soluble antigens from moldy hay produced symptoms in patients identical to those of acute farmer's lung disease(7).

Gregory *et al*(8-10) have studied moldy hays and found that 2 thermophillic actinomycetes, *Thermopolyspora polyspora* and *Micromonospora vulgaris,* are particularly characteristic of hay associated with farmer's lung. Pepys and coworkers(11) have shown that *T. polyspora* is the principal source of farmer's lung antigens. Emanuel *et al*(12) have isolated *T. polyspora* from lung tissue obtained by biopsy from a farmer's lung patient, and Pepys and Jenkins(13) have reported that inhalation of the TCA-soluble and TCA-insoluble antigens from *T. polyspora* produced symptoms of farmer's lung in affected subjects. Thus, it seems likely that *T. polyspora* is a causative agent of farmer's lung disease.

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Gregory(14) examined our H-1 moldy hay sample and found that it contained 28.361 million actinomycete and bacterial spores per g dry weight as compared to 1.883 million fungal spores/g. Isolates of microorganisms were obtained in culture by an Andersen sample(8), and 579 colonies of *T. polyspora* and 69 colonies of *M. vulgaris* were identified on 6 agar plates incubated at 60°C. These were the principal organisms identified. Two isolates of *T. polyspora,* A 91 from our H-1 moldy hay and A 94 from an English hay sample, were obtained from Dr. Gregory. This paper describes some of the properties of the TCA-soluble antigens from *T. polyspora* and their similarity to antigens from moldy hay.

Methods and results. The serum used in this study was the same as that described previously(6). Farmer's lung antigens were tested with this serum by double diffusion in agar gel(15) and by immunoelectrophoresis (16).

T. polyspora was grown for 48 hours at 60°C and at pH 7 on purified Difco Bacto agar containing mineral salts and 1% glucose. The agar slants were extracted with 10% TCA for several days. The TCA extracts were pooled, filtered, and dialyzed with distilled water and then freeze-dried. This material was dissolved in 50 ml of distilled water and fractionally precipitated by ethanol between the limits of 0 to 25%, 25 to 50%, and 50 to 75% alcohol. The antigens which were not precipitated by 75% ethanol were recovered by dialyzing the solution to remove the alcohol, and the preparation was freeze-dried. Most of the antigens were found in the material precipitated by 50 to 75% ethanol and in the material not precipitated by 75% ethanol.

The immunologic identity of these TCA-soluble antigens from isolate A 91 of *T. polyspora* and from H-1 moldy hay(6) is shown in Fig. 1. The fusion of the precipitin lines

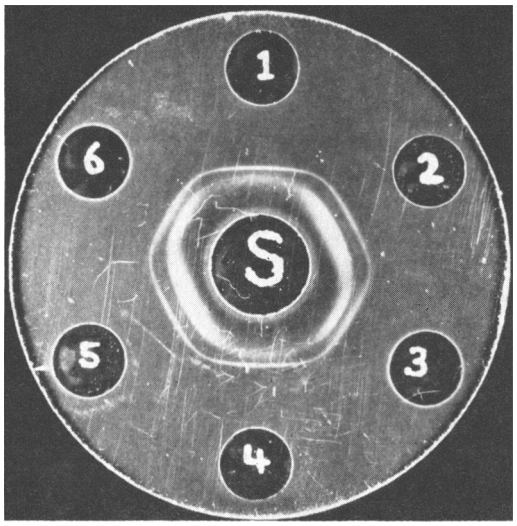

FIG. 1. Immunologic identity of the trichloroacetic acid (TCA-) soluble antigens from *T. polyspora* and H-1 moldy hay. The material extracted by TCA was fractionally precipitated by ethanol; 1 mg of each sample was placed in the outer wells. Well S contained 0.05 ml of farmer's lung serum; 1 and 4, antigens from moldy hay precipitated by 50 to 90% alcohol; 2 and 5, antigens from *T. polyspora* not precipitated by 75% alcohol; 3 and 6, antigens precipitated by 50 to 75% alcohol.

suggests that the antigens from moldy hay and from *T. polyspora* are identical serologically. The precipitin lines did not cross one another, nor were any spurs of partial identity observed.

The TCA-soluble material from *T. polyspora* which was not precipitated by 75% ethanol was further purified on a 1.9 × 56.5 cm column of diethylaminoethyl (DEAE-) cellulose equilibrated with 0.05 M Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) buffer, pH 8. The sample was eluted at a flow rate of 1 ml/min, and the effluent was monitored continuously at 220 mμ. As shown in Fig. 2, the column was washed with Tris-HCl buffer, then with 4 M sodium chloride, and then with 0.1 M piperazine-HCl buffer, pH 4.5, containing 20% sodium chloride. Antigens were detected in pooled fractions A and E which were eluted by Tris-HCl buffer and by sodium chloride, respectively. The properties of these antigens were similar to those from moldy hay which precipitated between the limits of 70 to 90% ethanol when these antigens were purified on DEAE-cellulose(6).

T. polyspora was grown at 60°C in 15 l of nutrient solution containing mineral salts, glucose and 0.3% yeast extract by Hartz (17). The mycelia were collected by centrifugation, washed 4 times with M/75 $Na_2HPO_4 \cdot H_2O$ using 2500 ml per wash, and the mycelia were freeze-dried. The freeze-dried mycelia (20.8 g) were suspended in 800 ml of cold phosphate buffer (pH 7.4, $\mu = 0.15$), and 50 ml portions of this suspension were sonicated for 5 min in a Raytheon sonic oscillator. The fragmented mycelia were centrifuged for 1 hr at 23,500 × *g*, and the sedimented material was washed twice with cold phosphate buffer. The washings were combined with the original super-

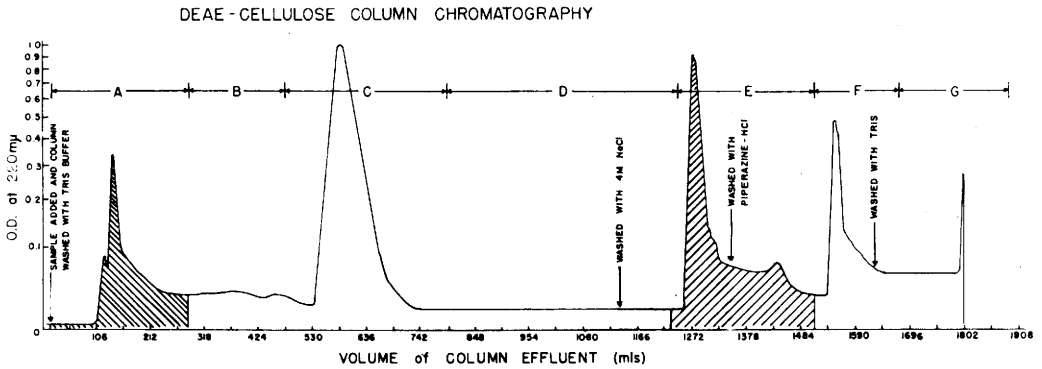

FIG. 2. DEAE-cellulose column chromatography of TCA-soluble material from *T. polyspora* which was not precipitated by 75% ethanol. Sample (100 mg) was eluted from a 1.9 x 56.5 cm column at a flow rate of 1 ml/min. Column was washed with 0.05 M Tris-HCl buffer (pH 8), 4 M sodium chloride, and 0.1 M piperazine-HCl buffer (pH 4.5) containing 20% NaCl as shown. Effluent was collected in 15 ml fractions which were pooled as indicated. Shaded pooled fractions A and E gave precipitins with farmer's lung serum.

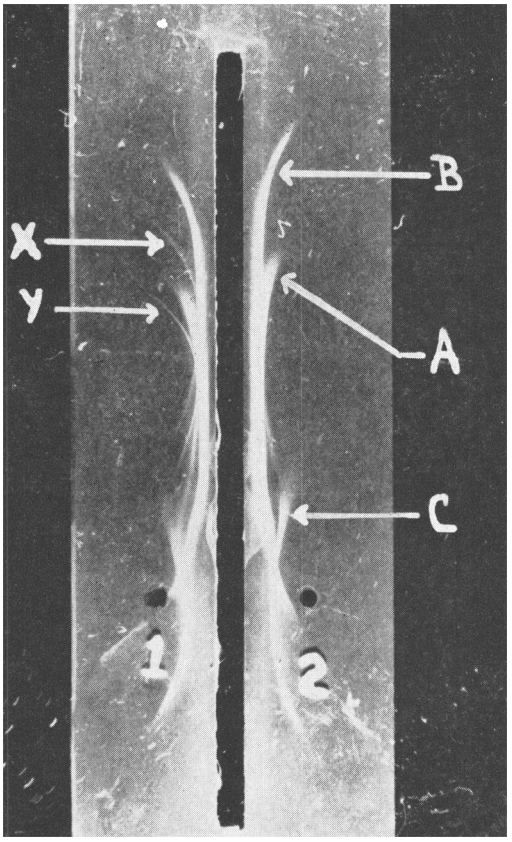

FIG. 3. Immunoelectrophoretic properties of antigens extracted from *T. polyspora* by pH 7.2 phosphate buffer and by cold TCA. Well 1 contained 125 μg of the material extracted by phosphate buffer, and well 2 contained 7.8 μg of the material extracted by cold TCA. The anode is at top of the gel. Both extracts contained 3 antigens, indicated by precipitin lines A, B, and C. The phosphate buffer extract contained 2 additional antigens shown by precipitin lines X and Y.

nate, dialyzed at 4°C against distilled water and freeze-dried.

The sedimented material was suspended in 1 liter of cold 10% TCA and stirred at 4°C for 20 hours. The suspension was centrifuged, and the sediment was washed twice with fresh 10% TCA. The supernates were combined, dialyzed at 4°C for 30 hours against distilled water and freeze-dried.

The immunoelectrophoretic properties of the antigens extracted by phosphate buffer and by cold TCA are shown in Fig. 3. Three precipitin lines, A, B, and C, were observed in each extract. The phosphate buffer extract had at least 2 additional antigens, shown by precipitin lines X and Y, which were not present in the TCA extract. These antigens could be removed from the phosphate buffer extract by adding TCA to the extract. The immunoelectrophoretic properties of the 3 TCA-soluble antigens were similar to the 3 TCA-soluble antigens present in H-1 moldy hay(6). Hot TCA removed an additional amount of antigen C from the residue which remained after cold TCA extraction, and more antigen C could be removed by treating the residue which remained after hot TCA extraction with 1 N NaOH.

A portion of the nutrient solution used for growing *T. polyspora* was dialyzed against distilled water, and an equal volume of 20% TCA was added to the non-dialyzable material. The precipitate was removed by centrifugation and the supernate was dialyzed against distilled water and freeze-dried.

Fig. 4 shows the immunologic identity of the antigens from *T. polyspora* and from the nutrient solution used for growing *T. poly-*

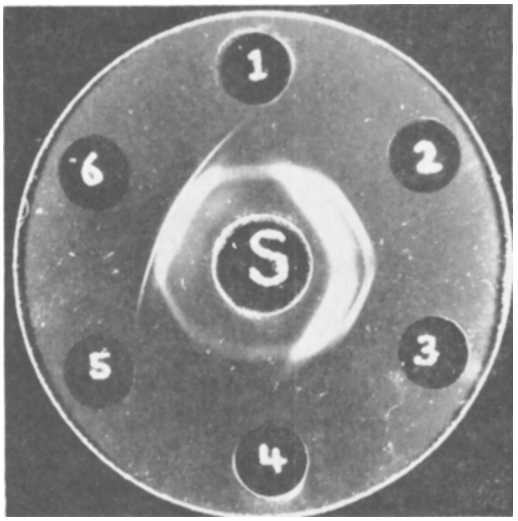

FIG. 4. Immunologic identity of the antigens extracted from *T. polyspora* and from the nutrient solution used for growing *T. polyspora*. In each case, the wells contained 0.02 ml of antigen extract, and the concentrations (shown in brackets) were so chosen that the samples were near antibody-antigen equivalence. Well S contained 0.05 ml of farmer's lung serum; 1 and 4, antigens extracted by NaOH (2000 μg); 2, antigens extracted by pH 7.2 phosphate buffer (200 μg); 3, antigens extracted by cold TCA (31.2 μg); 5, antigens extracted by hot TCA (2 μg); and 6, TCA-soluble, extracellular antigens (125 μg).

TABLE I. Effect of Pronase on TCA-Soluble Antigens. A 50 mg sample of TCA-soluble material was incubated for 24 hours at 37°C with 0.5 mg of Pronase. The Pronase was precipitated by TCA, and the filtrate was dialyzed to remove amino acids and peptides. Non-dialyzable material was hydrolyzed and analyzed for amino acids. Values are given as μg of amino acid/50 mg of antigen or as % of total amino acids.

Amino acid*	Antigen (50 mg)	Antigen and Pronase	Pronase (.5 mg)	Diff.	%
lysine	154.2	130.5	4.7	125.8	16.7
histidine	68.1	9.3	4.5	4.8	.6
arginine	28.2	—	—	—	—
aspartic acid	85.2	5.6	5.0	.6	.1
glutamic acid	344.9	188.3	4.6	183.7	24.4
threonine	44.0	2.8	2.5	.3	.04
serine	44.1	6.4	8.1	—	—
proline	109.9	12.2	—	12.2	1.6
glycine	44.7	12.2	4.5	7.7	1.0
alanine	184.0	160.0	4.0	156.0	20.7
valine	43.9	21.0	1.2	19.8	2.6
allo-isoleucine	249.1	243.2	1.0	242.2	32.2
isoleucine	17.7	—	1.4	—	—
leucine	31.5	—	1.8	—	—
Total	1,449.5			753.1	

* Tyrosine and phenylalanine were not determined.

spora when these preparations were tested with farmer's lung serum by double diffusion. The precipitin line nearest the outer walls is due to antigen C which is present in the phosphate buffer extract, cold TCA extract, hot TCA extract, and the NaOH extract. The nutrient solution contains a TCA-soluble, extracellular antigen which does not cross react with any of the other antigens.

Fifty mg of the material extracted from *T. polyspora* by cold TCA were dissolved in 1 ml of 0.05 M Tris-HCl buffer, pH 7.5. The sample was centrifuged to remove insoluble material. One ml of the proteolytic enzyme, Pronase (0.5 mg/ml Tris-HCl buffer), and a drop of toluene were added to the solution, and the tube was incubated for 24 hours at 37°C. Two control tubes were also incubated for 24 hours; one contained 50 mg of TCA-soluble material in 2 ml of Tris-HCl buffer, and the other contained 0.5 mg of Pronase in 2 ml of Tris-HCl buffer. After 24 hours, 2 ml of 20% TCA were added to each tube, and each solution was filtered through a 0.45 μ Millipore filter. The filtrates were dialyzed at 4°C against pH 7.2 phosphate buffer ($\mu = 0.15$). The samples were made to 5 ml; one ml was used to test for antigenicity, 4 ml of 12 N HCl were added to the remaining 4 ml of extract. These samples were hydrolyzed and analyzed for amino acids on a Beckman/Spinco Model 120 analyzer(18,19).

The antigens extracted by cold TCA were not destroyed after 24 hours digestion by Pronase. Table I shows the amino acid composition of this material before and after Pronase digestion. Prior to digestion, the antigenic material contained large amounts of lysine, glutamic acid, alanine and allo-isoleucine which comprised 10.6, 23.8, 12.7, and 17.2% of the total amino acids, respectively. Following Pronase digestion, these were virtually the only amino acids remaining, and they comprised 16.7, 24.4, 20.7, and 32.2% of the total amino acids, respectively.

The sugar content of the various extracts from the mycelia of *T. polyspora* was determined by means of thin layer and paper chromatography, using 80% phenol or ethyl acetate:acetic acid:water (3:1:3) as solvents and o-aminodiphenyl as the spray reagent (20). Galactose and arabinose were readily identified in all of the extracts. Glucosamine was also identified in these extracts using column chromatography on a 150 cm Dowex sulfonated resin operated at 50°C(18,19).

Discussion. We have examined the antigens from *T. polyspora* associated with farmer's lung disease and have found them to be similar to the antigens extracted from moldy hay. Trichloroacetic acid (TCA) extracts of moldy hay(6) and of *T. polyspora*

grown on synthetic media contained 3 antigens which produced 3 precipitin lines with the serum used in these studies. Fig. 3 shows the immunoelectrophoretic properties of these antigens. Only 2 precipitins could be distinguished by double diffusion (Fig. 1); the diffuse precipitin which occurred near the inner well probably was produced by 2 antigens.

The immunoelectrophoretic behavior of the antigens from moldy hay and from *T. polyspora* were similar, and the ethanol fractionated antigens from the two sources gave lines of identity on double diffusion plates (Fig. 1). Furthermore, the antigens from moldy hay and from *T. polyspora* which were not precipitated by 70 to 75% alcohol displayed the same behavior on DEAE-cellulose columns (Fig. 2). Thus, it seems likely that all of the farmer's lung antigens in our H-1 moldy hay sample were due to *T. polyspora*. *Micromonospora vulgaris*, which was also isolated from this hay, gave no precipitins with farmer's lung sera(21).

One of the problems concerning farmer's lung antigens from *T. polyspora* was whether they were produced extracellularly or were of cellular origin. This problem could best be solved by growing the organism in liquid culture, and this was done successfully by Hartz(17). Phosphate buffer extracts of mycelia produced 5 precipitins with the serum used in this study; 2 of these precipitins could be eliminated by treating the extract with TCA. Additional amounts of the 3 TCA-soluble antigens were extracted by cold TCA, and additional amounts of antigen C (Fig. 3) were extracted from the mycelial residue with hot TCA and with NaOH. Another TCA-soluble antigen was found in the growth medium which did not cross react with any of the other TCA-soluble antigens. This antigen was not observed in extracts of moldy hay or in extracts of *T. polyspora* grown on agar.

The 3 antigens extracted from *T. polyspora* by TCA were not destroyed by Pronase digestion. After digestion, the sample still contained a peptide composed primarily of lysine, glutamic acid, alanine and allo-isoleucine. This peptide appeared to be related to part of a cell wall glycopeptide which has been reported in many other Gram-positive organisms(22). Glycopeptides extracted from several Gram-positive organisms have been shown to be antigenic in rabbits(23). Antigenic preparations from *T. polyspora* which we have purified by DEAE-cellulose, Sephadex and Bio-Gel column chromatography(21) also contained from 1 to 8% protein or peptide in addition to large amounts of polysaccharide. The polysaccharide was composed primarily of galactose, arabinose and glucosamine. These sugars occur widely in the cell walls of Gram-positive organisms(22,24), and they were also detected in extracts of moldy hay(6). Our studies have indicated that the antigens responsible for farmer's lung disease are glycopeptides and that the peptide may be part of the structural cell wall of *T. polyspora*.

Summary. The trichloroacetic acid (TCA-) soluble antigens from *T. polyspora* had the same immunoelectrophoretic properties as the 3 farmer's lung antigens in a moldy hay sample from which this organism was isolated. The antigens cross reacted with one another and had similar properties on DEAE-cellulose columns. *T. polyspora* was grown in liquid culture. The mycelia were broken by sonication and extracted in order with pH 7.2 phosphate buffer, cold TCA, hot TCA and NaOH. Three antigens were extracted by cold TCA, and the phosphate buffer extract contained 2 additional TCA-insoluble antigens; the hot TCA and NaOH extracts were also antigenic. An extracellular, TCA-soluble antigen was found in the growth medium which did not cross react with the other TCA-soluble antigens. Pronase digestion did not destroy the 3 TCA-soluble antigens, but a residual peptide remained which was composed primarily of lysine, glutamic acid, alanine and allo-isoleucine. This peptide appeared to be part of a cell wall glycopeptide. The antigenic fractions were composed primarily of polysaccharides containing galactose, arabinose and glucosamine.

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Acceptance of Walker 256 Carcinosarcoma by Neonatally Thymectomized C57 BL/6 Mice. (30806)

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The significance of the thymus in the development of immunologic competence in many animals is well documented(1). The thymus also plays a role in the growth of certain tumors in rodents. Some experimental leukemias in mice are dependent on the presence of the thymus(2). Conversely, after neonatal thymectomy, the incidence of tumor formation in rats inoculated with the polyoma virus is increased(3,4), and the natural resistance of C57 BL mice to the oncogenic effects of this virus is decreased(5). Thymectomy performed in mice 3 days of age is followed by an increased incidence of skin tumors induced by benzopyrene(6). Neonatally thymectomized Z (C_3 H) mice accept allogeneic grafts of a mammary tumor from A strain mice despite histo-incompatibility between these strains at the H-2 locus(7).

The Jensen sarcoma, transplanted allogeneically to neonatally thymectomized Sprague-Dawley rats, "takes" more often and grows more rapidly than in control rats(8). Rejection of xenografts of the mouse sarcoma 1 ascites tumor by neonatally thymectomized inbred Lewis rats is delayed(9). On the other hand, neonatal thymectomy in Sprague-Dawley and Long-Evans rats does not alter the incidence, rate of growth, or development of metastases of transplanted Walker 256 tumor, a neoplasm that grows well in this species in any case(10).

Extracts of thymus have been shown to contain both tumor-promoting and tumor-inhibiting substances, the latter, at least, being not specific for thymic tissue(11).

The present work was undertaken in search of a combination of tumor and host that would delineate sharply and reasonably rapidly the role of the thymus in mice with