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THERMOACTINOMYCES CANDIDUS: EPIDEMIOLOGIC, ANTIGENIC, AND PATHOGENIC ASPECTS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

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GARY EUGENE HOLLICK

Norman, Oklahoma

1979

THERMOACTINOMYCES CANDIDUS: EPIDEMIOLOGIC, ANTIGENIC, AND PATHOGENIC ASPECTS

APPROVED BY

DISSERTATION COMMITTEE

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AEROBIOLOGY OF INDUSTRIAL PLANT AIR SYSTEMS: FUNGI AND RELATED ORGANISMS

CHAPTER I

INTRODUCTION

Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis is increasingly recognized as a significant health problem resulting from the inhalation of organic dusts found in home and occupational environments. The syndrome is a disabling pulmonary disease induced by antigenic exposure through inhalation resulting in a hypersensitivity reaction. Terminology in HP is extensive, e.g. a plethora of descriptions are used including farmer's lung, humidifier lung, bagassosis, berylliosis, etc. depending on the offending inhalant. Histological changes seen in these conditions are quite similar suggesting a common mechanism of injury. A specific infection is not involved, but cellular immune reactions do play a role in the pathogenesis of experimentally induced hypersensitivity pneumonitis (17, 25).

Recognition of the disease condition is credited to Campbell (5) who reported clinical histories of five farmers in England and correlated the condition to exposure to

"white mold" on their grain. Two previous reports (1, 22) describe similar respiratory illness but make no attempt to define the causative agent.

Fungi have been continually implicated as the offending inhalant in hypersensitivity pneumonitis (10, 30). The most frequently considered etiologic agents are fungal spores of *Alternaria*, *Aspergillus*, *Cryptostroma*, *Graphium*, and *Pullularia* (*Aureobasidium*), causing malt worker's lung, mushroom worker's disease, wood dust, and maple bark strippers lung (31). The thermophilic actinomycetes have been implicated as the sensitizing agents in farmer's lung, bagassosis, and humidifier lung (21, 26). Simple structural molecules such as cromolyn sodium (27), nitrofurantoin (13), and toluene diisocyanate (6, 7) also mediate a hypersensitivity reaction.

This diffuse interstitial lung disease is manifested by a general malaise, fever, chills and dyspnea. Onset is generally 4 to 8 hours following exposure (11). Peripheral blood analyses reveal a granulocytosis, specific antibody increase and sensitized T cell populations responsive to the etiologic agent (4). These tests can be adjunctly useful in diagnosis and establishment of the causative agent.

Pathogenetic mechanisms implicated in hypersensitivity pneumonitis include immune complex disease and/or cellular hypersensitivity (20). Clinical investigations also suggest as contributory, genetic predisposition and non-specific inflammation caused by the inhalant (12, 24).

An interstitial inflammatory process and alveolitis accompanied by non-caseating granulomas usually form the histologic basis for diagnosis (9). Occasionally symptomology is found only with acute inflammation. Identification of inhalants continues to expand the list of etiologic agents. Exposure to the agents is generally associated with occupation and, as such, is of economic import. Repeated exposures over an extended time period can result in end-stage lung disease (14) which is considered irreversible.

Industrial plant air systems can provide an environment for introduction of organisms into the air, circulation and perhaps concentration of particles. Aerosolization of common organisms from the environment may eventually reach employees resulting in "outbreaks" of hypersensitivity pneumonitis in sensitized individuals.

The current study was undertaken after six individuals working in an industrial plant in Southeastern United States were diagnosed as having hypersensitivity pneumonitis. Clinical diagnosis was based on symptoms, radiographic evidence and biopsy material. Serological tests for precipitins against commercially prepared antigens were positive. To determine the normal microorganism (bacteria and fungi) content of this controlled environment, impinger and regulated pore size samplers were used. Contributions of the industrial plant (such as flora from forced airheating, air conditioning or air circulating ducts) were determined over a one year

period to delineate areas of risk, to identify the isolates, and to find the frequency of occurrence of the organisms present.

CHAPTER II

MATERIALS AND METHODS

Sampling areas

Fifty locations within the industrial complex were initially sampled. These locations included the intake vents and pre- and post-air wash areas of 10 air conditioning systems as well as several positions along the lateral ducts of these systems. In addition, samples of air were taken under and within air conditioning vents in work areas where exposure to spores would be maximal. At three different subsequent time periods, 20 samples were taken randomly throughout the industrial complex.

Samples taken

Samples were coded and transported to the laboratory. Accumulated slime on the floors of the forced air-water cooling systems and in the water troughs was collected in sterile test tubes. Cooling water from the water troughs was filtered through Millipore (R) filters (0.45μ) and the filters placed on primary plating media. Air samples were taken with a modified Andersen sampler utilizing all 6 stages. The velocity of sampling was set at 1 cubic foot/min using

a sampling time of 4 min. Air samples within the air conditioning ducts were taken for 1-1.5 hr with Porton (all glass) impingers calibrated to sample between 1.5-2.0 liters/min. Scrapings of accumulated material from air filters and dirt deposits were extracted in 5.0 ml sterile water before plating onto primary isolation media.

Isolation procedure

Figure 1 outlines the sample processing. Bacteria, other than those isolated from slime, were not identified. Initial coded samples were plated onto blood agar, Sabourauds dextrose agar, Sabourauds dextrose agar with penicillin (20 µ/ml) and streptomycin (40 µg/ml), tryptic soy agar, and potato dextrose agar. Blood agar and tryptic soy agar plates were incubated at 55C while other plating media were incubated at 4, 25 and 37C. Plates were incubated for a total of 2 weeks. Colonies of different morphological appearance were transferred for isolation to potato dextrose agar and Sabourauds dextrose agar for fungi and to tryptic soy agar for thermophilic actinomycetes. Microcultures were established as described elsewhere using a block each of Sabaurauds agar and Czapeks agar (23).

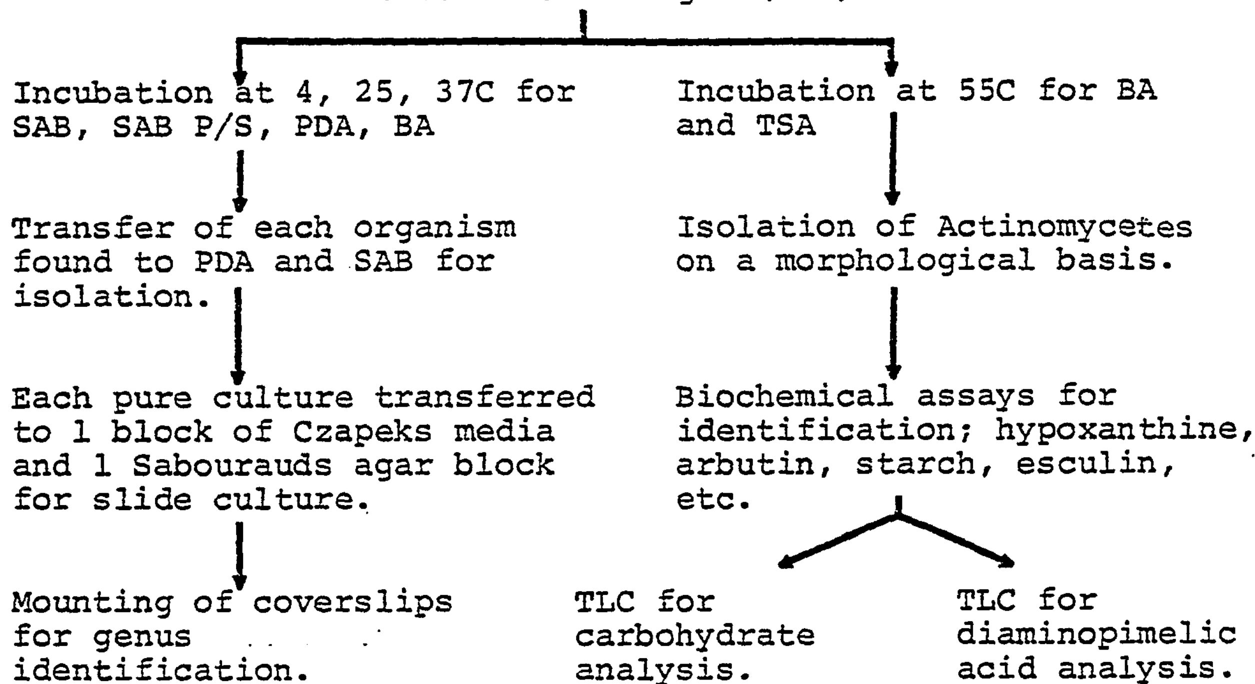
Isolate identification

Fungi were identified to the genus level by microscopic appearance in microculture using standard taxonomic keys (2). Thermophilic actinomycetes were identified by standard

Figure 1. Schematic diagram of sample processing.

Initial Coded Sample

(Air, dirt, water, etc.)
Plated on Blood Agar (BA),
Sabaurouds Dextrose Agar (SAB)
SAB + pen/strep
Tryptic Soy Agar (TSA) and
Potato Dextrose Agar (PDA)



biochemical tests (18) and by cell wall analysis for diagnostic carbohydrates and meso- or L-diaminopimelic acid using thin layer chromotography (TLC) (29). Bacteria isolated from slime were identified by standard biochemical criteria (3) and staining techniques.

CHAPTER III

RESULTS

As shown in Table 1 a total of 15 different genera were isolated on initial sampling. *Aspergillus*, *Cladosporium*, *Penicillium*, and *Alternaria* species predominated, being isolated from 72, 64, 56, and 26 percent of the sampled sites. The other genera of fungi listed in Table 1 were isolated from less than half of the locations sampled. Shown in Table 2 are the results of isolations of fungi in samples obtained at random sites throughout the industrial complex during 3 subsequent sampling times. *Aspergillus*, *Fusarium*, and *Penicillium* species were isolated most often at each sampling period. An additional 3 genera, which were not isolated during the initial study, were present during these sampling periods.

Since employees with a diagnosis of lymphocytic interstitial pneumonitis reacted in precipitin tests to extracts of the slime, a more thorough analysis of the slime was undertaken. Tables 3 and 4 give the identifications of the fungal and bacterial organisms recovered. It can be seen that *Aspergillus* was again the most prevalent fungus.

TABLE 1

Frequency of Isolation of the Most Common Genera of Fungi on Initial Visit

Genus	Frequency*
Aspergillus	72
Cladosporium	64
Penicillium	56
Alternaria	26
Nigrospora	22
Fusarium	20
Chaetomium	16
Epicoccum	14
Cephalosporium	12
Botrytis	10
Phoma	10
Rhizopus	6
Curvularia	4
Trichoderma	2
Stemphyllium	2

*Expressed as percent of sites which yielded a positive culture for a particular genus.

TABLE 2

Fungi Isolated at Random Locations on Three Different Occasions

Genus	Sampling Time		
	A	B	C
Aspergillus	8*	4	9
Penicillium	5	6	4
Fusarium	2	3	2
Cephalosporium	1	1	1
Curvularia	1	1	1
Nigrospora	1	0	1
Rhizopus	1	0	1
Geotrichum	1	0	1
Cladosporium	0	1	0
Alternaria	0	1	0
Epicoccum	0	1	0
Aureobasidium	0	1	0
Paecilomyces	0	1	0

*Number of isolates

TABLE 3

Fungi Recovered From Slime

Genus	Number of Isolates
Aspergillus	4
Penicillium	2
Cephalosporium	2
Alternaria	1
Fusarium	1
Rhizopus	1
Scopulariopsis	1

TABLE 4

Comparison of Bacterial Isolates from Slime in Two Industrial Plants

Plant A	Plant B
Achromobacter	*Aeromonas hydrophilia*
Bacillus	*Pseudomonas fluorescens* group
Enterbacter	*Pseudomonas putrefaciens*
Flavobacterium	*Serratia liquefaciens*
Pseudomonas	

An additional 6 genera of fungi were also isolated from this sample. Bacterial isolates from the slime are listed in Table 4. *Achromobacter*, *Bacillus*, *Enterobacter*, *Flavobacterium*, and *Pseudomonas* species predominated. These organisms appear to be peculiar to this sample of slime. An analysis of slime from a different plant manufacturing the same product yielded an entirely different microbial population. In this sample *Aeromonas*, *Pseudomonas*, and *Serratia* were the predominant bacteria.

Although several thermophilic bacteria were isolated, only *Thermoactinomyces candidus* and 1 thermophilic streptomycete were identified within the plant. *T. candidus* was isolated from 30 different sampling sites. The majority of isolates were obtained from samples taken from the air conditioning units. Dirt scrapings, air samples, and trough water all yielded positive cultures. Slime samples provided abundant colonies when incubated at 55C and it would appear that *T. candidus* is the major organism in this material. In fact, slime samples refrigerated for up to 3 months when cultured still gave positive isolations for *T. candidus*.

CHAPTER IV

DISCUSSION

The most prevalent fungal organism in our studies was *Aspergillus* (72%) followed by *Cladosporium* (64%) and *Penicillium* (56%). The occurrence of allergic bronchopulmonary aspergillosis has been well recognized (15). Although *Aspergillus* species are considered ubiquitous, only a select population of individuals becomes sensitized.

Penicillium cultures were numerous and have been implicated in hypersensitivity pneumonitis, i.e. cheese-washer's lung (31). *Cladosporium* is also a recognized etiological agent in hypersensitivity pneumonitis.

Similar distributions of organisms are commonly found in home and work environments. In several surveys, *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* were the most frequently found, ranging from frequencies of 25% to 70% (16, 28). *Aspergillus* was seen in much lower frequencies in a study by Lumpkins *et al*. (19) which considered seasonal variation in isolation. Collection of specimens in our study was limited in that initial sampling occurred during February (temperatures 19-22C) with

subsequent, smaller samplings in late spring and early summer. Sampling at different times during the year in the same locations may have revealed different relative frequencies of recovered organisms. Fluctuations of spore content in the environment are frequently dependent upon season and location of sampling (8, 19).

Fungi and thermophilic actinomycetes were found throughout the air conditioning system. *Aspergillus* and *T. candidus*, for example, were found at the intake vents, before and after the cool water wash, and in the air from the vents in employee working areas. Only part of the incoming air is taken from the outside; the remainder being recirculated air from within the plant. It was apparent in our study that the air wash system was ineffective in removing spores from the air. The slime forming in the air conditioning systems provided an abundant number of organisms and could, under proper conditions, serve as a growth medium for organisms or serve to concentrate trapped spores. This situation poses a severe threat to the employee population enjoying the conditioned air.

Characterization of the aerobiology of an industrial plant is an important step in defining areas of potential risk to employees. Exposure alone does not imply a host response since specific serum precipitins are frequently found in asymptomatic individuals (21, 25). However, repeated exposure in susceptible hosts may result in hypersensitivity pneumonitis which may lead to fibrotic, irreversible tissue

damage. Recognition of the existence of known sensitizing inhalants in the environment will simplify diagnosis and anticipate exposure problems of the employees.

Patients demonstrating clinical signs, typical x-ray patterns, histopathological changes and positive precipitins were monitored in home (results not shown), work and recreation environments. Sensitivity appeared and episodes worsened in the industrial plant. In a separate investigation, antigenic extracts from isolates obtained in this study were tested against sera from HP patients and normal employees. Crude preparations from the slime material and metabolic by-products from isolated cultures exhibited precipitin lines in routine Ouchterlony immunodiffusion tests (Charles E. Reed, personal communication). Correlation of the aerobiology and onset of disease has been suggested. Further studies are in progress to determine the incidence of precipitins in asymptomatic individuals using antigens prepared in our laboratory. Comparative studies of aerobiology of home and recreation environments have shown increased frequencies and total numbers of microorganisms in the air in the industrial system. Association between susceptible individuals and normal air flora will assist in recognition of the condition. By limiting exposure, hypersensitivity pneumonitis pathology is usually responsive to steroid therapy and is reversible. If these symptomatic individuals are not identified early in the disease course, they may progress to a non-responsive lung disease which is irreversible.

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CHEMICAL AND SEROLOGICAL COMPARISON OF TWO ANTIGEN EXTRACTS OF *THERMOACTINOMYCES CANDIDUS*

CHAPTER V

INTRODUCTION

The thermophilic actinomycetes have been implicated as sensitizing agents in a large number of cases of hypersensitivity pneumonitis. *Micropolyspora faeni*, *Thermoactinomyces vulgaris*, and *Thermoactinomyces sacchari* are most often associated with "farmer's lung" (6, 22) or bagassosis (17, 26), while *Thermoactinomyces candidus* is usually associated with "humidifier lung" or "air conditioner lung" (3, 10). The prevalence of these organisms in specific environments appears to parallel this association. *T. candidus* and *T. vulgaris* are ubiquitous, being isolated from air conditioners, humidifiers, wood dust, house dust, moldy grains and furnace filters (16). *M. faeni* is generally not found in air conditioning units, but mainly in wood dust, house dust, moldy grains and, in particular, moldy hay (16, 22).

The presence of precipitating antibodies in individuals exposed to thermophilic actinomycetes has been useful in the diagnosis of hypersensitivity pneumonitis (7, 11, 23, 25). A

positive precipitin test, however, indicates only exposure and not sensitivity since both symptomatic and asymptomatic individuals may possess precipitating antibody to these organisms (4, 12, 21). In addition, a retrospective study of 113 patients with farmer's lung by Wenzel, *et al*. (30) revealed that of the patients with positive precipitin tests, 44.2% reacted to more than one thermophilic actinomycete. Cross reactions to multiple actinomycete antigens in patients with farmer's lung have been reported by other investigators (1, 23, 30). Whether this is due to immunological cross reactivity of the antigens or to exposure to several actinomycetes remains unclear. However, Kurup *et al*. (13) demonstrated using double dialysis antigens from several actinomycetes that cross reactivity did exist particularly with *T. candidus* and *T. sacchari*. While many antigens from *M. faeni* have been isolated and characterized (11, 15, 19, 21, 25), relatively few reports have been concerned with the antigenic constitution of *T. candidus*.

This study was undertaken to serologically and chemically characterize two antigen extracts of *T. candidus*.

CHAPTER VI

METHODS AND MATERIALS

Organism

Thermoactinomyces candidus was isolated from an industrial air conditioning system. Cultures were maintained at 55C on tryptic soy agar slants and transferred at two week intervals. Identification was based on standard biochemical criteria (14) and by cell wall analysis for the presence of meso- or L-diaminopimelic acid (DAP) isomers and diagnostic carbohydrates according to the methods of Staneck and Roberts (27). Commercially available DAP (Sigma Chemical Co.) served as the standard.

Antigen preparation

A pyridine extract antigen (TPE) was isolated according to the procedure of Wenzel and Emanuel (29). The organism was grown in static culture in 500 ml of tryptic soy broth (TSB) (Difco) for 5 days at 55C. The cells were harvested, washed three times in sterile physiological saline and extracted for three days with stirring at 4C in 100 ml of pyridine buffer (0.2 M, pH 5.4). The supernatant was collected following centrifugation and dialyzed 5 days against distilled water, lyophilized

and reconstituted to 31 mg/ml in phosphate buffered saline (10^{-3} M, pH 6.8).

Double dialysis antigen (DDA) was obtained by the method of Edwards (9) as modified by Kurup *et al*. (13). The lyophilized antigen was reconstituted in sterile physiological saline to 66 mg/ml. Two batches of each antigen were prepared.

Chemical analysis

Protein estimation was performed by the method of Lowry *et al*. (18) using bovine serum albumin as standard. Carbohydrates were estimated by the phenol method of Dubois *et al*. (8) with alpha-D-glucose (Eastman) as the standard.

Production of rabbit antiserum

T. candidus spores were removed from growth on TSA slants and washed three times with sterile saline before adjusting to the proper concentration by plate counts. Two Deutch belt rabbits were inoculated with 1×10^6 spores intravenously on three successive days at weekly intervals. Animals were bled by cardiac puncture after 1 month and the serum tested for the presence of precipitins by microimmunoelectrophoresis (see below). Booster injections were given as needed at weekly intervals and the animals bled one week later.

Crossed immunoelectrophoresis

Glass plates (8 X 10 cm) were coated with 15 ml of 1% agarose (Microbiological Associates) in Veronal buffer (0.01M, pH 7.2). A well was punched into the gel using a 3 mm cork

bore one centimeter from the edge of the plate and filled with either full strength pyridine extract antigen or the double dialysis antigen. Electrophoresis was carried out in an electrophoresis chamber (Gelman) at 4C for 2 to 3 hours using 10V/cm. The buffer troughs were filled with high resolution buffer (5.778 g Tris (hydroxymethyl) aminomethane, 2.446 g barbituric acid, 9.756 g sodium barbital in 1 liter water, pH 8.8) and connected to the glass plates by means of a Whatman paper wick. After completion of electrophoresis in the first dimension, the agar was sliced 1.5 cm from the edge of the plate and the agar removed. A 1:5 dilution of rabbit antiserum in 12 ml of agarose was then layered over the plate. Electrophoresis was performed perpendicular to the previous migration (time and voltage as described above). The plates were incubated at 4C for 48 hours in a humidified chamber then dried by placing a moist piece of filter paper over the gel and incubating at 35C overnight. Bands were stained with Azocarmin B for 15 minutes. Destaining was achieved by rinsing the plates three times in methanol: acetic acid: water (45:10:45) for 15 minutes.

Disc gel electrophoresis

The method of acrylamide gel electrophoresis was essentially that described by Cooper (5). Lower 7.5% separator gels (100 mm) with upper 3.5% spacer gels (10 mm) were cast in glass columns measuring 5 X 120 mm. Columns were loaded by the layering of 35 μg protein of TPE and 1 mg protein DDA

suspended in 50% glycerol onto the spacer gels. The lower reservoir was filled with Tris buffer (pH 8.3). Electrophoresis was carried out in the cold at a constant current of 2 mA/gel until the tracking dye (bromphenol blue) had migrated to a point approximately 1 cm above the bottom of the gel. Protein bands were visualized by staining with Coomassie brilliant blue for 12 hours (2). Glycoproteins were stained with Alcian Blue according to the methods of Wardi and Michos (28). Gels were scanned at 600 nm for protein and 630 nm for glycoproteins in a recording spectrophotometer (Model 24 - Beckman). Rf values were calculated as the ratio of the distance of band migration to the distance of the tracking dye migration measured from the edge of the separator gel.

Fractions of the two antigen extracts were obtained by disc gel electrophoresis as described above. One gel was stained for protein while each of the remaining 17 gels were sliced into 1 cm segments, pooled and extracted in 5 ml of sterile water at room temperature. After 48 hours the gel slices were removed and the extract lyophilized. The fractions were reconstituted in 1 ml of sterile saline and tested for immunologic reactivity by immunoelectrophoresis.

Immunoelectrophoresis

Microscope slides were coated with 3 ml of 1% agarose (Microbiological Associates) in Veronal buffer (pH 7.2, 0.01M). A punch set (Gelman) was used to cut the electrophoretic pattern. Wells were filled with the extracts from disc gel electrophoresis

or with the crude antigens. The electrophoresis chamber was filled with high resolution buffer (0.045M, pH 8.8) and connected to the slides by means of Whatman paper wicks. Electrophoresis was carried out in the cold at a constant voltage of 6V/cm for 35 minutes. After electrophoresis, the troughs were filled with rabbit antisera and incubated in a humidified chamber for 48 hours. Precipitin bands were stained for 15 minutes in Coomassie brilliant blue after the gels had dried (2).

CHAPTER VII

RESULTS

Organism identification

Results of biochemical and physiological tests conformed exactly to the criteria outlined by Kurup and Fink (14) for the identification of *T. candidus*. Cell wall analysis revealed a Type III composition (14) characteristic for this organism. The identification of this organism has been confirmed by V. P. Kurup, (Veterans Administration Center, Wood, Wisconsin).

Chemical composition

The chemical composition of the DDA and TPE for both batches of antigen is given in Table 1. Some variation was noted in the protein and carbohydrate concentrations comparing batches, although the protein: carbohydrate ratio between batches did not appear to differ significantly. Protein and carbohydrate accounted for 29 to 41 percent of the total dry weight for DDA. By contrast, the TPE antigen contained considerably less protein and carbohydrate and accounted for only 0.3 or 3 percent of the total dry weight.

TABLE 1

Chemical Comparison of Two Batches of Double Dialysis and Pyridine Extract Antigens

ANTIGEN[a]	DRY WEIGHT MG	PROTEIN MG(%)	CARBOHYDRATE MG(%)	PROTEIN: CARBOHYDRATE
DDA 1	660	230(34.8)	42.5(6.5)	5.4
2	360	92.5(25.7)	14(3.9)	6.6
TPE 1	270	.78(.29)	.25(.09)	3.1
2	316	6.9(2.2)	2.6(0.8)	2.6

[a]DDA= Double Dialysis Antigen

TPE= Pyridine Extract Antigen

Crossed immunoelectrophoresis

Comparison of one batch of the TPE to the DDA using crossed immunoelectrophoresis is shown in Figures 1 and 2. Only one peak migrating toward the anode could be demonstrated for the TPE antigen, however, the DDA displayed two major peaks and one minor peak. It appears from these data that the antigenically reactive components in the TPE antigen and the DDA are not the same since they did not migrate to the same area. It should be mentioned that the antisera used was raised to whole spores and not the antigen extracts. There could, therefore, be antigenic components which are shared by the two antigens which are not present in intact spores.

Polyacrylamide gel electrophoresis

The results of polyacrylamide gel electrophoresis are depicted in Figures 3-6. A comparison of the two batches of DDA (Figures 3b, 4a, and 5) revealed a marked difference in the number of components with similar relative mobilities, the second batch of antigen contained six bands not present in the first. Components with an electrophoretic mobility of 0.96, 1.02, and 1.05 were not present in the first preparation. The position of the remaining three bands is difficult to ascertain but may correspond to proteins with a mobility of 0.64, 0.66, and 0.70. One component with a relative mobility of 0.40 was present in the first preparation but absent from the second.

A total of five bands was obtained when the gels were

Figure 1. Crossed immunoelectrophoresis of the double dialysis antigen. Electrophoresis in both the first and second dimension was carried out at 10V/cm for 2-3 hours. The anode is at the right of the figure.

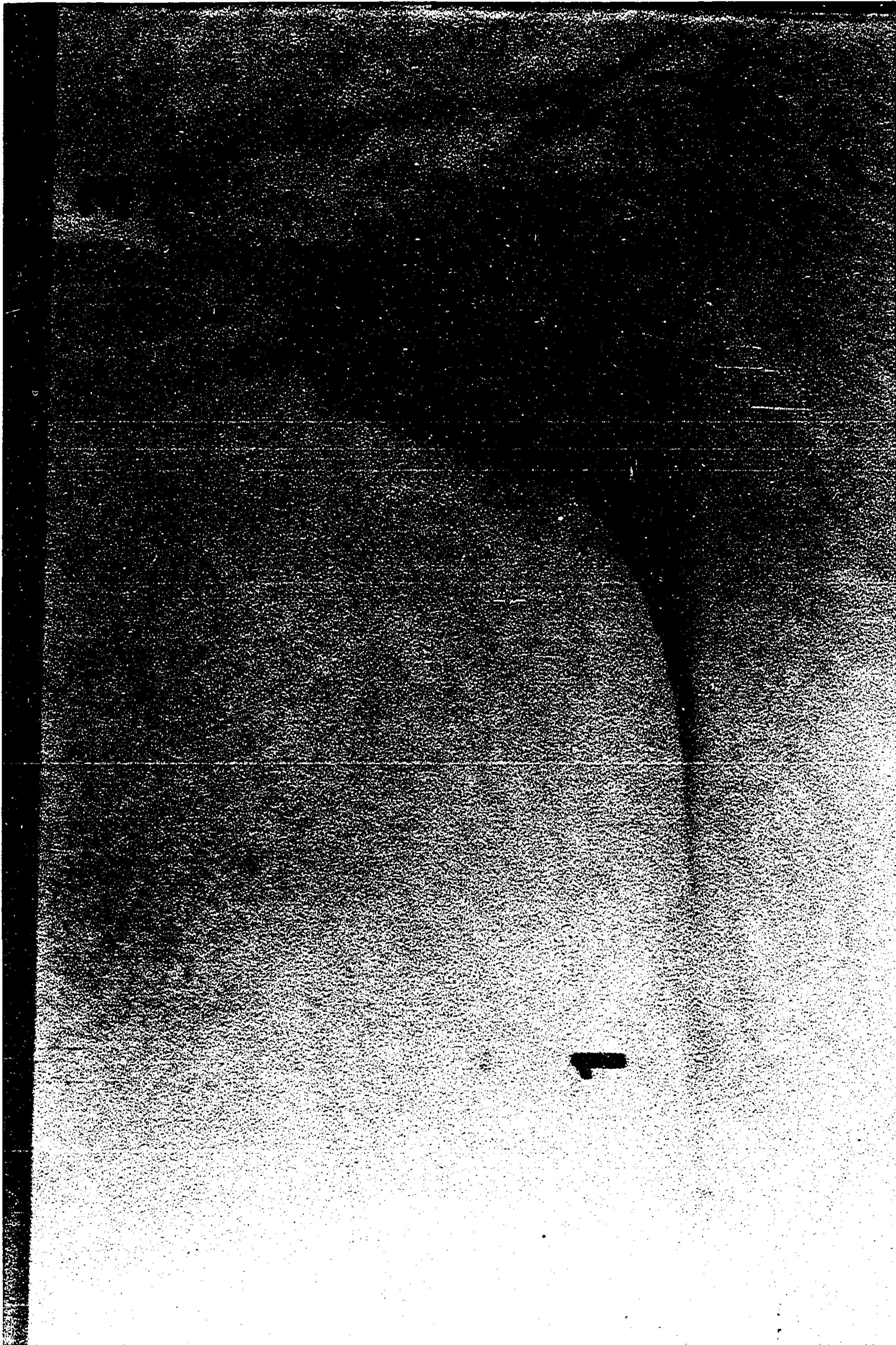


Figure 2. Crossed immunoelectrophoresis of the pyridine extract antigen. Electrophoresis in both the first and second dimension was carried out at 10V/cm for 2-3 hours. The anode is at the right of the figure.

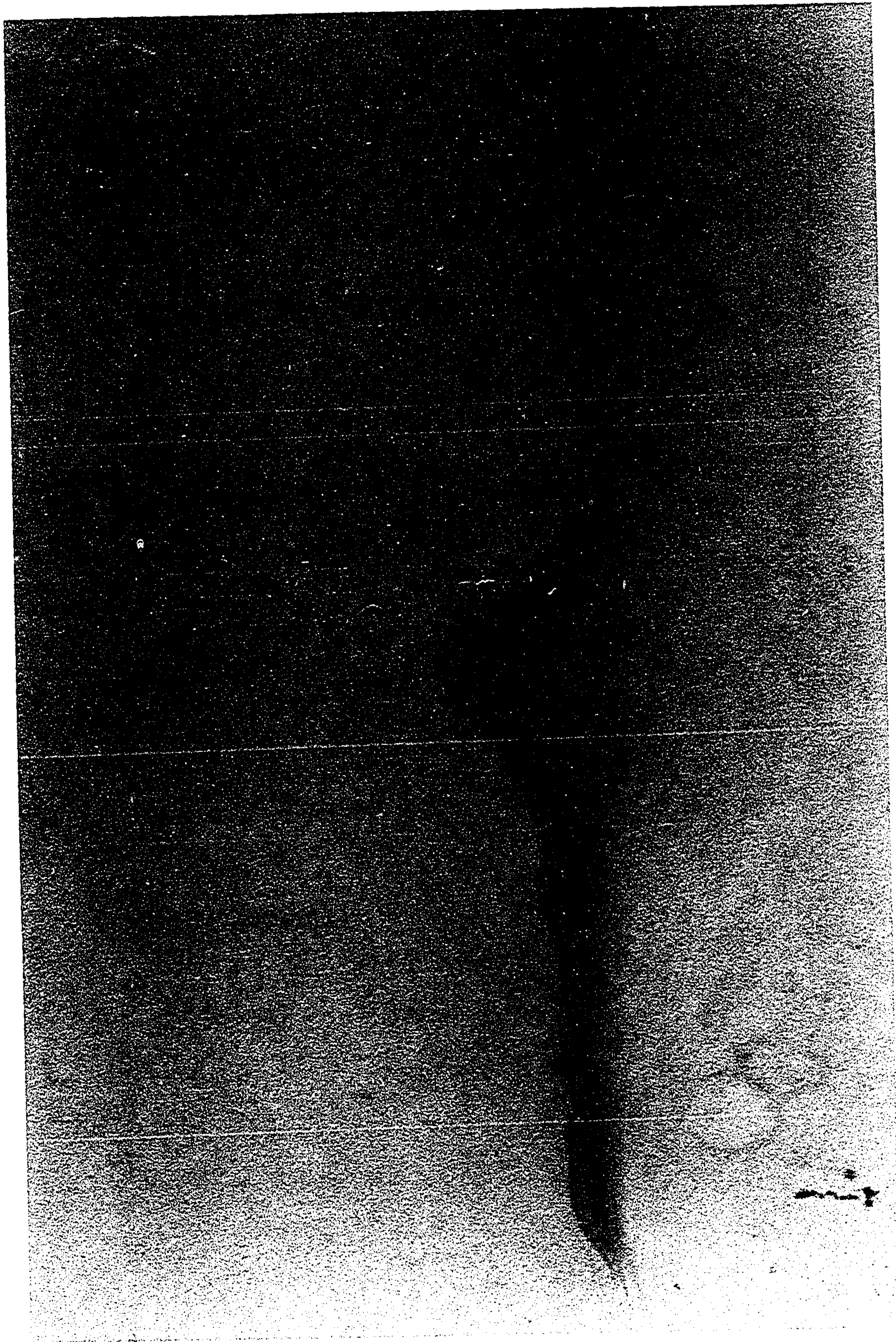
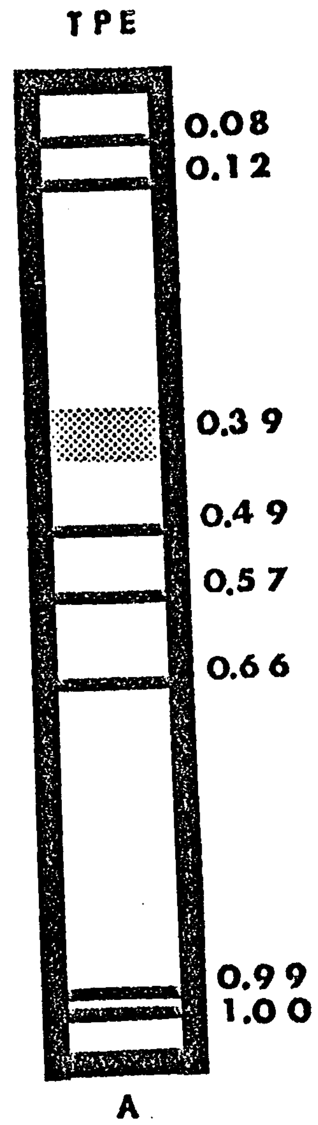


Figure 3. Comparative polyacrylamide gel electrophoretograms of the first batch of (A) the pyridine extract antigen and (B) the double dialysis antigen. Gels were loaded with 1 mg/ml protein of double dialysis antigen and 34 µg/ml protein pyridine extract antigen. The stippled area denotes a diffuse band. The anode appears at the bottom of the figure.

R_f



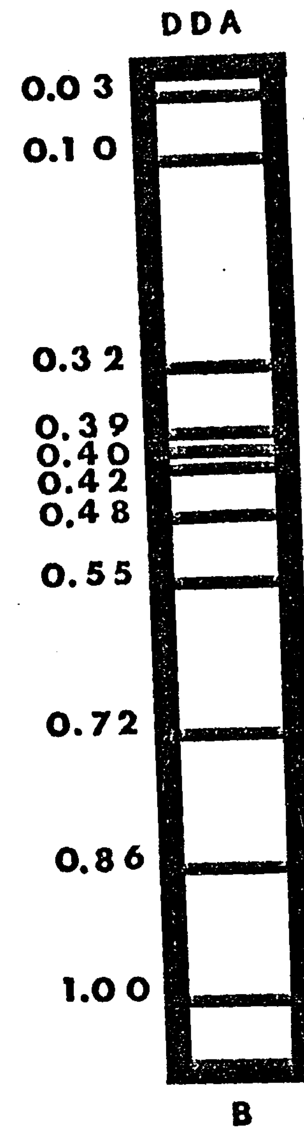
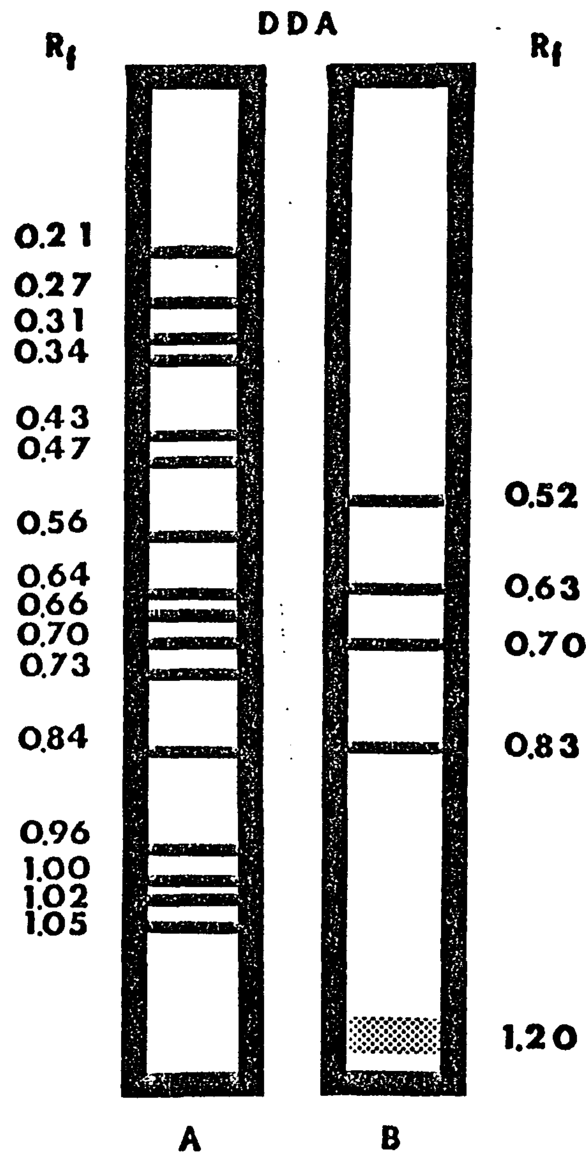


Figure 4. Comparative polyacrylamide gel electrophoretograms of the second batch of the double dialysis antigen and pyridine extract antigen. Gels were loaded with 1 mg/ml protein of double dialysis antigen and 34 μg/ml protein pyridine extract antigen. Coomassie Blue was used to stain gels (A) and (C) while gels (B) and (D) were stained with Alcian Blue. The stippled area denotes a diffuse band. The anode appears at the bottom of the figure.


DDA
Rf
Rf
0.21
0.27
0.31
0.34
0.43
0.47
0.56
0.64
0.66
0.70
0.73
0.84
0.96
1.00
1.02
1.05
0.52
0.63
0.70
0.83
1.20
A
B

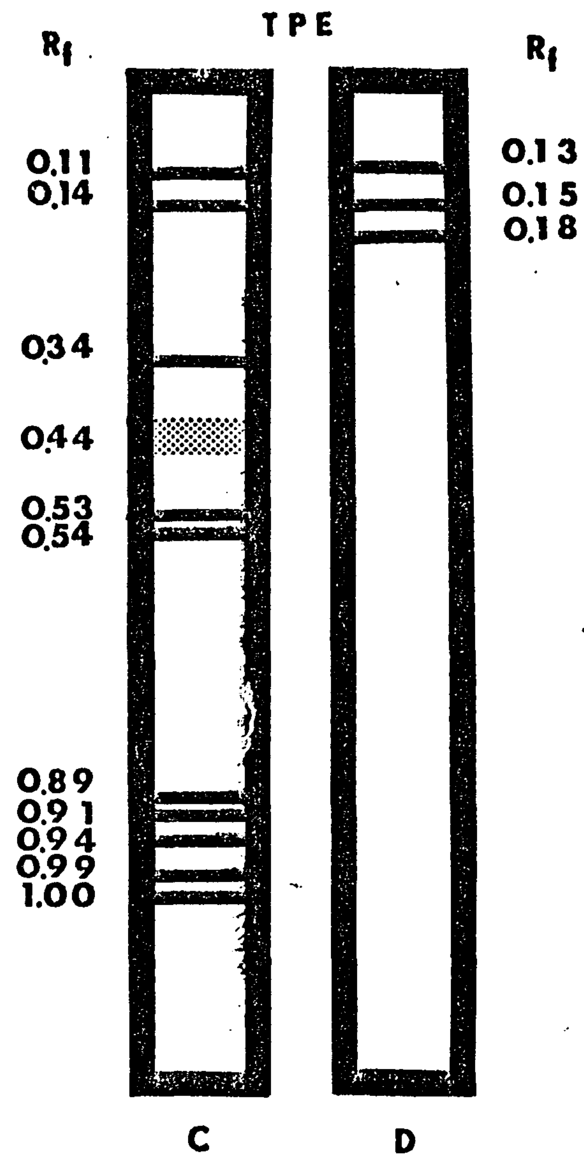
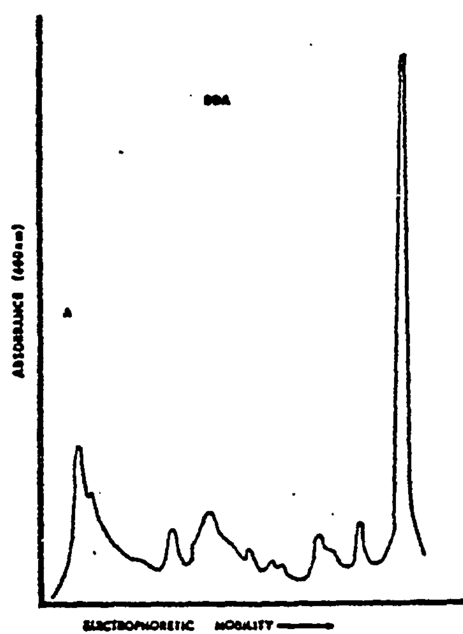
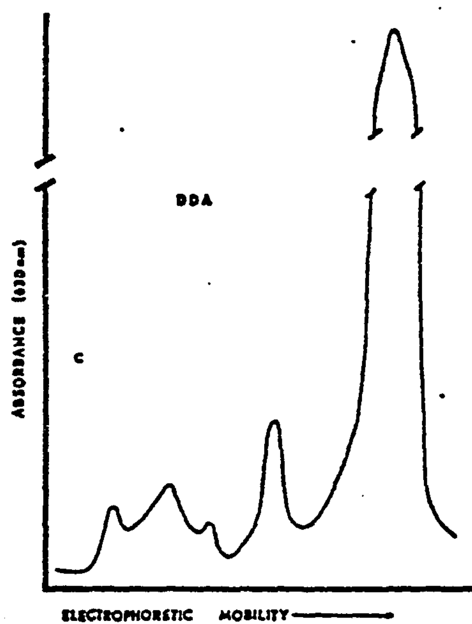

TPE
Rf
Rf
0.11
0.14
0.34
0.44
0.53
0.54
0.89
0.91
0.94
0.99
1.00
0.13
0.15
0.18
C
D

Figure 5. Recording spectrophotometric tracing of the first (A) and second (B and C) batches of the double dialysis antigen. Gels (A) and (B) were stained with Coomassie Blue. Gel (C) was stained with Alcian Blue.


DDA
A
ABSORBANCE (660nm)
ELECTROPHORETIC MOBILITY


DDA
C
ABSORBANCE (630nm)
ELECTROPHORETIC MOBILITY

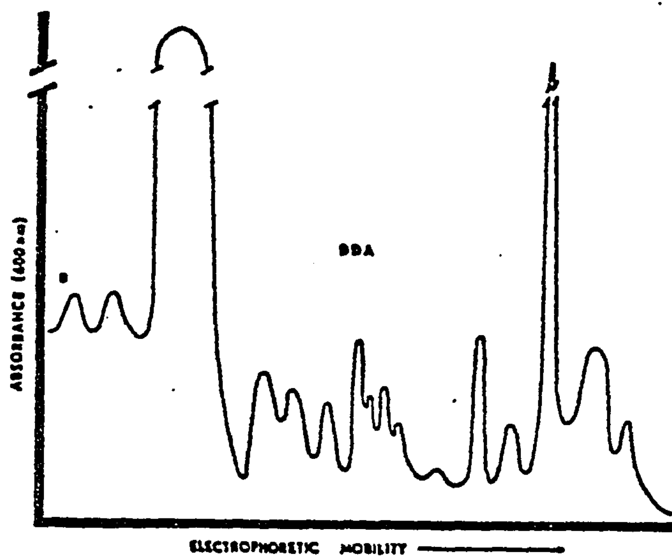
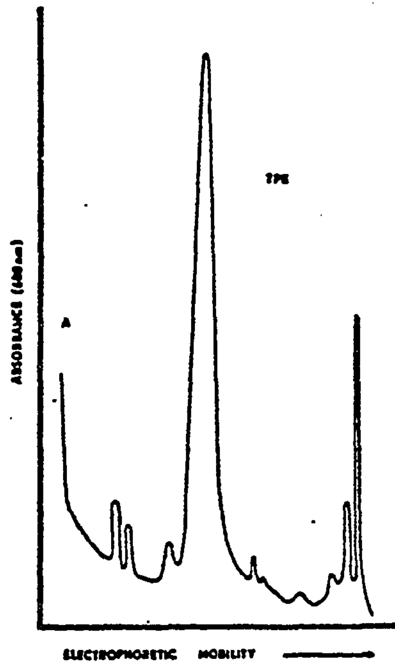
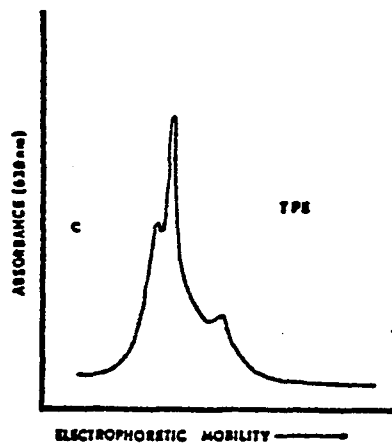
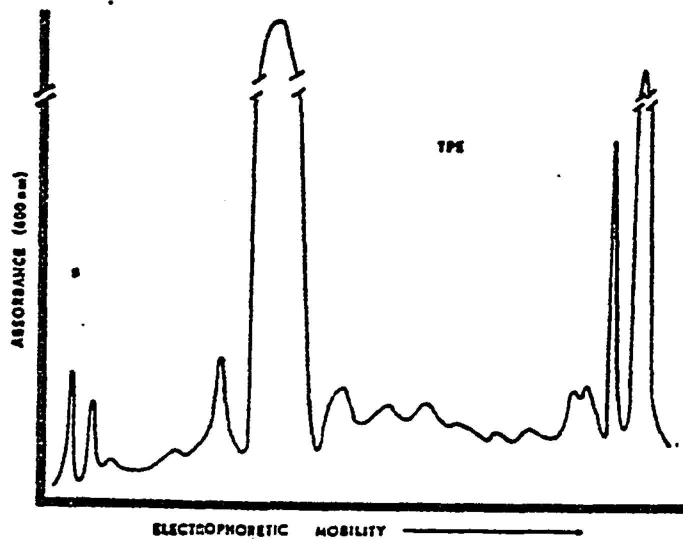

DDA
B
ABSORBANCE (600nm)
ELECTROPHORETIC MOBILITY

Figure 6. Recording spectrophotometric tracing of the first (A) and second (B and C) batches of the pyridine extract antigen. Gels (A) and (B) were stained with Coomassie Blue. Gel (C) was stained with Alcian Blue.


A
TPE
ELECTROPHORETIC MOBILITY


C
TPE
ELECTROPHORETIC MOBILITY


ABSORBANCE (600 nm)
TPE
ELECTROPHORETIC MOBILITY

stained with Alcian Blue. Figures 4b and 5c show the relative mobilities of these components. Bands with a mobility of 0.63, 0.70 and 0.83 probably represent glycoproteins as evidenced by their staining with Alcian Blue and Coomassie Blue. In addition, the DDA contained two components which only stained with the Alcian Blue. A dark staining large diffuse band was noted with an Rf of 0.52. Corresponding components were not seen when the gels were stained for protein.

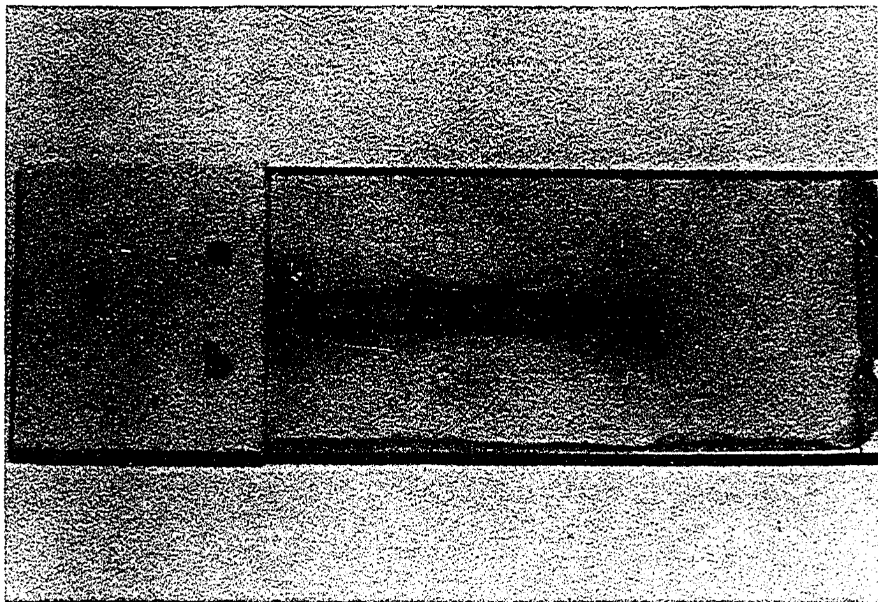
The number of components resolved in the TPE antigen varied depending on the batch of antigen analyzed. A comparison of Figures 3a, 4c, and 6 reveals at least four additional bands at Rf values of 0.34, 0.89, 0.91, and 0.94 which are present in the second preparation, but not in the first. The first TPE preparation contained a protein component (Rf 0.66) which was not present in the second antigen preparation. The presence of three glycoprotein components was revealed by Alcian Blue staining as seen in Figures 4d and 6c.

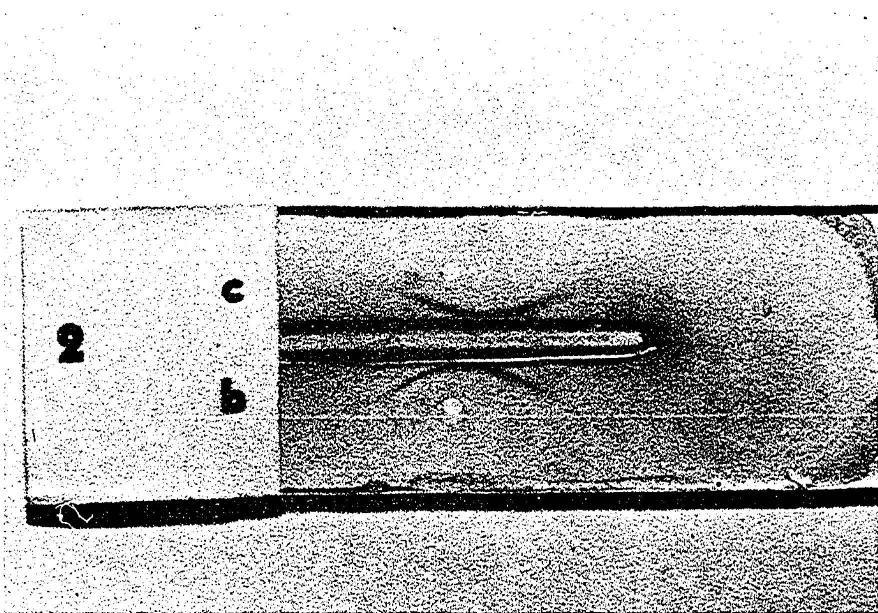
Comparison of the TPE and DDA antigens indicates that many protein components may be shared. However, it is readily apparent that the two antigen preparations do not share similar glycoprotein components.

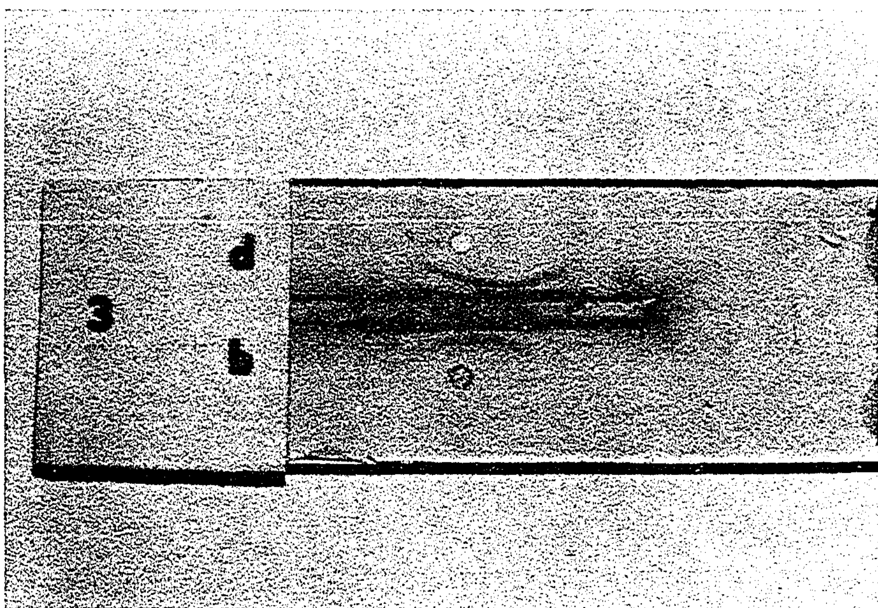
Fractionation of TPE and DDA antigens

The results of immunoelectrophoresis of the fractions obtained from DDA and TPE are given in Figure 7. An active fraction from both the TPE and DDA antigens was obtained in gel slice nine which corresponded to the last centimeter of

Figure 7. Immunoelectrophoresis of the crude double dialysis antigen (2c), crude pyridine extract antigen (1a), fraction 9 from the pyridine extract antigen (1b, 2b, 3b), and fraction 9 from the double dialysis antigen (3d). The trough was filled with rabbit antisera raised to _T. candidus_ spores. The anode is to the right of the figure.


a
b


2
c
b


3
d
b

the gel. Fraction 9 from the DDA antigen contained four protein bands and 1 glycoprotein component while the TPE antigen contained five protein bands. It is also apparent from Figure 7 that the reactive fraction obtained from the DDA and TPE antigens are not the same (compare 3d and 3b).

CHAPTER VIII

DISCUSSION

Definitive diagnosis of hypersensitivity pneumonitis is contingent upon the demonstration of the sensitizing agent in the environment, the presence of precipitating antibody to that agent, a clinical picture consistent with the disease and ultimately the results of insufflation studies. Immunodiffusion studies for the presence of precipitating antibody may lead to misleading or confusing results. It has been demonstrated that patients with disease can often show negative results in immunodiffusion studies (20) and that asymptomatic individuals may give positive tests (4, 7, 12, 23). Furthermore, cross reactivity between the thermophilic actinomycetes has been demonstrated (4, 7, 12, 23) which can lead to confusion about the identity of the offending organism. For this reason, many studies have been undertaken to isolate and analyze various components of the thermophilic actinomycetes. Most of these studies have centered on the antigens of *M. faeni* (11, 15, 21, 29).

The paucity of information on the chemical and immunological reactivity of antigens obtained from *T. candidus*

led to a comparative study of two antigen extracts from this organism. Roberts *et al.* (24) reported that the double dialysis antigen produced from *T. candidus* displayed 10-14 bands on polyacrylamide gel electrophoresis. Our results are similar in that we obtained 11-16 bands. In addition, we have demonstrated the presence of five glycoprotein bands.

Roberts *et al.* (24) also demonstrated with *M. faeni* antigens that variation in the number and intensity of bands occurred from batch to batch. Although the data were not given, it was stated that the same variation occurred for *T. candidus*. Our results have confirmed this observation.

It is interesting that our DDA protein values obtained by the Lowry method appeared high considering the concentration loaded onto the gels (1 mg). However, when the antigen was diluted 1:3 and 1:10 all but 2-3 of the bands disappeared. Roberts *et al.* (24) also noted the same phenomenon using the double dialysis antigen of *M. faeni*. They suggested that this is due to media components since uninoculated flasks treated in the same manner as the double dialysis antigen gave high protein values. On polyacrylamide gel electrophoresis this preparation gave a high degree of background staining.

Chemical and immunological analysis of TPE antigens from *T. candidus* have not appeared in the literature. Our results indicate that variation also occurs from batch to batch with the number of protein bands varying from 8-11.

The presence of 3 glycoprotein components also was demonstrated. These glycoproteins are different than those obtained from the double dialysis antigen.

Crossed immunoelectrophoresis studies revealed that the two antigen extracts did not contain the same immunogenic components when antisera to whole cells was used. The experiments described here, however, cannot discern whether the numerous bands resolved by polyacrylamide gel electrophoresis represent the same or cross reacting antigens. Antiserum raised in rabbits to the TPE and DDA antigens is now being tested by crossed immunoelectrophoresis to determine if the antigens contain similar immunogens.

Fractionation studies on polyacrylamide gels yielded one fraction from each of the antigen extracts that showed immunological reactivity. The reactive component obtained from the TPE antigen is probably protein in nature as no band reactive with Alcian Blue was noted in that region of the gel. It is not certain which of the five protein bands contained the antigen reactivity. The active fraction from the DDA antigen yielded two bands with the same mobility on immunoelectrophoresis. As both protein and glycoprotein bands were noted in this fraction, the exact nature of the reactive components are not known. Further chemical analysis of this fraction is now in progress.

Although immunologically reactive fractions have been isolated from the TPE and DDA antigens, the reactivity

was demonstrated using antisera to spores. These fractions may not be reactive with antisera from exposed individuals. Either antigenic preparation may be most suitable to detect precipitins in symptomatic patients. Extracts from whole organisms (29) and extracellular antigens (11, 15) have been used for this purpose. Antigens which are present in whole organisms were detected in this study. These may represent the major immunogenic stimulus on exposure. Current testing involves fraction 9 and the crude antigens in precipitin tests using sera obtained from guinea pigs exposed to aerosolized *T. candidus*. The use of antigenic fractions from thermophilic actinomycetes may negate the cross reactivity often observed in precipitin tests.

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CROSSED IMMUNOELECTROPHORETIC ANALYSIS OF TWO ANTIGEN EXTRACTS OF *THERMOACTINOMYCES CANDIDUS*

CHAPTER IX

INTRODUCTION

The association between the repeated inhalation of spores and mycelial fragments from thermophilic actinomycetes and the development of hypersensitivity pneumonitis has been well established (2, 4, 6, 14, 19, 20, 25). *Thermoactinomyces candidus* has been isolated from air conditioning units and home humidifier water (2, 6) where its presence has been implicated as the source of several outbreaks of hypersensitivity pneumonitis. *Micropolyspora faeni* and *Thermoactinomyces vulgaris* have been established as etiological agents of farmer's lung (4, 19, 20) while *Thermoactinomyces sacchari* has been shown to be associated with outbreaks of bagassosis (14, 25).

The accurate diagnosis of hypersensitivity pneumonitis induced by the thermophilic actinomycetes depends upon compatible clinical history, results of radiologic and pulmonary function tests, and the presence of precipitating antibodies directed against the offending inhalant. Several problems

are inherent however, when the results of precipitin tests are used in making a proper diagnosis. Healthy individuals may possess precipitating antibodies directed against the thermophilic actinomycetes. Roberts et al. (22) reported that 8.4% of 1,045 serum samples obtained from farmers had precipitating antibody directed against the thermophilic actinomycetes. Chmelik et al. (3) reported a 3% prevalence in 1,077 office workers and, by use of counterimmunoelectrophoresis, Kawai et al. (10) were able to demonstrate an 11.8% incidence of precipitins to several thermophilic actinomycetes. A study by Wenzel et al (30) revealed that 44.2% of patients with positive precipitin tests reacted to more than one thermophilic actinomycete. Positive precipitin tests to more than one thermophilic actinomycete have been reported by other investigators (1, 7, 11). Whether this is due to immunological cross reactivity of the antigens or to exposure to several actinomycetes remains unclear.

Because of the problems associated with precipitin tests, attempts have been made to isolate specific antigens which would not cross react and would serve to differentiate symptomatic from asymptomatic individuals. In this regard, the antigens obtained from M. faeni have been extensively studied (5, 8, 13, 17-19, 21, 23, 28, 29). Antigens obtained from T. candidus have received little attention (9, 24). In a preliminary study, we reported batch to batch variation in protein and carbohydrate content and in the number of components

that could be resolved by polyacrylamide gel electrophoresis (9). The present investigation was undertaken i) to determine the isoelectric points of the antigen components, ii) to determine the number of immunogenic components in the antigenic preparation as revealed by crossed immunoelectrophoresis, iii) to determine the heat and pronase sensitivity of the antigens, and iiii) to standardize a procedure which would allow a qualitative comparison of the reactivities of symptomatic and asymptomatic patients toward antigens derived from *T. candidus*.

CHAPTER X

MATERIALS AND METHODS

Organism

T. candidus was isolated from an industrial air conditioning system. Identification was based upon the biochemical tests outlined by Kurup and Fink (12). Analysis of whole cell hydrolysates by thin layer chromotography for the presence of meso or L-diaminopimelic (DAP) and diagnostic carbohydrates was performed as described by Staneck and Roberts (26). Commercially available DL-DAP (Sigma Chemical Co.) served as the standard. The organism was maintained on tryptic soy agar at 55C and transferred at two week intervals.

Antigen preparation

Initial studies utilized two different antigen preparations. A pyridine extract antigen (TPE) was prepared as previously described (9, 29) and adjusted to a concentration of 1.5 mg/ml protein in sterile saline. Double dialysis antigen (DDA) was prepared according to the procedure of Edwards (5) as modified by Kurup *et al*. (13) and adjusted to a concentration of 37 mg/ml protein. For some experiments the DDA was concentrated 2X in a Minicon^{R} B15 concentrator

(Amicon Corp., Lexington, Mass.).

Total protein was estimated by the method of Lowry *et al*. (16) using bovine serum albumin as the standard.

Production of antiserum

Antiserum to whole spores was raised as previously described (9). Two New Zealand white rabbits were inoculated in the nuchal area at weekly intervals with either the pyridine extract antigen (.1 mg/ml protein) or the DDA (2 mg/ml protein) suspended in Freunds incomplete adjuvant. Precipitins were monitored by immunoelectrophoresis as described previously (9). When the number of precipitin arcs reached their maximum number, the rabbits were bled by cardiac puncture. Serum was collected, portioned, and stored at -20C. For some experiments serum was concentrated 2X in a Minicon^R B15 concentrator (Amicon Corp., Lexington, Mass.).

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) was performed as previously described (9). Antiserum to the pyridine extract antigen or the DDA was diluted 1:10 in agarose for electrophoresis in the second dimension. The DDA was tested for heat lability by immersion in a boiling water bath for 30 min. The antigen was cooled, then subjected to CIE. Sensitivity to enzymatic digestion was assessed by treatment of the DDA with a final concentration of 50 µg/ml Protease (Repurified Type VI, Sigma Chemical Co.) for 1 hr at 37C. The

DDA was adjusted to its original volume in a Minicon^R B15 concentrator (Amicon Corp., Lexington, Mass.) then subjected to CIE.

Crossed immunoelectrophoresis with an intermediate gel was performed as described by Svedsen and Axelson (27). Electrophoretic conditions were as described for CIE. Anti-DDA or antispore serum was incorporated into the intermediate gel or reference gel at a dilution of 1:10. The addition of saline in the intermediate gel served as a control.

A piece of moist filter paper was placed over the gels and dried overnight at ambient temperature. Gels were stained with 0.1% Coomassie Brilliant Blue R250 (Bio-Rad, Richmond, Ca.) for 3-4 hr and destained by repeated rinses in water : ethanol : acetic acid (8:3:1).

Isoelectric focusing

Analytical electrofocusing in thin layers of polyacrylamide gel was performed using an LKB Multiphor apparatus and commercially available pH 3.5-9.5 Ampholine PAG plates (LKB Instruments Inc., Rockville, Md.). The anode solution consisted of 1 N H_3PO_4 and the cathode solution of 1 N NaOH. Gels were prefocused for 45 min at a constant voltage of 1000V. DDA (2X) was applied to the gels by means of a paper wick (approximate volume 10 µl) and electrophoresed for approximately 1.5 hr at a constant voltage of 1000V. Electrophoresis was terminated when a hemoglobin standard had migrated to its characteristic isoelectric point. The pH gradient was

determined by elution of 1 cm^2 blocks of gel in 1 ml double distilled water. Gels were placed in fixing solution (150 ml methanol and 350 ml distilled water, add 17.25 g sulfo-salicylic acid and 57.5 g trichloroacetic acid) overnight then stained with 0.1% Coomassie Brilliant Blue R250 for 6 hr. Destaining was acheived by successive rinses in water : ethanol : acetic acid (8:3:1). Gels were placed in a glycerol preserving solution (50 ml glycerol in 500 ml destaining solution) for 1 hr. The gels were allowed to dry in a dust free environment overnight and sealed with a sheet of plastic.

Crossed immunoelectrophoresis of crude DDA and heat inactivated DDA was also performed by using a "layering on" technique. Gels on glass plates (8 X 10 cm) were cast with a 2.5 cm strip of 1% agarose (Microbiological Associates) in veronals buffer (.01M, pH=7.2). The remainder of the plate was coated with 10 ml of a 1:10 dilution of anti-DDA (2X) also in 1% agarose. After termination of isoelectric focusing, a 1.5 cm strip containing the antigen sample was cut from the polyacrylamide gel. The strip was layered onto the 2.5 cm segment of gel. Electrophoresis in the second dimension was carried out at 4C on an LKB Multiphor apparatus. The buffer chambers were filled with half strength high resolution buffer (.02M, pH 8.8, 5.778 g Tris (hydroxymethyl) aminomethane, 2.446 g barbituric acid, 9.756 g sodium barbitol in 1 liter of water) and attached to the gels by means of a paper wick. Electrophoresis was performed at 4V/cm of gel

overnight. Gels were dried and stained as described above.

CHAPTER XI

RESULTS

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis of the TPE antigen, using antisera raised against the crude pyridine extract, revealed one mobile immunogenic component and one component which did not enter the gel in the first dimension (Figure 1). This result is similar to that obtained when antiserum to whole spores was used (9). For this reason, the TPE antigen was not characterized further.

When the DDA was subjected to crossed immunoelectrophoresis, a total of 15 peaks were obtained (Figure 2a). Figure 2b shows that the immunogenicity of peaks 2, 5, 6, 7, 8, and 12 was resistant to 100C for 30 min. As shown in Figure 2c, the immunogenicity also appeared to be totally resistant to digestion by pronase. The enzyme was active in our system since it inhibited the formation of an agglutination reaction between *Salmonella* H antigen and anti-H-antiserum.

Crossed immunoelectrophoresis with an intermediate gel

A previous study (9) had demonstrated 3 immunogenic components for the DDA using antispore serum. It was of

Figure 1. Crossed immunoelectrophoretic profile of the pyridine extract antigen of *T. candidus* using antiserum raised against the crude antigen. The anode appears to the right and top of this and the following figures.

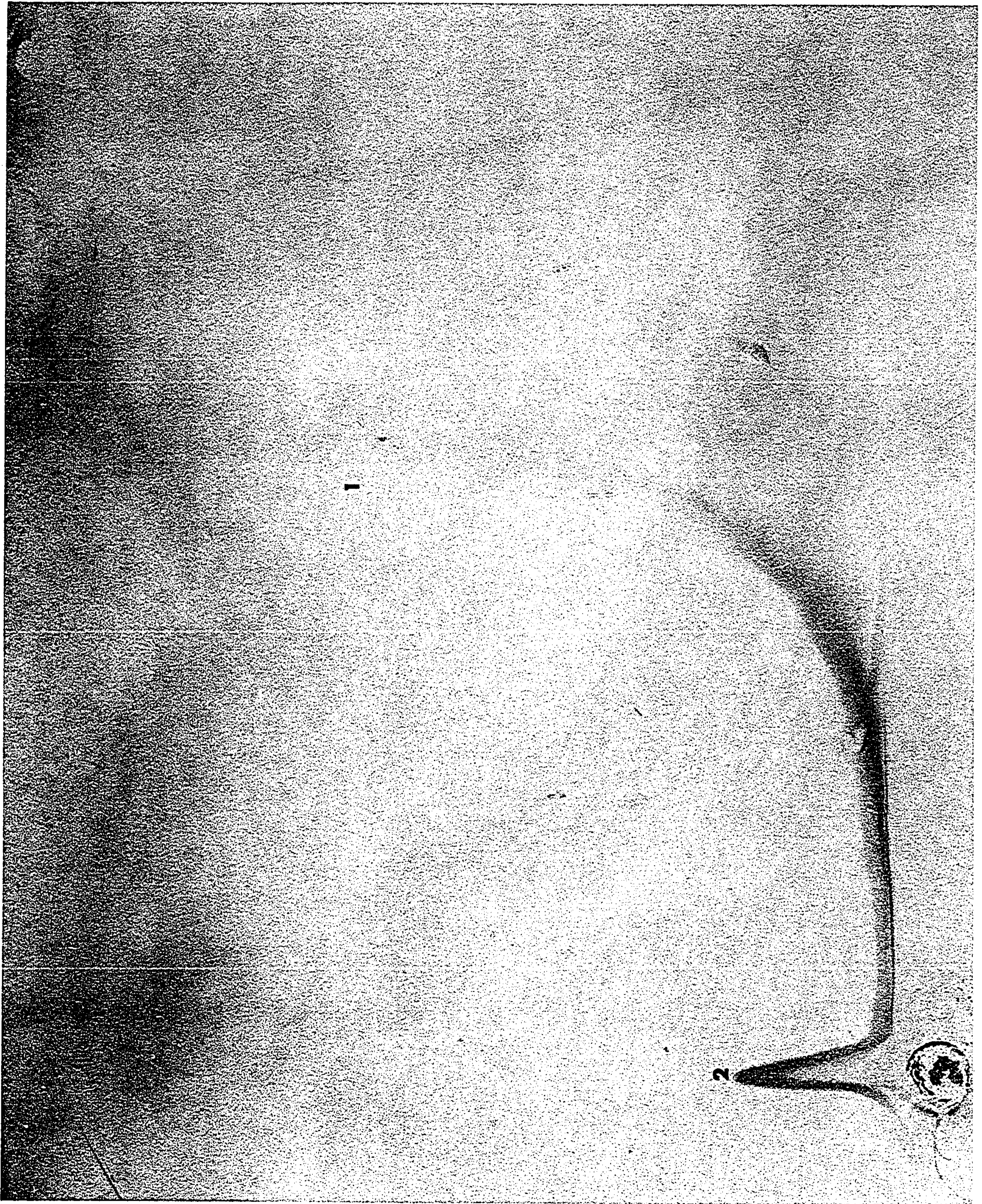
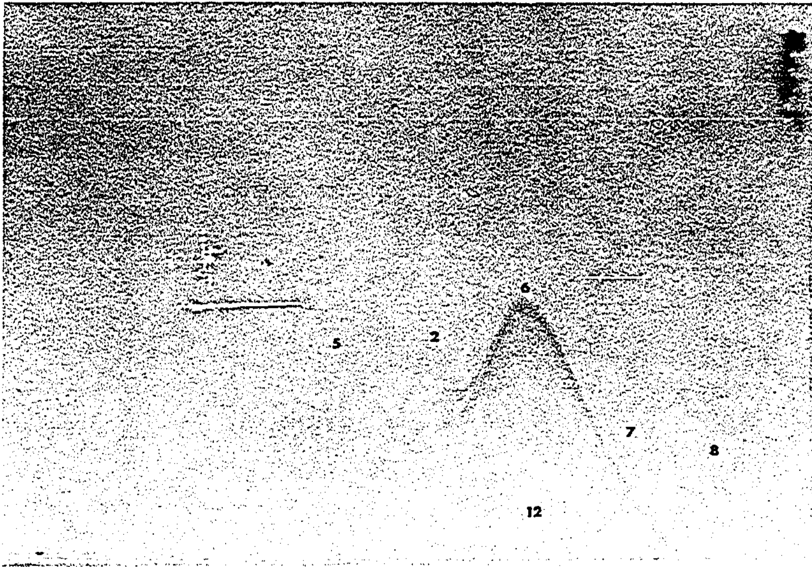
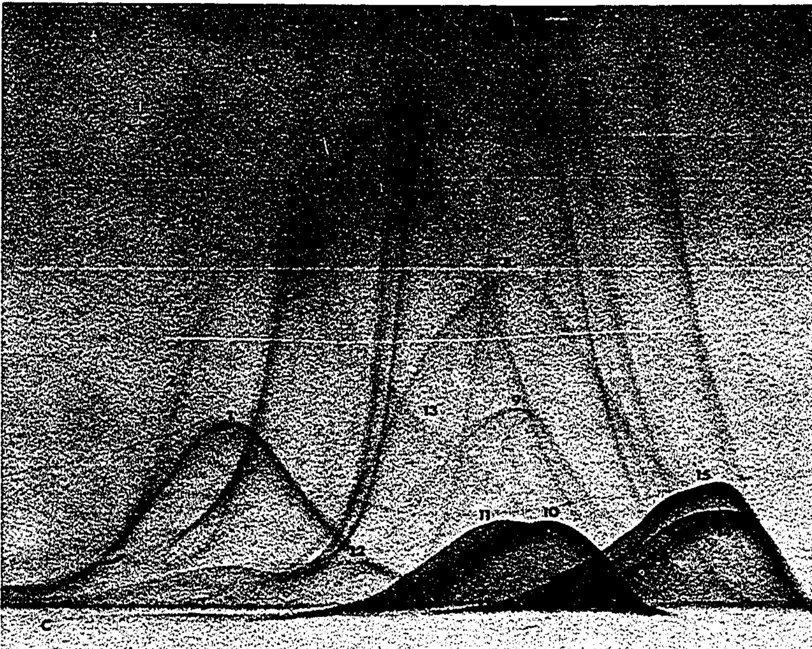


Figure 2. Crossed immunoelectrophoretic profile of A) the double dialysis antigen (DDA) from *T. candidus*, B) DDA treated at 100C for 30 min and, C) DDA treated with 50 ug pronase for 1 hr at 37C. The gel contained antibody raised to the crude DDA.



B

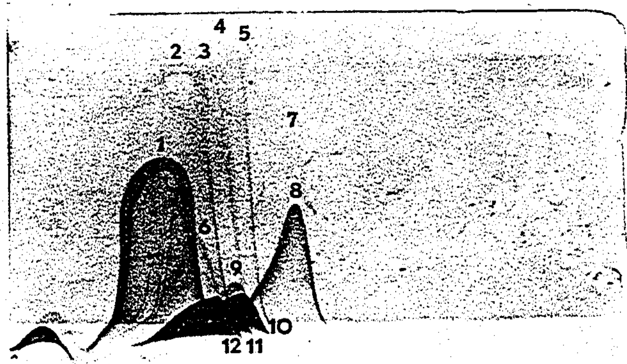


interest to determine if the antispore and the anti-DDA serum were detecting the same immunogens. Analysis by crossed immunoelectrophoresis with an intermediate gel showed that the two antisera were detecting 4 of the same components (Figure 3). This is evident by the absence of peaks 7, 9, and 12 in Figure 3b and a line of identity with peak 1a in the antispore antibody containing gel. Two immunogens, A and B, were detected only by the antispore serum. As shown in Figure 3c, most of the peaks do not have heads which also indicates that the antispore serum did not have antibody to these components. When saline was placed in the intermediate gel and anti-DDA serum in the reference gel, 15 peaks were obtained (Figure 3a). Only 2 peaks were obtained with the antispore antiserum in the reference gel (data not shown).

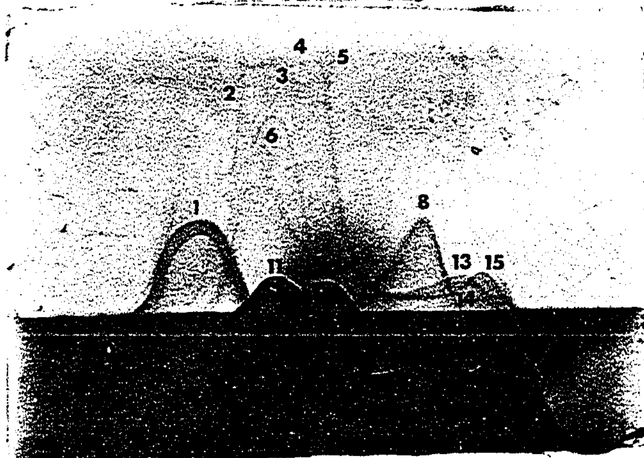
Isoelectric focusing of DDA

The results of isoelectric focusing studies are summarized in Table 1. Using a pH gradient of 3.5-9.5, a total of 19 components were resolved. When a pH gradient of 4.0-6.0 was used, only 12 components were found. All of the antigens banded in the acidic range with the majority of components migrating to a pH range of 4.5-5.8. Crossed immunoelectrophoresis using a "layering on" technique (Figure 4) yielded at least 16 immunogenic components. Table 1 also shows the pH range of the 6 heat stable immunogens. It can be seen that of the 10 components within the 5.2-5.8 range, only one was stable to heat. This is in contrast to those

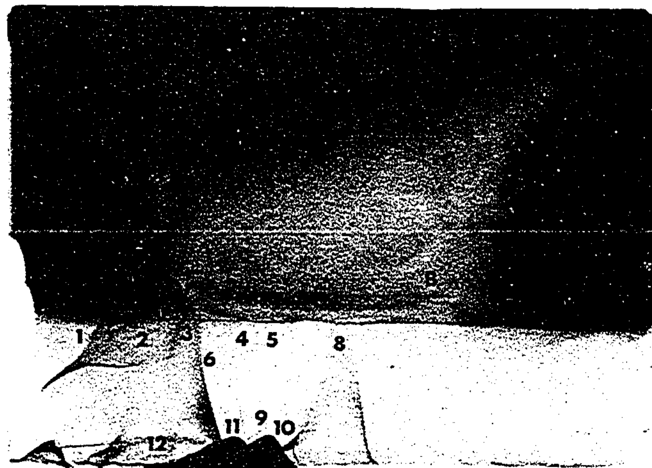
Figure 3. Crossed immunoelectrophoresis with an intermediate gel of A) saline, B) antispore antigen, and C) anti-DDA serum. The reference gel contained anti-DDA antisera in A and B while C contained antispore serum.



A



B



C

TABLE 1

Isoelectric Focusing of Crude DDA[a]

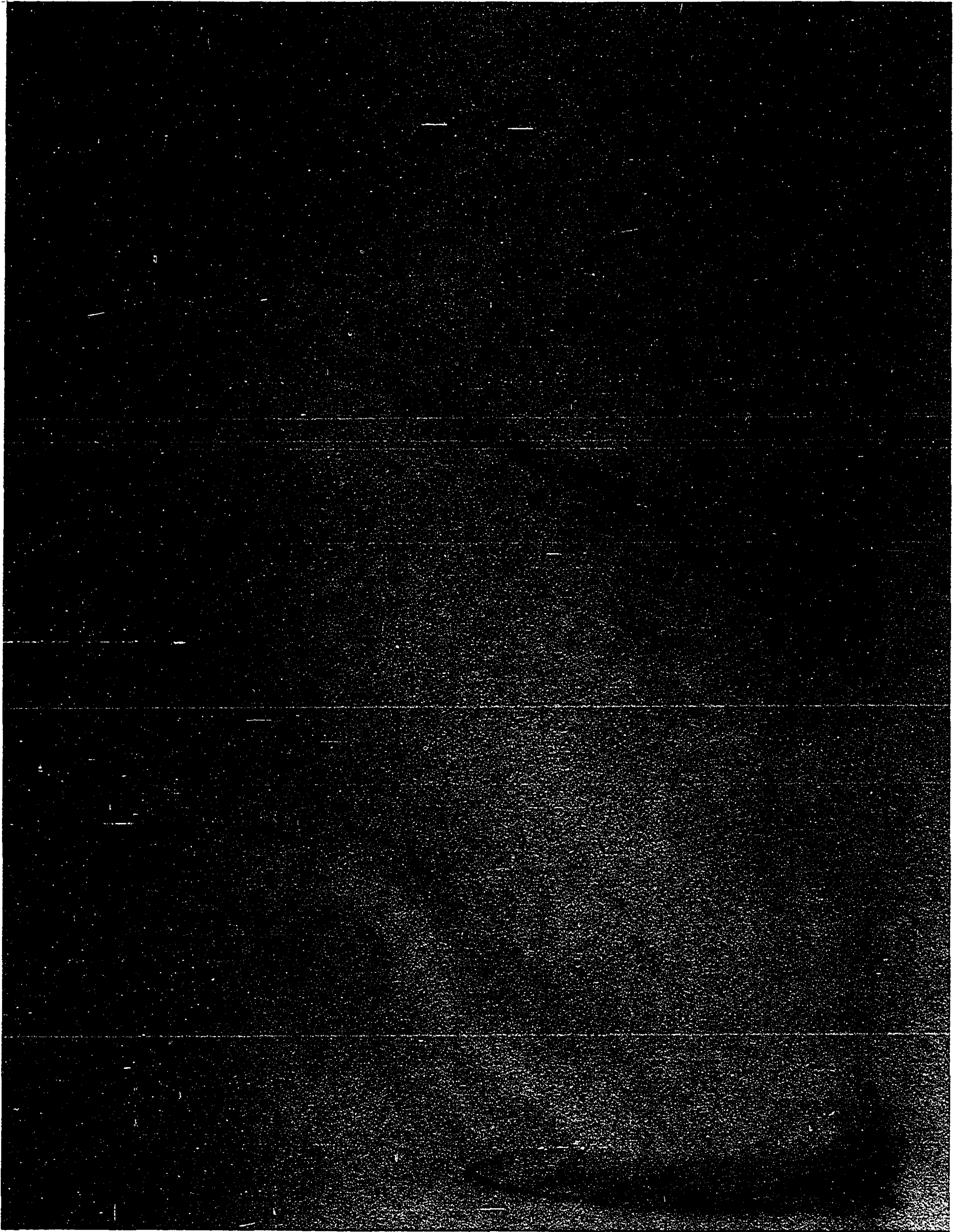
and Heat Inactivated DDA

	pH			
	6.4-5.8	5.8-5.2	5.2-4.5	4.5-3.6
number of components	1	10	7	1
number of heat stable immunogenic components[b]	1	1	4	0

a) DDA - double dialysis antigen

b) determined by crossed immunoelectrophoresis

Figure 4. Crossed immunoelectrophoretic profile of the DDA using a "layering on" technique. Isoelectric focusing was carried out in the first dimension on a pH 3.5-9.5 flatbed polyacrylamide gel. The gel was then sliced and layered onto agarose, which contained antibody directed against the DDA, and electrophoresed.



antigens migrating to a range of 4.5-5.2 where 4 of 7 components were heat stable. The component migrating to a pH range of 3.6-4.5 was heat labile while the component appearing in the range 5.8-6.4 was heat resistant.

CHAPTER XII

DISCUSSION

Many antigens from M. faeni have been isolated and partially characterized (5, 8, 13, 17, 19, 21, 23, 28, 29). The double dialysis antigen, however, has been reported to be more efficient in precipitin tests than most other preparations (5). This has led several investigators (13, 17, 18, 24) to initiate studies characterizing this antigenic preparation. There is a paucity of information regarding the double dialysis antigens obtained from T. candidus and T. sacchari (9, 15, 24). In this report and a previous study (9), we have initiated an electrophoretic and physiochemical analysis of the double dialysis antigen obtained from T. candidus.

The double dialysis antigen of M. faeni is complex. Polyacrylamide gel electrophoresis has shown 21-23 bands (24) while crossed immunoelectrophoresis has demonstrated 24 precipitin arcs (13). A study by Walbaum et al. (28) has demonstrated 75 bands by crossed immunoelectrophoresis. The double dialysis antigen of T. candidus appears to be somewhat less complex, in that 11-16 bands (9) and 10-14 bands (24) have been demonstrated by polyacrylamide gel electrophoresis.

Crossed immunoelectrophoretic studies reported here have shown 15 immunogenic components. The number of resolvable components can be increased by use of isoelectric focusing on flatbed polyacrylamide gels. Using this technique, a total of 19 bands were obtained of which 16 were found to be immunogenic. All of the antigens were acidic in nature with the pI's ranging from 3.5-5.7. Little information is available on the DDA of T. sacchari (15, 24). Roberts et al. (24) have shown 6 components by PAGE; two dimensional immunoelectrophoresis data are lacking.

Treatment of the DDA for 30 min at 100C removed 9 of the 15 precipitin arcs formed by crossed immunoelectrophoresis. Since it has been shown that the DDA contains at least 5 glycoproteins (9), it is possible that these heat resistant components are glycoprotein in nature. It has been reported by Lehrer and Salvaggio (15) that almost all the components of the DDA from T. sacchari are heat sensitive. Interestingly, pronase did not destroy any of the immunologically reactive components of the DDA. This is a curious finding because at least 9 of the components appear to be protein. It has been demonstrated, however, that the DDA of M. faeni, T. candidus, and T. vulgaris contain serine proteases (24) and, therefore, these enzymes could have degraded the pronase before digestion of any of the DDA components could occur. Alternatively, those proteases present in the antigenic preparation may have destroyed the proteins which otherwise would have been

susceptible. That the pronase was active in our system was indicated by its ability to inhibit an agglutination reaction between Salmonella H antigen and H antiserum. Our results are in contrast to those obtained by Lehrer and Salvaggio (15) for the DDA of T. sacchari which was partially sensitive to pronase. However, Roberts et al. (24) were unable to demonstrate any serine protease activity in the DDA obtained from T. sacchari.

Characterization of the TPE antigen from T. candidus has only recently been reported (9). Crossed immunoelectrophoretic analysis has shown that this antigenic preparation contains one mobile immunogenic component when antispore (9) or anti-TPE antiserum was used. The DDA contains the same immunogenic component since electrophoresis of the TPE antigen into a gel containing anti-DDA antiserum yielded 1 precipitin peak (unpublished observations). The immunogenic component of the TPE antigen has been isolated from fractionated polyacrylamide gels (9) and appears to be protein in nature.

It is difficult to decide which antiserum (against whole spores or the crude DDA) should be used in crossed immunoelectrophoretic studies when the goal is to characterize antigens which react with symptomatic patients sera. The disease is induced by the inhalation of spores and mycelial fragments. One would expect the primary humoral response to be to the spores. However, evidence has been presented that

symptomatic patients also react to metabolic antigens (17, 18). Another problem arises when cross reactivity between the thermophilic actinomycetes is encountered. This cross reactivity could be manifest by cross reacting antigens contained in the spores or in the double dialysis antigen itself. A comparison between the thermophilic actinomycetes would answer this question. Our data indicate that the anti-spore and anti-DDA antiserum detect 4 of the same components in the DDA. The antispore antiserum is able to detect 2 components (A and B) which the anti-DDA antiserum can not. The two components, however, are contained in the DDA. This is probably due to the lysis of the organism during the preparation of the DDA. The DDA, therefore, contains all of the immunogenic components which can be resolved using these two antisera.

The use of crossed immunoelectrophoresis and the "layering on" technique will be of value in comparing the patterns obtained with serum from symptomatic and asymptomatic patients. If a qualitative difference exists between these two patient populations, then the use of some of the physio-chemical properties of the DDA described in this report may be valuable in isolating a specific disease related antigen.

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PERIPHERAL AND ALVEOLAR RESPONSE IN GUINEA PIGS TO AN AEROSOL EXPOSURE OF *THERMOACTINOMYCES CANDIDUS* SPORES

CHAPTER XIII

INTRODUCTION

Hypersensitivity pneumonitis is a disabling pulmonary disease (interstitial pneumonia) induced by the repeated inhalation of mycelial fragments and spores from several species of thermophilic actinomycetes and fungi. Many small molecular weight inorganic compounds are also capable of inducing a pneumoconiosis (4, 5, 41). Since the symptoms and histopathology of the induced state are similar, regardless of the sensitizing agent, a common mechanism for disease production has been suggested. A specific infection is not involved, but cellular immunity does play a role in the pathogenesis of laboratory induced hypersensitivity pneumonitis (34). Many experimental animal models have been developed to study, in particular, these immunological mechanisms (reviewed in 27, 32, 33, 34, 37, 45). It is difficult to interpret and compare the results from these studies; the nature of the antigen used and the mode of exposure has resulted in seemingly conflicting

data. Many studies have used soluble antigens (2, 19, 31, 46-49) whereas a particulate antigen would more closely resemble the natural exposure situation. Bovine serum albumin (11, 50) and horseradish peroxidase (22) have been used to induce a hypersensitivity pneumonitis but are not generally described as etiologic agents of this disease process. Finally, many studies use an unnatural route of sensitization such as the parenteral injection of Freunds complete adjuvant containing the organism followed by aerosol exposure or intratracheal injection of spores (2, 17, 23-25, 36, 43, 47, 49, 51). In this regard, Brain _et al_. (3) have demonstrated that there is a significant difference in particle distribution depending upon intratracheal administration of the antigen or aerosol inhalation.

The pathogenesis of the disease has been ascribed to each of the four major types of immune injury (Type I-IgE anaphylaxis, Type II-lymphocyte cytotoxicity, Type III-immune complex disease and Type IV-classical delayed type hypersensitivity). This study evaluates the contribution of humoral and/or cellular immunity to the progression of hypersensitivity pneumonitis using a natural mode of exposure in the guinea pig. Clearance of the organism from the lung was assessed by cultural recovery.

CHAPTER XIV

MATERIALS AND METHODS

Spore preparation

T. candidus was maintained on tryptic soy agar (Difco) slants at 55C and transferred at 2 week intervals. Spore suspensions used for aerosal sensitization were prepared by washing the growth from 72 hr old slants with 5 ml of sterile saline. Growth from 40 slants was pooled, passed through 3 layers of cheese cloth and centrifuged (350 X g, 15 min). The pellet was washed 3 times and resuspended in 120 ml sterile saline. Microscopic examination indicated the suspension contained mainly spores with few mycelial fragments.

Experimental animals

Hartley strain guinea pigs (150-300 g) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). Animals were segregated according to sex and allowed to acclimate 2 weeks before use. Food and water were supplied ad libidum.

Production of aerosol

Groups of guinea pigs were sensitized to an aerosol

of *T. candidus* spores generated by a collision nebulizer attached to a modified Henderson apparatus (18). The nebulizer contributed 8 liters/minute of droplet particles of 10 microns (18) or less to the total air flow of 28 liters/minute through the Henderson apparatus. All animals were exposed a total of 3 times at 2 week intervals. Animals in experiment 1 (Figure 1) and the first two exposures in experiment 2 (Figure 2) were exposed for 15 min. The third exposure group of experiment 2 were subjected to 30 min of aerosolized spores. Control animals were exposed to saline.

Calculation of sensitizing dose

An aliquot from the nebulizer suspension was serially diluted in saline and plated in quadruplicate on TSA before and after exposure of the animals. The number of viable spores was determined after incubation of the plates at 55C for 3 days. Two-minute aerosol samples generated by the nebulizer were collected in Porton all-glass impingers (Ace Glass, Inc., Vineland, New Jersey, #DWG-B-2244) containing 10 ml of infusion broth (37 g Brain Heart Infusion (Difco); gelatin, 2 g; and Na_2HPO_4, 4 g in 1 liter distilled water). The number of viable spores per ml impinger was determined by plate counts as described above. Two or three impingers were used to sample the aerosol before, during, and after all of the animals were exposed. Spray factors were determined as described by Elberg and Henderson (10).

Figure 1. Protocol for the detection of peripheral cell mediated immunity.

EXPERIMENTAL DESIGN

EXPERIMENT 1

170 animals
Primary Exposure

84 animals for culture, including 9 controls

Samples: Lung, liver, kidney, spleen. Plated in duplicate on TSA at 55C. 5 animals/time sampling times at 1, 2, 3, 7, 14 days

↓ 2^0 aerosol on 50 animals

Same tests as above on 25 animals

↓ 3^0 aerosol on 25 animals

Same tests as above on 25 animals

74 animals for skin test (ST) and detection of Macrophage inhibition factor (MIF)

30 ST and 8 control animals. Same animals followed throughout exposure sequence ST read at 4, 16, 24 & 48 hours after test

36 animals for MIF. peritoneal washes at time 0, 2, & 7 days. 12 animals per exposure

12 animals for lymphocyte transformation (LT). Times: 0, 1, 2, 7, 14 days post exposure

↓ 2^0 aerosol

Same animals 10 LT, 2 control Times after primary exposure: 14, 15, 16, 21, 28 days

↓ 3^0 aerosol

Same animals 10 LT, 2 control Times after primary exposure: 28, 29, 30, 35, 42 days

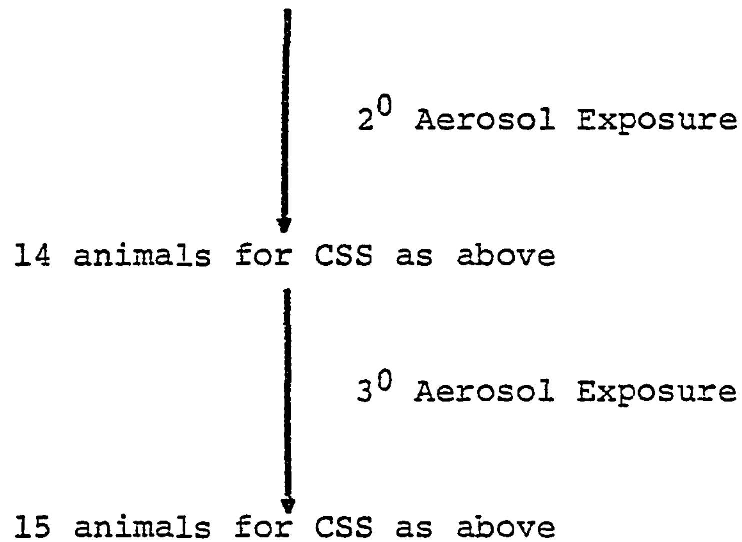
Figure 2. Protocol for the detection of alveolar cell mediated immunity.

EXPERIMENTAL DESIGN

EXPERIMENT 2

41 Animals
Primary Exposure

41 Animals, including 8 controls for cell stimulation studies (CSS). Sampling times at 1, 2, 3, 7, and 14 days post exposure.



The sensitizing dose was computed by the following equation:

(spray factor) (nebulizer concentration cfu/L) (k)

$$\text{where } k = \frac{\text{(exposure time) (respiratory volume)}}{\text{flow factor}}$$

The respiratory volume was taken to be 0.156 liters/minute (14). The flow factor was determined by dividing the total air flow (28 L/min) by the nebulizer air flow (8.4 L/min).

Cultural recovery

Lungs, livers, and spleens were removed from 5 animals at 1, 2, 3, 7, and 14 days after each exposure. Control animals exposed with saline were included at various time intervals throughout the study. Organs were removed, rinsed in sterile saline, and minced into separate grinding vessels which contained 3 ml of sterile saline. The organs were homogenized with a teflon pestle and 0.5 ml plated in duplicate onto tryptic soy agar. The plates were taped to prevent drying and incubated at 55C for 14 days. Plates were recorded as positive or negative.

Assessment of peripheral cell mediated immunity

Skin tests, lymphocyte transformation, and the production of macrophage inhibitory factor (MIF) were used to assess cell mediated immunity in the peripheral system. Figure 1 summarizes the number of animals exposed and the times after exposure the various parameters were measured. Antigens used

in these studies were a double dialysis (DDA) and a pyridine extract (TPE) antigen. The isolation and biochemical characterization of these antigens has been presented elsewhere (7, 20, 26, 44).

Skin tests were performed by intradermal injection of 100, 50, or 10 μg (final concentrations in 0.1 ml) DDA or TPE into the shaved flanks of guinea pigs. The antigens were alternated on the right or left side when skin tests were repeated. Erythema and induration were recorded 24 and 48 h later.

Lymphocyte transformation (LT) studies were performed exactly as described by Hall _et al_. (15). Phytohemagglutinin-M (Difco) at dilutions of 1:100, 1:500, and 1:1000 was used as a non-specific mitogen. DDA and TPE antigens were used at final concentrations of 100, 50, and 10 μg per ml. Results were calculated as the ratio of counts per minute of antigen-stimulated cultures to those of nonstimulated cultures (blastogenic index) and expressed as fold increase over control animals.

MIF production was assayed using the agarose-droplet method of Harrington and Statsny (16) as modified by Deighton _et al_. (6). Antigen concentrations were the same as those used in the LT studies. The ratio of the distance of macrophage migration in the presence of antigen to that of controls was used to calculate the percentage of migration inhibition.

Alveolar cell stimulation

The number of animals and the times after exposure cell mediated immune responses induced in the lung was monitored is summarized in Figure 2. At the indicated times after exposure animals were sacrificed by injection of 1-2 ml of Nembutol sodium-50 mg/ml (Abbott Laboratories) intraperitoneally. The animals were necropsied and the lungs removed en bloc, rinsed with sterile saline, then lavaged with calcium and magnesium free Hanks balanced salt solution (Gibco). Lavages were performed with a sterile 5 ml syringe to which a plastic canula had been attached. In most cases each lobe of the lung was lavaged at least two times (total volume approximately 50 ml) and the lavages pooled. Cells were centrifuged at 350 X g for 15 min and washed twice in TC 199 (Gibco) supplemented with L-glutamine, penicillin (100 μ/ml), and streptomycin (100 μg/ml). The resulting cell pellet was resuspended in TC 199 and adjusted by hemocytometer counts to a cell concentration of 2 X 10^6 cells/ml; cell viability, as assessed by Trypan Blue exclusion, was consistently greater than 90%. No attempt was made to differentiate macrophages from lymphocytes.

Preliminary experiments established as optimum a five day culture system with PHA-M added 72 hr after initiation of culture. Therefore, cell stimulation studies were performed exactly as described for the lymphocyte transformation assay. PHA-M was used at a final dilution of 1:10. The double dialysis antigen was used at 100, 50, and 10 μg/ml. Results were

calculated as a blastogenic index calculated as the ratio of counts in antigen stimulated cultures to counts in control cultures and expressed as fold increase over control animals.

Statistical analysis

A one-way analysis of variance was used to analyze the cell stimulation responses. Student's t test for unpaired data was used to determine the level of significance ($P < 0.05$).

CHAPTER XV

RESULTS

Sensitizing dose

Table 1 summarizes the sensitizing dose each group of animals received at the three exposure times. In both experiments the sensitizing dose increased with each exposure. These values represent the minimum number of spores each group received since only viable colony forming units were used in the calculation of the sensitizing dose.

Cultural recovery

T. candidus was isolated from lung homogenates at each sampling time. Cultures of spleen and liver were uniformly negative except in one instance where one colony was recovered from a liver homogenate. In Table 2 is shown the percent of lung homogenates which were positive following each exposure. After the first exposure, the percent of positive lungs decreased from 100 at days 1 and 2 to 20 percent at 14 days post exposure. Recovery of organisms following the second exposure displayed a different trend. At 1 and 2 days post exposure 40 and 80 percent of the lungs were positive. Lungs cultured at 3 and 7 days, however, were all positive. Fourteen

TABLE 1

Sensitizing Dose of Thermoactinomyces candidus Spores Given at Each Exposure Time

Experiment	EXPOSURE 1	2	3
1	3600[a]	6000	46000
2	490	7700	10000

a) Expressed as colony forming units/animal

TABLE 2

Percent of Lungs Culturally Positive After Aerosol Exposure to *Thermoactinomyces candidus* Spores

Exposure	% Positive Lungs				
	Days Post Exposure				
	1	2	3	7	14
1	100	100	80	20	20
2	40	80	100	100	20
3	60	80	40	40	80

days post exposure the percent positive decreased to 20. In no instances were 100 percent of the lungs positive after the third exposure. The values ranged from 40 percent positive at 3 and 7 days to 80 percent positive at 2 and 14 days post exposure.

Analysis of peripheral CMI

Lymphocyte transformation studies using peripheral cells were negative (results not shown). Blastogenic indices obtained for animals exposed to spores did not differ significantly from control animals which had received a saline aerosol. PHA responses for control animals averaged 5.1 for the three exposure times. The PHA blastogenic indices for the experimental animals ranged from 6.4 after the first exposure to 1.3 and 1.8 after the second and third exposures.

Skin tests using the DDA and pyridine extract antigen were uniformly negative. Several animals gave areas of erythema 10 mm with both the DDA and TPE antigen after the second and third skin test.

Assessment of CMI using the production of macrophage inhibition factor (MIF) were also unrewarding. MIF activity could not be demonstrated at any of the sampling times.

Alveolar cell stimulation

Figure 3 depicts the fold increase in blastogenic indices of experimental animals over those obtained in control animals. At 1, 2, 3, and 7 days post primary exposure no

Figure 3. Fold increase in blastogenic index of sensitized animals over control animals.

FOLD INCREASE IN BLASTOGENIC INDEX

6
5
4
3
2
1

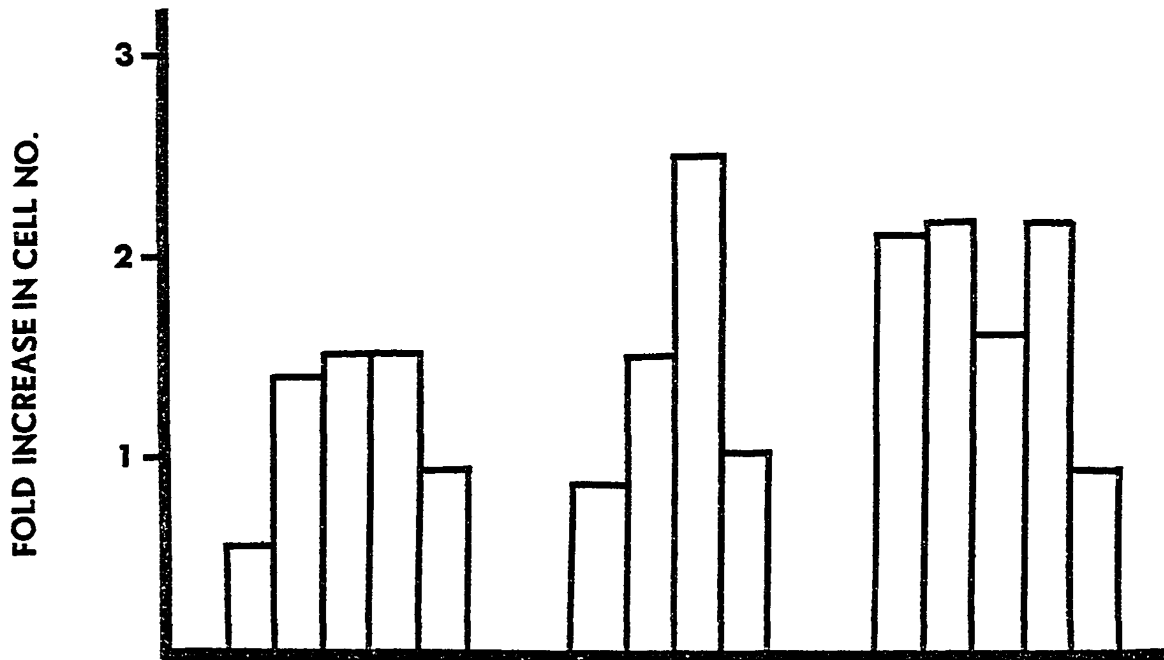
EXPOSURE 1 2 3 PHA

DAYS POST
EXPOSURE 1 2 3 7 14 1 2 3 14 1 2 3 7 14

significant increase in blastogenic indices over control animals was noted. Fourteen days post exposure the fold increase was increased significantly ($P < .05$). The fold increase remained significantly elevated following the second exposure. The blastogenic indices returned to control animal values (fold increase = 1) after the third exposure. The response of the alveolar cells to PHA averaged 5.6 throughout each of the three exposures.

The fold increase in the total number of cells per lavage of experimental animals over control animals is shown in Figure 4. The fold increase appeared to parallel the blastogenic index results in that the maximum fold increase occurred three days post secondary exposure. Unlike the fold increase in blastogenic index, the fold increase in cell number remained elevated at 1, 2, 3, and 7 days post exposure. The cell number returned to control animal values 14 days after sensitization.

Figure 4. Fold increase in total alveolar cells of sensitized animals over control animals.


FOLD INCREASE IN CELL NO.
3
2
1
EXPOSURE 1 2 3
DAYS POST EXPOSURE
1 2 3 7 14
1 2 3 14
1 2 3 7 14

CHAPTER XVI

DISCUSSION

Results obtained from animal studies have implicated all four types of immune injury in the pathogenesis of hypersensitivity pneumonitis. The majority of studies point to a mechanism of immune injury attributable to a Type III, Type IV, or a combination of III and IV reaction. The evidence for and against each type of immune injury has been amply reviewed (27, 32-34, 37, 45). The results of animal and human studies have led to a hypothesis concerning the pathogenesis of the disease. Schorlemmer *et al*. (39) and Salvaggio and Karr (35) have proposed similar mechanisms of pathogenesis. Initial response to the offending antigen is the activation of complement. Many studies have demonstrated the ability of spores and antigen extracts to activate the alternative and/or classical complement pathways (1, 8, 9, 28, 29, 39, 42). Liberated chemotactically active complement cleavage products (C3a, C5a, $\overline{C567}$) induce migration of polymorphonuclear leukocytes (PMN) to the area. An increase of this cell type in the lung with a concomitant decrease in the peripheral circulating PMN's is characteristic of the acute disease (13).

Additionally, particles ingested by the alveolar macrophages activate these cells to secrete hydrolytic enzymes (39). These enzymes can cleave C3 directly or by complex formation with activated factor B (39). (If sufficient antigen is inhaled, there would be a continued production of chemotactic factors. Accumulation of mononuclear phagocytes could induce the granulomatous inflammation as seen in hypersensitivity pneumonitis patients.) The inhaled antigen would also stimulate B and T lymphocytes. Antibodies formed by the B cells may combine with antigens to generate immune complexes. The ability of immune complexes of varying composition to induce lung damage has been previously shown (38). This pathogenic mechanism would account for the reports of a Type III etiology. Sensitization of T cells would also participate by secreting lymphokines such as MIF or blastogenic factor(s) which would account for reports of a Type IV etiology. Fibrosis, as seen in the end stage of the disease, might result through a macrophage-mediated influence (39).

Using a natural mode of exposure we have demonstrated the induction of a cell mediated immune response in alveolar cells after exposure to _T. candidus_ spores. Cell mediated immunity, using the production of MIF, lymphocyte transformation, or skin test results, could not be demonstrated in the peripheral system. Such compartmentalization or stimulation of "local" immunity for both cellular and humoral systems has been demonstrated with other organisms (40, reviewed in

21). There may be selection of peripheral system cells which would have been stimulated by our antigen. This is evidenced by the fact that the PHA blastogenic index from peripheral cells went from 6.4 after the first exposure to 1.3 and 1.8 after the second and third exposures. This corresponded to the time of appearance of antigen reactive cells in the alveolar system. As postulated by Schorlemmer et al. (39) the presence of high levels of organisms would provide a continued supply of chemotactic factors to mobilize cells from the peripheral system. In this regard, organisms were recovered from the lungs of sensitized animals throughout the experiment. Since the entire lung was not homogenized and the spore distribution throughout the lung not uniform, the percent positive lungs reported here are probably an underestimate.

Of special interest was the drop in fold increase in blastogenic index to control levels after the third exposure. This decrease occurred in spite of the fact that the total number of cells remained significantly above control cell numbers. Several possibilities could explain this apparent immune "paralysis". T cells in the alveoli could have become tolerant after the third exposure. The high spore dose and the continuous presence of antigen in the lung could induce a state of nonresponsiveness to the double dialysis antigen. The "paralysis" appeared to be antigen specific because the response to PHA was not diminished. Alternatively, the first

two exposures could have stimulated a clone of suppressor cells whose action was manifest only after the third exposure.

The hypothesis for the pathogenesis of hypersensitivity pneumonitis advanced by Schorlemmer et al. (39) and Salvaggio and Karr (35) does not deal with one important aspect of the disease, viz. the occurrence of asymptomatic individuals. It is well recognized that sensitization (as evidenced by the presence of precipitins) does not always lead to disease production. Furthermore, only a small percentage of individuals exposed to the same sensitizing does manifest the disease. Asymptomatic patients can possess precipitating antibody and may give positive lymphocyte transformation reactions (12, 30). Additionally, if the current hypothesis is correct, then one would expect to see a drop in complement levels due to specific or nonspecific activation of the complement levels due to specific or nonspecific activation of the complement cascade. Moore et al. (30), however, found that serum complement activity became depressed after aerosol exposure in asymptomatic patients. Our data may, in part, explain some of these difficulties. The third exposure group may represent the situation seen in asymptomatic individuals. If immune "paralysis" did occur one would not expect to see the Type III or Type IV reaction characteristic of the histopathological response. Histopathologic sections of apical lobes from guinea pigs in the third exposure group revealed no histopathologic change. Immune "paralysis" could also explain the

depressed complement levels in asymptomatic patients. Activated macrophages, seen in symptomatic disease (43), would not be available to clear the inhaled antigen. Thus the antigen could interact with complement components.

In conclusion, we have demonstrated that aerosolized *T. candidus* spores remains present in the lungs of guinea pigs at least 14 days post exposure. After two aerosol exposures alveolar cells are capable of responding to a metabolic antigen obtained from *T. candidus*. Stimulation of alveolar cells could not be demonstrated after the third exposure. Whether this immune "paralysis" is due to a state of tolerance or the presence of suppressor cells can not be discerned. In vitro mixed lymphocyte studies should resolve this question.

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SUMMARY

An outbreak of hypersensitivity pneumonitis at an industrial plant was investigated. Samples were taken throughout the plant to determine the resident fungal and actinomycete flora. A total of 15 different genera of fungi were isolated with *Aspergillus*, *Cladosporium*, *Penicillium*, and *Alternaria* species predominating. *Thermoactinomyces candidus* was the only thermophilic actinomycete isolated and was the predominant microorganism in the slime material accumulating on the floors of air conditioning units. The source of the sensitizing material appeared to be the slime material since all symptomatic patients gave positive precipitin tests utilizing an extract of the slime as antigen and the disease disappeared from the plant when the slime was removed from the air conditioning units.

Immunologically reactive fractions were isolated from two antigen extracts of *T. candidus* (*Thermoactinomyces* pyridine extract-TPE and double dialysis antigen-DDA). Activity was demonstrated using immunoelectrophoresis and crossed immunoelectrophoresis against rabbit antisera to *T. candidus* spores. Chemical analysis of DDA revealed 11 to 16 bands on polyacrylamide gel electrophoresis including five glycoprotein bands.

TPE contained 8 to 11 protein bands with 3 glycoprotein components. Both antigens showed variation in sequential preparations.

The DDA and TPE antigens were analyzed by crossed immunoelectrophoresis. In addition, the heat lability, pronase sensitivity, and isoelectric points of the components of the DDA were determined. Using antisera raised against crude TPE, 2 immunogenic components were resolved by crossed immunoelectrophoresis. A similar analysis of DDA using antisera raised against crude DDA revealed 15 immunogens. All but 6 components were heat labile whereas pronase had little effect on the number of resolvable components. Intermediate gel crossed immunoelectrophoresis using antiserum raised to whole spores detected 6 immunogenic components, 4 of which were also detected by the anti-DDA serum. A total of 19 bands were obtained when the DDA was subjected to flatbed isoelectric focusing on polyacrylamide gels. The isoelectric points (pI) for the various components were found to range from 3.5 to 5.7. Crossed immunoelectrophoresis using isoelectric focusing in the first dimension yielded at least 16 immunogenic components. Six components with pI's falling in the range of 4.5-6.4 were found to be resistant to heat.

The development of peripheral and alveolar cell mediated immunity (CMI) in guinea pigs was studied after an aerosol sensitization with T. candidus spores. Skin tests, the production of macrophage inhibition factor, and lymphocyte

transformation studies revealed no development of CMI in the peripheral system. Cell stimulation studies using alveolar cells revealed the development of CMI after the second exposure. However, the reactivity diminished following the third exposure. This occurred in spite of the fact that the total number of cells remained significantly above control animal cell counts.