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Micropolyspora faeni and farmer's lung disease

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SUMMARY

Several methods were used to obtain serologically active materials from cultures of *Micropolyspora faeni*. From the results of immunodiffusion and immunoelectrophoresis tests on these materials it is suggested that preparations for the laboratory diagnosis of farmer's lung disease (FLD) should contain concentrated culture supernatant (CS) and extracts of mycelium obtained by ultrasonic treatment (MU). Although CS and MU have many serological activities in common they also possess activities unique to each.

Extraction of mycelium with trichloracetic acid, boiling water or methanol yielded a product which gave simple patterns in immunodiffusion tests. The products contained little protein but were rich in carbohydrates, particularly arabinose, galactose and glucosamine. A similar material was obtained from a cell-wall preparation by treatment with lysozyme. Antibodies to the serologically active substances in these materials occurred more frequently in sera of patients with FLD than antibodies to any other *M. faeni* antigen.

Attempts to obtain serologically active materials from spores were unsuccessful. Moreover antibodies to *M. faeni* could not be removed from patients' sera by absorption with partially purified spore preparations. It is suggested that the hypersensitivity in FLD arises from exposure to mycelial antigens.

INTRODUCTION

Micropolyspora faeni is a thermophilic actinomycete found in mouldy hay. It is generally believed to be the causative organism of farmer's lung disease (FLD). Antibodies to this organism have been found in sera from most patients with FLD but also in sera from some apparently healthy people and patients with other respiratory diseases (Pepys *et al.* 1963). Antibodies have been found too in cattle suffering from fog-fever (Jenkins & Pepys, 1965).

Despite these limitations clinical diagnosis of FLD is often supported by the detection of humoral antibody to *M. faeni* using immunodiffusion tests. However no general agreement exists on the best method of detecting antibody or, more

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important, on the best method of extraction of antigens from cultures of the organism.

Early work was done with antigens extracted from defatted mouldy hay by 0·5% phenol-saline (Pepys *et al.* 1963) or by 10% trichloracetic acid (Kobayashi, Stahmann, Rankin & Dickie, 1964). In later work cultures of *M. faeni* on solid media were extracted with 10% trichloracetic acid (LaBerge & Stahmann, 1966) and treated by freezing and thawing (Pepys & Jenkins, 1965). From liquid cultures of *M. faeni* antigens have been obtained by concentration of culture supernatant (Jameson, 1968; Walbaum, Biguet & Tran Van Ky, 1969; Fletcher, Rondle & Murray, 1970) or by freezing and thawing cells. It is perhaps unfortunate that different strains of *M. faeni* were used by different workers so that comparison of results is difficult.

We have attempted to isolate different fractions of *M. faeni* using several extraction procedures and to determine the gross chemical composition and cytological origin of the antigens present in these fractions. We hoped to determine the best method of extraction of antigens and to identify the subcellular fraction which contains the antigens to which antibodies are most frequently detected.

MATERIALS AND METHODS

Growth of organisms

M. faeni, strain 1156, was grown in continuous culture using the conditions described by Fletcher *et al.* 1970. Culture from the fermentation vessel was centrifuged for 10 min. at 3,000 rev./min. The cell mass was washed twice with 0·9% (w/v) saline and dried from the frozen state. The supernatant was filtered through sintered glass and processed immediately.

Antigens from culture supernatant

Culture supernatant (CS) antigens were concentrated by dialysis against 40% polyethylene glycol (Carbowax 3000, Union Carbide Ltd.). Clarified supernatant was dialysed for 16 hr. against this solution and then for 36 hr. against 0·02 M phosphate buffer, pH 7·2. The procedure was repeated three times and the resulting concentrate (*approx.* 1/10 of original volume) was stored at 4° C. or dried from the frozen state.

In a few experiments CS antigens were prepared from other strains of *M. faeni*. These were strains 5280, 7760 and 9535 isolated from patients' sputum by Dr Moore, Public Health Laboratory, Exeter.

Attempts were made also to precipitate the antigens in culture supernatant using 2 vol. acetone at 4° C. (CA) and to absorb the antigens onto DEAE-Sephadex (Pharmacia Ltd., Uppsala, Sweden). Absorption was done with ion-exchanger equilibrated with 0·02 M phosphate buffer, pH 7·0, and elution was attempted with 2 M-NaCl. Dried eluted material was labelled CD.

Antigens from mycelium

Disruptive procedures

Suspensions of washed mycelium (250 mg./ml.) in 0·1 M phosphate buffer, pH 7·0 were treated at 4° C. for 2 min. at 17·5 kc/sec. using an MSE-Mullard ultrasonic disintegrator. The supernatant was recovered by centrifugation and the precipitate resuspended in buffer and treated again. The second supernatant was recovered, combined with the first and the pooled material dialysed to equilibrium against 0·02 M phosphate buffer, pH 7·0 and dried from the frozen state. This material was called MU.

Similar suspensions of organisms were treated five times by freezing to −25° C., forcing through an X-press (AB-Biox, Sweden; 1 mm. orifice) and warming to 4° C. Supernatants (MX) were recovered by centrifugation and dried from the frozen state.

Extraction procedures

Suspensions of washed mycelium (100 mg./ml.) in 5% (w/v) trichloracetic acid (TCA) were stirred at 4° C. for 24 hr. The deposit from centrifugation for 10 min. at 3000 rev./min. was re-extracted as before. After centrifugation the first and second supernatants were pooled, dialysed to equilibrium against phosphate buffer and dried from the frozen state to give the material MT.

A similar suspension was boiled for 4 hr. to give the extract MB and a suspension of mycelium (100 mg./ml.) in methanol was stirred 24 hr. at 4° C. to give the extract MM.

Enzyme treatment

Washed mycelium was suspended to a concentration of 100 mg./ml. in 0·1 M phosphate buffer, pH 7·0 containing 50 μg./ml. lysozyme. The suspension was incubated at 37° C. for 1 hr., the supernatant obtained by centrifugation, clarified by filtration through a Millipore filter (pore size 0·2 μm.) and dried from the frozen state. The material was labelled ML.

Antigens from cell walls

Cell wall preparation

The deposit from the preparation of MU antigens was subjected to further ultrasonic treatment until no intact mycelium could be seen by microscopical examination. The treated material was centrifuged, the deposit washed twice with phosphate buffer, pH 7·0 and twice with distilled water. Gram-positive *M. faeni* spores present in the preparation were almost entirely removed by centrifugation at 500*g* for 1 min. The final supernatant was dried from the frozen state.

Enzyme treatment

Cell wall preparations were treated with lysozyme as described for mycelium. The soluble material obtained was labelled CWL.

Preparation of spores

Cultures were kept at 55° C. for 2–3 weeks to obtain maximum spore production and lysis of mycelium. Centrifugation at 1000*g* for 10 min. gave a deposit which was suspended in 0·02 M phosphate buffer, pH 7·0 and subjected to 2 min. ultrasonic treatment. Four cycles of low-speed centrifugation and re-suspension in buffer without ultrasonic treatment gave a preparation which appeared microscopically to be essentially Gram-positive spores. The final preparation was suspended in distilled water and dried from the frozen state.

Attempts to obtain soluble extracts of spores by treatment with 10 % (w/v) TCA or lysozyme were unsuccessful and the preparations were used for serum absorption (*vide infra*).

Antisera used

The two sera used contained antibodies to more *M. faeni* antigens than did 50 others tested. Serum H1 was from a patient with FLD. Serum B1 was from a cow with 'fog-fever'. Both sera were originally investigated by Fletcher *et al.* (1970). They were virtually identical in antibody content.

Immunodiffusion tests and immunoelectrophoresis

Immunodiffusion tests were done as described by Fletcher *et al.* (1970). Immunoelectrophoresis was done essentially as described by Scheidegger (1955).

For test CS, MU and MX were used at 25 mg./ml. Other preparations were used at 15 mg./ml.

Chromatography

Extracts were hydrolysed by N-HCl or 0·5 N-H_2SO_4 for 10 hr. or 5 hr. respectively in sealed ampoules at 100° C. Cell walls were hydrolysed by 70 % H_2SO_4 for 16 hr. at 4° C. and, after dilution to 7 % H_2SO_4, for 4 hr. at 100° C. Hydrolysates were neutralized by an anion-exchange resin (De Acidite FF, carbonate form), clarified by centrifugation, evaporated to dryness *in vacuo* and redissolved in distilled water.

Materials were separated by descending chromatography on Whatman No. 4 paper using as solvent ethyl acetate, pyridine, water (2:1:2) as described by Jermyn & Isherwood (1949). Reducing sugars were detected by alkaline silver nitrate (Trevelyan, Proctor & Harrison, 1950) and amino sugars by the method of Partridge (1948).

RESULTS

Immunodiffusion tests on antigen preparations

The antigen preparations available are summarized in Table 1. Line pattern components (lpc) given in immunodiffusion tests by the preparations are shown in Table 2. Numbers given to the various lpc refer to the 'standard system' of Fletcher *et al.* (1970). Certain lpc are grouped together (3/4/5; 6/7; 8/9/10; 11/12; 13/14/15) because it was frequently not possible to differentiate the individual lpc involved. Some lpc (18–22; 27–29) are omitted because they were not regularly detected in these experiments.

Table 1. *Materials obtained from cultures of* M. faeni

Source	Method	Designation
Culture supernatant	Concentration	CS
	Acetone precipitation	CA
	DEAE absorption/elution	CD
Mycelium	Ultrasonic treatment	MU
	X-press degradation	MX
	TCA extraction	MT
	Boiling water extraction	MB
	Methanol extraction	MM
	Lysozyme treatment	ML
Cell wall	Lysozyme treatment	CWL

Table 2. *Immunodiffusion tests on materials obtained from* M. faeni

	Material tested									
Lpc*	CS†	CA	CD	MU	MX	MT	MB	MM	ML	CWL
1	+	+	+	−	−	−	−	−	−	−
2	+	+	+	−	−	−	−	−	−	−
3/4/5	+	+	+	+	+	±	+	±	±	+
6/7	+	+	+	+	+	−	−	−	−	−
8/9/10	+	+	±	+	+	+	+	+	+	+
11/12	+	+	+	+	±	−	−	−	−	−
13/14/15	+	+	+	+	−	−	−	−	−	−
16	+	+	+	+	+	−	−	−	−	−
17	+	+	−	+	±	−	−	−	−	−
23	+	+	+ +	−	−	−	−	−	−	−
24	+	+	+	−	−	−	−	−	−	−
25	+	+	+	−	−	−	−	−	−	−
26	+	+	+	−	−	−	−	−	−	−
30	−	−	−	+	−	−	−	−	−	−

* Lpc, line pattern components in immunodiffusion tests, the numbers as designated by Fletcher *et al.* (1970).

† The abbreviations are explained in Table 1.

It can be seen that CS gave more lpc (21) than any other preparation. The material CA was similar although some of the lpc were more diffuse and difficult to detect. The material CD was less complex than CS and was not considered further as a possible diagnostic reagent. Examination of CS antigens from other strains of *M. faeni* showed minor quantitative but no qualitative differences; this is shown in Pl. 1, fig. 2. Of the mycelial materials, MU was the most complex, giving 16 lpc. It lacked six of the serologically active materials present in CS but gave lpc 30 which was not given by CS. The material MX was less complex than MU and was not considered further. Differences between CS, MU and MX are shown in Pl. 1, fig. 1.

It was concluded that CS and MU were the most complex preparations and that some lpc given by the one were not given by the other and *vice versa*.

Mycelial preparations MT, MB, MM, ML and the cell wall preparation CWL gave essentially similar results. Each preparation contained only some part of the

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Table 3. *Rg values of materials detected in antigen hydrolysates*

Preparation	Rg values				
CA	8	22	65	86	111
MU	4	23	69	87	112
MT	8	—	66	87	113
CWL	9	22	65	86	111
Arabinose	—	—	—	—	111
Galactose	—	—	—	85	—
Glucosamine	—	—	67	—	—

lpc 3/4/5 'complex' and lpc 8/9/10. An immunodiffusion result with ML is shown in Pl. 1, fig. 1 and results with MM, MB and CWL are shown in Pl. 1, fig. 3. The significance of these results is considered in the Discussion.

Absorption of serum with spores

Several attempts were made to obtain soluble, serologically active materials from spore preparations. These were unsuccessful. Attempts were made therefore to absorb antibodies from antisera using purified spore suspensions. In a typical experiment 1 ml. antiserum was absorbed with 50 mg. spores for 4 hr. at room temperature. The result is shown in Pl. 1, fig. 4, where it can be seen that no antibodies were absorbed. Experiments using longer absorption times and different conditions (4° C. and 37° C.) confirmed this finding.

Immunoelectrophoresis

The material CS gave more precipitin arcs with serum H 1 than did any other extract. Using the nomenclature of Pepys *et al.* (1963) 3 of the arcs were in the A region and 4 in the C region. The material CA also gave arcs in the A, B and C regions but only 8 precipitin lines could be counted. Preparations MU and MX gave strong but diffuse arcs of precipitation in the A and C regions only. Preparations MT, MB and MM gave one clear arc in the A region and one or two indistinct, diffuse arcs in the C region. Materials ML and CWL gave diffuse arcs in the C region. Materials ML and CWL gave diffuse arcs in all three regions.

Chromatography

The Rg values of reducing materials found in hydrolysates of CA, MU, MT and CWL are given in Table 3. In all preparations arabinose, galactose and glucosamine were detected. Other substances with Rg values of 25 or less were also found. These were not identified.

DISCUSSION

This work shows that concentrated supernatants (CS) from cultures of *M. faeni* are serologically more complex than antigen preparations made in other ways. However, certain extracts of mycelium (MU) contain at least one serologically active substance (that giving lpc 30) that is not present in CS. For this reason it is

suggested that diagnostic antigens for FLD should be made by pooling preparations of CS and MU. Such preparations would contain not only materials giving all the known lpc of *M. faeni* extracts in immunodiffusion tests but also those materials giving precipitin arcs in the A, B and C regions in immunoelectrophoresis.

The close serological similarity between CS and MU is at variance with the finding of Walbaum *et al.* (1969). These authors detected only three antigens common to extracellular material and extracts of mycelium. However their results were obtained using sera produced in rabbits by inoculation of material from the different sources. They state that the antisera did not contain antibodies to materials migrating in the C region in immunoelectrophoresis, and this may account in part for the discrepancy between the results. Additionally we have found that rabbits respond poorly to inoculation with materials containing serologically active substances common to CS and MU.

Results with MT, MB, MM, ML and CWL were similar. Each material gave some of the lpc 3/4/5 and 8/9/10 in immunodiffusion tests and gave precipitin arcs in the A and C regions in immunoelectrophoresis. The materials ML and CWL also gave precipitin arcs in the B region. All the materials contained arabinose, galactose and glucosamine. They contained 2–6% 'protein' as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). They appear similar in composition and properties to the materials investigated by LaBerge & Stahmann (1966) and were crude fragments of mycelial cell wall. Materials giving lpc 3/4/5 and 8/9/10 in immunodiffusion tests are of interest because the corresponding antibodies are found in 50% and 57% respectively of sera from cases of FLD. This is more frequent than antibody to any other antigen.

The failure to extract serologically active material from spores or to absorb antibodies with spore preparations is of interest. It suggests that spore antigens *per se* might not be responsible for the hypersensitivity reactions associated with FLD. Lacey & Lacey (1964) have calculated that approximately $7{\cdot}5 \times 10^5$ actinomycete spores/min. are deposited in the lungs of men doing light work in a heavily contaminated area such as a hay barn. Despite this, spores have been isolated from lung biopsy material in four cases only (Wenzel, Emanuel, Lawton & Magnin, 1964). It is possible that hypersensitivity arises from mycelial fragments carried to the lung adsorbed on spores or that spores rapidly germinate and the mycelium is eliminated on the lung surface.

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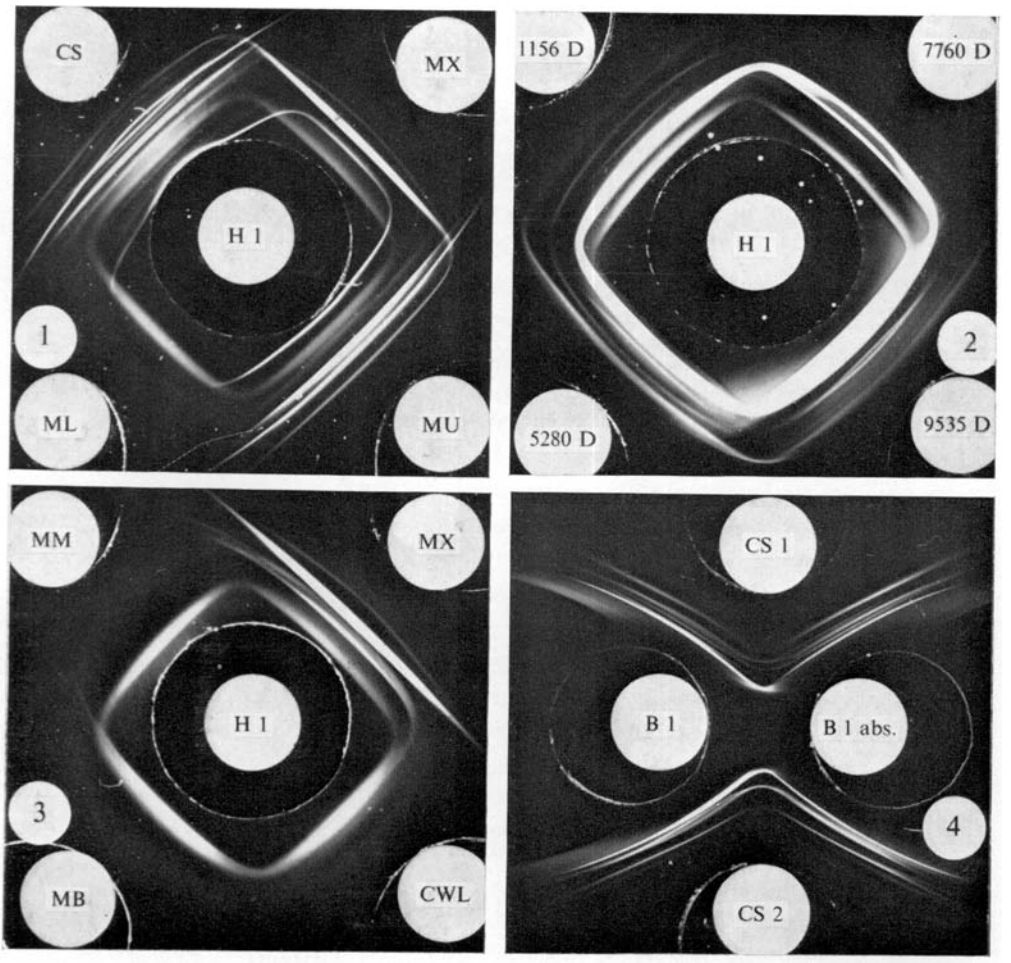
EXPLANATION OF PLATE

Fig. 1. The concentrated culture supernatant, CS, gave a complex pattern when tested against the serum H 1. The mycelial extract MU gave a less complex pattern, but both CS and MU contained more different serologically active materials than the mycelial extract MX. The lysozyme extract of mycelium, ML, gave only two or three lpc against serum H 1.

Fig. 2. This figure shows that ultrasonic extracts of four different strains of *M. faeni* gave essentially the same precipitation pattern when tested against serum H 1.

Fig. 3. The extracts MM, MB and CWL gave less complex patterns than the preparation MX. Materials MB and CWL gave three to four lpc whilst MM gave only two to three lpc.

Fig. 4. Absorption of serum B 1 with a spore suspension failed to remove antibodies to serologically active material present in the concentrated culture supernatants CS 1 and CS 2.



S. M. FLETCHER AND C. J. M. RONDLE (*Facing p.* 192)

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