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Antibody responses in patients with farmer's lung disease to antigens from *Thermoactinomyces vulgaris*

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SUMMARY

A serological analysis of mycelial antigens of *Thermoactinomyces vulgaris* in immunodiffusion with human sera revealed five individual antigens. Three antigens were proteins, sensitive to pronase and soluble in phenol. Two were cationic polysaccharides, sensitive to sodium periodate, and containing glucosamine and muramic acid.

Latex coated with mycelial antigens was compared with precipitin tests in detecting antibodies to *T. vulgaris*; the number of positive results detected by each test differed slightly, and a combination of the two tests detected the highest number. Counterimmunoelectrophoresis (CIE) was shown to be a very sensitive method for detecting precipitins, but not for their measurement. A prospective evaluation of immunodiffusion, latex agglutination and CIE as potential serodiagnostic techniques for farmer's lung disease is suggested.

INTRODUCTION

Farmer's lung disease (FLD) is an allergic alveolitis developing on exposure to mouldy farm produce, particularly hay containing the thermophilic actinomycetes *Micropolyspora faeni* or *Thermoactinomyces vulgaris*. The immunopathological response to *M. faeni* is probably complex, and precipitin-mediated Arthus type III (Pepys, 1969), cytotoxic type II (Wenzel, Emanuel & Gray, 1971; Hollingdale, 1974) and cell mediated (Kawai, Salvaggio & Harris, 1972; Wilkie, Pauli & Gygax, 1973) mechanisms have been suggested. *M. faeni* antigens have been enumerated (Fletcher & Rondle, 1973; Hollingdale, 1974). Surveys have shown that precipitins to *M. faeni* are the most frequent, but those to *T. vulgaris* occur in up to 50% of cases (Pepys & Jenkins, 1965). Wenzel, Emanuel & Lawton (1967) isolated *T. vulgaris* from lung biopsy from a patient with FLD, and precipitins only to *T. vulgaris* were found in the patient's serum. Hughes, Mattimore & Arbesman (1969) described a case of FLD where precipitins only to *T. vulgaris* were shown. *T. vulgaris* has also been isolated from mouldy bagasse and precipitins to it demonstrated in patients with bagassosis (Salvaggio *et al.* 1967).

Little, however, has been published on the antigenic composition of *T. vulgaris*. This study was undertaken to enumerate and chemically characterize antigens of *T. vulgaris*, and to develop sensitive serological tests for the estimation of serum antibodies to these antigens.

MATERIALS AND METHODS

Cultures

The strain of *T. vulgaris* examined was 1150, isolated from mouldy hay by Dr Maureen Lacey, Rothampsted Experimental Research Station. The liquid growth medium contained (per litre distilled water): casein hydrolysate (Hopkin & Williams Ltd), 10·0 g.; yeast extract (Difco Ltd), 5·0 g.; sodium glycerophosphate, 10·0 g.; sodium lactate, 5·0 g.; glucose, 2·0 g.; $MgSO_4.7H_2O$, 0·2 g.; $MnSO_4.4H_2O$, 0·02 g.; $FeSO_4.7H_2O$, 0·02 g. The pH was 7·2. In some batches, 2·0 g. sodium citrate replaced glucose. One volume of a 3-day culture was added to 30 vol. of medium and grown at 42° C. for 24 hr. with vigorous shaking. Cells were harvested at 20,000 ***g*** for 20 min., washed three times in 0·02 M phosphate-buffered saline, pH 7·2 (PBS), and stored at −30° C. until used.

Preparation of antigens

Mycelial (MU) antigen

Washed mycelium was disrupted at 4° C. for 10 min. at 17·5 kc./sec. using a MSE-Mullard Ultrasonifier, when microscopy showed that no intact mycelium was present. The supernatant after centrifugation at 10,000 ***g*** for 20 min. was dialysed against PBS and lyophilized.

Phenol extraction

Mycelium was extracted with 45% (w/w) aqueous phenol at 4° C. for 2 hr. as described by Hollingdale (1974). The aqueous and phenol phases were dialysed against distilled water to remove phenol. The aqueous phase was lyophilized (extract PA). The insoluble material from the phenol phase was also extracted at 37° C. for 30 min. with PBS, centrifuged at 10,000 ***g*** for 5 min. and the supernatant lyophilized (extract PP).

Trichloroacetic acid (TCA) extraction

Mycelium was extracted with 5% (w/w) TCA at 4° C. for 24 hr. and purified with acetone precipitation as described for *M. faeni* by Hollingdale (1974). The extract was dialysed against distilled water and lyophilized (extract TA).

Antigens of M. faeni

Mycelial MU antigen and PA and TA extracts of *M. faeni* were those described by Hollingdale (1974).

Analysis of extracts

Samples of mycelium, MU, phenol and TCA extracts were hydrolysed in 2 N-H_2SO_4 at 100° C. for 3 hr., saturated barium hydroxide added to pH 6·0, the mixture centrifuged, the supernatant lyophilized and resuspended to 0·25 ml. in distilled water. Liberation of amino sugars was performed in 6 N-HCl at 100° C. for 12 hr., hydrolysates dried *in vacuo* and resuspended in 0·25 ml. distilled water. Descending paper chromatography was on Whatman No. 1 paper with ethyl acetate–pyridine–water, 15:5:4 (v/v) as solvent. For the detection of sugars,

alkaline silver nitrate for reducing sugars and 0·2% ninhydrin in acetone for amino sugars, were used.

PA and TA extracts were also examined for heptoses, pentoses, hexoses, and methyl pentoses using cystein-H_2SO_4 (Kabat & Mayer, 1961), and lipopolysaccharide with carbocyanin dye (Janda & Work, 1971).

Treatment of antigens

MU, PP, PA and TA antigens were treated with pronase (Sigma Chemical Company, St Louis, Missouri, U.S.A.), or sodium metaperiodate, according to Hollingdale (1974). PA and TA antigens were also treated with α- or β-glucosidase (Sigma Chemical Company, St Louis, Missouri, U.S.A.) using the method for pronase treatment.

Antisera

For antigenic analysis, serum from a farmer with FLD and strongly reactive with *T. vulgaris*, and weakly with *M. faeni* in precipitin tests, was used throughout. This serum, HTv, contained antibodies to more *T. vulgaris* antigens than any other available. For comparison with *M. faeni* pooled human serum H2 (Hollingdale, 1974) was used. In some tests, serum from a sheep injected subcutaneously with *T. vulgaris* mycelium was used.

For a quantitative analysis of antibodies to *T. vulgaris*, sera received in this laboratory for routine testing were used. Each serum was initially tested for precipitating antibodies to *T. vulgaris* and *M. faeni* MU antigens. Control sera were from healthy urban dwellers with no history of exposure to mouldy hay.

Serological tests

Immunodiffusion

The method was that of Fletcher, Rondle & Murray (1970). MU and PP antigens were tested at 25 mg./ml. and PA and TA extracts at 2 mg./ml.

Immunoelectrophoresis

The method of Pepys & Jenkins (1965) was used. MU and PP antigens were tested at 40 mg./ml. and PA and TA extracts at 5 mg./ml.

Counterimmunoelectrophoresis

The method of Coonrod & Rytel (1973) was used. MU antigen was used in doubling dilutions between 15 mg./ml. and 0·05 μg./ml., and TA and PA extracts between 1·0 mg./ml. and 0·01 μg./ml.

Latex agglutination

Latex coated with *M. faeni* or *T. vulgaris* MU antigen was tested for agglutination by routine sera according to the method of Hollingdale (1974). For absorption of latex agglutinating (LA) antibody, 0·15 ml. of HTv or H2 sera was held with 1·5 mg. of antigen at 4° C. for 72 hr., and the precipitate removed by centrifugation. Control serum was held in the same way with PBS.

Table 1. *Latex agglutination titres of HTv and H2 sera*

Serum	Titre with latex coated with *T. vulgaris* MU	Titre with latex coated with *M. faeni* MU
HTv		
Unabsorbed	1024	64
Absorbed with *T. vulgaris*	< 8	64
Absorbed with *M. faeni*	512	< 8
H2		
Unabsorbed	< 8	1024
Absorbed with *T. vulgaris*	< 8	512
Absorbed with *M. faeni*	< 8	< 8

Table 2. *Number of sera from patients with farmer's lung disease reacting with* Thermoactinomyces vulgaris *and* Micropolyspora faeni *MU antigens in precipitin and latex agglutination (LA) tests*

Precipitins to	No.	LA antibodies to *M. faeni*	LA antibodies to *T. vulgaris*
M. faeni only	50	50	7
M. faeni and *T. vulgaris*	50	45	43
T. vulgaris only	10	4	10

RESULTS

Immunodiffusion

In immunodiffusion tests using HTv serum and MU antigens five lines were present, numbered 1–5 from the antiserum well outwards. After treatment of MU with pronase, two lines, 2 and 3 remained, whereas these lines were abolished after treatment with sodium periodate, leaving lines 1, 4 and 5. Neither α- nor β-glucosidase had any effect on the line patterns. The PA and TA extracts reacted identically and gave lines 2 and 3, and PP extract gave lines 4 and 5. With the sheep antiserum, the precipitin reaction of MU, PP and TA extracts was similar to that with HTv serum. No cross-reaction was seen with *M. faeni* MU or PA antigens using H2 serum, nor was it demonstrated by cross-absorption.

Immunoelectrophoresis

In immunoelectrophoresis tests using HTv serum, 5 arcs were given by MU antigen – 2 adjacent to the antigen well, 2 towards the anode and 1 towards the cathode. Two arcs towards the anode were given by the PA and TA extracts.

Counterimmunoelectrophoresis

MU antigen reacted with HTv serum, giving at least one line over the range 15 mg./ml. to 1·8 μg./ml. However, TA extract reacted to a dilution of 50 ng./ml. Dilutions of HTv serum were unreactive with undiluted serum. CIE, therefore, is highly sensitive in detecting the presence of antibodies to *T. vulgaris* antigens, particularly polysaccharides, but has little potential to quantitate antibodies. Its

much greater sensitivity than immunodiffusion and its rapid application may prove its usefulness.

Latex agglutination

The results of LA tests with HTv and H2 sera with latex coated with *T. vulgaris* or *M. faeni* MU antigens, and the titres of these sera after absorption are shown in Table 1. HTv reacted more strongly with *T. vulgaris* than with *M. faeni*, whereas H2 only reacted with *M. faeni*. Absorption of HTv or H2 with *T. vulgaris* MU antigen abolished the reaction with latex coated with *T. vulgaris* MU, but did not affect reaction with latex coated with *M. faeni* MU. Similarly absorption of these sera with *M. faeni* MU antigen abolished the reaction with latex coated with *M. faeni* MU, and did not affect the reaction with latex coated with *T. vulgaris* MU. Like immunodiffusion, LA tests do not show any cross-reactivity between *T. vulgaris* and *M. faeni*.

Sera from patients without FLD but with precipitins to *T. vulgaris* or *M. faeni*, or sera from healthy urban dwellers, did not contain LA antibodies to these organisms. The incidence of precipitins and LA antibodies in the sera from patients with FLD received in this laboratory is shown in Table 2. Almost all sera with precipitins to *M. faeni* contained LA antibodies to *M. faeni*, and almost all sera with precipitins to *T. vulgaris* contained LA antibodies to *T. vulgaris*. However, 7/50 sera with precipitins only to *M. faeni* contained in addition LA antibodies to *T. vulgaris* and 4/10 sera with precipitins only to *T. vulgaris* contained in addition LA antibodies to *M. faeni*. Of the 50 sera with precipitins to both *M. faeni* and *T. vulgaris*, 45 contained LA antibodies to *M. faeni* and 43 contained LA antibodies to *T. vulgaris*, showing that 12 sera only contained LA antibodies to one organism.

Generally the titre of the serum in the LA test reflected its reactivity in precipitin tests, that is strongly precipitating sera gave the highest LA titres of about 1/1024. However, several weakly precipitating sera also gave high LA titres, and several strongly precipitating sera gave low LA titres. In general LA titres were a more sensitive quantitative estimation of the level of antibodies to *T. vulgaris* or *M. faeni* than precipitin tests.

Analysis of T. vulgaris *antigens*

Paper chromatography of hydrolysates of *T. vulgaris* mycelium, PA or TA extracts gave two spots reactive with ninhydrin or silver nitrate, corresponding to glucosamine and muramic acid. Ribose was detected in mycelial and PA hydrolysates but not TA hydrolysates and is probably derived from ribonucleic acid. Hydrolysates of mycelium, PA or TA extracts of *T. vulgaris* grown in culture medium containing glucose, also gave a trace reaction for glucose. However, extracts from *T. vulgaris* grown in culture medium where sodium citrate replaced glucose did not contain glucose, although serologically such extracts were similar. It is probable, therefore, that the trace of glucose was derived from contamination with the culture medium and that glucose is not a constituent sugar of *T. vulgaris*.

Applications of the cystein-H_2SO_4 method did not demonstrate additional

sugars, though a peak corresponding to pentose, presumably ribose, was formed. The extracts were unreactive with the carbocyanin dye, indicating the absence of lipopolysaccharide structure.

DISCUSSION

These results show that the precipitating antibody response to *T. vulgaris* in patients with FLD is much less complex than that to *M. faeni*. With *T. vulgaris* MU antigen only five lines of precipitation were demonstrated, whereas 16 lines were demonstrated with a sonicated *M. faeni* MU antigen (Hollingdale, 1974), and up to 29 lines under optimal conditions with *M. faeni* culture medium antigens (Fletcher *et al.* 1970). It is possible that additional precipitins may be demonstrated in other human sera, though serum HTv used here contained more precipitins than 50 other sera examined. It is noteworthy that the experimentally produced sheep antiserum gave similar precipitin reactions to those given by HTv serum. The suggested role of precipitins inducing an Arthus type III hypersensitivity in response to exposure to *M. faeni* (Pepys, 1969) may, therefore, be less important in the response to *T. vulgaris* than non-precipitin-mediated mechanisms. Indeed, Pepys & Jenkins (1965) found that inhalation of *M. faeni* antigens induced pulmonary responses several hours later indicating an Arthus mechanism, whereas inhalation of *T. vulgaris* induced an acute asthmatic response.

Like *M. faeni*, *T. vulgaris* contains two groups of antigens: pronase-sensitive proteins, extractable with aqueous phenol; and polysaccharides sensitive to sodium periodate and extractable with aqueous phenol or TCA. No cross-reaction was demonstrated between antigens of *T. vulgaris* and *M. faeni*. This agrees with Seabury *et al.* (1973), who showed no cross-reaction in precipitin tests between isolates from humidification systems identified as *T. vulgaris* and *M. faeni*. This differing antigenicity from *M. faeni* is reflected in the very different polysaccharide structures of the two organisms. The two polysaccharides from *T. vulgaris* are negatively charged and contain glucosamine and muramic acid, reflecting the type III classification of *T. vulgaris* of Becker, Lechevalier & Lechevalier (1965) based on the presence of these amino sugars in the cell wall. However, those from *M. faeni* are uncharged lipopolysaccharides resembling those from Enterobacteriacae (Hollingdale, 1974). It has been suggested that *M. faeni* polysaccharides may adhere to tissue cells inducing a cytotoxic response (Wenzel *et al.* 1971; Hollingdale, 1974). It is possible that *T. vulgaris* polysaccharides act similarly, but their avidity for tissue cells will require investigation.

Latex agglutination appears to be more sensitive for detecting antibodies to *T. vulgaris* than precipitin tests, though a combination of the two gives an even higher number of positive results. This agrees with Murray, Pepys & Brighton (1967), who compared latex agglutination tests with precipitin tests for detecting antibodies to *M. faeni*. This increase in number of positive sera may reflect the types of antibodies reactive in these tests. Hollingdale (1974) showed that IgG antibodies reacted in precipitin tests, but IgG, IgA and particularly IgM antibodies reacted in agglutination. Such tests appear particularly useful in quantitating the antibody response to *T. vulgaris* and *M. faeni*. CIE, however, appears

highly sensitive in detecting precipitins to *T. vulgaris* antigens, particularly polysaccharides, though its use is limited for *M. faeni* where the polysaccharides are neutral and do not migrate. A prospective evaluation of CIE, latex agglutination and precipitin tests would evaluate the role of each as a diagnostic procedure.

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REFERENCES

Becker, B., Lechevalier, M. P. & Lechevalier, H. A. (1965). Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. *Applied Microbiology* **13**, 236.

Coonrod, J. D. & Rytel, M. W. (1973). Detection of type-specific pneumococcal antigens by counterimmunoelectrophoresis. 1. Methodology and immunologic properties of pneumococcal antigens. *Journal of Laboratory and Clinical Medicine* **81**, 770.

Fletcher, S. M. & Rondle, C. J. M. (1973). *Micropolyspora faeni* and farmer's lung disease. *Journal of Hygiene* **71**, 185.

Fletcher, S. M., Rondle, C. J. M. & Murray, I. G. (1970). The extracellular antigens of *Micropolyspora faeni*: their significance in farmer's lung disease. *Journal of Hygiene* **68**, 401.

Hollingdale, M. R. (1974). Antibody responses in patients with farmer's lung disease to antigens from *Micropolyspora faeni*. *Journal of Hygiene* **72**, 79.

Hughes, W. F., Mattimore, M. D. & Arbesman, C. E. (1969). Farmer's lung in an adolescent boy. *American Journal of Diseases of Children* **118**, 777.

Janda, J. & Work, E. (1971). A colorimetric estimation of lipopolysaccharides. *FEBS Letters* **16**, 343.

Kabat, E. A. & Mayer, M. M. (1961). *Experimental Immunochemistry*, pp. 526–41. Springfield: Thomas.

Kawai, T., Salvaggio, J. & Harris, J. (1972). Cell-mediated immunity in experimental hypersensitivity pneumonitis. *Clinical Research* **20**, 511.

Murray, I. G., Pepys, J. & Brighton, W. D. (1967). The latex agglutination test in farmer's lung. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **26**, 96.

Pepys, J. (1969). Hypersensitivity diseases of the lungs due to fungi and organic dusts. *Monographs in Allergy*, no. 4. Basel: Karger.

Pepys, J. & Jenkins, P. A. (1965). Precipitin (F.L.H.) test in farmer's lung. *Thorax* **20**, 21.

Salvaggio, J., Buechner, H. A., Domer, J., Fink, J. & Kawai, I. (1967). Demonstration of precipitins against extracts of thermophilic actinomyces in the sera of affected individuals. *Journal of Allergy* **39**, 106.

Seabury, J., Salvaggio, J., Domer, J., Fink, J. & Kawai, I. (1973). Characterisation of thermophilic actinomycetes isolated from residential heating and humidification systems. *Journal of Allergy and Clinical Immunology* **51**, 161.

Wenzel, F. J., Emanuel, D. A. & Gray, B. S. (1971). Immunofluorescent studies in patients with farmer's lung. *Journal of Allergy and Clinical Immunology* **48**, 224.

Wenzel, F. J., Emanuel, D. A. & Lawton, B. R. (1967). Pneumonitis due to *Micromonospora vulgaris* (farmer's lung). *American Review of Respiratory Diseases* **95**, 652.

Wilkie, B., Pauli, B. & Gygax, M. (1973). Hypersensitivity pneumonitis: experimental production in guinea pigs with antigens of *Micropolyspora faeni*. *Pathologia et Microbiologia* **39**, 393.