Cross-reacting antibodies to Micropolyspora faeni in Mycoplasma pneumoniae infection:

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Summary
Two cases are described of severe Mycoplasma pneumoniae infection in whom cross-reacting antibodies developed to a glycopeptide antigen of Micropolyspora faeni. The antibodies were predominantly IgG, although some IgM was detected. Precipitin responses to M. faeni were detected for 4 months after the initial illness in one case, and 1 month in the second case. The cross-reacting antibodies were closely related to the antibodies responsible for the complement fixation reaction to M. pneumoniae. It is suggested that a common polysaccharide antigen may exist on the surface membranes of M. pneumoniae and M. faeni. Initial studies of the frequency of the development of cross-reacting antibodies in M. pneumoniae infection suggest it is uncommon. Atypical cases of farmer’s lung should be investigated to exclude M. pneumoniae infection.

Introduction
Mycoplasma pneumoniae is now recognized as a common and important pathogen of the respiratory tract (Jones, 1971). The true incidence of M. pneumoniae infection is not known; many are subclinical and the incidence of pneumonia in adults is small (Noah, 1974). Bizarre complications occasionally occur, with the development of a cold agglutinin positive haemolytic anaemia, encephalitis, erythema multiforme, arthralgia and myringitis having been documented (Thomas, 1970). Cross-reacting antibodies directed towards the host’s tissues have been isolated and it has been suggested that the complications of M. pneumoniae infection may have an immunological basis (Sterner & Biberfeld, 1969; Biberfeld, 1971a).

Subjects with hypersensitivity pneumonitis such as farmer’s lung often show precipitating antibodies to antigens derived from thermophilic actinomycetes (Roberts, 1974). The actinomycete most frequently associated with farmer’s lung is Micropolyspora faeni and precipitating antibodies to this organism have been regarded as evidence of exposure to mouldy hay (Pepys, 1974). We wish to report the develop-
ment of cross-reacting antibody to *M. faeni* in two subjects with severe *M. pneumoniae* infection but no history of previous exposure to mouldy hay.

**Case reports**

**Case 1**

A 30-year-old male presented with a prodromal illness of dry cough and fever, progressing to dyspnoea and dull right anterior chest pain. Ampicillin therapy was ineffective. No significant occupational history was obtained and no exposure to any known cause of an allergic alveolitis was found. On examination he was toxaemic, with pyrexia, and there were many crackles over the lung bases. A myringitis was noted. Chest radiograph (Fig. 1) showed bilateral fine nodular shadowing in both lung fields with a lower zone predominance. Blood gases showed hypoxaemia, $PO_2$ 67 mmHg, and hypocapnia, $PCO_2$ 25 mmHg, breathing air. The haemoglobin was normal, 14·6 g%, the WCC 6200/mm$^3$, normal differential count. Lung function tests showed a reduction in the transfer factor to 22 ml CO/min/Torr (67% of predicted normal). Therapy with tetracycline was effective. Serial immunoglobulins (Hyland Immunoplates) and complement fixation tests to *M. pneumoniae* are illustrated in Table 1.

**Skin testing.** The patient was skin tested with purified glycopeptide antigens and protein antigens of *M. faeni* at 100 µg/ml, and a control saline solution. An immediate weal with erythema was noted to the glycopeptide antigens which subsided, to be followed by slight induration at 6 hr. No reaction was observed to protein antigens or saline control.

![Fig. 1. Chest X-ray at presentation showing fine nodular infiltration.](image-url)
Table 1. Sequential immunoglobulin levels, IgG, IgM, IgA (g/l) related to *Mycoplasma pneumoniae* complement fixation titres (MCFT)

<table>
<thead>
<tr>
<th>Time after onset of illness (weeks)</th>
<th>Case 1</th>
<th>Case 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td></td>
<td>(6–16)</td>
<td>(0–5–2–0)</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>1·9</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>2·1</td>
</tr>
<tr>
<td>12</td>
<td>21</td>
<td>0·9</td>
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<tr>
<td>5</td>
<td>16</td>
<td>5·5</td>
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<tr>
<td>10</td>
<td>17</td>
<td>6·2</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>3·6</td>
</tr>
</tbody>
</table>

Case 2
A 29-year-old female presented with a prodromal illness of cough, dyspnoea and fever. Confusion and right-sided chest pain developed and ampicillin therapy was ineffective. No exposure to any known cause of an allergic alveolitis was obtained. Her daughter had suffered a pneumonia 3 weeks prior to the mother’s illness. She was pyrexial, pale and cyanosed, with crackles over the basal pulmonary areas. Chest radiograph (Fig. 2) showed bilateral basal consolidation. Blood gases showed hypoxaemia, $PO_2$ 43 mmHg, and hypocapnia, $PCO_2$ 24 mmHg breathing air. The haemoglobin was 13·3 g%, the WCC was 4100/mm$^3$, normal differential count. Lung function tests performed during the convalescence showed a reduction in the transfer factor to 13 ml CO/min/Torr (52% of predicted normal). Serial immunoglobulins and complement fixation tests to *M. pneumoniae* are illustrated in Table 1.

Skin testing. Skin testing, as detailed for Case 1, showed an immediate weal and erythema to the glycopeptide antigen only. This persisted for 30 min and no further reaction was noted.

Fig. 2. Chest X-ray at presentation showing bilateral basal consolidation.
Immunological studies

Materials and methods

*M. faeni* antigens were prepared as previously described (Edwards, 1972) and used at 1 mg/ml in saline. Protein antigens were precipitated with ethanol and purified by chromatography on diethylaminoethyl-cellulose (DEAE) and gel filtration on Sephadex G200. *M. faeni* glycopeptide antigens (MFG) were extracted with 5% trichloroacetic acid and purified by chromatography on DEAE and gel filtration on Sephadex G200. Extracts of human fetal brain, lung, intestine and adult brain were prepared by extraction of the homogenized tissue with 1 M perchloric acid for 30 min at room temperature. The suspension was centrifuged at 10,000 g for 10 min and the supernatant dialysed against water, the final concentration being adjusted to 5 mg/ml, except for fetal brain (concentration 30 mg/ml).

Immunoglobulin G (IgG) preparation from Case I was prepared by the technique of Levy & Sober (1960) and concentrated to 30 mg/ml with Lyphogel (Gelman Instruments Ltd, Lancing, Sussex). The purity was assessed by immunoelectrophoresis against anti-whole human serum and by gel diffusion against specific antisera to IgG, IgM and IgA. The sample contained only IgG.

Gel diffusion and immunoelectrophoresis

Gel diffusion was carried out in 1.5% Ionagar no. 2 (Oxoid) in citrate phosphate buffer, pH 7-2. Central wells, 1 cm diameter, and peripheral wells, 4 mm diameter, in hexagonal arrangement, were used with a 1 cm, centre to centre, spacing. Immunoelectrophoresis (in 1.5% Ionagar no. 2 gel in saline) was performed, with MFG at 1 mg/ml in 4 mm diameter wells and serum in 1 cm width troughs for direct immunoelectrophoresis; for reverse immunoelectrophoresis serum was run into 1 cm diameter wells and MFG in 4 mm troughs. Large scale wells and troughs were necessary for positive reactions.

Cold agglutinins

Cold agglutinins were determined by incubating serial twofold dilutions of serum with thrice washed human group O red blood cells at 2% concentration in phosphate buffered saline pH 7-2 (PBS). The effect of mercaptoethanol (2ME) was assessed by repeating in the presence of 0-2 M 2ME.

Complement fixing antibody to *M. faeni*

To 400 µl of serum from Case I were added 400 µl fresh guinea-pig serum (previously absorbed with sheep red blood cells). Two hundred microlitre aliquots of the mixture were dispersed into three tubes and to these were added 100 µl of MFG at 0, 10 and 100 µg/ml (antigens filtered less than 220 µm). After incubation for 1 hr at 37°C, 4-7 ml complement diluent was added and each tube titrated for residual complement activity using optimally sensitized sheep red blood cells (Kabat & Mayer, 1961). The experiment was duplicated using normal human serum as a control.

Cold agglutinin cross-reactivity and absorption by various extracts

To 5 ml of serum from Case 1, 1 ml of packed washed human group O red blood cells was added and incubated at 4°C. After overnight incubation the supernatant was removed and tested for precipitin activity to MFG by gel diffusion. The red blood
cells were washed three times in cold PBS and after resuspension in 2.5 ml PBS incubated for 30 min at 37°C. After centrifuging the supernatant was tested for cold agglutinin activity and presence of antibody to MFG and fetal extracts.

Aliquots of serum from Case 1 were absorbed by strain ‘H’ and strain ‘3528’ *Staphylococcus aureus* teichoic acids (kindly provided by Dr R. A. Archibald, Newcastle University), also fetal brain extract at 10 mg/ml serum, and alcohol killed *M. faeni* organisms at 50 mg/ml serum. After 2-5 hr at 37°C and 16 hr at 4°C supernatants were tested by double gel diffusion against MFG and fetal extracts.

**Cryoglobulin activity**

Serum from Case 2 was studied. Five millilitres were filtered less than 220 μm, and incubated at 0°C for 16 hr. The precipitate was centrifuged and washed once with saline at 0°C; its buoyancy preventing a meaningful yield, if washed further. The precipitate was resuspended in 1 ml PBS and tested for cold agglutinin activity, precipitins to MFG and the presence of IgG, IgM and IgA by gel diffusion using specific antisera.

**Results**

**Immunological reactivity of sera**

*M. pneumoniae* complement fixing titre was 1/4096 in Case 1 and 1/2048 in Case 2 at the onset of investigation and both had precipitins to MFG but not protein antigens (Fig. 3). Cold agglutinin titre of Case 1 was 1/64 and Case 2, 1/64. These were abolished in the presence of 0.2 M 2ME although the 2ME treated sera still reacted with MFG on gel diffusion. The antibody to MFG was complement fixing. Of forty-seven C'H_{50} units available per reaction mixture twenty C'H_{50} units were used at 10 μg MFG/ml serum and forty C'H_{50} units at 100 μg MFG/ml serum compared with one and five C'H_{50} units utilized by normal serum/ml at 10 and 100 μg/ml respectively. The avidity of the antibody for MFG was of a low order, part of the precipitate dissolving from the gel during saline washing. The reaction of isolated IgG with MFG confirmed

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**Fig. 3.** Gel diffusion reaction between serum from Case 1 and *M. faeni* antigens. MF, total antigenic complex; G, glycopeptide antigens; P, protein antigens. Serum from Case 2 gave an identical result.
this immunoglobulin as having antibody activity. However, reverse immunoelectrophoresis did not produce a characteristic IgG arc. The precipitation arc indicated a mobility close to that of IgM or a fast moving IgG. Treatment of well-washed gel precipitin lines formed between Case 1 serum and MFG with fluorescein coupled anti-IgG and anti-IgM indicated the presence of both immunoglobulins. Direct immunoelectrophoresis of MFG and development with Case 1 serum showed that the reacting glycopeptide had the mobility of \textit{M. faeni} antigen Ia or Ib (Edwards, 1972). Absorption of serum by \textit{M. faeni} organisms produced no gel diffusion reaction against MFG indicating the glycopeptide antigen to be located on the cell or spore surface of \textit{M. faeni}.

**Cross-reactivity**

Serum from Case 1 absorbed with MFG reduced the complement fixing titre to \textit{M. pneumoniae} from 1/4096 to 1/512 but failed to reduce the cold agglutinin titre. That the cold agglutinins and MFG precipitins were not related was confirmed by the failure of group O human red blood cells to absorb out precipitin activity to MFG. The agglutinins eluted from the surface of the group O red blood cells had a cold agglutinin titre of 1/32 and specific antiserum detected mainly IgM with a trace of IgG. These eluted agglutinins failed to give a gel diffusion reaction with MFG even when concentrated ×10.

Tissue cross-reactivity was demonstrated in Case 1. Reactions were observed to fetal brain, intestine, liver and adult brain with whole serum but no reaction was observed with isolated at IgG 30 mg/ml. Antibody activity to MFG was not absorbed out by teichoic acids nor fetal brain extracts. Teichoic acids also failed to absorb out activity to fetal brain extract.

**Cryoglobulin activity**

IgM and IgG, but not IgA, were detected in the cryoprecipitate from Case 2. The O.D.\textsubscript{280} of the dissolved precipitate was approximately equivalent to 17 mg/ml protein. The cold agglutinin titre was 1/64 but there was no activity towards MFG nor fetal extracts on gel diffusion.

**Discussion**

Our serological studies have shown specific antibodies and cross-reacting antibodies to be produced in \textit{M. pneumoniae} infection. In the cases studied we have found both IgG and IgM to be reactive. IgM was largely responsible for the cold agglutinins associated with the disease, as determined by 2ME activity, and IgG for the gel diffusion reaction against MFG as determined by the reactivity of the isolated IgG from Case 1. There was no cross-reactivity between the cold agglutinin antigenic site and the MFG whereas MFG antigens absorbed most of the reactivity to \textit{M. pneumoniae} antigen. The cross-reacting antibodies persisted for 4 months in Case 1 and 1 month in Case 2.

The cases studied have been examples of severe infection with bilateral pulmonary consolidation, severe toxaemia and in whom cold agglutinins developed. This form of disease is uncommon and probably accounts for less than 8% of all cases of pneumonia due to \textit{M. pneumoniae} (Foy & Alexander, 1969). The severity of the pneumonia in our cases as shown by the clinical features, suggests that it will be encountered uncommonly in clinical practice.
The antibody response to *M. pneumoniae* is now well documented (Biberfeld, 1968, 1971b; Schleuderberg, 1965; Jones & Stewart, 1974). Antibody begins to appear in the second week, reaches a maximum in the third or fourth week, and thereafter, declines slowly. The complement fixing antibodies to *M. pneumoniae* belong to both the IgM and IgG class, the proportion of IgG to IgM antibodies increasing with time after the onset of infection. In cases of reinfecion the antibody response is predominantly IgG (Biberfeld, 1971b).

IgM antibodies, directed specifically against the I antigen of the red cell are responsible for cold agglutinin activity, the level of cold agglutinins correlating well with the severity of the illness. The I antigen is thought to be modified by hydrogen peroxide, produced by *M. pneumoniae*, eliciting an antibody response (Smith, McGinniss & Schmidt, 1967). The cold agglutinins are unrelated to the complement fixing antibodies which are thought to be directed to antigens of the limiting membrane of *M. pneumoniae*. The main serologically active *M. pneumoniae* antigens are glycocolipids, with potentiation by a haptenic phospholipid, coupled to proteins in the limiting membrane of the organism (Lemcke, 1973). Cross-reacting antibodies to brain have previously been described in *M. pneumoniae* infection (Biberfeld, 1971a) and it has been suggested that a common lipid antigenic structure may be present in brain tissue and the membrane of *M. pneumoniae*. We have confirmed this finding but absorption with fetal brain antigen of positive MFG sera in Case I did not abolish the precipitin reaction suggesting that a different common antigen may be involved in the latter reaction. Gourlay & Shifrine (1966) have described cross-reacting antibodies in *Mycoplasma mycoides* infection in cattle. Cross-reactions were observed to polysaccharides of various micro-organisms, e.g. *Cryptococcus neoformans*, *Acrosiphonia* and also to a pneumogalactan from normal bovine lung. We suggest that a similar cross-reaction may occur in *M. pneumoniae* infection to a polysaccharide residue present on the cell wall of *M. faeni*.

We have not been able to establish a pathogenic role to the cross-reacting antibodies detected but precipitins to *M. faeni* have previously been regarded as evidence of exposure to mouldy hay (Pepys, 1974) and have been associated with pulmonary disease in most subjects. The gel diffusion and skin test reactions of our subjects to MFG suggest that the capacity to respond immunologically in a manner suggestive of prior exposure to mouldy hay has been conferred non-specifically by the *M. pneumoniae* infection. False-positive precipitin responses to *M. faeni* have been recorded previously. Faux et al. (1970) described false-positive precipitation reactions to extracts of organic dusts due to a teichoic acid derived from *St. aureus*. Although one of the major antigenic lipid fractions of the limiting membrane of *M. pneumoniae* is glycerophosphoryl diglucosyl diglyceride which is chemically closely related to teichoic acid, this was not the mechanism in our cases.

The development of false-positive precipitin reactions in *M. pneumoniae* infection has clinical significance in that the presentation with toxaemia, dyspnoea and a radiological pulmonary infiltration, may simulate the clinical picture of acute farmer's lung. The case reported by Ansari & Carter (1974) as an unusual example of farmer's lung in childhood was probably an example of cross-reacting antibodies to *M. faeni*, the titre of complement-fixing antibodies to *M. pneumoniae* rising from 1/640 to 1/2560 within 5 days. The fatal acute case of Barrowcliff & Arblaster (1968) may possibly be a further example. Initial studies of the frequency of this cross-reaction suggest that it is uncommon in *Mycoplasma* infection. We have studied nine further
cases with *Mycoplasma* complement fixing titres ranging from 1/256 to 1/4096, and have obtained negative results to MFG.

It is suggested that if an atypical case of farmer’s lung occurs, *M. pneumoniae* infection be considered and the appropriate investigations performed.

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**References**


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