A search for mycoplasma infections in patients with chronic bronchitis

J. D. Cherry\textsuperscript{1}, D. Taylor-Robinson, H. Willers\textsuperscript{2}, and A. C. Stenhouse

Clinical Research Centre, Harvard Hospital, Salisbury, Wilts, and the University of Sheffield, Virus Research Laboratory, Lodge Moor Hospital, Sheffield

Throat and bronchoscopy specimens for mycoplasma isolation studies were collected from 22 patients with chronic bronchitis and 20 patients without chronic bronchitis. Twenty-six of 50 patients attending a chronic bronchitis clinic had throat, nasal, or sputum specimens collected for attempted mycoplasma isolation, and all of these patients had multiple serum samples taken for mycoplasma antibody studies. Mycoplasmas were recovered from throat and bronchoscopy specimens of the chronic bronchitic and non-bronchitic patients with about equal frequency. The concentration of organisms in the bronchoscopy specimens of two patients with chronic bronchitis was greater than in their throat specimens, suggesting downward spread and multiplication of mycoplasmas rather than contamination by passage of the bronchoscope. Eighty-three per cent of the rises in mycoplasma antibody titre in chronic bronchitic patients occurred during or immediately after an acute respiratory illness, and this relationship of rise in antibody titre to acute illness was significantly more frequent than rises in antibody titre not associated with illness. We suggest that mycoplasmas may be present in the bronchi of some patients suffering from chronic bronchitis and that, while such mycoplasma infections are often silent, they may become sufficiently active during infection by other agents to stimulate a mycoplasma antibody response.

Mycoplasmas in animals are frequently involved in chronic illnesses. In particular, Mycoplasma gallisepticum is one of the agents responsible for chronic respiratory disease of chickens (Fabricant, 1969) and M. pulmonis, while probably not having a primary aetiological rôle, is certainly involved in the murine chronic respiratory syndrome (Tully, 1969). Because these animal illnesses are somewhat similar to chronic bronchitis in man we thought that a mycoplasma might have some rôle in the human disease. The present study is a search for mycoplasma infections in patients with chronic bronchitis and in subjects without this disease.

MATERIALS AND METHODS

PATIENT POPULATION Two groups of patients were studied. Group 1 comprised 42 adults who underwent therapeutic and/or investigative bronchoscopy in Sheffield during September and October 1965. Of these persons, 22 had chronic bronchitis and the remaining 20 without chronic bronchitis served as a control group. Group 2 consisted of 50 patients who attended the bronchitis clinic of the Royal Hospital, Sheffield during the period July 1964 to July 1965. Multiple serum samples were collected from these patients at times of quiescence and during exacerbations of respiratory illness. Of this group, 25 patients also had swabs taken on one or more occasion from the throat and nose, or sputum collected for attempted mycoplasma isolation; specimens were collected during periods of both quiescence and exacerbation of clinical illness.

COLLECTION OF SPECIMENS Throat swabs (cotton-wool) were obtained from group 1 bronchoscopy patients after the sucking of a benzocaine lozenge. Prilocaine anaesthetic was then sprayed in the mouth and larynx and bronchoscopy was performed. Bronchial aspirate was collected directly into screw-capped vials or into sterile U-tube side arms of a suction apparatus. When difficulty in clearing thick aspirate was encountered, clearance was effected using sterile phosphate-buffered saline.
Specimens were collected in duplicate and placed in bottles which contained 4 ml of mycoplasma liquid medium. Single specimens from group 2 patients were also placed in mycoplasma liquid medium contained in bottles. All specimens were kept at 4°C immediately after collection, were transported to Salisbury at −70°C, and stored at this temperature until laboratory investigations were performed during May to December 1969.

**ISOLATION OF MYCOPLASMAS** The medium used for the culture of mycoplasmas has been described previously (Manchee and Taylor-Robinson, 1968). Frozen specimens were thawed at 37°C and then 0.1 ml of each was inoculated on agar medium (pH 7.8) and 0.2 ml aliquots of each were inoculated into 1.8 ml of liquid medium contained in screw-capped vials of 2.5 ml capacity. The liquid medium was supplemented with 0.1% of either glucose, arginine, or t-1ea. Cultures were incubated at 37°C, the agar media in an atmosphere of 5% CO₂ and 95% N₂. Vials which contained media supplemented with arginine or urea were observed for two weeks and those with glucose for three weeks. If during these times a change of colour was observed in the media, they were subcultured into liquid media and onto agar medium. In addition, medium which contained arginine was routinely subcultured onto agar medium at 4 and 10 days of incubation.

Agar media were incubated for three weeks and observed at intervals for the development of colonies. Such colonies were cloned to agar medium and then grown in liquid medium containing arginine. Isolates from liquid medium cultures were cloned in agar and finally grown in liquid medium for identification.

**IDENTIFICATION OF ISOLATES** This was done by the disc growth-inhibition technique (Clyde, 1964). Rabbit antisera prepared against the following mycoplasmas were used: *M. salivarium* (strain PG20), *M. orale* type 1 (strain CH 19299), and *M. hominis* (strain V2785).

**SEROLOGICAL STUDIES** Antibody in the sera of group 2 patients was titrated by the metabolic-inhibition (MI) technique as described by Taylor-Robinson, Purcell, Wong, and Chanock (1966) and Purcell, Taylor-Robinson, Wong, and Chanock (1966). The following mycoplasmas were used: *M. salivarium* (strain SP425H), *M. orale* type 1 (strain CH 19299), *M. hominis* (strain SP425B), and *M. pneumoniae* (strain FH).

**RESULTS**

**MYCOPLASMA ISOLATIONS** One hundred and thirty-three specimens from 68 patients in groups 1 and 2 were examined and 43 mycoplasma strains were recovered (Table I). The majority of the strains were isolated from the throat (isolation rate 52%).

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### Table I

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of Specimens</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Nose</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Throat</td>
<td>63</td>
<td>34</td>
</tr>
<tr>
<td>Sputum</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Bronchoscopy</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>43</td>
</tr>
</tbody>
</table>

*Both *M. salivarium* and *M. hominis* were found in one specimen from the throat.*

Five strains were isolated from bronchoscopy specimens (isolation rate 12%). Of the 43 strains isolated, 28 were *M. salivarium*, 12 were *M. hominis*, and 3 were *M. orale* type 1; *M. pneumoniae* was not isolated.

The number of organisms present in each specimen containing mycoplasmas was assessed and is recorded in Table II. Sixty-two per cent of the specimens known to have mycoplasma organisms contained 10 or more colony-forming units (CFU)/0.1 ml.

**Isolations from patients undergoing bronchoscopy (group I)** The source and type of mycoplasma isolated are recorded in Table III. Eleven myco-

### Table II

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of Specimens with Indicated No. of Colonies/0.1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10</td>
</tr>
<tr>
<td>Nose</td>
<td>14*</td>
</tr>
<tr>
<td>Throat</td>
<td>14*</td>
</tr>
<tr>
<td>Sputum</td>
<td>2</td>
</tr>
<tr>
<td>Bronchoscopy</td>
<td>2</td>
</tr>
</tbody>
</table>

*Includes two isolates that were recovered initially only in liquid medium containing 0.1% arginine.*

*One throat specimen contained mycoplasmas of two types.*

### Table III

<table>
<thead>
<tr>
<th>Source and Type of Mycoplasma Isolated</th>
<th>No. of Patients</th>
<th>Non-bronchitic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchitic</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Non-bronchitic</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

*Two organisms were recovered from one non-bronchitic patient.*

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\(A \text{ search for mycoplasma infections in patients with chronic bronchitis}\)

63
plasmas (50%) were isolated from the chronic bronchitic patients and 15 (75%) from the non-bronchitic subjects. The concentration of organisms in the throats of the subjects in each group was about the same. Further, mycoplasmas were isolated no more frequently from the bronchoscopy specimens of chronic bronchitic patients than from patients without bronchitis. All those patients who had a bronchoscopy specimen from which a mycoplasma was isolated also had the organisms in their throat specimens (Table IV).

Isolations from chronic bronchitic patients (group 2) Twenty-six patients in this group had one or more specimens submitted for attempted mycoplasma isolation. Thirty-eight specimens were obtained during acute respiratory illnesses and 12 during quiescent periods. As noted in Table V, the majority of mycoplasmas were isolated from the throat specimens and isolations were more common during quiescence than during acute respiratory illness.

### TABLE IV

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Source</th>
<th>Mycoplasmas</th>
<th>Polymorphonuclear Cells Seen on Smear of Bronchial Aspirate</th>
<th>Diplococcus pneumoniae on Routine Culture of Bronchial Aspirate</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Throat</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Carcinoma bronchus</td>
</tr>
<tr>
<td></td>
<td>Bronchoscopy</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Carcinoma bronchus</td>
</tr>
<tr>
<td>2</td>
<td>Throat</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Suspected carcinoma—not confirmed</td>
</tr>
<tr>
<td></td>
<td>Bronchoscopy</td>
<td>M. hominis</td>
<td>-</td>
<td>-</td>
<td>Chronic bronchitis</td>
</tr>
<tr>
<td>3</td>
<td>Throat</td>
<td>M. salivarium</td>
<td>+</td>
<td>+</td>
<td>Chronic bronchitis</td>
</tr>
<tr>
<td></td>
<td>Bronchoscopy</td>
<td>M. salivarium</td>
<td>+</td>
<td>+</td>
<td>Chronic bronchitis</td>
</tr>
<tr>
<td>4</td>
<td>Throat</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Chronic bronchitis</td>
</tr>
<tr>
<td></td>
<td>Bronchoscopy</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Chronic bronchitis</td>
</tr>
<tr>
<td>5</td>
<td>Throat</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Chronic bronchitis</td>
</tr>
<tr>
<td></td>
<td>Bronchoscopy</td>
<td>M. salivarium</td>
<td>-</td>
<td>-</td>
<td>Chronic bronchitis</td>
</tr>
</tbody>
</table>

### TABLE V

<table>
<thead>
<tr>
<th>Source</th>
<th>Acute Respiratory Illness (no. isolations/ no. specimens)</th>
<th>Quiescent Phase (no. isolations/ no. specimens)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nose</td>
<td>2/15</td>
<td>0/4</td>
</tr>
<tr>
<td>Throat</td>
<td>7/14</td>
<td>6/7</td>
</tr>
<tr>
<td>Sputum</td>
<td>2/9</td>
<td>0/1</td>
</tr>
<tr>
<td>Total</td>
<td>11/38</td>
<td>6/12</td>
</tr>
</tbody>
</table>

However, the concentration of organisms in the bronchoscopy specimens from the two patients with chronic bronchitis was greater than in the corresponding throat specimens, whereas the converse was the case for the non-bronchitic patients. It is of interest that in one non-bronchitic patient (No. 2) the type of mycoplasma isolated from the bronchoscopy specimen was different from the types recovered from the throat. In addition to a large number of mycoplasma organisms, both chronic bronchitic patients had polymorphonuclear cells in smears and pneumococci were recovered on culture of aspirated bronchial material whereas this was not so in the non-bronchitic patients.

### TABLE VI

<table>
<thead>
<tr>
<th>Mycoplasma Used in Test</th>
<th>No. of Four-fold or Greater MI Antibody Titre Rises</th>
<th>After Acute Respiratory Illness</th>
<th>During Acute Respiratory Illness</th>
<th>Not Associated with Acute Respiratory Illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. salivarium</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M. hominis</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>M. orale type 1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>M. pneumoniae</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total no. rises/ total no. possible rises</td>
<td>13/54 (24%)</td>
<td>12/45 (27%)</td>
<td>5/50 (10%)</td>
<td></td>
</tr>
</tbody>
</table>

1. Total no. of possible rises was calculated by observing the no. of clinical exacerbations during the time of serological study.
2. P<0.05 when compared with the combined results of antibody rises noted after and during acute respiratory illness.
body titre to the clinical condition of the patient is recorded in Table VI. Eighty-three per cent of the rises in antibody titre, as measured by the MI technique, occurred during or immediately after an episode of acute respiratory illness. The antibody titre rises in ‘illness’ and in ‘non-illness’ groups were compared with the total antibody titre rises that were possible in each group. When this was done it was seen that the relationship of rise in antibody titre to illness was significantly more frequent (p<0.05) than rises in antibody titre not associated with illness. On the other hand, there was no correlation between the rise in titre of antibody to a particular mycoplasma and the isolation of that mycoplasma from the patient. Furthermore, in many cases in which a mycoplasma was isolated antibody was not detected in the serum from the patient.

**DISCUSSION**

Chronic bronchitis is characterized clinically by chronic or recurrent productive cough as a result of chronic inflammation of the bronchial system. In addition to chronic respiratory symptoms, acute exacerbations of illness occur in which there is an increase in either the quantity or purulence of sputum. The excessive production of mucus is associated with hyperplasia of the mucous glands. The primary factor(s) which initiate(s) this change is obscure, although many aspects of the pathogenesis of the disease and its exacerbations are known. Acknowledged factors concerned in the progression of the disease include irritants in the inspired air, stagnation of mucus, inhibition of ciliary activity, and infection by various microorganisms (Chodosh and Segal, 1964; Stuart-Harris, 1968).

It has been shown (Sommerville, 1963; Carilli, Gohd, and Gordon, 1964; Stark, Heath, and Curwen, 1965; Eadie, Stott, and Grist, 1966; Ross, McMichael, Eadie, Lees, Murray, and Pinkerton, 1966; Stenhouse, 1967; Stott, Grist, and Eadie, 1968) that many virus infections can cause clinical exacerbations of chronic bronchitis and that *Haemophilus influenzae* and possibly *Diplococcus pneumoniae* are important in the chronic quiescent disease as well as in exacerbations (Hers, 1961; Lees and McNaught, 1959; Stuart-Harris, 1968).

Mycoplasmas might also play a role in chronic bronchitis in one or more of the following ways. First, mycoplasma infection could be the primary aetiological factor, the organisms being a constant irritant to the epithelium resulting in excessive mucus production. If this were the case, it could be postulated that other irritants, including bacteria, were secondary factors contributing more to the periodic exacerbations. A precedent for this hypothesis is found in chronic respiratory disease of chickens in which *M. gallisepticum* may cause a chronic infection of the respiratory tract which is made worse by secondary infection with viruses and bacteria (Fabricant, 1969). Secondly, mycoplasma infection of the human respiratory tract could be silent, the organisms not causing an effect by themselves. However, acute irritation by other factors such as viruses might cause activation to produce disease. If this were the case, a single mycoplasma infection could be related to the symptomatology of multiple exacerbations.

**Table VII**

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morton, Smith and Keller (1952)</td>
<td>10 bronchoscopy samples were examined and none yielded mycoplasmas</td>
</tr>
<tr>
<td>Carilli <em>et al.</em> (1964)</td>
<td>4 of 46 (8.7%) acute respiratory illnesses in chronic bronchitis patients were associated with probable <em>M. pneumoniae</em> infection</td>
</tr>
<tr>
<td>Rytel, Conner, Welch, Kraybill, Edwards, Rosenbaum, Frank, and Miller (1964)</td>
<td>173 patients were bronchosoped and 45 mycoplasma strains were isolated. These comprised 35 from 108 bronchectasis patients, 6 from 42 bronchopneumonia patients, 2 from 7 chronic bronchitic patients, and 2 from 11 patients with other pulmonary conditions</td>
</tr>
<tr>
<td>Ross <em>et al.</em> (1966)</td>
<td>No rises in titre of complement-fixing antibody to <em>M. pneumoniae</em> were noted in 125 acute exacerbations of chronic bronchitis</td>
</tr>
<tr>
<td>Hers and Masurel (1967)</td>
<td>26 exacerbations of chronic bronchitis and emphysema were associated with <em>M. pneumoniae</em> infection</td>
</tr>
<tr>
<td>Organick and Worman (1967)</td>
<td>78 patients underwent bronchoscopy and mycoplasmas were isolated from 11.5% and from pharyngeal swabs of 33%. Mycoplasmas were also recovered from the pharynx of 28 of 50 normal subjects. They were not recovered from bronchoscopy specimens of 15 patients with chronic bronchitis</td>
</tr>
<tr>
<td>Lambert (1968)</td>
<td>Complement-fixing antibody to <em>M. pneumoniae</em> was noted in 16.4% of 566 normal subjects and in 35.5% of 137 chronic bronchitic patients</td>
</tr>
<tr>
<td>Inglis, Davies and Andrews (1969)</td>
<td>100 patients underwent bronchoscopy and mycoplasmas were recovered from 25 pharyngeal swabs and 8 bronchoscopy specimens. The bronchoscopy-positive specimens were from the following clinical categories: carcinoma, 4; infective process, 1; non-infective process, 1; unknown, 2</td>
</tr>
</tbody>
</table>
third possibility is that mycoplasmas have no
great part in the long-term chronic bronchitis
syndrome but that infection with a specific myco-
plasma might be responsible for a particular
exacerbation. This situation would seem to occur
when chronic bronchitic patients are initially in-
fected with *M. pneumoniae*. In Table VII a
summary is presented of mycoplasma studies in
chronic bronchitis and some other respiratory ill-
nesses. The findings of Carilli et al. (1964) and
Hers and Masurel (1967), as well as those of the
present study, suggest that *M. pneumoniae* in-
fection in a patient with chronic bronchitis is
frequently associated with an acute clinical
exacerbation. Whether other mycoplasma sero-
types play a part in chronic bronchitis and its
acute exacerbations is less clear. In the present
study, as well as in those recorded in Table VII,
there is no apparent meaningful relationship
between the presence of mycoplasmas in throat
or bronchoscopy specimens and the presence of
chronic bronchitis or exacerbations thereof. Of
course the low rate of isolation from bronchi may
not be a true reflection of the presence of myco-
plasmas. A single specimen taken at bronchoscopy
is hardly likely to give a precise picture of the
mycoplasma flora throughout the bronchial
system. The low isolation rate is probably not
the result of storing specimens at $-70^\circ$ C for four
years. The observations of Addey, Taylor-Robin-
son, and Dimic (1970) indicated that mycoplasmas
survived for years at $-70^\circ$ C. Furthermore, the
isolation rate of 52% from throat specimens in
the present study is similar to that noted by
Organick and Worman (1967), although the iso-
lration rate for *M. orale* type 1 was lower than
expected (Hendley and Jordan, 1968).

An interesting finding in the present study was
that the mycoplasma organisms in bronchoscopy
specimens taken from the chronic bronchitic
patients were in a greater concentration than
those in corresponding throat specimens. This
suggests at least that the mycoplasmas found in
the bronchial secretions spread from the throat
to the bronchi and multiplied there and that their
presence was not just the result of contamination
of the lower respiratory tract by passage of the
bronchoscope. The rises in the titre of antibody
(Table VI) are also of interest. These rises were
detected against mycoplasmas which were not
always recoverable from the patient. This could
be due to the inefficiency of mycoplasma isolation
or to a lack of specificity of the MI technique.
The latter is extremely specific for the measure-
ment of antibody in rabbit antisera but less so
with acute infection sera (Purcell, Wong, Chanock,
Taylor-Robinson, Cancola, and Valdesuso, 1967). The results of many studies have shown
that mycoplasmas may be isolated repeatedly
from the oropharynx of persons without disease.
They were recovered from the throat, nose, or
sputum of more than 50% of all the patients we
studied, including those without chronic bron-
chitis. In view of the existence of such chronic
infection, it would not seem plausible that many
of the rises of antibody titre were the result of
acute infections by mycoplasmas. As myco-
plasmas of human origin other than *M. pneumoniae* have rarely, if ever, been found to
be the cause of respiratory illness, it would be
difficult to accept them as a primary cause of
acute exacerbations in chronic bronchitis.

The facts at hand suggest that mycoplasmas
may well be present in the bronchi of some
patients suffering from chronic bronchitis for
considerable periods of time; whether in such
cases they contribute to the pathogenesis of the
condition is difficult to say. However, the fact that
25 acute exacerbations of chronic bronchitis were
associated with four-fold rises in the titre of
mycoplasma antibody suggests that at these times
the organisms of silent infections were suddenly
more antigenic, possibly by more active participa-
tion in tissue damage. It could be postulated that
these periods of increased mycoplasma activity
were the secondary result of infections with other
agents such as viruses or bacteria. The work of
Jordan and Kulasegaram (1968) tends to support
this hypothesis. They noted that chickens chroni-
cally infected by *M. gallisepticum* had rises in the
titre of antibody to this mycoplasma during acute
infections with infectious bronchitis virus. Fur-
thermore, Reed (1971) has found an increased
multiplication of *M. hyorhinis* in pig and calf
tracheal organ cultures infected by swine influ-
enza virus.

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