

A search for mycoplasma infections in patients with chronic bronchitis

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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, 26 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.

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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 urea. 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%).

TABLE I
SOURCE AND TYPE OF MYCOPLASMAS ISOLATED

Source	No. of Specimens	Isolates			
		Total	<i>M. salivarium</i>	<i>M. hominis</i>	<i>M. orale</i> type 1
Nose	18	2	2		
Throat ¹	63	34	20	12	2
Sputum	10	2	2		
Bronchoscopy	42	5	4		1
Total	133	43	28	12	3

¹ 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 1) The source and type of mycoplasma isolated are recorded in Table III. Eleven myco-

TABLE II
CONCENTRATION OF MYCOPLASMA ORGANISMS IN 42 POSITIVE SPECIMENS

Source	No. of Specimens with Indicated No. of Colonies/0.1 ml		
	< 10	10-100	> 100
Nose	1	1	
Throat	14 ^{1,2}	8 ²	12
Sputum		1	1
Bronchoscopy	2	3	

¹ Includes two isolates that were recovered initially only in liquid medium containing 0.1% arginine.

² One throat specimen contained mycoplasmas of two types.

TABLE III
DISTRIBUTION OF MYCOPLASMAS IN 22 CHRONIC BRONCHITIC AND 20 NON-BRONCHITIC PATIENTS (BRONCHOSCOPY PATIENTS OF GROUP 1)

Source and Type of Mycoplasma Isolated	No. of Patients	
	Bronchitic	Non-bronchitic
Throat isolates ¹	9	12
<i>M. salivarium</i>	9	8
<i>M. hominis</i>		2
<i>M. orale</i> type 1		2
Bronchoscopy isolates	2	3
<i>M. salivarium</i>	2	2
<i>M. orale</i> type 1		1
Total no. of mycoplasmas isolated	11	15
Total no. of patients examined	22	20

¹ Two organisms were recovered from one non-bronchitic patient.

TABLE IV
FINDINGS IN FIVE PATIENTS WHOSE BRONCHOSCOPY SPECIMENS YIELDED MYCOPLASMAS

Patient No.	Source	Mycoplasmas		Polymorpho- nuclear Cells Seen on Smear of Bronchial Aspirate	<i>Diplococcus pneumoniae</i> on Routine Culture of Bronchial Aspirate	Diagnosis
		Colonies/0.1 ml. of Specimen	Identification			
1	Throat Bronchoscopy	73 16	<i>M. salivarium</i> <i>M. salivarium</i>	—	—	Carcinoma bronchus
2	Throat Bronchoscopy	2 14 3	<i>M. salivarium</i> <i>M. hominis</i> <i>M. orale type 1</i>	—	—	Carcinoma bronchus
3	Throat Bronchoscopy	96 1	<i>M. salivarium</i> <i>M. salivarium</i>	—	—	Suspected carcinoma —not confirmed
4	Throat Bronchoscopy	3 35	<i>M. salivarium</i> <i>M. salivarium</i>	+	+	Chronic bronchitis
5	Throat Bronchoscopy	2 87	<i>M. salivarium</i> <i>M. salivarium</i>	+	+	Chronic bronchitis

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).

TABLE V
RELATIONSHIP OF CLINICAL STATUS OF 26 CHRONIC BRONCHITIC PATIENTS (GROUP 2) TO ISOLATION OF MYCOPLASMAS FROM THEM

Source	Acute Respiratory Illness (no. isolations/ no. specimens)	Quiescent Phase (no. isolations/ no. specimens)
Nose	2/15	0/4
Throat .. .	7/14	6/7
Sputum .. .	2/9	0/1
Total	11/38	6/12

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.

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.

SEROLOGICAL STUDIES The 50 patients in group 2 who suffered from chronic bronchitis each had two or more serum specimens submitted for mycoplasma antibody studies. A total of 205 sera were examined and, of these, 70 were obtained during episodes of acute respiratory illness and 129 during quiescent periods (the clinical condition was not recorded at the time of collection of six specimens). The relationship of the rise in anti-

TABLE VI
RELATIONSHIP OF RISE IN MYCOPLASMA ANTIBODY TITRE TO CLINICAL CONDITION OF CHRONIC BRONCHITIC PATIENTS

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

¹Total no. of possible rises was calculated by observing the no. of clinical exacerbations during the time of serological study.

²P < 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. A

TABLE VII

MYCOPLASMA STUDIES IN CHRONIC BRONCHITIS AND OTHER RESPIRATORY ILLNESSES

Investigators	Findings
Morton, Smith and Keller (1952) Carilli <i>et al.</i> (1964)	10 bronchoscopy samples were examined and none yielded mycoplasmas 4 of 46 (8.7%) acute respiratory illnesses in chronic bronchitis patients were associated with probable <i>M. pneumoniae</i> infection
Rytel, Conner, Welch, Kraybill, Edwards, Rosenbaum, Frank, and Miller (1964)	173 patients were bronchoscoped and 45 mycoplasma strains were isolated. These comprised 35 from 108 bronchiectasis patients, 6 from 42 bronchopneumonia patients, 2 from 7 chronic bronchitic patients, and 2 from 11 patients with other pulmonary conditions
Ross <i>et al.</i> (1966)	No rises in titre of complement-fixing antibody to <i>M. pneumoniae</i> were noted in 125 acute exacerbations of chronic bronchitis
Hers and Masurel (1967)	26 exacerbations of chronic bronchitis and emphysema were associated with <i>M. pneumoniae</i> infection
Organick and Worman (1967)	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
Lambert (1968)	Complement-fixing antibody to <i>M. pneumoniae</i> was noted in 16.4% of 566 normal subjects and in 35% of 137 chronic bronchitic patients
Inglis, Davies and Andrews (1969)	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

third possibility is that mycoplasmas have no great part in the long-term chronic bronchitis syndrome but that infection with a specific mycoplasma might be responsible for a particular exacerbation. This situation would seem to occur when chronic bronchitic patients are initially infected with *M. pneumoniae*. In Table VII a summary is presented of mycoplasma studies in chronic bronchitis and some other respiratory illnesses. The findings of Carilli *et al.* (1964) and Hers and Masurel (1967), as well as those of the present study, suggest that *M. pneumoniae* infection in a patient with chronic bronchitis is frequently associated with an acute clinical exacerbation. Whether other mycoplasma serotypes 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 mycoplasmas. 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°C for four years. The observations of Addey, Taylor-Robinson, and Dimic (1970) indicated that mycoplasmas survived for years at -70°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 isolation 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 measurement of antibody in rabbit antisera but less so

with acute infection sera (Purcell, Wong, Chanock, Taylor-Robinson, Canchola, 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 bronchitis. 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 mycoplasmas 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 participation 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 chronically infected by *M. gallisepticum* had rises in the titre of antibody to this mycoplasma during acute infections with infectious bronchitis virus. Furthermore, Reed (1971) has found an increased multiplication of *M. hyorhinae* in pig and calf tracheal organ cultures infected by swine influenza virus.

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