Mycoplasmas and rheumatoid arthritis

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The view that rheumatoid arthritis may have an infective aetiology dates from the 1930s at least, and enjoys a fluctuating popularity. It is based on some similarities between rheumatoid arthritis and diseases known to be caused by infective agents (Hill, 1968; Ford, 1963, 1968; *Lancet*, 1970; Buchanan and Dick, 1972) or on the similarity of the pathological changes in human rheumatoid arthritis compared with those in arthritis of animals known to be caused by mycoplasmas (Sabin, 1939a,b; Sharp, 1964; Sharp and Riggs, 1967; Ross and Switzer, 1968). The response of mycoplasmal arthritis in mice (Sabin and Warren, 1940; *British Medical Journal*, 1965) and of rheumatoid arthritis in man (Ford, 1968) to treatment with gold salts has been taken as suggesting a mycoplasmal cause of the latter disease. Recent work (Smiley, Sachs, and Ziff, 1968) has shown a substantial local, committed, synthesis of immunoglobulins in the synovium, but the nature of the stimulating antigen is unknown.

Many attempts have been made to isolate mycoplasmas from specimens of synovial fluid or synovial membrane from patients with rheumatoid arthritis. A summary of some attempts over the last 12 years is shown in Table I. It will be seen that results were variable, sometimes with higher isolation rates from non-RA cases or controls. Those series with high rates of isolation in cell-free media are Fahlberg, and others (1966), Williams (1968), Marcolongo and others, (1969), and Jansson and others (1971a,b). Other workers used cell culture inoculation as a preliminary step to cultivation on cell-free media. Williams (1968) identified his isolates as strains of *Mycoplasma fermentans*, and Jansson and her co-workers (1971a) showed their small-colony mycoplasmas to be related serologically to *Mycoplasma arthritidis*.

The existence of the more striking positive reports made it imperative to try and repeat the results. The present report describes a series of attempts to isolate mycoplasma from specimens of rheumatoid synovial membrane and synovial fluid over a period of 8 years from 1964 to 1972. A wide variety of techniques and media was used.

Materials

The synovial fluids were collected at the Rheumatic Diseases Unit, Northern General Hospital, Edinburgh. The specimens of synovial membrane were taken at operation at the Princess Margaret Rose Hospital, Edinburgh. Patients were considered to have rheumatoid arthritis if they fulfilled the criteria of the American Rheumatism Association (1959), with or without the demonstration of rheumatoid factor in the serum. 176 patients with rheumatoid arthritis were examined; in 108 the sensitized sheep cell test was positive, and in 56 it was negative; in twelve the test had not been carried out. Any patients with 'doubtful' rheumatoid arthritis or juvenile rheumatoid arthritis have been excluded. Control material from 47 patients with non-rheumatoid conditions included specimens of normal synovial membrane removed at meniscectomy (fourteen patients); membrane removed from two patients with osteoarthritis and one with osteochondritis; also synovial fluids from nineteen patients with osteoarthritis, four with psoriatic arthropathy, three with polymyalgia rheumatica, one with intermittent hydrarthrosis, one with rheumatic fever, one with ankylosing spondylitis, and one with erythema nodosum. Lastly, 15 fluids from twelve patients with Reiter's disease were examined.

Methods

The synovial fluids were mostly inoculated into media in the undiluted form; in the majority of experiments large sampling volumes were used, but in some, dilutions of the fluids were also inoculated. The synovial membranes were originally homogenized by a mechanical blender, but were later minced finely with sterile instruments before being added to the appropriate medium.

The technique consisted of culturing the clinical samples in various liquid or semi-solid media, with or without inhibitors, and then subculturing onto supplemented PPLO agar. Some preparations were also inoculated directly onto supplemented PPLO agar. Some sets of specimens were cultured aerobically, some anaerobically, and some in 10% CO₂ in air. Specimens in series 1 were incubated for 8 weeks before being discarded as negative; in later series this period was reduced to 3 weeks, except for series 4 in which the plates were incubated for 6 weeks.

The liquid media used were the following:

- (a) Eagle-Hanks tryptose phosphate broth (Mackay, 1966).

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- (b) Supplemented semi-solid PPLO agar (Stewart and Chowdry, 1968).
- (c) Supplemented PPLO broth at $\times 2$ normal concentration to allow the addition of an equal quantity of synovial fluid (Stewart, Alexander, and Duthie, 1969).
- (d) As (c) but with the addition of 2% (w/v) NaCl to detect possible L-forms (Stewart and others, 1969).
- (e) Heated blood broth (Stewart and others, 1969).
- (f) Bullock heart broth (based on Williams, 1968) with supplements as for (c) but at half concentrations.
- (g) Diphasic medium prepared from single strength PPLO broth/agar supplemented as for (c) but at half strength and with the addition of 20% (v/v) of a pancreatic digest of bovine lung (S. M. Stewart, unpublished data).
- (h) Bovine heart infusion broth and agar (Jansson, 1971) which included 1% (v/v) pasteurized egg yolk.

Table I Reports of the isolation of mycoplasmas from patients with rheumatoid arthritis, Reiter's syndrome, and from control materials

Reference	Isolation method	Specimen*	Diagnosis†	Isolation rate‡	Identity of mycoplasma
Jonsson (1961)	Cell free (CF) culture	S/F S/F	RA Control	0/12 12/25	— Unidentified
Arai, Ishikawa, and Hotta (1964)	CF culture	S/F S/F	RA Control	8/12 0/19	Unidentified —
Bartholomew and Himes (1964)	Cell culture	S/F S/F S/F	RA Reiter's Control	5/7 3/4 0/6	At least 4 antigenic groups —
Claus, McEwen, Brunner, and Tsampanlis (1964)	CF culture	S/F	RA	0/8	—
		S/F	Reiter's	0/11	—
		S/F	Control	0/11	—
		S/M	RA	0/6	—
		S/M S/M	Reiter's Control	2/8 0/10	Unidentified —
Bartholomew (1965, 1967)	Cell culture	S/F	RA	6/7	2 <i>M. hyorhina</i> 1 <i>M. hominis</i> 1 <i>M. arthritidis</i>
		S/F	Reiter's	3/4	
		S/F	Control	0/7	
Barnett, Balduzzi, Vaughan, and Morgan (1966)	Cell culture	S/F and S/M	RA	0/17	—
Fahlberg, Moore, Redmond, and Brewer (1966)	CF culture	S/F	RA	22/24	Unidentified—10 antigenically homogeneous
		S/M	Control	2/14	
Williams (1968)	CF culture	S/F	RA	36/90	<i>M. fermentans</i> 1 <i>M. fermentans</i> 2 <i>M. hominis</i> 2 Unidentified
		S/F	Control	5/26	
Marcolongo, Carcassi, Bianco, Bravi, Di Paolo, and Lunghetti (1969)	CF culture	S/F	RA	1/60	Unidentified
		S/F	Control	0/10	—
		S/M	RA	43/58	Unidentified
		S/M	Control	0/10	—
Fraser, Shirodaria, Haire, and Middleton (1971)	Cell culture	S/M	RA	10/17	Unidentified
	CF culture	S/M S/M	Control RA	1/5 0/15	Unidentified —
Jansson, Mäkisara, Vainio, Vainio, Snellman, and Tuuri (1971a)	CF culture and egg inoculation	S/F and S/M	RA	40/88	Mostly small colonies serologically related to <i>M. arthritidis</i>
		S/F and S/M	Control	0/24	
Jansson, Wallgren, Wegelius, and Tuuri (1971b)		S/F	JRA	2/2	

* S/F = synovial fluid; S/M = synovial membrane.

† RA = rheumatoid arthritis; JRA = juvenile rheumatoid arthritis.

‡ No. positive/total.

All the media, excluding (a) and (e), contained yeast extract prepared according to the method of Hers (Marmion, 1967). Media (a), (b), (c), (d), (e), and (f) were subcultured onto PPLO agar supplemented as for liquid medium (f). The solid medium used for cultures from medium (g) also contained 20% (v/v) pancreatic digest of bovine lung; that from (h) was equivalent to the broth with the exclusion of the egg yolk.

Details of the serum and atmospheric conditions of incubation for the various series of specimens are shown in Table II. The media used in series 2 and 3 did not contain any inhibitors.

These media were designed primarily to grow 'large colony' mycoplasmas rather than T-strains; the latter would have grown on some of the media without inhibitors and with anaerobic incubation.*

Other tests

(1) Some suspensions of synovial membrane in series 1 were inoculated directly onto supplemented PPLO agar plus horse serum and incubated aerobically, anaerobically, or in 10% CO₂ in air.

(2) 15 specimens of synovial fluid were frozen and thawed before inoculation into liquid medium (series 2).

(3) Cell cultures of synovial fluids (Mackay, Panayi, Neill, Robinson, Smith, Marmion, and Duthie, 1974) and cell cultures of synovial membranes prepared by trypsinization, in a similar manner to that described by Grayzel and Beck (1970), were also examined for mycoplasmas (series 4).

(4) Metabolic inhibition tests were carried out on sera from patients with rheumatoid arthritis and, as controls, on sera from patients with chronic bronchitis. The controls were matched for age and sex, and the blood samples

* Detailed information regarding the media may be obtained from S.M.S.

were collected shortly after those from the rheumatoid patients. *Mycoplasma hyorhinis* and *M. fermentans* were used as the test organisms.

Results

Liquid or semi-solid medium cultures of synovial membrane and synovial fluid

Table II shows the type of media and the atmospheric conditions of incubation of the subcultures on solid medium, and the number of specimens examined from patients with rheumatoid arthritis, with Reiter's syndrome, and with other conditions (non-RA) in each of the 5 series of experiments together with the dates of starting and finishing.

Of a total of 133 examinations of 88 synovial membranes and 139 examinations of 119 synovial fluids from RA patients, only one yielded a culture of mycoplasma. This was a specimen of synovial membrane from a seropositive patient and it had been cultured in Eagle-Hanks tryptose phosphate broth with swine serum. The isolate was shown to be *M. hyorhinis*, a species from swine that could have been introduced with the swine serum used in the medium. No mycoplasmas were isolated from any of the other examinations of specimens from patients with rheumatoid arthritis, nor from the 18 examinations of 15 fluids from twelve patients with Reiter's syndrome, nor from the 28 examinations of 19 membranes and 44 examinations of 34 fluids from non-rheumatoid patients.

Small bodies were seen on and in the egg yolk PPLO agar medium used in series 5, recalling the colonies

Table II *Details of liquid and semi-solid media and number of specimens tested in the 5 series*

Series no.	Liquid or semi-solid medium	Serum in medium	Atmosphere*	Synovial membranes		Synovial fluids			Period of investigation
				RA†	Non-RA†	RA†	Reiter's	Non-RA†	
1	Eagle-Hanks Semi-solid agar	Swine Swine	Aerobic Aerobic	12 9	1 0	0 0	0 0	0 0	25 Feb 1964– 30 June 1964
2	Eagle-Hanks Eagle-Hanks Eagle-Hanks PPLO broth × 2 PPLO broth × 2 + 2% NaCl Heated blood broth Bullock heart Semi-solid agar	Horse Swine Human Human Human Horse (blood) Horse Human	10% CO ₂ /air 10% CO ₂ /air 10% CO ₂ /air 10% CO ₂ /air 10% CO ₂ /air 10% CO ₂ /air 10% CO ₂ /air 10% CO ₂ /air	7 18 22 0 14	6 4 9 0 3	0 0 0 54 22	0 0 0 10 3	0 0 0 19 7	16 Nov 1965– 13 Feb 1968
3	Eagle-Hanks PPLO × 2 + 0.5% Tween 80	Human Human	10% CO ₂ /air 10% CO ₂ /air	17 10	0 0	0 0	0 0	0 0	1 Oct 1969– 11 Feb 1970
4	Diphasic	Horse	Aerobic and anaerobic	0	0	12	1	4	5 Jan 1972– 22 Feb 1972
5	Brain heart infusion broth + egg yolk	Human	Anaerobic	0	0	7	0	1	12 Apr 1972– 28 Apr 1972
Total no. of examinations				133	28	139	18	44	
Total no. of specimens examined				88	19	119	15	34	

* Atmospheric condition of incubation of subcultures on solid medium.

† RA = rheumatoid arthritis; Non-RA = conditions other than rheumatoid arthritis or juvenile rheumatoid arthritis (for diagnoses, see Materials).

described by Jansson (1971); but similar bodies were seen on and in medium inoculated from control egg yolk broth.

Direct inoculation of synovial membrane suspension onto supplemented PPLO medium

In series 1 and 3, 59 suspensions of synovial membrane from patients with rheumatoid arthritis and 12 from patients with non-rheumatoid conditions were inoculated directly onto plates containing supplemented PPLO medium with horse serum (series 1) or human serum (series 3). Of the 59 rheumatoid specimens, 26 were incubated aerobically, 8 anaerobically, and 25 in 10% CO₂ in air; 8 of the non-rheumatoid specimens were incubated aerobically and 4 anaerobically. In no case was a mycoplasma isolated.

Effect of freezing and thawing on the isolations from synovial fluids

Fifteen specimens of synovial fluid (in series 2) from patients with rheumatoid arthritis were frozen and thawed before being inoculated. 4 of these were inoculated into double strength supplemented PPLO broth and 11 into semi-solid PPLO agar. In all cases human serum was used and subcultures were incubated in 10% CO₂ in air. No mycoplasmas were isolated.

Aerobic and anaerobic cultures of cells and supernatants from cell cultures from synovial fluids and synovial membranes

No mycoplasmas were isolated from cultures of the cells or the fluid phase of cell cultures established from 9 synovial fluid specimens from patients with rheumatoid arthritis, nor from two sets of specimens from non-rheumatoid patients. Direct inoculation of the cell deposits from the synovial fluids onto supplemented PPLO medium also failed to yield mycoplasmas. Similarly, no mycoplasmas were isolated from the cell cultures of 7 synovial membranes from six patients with rheumatoid arthritis.

Subcultures from PPLO egg yolk broth into fresh egg yolk broth

No mycoplasmas were isolated on solid medium from PPLO egg yolk broth subcultures from the initial PPLO egg yolk broths inoculated with synovial fluid

from four patients with rheumatoid arthritis (series 5).

Metabolic inhibition tests

Table III shows the metabolic inhibiting titres in the sera from rheumatoid and non-rheumatoid patients using *M. hyorhinis* and *M. fermentans* as antigens. There was no significant difference in the distribution of antibody titres between the sera originating from rheumatoid and non-rheumatoid patients.

Discussion

Mycoplasmas are present as commensals in humans (Edward and Freundt, 1969) and at least one strain (*Mycoplasma pneumoniae*) is known to be pathogenic. They also occur widely both as commensals and as pathogens in a variety of animals (Edward and Freundt, 1969).

They are frequently found as contaminants in cell cultures (Hayflick, 1965; Stanbridge, 1971). They may come from the medium constituents, from the original cell line, from contamination of the cell line with other cells carrying mycoplasmas, or from the handlers. Therefore, the reports of isolations of mycoplasmas after the inoculation of cell cultures with specimens from patients with rheumatoid arthritis must be treated with reserve. Most authors who have used cells for isolation of mycoplasmas acknowledge this fact. Even though control cell cultures do not yield mycoplasmas, it is possible that the addition of clinical tissue specimens may increase the population of mycoplasmas already present in the cell culture and allow growth on sub-cultivation to cell-free medium. Fraser and others (1971) isolated mycoplasmas from 10 synovial cell cultures from RA patients and from one cell culture from a patient with another condition. The isolates were tentatively identified as *Mycoplasma laidlawii* and *M. hyorhinis*. These species were found as cell culture contaminants (Barile and Kern, 1971; Stanbridge, 1971). A subsequent epidemiological analysis of the dates of isolation of the mycoplasmas provided circumstantial evidence of cross-contamination from cell lines introduced into the laboratory, unsuspectingly carrying these mycoplasmas (K. B. Fraser, personal communication, 1973). Nevertheless, it is recognized that

Table III Results of metabolic inhibition tests on sera from patients with rheumatoid arthritis (RA) and non-rheumatoid conditions (non-RA)

Test organism	Diagnosis	No. of sera tested	Metabolic inhibition titre—reciprocal						
			<6	6	12	24	48	96	>96
M. hyorhinis	RA	20	4	5	6	2	0	3	0
	Non-RA	20	7	5	2	2	1	1	2
M. fermentans	RA	12	12	0	0	0	0	0	0
	Non-RA	7	6	0	1	0	0	0	0

there are mycoplasmas that will grow in cell culture systems and only poorly or not at all in cell-free media; see for example the history of the isolation of *Mycoplasma suipneumoniae* (Goodwin and Whittlestone, 1966).

Most of the investigations with cultures of rheumatoid synovial fluid or membrane direct onto cell-free culture media have been concerned with small numbers of specimens; in most the few isolates were not identified (see Table 1). The synovium and its blood vessels are recognized to be effective traps or filters for particles circulating in the blood, e.g. immune complexes, bacteria, and the presence of antigenically diverse mycoplasmas in small numbers of specimens might be explained on that basis. However, the findings in two published series, namely those of Williams (1968) and Jansson and her co-workers (1971a), of a single antigenic type of mycoplasma in a high proportion of RA cases compared with controls is not easily dismissed with such an explanation.

Williams (1968) isolated *M. fermentans* from 40% of 90 specimens from patients with rheumatoid arthritis, but from only one of 26 specimens of control material, though other serotypes of mycoplasma were isolated from 4 other specimens of control material. He also reported metabolic inhibiting antibodies to *M. fermentans* in 72% of rheumatoid patients with rheumatoid factor, and in 36% of seronegative rheumatoid patients, and 28% of persons without RA. Further presumptive evidence of the involvement of *M. fermentans* in rheumatoid arthritis was that the migration of leucocytes taken from rheumatoid patients was inhibited in the presence of *M. fermentans* antigen (Williams, Brostoff, and Roitt, 1970; Williams and Bruckner, 1971).

The present work included methods that would be expected to isolate strains of *M. fermentans*, though the method of sucrose density gradient treatment of the specimens was not used in our investigations. The failure to isolate *M. fermentans* is in accord with the recent negative reports of Cole, Ward and Smith (1973), and Windsor, Nicholls, Maini, Edward, Lemcke, and Dumonde (1974), who did use a preliminary gradient centrifugation, and also those of Mardh, Nilsson, and Bjelle (1973). We have no explanation for the discrepancy between the original positive reports of Williams and the negative findings in this and other studies, but the role of *M. fermentans* in RA must surely be regarded as 'non proven'.

Jansson's early work (Jansson and Wager, 1967) reported the isolation of large-colony strains of *M. arthritidis*. In later series, small-colony isolates were reported (Jansson and others 1971a); these were serologically related to *M. arthritidis*. Similar small-colony mycoplasmas were isolated from leukaemic bone marrow (Jansson, Vainio, and Tuuri, 1971c). The small colonies described by Jansson are of a very special nature and are unlikely to be detected with

conventional media examined under the low power of the microscope. It would probably be necessary to have greater experience of handling these special variants before recognizing them on isolation from specimens. Though we used a similar method and medium as Jansson for the examination of some of our specimens, we did not see any clear-cut examples of colonies similar to those described by her. Though there were structures that resembled small-colony mycoplasmas, these did not produce colonies on subculture, and similar bodies were seen both on the surface and in the medium of plates inoculated with control uninfected broths. The results described by Jansson are interesting, but our methods and experience were not adequate to confirm or refute Jansson's findings; we feel that some additional attribute to simple morphological identification, e.g. incorporation of radioactive isotope labelled precursors into the colonies or immunofluorescence with *M. arthritidis* antiserum, would be required for a confident identification of these special structures as mycoplasmas.

The establishment of cells from synovial membranes and fluids in culture may have the advantage of diluting antibody in the tissues which may be inhibitory to the mycoplasmas. But in spite of using this method in some of the present series and also culturing dilutions of synovial fluids and synovial membrane suspensions, no mycoplasmas were isolated.

The failure to show any difference in the range of metabolic-inhibiting antibodies against *M. fermentans* in patients with rheumatoid arthritis and in control subjects is in accord with results reported by Chanock, Purcell, and Decker (1967) and Mardh and others (1973), though they are at variance with those reported by Williams (1968). The range of titres in sera from control subjects in the present series was similar to that found by Mardh and others (1973). Chanock and others (1967) also found no difference in the two series of sera using *M. hyorhinis* as the test organism.

Since Sabin's early report of the association of mycoplasmas with a chronic arthritis in mice (1939a), many attempts have been made to show that these organisms play an aetiological role in rheumatoid arthritis in humans. The fact that many mycoplasmas are difficult to isolate on artificial culture medium means that negative results must be treated with caution. Nevertheless, the absence of confirmation, in this and other series, of the findings of the more impressive series reported in the literature casts much doubt on the aetiological role of conventional mycoplasmas in rheumatoid arthritis.

Summary

Attempts were made, using a variety of culture media, to isolate mycoplasma from 88 synovial membranes and 119 synovial fluids from patients with definite rheumatoid arthritis. *M. hyorhinis* was isolated from one culture of a synovial membrane, but the medium,

which contained swine serum, may have been the source of the organism. No other mycoplasmas were isolated from the rheumatoid specimens examined, nor from 19 membranes and 34 fluids from patients with non-rheumatoid conditions, nor from 15 fluids from patients with Reiter's disease. Though an unconventional type of mycoplasma may be shown to be involved in the aetiology of rheumatoid arthritis, this has yet to be substantiated.

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