Search for mycoplasma in synovial fluids from patients with rheumatoid arthritis

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The isolation of *Mycoplasma fermentans* from over 40 per cent. of synovial fluids taken from patients with rheumatoid arthritis has been reported by Williams (1967a, b; 1970). Although other studies have suggested an association between this disease and other species of mycoplasma (Bartholomew, 1965; Jansson and Wager, 1967; Jansson, Mäkisara, Vainio, Vainio, Snellman, and Tuuri, 1971; Fraser, Shirodaria, Haire, and Middleton, 1971), the possibility that *M. fermentans* was associated with rheumatoid arthritis was supported by the finding that there was inhibition of peripheral leucocyte migration in the presence of extracts of *M. fermentans*, indicating the existence of a specific immunological response (Williams, Brostoff, and Roitt, 1970). In view of the importance of such an association between *M. fermentans* and rheumatoid arthritis, we have attempted to isolate mycoplasmas from synovial fluids by techniques including those described by Williams.

Methods

Source of synovial fluids

Synovial fluids were obtained from knee effusions by needle aspiration under aseptic conditions from 43 patients with rheumatoid arthritis and five non-rheumatoid patients (Table I). The patients with rheumatoid disease were categorized according to the American Rheumatism Association criteria (1959) as follows: 'Classical' 28; 'Definite' 10; 'Probable' 5. All patients had active polyarthritis and the duration of disease in ten was less than 1 year; in twelve between 1 and 5 years; and in 21 greater than 5 years. At the time of the aspiration no patients were receiving antibiotics and none had been treated with gold or chloroquine in the preceding 12 weeks. Altogether 68 synovial fluids were obtained from 43 rheumatoid patients, in many instances by aspiration of both knees during the same session, and in a few on a second aspiration following reaccumulation of fluid. Synovial fluids from knee joints were also obtained from five non-rheumatoid patients whose diagnosis is detailed in Table I.

The fluids were placed in sterile bijoux bottles and

Table I Rheumatoid arthritis patients (total 43)

(1) Disease classification (A.R.A.)*:	Classical	28
	Definite	10
	Probable	5
(2) Duration of disease (yrs):	Less than 1	10
	1-5	12
	>5	21
(3) Total number of synovial fluids		68

Non-rheumatoid patients (total 5)

Osteoarthritis	3
Reiter's syndrome	1
Acromegaly	1

* American Rheumatism Association criteria (see reference).

transported at ambient temperature for direct inoculation into a variety of media (Table II) and for Series I of the density gradient separations (Table III). For Series II and III of the gradient separations, the aspirates were transported on ice. The duration between aspiration and setting

Table II Direct inoculation and culture of synovial fluids in mycoplasma media: cultural procedures employed in two independent laboratories

Inoculation*	Synovial fluids (neat and 1/10 dilution)		
	Agar plates (AER and AN)†	Semi-solid medium	T-broth
Observation period (days)	14-28	14-28	14
Subcultures	—	Broth to plates (AN) on 7th and 14th day	—
Results	Eleven rheumatoid and three non-rheumatoid fluids examined: No mycoplasmas isolated		

* Details of media in text.

† AER = Aerobic culture; AN = Anaerobic (95 per cent. N₂/5 per cent. CO₂) culture.

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Table III Culture of synovial fluids separated on sucrose gradients: (details of procedure)

Series	I	II	III
Transport (temperature)	Bijou (ambient)	Bijou (ice)	Bijou in sachet (ice)
Inoculation of visible bands (a) (b)	Plate (AN) Semisolid (0.1 ml.)*	Plate (AN) Broth (0.2 ml.)	Plates (AER and AN)† Broth (0.2 ml.)
Subcultures	Semisolid culture onto Plates (AN) on Day 4, 7, and 14	Original broth culture into broth and onto Plates (AN) Day 7, 14, and 21	

* Volume of gradient inoculated into liquid medium.

† AER = Aerobic culture; AN = Anaerobic (95% N₂/5% CO₂) culture.

up of density gradients or direct inoculation into media was 2 to 4 hrs, except for 22 out of 33 synovial fluids in Series I (Table IV) which were stored at -70°C. before density gradient separation and subsequent culture.

Table IV Cultural results on synovial fluids separated on sucrose gradients

Series	I	II	III	Total
No. of fluids from (a) Rheumatoid patients (b) Non-rheumatoid patients	29 4	17 1	18 0	64 5
No. of bands cultured	65	41	43	149
Acid colour changes	3	15	4	22
Evidence of bacterial contamination*	1	16	4	21
Mycoplasma isolations	0	0	0	0

Series I, II, and III refer to classification in Table III.

* See text.

Media

The composition of the media was basically that described by Chanock, Hayflick, and Barile (1962). For liquid and semi-solid media (the latter containing 0.07 per cent. Oxoid Ionagar No. 2), the serum supplement was horse serum (20 per cent., v/v). Glucose (0.1 per cent, w/v) and phenol red (0.002 per cent., w/v) were included, as well as penicillin (200 units/ml.) and thallous acetate (1/4,000) as bacterial inhibitors. Three types of solid medium were used, one containing horse serum and inhibitors at the concentrations given above, a second containing horse serum but no inhibitors, and a third with pig serum (20 per cent., v/v) and inhibitors. In addition, T-broth, a medium for T-strain mycoplasmas based on the formula of Csonka, Williams, and Corse (1966), with added urea (0.1 per cent., w/v) and phenol red was used. Horse sera, yeast extract, and agar constituents were pre-tested for their ability to support growth by counting colony-forming units in cultures of *M. pneumoniae*, *M. gallisepticum*, and *M. synoviae*. Other constituents were tested in liquid and solid media by passage of *M. fermentans* (PG18) and seven other stock strains.

The composition of the sucrose density gradients was similar to that described by Williams (1970) and comprised—1 ml. 90 per cent., 0.5-ml. amounts of 85, 80, 75,

and 70 per cent., and 1 ml. 50 per cent. sucrose. After equilibration overnight at 4°C., 1 ml. synovial fluid was layered onto the gradient and centrifuged for 2 hrs at 20,000 r.p.m. in a Spinco SW39 rotor. The centrifuge tube containing the separated synovial fluid was placed over a light source and each visible band (usually two or more per specimen) was removed separately, from the top downwards, in approximately 0.2-ml. amounts drawn up into a 1-ml. syringe through a blunt needle with the tip bent at an angle of 90° (Williams, personal communication).

Results

(i) Direct inoculation of synovial fluids into culture media

Fourteen synovial fluids (11 rheumatoid and 3 non-rheumatoid) were each divided into two, and one each was cultured in the laboratories at Beckenham and at County Hall, London, by direct inoculation into solid and liquid mycoplasma media (Table II). Each synovial fluid was plated onto three different agar media, into semi-solid medium, and T-broth. In both laboratories, semi-solid cultures were further sub-cultured onto agar plates after 7 and 14 days' incubation and in one laboratory additional sub-cultures into semisolid medium were also performed. No mycoplasmas were isolated.

(ii) Culture of synovial fluids after density gradient centrifugation

The 69 synovial fluids cultured after separation on density gradients can be separated into three series (Tables III and IV).

SERIES I

33 fluids were transported at ambient temperature and 0.1-ml. amounts from each visible band were inoculated into solid and semisolid media. From these fluids a total of 65 bands were cultured (Table IV).

SERIES II

The fluids were transported on ice and the cultural methods were more closely aligned with those of Williams (personal communication) in that 0.2 ml. of each visible band was inoculated into 2 ml. broth

contained in a 2-ml. vial. In contrast to Series I, there were acid colour changes in the majority of the inoculated broths (Table IV) and these were associated with the subsequent culture on mycoplasma agar of *Achromobacter* species. The colour changes were detected between 10 and 25 days after inoculation and were not associated with the turbidity usually encountered with bacterial contamination. The colour changes were not obtained in further passages.

SERIES III

To minimize possible sources of bacterial contamination, centrifuge tubes were gamma-irradiated and the bijou bottles containing the synovial fluids were transported in watertight sachets on ice. The cultural procedures were similar to those in Series II (Table III) but there was a marked reduction in the number of bacterial isolations from the bands cultured in Series III (Table IV). All four isolates from Series III were characterized as *Flavobacterium aquatile*.

No mycoplasmas were isolated from the synovial fluids in any of the three series.

(iii) Deliberate inoculation and recovery of *M. fermentans* from synovial fluids

To confirm that our techniques were capable of isolating *M. fermentans*, frozen broth cultures of the organism were placed on density gradients and centrifuged. The gradients were then sampled either by collecting fractions of the gradient allowed to drip through the punctured base of the centrifuge tube, or with a syringe by inserting a needle through the side wall of the tube containing the gradient at five different levels. Culture of these samples showed that colony-forming units of mycoplasma were distributed throughout the gradient but were most concentrated when taken from one of the visible bands (see Figure, band B). A total of six rheumatoid synovial fluids stored at -70°C . from which no *M. fermentans* had been previously isolated were deliberately inoculated with *M. fermentans* cultures; three were incubated for 1 hr at 37°C . and three were placed on ice for $2\frac{1}{2}$ hrs before layering on gradients. *M. fermentans* was recovered from the gradients after centrifugation in all six instances. The number of organisms was pre-determined in the three fluids

FIGURE Photograph of sucrose density gradients after centrifugation at 20,000 r.p.m. for 2 hrs, each overlaid with 1 ml. of the following:

- (1) Synovial fluid from a patient with rheumatoid arthritis
- (2) Frozen and thawed broth culture of *M. fermentans* (line B is associated with a peak in the number of colony forming units; line A is produced in uninoculated media by freezing and thawing)
- (3) Uninoculated broth

kept on ice, and it was estimated that 4×10^2 to 4×10^3 colony-forming units had been applied to the gradient.

Discussion

Reports on the recovery of mycoplasmas from human synovial fluids need to be interpreted with caution. When mycoplasmas have been isolated from synovial fluids or membranes inoculated into tissue cultures (Bartholomew, 1965; Fraser and others, 1971), or from primary cell cultures initiated from synovial membranes (Fraser and others, 1971), there are several possible sources of contamination, including commercial bovine serum (Barile and DelGiudice, 1972). Tissue cultures are notoriously prone to mycoplasma contamination (Stanbridge, 1971) and the incidence has been noted in some surveys to be as high as 70 to 80 per cent. (Sethi, 1972). *M. hyorhinis* was the organism most frequently isolated by Bartholomew (1967), and this mycoplasma (of porcine origin) is particularly regarded as a tissue culture contaminant (Sabin, 1967). It is noteworthy that some of the strains isolated by Fraser and others (1971) were also identified as *M. hyorhinis* by Taylor-Robinson (1972) and another strain was identified by themselves as *M. laidlawii*, another known tissue culture contaminant (Barile and Kern, 1971). Jansson and Wager (1967) reported the isolation of *M. arthritidis* (a rat mycoplasma) from two patients with rheumatoid arthritis. The minute colonies obtained by Jansson and others (1971) from synovial fluids plated on media containing egg yolk have not yet been unequivocally shown to be mycoplasmas.

The isolation by Williams in artificial media of *M. fermentans* from 40 per cent. of rheumatoid synovial fluids prompted the present investigation. Nevertheless, using media pre-tested for their ability to support growth of mycoplasmas and the technique described by Williams, we have failed to confirm his findings. Visible bands were consistently seen when synovial fluids or broth cultures of *M. fermentans* (PG18) were centrifuged on sucrose density gradients (see Figure); but we doubt whether this indicates the presence of mycoplasmas in synovial fluid as suggested by Williams (1970). The particles found in rheumatoid synovial fluids by electron microscopy by Williams (1967a, b) and regarded by him as minimal reproductive units of a mycoplasma, fall below the smallest size generally accepted for mycoplasmas (Maniloff, 1969; Lemcke, 1971). Our failure to isolate mycoplasmas seems all the more significant, since a stock strain of *M. fermentans* (PG18) was shown to be capable of surviving sucrose gradient centrifugation and could be consistently recovered from deliberately inoculated synovial fluids.

A fairly high incidence of water-borne species of *Achromobacter* and *Flavobacterium* was noted in this

study. These organisms were especially prevalent when synovial fluids were transported on ice in Series II (Table III). When special precautions were taken to avoid contamination of the outside of the bijou bottles in Series III (Table III), the isolation of these organisms was markedly reduced, and it is therefore likely that the ice was the source of the bacteria. The acid colour changes observed in some broth media (Table IV) were associated with bacteria, but the colour change could not be passaged in broth and no mycoplasmas were detected on subculture on the usual mycoplasma agar plates. The colour changes were probably caused by bacteria although it is possible that synovial exudate cells were concentrated in the visible bands of the density gradient and produced a colour change due to metabolic activity when transferred to the mycoplasma media.

It has been suggested that mycoplasmas may be present only in early cases of rheumatoid arthritis. This study included several patients whose active disease was recent and who were examined at the earliest stage which is likely to present for hospital treatment. Patients receiving any antimycoplasma drugs were excluded from the study.

The detection of cellular hypersensitivity to *M. fermentans* in the leucocyte migration inhibition test (Williams and others, 1970) has been re-examined by us; using similar mycoplasma antigen preparations we have failed to reproduce their finding of inhibition of cell migration only of rheumatoid leucocytes (Maini, Lemcke, Windsor, and Dumonde, 1973). Although inhibition of leucocytes was observed in a large number of rheumatoid subjects, it was also apparent to a similar degree in some entirely healthy individuals and several hospital patients without joint disease. Our findings cast doubt on the assumption that the cell migration results indicate a causal relationship between rheumatoid arthritis and *M. fermentans* infection.

Summary

73 fluids from 43 patients with rheumatoid arthritis and five from non-rheumatoid subjects were examined for mycoplasmas. None was isolated from eleven fluids from rheumatoid joints or three fluids from non-rheumatoid joints by direct culture into a variety of suitable media in two independent laboratories. Visible bands obtained by separation of 64 rheumatoid fluids and five non-rheumatoid fluids on sucrose density gradients (after the technique of Williams) similarly yielded no evidence of mycoplasmas on culture.

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