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Mycoplasmas in cell cultures from rheumatoid synovial membranes

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SUMMARY

Ten strains of myocoplasmas were recovered from cultures of synovium or cultures inoculated with synovial fragments from rheumatoid arthritis and one from osteo-arthritis. The source of the organisms is not known. Patients with rheumatoid arthritis had no complement-fixing antibody and no fluorescent staining antibody against the mycoplasmas isolated and no mycoplasma antigen was detected by immunofluorescence in sections of synovia and in synovial fluids.

The strains isolated were of two main serological types and could be distinguished by direct fluorescent antibody staining from standard types of human commensals and the common tissue-culture contaminants. One may be *Mycoplasma laidlawii*.

INTRODUCTION

Whilst searching in synovium from rheumatoid arthritis for signs of infection by certain enveloped viruses, we noticed that pieces of such synovium always induced acidity in cell cultures into which they were introduced and that mycoplasmas could eventually be recovered from these. Since we had thus inadvertently confirmed observations by Bartholomew (1965) we give our findings here along with some attempts to ascertain the source of the mycoplasmas which were found.

METHODS

Material

Synovium, taken at therapeutic synovectomy from patients with rheumatoid arthritis and from a few operations on osteo-arthritic or injured, non-diseased joints, was placed directly into serum-free sterile cell-culture medium and dealt with in the laboratory within 1 hr. Samples of serum were obtained from the same patients.

Cell cultures

Four systems of cell-culture were inoculated with 10–20 fragments of synovium each about 2 mm. in diameter, because digestion of synovial membrane by trypsin, papain or collagenase did not give a usable cell suspension. Some synovia were put into two or more systems.

System 1. *Inoculation of animal cells.* Fragments of synovium were placed in rabbit, RK 13, and monkey, Vero, cells known to be free of mycoplasmas by staining and by culture and shown subsequently by fluorescent antibody staining to be free also of the types of mycoplasma found in our experiments. Inoculated cells and controls were kept 6 days in growth medium or 14 days in maintenance medium, then scraped off the glass and one-tenth the quantity passed into a fresh monolayer. Six or seven passes were made, the last culture being grown on cover-slips for staining with fluorescent antibodies.

System 2. *Mixed culture with animal cells.* Semi-confluent layers of RK 13 and Vero cells were again used. Since, after 4 days, some outgrowth of cells was visible from synovial fragments, cell suspensions were prepared by trypsinization, divided into three equal volumes and passed in growth medium by conventional methods. At each pass inoculated and control cultures were placed also in maintenance medium and left 12–14 days. Seven, eight or nine such passes were made of various synovial membranes, cover-slips being made of the final culture as in system 1.

System 3. *Human synovial cell cultures.* Synovium did not grow readily in our standard medium, but one of us (P.V.S.) succeeded in initiating cultures in medium containing 30 % foetal calf serum and maintaining them for long periods in Eagle's medium plus 20 % foetal calf serum which was changed at intervals of 4 days for 2 months, then of 10 days for 6 months.

System 4. *Mixed cultures with human cells.* Synovial tissue and cells or supernatant fluids from specimens in System 3 were also added to cultures of human embryo kidney cells and human embryo lung cells prepared in the laboratory by trypsinization of fresh material. Synovial tissue is toxic to these cultures, so the mixed synovial and lung cultures were passed at 4-day intervals in growth medium along with an equal number of fresh lung cells. After 6 or 7 passes, cultures were tested for mycoplasmas.

Culture media

Cells. Eagle's medium (Macpherson & Stoker, 1962) was used throughout. Penicillin only, 100 units/ml., was present in systems 1–3 but mycostatin, 50 units/ml., was added to System 4. Foetal calf serum, tested in liquid medium for absence of mycoplasmas, was used at 2 % concentration for maintenance cultures, 10 % for growth experiments and 20 % or 30 % for synovial cultures. Cells were incubated in 5 % CO_2 at 36° C.

Mycoplasmas. Strains were grown in liquid medium, for inoculation into tissue culture and for use as antigen, and on solid medium for recovery, identification and purification.

Liquid medium was prepared from Difco brain–heart infusion broth, 18·5 g./350 ml. distilled water, autoclaved at 15 lb./in.^{2} and allowed to cool. Sterile, non-inactivated, horse serum No. 3 (Burroughs Wellcome) was added to give a concentration of 20 %, and yeast extract to 10 %. Rabbit serum was used in place of horse serum, when preparing antigen. The yeast extract was prepared from fresh baker's yeast extracted for 30 min. with an equal weight of distilled water at 75° C. at pH 4·5. After centrifugation the supernatant was adjusted to pH 8 and

sterilized by Seitz filtration. Solid medium was prepared from Difco PPLO agar, 17 g./350 ml. distilled water, treated similarly to the liquid medium and supplemented with the same concentration of horse serum and yeast extract.

Ampicillin to a concentration of 1 mg./ml. was added to both media (Hutchinson, 1969).

Attempts were made to isolate mycoplasmas by growing synovial cell colonies in soft agar (Macpherson & Montagnier, 1964) and over cell layers in agarose as described by Zgorniak-Nowosielska, Sedwick, Hummeler & Koprowski (1967).

Strains of mycoplasmas for serological comparison were obtained from the National Collection of Type Cultures as follows: *M. dispar*, NCTC 10125; *M. fermentans*, NCTC 10117; *M. hominis*, NCTC 10111; *M. laidlawii*, NCTC 10116; *M. orale*, Type I, NCTC 10112; *M. pneumoniae*, NCTC 10119; *M. pulmonis*, NCTC V.1145-64. The strain number of *M. agalactiae* is not known. *M. arthritidis* is strain 'Arthr' of Dr Ruth Lemcke and *M. orale II* was provided by Dr B. E. Andrews.

Histology

Sections of synovium were cut in a cryostat and stained conventionally by haematoxylin and eosin, by Giemsa's stain and, after acetone fixation, by fluorescent antibody. Antisera against viruses and mycoplasmas were conjugated by Rinderknecht's method (1962) and applied directly because indirect staining gave interesting, but unwanted complications.

Cytology. Cell cultures on cover-slips were stained by May–Grunwald Giemsa's stain, by prolonged staining with Giemsa's stain at 37° C. or with orcein, and also by the direct method with fluorescent antibody specific for viruses and mycoplasmas. Indirect staining by conjugated anti-human globulin was used on virus and mycoplasma-infected cell cultures, to test for antibody in patient's sera.

Serology of mycoplasmas

Antisera were prepared by subcutaneous inoculation into rabbits of deposits of purified mycoplasma strains grown in medium containing rabbit serum, thrice washed in 0·01 M phosphate buffered saline solution (P.B.S.) and made up in complete Freund's adjuvant. Sera were tested by overnight complement fixation and, after conjugation, by fluorescent staining. Inhibition of growth of mycoplasmas by standard antisera was tested by the disk method on agar (Clyde, 1964).

RESULTS

The isolation of mycoplasma strains

System 1

Of six synovia so treated only one yielded virus or signs of virus antigen. This was a strain of rubella virus, presumed to be a laboratory contaminant.

All cultures inoculated with rheumatic tissue became very acid after 48 hr. incubation. This propensity to form more acid than control cultures was found also in all subcultures after 4 days in growth medium, or 10–12 days in maintenance medium.

Two cultures in maintenance medium showed Giemsa-stained granules in a centrifuged deposit (7000 *g* for 10 min.), and yielded mycoplasmas, but no bacteria, on suitable media. These mycoplasma strains were provisionally designated Belfast one (Bel_1) and Belfast two (Bel_2).

System 2

In nine inoculated cultures no cellular changes were seen that did not also appear in controls. No viruses were recovered. The amount of acid produced by inoculated cultures remained above that of control cultures for three passes and then became similar to controls.

Mycoplasmas were not sought in this series of tests because those found in system 1 were at this time regarded as contaminants, but much later, immunofluorescence tests on stored cover-slips from the seventh pass of two synovia of the series showed mycoplasma antigens of type Bel_1. Corresponding control, seventh pass, uninoculated cells had no stainable mycoplasmas.

System 3

Synovial cultures were prepared by one of us (P.V.S.) and maintained in a separate laboratory from the experiments on animal cells. The supernatant fluids of five synovial cell cultures were tested for virus activity by inoculation into fresh cultures of human embryo kidney cells. No virus was found, but the cultures became very acid. Four out of five cultures yielded mycoplasmas – one cultivated directly from synovial culture, three grown in abundance from the first pass in human embryo kidney cells which, uninoculated, remained free of mycoplasmas. The strain grown directly from synovium was similar to Bel_1 and was isolated from a culture of the same patient's synovium. Two other strains came from the same patients in whom the mixed animal-cell cultures of system 2 ultimately showed type Bel_1 antigen.

System 4

Inoculated and control cultures were tested for the presence of bacteria and mycoplasmas after six or seven passes.

Six more mycoplasma strains were found in inoculated cultures, but no bacteria. Control cultures were free of organisms. The combined recovery rates in the three sorts of experiments are shown in Table 1, the two animal systems being one sort.

Other tests

Five specimens of synovial cells and fragments grown in soft agar and three grown in agarose containing RK 13 cells showed no growth of mycoplasmas. Fifteen other synovia and synovial fluids gave no growth when inoculated directly into mycoplasma medium, yet seven of these gave mycoplasmas in the tissue culture systems.

Table 1. *The proportion* of mycoplasma infections in cultures of joint tissue*

System	Inoculum	No. of cultures	No. yielding mycoplasmas
(1, 2) Animal cells	Rheumatoid synovium	6	2
	None	6	0
(3) Rheumatoid synovial cultures	None	6	5†
(4) Human embryo cells	Rheumatoid synovium	7	5
	Osteo-arthritic synovium	2	1
	Normal synovium	3	0
	None	6	0

* Excludes two fluorescence identifications in system 2.
† Includes the two 'positive' sources of system 1.

Table 2. *Properties of mycoplasma strains Bel_1 and Bel_2*

	Strain	
	Bel_1	Bel_2
Growth rate	Slow. Not on blood agar	Rapid. Adaptable to blood agar
Colony type	Small, granular, indefinite central opacity	Large, clear, marked central opacity
Yeast extract	Required	Not required
Fermentation of glucose in Eagle's medium	Slow	Rapid
Haemolysis of sheep cells	Absent	Present, alpha type
Growth in cell culture	Continuous coating of organism on cell	Lumpy aggregates on cell membrane
Cytopathic effect on human embryo cells and RK13 cells	Very little	Marked destruction
Survival on PPLO agar		
37° C.	14 days	48 days
4° C.	30 days	> 300 days
Antigenicity	Distinct from Bel_2	Distinct from Bel_1

The properties of the mycoplasma strains

Mycoplasma strains were recognized by certain characteristics: the formation of polymorphic structures in liquid media; typical embedded colonies on agar media, suitably enriched; poor staining with Gram's stain; granule-formation with Dienes's stain; and lastly, sensitivity to certain broad-spectrum antibiotics, but not to penicillin (see Marmion, 1967). Their appearance on electron microscopy when stained by negative contrast and also when pelleted and sectioned was also taken into account. The properties of strains Bel_1 and Bel_2 from system 1 were first examined in preparation for comparative studies of the other strains.

It will be seen from Table 2 that the cultural characteristics of Bel_1 and Bel_2 are markedly different. In tissue culture alone, the different forms of adhesion to the cell and the pronounced cytopathic effect of Bel_2 were useful and reproducible

attributes which were always correlated with a clear distinction between Bel_1 and Bel_2 by direct fluorescent staining.

Nevertheless, the organisms did share antigens, as is seen in Table 3. No detailed analysis has been attempted, but guinea-pig antisera (unpublished data) indicate much more antigenic overlap between Bel_1 and Bel_2. The organisms are poor inducers of complement-fixing antibody, for seven rabbits given a course of antigen failed to develop detectable antibody. Four rabbits, however, given antigen in Freund's adjuvant developed antibody after two courses, 5 months apart, of 3 and 4 weekly injections.

Table 3. *Antigenic relationship between mycoplasma strains Bel_1 and Bel_2*

Test and titre	Serum	Bel_1	Bel_2
Complement fixation	Anti-Bel_1	256	32
	Anti-Bel_2	Nil	128
Indirect immunofluorescence	Anti-Bel_1	256	32
	Anti-Bel_2	16	> 256
Direct immunofluorescence	Anti-Bel_1	12	< 3
	Anti-Bel_2	< 3	> 24

Table 4. *Serological relationship of Bel_1 and Bel_2 to other mycoplasmas, tested by direct immunofluorescence, in tissue culture*

Rheumatoid mycoplasma strains	Serum: Anti-Bel_1	Serum: Anti-Bel_2	Standard strains	Serum: Anti-Bel_1	Serum: Anti-Bel_2
LM (2 isolates)	+	0	*M. pneumoniae*	0	0
BW	+	0	*M. hominis*	0	0
JM (2 isolates)	+	0	*M. fermentans*	0	0
TP	+	0	*M. salivarium*	–	–
SS	0	0	*M. orale* (I)	0	0
SO	0	+	*M. orale* (II)	0	0
DG	0	+	*M. arthritidis*	0	0
SM	0	+	*M. pulmonis*	0	0
FL	0	+	*M. agalactiae*	0	0
MC	0	+	*M. dispar*	0	0
Original Bel_1	+	0	*M. laidlawii*	0	+
Original Bel_2	0	+			

+ = Specifically stained; 0 = not stained; – = not done.

Using the direct fluorescent antibody test, we obtained the cross-reactions, shown in Table 4, between antisera to Bel_1 and Bel_2 and the remaining strains, all tested in tissue culture of human lung cells. Four strains resemble Bel_1 and are antigenically unlike five other strains which are similar to Bel_2. One strain was not stained by either serum. The same antisera do not react in the direct test with standard 'human' mycoplasmas nor with some others that have been implicated from time to time as contaminants in tissue-culture experiments. Antiserum to

Bel_2 did react with *M. laidlawii*, which stained more intensely than does Bel_2 itself. The growth of our strains Bel_1 and Bel_2 was not inhibited by standard test antisera to the five human strains tested in Table 4.

Clinical immunology

In order to investigate the clinical significance of strains Bel_1 and Bel_2, sera of patients were examined for specific antibody by indirect immunofluorescence and by complement fixation. In addition, sections of synovial membranes were examined by fluorescent antibody staining for antigen to Bel_1 and Bel_2. Sera, which included all those whose tissues gave mycoplasma-positive cultures, were tested at 1/5 dilution on cell-cultures infected with Bel_1 and Bel_2. Sections of synovia were stained with four times the minimal staining concentration of each conjugate of anti Bel_1 and anti Bel_2 sera.

Table 5. *Clinical immunology of mycoplasma isolates*

	Tests for antibody			Tests for antigen		
		No. positive to:			No. positive to:	
Material tested	No. tested	Bel_1	Bel_2	No. tested	Anti-Bel_1	Anti-Bel_2
	Method: immunofluorescence					
Rheumatoid sera	32	1	1	–	–	–
Rheumatoid synovial fluid	4	0	0	6	0	0
Non-rheumatoid sera	21	0	2	–	–	–
Synovial membranes	—	–	–	20	0	0
	Method: complement fixation					
Rheumatoid sera	14	0	0	–	–	–
Rheumatoid synovial fluid	3	0	0	4	0	0
Non-rheumatoid sera	12	0	0	–	–	–

– = Not done.

Table 5 shows that antibody to Bel_1 and Bel_2 is not characteristic of rheumatoid sera, nor do rheumatoid synovia, even allowing for errors of sampling bulky tissue, contain Bel_1 and Bel_2 antigens. No inhibitors of specific fluorescent staining were present in a few sera and a few synovial fluids that were tested.

In addition to the absence of mycoplasma antigens from synovial tissue, we observed that potent specific antisera against the viruses of influenza A, mumps, measles, German measles, respiratory syncytial disease and herpes simplex produced no specific staining of seven rheumatoid synovia, nor any staining of cells into which the same rheumatoid tissues had been inoculated (systems 1 and 2).

DISCUSSION

Mycoplasmas have grown only in cultures of rheumatic synovium or in cultures inoculated with pieces of synovium and not in uninoculated cultures. The circumstances in which they were recovered simulate those which give rise also to

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growth of diphtheroids (Duthie, Stewart, Alexander & Dayhoff, 1967) and L-forms of bacteria (Pease, 1969) from similar material; that is to say, prolonged cultivation and repeated passage. Contamination, the first likely source, cannot be ruled out, but this explanation can apply only to rheumatoid tissue and not the cell cultures. The sources of contamination common to all procedures were surgical operation, the initial dissection of the tissue in the laboratory and animal culture media, namely trypsin and calf serum: yet the same trypsin and calf serum were used in control cultures which remained mycoplasma-free.

The absence of mycoplasmas from control experiments, the fact that they are not strains of the common type of laboratory contaminants (Macpherson, 1966), and the duplication of four recoveries in separate rooms by separate workers, are in favour of a synovial origin of the mycoplasma isolates.

Against this conclusion are: failure to find mycoplasmas in primary cell-cultures, or by direct inoculation of mycoplasma culture medium, absence of antibody response in the patients, and lack of mycoplasma antigen in sections of rheumatoid joints.

If our results and those of Bartholomew (1965) are taken first at face-value, we must believe that rheumatoid tissue is apt to carry very small numbers of mycoplasma organisms. If so, the organisms must be practically non-antigenic. They are poor inducers of complement-fixing antibody in rabbits, as we have stated, and in guinea-pigs (unpublished data). More sensitive methods of assay (Taylor-Robinson *et al.* 1965) might reveal specific antibody in the patients.

No occult variety of cellular infection is known which would account for our complete failure to find mycoplasma antigen in synovia by immunofluorescence. There is thus nothing to suggest that the recovered organisms are responsible for the immunological features of the rheumatoid joint. Although some of our specimens contained very numerous foci of large and small mononuclear cells, and also collections of plasma cells, no mycoplasma antigen was found in or near them.

If the mycoplasma strains which we have isolated are contaminants, the fluorescent antibody tests give little indication of their origin. The cross-reaction between Bel_2 and *M. laidlawii* was unexpected and the two organisms have got very similar cultural properties. If Bel_2 is *M. laidlawii*, it should have grown readily in mycoplasma PPLO medium from the original tissue. Although our strains are distinguishable from standard ones by immunofluorescence, they may well be variants of common mycoplasmas, selected by long passage in tissue culture.

It remains possible that poorly antigenic organisms, by persisting somewhere in the immune system, alter immunological function in some way other than by inducing a specific immune response. Our fluorescence tests did not reveal any cross-reaction between specific mycoplasma antibody and joint tissue; tests of cellular hypersensitivity to our strains have not yet been carried out; the effect of the organisms on the patients' responses to other antigens is also unknown. We consider that such possibilities should be investigated before discarding the mycoplasma strains as mere commensals in a tissue whose resistance to infection is somewhat impaired (Editorial, 1967).

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