Failure to show mycoplasmas and cytopathogenic virus in rheumatoid arthritis

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Middleton, P. J., and Highton, T. C. (1975). *Annals of the Rheumatic Diseases*, **34**, 369–372. Failure to show mycoplasmas and cytopathogenic virus in rheumatoid arthritis. Synovial needle biopsies, joint aspirates, and joint tissue obtained at open operation from 41 cases of rheumatoid arthritis were inoculated onto PPLO media, L-form medium, and cell cultures for the isolation of mycoplasmas, L-form bacteria, and viruses. Medium suitable for the isolation of 'T' strain mycoplasmas was not employed. No mycoplasmas, L-form bacteria, or cytopathogenic viruses were shown. Similar specimens from nine patients diagnosed as having Reiter's disease were examined in a like manner and yielded only one *Mycoplasma hominis* type 1 isolate from a knee joint biopsy.

It is concluded that known strains of mycoplasma and bacterial L-forms do not play a direct role in early and established cases of rheumatoid arthritis. Some of the cell cultures used in this study contained mycoplasma contaminants. Bacterial contaminants were also encountered in occasional batches of L-form medium.

The aetiology of rheumatoid arthritis has still to be elucidated (Denman, 1970). The possible role of infectious agents in this disease process have been the subject of investigation reported in several papers but with conflicting results (Smith and Ward, 1971; Ford, 1968).

Our aim was to re-examine the question of the possible role of infectious agents in arthritis, particularly in rheumatoid arthritis by attempting to isolate mycoplasmas, L-form bacteria, and cytopathogenic virus from an adequate number of diseased joint specimens. We also sought to reduce the likelihood of 'isolating' contaminant micro-organisms by the provision of sufficient control cultures and by the practice of good aseptic technique.

Patients and methods

The patients studied included 41 subjects with rheumatoid arthritis according to the diagnostic criteria of the American Rheumatism Association (1959), except for two patients whose illnesses were of short duration at the time of the tests but whose subsequent history was that of rheumatoid arthritis. Duration of illness varied between 2 weeks and 27 years. There were nine patients diagnosed as Reiter's disease on the bases of a history or witnessed

conjunctivitis, urethral discharge, joint symptoms, skin rash, and a negative sheep cell agglutination test (SCAT). The average age of the Reiter's disease patients was 34 years and duration of illness varied from 2 weeks to 12 years. Other patients in the study group comprised four with ankylosing spondylitis (sacroiliitis and negative SCAT); three with psoriasis (negative SCAT, active polyarthritis involving terminal interphalangeal joints, skin and nail manifestations, duration of disease 2–12 years); three with Still's disease (negative SCAT, polyarthritis, aged less than 16 years, duration of disease 2 months to 9 years); one each of osteoarthritis, gout, tuberculosis, systemic lupus erythematosus, a normal, and two with polyarthritis who were not diagnosed by the end of our study period.

Synovial tissue for isolation of mycoplasmas was obtained either in the course of diagnostic and therapeutic work or for investigative purposes only. Informed consent was given when the procedure was done on this latter basis. Polley-Bickel needle biopsies of synovium, tissues obtained at open operation for therapeutic or exploratory reasons, and joint aspirates were either processed immediately or held at 4°C for 1–2 hours before inoculation. Needle biopsy and surgical specimens were chopped finely with scissors and two-thirds of the minced tissue ground in a mortar with sterile sand and 0.01 mol/l sucrose or brain-heart infusion broth (Difco). After a

settling period of 5 min the supernatant fluid was collected and 0.05 ml volume spread over the surfaces of 50 mm PPLO and L-form agar plates. Cell cultures received 0.1 ml supernatant fluid per tube. PPLO diphasic medium or broths and L-form medium broths received 0.2 ml inoculum. Minced, unground tissues were, in addition, added to cell cultures and the broth media.

Solid and liquid mycoplasma media were made according to Marmion (1967). A modified L-form medium based on the formula of Gutman, Turck, Petersdorf, and Wedgwood (1965) was used. Primary human amnion cell cultures were prepared by the method of Duncan and Bell (1961). Strains of diploid human embryo fibroblasts were established from lung or skin and muscle by the procedure of Hayflick and Moorhead (1961). Inoculated cell cultures for subsequent subculture to PPLO agar plates were maintained in Eagle's minimal essential medium with 15% fetal calf serum, yeast extract, and penicillin 100 units/ml. Cell cultures employed for the attempted demonstration of cytopathogenic viruses were maintained in Eagle's medium with 2% serum plus penicillin 100 units/ml and streptomycin 100 µg/ml.

Inoculated PPLO and L-form agar plates were incubated aerobically in humidified plastic boxes at 37°C and anaerobically at 37°C in Baird and Tatlock anaerobic jars containing an atmosphere of 5% CO₂ and 95% H₂. Plates were inspected once or twice weekly with an inverted microscope at ×40 magnification. After 15–20 days of incubation agar blocks were cut from inoculated plates for passage to fresh plates by means of a push-block technique. Diphasic PPLO medium or broths were subcultured to PPLO agar plates at 1–2 weeks and again at 3–4 weeks after inoculation. L-form medium broths were sub-

cultured to solid phase L-form plates and blood agar plates 1–2 weeks after inoculation. Inoculated and uninoculated control test tube monolayer cell cultures were observed for 1–4 weeks for cytopathic effect. Subcultures from inoculated and control cell cultures to PPLO agar plates were made after 1 cycle of freezing and thawing. Mycoplasma-like colonies were stained and examined by the method of Dienes (1945). Fibroblast-like cell cultures were established from 4 rheumatoid arthritis operative specimens. These were observed for 3–5 weeks for cytopathic effect and later subcultured to PPLO medium.

Results

The Table sets out the number of patients in each diagnostic category, the type of specimens obtained, and the nature and number of the isolation systems employed. With the exception of a single mycoplasma isolate from a knee joint needle biopsy, no other mycoplasmas or L-form bacteria were observed in the inoculated cell cultures. The single mycoplasma isolate was obtained from a 30-year-old male during the acute phase of a recurrent episode of Reiter's disease. Colonies appeared in both anaerobic and aerobic PPLO agar plates. The isolate was identified as *M. hominis* type 1. A needle biopsy taken at the same time from this particular patient's opposite knee joint which had been involved several months previously failed to yield any micro-organism.

During this study contaminant micro-organisms

Table *Diagnostic category of patients, type of specimens obtained, and nature of isolation systems used*

Definitive diagnosis	No. of patients	Nature of specimen	Total specimens	No. specimens examined according to isolation system			Result
				PPLO*	L-form†	Cell culture	
Rheumatoid arthritis	41	4 fluid (joint aspirate) 31 open biopsy 15 needle biopsy	50	50	18	35	Neg
Reiter's disease	9	4 fluid (joint aspirate) 1 open biopsy 10 needle biopsy 1 urethral swab 2 prostatic fluid	18	18	3	14	Pos (<i>M. hominis</i>)
Ankylosing spondylitis	4	1 open biopsy 3 needle biopsy	4	4	2	4	Neg
Psoriasis	3	2 open biopsy 1 needle biopsy	3	3	2	3	Neg
Osteoarthritis	1	1 needle biopsy	1	1	0	1	Neg
Gout	1	1 needle biopsy	1	1	0	0	Neg
Polyarthritis (not yet diagnosed)	2	1 fluid (joint aspirate) 1 needle biopsy	2	2	1	2	Neg
Tuberculosis	1	1 open biopsy	1	1	0	1	Neg
Still's disease	3	1 open biopsy 2 needle biopsy	3	3	0	3	Neg
Systemic lupus erythematosus	1	1 needle biopsy	1	1	0	1	Neg
Normal	1	1 open biopsy	1	1	0	1	Neg

*PPLO = solid-phase mycoplasma medium.

† L-form = solid-phase L-form medium.

were frequently encountered. Mycoplasma-like colonies were isolated from 14 inoculated and uninoculated cell cultures from one particular strain of human embryo fibroblasts. Similar organisms were not recovered from broth or solid phase PPLO media inoculated with the same specimen's material. The identity of these mycoplasma-like colonies was not established. Bacteria were isolated from 3 inoculated L-form cultures. However, similar bacteria were also recovered from uninoculated L-form broth medium which had been prepared and incubated at times identical with the inoculated broths. Mould contaminants upon aerobic PPLO agar plates occasionally necessitated terminating such cultures during the initial passage.

Discussion

Our failure to show mycoplasma, L-form bacteria, and viral cytopathogenic agents with one exception in this series of patients admits to several possibilities: that either these organisms play no part in joint disease or they were not present in a persistent infective form, or the isolation systems we employed were inappropriate. Other workers were also unable to isolate mycoplasma or viruses in rheumatoid arthritis (Barnett, Balduzzi, Vaughan, and Morgan, 1966; Ford and Oh, 1965; Stewart, Duthie, MacKay, Marmion and Alexander, 1974). It also seems unlikely that our negative results could be accounted for on a basis of inadequate tissue samples since 31 open operation specimens were obtained from patients with rheumatoid arthritis and most of the 15 needle biopsies showed histological evidence of an inflammatory process. The virological studies were admittedly very basic. Interference tests and electron microscope examination of inoculated cell cultures might have indicated noncytopathic viruses. Although Highton, Caughey, and Rayns (1966) described an inclusion body in the endothelial cells of small blood vessels of the rheumatoid synovium,

it could not be definitely identified as virus or bacterial in nature and the inclusions were not enclosed in a membrane and therefore unlikely to have been ingested. Describing the ultrastructural appearances of cultured rheumatoid synovial cells, Highton and Palmer (1971) stated, 'We did not see any inclusions that we could unequivocally regard as viruses'.

The mycoplasma medium we used readily supported the growth of reference strains including *Mycoplasma pneumoniae*. Even so, such medium might have been unsatisfactory for the isolation of highly fastidious mycoplasmas (Shepard and Luncford, 1965; Moore, Redmond, and Livingston, 1966). Moreover, we did not use medium suitable for the isolation of T-strain mycoplasma or medium similar to that employed in the work of Jansson, Mäkisara, Vainio, Snellman, and Tuuri (1971). Our specimen material did not yield diphtheroids (Duthie, Stewart, Alexander, and Dayhoff, 1967) or L-form bacteria. None of our patients studied for L-form bacteria had been receiving antibiotics at the time of biopsy.

In a study of this kind involving the use of complex inanimate media, cell cultures, long-term cultivation, passage and subculture manipulations, the chances of encountering contaminant micro-organisms must be regarded as likely. We in fact encountered mycoplasma, bacterial, and mould contaminants in cell cultures, L-form medium, and PPLO agar plates, respectively.

We thank Professor J. A. R. Miles who encouraged and fostered this work; Professor B. P. Marmion who supplied both advice and reference strains of mycoplasmas and who typed the *M. hominis* isolate; Miss Pamela West for technical assistance. The Rheumatic Diseases Research Laboratories were assisted by a grant from the Golden Kiwi Medical Distribution Committee and more recently from the Medical Research Council of New Zealand, and this financial help is gratefully acknowledged. The help and co-operation of our orthopaedic colleagues was essential.

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