SYSTEMATIC DETECTION OF MYCOPLASMAS BY CULTURE AND POLYMERASE CHAIN REACTION (PCR) PROCEDURES IN 209 SYNOVIAL FLUID SAMPLES

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SUMMARY

The objective was to investigate the presence of mycoplasmas in rheumatoid arthritis (RA) and other chronic arthritides. Samples of synovial fluid (SF) were systematically collected from all patients presenting with an articular effusion. Each sample was divided into three parts. The first was kept for cytological count and culture on standard media for pyogens and mycobacteria, the second was cultivated on specific media for mycoplasmas and the third frozen for subsequent study by polymerase chain reaction (PCR). A total of 209 samples were studied. Half of the patients had inflammatory rheumatic diseases: RA (27), spondyloarthropathy (28), connective tissue disease (5), unclassified arthritis (45). The remaining suffered from other conditions, including osteoarthritis (60), gouty arthritis (19), haemarthrosis (5), post-traumatic effusion (2). Eight samples were positive by culture, two for Mycoplasma hominis, three for M. fermentans, one for M. salivarum, one for M. orale and one for Ureaplasma urealyticum. All the patients concerned had an inflammatory rheumatic disease: five had RA, one had psoriatic arthritis and two had unclassified arthritis. These results were confirmed by PCR in two cases (one M. fermentans, one U. urealyticum). The lack of sensitivity of the conventional PCR assay on SF is discussed. Mycoplasmas were mainly detected in SF of RA patients. These results raise the question of the possible role of mycoplasmas in the triggering and maintenance of inflammatory rheumatic diseases, especially RA.

KEY WORDS: Rheumatoid arthritis, Mycoplasma, Synovial fluid.

PATIENTS AND METHODS

Patients

Over a 2.5 yr period (30 months), 209 samples of SF were collected from 201 patients: 32 SF from 27 patients with RA, 29 SF from 28 patients with spondyloarthropathy (six of whom had reactive arthritis and five psoriatic spondyloarthropathy), five SF from five patients with connective tissue disease, 46 SF from 45 patients with unclassified arthritis, 60 SF from 60 patients with osteoarthritis, 20 SF from 19 patients with gouty arthritis or chondrocalcinosis, one SF from a patient with villonodular synovitis, two SF from two patients with amyloidosis arthropathy, five SF from five patients with haemarthrosis, two SF from two patients with post-traumatic joint effusion, two SF from two patients with effusion due to a neighbouring metastasis, two SF from two patients with neuroarthropathy, two SF from two patients with arthropathy associated with dialysis and one SF from a patient with hypertrophic osteoarthropathy.

SF specimens

Each sample of SF was divided into three parts. The first part was used for cytological count and culture on standard media for pyogens and mycobacteria. The second, 1 ml SF, was added to 1 ml 2SP (sucrose phosphate) transport medium [11], and immediately brought to the laboratory to be cultured on specific media for mycoplasmas. The third was frozen (−80°C) for subsequent study by PCR.

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Mycoplasma culture conditions
The mixture of SF and 2SP was incubated on different specific media: 200 μl in 2 ml of liquid Edward modified medium supplemented with arginine, 100 μl in 1 ml of liquid Edward modified medium supplemented with glucose and 100 μl on agar Edward modified medium for mycoplasmas [12], 100 μl in 1 ml of liquid Shepard medium and 100 μl on agar Shepard medium for ureaplmas [13]. These media were incubated for 1 month at 37°C under a carbon dioxide atmosphere. Species were identified according to the appearance of colonies, their biochemical properties, growth-inhibition tests [14] and, when possible, by PCR.

Preparation of sample for PCR
After thawing, 500 μl of SF samples were centrifuged for 60 min at 14 000 g. The pellet was resuspended in TE buffer [10 mm Tris–HCl (pH 8.0), 1 mm EDTA], incubated for 1 h at 56°C with 1% sodium dodecyl sulphate (SDS) and 100 μg/ml proteinase K (Sigma), and then heated at 95°C for 15 min to inactivate proteinase K. Nucleic acids were extracted once with phenol–chloroform–isoamyl alcohol (25:24:1), and once with chloroform–isoamyl alcohol (24:1). The aqueous phase was precipitated at −20°C for 18 h by the addition of 2 vols of 95% ethanol and 0.1 M sodium acetate. DNA was recovered by centrifugation at 14 000 g for 30 min and the pellet resuspended in 100 μl distilled water. A volume of 10 μl of the DNA preparation was used for the PCR assay.

PCR assay
PCR reactions were performed with an automated thermocycler (Perkin-Elmer Cetus 480, Norwalk, CT, USA). Specific oligonucleotide primers were used for the different species of mycoplasma affecting humans: *M. hominis*, *M. fermentans*, *M. pneumoniae*, *M. genitalium* and *Ureaplasma urealyticum*. These were chosen in published nucleotide sequences: in the 16S rRNA gene for *M. hominis* [15], in an insertion sequence for *M. fermentans* [16], in the adhesin gene for *M. pneumoniae* and *M. genitalium* [17], and in the urease gene for *U. urealyticum* [18]. To evaluate the presence of PCR inhibitors, each sample was also tested with primers settling to a fragment of the DQα human leucocyte antigen gene [19]. All these oligonucleotides were synthesized by the methoxyphosphoramidite method on an Applied Biosystems Model 38SA DNA synthesizer (Applied Biosystems, San Jose, CA, USA) at the Institut de Biochimie Cellulaire et de Neurochimie (Bordeaux, France). Amplification was performed in a final volume of 50 μl containing 5 μl of 10× assay buffer, 1.5 mm MgCl₂, 200 μM each of dATP, dCTP, dTTP and dGTP, 1 μM of each primer and 1.5 U of Taq DNA polymerase (Promega Corporation, Madison, WI, USA). Amplification consisted of a 3 min thermal delay step at 94°C followed by 35 cycles comprising a 1 min denaturation step at 94°C, a 1 min annealing step at 55°C and a 1 min elongation step at 72°C. The products of PCR were analysed by 2% agarose gel electrophoresis and ethidium bromide staining. Samples containing a band of the expected size were controlled by hybridization. After Southern blotting, filters were hybridized with a 32P-radiolabelled internal specific probe at 55°C overnight, then washed under highly stringent conditions before autoradiography.

Statistical analysis
Fisher’s exact test was used to compare proportions and Student’s *t*-test for the comparison of means between the two groups.

RESULTS
Results are summarized in Tables I and II.

<p>| TABLE I |
| Results of the cultures and data of the corresponding patients |</p>
<table>
<thead>
<tr>
<th>No.</th>
<th>Organism</th>
<th>Culture</th>
<th>PCR</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>Features</th>
<th>Disease duration (months)</th>
<th>Cell count (mm³)</th>
<th>Neutrophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>M. hominis</em></td>
<td>+</td>
<td>–</td>
<td>F</td>
<td>59</td>
<td>RA</td>
<td>Symmetrical polyarthritis</td>
<td>RF –</td>
<td>6</td>
<td>11 400</td>
</tr>
<tr>
<td>2</td>
<td><em>M. hominis</em></td>
<td>+</td>
<td>–</td>
<td>M</td>
<td>21</td>
<td>PsA</td>
<td>Monoarthritis</td>
<td>HLA B27 + psoriasis</td>
<td>24</td>
<td>5000</td>
</tr>
<tr>
<td>3</td>
<td><em>M. fermentans</em></td>
<td>+</td>
<td>+</td>
<td>F</td>
<td>69</td>
<td>Unclas A</td>
<td>Monoarthritis</td>
<td>jur–A in past history</td>
<td>1</td>
<td>5000</td>
</tr>
<tr>
<td>4</td>
<td><em>M. fermentans</em></td>
<td>+</td>
<td>+</td>
<td>F</td>
<td>55</td>
<td>RA</td>
<td>Destructive polyarthritis</td>
<td>RF +</td>
<td>61</td>
<td>20 000</td>
</tr>
<tr>
<td>5</td>
<td><em>M. fermentans</em></td>
<td>+</td>
<td>inh</td>
<td>F</td>
<td>48</td>
<td>Unclas A</td>
<td>Non-erosive oligoarthritis</td>
<td>RF-ANA 1/160</td>
<td>6</td>
<td>15 000</td>
</tr>
<tr>
<td>6</td>
<td><em>M. salivarium</em></td>
<td>+</td>
<td>NA</td>
<td>M</td>
<td>69</td>
<td>RA</td>
<td>Chronic erosive polyarthritis</td>
<td>RF +</td>
<td>58</td>
<td>60 000</td>
</tr>
<tr>
<td>7</td>
<td><em>M. orale</em></td>
<td>+</td>
<td>NA</td>
<td>F</td>
<td>60</td>
<td>RA</td>
<td>Symmetrical polyarthritis</td>
<td>RF –</td>
<td>9</td>
<td>19 000</td>
</tr>
<tr>
<td>8</td>
<td><em>U. urealyticum</em></td>
<td>+</td>
<td>+</td>
<td>M</td>
<td>72</td>
<td>RA</td>
<td>Symmetrical polyarthritis</td>
<td>RF +</td>
<td>2</td>
<td>16 000</td>
</tr>
</tbody>
</table>


<p>| TABLE II |
| Comparisons between the mycoplasma-positive and -negative groups of patients with inflammatory rheumatic diseases |</p>
<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Disease duration (months)</th>
<th>ESR (mm/h)</th>
<th>Cell count (mm³)</th>
<th>Neutrophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>57</td>
<td>21</td>
<td>48</td>
<td>19 000</td>
</tr>
<tr>
<td>± s.d.</td>
<td>17</td>
<td>25</td>
<td>34</td>
<td>18 000</td>
</tr>
<tr>
<td>Negative group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>49</td>
<td>61</td>
<td>50</td>
<td>19 500</td>
</tr>
<tr>
<td>± s.d.</td>
<td>16</td>
<td>92</td>
<td>39</td>
<td>20 000</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.12</td>
<td>0.14</td>
<td>0.87</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Standard cultures

All standard cultures remained negative.

Culture on specific media for mycoplasmas

Eight cultures were positive: two for *M. hominis*, three for *M. fermentans*, one for *M. salivarium*, one for *U. urealyticum* and one for *M. orale*. In each case, the culture was confirmed on both solid and liquid media.

PCR study

The presence of inhibitors of DNA polymerase was demonstrated in 30% of the samples studied by a negative reaction of the PCR assay with human HLA primers. PCR with HLA primers were positive for all but one (no. 5) of the eight SF from which mycoplasmas had been cultured. Only two fluids were positive, thus confirming two of the culture results: one sample positive for *U. urealyticum*, one for *M. fermentans*. Regarding the latter case, three SF samples had been collected at 6 monthly intervals from the same patient. Interestingly, the first SF was culture positive and PCR negative, the second culture negative and PCR positive, and the third negative for both culture and PCR. The patient in question, who had classic RA, was given no antibiotics during this period but was treated with methotrexate and low-dose prednisone.

Relationship between the presence of mycoplasmas and the different diseases

All the mycoplasmas were detected in the SF of patients (five women and three men) who had an inflammatory rheumatic disease: RA (*n* = 5), psoriatic spondyloarthropathy (*n* = 1) and unclassified arthritis (*n* = 2).

Comparison between mycoplasma-positive and -negative patients

No significant difference was found between the age of the eight patients whose SF was culture positive (positive group) and that of the other patients with inflammatory rheumatic conditions (negative group). There was also no difference regarding the duration of illness between the positive and the negative groups. In four of the eight positive cases, however, onset of the illness was recent, with the first articulatory manifestations dating from less than 6 months before SF was collected. The erythrocyte sedimentation rate (ESR), as well as cell and neutrophil counts in SF, did not differ significantly between the two groups (see Table II).

Antibiotic treatment

Amongst the eight patients from whose SF a mycoplasma was isolated, six were treated with cyclohexadine once the results of the culture study were known (doxycycline 200 mg/day for 2 months), in addition to their previous treatment: NSAID or prednisone and a slow-acting anti-rheumatic drug (SAARD). Only one patient, with unclassified arthritis, improved significantly. Twenty months after the cyclohexadine treatment was stopped, she was still in subcomplete remission. She is still receiving hydroxychloroquine.

DISCUSSION

The possibility of mycoplasmas being isolated from synovial specimens of patients with various rheumatic disorders, including RA, was largely debated in the 1960s and the early 1970s, but, as a matter of fact, very few teams reported such isolation, and these results were sometimes contested. As mycoplasmas are common cell culture contaminants, the studies in which cell cultures were used to isolate mycoplasmas from synovial specimens were not reliable [20–23]. Like Williams *et al.* [7, 8], Märdh *et al.* [9] and Jansson *et al.* [24], we used cell-free media, thus excluding theoretically the principal risks of contamination. Jansson *et al.* could isolate mycoplasmas from synovial samples of patients with rheumatic disorders, but could not clearly identify these isolates, concluding that they were serologically related to *M. arthritidis*, which is known to be a pathogen in rodents [24, 25]. We were able to identify all strains isolated from our clinical samples: according to the appearance of colonies, biochemical properties and growth-inhibition test for all species, and with confirmation by PCR assays when available (for *M. fermentans*, *M. hominis* and *U. urealyticum*). Williams *et al.* claimed to have isolated *M. fermentans* in 40%, using a sucrose density gradient to separate the organisms from the cells contained in the synovial effusion before cultivating them in conventional liquid medium. However, this technique has been criticized because the sucrose density gradient was charged with inducing colour changes of the culture medium that could have been considered as a positivity of the culture. Williams admitted that the organisms could not be grown in all cases on solid medium after the change in colour was observed in liquid medium [26]. Moreover, such isolations were not confirmed by numerous later studies [27–29], despite using 12 different culture media in one of them [29].

So, our culture study confirmed that different human mycoplasmas can occasionally be cultured from the SF of patients with RA and other inflammatory rheumatic arthritides. Our results cannot be explained by contamination. First, as mentioned above, we used cell-free media. Second, mycoplasmas were only recovered from SF of patients with inflammatory rheumatic diseases. No mycoplasma was isolated from the SF of patients suffering from other conditions, including osteoarthritis, gouty arthritis, chondrocalcinosis and post-traumatic joint effusion. A contamination should have led to a random distribution of the results. Moreover, two of the positive cultures were confirmed by PCR. In one of these patients, the sample positive by PCR was different from the one which was culture positive. Furthermore, the recovered mycoplasmas have been undoubtedly identified as *M. fermentans* (*n* = 3), *M. hominis* (*n* = 2), *M. salivarium* (*n* = 1), *M. orale* (*n* = 1) and *U. urealyticum* (*n* = 1), which are all human mycoplasmas.

Our PCR results are perhaps more surprising. Since PCR is considered to be a very sensitive method, we should have obtained better results with PCR than with...
cultures. In fact, we simply obtained confirmation of two positive culture results. We did not, however, use specific primers for \textit{M. salivarium} and \textit{M. orale} which were cultured from two patients, and PCR was inhibited (PCR with HLA primers negative) in one of the SF from which \textit{M. fermentans} had been isolated (no. 5). Nevertheless, the sets of primers we used were considered to be specific and sensitive, as they were validated on other kinds of clinical samples (urine, genital swabs, endotracheal aspirates, etc.). Moreover, the DNA was extracted according to standard protocols and the conditions of amplification were those commonly used in the laboratory for the identification of culture products as well as detection in other clinical samples. However, PCR sensitivity would appear to be relatively poor in SF, due to the presence of many inhibitors of the reaction (DNases, proteases, hybridization with partially complementary DNA facilitated by the disproportion between human and bacterial DNA, etc.). By voluntary contamination of SF samples with different concentrations of bacterial cultures, we have been able to evaluate the sensitivity of our PCR protocol at 10^{-9} to 10^{-10} colour change units per millilitre (personal unpublished data). This is less than that attributed to the culture assay. Thus, we may have failed to detect low amounts of mycoplasma DNA. The limits of the PCR technique in some clinical samples have already been reported [30, 31]. Moreover, after we did this study, we tried a semi-nested PCR assay developed for \textit{M. fermentans} [32] in another series of synovial specimens. With this assay we could detect the presence of \textit{M. fermentans} in synovial specimens of eight (21\%) patients with RA, two (20\%) patients with spondyloarthopathy, one (20\%) patient with psoriatic arthritis and four (13\%) patients with undifferentiated arthritis [33]. Thus, conventional PCR is probably not sensitive enough in synovial specimens. Other PCR assays, such as nested PCR or reverse transcriptase PCR (RT-PCR), would probably improve the sensitivity of detection studies in synovial specimens, but still have not been developed for most human mycoplasmas.

So, we have shown that mycoplasmas were present in the SF of eight patients presenting with several inflammatory rheumatic diseases of unknown cause, including RA. Such results raise the question of the possible role of mycoplasmas in the triggering and maintenance of these diseases. Further studies are required to answer this question. Improvement of the PCR procedures and adaptation to synovial specimens should facilitate the task.

Acknowledgement

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References


