Detection of mycoplasmal infections in blood of patients with rheumatoid arthritis

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Abstract

Objective. Mycoplasmal infections are associated with several acute and chronic illnesses. Some mycoplasmas can enter a variety of tissues and cells, and cause system-wide or systemic signs and symptoms.

Methods. Patients (14 female, 14 male) diagnosed with rheumatoid arthritis (RA) were investigated for mycoplasmal infections in their blood leucocytes using a forensic polymerase chain reaction (PCR) procedure. Amplification was performed with genus- and species-specific primers, and a specific radiolabelled internal probe was used for Southern hybridization with the PCR product. Patients were investigated for the presence of Mycoplasma spp., and positive cases were further tested for infections with the following species: M. fermentans, M. hominis, M. pneumoniae and M. penetrans.

Results. The Mycoplasma spp. sequence, which is not entirely specific for mycoplasmas, was amplified from the peripheral blood of 15/28 patients (53.6%) and specific PCR products could not be detected in 13 patients (46.4%). Significant differences (P < 0.001) were found between patients and positive healthy controls in the genus test (3/32) and in the specific tests (0/32). Moreover, the incidence of mycoplasmal infections was similar in female and male patients. Using species-specific primers, we were able to detect infections with M. fermentans (8/28), M. pneumoniae (5/28), M. hominis (6/28) and M. penetrans (1/28) in RA patients. In 36% of the patients, we observed more than one Mycoplasma species in the blood leucocytes. All multiple infections occurred as combinations of M. fermentans with other species.

Conclusions. The results suggest that a high percentage of RA patients have systemic mycoplasmal infections. Systemic mycoplasmal infections may be an important cofactor in the pathogenesis of RA, and their role needs to be explored further.

Key words: Rheumatoid arthritis, Chronic infections, Mycoplasma, Polymerase chain reaction.

Mycoplasmas are the smallest self-replicating, pleomorphic bacteria that lack cell walls [1, 2]. The largest group of the class Mollicutes is divided into >100 Mycoplasma species, which are further subclassified into various strains. Mycoplasmas are often found as extracellular parasites attached to the external surfaces of host cells, but some species invade host tissues and cells, and replicate intracellularly. These microorganisms can produce a variety of effects on host cells and tissues. Besides affecting cell growth and morphology, mycoplasmas are able to alter metabolic, immunological and biochemical functions [3].

Mycoplasmas are commonly found in the oral cavity and as symbiotic gut flora. Formerly, mycoplasmas were considered as relative benign microorganisms with a low pathogenic potential. When they penetrate into blood vessels and colonize major organs, certain species can, however, cause acute and chronic illnesses. Some mycoplasmas, such as M. penetrans, M. fermentans and M. pirum, can enter a variety of tissues and cells, and cause a broad spectrum of signs and symptoms [3]. Mycoplasmas have also been shown to have a complex relationship with the immune system [4]. They are very effective at evading host immune responses, and synergism with other infectious agents has been seen. The best known species is M. pneumoniae, which can cause atypical pneumonia [5, 6]. Mycoplasmal infections can be present as different clinical disorders with acute and chronic signs and symptoms. Although many of these signs and symptoms are non-specific, they seem to be related, in part, to immunological or autoimmunological responses. For example, using culturing techniques, Ureaplasma urealyticum, M. pneumoniae and M. salivarium have been localized in the joint tissues of patients with rheumatoid diseases [7]. Hoffman et al. [8] found serological evidence for active and inactive mycoplasmal infections in patients with rheumatoid arthritis (RA) and juvenile RA, but they could not detect mycoplasmal DNA in the synovial fluid of these
patients using the polymerase chain reaction (PCR). Other studies observed immunological evidence for mycoplasmal infections in RA patients [9, 10].

We have begun to examine patients with chronic illnesses for the presence of systemic mycoplasmal infections. In recent studies, we have shown that patients with chronic fatigue syndrome (CFS) and/or fibromyalgia syndrome (FMS) have a much higher incidence of mycoplasmal infections in their blood leukocytes than healthy controls without clinical signs and symptoms [11–13]. We hypothesized that chronic mycoplasmal infections might also be related to the pathogenesis of other chronic illnesses, such as RA.

Mycoplasmal infections are usually diagnosed by serological procedures or culture techniques [14, 15]. Both of these techniques are very limited in their sensitivity, and thus mycoplasmal infections are often under-diagnosed or misdiagnosed [16]. The introduction of Mycoplasma-specific primers in PCR enables sensitive and specific detection of mycoplasmal infections, and discrimination between different Mycoplasma species. Using PCR techniques, the presence of mycoplasmas was investigated in synovial fluids of patients with RA and other chronic arthritis. Schaeverbeke et al. [17] showed that M. fermentans, but not M. penetrans, was detectable in 20% of these patients and other types of arthritis of unknown causes, but not in patients with reactive, post-traumatic or chronic juvenile arthritis. Additionally, M. genitalium was found in some RA patients [18]; however, the sensitivity of the conventional PCR procedures was not satisfactory [19]. The forensic PCR method that we use to identify mycoplasmal infections is very sensitive and highly specific [11].

In this preliminary study, we report on the detection of mycoplasmas in blood leucocytes of patients with RA. Using a sensitive forensic PCR method with genus-specific primers, we investigated blood samples for the presence of any type of mycoplasmal infection. Using species-specific primers, we then tested for the presence of several Mycoplasma species.

Materials and methods

Patients

Blood samples from 28 patients (14 female, 14 male), diagnosed with RA, were investigated for mycoplasmal infections in their blood leucocytes. The American College of Rheumatology modified criteria were used for diagnosis [20]. All patients were examined by a rheumatologist (ARF) and all fulfilled the ACR classification criteria for RA. Patients’ ages ranged between 22 and 65 yr (median 42 yr). The duration of RA history was 16–300 months (median = 149 months). All patients had had no antibiotic treatments for at least 6 weeks before the blood was drawn.

Specimens

Specimens were collected and treated as previously described [11]. Briefly, blood was collected in citrate-containing tubes and immediately brought to ice bath temperature. Samples were shipped refrigerated or on wet ice by overnight courier. Whole blood (50 µl) or blood leucocytes were used for the preparation of DNA using Chelex (Biorad) as follows. Blood cells were lysed with nanopure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13 000 g for 2 min, the supernatants were discarded. Chelex solution (200 µl) was added, and the samples were incubated at 56°C and 100°C for 15 min each. Aliquots from the centrifuged samples were used immediately for PCR or stored at −70°C until use.

Amplification

Amplification of the target sequences (Table 1) was performed in a total volume of 50 µl PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 9) containing 0.1% Triton X-100, 200 µM each of dATP, dTTP, dGTP and dCTP, 100 pmol of each primer, and 0.5–1 µg of chromosomal DNA. Purified mycoplasmal DNA (0.5–1 ng of DNA) was used as a positive control for amplification. The amplification was carried out for 40 cycles with denaturation at 94°C. Annealing was performed at 60°C (genus-specific primers and M. penetrans) or 55°C (M. pneumoniae, M. hominis and M. fermentans). The extension temperature was 72°C in all cases. Finally, product extension was allowed at 72°C for 10 min [21–23]. Negative and positive controls were used in each experimental run.

Southern blot confirmation

The amplified samples were run on a 1% agarose gel containing 5 µl/100 ml of ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). After denaturing and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking was performed. Membranes were pre-hybridized with hybridization buffer consisting of Denhardt’s solution and 1 mg/ml salmon sperm as blocking reagent. Membranes were then hybridized with 32P-labelled corresponding internal probe (107 c.p.m./bag) (see Table 1). After hybridization and washing to remove unbound probe, the membranes were exposed to autoradiography film for 7 days at −70°C.

Results

For the detection of mycoplasmal infections in blood leucocytes, we first used genus-specific primers. The Mycoplasma spp. sequence was amplified from DNA extracted from the peripheral blood of 15/28 (53.6%) patients, whereas specific PCR products were not detected in the 13 negative patients (46.4%). Results were similar in female and male patients. In 32 healthy controls without any clinical signs and symptoms, positive results were shown in three cases (9.4%) for Mycoplasma spp. test, but not for the other species-specific tests (0/32).

Specific primers for M. fermentans, M. pneumoniae, M. penetrans and M. hominis were used to detect species-
specific mycoplasmal DNA by PCR. In 10/15 patients with a positive signal for *Mycoplasma* spp., we detected one or more *Mycoplasma* species, but in five positive patients we were unable to find at least one of the four tested species. The incidences of infections with *M. fermentans* (8/28), *M. pneumoniae* (5/28) and *M. hominis* (6/28) were similar. *Mycoplasma penetrans* was found in only one patient. In 36% of the patients who tested positive for the general mycoplasmal infection, we observed more than one species in the blood leucocytes. These multiple infections occurred as combinations of *M. fermentans* with other species. Single infections were found in five patients (*M. fermentans*, n = 2; *M. hominis*, n = 2; *M. pneumoniae*, n = 1), but were not observed with *M. penetrans*. All four species were detected in one patient.

Although the GPO1 and UNI sequences are capable of a few possible cross-reactions with *Mycoplasma*-related organisms, the conditions used yielded specific products for mycoplasmas as shown by van Kuppeveld et al. [24] and Dussurget and Roulland-Dussoix [25]. That the patients we examined had mycoplasmal infections was confirmed by species analysis using PCR. Using the *M. fermentans*-specific primers SB1 and SB2 from the *tuf* gene, we found a single band of 850 bp that hybridized only with the 32P-labelled internal probe SB3. Similar results were obtained for the other *Mycoplasma* species (Fig. 1). To examine the reliability of the method, we performed multiple assays (repeated 3–7 times) on 40 samples with other diagnoses. All results were completely reproducible. In three cases, the sixth and seventh repeat of an initial positive result produced only a weak but positive signal due to degradation of DNA.

Fresh blood and immediate DNA preparation resulted in better results than blood that was processed after a period of time at room temperature. Six positive blood samples were divided into five aliquots each and stored at room temperature for different time intervals (processed immediately or after 1, 2, 4 or 7 days). Over time, the PCR signal decreased. In all samples that showed positive results in fresh DNA preparations, the PCR signal became weak after 2 days of blood storage at room temperature. After 4 days, negative results were obtained in four cases, whereas the other two samples showed very faint bands. No specific PCR product was

**Table 1.** Sequences from mycoplasmal DNA used for *Mycoplasma* genus-specific and species-specific PCR. The specificity of each primer was evaluated using the Blast-Search program on GenBank [44]

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Sequence</th>
<th>Target</th>
<th>Size (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPO1 primer</td>
<td>ACT CCT ACG GGA GGC AGC AGT A</td>
<td>16S mRNA</td>
<td>717</td>
<td>Van Kuppeveld et al., 1992 [24]</td>
</tr>
<tr>
<td>MGSO primer</td>
<td>TGC ACC ATC TGT CAT TCT GTT ACG CTC</td>
<td>Genus</td>
<td>850</td>
<td>Berg et al., 1996 [23]</td>
</tr>
<tr>
<td>UNI- probe</td>
<td>TAA TCC TGT TTT TCT CCC AC</td>
<td><em>tuf</em> gene</td>
<td>144</td>
<td>Bernet et al., 1989 [45]</td>
</tr>
<tr>
<td>SB 1 primer</td>
<td>CAG TAT TATCAA AGA AGG</td>
<td><em>tuf</em> gene</td>
<td>281</td>
<td>Van Kuppeveld et al., 1992 [24]</td>
</tr>
<tr>
<td>SB 2 primer</td>
<td>TCT TTT GTT ACTT GAA ATT CTT TCA</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>SB 3 probe</td>
<td>TTT TTC AGT TTC GTA TTC GAT G</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>MP5-1 primer</td>
<td>GAA GCC GCCT TTT TCC ACG GTG TTG</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>MP5-2 primer</td>
<td>ATT ACC ATC TTT TTT GAA AGG</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>MP5-3 primer</td>
<td>CAG TAT TATCAA AGA AGG</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>Mhom1 primer</td>
<td>TTT TTC AGT TTC GTA TTC GAT G</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>Mhom2 primer</td>
<td>GTC TGC AAT CAT TTT CTA TTT TAA A</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>GPO1 probe</td>
<td>ACT CCT ACG GGA GGC AGC AGT A</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>IMM-7 primer</td>
<td>GGA AAC GGG AAT GGT GGA ACA GAT</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>IMM-5 primer</td>
<td>TTT TTC AGT TTC GTA TTC GAT G</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
</tr>
<tr>
<td>IMM-3 probe</td>
<td>AGG GAA TCT GTG ATT CAA A</td>
<td><em>tuf</em> gene</td>
<td>704</td>
<td>Nasralla et al. (submitted)</td>
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</table>

**Fig. 1.** Detection of different *Mycoplasma* species in control samples. Each sample was prepared as a positive control containing species-specific DNA and a negative control containing water. Control DNA was detected using primer pairs as described in Table 1. Electrophoresis was carried out on agarose gel containing ethidium bromide. Bands were visualized using UV light. Lane 1, *M. fermentans* (negative control); lane 2, *M. fermentans* (positive control); lane 3, *M. penetrans* (negative control); lane 4, *M. penetrans* (positive control); lane 5, *M. pneumoniae* (negative control); lane 6, *M. pneumoniae* (positive control); lane 7, *M. hominis* (negative control); lane 8, *M. hominis* (positive control). Inverted figure.
detectable after 1 week. Additionally, blood collected in tubes containing citrate gave better results than blood collected in acid-EDTA.

The sensitivity of Mycoplasma detection by the described method was assessed by the detection of control Mycoplasma DNA and by internal Southern hybridization using Mycoplasma-specific probes. Using serial dilutions of Mycoplasma DNA, the method was able to detect as little as 10 fg of DNA [11]. In other experiments, M. fermentans was added to control blood samples at various concentrations. We were able to detect specific products down to 10 ccu/ml blood. Thus, with the use of specific Southern hybridization, this PCR procedure can result in specific test results of high sensitivity, down to the presence of approximately a single microorganism in a clinical sample.

In our experience, conventional PCR yields similar results to forensic PCR with extracellular Mycoplasma, but not with clinical samples that contain intracellular mycoplasmas. The reason for this is not known, but it could be due to inhibitors present in the clinical samples or to loss of Mycoplasma DNA in the conventional extraction procedures due to protein complexing.

Discussion

Although the underlying causes of RA are not known, RA and other autoimmune diseases could be triggered, at least in part, by infectious agents. The remarkable clinical and pathological similarities between certain infectious diseases in animal species and those of some human rheumatic illnesses, such as RA, have encouraged the search for a microbial aetiology for these syndromes. A long list of microorganisms, including aerobic and anaerobic intestinal bacteria, several viruses and mycoplasmas, have been proposed as important in these illnesses [26]. Although several initial findings on many aetiological agents were not corroborated by further studies, the concept of a microbial trigger for RA is attractive. Recently, there has been increasing evidence that mycoplasmas may, in part, play a role in the genesis of arthritis [27].

In the present pilot study, we detected several Mycoplasma species in blood leucocytes of patients suffering from RA. Although the patient numbers in these studies were not large, using a highly sensitive and specific PCR technique we were able to detect mycoplasmal DNA in > 50% of patients. Mostly we detected M. fermentans, and M. penetrans was found in only one patient with multiple mycoplasmal infections. Recently, similar findings were published using synovial fluids and joint tissue specimens [17]. Additionally, we observed infections with M. pneumoniae and M. hominis. The presence of trace amounts of mycoplasmal antigens for these species or specific antibodies against Mycoplasma species were found in other studies using immunological methods [10, 14]. Interestingly, we detected multiple infections with several Mycoplasma species in a high percentage of our patients, but these multiple infections were seen only in combination with M. fermentans infections. The UNI- and GPO1 primers are not totally genus specific. However, the conditions used for PCR yield amplification products with a high degree of specificity and sensitivity [24, 25]. To overcome the problems regarding the limited specificity, we confirmed the results for the Mycoplasma assay with highly species-specific assays. We were able to identify at least one Mycoplasma species in 10 of 15 patients where the general test was positive. In the remaining five patients, it is more likely that other Mycoplasma species were responsible for the positive amplification signal, such as M. arthritidis, rather than cross-reactions with other closely related microorganisms. However, the limited specificity of the general test cannot completely rule out such cross-reactivity. Future studies will include more mycoplasmal species using highly species-specific primers.

Since little is known about the possible involvement of mycoplasmas in the pathogenesis of chronic diseases, it remains uncertain whether our findings represent a causal agent, cofactor or secondary superinfection in patients with immune disturbances. However, mycoplasmas are able to induce immune dysfunctions and autoimmune reactions. Thus, mycoplasmal infections may, in part, be involved in the pathogenesis of RA.

Mycoplasmal infections were reported in patients with various inflammatory diseases, such as endocarditis [28], pericarditis [29] or encephalomyelitis [30], where immunological or autoimmune phenomena co-exist. Although the basis for these infections is not well understood, it is apparent that several species of pathogenic mycoplasmas are endowed with a sophisticated genetic machinery for altering their surface attributes. This surface phenotypic variation is thought to play a key role in the establishment and persistence of Mycoplasma infections by enabling evasion of host defences and by ensuring adaptation to the rapidly changing microenvironmental conditions encountered in the host [31]. Non-specific interactions between mycoplasmas and B lymphocytes have been implicated in disease pathogenesis, possibly leading to autoimmune reactions, modulation of immunity and/or promotion of lesion development [32]. The potential role of mycoplasmas in various joint diseases remains unknown, but they could be an important factor or cofactor. Thus, the complex relationship between mycoplasmal infections and the immune system of the host may, in part, be responsible for the pathogenesis of rheumatological inflammatory diseases. For example, M. arthritidis-related superantigens were found to compromise T cells [33], and they can trigger and exacerbate autoimmune arthritis in animal models. Furthermore, this Mycoplasma species releases substances that act on polymorphonuclear granulocytes, such as oxygen radicals, and chemotactic and aggregating substances [34]. Several studies have shown that mycoplasmal infections lead to increased levels of pro-inflammatory cytokines, such as interleukin-1, -2, -4 and -6 [35, 36]. Therefore, M. arthritidis and possibly other species may be responsible, in part, for autoimmune phenomena in the early stages of RA, and their progression. Deficient
or aberrant immune responses (or other underlying diseases) might be necessary for the development and progression of RA and other rheumatological illnesses. Other microorganisms are still under investigation as causative agents or important cofactors for these chronic diseases. Reports about the detection of Epstein–Barr virus or cytomegalovirus in synovial fluids are controversial [37–39]. Furthermore, retroviruses and enteropathogenic bacteria continue to be intensively discussed as possible aetiological factors of RA [40, 41]. The identification of mycoplasmal infections in the leucocyte blood fractions of a rather large subset of RA patients supports the hypothesis that mycoplasmas, and probably other chronic infections as well, may be an important source of, or cofactor for, morbidity in these patients. Further investigation of the potential role of mycoplasmas in RA patients will require comparison with other forms of arthritis and chronic inflammatory diseases.

Recently, it was found that minocycline is an interesting new drug for the treatment of RA. Tetracycline compounds have long been used by rheumatologists, and their anti-rheumatic activity has been demonstrated [42]. The reason why minocycline alleviates the clinical signs and symptoms of RA is unclear, but the responses of some patients with RA to minocycline might be due to the susceptibility of mycoplasmas to tetracyclines [43].

References

28. Prattichizzo FA, Simonetti I, Galetta F. Carditis associated with Mycoplasma pneumoniae infection. Clinical aspects


35. Mühlradt PF, Quentmeier H, Schmitt E. Involvement of interleukin-1 (IL-1), IL-6, IL-2 and IL-4 in generation of cytolytic T cells from thymocytes stimulated by a Mycoplasma fermentans-derived product. Infect Immun 1991;59:3962–8.


