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Investigation of infectious agents associated with arthritis by reverse transcription PCR of bacterial rRNA

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Abstract

In reactive and postinfectious arthritis the joints are generally sterile but the presence of bacterial antigens and nucleic acids has been reported. To investigate whether organisms traffic to affected joints in these conditions, we performed reverse transcription PCR using universal primers to amplify any bacterial 16S rRNA sequences present in synovial fluid. Bacterial sequences were detected in most cases, even after treatment of the synovial fluid with DNase, implying the presence of bacterial RNA and therefore of transcriptionally active bacteria. Analysis of a large number of sequences revealed that, as reported in rheumatoid arthritis, most were derived from gut and skin commensals. Organisms known to have triggered arthritis in each case were not found by sequencing the products obtained using universal primers, but could in some cases be shown to be present by amplifying with species specific primers. This was the case for *Yersinia pseudotuberculosis* and *Chlamydia trachomatis*. However, in arthritis thought to be related to *Campylobacter* infection the sequences obtained were not from *Campylobacter jejuni* or *C. coli*, but from other *Campylobacter* spp. that are not known to be associated with reactive arthritis and are probably present as commensals in the gut. We conclude that although rRNA from reactive arthritis associated organisms can be detected in affected joints, bacterial RNA from many other bacteria is also present, as was previously noted in studies of other forms of inflammatory arthropathy.

Keywords: bacterial rRNA, *Campylobacter*, *Chlamydia*, reactive arthritis, *Yersinia*

Introduction

Several forms of inflammatory arthritis are triggered by infection with bacteria, including reactive arthritis (ReA) following certain gastrointestinal or urogenital infections [1], Lyme disease [2] and the arthritis associated with streptococcal or neisseria infection. Each of these differs from classical septic arthritis in that bacteria cannot readily be isolated from the affected joints, although occasionally *Borrelia burgdorferi* has been cultured from the synovial fluid (SF) of some patients with Lyme arthritis [2,3], and gonococci or meningococci can be cultured from joints in the early phase of disease [4]. Because the joint is usually sterile, these forms of arthritis have been considered to be the result of some form of autoimmune reaction that was triggered by an infection at a site distant from the joint. However, in more recent studies bacterial antigens [5–7] and, in several cases, bacterial DNA and/or RNA have been identified in synovium or SF from selected patients with ReA [8–10] and Lyme arthritis [11]. This implies that organisms associated with these forms of arthritis do reach the joint, although they may be present there in an uncultivable state, and raises the possibility that joint inflammation is driven by immune responses to bacterial antigens without the need to evoke autoimmune mechanisms. In Lyme disease both mechanisms have been suggested for different phases of disease [12,13]: arthritis driven by bacterial antigens in early disease that is responsive to antibiotic treatment; and autoimmunity in antibiotic-resistant, chronic disease caused by cross-reactive T cells that are responsive to *Borrelia* OspA

bp = base pairs; RA = rheumatoid arthritis; ReA = reactive arthritis; RT = reverse transcription; SAPHO = synovitis, acne, pustulosis, hyperostosis, osteitis; SF = synovial fluid.

protein and self-antigens such as CD11a. However, the precise role of bacterial antigens in pathogenesis has not been defined, and it is unclear whether bacterial antigens reach the joint in sufficient quantities to account for the major sustained inflammation that can characterize ReA.

Using a reverse transcription (RT)-PCR technique to detect bacterial 16S rRNA, we recently demonstrated that transcriptionally active bacteria are present in synovial tissue from patients with rheumatoid arthritis (RA) and other arthropathies [14]. These bacteria were mainly derived from commensals that are normally present in the skin and gut, and we concluded that bacteria engulfed by macrophages can reach the joint when macrophages are recruited to the synovial membrane, particularly when recruitment is increased by joint inflammation. We have now applied this general technique for the detection of bacteria to SF samples from patients with ReA and other forms of postinfectious arthritis. The data show that the same commensal bacteria can also be detected in SF from these patients, whereas specific disease associated bacteria can also be demonstrated in some cases. However, bacterial rRNA sequences from disease associated bacteria were detected relatively infrequently as compared with those from other bacteria also detected in the joint. These findings have implications for our understanding of the pathogenesis of postinfectious arthritis.

Materials and method

Patients

SF samples were collected by routine aspiration, mainly from patients attending the Department of Medicine, Addenbrooke's Hospital, Cambridge, UK. Diagnoses were established by history, serology and culture, as appropriate (Table 1).

Isolation of RNA from synovial fluid and reverse transcriptase PCR amplification of bacterial rDNA

Total RNA was isolated from 200 µl untreated centrifuged SF using lysis in the presence of 500 µl phenol pH 4.0 and 100 µl chloroform isoamyl alcohol in a Hybaid Ribolyser™ (Hybaid, Teddington, Middlesex, UK) according to the manufacturer's instructions, and recovered by precipitation with propan-2-ol, dried under appropriate sterile conditions, and then dissolved in diethylene pyrocarbonate-treated water containing 0.1 mmol/l EDTA. Before the RT step, an aliquot of total RNA was subjected to DNase treatment with 1 unit DNase I (Cambridge Bioscience, Cambridge, UK), involving incubation at 37°C for 30 min followed by denaturation of the enzyme by incubation at 75°C for 15 min, whereas the remaining aliquot was not treated. This was to allow comparisons between DNase treated and untreated RNA.

Control bacterial rRNAs and total SF RNA were reverse transcribed using the same method, as previously described [14]. To eliminate the risk of contamination with bacterial nucleic acids from external sources, all reagents were prepared using distilled water irradiated with ultraviolet at 254 nm for 2 min. Negative controls were included at each stage of the RT-PCR procedure in order to ensure that no contamination of samples occurred during protocol implementation.

Bacterial ribosomal DNA fragments were amplified from total cDNA and bacterial genomic DNA by PCR amplification using universal, bacterial rRNA specific oligonucleotide primers R1 and R2, as previously described in detail [14]. All PCR negative samples were tested for potential PCR inhibitors by the addition of 5 ng *Escherichia coli* genomic DNA, to their cDNA, and retested as described above. If PCR products of the expected size (approximately 400 bp) were detected, then these products were cloned into the PCR product cloning vector pT7-blue (Novagen, Madison, Wisconsin, USA), according to the manufacturer's instructions, for sequencing and analysis as previously described [14].

Development of specific PCR tests

Having designed specific oligonucleotides that were unique for a given bacteria sequence, these were tested against a panel of 2 µl aliquots from 96 sequenced bacteria PCR products inserted into recombinant *E. coli*.

Using standard reaction mixes and standard cycling times (see above), different primers were tested at annealing temperatures of 55, 60, 63 and 65°C. PCR products were then visualized using gel electrophoresis. When the only PCR product detected was from the positive control DNA and the other 95 wells remained negative, a set of primers was considered to be specific. If, however, the positive control also failed before specificity was reached, then the $MgCl_2$ concentration was raised by 0.5 mmol/l increments at the first temperature at which the positive control was seen to fail. If this failed to get the positive control to work before loss of specificity was reached, then the primers were redesigned and the process repeated.

Results

SF samples were collected from 12 patients with various forms of postinfectious arthropathy; 10 had clinical features of ReA and two had postmeningococcal or poststreptococcal arthritis. An additional two patients were studied with undifferentiated spondyloarthropathy in which a diagnosis of chronic ReA was considered possible. Patient details are given in Table 1, along with the basis on which the diagnosis was made. Total RNA was extracted from the SF samples and approximately half was then treated with DNase I, and then aliquots of both were subjected to RT-PCR using universal primers R1 and R2 for detection of bacterial 16S rRNA sequences. Of DNase treated samples 11 out of 14 tested positive, as

Table 1

Clinical features of patients included in the study

		Reverse transcription PCR using						
		Universal primers		Specific primers				
Patient	Bacteria implicated	DNase	Non-DNase	*Yer*	*Chlam*	*E. coli*	*Camp*	Clinical details
A	*Yersinia*	6/20	2/20	+		+		Acute ReA (see [10]); positiveve serology (agglutination 1:2500) and T cell responses
B	Enteric	10/20	7/20			+		Acute ReA following gastroenteritis; organism unknown
C	Meningococci	Negative	2/20			+		Acute postinfective arthritis; sore throat, rash, positive serology; daughter had meningococcal purpura and arthritis
D	*Streptococcus*	17/20	5/20			+		Chronic erosive seronegative oligoarthritis; persistent positive serology – very high ASOT (>1200 U) and anti-Dnase
E	*Chlamydia*	5/20	5/20		+	+		Acute sexually acquired ReA; high T-cell mediated responses to CT
F	Yersinia (?)	7/20	6/20			+		Acute (?) ReA; positive serology, B27+ and T-cell responses; no enteritis
G	Unknown	12/20	1/20			+		Chronic seronegative oligoarthritis; culture positive *Campylobacter* infection 2 years pre-arthritis
H	Unknown	11/20	5/20			+		Chronic seronegative oligoarthritis
I	*Yersinia*	8/20	9/20			+		Acute ReA following gastroenteriitis; positive serology (IgM 1:640, IgG 1:2560, IgA <1:80) and T-cell responses
J	*Chlamydia*	7/20	3/20			+		Acute sexually acquired ReA; high T-cell mediated responses to CT
K	*Campylobacter*	19/20	20/20			+		Psoriatic arthritis complicated by culture positive enteritis and flare in joint symptoms (?ReA)
L	*Campylobacter*	19/20	16/20			+		Acute ReA following enteric infection
M	*Campylobacter*	Negative	Negative					Acute seronegative oligoarthritis and vasculitis; positiveve serology (IgM <1:80, IgG 1:640, IgA 1:160)
N	*Campylobacter*	Negative	Negative					Sero-negative oligoarthritis; positive serology (IgM 1:640, IgG 1:640, IgA 1:160) and T-cell responses

Universal (DNase and non-DNase) indicates the number of clones sequenced giving good quality sequence from PCR products obtained using universal primers R1 and R2 from each patient, with and without DNase treatment. Specific indicates results of subsequent *Yersinia* (*Yer*), *Chlamydia* (*Chlam*), *Escherichia coli*, and *Campylobacter* (*Camp*) specific PCR performed on the products obtained using universal primers. ASOT, antistreptolysin O titre; CT, *Chlamydia trachomatis*.

compared with 12 out of 14 of the untreated samples. SF from patient C was the only sample that tested positive without DNase treatment and negative following DNase treatment, indicating that bacterial 16S DNA was most likely being detected rather than RNA. Although samples from patients M and N were negative with and without DNase treatment, all other samples had evidence of bacterial 16S rRNA, indicating the presence of 'live' transcribing bacteria in the inflamed joint.

Sequencing of the PCR products was then performed. Twenty clones were picked from each PCR product and sequenced. Only good quality, full-length (350–400 bp) sequences were analyzed in detail; short sequences (less than 200 bp) were ignored because the species from which they came could not be accurately identified. A summary of the total number of sequences analyzed from each patient is detailed in Table 2. (Note that the primers R1 and R2 fail to amplify *Chlamydia* 16S rRNA, and so

Table 2

Bacterial sequences identified in synovial fluid from reactive arthritis and postinfectious arthritis patients

Bacteria (Non-DNase)	Bacteria (DNase)	Bacteria (Non-DNase)	Bacteria (DNase)
Patient A			1 × *Neisseria* (3)
1 × *E. coli* (2)	2 × *E. coli* (5)		1 × *Lactobacillus* (3)
1 × *Abiotrophia adiceins*	1 × *Pseudomonas* (1)		1 × *Peptostreptococcus* (2)
	1 × *Pseudomonas* (4)		
	1 × *Streptococcus* (9)	**Patient H**	
	1 × *Anabaena* (1)	3 × *Deinococcus* (1)	3 × *Staphylococcus* (1)
		2 × *Neisseria* (1)	2 × *Lactobacillus* (1)
Patient B			1 × *Bradyrhizobium* (1)
2 × *Propionibacterium acnes* (1)	3 × *Anabaena* (1)		1 × *Bacillus* (5)
1 × *Streptococcus* (7)	1 × *Mycobacterium* (1)		1 × *Peptostreptococcus* (4)
1 × *Anabaena* (1)	1 × *Staphylococcus* (1)		1 × *Anabaena* (1)
1 × *Lactobacillus* (2)	1 × *Peptostreptococcus* (3)		1 × *Taxeobacter*
1 × *Actinomycetes* sp.	1 × *Bacillus* (3)		1 × *Acinetobacter*
1 × Unidentified *Eubacterium*	1 × *Neisseria* (1)		
	1 × *Desulfovibrio* sp.	**Patient I**	
	1 × *Brevundimonas* sp.	2 × *Prevotella* (2)	1 × *Curtobacterium* (1)
		1 × *Bacillus* (4)	1 × *Curtobacterium* (2)
Patient C		1 × *Methylobacterium* (1)	1 × *P. acnes* (1)
1 × *P. acnes* (1)		1 × *Neisseria* (1)	1 × *Bradyrhizobium* (1)
1 × *Streptococcus* (2)		1 × *P. acnes* (1)	1 × *Mycobacterium* (2)
		1 × *Bradyrhizobium* (1)	1 × *Anabaena* (1)
Patient D		1 × *Pseudomonas* (2)	1 × *Pseudomonas* (2)
1 × *Staphylococcus* (1)	2 × *P. acnes* (1)	1 × *Dunganella*	1 × Unidentified *Eubacterium*
1 × *Pseudomonas* (6)	1 × *Staphylococcus* (2)		
1 × *Corynebacterium* (3)	1 × *Staphylococcus* (3)	**Patient J**	
1 × *Gamella* (2)	1 × *Corynebacterium* (2)	1 × *Corynebaterium* (1)	1 × *Corynebacterium* (4)
CC29 *Agrobacterium*	1 × *Nevskia ramosa* (1)	1 × *Staphylococcus* (3)	1 × *Staphylococcus* (2)
	1 × *Anabaena* (1)	1 × *Paracoccus* sp.	1 × *Pseudomonas* (1)
	1 × *Lactobacillus* (2)		1 × *Streptococcus* (1)
	1 × *Methylobacterium* (2)		1 × *P. acnes* (1)
	1 × *Streptococcus* (2)		1 × *Bradyrhizobium* (1)
	1 × *Rhodococcus* (2)		1 × *Nevskia ramosa* (1)
	1 × *Timone* (2)		
	1 × Unidentified	**Patient K**	
	1 × *Tetracoccus*	3 × Unidentified *Eubacterium* (1)	3 × *Anabaena* (2)
	1 × *Comamonas*	3 × *Anabaena* (2)	1 × *Anabaena* (3)
	1 × *Frankia*	2 × *P. acnes* (1)	3 × *P. acnes* (1)
	1 × Actinomycetes	2 × *Alacaligenes* (1)	2 × *Lactobacillus* (2)
		2 × *Rhodococcus* (1)	2 × *Nocardioides* (1)
Patient E		1 × *Streptococcus* (6)	1 × *Neisseria* (2)
3 × *Staphylococcus* (1)	1 × *Anabaena* (1)	1 × *Neisseria* (2)	1 × *Timone* (1)
1 × *Staphylococcus* (3)	1 × *Streptococcus* (1)	1 × *Staphylococcus* (1)	1 × *Prevotella* (1)
1 × *Streptococcus* (8)	1 × *Pseudomonas* (1)	1 × β-Proteobacterium	1 × Unidentified *Eubacterium* (1)
	1 × *Neviskia ramosa* (1)	1 × Unidentified *Eubacterium*	1 × *Staphylococcus* (1)
	1 × Unidentified	1 × Brevibacterium	1 × *Burkholderia*
		1 × *Burkholderia*	1 × *Kineosporia*
Patient F		1 × *Nitrosomonas*	1 × *Spirochaeta*
1 × *Propionibacterium* (2)	2 × *Pseudomonas* (2)		
1 × *Streptococcus* (3)	1 × *Propionibacterium* (2)	**Patient L**	
1 × *Anabaena* (1)	1 × *Anabaena* (1)	4 × Unidentified *Eubacterium* (1)	9 × Unidentified *Eubacterium* (1)
1 × *Stenotrophomonas*	1 × *Corynebacterium* (1)	3 × *Gamella* (1)	3 × *Micrococcus* (1)
1 × *Burkholderia*	1 × *Streptococcus* (4)	1 × *Staphylococcus* (1)	1 × *Micrococcus* (2)
1 × *Actinomyces*	1 × *Afipia*	1 × *P. acnes* (1)	1 × *Methylobacterium* (1)
		1 × *Pseudomonas* (3)	1 × *Methylobacterium* (2)
Patient G		1 × *Corynebaterium* (1)	1 × *Alcaligenes* (2)
1 × *Pseudomonas* (5)	3 × *P. acnes* (1)	1 × *Timone* (1)	1 × *Staphylococcus* (1)
	3 × *Pseudomonas* (5)	1 × *Rhodococcus* (1)	1 × *Afipia*
	1 × *Streptococcus* (2)	1 × *Peptostreptococcus* (1)	1 × *Pinus strobus*
	1 × *Streptococcus* (5)	1 × *Neisseria* (2)	
	1 × *Gamella* (3)	1 × *Methylobacterium* (1)	

Details of the bacterial species identified by sequencing clones obtained following reverse transcription PCR. Twenty clones were picked from each PCR product. Only good quality, full-length sequences (350–400 bp) were analyzed in detail; shorter sequences were ignored because the species from which they came could not be accurately identified. Each column shows the identity and the number of sequences obtained. The numbers in brackets after species names serve to identify different sequences from the same organism. Full details of the sequences can be obtained from the authors on request.

Table 3

Primers and amplification conditions used in the study

Forward primer (5′–3′)	Reverse primer (5′–3′)	Specific for	Annealing temperature /time	$MgCl_2$ concentration (mmol/l)	Size of product (bp)
AGTAGTTTACTACTTTGCCG	ACTGCTGCCTCCCGTAGGAG	Universal (R1 and R2)	58°C/60 secs	1.5	350
CATAACGTCGCAAGACCAAA	GTGCAATATTCCCCACTGCT	*E. coli*	58°C/45 secs	1.5	187
TTGGGAATAACGGTTGGAAA	TGTCTCAGTCCCAGTGTTGG	*Chlamydia* spp.	59°C/30 secs	1.5	203
CGCACGGGTGAGTAAGGTA	GCGTCATAGCCTTGGTAAGC	*Campylobacter* spp.	66°C/1 min	2.5	170
AGTAGTTTACTACTTTGCCG	CCGATGGCGTGAGGCCCTAA	*Yersinia* spp.	65°C/30 secs	3.5	154
CGCACGGGTGAGTAAGGTA	GCTTAACACAAGTTGACTAG	*Campylobacter jejuni*	63°C/30 secs	2.5	70
CGCACGGGTGAGTAAGGTA	GTCTTACATAAGTTAGATA	*Campylobacter concisus*	55°C/30 secs	2.0	70
CGCACGGGTGAGTAAGGTA	ATACCTCATACTCCTATTTAAC	*Bacteroides ureolyticus*	55°C/30 secs	2.0	70

Specific oligonucelotides were designed as described here. Standard reaction mix was used with each set of oligonucleotides (see Materials and method), although annealing conditions varied, as did $MgCl_2$ concentration. Denaturation conditions were always 94°C for 1 min and extension was performed at 72°C for 1 min (10 min final extension). Typically 35–40 cycles were performed. If no PCR product was detected, then a second round of amplification was performed using 2 µl of PCR product as template.

Chlamydia sequences would not be expected to be found in this analysis; their presence is investigated below when *Chlamydia*-specific primers were developed and used.)

As previously noted in the study of bacterial rRNA sequences amplified from synovium of RA patients [14], each patient's SF contained a diverse range of bacteria with no one species dominating the population detected. Interestingly, with the possible exception of patient D, in whom *Streptococcus* spp. were detected, no evidence of the bacteria thought to be the causative agent for arthritis was detected in any of the samples. Although there was a reasonable correlation between the bacteria detected with and without DNase treatment (e.g. patient K), some samples showed the presence of quite different bacteria in the DNase treated and untreated samples (e.g. patient H). This probably reflects the relatively small number of sequences generated from each sample. In our previous study in which 46 sequences from each tissue sample were analysed [14], there was only approximately 80% correlation between the bacteria detected when the same PCR product was cloned and sequenced on separate occasions. This indicates that even with a large amount of sequencing only a relatively small proportion of the total bacteria present within the joint are detected.

Detection of specific disease associated bacteria

Having failed to detect evidence of bacterial 16S rRNA from any of the causative agents in any of the SF samples tested, primers were designed that were specific for *Yersinia* spp., *Campylobacter* spp. and *Chlamydia* spp.; as a control, primers to detect *E. coli* sequences were also used because *E. coli* rRNA had been detected in SF samples in this and previous studies (Table 2). As a rapid means of testing the specificity of these oligonucleotides, a 96-well plate was set up in which each well contained 200 µl recombinant *E. coli* with an inserted cloning vector containing the 16S rRNA sequence of one of the bacteria detected during the study. This allowed a diverse range of different bacteria to be screened using various specific oligonucleotides. In addition, because the 16S rRNA sequences expressed in *E. coli* were from bacterial products actually isolated from synovial tissue and SF, they were a valid test of the ability of the oligonucleotides to distinguish between different bacteria likely to be present in the joint.

Using this technique we were able to generate oligonucleotides (Table 3) that could specifically detect *Yersinia* spp., *Campylobacter* spp., *Chlamydia trachomatis* and *E. coli* sequences. When these primers were tested in the 14 patients described in Table 1, positive results were obtained in only two cases. In patient A with *Yersinia*-induced ReA a product was obtained using *Yersinia*-specific primers, whereas for patient E with *Chlamydia*-induced ReA a product was obtained with *Chlamydia*-specific primers. Subsequent cloning and sequencing of the specific PCR products generated indicated that the *Yersinia* sp. detected was either *Yersinia pseudotuberculosis* or *Y. pestis* (very closely related). Serology indicated that this patient had indeed been infected with *Yersinia pseudotuberculosis* [10]. Likewise, the products obtained with *Chlamydia*-specific primers were from *C. trachomatis*.

Use of *Campylobacter*-specific primers also produced products in patients K and L, both of whom were thought to have *Campylobacter*-triggered ReA. However, subse-

quent sequencing of the *Campylobacter*-specific products did not reveal the presence of *Campylobacter jejuni* or *Campylobacter coli*, the bacteria most commonly associated with ReA. SF from patient K was found to contain a *Campylobacter concisus* sequence, whereas that from patient L contained a different *Campylobacter concisus* sequence and a *Bacteroides ureolyticus* sequence (a species with rRNA very closely related to that of *Campylobacter* spp.). Neither of these gut and urogenital tract bacteria has previously been reported to be associated with ReA.

Further investigations of *Campylobacter* sequences detected

The detection of *Campylobacter*-related sequences, which appeared to be specific for samples taken from patients with *Campylobacter*-associated ReA, raised the possibility that we had identified new *Campylobacter* spp. that might be associated with disease. To address this issue further, efforts were made to gather more sequence data on the *Campylobacter* spp. identified. The *Campylobacter*-specific oligonucleotides only generate a 150 bp fragment for sequence, whereas identification of the PCR product generated using universal primers R1 and R2 that contained the *Campylobacter* sequences should reveal 400 bp of sequence from that bacterium. However, in spite of isolating more than 1000 individual clones from each of the PCR products obtained using 'universal' primers, and screening using *Campylobacter*-specific primers, we were unable to identify a single clone that contained the *Campylobacter* sequence (but note that amplification of the 'universal' PCR product using the *Campylobacter*-specific primers confirmed that the *Campylobacter* sequence was present within the population). This would appear to indicate that the *Campylobacter* spp. detected by specific PCR within the product obtained using 'universal' PCR represent less than 0.1% of the total bacteria found within the inflamed joints of these patients. However, the copy number of 16S rRNA per cell can vary enormously from bacteria to bacteria, and this low frequency of *Campylobacter* sequences could reflect less transcriptionally active bacteria being swamped by more active bacteria.

Specific oligonucleotides were then designed to distinguish between *Campylobacter concisus*, *Campylobacter jejuni* and *B. ureolyticus*. Although these species are very closely related, oligonucleotides were developed that could differentiate between the three (Table 3). Large scale screening of 'universal' 16S rRNA PCR products generated from SF and synovial tissue from RA, non-RA and ReA patients (48 samples tested in total) revealed that *Campylobacter concisus* and *B. ureolyticus* could be detected in five samples (two *Campylobacter*-associated ReA, one *Chlamydia*-associated ReA, one RA sample and one undifferentiated arthritis sample). In contrast, *Chlamydia trachomatis* sequences were only ever detected in samples taken from patients with *Chlamydia trachomatis* associated ReA (four out of six samples from patient E, obtained at different time points over 3 years of active arthritis, and additional patients subsequent to this study), whereas 41 non-*Chlamydia* associated samples were negative. In addition *Y. pseudotuberculosis* sequences were only detected in samples from patients with *Y. pseudotuberculosis* associated ReA (one out of three samples tested), whereas all controls were negative. This would imply that the *Campylobacter* sequences we detected in ReA SF were the result of very low levels of commensal *Campylobacter* spp., rather than specific disease causing bacteria. *Campylobacter jejuni* sequences were not detected in any samples, implying that live *Campylobacter jejuni* does not reach the inflamed joint.

Discussion

Although bacterial antigens, DNA and RNA from the arthritis-triggering organism have previously been reported in SF and tissue from ReA patients, the presence of a wide variety of bacterial species was unexpected, but we reported very similar findings in our previous study of RA and other forms of chronic arthritis [14]. The source of these organisms is not known but may be environmental or from the indigenous microflora. As noted, we took stringent precautions to avoid laboratory contamination, and the organisms identified cannot all be accounted for by skin bacteria introduced into the SF at the time of percutaneous aspiration. We did not surgically remove the skin at the aspiration site to avoid contamination with skin flora, but our results are rather similar to those obtained by others who took this precaution [15]. As tests of the effectiveness of our precautions, reagent controls were always negative, and when normal synovium was tested (in a previous study) no bacterial rRNA was obtained. In the present study two SF samples were also negative when DNase was used. In addition, the sequences obtained varied between patients (including the *E. coli* sequences), arguing against laboratory contamination. Together, this constitutes strong evidence of the ability of commensal organisms such as *E. coli* to colonize inflamed joints; the gut in different patients would be expected to contain a range of *E. coli* 'subspecies', with minor variant rRNA sequences.

Our results are similar to those detailed in other recently published reports [16–18], although our use of RT-PCR rather than PCR is likely to be responsible for the high proportion of SF samples in which we could detect bacteria. Nevertheless, all of the studies have emphasized that bacterial nucleic acids can be detected in many forms of inflammatory arthropathy (RA, inflammatory osteoarthritis, crystal arthropathy [18]) in addition to ReA, and that organisms associated with ReA can be found in synovium/SF of patients with other diagnoses [16]. Many

species that we found in ReA SF (e.g. *Propionibacterium acnes* and *Streptococcus epidermidis*) were seen in our own previous study, implying that their presence in the joint is not disease specific and that they are likely to be opportunistic colonizers of inflamed joints. *P. acnes* has also be implicated by some investigators in the pathogenesis of SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis) [19]; our data confirm that the organism can access joints, but if it plays a role in SAPHO then this must reflect an abnormal response to the organism in that disease because it is clearly present in synovium from other conditions. Likewise, although streptococci were identified in a patient with seronegative erosive arthritis and persistent high titres of antistreptococcal antibodies, the role of streptococci in this case must remain speculative because streptococcal rRNA was amplified from six other ReA and several RA patients in the previous study.

The present analysis of SF from ReA patients indicates that, even in patients in whom an infectious agent is known to be involved in the pathogenesis of the arthritis, detection of that infectious agent by universal PCR can be masked by the high level of commensal bacteria present. Their presence suggests trafficking from sites such as the gut (an idea strengthened by the detection of *E. coli* sequence in all samples). In enteric ReA active bowel inflammation will inevitably affect the barrier function of the gut wall, allowing systemic access by gut flora. Nonsteroidal drugs have also been reported to impair gut permeability and mucosal competence [20,21], and most of our patients were taking such drugs. The fact that sequences from species of bacteria that definitely play a role in triggering arthritis are in a distinct minority among all of the sequences detected within ReA SF raises questions about the role of intra-articular bacteria in the pathogenesis of ReA, and questions the practical use of universal PCR to diagnose ReA. Although the technique shows increased sensitivity over detection of 16S rDNA, this increased sensitivity may also mask detection of the causative agent. However, the use of specific primers showed complete specificity (*Yersinia* and *Chlamydia* rRNAs were only detected in patients in whom other evidence pointed to the involvement of these organisms) but low sensitivity in the case of *Yersinia*, because only one out of three patients with ReA thought to be related to *Yersinia* infection had evidence of the specific rRNA in SF.

The findings from investigations in patients with *Campylobacter*-induced ReA were unexpected. Although *Campylobacter* sequences were isolated, *Campylobacter jejuni* was never detected in any of the samples tested. Our data are unable to distinguish clearly between the possibility that certain *Campylobacter* spp. other than *Campylobacter jejuni* can sometimes be associated with ReA, and the dissemination of commensal *Campylobacter* spp. to inflamed joints along with other components of gut flora. The latter is more likely in view of the isolation of *Campylobacter* sequences from patients with diagnoses other that ReA, but it is not inconceivable that a normal member of the gut flora could, under the appropriate circumstances, be involved in triggering ReA; ReA secondary to *Clostridium difficile* infection falls into this category [22].

In conclusion we have shown that, in forms of arthritis associated with preceding infection, rRNA from the organism responsible can sometimes be demonstrated within SF. However, rRNAs from the commensal flora of gut and skin are even more easily detected. What are the implications of these findings for our understanding of the pathogenesis of postinfectious arthritis? First, the fact that rRNA sequences from disease associated organisms are a minority of those detected in the joint does not necessarily imply that the disease associated organisms have no role in pathogenesis locally. They may synthesize antigens that evoke vigorous T-cell mediated immune responses, as suggested by the consistent finding of prominent responses to triggering organisms in ReA SF T cells [23,24]. In contrast, the immune system is usually tolerant of gut flora [25]. It is clear that the traffic of organisms to the joint does not lead to their replication and sepsis, because the joints remain sterile when culture studies are carried out. Presumably, they are controlled by innate immune mechanisms, including macrophages and polymorphs; indeed, it is likely that many of the organisms detected were engulfed by phagocytes in the periphery that were then recruited to the inflamed joint. Nevertheless, although the organisms may be rapidly eliminated or contained, and may not elicit specific immune responses, they may have a proinflammatory effect because of the effects of component such as lipopolysaccharide, bacterial DNA and bacterial heat shock proteins on the innate immune system [26–29]. These effects may amplify inflammation initially triggered by an immune response to an ReA-associated organism. The duration and severity of arthritis following infection could reflect the efficiency with which these generic proinflammatory mechanisms are brought under control.

Conclusion

RT-PCR using universal primers reveals the presence of bacterial 16S rRNA in SF from patients with ReA or postinfectious arthritis. However, the majority of the sequences detected are derived from commensal bacteria that traffic, probably in macrophages, to the inflamed joint. 16S rRNA from organisms that are responsible for causing arthritis can also be detected but only in a minority of cases, and only by using species-specific primers for amplification. This might reflect low abundance of these bacteria in SF, but truly quantitative studies targeting organism chromosomal and DNA and using real-time PCR would be required to confirm this idea.

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References
1. Gaston J: **Symposium: reactive arthritis**. *Rheumatol Europe* 1995, **24**:5-22.
2. Nocton JJ, Steere AC: **Lyme disease.** *Adv Intern Med* 1995, **40**: 69-117.
3. Johnston YE, Duray PH, Steere AC, Kashgarian M, Buza J, Malawista SE, Askenase PW: **Lyme arthritis: spirochaetes found in synovial microangiopathic lesions.** *Am J Pathol* 1986, **118**:26.
4. Mahowald M: **Gonococcal arthritis.** In *Rheumatology*, vol 2. Edited by Klippel J, Dieppe PA. London: Mosby; 1998: 6.3.1-6.3.8
5. Keat A, Thomas B, Dixey J, Osborn M, Sonnex C, Taylor-Robinson D: ***Chlamydia trachomatis* and reactive arthritis: the missing link.** *Lancet* 1987, **i**:72-74.
6. Granfors K, Jalkanen S, von Essen R, Lahesmaa-Rantala R, Isomaki O, Pekkola-Heino K, Merilahti-Palo R, Saario R, Isomaki H, Toivanen A: ***Yersinia* antigens in synovial fluid cells from patients with reactive arthritis.** *N Engl J Med* 1989, **320**:216-221.
7. Granfors K, Jalkanen S, Lindberg AA, Maki-Ikola O, von Essen R, Lahesmaa-Rantala R, Isomaki H, Saario R, Arnold WJ, Toivanen A: ***Salmonella* lipopolysaccharide in synovial cells from patients with reactive arthritis.** *Lancet* 1990, **335**:685-688.
8. Taylor-Robinson D, Gilroy C, Thomas B, Keat A: **Detection of *Chlamydia trachomatis* DNA in joints of reactive arthritis patients by polymerase chain reaction.** *Lancet* 1992, **340**:81-82.
9. Schumacher H, Arayssi T, Branigan P, Gerard H, Klippel J, Pando J, Getsy J, Bulbul R, Yarboro C, Rothfuss S, *et al.*: **Surveying for evidence of synovial Chlamydia trachomatis by polymerase chain reaction (PCR). A study of 411 synovial biopsies and synovial fluids**. *Arthritis Rheum* 1997, **40**:S270.
10. Gaston JSH, Cox C, Granfors K: **Clinical and experimental evidence for persistent Yersinia infection in reactive arthritis.** *Arthritis Rheum* 1999, **42**:2239-2242.
11. Nocton JJ, Dressler F, Rutledge BJ, Rys PN, Persing DH, Steere AC: **Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis**. *N Engl J Med* 1994, **330**:229-234.
12. Gross DM, Forsthuber T, Tarylehmann M, Etling C, Ito K, Nagy ZA, Field JA, Steere AC, Huber BT: **Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis.** *Science* 1998, **281**:703-706.
13. Hemmer B, Gran B, Zhao YD, Marques A, Pascal J, Tzou A, Kondo T, Cortese I, Bielekova B, Straus SE, *et al.*: **Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease.** *Nature Med* 1999, **5**:1375-1382.
14. Kempsell KE, Cox CJ, Hurle M, Wong A, Wilkie S, Zanders ED, Gaston JSH, Crowe JS: **Reverse transcriptase-PCR analysis of bacterial rRNA for detection and characterization of bacterial species in arthritis synovial tissue.** *Infection Immun* 2000, **68**: 6012-6026.
15. Wilbrink B, Vanderheijden IM, Schouls LM, Vanembden JDA, Hazes JMW, Breedveld FC, Tak PP: **Detection of bacterial DNA in joint samples from patients with undifferentiated arthritis and reactive arthritis, using polymerase chain reaction with universal 16S ribosomal RNA primers.** *Arthritis Rheum* 1998, **41**:535-543.
16. Olmez N, Wang GF, Li Y, Zhang HD, Schumacher HR: **Chlamydial nucleic acids in synovium in osteoarthritis: what are the implications?** *J Rheumatol* 2001, **28**:1874-1880.
17. Gerard HC, Wang Z, Wang GF, ElGabalawy H, GoldbachMansky R, Li Y, Majeed W, Zhang H, Ngai N, Hudson AP, *et al.*: **Chromosomal DNA from a variety of bacterial species is present in synovial tissue from patients with various forms of arthritis.** *Arthritis Rheum* 2001, **44**:1689-1697.
18. van der Heijden IM, Wilbrink B, Tchetverikov I, Schrijver IA, Schouls LM, Hazenberg MP, Breedveld FC, Tak PP: **Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides.** *Arthritis Rheum* 2000, **43**:593-598.
19. Kotilainen P, Merilahti-Palo R, Lehtonen OP, Manner I, Helander I, Mottonen T, Rintala E: ***Propionibacterium acnes* isolated from sternal osteitis in a patient with SAPHO syndrome.** *J Rheumatol* 1996, **23**:1302-1304.
20. Mielants H, Goemaere S, De Vos M, Schelstraete K, Goethals K, Maertens M, Ackerman C, Veys EM: **Intestinal mucosal permeability in inflammatory rheumatic diseases. I. Role of antiinflammatory drugs.** *J Rheumatol* 1991, **18**:389-393.
21. Bjarnason I: **Intestinal permeability.** *Gut* 1994, **35(suppl)**:S18-S22.
22. Cope A, Anderson J, Wilkins E: ***Clostridium difficile* toxin induced reactive arthritis in a patient with chronic Reiter's syndrome.** *Eur J Clin Microbiol Infect Dis* 1992, **11**:40-43.
23. Gaston JSH, Life PF, MerilahtiPalo R, Bailey L, Consalvey S, Toivanen A, Bacon PA: **Synovial T lymphocyte recognition of organisms that trigger reactive arthritis.** *Clin Exp Immunol* 1989, **76**:348-353.
24. Sieper J, Braun J, Wu P, Kingsley G: **T-cells are responsible for the enhanced synovial cellular immune response to triggering antigen in reactive arthritis**. *Clin Exp Immunol* 1993, **91**:96-102.
25. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Meyerzum-Buschenfelde K: **Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease.** *Clin Exp Immunol* 1995, **102**:448-455.
26. Medzhitov R, Prestonhurlburt P, Janeway CA: **A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.** *Nature* 1997, **388**:394-397.
27. Deng GM, Nilsson IM, Verdrengh M, Collins LV, Tarkowski A: **Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis.** *Nat Med* 1999, **5**:702-705.
28. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, *et al.*: **A Toll-like receptor recognizes bacterial DNA.** *Nature* 2000, **408**:740-745.
29. Kol A, Lichtman AH, Finberg RW, Libby P, Kurt-Jones EA: **Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells.** *J Immunol* 2000, **164**:13-17.

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