Detection of Bacterial DNA in Latin American Patients with Reactive Arthritis by Polymerase Chain Reaction and Sequencing Analysis

RAQUEL CUCHACOVICH, SHANKAR JAPA, WEN QUN HUANG, ARMANDO CALVO, LUIS VEGA, RUBEN BURGOS VARGAS, RANJU SINGH, DIANA FLORES, IVETTE CASTRO, and LUIS R. ESPINOZA

ABSTRACT. ***Objective.*** Bacteria and/or their antigens are thought to play a role in the pathogenesis of reactive arthritis (ReA). Polymerase chain reaction (PCR) using the 16S ribosomal RNA-PCR method was used to identify bacterial DNA in synovial fluid (SF) and tissue (ST) in a well defined group of patients with chronic ReA. In addition, species found were identified by means of sequence analysis.

Methods. We examined 15 ST and 5 SF samples of 15 patients with ReA, 5 ST samples of 5 patients with osteoarthritis (OA), and 8 SF from 8 patients with closed traumatic knee injuries using a nested PCR with universal 16S rRNA primers. In addition, a nested PCR was developed to detect DNA sequences of *Salmonella* sp. and *Mycoplasma* sp. Automated sequencing and comparative data analysis (GenBank) were also performed to identify the species.

Results. Bacterial DNA was identified in 8 cases, 5 ST and 3 SF; *Chlamydia trachomatis* (n = 2), *Pseudomonas* sp. (n = 3), and *Bacillus cereus* (n = 2) were the most common microorganisms identified. A variety of microorganisms including *Clostridium* sp., *Lactobacillus* sp., *Pseudomonas migulae*, *P. fluorescens*, and *P. putida*, and *Neisseria meningitidis* serogroup B were also identified. In half of the cases (4/8) 2 to 3 bacterial antigens were identified simultaneously.

Conclusion. Bacterial DNA is present in the joints in patients with chronic ReA. A wide spectrum of bacteria including some not previously associated with ReA were identified. Further studies are needed to establish their exact role in the pathogenesis of ReA and related arthritides. (J Rheumatol 2002;29:1426–9)

Key Indexing Terms:
REACTIVE ARTHRITIS PSORIATIC ARTHRITIS OLIGOARTHRITIS
ANKYLOSING SPONDYLITIS POLYBACTERIAL DNA

Spondyloarthropathies (SpA) constitute a clinical spectrum of inflammatory musculoskeletal disorders of special interest due to their close association with MHC class I molecule HLA-B27 and with a variety of triggering microorganisms[1,2]. This association is not clearly defined, although experimental animal models support an active role for HLA-B27 antigen[3]. It has also been postulated that the expression of HLA-B27 can modify the response to bacterial contact and modulate bacteria invasion and survival, but there is no universal agreement on these proposed mechanisms[2].

On the other hand, the increasing use of the molecular biological techniques such as polymerase chain reaction (PCR) has facilitated the identification of bacterial DNA and RNA of multiple species in synovial fluid (SF) and synovial tissue (ST) of patients with different forms of SpA[4-7]. The identification of persistent bacterial antigens within the joint has strengthened the notion for an active role of microorganisms in the pathogenesis of this group of disorders.

We investigated the presence of bacterial DNA in SF and ST using the 16S ribosomal RNA-PCR method, and sought to identify the species of the bacteria using sequence analysis in a group of patients with longstanding SpA.

MATERIALS AND METHODS

Patients. We examined ST and SF samples from knee effusions of 15 Peruvian patients presenting to the outpatient arthritis clinic at the University Hospital, Cayetano Heredia University, Lima, Peru. All patients were men, with a mean age of 31.9 ± 16.4 (range 11–64 yrs), mean disease duration of 12.4 ± 8.4 (6–30 mo), exhibiting an oligoarticular/monoarticular clinical picture, with the knee joint involved in all cases. All patients were symptomatic, without antibiotic therapy, and receiving nonsteroidal antiinflammatory therapy (Table 1). Clinical diagnosis of reactive arthritis (ReA) was established using diagnostic criteria[8]. In addition, ST was obtained from 5 patients with osteoarthritis undergoing routine arthro-

From the Department of Medicine, Section of Rheumatology, LSU Health Science Center, New Orleans, Louisiana, USA; Cayetano Heredia University, Lima, Peru; and Hospital General de Mexico, Mexico DF, Mexico.

R. Cuchacovich, MD, Associate Professor; W.Q. Huang, MD; R. Singh, MD, Rheumatology Fellow; D. Flores, MD, Research Fellow; I. Castro, MD, Research Fellow; L.R. Espinoza, MD, Professor and Chief, LSU Medical Center; S. Japa, PhD, Assistant Professor, Pathology Department, Tulane University, New Orleans; A. Calvo, MD, Associate Professor; L. Vega, MD, Assistant Professor, Cayetano Heredia University; R. Burgos-Vargas, MD, Professor, Hospital General de Mexico.

Address reprint request to Dr. L.R. Espinoza, LSU Medical Center, 1542 Tulane Ave., New Orleans, LA 70112.

Submitted September 19, 2001; revision accepted January 9, 2002.

Table 1. Clinical features of patients with reactive arthritis.

Clinical Features	Bacterial DNA Positive, n = 8	Bacterial DNA Negative, n = 7
Mean age, yrs	33.1 (SD 16.5)	30.6 (SD 17.4)
Mean disease duration, mo	11 (SD 8.7)	13.857 (SD 8.113)
History of genitourinary infection	2	2
History of gastrointestinal infection	1	1
Joint pattern		
Monoarticular	4	5
Oligoarticular	4	2
Sacroiliac involvement	–	–
Extraarticular manifestations		
Ocular	–	–
Urethritis	+	+
Rash	–	–
Diarrhea	+	+
HLA-B27	1	0
Mean sedimentation rate	42.8 (SD 10.3)	36.4 (SD 10.0)

scopic knee surgery and SF from 8 closed traumatic knee injuries. Particular care was taken to maintain a sterile environment and avoid contamination when obtaining the samples.

Methods. ST and SF were obtained from the knee joint in all cases. ST samples were obtained with a Parker Pearson needle; for SF the second portion was used for analysis to reduce the chance of contamination by skin flora. Microbiologic cultures and screening for crystals were performed routinely. All SF samples were also cultured for aerobic and anaerobic microorganisms, *Mycobacteria* and *Mycoplasma* spp. using established culture techniques.

HLA-B27 positivity was determined using a microcytotoxicity assay.

Genomic DNA was isolated from the samples using a QIA amp DNA mini kit (Qiagen Inc., Chatsworth, CA, USA). Determination of DNA concentration, yield, and purity was done by measuring the absorbance at 260 nm. The DNA was amplified using the primers (Table 1) as described[9]. The PCR reaction mixture consisted of 8FPL/806R and 515FPL/13B, 1× PCR buffer (100 mM Tris-HCL, pH 8, 3–500 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM PCR primers, 2.5 U AmpliTaq DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), 1 μg DNA template in a final volume of 100 μl reaction mixture. Primers were synthesized by Bio Source international (Camarillo, CA, USA).

Two sets of primers [8FPL/806R (834 bp) and 515FPL/13B (904 bp); Table 2] were used in a one step PCR, and the amplification consisted of initial denaturation step at 94°C for 5 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min. A final extension step at 72°C for 10 min was also performed.

Positive samples for eubacterial 16S rRNA gene were reamplified performing a nested PCR using the 515FPL/806 primers (328 bp). Amplification consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. All PCR steps were carried out in a Perkin-Elmer Gene Amp PCR System 2400. Also, all samples were analyzed by one step PCR with 2 sets of *Salmonella* sp. primers [ST15/ST11 (429 bp)[10] and INV-A/INV-E (457 bp)[11,12]], which cover more than 19 serovars of *Salmonella*.

The 16S rRNA-PCR amplicons were detected by ethidium bromide stained 3% Nusieve agarose gels (FMC, Gibco BRL, NY, NY, USA). To avoid contamination, the sample preparation, pipetting of reaction tubes, and amplification were done in 3 different laboratory rooms, under safety and sterilized hoods.

Sequence analysis. The 328 bp PCR product identified by agarose gel electrophoresis was purified using a QIA quick purification kit (Qiagen, Chatsworth, CA, USA) and then subjected to automated sequencing (DNA Sequencing Laboratory, Yale University). The DNA sequences were compared for similarity using the Fast A program on the US National Center for Biotechnology Information database BLAST search.

RESULTS

Routine bacteriologic cultures including cultures for mycoplasma were negative.

PCR analysis of patient samples. Bacterial DNA, using broad range primers, was detected in the ST of 5 patients and in 3 SF samples (53.3%).

The bacterial species from all ReA patients were identified after sequencing and comparative data analysis of the 16S amplicons from the ST and SF positive samples. *Chlamydia trachomatis*, *Pseudomonas migulae*, *P. fluorescens*, and *P. putida* were found in ST; *Clostridium* sp., *Lactobacillus*, *Neisseria meningitidis* serogroup B, and *Bacillus cereus* in SF. One class of bacterial DNA was identified in 4/8 samples, and *C. trachomatis* and *Pseudomonas* sp. were the most common bacterial DNA identified in this group. In 4/8 (50%) of the PCR positive samples, 2 to 3 different classes of bacterial DNA were identified and these were *P. fluorescens*, *P. putida*, and *P. migulae*. *Bacillus*

Table 2. Primers for broad range PCR amplification of bacterial 16S rDNA sequences.

Primer	Nucleotide Sequence, 5′ to 3′											16S rRNA Positions*
Forward												
8FPL	GCG GCT	GAT CAG	CCG	CGG	CCG	CTG	CAG	AGT	TTG	ATC	CTG	8–27
515FP**	GCG GGT	GAT AA	CCT	CTA	GAC	TGC	AGT	GCC	AGC	AGC	CGC	515-533
Reverse												
806R	GCG AAT	GAT	CCG	CGG	CCG	CGG	ACT	ACC	AGG	GTA	TCT	806–787
13B	CGG	GAT	CCC	AGG	CCC	GGG	AAC	GTA	TTC	AC		1390-1371

* *E. coli* 16S rRNA positions that correspond to the 5' and 3' ends of each of the primers. ** These primers are homologous to 16S-like rRNA sequences from many organisms in all 3 domains.

cereus was present with *Clostridium* sp. and *Lactobacillus* in 2 cases (Table 3).

The validity of our data is emphasized by the fact that no concomitant bacterial DNA was detected in patients with definite osteoarthritis or in healthy controls.

Correlation HLA-B27 and bacterial DNA. Among the 15 patients studied, one was HLA-B27 positive. Correlation between HLA-B27 and presence of bacterial DNA could not be ascertained due to the low prevalence of HLA-B27 in this population.

DISCUSSION

Our findings demonstrate the presence and identification of bacterial species in SF and ST samples of patients with chronic ReA. The use of a sensitive method to amplify eubacterial 16S rDNA confirmed the presence or absence of bacterial pathogens at normally sterile body sites. This was accomplished by amplifying 16S rRNA gene using PCR and subsequent sequence analysis. It was also possible in SF and ST samples to analyze the bacterial species involved in our patients with ReA, by following stringent techniques for both sample collection and subsequent molecular microbiologic analysis. The bacterial DNA products identified appeared to be derived from several bacterial species, although all of them can be found in the human intestinal, urogenital, and respiratory tracts. Further, PCR products were found with similar frequency in SF and ST samples, although they were not often simultaneously detected in all of them. Of interest, bacterial DNA was detected in patients with established disease of long duration of up to 10 years.

Our data are relevant and interesting in view of the low prevalence of seronegative SpA in Latin American countries[13], particularly in Peru. This has been thought to be secondary to the low prevalence of HLA-B27. Indeed, Peruvian Mestizos have a frequency of HLA-B27 of only 1.1%, and similar figures are also described in Chile. On the other hand, there is a high prevalence of endemic dysentery in these populations, and Gram negative enteral microorganisms including *Shigella*, *Campylobacter*, and *Salmonella* spp. are the most prevalent etiologic agents[14].

Table 3. Distribution of bacterial species identified.

Sample	Index Bacterial DNA	Additional Bacterial DNA
ST 1	*C. trachomatis*	
ST 2	*C. trachomatis*	
ST 3	*Pseudomona spp.*	
ST 4	*Pseudomona spp.*	*P. fluorescens, P. putida, P. migulae*
ST 5	*Pseudomona spp.*	*P. fluorescens, P. migulae*
SF 1	*Lactobacillus/Clostridium*	*B. cereus*
SF 2	*Lactobacillus/Clostridium*	*B. cereus*
SF 3	*N. miningitidis* Serogroup B	

ST: synovial tissue; SF: synovial fluid.

Nevertheless, SpA and ReA are not common. A similar phenomenon used to be seen in sub-Saharan Africa prior to the advent of the HIV pandemic, which has resulted in a significant increase in the prevalence of these rheumatic disorders despite a very low prevalence of HLA-B27[15-17]. Sequence analysis of the PCR positive samples revealed multiple microorganisms, with the majority being of enteral origin. This is the first time that the presence of *Bacillus cereus* DNA has been found in ST; *B. cereus* and *Clostridium perfringens* can cause a similar clinical picture of food poisoning, characterized by diarrhea without fever in association with lower abdominal cramps, with a disease duration from 20 to 36 h, and which is self-limited. The latter finding appears to support a role for the gastrointestinal tract as an important portal of entry in the etiopathogenesis of these disorders in these populations.

The genuses most frequently identified were *C. trachomatis* and *Pseudomonas* spp. In most patients a single bacterial species was identified, although in some cases there was a mixture of bacterial species. In this regard, our findings corroborate reports in other populations using a 16S rDNA universal primer to analyze joint samples from patients with similar rheumatic disorders[18,19].

Most of the microorganisms identified in the joint to date have a reported association with arthritis, but an increasing number including *Pseudomonas* spp. and certain *Salmonella* spp. have not been thought to be pathogenic to humans. It is clear that a better understanding of the contributions of our intestinal microflora to our biology is needed for insights into the potential role of microflora in the pathogenesis of ReA. In this regard, increasing attention is being paid to the role of nonpathogenic bacteria in maintaining normal immune activation in the gut. It has been recently shown that an avirulent strain of *Salmonella* abrogates production of inflammatory cytokines in cultured human epithelial cells[20]. This is accomplished by inhibiting ubiquitination and degradation of IκB, thus blocking nuclear factor-κB directed transactivation of the genes encoding inflammatory mediators[20]. In addition, it has been shown that loss of tolerance to commensal gut flora results in inappropriate activation of the mucosal immune system in patients with inflammatory bowel disease, and that both Crohn's disease and ulcerative colitis respond to treatment with broad spectrum antibiotics[21-23].

Experimental animal models also provide support for a role of the commensal microflora in the pathogenesis of inflammatory intestinal disease. HLA-B27/ß$_2$-microglobulin transgenic rats and knockout mice that lack interleukin 2 (IL-2) or T cell receptor, or conventionally raised mice deficient in IL-10 develop colitis, which is abrogated when these animals are raised in a germ-free environment[24]. However, the exact role and/or interrelationship between the microflora and the compromised intestinal barrier function need to be established. This is of relevance to the SpA,

considering that a large proportion of patients exhibit subclinical gut inflammatory changes[25].

Like Gerard, *et al*[19], we could not establish significant clinical differences between patients with bacterial DNA in the joint and those without. In addition, no patient exhibited clinical characteristics consistent with septic arthritis. Whether microbial DNA bacteria and/or virus identified in the synovium plays a role in the etiopathogenesis or is an innocent bystander remains to be established[26-29]. Further, no differences were observed in the clinical picture between patients in whom only one bacterial species was identified and those with more than one species.

Our study confirms the presence of bacterial DNA by PCR in a group of patients with chronic SpA. Microorganisms identified are similar to those reported in other geographic locations, and provide further documentation for the presence of nonpathogenic and pathogenic bacterial species in the joints of patients with ReA. Their exact role in disease pathogenesis requires further investigation.

REFERENCES

1. Lopez de Castro JA. The pathogenic role of HLA-B27 in chronic arthritis. Curr Opin Rheumatol 1998;10:59-66.
2. Ikeda M, Yu DTY. The pathogenesis of HLA-B27 arthritis: role of HLA-B27 in bacterial defense. Am J Med Sci 1998;316:257-63.
3. Taurog JD, Richardson JA, Croft JT, et al. The germ free state presents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J Exp Med 1994;180:2359-64.
4. Braun J, Tuszewski M, Eggens U, et al. Nested polymerase chain reaction strategy simultaneously targeting DNA sequencing of multiple bacterial species in inflammatory joint diseases. I. Screening of synovial fluid samples of patients with spondyloarthropathies and other arthritides. J Rheumatol 1997;24:1092-100.
5. Grangors K, Jalkanen S, von Essen R, et al. Yersinia antigens in synovial fluid cells from patients with reactive arthritis. N Engl J Med 1989;320:216-21.
6. Branigan PJ, Gerard HC, Hudson AP, Schumacher HR, Pando J. Comparison of synovial tissue and synovial fluid as the source of nucleic acids for detection of Chlamydia trachomatis by polymerase chain reaction. Arthritis Rheum 1996;39:1740-6.
7. Gerard HC, Branigan PJ, Shumacher HR, Hudson AP. Synovial Chlamydia trachomatis in patients with reactive arthritis/Reiter's syndrome are viable but show aberrant gene expression. J Rheumatol 1998;25:734-42.
8. Sieper J, Kingsley G. Report on the 3rd Workshop in Reactive Arthritis. Ann Rheum Dis 1996;55:564-70.
9. Relman DA. The identification of uncultured microbial pathogens. J Infect Dis 1993;168:1-8.
10. Aabo S, Rasmussen OF, Rossen L, et al. Salmonella identification by the polymerase chain reaction. Mol Cell Probes 1993;7:171-8.
11. Rhan K, De Grandis A, Clarke RC, et al. Amplification of an inv A gene sequence of Salmonella typhimurium by the polymerase chain reaction as a specific method of detection of Salmonella. Mol Cell Probes 1992;6:271-9.
12. Stone G, Oberst R, Hays M, et al. Combined PCR-oligonucleotide ligation assay for rapid detection of Salmonella serovars. J Clin Microbiol 1995;33:2888-93.
13. Angulo J, Castro F, Quispe E, Espinoza LR. A clinical profile of reactive arthritis in a Peruvian series: a pilot study. J Clin Rheumatol 2000;6:128-35.
14. Schumacher R Jr. Reactive arthritis. Rheum Dis Clin N Am 1998;24:261-73.
15. Lutalo SK. Chronic inflammatory rheumatic diseases in black Zimbabweans. Ann Rheum Dis 1985;44:121-5.
16. Stein M, Davis P. Rheumatic disorders in Zimbabwe: a prospective analysis of patients attending a rheumatic disease clinic. Ann Rheum Dis 1990;49:400-3.
17. Burch VC, Isaacs S, Kalla AA. Ethnicity and patterns of spondyloarthritis in South Africa — analysis of 100 patients. J Rheumatol 1999;26:2195-200.
18. Wilbrink B, van der Heijden IM, Schouls LM, et al. 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;41:535-43.
19. Gerard HC, Wang Z, Wang GF, 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;44:1689-97.
20. Neish AS, Gerwitz AT, Zeng H, et al. Prokaryotic regulation of epithelial responses by inhibition of I kappa B-alpha ubiquitination. Science 2000;289:1560-3.
21. Duchmann R, Kaiser I, Herman E, Mayet W, Ewe K. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease. Clin Exp Immunol 1995;102:448-55.
22. Hooper L, Gordon J. Commensal host-bacterial relationships in the gut. Science 2001;292:1115-8.
23. Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. Mucosal antibodies in inflammatory bowel disease are direct against intestinal bacteria. Gut 1996;38:365-75.
24. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon ATR. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 1999;354:635-9.
25. Mielants H, Veys EM, Goemaere S, Goethals K, Cuvelier C, De Vos M. Gut inflammation in the spondyloarthropathies: clinical, radiologic, biologic and genetic features in relation to the type of histology: a prospective study. J Rheumatol 1991;18:1542-51.
26. Sieper J, Braun J. Pathogenesis of spondyloarthropathies: persistent bacterial antigen, autoimmunity or both? Arthritis Rheum 1995;38:1547-54.
27. Kingsley G. Microbial DNA in the synovium — a role in etiology or mere bystander? Lancet 1997;349:1038-9.
28. Sieper J, Braun J, Kingsley G. Report on the Fourth International Workshop on Reactive Arthritis. Arthritis Rheum 2000;43:720-39.
29. Detley H, Hubner B, Seidl B, et al. Detection of multiple viral DNA species in synovial tissue and fluid of patients with early arthritis. Ann Rheum Dis 2000;59:342-6.