Undifferentiated arthritis is common and poses an important pathogenic and diagnostic dilemma, especially in persistent form. The term undifferentiated arthritis was introduced to describe those forms of synovitis that cannot be classified into an established disease category [1, 2]. The reported frequency is 20%–55% in early arthritis clinics and has been noted to become chronic in at least 15%–25% of cases [1, 3–5].

Reactive arthritis is characterized by a sterile, usually self-limiting arthritis, in particular following a gastrointestinal, urinogenital, or pharyngeal infection. *Yersinia enterocolitica* is one of the microorganisms associated with reactive arthritis [6, 7]. The incidence of *Yersinia*-associated arthritis has been shown to be underestimated when conventional diagnostic techniques are used: stool cultures are often negative after the acute phase, and the conventional serum agglutination test is of limited value because of lack of sensitivity and specificity, especially in cases of chronic infection [8–10]. Furthermore, the association of arthritis with symptoms of a prior infection, which may be mild or may have occurred a long time previously, can be unclear [11]. When the association of arthritis with a recent infection remains undetected, the patient is likely to be classified as having undifferentiated arthritis. Therefore, it is conceivable that forms of *Yersinia*-induced reactive arthritis underly some subsets of unclassified joint disease.

In order to gather evidence implicating *Yersinia* in persistent undifferentiated arthritis, circulating class-specific antibodies to *Yersinia* outer proteins were detected with use of an immunoblotting technique [12]. This technique has the advantage of being more sensitive and specific than the still regularly used agglutination assay [12]. Using immunohistologic techniques, we then tried to demonstrate the presence of *Y. enterocolitica* antigens in colonic and synovial tissue specimens from two patients with circulating IgA and IgG antibodies to *Yersinia* outer proteins. In addition, efforts were made to propagate *Y. enterocolitica*–specific T-cell clones from the synovial tissue. Finally, using a *Yersinia*-specific PCR, we attempted to amplify *Yersinia* DNA from synovial biopsy specimens to investigate the role of *Y. enterocolitica* in the pathogenesis of chronic undifferentiated polyarthritis.

### Patients and Methods

#### Patients

**Patient 1.** A 57-year-old woman who had type II diabetes mellitus was referred to the department of rheumatology for analysis of undifferentiated polyarthritis. During the previous 4 years she had developed progressive pain and swelling of knees and elbows. No other complaints were reported.

On physical examination, joint swelling with synovial effusions in both elbows and knees and evident functional impairment were observed. No cutaneous lesions or lymphadenopathy were found.

Synovial fluid analysis of knee joint fluid revealed a WBC count of 6,500 cells/mm³. Crystalline material was absent and bacterial cultures were negative. Laboratory data showed an erythrocyte sedimentation rate of 14 mm/h and serum levels of C-reactive protein of 17 mg/L. Tests for serum antibodies to *Borrelia burgdorferi*, *Chlamydia*, human parvovirus, and rheumatoid factor and for antinuclear antibodies were negative.
The patient was negative for human leukocyte antigen (HLA)-B27 (HLA typing: A24, A9, A29, A19, B7, B44, B12, Bw4, Bw6, Cw7, DR4, DR7, DR53, DQ2, DQ7, DQ3). On the initial radiographs of elbows and knees, only minor degenerative features were noted. After 2 years of follow-up, radiographs of the elbows showed no changes, but those of the knees revealed some cartilage loss in both knees and a tiny erosion on the medial tibia plateau of the left knee.

Patient 2. A 44-year-old woman presented with an 18-month history of polyarthritis of shoulders, knees, ankles, and fingers, morning stiffness lasting a few hours, and recurrent episodes of diarrhea. The stools were not bloody. About 6 months before her first visit to our department she had experienced a period of fever, which had persisted for several weeks. Further complaints were low backache and episodic hindfoot pain and swelling.

On physical examination, swelling of the right knee and both ankles, as well as tenderness along the base of the Achilles tendon, was noted.

The erythrocyte sedimentation rate was 30 mm/h. Tests for serum antibodies to B. burgdorferi, Chlamydia, human parvovirus, and rheumatoid factor and for antinuclear antibodies were negative. The patient was negative for HLA-B27 (HLA typing: A24, A9, A29, A19, B7, B44, B12, Bw4, Bw6, Cw7, DR4, DR7, DR53, DQ2, DQ7, DQ3). On the initial radiographs of elbows and knees, only minor degenerative features were noted. After 2 years of follow-up, radiographs of the elbows showed no changes, but those of the knees revealed some cartilage loss in both knees and a tiny erosion on the medial tibia plateau of the left knee.

Generation of T Cell Clones from Synovial Tissue

Biopsy fragments were divided and digested at 37°C by the addition of collagenase (0.15 g/50 mL) (Sigma, Bornheim, Breda, The Netherlands) and hyaluronidase (0.005 g/50 mL) (Sigma) in Iscove’s modified Dulbecco’s modified Eagle’s medium (IMDM; Gibco-BRL, Breda, The Netherlands). T cells were subsequently cloned (seeded at 1 cell/well) in an aspecific manner, with use of irradiated (3,000 rad) feeder cells from allogeneic peripheral blood lymphocytes (2 × 10^6/mL) in IMDM with 10% normal human serum (NHS) containing phytohemagglutinin (0.5 µg/mL; Murex Diagnostic Limited, Dartford, UK) and 25 U of recombinant interleukin-2 (rIL-2; Biotech AG, Dreieich, Germany), in a total volume of 100 µL. Outgrowth of clones was noted from 7 days onward. The clones were expanded by another round of restimulation. Fresh IL-2-containing medium was added every 3–4 days if necessary.

T Cell Proliferation Assays

Suspensions of a mix of heat-killed Y. enterocolitica O3, O5, O8, and O9, Salmonella enteritidis, and Chlamydia trachomatis (kindly supplied by the Department of Bacteriology, Leiden University Hospital) were prepared. Phytohemagglutinin and IL-2 were used as soluble mitogens.

Thirty-seven clones from patient 1 and 53 clones from patient 2 were screened for their proliferative responses to a mix of Yersinia bacilli. Proliferative responses were determined in triplicates in 200 µL of culture medium. The medium consisted of IMDM supplemented with 10% normal human serum, streptomycin (100 µg/mL); Boehringer Mannheim, GmbH, Mannheim, Germany), penicillin (100 units/mL; Boehringer), and 2 mM of glucose (Boehringer) containing 2 × 10^5 T cells as responders and 5 × 10^6 autologous peripheral blood mononuclear cells as antigen-presenting cells, in 0.2-mL round-bottom 96-well tissue culture plates (Costar, Cambridge, MA). The bacterial antigens were diluted to a final concentration of 10^7 bacteria/mL.

Background proliferation was measured in the absence of antigen. Phytohemagglutinin and IL-2 were used as controls for T cell stimulation.

The cultures were pulsed with ^3H-thymidine (1 µCi/well) (REN, DuPont, Dordrecht, The Netherlands) after 72 hours and harvested after an additional 18 hours. The cells were transferred onto nitrocellulose filters with an automated cell harvester (Skaton, Lier, Norway), and ^3H-thymidine incorporation was measured with a beta-scintillation counter (1217 Rackbeta liquid scintillation counter, LKB, Wallace, Turku, Finland). Results are expressed as a stimulation index (SI). The SI was defined as proliferation induced by antigen divided by
background proliferation. An SI of $\geq 5$ was considered a positive response.

**Phenotypic Analysis of T Cell Clones**

Cells were cytopun (Cytofuge, Nordic Immunological Laboratories, Tilburg, The Netherlands) onto Mattrand slides (Knittelgläser, Braunschweig, Germany) and stained with the following mouse monoclonal antibodies: anti-CD3 (Becton Dickinson, San Jose, CA), anti-CD4 (Becton Dickinson), and anti-CD8 (Dako, Glostrup, Denmark). This was followed by incubation with affinity-purified horseradish-peroxidase-conjugated goat antibodies to mouse Ig (Dako). Horseradish-peroxidase activity was detected with use of hydrogen peroxide as substrate and amino ethylcarbazole (AEC, Sigma) as dye.

**Yersinia PCR**

**Extraction of DNA.** Synovial biopsy specimens (25–50 mg) were incubated in 1 mL of digestion buffer (TE: 500 mM of TRIS [pH, 9] and 20 mM of EDTA; 10 mM of NaCl; 1% SDS; and a 0.5-mg/mL concentration of proteinase K) at 60°C for 18 hours. The digested material was extracted twice with TE-buffered phenol, precipitated with ethanol, and washed with 70% ethanol. The DNA pellet was resuspended in 25–100 μL of sterile water, depending on the viscosity of the DNA extract.

**PCR.** The primers (5'-GAACTCGATGATAACTGGG-3' and 5'-GCAATTCAACCCACTTCAA-3') directed toward the ail-gene of all pathogenic Y. enterocolitica strains, as described by Kwaga et al., were used for the PCR reaction [14]. Primers were synthesized by Applied Biosystems (Perkin Elmer, Gouda, The Netherlands). DNA amplification was done in 25-μL reaction volumes. Each reaction contained 12.5 pmol of each primer, 0.25 units of SuperTth DNA polymerase (HT Biotechnology Ltd., Cambridge, UK), and standard amounts of amplification reagents (200 mM of each dNTP; 10 mM of Tris-HCl [pH, 9], 1.5 mM of MgCl₂; 50 mM of KCl; 0.1% Triton X-100; and 0.01% gelatin). A 40-mL overlay of sterile mineral oil was added to the tubes, followed by 5 μL of DNA extract.

To minimize nonspecific amplification, a “touchdown” PCR program was used: 3 minutes at 95°C, followed by 2 cycles of 30 seconds at 95°C, 1 minute at 66°C, and 1 minute at 72°C, and then 2 cycles identical to the previous 2 cycles, but with an annealing temperature of 64°C; every 2 cycles, the annealing temperature was lowered by 2°C until the annealing temperature was 56°C [15]. At this temperature an additional 30 cycles were run, and the program ended with 7 minutes at 72°C. All PCRs were carried out in an OmniGene Thermal Cycler (Hybaid Ltd, Teddington, Middlesex, UK).

Positive as well as negative controls were included in each experiment. Positive controls consisted of reaction mixtures, spiked with 10 ng of pVM103cc (a kind gift of Dr. V. Miller, UCLA, Los Angeles) in water, and in different dilutions of the clinical DNA extracts. Negative controls to check for cross-contamination of sample extractions were included with every clinical sample (extraction controls). These negative controls consisted of digestion buffer without clinical material and were handled in the same way as the clinical samples. To test the PCR reaction mixture itself for contamination, a negative control consisting of reaction mixture without DNA template was also included in each experiment (reaction-mix control).

**Detection of amplified product.** PCR products were separated by electrophoresis of 10 μL of the reaction mixture in an ethidium bromide–stained 2% agarose gel. A 1-kb DNA ladder (Gibco BRL, Life Technologies B. V., Breda, The Netherlands) was used as a DNA size standard. The DNA was visualized on an ultraviolet transilluminator and photographed.

**Results**

**Serology.** Immunoblotting of serum samples of patient 1 demonstrated IgA and IgG antibodies to the 36-kD (Yop D), 38-kD (Yop B), and 46-kD (Yop H) proteins as well as IgG antibodies to the 58-kD (Yop M) protein. Patient 2 had serum IgA and IgG antibodies to the 36-kD, 38-kD, and 46-kD proteins (figure 1).

**Immunofluorescence.** Y. enterocolitica antigens were demonstrated by indirect immunofluorescence in colonic and synovial biopsy specimens from both patients (figure 2). The particles exhibited membranous fluorescence and were oval. Only a few positive antigens were seen per tissue section. It was
not possible to determine whether the particles were localized intracellularly or extracellularly.

*Proliferative responses of synovial tissue T cell clones to Y. enterocolitica.* Because of the presence of the mitogen phytohemagglutinine in the cloning medium, the growth of T cells occurred randomly, without prior in vitro selection of Y. enterocolitica-reactive T cells. The cloning efficiency of T lymphocytes from the synovial tissue of patient 1 was 3.7%, and from patient 2, 5.8%.

For patient 1, 37 clones were propagated and screened for antigen-specificity. Four CD4+ clones (11.7%) proliferated in response to Y. enterocolitica in the presence of autologous antigen presenting cells (table 1). None of the clones cross-reacted with S. typhymurium or C. trachomatis.
Figure 1. Proliferative responses of synovial tissue T cell clones to Y. enterocolitica.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clone</th>
<th>Proliferative response (SI) to Yersinia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W-1</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>W-3</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>W-13</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>W-29</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>A-1</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>A-7</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>A-30</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>A-33</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>A-40</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>A-44</td>
<td>6</td>
</tr>
</tbody>
</table>

NOTE: The stimulation index (SI) was defined as proliferation induced by antigen, divided by background proliferation. An SI of ≥5 was considered a positive response. Clone A-30 was found to cross-react with C. trachomatis (SI = 3).

For patient 2, 53 clones were tested, and six CD4+ clones (11.3%) proliferated in response to Y. enterocolitica (table 1). Clone A-30 was the only clone that cross-reacted with C. trachomatis (table 1).

Yersinia PCR. No Yersinia DNA was detected in the synovial biopsy specimens of either patient, as determined by a PCR that was designed to amplify the ail-gene, present on all pathogenic Y. enterocolitica strains.

Disease course. In the assumption that these two patients had persistent yersinia infections, treatment with ciprofloxacin (500 mg twice daily over a period of 3 months) was initiated. At the end of the treatment period, patient 1 reported no abatement of symptoms. During the course of 2 years' follow-up, the objective arthritis disappeared, leaving arthralgia (especially of the knees) as the residual complaint. Patient 2 had temporary abatement of symptoms during and in the initial period following treatment. This improvement consisted of a decrease in joint swelling and in bowel symptoms. After 2 years' follow-up, however, she again had active arthritis of both knees, arthralgia of both hip joints, and recurring bouts of diarrhea. The Yersinia serology did not change in either patient.

Discussion

The results of this study suggest that chronic undifferentiated polyarthritis can be caused by Y. enterocolitica. In both patients the presence of IgA antibodies to Yersinia outer proteins indicates chronic stimulation by plasmid Yersinia antigen [8, 11, 16]. The presence of Yersinia antigens in the colon and synovial tissue and the propagation of Y. enterocolitica—specific T cells from the synovial tissue of both patients further substantiate the role of yersinia infections in the pathogenesis of at least some forms of chronic undifferentiated arthritis.

Several investigators have tried to elucidate the relationship between undifferentiated arthritis and reactive arthritis, as data presented in various studies demonstrated that infections associated with reactive arthritis may have an asymptomatic course [6, 7]. Such a relationship was highly suggested when different methods were tested in groups of patients with undifferentiated arthritis: (1) detection of antibody against arthritis-associated bacteria, (2) detection of lymphocyte proliferation, and (3) detection of antigen [7, 17–21].

In chronic infection, the agglutination test still often used to detect yersinia infections is negative in 80% of the cases [11–13, 22]. Moreover, the specificity of this test is reduced by cross-reactivity of Yersinia O antigens with diverse gram-negative bacteria. This has resulted in the use of other serological techniques, such as the detection of class-specific antibodies to plasmid-encoded virulence proteins by immunoblotting, which has proved to be more sensitive and specific than the routine agglutination test [12, 13, 23].

In a surveillance study in the Netherlands of 355 patients in whom a diagnosis of yersiniosis was considered on clinical grounds (215 patients were culture-positive and 140 culture-negative), the sensitivity of the agglutination test for the diagnosis of chronic yersiniosis was found to be 18%, whereas the sensitivity of the immunoblotting technique was 83% [23]. The specificities of these tests were 29% and 82%, respectively. The prevalence of positive IgA and IgG immunoblots for Yersinia outer protein has been shown to be 2% in the normal population, 0% in a population of patients with rheumatoid arthritis, and 10% in a population with seronegative spondyloarthropathy [8, 16].

We chose to use the immunoblot technique to initially screen sera from patients with undifferentiated arthritis for primary asymptomatic yersinia infection. In two patients with undifferentiated arthritis, who were found to have circulating IgA and IgG antibodies to Yersinia outer proteins, three other techniques were subsequently employed to substantiate the role of Yersinia in the pathogenesis of these two undifferentiated polyarthritides.

Previous studies have demonstrated the significance of the detection of Yersinia outer proteins by means of an indirect immunofluorescence technique in the diagnosis of chronic yersiniosis; the sensitivity and specificity were found to be 94% and 93%, respectively [23]. The correlation between indirect immunofluorescence and the immunoblotting technique was 85% [23]. The persistence of Yersinia antigens in the intestinal tissue is closely associated with elevated serum levels of IgA antibodies against Yersinia outer proteins in patients with Yersinia-induced arthritis [8, 11, 16]. Yersinia antigens have not been detected in any of the studies of synovial fluid and tissue from patients with reactive arthritis induced by agents other than Y. enterocolitica or patients with other rheumatic diseases (for example, septic arthritis or rheumatoid arthritis) [24–29].

In the present study, rod-shaped particles were detected in the synovial tissue and bowel specimens from both patients with immunohistologic staining with polyclonal antibodies to Yersinia adhesin A. In agreement with other studies, only a
few positive staining antigens were observed per tissue section [24–26]. Because of the staining technique used it was not possible to discern whether antigens were located intracellularly or extracellularly.

Controversy still exists about the structure of bacterial antigens detected by immunohistologic techniques in Yersinia-induced reactive arthritis. Predominantly, a granular staining pattern of the cytoplasm of either polymorphonuclear cells or mononuclear cells is found in both synovial fluid and tissue, suggesting the presence of degraded bacteria [24, 25, 27–29]. Nevertheless, as in our study, rod-shaped particles in synovial tissue have also been described [26, 29].

As no Yersinia DNA has as yet been detected in cases of Yersinia-induced arthritis, PCR has not been implemented in the diagnostic workup of these forms of arthritis [27, 28]. No Yersinia-specific PCR product was detected in this study, corroborating these findings. A possible explanation, reconciling the data from antigen and Yersinia DNA detection studies, can be inferred from a recent report on a synovial cell infection model, suggesting that Yersinia can lose its DNA when residing intracellularly in long-term synovial cultures [30].

The demonstration of bacterial antigens in both colonic and synovial tissue specimens supports the hypothesis that the bowel is the reservoir of bacteria that can subsequently disseminate into the joint [8, 31]. As has been postulated before, patients’ defense against invading microorganisms may fail, resulting in persistence of bacteria close to the intestinal epithelium [8]. Subsequently, microbial antigens, perhaps as part of immune complexes or within cells, may enter the synovial tissue and trigger an immune-mediated process [32–34].

As the “gold standard” for chronic forms of Yersinia-induced reactive arthritis still has to be defined, and as T cell cloning techniques are very labor intensive, no extensive studies have been performed to determine the sensitivity and specificity of the clonal responses for diagnostic purposes. Bulk-culture proliferation studies have demonstrated that although T cell responses were not solely confined to the inciting agent, the proliferative responses in patients with enteric and urogenital reactive arthritis were significantly higher to the inciting agent than to other bacterial agents [35–37]. Similar results have been found using T cell cloning techniques [38, 39]. Yersinia-specific proliferative responses of synovial fluid mononuclear cells have also been noted in patients with other forms of arthritis of unknown etiology, for example, undifferentiated arthritis and juvenile chronic arthritis [40, 18].

T cell clones specific for Y. enterocolitica have been propagated from synovial fluid and tissue from patients with postenteric reactive arthritis, confirming the presence of T cells with specificity for the inciting antigen at the clonal level [38, 39]. The outgrowth of Y. enterocolitica–specific T cell clones from synovial tissue implies the potential for direct triggering of an inflammatory response by presentation of bacterial antigen locally in the joint. The finding in the present study that synovial T cells in the patients specifically recognized Y. enterocolitica supports this hypothesis. Special care was taken to avoid antigen selection during the cloning procedure, by using phytohemagglutinine as an initial stimulus [41]. The relatively high frequency of Y. enterocolitica–specific T cell clones cultured strongly supports the hypothesis that we are dealing here with previously unrecognized forms of reactive arthritis.

In conclusion, in the present study four different techniques were used to investigate the role of Y. enterocolitica in the pathogenesis of subsets of undifferentiated arthritis. This is the first report in which circulating antibodies to Yersinia outer proteins, Yersinia-specific T cell clones cultured from the synovial tissue, and Yersinia virulence antigens in both colon and synovial tissue have been detected simultaneously in patients with undifferentiated arthritis. The findings support the idea that at least some forms of chronic undifferentiated polyarthritis are in fact forms of reactive arthritis, although as yet an intercurrent infection cannot be wholly excluded.

In these patients a primary asymptomatic bowel infection could result in persistence of bacteria, viable or nonviable, close to the intestinal epithelium, from where dissemination into the joint might take place. The finding that Yersinia antigens are present in synovial tissue, whereas Yersinia DNA cannot be detected, could be the result either of the deposition of parts of Yersinia bacteria or of intact Yersinia bacteria that are not able to survive because of growth restriction and apoptosis [30]. The prolonged persistence of Yersinia antigens in the synovial compartment may be the cause of the maintenance of the inflammatory host responses in the joints of patients with reactive arthritis due to Yersinia. Further studies in larger groups of patients with various forms of arthritis are needed to either corroborate orcontest these findings.

References


Figure 1. Alexandre Jean Emile Yersin (1863–1943), Swiss bacteriologist, is best known for his isolation of Yersinia pestis, the cause of bubonic plague. This stamp was issued in 1943 to honor Yersin for his introduction of the rubber tree into Indochina. (From the medical philately collection of J. N. Shanberge, MD, University of Michigan.)