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Experimental *Yersinia* Infection of Human Synovial Cells: Persistence of Live Bacteria and Generation of Bacterial Antigen Deposits Including "Ghosts," Nucleic Acid-Free Bacterial Rods

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***Yersinia enterocolitica* O:3 was maintained in primary cultures of human synovial cells for 6 weeks as cultivable organisms and thereafter for 2 more weeks as antigen aggregates containing specific lipopolysaccharides (LPS). Some seemingly intact bacteria were "ghosts," bacterial rods possessing LPS but not DNA. The prolonged persistence of yersiniae, and consequently of *Yersinia* antigens, in synovial cells may be the cause of the maintenance of the inflammatory host responses in the joints of patients with reactive arthritis due to *Yersinia* infection.**

Reactive arthritis triggered by infection with *Yersinia enterocolitica* is a frequent cause of acute arthritis in humans (17). One to 3 weeks after infection with or without gastroenteritis, patients may suffer from mild arthralgias to severe polyarthritis. After a few weeks to months, most patients recover, but a subset will develop chronic arthritis (14). It has been claimed that yersiniae may persist in the gut and that only bacterial products, mainly lipopolysaccharides (LPS), are transported into the joint where they induce arthritis (2). Synovial fluids of four patients with *Yersinia* reactive arthritis yielded yersinial antigens, but no yersinial DNA, at a median of 3 months after the beginning of arthritis (18). *Yersinia* antigen was found mainly in neutrophils; these cells have a short life span of a few hours, which suggests that they might have taken up *Yersinia* antigens only recently (6). Examination of synovial tissue obtained several weeks to months after the beginning of arthritis demonstrated *Yersinia* antigen but no intact bacteria (15). In contrast, another group found oval particles with a rod-like morphology in synovial tissue, suggesting the presence of live bacteria; however, attempts to cultivate yersiniae from these tissues or from joint fluids failed (7).

Interestingly, these rod-like particles were found in synovial tissue but not in synovial fluid. Comparable results were obtained for patients with arthritis due to *Chlamydia trachomatis* (1) and *Borrelia burgdorferi* (16). This might be due to the persistence of these microorganisms in the resident cells of the joint tissue, as opposed to the migrating immunocompetent cells.

Thus, although the etiology of *Yersinia* reactive arthritis is known, the pathogenesis is far from clear, and it is not known how yersiniae might reach the joint. Recent studies using the *Yersinia*-induced rat arthritis model revealed that yersiniae are able to invade synovial tissue during the first week after infection without vigorous multiplication and consequently without induction of microabscesses and without arthritis (5). When arthritis developed 1 week after infection, yersiniae could only rarely be cultivated from the affected joint tissue. These data suggest that yersiniae might be able to invade and survive in human joint tissue prior to the onset of the host response killing the pathogen and inducing arthritis (8).

The target cell of persisting bacteria is unknown, but the cell is most probably resident and long-living. In an attempt to imitate the synovial environment, we established a model using normal human synovial cells (SC) experimentally infected with yersiniae and studied the intracellular survival and degradation of the bacteria.

SC were prepared from normal human joint tissues of 12 cadavers by tryptic digestion and propagated, as described previously (11). SC are derived from the synovial membrane, including the lining cells and the sublining cells. SC were used for experiments during passages 3 to 10, when immunocompetent cells, including macrophages, had disappeared. Cells were grown on circular glass slides in 24-well plates in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (complete medium) at 37°C in 5% CO_2 and passaged with trypsin (11).

A patient isolate of *Y. enterocolitica* O:3, strain 108-P, harboring the virulence plasmid pYV, was grown for 24 h at 27°C in tryptone-yeast broth (9).

Subconfluent cells were infected at 37°C with a bacterial suspension with an optical density of 0.1 (at 600 nm), diluted 1:20 in RPMI 1640, at a multiplicity of infection of 50 yersiniae per cell (5×10^6 yersiniae per 10^5 SC). After the specified period, cells were washed and incubated for 2 h in complete medium containing 25 μg of gentamicin (Merck, Darmstadt, Germany) per ml to kill extracellular bacteria. After a further washing step, cells were grown in medium with 10 μg of gentamicin per ml. The MIC of gentamicin was 2.5 μg/ml for the tested *Yersinia* strain. At gentamicin concentrations of 25 μg/ml or greater, the number of yersiniae that could be grown from lysed cell monolayers diminished. Pretreatment of SC with 25 μg of gentamicin per ml for 5 days before infection followed by the standard protocol diminished intracellular survival of yersiniae; pretreatment with gentamicin at a concentration of 10 or 5 μg/ml or no pretreatment with gentamicin had no effect. Therefore, the standard protocol allowed protection of internalized yersiniae from gentamicin treatment and killing of extracellular yersiniae. In some experiments infected cells were propagated with trypsin as described above for noninfected SC.

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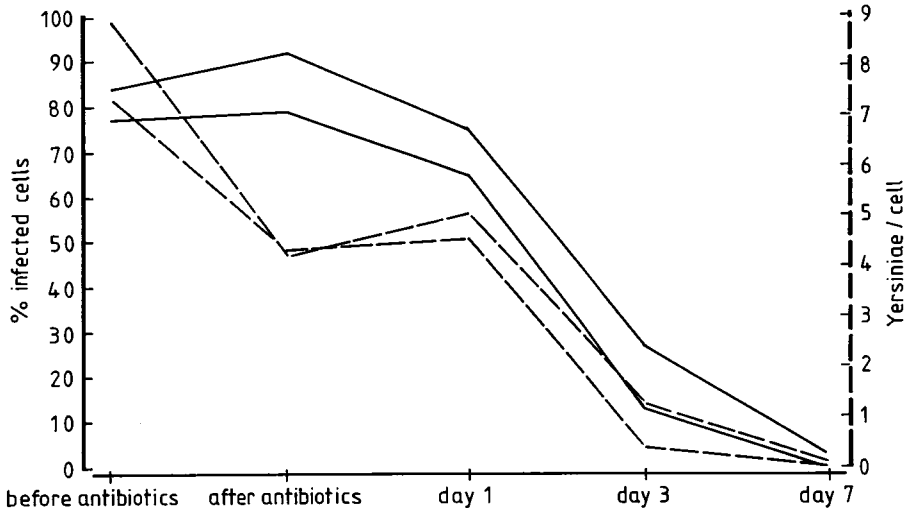

FIG. 1. Early infection (first week) by *Y. enterocolitica* O:3 in normal human SC (two independent experiments with two different cell lines). Dashed lines, average number of yersiniae per cell; solid lines, percentage of infected cells as determined by Giemsa staining. The number of bacteria was determined first after 30 min of infection and thorough washings (before antibiotics) and then again after 2 h in the presence of 25 μg of gentamicin per ml, which killed extracellular bacteria. Further determinations were performed after 1, 3, and 7 days. At day 7, cultures yielded 2.7×10^4 and 1.6×10^4 CFU per monolayer.

Giemsa staining was performed with a freshly filtered staining solution (Merck) for 20 min after fixation for 10 min with ice-cold methanol. *Yersinia* LPS (serotype O:3 antigen) was detected by direct immunofluorescence staining with a monoclonal fluorescein isothiocyanate-tagged anti-LPS antibody (clone 2D8; Progen, Heidelberg, Germany). Staining for DNA in methanol-fixed monolayers was performed with propidium iodide (Sigma, Deisenhofen, Germany) at 10 μg/ml in phosphate-buffered saline for 10 min (13). Cultivable bacteria were detected as CFU (reported as CFU per monolayer) after lysis of cells in 1 ml of 0.5% tergitol in 0.5% bovine serum albumin–phosphate-buffered saline (4).

Infection of SC with *Y. enterocolitica* O:3 for 30 min resulted in attachment and internalization of bacteria, as could be seen by Giemsa staining before and after treatment with gentamicin: extracellular yersiniae were rapidly cleared after incubation with gentamicin. In the absence of gentamicin, multiplying extracellular yersiniae were highly toxic for the cells, which rounded up and detached after a few hours.

To determine if the expression of plasmid-encoded virulence factors, including the adhesion factor YadA and the secreted *Yersinia* proteins (Yops), influenced the infection, SC were infected for 30 min with yersiniae that had been grown at 37°C (expression of virulence factors) or at 27°C (no expression) before infection. The percentage of infected cells, the average number of yersiniae per cell, and the number of CFU of cell-associated yersiniae were not different after 2 h in the presence of gentamicin. These results showing no influence of the precultivation temperature were confirmed by the finding that yersiniae cured of the virulence plasmid were not altered in their ability to enter SC in comparison with the parental strain. In all further experiments, virulent yersiniae were precultivated at 27°C.

When SC were infected for 15, 30, or 60 min with yersiniae, the percentage of infected cells, the average number of yersiniae per cell, the percentage of cells containing *Yersinia* LPS, and the number of CFU per monolayer were increased with prolonged infection time. When the time of infection was increased beyond 60 min, cells were detached (cytotoxic effect). In further experiments, SC were infected with yersiniae for 30 min.

When SC were incubated for 7 days after infection in the presence of 10, 5, or 1% FCS and 10 μg of gentamicin per ml in RPMI 1640, the percentage of cells containing *Yersinia* LPS and the number of CFU did not vary as a result of the different concentrations of FCS. However, SC did not grow well and looked unhealthy with 1% FCS. Therefore, further experiments were carried out with 10% FCS unless otherwise specified.

Infection of SC with yersiniae at a multiplicity of infection of 50 for 30 min resulted in attachment of about eight bacteria per cell as revealed by Giemsa staining after intense washing (Fig. 1). After 2 h in the presence of 25 μg of gentamicin per ml, killing extracellular bacteria, internalization of four bacteria per cell was found (Fig. 1). After 24 h in the presence of 10 μg of gentamicin per ml, there were about five bacteria per cell. Thereafter, the number of bacteria per cell dropped rapidly, and after 7 days, yersiniae could only rarely be seen by Giemsa staining. However, at this time there were still about 10^4 CFU per monolayer in the SC cultures, which corresponds to the infection of approximately 5% of the cells with one cultivable yersinia.

Since SC grow slowly in cell culture, it was possible to study the intracellular persistence of yersiniae for several weeks after infection. Bacteria could not be identified by Giemsa staining after 14 days or later. However, by immunofluorescence staining with a monoclonal antibody to serotype O:3 LPS, cells showed bacterial antigens of diverse shapes for longer periods. During the first week after infection, SC contained morphologically intact rod-shaped bacteria as demonstrated by immunofluorescence (not shown). Thereafter, the number of intact bacteria decreased, and the antigen detected within the cytoplasm appeared as irregularly shaped dots, indicating the formation of bacterial membrane vesicles. During the next few weeks, the sizes of the antigen deposits decreased, but they were still clearly detectable after 7 weeks in comparison with mock-infected cells.

Determination of the number of cultivable yersiniae per monolayer showed an increase during the first few days after infection followed by a slow drop in the CFU per monolayer. Cultivable yersiniae could be detected for at least 3 to 4 weeks (Fig. 2), and withdrawal of gentamicin led to rapid overgrowth

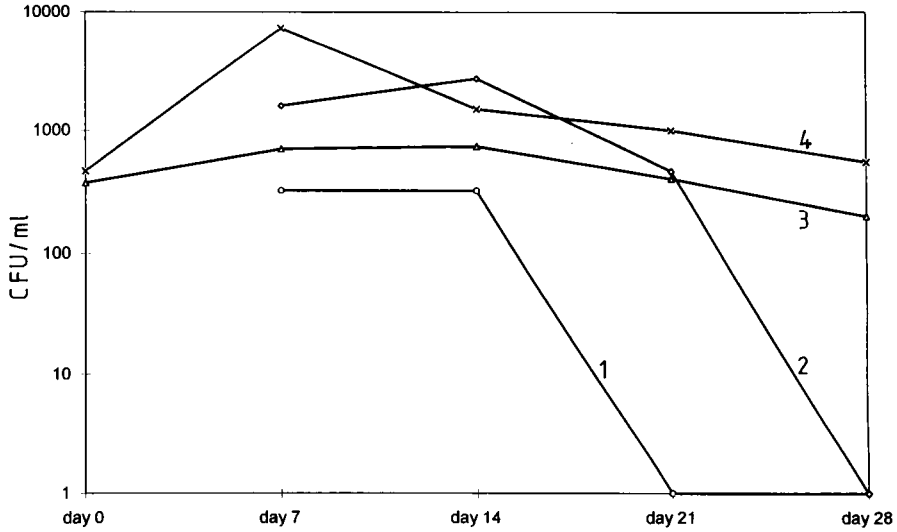

FIG. 2. Persistent infection by *Y. enterocolitica* O:3 in normal human SC. CFU per milliliter of lysate of the whole monolayer are shown. Values are the averages of two parallel determinations which did not differ by more than 10%. In experiments 1 and 2, cells were cultured in the presence of 10% FCS and propagated weekly. In experiments 3 and 4, cells were maintained at 3% FCS, thus curtailing proliferation of cells. Although in patients with rheumatoid arthritis there is synovial proliferation, SC do not proliferate in reactive arthritis; thus, growth conditions at 3% FCS resemble more closely the conditions found in vivo. A repeat experiment yielded very similar results.

of cells by yersiniae. The time that cultivable yersiniae were present in the cultures was increased when cells still looked healthy, but they remained in stationary phase because of the low concentration of 3% FCS. In contrast, proliferating cells that were propagated weekly no longer harbored yersiniae after about 3 weeks, and when gentamicin was withdrawn, cultivable yersiniae could not be recovered. These data are consistent with the conclusion that yersiniae ceased proliferating in SC after the first week of infection. However, yersiniae were able to survive intracellularly for at least 4 weeks.

The percentage of cells positive for *Yersinia* antigen and the concentration of cultivable yersiniae declined during the first 3 weeks of infection, but when proliferating cell cultures became sterile after 3 to 4 weeks, *Yersinia* antigen was still detectable in 10 to 20% of SC. In another series of experiments, SC contained live yersiniae until day 42 after infection but were sterile at days 56 and 70. In parallel cultures, LPS antigen could be found in 20, 5, and 5% of the cells, respectively.

When SC were stained simultaneously for LPS and DNA, yersiniae appeared as double-stained, rod-shaped bacteria after 1 day. After 5, 8, and 14 days, only approximately 40, 30, and 30%, respectively, of the yersiniae were found to be positive for LPS and DNA. The remaining 60, 70, and 70%, respectively, of the yersiniae were detectable only by LPS staining, indicating the loss of bacterial DNA and the generation of bacterial "ghosts" (Fig. 3). After 35 days, only about 5% of the few remaining rod-shaped bacteria still contained DNA.

To study systematically the invasion and persistence of yersiniae inducing reactive arthritis, we have established a cell culture system with SC, the resident joint cells. This SC infection model has been successfully exploited in investigations of virus-induced arthritis showing a species-specific interaction and a persistent infection by several arthritogenic viruses (11, 12). Further results showed a reduced expression of adhesion molecules and HLA class II molecules on virus-infected cells in comparison with noninfected cells, which might be important for maintaining the persistent infection (10).

Here, we have shown that yersiniae can invade and persist in vitro in SC for at least 1 month. A few days after infection, multiplication of yersiniae in SC abated and bacterial antigen was deposited in the cytoplasm. In the presence of gentamicin, killing extracellular bacteria, there was no evidence for cytopathogenicity of the yersiniae internalized in SC. In contrast, HeLa cells reduced the number of intracellular yersiniae during only the first few hours of cultivation by more than 99% (3). Long-term incubation could not be performed in two other in vitro infection models: HeLa cells have a proliferation rate too high for the study of persistence (3), and macrophages have a relatively short life span (19). Therefore, we chose SC to study the interaction of joint cells and yersiniae.

Remarkably, after 1 week of infection, Giemsa staining became negative for rod-shaped yersiniae, but *Yersinia* antigen was still detected, similar to what was found in the joints of patients with reactive arthritis (6). However, at this time there were still viable bacteria in SC, in contrast to the case for patients with reactive arthritis, in whom cultivable yersiniae were not found (7). These results do not contradict the assumption that yersiniae are able to survive in joint tissue, because it is important to consider the interval of time between infection of the human host and sampling. Reactive arthritis appears a few days to weeks after infection, and it often takes several more days to weeks until sampling of synovial fluid or tissue specimens is done. If yersiniae reach the joint early during the prearthritic phase of the infection, sampling may occur 1 month or more after infection. After that period of time, the number of yersiniae in SC infected in vitro also had decreased. In addition, host defense mechanisms which are not included in our SC model may shorten the period of persistence of living yersiniae in vivo.

Synovial fluids of patients with reactive arthritis do not contain *Yersinia* DNA as determined by PCR (18) or *Yersinia* rod-like structures as determined by immunofluorescence (6). According to the results of this study, yersiniae persist and deposit antigen in the resident cell population of the synovial tissue. At later times, when no more yersiniae could be grown from SC, LPS antigen persisted in these cells. Probably *Yersinia* antigen released from the resident joint cells in patients with reactive arthritis can be taken up and be further processed by

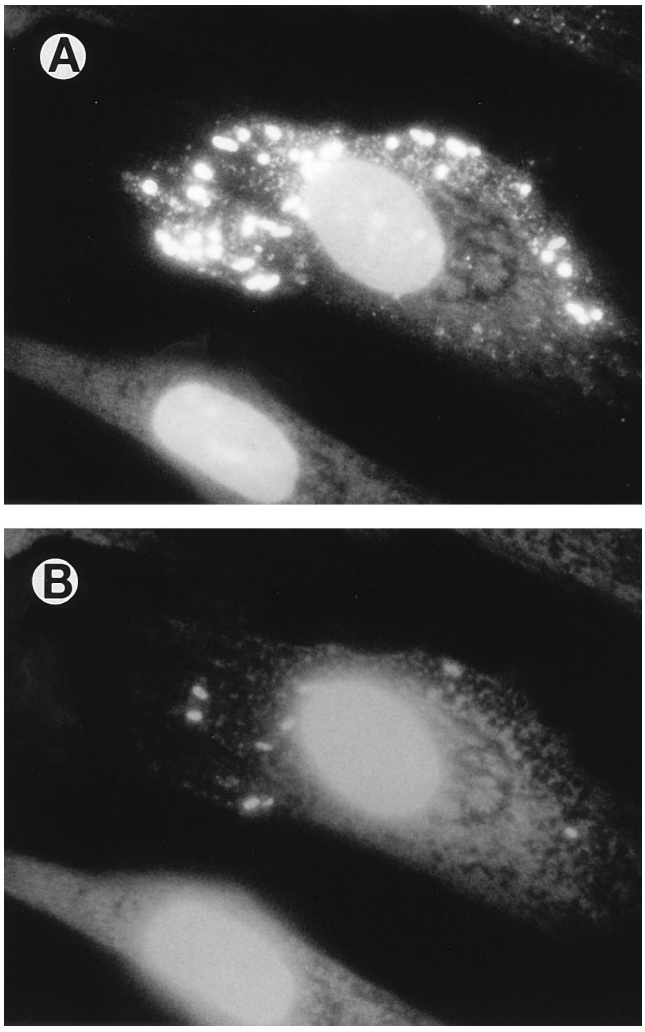

FIG. 3. Human SC after 14 days of infection with *Y. enterocolitica* O:3 in the presence of gentamicin. (A) LPS staining with a monoclonal fluorescein isothiocyanate-conjugated antibody against *Y. enterocolitica* O:3 LPS (filter, 495 nm). Multiple bacterial rods and dots of processed antigen are seen. (B) Additional staining of the same monolayer for DNA with propidium iodide (filter, >650 nm). Only a few of the yersiniae seen in panel A still contain DNA; the other yersiniae are ghosts.

professional phagocytes in the synovial fluid, where it can be detected by immunofluorescence (6).

Noncultivable rod-shaped particles resembling intact yersiniae were detected in synovial tissues of patients with reactive arthritis due to yersiniae by *Yersinia*-specific immunofluorescence (7). This observation can be explained by the results of this study: by simultaneous staining for LPS and DNA, we identified bacterial ghosts, i.e., membrane structures that retained the rod shape of a *Yersinia* bacterium but did not contain DNA and were not viable.

Thus, the SC infection model is able to explain and reconcile diverse data derived from patients with reactive arthritis due to yersiniae by several groups of investigators. The nonprofessional phagocyte population of the resident SC might represent the niche required for prolonged survival of yersiniae in the host. Moreover, this model allows for studying further aspects of the interaction of yersiniae and joint cells.

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REFERENCES

1. **Branigan, P. J., H. C. Gerard, D. Saaibi, A. P. Hudson, and H. R. Schumacher.** 1995. Screening of synovial tissue versus fluid from patients with Reiter's syndrome, other spondyloarthropathies, or reactive arthritis for chlamydia via polymerase chain reaction. Arthritis Rheum. **38**(Suppl.)**:**R20. (Abstract.)
2. **DeKoning, J., J. Heesemann, J. A. A. Hoogkamp-Konstanje, J. J. M. Festen, P. M. Houtman, and P. L. M. vanOijen.** 1989. Yersinia in intestinal biopsy specimens from patients with seronegative spondylarthropathy: correlation with specific serum IgA antibodies. J. Infect. Dis. **159:**109–112.
3. **Denenish, J. A., and D. A. Schiemann.** 1981. HeLa cell infection by *Yersinia enterocolitica*: evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. Infect. Immun. **32:**48–55.
4. **Ewald, J. H., J. Heesemann, H. Rüdiger, and I. B. Autenrieth.** 1994. Interaction of polymorphonuclear leukocytes with *Yersinia enterocolitica*: role of the yersinia virulence plasmid and modulation by the iron-chelator desferrioxamine B. J. Infect. Dis. **170:**140–150.
5. **Gaede, K. I., and J. Heesemann.** 1995. Arthrogenicity of genetically manipulated *Yersinia enterocolitica* serotype O8 for Lewis rats. Infect. Immun. **63:** 714–719.
6. **Granfors, K., S. Jalkanen, R. von Essen, O. Lahesmaa Rantala, O. Isomaki, K. Pekkola Heino, R. Merilahti Palo, R. Saario, H. Isomaki, and A. Toivanen.** 1989. Yersinia antigens in synovial fluid cells from patients with reactive arthritis. N. Engl. J. Med. **320:**216–221.
7. **Hammer, M., H. Zeidler, S. Klimsa, and J. Heesemann.** 1990. *Yersinia enterocolitica* in the synovial membrane of patients with yersinia-induced arthritis. Arthritis Rheum. **33:**1795–1800.
8. **Heesemann, J., K. Gaede, and I. B. Autenrieth.** 1993. Experimental *Yersinia enterocolitica* infection in rodents: a model for human yersiniosis. APMIS **101:**417–429.
9. **Heesemann, J., and R. Laufs.** 1985. Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. J. Clin. Microbiol. **22:**168–175.
10. **Huppertz, H. I.** 1994. How could infectious agents hide in synovial cells? Possible mechanisms of persistent viral infection in a model for the etiopathogenesis of chronic arthritis. Rheumatol. Int. **14:**71–76.
11. **Huppertz, H. I., and J. K. Chantler.** 1991. Restricted mumps virus infection of cells derived from normal human joint tissue. J. Gen. Virol. **72:**339–347.
12. **Huppertz, H. I., N. P. Miki, and J. K. Chantler.** 1991. Susceptibility of normal human joint tissue to viruses. J. Rheumatol. **18:**699–704.
13. **Jernaes, M. W., and H. B. Steen.** 1994. Staining of *Escherichia coli* for flow cytometry: influx and efflux of ethidium bromide. Cytometry **17:**302–309.
14. **Leirisalo-Repo, M., and H. Suoranta.** 1988. Ten year follow-up study of patients with yersinia arthritis. Arthritis Rheum. **31:**533–537.
15. **Merilahti-Palo, R., K. O. Söderström, R. Lahesmaa-Rantala, K. Granfors, and A. Toivanen.** 1991. Bacterial antigens in synovial biopsy specimens in yersinia triggered reactive arthritis. Ann. Rheum. Dis. **50:**87–90.
16. **Valkenaere, I., B. Jaulhac, J. Pourel, P. Fener, J. L. Kuntz, Y. Piémont, and H. Monteil.** 1993. Improved detection of *Borrelia burgdorferi* DNA by synovial tissue rather than synovial fluid testing in Lyme arthritis. Arthritis Rheum. **36**(Suppl.)**:**S41. (Abstract.)
17. **Valtonen, V. V., M. Leirisalo, P. J. Pentikäinen, T. Räsänen, I. Seppälä, U. Larinkari, M. Ranki, S. Koskimies, M. Malkamäki, and P. H. Mäkelä.** 1985. Triggering infections in reactive arthritis. Ann. Rheum. Dis. **44:**399–405.
18. **Viitanen, A. M., P. Arstila, R. Lahesmaa, K. Granfors, M. Skurnik, and P. Toivanen.** 1991. Application of the polymerase chain reaction and immunofluorescence techniques to the detection of bacteria in yersinia-triggered reactive arthritis. Arthritis Rheum. **34:**89–96.
19. **Wuorela, M., S. Jalkanen, P. Toivanen, and K. Granfors.** 1993. Yersinia lipopolysaccharide is modified by human monocytes. Infect. Immun. **61:** 5261–5270.

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