Infection of Synovial Fibroblasts in Culture by Yersinia enterocolitica and Salmonella enterica Serovar Enteritidis: Ultrastructural Investigation with Respect to the Pathogenesis of Reactive Arthritis

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Synovial fibroblasts were infected with Yersinia enterocolitica or Salmonella enterica serovar Enteritidis and analyzed by electron microscopy and fluorescence in situ hybridization. Intracellular bacterial replication was followed by degradation leading to “ghosts” possessing lipopolysaccharides but not DNA. However, single bacteria survived for more than 2 weeks. Therefore, transient intra-articular infection might be the missing link between initial intestinal infection and late synovial inflammation in the pathogenesis of reactive arthritis.

Reactive arthritis due to infection with yersiniae or salmonellae is a joint inflammation that develops 1 to 3 weeks following gastrointestinal infection with or without diarrhea. Usually arthritis is self-limiting, but a subset of patients develop chronic arthritis (15). Originally, reactive arthritis was supposed to occur in the absence of bacteria from the joint. However, ever since the bacterial antigen was found in the inflamed joints, it has been acknowledged that the presence of the bacterial antigen or even intact bacteria within the joint might be important for the pathogenesis of reactive arthritis, at least in the early phases of the disease. Lipopolysaccharides (LPS) from yersiniae and salmonellae were detected by immunofluorescence (IF) in synovial tissue and fluid cells (4–6, 18). In synovial fluid cells, the yersinial antigen was also found in neutrophils, cells with a short life span, suggesting that they might have taken up this antigen shortly before being tested (4). Oval particles with a rod-like morphology were identified by IF in synovial tissue from patients with yersinia-induced arthritis. However, attempts to cultivate yersiniae or salmonellae from the affected joints usually failed (4–6). The identification of yersinial DNA sequences was always unsuccessful (19, 26), but there were some reports about the detection of salmonella DNA in synovial fluid cells (C. O. Garcia, S. Paira, R. Burgos, J. Molina, J. F. Molina, and C. Calvo. Scientific Program, 60th Natl. Sci. Meet. Am. Coll. Rheumatol., 18 to 22 October 1996, Orlando, Fla., abstr. 948; and S. Nikkari, T. Mońtońen, R. Saario, U. Yli-Kerittula, M. Leirisalo-Repo, P. Laitio, and P. Toivanen. Scientific Program, 60th Natl. Meet. Am. Coll. Rheumatol., 18 to 22 October 1996, Orlando, Fla., abstr. 950). These data suggest that yersiniae or salmonellae may invade joint tissue and that, after eradication of living or cultivable bacteria, pathogenic bacterial components, such as

LPS, may persist in the joint and lead to the induction and perpetuation of arthritis.

Recently the generation of “bacterial ghosts” by in vitro infection of synovial fibroblasts (SF) with yersiniae and IF staining has been reported. These ghosts were seemingly intact bacteria possessing LPS but lacking DNA and were detectable for at least 7 weeks after infection (11).

To investigate invasion, degradation, and persistence of bacteria in SF, we infected primary human SF with yersiniae or salmonellae and analyzed infected cells by transmission electron microscopy, immunogold electron microscopy, and fluorescence in situ hybridization (FISH).

SF were derived by trypsin digestion from the knee joint synovial membranes of human cadavers free from infectious or rheumatic diseases. Cells were analyzed for the presence of HLA B27 by PCR and IF and found to be negative (8, 12). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and used during passages 5 to 15. Confluent monolayers of synovial cells were infected with patient isolates of either Yersinia enterocolitica 0:3 or Salmonella enterica serovar Enteritidis at a multiplicity of infection of 300 for 30 min (11, 13). Then, cells were washed, and 25 µg of gentamicin/ml was added to the culture medium to kill extracellular bacteria. After 2 h, cells were washed again and grown in culture medium containing 4 µg of gentamicin per ml. Gentamicin used at this concentration killed extracellular bacteria but did not affect intracellular bacteria (11–13).

Cells were prepared for transmission electron microscopy after a period ranging from 1 h to 2 weeks. After being trypsinated to obtain single-cell suspensions or monolayers, fibroblasts were fixed in 2.5% glutaraldehyde at 4°C for at least 2 h. This was followed by postfixation with immersion in osmium tetroxide and dehydration in graded ethanol. Cells were finally embedded in Epon 812 (Roth) via propylene oxide. Ultrathin sections were cut with an OMU 2 microtome (Reichert). For contrast, sections were incubated with lead citrate for 8 min, washed in distilled water, and incubated with uranyl acetate for 8 min (3). All sections were examined with a Zeiss EM 10 transmission electron microscope.
For immunogold labeling, infected fibroblasts were trypsinized and fixed in 2% paraformaldehyde containing picric acid for 4 h at 4°C as described previously (24). This was followed by dehydration in graded ethanol. Preparations were then embedded in London Resin White (London Resin Company, London, Great Britain), and ultrathin sections were cut. To inactivate residual aldehyde groups, sections were incubated in 0.05 M glycine in Tris buffer. Sections were incubated with primary antibodies for 1 h using either mouse monoclonal antibodies to LPS of \textit{Y. enterocolitica} O:3 (ProGen, Heidelberg, Germany) or rabbit monospecific antibodies to \textit{S. enterica} serovar Enteritidis (10) and with secondary antibodies using either goat anti-mouse immunoglobulin G (IgG) and IgM antibodies conjugated to 10-nm gold particles or goat anti-rabbit IgG and IgM antibodies conjugated to 15-nm gold particles, in accordance with the manufacturer’s instructions (Aurion, Wageningen, The Netherlands) (17). For contrast, sections were then incubated with lead citrate and uranyl acetate as described above. Uninfected control cultures were treated identically.

To inactivate bacteria, suspensions of bacteria were irradiated by UV light for 30 min at a wavelength of 302 nm (UV Transilluminator TM36, UVP, Cambridge, England) or kept in RPMI 1640 medium containing 25 μg of gentamicin per ml at 37°C. Infected cells were also incubated with recombinant human gamma interferon (IFN-γ; a kind gift from Rentschler Pharma) at a concentration of 1,000 U/ml (9, 12). Bacteria and infected SF were fixed in 2.5% glutaraldehyde after various periods of time and processed for electron microscopy.

For IF staining and FISH, SF were grown on coverslips to confluent monolayers and infected with \textit{Y. enterocolitica} O:3 at a multiplicity of infection of 50 for 30 min. Cells were washed, and extracellular bacteria were killed by adding 25 μg of gentamicin/ml for 2 h. Subsequently, cells were grown in RPMI medium supplemented with 10% FCS and 4 μg of gentamicin/ml. Fibroblasts were fixed in methanol for 10 min at room temperature after 4, 12, and 24 h and after 3, 7, 14, 21, 60, and 90 days.

Yersinial LPS was detected by direct IF using a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody to \textit{Y. enterocolitica} O:3 LPS (ProGen, 2D8-FITC). Staining of DNA was performed with 1 μg of DAPI (4’,6’-diamidino-2-phenylindole)/ml for 20 min. FISH was performed by hybridizing three yersinia-specific oligonucleotides encoding yersinial 16S rRNA, i.e., Y.16S-69, Y.ent16S-184, and Y.16S-1241 (25), to the different samples. All oligonucleotides were synthesized and labeled with 5’-Cy3 by a commercial supplier (Metabion, Munich, Germany). Oligonucleotides were hybridized to yersinia-
infected fibroblast cells. Since metabolic activity correlates to the rRNA content of cells (1, 21), it has been suggested that signal intensity after in situ hybridization reflects the physiological activity of bacterial cells. On the basis of the obtained signals, physiological activity was graded highly active, active, or not active. IF-, DAPI-, and FISH-stained bacteria were counted, and relative numbers were determined.

Ultrastrastructural results for yersiniae and salmonellae were not different, so they will be presented together.

Bacteria were easily recognized by transmission electron microscopy in cell monolayers by their characteristic morphology in comparison to bacterial pellets prepared in parallel. Infection of SF with yersiniae or salmonellae started with adhesion to the cell membrane (Fig. 1). Within a few minutes to several hours after infection, bacteria were seen to be attached to the cell surface. Adhesion was followed by uptake of the bacteria into the cells (Fig. 2A). One hour after infection, about 20 to 30% of cells were infected as judged by electron microscopy. At this time, intracellular bacteria seemed to be morphologically intact, showing their typical structure: the electron-dense bacterial wall surrounding the less-electron-dense bacterial cytosol and containing characteristic electron-dense granula (Fig. 1). Bacteria were observed in the cytoplasm of the synovial cells, surrounded closely by a cellular membrane.

After a few hours, many bacteria were engulfed in vacuoles. Increasing numbers of yersiniae and salmonellae within these vacuoles suggested intracellular bacterial replication, leading to large aggregations of bacteria in SF with a maximum frequency 24 h after infection (Fig. 2B). Morphologically intact bacteria were seen within these vacuoles for over 7 days after infection.

Four hours after infection, almost all bacteria were positive for DNA by DAPI staining but only 70% were positive for LPS by IF staining. At this point of time, bacteria showed very low metabolic activity as demonstrated by FISH analysis. Twelve hours after infection, DAPI still stained nearly 100% of bacteria but the LPS-specific antibody detected less than 10% of cells (Fig. 3). However, bacterial metabolic activity determined by FISH for yersinial rRNA was high in nearly all bacteria. It decreased 1 day after infection negligibly.

The number of morphologically intact bacteria within SF began to decrease approximately at day 1 after infection due to degradation. One day after infection degraded bacteria were seen in about 10 to 15% of cells. The number of degrading bacteria decreased gradually in the following days. Degradation proceeded in a characteristic manner (Fig. 4A). The bacterial cytosol became less electron dense. In yersiniae, a temporary increase of electron-dense granula was observed. This was followed in both bacterial species by loss of their electron-dense granula. The DNA of the microorganisms condensed and was seen as electron-dense deposits inside the bacterial cytosol. Moreover, the formation of outer membrane blebs was observed.

These processes finally led to the total disappearance of the bacterial cytosol, the remnants being “empty” bodies consisting of bacterial walls only (Fig. 4B).

Comparison of FISH and fluorescence stainings of yersiniae showed that, 3 days after infection, only 20% of yersiniae could be visualized by FISH. The absolute number of bacteria gradually decreased to the end of this study (day 90 after infection). After day 7 after infection bacteria were mostly FISH negative, suggesting no metabolic activity. The percentage of bacteria stained by IF increased to 50%. The percentage of LPS-positive but DNA-free yersiniae increased significantly. There were still DAPI-positive cells without IF staining. Moreover, in a few SF, large agglomerations of yersiniae were found. These bacteria were DAPI positive but FISH negative. IF staining was diffuse and not directly associated with yersiniae (Fig. 5).
FIG. 3. Detection of *Y. enterocolitica*-infected SF cells 12 h after infection by fluorescence microscopy. (A) Section shown after staining with nucleic acid stain DAPI. (B) Same section after FISH with probes Y.16S-69-Cy3, Y.ens16S-184-Cy3, and Y.16S-1241-Cy3. (C) Same section after immunolabeling with a FITC-labeled monoclonal antibody targeted to *Y. enterocolitica* O:3 LPS. (D) Superimposition of images in panels A to C demonstrating the presence of yersinia cells that are DAPI and FISH positive but IF negative (arrows with dots) occurring together with bacterial cells positive by all three staining techniques (arrows).

FIG. 4. (A) Yersinia in SF 2 days after infection with *Y. enterocolitica*. Shown is the beginning degradation, with formation of outer membrane blebs and condensation of DNA, but a still-intact bacterial wall. (B) Yersinia in SF 2 weeks after infection with *Y. enterocolitica*. Only the characteristic bacterial wall remains intact, and the typical rod-like morphology suggests that this is indeed yersinia, probably the equivalent of the ghost, seen by IF. The cytosol is almost entirely dissolved.
Electron microscopy showed comparable aggregations of bacteria several days after infection.

Since bacteria lost their characteristic morphology due to degradation, we identified degraded bacteria by immunogold electron microscopy using primary antibodies to yersiniae or salmonellae, followed by gold-labeled secondary antibodies. Antibodies bound specifically to bacterial cells in bacterial pellets or in SF in the early phase after infection with nearly no background staining (Fig. 6). Immunogold labeling was also seen on degraded bacteria marking the bacterial walls of empty bodies a few days after infection. Positive staining could be found during the early period of infection within vacuoles containing intact bacteria as well as amorphous material (Fig. 6A). Positive staining was also found on debris situated directly in the cytoplasm of SF at later time points after infection, suggesting that this material was derived from degraded bacteria.

To compare intracellular with extracellular bacterial degradation, salmonellae and yersiniae were inactivated by irradiation with UV light for 30 min or by treatment with 25 μg of gentamicin/ml. Bacterial pellets were prepared for electron microscopy evaluation. Immediately after UV light irradiation, these bacteria could not be distinguished from untreated living bacteria, but it was not possible to subculture them. After 2 days, all stages of degraded bacteria could be observed, with loss of electron-dense granula, brightening of the bacterial cytosol, formation of outer membrane blebs, and presence of cell walls with empty cytoplasm. Treatment of yersiniae or salmonellae with 25 μg of gentamicin/ml led to bacterial degradation in the same way within 3 days. Subcultivation was not possible already 1 day after antibiotic treatment. After incubation of infected cells with IFN-γ, degradation was seen earlier than in uninfected cells but degradation occurred in the same way.

The pathogenesis of reactive arthritis includes the presence of bacterial products and probably even living organisms. However, the possible location of persisting bacteria is under debate. There is strong evidence that bacteria may persist in more than one location in the body.

As a possible model for persistence of bacteria in reactive arthritis, infection of SF with yersiniae has been investigated previously (11). Living yersiniae persisted for at least 4 weeks...
within SF, and the generation of ghosts, nucleic acid-free bacterial rods, was described. The present study was performed to investigate ultrastructurally the infection of SF with yersiniae and salmonellae and bacterial degradation leading to ghosts. Adhesion and uptake of bacteria were followed by bacterial replication within SF and thereafter degradation. During degradation, the formation of outer membrane blebs, which are typically seen in the process of bacterial degradation, was observed as described by Robinson et al. (20). Finally, degraded particles consisted of the bacterial envelope only, presumably the equivalent of the ghosts. The formation of ghosts could also be observed after bacterial treatment with high doses of gentamicin and after UV light irradiation. We therefore conclude that bacterial degradation, including the generation of ghosts, is a nonspecific process which can be triggered under different conditions. Degradation of intracellular yersiniae due to treatment with IFN-γ has been recently described: IFN-γ led to reduced titers of live intracellular bacteria (9, 12, 14). On the other hand, we could show that some cytokines including interleukin-4 may enhance bacterial proliferation in infected SF (14) and that dendritic cell function is perturbed by Y. enterocolitica infection in vitro (22, 23). Thus, Y. enterocolitica seems to be able to delay the immune response until enough bacteria have multiplied.

The formation of ghosts was also confirmed in the present study by positive IF (LPS staining) staining and negative DAPI staining. The percentage of LPS-positive, but DNA- and ribosome-negative, bacterial rods increased to the end of the study.

Although ghosts were detected several weeks after infection by IF as well as electron microscopy, single bacteria seemed to have survived. The complete degradation of the microorganisms by SF takes a long time, and bacterial antigens, such as LPS, which plays a crucial role in the pathogenesis of reactive arthritis (4, 5, 16, 18), can persist within the cells for even longer periods.

Immunogold electron microscopy using anti-LPS antibodies resulted in the staining of not only whole bacteria and ghosts but also amorphous material. These might be bacterial components, including LPS, which have been released from intracellular bacteria. In epithelial cells, Salmonella enterica serovar Typhimurium was found to release LPS to vesicles of the host cell (2). In our study, a few hours after infection, we found vacuoles that contained intact yersiniae and salmonellae as well as antigenic bacterial material. The amorphous material might also consist of residual particles of degraded bacteria, since it was found many days after infection directly in the cytoplasm of SF outside vesicles.

In the first hours after infection, metabolic activity of the intracellular microorganisms can be assumed to be low as shown by FISH for yersinial 16S rRNA (weak hybridization signal). However, it increased significantly during the following hours, when bacteria started to replicate. Unexpectedly, although bacteria showed high numbers of ribosomes and a positive DAPI stain for DNA, less than 10% of DAPI-positive cells stained positive for LPS by IF with a monoclonal antibody. The most likely explanation for this unusual phenomenon is that yersiniae may express a high density of YadA molecules on the surface and that these molecules mask LPS. Alternatively, modification of the O-specific side chain of LPS might lead to the loss of the epitope for the O:3-specific monoclonal antibody when yersiniae are grown intracellularly.

Transient infection of synovial cells of the joints might be the
missing link between the initial intestinal infection and the late synovial inflammation caused by degraded bacterial material.

After intestinal infection, bacteria gain access to the circulation and might be transported, either in plasma or in lymphatic cells, to the joint, where they enter synovial cells and replicate. Although many bacteria are killed, some survive, and the dead bacteria release arthritogenic material, which is slowly degraded by the host cells. In the presence of further host factors synovitis is induced. The longer this process drags on the more arthritogenic material accumulates. After several weeks, at the time of clinical sampling of synovial fluid or tissue for the diagnosis of reactive arthritis, living bacteria are no longer present or at least are no longer cultivable. When, after several more weeks to months, all bacterial products have been eliminated from the host, arthritis disappears; this constitutes the self-limiting course.

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REFERENCES


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