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# *Yersinia* Lipopolysaccharide Is Modified by Human Monocytes

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**Reactive arthritis is usually self-limiting polyarthritis, which develops after certain gastrointestinal or urogenital tract infections, mostly in susceptible HLA B27-positive individuals. In the pathogenesis of this arthritis, it is probably important that structures of the causative bacteria are found in the affected joints. The structure found in the synovial fluid phagocytes of the patients with reactive arthritis after *Yersinia*, *Salmonella*, and *Shigella* infections has always been lipopolysaccharide (LPS) of the causative bacteria. It has been in a highly processed form but still immunoreactive. To follow the degradation process of LPS, we fed peripheral blood monocytes of healthy blood donors with heat-killed *Yersinia enterocolitica* O:3 bacteria in vitro and monitored the fate of LPS by immunofluorescence and immunoblotting methods. Heat-killed bacteria were used since *Y. enterocolitica* O:3 bacteria are able to live inside monocytes in vitro and dividing intracellular bacteria would have made it impossible to monitor the degradation process of LPS with these methods. Both the core region and the O-polysaccharide chain of LPS persisted in cytoplasmic vacuoles and on plasma membrane of monocytes through the 7-day follow-up time. Migration properties of processed LPS in sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggested structural modifications of LPS. We also demonstrated that core epitopes appearing on the surface of *Yersinia*-fed monocytes on day 4 of incubation were processed intracellularly, suggesting that LPS-containing phagocytes are a constant source of membrane-active LPS in their microenvironment as well as in the joints of arthritic patients.**

Reactive arthritis is usually self-limiting polyarthritis, which develops after gastrointestinal or urogenital infections caused by yersiniae, salmonellae, shigellae, campylobacters, or chlamydiae. Pathogenetic mechanisms leading to arthritis after these infections are not known, but the disease is closely linked to human leukocyte antigen (HLA) B27 tissue antigen. Arthritis usually develops 1 to 4 weeks after the initial infection. The immune responses of patients developing reactive arthritis have been studied in many laboratories. These patients generally show weak cell-mediated and strong and long-lasting immunoglobulin A and G antibody responses against the causative organisms (13, 21). Both of these characteristics suggest prolonged persistence of microbes and microbial structures. The antibody response is directed mostly against lipopolysaccharide (LPS), an outer membrane structure common to all arthritis-inducing pathogens. Recently, structures (LPS) of the causative agent have been shown to exist in the affected joints, e.g., in synovial fluid phagocytes. However, no live organisms or bacterial DNA has been detected (13, 14–16, 34).

Mononuclear phagocytes are known to harbor pathogens causing many persistent infections in the body, and the arthritis-triggering pathogens also can survive in these cells (8, 31). Monocytes and macrophages also have the machinery to degrade antigens. They, for example, are able to remove fatty acids by enzymatic deacylation and dephosphorylate the lipid A region of LPS (32, 39). Several reports have suggested that the O-polysaccharide chain of LPS as well is partially degraded by these cells (9, 10, 14, 16). In vivo studies have shown, however, that it is the polysaccharide portion of LPS which is retained after partial degradation, presumably in a compartment where only slow or nondegradable molecules are present (16, 28, 44). As long-living highly mobile cells, phagocytes could transport LPS from the site of primary infection to other parts of the body such as the inflamed joints of arthritic patients.

The purpose of the present work was to study the capacity of mononuclear phagocytes to degrade *Yersinia* LPS. We demonstrated that structures of LPS persist and are altered in monocytes during the first week of incubation in vitro. We also demonstrated the persistence of LPS-derived carbohydrates on the plasma membrane of *Yersinia*-fed monocytes and the appearance of intracellularly processed LPS molecules on the surface of these cells after prolonged incubation.

## MATERIALS AND METHODS

**Bacteria.** The strain of *Yersinia enterocolitica* serotype O:3 used (4147/83) was a stool isolate from a patient developing reactive arthritis as a result of infection. The strain contains a virulence-associated 72-kb plasmid. A plasmid-cured derivative of the strain was obtained by cultivating the bacteria on magnesium-oxalate agar at 37°C (11). The presence or absence of the virulence plasmid was verified by autoagglutination (27). Stock cultures were maintained at −40°C in 20% (vol/vol) glycerol-Trypticase soy broth. The bacteria with and without virulence-associated plasmid were grown under conditions favoring the expression of virulence plasmid-encoded outer membrane proteins (43). The resulting bacterial cultures were suspended in saline, harvested by centrifugation (20 min, 3,000 × *g*), and washed three times in saline. The bacteria were killed with heat (1 h, 100°C) and stored in phosphate-buffered saline (PBS) at 4°C. Since some of the virulence plasmid-encoded outer membrane proteins may be lost when bacterial cells are washed, a batch of the same virulence plasmid-bearing *Yersinia* bacteria was grown

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in RPMI 1640 medium with L-glutamine (RPMI; Gibco, Paisley, Scotland) and stored in that medium after killing without any washes.

**Isolation of peripheral blood monocytes and incubation with bacteria.** Buffy coats of healthy blood donors were obtained from the Finnish Red Cross Blood Donation Center in Turku. Peripheral blood mononuclear cells were prepared by using Ficoll-Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) centrifugation. Monocytes were allowed to adhere to six-well plastic tissue culture plates (Costar, Cambridge, Mass.) in the presence of 10% heat-inactivated fetal calf serum for 1 h. Nonadherent cells were removed by washing the plates three times with Hanks' balanced salt solution (HBSS; prepared in our laboratory). Adherent cells, mostly monocytes as determined by morphological characteristics, were ≥95% viable.

Monocytes were overlaid with prewarmed culture medium (HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered [20 mmol/liter] RPMI supplemented with 10% heat-inactivated human AB serum [Finnish Red Cross]). Bacteria suspended in the same medium (about 200 bacteria per monocyte) were overlaid. The tissue culture plates were then centrifuged at 1,700 × *g*, 20°C, for 10 min to get the bacteria into contact with the monocytes on the bottom of the wells. The monocytes were allowed to ingest the bacteria for 1 h, and then extracellular bacteria were removed by washing the plates three times with HBSS. Incubation was continued for different periods of time (1 or 3 h or 1, 2, 4, or 6 to 7 days) in culture medium (HEPES-buffered RPMI) with 50 mg of gentamicin (G-mycin; Orion, Espoo, Finland) per ml supplemented with 10% fetal calf serum. Monocytes were harvested by incubating the plates in prewarmed EDTA (5 mmol/liter; Sigma Chemical Co., St. Louis, Mo.) in $Ca^{2+}$-$Mg^{2+}$-free HBSS for 10 min and by scraping the plates with a rubber policeman after different incubation times.

**MAbs.** Mouse monoclonal antibodies (MAbs; immunoglobulin G1) specific for the core oligosaccharide, more specifically for the outer core (designated 2B5) (42a) (38) and for the O-polysaccharide chain (designated A6) of *Y. enterocolitica* O:3 (38), were used for detection of bacterial antigens. A subclass-matched MAb specific for chicken T cells (3G6) was used as a negative control. Biotinylated and fluorescein isothiocyanate-conjugated MAbs against vascular adhesion protein (41) were used as negative controls in double staining experiments. All MAbs were culture supernatants.

**Immunofluorescence staining.** Peripheral blood monocytes which had ingested and processed *Y. enterocolitica* O:3 for different periods of time were cytocentrifuged onto microscope slides (50,000 cells per slide), air dried, and fixed with cold acetic acid-ethanol (5%:95% [vol/vol]) at −20°C for 10 min. Slides were then stored at −20°C until used. For staining, the slides were overlaid with mouse MAbs diluted in PBS containing 0.2% bovine serum albumin (BSA; fraction V; INC Biomedicals Ltd., Buckinghamshire, England; mixture designated BSA-PBS) and incubated at room temperature for 30 min. The slides were then washed three times with BSA-PBS and stained with fluoresceinated $F(ab')_2$ fragments of anti-mouse immunoglobulin G (1:200) (Sigma) at room temperature for 30 min. After three washes, the slides were dried, mounted with PBS-glycerol (1:9 [vol/vol]) which contained 1 mg of *p*-phenylenediamine (Sigma) per ml, and finally read under a Leitz diaplan-incidence light-fluorescence microscope (SM-Lux; Leitz, Wetzlar, Germany) with an Osram HBO 100-W mercury lamp or with a confocal microscope (CCM; EMBL, Heidelberg, Germany). Monocytes of 10 individuals were studied.

For flow cytometry, monocytes of the same individual were divided into three groups and incubated for 1 h, 1 or 2 days, and 7 days. Twenty-six individuals were studied. Cells were stained in suspension by using the same antibodies and incubation times as those used for immunofluorescence on slides. Cells were fixed with 1% formaldehyde (Merck, Darmstadt, Germany) in PBS before analyses.

Slides for double staining were incubated with fluorescein isothiocyanate-conjugated mouse MAb against the O-polysaccharide chain of LPS (A6) for 30 min and washed three times. The slides were then overlaid with biotinylated mouse MAb against the outer core of LPS (2B5) and incubated for 30 min. After three washes, the slides were overlaid with streptavidin Texas red conjugate (Becton-Dickinson, San Jose, Calif.) and incubated for 30 min. After three washes, the slides were air dried, mounted, and studied under a fluorescence microscope as described earlier.

**Flow cytometry.** Analyses were performed with a FACScan flow cytometer (Becton-Dickinson & Co., Mountain View, Calif.).

**Electrophoresis and Western blotting (immunoblotting).** The cell deposits were solubilized in Laemmli's sample buffer under mild reducing conditions (0.1% β-mercaptoethanol) (26) and separated by using vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a stacking gel of 5% acrylamide and a resolving-gradient gel of 5 to 12.5 or 10 to 12.5% acrylamide. Standards of known molecular weight were included in each gel run (Rainbow protein molecular weight markers; Amersham, Buckinghamshire, England). Western blotting was performed by a modification of the method of Towbin et al. (45). The separated components in the gel were immediately transferred electrophoretically onto nitrocellulose sheets (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany) by using a Transphor apparatus (LKB-Bromma, Bromma, Sweden) and prechilled Tris-glycine buffer (pH 8.3) at 1.0 A for 1 h 15 min. Nonspecific binding sites of the nitrocellulose were blocked by incubation in PBS containing 1% nonfat milk at 20°C for 2 h. After three washes with PBS, the nitrocellulose sheets were cut into strips, and each strip was stained separately. The strips were allowed to react overnight at 4°C with a 1:2 (2B5) or a 1:50 dilution (A6) of mouse MAbs in PBS with 1% nonfat milk or with undiluted negative control (3G6). The strips were then rewashed and incubated at room temperature for 3 h with horseradish peroxidase-labeled antimouse antibodies (DAKO Immunoglobulins, Copenhagen, Denmark). After three washes, the strips were developed with dioctyl sulfosuccinate substrate (Sigma) (42).

**Immunoprecipitation.** Peripheral blood monocytes fed with *Y. enterocolitica* O:3 were incubated at 4°C for 1 h with the mouse MAb specific for the outer core of LPS (2B5) to cover the cell surface-bound LPS molecules with antibodies. The same bacteria alone, without monocytes, were treated in the same way to serve as a control. *Yersinia*-fed monocytes and control bacteria were lysed with lysis buffer (0.1 M NaCl, 0.01 M sodium phosphate, 1% Triton X-100, 0.5% deoxycholate [pH 7.4]; 1.5 ml/$10^7$ cells), and the cell lysate was cleared by centrifugation at 10,000 × *g* for 15 min. Cell surface-bound anti-LPS antibodies, which were attached to the LPS molecules on the plasma membrane of *Yersinia*-fed monocytes or control bacteria prior to lysis, were precipitated with Protein A-Sepharose 4B beads (Pharmacia LKB) conjugated with rabbit anti-mouse immunoglobulin (DAKO Immunoglobulins; cell surface LPS). The cellular fragments

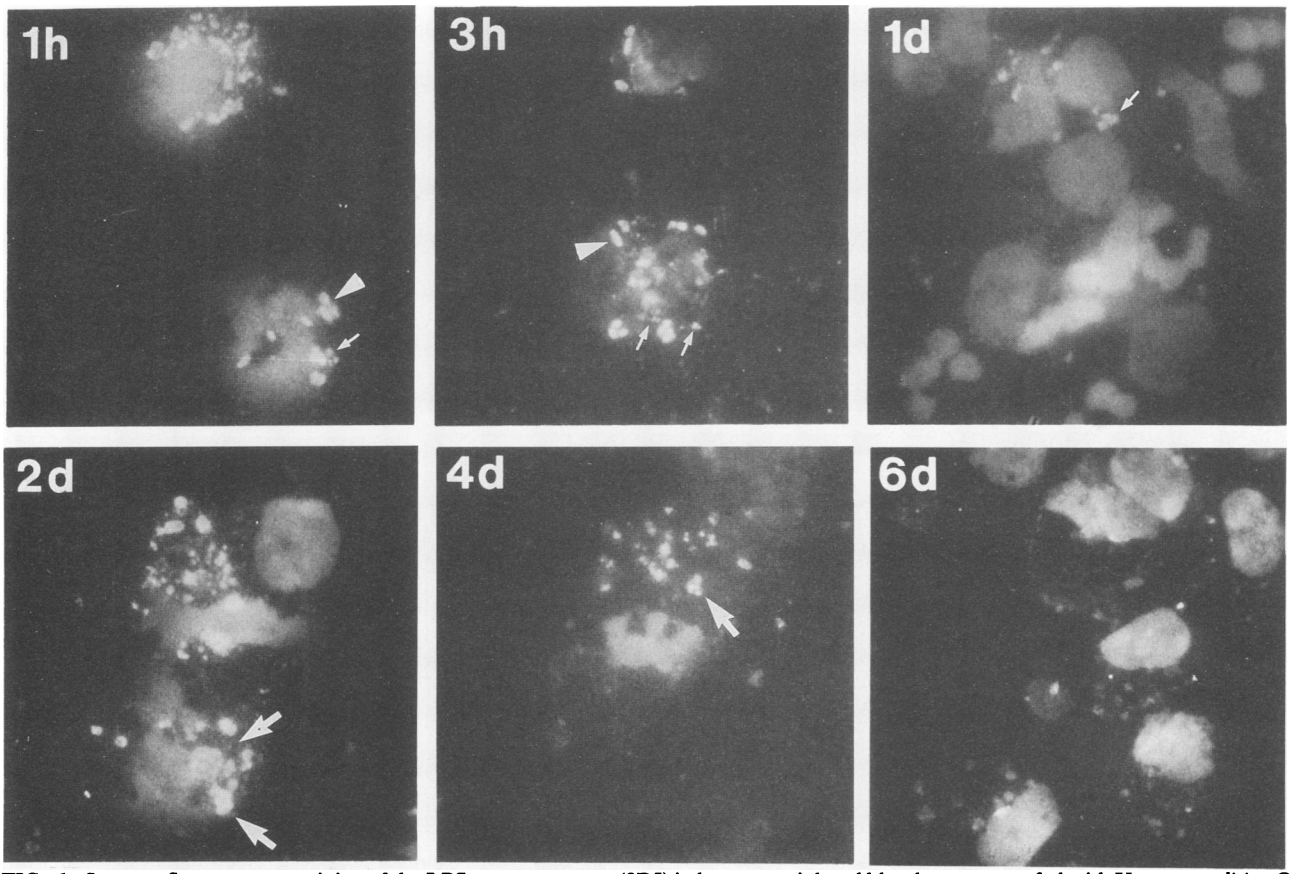


FIG. 1. Immunofluorescence staining of the LPS core structures (2B5) in human peripheral blood monocytes fed with *Y. enterocolitica* O:3 in vitro. Incubation was continued for different periods of time after feeding as indicated in the figure. d, day. Arrowheads indicate intact bacteria, small arrows indicate small vacuoles containing LPS, and large arrows indicate loosely packed big vacuoles.

that survived the first round of lysis were lysed further with lysis buffer containing 0.1% SDS and DNase I, grade II (Boehringer GmbH, Mannheim, Germany) at 4°C overnight. The lysate, which no longer contained plasma membrane-bound LPS molecules, was then cleared by centrifugation. The lysates were again subjected to immunoprecipitation with Protein A-Sepharose 4B beads conjugated with MAb 2B5 (intracellular antigen fraction I). The cell debris from the second round of lysis was stored at −20°C until used. Intracellular antigen fraction II was collected by boiling the remnants of monocytes in Laemmli's sample buffer after two rounds of lysis. *Yersinia* bacteria which were used as controls were incubated and lysed as monocytes. All of the isolated LPS fractions were then subjected to electrophoresis and Western blotting as described in the previous section. Each strip was divided into two pieces, and one was stained with 2B5 and the other was stained with negative control (3G6).

**Tissue typing.** HLA B27 antigen was determined by using a cytotoxic assay (Histognost; Behringwerke AG, Marburg, Germany).

## RESULTS

**Distribution of LPS in monocytes.** The fate of LPS was monitored to determine how LPS is released from bacteria and where it goes in the monocytes. Degradation of *Yersinia* bacteria in monocytes began within 1 h. Some bacteria which had maintained their integrity were still detected by immunofluorescence after 3 h of incubation (Fig. 1 and 2). All bacteria started to degrade by the first day of incubation (Fig. 1 and 2). Small, LPS-containing, vacuole-like structures dominated during the early hours of incubation. The size of the LPS clusters increased by day 2 of incubation, and at the same time, the number of vacuoles diminished. The O-polysaccharide chain of LPS especially was loosely packed in those vacuoles during days 2 to 4 of incubation (Fig. 2). The vacuoles became more tightly packed when incubation was continued (Fig. 1 to 3). Double staining experiments revealed that both the core and the O-polysaccharide chain of LPS stayed in the same vacuoles throughout the incubation time tested (7 days; Fig. 4). Structures recognized by our MAb against the core part of LPS seemed to have affinity also for nuclear membranes (Fig. 4). No significant variation in the degradation process between bacteria with or without virulence plasmid-encoded proteins or bacteria which had not been washed after growing was observed. The monocytes of five HLA B27-positive individuals degraded LPS like monocytes of five HLA B27-negative persons.

**Monocyte-induced modifications of LPS structure.** Highly processed forms of LPS are found in phagocytic cells in the joints of patients with reactive arthritis. We wanted to monitor the degradation process of LPS which is derived from *Yersinia* bacteria. The antigenic epitopes of both the core region and the O-polysaccharide chain of LPS persisted in monocytes for 7 days in ample amounts when tested by Western blotting (Fig. 5 and 6). The incubation of *Yersinia*

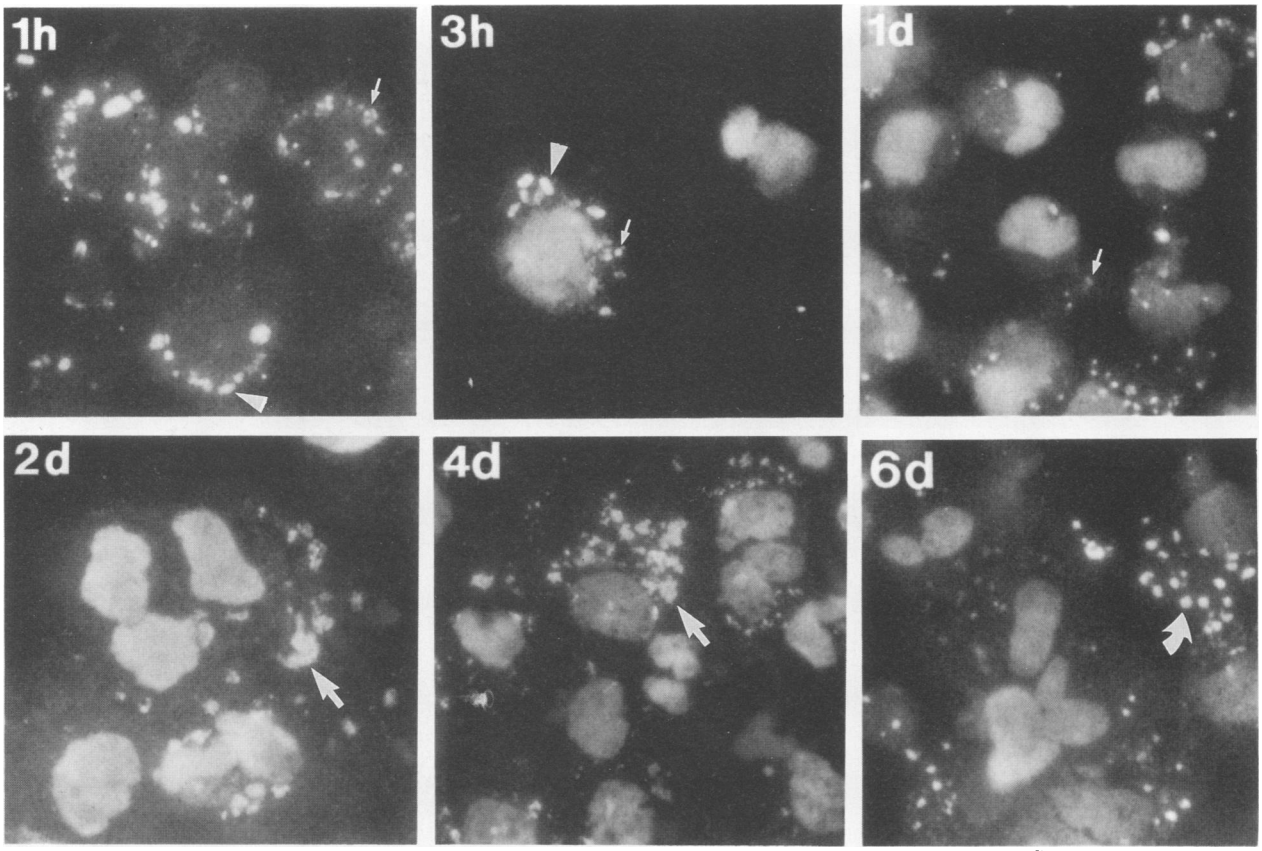


FIG. 2. Immunofluorescence staining of the O-polysaccharide chain of LPS (A6) in human peripheral blood monocytes fed with *Y. enterocolitica* O:3 in vitro. Incubation was continued for different periods of time after feeding as indicated in the figure. d, day. Arrowheads indicate intact bacteria, small arrows indicate small vacuoles containing LPS, large arrows indicate loosely packed big vacuoles, and the curved arrow indicates tightly packed LPS vacuoles.

bacteria within monocytes resulted in an overall decrease in the electrophoretic mobility of both the core part and the O-polysaccharide chain of LPS. Decreased mobility was seen as early as after 1 h of incubation, and it was especially prominent on days 2 to 4 of incubation. An increase in the migration of both LPS structures was seen on day 6 of incubation.

When monocytes had started to process the bacteria, new bands with higher and lower apparent molecular masses emerged to the core region as early as during the first hour of incubation as detected with MAb against the outer core. The slower migrating bands were most prominent on days 1 to 4 of incubation.

A novel band with an apparent molecular mass of about 33 kDa was detected with the MAb against the O-polysaccharide chain of LPS (A6) in *Yersinia*-fed monocytes. This band became visible on the first day of incubation, and it was never seen in control cells or bacteria. The band always appeared at the same position, regardless of the shift in electrophoretic migration of other bands which correspond to the repeating units of O-polysaccharide.

**Cell surface expression of LPS antigens.** LPS can bind to plasma membrane via several receptor-mediated and unspecific mechanisms. We monitored the expression of carbohydrate epitopes of LPS on *Yersinia*-fed monocytes to determine how long immunologically active LPS would stay on the plasma membrane and presumably disturb the plasma membrane-associated functions of the phagocyte. Immunofluorescence staining of cell surfaces revealed that LPS antigens were also attached to the plasma membrane of monocytes during ingestion of bacteria (Table 1; Fig. 7). More than 70% of *Yersinia*-fed monocytes had an immunologically detectable core region of LPS on their plasma membrane after 1 h of incubation. The number of cells with the core sugars of LPS on their surface stayed at almost the original level during the first 2 days of incubation (Table 1). Furthermore, the intensity of staining also remained high for 2 days (Fig. 7). Forty percent of monocytes were still positive on day 7 of incubation (Table 1).

The picture was different when *Yersinia*-fed monocytes were stained with the MAb against the O-polysaccharide chain of LPS (Table 1; Fig. 7). Thirty-seven percent of *Yersinia*-fed monocytes had O-polysaccharide-derived material on their surface after 1 h of incubation. Although the number of positive cells stained with the MAb against the O-polysaccharide chain of LPS remained quite constant during the first day of incubation, the fluorescence intensity started to decrease already during that time, suggesting that the number of LPS molecules with detectable O-side chains per monocyte diminished (Fig. 7). A clear decline in the number of positive monocytes was seen after day 2 of incubation.

Eight of the 26 individuals studied were HLA B27 positive. The expression of LPS epitopes on the surface of *Yersinia*-fed monocytes was similar in these two groups.

**Immunoprecipitation.** Immunoprecipitation experiments

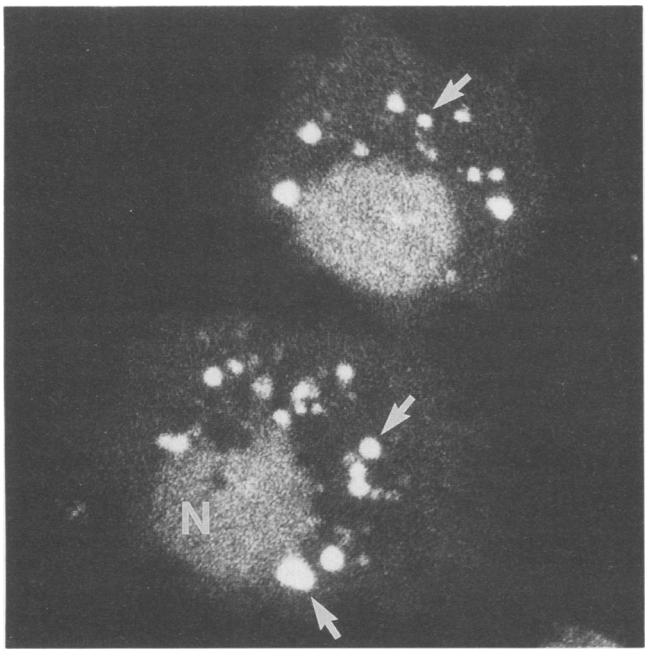


FIG. 3. Confocal microscopy of human peripheral blood monocytes showing the condensed vacuole-like structures of different sizes (arrows). The nucleus (N) stained because of the presence of *p*-phenylenediamine. The monocytes were fed with *Y. enterocolitica* O:3 in vitro and incubated for 6 days after feeding. Immunofluorescence staining was with the MAb against the O-polysaccharide chain of LPS.

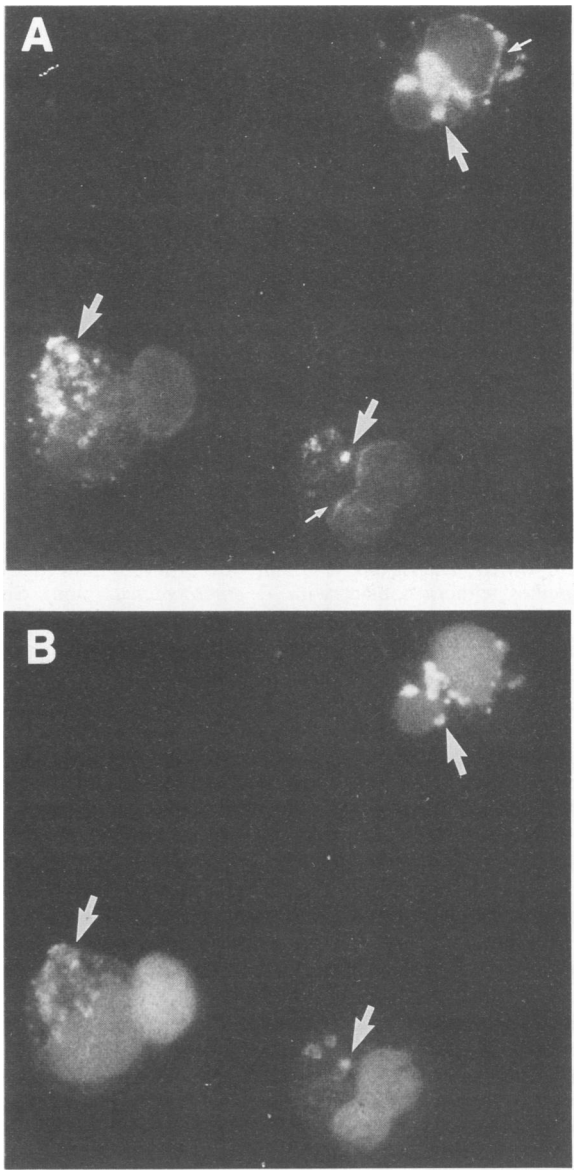


FIG. 4. Double-immunofluorescence staining of human peripheral blood monocytes showing the colocalization of the core epitopes (A; Texas red labeled) and the O-polysaccharide chain (B; fluorescein isothiocyanate labeled) of LPS (large arrows). Core-specific MAb also has affinity for nuclear membranes (small arrows).

were carried out to determine whether some of the cell surface-bound LPS could be derived from the intracellular LPS pool. The electrophoretic migration of LPS which was immunoprecipitated from the control bacteria incubated under the same conditions as the monocytes fed with the bacteria did not change in the course of the 4-day incubation (Fig. 8 and 9). Figures 8 and 9 represent results of two individual electrophoresis runs. The same 0-h incubated monocytes serve as an internal control to the relative migration of monocyte-derived as well as control bacterium-derived LPS because the core bands migrate near the leading edge of the electrophoresis and protein molecular weight standards as such cannot be used to compare the relative migration of LPS bands in this area. The migration of monocyte-associated LPS was similar to that of the control bacteria during the first 2 days of incubation (Fig. 8). On day 4 of incubation, the LPS precipitated from monocytes had not migrated upon SDS-PAGE like LPS isolated by similar methods from control bacteria. The faster-moving LPS band immunoprecipitated from the surface of the monocytes corresponded to the one obtained from the monocyte cytoplasm and not to that of control bacteria (Fig. 9). This suggests that part of the cell surface-bound LPS had been processed inside monocytes and then transferred to the surface of monocytes.

## DISCUSSION

Earlier studies by our group have demonstrated the existence of *Yersinia* LPS in synovial samples of patients with *Yersinia*-triggered reactive arthritis for several weeks, in a few cases for several years, after the original infection. The LPS in synovial fluid phagocytes is in a highly processed form, but it is still immunoreactive. In search of reasons for the long persistence of *Yersinia* structures in the bodies of patients developing reactive arthritis, we started to study the capacity of mononuclear phagocytes to process LPS.

Most of the patients who develop reactive arthritis (70 to 90%) after triggering infections are HLA B27 positive. The exact role of this tissue antigen in the pathogenesis of seronegative spondyloarthropathies is unknown. On the other hand, most of the HLA B27-positive individuals are healthy. By our methods, we could not see any difference in the capacity of peripheral blood monocytes from HLA B27-positive and -negative persons to process LPS. This does not, however, rule out the possibility that there might

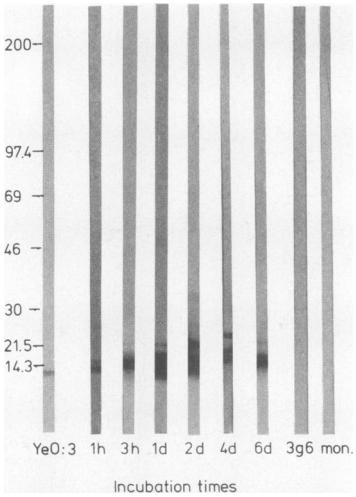


FIG. 5. Western blot of control bacteria (YeO:3), human peripheral blood monocytes incubated for different periods of time after feeding with *Y. enterocolitica* O:3 in vitro (1 h to 6 days [d]), and monocytes without contact with *Y. enterocolitica* (mon). Strips were stained with the MAb against the core region of LPS (2B5). As a negative control, a strip containing *Y. enterocolitica* O:3-fed monocytes (incubated for 1 day after feeding) was stained with the MAb against chicken T cells (3g6).

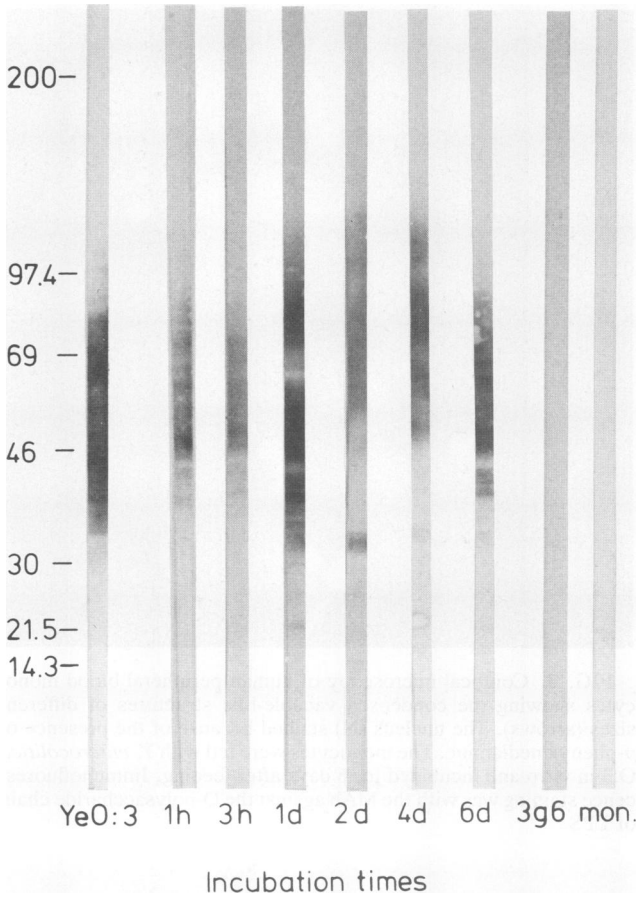


FIG. 6. Western blot of control bacteria (YeO:3), human peripheral blood monocytes incubated for different periods of time after feeding with *Y. enterocolitica* O:3 in vitro (1 h to 6 days [d]), and monocytes without contact with *Y. enterocolitica* (mon). Strips were stained with the MAb against the O-polysaccharide chain of LPS (A6). As a negative control, a strip containing *Y. enterocolitica* O:3-fed monocytes (incubated for 1 day after feeding) was stained with the MAb against chicken T cells (3g6).

exist some difference between healthy and arthritic HLA B27-positive individuals.

Immunofluorescence staining showed that the detaching of LPS from heat-killed *Y. enterocolitica* O:3 bacteria inside monocytes began very rapidly so that within 1 h of incubation some bacteria had lost their integrity and LPS was gathered to small, cytoplasmic vacuole-like structures. Most of the bacteria appeared to be, however, still intact at that time. The fast onset of the degradation process was most probably due to the use of heat-killed bacteria since autoclaving is known to make bacteria more susceptible to the degrading enzymes of phagocytes (37). Dacosta et al. have shown that the degradation of live *Shigella dysenteriae* starts after 3 h of incubation (4).

Both the core region and the O-polysaccharide chain of LPS released from ingested bacteria were first detected in small vacuoles, which apparently fused and formed larger condensing vacuoles by the end of the 1-week follow-up period. The formation of large vacuole-like structures may also result from ingestion of LPS processed and excreted earlier by other monocytes. In vitro studies with isolated LPS have shown that disruption of the plasma membrane by insertion of the lipid A into the phospholipid bilayer of the plasma membrane facilitates massive migration of LPS bilayers into the cytoplasm of monocytes by passive diffusion (24, 25, 40). LPS entering the cells via this mechanism often appears as a large aggregate in the cytoplasm without a surrounding limiting membrane (24, 25). The vacuoles we saw resembled the ones seen in synovial fluid phagocytes in some patients with *Yersinia*-triggered reactive arthritis (16). These data suggest that LPS which is released from bacteria obviously ends up in corresponding compartments of the cell as isolated LPS.

The migration of both the core region and the O-polysaccharide chain of LPS processed inside monocytes decreased in SDS-PAGE in comparison with native bacteria. A corresponding overall shift in the migration pattern of macrophage-processed LPS in SDS-PAGE in the direction of higher-molecular-mass components has also been found by Duncan et al. with radiolabeled LPS of *Escherichia coli* (7). Although most reports suggest that migration characteristics of LPS in SDS-PAGE depend upon size (2, 12, 20, 33, 46), electrophoretic conditions (19) and biochemical characteristics of this complex macromolecule also may affect its electrophoretic mobility in SDS-polyacrylamide gels. Furthermore, Hitchcock and Brown have shown that the coelectrophoresis of proteins with LPS influences the migration characteristics of the LPS (19).

Our methods did not allow the exact characterization of the reasons for the altered electrophoretic migration, but structural changes of the LPS molecule are suggested. Aggregate formation of dissociated LPS due to polysaccharide-polysaccharide interaction (30) or through interactions mediated by the lipid A region of LPS (20) is unlikely, since treatment of extracted endotoxin with SDS results in reversible dissociation of the aggregates into subunits (20, 35). SDS has also been shown to dissociate LPS from other bacterial outer membrane components such as porins (36). Previously, it has been shown that the lipid A content alters the

TABLE 1. Percentage of monocytes expressing LPS antigens after the cells have ingested and degraded *Y. enterocolitica* O:3 in vitro

| Antibodies | % Monocytes expressing LPS antigens after incubation time of[a]: | | | |
|---|---|---|---|---|
| | 1 h | 1 day | 2 days | 7 days |
| MAb against the O-polysaccharide chain of LPS (A6) | 37.0 ± 4.5 (26) | 35.4 ± 6.8 (12) | 19.7 ± 4.6 (10) | 18.2 ± 5.4 (17) |
| MAb against the outer core part of LPS (2B5) | 72.9 ± 2.0 (26) | 63.9 ± 6.1 (11) | 66.8 ± 7.9 (10) | 40.1 ± 8.2 (17) |

[a] The results represent the mean ± standard error of the mean of the number of experiments shown in parentheses. The background fluorescence determined with the negative control antibody (3G6) and the fluorescence of control cells stained with either A6 or 2B5 were subtracted from the readings shown.

electrophoretic migration of LPS in SDS-PAGE (22, 36). The lipid A region has been suggested to be the SDS binding component of LPS (35), and diminished fatty acid content of LPS leads to slower migration in SDS-PAGE. Modifications in the O-polysaccharide chain of LPS can also cause aberrant migration of LPS in SDS-PAGE (19, 23). The shift towards a high-molecular-mass form of LPS in our system may be due to the deacylation of the fatty acids by monocytes. Enzymatic deacylation was likely to occur under the conditions we used, and the migration of LPS might have been retarded because SDS-binding fatty acids of the lipid A region were cleaved.

The novel bands which we detected with the MAb against the core region presumably resulted also from changes both in the lipid A and in the polysaccharide parts of LPS. The migration profile of the core region of processed LPS in our experiments corresponded to semirough (SR) and rough (R) LPS profiles published before (19, 23, 36, 46). This suggests that the novel slower- and faster-migrating bands of the processed LPS we detect with the MAb against the core region result from core epitopes which have become uncovered because of the removal of repeating units from the O-polysaccharide chain. The core oligosaccharides of some LPS molecules may also be modified to some extent by monocytes. The disappearance of the fastest-migrating material after day 4 of incubation may result from the loss of our antigenic epitope due to degradations of the outer oligosaccharide core of processed LPS.

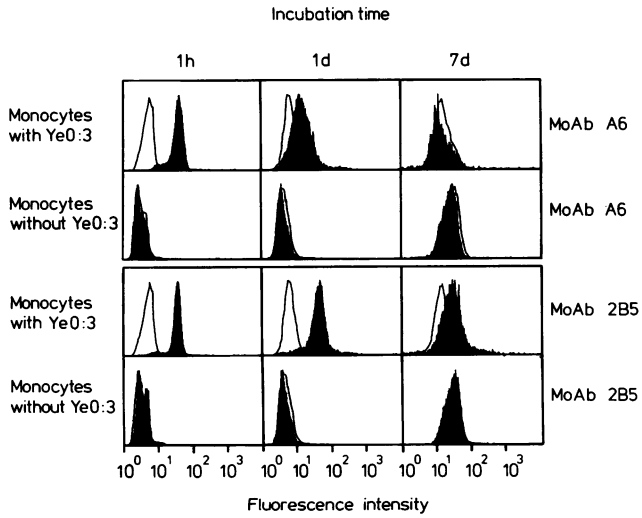


FIG. 7. Immunofluorescence staining of cell surface-bound LPS antigens of *Yersinia*-fed and control monocytes. Cells from the same individual were incubated for different periods of time (after feeding or without contact with bacteria) in vitro as indicated in the figure. d, day. Fluorescence profiles of monocytes stained with either the MAb against the O-polysaccharide of LPS (A6; black histograms) or with the MAb against the core polysaccharide of LPS (2B5; black histograms) are shown. Monocytes were stained with the MAb against chicken T cells (3G6; white histograms) as a control.

The degradation of the O-polysaccharide region of LPS is supported by the fact that, in LPS processed by monocytes, an extra 33-kDa band reactive with our MAb against the O-polysaccharide chain of LPS could be detected. This band may represent the same material that we demonstrated to persist in synovial fluid phagocytes in patients with *Yersinia*-triggered reactive arthritis (16). The molecular mass of the O-polysaccharide antigen in synovial fluid phagocytes of the arthritic patients is about 29 kDa, but in this study, the electrophoresis was run under somewhat different conditions and the percentage of acrylamide in the resolving gradient gel was different. These differences may account for the 4-kDa discrepancy in the apparent molecular mass.

We could demonstrate that 73% of *Yersinia*-fed monocytes had epitopes recognized by our MAb against the core region of LPS on their plasma membrane after 1 h of incubation. The corresponding number of positive cells having the O-polysaccharide chain of LPS on their surface was 37%. These values dropped to 40 and 18%, respectively, after 7 days of incubation. Because ingestion of *Yersinia* bacteria took place in the presence of human serum, it is very likely that the specific LPS receptors mediate the binding of *Yersinia* LPS to mononuclear phagocytes (47, 48). The persistence of LPS on the surface of monocytes, and especially the predominance of LPS molecules without the O-polysaccharide chain, however, favor the interpretation that at least part of the cell surface-bound LPS has bound to plasma membrane via nonspecific interactions between the lipid A region and plasma membrane phospholipids (29).

The core region of LPS was expressed on the surface of *Yersinia*-fed monocytes without any significant decrease in either the number of positive cells or fluorescence intensity (representing the number of the core structures per monocyte) during the first 2 days of incubation. In the case of the O-polysaccharide chain of LPS, a gradual loss of molecules was seen. This means that either LPS molecules without O-polysaccharide chain stay on the surface of monocytes for considerably longer than molecules with it or that monocytes repeatedly bind LPS which has already been processed and excreted by other cells. Davies et al. (6) have shown that the association of LPS to cells follows a consistent pattern of cyclic fluctuation between adsorption and desorption. The results of our immunoprecipitation studies support the hypothesis of constant excretion and binding and/or ingestion of LPS to monocytes because the migration pattern of the cell surface-bound LPS corresponded to LPS isolated from the intracellular fraction and not to that of the control bacteria on day 4 of incubation. Our MAb against core oligosaccharides of *Y. enterocolitica* O:3 consistently recognizes two bands which presumably represent two different forms of the core oligosaccharide (1). Several bacteria including *Yersinia pestis* have been shown to make two types

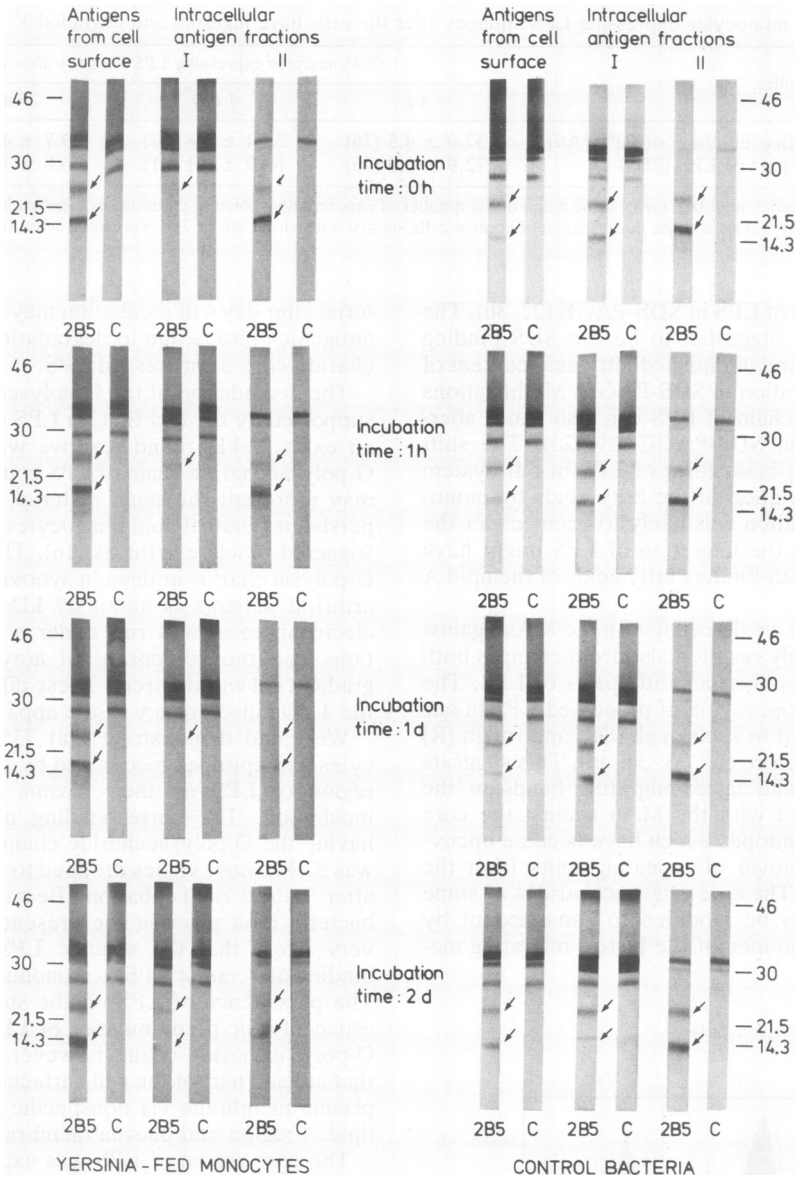


FIG. 8. Western blot of the core polysaccharides immunoprecipitated from either *Yersinia*-fed monocytes or control bacteria (antigens from cell surface, intracellular antigen fraction I). Intracellular antigen fraction II was collected by boiling the remnants of monocytes or control bacteria in Laemmli's sample buffer with 10% SDS after two rounds of lysis. Incubation times were from 0 h (feeding without further incubation) to 2 days (d) as indicated in the figure. Strips were stained with the MAb against the outer core of LPS (2B5) or with the control antibody (C) against chicken T cells (3G6). Core-specific bands are indicated with arrows. Unspecific staining, which is also visible in the controls, is due to cross-reactions between different immunoglobulins during precipitation.

of core molecules (5, 18). The faster-migrating LPS band has inevitably been processed before excretion to the cell surface. LPS in culture supernatants of LPS-ingested macrophages has been shown to be similar to LPS in macrophage lysates and different from native LPS (7). Earlier studies have shown that treatment with alkali, which removes fatty acids from LPS, makes it a more polar molecule and, thus, 10 to 15 times more surface active in its ability to penetrate surface monolayers than the original LPS (3). Enzymatically deacylated LPS is also suggested to be more membrane active than the native LPS (17). Thus, it is tempting to speculate that the modified LPS we detect on the surface of *Yersinia*-fed cells may be in the deacylated form.

These results indicate that, although LPS is retained in an immunologically detectable form in mononuclear phagocytes for considerably long periods of time, some modifications of the LPS molecule clearly take place. Our results support earlier reports suggesting that, in addition to processing of the lipid A region of LPS, the degradation of the immunologically active O-polysaccharide and the core polysaccharide of LPS also takes place in phagocytes (7, 9, 10, 14, 16). During the first week of incubation, LPS molecules do not, however, achieve an extensively processed form seen in the joints of arthritic patients, although the appearance of a novel band in SDS-PAGE corresponding to the O-antigenic material in the joints of arthritic patients sug-

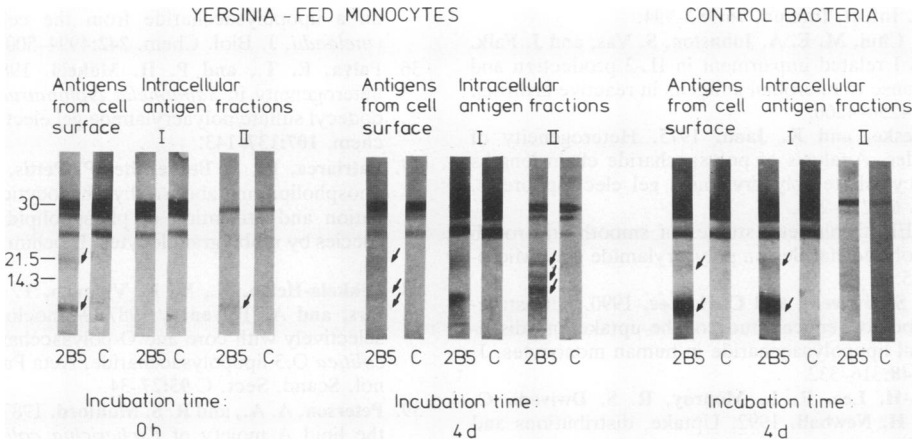


FIG. 9. Western blot of the core polysaccharides immunoprecipitated from *Yersinia*-fed monocytes or control bacteria immediately after feeding (0 h; the same sample as that described in the legend to Fig. 8) or after 4 days (d) of incubation. Isolation of antigens and staining procedures were performed as described in the legend to Fig. 8.

gests that the degradation process in vitro mimics that in vivo. We have also demonstrated that intracellularly processed LPS epitopes appear on the surface of *Yersinia*-fed monocytes after prolonged incubation. The persistence of LPS structures, especially on the surface of *Yersinia*-fed monocytes, can significantly alter the ability of LPS-containing phagocytes to communicate with other cells of the immune system, vascular endothelium, or extracellular matrix molecules. These findings suggest that LPS-containing phagocytes are a constant source of membrane-active LPS in their microenvironment, as in the joints of arthritic patients.

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