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Antibody and Delayed-Type Hypersensitivity Responses to *Ochrobactrum anthropi* Cytosolic and Outer Membrane Antigens in Infections by Smooth and Rough *Brucella* spp.

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Immunological cross-reactions between *Brucella* spp. and *Ochrobactrum anthropi* were investigated in animals and humans naturally infected by *Brucella* spp. and in experimentally infected rams (*Brucella ovis* infected), rabbits (*Brucella melitensis* infected), and mice (*B. melitensis* and *Brucella abortus* infected). In the animals tested, *O. anthropi* cytosolic proteins evoked a delayed-type hypersensitivity reaction of a frequency and intensity similar to that observed with *B. melitensis* brucellin. *O. anthropi* cytosolic proteins also reacted in gel precipitation tests with antibodies in sera from *Brucella* natural hosts with a frequency similar to that observed with *B. melitensis* proteins, and absorption experiments and immunoblotting showed antibodies to both *Brucella*-specific proteins and proteins common to *Brucella* and *O. anthropi*. No antibodies to *O. anthropi* cytosolic proteins were detected in the sera of *Brucella*-free hosts. Immunoblotting with sera of *Brucella*-infected sheep and goats showed immunoglobulin G (IgG) to *Brucella* group 3 outer membrane proteins and to *O. anthropi* proteins of similar molecular weight. No IgG to the O-specific polysaccharide of *O. anthropi* lipopolysaccharide was detected in the sera of *Brucella*-infected hosts. The sera of sheep, goats, and rabbits infected with *B. melitensis* contained IgG to *O. anthropi* rough lipopolysaccharide and lipid A, and *B. ovis* and *O. anthropi* rough lipopolysaccharides showed equal reactivities with IgG in the sera of *B. ovis*-infected rams. The findings show that the immunoresponse of *Brucella*-infected hosts to protein antigens is not necessarily specific for brucellae and suggest that the presence of *O. anthropi* or some related bacteria explains the previously described reactivities to *Brucella* rough lipopolysaccharide and outer membrane proteins in healthy animals.

Members of the genus *Brucella* are gram-negative bacteria that cause brucellosis, an infectious disease affecting livestock and humans, and whose laboratory diagnosis is usually performed by bacteriological and serological tests (4, 15). Whereas standard tests for infections caused by smooth (S) *Brucella* spp. (*Brucella melitensis*, *Brucella abortus*, and *Brucella suis*) detect antibodies to the outer membrane (OM) S-lipopolysaccharide (S-LPS) (4, 15, 50), tests used for infections caused by the rough (R) species *Brucella ovis* detect antibodies to the R-LPS and to some OM proteins (39). *Brucella* spp. also elicit an immune response to soluble protein antigens which can be detected in serological (14, 15, 17, 28, 37, 42, 43, 46, 51) and delayed-type-hypersensitivity (DTH) tests (7, 20).

Serological cross-reactions between the S-LPSs of *Brucella* and of some gram-negative bacteria, most notably *Yersinia enterocolitica* O:9, have been described and characterized at the molecular level (38) and are a cause of false-positive results in standard serological tests for brucellosis (4). On the other hand, an immunological cross-reactivity between proteins of *Brucella* spp. and of other bacteria has never been reported with sera from naturally infected hosts or in studies with hyperimmune sera (11, 13). For this reason, and because protein antigens are largely shared by all *Brucella* spp. (13), tests detecting antibodies or DTH reactions to *Brucella* proteins are considered specific for the genus (15, 18).

Ochrobactrum anthropi is a gram-negative bacterium which encompasses strains formerly included in the Centers for Disease Control and Prevention Vd group (27) and which is isolated with increasing frequency from immunocompromised patients as well as in other situations (2, 9, 19, 25, 29, 47). Analysis of the 16S rRNA has placed *O. anthropi* in the alpha subdivision of *Proteobacteria*, with a close proximity to *Brucella* (34). Moreover, some *O. anthropi* strains are positive in a PCR test for the diagnosis of *Brucella* infections (41). Thus, the possibility that *Brucella* and *O. anthropi* show a hitherto-unnoticed antigenic cross-reactivity exists. Immunological cross-reactivities between taxonomically related bacteria have been observed by using hyperimmune sera (8), but it is possible that such cross-reactions could also be observed with sera from naturally infected hosts. In fact, we have observed that sera from brucella-free hosts show unexpectedly high reactivities with R-LPS and some OM proteins (21, 23, 39) and that immunocompromised patients who become infected by *O. anthropi* develop antibodies to *Brucella* proteins (33). Since anti-protein tests are receiving increasing attention for the diagnosis of brucellosis (5, 10, 24, 26, 43, 44), it is appropriate to study whether such a cross-reactivity can be observed in naturally *Brucella*-infected hosts and whether it can affect the specificity of anti-*Brucella* protein tests. The results of such a study are reported here.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *O. anthropi* LMG 3331 and LMG 3301 were obtained from the Laboratorium voor Microbiologie Gent Culture Collection (University of Ghent, Ghent, Belgium). *B. melitensis* 16M (S, virulent), *B. melitensis* 115 (R mutant, avirulent), *B. ovis* PA (virulent), *B. ovis* Reo 198 (CO_2 independent), *B. abortus* 2308 (S, virulent), and *B. abortus* RB51 (R) are strains which have been described previously (4, 7, 45). For antigen extraction, *O. anthropi* and *B. melitensis* were grown in a medium consisting of 1.7% Casitone (E. Merck, Darmstadt, Germany), 0.3% Soytone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Merck), 0.25% K_2HPO_4, 2% glucose, 0.5% NaCl, and 0.01% antifoam A–butyl acetate (1:3) (Sigma Chemical Co., St. Louis, Mo.) in a 15-liter Biostat fermentor (B. Braun Melsungen AG, Leinfelden, Germany) for 36 h at 36°C and 35% O_2 saturation. *B. ovis* Reo 198 was grown in tryptic soy broth (Difco Laboratories) supplemented with 2.5% yeast extract in 2-liter flasks on an orbital shaker. Cells were harvested by tangential flow filtration (Omega 100K filter; Filtron Technology Corp., Northborough, Mass.) or by centrifugation (7,000 × *g*, 15 min) and washed twice with 0.8% NaCl (*B. melitensis* 16M was inactivated with phenol [0.5%, 36°C, 48 h] before harvesting). For animal immunization or experimental infection, the appropriate strains were grown on tryptic soy agar slopes with (*B. ovis*) or without 2.5% yeast extract.

Cytosol and cell envelope fractions. *B. melitensis* 115, *B. ovis* Reo 198, and *O. anthropi* LMG 3301 cells were disintegrated in the presence of nucleases with a 40K French pressure cell (SLM Instruments Inc., Urbana, Ill.) operating at an internal pressure of 35,000 lb/in^2 (7). The cell envelope fraction was sedimented (80,000 × *g*, 2 h, 4°C), and the supernatant was held at 4°C for 24 h before being ultracentrifuged again under the same conditions. The new supernatant (cytosol) was dialyzed against 10 mM phosphate buffer (pH 7.2) (two changes of buffer in 48 h) at 4°C and freeze-dried. Cytosols were free of cell envelope components as judged by the absence of inner membrane enzymatic markers (36), 3-deoxy-D-manno-2-octulosonic acid (Kdo) measured colorimetrically (16), or LPS detectable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by periodate-silver staining (39). A fraction rich in OM proteins was obtained from cell envelopes by extraction with Sarkosyl (Sigma) and Zwittergent 316 (Calbiochem-Behring, San Diego, Calif.) (36). The Zwittergent 316 extract was precipitated with acetone and solubilized in 0.7 M 2-mercaptoethanol–10% glycerol–10 mM Tris-HCl (pH 6.8) for SDS-PAGE and Western blot analyses.

LPSs and *O. anthropi* O-specific polysaccharide. *B. melitensis* 16M crude S-LPS was obtained by methanol precipitation of the phenol phase of a water-phenol extract (31), and *O. anthropi* S-LPS was obtained from strain LMG 3331 by the standard water-phenol method (48). The crude fractions were digested with nucleases and proteinase K, sedimented by ultracentrifugation under the conditions described previously (16), and freeze-dried. The O-specific polysaccharide of *O. anthropi* was obtained by mild acid hydrolysis of the S-LPS (48). *O. anthropi* LMG 3301 and *B. ovis* Reo 198 R-LPSs were obtained by the petroleum ether-chloroform-phenol method (22). After flash evaporation of the petroleum ether and chloroform, the phenol phase was saturated with water and the precipitate was sedimented (7,000 × *g*, 20 min, room temperature) and processed as in the standard protocol to yield the R-LPS. In addition, for *O. anthropi* LMG 3301, a lipid A-rich fraction (49) was obtained by precipitating the water-saturated phenol with 10 volumes of acetone. This precipitate was sedimented (7,000 × *g*, 20 min, room temperature), washed with acetone, dialyzed, and freeze-dried. Kdo contents for the above-described preparations were as follows: *B. melitensis* S-LPS, 0.9%; *O. anthropi* S-LPS, 0.52%; *O. anthropi* R-LPS, 1.38%; and *O. anthropi* lipid A-rich fraction, 0.20%. The protein content of these preparations (determined by the modified Lowry method [16]) was less than 0.2%, except for the lipid A-rich fraction, which contained 4.5% protein.

Immunological tests. (i) Gel immunoprecipitations. Counterimmunoelectrophoresis (CIEP) was carried out in 1% agarose (Indubiose A37HAA; IBF-Biotechnics, Villeneuve la Garenne, France) gels in 0.04 M sodium barbital-HCl buffer (pH 8.6) (14, 17). Two rows of 3-mm-diameter wells were punched 1 cm apart, anodic wells were filled with the sera, and 20 μl of cytosol (2 mg [dry weight] per ml) was dispensed into the cathode wells. After electrophoresis (1 h at 2 V/cm), the plates were incubated for 1 h in 5% sodium citrate and washed with distilled water for 24 h. Immunoelectrophoresis was carried out in 1.8% agarose–0.05% NaN_3–0.04 M sodium diethylbarbiturate-HCl (pH 8.6) at 6 V/cm for 2 h (12, 13). After diffusion of the sera and immunoprecipitation (18 h at 25°C), the plates were soaked for 24 h in saline, washed with distilled water for 24 h, air dried, and stained with 1% Coomassie R-blue in water-ethanol-acetic acid (9:2:9). The reverse single radial diffusion test was carried out by including the proteins (1,500 to 200 μg per ml) in agarose-glycine buffer gels (28). Double gel immunodiffusion was carried out in the above-described agarose-barbital gels.

(ii) Western blotting. After SDS-PAGE, proteins were electroblotted onto Immobilon P membranes (Millipore Corp., Bedford, Mass.) by the semidry method by using a Trans-Blot SD apparatus (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturers. For LPS gels, electroblotting was performed on nitrocellulose sheets (type HA, 0.45-μm pore size; Millipore) in a Trans-Blot cuvette (Bio-Rad Laboratories) containing 20% methanol, 192 mM glycine, and 20 mM Tris-HCl (pH 8.3) at 200 mA for 5 h. After incubation with the appropriate sera, blots were developed with peroxidase-conjugated protein G (Pierce Chemical Co., Rockford, Ill.) and 4-chloro-1-naphthol-H_2O_2 (39).

(iii) Indirect ELISA. Standard 96-well polystyrene plates (Inotech-ELISA; Bioreba, Basel, Switzerland) were coated with 100 μl of the appropriate antigen (S-LPS, R-LPS, or lipid A-rich fraction) per well at 2.5 μg (dry weight) per ml in 10 mM phosphate buffer (pH 7.2). After overnight incubation at 4°C, nonadsorbed material was removed with four washings of the same buffer solution containing 0.05% Tween 20, and the plates were stored dry at 4°C until used. The remaining enzyme-linked immunosorbent assay (ELISA) steps were performed as described previously (17) with peroxidase-protein G (Pierce) as the immunoglobulin G (IgG)-specific conjugate and 0.1% 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (diammonium salt; ABTS [Sigma]) in 0.05 M citrate buffer (pH 4) with 0.004% H_2O_2 as the developing substrate.

(iv) DTH tests. Two experiments were conducted. In the first experiment, 6-week-old BALB/c mice were used. Groups of five mice were inoculated intraperitoneally with either 5×10^4 CFU of *B. melitensis* 16M or 5×10^4 CFU of *B. abortus* 2308 or with saline (0.1 ml) as a control. Five weeks later, all mice were injected intradermally in the rear footpads with 20 μl of a 0.8-mg/ml solution of cytosols from *B. melitensis* 115 (right footpad) or *O. anthropi* (left footpad). All mice were killed 48 h later, and the whole footpads were fixed in Bouin's solution, embedded in paraffin, and processed for microscopic examination of lesions.

In the second experiment, a total of 14 *Brucella*-free rams were inoculated by the conjunctival and preputial routes with 1.2×10^9 CFU of *B. ovis* PA. Two months later, all rams were tested by intradermal inoculation of *O. anthropi* cytosol (150 μg [dry weight] in 0.1 ml of 0.15 M NaCl) in the skin of the tail and, simultaneously, by subcutaneous inoculation of brucellin (brucellin OCB, batch 26 N 151; Rhone-Merieux, Lyon, France) in the right lower eyelid at the dose recommended by the manufacturer. After 72 h, the intensity of the reactions was evaluated by microscopic examination of samples taken at necropsy from inoculation sites (7). All of the rams were found to be infected with *B. ovis* by bacteriological culture of necropsy specimens.

Immune and control sera. Sera were obtained as follows.

(i) Humans. Sera were obtained from 24 brucellosis patients who were blood culture positive for *B. melitensis* and who had serum agglutination titers of ≥320 and from 10 healthy donors.

(ii) Cattle. Sera were drawn from 20 cattle which were positive in both the rose bengal and complement fixation tests (4) for brucellosis (titers > 256) from herds in which *B. abortus* had been isolated and also from 66 *Brucella*-free cattle.

(iii) Sheep. Sera were taken from 36 ewes bacteriologically positive for *B. melitensis* and also positive in both the rose bengal and complement fixation tests (titers ≥ 32) and from 66 *Brucella*-free sheep. In addition, sera from 14 rams which had been inoculated with 1.2×10^9 CFU of *B. ovis* PA and which were found to be infected at necropsy and from 15 *Brucella*-free rams were used.

(iv) Goats. Sera were drawn from 36 goats which were milk culture positive for *B. melitensis* and positive in the rose bengal and complement fixation tests (titers ≥ 32) and from 66 *Brucella*-free goats.

(v) Rabbits. New Zealand White female rabbits were inoculated with 10^9 CFU of *B. melitensis* 16M by the intravenous route (12) and bled 15 days and 1, 3, and 6 months after inoculation. A second group of animals was immunized by intraperitoneal injection of live cells of *O. anthropi* LMG 3301 (10^9 CFU, nine injections at 2-day intervals). In addition, groups of rabbits were hyperimmunized with acetone-killed cells of either *B. melitensis* 115 or *O. anthropi* LMG 3331 by the intramuscular route by following a protocol described previously (13). Sera taken before immunization were used as negative controls.

Serum absorption. Absorbed sera for ELISA and immunoelectrophoresis were obtained by mixing sera (100 μl) with 2.5 mg of freeze-dried cells or cytosol, respectively, and incubating them for 2 h at 37°C followed by 24 h at 4°C. Immunoprecipitates were removed by centrifugation (12,000 × *g*, 15 min), and supernatants were collected.

RESULTS

Immunological cross-reactivity between *Brucella* and *O. anthropi* cytoplasmic proteins. Figure 1 shows the results of Western blot and double gel immunodiffusion analyses carried out with *B. melitensis* and *O. anthropi* cytosols. In Western blots, up to 10 *Brucella* and *O. anthropi* cytosolic components were detected with the heterologous hyperimmune sera (Fig. 1, lanes Oa and Bm). As expected, the serum from a healthy human donor gave negative results with both cytosols (Fig. 1, lanes 1). In contrast, the serum of a human patient infected with *B. melitensis* contained antibodies to both the homologous and heterologous cytosols (Fig. 1, lanes 2). A *Brucella*-specific band with an apparent molecular weight of about 16,000 (16K) was detected in the cytosol of *B. melitensis* (Fig. 1, lanes 2), but antibodies to this component were not always present in eight other human sera tested (not shown). In addition, fainter

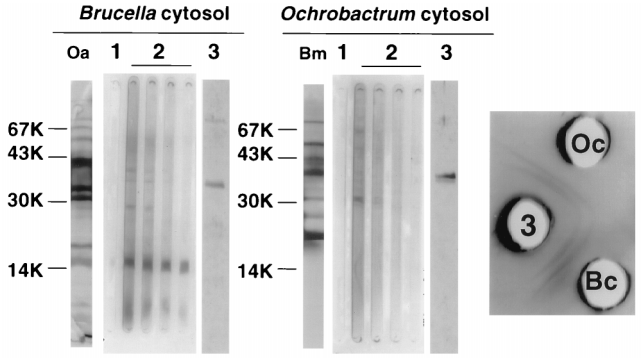

FIG. 1. Western blots (left and center) and double gel immunodiffusion (right) with cytosols from *B. melitensis* (Bc) or *O. anthropi* (Oc). The sera used were from rabbits hyperimmunized with *B. melitensis* 115 (Bm), rabbits hyperimmunized with R *O. anthropi* (Oa), a healthy human donor (1), and a brucellosis patient (2) and pooled sera from six infected goats (3). Serum dilutions in Western blots were 1:25 (lanes Bm, Oa, 1, and 3) or 1:25 to 1:200 (lanes 2).

bands common to *O. anthropi* and *Brucella* cytosols were detected with the same patient serum, and titers were similar for the common components (Fig. 1, lanes 2). A pool of six *B. melitensis*-positive goat sera detected only a major band of about 35K in both cytosols, although the apparent molecular weight of the *B. melitensis* band was slightly lower than that of the *O. anthropi* band (Fig. 1, lanes 3). The cross-reactivity between *Brucella* and *O. anthropi* cytosols was also observed in gel precipitation (Fig. 1, right panel, and Fig. 2), and more common components were clearly detected by this method than by immunoblotting. Moreover, reactions of total identity were observed in double gel immunodiffusion (Fig. 1, right panel), and as illustrated in Fig. 2, absorption of pools of sera from *Brucella*-infected hosts with the *O. anthropi* cytosol removed most, but not all, of the antibodies reacting with the homologous antigen.

These findings were extended by CIEP, a simple and sensitive test which allows the screening of large numbers of sera. Table 1 summarizes the CIEP results. As can be seen, the homologous and heterologous antigens had the same sensitivity in the detection of *Brucella*-infection of humans, goats, and cattle, and only 1 of the 35 sheep sera that developed precipitin lines with the *B. melitensis* cytosol failed to react with the *O. anthropi* cytosol. Sera from 208 *Brucella*-free hosts tested did not give positive results in CIEP with either cytosol. Since digestion of cytosols with proteinase K abrogated the reactivities, they were unequivocally assigned to protein antigens. The CIEP analyses also showed that the number of precipitin lines produced by a given serum was most often the same with both the homologous and heterologous cytosols, varying from one to four. These results were confirmed by reverse single radial diffusion.

TABLE 1. Results of CIEP with sera from hosts naturally infected with S *Brucella* spp.

Host	Status	Total no. of sera	No. of sera precipitating cytosol of:	
			B. melitensis	*O. anthropi*
Humans	Healthy	10	0	0
	B. melitensis infected	24	22	22
Goats	*Brucella* free	66	0	0
	B. melitensis infected	36	34	34
Sheep	*Brucella* free	66	0	0
	B. melitensis infected	36	35	34
Cows	*Brucella* free	66	0	0
	B. abortus infected	20	20	20

Photomicrographs of the skin reactions at 72 h evoked by brucellin and the *O. anthropi* cytosolic fraction in the same *B. ovis*-infected ram are shown in Fig. 3. As can be seen, both showed the macrophage and lymphocyte infiltrations around dermis vessels characteristic of DTH reactions. Consistent with the presence of cross-reacting antibodies to *O. anthropi* and

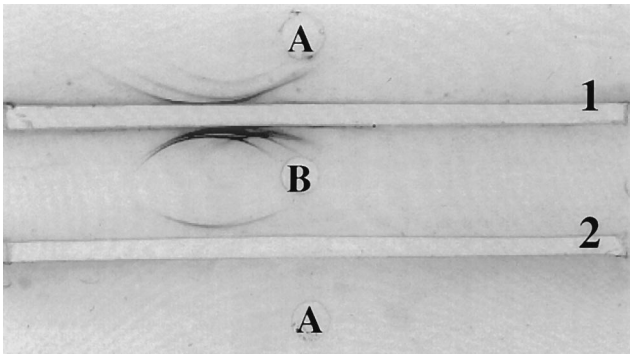

FIG. 2. Immunoelectrophoretic analysis of *O. anthropi* and *B. melitensis* cytosols (wells A and B, respectively). Trough 1 contained a pool of sera from *B. melitensis*-infected goats, and trough 2 contained the same pool absorbed with *O. anthropi* cytosol. The cathode is on the right.

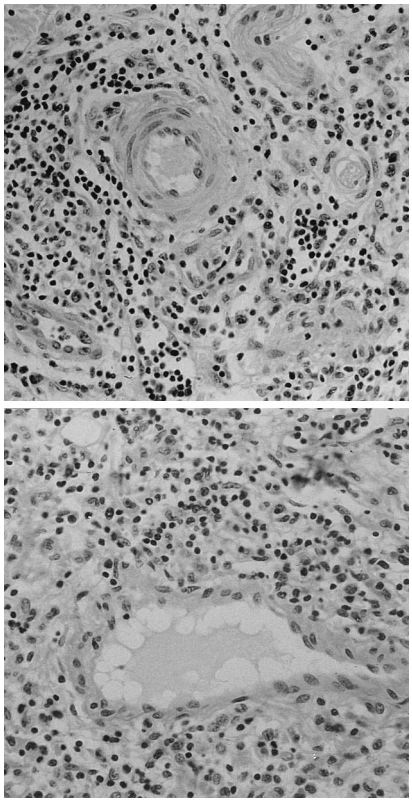

FIG. 3. Hematoxylin and eosin stain showing DTH reactions evoked by brucellin (top) and by the cytosol of *O. anthropi* (bottom) after intradermal inoculation into the same *B. ovis*-infected ram. Magnification, ×17.5.

TABLE 2. DTH responses to brucellin and to *O. anthropi* cytosol in mice infected with S *Brucella* and in rams infected with *B. ovis*

Group	No. of animals	No. of animals showing DTH to:	
		Brucellin	*O. anthropi* cytosol
Mice inoculated with *B. abortus* 2308	5	3	3
Mice inoculated with *B. melitensis* 16M	5	5	3
Brucella-free mice	5	0	0
Rams infected with *B. ovis*	14	14	14
Brucella-free rams	15	0	0

Brucella proteins, leukocytes indicative of a mixed-type reaction were also detected in some cases (not shown). When DTH test results for *B. melitensis*- or *B. abortus*-infected mice and *B. ovis*-infected rams were compared, a close parallelism in the number of animals showing DTH to brucellin and to *O. anthropi* cytosol was found (Table 2).

Immunological cross-reactivity between *Brucella* and *O. anthropi* LPSs. Sera from *B. melitensis*-infected rabbits or naturally infected hosts failed to react significantly with the S-LPS and the O-specific polysaccharide of *O. anthropi* in ELISA, although sera from rabbits immunized with the homologous strain showed a strong reactivity (not shown). However, some reactivity was observed in Western blots with the *O. anthropi* S-LPS and the sera of animals infected with S *Brucella* spp. (not shown), suggesting cross-reactions at the core and/or lipid A level.

SDS-PAGE analysis showed predominantly R-LPS in the petroleum ether-chloroform-phenol extract of *O. anthropi* LMG 3301 (Fig. 4A, lane 1), and consistent with the nature of the extract, no material was detected in the lipid A-rich fraction by silver-periodate staining (Fig. 4A, lane 2). As determined by Western blotting, sera from rabbits immunized with live R *O. anthropi* contained IgG to the homologous R-LPS (Fig. 4B, lane 1) but not to the lipid A-rich fraction (Fig. 4B, lane 2). However, the sera from S *B. melitensis*-infected rabbits which were bled 45 days or 3 months after infection (but not 15 days or 6 months after infection) had IgG to both the R-LPS and the lipid A-rich fraction (Fig. 4C, lanes 1 and 2). Consistent with these results, ELISAs showed similar levels of IgG (optical density readings from 1.6 to 1.2 for the 1:50 serum dilution) reacting with *O. anthropi* R-LPS or with the lipid A-rich fraction in the sera of rabbits infected with *B. melitensis* or immunized with live *O. anthropi*. Low and variable levels of IgG (optical density readings from 0.6 to 0.2 for the 1:50 serum dilution) to these extracts were detected in the sera from *B. abortus*-infected cattle and *B. melitensis*-infected sheep, goats, and humans (not shown). All the above-mentioned reactivities were abrogated by absorbing the sera with whole R cells (*B. abortus* RB51 or *O. anthropi* LMG 3301) (not shown).

Since the homologous R-LPS is an immunodominant antigen in *B. ovis* infections (21, 39), the reactivities of the *O. anthropi* and *B. ovis* R-LPSs were compared by using sera from rams experimentally infected with *B. ovis* (Fig. 5A and B). These sera showed similar reactivities with the homologous and heterologous R-LPS, and reactivity with the lipid A-rich fraction of *O. anthropi* was also detected (Fig. 5C).

Immunological cross-reactivity between *Brucella* and *O. anthropi* OM proteins. Figure 6 shows representative results of Western blot analyses performed with extracts rich in OM proteins and pools of sera from six *B. ovis*-infected rams (Fig. 6A) and from six *B. melitensis*-infected goats (Fig. 6B). Both pools contained IgG to *B. melitensis* (Fig. 6, lanes 1) and *B. ovis* (lanes 4) OM proteins with apparent molecular weights close to 38K and 28K, but no reaction was observed with an *Escherichia coli* OM protein extract (lanes 3) obtained as described elsewhere (36). The same sera recognized a protein of about 28K present in the *O. anthropi* OM extract (lanes 2). A pool of sera from *B. abortus*-infected cattle showed a much weaker reactivity at the same dilution (not shown). The bands corresponding to the R-LPS were clearly detected with the ram sera and the *Brucella* OM extracts (Fig. 6A, lanes 1 and 4), but consistent with the ELISA results, the sera from *B. melitensis*-infected goats did not reveal the R-LPS in the *O. anthropi* extract (Fig. 6B, lane 2).

DISCUSSION

The results presented show that an immune response to *O. anthropi* antigens is consistently elicited by experimental and natural infections with *B. ovis*, *B. melitensis*, and *B. abortus* in a variety of hosts. As illustrated by some of the Western blotting results, serological cross-reactions among taxonomically related bacteria can be shown with sera from hyperimmunized animals (8). However, in this study the cross-reactivity was observed not only with hyperimmune sera but also with sera from hosts with natural or experimental brucellosis, and cross-reactivity was also detected in the DTH skin test. In this regard, *Brucella* proteins elicit cell- and antibody-mediated immunoresponses which have been investigated for diagnostic purposes (4, 7, 10, 14, 17, 20, 28, 30, 32, 37, 39, 42–44, 46, 50, 51). Therefore, it is noteworthy that the diagnostic sensitivity obtained with the homologous and heterologous cytosols was the same and that cross-absorption removed most of the antibodies to the homologous cytosol. It is also interesting that, compared with gel immunoprecipitation, immunoblotting with cytosols detected fewer antigens shared by *O. anthropi* and *Brucella*, suggesting the importance of conformational epitopes in the cross-reactions.

The above-discussed observations show that the *O. anthropi* antigens detected by the sera from *Brucella*-infected hosts are of diagnostic significance in brucellosis. Since cell-mediated immunoresponses to proteins are important in brucellosis (7, 20), this conclusion is further supported by the fact that *O. anthropi* cytosolic proteins evoked a DTH response of a frequency and intensity similar to that evoked by *Brucella* proteins. Infections by *Y. enterocolitica* O:9 and a few other gram-negative bacteria cause false-positive reactions in tests detect-

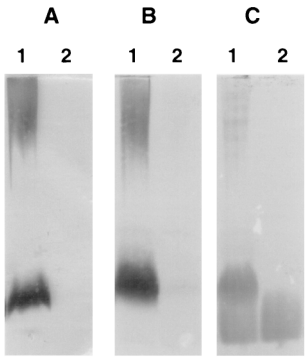

FIG. 4. SDS-PAGE for LPS (A) and Western blot (B and C) analyses of *O. anthropi* R-LPS (lanes 1) and lipid A-rich fraction (lanes 2). The sera used (1:50 dilution) were from rabbits immunized with live R *O. anthropi* (B) or infected with S *B. melitensis* (C).

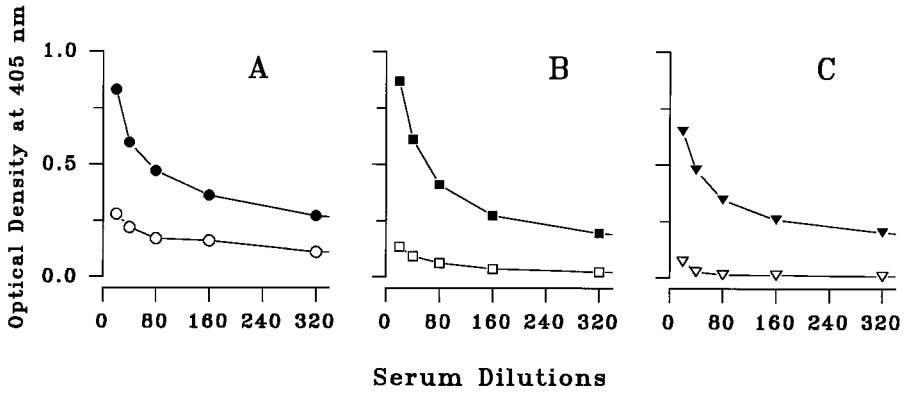

FIG. 5. Analysis by ELISA of the presence of IgG to *B. ovis* R-LPS (A), to *O. anthropi* R-LPS (B), and to an *O. anthropi* lipid A-rich fraction (C). Filled symbols represent results with the sera of 21 *B. ovis*-infected rams, and open symbols represent results with the sera of 21 *Brucella*-free rams.

ing antibodies to *Brucella* S-LPS (4, 5). On the other hand, no cross-reactivity with proteins of other bacteria has been reported (11), and this is one reason for developing tests with *Brucella* proteins (5, 15, 18). Although some of the more promising *Brucella* proteins are cytosolic (26, 44), and the cross-reactivity described above could suggest that *Brucella* antiprotein tests are less specific than previously thought, the fact is that we did not detect antibodies to *O. anthropi* proteins in the 208 sera from *Brucella*-free hosts tested or DTH responses in the *Brucella*-free rams. Because of the bacterium's multiple and variable antibiotic resistance patterns (6, 29) and reported isolation from a soil invertebrate (1), it seems likely that the primary habitat of *O. anthropi* is the soil. To the best of our knowledge, isolation of *O. anthropi* from livestock has not been attempted, but this bacterium has been repeatedly described as an opportunistic pathogen of humans (9, 27), and some of these patients develop antibodies cross-reacting with *Brucella* antigens (33). Thus, although our results with the *Brucella*-free animals show that it is unlikely, the possibility that *O. anthropi* represents a source of nonspecificity in antiprotein tests for brucellosis cannot presently be excluded (see below).

Research carried out with sera from humans and animals infected with S *Brucella* spp. has shown that the S-LPS, and to a much lesser extent several OM proteins, is the major antigen of the *Brucella* cell surface (10, 12, 15, 17, 18, 23, 39, 50). No serological reactivity between sera from *Brucella*-infected animals and the S-LPS of *O. anthropi* was observed. This is in agreement with the characterization of the corresponding O chains as polymers of *N*-formylperosamine (38) and of a glucosamine-rhamnose disaccharide (48). However, a significant cross-reactivity between some sera from hosts infected with *Brucella* spp. and both the *O. anthropi* R-LPS and the lipid A-rich fraction was observed. In particular, comparison of *O. anthropi* and *B. ovis* R-LPSs in ELISAs with sera from rams experimentally infected with *B. ovis* showed that both antigens would have the same diagnostic value in this assay. The LPS core of *O. anthropi* contains galacturonic acid (49), which is not found in *Brucella*, but also Kdo, glucose, and glucosamine (49), which are present in *Brucella* LPS (35). Although the cross-reactivity observed can be accounted for by core epitopes, the reactivity observed with the lipid A-rich fraction shows that lipid A antigenic determinants (40) also contribute significantly. Antibodies to the homologous core and lipid A determinants in the sera of S *Brucella*-infected animals have been reported previously (3, 40).

It has been a constant observation that *B. ovis*-infected sheep show high background reactivities with the homologous R-LPS when tested in ELISA with conjugates of heavy- and light-chain specificity, and this reactivity can be removed in part by using IgG-specific conjugates (21). Moreover, previous comparative studies have shown that although the antibody response to group 3 OM proteins is more intense in *B. ovis*- than in *B. melitensis*-infected sheep (39), sera from *Brucella*-free sheep often contain low titers of antibodies reacting with group 3 OM proteins (23, 39). We have hypothesized that such antibodies could be elicited by some unknown ubiquitous bacteria (23), and the data presented here point to *O. anthropi*, and perhaps to some unknown taxonomically related bacteria, as the logical candidates.

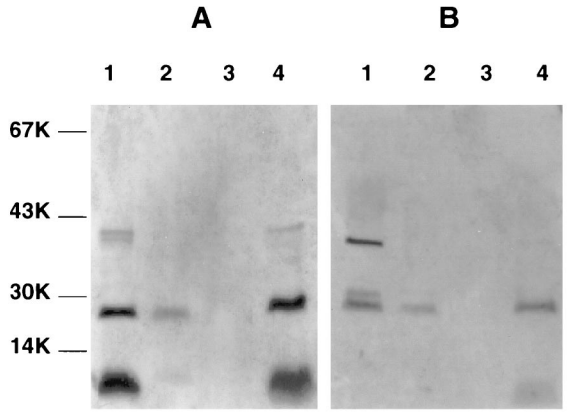

FIG. 6. Western blots with OM-rich extracts from R *B. melitensis* (lanes 1), R *O. anthropi* (lanes 2), *E. coli* O111 (lanes 3), and *B. ovis* (lanes 4). The sera used (1:50 dilution) were pools from six infected rams (A) or from six infected goats (B).

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