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Effectiveness of Natural and Synthetic Complexes of Porin and O Polysaccharide As Vaccines against *Brucella abortus* in Mice

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A single vaccination of mice with a complex of porin and smooth lipopolysaccharide (porin-S-LPS) extracted from virulent *Brucella abortus* 2308 provided significant protection ($P < 0.01$ to $P < 0.001$) against challenge with the same strain, equivalent to that achieved by vaccination with living attenuated *B. abortus* 19. The porin-S-LPS vaccine given without adjuvant or in several adjuvants (trehalose dimycolate and muramyl dipeptide; the pluronic polymer L-121 and muramyl dipeptide; or complexed with Quil A in immunostimulating complexes) provided equivalent protection. In contrast, one vaccination with porin complexed with rough LPS (porin-R-LPS) from a rough mutant of strain 2308 provided no protection with any adjuvant tested. In one experiment, two inoculations with the porin-R-LPS resulted in a low level of protection, probably owing to priming of the animals for production of O-polysaccharide-specific antibodies. However, one vaccination with rough-strain porin covalently bound to purified O polysaccharide conferred protection equal to that obtained with natural complexes of porin-S-LPS or with living strain 19. A synthetic vaccine containing long chains of O polysaccharide was more effective than one prepared with short chains. Protective vaccines caused the formation of increased concentrations of circulating O-polysaccharide-specific antibodies, although there were individual exceptions to the quantitative association between O-polysaccharide-specific antibodies and protection. Antibodies specific for porin or R-LPS were found in negligible quantities in vaccinated mice. These results provide additional evidence that the O polysaccharide will constitute an essential component of an effective subcellular vaccine against *B. abortus* and that O-polysaccharide-specific antibodies play an important role in protective immunity in brucellosis.

Disease caused by *Brucella abortus* affects humans and a number of animal species, particularly cattle (43). Attenuated *B. abortus* 19 has been used for many years to vaccinate cattle against brucellosis. Among the major drawbacks of this vaccine are its ability to cause disease in cattle (14) and humans (56) and to induce O-polysaccharide (OPS)-specific antibodies (O antibodies) which interfere with the interpretation of serodiagnostic tests (43).

A principal goal of research in this laboratory (31, 53–55) and others (5, 6, 13, 15, 47, 48) is the development of a subcellular vaccine for bovine brucellosis. Such a vaccine would not be commercially acceptable unless it provided an advantage over strain 19 such that the antibody response which it induced could be clearly distinguished from that evoked by virulent field strains. This could be accomplished easily if it was possible to design an effective vaccine which excluded the OPS. However, recent studies with the murine model system have provided evidence by active (31) and passive (26, 31, 32) immunizations that O antibodies play an important role in protection.

It is not yet certain, however, that the OPS of *B. abortus* represents an indispensable element of an effective vaccine. This question must be resolved to provide direction for future efforts on development of subcellular vaccines. The experiments reported here were done in pursuit of that objective. We performed active immunizations with simple and defined vaccines with or without the OPS. The design of the experiments was based on a hypothesis proposed by one of us (52) that the simplest vaccine which could provide protection equivalent to strain 19 would have to include the OPS and a protein such as porin to which smooth lipopolysaccharide (S-LPS) was bound in the living bacterium. The protein would at once serve as a carrier for the induction of O antibodies and, in the presence of appropriate adjuvants, induce cell-mediated immune responses required for protection (52).

(The data in this report are taken in part from the Ph.D. thesis of M. J. Eis, Cornell University, Ithaca, 1988.)

MATERIALS AND METHODS

Mice. Five-week-old female BALB/cByJ mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and held for 1 week before use.

Bacterial strains for vaccination and challenge. *B. abortus* smooth strain 2308 of known virulence was used for challenge infections. In some experiments, vaccination or challenge was performed with vaccine strain 19 (Biologics Division, U.S. Department of Agriculture). Stock cultures were prepared and stored as described previously (31), and inocula were prepared from a freshly thawed vial which was diluted in a predetermined manner in sterile phosphate-buffered saline (PBS) to yield an infecting dose of 5×10^4 bacteria per 0.1 ml.

Rough stains of 2308 were provided by G. G. Schurig (Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Va.) and B. L. Deyoe (National Animal Disease Center, Ames, Iowa). These are referred to as strains RB51 and NADC, respectively.

Antigens for vaccines and immunoassays. (i) Porins. Native porin proteins were extracted and purified from smooth

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strain 2308 and rough strain NADC by a modification of previously described methods (51). Porins were freed of detergents, lyophilized, and stored in a desiccator at room temperature (55). Porin from rough strain RB51, which was used for production of synthetic vaccines, was produced by a modification of the methods of Verstreate et al. (51) in which bacterial cells were disrupted by vortexing with glass beads for 5 min at 0°C (Bead-Beater; Biospec Products, Bartlesville, Okla.) and proteins separated by ion-exchange chromatography were subjected to chromatofocusing (PBE 94; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) prior to gel filtration. Denaturation of the porin was required for successful coupling to the OPS. This was accomplished by heating the protein at 100°C for 15 min in 10 mM Tris buffer (pH 8.0) containing 0.1 M NaCl, 0.1% Zwittergent 3-14 (Calbiochem-Behring, La Jolla, Calif.), 0.02% NaN_3, and 2% sodium dodecyl sulfate. Sodium dodecyl sulfate and NaN_3 were removed by gel filtration on Sephacryl S-300 (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 7.5) containing 0.1 M NaCl and 0.015% Zwittergent 3-14. Porin-containing fractions were pooled and concentrated by ultrafiltration during which the buffer was replaced with carbonate buffer (0.1 M, pH 9.2) used in the coupling reaction.

(ii) S-LPS. S-LPS (fraction f6) was produced from smooth strain 2308 by extraction in hot phenol followed by treatment with guanidinium thiocyanate (38).

(iii) OPS. LPS was extracted from cells of *B. abortus* smooth strain 2308 by the method of Moreno et al. (35). A solution of purified LPS in 2% acetic acid was refluxed at 100°C (8 h), cooled to 4°C, and diluted with an equal volume of 70% trichloroacetic acid to precipitate proteins. After centrifugation (17,000 × *g*, 15 min), the supernatant was combined with methanol (5 volumes) at 4°C, stirred (16 h), and centrifuged (17,000 × *g*, 20 min). The resulting pellet was dissolved in distilled deionized water (DDW) (100 ml), dialyzed against DDW for 2 days, and lyophilized. The product was suspended in 20 mM Tris chloride buffer (pH 8.0, 100 ml) and centrifuged (17,000 × *g*, 10 min), and the supernatant was applied to a column of DEAE-Sephadex A-50 equilibrated with the same buffer. Eluted fractions (absorbing at 210 nm) were combined, and OPS was precipitated by the addition of 5 volumes of methanol at 4°C. After centrifugation (17,000 × *g*, 15 min), the pellet was dissolved in DDW, dialyzed against DDW, and lyophilized, affording pure OPS whose ^{13}C and ^{1}H nuclear magnetic resonance and infrared spectra were in agreement with published values (10).

(iv) Preparation of long and short OPS oligomers. Anhydrous HF (15 ml) was added to a 2-oz (59-ml) polyethylene bottle with a Teflon stir bar under argon charged with 775 mg of purified OPS. The bottle was tightly capped, and the colorless solution was stirred at −20°C for 2.5 h. The HF was removed under anhydrous conditions with a stream of argon and then under vacuum (0.1 torr [13.33 Pa], minimum 1 h) until the reddish solid became colorless. This residue was dissolved in DDW (10 ml) and submitted to gel filtration on a column (2.6 by 95 cm) of Bio-Gel P-4 (200/400 mesh) at a flow rate of 10 ml/h and with UV detection at 210 nm. Fractions were collected at ca. 190 to 200 ml (void volume), ca. 200 to 300 ml, and ca. 300 to 400 ml to afford, after lyophilization, unreacted OPS (42 mg, 6%), long α-1-fluoro-OPS oligomers (306 mg, 41%), and short α-1-fluoro-OPS oligomers (214 mg, 28%), respectively. Short-chain oligomers were calculated to contain between 5 and 20 sugar residues, while the size of long-chain oligomers varied from about 20 sugar residues to native length.

Long (382 mg) and short (219 mg) α-1-fluoro-OPS oligomers were suspended in 2.6 and 1.4 ml, respectively, of freshly distilled 2-azidoethanol. (It must be emphasized that 2-azidoethanol is explosive. Preparations of this material should never be heated above 60°C, and all distillations must be performed behind a safety shield.) Additional 2-azidoethanol (2.6 and 1.4 ml, respectively) saturated with anhydrous HCl was added to each vessel. After being capped tightly, the solutions were stirred at 25°C for 12 h. Hydrogen chloride was removed in vacuo at 20°C and residual 2-azidoethanol was removed under vacuum (0.1 torr) at 25°C over ca. 24 h. Each crude product was dissolved in ca. 5 ml of DDW and applied to a Bio-Gel P-4 column (2.6 × 95 cm) (200/400 mesh) with a flow rate of ca. 8 ml/h. Lyophilization afforded long azidoethyl-OPS (300 mg, 79%) and short azidoethyl-OPS (200 mg, 91%).

Catalytic hydrogenation of the long (317 mg) and short (324 mg) azidoethyl-OPS was done in separate 100-ml round-bottom flasks by adding 10% Pd on C (150 mg) to solutions of each sample in ethanol-water (75 ml:15 ml). The mixtures were stirred while H_2 was bubbled through E-fritted tubes for 36 to 48 h. Ethanol was removed in vacuo and was replaced by 100 ml DDW. Catalyst was removed by filtration through a 4-mm pad of Celite, and the solutions were lyophilized to afford both long 2-aminoethyl-OPS (286 mg, 90%) and short 2-aminoethyl-OPS (260 mg, 80%).

A 100-mg sample of either long or short 2-aminoethyl-OPS in DDW (2.5 ml) was added to a solution of di-2-hydroxypyridyl thionocarbonate (23) (100 mg, 0.43 mmol) in DMF (5 ml) at 15°C. The solution was stirred at 25°C (1 h) and then diluted with 100 ml of DDW, washed with CH_2Cl_2 (20 ml, five times) and lyophilized. The residue was dissolved in DDW (5 ml) and centrifuged and the supernatant was passed through a column (2.6 by 100 cm) of Bio-Gel P-4 (200/400 mesh) at 8 ml/h, with UV detection at 248 nm. Lyophilization afforded long α-1-O(2-isothiocyanatoethyl)-OPS (NCS-OPS) (86 mg, 86%) or short NCS-OPS (75 mg, 75%).

Satisfactory infrared spectra were obtained from the compounds at each stage of the reaction.

(v) Long and short porin-OPS glycoprotein conjugates. Long NCS-OPS (50 mg) in 0.230 ml of 0.1 M carbonate buffer (pH 9.2) was added to a 2-ml polyethylene centrifuge tube containing 5 mg of denatured porin in 0.125 ml of the above buffer. The tube was capped, and the solution was gently vortexed for 15 s. After standing at room temperature for 36 h, the solution was applied directly to the top of a gel filtration column (2.6 by 96 cm) of Sephacryl S-300 equilibrated with 50 mM phosphate buffer (pH 7.5)–0.1 M NaCl–0.015% Zwittergent 3-14. The flow rate was adjusted to 18 ml/h with collected fractions of 6 ml and UV detection at 280 nm. Pooled fractions (120 ml) containing glycoprotein were concentrated by ultrafiltration on a YM-10 membrane, followed by buffer exchange with 0.005% Zwittergent in DDW to produce a final volume of 4.75 ml (0.66 mg of protein per ml by Lowry assay) which was made 0.02% in NaN_3 and stored at 4°C. Short-chain porin-OPS conjugate (4 ml [0.41 mg/ml]) was produced by identical methods from a mixture of 35 mg of short NCS-OPS and 5 mg of denatured porin. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses of long- and short-chain conjugates demonstrated little or no native porin. Whereas porin produced two closely spaced, well-resolved bands, the conjugates produced an unresolved smear of higher-molecular-weight materials which terminated just above the principal porin band (M. J. Eis, Ph.D. thesis).

(vi) Conversion of α-1-O-(2-aminoethyl)-OPS oligomers to

α-1-O-(*N*-stearoyl-2-aminoethyl)-OPS glycolipid. Stearoyl chloride (86 mg, 0.28 mmol) in DMF (3 ml) was added dropwise to a solution of long 2-aminoethyl-OPS (125 mg) in DMF (6 ml) and pyridine (4.5 ml). The reaction mixture was stirred at 25°C for 6 h and then diluted with methanol (5 ml) and stirred for an additional 30 min. The bulk of solvent was removed in vacuo at 45°C. The residue was dissolved in DDW (200 ml), and the solution was adjusted to pH 8.0 with 1 M NaOH and lyophilized. The resulting waxy white solid was collected on an E-fritted glass filter and washed with CH_2Cl_2 (100 ml twice). Rinsing of the frit with DDW (100 ml once) and lyophilization of the resulting soapy solution afforded 119 mg of crude N,O-stearoylated glycolipid. To hydrolyze O stearoyl groups, 100 mg of this material was redissolved in 10 ml of 0.1 M NaOH and stirred at 55°C for 3.5 h. The solution was cooled, diluted to 100 ml with DDW, adjusted to pH 5.0 with 5% HCl, and lyophilized to yield a waxy solid. This was washed with CH_2Cl_2 (100 ml twice) atop an E-fritted glass filter and dissolved in DDW (20 ml), and the resulting solution was dialyzed in 3,200-molecular-weight-cutoff dialysis tubing against two 1,000-ml volumes of DDW over 2 days. Lyophilization of the dialyzed solution afforded 40 mg of glycolipid which produced satisfactory nuclear magnetic resonance and infrared spectra.

Adjuvants. (i) TDM and MDP. Trehalose dimycolate (TDM) and *N*-acetylmuramyl-L-α-aminobutyryl-D-isoglutamine (bMDP) were preparations used previously (31). Preparation of vaccines with these adjuvants was as described previously (31) except that squalane (Sigma Chemical Co., St. Louis, Mo.) was used in place of mineral oil.

(ii) ISCOMs. Immunostimulating complexes (ISCOMs) were prepared by published methods by Morein and colleagues (34, 45) with porins of the smooth and the NADC rough strain of *B. abortus* 2308.

(iii) L-121 and MDP. The method of vaccine preparation for L-121 and MDP was kept as similar as possible to that used for TDM and MDP. bMDP solubilized in methanol was added to dried antigen in a glass homogenizer. After the methanol was completely evaporated, the pluronic polyol L-121 (20, 21) (BASF Wyandotte, Co., Parsippany, N.J.) and squalane were added and homogenized as previously described (55). A solution of PBS with 0.2% Tween 80 (PBS-Tween) was then added and emulsified with the oil mixture (55). Each mouse received 5 μg of antigen, 10 μg of bMDP, 2.5 μg of L-121, 5 μl of squalane, and 95 μl of PBS-Tween.

Blood sampling. Blood samples were taken retro-orbitally and in most experiments were pooled within treatment groups. Sera were stored at −20°C. Thawed samples were diluted 1:25 in PBS and passed through a bacterial filter before use.

Experimental design. Unless otherwise stated, 6-week-old mice were vaccinated subcutaneously (s.c.) 4 weeks prior to intravenous (i.v.) challenge with approximately 5×10^4 live *B. abortus* cells. Blood samples were taken on one or more occasions during the course of some experiments. Quantitative cultures for viable *B. abortus* were performed on spleens at 1 and 4 weeks postinfection (p.i.) by the methods of Montaraz and Winter (31). Spleens from mice vaccinated with live strain 19 before challenge with strain 2308 were cultured on plates containing 0.1% erythritol to inhibit the growth of strain 19 (31).

Monoclonal antibody. A monoclonal antibody specific for the A epitope of the *B. abortus* OPS (8, 10) was provided as ascites fluid by James Douglas (University of Hawaii, Honolulu). The titer of this antibody with the f6 fraction in a standard plate enzyme-linked immunosorbent assay (ELISA) was 25,160.

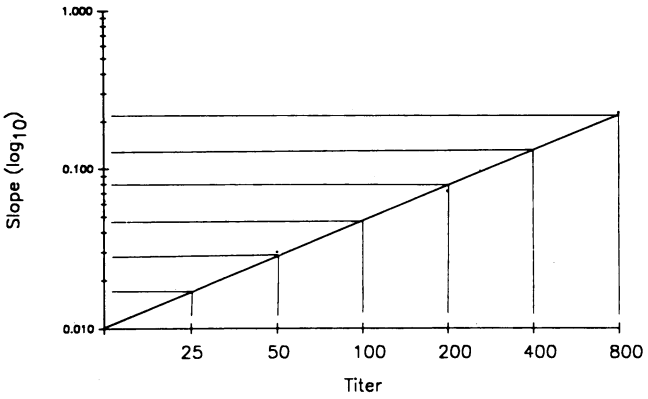

FIG. 1. Linear relationship between ELISA antibody titers and log of slope values.

Enzyme immunoassays. Antibodies were measured by an indirect kinetics-based ELISA (55). Plates were incubated overnight at 4°C with 500 ng of antigen per well of carbonate buffer (pH 9.6). After four washes with PBS containing 0.05% Tween 20, each well received 100 μl of serum diluted 1:25 in PBS. Plates were incubated for 60 min at 37°C and then washed four more times. A 100-μl portion of Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy and light chains) (Organon Teknika, West Chester, Pa.) diluted 1:1,000 in PBS-Tween 20 was added to each well followed by incubation for 30 min at room temperature. After final washing, 100 μl of 2,2′-azino-di-(3-ethylbenzthiazolinesulfonic acid) and hydrogen peroxide substrate solution was added. The slope of colored product development was measured in the linear response phase of enzyme-substrate interaction and was determined by computer-timed absorbance readings at 410 nm 2, 4, and 6 min after substrate addition. Standard plate ELISA was conducted by the same procedure except that plates were incubated for 30 min at 37°C with sera and 20 min at 37°C after conjugate addition. Colored product development was terminated after 20 min at room temperature by the addition of 100 μl of 0.1 M HF per well. Absorbance was read at 410 nm on a Minireader II (Dynatech Corp., Chantilly, Va.). Antibody titer was considered to be the highest dilution at which absorbance readings were 0.2 units greater than the control.

The kinetics-based ELISA was used because it enables more precise comparisons to be made among samples (22). For ease of understanding, however, the approximate conversions of the data from slope values to titers are included in the tables and will be used in referring to antibody responses. To obtain these conversions, we prepared samples from a positive control serum which gave titers of 50, 200, and 800, respectively, in the standard plate ELISA. Slope values were then derived from 1:25 dilutions of each of the three samples, because 1:25 was standard dilution used for antibody determinations by kinetics-based ELISA. A nomograph established by regression analysis demonstrated a linear relationship between the log of slope values and antibody titers ($r^2 = 1.0$) (Fig. 1).

A competitive assay was developed to determine the comparative amounts of antibody-reactive OPS on the antigens used for vaccination. Antigen-coated microtiter wells were prepared by adding 100 μl of f6 (2.5 μg/ml in carbonate buffer [pH 9.6]) per well and freezing the plate at −20°C. Plates were thawed 1 to 2 h before use. Well-washing

TABLE 1. Estimation of S-LPS and OPS content of vaccines by a competitive inhibition test

Vaccine	% S-LPS[a]	% OPS[a]
Porin-S-LPS[b]	15.0	10.7
Porin-R-LPS (NADC)[c]	0.01	0.007
Porin-R-LPS (RB51)[c]	0.01	0.007
R-porin-OPS (long)[d]		58.2
R-porin-OPS (short)[d]		10.7

[a] S-LPS fraction of f6 was used as a standard. Calculations were based on the content of S-LPS and OPS in the f6 sample as 94% (35, 38) and 67% (35), respectively.
[b] Native porin from smooth strain 2308.
[c] Native porin from rough 2308 strains NADC and RB51.
[d] Short- or long-chain OPS covalently linked to denatured porin from strain RB51.

procedures were as described above. Duplicate external dilutions of 100-μl samples of test antigen were incubated with 100 μl of a 1:800 dilution of monoclonal antibody for 3 h prior to the addition of 100 μl of the mixture to a washed antigen-coated well for 1 h. After washing, 100 μl of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Organon Teknika) was added for a 3-h incubation. After washing, 100 μl of *p*-nitrophenyl disodium phosphate was added at 1 mg/ml in diethanolamine buffer (pH 9.8), and slope values were determined from readings at 405 nm 2, 4, and 6 min later. A standard curve was prepared with samples of f6 in twofold dilutions from 20 to 0.31 μg. Test samples were assayed at concentrations of 40, 20, and 10 μg. An equation for the standard curve was determined by linear regression and then used to calculate the concentration of OPS in the test samples. An r^2 of 0.97 was found for the regression after log transformation of the antigen concentrations. The f6 standard was assumed to contain 94% S-LPS (35, 38) and 67% OPS (35).

Statistical methods. A log value of bacteria in each spleen was obtained by averaging the triplicate counts following log conversion (31). Statistical significance was determined by Fisher's protected least-significant-difference test (42). Correlations were analyzed by Pearson's product moment test (42).

RESULTS

OPS content of antigens. Porin extracted from smooth strain 2308 contained an estimated 15% associated S-LPS (Table 1), consistent with prior data (51). The two preparations of porin derived from rough strains of 2308 contained trace quantities of S-LPS according to the competitive inhibition assay (Table 1). However, this interpretation must be qualified because these values lie within the range of experimental error of the test. The synthetic vaccines prepared by binding long or short OPS chains to porin of rough strain RB51 were composed of approximately 58.2 and 10.7% OPS, respectively (Table 1). Both of these preparations when used as antigens in ELISAs produced reactions with monoclonal O antibodies equivalent in titer to that obtained with the O glycolipid (data not shown).

Vaccination trials with natural complexes of porin and LPS. Vaccination with 30 μg of porin-S-LPS conferred significant protection against challenge infections with either *B. abortus* 2308 or 19 at both 1 and 4 weeks p.i. (Table 2). The magnitude of protection was not significantly enhanced at either time period by the inclusion of TDM and MDP adjuvant in the vaccine. In contrast, vaccination with the same quantity of porin-R-LPS with or without adjuvant provided no significant protection against either challenge strain (Table 2).

Vaccination trials with porins incorporated into ISCOMs produced the same results. Porin-S-LPS ISCOMs provided significant protection against strain 2308 at 1 and 4 weeks p.i. even at the lowest dose tested (40 ng) (Table 3). At 1 week p.i., protection provided by 30 μg of porin-S-LPS in PBS was not different from that produced by 5 μg of ISCOMs, but was significantly greater ($P < 0.001$ to $P < 0.01$) than protection provided by all lower doses. Decreasing doses of ISCOMs produced successively lower levels of protection (Table 3) which were significantly different from each other ($P < 0.001$ to $P < 0.05$) except for the 1-μg and 200-ng doses. At 4 weeks p.i., no significant differences in protection could be ascribed to dose of vaccine or presence of adjuvant. Again, porin-R-LPS in ISCOMs produced no significant protection at any dose used (Table 3).

An experiment was next performed to test the effect of different adjuvants in enhancing protection provided by a uniform dose of porin-S-LPS. A 5-μg dose of porin-S-LPS conferred significant protection against strain 2308 at 1 and 4 weeks p.i. whether given as ISCOMs, with TDM and MDP, with L-121 and MDP, or without adjuvant (Table 4). The incorporation of adjuvants did not increase protection except at 1 week p.i. when the vaccine with TDM and MDP

TABLE 2. Vaccination with porin from smooth or rough strains of *B. abortus* 2308 with or without adjuvant[a]

Expt	Vaccine (μg)[b]	Adjuvant	Challenge strain	Log_{10} brucellae in spleens (mean ± SD)	
				1 wk p.i.	4 wk p.i.
1	None	None	2308	6.57 ± 0.94	7.01 ± 0.64
	Porin-S-LPS (30)	None	2308	4.18 ± 0.82***	5.77 ± 1.25*
	Porin-S-LPS (30)	TDM-MDP	2308	3.88 ± 0.81***	5.34 ± 1.08**
	Porin-R-LPS (30)	None	2308	7.20 ± 0.15 (NS)	7.11 ± 0.25 (NS)
	Porin-R-LPS (30)	TDM-MDP	2308	6.00 ± 1.33 (NS)	6.97 ± 0.28 (NS)
	None	TDM-MDP	2308	7.14 ± 0.10 (NS)	6.80 ± 0.32 (NS)
2	None	None	19	6.83 ± 0.45	6.37 ± 0.29
	Porin-S-LPS (30)	None	19	3.16 ± 0.68***	3.69 ± 1.45***
	Porin-S-LPS (30)	TDM-MDP	19	3.43 ± 1.45***	3.72 ± 1.68***
	Porin-R-LPS (30)	None	19	7.20 ± 0.16 (NS)	6.25 ± 0.27 (NS)
	Porin-R-LPS (30)	TDM-MDP	19	5.93 ± 1.60 (NS)	5.60 ± 1.36 (NS)
	None	TDM-MDP	19	6.95 ± 0.27 (NS)	5.79 ± 0.66 (NS)

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308 or strain 19. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; NS, not significant.
[b] Porin from smooth strain (S) or from rough strain NADC (R) 2308.

TABLE 3. Vaccination with porin from smooth or rough strains of *B. abortus* 2308 in ISCOMs[a]

Expt	Vaccine (μg)[b]	Adjuvant	Log_{10} brucellae in spleens (mean ± SD)	
			1 wk p.i.	4 wk p.i.
1	None	None	7.14 ± 0.12	6.99 ± 0.37
	Porin-S-LPS (30)	None	3.81 ± 0.10***	5.57 ± 0.96*
	Porin-S-LPS (5)	ISCOM	3.39 ± 0.22***	4.38 ± 0.85***
	Porin-S-LPS (1)	ISCOM	4.59 ± 0.54***	5.41 ± 0.84**
	Porin-S-LPS (0.2)	ISCOM	4.86 ± 0.39***	4.61 ± 0.85***
	Porin-S-LPS (0.04)	ISCOM	5.40 ± 0.56***	5.20 ± 1.11**
2	None	None	7.17 ± 0.16	7.04 ± 0.48
	Porin-R-LPS (30)	None	7.17 ± 0.13 (NS)	6.97 ± 0.53 (NS)
	Porin-R-LPS (5)	ISCOM	7.41 ± 0.08 (NS)	7.42 ± 0.14 (NS)
	Porin-R-LPS (1)	ISCOM	7.09 ± 0.75 (NS)	6.98 ± 0.31 (NS)
	Porin-R-LPS (0.2)	ISCOM	7.03 ± 0.40 (NS)	6.56 ± 1.04 (NS)
	Porin-R-LPS (0.04)	ISCOM	7.23 ± 0.28 (NS)	6.99 ± 0.59 (NS)

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.
[b] Porin from smooth strain 2308 (S) or rough strain NADC (R) 2308.

provided greater protection than antigen in PBS ($P < 0.05$). The efficacy of vaccines containing different adjuvants did not differ significantly among each other.

Vaccination trials with synthetic complexes of porin and OPS. The results obtained with natural complexes of porin and LPS supported the hypothesis that the OPS was an essential component of an effective vaccine. We therefore proceeded to determine whether porin-R-LPS, an ineffective vaccine, could be converted into an effective one by the addition of OPS.

In the first experiment, mice were vaccinated with 30 μg of synthetic complexes of R-porin coupled with long (R-porin-OPS-long) or short (R-porin-OPS-short) chains of OPS in the TDM and MDP adjuvant. The synthetic complexes conferred significant protection against challenge infection with strain 2308 at 1 week p.i. ($P < 0.001$) (Table 5). Protection was comparable to that obtained in mice vaccinated with porin-S-LPS (Table 5) and was significantly greater than that due to adjuvant alone (Table 5) ($P < 0.001$). Mice vaccinated with porin-R-LPS were not protected (Table 5). In another trial, protection was demonstrated at 1 week ($P < 0.001$) and 4 weeks ($P < 0.05$) in mice vaccinated with 30 μg of R-porin-OPS-long without adjuvant, whereas no protection was afforded to mice vaccinated with the same lot of denatured porin which had not been conjugated to OPS (Table 6).

Despite initial success achieved with the R-porin-OPS short-chain vaccine (Table 5), two attempts to reproduce protection with this preparation failed (data not shown). An experiment was therefore performed to determine whether revaccination would improve the effectiveness of this vaccine. At the same time, a direct comparison was made between the natural and synthetic porin vaccines and living strain 19. Mice vaccinated twice with porin-S-LPS or R-porin complexed with either long- or short-chain OPS were protected following challenge with strain 2308 ($P < 0.01$ to $P < 0.001$) (Table 7). Levels of protection obtained with the subcellular vaccines were in no instance significantly different from that achieved with strain 19. In this experiment, low (0.74 logs) but significant ($P < 0.05$) protection was also achieved with the porin-R-LPS vaccine (Table 7). Protection achieved with the porin-R-LPS vaccine was, however, significantly below that obtained with strain 19, porin-S-LPS, or R-porin-OPS-long vaccines ($P < 0.01$).

Association of protection with induction of O antibodies. Pooled sera taken just prior to challenge infection from mice vaccinated 4 weeks earlier with 5 μg of porin-S-LPS in PBS contained O antibodies at a titer of 100, and incorporation of adjuvants increased the titers four- to eightfold (Table 4). All these vaccines conferred high levels of protection, as noted above (Table 4).

In the experiment reported in Table 7, pooled sera were collected from each group prior to the first and second vaccinations and prior to challenge infection. Individual samples were taken prior to killing the mice at 1 week p.i. A single vaccination with porin-S-LPS resulted in a titer of O antibodies of 200 4 weeks later (Table 7). This was increased to 800 as a result of revaccination and remained at this level

TABLE 4. Vaccination with porin from smooth strain 2308 in different adjuvants[a]

Vaccine (μg)[b]	Adjuvant	Antibody level[c]		Log_{10} brucellae in spleens (mean ± SD)	
		Slope (10^3)	Titer	1 wk p.i.	4 wk p.i.
None	None			7.20 ± 0.15	7.17 ± 0.48
Porin-S-LPS (5)	None	62	100	3.90 ± 0.45***	5.55 ± 0.66**
Porin-S-LPS (5)	ISCOM	241	800	3.46 ± 0.83***	5.29 ± 1.07**
Porin-S-LPS (5)	TDM-MDP	242	800	3.25 ± 0.39***	4.71 ± 0.83***
Porin-S-LPS (5)	L-121-MDP	134	400	3.47 ± 0.20***	4.25 ± 0.68***

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P \leq 0.01$.
[b] Porin from smooth strain 2308.
[c] Mice were bled retro-orbitally prior to challenge. Pooled serum samples were tested in kinetics-based ELISA against fraction f6. Numbers reported are slopes, and titers were estimated from slopes (Fig. 1).

TABLE 5. Vaccination with naturally occurring porins or synthetic porin-OPS complexes[a]

Vaccine (μg)	Adjuvant	Log_{10} brucellae in spleens (mean ± SD) (1 wk p.i.)
None	None	6.86 ± 0.08
Porin-S-LPS[b] (30)	TDM-MDP	4.01 ± 1.07***
Porin-R-LPS[b] (30)	TDM-MDP	6.81 ± 0.38 (NS)
R-porin-OPS (short)[c] (30)	TDM-MDP	3.57 ± 0.23***
R-porin-OPS (long)[c] (30)	TDM-MDP	3.22 ± 0.31***
None	TDM-MDP	5.34 ± 0.84**

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; **, $P \leq 0.01$; NS, not significant.

[b] Porin from smooth (S) or rough strain NADC (R) 2308.

[c] Short- or long-chain OPS covalently linked to porin from rough strain RB51.

after challenge infection (Table 7). Although vaccination with porin-R-LPS produced only the most marginal increase after the second inoculation, it is notable that the titer in this group following challenge was fourfold greater than that in the nonvaccinated group (Table 7). In contrast with the high antibody response (titer of 400) observed after the first vaccination with the synthetic long-chain vaccine, a single vaccination with the short-chain vaccine produced no increase in O antibodies (Table 7). A second inoculation of the short-chain vaccine caused the appearance of circulating O antibodies (titer of 100), and the titer of antibodies in this group following challenge was fourfold greater than that in the nonvaccinated group (Table 7). Antibody levels (measured as slope values) at 42 days were correlated in an inverse fashion with mean log numbers of bacteria cultured from the spleens of each treatment group ($r^2 = -0.828$; $P < 0.05$).

Marginal increases in antibodies specific for the porin-R-LPS complex were detected in groups immunized with subcellular vaccines (Table 7). In only one instance did these attain a titer of 25 (Table 7).

Further analyses were performed on individual sera of four of the groups to determine whether levels of O antibodies at 1 week p.i. were correlated with protection in individual animals. For this purpose, the O glycolipid was used as the antigen, primarily to ensure that antibody responses produced by porin-R-LPS were not directed against porin or lipid A determinants. Analyses of the 35-day and 42-day serum pools with f6 and O glycolipid antigens demonstrated comparable reactions (Table 8) which were highly correlated ($r^2 = 0.968$; $P < 0.01$). Slopes of reactions against O glycolipid were on the average 12.5% lower than those with f6. Levels of O antibodies were very similar in mice numbers 13 to 18, immunized with the long-chain synthetic vaccine, in which spleen counts showed little variation (Table 9). In the other groups, selected because of the higher standard deviations in spleen counts, a range of antibody levels occurred within each group and in no group was there a significant correlation between antibody levels and spleen counts. In a few animals (numbers 4 and 19), high levels of protection occurred despite levels of O antibodies no different from the mean level in the nonvaccinated controls (Tables 7 and 9).

DISCUSSION

B. abortus is a facultative intracellular parasite (11, 28), and evidence from the murine model of infection indicates that humoral (3, 26, 29, 32, 36, 39, 44) as well as cell-mediated (31, 37, 40) immune responses contribute to protection. The same views are currently held in respect to immunity against *Salmonella typhimurium* (16). In murine infections with both *B. abortus* (26, 32) and *S. typhimurium* (12), monoclonal antibodies specific for the OPS have been shown to confer protection passively. The data presented here complement and extend these findings with *B. abortus* by demonstrating that an acquired immune response to the OPS provides protection equivalent to that obtained with a living vaccine (Tables 7 and 9). It must be emphasized in this connection that in the model system we used, critical comparisons in logs of protection (i.e., the difference in mean log numbers of brucellae in the spleens of vaccinated and control groups) achieved by various vaccines must be restricted to comparisons made within the same experiment. For example, in experiments performed 15 months apart which are reported in Tables 3 and 7, vaccination of mice with 30 μg of the same preparation of porin-S-LPS in PBS produced logs of protection at 1 week p.i. of 3.33 and 1.74, respectively. Each result reflected a highly significant difference from control values ($P < 0.001$). Different lots of challenge strain 2308 were used in the two experiments. Small differences in lots of *B. abortus* challenge strains and in lots of experimental animals probably account for most of these variations. We have also noted that protection achieved at 1 week p.i. is a reliable predictor of protection at later time intervals. With infrequent exceptions, protection following vaccination with either living strain 19 or subcellular vaccines decreases between 1 and 4 weeks p.i. (Tables 2, 3, 4, and 6) (31), and long-term studies have demonstrated that even in mice vaccinated with strain 19, chronic infection develops following challenge infection with 5×10^4 CFU of strain 2308 and the protective effects of vaccination have disappeared by 12 weeks p.i. (A. J. Winter and G. E. Rowe, unpublished data).

The synthetic vaccine which contained long OPS chains proved better than the one with short OPS chains. This was associated in the long-chain vaccine with a higher apparent content of OPS (Table 1) and a more effective stimulation of O antibodies, particularly evident following a single vaccination (Table 7, day 28). The interpretation of data from the competitive inhibition assay must, however, be qualified in comparing preparations with long and short OPS chains. The short oligosaccharides may have combined less effectively with the monoclonal antibody used in the assay, which would have resulted in an underestimation of carbohydrate content. The quantity of O saccharide measured in the short-chain synthetic vaccine was nevertheless equal to that in the natural complex (Table 1), so that the differing qualities of the synthetic vaccines more likely reflected differences in OPS chain length. In a similar study performed with *S. typhimurium*, Svenson et al. (46) protected mice

TABLE 6. Vaccination with rough strain porin alone or complexed with long-chain OPS[a]

Vaccine (μg)	Log_{10} brucellae in spleens (mean ± SD)	
	1 wk p.i.	4 wk p.i.
None	6.17 ± 0.12	7.19 ± 0.43
R-porin[b] (30)	6.43 ± 0.47 (NS)	7.26 ± 0.14 (NS)
R-porin-OPS (long)[c] (30)	4.48 ± 0.25***	5.77 ± 0.90*

[a] Vaccination was performed s.c. 4 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. In comparisons with untreated control groups, ***, $P \leq 0.001$; *, $P \leq 0.05$; NS, not significant. No adjuvant was used.

[b] Porin from rough strain RB51, denatured in sodium dodecyl sulfate.

[c] Long-chain OPS covalently linked to porin from strain RB51.

TABLE 7. Relationship between serum antibodies and protection against *B. abortus* in mice immunized with living or subcellular vaccines[a]

Vaccine (μg)	Antigen[b]	Antibody level (10^3) on expt day[c]:				Log_{10} brucellae in spleens (mean ± SD) (1 wk p.i.)
		0	28	35	42	
None	f6	2 (<)	2 (<)	3 (<)	36 (50)	6.39 ± 0.18
	Porin-R-LPS	4 (<)	3 (<)	6 (<)	6 (<)	
Strain 19[d]	f6	2 (<)	48 (100)	84 (200)	142 (400)	4.53 ± 0.84***
	Porin-R-LPS	3 (<)	3 (<)	5 (<)	6 (<)	
Porin-S-LPS[e] (30)	f6	2 (<)	98 (200)	233 (800)	229 (800)	4.64 ± 0.20***
	Porin-R-LPS	3 (<)	7 (<)	20 (25)	10 (<)	
Porin-R-LPS[e] (30)	f6	2 (<)	4 (<)	9 (<)	117 (200)	5.66 ± 0.87*
	Porin-R-LPS	4 (<)	5 (<)	9 (<)	13 (<)	
R-porin-OPS (long)[f] (30)	f6	2 (<)	137 (400)	190 (400)	235 (800)	4.54 ± 0.14***
	Porin-R-LPS	6 (<)	8 (<)	10 (<)	12 (<)	
R-porin-OPS (short)[f] (30)	f6	3 (<)	4 (<)	57 (100)	97 (200)	5.06 ± 0.78***
	Porin-R-LPS	4 (<)	5 (<)	9 (<)	10 (<)	

[a] Vaccination with subcellular products was performed s.c. 1 and 5 weeks prior to challenge i.v. with approximately 5×10^4 cells of strain 2308. Strain 19 was inoculated 5 weeks before challenge. In comparisons with untreated control groups, ***, $P \leq 0.001$; *, $P \leq 0.05$.
[b] Antibodies were measured against either *B. abortus* S-LPS (f6) or rough strain RB51 native porin (porin-R-LPS).
[c] Mice were bled retro-orbitally prior to vaccination (day 0), revaccination (day 28), challenge (day 35), and spleen culture (day 42). Pooled serum samples were tested in kinetics-based ELISA. Numbers outside parentheses represent slope values. Numbers in parentheses are titers estimated from slopes (Fig. 1). <, titer < 25.
[d] Living *B. abortus* 19 at approximately 5×10^4 CFU per mouse.
[e] Undenatured porins from smooth strain (S) or rough strain RB51 (R) of 2308.
[f] Short- or long-chain OPS covalently linked to porin from strain RB51.

against lethal infection with synthetic vaccines containing octasaccharides of the OPS. However, no comparison was made in that study with vaccines containing longer OPS chains.

The weight of evidence supports the view that protection induced by the subcellular vaccines resulted from the induction of O antibodies. In the first instance, highly effective protection could be induced without the requirement for adjuvants which enhance cell-mediated immune responses (Tables 2, 3, 4, 6, and 7). Second, antibodies specific for moieties other than the OPS (e.g., porin, lipid A, or LPS core epitopes) were found in extremely low concentrations in sera of immune mice (Table 7). Third, there was a proportional relationship between the capacity of a vaccine to induce O antibodies and its capacity to induce protective immunity. This was evident in assays performed both before (Tables 4, 7, and 8) and after (Tables 7 and 8) challenge infection. Although a significant inverse correlation occurred within treatment groups between mean concentrations of O antibodies and mean numbers of *B. abortus* in spleens ($P < 0.05$) (Table 7), analysis of individuals within treatment groups failed to demonstrate such a relationship in all cases (Table 9). Examples are evident of both mice with a high level of protection in the absence of increased O antibodies (Table 9, mice 4 and 19) and others in which protection was marginal or absent despite raised levels of O antibodies (Table 9, mice 6, 10, 11, 12, and 24). Such exceptions do not, however, invalidate the hypothesis that O antibodies have a

TABLE 8. Comparison of antibody levels measured in ELISA with S-LPS (f6) and O glycolipid (O gly) antigens[a]

Vaccine (μg)	Antibody level (10^3) on expt day[b]:			
	35		42	
	f6	O gly	f6	O gly
Strain 19 (5×10^4 CFU)	84	70	142	93
Porin-S-LPS (30)	233	215	229	177
Porin-R-LPS (30)	9	8	117	80
R-porin-OPS (long) (30)	190	213	235	198
R-porin-OPS (short) (30)	57	50	97	77
None (PBS)	3	4	36	28

[a] Serum pools tested were from the same experiment reported in Table 7.
[b] Slope values from kinetics-based ELISA.

TABLE 9. Serum antibodies specific for OPS and numbers of *B. abortus* in spleens of individual mice 1 week after challenge infection[a]

Vaccine (μg)	Mouse no.	Brucellae in spleens (10^4)[b]	Antibody levels[c]	
			Slope (10^3)	Titer
Strain 19 (5×10^4 CFU)	1	0.6	50	100
	2	1.3	165	400
	3	1.4	103	200
	4	1.4	30	50
	5	8.3	32	50
	6	120.0	104	200
Porin-R-LPS (30)	7	5.6	55	100
	8	8.5	82	200
	9	10.0	75	200
	10	160.0	68	100
	11	180.0	131	200
	12	630.0	89	200
Porin-OPS (long) (30)	13	1.8	196	400
	14	3.3	178	400
	15	3.7	158	400
	16	4.0	196	400
	17	4.1	205	400
	18	4.7	209	400
Porin-OPS (short) (30)	19	2.7	18	25
	20	2.9	64	100
	21	5.5	112	200
	22	6.4	77	200
	23	25.0	25	25
	24	310.0	75	200

[a] Sera were those taken on day 42 in the experiment reported in Table 7.
[b] Mean number of *B. abortus* in spleens of the control group was 245×10^4.
[c] Slope values from kinetics-based ELISA with O glycolipid as the antigen. Titers were estimated from slopes (Fig. 1).

critical role in protective immunity. A more critical test of this hypothesis would be to determine the correlation between antibody titers just prior to challenge and protection. Moreover, the resolution of this question will require not only quantitative measurements but also comparisons of isotype distribution and functional assessments of O antibodies from mice exhibiting different levels of immunity.

There is growing evidence that porins or other outer membrane proteins will prove to be useful as vaccines capable of inducing protective antibodies against *Neisseria meningitidis* (7, 17), *Pseudomonas aeruginosa* (18, 30), and *Haemophilus influenzae* (19, 27). A protective role of porin antibodies against *S. typhimurium* has been proposed in several studies (4, 24, 49). Although some workers have now concluded that protection against *S. typhimurium* attributed to porin antibodies was due to O antibodies (41), the issue remains unresolved (49). Data reported here offer no evidence for the participation of porin antibodies in protection against *B. abortus*. The antibody response of mice to porin was almost negligible, even when porin vaccines lacked OPS (Table 7). The protection produced in mice following two vaccinations with porin from rough strain RB51 was almost certainly due to O antibodies. The data indicate that two inoculations of this vaccine primed the mice to produce an accelerated O antibody response to the challenge infection (Tables 7 and 9). It cannot be excluded that some of the antibodies reactive with f6 or O glycolipid were directed at core determinants. However, the marginal reactivity of these sera with porin-R-LPS (Table 7) indicates that the majority of antibodies were specific for OPS. Priming could have been a response to the porin and to trace quantities of OPS in the LPS of the rough strain or, in the absence of S-LPS, to the porin carrier alone. Whereas antibodies specific for porin and the group 3 outer membrane protein of *B. abortus* can be induced in cattle vaccinated with a rough strain of *B. abortus* such as 45/20 in TDM and MDP adjuvants (53, 55), experimental infection with smooth virulent strains fails to evoke an appreciable increase in outer membrane protein-specific antibodies in cattle (C. L. Baldwin, Ph.D. thesis, Cornell University, Ithaca, 1983) or mice (Table 7). This suggests that in the cell envelope of *B. abortus*, epitopes of OPS are strongly dominant over those of the associated outer membrane proteins. Moreover, the failure of monoclonal antibodies specific for the porin of *B. abortus* to protect mice (32) and of polyvalent antisera specific for porin or group 3 antibodies to agglutinate smooth whole *B. abortus* cells (A. J. Winter, unpublished data) suggest that, as in the members of the family *Enterobacteriaceae* (50), the outer membrane proteins of *B. abortus* are in their native state inaccessible to antibodies because of the long OPS chains. If this was so, antibodies protective against *B. abortus* would be limited to those with specificity for the OPS.

Although under natural conditions of infection the porin of *B. abortus* is ineffective in inducing antibodies, it does stimulate T-cell responses. Thus, peripheral blood lymphocytes of cattle infected with strain 2308 underwent blastogenic transformation when exposed to highly denatured porin proteins largely depleted of LPS (2). Denatured porin proteins also induced delayed-type hypersensitivity in cattle infected with strain 2308 or in mice infected with strain 19 (A. J. Winter, G. E. Rowe, and W. L. Castleman, unpublished data). The data from the present study do not, however, serve to advance the hypothesis that the porin of *B. abortus* can induce a protective cell-mediated immune response. Adjuvants were selected which enhanced cell-mediated as well as humoral immune responses (1, 9, 25, 33), and the combinations of TDM and MDP (53–55) and L-121 and MDP (A. J. Winter and G. E. Rowe, unpublished data) were already known to be potent in induction of cell-mediated immune responses against porins of *B. abortus* in cattle. These adjuvants did enhance the formation of O antibodies (Table 4), but evidence of protection attributable to cell-mediated immunity could not be inferred from any of these data. Although adoptive transfer experiments conducted in several laboratories have provided evidence for the existence of immune T cells which confer protection against *B. abortus* (31, 37, 40), the antigenic specificities of these T cells have not been established. A rigorous examination of the hypothesis that vaccination of mice with porins or other purified cell envelope proteins in conjunction with selected adjuvants can induce T cells capable of conferring protective immunity against *B. abortus* is currently under way in our laboratory.

The application of these findings from the mouse model to the construction of subcellular vaccines for cattle is still premature. Two recent well-controlled studies have failed to demonstrate protection against brucellosis in pregnant heifers by immunization with subcellular vaccines with or without S-LPS. Confer et al. (13) vaccinated cattle with a mixture of salt-extractable proteins (47) which did contain S-LPS as evidenced by the development of positive serological tests (13), whereas Adams et al. (L. G. Adams, R. P. Crawford, T. A. Ficht, R. Smith III, B. A. Sowa, J. W. Templeton, J. D. Williams, and A. M. Wu, 40th Annual Brucellosis Conference, Chicago, Ill., Nov. 14 and 15, 1987) used LPS-free recombinant outer membrane proteins of *B. abortus*. The failures of these vaccines may have had multiple causes and do not resolve the question of the importance of the OPS in an effective vaccine against bovine brucellosis.

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