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Vaccination with *Brucella abortus* Rough Mutant RB51 Protects BALB/c Mice against Virulent Strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*

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Vaccination of BALB/c mice with live *Brucella abortus* RB51, a stable rough mutant, produced protection against challenge with virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Passive-transfer experiments indicated that vaccinated mice were protected against *B. abortus* 2308 through cell-mediated immunity, against *B. ovis* PA through humoral immunity, and against *B. melitensis* 16M through both forms of immunity. Live bacteria were required for the induction of protective cell-mediated immunity; vaccination with whole killed cells of strain RB51 failed to protect mice against *B. abortus* 2308 despite development of good delayed-type hypersensitivity reactions. Protective antibodies against the heterologous species were generated in vaccinated mice primarily through anamnestic responses following challenge infections. Growth of the antigenically unrelated bacterium *Listeria monocytogenes* in the spleens of vaccinated mice indicated that nonspecific killing by residual activated macrophages contributed minimally to protection. These results encourage the continued investigation of strain RB51 as an alternative vaccine against heterologous *Brucella* species. However, its usefulness against *B. ovis* would be limited if, as suggested here, epitopes critical for protective cell-mediated immunity are not shared between *B. abortus* and *B. ovis*.

Brucellosis affects many animal species as well as humans (49). *Brucella abortus* is the most important cause of bovine brucellosis, while both *Brucella ovis* and *Brucella melitensis* cause brucellosis in sheep (49). Whereas the lipopolysaccharides (LPS) of wild-type strains of *B. abortus* and *B. melitensis* contain O polysaccharide side chains (OPS) that give colonies a smooth phenotype, *B. ovis* occurs naturally in the rough phenotype because its LPS is devoid of OPS (49). The OPS of *B. abortus* and *B. melitensis* contain various proportions of two type-specific epitopes, designated A and M (12), and other epitopes common to the OPS of both species (20, 43). In the BALB/c mouse model, protection against *B. abortus* (2) and *B. ovis* (26) is mediated by both humoral and cell-mediated forms of immunity. Antibodies protective to *B. abortus* are specific predominantly for the OPS (31, 35), while those protective to *B. ovis* are directed primarily against outer membrane proteins (26), which are prominently exposed on the cell surface in the absence of OPS (38).

Vaccination represents an essential element in the control of bovine and ovine brucellosis. Live attenuated *B. abortus* 19 and *B. melitensis* Rev 1 have served as efficacious vaccine strains for cattle and sheep, respectively (5, 46). However, both vaccines have the disadvantages of inducing OPS-specific antibody responses that interfere with serological diagnosis of disease (7, 24, 28, 40, 46), of causing abortion in vaccinated animals under some circumstances (19, 27), and of being pathogenic for humans (6, 53). Alternative vaccines to strains 19 and Rev 1 have been sought for many years, with limited success (1, 8, 15, 18, 32, 37, 47).

In recent years, Schurig and his coworkers produced a stable rough variant of virulent *B. abortus* 2308 that was designated RB51 (44). Strain RB51 had diminished virulence in comparison with strains 2308 and 19 (41, 42, 50) and did not induce the formation of OPS-specific antibodies (13, 44, 50). Preliminary data demonstrated that vaccination with live strain RB51 provided protection against challenge with strain 2308 in mice (4, 44), and recent experiments have indicated that strain RB51 may serve as an alternative vaccine for cattle (14). Because of the close genetic relationship among species of the genus *Brucella* (33), we performed experiments in the mouse model to test the hypothesis that strain RB51 might serve as an effective vaccine not only against *B. abortus*, but also against heterologous *Brucella* species.

MATERIALS AND METHODS

Mice. Female BALB/cByJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) at 9 weeks of age and were used 1 week later.

Bacterial strains. Stock cultures of *B. abortus* vaccine strain 19 and virulent strain 2308 had been used previously and were known to give typical growth curves in BALB/c mice (2, 21, 22). Strain RB51 was isolated from the draining lymph node of a heifer that had been vaccinated with a live culture several weeks previously. This isolate, as well as *B. melitensis* attenuated strain Rev 1 and virulent strain 16M, were obtained from G. G. Schurig. *B. ovis* PA was supplied by J. M. Verger (Station de Pathologie Infectieuse et Immunité, INRA, Nouzilly,

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France). *B. melitensis* 16M and *B. ovis* PA were used after two serial passages in BALB/c mice and isolation in pure culture from spleens (26). *Listeria monocytogenes* EGD is a virulent strain used previously in our laboratory (2). Stock cultures of all *Brucella* strains were prepared after 72 h of growth on Schaedler blood agar plates. Plate growth was suspended in Albimi broth (Difco Laboratories, Detroit, Mich.), aliquoted and snap frozen in a dry ice-alcohol mixture, and stored at −70°C. *L. monocytogenes* was grown to log phase in tryptic soy broth (Difco), aliquoted, snap frozen, and stored at −70°C. For mouse inoculation, contents of freshly thawed vials were diluted with sterile phosphate-buffered saline (PBS) to the desired concentration. Exact numbers were determined retrospectively by viable counts (34).

Antigens for vaccines, hypersensitivity tests, and immunoassays. Whole killed cells (WKC) of strain RB51 were prepared from plate-grown cells that were killed with methanol (48), dialyzed exhaustively in distilled water, and lyophilized. A whole-cell extract (WCE) of strain RB51 was obtained by boiling WKC for 3 min in Laemmli sample buffer (44). The LPS of *B. abortus* RB51 and 2308 (44), *B. melitensis* 16M (36), and *Yersinia enterocolitica* O:9 (25) was extracted from whole cells with hot phenol. The rough LPS of strain RB51 was purified from the aqueous phase, while the LPS of the smooth strains was obtained from the phenol phase. Live cells of strain RB51 were suspended in PBS and autoclaved to extract *B. abortus* soluble antigen (BASA) (30). A hot saline (HS) extract of *B. ovis* REO 198, which contains rough LPS complexed with outer membrane proteins, was obtained by methods described previously (39).

Blood sampling. Mice were bled under anesthesia from the retroorbital sinus or the heart. Sera were stored at −70°C unless used on the same day.

DTH. Delayed-type hypersensitivity (DTH) reactions were determined by the method described previously (2). Mice were injected with BASA (20 μg in 20 μl of PBS) in the right footpad and 20 μl of PBS in the left footpad. Footpad thicknesses were measured 48 h later with a Hauptner dial caliper (Jorgenson Laboratories, Loveland, Colo.). A difference in footpad thickness of ≥2 U (1 U = 0.1 mm) was regarded as a positive reaction. Lesions were confirmed as DTH reactions by histological examination (2).

Quantitation of bacterial numbers. Mice were killed by CO_2 asphyxiation. Spleens were homogenized, diluted serially, and plated (34). *Brucella* colonies were counted after incubation for 3 days (*B. abortus* and *B. melitensis*) or 5 days (*B. ovis*) at 37°C under 10% CO_2. *Listeria* colonies were counted after aerobic incubation at 37°C for 1 day.

T-lymphocyte enrichment. B-cell depletion was accomplished by a single negative panning step, using the technique described by Araya et al. (2). Percentages of B cells (surface immunoglobulin [Ig] positive), $CD4^+$, $CD8^+$, and Mac-1^+ cells were determined by immunofluorescence procedures (2). After panning, B cells represented <1% of the total cell population.

Experimental design. (i) Time course of infection. Mice (10 weeks old) were inoculated intraperitoneally (i.p.) with 3×10^8 CFU of strain RB51 in 0.1 ml of PBS. At selected intervals postinoculation (p.i.), groups of mice ($n = 5$) were killed, and spleens were processed for quantitative counts.

(ii) Vaccination and challenge trials. Mice (10 weeks old) in groups of five were vaccinated by i.p. or subcutaneous (s.c.) inoculation of selected dosages of live strain RB51. In some experiments, mice were injected s.c. twice at a 4-week interval with WKC (20 or 30 μg, dry weight) in QS-21 adjuvant (Cambridge Biotech. Corp., Worcester, Mass.) using a formulation described previously (26). Five weeks after the first vaccination, mice were challenged by intravenous (i.v.) inoculation of *B. abortus* 2308 (5×10^4 CFU), *B. melitensis* 16M (5×10^4 CFU), *B. ovis* PA (5×10^3 CFU), or *L. monocytogenes* EGD (1×10^5 CFU). Mice challenged with *Brucella* spp. were killed for spleen counts 1 week (*B. abortus*) or 2 weeks (*B. melitensis* and *B. ovis*) later. Appropriate challenge doses and challenge-to-sacrifice intervals had been established previously for each strain (26, 34). Spleen counts on mice challenged with *L. monocytogenes* were performed 1 day later (2). Blood samples were taken prior to vaccination, immediately before challenge in most experiments, and at death. When DTH was tested, BASA was injected 48 h before sacrifice, and footpad thicknesses were measured just before death (2).

(iii) Passive-transfer assays. Experiments were performed to determine the protective capacity of splenic T cells or serum from donor mice vaccinated with RB51 against virulent strains of *B. abortus*, *B. melitensis*, and *B. ovis*. When *B. abortus* 2308 was the challenge strain, additional groups of recipients were injected with immune T cells or serum from donors vaccinated with *B. abortus* 19 to serve as positive controls. In challenge experiments with *B. melitensis* 16M or *B. ovis* PA, T cells and serum from donors vaccinated with *B. melitensis* Rev 1 served as the positive control. Negative control groups received PBS, since, in accord with prior findings (2, 26, 34), it was established with all three challenge strains that there were no significant differences in splenic counts between groups that were injected with 0.1 ml of PBS and those that received either 0.1 ml of normal mouse serum or 3×10^7 normal splenic T cells prior to inoculation of the challenge strain.

Donor mice (10 weeks old) were vaccinated i.p. with 3×10^8 CFU of strain RB51. Other groups were vaccinated by i.v. inoculation of 5×10^4 CFU of *B. abortus* 19 (34) or 5×10^4 CFU of *B. melitensis* Rev 1 (26). Five weeks later, donor groups were exsanguinated, and T-lymphocyte suspensions were prepared from their spleens. Groups of five recipients (10 weeks old) were injected i.v. with pooled suspensions of lymphocytes (3×10^7 viable cells in 0.4 ml of Dulbecco's modified Eagle's medium) or pooled antisera (0.1 ml) from a donor group (2). After 1 h, groups of recipients were challenged i.v. with 5×10^4 CFU of *B. abortus* 2308, 5×10^4 CFU of *B. melitensis* 16M, or 5×10^3 CFU of *B. ovis* PA. Spleen counts were performed at the postchallenge intervals designated above. Recipient groups receiving T cells were bled prior to death. These sera were tested for antibody responses against the challenge strain to ensure that protection had not resulted from anamnestic antibody responses due to residual B cells in the transferred population (2).

KELA. A previously described (26, 52) kinetics-based enzyme-linked assay (KELA) with a goat anti-mouse IgM, IgG, and IgA conjugated to horseradish peroxidase (Cappel, Organon Teknika, Durham, N.C.) was used to quantitate antibodies following vaccination and challenge. Because the A epitope is dominant in the OPS of *B. abortus* 2308 and 19 (12, 49) and is shared by the OPS of *Y. enterocolitica* O:9 (9, 10), the LPS of *Y. enterocolitica* O:9 was used to test sera from mice challenged with *B. abortus* 2308. LPS of *B. melitensis* 16M and the HS extract of *B. ovis* REO 198 were used as antigens when mice were challenged with *B. melitensis* 16M and *B. ovis* PA, respectively. Sera from mice vaccinated with strain RB51 were also tested with RB51 WKC. The rate of reaction between substrate solution and enzyme, expressed as slope, was determined from linear regression analysis of time versus absorbance and is directly proportional to the amount of antibody in the sample (51). Based on the linear relationship between the log of slope values and antibody titers (52), slope values (10^3)

of >150 slope units correspond to high antibody titers, 50 to 150 slope units correspond to moderate titers, and slopes of <50 units correspond to low titers. Data from assays performed on separate days were normalized by regression analysis from a standard curve obtained by the inclusion of the same set of standards on each plate. Prior to treatment, slope values (10^3) averaged <14 with any of the antigens employed.

Western (immuno) blots. Western blots were performed as described previously (44). Briefly, antigens were electrophoresed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a resolving gel containing 12.5% acrylamide. Preparations were transferred to nitrocellulose membranes which were blocked with 2% bovine serum albumin (BSA) and then incubated overnight at room temperature with 1:50 or 1:100 dilutions of sera. Following incubation and washings, membranes were treated with the appropriate horseradish peroxidase-labeled anti-IgG conjugate (heavy- and light-chain specific; Cappel) and developed with 4-chloro-1-naphthol.

Bru 38, a rat monoclonal antibody specific for an epitope common to the OPS of *B. abortus* and *B. melitensis* (43), produced a typical smear ranging from above 94 to about 25 kDa with LPS from both *B. abortus* 2308 and *B. melitensis* 16M (see Fig. 2, lanes 1 and 2).

Bru 48, a monoclonal antibody derived from B cells of a mouse immunized with strain RB51, is specific for the rough LPS of *Brucella* spp. (G. G. Schurig, unpublished data). Reaction of strain RB51 LPS with Bru 48 produced a broad band from 35 to 21 kDa (see Fig. 2, lane 3), instead of in the typical location between 20 and 14 kDa (17). This was attributable to protein contamination of the rough LPS preparation, since it gave no reaction with Bru 38 and produced a band with Bru 48 of 18 to 12 kDa following digestion with proteinase K (data not shown). Bru 48 cross-reacted strongly with rough LPS in the HS extract of *B. ovis* to produce a series of diffuse bands extending from 94 to below 14 kDa (see Fig. 2, lane 5), reflecting the binding of LPS to proteins in this extract (23, 39). Protein antigens in the HS extract are readily detected by KELA (26) but not in Western blots, in which protein-antibody interactions are either poorly resolved or completely inhibited by the presence of LPS (23).

Goat serum 48, a polyclonal antiserum obtained after hyperimmunization with a sonicated extract of strain RB51, produced discrete bands with a variety of antigens presumed to be proteins in strain RB51 WCE (see Fig. 2, lane 4). The lowermost band (13 to 14 kDa) probably represented the rough LPS, since Bru 48 produced a single band with WCE at the same position (data not shown).

Statistical methods. A mean value for each spleen count was obtained following log conversion (34). Statistical analyses were performed with Student's *t* test, which was modified when required for the occurrence of unequal variances between experimental groups (45). Log units of protection were obtained by subtracting mean counts for the principal group from the mean for the corresponding control group.

RESULTS

Properties of strain RB51 as a vaccine in mice. Experiments were performed to confirm reports (4, 44) that optimal protection against *B. abortus* in mice required i.p. vaccination of strain RB51 in numbers exceeding 10^8 CFU. When mice were inoculated i.p. with 3×10^8 CFU of RB51, there was a steady decline in numbers, so that by week 5 p.i., counts averaged well below 100 CFU per spleen (Fig. 1). An optimal vaccine dose was established by testing protection against

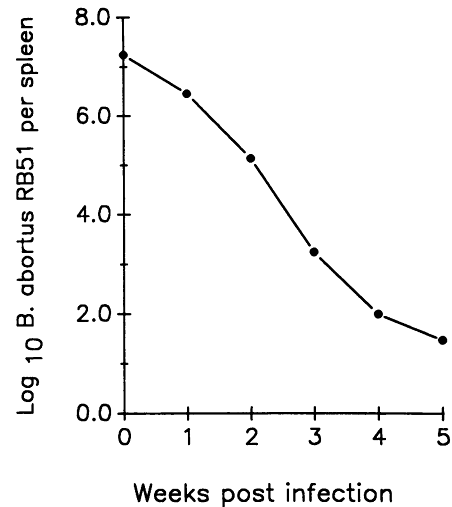

FIG. 1. Growth curve of *B. abortus* RB51 in spleens of BALB/c mice following i.p. inoculation of approximately 3×10^8 CFU. The first interval tested was at 1 h p.i. ($n = 5$). Standard deviations did not exceed 0.11 and are not shown.

strain 2308 in mice vaccinated i.p. with graded quantities of strain RB51. Protection decreased progressively from the highest vaccine dose (3×10^8 CFU) to the lowest (3×10^6 CFU) (Table 1, experiment 1). In another experiment, vaccination i.p. with 3×10^8 CFU of strain RB51 provided much better protection against strain 2308 than s.c. vaccination with the same dose either once or twice (Table 1, experiment 2). Finally, one vaccination i.p. with live RB51 (3×10^8 CFU) provided good protection against strain 2308 in the absence of DTH responses, whereas two vaccinations s.c. with 30 μg of strain RB51 WKC (ca. 1.5×10^9 bacteria per dose) in QS-21 adjuvant induced DTH but provided no protection (Table 1, experiment 3). Levels of OPS antibodies in both vaccinated groups were extremely low (mean KELA slopes, <10) and did not differ significantly from those of the control group (Table 1, experiment 3). A repetition of this experiment yielded identical results. The vaccination protocol was therefore set at one inoculation of 3×10^8 CFU of strain RB51, followed by challenge infection 5 weeks later.

Vaccination trials with heterologous challenge strains. Vaccination with strain RB51 not only provided protection against *B. abortus* 2308, but also conferred over 2 log units of protection ($P < 0.01$) to mice challenged with *B. melitensis* 16M or *B. ovis* PA (Table 2). These results are representative of four experiments. The occurrence of larger variances in splenic counts of vaccinated groups than in those of control groups (Table 2) has been noted previously (26, 34, 52). However, in the data in Table 2, there were no overlaps between individual counts of vaccinated and control groups, and individual counts for vaccinated mice were in every instance more than 10-fold lower than the mean count for the corresponding control group. Vaccination with strain RB51 caused no increase over control values of antibodies specific for the A epitope of OPS, following challenge with *B. abortus* 2308 (Table 2, groups 1 and 2). However, vaccination did cause anamnestic antibody responses following challenge with both *B. melitensis* 16M (Table 2, groups 3 and 4) and *B. ovis* PA (Table 2, groups 5 and 6). In mice vaccinated with strain RB51 and challenged with *B. melitensis* 16M (group 3), antibodies in terminal bleeds were demonstrable by Western blots against

TABLE 1. Effects of antigen dose, route of inoculation, and live versus killed bacteria on protection provided to BALB/c mice against *B. abortus* 2308 by vaccination with strain RB51[a]

Expt no.	Treatment group ($n = 5$)	Vaccine and dose (CFU)	Route of vaccination	Log_{10} *B. abortus* 2308 in spleen[b] ($\bar{x} \pm$ SD)	Log units of protection
1	1	RB51 live, 3×10^8	i.p.	4.77 ± 0.37***	1.42
	2	RB51 live, 3×10^7	i.p.	5.21 ± 0.21**	0.98
	3	RB51 live, 3×10^6	i.p.	5.46 ± 0.37*	0.73
	4	None (PBS)	i.p.	6.19 ± 0.51	
2	1	RB51 live, 3×10^8	i.p.	4.94 ± 0.58**	1.72
	2	RB51 live, 3×10^8	s.c., once	6.21 ± 0.35*	0.45
	3	RB51 live, 3×10^8	s.c., twice	5.97 ± 0.46*	0.69
	4	None (PBS)		6.66 ± 0.16	
3[c]	1	RB51 live, 3×10^8	i.p.	4.95 ± 0.68**	1.61
	2	RB51 killed, 30 μg + adjuvant	s.c., twice	6.34 ± 0.11†	0.22
	3	None (PBS)		6.56 ± 0.12	

[a] Mice were vaccinated once with live bacteria or twice at an interval of 4 weeks with killed cells. Five weeks after the first vaccination, mice were challenged i.v. with *B. abortus* 2308 (5×10^4 CFU), and spleens were cultured 1 week later. Footpad reactions for DTH were read just before death, 48 h after the injection of BASA.
[b] In comparison with control groups: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; †, not significant.
[c] DTH reactions were as follows (mean units of difference in footpad thickness ± standard deviation): group 1, 1.7 ± 0.8; group 2, 3.9 ± 0.7; group 3, 1.4 ± 0.8. KELA slope (10^3) of terminal bleeds with RB51 WKC as antigen: group 1, 45.8 ± 5.0; group 2, 65.6 ± 7.9; group 3, 11.0 ± 14.2. KELA slope (10^3) of terminal bleeds with *Y. enterocolitica* O:9 as antigen: group 1, 9.6 ± 7.4; group 2, 8.2 ± 1.3; group 3, 5.4 ± 2.8.

the OPS of *B. melitensis* and to two high-molecular-weight protein antigens of strain RB51 (Fig. 2, lanes 10 and 11). Mice vaccinated with strain RB51 and challenged with *B. ovis* (group 5) displayed more extensive antibody responses to protein antigens of strain RB51 (Fig. 2, lane 7; most bands are poorly reproduced at only one end of the blot) and a strong response to the rough LPS of *B. ovis* (Fig. 2, lane 6). Responses were minimal or absent in sera of unvaccinated mice in groups 4 and 6 (Fig. 2, lanes 8, 9, 12, and 13).

Since activated macrophages have an essential role in protection against *B. abortus* (11, 29), we determined the possible contribution to protection of residual activated macrophages in spleens of vaccinated mice at the time of challenge, using the antigenically unrelated intracellular parasite *L. monocytogenes*. Mice vaccinated 5 weeks earlier with strain RB51 and unvaccinated controls were challenged i.v. with *L. monocytogenes*, followed 1 day later by enumeration of *Listeria* organisms in the spleens. In two experiments, the log units of protection were extremely low (0.12, not significant; 0.13, $P < 0.01$) (Table 3), in comparison with 2 to 3 log units of protection against *L. monocytogenes* observed in spleens of mice undergoing acute infections with *B. abortus* 19 or 2308 (2, 21).

Passive transfer of immunity. Vaccination with both *B. abortus* RB51 and 19 induced T cells that were protective against *B. abortus* 2308 (Table 4). Although log units of protection obtained with T cells of strain 19 were higher, protection provided by both preparations was highly significant ($P < 0.001$). Sera from donors vaccinated with strain 19 contained high concentrations of OPS-specific antibodies (KELA slope [10^3] = 257) that were protective against 2308, in accord with prior results (3), whereas vaccination with strain RB51 failed to induce antibodies protective against smooth strain 2308 (Table 4).

T cells from donors vaccinated with strain RB51 provided significant protection against *B. melitensis* 16M (0.64 log units, $P < 0.01$), and yet the same preparation of T cells was completely ineffective against *B. ovis* PA (Table 4). In contrast, T cells from Rev 1-vaccinated donors provided protection against both strains (Table 4). This result was repeated in another experiment in which strain Rev 1-induced T cells were

TABLE 2. Protection against *B. abortus*, *B. melitensis*, and *B. ovis* provided to BALB/c mice by vaccination with live strain RB51[a]

Treatment group ($n = 5$)	Vaccine[b]	Challenge strain	KELA slope (10^3) of terminal bleeds[c] ($\bar{x} \pm$ SD)	Log_{10} brucellae in spleen ($\bar{x} \pm$ SD)[d]	Log units of protection
1	+	*B. abortus* 2308	18 ± 2	4.70 ± 0.49***	1.72
2	–	*B. abortus* 2308	16 ± 4	6.42 ± 0.06	
3	+	*B. melitensis* 16M	114 ± 35	4.70 ± 0.63**	2.03
4	–	*B. melitensis* 16M	42 ± 8	6.73 ± 0.17	
5	+	*B. ovis* PA	139 ± 16	4.88 ± 1.16**	2.30
6	–	*B. ovis* PA	39 ± 13	7.18 ± 0.40	

[a] Groups 1, 3, and 5 were vaccinated i.p. with 3×10^8 CFU of strain RB51. Five weeks later, mice were challenged i.v. with *B. abortus* 2308 (5×10^4 CFU), *B. melitensis* 16M (5×10^4 CFU), or *B. ovis* PA (5×10^3 CFU). Spleens of groups 1 and 2 were cultured at 1 week and spleens of groups 3 to 6 were cultured at 2 weeks after challenge. Results are representative of four experiments.
[b] +, vaccinated; –, injected with PBS.
[c] KELA data represent responses to *Y. enterocolitica* O:9 LPS (groups 1 and 2), *B. melitensis* 16M LPS (groups 3 and 4), and *B. ovis* HS (groups 5 and 6). When strain RB51 WKC was used as the antigen, slopes (10^3) were as follows: group 1, 53 ± 5; group 2, 12 ± 2; group 3, 72 ± 9; group 4, 11 ± 3; group 5, 53 ± 5; group 6, 17 ± 4.
[d] In comparison with control groups: ***, $P \leq 0.001$, **, $P \leq 0.01$.

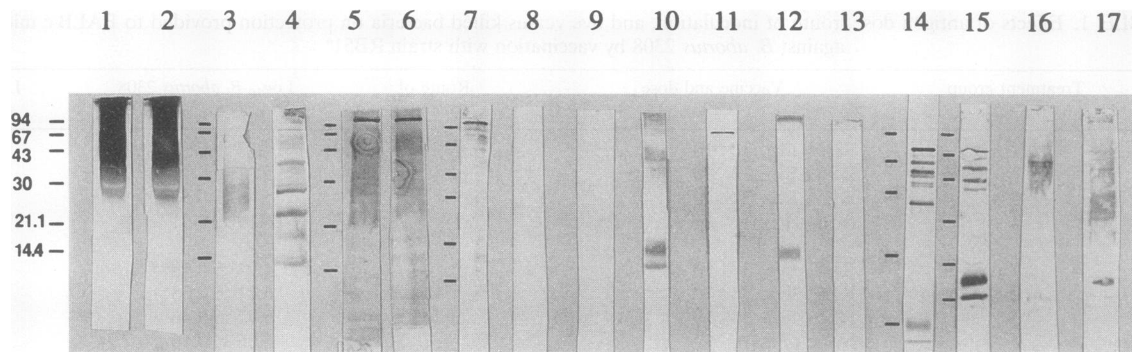

FIG. 2. Western blot analyses of serum pools from principal and control groups that were vaccinated with strain RB51 and challenged with heterologous *Brucella* spp. (Table 2, groups 3, 4, 5, and 6), and immune serum pools used in passive transfer experiments (Table 4). Lanes: 1, *B. abortus* 2308 LPS/Bru 38; 2, *B. melitensis* 16M LPS/Bru 38; 3, strain RB51 LPS/Bru 48; 4, strain RB51 WCE/goat 48; 5, *B. ovis* HS/Bru 48; 6, *B. ovis* HS, group 5; 7, strain RB51 WCE/group 5; 8, *B. ovis* HS/group 6; 9, strain RB51 WCE/group 6; 10, *B. melitensis* 16M LPS/group 3; 11, strain RB51 WCE/group 3; 12, *B. melitensis* 16M LPS/group 4; 13, strain RB51 WCE/group 4; 14, strain RB51 WCE/strain RB51 immune serum; 15, *B. melitensis* 16M LPS/strain RB51 immune serum; 16, strain RB51 LPS/strain RB51 immune serum; 17, *B. melitensis* 16M LPS/strain Rev 1 immune serum. Standard size markers (in kilodaltons at left) are indicated for each separate gel.

protective against *B. ovis* PA (0.47 log units, $P < 0.01$), while strain RB51-induced T cells were not (−0.05 log units).

Vaccination with strain RB51 induced only low levels of antibodies (KELA slope [10^3] = 54) that conferred low levels of protection against *B. melitensis* 16M and *B. ovis* PA (Table 4). Western blot analysis of this serum pool revealed antibodies against protein antigens of strain RB51 and *B. melitensis* 16M (Fig. 2, lanes 14 and 15). Antibodies specific for the rough LPS of strain RB51 were also detected (Fig. 2, lane 16), but antibodies for the OPS of *B. abortus* 2308 (data not shown) or *B. melitensis* 16M (Fig. 2, lane 15) were not. High concentrations of antibodies (KELA slope [10^3] = 169) from strain Rev 1-vaccinated mice, which were strongly protective against *B. melitensis* 16M (Table 4), were specific predominantly for the OPS (Fig. 2, lane 17), and therefore failed to protect against *B. ovis* PA (Table 4), which lacks OPS.

DISCUSSION

Vaccination with *B. abortus* rough mutant RB51 consistently protected BALB/c mice against challenge with virulent strains of homologous as well as heterologous *Brucella* species (Table 2). Passive-transfer experiments demonstrated that protection was immunologically specific (Table 4), although nonspecific effects due to residual activated macrophages in the spleen at the time of challenge may have contributed marginally to protection (Table 3).

The same preparation of immune T cells from strain RB51-vaccinated donors donors which failed to protect against *B. ovis* PA protected against a 10-fold-higher challenge dose of *B. melitensis* 16M as effectively as did T cells from donors vaccinated with *B. melitensis* Rev 1 (Table 4). Epitopes critical for protective cell-mediated immunity were thus shared between strain RB51 and *B. melitensis* 16M but may not have been shared between strain RB51 and *B. ovis* PA. If this were so, the potential usefulness of strain RB51 as a vaccine against *B. ovis* would be in question, assuming that strain PA was representative of the species. The antigens of strain RB51 responsible for the induction of protective T cells are unknown. However, the generation of protective, DTH-negative T cells with live bacteria and nonprotective, DTH-positive T cells with killed organisms (Table 1, experiment 3) suggests that protective antigens are induced during the course of infection and are lost upon cultivation in vitro.

The modest levels of protection transferrable against *B. melitensis* 16M and *B. ovis* PA from donors vaccinated with strain RB51 (Table 4) contrast with the high levels of protection afforded to vaccinated mice challenged directly with these strains (Table 2). A partial explanation for this difference may be the fact that vaccination with strain RB51 in itself induced only low levels of protective antibodies against the heterologous strains (Table 4) but primed the mice to produce anamnestic antibody responses following challenge with *B. melitensis* 16M or *B. ovis* PA (Table 2). The passive-transfer experiments conducted here (Table 4) would not have detected antibody-mediated protection dependent to such a pronounced degree on anamnestic reactions. To have done so would have required transfers of both B cells and T cells from vaccinated donors or of antisera taken after the anamnestic response had been generated.

While sera of mice vaccinated with strain RB51 provided a low level of protection against *B. melitensis* 16M (Table 4) in the absence of detectable OPS-specific antibodies (Fig. 2, lane 15), challenge with *B. melitensis* 16M caused an anamnestic response, with the formation of OPS-specific antibodies which were probably highly protective (Table 2, group 3; Fig. 2, lane 10). Trace quantities of OPS, which may occur in strain RB51, would have sufficed to prime the mice. Strain RB51 vaccination sometimes primed mice for secondary responses to the OPS of *B. abortus* (data not shown). However, such priming was not

TABLE 3. Effects of vaccinating BALB/c mice with strain RB51 on clearance of *L. monocytogenes* from the spleen[a]

Expt no.	Treatment group	Vaccination with strain RB51[b]	Log_{10} *L. monocytogenes* in spleen ($\bar{x} \pm SD$)[c]	Log units of protection
1	1	+	5.92 ± 0.17†	0.12
	2	−	6.04 ± 0.16	
2	1	+	6.24 ± 0.08**	0.13
	2	−	6.37 ± 0.06	

[a] Mice were vaccinated i.p. with strain RB51 (3×10^8 CFU) 5 weeks prior to challenge i.v. with 1×10^5 CFU of *L. monocytogenes* EGD, a dose which is lethal by day 4 p.i. (2). *L. monocytogenes* was enumerated in the spleens 1 day after challenge infection.

[b] +, vaccinated; −, injected with PBS.

[c] In comparison with control groups: **, $P < 0.01$; †, not significant.

TABLE 4. Passive transfer of protection to BALB/c mice against *B. abortus* 2308, *B. melitensis* 16M, and *B. ovis* PA by T cells and serum from donors vaccinated with live strain RB51, 19, or Rev 1

T cells or serum transferred[a]	Challenge strain	Passive transfer of protection[b]			
		T cells		Serum	
		Log_{10} brucellae in spleen ($\bar{x}$ ± SD)[c]	Log units of protection	Log_{10} brucellae in spleen ($\bar{x}$ ± SD)	Log units of protection
RB51	*B. abortus* 2308	5.86 ± 0.10***	0.57	6.41 ± 0.12†	0.02
Strain 19	*B. abortus* 2308	5.39 ± 0.22***	1.04	4.78 ± 0.16***	1.65
None	*B. abortus* 2308	6.43 ± 0.05		6.43 ± 0.05	
RB51	*B. melitensis* 16M	6.08 ± 0.11**	0.64	6.45 ± 0.06**	0.35
Rev 1	*B. melitensis* 16M	6.25 ± 0.10**	0.47	3.78 ± 0.24***	3.02
None	*B. melitensis* 16M	6.72 ± 0.23		6.80 ± 0.15	
RB51	*B. ovis* PA	7.52 ± 0.13	0.00	6.98 ± 0.39*	0.49
Rev 1	*B. ovis* PA	7.19 ± 0.21*	0.33	7.46 ± 0.36†	0.01
None	*B. ovis* PA	7.52 ± 0.13		7.47 ± 0.17	

[a] Donor groups were vaccinated i.p. with strain RB51 (3×10^8 CFU) or i.v. with strain 19 (5×10^4 CFU) or Rev 1 (5×10^4 CFU) 5 weeks prior to sacrifice. One group of donors and recipients were used to test protection against strain 2308. Another group of donors provided T cells and antisera for the *B. melitensis* 16M and *B. ovis* PA challenge trials, with T cells being assessed against both strains in the same experiment and antisera in a later experiment. The compositions of the transferred cell populations were as follows. Strain RB51 donors (*B. abortus* 2308 challenge): $CD4^+$, 58%; $CD8^+$, 22%; surface Ig^+, 0.8%, Mac-1^+, 11%. Strain 19 donors (*B. abortus* 2308 challenge): $CD4^+$, 64%; $CD8^+$, 23%; surface Ig^+, 0.7%; Mac-1^+, 6%. Strain RB51 donors (*B. melitensis* 16M and *B. ovis* PA challenge): $CD4^+$, 59%; $CD8^+$, 24%; surface Ig^+, 0.4%; Mac-1^+, 12%. Strain Rev 1 donors (*B. melitensis* 16M and *B. ovis* PA challenge): $CD4^+$, 58%; $CD8^+$, 31%; surface Ig^+, 0.4%; Mac-1^+, 10%. KELA slopes (10^3) of the donor serum pools were as follows: strain RB51 donors (*B. abortus* 2308 challenge); 58 using strain RB51 WKC as antigen; strain 19 donors (*B. abortus* 2308 challenge), 257 using *Y. enterocolitica* O:9 as antigen; strain RB51 donors (*B. melitensis* 16M and *B. ovis* PA challenge), 54 using strain RB51 WKC as antigen; strain Rev 1 donors (*B. melitensis* 16M and *B. ovis* PA challenge), 169 using *B. melitensis* 16M LPS as antigen.

[b] Recipient groups ($n = 5$) received T cells (3×10^7) or antiserum (0.1 ml) i.v. 1 to 2 h before i.v. challenge with *B. abortus* 2308 (5×10^4 CFU), *B. melitensis* 16M (5×10^4 CFU), or *B. ovis* PA (5×10^3 CFU). Spleens were cultured 1 week (*B. abortus*) or 2 weeks (*B. melitensis* and *B. ovis*) later. None of the principal groups that received T cells developed antibody responses against the challenge strain that exceeded those of the corresponding control group.

[c] In comparison with control groups: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; †, not significant.

noted in rabbits, goats, or cattle (13, 44) and was not usual in mice (Table 1, experiment 3; Table 2, group 1). In mice vaccinated with strain RB51 and challenged with *B. ovis* PA (Table 2, group 5), protective antibodies would presumably have been specific for outer membrane proteins, and possibly the rough LPS, in accord with prior findings (26). Low levels of protection against a smooth strain of *B. abortus* by antibodies specific for outer membrane proteins (16) or rough LPS (17) have previously been noted, which may explain the limited protection provided against *B. melitensis* 16M by strain RB51 antiserum (Table 4).

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