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Protective Immunity to *Brucella ovis* in BALB/c Mice following Recovery from Primary Infection or Immunization with Subcellular Vaccines

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Experiments were performed with BALB/c mice to elucidate the roles of humoral and cell-mediated immune responses in the acquisition of protective immunity to *Brucella ovis* and to compare infection immunity with immunity developed through vaccination with a hot saline extract (HS) of *B. ovis*. Mice convalescing from a primary infection with *B. ovis* displayed a high level of resistance to reinfection, as evidenced by splenic bacterial counts decreased over 10,000-fold from control groups at 2 weeks after challenge. Passive transfer assays revealed that protection was mediated by both T lymphocytes and antibodies but that antibodies had a substantially greater role on the basis of log units of protection that were transferred. Antibodies specific for HS proteins in sera from convalescent mice were predominantly of the immunoglobulin G 2a and 3 isotypes. Vaccination with HS conferred good protection against *B. ovis*, but protection was greatly enhanced by the incorporation of QS-21 or other adjuvants. Protection provided by the HS vaccine resulted largely from immune responses to its protein moieties. A critical evaluation of the protective efficacy of the rough lipopolysaccharide component of HS was precluded by its poor immunogenicity in BALB/c mice. HS–QS-21 afforded protection against challenge infection with *B. ovis* as good as that which developed after a primary infection and as good as or better than that provided by attenuated *Brucella melitensis* vaccine strain Rev 1. Passive transfer experiments confirmed that the magnitudes of both humoral and cell-mediated forms of protective immunity were equivalent in mice vaccinated with HS–QS-21 and those recovering from a primary infection. Protective immunity to *B. ovis* in mice therefore resembled that to *Brucella abortus*, except that the relative roles of humoral and cell-mediated immunity, rather than being equivalent, were shifted toward a greater role for antibodies.

Brucella ovis causes a disease in sheep characterized by epididymitis and reduced fertility in rams (12, 13, 39) and by abortion in ewes (28, 32, 35). Ovine brucellosis is recognized as the cause of major economic losses in countries in which sheep husbandry is an important industry (14). Vaccination is the only method of control suitable for countries with a high incidence of this disease (6), and at present, live attenuated *Brucella melitensis* Rev 1 is still the strain most widely used for this purpose (9, 21, 25, 33). However, Rev 1 produces serological responses that interfere with the diagnosis of both *B. ovis* and *B. melitensis* infections (9, 26, 42) and is pathogenic for humans (7). Moreover, its use is now prohibited in countries free of *B. melitensis*. Numerous attempts to develop killed vaccines that are as effective as Rev 1 have met with limited success (1, 11, 15, 34, 50).

B. ovis is a natural rough species and so lacks the O polysaccharide that constitutes the principal surface antigen of smooth *Brucella* species (17). A hot saline extract (HS) obtained from *B. ovis* REO 198 contained rough lipopolysaccharide (R-LPS) and several proteins, including abundant group 3 outer membrane proteins (16, 24, 41). Rams infected with *B. ovis* produced antibody responses to the R-LPS and proteins in the HS (24, 40, 41). Therefore, we believed that one or more HS antigens, particularly the surface-exposed group 3 protein, might induce a protective immune response to *B. ovis*. In fact, we have preliminary evidence that HS incorporated with an adjuvant induced significant levels of protection in rams against *B. ovis* (8).

Despite the recognized economic importance of ovine brucellosis and ongoing efforts to develop alternative vaccines to Rev 1, nothing is known about the nature of protective immunity to this disease. This stands in marked contrast to *Brucella abortus*, for which the mouse model has been extensively employed in recent years to study a variety of host-parasite interactions and the development of protective immunity (5). The objectives of the present work were, first, to use the mouse model in order to define the roles of humoral and cell-mediated immune responses in the elimination of *B. ovis* in a primary infection. We then wished to evaluate the protective qualities of the HS vaccine and to compare the magnitude of protective immunity acquired through vaccination with that which developed during a primary infection. Our selection of a vaccine for a future field trial with rams would depend upon its ability to induce protection in both humoral and cell-mediated compartments at least as good as that which followed recovery from disease.

MATERIALS AND METHODS

Mice. Female BALB/cByJ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) 1 week before use. Mice to be used in vaccination experiments were shipped at 5

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weeks of age, while animals scheduled for passive transfer experiments were shipped at 9 weeks of age.

Bacterial strains. *B. ovis* PA was obtained from J. M. Verger (Station de Pathologie Infectieuse et Immunité, Institut National de la Recherche Agronomique, Nouzilly, France). *B. melitensis* 16M and Rev 1 were supplied by G. G. Schurig (Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Va.). *B. ovis* PA and *B. melitensis* 16M were used as challenge strains after two serial passages in BALB/c mice and isolation in pure culture from spleens. Stock cultures were prepared from 48-h growth on Schaedler blood agar plates and were stored at −70°C in Albimi broth (Difco Laboratories, Detroit, Mich.). For infection, contents of freshly thawed vials were diluted with sterile phosphate-buffered saline (PBS) to the desired concentration. Exact numbers of cells were established retrospectively by viable counts (36).

Antigens for vaccines and immunoassays. HS from *B. ovis* REO 198 was obtained as described previously (17, 41) by suspending live cells in physiological saline (10 g of packed cells per 100 ml) and heating the suspensions in flowing steam for 15 min. After centrifugation at 12,000 × *g* for 15 min, the supernatant was dialyzed for 2 days at 4°C against several changes of distilled deionized water. The dialyzed material was centrifuged for 5 h at 100,000 × *g*, and the pellet (HS) was suspended in distilled deionized water and lyophilized. The product produced a typical profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with group 3 outer membrane proteins by far the dominant component (data not shown). HS proteins (HS-PCP) were purified by extraction of 2 mg of HS with 7 ml of a mixture of petroleum ether, chloroform, and phenol (PCP) (23) for 1 min at 16,000 rpm in a homogenizer (Sorvall Omnimixer; Dupont Co., Wilmington, Del.). After centrifugation at 12,000 × *g*, the sediment (HS-PCP) was washed once and then suspended in distilled deionized water and lyophilized (41).

R-LPS was obtained from HS by elimination of proteins with the PCP protocol followed by precipitation of R-LPS from the phenol phase with water (41). The final product had a protein content of 0.6% (dry weight) and a 2-keto-3-deoxyoctulosonic acid content of 2.8% (dry weight), which represented an LPS content of approximately 51% (37).

Adjuvants. QS-21, a purified derivative of saponin (31), was a gift from Cambridge Biotech Corp. (Worcester, Mass.). The pluronic polyol L-121 was supplied by the BASF Wyandette Co. (Parsippany, N.J.), and the purified oleic ester Montanide 888 was a gift from Seppic, Inc. (Paris, France). *N*-Acetylmuramyl-L-threonyl-D-isoglutamine (tMDP) was a gift from Syntex, Inc. (Palo Alto, Calif.). Squalane was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Preparation and administration of vaccines. Subcellular vaccines were solubilized in PBS and injected subcutaneously (s.c.) over the back or into the hind footpads, with or without adjuvant, in a final volume of 0.1 ml. QS-21 in aqueous solution was mixed with the antigen to a concentration of 150 μg/ml (15 μg per dose). An emulsion of L-121, squalane, and antigen in PBS with 0.2% Tween 80 (PBS-Tween) was prepared as described previously (53). A dose of vaccine contained 20 μg of antigen, 2.5 μg of L-121, 5 μl of squalane, and 95 μl of PBS-Tween. Stable emulsions of antigen with Montanide 888 were produced by vortexing the adjuvant with squalane and antigen solution for 7 min. A dose of vaccine contained 20 μg of antigen, 5 μl of Montanide 888, 45 μl of squalane, and 50 μl of PBS. tMDP was incorporated into some of the vaccines to a concentration of 50 μg per mouse.

B. melitensis Rev 1 was inoculated s.c. in a volume of 0.1 ml containing 5×10^4 CFU.

Blood sampling. Mice were bled under light anesthesia from the retroorbital sinus or the heart. Serum samples were stored at −70°C unless used for passive transfer assays immediately upon separation.

Quantitation of bacterial numbers in the spleen. Mice were killed by CO_2 asphyxiation. Spleens were homogenized, diluted serially, and plated (36). Colonies were counted after incubation for 3 days (*B. melitensis*) or 4 to 5 days (*B. ovis*) at 37°C under 10% CO_2. In groups vaccinated with Rev 1, numbers of Rev 1 cells were determined by doing counts on parallel sets of plates that were either incubated without CO_2 (to exclude *B. ovis*) or contained 2.5 μg of streptomycin per ml (to exclude *B. melitensis* 16M) (2).

T-lymphocyte enrichment. B cells were depleted by a single negative panning step by using the technique described by Araya et al. (3) without modification. Percentages of B cells (surface immunoglobulin [Ig] positive) and of $CD4^+$, $CD8^+$, and Mac-1^+ cells were determined by immunofluorescence assays (3).

Experimental design. (i) Time course of infection. Mice (6 weeks old) were inoculated intravenously (i.v.) with 5×10^3 CFU of *B. ovis* PA or 5×10^4 CFU of *B. melitensis* 16M in 0.1 ml of PBS. At selected times postinoculation (p.i.), groups of mice ($n = 5$ unless noted otherwise) were killed, and spleens were processed for quantitative counts.

(ii) Vaccination and challenge trials. Mice (6 weeks old) in groups of five were vaccinated either once with live Rev 1 or twice at an interval of 4 weeks with subcellular vaccines. Five weeks after the first vaccination, mice were challenged by i.v. inoculation of *B. ovis* PA or *B. melitensis* 16M. Two weeks after challenge, mice were killed and spleen counts were performed. In most experiments, blood samples were taken prior to vaccination, immediately before challenge, and at the time of sacrifice.

(iii) Passive transfer assays. Donor mice at 10 weeks of age were either vaccinated twice with HS–QS-21 or inoculated i.v. with 5×10^3 CFU of *B. ovis* PA. Five weeks after the first vaccination and at selected times after infection, donor groups were exsanguinated and T-lymphocyte suspensions were prepared from their spleens or popliteal lymph nodes. 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 (3). After 1 h, recipients were challenged i.v. with 5×10^3 CFU of *B. ovis* PA, and 2 weeks thereafter, mice were killed and spleen counts were made. Control groups received PBS since it had been established that there were no differences in splenic numbers of *B. ovis* 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 T cells prior to inoculation with *B. ovis* (difference in mean counts, <0.1 $\log_{10}$ unit). Recipient groups receiving T cells were bled prior to sacrifice.

KELA. Antibodies against HS and R-LPS were measured in an indirect, computer-assisted kinetics-based enzyme-linked assay (KELA), as described previously (53), with a goat anti-mouse IgM, IgG, and IgA horseradish peroxidase conjugate (Cappel, Organon Teknika, Durham, N.C.). In selected tests, goat anti-mouse horseradish peroxidase conjugates specific for individual isotypes (IgM, IgG1, IgG2a, IgG2b, and IgG3) (Southern Biotechnology Associates, Birmingham, Ala.) were used under the same conditions. The rate of the reaction between substrate solution and enzyme, expressed as slope, is directly proportional to the amount of antibody in the sample (51) and was determined from linear regression analysis of time versus absorbance. Data from assays performed on sep-

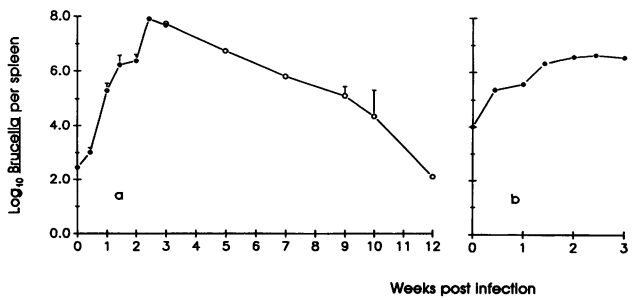

FIG. 1. (a) Growth curve of twice-passaged *B. ovis* PA in spleens of BALB/c mice following i.v. inoculation of approximately 5×10^3 CFU. Solid and open circles represent separate experiments for which *n* at each time point was 5 and 3. The first interval tested was at 1 h p.i. Growth was not detected in any of the spleens at 12 weeks p.i., so a $\log_{10}$ value of 2.12 was assigned to the group according to the previous convention (36). (b) Growth curve of twice-passaged *B. melitensis* 16M in spleens of BALB/c mice following i.v. inoculation of approximately 5×10^4 CFU. The first interval tested was at 1 h p.i. ($n = 5$). Bars, standard deviations.

arate plates and on different days were normalized by regression analysis from a standard curve obtained by inclusion of the same set of standards on each plate. On the basis of the linear relationship between the log of slope values and antibody titers (53), slope values (×1,000) of >150 corresponded to high antibody titers, 50 to 150 slope units corresponded to moderate titers, and 15 to 50 units corresponded to low titers. Prior to infection or vaccination, slope values (×1,000) for HS and R-LPS averaged 10 and 12, respectively.

Statistical methods. A mean value for each spleen count was obtained by averaging the triplicate values following log conversion (36). Log units of protection were calculated by subtracting individual counts of the principal group from the mean count of the corresponding control group (3). Statistical analyses were performed with Student's *t* test (48).

RESULTS

Growth patterns of *B. ovis* PA and *B. melitensis* 16M. After two passages in mice, *B. ovis* PA grew readily in the spleen. Low inoculum numbers became established and increased by approximately 5 logs during the first 2 1/2 weeks p.i., followed by a gradual decline (Fig. 1a). The plateau at 2 weeks p.i. (Fig. 1a) was not reproduced in subsequent experiments in which counts at 2 weeks p.i. were at least 1 log higher (Table 1 and see Tables 4 to 6). The challenge dose selected for most experiments was 5×10^3 CFU, with an interval of 2 weeks between challenge and sacrifice. In some experiments, a 10-fold-higher challenge dose was used, resulting in splenic cell numbers exceeding 10^8 by 2 weeks p.i. (see Tables 3 and 5).

Twice-passaged *B. melitensis* 16M produced a growth curve over the first 3 weeks p.i. resembling that of virulent *B. abortus* 2308 (36). Numbers increased during the first 1 1/2 weeks p.i., peaked at levels much lower than those of *B. ovis* PA, and then plateaued (Fig. 1b). A challenge dose of 5×10^4 CFU of strain 16M was used with a challenge-to-sacrifice interval of 2 weeks.

Rev 1 replicated actively in the spleens of BALB/c mice during the first 2 weeks p.i. (data not shown), in accord with prior observations (10).

None of these strains produced clinical signs of disease.

Infection immunity. Mice convalescent from infection with *B. ovis* PA were highly resistant to reinfection with the homologous strain. In separate experiments, groups of mice challenged with *B. ovis* PA (5×10^3 CFU) 10 1/2 or 13 weeks after an initial infection exhibited 4.57 and 5.39 log units of protection ($P < 0.001$), respectively, compared with control groups of naive mice.

Immunity through vaccination with HS. Mice were vaccinated with graded quantities of HS, with and without QS-21. High levels of protection were obtained with 20 or 2 μg of HS incorporated with QS-21, whereas only the higher dose of antigen was protective in the absence of adjuvant (Table 1). A vaccine composed of 20 μg of HS plus QS-21 was selected for subsequent studies. A clear association existed among the groups between the concentration of circulating HS-specific antibodies at the time of challenge and the level of protection obtained (Table 1). The presence of QS-21 resulted in large and highly significant ($P < 0.01$ to $P < 0.001$) increases in antibodies of the IgG1, IgG2a, and IgG2b isotypes (Table 2). A similar distribution of isotypes occurred in three other serum pools of mice that had been vaccinated with HS–QS-21, except that the level of IgG3 antibodies was substantially higher (Table 2). Whereas IgG2a and IgG2b antibodies predominated in mice vaccinated with HS–QS-21, infection with *B. ovis* induced predominantly IgG2a and IgG3 antibodies, with IgG1 at the lowest level (Table 2).

In another experiment with a 10-fold-higher challenge dose, inclusion of QS-21 with HS resulted in significantly higher antibody levels ($P < 0.001$) and significantly better protection than that provided by HS alone ($P < 0.01$) or Rev 1 ($P < 0.01$) (Table 3). Incorporation of adjuvant L-121 or Montanide 888, with or without tMDP, also induced high concentrations of

TABLE 1. Protection against *B. ovis* provided by vaccination with graded quantities of HS with and without QS-21 adjuvant

Treatment group ($n = 5$)[a]	HS (μg)	QS-21[b]	KELA slope (10^3) at challenge ($\bar{x} \pm$ SD)[c]	Log_{10} units of *B. ovis* in spleen ($\bar{x} \pm$ SD)	Log units of protection[d]
1	20	+	406 ± 21	3.10 ± 0.01	4.11***
2	20	−	201 ± 50	4.52 ± 1.38	2.69**
3	2	+	282 ± 24	3.50 ± 0.65	3.71***
4	2	−	79 ± 23	6.97 ± 1.15	0.24†
5	0.2	+	60 ± 30	7.39 ± 0.20	−0.18†
6	0.2	−	25 ± 19	6.67 ± 1.34	0.54†
7	None (PBS)	−	4 ± 1	7.21 ± 0.46	

[a] Mice were vaccinated twice over a 4-week interval and challenged i.v. with 5×10^3 CFU of *B. ovis* PA 1 week after the second vaccination. Spleens were cultured 2 weeks later.

[b] +, with QS-21; −, without QS-21.

[c] KELA analyses were performed with HS antigen.

[d] ***$P \leq 0.001$ and **$P \leq 0.01$ compared with PBS control group; †, not significant.

TABLE 2. Isotype distribution of antibodies specific for HS antigens in sera of mice vaccinated with HS or infected with *B. ovis*

Treatment	Antibody isotype (slope [10^3])[a]				
	IgM	IgG3	IgG2b	IgG1	IgG2a
Vaccination					
HS (20 μg)–QS-21[b]	133 ± 104†	118 ± 54†	299 ± 73**	196 ± 53**	307 ± 44***
HS in PBS[c]	70 ± 35	101 ± 80	142 ± 77	91 ± 45	97 ± 54
HS (20 μg)–QS-21[d]	188 ± 48	240 ± 55	327 ± 75	179 ± 65	289 ± 60
B. ovis infection (5×10^3 CFU)[e]	51 ± 13	173 ± 34	111 ± 17	39 ± 17	221 ± 9

[a] KELA assays with HS antigen. Values are means ± standard deviations. †, not significant. **$P < 0.01$ and ***$P < 0.001$ compared with the group receiving HS in PBS.

[b,c] Treatment groups 1 and 2, respectively, from Table 1. The mice in each group were bled 1 week after the second vaccination.

[d] Three serum pools used in passive transfer assays. Mice were bled 1 week after the second vaccination. Pools were composed of serum samples from 10 to 20 donor mice.

[e] Three serum pools used in passive transfer assays. Mice were bled at 9, 10, and 13 weeks p.i., respectively. Each pool was composed of serum samples from 10 donor mice.

antibodies and high levels of protection (Table 3) that did not differ significantly from the values recorded for QS-21.

Both HS–QS-21 and live Rev 1 vaccines produced significant protection against *B. ovis* ($P < 0.001$ and $P < 0.01$, respectively) (Table 4), but protection provided by HS–QS-21 was significantly greater ($P < 0.01$). In contrast, the HS–QS-21 vaccine failed to provide any protection against *B. melitensis* 16M, while Rev 1 was highly protective (Table 4). A repetition of this experiment produced the same results, except that HS–QS-21 and Rev 1 gave equivalent protection against *B. ovis* (data not shown). In two further comparisons, protection against *B. ovis* PA provided by vaccination with HS–QS-21 was equivalent to, or significantly greater than, that which resulted from vaccination with Rev-1 (data not shown).

Vaccination with HS-PCP, the purified protein fraction of HS, provided protection against *B. ovis* as good as that with HS when a high challenge dose (5.8×10^4 CFU) was used, whereas purified R-LPS was completely ineffective (Table 5). The same results were obtained with HS and HS-PCP in two repetitions of this experiment with a 10-fold-lower challenge dose. With the lower challenge dose, R-LPS was also protective, although at levels 10-fold or more below those of HS-PCP (data not shown). Mice vaccinated with R-LPS produced extremely low antibody responses to that antigen, and in mice vaccinated with intact HS, the R-LPS-specific antibodies represented a negligibly small proportion of the total antibody response (Table 5 [other data not shown]). The immunogenicity of R-LPS could not be improved by coupling it to bovine serum albumin (BSA) (data not shown).

Passive transfer of immunity. T cells from spleens of convalescent donors (Table 6, group 1) provided significant protection against the homologous challenge strain (0.80 log unit, $P < 0.01$), and serum antibody levels in recipient group 1 were no different from those of the control group 7, thus ensuring that protection was not the result of antibodies produced by residual B cells in the transferred population. This result was repeated in a separate experiment with two other T-cell preparations (log units of protection, 1.17 [$P < 0.001$] and 0.92 [$P < 0.01$]). No protection was obtained in mice receiving splenic T cells from donors vaccinated s.c. over the back with HS–QS-21 (Table 6, group 3), although in a repetition of this experiment, some protection was demonstrated (0.58 log unit, $P < 0.05$). In contrast, popliteal lymph node T cells from mice vaccinated in the hind footpads provided excellent protection (1.32 log units, $P < 0.001$)(Table 6, group 5), and the low number of residual B cells that were transferred (0.9%) precluded a significant contribution from antibodies.

Antiserum pools from all three donor groups provided significant levels of protection ($P < 0.001$ to $P < 0.05$) that were much higher in log units than protection given by the corresponding pools of T cells (Table 6). In another experiment with the same antiserum pools, results were very similar (data not shown), except that protection with the group 4 serum (HS, s.c. vaccination) was higher and more consistent (3.31 log units, $P < 0.01$). In a separate experiment, antisera from convalescent or vaccinated mice again provided high levels of protection (ranging from 2.36 [$P < 0.01$] to 4.31 [$P < 0.001$] log units) that exceeded the protection obtained from the corresponding pools of T cells by 1.39 to 3.39 log units (data not shown).

TABLE 3. Protection against *B. ovis* provided by vaccination with HS in selected adjuvants

Treatment group ($n = 5$)[a]	Antigen	Adjuvant	KELA slope (10^3) at challenge ($\bar{x}$ ± SD)[b]	Log_{10} units of *B. ovis* in spleen ($\bar{x}$ ± SD)	Log units of protection[c]
1	HS (20 μg)	QS-21	425 ± 26	4.00 ± 1.07	4.10***
2	HS (20 μg)	L-121	384 ± 28	5.28 ± 1.40	2.82**
3	HS (20 μg)	L-121–tMDP	392 ± 38	4.14 ± 2.71	3.96*
4	HS (20 μg)	Montanide 888	309 ± 35	3.63 ± 1.92	4.47**
5	HS (20 μg)	Montanide 888-tMDP	328 ± 64	2.87 ± 1.93	5.23**
6	HS (20 μg)	None	186 ± 56	6.42 ± 0.88	1.68**
7	Rev 1 (live)	None	13 ± 6	7.08 ± 0.31	1.02**
8	None (PBS)	None	10 ± 2	8.10 ± 0.15	

[a] Groups 1 to 6 were vaccinated s.c. twice over a 4-week interval. Group 7 was inoculated s.c. on day 0 with 5×10^4 CFU of Rev 1. All mice were challenged i.v. with 5×10^4 CFU of *B. ovis* PA 5 weeks after the first treatment, and spleens were cultured 2 weeks after challenge.

[b] KELA analyses were performed with HS antigen.

[c] ***$P \leq 0.001$, **$P \leq 0.01$, and *$P \leq 0.05$ compared with the PBS control group.

TABLE 4. Comparison of vaccinations with HS and Rev 1 against challenge with *B. ovis* and *B. melitensis*

Treatment group ($n = 5$)[a]	Vaccine	Challenge	Log_{10} units of brucellae in spleen ($\bar{x} \pm SD$)	Log units of protection[b]
1	HS (20 μg)–QS-21	*B. ovis* PA	2.65 ± 1.14	4.69***
2	Rev 1 (live)	*B. ovis* PA	5.57 ± 0.82[c]	1.77**
3	None (PBS)	*B. ovis* PA	7.34 ± 0.12	
4	HS (20 μg)–QS-21	*B. melitensis* 16M	6.64 ± 0.30	0.04†
5	Rev 1 (live)	*B. melitensis* 16M	3.78 ± 0.73[c]	2.90***
6	None (PBS)	*B. melitensis* 16M	6.68 ± 0.10	

[a] Groups 1 and 4 were vaccinated twice over a 4-week interval. Groups 2 and 5 were vaccinated s.c. on day 0 with 5×10^4 CFU of Rev 1. Challenge inoculations of 5×10^3 CFU of *B. ovis* PA and 5×10^4 CFU of *B. melitensis* 16M were made i.v. Spleens were cultured 2 weeks after challenge.
[b] ***$P \leq 0.001$ and **$P \leq 0.01$ compared with PBS control groups. †, not significant.
[c] No Rev 1 colonies were detected in the spleens.

DISCUSSION

The data presented here demonstrate that the BALB/c mouse, recognized as a useful model for brucellosis caused by *B. abortus* (5), may also be used to advantage for studying *B. ovis*. Thus, *B. ovis* replicated readily in the spleen (Fig. 1a) and liver (29a) to produce a chronic and asymptomatic infection which was overcome through a potent immune response (Table 6). In a recent study with *B. ovis* PA in outbred CD1 mice, bacterial replication was limited or failed to occur (29). This was probably due in part to greater inherent resistance of the CD1 strain, which was also more resistant than BALB/c mice to *B. abortus* (52). In addition, it is likely that the twice-passaged strain used in this study was more virulent.

Although the status of *B. ovis* as a facultative intracellular bacterium capable of survival in macrophages has yet to be established, passive transfer experiments now demonstrate that, in mice, protection is partially attributable to cell-mediated immunity (Table 6). The identity of the protective cell type or types is not yet known. The induction by *B. ovis* of a dominant $CD4^+$, T_H1 population known to be protective against several protozoan agents (45) was suggested by the predominance in infected mice of IgG2a and IgG3 antibodies, coupled with minimal IgG1 responses (Table 3). *B. abortus*, which stimulates a polyclonal T_H1 response (22, 49), induces primarily IgG2a and IgG3 antibodies specific for covalently bound haptens (46) or *B. abortus* outer membrane proteins (19). Recent evidence (47) indicates that gamma interferon exerts control over IgG3 as well as IgG2a synthesis. Since there is now strong support for a protective function of natural killer cells (18) and γδ T cells (27, 43) in murine listeriosis, the involvement of one or both of these cell types in protection against *B. ovis* is also possible. However, excellent protection was transferred by lymph node lymphocytes in which $CD4^+$ and $CD8^+$ cells accounted for the total cell population (Table 6), indicating that these cell populations had an important role in providing cell-mediated immunity to *B. ovis*.

Whereas immune T cells were largely absent from the spleens of HS-vaccinated mice prior to challenge infection, T cells from the popliteal nodes of mice vaccinated in the hind footpads provided the highest level of protection of any T-cell preparation tested (Table 6 and other data). We believe that the paucity of immune T cells in the spleens of HS-vaccinated mice prior to challenge infection occurred because, during the first weeks after the two vaccinations, these cells would have localized predominantly in the inflamed draining lymph nodes, which would have contained substantial quantities of antigen absorbed from the injection sites. In like fashion, immune T cells would have localized in the spleens of mice undergoing active infection in that organ, including the spleens of the HS-vaccinated mice following i.v. challenge infection.

The remarkably high levels of protection obtained with antibodies in passive transfer assays (Table 6), orders of magnitude higher than those achieved against *B. abortus* (3, 4, 20), may have several explanations. In *B. abortus* infection, for example, passive protection by IgG antibodies against attenuated vaccine strain 19, which is readily killed within macrophages when opsonized by IgG antibodies, is consistently 1 log unit greater than that against virulent strain 2308, which is more resistant to killing when comparably opsonized (20, 30). The protective functions of antibodies against *B. ovis* could have included opsonization with enhancement of intracellular killing or mediation of extracellular killing by complement or through antibody-dependent cellular cytotoxicity by natural killer or other effector cells.

Protection transferred by three antiserum pools from convalescent mice ranged from 2.36 to 5.25 log units, while that from HS-vaccinated donors ranged from 2.68 to 5.10 log units. It is unclear why some antiserum pools conferred higher levels

TABLE 5. Protection against *B. ovis* provided by vaccination with HS or its protein and LPS fractions

Treatment group ($n = 5$)[a]	Vaccine[b]	KELA slope (10^3) at challenge ($\bar{x} \pm SD$)		Log_{10} units of *B. ovis* in spleen ($\bar{x} \pm SD$)	Log units of protection[c]
		HS	R-LPS		
1	HS	451 ± 11	43 ± 19	5.66 ± 1.27	2.54**
2	HS-PCP	259 ± 15	20 ± 4	5.63 ± 1.44	2.57**
3	R-LPS	38 ± 20	18 ± 12	8.21 ± 0.07	−0.01†
4	None (PBS)	19 ± 5	ND	8.20 ± 0.15	

[a] Mice were vaccinated twice over a 4-week interval and were challenged i.v. with 5.8×10^4 *B. ovis* PA cells 1 week after the second vaccination. Spleens were cultured 2 weeks later.
[b] All vaccines contained 20 μg of antigen combined with QS-21. The control group received PBS in place of vaccine.
[c] **$P \leq 0.01$ compared with the PBS control group. †, not significant.

TABLE 6. Passive transfer of protection against *B. ovis* by immune T cells and antiserum

Recipient group ($n = 5$)[a]	T cells or serum transferred[b]	KELA slope (10^3) of terminal bleeds ($\bar{x} \pm$ SD)[c]	Log_{10} units of *B. ovis* in spleen ($\bar{x} \pm$ SD)	Log units of protection[d]
1	*B. ovis*, 10 wk, T cells	37 ± 16	6.57 ± 0.26	0.80**
2	*B. ovis*, 10 wk, serum	ND	2.12 ± 0[e]	5.25***
3	HS, s.c. vaccination, T cells	74 ± 16	7.40 ± 0.22	−0.03†
4	HS, s.c. vaccination, serum	ND	4.69 ± 1.69	2.68*
5	HS, footpad vaccination, T cells	NA	6.05 ± 0.17	1.32***
6	HS, footpad vaccination, serum	ND	2.27 ± 0.36	5.10***
7	None (PBS)	33 ± 12	7.37 ± 0.42	

[a] Recipients received T cells (3×10^7) or antiserum (0.1 ml) i.v. 1 to 2 h before i.v. challenge with 5×10^3 CFU of *B. ovis* PA. Spleens were cultured 2 weeks later.

[b] Donor groups were as follows: *B. ovis*, 10 wk, group inoculated i.v. with 5×10^3 CFU of *B. ovis* PA 10 weeks previously; HS, s.c. vaccination, group vaccinated s.c. over the back twice over a 4-week interval with HS (20 μg)–QS-21 and sacrificed 1 week after the second vaccination; HS, footpad vaccination, group vaccinated in each hind footpad twice over a 4-week interval with HS (20 μg)–QS-21 and sacrificed 1 week later. Spleen cells were harvested from *B. ovis*, 10 wk, and HS, s.c. vaccination, donors, and popliteal lymph nodes were harvested from HS, footpad vaccination, donors. The compositions of the transferred cell populations were as follows: *B. ovis*, 10 wk, $CD4^+$, 64%; $CD8^+$, 24%; secretory Ig^+, 2.5%; Mac-1^+, 9.0%; HS, s.c. vaccination, $CD4^+$, 63%; $CD8^+$, 22%; secretory Ig^+, 2.0%; Mac-1^+, 25%; HS, footpad vaccination, $CD4^+$, 84%; $CD8^+$, 16%; secretory Ig^+, 0.9%; Mac-1^+, 0%. KELA slopes (10^3) of the donor serum pools tested against HS antigen: *B. ovis*, 10 wk, 266; HS, s.c. vaccination, 455; HS, footpad vaccination, 415.

[c] KELA analyses were performed with HS antigen. ND, not done. NA, not available because of technical error.

[d] $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{*}P < 0.05$ compared with PBS control group. †, not significant.

[e] *B. ovis* was not detected in any of the spleens, so a log_{10} value of 2.12 was assigned to the group according to the previous convention (36).

of protection than others. Such differences were not associated with total antibody concentrations determined by KELA (Table 6). In fact, antiserum pools from convalescent mice were overall as protective as those from vaccinated groups, despite lower levels of antibodies of all isotypes (Tables 2 and 6). Even though there was no difference in the recognition of antigens on methanol-killed whole *B. ovis* cells by convalescent phase sera and sera induced by the heat-treated HS antigens as judged by KELA (28a), some differences in the fine specificity of antibodies that could have affected protection may have existed between them.

The importance of the protein constituents of HS in accounting for protective immunity was illustrated by the induction of equivalent protection by purified proteins (HS-PCP) and intact HS (Table 5). Purified R-LPS also induced low levels of protection against a lower challenge dose, but the poor immunogenicity of R-LPS in BALB/c mice (Table 5) precludes an unqualified judgment of its protective efficacy. Although outer membrane protein antigens of *B. ovis* and *B. melitensis* are antigenically related (24, 41, 44), HS-specific antibodies would probably not have been protective against *B. melitensis* with its surface-exposed O polysaccharide, and cross-protective cell-mediated immune responses were apparently not induced by HS (Table 4).

The combination of HS and QS-21 fulfills our criteria for selection of an appropriate vaccine for a field trial with rams. Aside from its potency in inducing antibodies (Tables 1 to 3 and 5) that were demonstrated to be protective (Table 6), HS–QS-21 evoked a protective cell-mediated immune response as good as that obtained from the T cells of convalescent mice (Table 6). It remains to be seen whether the protective T cells induced by vaccination with HS–QS-21 and through primary infection with *B. ovis* are distributed into the same subsets. However, the ability of QS-21 to stimulate the formation of a major histocompatibility complex class I-restricted CD8 T-cell response to a simple protein antigen has already been established (38) and represents an essential attribute of an adjuvant for subcellular vaccines against intracellular parasites. An additional important advantage of QS-21 lies in the extreme simplicity of vaccine preparation and administration, coupled with the absence of oils and emulsifying agents from the injected product.

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