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Variation in *Brucella abortus* 2308 Infection in BALB/c Mice Induced by Prior Vaccination with Salt-Extractable Periplasmic Proteins from *Brucella abortus* 19

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The study compared the immune and protective responses induced in BALB/c mice vaccinated with six salt-extractable periplasmic protein fractions (*Brucella* cell surface proteins [BCSP]) of *Brucella abortus* 19 and later challenge exposed with *B. abortus* 2308. BCSP70 was precipitated with ammonium sulfate at 70% saturation, and BCSP100 was precipitated with ammonium sulfate at 100% saturation by use of supernatant fluid of BCSP70 that had been precipitated with 70% ammonium sulfate. Four subfractions were separated from BCSP100 by anion-exchange high-performance liquid chromatography (HPLC). Monophosphoryl lipid A (MPL) from *Salmonella typhimurium* Re mutant strain was used as a potential immune response modifier in some vaccines. Reduced or increased numbers of CFU and increased spleen size in the principal groups of mice relative to that of the nonvaccinated control group were considered protectiveness or virulence (survival) criteria. Results indicated that vaccines prepared from BCSP70 and BCSP100 were moderately protective and immunogenic. The subfractions designated BCSP100-A through BCSP100-D purified by anion-exchange HPLC were not protective when MPL was not used as an immune response modifier. However, two subfractions were associated with significant ($P < 0.05$) increases in CFU per spleen and splenomegaly in vaccinated mice compared with those in nonvaccinated challenge-exposed mice. MPL enhanced protection or was neutral when used with BCSP70, BCSP100, BCSP100-C, and BCSP100-D. Serologic results of an enzyme-linked immunosorbent assay indicated that MPL modulated the immunoglobulin G responses induced by BCSP70, BCSP100, and subfraction BCSP100-B vaccines only. The overall results suggest that certain proteinaceous periplasmic fractions might serve as virulence or survival factors in *B. abortus* infections.

In a normal animal, an equilibrium exists between the immune system of the host and its microbial environment (1, 3, 11, 13, 14, 18, 19, 26, 28, 42). Some diseases occur only when the balances are disrupted either by failure of the host's immune surveillance system or because the microbe develops special factors or attributes that enable it to circumvent the host's defenses. In that context, *Brucella* infection of the host animal and the putative disease and immunity induced are thought to be due to specific virulence factors produced by *Brucella* species constitutively or in response to the host defenses (6, 8, 9, 17, 25, 37, 38, 41, 57). The severity of the disease produced and the subsequent immune responses induced are dependent on the biochemical nature of the bacterial factors involved and their interactions (2, 3, 10, 12, 14–16, 21–25, 30–37, 40–43, 54, 57, 59, 60). Many of these factors are proteins, while others are simple or complex polysaccharides, lipopolysaccharides (LPS), or lipoproteins.

Although recently there are reports of a potentially useful vaccine involving the use of a rough mutant (RB51) of *Brucella abortus*, there are serious problems associated with existing brucellosis vaccines (unpublished data). Therefore, development of a superior vaccine(s) is a worthwhile endeavor (unpublished data). Previously, in attempts to develop better nonliving vaccines against *B. abortus* infections, killed attenuated cultures, crude fractions of whole cells, and partially purified cellular fractions of the members of the *Brucella* genus have been tested as vaccines (2, 6, 14, 28, 31). More recently, however, studies were directed at the use of outer membrane proteins (including porins), cell surface proteins, polysaccharides, and LPS (2, 10, 12, 23, 24, 30–34, 41, 53, 54, 59, 60). We have been able to show that some of the *Brucella* cell surface proteins (BCSP) are immunogenic (37, 40, 51, 53) and that others may serve as virulence factors in the disease process (20, 37, 38, 57).

The periplasmic proteins BCSP70 (4, 5, 38, 40, 51, 53, 54) and BCSP100 (37) were isolated from *B. abortus* 19 by differential ammonium sulfate precipitation at 70 and 100% saturation, respectively, from supernatant fluid of a high-salt extract of *B. abortus*. Three subfractions, BCSP20, BCSP31, and BCSP45, were isolated from protein BCSP70 and have been studied (4, 7, 20, 37–39, 45, 46, 49, 53, 57). The immunogenic, pathogenic, and protective properties of BCSP100 and its subfractions BCSP100-A, BCSP100-B, BCSP100-C, and BCSP100-D have not been studied previously (unpublished data).

The objective of the study was to determine the efficacy of vaccines prepared from subfractions of BCSP100 relative to that of nonfractionated BCSP100 and BCSP70 vaccines with monophosphoryl lipid A (MPL) from *Salmonella typhimurium* Re mutant strain as an immune response modifier (37, 53, 54).

MATERIALS AND METHODS

Experimental design. The study was divided into two experiments. Experiment 1 was designed to determine the relative efficacies of vaccines prepared from four subfractions of protein BCSP100 and to compare them with the efficacy of vaccine prepared from BCSP70 and BCSP100. Experiment 2 was designed to determine whether MPL would improve the efficacy of the vaccines used in experiment 1. Mice were given one vaccinal inoculation on day 1 of each experiment, and immunity was challenged with a virulent culture of *B. abortus* 2308 on day 28 (see Tables 1 and 2). Necropsy examination was conducted on days 42 to

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44. Blood samples were taken on day 28 (prechallenge exposure sera) immediately before challenge exposure of immunity and on days 42 to 44 at necropsy examination (postchallenge exposure sera). Sera were tested for antibodies against each antigen by an enzyme-linked immunosorbent assay (ELISA).

Mice. Female BALB/cANCrLBR mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) less than 2 months old and weighing between 15 and 20 g were used in the experiments. The mice were placed in mouse cages with no more than four mice per cage and given feed (mouse-rat diet [4% fat] Teklad-A; Harlan Sprague Dawley, Inc., Winfield, Iowa) and water ad libitum. The mice were acclimated for 1 week before vaccination and housed in an air-conditioned, air-filtered room that contained only mice.

Bacteria. Stock cultures of virulent *B. abortus* 2308 were isolated from an aborted bovine fetus and stored at −70°C. The thawed culture was streaked on potato infusion agar and incubated for 40 to 44 h at 37°C. Colonies were washed from the agar surface with saline solution (0.15 M NaCl) and adjusted to an optical density of 0.125 at 600 nm with a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Analytical System Division, Rochester, N.Y.). This optical density reading represented approximately 10^9 CFU/ml of suspension. Appropriate dilutions in 0.15 M NaCl were made to yield an inoculum containing approximately 10^4 CFU/0.2 ml for challenge exposure of mice. The *B. abortus* 19 cultures that were used to prepare BCSP and derivatives were stored in 0.15 M NaCl at −70°C.

Isolation of BCSP70. BCSP70 was isolated from washed methanol-inactivated *B. abortus* 19 (5). Cultures were inoculated into a fermentor containing 12 liters of medium composed of Bacto Dextrose (30 g/liter), peptone M (30 g/liter), Bacto Yeast Extract (10 g/liter; all from Difco, Detroit, Mich.), NaH_2PO_4 (9 g/liter), Na_2HPO_4 (3.3 g/liter), and distilled water. The culture was supplied with a source of filter-sterilized air. After incubation for 48 h at 37°C, the cells were collected by filtration, washed three times with sterile 0.15 M NaCl solution, centrifuged at 18,000 × *g* for 20 min, and inactivated by the addition of 2 volumes of methanol to 1 volume of cell suspension. The cell suspension was stirred overnight at 5°C and allowed to stand at 5°C for 1 week with occasional stirring to ensure complete inactivation of the cells. The suspension was centrifuged at 12,000 × *g* for 1 h, and the cells were resuspended in the hypertonic salt solution. The proteins were extracted at 5°C by gently stirring for 16 h. BCSP70 and BCSP100 were obtained from supernatant fluids by sequential ammonium sulfate precipitation at 70 and 100% saturation, respectively. The extraction procedure was repeated, and extracts were combined, dialyzed against 5 mM NH_4CO_3, and concentrated by freeze-drying. The lyophilized proteins were dissolved in 5 mM NH_4HCO_3, and samples were loaded on denaturing gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels (2 to 15% polyacrylamide; Pharmacia, Uppsala, Sweden) and run as described in the manufacturer's instructions (see Fig. 1a). The protein concentration was determined by the procedure of Lowry et al. (27). The preparations were stored at −70°C until used.

Isolation of subfractions of BCSP100. The BCSP100 subfractions were prepared from frozen BCSP100 by anion-exchange high-performance liquid chromatography (HPLC) with a Synchropak AX-300 column (4.6 by 250 mm; SynChrom, Inc., Linden, Ind.). The column was equilibrated in 10 mM sodium phosphate buffer (pH 7.2). Two hundred fifty microliters (approximately 250 μg) of protein solution was injected onto the column. The proteins were eluted with a two-step linear gradient from 0 to 0.5 M and from 0.5 to 1.0 M sodium chloride in equilibration buffer. The elution of proteins was monitored by absorption at 214 nm. Fractions (0.5 ml) were pooled as indicated below (see Fig. 1b), concentrated by lyophilization, and dialyzed against 5 mM ammonium bicarbonate. Dialyzed fractions were marked as peaks 1 to 4 and designated BCSP100-A through -D on the basis of their order of elution from the column. The fractions were stored at −70°C.

Isolation of LPS. The LPS used as antigen in the ELISA was extracted from *B. abortus* 2308 by a butanol extraction procedure followed by digestion with proteinase K (E. Merck, Darmstadt, Germany) as described previously (33, 34). The preparation contained less than 1% protein.

Adjuvant. Nontoxic MPL was obtained from a commercial source (Ribi Immunochem Research, Inc., Hamilton, Mont.) and isolated from *S. typhimurium* Re mutant strain. The MPL was stored at 4°C until used in the vaccine at a dose of 50 μg per dose.

Vaccination. Before inoculation of mice, the frozen vaccines were thawed and diluted with 0.15 M NaCl solution to get the desired concentration of each vaccine (see Tables 1 and 2). Each mouse was vaccinated with 0.2 ml of the respective vaccine intraperitoneally. Control mice were inoculated with 0.15 M NaCl (placebo).

Challenge exposure. Four weeks after vaccination, the immunity of mice was challenged with a virulent culture of *B. abortus* 2308. Each mouse was given approximately 10^4 CFU intraperitoneally in 0.2 ml of saline solution. The challenge inoculum of approximately 10^4 CFU of *B. abortus* 2308 per 0.2 ml was used because results of a previous study in our laboratory indicated that this dose consistently caused moderate signs of brucellosis in nonvaccinated BALB/c mice within 1 month of exposure. Challenge exposure of immunity with an inoculum of $\leq 10^3$ CFU caused only mild or inconsistent signs of brucellosis within the same time period. The retrospective numbers of CFU for challenge exposure were 1.2×10^4 and 1.8×10^4 in experiments 1 and 2, respectively.

Serologic examination. Specific serum immunoglobulin M (IgM) and IgG antibody concentrations against the antigens were determined on day 28 when the immunity of mice was challenged (prechallenge) and 14 to 16 days later at necropsy examination (postchallenge) by an ELISA (37, 50, 58). The LPS results were determined with 1:400 dilutions of serum and 1 μg of LPS per ml as the solid-phase antigen. The BCSP70 and BCSP100 assays were performed with 1:100 dilutions of serum with 0.1 μg of BCSP70 or BCSP100 as the test antigen (37, 39, 54). Results of the IgM and IgG antibody concentration determinations were expressed as absorbance units (A_{410}/A_{450}) and obtained with an ELISA plate reader (Dynatech Laboratories, Alexandria, Va.).

Bacterial culture of the spleen. The numbers of CFU of *B. abortus* per spleen and the spleen weights were determined as described previously (40, 41).

Analysis of data. Statistical differences in CFU, spleen weights, and specific IgM and IgG concentrations were determined and analyzed with Tukey-Kramer multiple-comparison tests with the software program InStat (GraphPad Software, San Diego, Calif.).

RESULTS

Isolation of protein fractions. A representative pattern of BCSP70 and BCSP100 on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel is shown in Fig. 1a. Lanes 2 and 3 contained 2 μg of BCSP70, and lanes 4 and 5 contained 2 μg of BCSP100. BCSP100 shows protein bands at 34, 31 to 32, 24, 19, 16.5, 14, and 12 kDa, whereas BCSP70 has all of these plus a doublet at 38 kDa and a band at 45 kDa. Some preparations of BCSP100 also contain minor amounts of these additional proteins. The difference between BCSP70 and BCSP100 is the enrichment of the 16-kDa band (the Cu-Zn superoxide dismutase [Cu-Zn SOD]). BCSP100 is usually devoid of proteins larger than 38 kDa. A representative HPLC anion-exchange chromatogram is shown in Fig. 1b. Peaks 1 and 2 (BCSP100-A and -B) contained proteins with molecular weights of 16,500 and 31,000, respectively (Fig. 1b, inset). Peak 3 (BCSP100-C) contained proteins with molecular weights of 12,000, 19,000, and 32,000. Peak 4 (BCSP100-D) contained proteins with molecular weights of 24,000 and 34,000. The concentrations of BCSP100-C and -D were lower, which is reflected by the lower intensity of the stained protein bands.

Experiment 1. CFU and splenic weights in mice vaccinated with subfractions of BCSP100. Analysis of the data from experiment 1 showed a dose-dependent significant decrease in CFU and spleen weights (Table 1). When the CFU of control mice (Table 1, group I-1) were compared with those of the vaccinated mice, only mice given 1.0 (group I-3) or 30 (group I-5) μg of BCSP70 and 10.0 (group I-20) or 30.0 (group I-21) μg of BCSP100 had decreased numbers of CFU ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.001$, respectively). Mice in groups I-6, -7, -9, and -10 had higher numbers of CFU than did control mice ($P < 0.05$). When the spleen weights of control mice (group I-1) and vaccinated mice were compared, only values for mice given 30.0 μg of BCSP70 (group I-5) and 0.1 μg of BCSP100-C (group I-12) were different ($P < 0.05$).

Mice in most groups developed increased IgM antibody concentrations before challenge of immunity with *B. abortus* 2308. This increase in IgM antibody concentration was dependent on the antigen used in the ELISA (Fig. 2). When the prechallenge exposure serum IgM antibody concentrations with LPS as the test antigen were compared, none of the groups had meaningful increases in IgM antibody concentrations (Fig. 2a). When the prechallenge serum IgM antibody concentrations with BCSP70 as the test antigen were compared, only values for mice in group I-7 were different from those for control mice ($P < 0.05$) (Fig. 2c). When the prechallenge serum IgM antibody concentrations with BCSP100 as the test antigen were compared, only values for mice in group I-20 were different from those for control mice ($P < 0.001$) (Fig. 2e). When the postchallenge serum IgM antibody concentrations with LPS as the test antigen were compared, values for mice in groups I-2 and I-18 ($P < 0.05$), groups I-11, -17, and -19 ($P < 0.01$), and groups I-4 and I-14 ($P < 0.001$) were different from those for

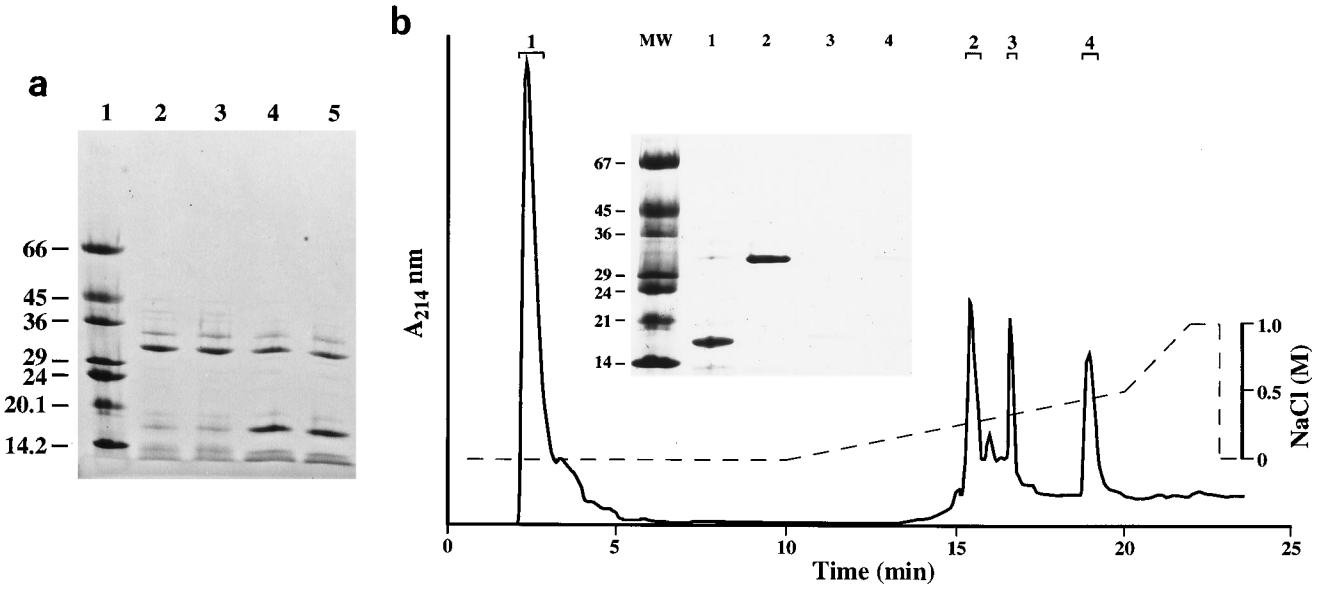

FIG. 1. (a) SDS-PAGE of *B. abortus* 19 BCSP70 and BCSP100. Lanes: 1, molecular mass standards, 11.6 μg (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 21 kDa; α-lactalbumin, 14.2 kDa); 2 and 3, 2 μg of BCSP70; 4 and 5, 2 μg of BCSP100. (b) Anion-exchange HPLC of salt-extractable protein fraction precipitated with ammonium sulfate at 70 to 100% saturation. Protein (approximately 250 μg) was loaded onto the column, which was previously equilibrated with 50 mM sodium phosphate (pH 7.2). Proteins were eluted with a two-step linear gradient from 0 to 0.5 M and 0.5 to 1.0 M sodium chloride in equilibration buffer. Protein elution was monitored at 214 nm. Fractions of 0.5 ml were collected and pooled. Inset, SDS-PAGE of each peak sample (1 μl) denatured with an equal volume of sample buffer and loaded onto a 10 to 15% gradient gel. Lanes: 1, peak 1; 2, peak 2; 3, peak 3; 4, peak 4. MW, molecular weight markers (in thousands).

control mice (Fig. 2b). When the postchallenge serum IgM antibody concentrations with BCSP70 as the test antigen were compared, values for all mice except those in groups I-5 and I-10 were different from those for control mice ($P > 0.05$) (Fig. 2d). When the postchallenge serum IgM antibody concentrations with BCSP100 as the antigen were compared, values for mice in groups I-13 ($P < 0.05$) and I-4 and -14 ($P < 0.001$) were different from those for control mice (Fig. 2f).

There were increases in some of the postchallenge serum IgG antibody concentrations with the specific antigen in comparison with those of the prechallenge sera (Fig. 3). When the prechallenge exposure serum IgG antibody concentrations with LPS as the antigen were compared, only values for mice in groups I-5 and -21 were different from those for control mice ($P < 0.01$) (Fig. 3a). When the IgG antibody concentrations with BCSP70 as the antigen were compared, only values for mice in groups I-8 and I-21 were different from those for control mice ($P < 0.01$) (Fig. 2c). When the IgG antibody concentrations with BCSP100 as the antigen were compared, only group I-21 values were different from those of control mice ($P < 0.001$) (Fig. 2e). When the postchallenge serum IgG antibody concentrations with LPS as the antigen were compared, only values for mice in groups I-4 and -20 were different from those for control mice ($P < 0.001$) (Fig. 3b). When the values for IgG antibody concentrations with BCSP70 as the antigen were compared, only values for mice in group I-21 ($P < 0.01$) and groups I-4 and -20 ($P < 0.001$) were different from those for control mice (Fig. 3d). When the IgG antibody concentrations with BCSP100 as the antigen were compared, only values for mice in group I-14 were different from those for control mice ($P < 0.001$) (Fig. 3f).

Experiment 2. CFU and splenic weights in mice vaccinated with subfractions of BCSP100 with MPL as an immune response modifier. Analysis of data from experiment 2 showed variable responses, with MPL not always enhancing the protectiveness of the vaccine (Table 2). When the numbers of CFU of mice were compared, the values for mice in groups II-3 to -6 were approximately 3 logs lower than those of control mice (group II-1) ($P < 0.001$). When the spleen weights of mice were compared, only the values for mice in groups II-3, -9, -12, and -14

TABLE 1. Comparison of postchallenge exposure CFU numbers and splenic weights of mice vaccinated with BCSP and subfractions

Group[a]	Vaccine	Dose (μg of protein)/0.2-ml inoculation	CFU ($\log_{10}$)[b]	Spleen wt (mg)[b]
I-1	Placebo (0.15 M NaCl)	0	6.36 ± 0.79[a]	200 ± 40[f,g]
I-2	BCSP70	0.1	6.27 ± 0.96[a]	239 ± 55[f]
I-3	BCSP70	1.0	5.31 ± 0.68[b]	189 ± 43[f,g]
I-4	BCSP70	10.0	5.71 ± 0.39[a,b]	160 ± 34[g]
I-5	BCSP70	30.0	5.20 ± 0.72[b]	123 ± 23[h]
I-6	BCSP100-A	0.1	7.46 ± 0.21[c]	243 ± 29[f]
I-7	BCSP100-A	1.0	7.13 ± 0.25[c]	224 ± 48[f]
I-8	BCSP100-A	10.0	6.10 ± 0.69[a,b]	204 ± 18[f]
I-9	BCSP100-B	0.1	7.22 ± 0.20[c]	271 ± 38[f]
I-10	BCSP100-B	1.0	7.18 ± 0.24[c]	258 ± 37[f]
I-11	BCSP100-B	10.0	6.55 ± 0.35[a,d]	210 ± 30[f]
I-12	BCSP100-C	0.1	6.83 ± 0.20[a,d]	280 ± 67[f]
I-13	BCSP100-C	1.0	6.60 ± 0.36[a,d]	250 ± 39[f]
I-14	BCSP100-C	10.0	6.57 ± 0.29[a,b]	253 ± 24[f]
I-15	BCSP100-D	0.1	6.96 ± 0.44[a,d]	226 ± 50[f]
I-16	BCSP100-D	1.0	6.76 ± 0.39[a,d]	204 ± 62[f,g]
I-17	BCSP100-D	10.0	6.56 ± 0.46[a,d]	216 ± 40[f,g]
I-18	BCSP100	0.1	6.74 ± 0.44[a,d]	224 ± 48[f,g]
I-19	BCSP100	1.0	6.08 ± 0.38[a,b,d]	180 ± 37[f,g]
I-20	BCSP100	10.0	5.52 ± 0.46[b]	133 ± 17[g,h]
I-21	BCSP100	30.0	4.54 ± 0.95[b,e]	135 ± 24[g,h]

[a] Eight mice were included in each group (each group initially contained 10 mice, but 2 mice from each group were euthanized before challenge exposure). Each mouse was challenged with 10^4 CFU of *B. abortus* 2308 intraperitoneally.

[b] CFU or splenic weights with the same superscript letters are not significantly ($P > 0.05$) different. Values are means ± standard deviations.

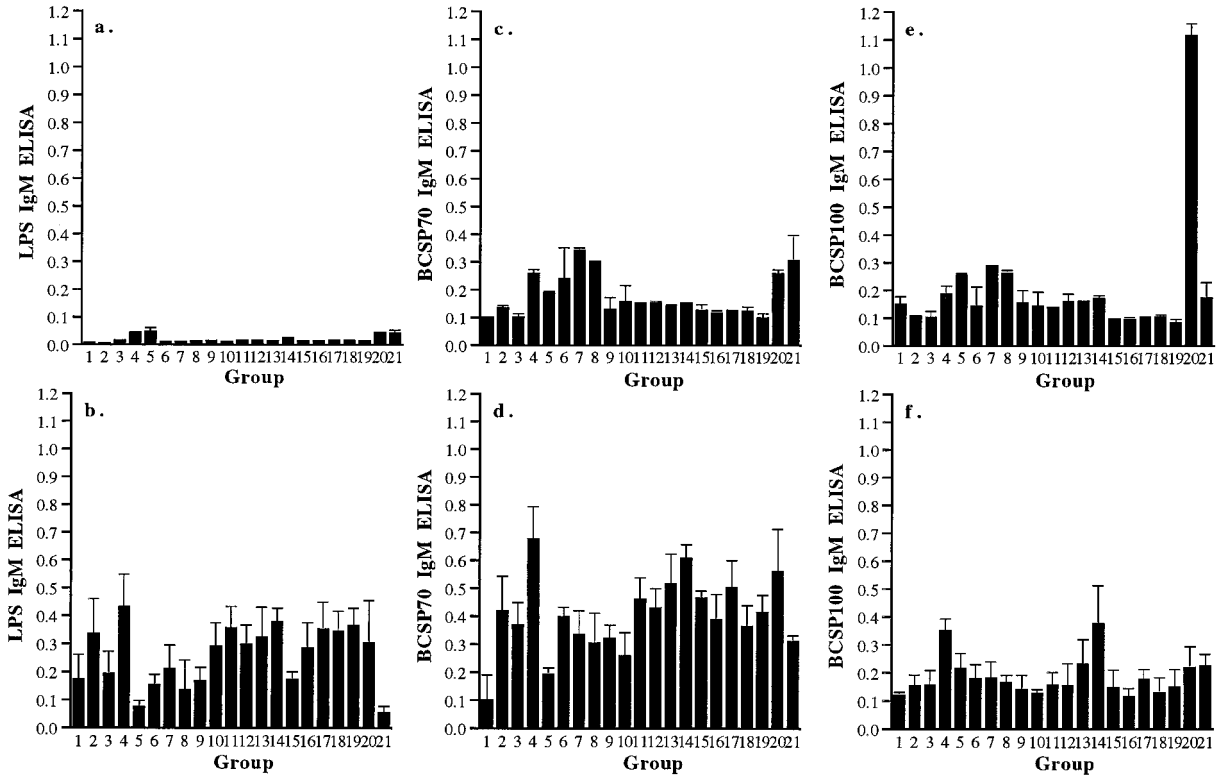

FIG. 2. Prechallenge (a, c, and e) and postchallenge (b, d, and f) exposure ELISA IgM antibody results for mice vaccinated with BCSP70, BCSP100, and subfractions of BCSP100. Mice in group I-1 were given 0.2 ml of 0.15 M NaCl. Mice in groups I-2, -3, -4, and -5 were given 0.1, 1.0, 10.0, and 30.0 μg of BCSP70, respectively. Mice in groups I-6, -7, and -8 were given 0.1, 1.0, and 10.0 μg of BCSP100-A, respectively. Mice in groups I-9, -10, and -11 were given 0.1, 1.0, and 10.0 μg of BCSP100-B, respectively. Mice in groups I-12, -13, and -14 were given 0.1, 1.0, and 10.0 μg of BCSP100-C, respectively. Mice in groups I-15, -16, and -17 were given 0.1, 1.0, and 10.0 μg of BCSP100-D, respectively. Mice in groups I-18, -19, -20, and -21 were given 0.1, 1.0, 10.0, and 30.0 μg of BCSP100, respectively. The results are expressed as ELISA absorbance (10^{-3}) at A_{410}/A_{450} with the specific antigen.

($P < 0.01$) and in groups II-4 to -6 ($P < 0.001$) were different from those for control mice (group II-1).

The prechallenge exposure serum IgM antibody concentrations were similar to the postchallenge antibody concentrations (Fig. 4). When the prechallenge serum IgM antibody concentrations with LPS as the test antigen were compared, values for mice in groups II-4 and -12 ($P < 0.05$) and in groups II-5 and -6 ($P < 0.001$) were different from those for control mice (Fig. 4a). When the prechallenge serum IgM antibody concentrations with BCSP70 as the test antigen were compared, values for mice in groups II-3, -13, and -14 ($P < 0.01$) and in groups II-4, -5, and -6 ($P < 0.001$) were different from those for control mice (Fig. 4c). When the prechallenge serum IgM antibody concentrations with BCSP100 as the test antigen were compared, values for mice in groups II-8 and -13 ($P < 0.05$), in group II-14 ($P < 0.01$), and in groups II-4 to -6 ($P < 0.001$) were different from those for control mice given the placebo (Fig. 4e). When the postchallenge serum IgM antibody concentrations with LPS as the test antigen were compared, values for mice in groups II-10 and -12 ($P < 0.05$) and in groups II-2 and -8 ($P < 0.01$) were different from those for control mice (Fig. 4b). When the postchallenge serum IgM antibody concentrations with BCSP70 as the test antigen were compared, no differences existed between the values of the groups ($P > 0.05$) (Fig. 4d). When the postchallenge serum IgM antibody concentrations with BCSP100 as the test antigen were compared, values for mice in groups II-3 ($P < 0.01$) and in groups II-4 to -6 and in group II-13 ($P < 0.001$) were different from those for control mice (Fig. 4f).

The postchallenge serum IgG antibody concentrations were increased in comparison with those of the prechallenge serum (Fig. 5). When the prechallenge serum IgG antibody concentrations with LPS as the antigen were compared, values for mice in groups II-4, -5, and -6 were different from those for control mice ($P < 0.001$) (Fig. 5a). When the IgG antibody concentrations with BCSP70 as the antigen were compared, values for mice in group II-3 ($P < 0.05$) and in groups II-4 to -6 ($P < 0.001$) were different from those for control mice (Fig. 5b). When the IgG antibody concentrations with BCSP100 as the antigen were compared, values for mice in groups II-6 and -10 were different from those for control mice ($P < 0.001$) (Fig. 5e). When the postchallenge serum IgG antibody concentrations with LPS as the antigen were compared, values for mice in groups II-3 and -4 ($P < 0.01$) and in groups II-5 and -6 ($P < 0.001$) were different from those for control mice (Fig. 5b). When the IgG antibody concentrations with BCSP70 as the antigen were compared, values for mice in groups II-3 to -6 were different from those for control mice ($P < 0.001$) (Fig. 5d). When the IgG antibody concentrations with BCSP100 as the antigen were compared, values for mice in groups II-4 ($P < 0.05$) and in groups II-5, -6, and -10 ($P < 0.001$) were different from those for control mice (Fig. 5f).

DISCUSSION

In experiment 1, the CFU and splenic weight results indicated that BCSP70 and BCSP100 induced protection in mice when inoculated at doses of 1.0, 10, and 30 μg, but the use of

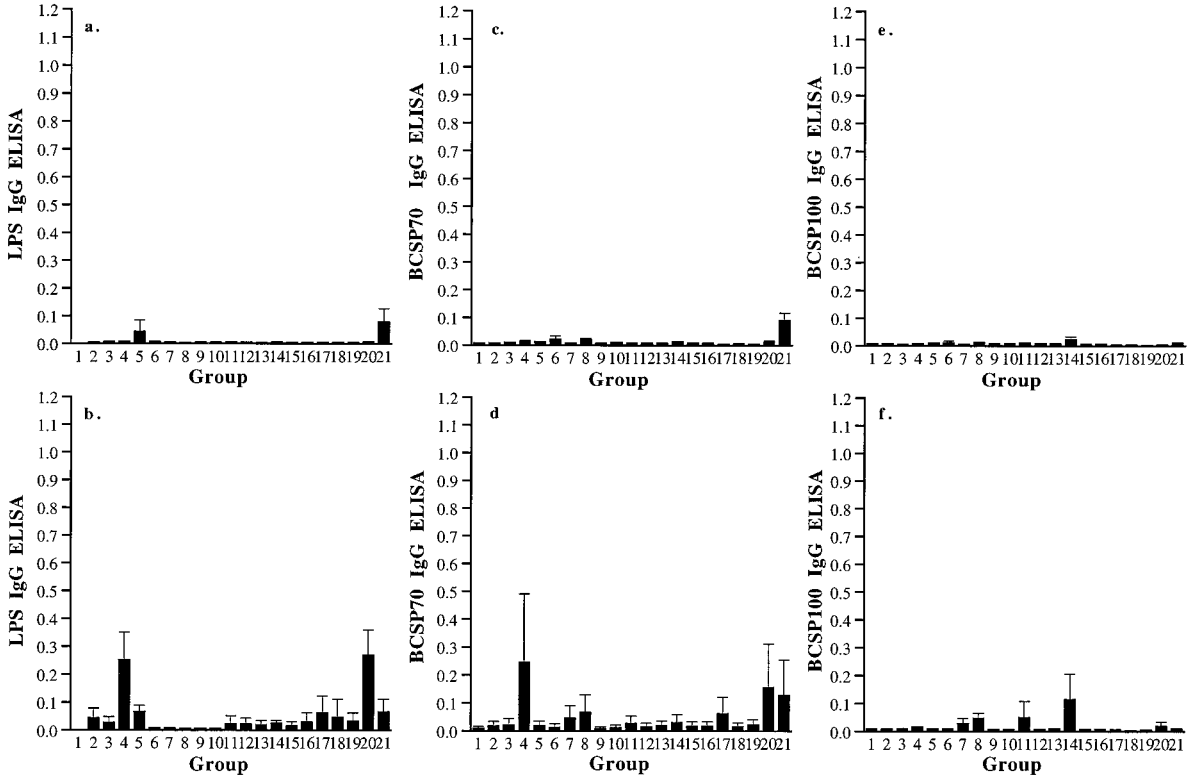

FIG. 3. Prechallenge (a, c, and e) and postchallenge (b, d, and f) exposure ELISA IgG antibody results for mice vaccinated with BCSP70, BCSP100, and subfractions of BCSP100. Mice in the various groups were given the amounts of placebo and proteins as described in the legend to Fig. 2. The results are expressed as ELISA absorbance (10^{-3}) at A_{410}/A_{450} with the specific antigen.

MPL did not enhance that protection. The results also indicated that mice given BCSP100-A, BCSP100-B, BCSP100-C, and BCSP100-D vaccines without MPL were not protected compared with nonvaccinated mice. The results indicated that MPL enhanced BCSP100-C and BCSP100-D vaccines. Results in previous studies indicated that MPL enhanced BCSP70 immunity and protectiveness in BALB/c mice exposed to *B. abortus* and that the relative protectiveness was related to the optimum dose of protein in the vaccine (37, 40, 54). High doses of BCSP70 neutralized or masked the potential enhancing effects of the MPL (40, 54).

TABLE 2. Comparison of postchallenge exposure CFU and splenic weights of mice vaccinated with subfractions of BCSP plus MPL as an adjuvant

Group[a]	Vaccine	Dose (μg of protein)/0.2-ml inoculation		CFU ($\log_{10}$)[b]	Spleen wt (mg)[b]
		Vaccine	MPL		
II-1	Placebo (0.15 M NaCl)	0		7.45 ± 0.26^{a}	274 ± 42^{e}
II-2	MPL	50.0		$7.28 \pm 0.21^{a,b}$	$284 \pm 93^{e,f}$
II-3	BCSP100	30.0		4.55 ± 0.49^{d}	$129 \pm 66^{f,g}$
II-4	BCSP100 + MPL	30.0	50.0	4.13 ± 0.75^{d}	104 ± 9^{g}
II-5	BCSP70	30.0		3.84 ± 0.66^{d}	111 ± 11^{g}
II-6	BCSP70 + MPL	30.0	50.0	4.13 ± 0.29^{d}	107 ± 9^{g}
II-7	BCSP100-A	10.0		$7.00 \pm 0.37^{b,c}$	$280 \pm 97^{e,f}$
II-8	BCSP100-A + MPL	10.0	50.0	$7.01 \pm 1.15^{a,b,c}$	$255 \pm 66^{e,f}$
II-9	BCSP100-B	10.0		$7.08 \pm 0.68^{a,b,c}$	208 ± 57^{f}
II-10	BCSP100-B + MPL	10.0	50.0	$7.02 \pm 0.56^{a,b,c}$	$226 \pm 67^{e,f}$
II-11	BCSP100-C	10.0		6.31 ± 0.97^{c}	$225 \pm 66^{e,f}$
II-12	BCSP100-C + MPL	10.0	50.0	$6.95 \pm 0.49^{b,c}$	221 ± 45^{f}
II-13	BCSP100-D	10.0		$5.55 \pm 1.33^{c,d}$	$210 \pm 90^{e,f}$
II-14	BCSP100-D + MPL	10.0	50.0	$6.86 \pm 0.35^{b,c}$	190 ± 66^{f}

[a] Eight mice were included in each group (each group initially contained 10 mice, but 2 mice from each group were euthanized before challenge exposure). Each mouse was challenged with 10^4 CFU of *B. abortus* 2308 intraperitoneally.

[b] CFU or splenic weights with the same superscript letters are not significantly ($P > 0.05$) different. Values are means ± standard deviations.

The results also suggested that two of the subfractions, BCSP100-A and BCSP100-B, enhanced the severity of infection or the survival of *B. abortus* as manifested by increased CFU. This finding was not unexpected because unpublished data with antisera to the 20-kDa (20 kDa on homogeneous gels and 16.5 kDa on gradient gels) and the 31-kDa proteins in a Western blot (immunoblot) showed that BCSP100 contained subfractions that were identical to the 31-kDa and 20-kDa proteins, both of which have been suggested as virulence factors in brucellosis. BCSP100-A contained a 16-kDa band (Fig. 1b) and is similar to BCSP20 and its derivative rBCSP20, previously identified as the Cu-Zn SOD when obtained from BCSP70 (4, 37–39, 44–46, 48, 53, 57). Although the exact role of BCSP20 and rBCSP20 in *B. abortus* infection has not been elucidated, *Brucella* SOD might be a survival or virulence factor (survival factor for the bacteria or a virulence factor for the host). Questions surrounding the role of SOD in brucellosis may arise because SOD may occur in three forms, namely, Cu-Zn, Mn, and Fe SODs (25, 44). Two of the enzymes (Mn and/or Fe SOD) are thought to have a role as housekeeping enzymes to remove harmful superoxide radicals produced during metabolism. The Cu-Zn SOD concentration might vary at different times during infection (57).

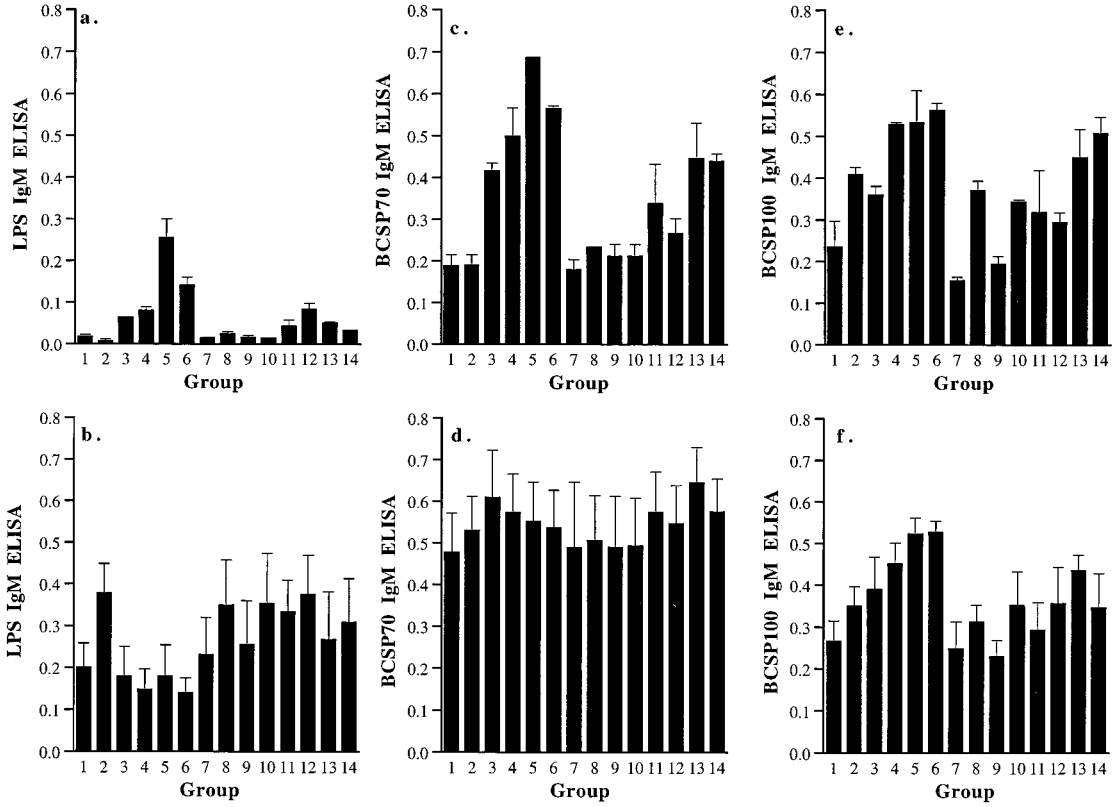

FIG. 4. Prechallenge (a, c, and e) and postchallenge (b, d, and f) exposure ELISA IgM antibody results for mice vaccinated with BCSP70, BCSP100, and subfractions of BCSP100 with MPL as an adjuvant. Mice in group II-1 were given 0.2 ml of 0.15 M NaCl. Mice in group II-2 were given 50.0 μg of MPL. Mice in group II-3 were given 30.0 μg of BCSP100. Mice in group II-4 were given 30.0 μg of BCSP100 plus 50.0 μg of MPL. Mice in group II-5 were given 30.0 μg of BCSP70. Mice in group II-6 were given 30.0 μg of BCSP70 plus 50.0 μg of MPL. Mice in group II-7 were given 10.0 μg of BCSP100-A. Mice in group II-8 were given 10.0 μg of BCSP100-A plus 50.0 μg of MPL. Mice in group II-9 were given 10.0 μg of BCSP100B. Mice in group II-10 were given 10.0 μg of BCSP100-B plus 50.0 μg of MPL. Mice in group II-11 were given 10.0 μg of BCSP100-C. Mice in group II-12 were given 10.0 μg of BCSP100-C plus 50.0 μg of MPL. Mice in group II-13 were given 10.0 μg of BCSP100-D. Mice in group II-14 were given 10.0 μg of BCSP100-D plus 50.0 μg of MPL. The results are expressed as ELISA absorbance (10^{-3}) at A_{410}/A_{450} with the specific antigen.

Immunogenic and/or protective peptides were synthesized on the basis of the structure of the Cu-Zn SOD. By using predicted hydrophilicity, flexibility, and antigenicity as selection factors for vaccine candidates, two of three synthetic peptides induced protective and or immune responses that were commensurate with both B-cell and T-cell epitopes (49, 53). In another study, we were able to show that serologic responses of SOD could be modified chemically (37). On the basis of the results reported in those studies (37, 49, 53), it is likely that the Cu-Zn SOD contained both B-cell and T-cell epitopes.

Subfraction BCSP100-B contained a 31-kDa band (Fig. 1b) previously identified as BCSP31 and its derivative rBCSP31 (7, 20, 37–39, 57) and were considered survival or virulence factors for *B. abortus*. Like SOD, if rBCSP31 and BCSP100-B are virulence factors, they would be expected to induce protective responses if administered before challenge. However, this might not always be true because mice that were administered BCSP100-B in the present study had increased numbers of CFU when compared with those of nonvaccinated control mice, and the administration of rBCSP31 induced similar results in other studies (38, 39). The results were not unexpected because, as reported earlier, vaccination of mice with SOD may result in different responses depending on the time it is given to mice (38). In that study (38), rBCSP31 induced major increases in antibody production in mice, but the mice were not protected and the administration of rBCSP31 vaccine reduced the protectiveness of the previously acquired LPS immunity. These results were as expected because immunity in brucellosis is due mainly to cellular immune mechanisms, and the induction of increased antibody does not correlate with cellular immunity and could act antagonistically (36, 38, 40).

We would like to emphasize that the results obtained with subfractions BCSP100-B, like those obtained with BCSP100-A, appear to be meaningful and are comparable to those obtained in other studies with rBCSP20 and rBCSP31. The subfractions used in these studies were not recombinant proteins produced in *Escherichia coli*, and both probably contain minor amounts of additional proteins (Fig. 1b). Therefore, it is possible that the results obtained in the present experiment with BCSP100-A and BCSP100-B might be due to the interaction of multiple protein components. Results in previous studies indicated that different fractions and possibly different epitopes, whether induced chemically or extracted from *Brucella* cells, can act antagonistically (9, 35, 37, 38).

Subfraction BCSP100-C contained a protein with a molecular mass of 35 kDa, and BCSP100-D contained two proteins with molecular masses of 18 and 13 kDa (Fig. 1b). The identities of these proteins have not been established. The use of pure subfractions of BCSP100 in combination with each other or in conjunction with an immune response modifier such as MPL could possibly serve as a worthwhile scheme for studying immune mechanisms which could expedite the development of

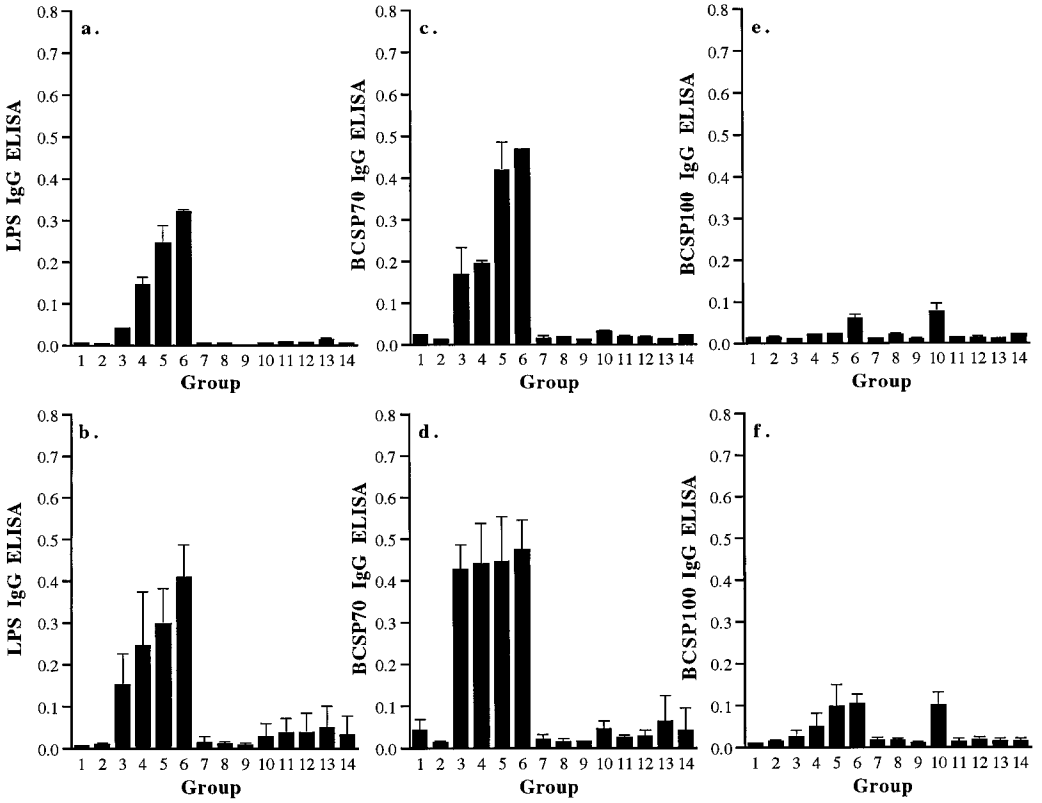

FIG. 5. Prechallenge (a, c, and e) and postchallenge (b, d, and f) exposure ELISA IgG antibody results for mice vaccinated with BCSP70, BCSP100, and subfractions of BCSP100 with MPL as an adjuvant. Mice in the various groups were given the amounts of placebo, proteins, and MLP described in the legend to Fig. 4. The results are expressed as ELISA absorbance (10^{-3}) at A_{410}/A_{450} with the specific antigen.

highly effective and specific synthetic vaccines for brucellosis (53).

The dose-response relationships in which increased doses result in increased protection as described previously (6, 21, 22, 36, 40) could explain the results in the present study. Compared with control mice, mice vaccinated with lower doses (0.1 and 1.0 μg) of BCSP100 and its subfractions had higher CFU and splenic weights, while mice vaccinated with 10.0 μg of BCSP100 or more and mice vaccinated with more than 1.0 μg of BCSP70 had significantly ($P < 0.05$) reduced numbers of CFU and splenic weights. The finding that BCSP70 vaccines induced better protective and serologic responses than did BCSP100 at all doses probably indicated that BCSP70 contained a larger proportion of protective epitopes per dose than BCSP100 did (40).

Although the prechallenge serologic results with the three antigens in the ELISA were similar to those in another study (37), the postchallenge exposure serologic results suggested that *B. abortus* enhanced the IgM immune responses but induced variable IgG responses (Fig. 3). Possibly, the serologic results obtained when a particular antigen was used, apart from the lowered specificity, were due to the time or stage of infection during which the serum samples were taken. Results in other studies indicated that the serum antibody concentrations were dependent on the stage of infection or the length of time after vaccination in which the serum samples were taken (1, 6, 15, 34, 47, 48).

The overall results from experiment 1 probably mean that proportionality (21, 22, 29, 36, 37, 40, 54) was very important in the BALB/c mouse model to ensure proper immune functions and that a low dose of antigen may be more effectively processed by the mouse than a larger dose of the antigen. We suggest that larger doses of antigen perturbed the host normal cellular responses and thereby suppressed the overall host immune system (21, 22, 29, 40). Also, a larger dose of the specific antigen could neutralize or mask a specific effect such as IgG production (an increase in immunoglobulin subclass G3 that is associated with enhanced protection), as seen with LPS vaccines (15, 24, 30, 32, 34), while stimulating cellular immunity that was primarily responsible for the reduced numbers of CFU (1, 8, 9).

In experiment 2, the CFU and splenic weight results indicated that BCSP70, BCSP100, and subfractions BCSP100-C and BCSP100-D were capable of inducing partial protection against *B. abortus* infection. As shown by the results of experiment 1, the unequal reductions in CFU and spleen weights induced by 30 μg of BCSP70 and by the same dose of BCSP100 indicate that BCSP70 was more effective than BCSP100 in inducing protection at the same dose (37).

The fact that MPL did not enhance the protective responses in mice given 30 μg of BCSP70 or BCSP100 was not surprising. Results in other studies (references 37 and 54 and unpublished results) indicated that the apparent enhancement seen when MPL was used can be neutralized or masked by large antigen doses. This probably meant that once the optimal concentration and proportion of relevant epitopes were reached, additional amounts caused perturbation of either the protective or the serologic response, or both, even when a constant dose of MPL was used. Results in another study (37) indicated that approximately 5.0 μg of BCSP70 and 50.0 μg of MPL per

mouse are the optimal doses and proportions of protective epitopes for a BCSP70 vaccine. Interestingly, MPL did not enhance LPS protection at any dose ($\geq$0.01 and $\leq$5.0 μg) but was an enhancer for BCSP70 at doses of $\geq$0.01 and $\leq$10.0 μg and for BCSP100 at doses of 10.0 and 30 μg.

The results also indicated that both subfraction BCSP100-C and BCSP100-D vaccines induced protection in mice compared with mice that were nonvaccinated. The results suggested, however, that the protection was not enhanced by MPL. This lack of enhancement might be due to the large doses (10 μg) of BCSP100-C and BCSP100-D used to vaccinate mice as discussed previously. Possibly, both BCSP100-C and BCSP100-D represented at least the optimal concentrated protective epitopes of nonfractionated BCSP100 that negated MPL potential modulation. Results indicating that subfractions BCSP100-A and BCSP100-B did not induce protection were not unexpected. These results are in agreement with those of experiment 1 and other studies in which both BCSP100-A, i.e., a protein identified as BCSP20 (Cu-Zn SOD), and BCSP100-B, i.e., a protein identified as BCSP31, appeared to act as virulence or survival factors in *B. abortus* infection (4, 7, 20, 37, 54).

The prechallenge exposure serologic results indicated that MPL slightly enhanced both the IgM and IgG responses. However, no direct relationships were established between the enhancements and the protective responses of reduced CFU numbers and spleen weights found in the postchallenge exposure results. The lack of increased IgM and IgG concentrations in BCSP100-B-vaccinated mice was noteworthy because results from experiment 1 and elsewhere (37) suggested that rBCSP31, which seems to be similar to the active factor in BCSP100-B, perturbed the immune systems of the BALB/c mice by enhancing CFU while causing increased IgG concentrations. This apparent contradiction suggested that natural BCSP100-B probably contained interfering epitopes not present in the recombinant rBCSP31.

The postchallenge exposure serologic results that showed high IgG concentrations with correspondingly reduced numbers of CFU were expected in mice vaccinated with 30 μg of BCSP70 and BCSP100. The relatively increased IgG response in mice vaccinated with BCSP100-B (the BCSP31 protein) could be due to protection (reduced CFU) induced by contaminating LPS negated by BCSP31 and accompanied by a corresponding increase in the IgG antibody concentration (38).

The combined results of experiments 1 and 2 (Tables 1 and 2) were different for the groups of mice vaccinated with 30-μg doses of BCSP70. This could be due to different numbers of CFU in the challenge inocula or to variations in the two cultures and different doses used. It should be noted, however, that the mice in group 5 (Tables 1 and 2) of both experiments had reduced ($P < 0.05$) CFU and spleen weights compared with those of nonvaccinated mice (group 1 in both experiments). This probably indicated that unintentional and unavoidable variations in materials and methods occurred when experiments were done individually and separately.

The results from the two experiments indicated that (i) BCSP70 and BCSP100 are immunogenic and partially protective when used to vaccinate BALB/c mice against *B. abortus*, (ii) the relative protectiveness based on the dose of immunogen used was in the order of BCSP70 > BCSP100, (iii) two subfractions, BCSP100-C and BCSP100-D, appeared to be partially protective, while subfractions BCSP100-A and BCSP100-B appeared to be virulence or survival factors, and (iv) MPL enhanced the immune and protective responses when used as an adjuvant with low doses of BCSP70 and BCSP100 and with BCSP100-C and BCSP100-D. However, MPL did not enhance the protective responses of large doses of BCSP70 and BCSP100 nor did it enhance 10-μg doses of BCSP100-A and BCSP100-B.

We suggest that our results and those of others permit development of a practical systematic procedure for isolating, identifying, and testing specific immune epitopes associated with *B. abortus* periplasmic proteins as potential vaccines. Briefly, the schematic model for the development of a multiple-epitope vaccine would be as follows: (i) isolate BCSP from the periplasmic space of *B. abortus* cells (52, 59); (ii) extract, characterize, and test the immunogenicity of the protein-containing subfractions (5, 37–40); (iii) fractionate the proteins by ion-exchange HPLC and produce antibodies to the purified proteins; (iv) clone the genes coding for the proteins by use of antibody detection methods; (v) test the immunogenicity of the proteins (7, 20, 38, 39, 45, 46, 54, 57); (vi) determine the amino acid sequence by conventional solid-phase protein sequencing or by DNA sequencing of the cloned genes; (vii) determine relevant peptide structures and identify potentially useful peptides (53, 55, 56); (viii) synthesize selected peptide epitopes (53, 55, 56) and test synthetic epitopes for immunogenicity in tissue culture and animal models (49, 53); and (ix) synthesize multiple-epitope vaccines by selecting epitopes with specific propensities relevant to humoral and cell-mediated immune responses (53, 55, 56). Perhaps this model can be extrapolated for use in studies on the immunogenesis and pathogenesis associated with immunoreactive proteins of other bacteria.

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