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Alteration of Protective and Serologic Responses in BALB/c Mice Vaccinated with Chemically Modified versus Nonmodified Proteins of *Brucella abortus* 19

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A study was conducted to determine whether the covalent chemical modification of *Brucella abortus* 19 salt-extractable proteins (BCSP) and BCSP derivatives would modulate the immune responses in BALB/c mice. Salt-extractable proteins BCSP 0-70 and BCSP 70-100 were modified with acetoacetic anhydride, and recombinant proteins rBCSP20 (20 kDa), rBCSP31 (31 kDa), and rBCSP45 (45 kDa) were modified with succinic and dodecanoyl anhydrides. Four weeks after mice were vaccinated with the different preparations, principal and control mice were challenge exposed with a virulent culture of *B. abortus* 2308, and mice were necropsied 2 weeks later. Serum samples were obtained immediately before mice were challenge exposed and at necropsy. Sera were tested for specific immunoglobulin M (IgM) and G (IgG) antibodies by using an enzyme-linked immunosorbent assay. Acylation decreased the immune responses (increased IgG antibodies and reduced spleen CFU and splenomegaly) induced by both BCSP 0-70 and BCSP 70-100. Modification of the recombinant proteins by dodecanoyl and succinic anhydrides had no effect on the protection induced; however, the IgG serologic responses to the homologous and heterologous proteins were altered. Monophosphoryl lipid A markedly enhanced the immunogenicity of BCSP 0-70.

Brucellosis is an important zoonotic disease (35, 42). In addition to causing health problems in humans, certain members of the genus *Brucella* are of tremendous economic importance to the food animal industry (35). For example, *Brucella abortus*, which causes abortion and infertility in cattle, was once possibly the most costly disease to the cattle industry in the United States. Although large sums of money and many different approaches have been directed at prevention, control, and eradication of brucellosis, the disease still is a problem. The most important need at present is a highly (>90%) effective and safe one-dose vaccine.

Effective vaccines presently used to control and prevent infection in cattle consist of either whole-cell killed preparations or attenuated live cultures. Subunit vaccines have been used but are not highly effective in protecting cattle from subsequent infection and disease (8, 41). Factors which usually influence the development of immunity against brucellosis in mice are related to the nature and dose of immunogen and the manner in which the immunizing epitopes are presented to the host's immune surveillance system (2, 3, 5, 10, 19, 20, 27, 31, 32). The overall immunity induced involves both cell-mediated and humoral antibody effects, which probably act together in preventing and eliminating infection. However, the effects might not always act together and sometimes may be antagonistic to each other, as has been reported for other diseases (12, 15).

It is generally accepted that immunity in brucellosis is mostly due to cellular immunity mechanisms (2, 7, 13, 27). The reason subunit vaccines are less effective than whole-cell vaccines may be due to the lack of constitutive cofactors of the former that are inherent in whole-cell vaccines. The extracting process used for fractional vaccines may also cause chemical and physical rearrangements of the immunizing epitopes. These induced rearrangements might mask or otherwise inactivate the protective epitopes in subunit vaccines, thereby making them ineffective for cell-mediated or humoral antibody responses.

Because immunity in brucellosis is due to both cell-mediated and humoral effects, both B- and T-cell responders must be considered when subunit vaccines are used (2, 7, 27, 34). Because of this, an important question in the development of subunit vaccines is whether the different components can be manipulated to give specific epitopic responses. Some investigators (1, 15, 18, 22, 32–34) suggest that chemical modifications of nonimmunogenic or poorly immunogenic substances can convert them to highly effective immunogens, while others (9, 22–24) reported that immunization with chemically modified antigens favors cell-mediated responses whereas unmodified antigens tend to elicit humoral responses. In our laboratory, unmodified protein antigens were not highly effective against infection by *B. abortus* although they usually induced excellent humoral antibody responses (28–30, 39, 40).

The objective in this study was to determine whether covalent chemical modification of *B. abortus* salt-extractable and recombinant derived proteins would enhance the immune responses of BALB/c mice against challenge exposure with a virulent culture of *B. abortus* 2308.

MATERIALS AND METHODS

Experimental design. The study consisted of two experiments. In experiment 1, the immune responses induced in principal mice by different doses of acylated and nonacylated salt-extractable *Brucella* cell surface proteins (BCSP 0-70 and BCSP 70-100) were determined. In experiment 2, the immune responses induced by succinic and dodecanoyl anhydride-modified recombinant proteins rBCSP20, rBCSP31, and rBCSP45 were determined.

Experiment 1 was designed specifically to compare the immunity induced by BCSP 0-70 and BCSP 70-100. These salt-extractable proteins were modified with acetoacetic anhy-

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TABLE 1. Postchallenge exposure spleen CFU and spleen weights in mice vaccinated with different concentrations of acylated and nonacylated *Brucella* cell surface proteins (BCSP 0-70 and BCSP 70-100)

Group	No. of mice[a]	Vaccine	Dose	CFU (log_{10}) (mean ± SD)[b]	Spleen wt (mg) (mean ± SD)[b]
1	7	Placebo (0.15 M NaCl)	0.2 ml	7.01 ± 0.12[a]	324 ± 50[e]
2	7	BCSP 0-70	0.1 μg	5.11 ± 0.89[b]	114 ± 13[f,g]
3	8	BCSP 0-70-AC[c]	0.1 μg	6.83 ± 0.25[a]	211 ± 44[f]
4	7	BCSP 0-70	1.0 μg	4.44 ± 0.34[b]	111 ± 6[f,g]
5	8	BCSP 0-70-AC	1.0 μg	5.32 ± 1.21[b]	148 ± 35[f]
6	7	BCSP 0-70	10.0 μg	4.64 ± 0.64[b,c]	111 ± 16[f,g]
7	8	BCSP 0-70-AC	10.0 μg	4.96 ± 0.86[b,c]	130 ± 30[f,g]
8	7	BCSP 0-70	30.0 μg	4.59 ± 0.81[b,c]	100 ± 5[f,g]
9	8	BCSP 0-70-AC	30.0 μg	5.02 ± 1.31[b,c]	135 ± 27[f]
10	7	BCSP 70-100	0.1 μg	6.51 ± 0.35[b]	181 ± 35[f]
11	8	BCSP 70-100-AC	0.1 μg	7.05 ± 1.96[a]	311 ± 76[e]
12	7	BCSP 70-100	1.0 μg	6.04 ± 0.05[b]	157 ± 22[f]
13	8	BCSP 70-100-AC	1.0 μg	6.76 ± 0.52[a]	206 ± 60[f]
14	7	BCSP 70-100	10.0 μg	4.67 ± 0.88[b,c]	116 ± 18[f,g]
15	8	BCSP 70-100-AC	10.0 μg	4.98 ± 1.54[b,c]	170 ± 54[f]
16	7	BCSP 70-100	30.0 μg	4.71 ± 0.80[b,c]	130 ± 34[f,g]
17	8	BCSP 70-100-AC	30.0 μg	5.81 ± 0.30[b]	155 ± 27[f]
18	9	MPL	50.0 μg	6.59 ± 0.30[a]	307 ± 55[e]
19	8	BCSP 0-70	5.0 μg	4.41 ± 0.76[c]	99 ± 15[g]
20	8	BCSP 0-70 + MPL	5.0 μg	0.19 ± 0.50[d]	99 ± 6[g]

[a] Groups of mice were vaccinated intraperitoneally. Four weeks later, two or three mice from each group were euthanatized, and blood was collected for serum antibody analysis by ELISA. The remainder of the mice were challenge exposed intraperitoneally with *B. abortus* 2308. Mice were necropsied 2 weeks after challenge exposure.
[b] Values with the same superscript letters are not different ($P > 0.05$).
[c] BCSP 0-70-AC, acetoacetyl BCSP 0-70.

dride because results from other studies (21–24) indicated that acylation of certain proteins enhances their immunogenicity. In our laboratory, results indicated that dodecanoyl anhydride modification enhanced the immunogenicity of BCSP 0-70 in lemmings, guinea pigs, and cattle (8, 38, 39). The effects of acylation of BCSP 0-70 on the immunogenicity in mice were not determined, and the effects of modification on BCSP 70-100 immunogenicity have not been studied.

Groups of principal mice were given different doses of either unmodified BCSP 0-70 and BCSP 70-100 or corresponding doses of acylated BCSP 0-70 and BCSP 70-100 (Table 1). Nonvaccinated control mice were given a 0.15 M NaCl solution (placebo). One group of mice was given 5.0 μg of BCSP 0-70, one group was given 50.0 μg of monophosphoryl lipid A (MPL), and one group was given 5.0 μg of BCSP 0-70 plus 50.0 μg of MPL. These three groups served as positive vaccinated controls, because results from an earlier study (40) indicated that MPL was a good immune response modifier when used with BCSP 0-70.

Experiment 2 was designed to compare the immunogenicity of chemically modified recombinant proteins with that of nonmodified proteins. The immune responses induced by succinic and dodecanoyl anhydride-modified recombinant proteins rBCSP20, rBCSP31, and rBCSP45, which were derived in *Escherichia coli* from BCSP 0-70 extracts, were determined. Results in other studies (38, 39) indicated that the extractable proteins BCSP20, BCSP31, and BCSP45 which were isolated from BCSP 0-70 were partially immunogenic in laboratory animals. However, questions arose concerning possible contamination of the proteins by brucellar lipopolysaccharide (LPS) that was not eliminated during the isolation process. The recombinant proteins, derived from the extracted proteins of BCSP 0-70 and used in the present study, are presumed to be free of brucellar LPS. These proteins did not induce protection in mice when used without adjuvant or chemical modification (28, 29). The immunity induced by BCSP 0-70, the progenitor of the subfractional proteins, was enhanced by modification with dodecanoyl anhydride (8, 39). Results in other reports (22, 32, 33) indicated that succinic anhydride modification induces results similar to those of dodecanoyl anhydride modification. Nonvaccinated control mice were given a 0.15 M NaCl solution (placebo). For each recombinant protein, a group of eight mice was given 10.0 μg of the respective modified or nonmodified protein. One group of eight mice was vaccinated with 0.5 μg of LPS as a positive control.

In both experiments, mice were given vaccinal inoculations on day 0, and 28 days later all mice were challenge exposed with a virulent culture of *B. abortus* 2308. Blood samples were taken on day 28 (prechallenge exposure sera) immediately before challenge exposure and 2 weeks (day 42) after challenge exposure at necropsy (postchallenge exposure sera). Sera were tested for antibodies against the different antigens by using an enzyme-linked immunosorbent assay (ELISA) (28, 30, 36).

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

Bacteria. Stock cultures of a virulent *B. abortus* 2308 were isolated from an aborted bovine fetus and were stored at −70°C. The thawed culture was streaked on potato infusion agar and incubated for 40 to 44 h at 37°C, then washed from the agar surface with saline solution (0.15 M NaCl), and adjusted to an optical density of 0.125 at 600 nm by using a colorimeter (Spectronic 20 colorimeter; Baush & 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.

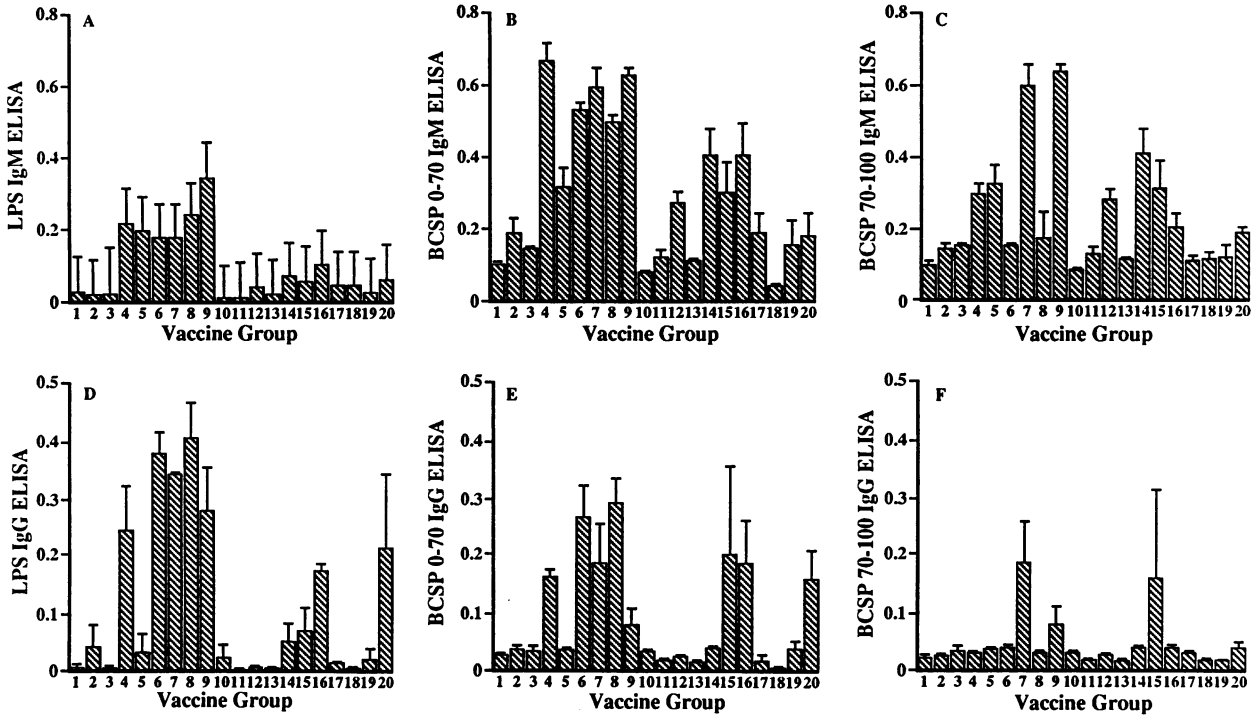

FIG. 1. Prechallenge exposure ELISA results for mice vaccinated with different doses of acetoacetic anhydride-modified *Brucella* cell surface proteins (BCSP 0-70 and BCSP 70-100) and with nonmodified proteins. Mice in group 1 were given 0.2 ml of 0.15 M NaCl. Mice in groups 2, 4, 6, and 8 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of nonmodified BCSP 0-70, respectively. Mice in groups 3, 5, 7, and 9 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of acetoacetic anhydride-modified BCSP 0-70, respectively. Mice in groups 10, 12, 14, and 16 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of nonmodified BCSP 70-100, respectively. Mice in groups 11, 13, 15, and 17 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of acetoacetic anhydride-modified BCSP 70-100, respectively. Mice in group 18 were given 50.0 μg of MPL. Mice in group 19 were given 5.0 μg of nonmodified BCSP 0-70, mice in group 20 were given 5.0 μg of nonmodified BCSP 0-70, and mice in group 20 were given 5.0 μg of nonmodified BCSP 0-70 plus 50.0 μg of MPL. The results are depicted as ELISA absorbance (10^3) at A_{410}/A_{450}, using the specific antigen.

Isolation of BCSP. BCSP 0-70 was isolated from washed methanol-inactivated *B. abortus* 19 as previously described (37–39) and modified (4). Briefly, cells were grown in liquid culture, collected by filtration, washed with 0.15 M NaCl solution, and inactivated by addition of 2 volumes of methanol to 1 volume of cell suspension. The cell suspension was stirred overnight at 5°C and then allowed to stand at 5°C for 1 week, with occasional stirring. The suspension was centrifuged at 12,000 × *g* for 1 h, the cells were resuspended in a hypertonic salt solution, and the proteins were extracted at 5°C by gently stirring for 16 h. The extraction procedure was repeated, and the extracts were combined, concentrated by freeze-drying, dialyzed, and precipitated by ammonium sulfate at 70% saturation. BCSP 70-100 was prepared in the same way as was BCSP 0-70 except that it was precipitated by using ammonium sulfate at 100% saturation, using the supernatant solution from the 70% ammonium sulfate precipitation step. The precipitated proteins were dissolved in and dialyzed against 5 mM NH_4HCO_3. Protein concentration was determined by the Lowry procedure (14). The BCSP was stored at −70°C until used.

Isolation of recombinant proteins. BCSP31 was isolated and purified as previously described (6, 17), using a two-step procedure consisting of detergent extraction followed by ion-exchange chromatography. The other recombinant proteins (rBCSP20 and rBCSP45) were isolated and purified by a similar procedure that was modified so that the recombinant protein was bound to the ion-exchange resin at low ionic strength (5 to 10 mM NaCl) and was specifically eluted in an increasing salt gradient.

Isolation of LPS. The LPS used for the vaccine and antigen in the ELISA was extracted from *B. abortus* 2308 by a butanol extraction procedure followed by proteinase K (E. Merck, Darmstadt, Germany) digestion as described previously (26). The preparation contained less than 1% protein.

Chemical modification of proteins. The BCSP 0-70 and BCSP 70-100 proteins were modified with acetoacetic anhydride, and the rBCSP fractions were modified with dodecanoyl anhydride and succinic anhydride (11, 16, 18, 32, 33). The extent of amino group modification was determined with trinitrobenzene sulfonic acid (11).

Adjuvant. Nontoxic MPL was obtained from a commercial source (Ribi Immunochem Research, Inc., Hamilton, Mont.) and was isolated from a *Salmonella typhimurium* Re mutant. The MPL was stored at 4°C until thawed and used in the vaccines 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 obtain the desired concentration of each vaccine (Tables 1 and 2). Mice were vaccinated by inoculating each mouse with 0.2 ml of the respective vaccine intraperitoneally. Control mice were inoculated with 0.15 M NaCl as the placebo.

Challenge exposure. Four weeks (day 28) after vaccination, mice were challenge exposed with a virulent culture of *B. abortus* 2308. Each mouse was given approximately 10^4 CFU intraperitoneally in 0.2 ml of saline solution.

Serologic examination. Serum immunoglobulin M (IgM) and G (IgG) antibody concentrations against the different antigens were determined by using sera from mice (Tables 1

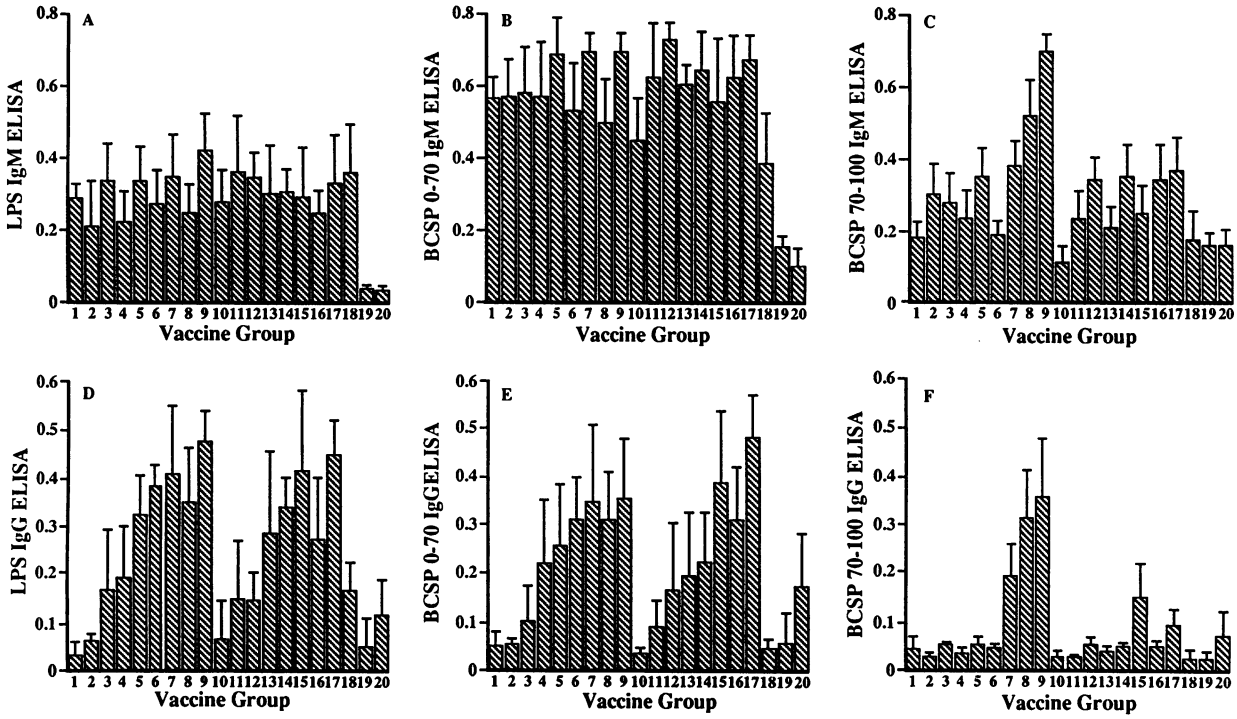

FIG. 2. Postchallenge exposure ELISA results for mice vaccinated with different doses of acetoacetic anhydride-modified *Brucella* cell surface proteins (BCSP 0-70 and BCSP 70-100) and with the corresponding nonmodified proteins. Mice in group 1 were given 0.2 ml of 0.15 M NaCl. Mice in groups 2, 4, 6, and 8 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of nonmodified BCSP 0-70, respectively. Mice in groups 3, 5, 7, and 9 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of acetoacetic anhydride-modified BCSP 0-70, respectively. Mice in groups 10, 12, 14, and 16 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of nonmodified BCSP 70-100, respectively. Mice in groups 11, 13, 15, and 17 were given doses of 0.1, 1.0, 10.0, and 30.0 μg of acetoacetic anhydride-modified BCSP 70-100, respectively. Mice in group 18 were given 50.0 μg of MPL. Mice in group 19 were given 5.0 μg of nonmodified BCSP 0-70. Mice in group 20 were given 5.0 μg of nonmodified BCSP 0-70 plus 50.0 μg of MPL. The results are depicted as ELISA absorbance (10^3) at A_{410}/A_{450}, using the specific antigen.

and 2) on the day (day 28) mice were challenge exposed and 2 weeks later (day 42) at necropsy, using an ELISA (29, 30, 36, 40). Results of BCSP, LPS, and recombinant protein-specific IgM and IgG antibody concentrations are expressed as absorbance units (A_{410}/A_{450}) and were obtained by using an ELISA plate reader (Dynatech Laboratories, Alexandria, Va.).

Culture examination of the spleen. The CFU of *B. abortus* per spleen and mean spleen weights were determined as previously described (28–30).

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

RESULTS

The results of two experiments are presented. In experiment 1, BCSP 0-70 and BCSP 70-100 were compared with their respective acetoacetylated proteins as immunogens for murine brucellosis. In experiment 2, the recombinant proteins rBCSP20, rBCSP31, and rBCSP45, which constitute BCSP 0-70 and BCSP 70-100, are compared with succinylated and dodecanoylated recombinant proteins as vaccines.

Experiment 1. Mice in groups 4, 6, 8, 14, 16, 19, and 20 ($P < 0.001$), groups 7, 9, and 15 ($P < 0.01$), and groups 2 and 5 ($P < 0.05$) had CFU that were less than that of mice (group 1) given the placebo (Table 1). The CFU of other mice (groups 3, 10 to 13, 17, and 18) were not significantly ($P > 0.05$) different from CFU of mice given the placebo. As indicated by the P values, the CFU of mice given BCSP 0-70 or BCSP 70-100 was lower than the CFU of mice given acylated BCSP 0-70 or acylated BCSP 70-100. When the spleen weight of mice given placebo (group 1) was compared with spleen weights of other mice, all groups were different ($P < 0.001$) except groups 11 and 18 ($P > 0.05$). The greatest CFU reduction occurred in mice given 5.0 μg of BCSP 0-70 plus MPL (group 20), but the spleen weights of the two groups (groups 19 and 20) which were given 5.0 μg of BCSP 0-70 were similar.

The IgM and IgG antibody concentrations in prechallenge exposure sera were determined by ELISA using LPS, BCSP 0-70, and BCSP 70-100 antigens (Fig. 1). When LPS was used as the test antigen for IgM antibody concentrations, only groups 8 and 9 ($P < 0.001$), 4 and 6 ($P < 0.01$), and 7 ($P < 0.05$) were different from mice given the placebo (Fig. 1A). When the IgG antibody concentration of group 1 mice was compared with those of other groups, only groups 6 to 9 ($P < 0.001$), 4 ($P < 0.01$), and 20 ($P < 0.05$) were different (Fig. 1D). When BCSP 0-70 was used as the test antigen for IgM antibody concentrations, only groups 4 and 6 to 9 ($P < 0.001$) and groups 14 and 16 ($P < 0.05$) were different from mice given the placebo (Fig. 1B). When the IgG antibody concentrations were compared, only groups 6 and 8 ($P < 0.01$) were different from mice given the placebo (Fig. 1E). When BCSP 70-100 was used as the test antigen for IgM antibody concentration, groups 5, 7, 9, and 14 ($P < 0.001$), 4 and 15 ($P < 0.01$), and 12 ($P < 0.05$) were different from mice given the placebo (Fig. 1C). When the IgG antibody concentrations were compared, only group 7 ($P < 0.05$) was different from mice given the placebo (Fig. 1F).

TABLE 2. Postchallenge exposure CFU and spleen weights in mice vaccinated with nonmodified and chemically modified recombinant proteins rBCSP20, rBCSP31, and rBCSP45 which were derived from salt-extractable *Brucella* cell surface protein BCSP 0-70

Group	No. of mice[a]	Vaccine[b]	Dose	CFU (log_{10}) (mean ± SD)[c]	Spleen wt (mg) (mean ± SD)[c]
1	6	Placebo (0.15 M NaCl)	0.2 ml	6.16 ± 0.22[a]	193 ± 23[c]
2	8	LPS	0.5 μg	3.72 ± 0.81[b]	112 ± 20[d]
3	8	rBCSP20	10.0 μg	6.04 ± 0.23[a]	180 ± 15[c]
4	8	S-rBCSP20	10.0 μg	5.91 ± 0.19[a]	169 ± 24[c]
5	8	D-rBCSP20	10.0 μg	6.41 ± 0.19[a]	179 ± 20[c]
6	8	rBCSP31	10.0 μg	6.07 ± 0.03[a]	184 ± 13[c]
7	8	S-rBCSP31	10.0 μg	5.90 ± 0.73[a]	166 ± 26[c]
8	8	D-rBCSP31	10.0 μg	6.18 ± 0.31[a]	180 ± 13[c]
9	8	rBCSP45	10.0 μg	6.07 ± 0.26[a]	199 ± 21[c]
10	8	S-rBCSP45	10.0 μg	6.20 ± 0.48[a]	184 ± 17[c]
11	8	D-rBCSP45	10.0 μg	5.95 ± 0.37[a]	188 ± 39[c]

[a] Groups of mice were vaccinated intraperitoneally. Four weeks later, two mice from each group were euthanatized, and blood was collected for serum antibody analysis by ELISA. The remainder of the mice were challenge exposed intraperitoneally with *B. abortus* 2308. Mice were necropsied 2 weeks after challenge exposure.

[b] S- or D- indicates that the rBCSP protein was modified with succinic anhydride or dodecanoyl anhydride, respectively.

[c] Values with the same superscript letters are not different ($P > 0.05$).

The IgM and IgG antibody concentrations in postchallenge exposure sera were determined with ELISA using LPS, BCSP 0-70, and BCSP 70-100 as test antigens (Fig. 2). When LPS was used as the test antigen to compare the IgM antibody concentrations, only groups 19 and 20 ($P < 0.001$) were different from mice given the placebo (Fig. 2A). When the IgG antibody concentrations were compared, groups 5 to 9 and 13 to 17 ($P < 0.001$) were different from mice given the placebo (Fig. 2D). When BCSP 0-70 was used as the test antigen to compare the IgM antibody concentrations, only groups 19 and 20 ($P < 0.001$) were different from mice given the placebo (Fig. 2B). When the IgG antibody concentrations were compared, groups 6 to 9 and 15 to 17 ($P < 0.001$) and group 5 ($P < 0.05$) were different from mice given the placebo (Fig. 2E). When BCSP 70-100 was used as the test antigen to compare the IgM antibody concentrations, groups 5, 7 to 9, 14, and 17 ($P < 0.001$) and groups 12 and 16 ($P < 0.01$) were different from mice given the placebo (Fig. 2C). When the IgG antibody concentrations were compared, groups 7 to 9 ($P < 0.001$) and group 15 ($P < 0.01$) were different from mice given the placebo (Fig. 2F). The concentrations of IgG and IgM were higher when the homologous antigen was used in ELISA than when the heterologous antigens were used (Fig. 2).

Experiment 2. When the spleen CFU and splenic weight values of mice vaccinated with modified or nonmodified recombinant protein vaccines were compared with those of mice given the placebo, there were no significant ($P > 0.05$) differences (Table 2). Mice vaccinated with LPS, which was used as a positive control, had CFU and splenic weights significantly ($P < 0.001$) lower than those of other mice.

When the prechallenge exposure sera were examined by ELISA, there were increases in IgG and IgM concentrations in all vaccinated mice, especially against the homologous antigens, compared with mice given the placebo (Fig. 3). The highest IgG antibody concentrations were in sera of mice vaccinated with nonmodified homologous protein. Some sera had higher IgM concentrations against heterologous antigens than against the homologous antigens (Fig. 3C and F).

When LPS was used as the test antigen to compare the IgM antibody concentrations, only group 2 ($P < 0.001$) was different from mice given the placebo (Fig. 3A). When the IgG antibody concentrations were compared, group 2 ($P < 0.001$) and groups 3, 5, and 7 to 9 ($P < 0.05$) were different from mice given the placebo (Fig. 3D). When BCSP 0-70 was used as the test antigen to compare the IgM concentrations, group 10 ($P < 0.01$) was different from mice given the placebo, and groups 3, 5, 7, and 9 ($P < 0.01$) and groups 4, 6, 8, and 10 ($P < 0.05$) were different from group 11 (Fig. 3B). When the IgG antibody concentrations were compared, only group 2 ($P < 0.05$) was different from mice given the placebo (Fig. 3E). Groups 3 to 5, 7, and 9 ($P < 0.05$) were different from mice given LPS. When rBCSP20 was used as the test antigen for the IgM and IgG antibody concentrations, none ($P > 0.05$) were different (Fig. 3C). When rBCSP31 was used as the test antigen for IgM and IgG antibody concentrations, none ($P > 0.05$) were different. When rBCSP45 was used as the test antigen to compare the IgM antibody concentrations, group 2 ($P < 0.01$) was different. Groups 4, 5, 7, and 9 ($P < 0.01$) and group 3 ($P < 0.05$) were different (Fig. 3C). When rBCSP45 was used as the test antigen to determine the IgG antibody concentrations the P values were greater than 0.05.

The IgM and IgG antibody concentrations of postchallenge exposure sera were determined by ELISA (Fig. 4). When LPS was used as the test antigen for IgM antibody concentrations, only groups 2, 3, and 6 ($P < 0.001$), 4 ($P < 0.01$), and 5 and 7 ($P < 0.05$) were different from mice given the placebo (Fig. 4A). Groups 2, 3, and 6 ($P < 0.01$) were different from group 9 (vaccinated with rBCSP45). When the IgG antibody concentrations were compared, groups 3 to 8 and 10 ($P < 0.001$) and groups 2 and 9 ($P < 0.01$) were different from mice given the placebo (Fig. 4D). When BCSP 0-70 was used as the test antigen for IgM antibody concentrations, group 7 ($P < 0.01$) and groups 4 and 6 ($P < 0.05$) were different from mice given the placebo (Fig. 4B). Group 9 was different from groups 7 ($P < 0.01$) and 6 ($P < 0.05$). When the IgG antibody concentrations were compared, no group ($P > 0.05$) was different from mice given the placebo (Fig. 4E). Groups 8 and 9 ($P < 0.05$) were different from group 6. When rBCSP20 was used as the test antigen to compare the IgM antibody concentrations, no group ($P > 0.05$) was different from mice given the placebo (Fig. 4C). Group 9 differed from groups 4 and 6 ($P < 0.01$) and group 7 ($P < 0.05$). When rBCSP20 was used to compare the IgG antibody concentrations, groups 3 and 9 ($P < 0.001$), 6 ($P < 0.01$), and 2 ($P < 0.05$) were different (Fig. 4F). When rBCSP31 was used as the test antigen to compare the IgM antibody concentrations, the P values for all groups were greater than 0.05 (Fig. 4C). When rBCSP31 was used to compare the IgG antibody concentrations, no group ($P > 0.05$) was different from mice given the placebo (Fig. 4F). The groups ($P > 0.05$) were also not different from each other. When rBCSP45 was used as the test antigen to compare the IgM antibody concentrations, all groups ($P < 0.001$) were different from mice given the placebo (Fig. 4C). There were no differences ($P > 0.05$) between the other groups. When rBCSP45 was used to compare the IgG antibody concentrations, groups 8 and 11 ($P < 0.001$) and group 5 ($P < 0.01$) were different from mice given the placebo (Fig. 4F). Group 2 was different from group 8 ($P < 0.01$). Group 3 was different from groups 7 and 11 ($P < 0.001$). Group 4 was different from groups 5, 8, and 11 ($P < 0.001$). Group 5 was different from groups 7, 9, and 10 ($P < 0.001$) and 6 ($P < 0.01$). Group 6 was different from groups 9 and 11 ($P < 0.001$). Group 7 was different from groups 8 and 11 ($P < 0.001$). Group 8 was different from groups 9 and 10 ($P < 0.001$). Group 9 was

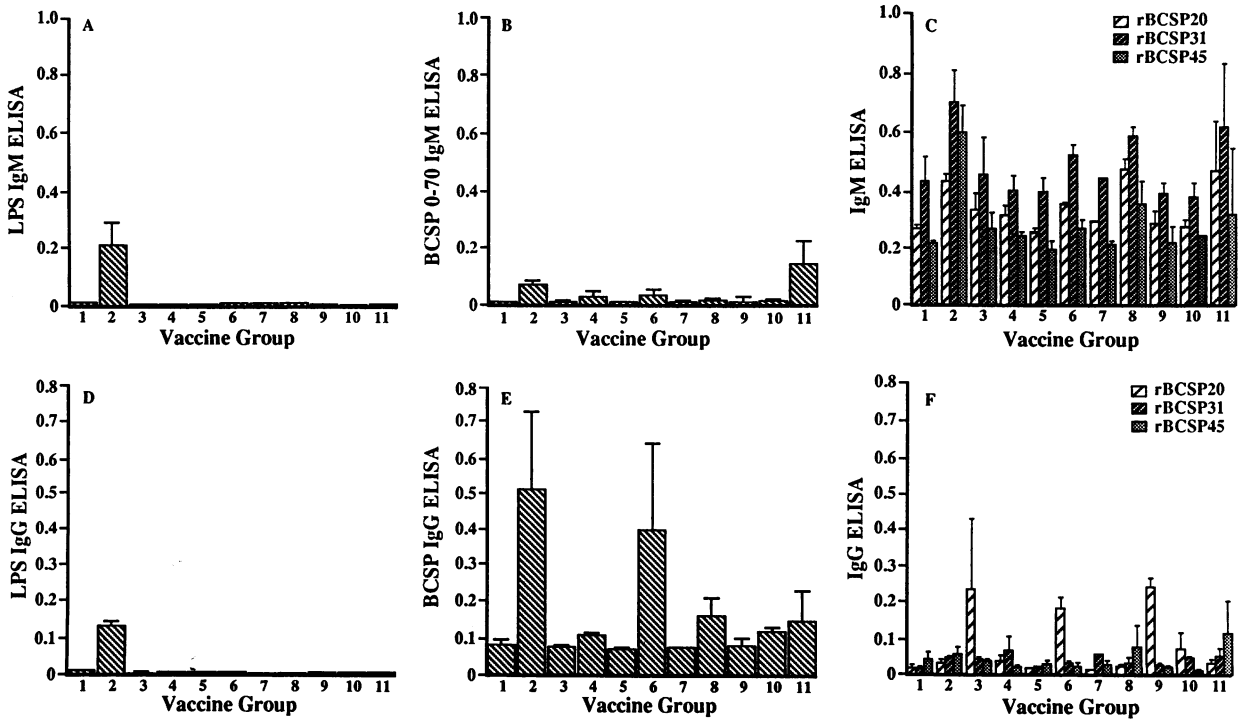

FIG. 3. Prechallenge exposure ELISA results for mice vaccinated with dodecanoyl anhydride- and succinic anhydride-modified recombinant proteins rBCSP20, rBCSP31, and rBCSP45 and the corresponding nonmodified recombinant proteins. Mice in group 1 were given 0.2 ml of 0.15 M NaCl. Mice in group 2 were given 0.5 μg of LPS. Mice in groups 3, 4, and 5 were given 10.0 μg of nonmodified rBCSP20, succinic anhydride-modified rBCSP20, and dodecanoyl anhydride-modified rBCSP20, respectively. Mice in groups 6, 7, and 8 were given 10.0 μg of nonmodified rBCSP31, succinic anhydride-modified rBCSP31, and dodecanoyl anhydride-modified rBCSP31, respectively. Mice in groups 9, 10, and 11 were given 10.0 μg of nonmodified rBCSP45, succinic anhydride-modified rBCSP45, and dodecanoyl anhydride-modified rBCSP45, respectively. The results are depicted as ELISA absorbance (10^3) at A_{410}/A_{450}, using the specific antigen.

different from group 11 ($P < 0.001$), and group 10 was different from group 11 ($P < 0.001$).

DISCUSSION

The results obtained in experiment 1 indicate that acylation of BCSP 0-70 or BCSP 70-100 does not enhance their immunogenicity for mice against *B. abortus* infections. Contrariwise, acylation caused decreases in the immune responses of reduced spleen CFU and splenomegaly compared with mice that received nonacylated proteins. The greatest reduction in protection occurred in mice that received low doses of the acylated proteins compared both with mice given nonacylated proteins at the same concentration and with mice given the placebo. Possibly, at low doses, the concentrations of unmodified epitopes were too low to induce protective immunity or the epitopes interfered with the immunogenesis, whereas at higher doses, the number of nonmodified protective epitopes was sufficient to induce protective immunity in spite of the presence of acylation effects. In each case in which equal doses of acylated and nonacylated proteins were given, the group that received the acylated protein, regardless of the dose, had higher CFU and spleen weights. This finding also might mean that the number of unmodified epitopes was the critical factor which determined the difference; unfortunately, we did not titrate the dose range for these acylated proteins. Acylation of proteins was reported to increase the protective immunity of BCSP vaccines, but apparently the protective immunity was dependent on the percent modification of available primary amino groups of the proteins (39).

The linear increases in the protection corresponding to increased doses of both BCSP 0-70 and BCSP 70-100 whether the proteins were acylated or not indicate that dose-response relationships are important with *Brucella* protein vaccines (5, 30, 40). The results also indicate that BCSP 0-70, at the same dose, is 10 times more effective than BCSP 70-100. The protective epitopes involved with both BCSP 0-70 and BCSP 70-100 immunity are presumed to be identified closely with similar epitopes found in LPS. Although we assume that our method of preparing the proteins eliminated LPS and given the fact that the proteins were free of 3-deoxy-D-manno-octulosonic acid, a prequisite for LPS presence, it is still possible that one or more common but unidentified immunizing epitopes were present in these vaccines. We feel, however, that the common protective epitopes in LPS and the proteins might not have been responsible for the serologic responses seen in experiment 1. The serologic results in experiment 2, in which LPS and BCSP 0-70 were used as the antigens in ELISA and vaccinated mice were not protected, gave results similar to the serological results in experiment 1, in which vaccinated mice were protected.

The prechallenge serologic responses were as expected and indicate that increased IgG antibody is, in an unknown way, associated with increased protection. Substantiating this is the fact that at all dose levels, as would be expected, acylation caused a reduction in IgG antibody concentration accompanying the increased CFU and spleen weight values compared with the corresponding nonacylated protein dose levels. This seems reasonable because others have reported that immuni-

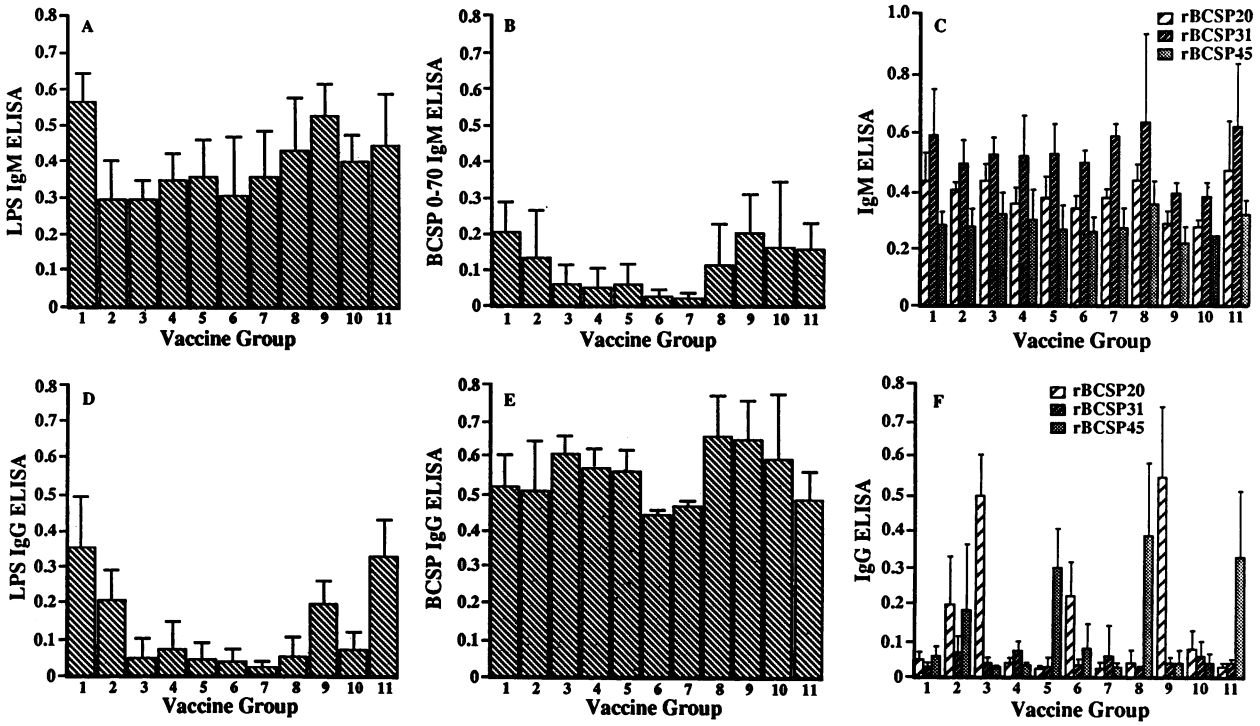

FIG. 4. Postchallenge exposure ELISA results for mice vaccinated with dodecanoyl anhydride- and succinic anhydride-modified recombinant proteins rBCSP20, rBCSP31, and rBCSP45 and the corresponding nonmodified recombinant proteins. Mice in group 1 were given 0.2 ml of 0.15 M NaCl. Mice in group 2 were given 0.5 μg of LPS. Mice in groups 3, 4, and 5 were given 10.0 μg of nonmodified rBCSP20, dodecanoyl anhydride-modified rBCSP20, and succinic anhydride-modified rBCSP20, respectively. Mice in groups 6, 7, and 8 were given 10.0 μg of nonmodified rBCSP31, dodecanoyl-modified rBCSP31, and succinic anhydride-modified rBCSP31, respectively. Mice in groups 9, 10, and 11 were given 10.0 μg of nonmodified rBCSP45, dodecanoyl anhydride-modified rBCSP45, and succinic anhydride-modified rBCSP45, respectively. The results are depicted as ELISA absorbance (10^3) at A_{410}/A_{450}, using the specific antigen.

zation with chemically modified antigens favors cell-mediated responses, while unmodified antigens tend to elicit humoral antibody responses (9, 21–24). Consequently, these results suggest that although acylation lowered IgG antibody concentrations, the lowering was not directly related to protection because in brucellosis, protection is mainly dependent upon cell-mediated immunity.

We suggest that the lack of direct relationship between increased protection and increased IgG antibody concentrations is due to the fact that the protective subclass found in IgG that affects brucellar immunity in BALB/c mice is IgG3 and the ELISA results involve, in addition to IgG3, subclasses IgG1, IgG2a, and IgG2b. Unpublished results in our laboratory indicated that the better-protected ($P < 0.001$) vaccinated mice had higher IgG3 antibody concentrations in their prechallenge exposure sera than did vaccinated and partially protected ($P < 0.05$) mice with lower IgG3 antibody concentrations or nonprotected ($P > 0.05$) nonvaccinated mice. Another report (25) of results from our laboratory indicated that the injection of monoclonal IgG3 antibodies into BALB/c mice provided partial protection against *B. abortus* infection.

When the IgG isotype distribution and concentrations in the prechallenge exposure sera were determined (data not shown), the sera of mice (groups 19 and 20) given MPL had the highest IgG3 concentrations and the mice (group 20) also given BCSP 0-70 (experiment 1, Table 1) were the best-protected mice; group 20 mice had significant ($P < 0.001$) reduction in CFU and splenomegaly compared with other mice in experiment 1. Results in another study (40) indicated that MPL was an excellent immune response modifier in BCSP 0-70 vaccines but MPL did not enhance the immunogenicity of LPS vaccines. The role of IgG3 and the mechanisms involved when MPL is used as an immune response modifier in vaccines are beyond the scope of this report.

The results from experiment 2 indicated that the modification of the recombinant proteins rBCSP20, rBCSP31, and rBCSP45 by succinic and dodecanoyl anhydrides altered their induction of serologic responses to homologous as well as heterologous antigen as determined by ELISA. Others (18, 21, 22, 33) have reported that modification by these chemical compounds cause changes in the immune responses induced. The vaccines prepared from recombinant proteins, both modified or nonmodified, however, although inducing increased IgG and IgM antibody concentrations in the sera of vaccinated mice, did not enhance the protective responses of reduced spleen CFU and splenomegaly in challenge-exposed mice compared with mice given the placebo. This lack of correlation might indicate that the proteins do not contain specific epitopes for cell-mediated immune functions that are found in LPS or BCSP fractions although the modifications enhanced the recombinant protein to produce antibodies. Results in a related study (unpublished data) in our laboratory indicated that rBCSP45 derived from BCSP 0-70 and an uncharacterized subfraction from BCSP 70-100 induced significant ($P < 0.01$) protection in mice when MPL plus these proteins were used in vaccines. This probably means that increased antibody concentrations alone are not able to cause reduced CFU and splenomegaly significantly and some additional factor such as LPS or

MPL is also needed to modulate the cell-mediated protective effects. It is well established that the immunity in brucellosis is due to cell-mediated responses (2, 7, 13).

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