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Cattle Serologically Positive for *Brucella abortus* Have Antibodies to *B. abortus* Cu-Zn Superoxide Dismutase

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In this study, we demonstrated by a Cu-Zn superoxide dismutase-specific enzyme-linked immunoassay that cattle that are serologically positive for *Brucella abortus* have serum immunoglobulin G antibodies to *B. abortus* Cu-Zn superoxide dismutase. The specificity of the antibody reactivity was confirmed by Western blot (immunoblot) analysis with *B. abortus* salt-extractable proteins containing native Cu-Zn superoxide dismutase and with recombinant *B. abortus* Cu-Zn superoxide dismutase. The results represent a first step in the direction of the development of a multiprotein diagnostic reagent for bovine brucellosis.

Brucellosis is an acute and chronic infectious disease of domestic animals that is transmissible to humans (10). Animals infected with *Brucella abortus* produce antibodies to numerous bacterial components (9), including O-chain polysaccharide consisting of repeating units of 4-formamido-4,6-dideoxy-D-mannose (7, 8, 11). We had observed that infected animals also produce antibodies to a high-salt protein extract of *B. abortus* (5, 15). Recently, we cloned the gene coding for one of the salt-extracted proteins that has an apparent molecular mass of approximately 20,000 Da (6). The identity of this protein was established from the protein sequence of the recombinant form of the 20,000-Da protein, which shared a 57% identity with the enzyme Cu-Zn superoxide dismutase from *Photobacterium leiognathi* (4). Subsequent enzyme assays established that the *Brucella* enzyme is a Cu-Zn superoxide dismutase (SOD) with a molecular weight of 16,071 (based on the amino acid sequence) (4).

In this article, we demonstrate that sera obtained from cattle that are serologically positive for *B. abortus* have serum immunoglobulin G (IgG) antibodies to *B. abortus* Cu-Zn SOD.

MATERIALS AND METHODS

Protein preparations. The *Brucella* cell surface proteins used in the enzyme-linked immunosorbent assay (ELISA) and Western blotting (immunoblotting) procedures were the high-salt protein extract (BCSP) and the recombinant Cu-Zn SOD. BCSP from methanol-killed *B. abortus* 19 was prepared by a method described previously (5, 15) and modified as follows. *B. abortus* was grown in filter-sterilized Trypticase soy broth (Difco, Bedford, Mass.) (1). Cells were harvested, washed free of medium, and methanol inactivated as described previously (5, 15). The cell pellet was extracted twice with 1 M NaCl–0.1 M sodium citrate for 16 h at 5°C with gentle stirring. The proteins were recovered from the extract by dialysis and clarification of the supernatant and by ammonium sulfate precipitation at 70% saturation and another dialysis.

The SOD was prepared from recombinant *Escherichia coli* containing plasmid pUC9:pBA1527 coding for the *Brucella* SOD, as described previously (6). Briefly, a Triton X-100 extract of a 20-h culture grown in Luria broth containing 0.005% ampicillin was dialyzed, clarified, and applied to a CM-Sepharose column. The SOD was eluted with a linear gradient of 5 to 500 mM NaCl in 10 mM phosphate buffer, pH 6.5. Tubes containing the SOD were combined and lyophilized.

Polyacrylamide gel electrophoresis and immunoblotting. Samples (5 μg per lane) were applied to a 10-to-15% gradient polyacrylamide gel (8 cm by 10 cm by 1 mm) (Integrated Separation Systems, Hyde Park, Mass.), and electrophoresis was performed under denaturing conditions at 20 mA per gel (5). The proteins were electrophoretically transferred to 0.45-μm-pore-size nitrocellulose (Schleicher & Schuell, Keene, N.H.) at 30 V and 0.11 A for 16 h, as described by Towbin et al. (16). A portion of the blot was stained for 2 min with 0.1% amido black in 25% isopropanol–10% acetic acid and destained in 25% isopropanol–10% acetic acid. The remainder of the blot was cut into strips and incubated for 15 min with 1% fish gelatin (Norland Products, Inc., New Brunswick, N.J.) in 0.1 M phosphate-buffered saline–0.5% Tween 80 (PBS-Tween) (Difco, Detroit, Mich.). The strips were incubated for 2 h at room temperature with bovine sera diluted 1:100 in PBS-Tween; the strips were then washed with PBS-Tween and incubated for 16 h at 5°C with a 1:3,200 dilution of horseradish peroxidase-conjugated rabbit anti-bovine IgG (gamma-chain specific) (Kirkegaard & Perry, Gaithersburg, Md.). The strips were washed with PBS-Tween, rinsed with distilled water, and incubated with a solution containing 20 ml of 0.1 M PBS (pH 7.2), 4.0 ml of 0.3% 4-chloronaphthol in methanol, and 10 μl of 30% H_2O_2. After color development was complete, the strips were rinsed with distilled water.

Enzyme-linked immunoassay. Enzyme-linked immunoassay was performed essentially as described previously (14). BCSP at a concentration of 0.1 μg per well and recombinant SOD at a concentration of 0.2 μg per well were incubated overnight at 5°C in microtiter plates (ProBind, Falcon no. 3915; Becton Dickinson, Lincoln Park, N.J.). Plates were washed, incubated with control and test sera diluted 1:100, incubated with rabbit anti-bovine horseradish peroxidase-conjugated IgG (gamma-chain specific) diluted at 1:3,200, and developed. The results were recorded as $A_{410/450}$, as described previously (14).

Sera. Twenty-one test serum samples from serologically positive cattle and one serologically negative serum sample (no. 22) were obtained from a serum bank maintained at the National Veterinary Services Laboratories, Ames, Iowa. All samples (1-ml aliquots) had been lyophilized, stored at 5°C, and reconstituted to the original volume with sterile distilled

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TABLE 1. History of bovine sera[a]

Serum sample[b]	Age of cow[c]	Vaccination status[d]	*B. abortus* biovar isolated	Source of isolation[e]
1	Adult	Vac. calf	1	NA (DBL)
2	Adult	Vac. calf	1	NA (DBL)
3	Adult	Vac. calf	1	NA (DBL)
4	Adult	Vac. calf	1	NA (DBL)
5	Adult	Vac. calf	1	NA (DBL)
6	Adult	Vac. calf	No sample	ND
7	Adult	Vac. calf	1	NA (DBL)
8	Adult	Not vac.	1	NA (DBL)
9	Adult	Not vac.	1	M, V, P (DBL)
10	Adult	Vac. calf	1	M, V, P (DBL)
11	Adult	Not vac.	1	M, V, P (DBL)
12	Adult	Vac. calf	Strain 19	NA (OK)
13	3 yr	Vac. calf	1	LN, M (DBL)
14	7 yr	Vac. calf	1	LN (DBL)
15	6 yr	Vac. calf	1	M (DBL)
16	12 yr	Vac. calf	4	M (DBL)
17	8 yr	Not vac.	None	NI (DBL)
18	9 yr	Not vac.	None	NI (DBL)
19	10 yr	Vac. calf	4	M (DBL)
20	Adult	Vac. calf	1	LN (DBL)
21	4 yr	Vac. calf	Strain 19	LN (DBL)
22	Adult	Vac. calf	No sample	ND

[a] Sera were obtained from a serum bank maintained at the National Veterinary Services Laboratories.

[b] Serum identification number. Origins of serum samples were as follows: samples 1 through 7, Missouri (farm); samples 9 through 12, National Veterinary Services Laboratories (experimentally infected); sample 13, Oklahoma (farm); samples 14 through 16 and 18 through 24, Tennessee (farm).

[c] Age of animal (if known) when serum was obtained.

[d] Vac. calf, calfhood-vaccinated calf; Not vac., not calfhood vaccinated.

[e] NA, information not available; DBL, Diagnostic Bacteriology Laboratory of the National Veterinary Services Laboratories (state-federal laboratory where culture work was performed); ND, not done; M, milk; V, vaginal smear; P, placenta; LN, lymph node; NI, no isolation; OK, Oklahoma.

water before use. Information on the origins of the sera and *Brucella* isolation from tissues is presented in Table 1. Tissues, when available (Table 1), were cultured as described by Alton et al. (1). The serologic tests (buffered antigen plate agglutination test, Card test, complement fixation [CF] test, standard plate test, Rivanol test, and standard agglutination test) were performed as described by Alton et al. (1). The particle concentration fluorescence immunoassay (PCFIA) was performed as recommended by the manufacturer (IDEXX, Portland, Maine). The positive control serum for the ELISA and Western blot experiments was obtained from a cow that had been experimentally infected with *B. abortus* 2308 (cow 47), and the serum had been collected 4 months after infection; the negative control serum for the ELISA and Western blot experiments was a pooled serum composed of sera from 73 nonvaccinated *Brucella*-free cows provided by the Animal Services Section, National Animal Disease Center. All sera were used at a dilution of 1:100.

Statistical analysis. The data were analyzed by Tukey's test with GraphPad software (InStat, San Diego, Calif.).

RESULTS

Table 2 shows the IgG ELISA values obtained with BCSP and recombinant Cu-Zn SOD and the results of the standard serologic tests. Generally, the standard serologic tests (the standard plate test, the Rivanol test, and the standard agglutination test), which are indicative of IgG and IgM antibody reactions with *B. abortus* lipopolysaccharide, have results similar to each other and comparable to the results of the CF test, which measures anti-lipopolysaccharide IgG (and IgM) antibodies. The CF test is considered the most reliable (sensitive and specific) test for brucellosis (1). The PCFIA, which measures the inhibition by anti-*Brucella* IgG antibodies of the test serum of fluorescent-label-tagged antibodies to lipopolysaccharide-labeled particles, had results that corresponded well with the results of the CF test (Table 2), i.e., a high CF titer generally corresponded to a low PCFIA value (serum samples 4, 6, 13, 15, and 19). A PCFIA value of ≥ 0.701 is considered negative, and values of ≤ 0.700 are considered positive. The BCSP-specific and the SOD-specific ELISAs measure antibodies to protein rather than to lipopolysaccharide; titers of IgG antibodies to BCSP and SOD would not necessarily be the same as those of antibodies to lipopolysaccharide. Therefore, titer increases in the CF test or the agglutination test are not necessarily associated with increases in ELISA values, as is exemplified by serum samples 1 and 2 (Table 2). This is presumably due to different concentrations of antibodies to distinct antigens of *B. abortus*. The BCSP ELISA results vary from 0.145 (0.142 is considered negative [14]) to 0.321, whereas the SOD ELISA results vary from 0.088 (0.121 is considered negative; see below) to 0.214 (Table 2). The average ELISA results for the 21 serologically positive animals (serum sample 22 is serologically negative) are 0.275 ± 0.041 with BCSP and 0.156 ± 0.047 with SOD (Table 3). From the results with the negative control sera, a conservative ELISA cutoff value (the mean plus 3 standard deviations) of 0.121 can be calculated for the SOD-specific ELISA. On the basis of this value, 17 of 21 (81%) serologically positive animals contain antibodies to SOD as detected by the ELISA. Four serum samples (no. 9, 10, 18, and 20) were negative by the SOD ELISA but positive by the standard serologic tests and the BCSP ELISA. One serum sample (no. 22) was considered negative by both the standard serologic tests and the ELISAs. These results show that not all of the animals tested have antibodies to SOD that are detectable under the ELISA test conditions used.

Since SOD-specific IgG was also present in sera from two animals infected with strain 19 (Table 1), it appears that infection or prolonged exposure of the animal to the organism may be a prerequisite for antigen processing and subsequent production of antibodies to SOD. Although the SOD-specific IgG absorbance values were lower than the BCSP-specific IgG absorbance values (Table 3), both sets of values were significantly different ($P < 0.001$) from the values for the negative control group. Evidently, cattle sera contain antibodies to additional components as measured by the BCSP-specific ELISA which are not present in the SOD antigen.

The specificity of the ELISA reactions obtained with BCSP and with recombinant SOD was examined by Western blotting. Figure 1 shows Western blots with BCSP (lanes 1 to 3) and recombinant SOD (lanes 4 to 6). Lanes 1 and 4 are amido black-stained protein blots. Lanes 2 and 4 show immunoblots with the *Brucella*-positive serum from cow 47, whereas lanes 3 and 6 show immunoblots with the pooled *Brucella*-negative serum. The positive control serum reacts with a large number of BCSP bands, including a protein with a molecular weight of 20,000 that corresponds to the SOD band shown on the protein blot (lane 4) and the immunoblot (lane 5) with the purified SOD. Figure 2 shows the immunoblots with the Cu-Zn SOD as the antigen for 21 of the 22 serum samples described in Table 2. Lane 1 is the stained protein blot; lanes 2 and 3 are the immunoblots with the positive and negative control sera, respectively. Lanes 4 through 24 correspond to serum samples

TABLE 2. Serologic data for bovine sera

Serum sample	Result of test							IgG ELISA value with:	
	BAPA[a]	Card	CF[b]	PCFIA[c]	SPT[d]	RIV[e]	SAT[f]	BCSP[g]	Cu-Zn SOD[h]
1	+	+	1/20	0.827	I100	N25	I50	0.307	0.204
2	+	+	4/320	0.275	200	200	200	0.301	0.163
3	+	+	4/20	0.750	100	I25	100	0.223	0.165
4	+	+	4/320	0.171	200	200	200	0.286	0.214
5	+	+	4/10	0.802	50	N25	I50	0.243	0.140
6	+	+	4/320	0.197	200	200	200	0.305	0.127
7	+	+	4/40	0.300	I100	I50	I100	0.300	0.185
8	+	+	>4/160	ND	200	200	200	0.257	0.152
9	+	+	>4/160	ND	200	200	200	0.278	0.088
10	+	+	>4/160	0.184	100	I100	200	0.265	0.107
11	+	+	>4/160	0.188	200	I200	200	0.283	0.176
12	+	+	3/160	ND	25	50	25	0.292	0.143
13	+	+	4/160	0.118	I100	I100	200	0.316	0.311
14	+	+	4/160	0.168	I100	I200	200	0.301	0.196
15	+	+	4/160	0.106	200	200	200	0.306	0.178
16	+	+	2/40	0.528	I200	50	I200	0.217	0.137
17	+	+	4/640	0.320	200	100	200	0.321	0.155
18	+	+	3/160	0.386	200	100	200	0.271	0.096
19	+	+	4/640	0.153	200	50	200	0.316	0.124
20	+	+	1/320	0.227	200	50	200	0.238	0.115
21	+	+	2/80	0.401	200	50	200	0.290	0.152
22	−	−	N10	0.990	N25	N25	N25	0.145	0.113

[a] BAPA, buffered antigen plate agglutination (1); +, positive; −, negative.
[b] N, no reaction. The numerator indicates the degree of fixation, and the denominator indicates the highest dilution showing fixation (1).
[c] Results of ≤0.700 are considered positive; results of ≥0.701 are considered negative. ND, not done.
[d] SPT, standard plate test; I, incomplete agglutination; N, no reaction (1).
[e] RIV, Rivanol test; I, incomplete agglutination; N, no reaction (1).
[f] SAT, standard agglutination test; I, incomplete agglutination; N, no reaction (1).
[g] BCSP was the solid-phase antigen. An ELISA value of ≤0.142 (negative, plus 3 standard deviations) is considered the cutoff value, as previously determined (14).
[h] Cu-Zn SOD was the solid-phase antigen. No criteria have been established for a negative cutoff value, although the mean ELISA values plus 2 and 3 standard deviations for the *Brucella*-negative serum are 0.096 and 0.121, respectively (Table 3).

1 through 8 and 10 through 22. Strong reactions were observed with the known positive serum (lane 2), and somewhat variable reactions were observed with the test sera. For example, a relatively strong reaction is observed with serum sample 18 (lane 20), whereas the ELISA value is low (Table 2). Nevertheless, it is apparent that cattle sera contain antibodies to SOD.

DISCUSSION

Cattle infected with *B. abortus* produce antibodies to *Brucella* proteins (3, 14). We previously demonstrated that one of the major proteins of the BCSP preparation, with a molecular mass of approximately 20,000 Da, is a Cu-Zn SOD (4, 6). The results presented in this article demonstrate that serologically positive cattle contain serum antibodies to SOD as measured by enzyme immunoassay and Western blotting. We observed that SOD antibody concentrations measured by ELISA do not necessarily correspond to concentrations measured by the standard serologic tests for brucellosis. This is not entirely surprising, because the standard serologic tests measure primarily IgG and IgM antibodies to lipopolysaccharide (1) whereas the ELISA measures IgG antibodies to recombinant SOD that is devoid of *Brucella* lipopolysaccharide. The concentration of SOD antibodies in the sera as measured by ELISA varied somewhat and was not always consistent with the BCSP ELISA or with the Western blot results. We observed that four serum samples (no. 9, 10, 18, and 20) had relatively low SOD ELISA values. Yet all four samples had relatively high titers in the standard serologic tests. Three of these four samples (no. 10, 18, and 20) also had relatively strong reactions in the immunoblots (Fig. 2, lanes 12, 20, and 22, respectively). The difference in these results may be a reflection of antibody binding to SOD in the native and denatured states. In addition, binding of SOD (in the native and denatured states) to the solid phase would also affect antibody binding by affecting the exposure of relevant epitopes. Suppression of IgG formation in *Brucella* infections has been observed by others (13); however, this may not be responsible for the low ELISA values reported here for serum samples 9, 10, 18, and 20, although concentrations of SOD-specific IgG antibodies in serum are an important factor. When cattle sera were diluted 500-fold instead of 100-fold, no reactions with a 20-kDa band were observed in immunoblots with sera from infected cattle (5). Because a 27% identity between *Brucella* and bovine SODs exists (4), some interfer-

TABLE 3. IgG ELISA and CF test results for sera from seropositive cattle

Group (n)[a]	IgG ELISA absorbance values (mean ± SD) with[b]:		CF result[c]
	BCSP	Cu-Zn SOD	
Naturally infected seropositive (21)	0.275 ± 0.041 A	0.156 ± 0.047 C	4/10–4/640
Positive control (6)	0.310 ± 0.077 A	0.234 ± 0.019 D	4/640
Negative control (6)	0.045 ± 0.021 B	0.046 ± 0.025 E	N[d]

[a] Positive and negative control sera are described in Materials and Methods.
[b] All ELISA values followed by different letters are significantly different.
[c] The denominator is the highest dilution showing fixation, and the numerator indicates the degree of fixation.
[d] N, negative at a dilution of 1:10.

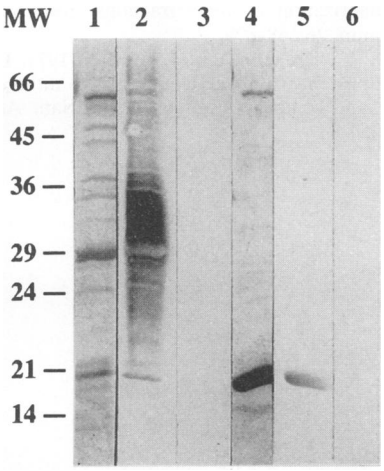

FIG. 1. Western blot with positive and negative control sera. BCSP (5 μg) and recombinant Cu-Zn SOD (5 μg) were separated on a 10-to-15% sodium dodecyl sulfate gradient gel under denaturing conditions. The blot was incubated with positive and negative control sera (diluted 1:100). Lane 1, amido black-stained blot of BCSP; lane 2, immunoblot incubated with positive control serum from an experimentally infected cow (cow 47); lane 3, immunoblot with a negative control serum prepared from pooled serum from *Brucella*-free cows; lane 4, amido black-stained blot of recombinant Cu-Zn SOD; lane 5, immunoblot with positive control serum (cow 47); lane 6, negative control serum. MW, molecular weight (in thousands).

ence from endogenous bovine erythrocyte Cu-Zn SOD in serum may be expected in measurements of *Brucella* SOD-specific antibodies, especially when low levels of *Brucella* antibodies are present. This interference may account for the low reactivity of the four serum samples (no. 9, 10, 18, and 20) in the SOD-specific ELISA. Generally, however, the mean SOD ELISA values are significantly higher than those for the control sera.

To our knowledge, this is the first report describing anti-SOD antibodies in cattle infected with pathogenic gram-negative microorganisms. The significance of an SOD antibody response in pathogenesis is not known. Although others (2, 3) have postulated a role for SOD in pathogenesis, the significance of the *Brucella* Cu-Zn SOD in pathogenesis or in evasion by *B. abortus* of the host's immune surveillance system has not yet been evaluated. Recently, Cu-Zn SODs were detected in whole-cell lysates in virulent as well as avirulent *B. abortus*

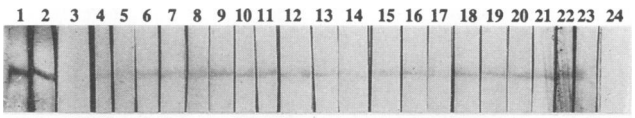

FIG. 2. Western blot with sera from serologically positive animals with recombinant *B. abortus* 19 Cu-Zn SOD. The blot was incubated with sera diluted 1:100. Lane 1, amido black-stained blot of Cu-Zn SOD; lane 2, immunoblot with serum from cow 47 (positive control); lane 3, immunoblot with pooled sera from *Brucella*-free cows; lanes 4 through 24, immunoblots with sera from serologically positive cows 1 through 8 and 10 through 22 (lane 4, serum 1; lane 5, serum 2; lane 6, serum 3; lane 7, serum 4; lane 8, serum 5; lane 9, serum 6; lane 10, serum 7; lane 11, serum 8; lane 12, serum 10; lane 13, serum 11; lane 14, serum 12; lane 15, serum 13; lane 16, serum 14; lane 17, serum 15; lane 18, serum 16; lane 19, serum 17; lane 20, serum 18; lane 21, serum 19; lane 22, serum 20; lane 23, serum 21; lane 24, serum 22).

(12), but there are no published reports to indicate if the Cu-Zn SOD plays a role in the organism's survival within the host's phagocytic cells.

We have demonstrated that 17 of 21 serum samples (81%) that are serologically positive for *B. abortus* contain antibodies to a characterized *Brucella* protein that has been previously shown to be Cu-Zn SOD. For a diagnostic test to be considered effective, preferably nearly 100% of serologically positive sera should be detected by the test. A good diagnostic reagent would most likely contain more than one immunoreactive protein to cover the spectrum of protein antibody response by cattle of different genetic backgrounds. The results shown here represent a first step in the direction of the development of a multi-protein diagnostic reagent for bovine brucellosis.

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