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Immune Responses and Resistance to Brucellosis in Mice Vaccinated Orally with *Brucella abortus* RB51

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Immune responses and resistance to infection with *Brucella abortus* 2308 (S2308) were measured in mice following oral or intraperitoneal (i.p.) vaccination with strain RB51 (SRB51). Bacteria persisted in the parotid lymph node for 4 weeks following oral vaccination of mice with 5×10^8 or 5×10^6 CFU of SRB51. Bacteria did not appear in the spleen during 12 weeks after oral vaccination, whereas they did appear in the spleen for 8 weeks following i.p. vaccination of mice with SRB51 (5×10^8 or 5×10^6 CFU). Increased resistance to S2308 infection occurred at 12 to 20 weeks in mice vaccinated i.p. with SRB51 (5×10^8 or 5×10^6 CFU) but occurred at 12 weeks only in mice vaccinated orally with SRB51 (5×10^8 CFU). Oral SRB51 vaccination induced lower levels of antibodies to the surface antigens of intact SRB51 bacteria than did i.p. vaccination. However, neither route of vaccination induced anamnestic antibody responses to the surface antigens of intact S2308 bacteria after challenge infection of the vaccinated mice with S2308. Mice vaccinated orally with SRB51 and challenged with S2308 at 12 to 20 weeks had lower and less persistent spleen cell proliferation and production of gamma interferon in response to S2308 and certain immunodominant S2308 proteins (32 to ≤18 kDa) than did mice vaccinated i.p. with SRB51. However, mice vaccinated orally or i.p. with SRB51 and challenged with S2308 had similar spleen cell tumor necrosis factor alpha production. These results indicate that oral vaccination of mice with SRB51 was effective in inducing protective immunity to S2308 infection, although the immunity was lower and less persistent than that induced by i.p. vaccination. The lower protective immunity induced by oral vaccination may have resulted from lower and less persistent cell-mediated immunity and gamma interferon production in response to S2308 and S2308 proteins.

Brucella abortus RB51 (SRB51) is a lipopolysaccharide (LPS) O-antigen-deficient mutant of the virulent strain *B. abortus* 2308 (S2308) (24). SRB51 is being evaluated as an alternative to using the *B. abortus* 19 (S19) vaccine for preventing brucellosis and abortions in cattle, because, unlike S19, it does not induce antibodies to the *Brucella* LPS O antigens that are detected by serodiagnostic tests for brucellosis (6, 7, 25, 28). In addition, cattle vaccinated with SRB51 have both increased resistance to infection and fewer abortions that are induced by S2308 (6, 7). Consequently, SRB51 appears to be an effective vaccine which may facilitate the identification and removal of cattle with brucellosis from vaccinated herds. The U.S. Department of Agriculture Animal Plant and Health Inspection Service has recently designated SRB51 as an official calfhood brucellosis vaccine for cattle (30).

The SRB51 vaccine is effective in preventing brucellosis when given subcutaneously to cattle (6, 7) and intraperitoneally (15, 32) or subcutaneously (15) to mice. Certain wild ruminants such as bison and elk are susceptible to brucellosis (8, 9, 22, 38, 40), and the disease in these animals can be experimentally transmitted to cattle (9, 37). Free-roaming bison and elk in Yellowstone National Park and the surrounding area are heavily infected with *B. abortus* (8, 11, 38). Preventing brucellosis in these animals is considered important in eliminating potentially serious sources of infection for neighboring cattle herds (8, 36, 40). Administering the SRB51 vaccine parenterally to individual free-roaming wild ruminants in large herds would probably be highly impracticable, and a less tedious method such as oral vaccination of entire herds with SRB51 in feed may be more beneficial in preventing brucellosis in these animals. Oral vaccination with SRB51 may be efficacious, since other vaccines such as S19 and *B. suis* 2 are effective when given orally to cattle (16, 41). In addition, preliminary evidence indicates that oral SRB51 vaccination of monogastric animals (feral pigs) induces partial protection from abortion following experimental infection with *B. suis* (12).

Most of the knowledge about the immunology and protective immunologic mechanisms associated with SRB51 vaccination has been derived from studies with mice (15, 24, 31, 32). Furthermore, mice have served as a useful animal model in testing the SRB51 vaccine because they exhibit immune responses to SRB51 and SRB51 antigens (15, 24, 31, 32) which are similar to the responses which occur in SRB51-vaccinated ruminants such as cattle (26–29) and goats (23). In this study, immune responses and protective immunity were characterized in oral SRB51-vaccinated mice as an animal model for designing oral SRB51 vaccination protocols for use in ruminants.

MATERIALS AND METHODS

Culture medium. All cell culture experiments were performed with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5×10^{-5} M 2-mercaptoethanol. This supplemented medium is referred to below as RPMI.

***B. abortus* cultures and proteins.** The lyophilized live SRB51 vaccine was prepared by the Colorado Serum Co. (Denver, Colo.). Live cultures of S2308 and killed cultures of SRB51 and S2308 (killed by γ-irradiation at 1.4×10^6 rads) were prepared as described previously (28). Whole-cell lysates were prepared from killed S2308 cultures and separated into 106- to 18-kDa proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (28). The S2308 proteins were eluted from the gel into 22 fractions by using a Blote-

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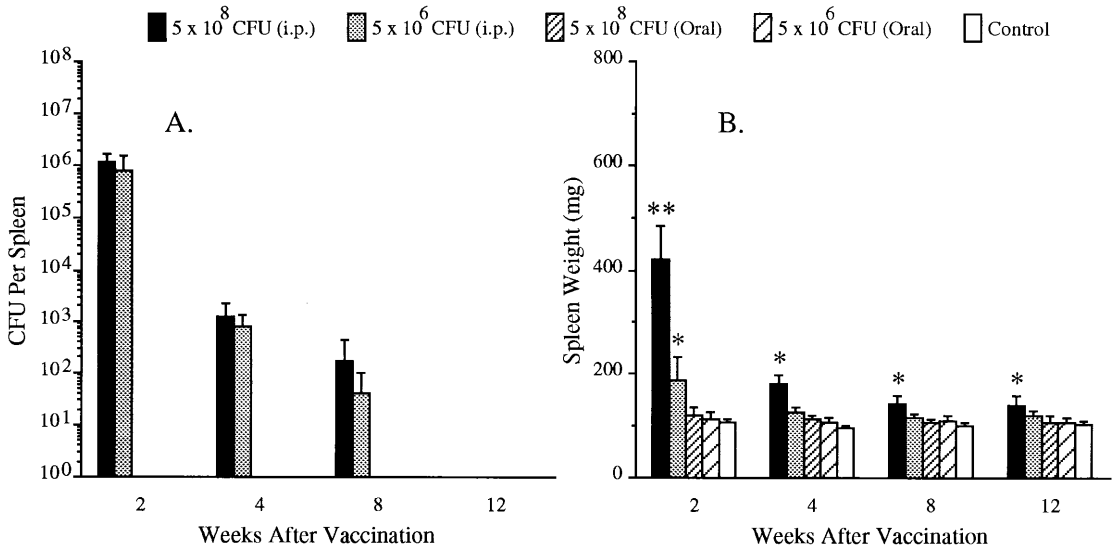

FIG. 1. Persistence of SRB51 in spleen and spleen weights in SRB51-vaccinated mice. Mice were vaccinated i.p. or orally with SRB51 (5×10^8 or 5×10^6 CFU). Numbers of SRB51 CFU per spleen (A) and spleen weights (B) were measured at 2, 4, 8, and 12 weeks after vaccination. Results are expressed as mean ± SEM ($n = 5$). $P \leq 0.001$ (**) or $P \leq 0.01$ (*) versus controls.

lutor B35 (Biometra, Göttingen, Germany). Each fraction was concentrated and filter sterilized as described previously (28) and contained between 25 and 5 μg of protein per ml. A 50-μl aliquot of each of the 22 protein fractions of S2308 (undiluted or at a 1:4 dilution) was added to two separate wells of 96-well flat-bottom microtiter plate, and plates were stored at −70°C.

Vaccination and challenge infection of mice with *B. abortus*. Female 8-week-old BALB/c AnNHsD mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.) and were used in the experiments when they were 10 weeks old. The lyophilized SRB51 vaccine was reconstituted in a 0.15 M NaCl saline solution. Mice were vaccinated orally by placing 20 μl of saline containing 5×10^8 or 5×10^6 CFU onto the pharyngeal mucosa. Additional groups of mice were vaccinated intraperitoneally (i.p.) with 0.2 ml of saline containing 5×10^8 or 5×10^6 CFU of SRB51. These mice served as positive controls because we previously determined that mice vaccinated i.p. with 5×10^8 or 5×10^6 CFU of SRB51 have increased resistance to infection with S2308 (32). Nonvaccinated control mice were injected i.p. with 0.2 ml of saline alone. Nonvaccinated control mice and vaccinated mice at 12, 16, or 20 weeks after vaccination were challenged with S2308 by an i.p. injection of 2×10^4 CFU in 0.2 ml of 0.15 M NaCl saline solution.

Histopathology and immunoperoxidase staining. The right and left parotid lymph nodes were collected from nonvaccinated control mice and from orally SRB51-vaccinated mice at 2, 4, 8, or 12 weeks after vaccination. The lymph nodes were fixed in 10% neutral buffered formalin, processed by routine paraffin embedding, and sectioned at 4 to 6 μm. Sections were stained with hematoxylin and eosin and examined by light microscopy. Unstained sections were processed for immunoperoxidase detection of SRB51 antigens with hyperimmune rabbit SRB51 antisera as described previously (33).

Collection of tissues. Blood samples and spleens were obtained from age-matched nonvaccinated control mice ($n = 5$) and from vaccinated mice ($n = 5$) at 2, 4, 8, or 12 weeks after vaccination. In addition, blood and spleens were obtained from nonvaccinated control mice ($n = 12$) and from vaccinated mice ($n = 12$) at 2 weeks after they were challenged with S2308. The blood was allowed to clot for 6 h at 25°C before centrifugation, and serum samples were then stored at −70°C. The spleens were weighed, approximately one-third of the spleen was excised, and the excised portion was weighed. The excised portion of the spleen was used for bacterial culture, and the remaining portion was used to prepare spleen cell suspensions.

Serology. Thawed serum samples were measured for antibody to γ-irradiated killed SRB51 or S2308 by a dot enzyme-linked immunosorbent assay (ELISA) with a goat anti-mouse immunoglobulin G (H- and L-chain specific)-horseradish peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, Pa.) as described previously (7). Results of the dot ELISA were expressed as mean $\log_{10}$ titer ± standard error of the mean (SEM).

Culture analysis. The excised portion of the spleen was processed to form a cell lysate by using a tissue grinder, and the number of *B. abortus* CFU in the lysate was determined by plating dilutions onto tryptose agar plates as described previously (21). The number of CFU per total spleen was determined by the following formula: (total spleen weight/spleen portion weight) × number of CFU in spleen portion. The results were expressed as mean $\log_{10}$ CFU per total spleen ± SEM.

Preparation of spleen cell suspensions. After removal of part of the spleen for culture analysis, the remaining portion was placed on a sterile 60-mesh stainless steel screen, minced with scissors, and processed to form a spleen cell suspension as described previously (33). The spleen cells were then placed in RPMI. Cells from each mouse ($n = 5$) in the nonvaccinated and SRB51-vaccinated groups of mice were kept separate following collection of the spleens at 2 to 12 weeks after vaccination. However, the spleen cells were pooled following challenge infection of mice ($n = 12$) in these groups with S2308 at week 12, 16, or 20. Spleen cell suspensions from 12 mice in each group were divided into six separate pairs of samples, and each pair was pooled to form six samples per group.

Measurement of spleen cell proliferation to S2308 and S2308 proteins. RPMI containing 3×10^5 spleen cells (50 μl) was added to each of two separate flat-bottom wells of a 96-well microtiter plate that contained 100 μl of various concentrations of γ-irradiated S2308 (10^8 to 10^5 bacteria per well). Plates containing the 22 isolated S2308 protein fractions (106 to 18 kDa) were thawed, and 50 μl of RPMI containing 3×10^5 spleen cells was added to each well. All spleen cell cultures were incubated with S2308 or S2308 protein for 5 days at 37°C in 5% CO_2. Cell cultures were mixed on days 2 and 4 by shaking the microtiter plates for 1 min on a Micro Shaker II (Dynatech Laboratories Inc., Alexandria, Va.) with an instrument setting of 3.5. After the 5-day incubation, cell cultures were pulsed for 18 h with 1.0 μCi of [^{3}H]thymidine per well. The cells were then harvested and measured for radioactivity (in counts per minute) in a liquid scintillation counter. Cell proliferation results were expressed as mean counts per minute ± SEM incorporated by duplicate cultures.

Measurement of IFN-γ and TNF-α production by spleen cells in response to S2308 or S2308 proteins. Supernatants were removed from a second set of spleen cell cultures which had been incubated for 5 days with S2308 (10^8 to 10^5 bacteria per well) or the 22 protein fractions of S2308. Concentrations of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) in the supernatants were measured with mouse ELISA kits as described by the manufacturer (Genzyme, Cambridge, Mass.). The results were expressed as nanograms of the cytokine per milliliter ± SEM.

Statistical analysis. Statistical differences among nonvaccinated control mice or mice vaccinated orally or i.p. with SRB51 were determined by analysis of variance and Fisher's protected least significant difference.

RESULTS

Persistence of SRB51 in spleen and spleen weights. Mice vaccinated i.p. with 5×10^8 or 5×10^6 CFU of SRB51 exhibited a similar and rapid decline in the number of bacteria in their spleens between 2 and 8 weeks, and no bacteria were cultured from the spleens at 12 weeks after vaccination (Fig. 1A). In contrast, no bacteria were cultured from the spleens from 2 to 12 weeks after mice were vaccinated orally with 5×10^8 or 5×10^6 CFU of SRB51 (Fig. 1A). Spleen weights in mice vaccinated i.p., but not orally, with SRB51 were higher ($P \leq 0.001$ or $P \leq 0.01$) than were spleen weights in nonvaccinated control mice (Fig. 1B).

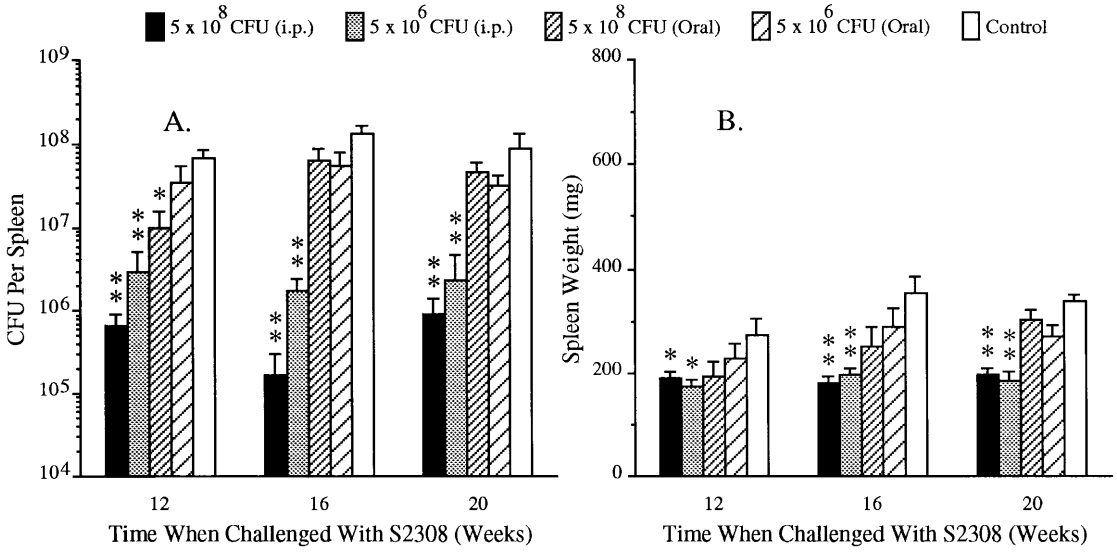

FIG. 2. Resistance to infection and spleen weights in SRB51-vaccinated mice following challenge infection with S2308. Mice were challenged with S2308 at 12, 16, or 20 weeks after i.p. or oral vaccination with SRB51 (5×10^8 or 5×10^6 CFU). Numbers of S2308 CFU per spleen (A) and spleen weights (B) were measured at 2 weeks after challenge. Results are expressed as mean ± SEM ($n = 12$). $P \leq 0.01$ (**) or $P \leq 0.05$ (*) versus controls.

Histopathology and immunoperoxidase staining. Well-developed germinal centers containing numerous mitotic figures, tingible body macrophages, and residual bodies were found in the parotid lymph nodes at 2 to 8 weeks after oral vaccination of mice with 5×10^8 or 5×10^6 CFU of SRB51 (results not shown). The germinal centers were most common in mice vaccinated with the highest dose of SRB51. They were not present in nonvaccinated control mice and in mice at 12 weeks after oral SRB51 vaccination. Immunoperoxidase staining revealed the presence of SRB51 antigens in the parotid lymph nodes at 2 and 4 but not at 8 or 12 weeks after oral vaccination of mice with SRB51 (5×10^8 or 5×10^6 CFU). The antigens were located mainly in macrophages, although they were also occasionally found in neutrophils (results not shown).

Resistance to infection and spleen weights. Mice vaccinated i.p. with SRB51 (5×10^8 or 5×10^6 CFU) had higher ($P \leq 0.01$) resistance to infection with S2308 at 12, 16, and 20 weeks after vaccination than did nonvaccinated control mice (Fig. 2A). Mice vaccinated orally with 5×10^8 but not with 5×10^6 CFU of SRB51 had increased ($P \leq 0.05$) resistance to infection at 12 weeks. However, neither dose of the oral vaccine increased the resistance of mice to S2308 infection at 16 and 20 weeks (Fig. 2A). After challenge infection with S2308, spleen weights in mice vaccinated i.p. but not orally with SRB51 (5×10^8 or 5×10^6 CFU) were lower ($P \leq 0.01$ or $P \leq 0.05$) than were spleen weights in S2308-challenged, nonvaccinated control mice (Fig. 2B).

Spleen cell proliferation in response to S2308. Spleen cells obtained from mice from 4 to 12 weeks after i.p. vaccination with SRB51 (5×10^8 or 5×10^6 CFU) had higher ($P \leq 0.05$) proliferation in response to S2308 than did spleen cells from nonvaccinated control mice (Fig. 3A). Spleen cells from mice vaccinated orally with 5×10^8 CFU of SRB51 had increased ($P \leq 0.05$) proliferation in response to S2308 at 8 weeks; however, the response was lower ($P \leq 0.05$) than the response in mice vaccinated i.p. with 5×10^8 CFU of SRB51 (Fig. 3A). Mice vaccinated i.p. (5×10^8 or 5×10^6 CFU) or orally (5×10^8 CFU) with SRB51 and challenged with S2308 at week 12, 16, or 20 had higher ($P \leq 0.05$) spleen cell proliferation in response to S2308 than did S2308-infected, nonvaccinated control mice (Fig. 3B). During these times, the responses in the orally vaccinated mice were either the same as or lower than ($P \leq 0.05$) those in mice vaccinated i.p. with SRB51.

Spleen cell proliferation in response to S2308 proteins. Throughout the study, each group of vaccinated mice had spleen cell proliferative responses to the 22 undiluted S2308 protein fractions (106 to 18 kDa) which were similar to the responses when the proteins were diluted fourfold. Therefore, only responses to the undiluted proteins are presented here. Mice vaccinated i.p. with SRB51 (5×10^8 or 5×10^6 CFU) and challenged at week 12 with S2308 had spleen cells which proliferated most in response to S2308 proteins of 32, 27, 18, and <18 kDa (Fig. 4). These proteins also induced the greatest proliferation by spleen cells from mice which were challenged with S2308 at 16 or 20 weeks after i.p. vaccination with SRB51 (data not shown). Mice vaccinated orally with 5×10^8 but not 5×10^6 CFU of SRB51 and challenged at 12 weeks with S2308 had spleen cells which proliferated in response to most of the same S2308 proteins (27, 18, and <18 kDa) as did spleen cells from mice vaccinated i.p. with SRB51 after challenge infection with S2308 (Fig. 4). However, all groups of mice vaccinated orally with SRB51 had low or no spleen cell proliferation in response to the 27-, 18-, and <18-kDa proteins following challenge infection with S2308 at 16 or 20 weeks (data not shown).

Serologic responses. Mice vaccinated i.p. with SRB51 (5×10^8 or 5×10^6 CFU) had higher ($P \leq 0.05$) serum antibody titers to the surface antigens of intact SRB51 bacteria from weeks 2 to 12 than did mice vaccinated orally with SRB51 (Table 1). In addition, SRB51 antibodies in serum were present at 2 weeks in mice vaccinated i.p. with SRB51 whereas these antibodies did not appear until 4 to 8 weeks in mice vaccinated orally with SRB51. All groups of mice vaccinated orally or i.p. with SRB51 did not have measurable antibody titers to the surface antigens of intact S2308 bacteria from weeks 2 to 12 after vaccination (data not shown). In addition, all groups of SRB51-vaccinated mice did not have significantly increased antibody titers to S2308 surface antigens at 2 weeks after challenge infection with S2308 (week 12, 16, or 20) when compared with S2308-infected, nonvaccinated control mice (data not shown).

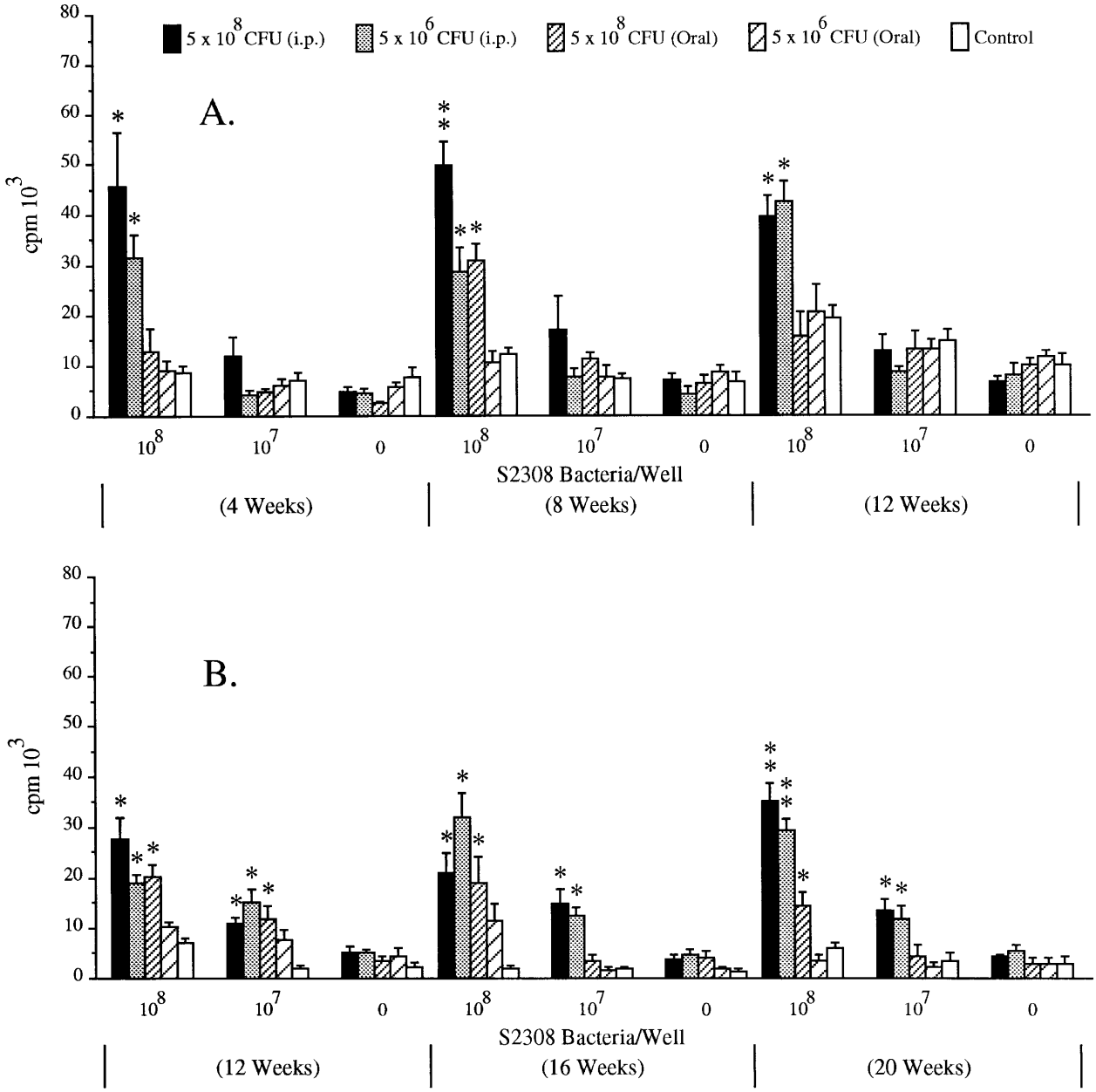

FIG. 3. Spleen cell proliferation in response to S2308 in SRB51-vaccinated mice before and after challenge infection with S2308. Mice were vaccinated i.p. or orally with SRB51 (5×10^8 or 5×10^6 CFU). Spleen cell suspensions were prepared from vaccinated mice at 4, 8, or 12 weeks (A) and from vaccinated mice at 2 weeks after challenge with S2308 at 12, 16, or 20 weeks (B). Cells (3×10^5 cells per well) were incubated for 5 days with 10^8 or 10^7 γ-irradiated killed S2308 bacteria and then pulsed for 18 h with [^{3}H]thymidine. Results are expressed as mean ± SEM ($n = 6$). $P \leq 0.05$ for ** or * versus controls and for ** versus *.

Spleen cell IFN-γ and TNF-α production. Mice vaccinated i.p. (5×10^8 or 5×10^6 CFU) or orally (5×10^8 CFU) with SRB51 and challenged 12 weeks later with S2308 had higher ($P \leq 0.01$ or $P \leq 0.05$) spleen cell IFN-γ production in response to S2308 than did spleen cells from S2308-infected, nonvaccinated control mice (Fig. 5A). In addition, spleen cells from mice vaccinated i.p. with SRB51 (5×10^8 or 5×10^6 CFU) produced larger ($P \leq 0.01$) amounts of IFN-γ in response to two groups of S2308 proteins (32- to 27-kDa, fractions 9 to 14; ≤18 kDa, fractions 15 to 22) than did spleen cells from S2308-infected, nonvaccinated control mice (Fig. 5B). In contrast, spleen cells from mice vaccinated orally with SRB51 (5×10^8 CFU) produced increased ($P \leq 0.05$) amounts of IFN-γ in response to only one group of S2308 proteins (≤18 kDa, fractions 15 to 22). Mice vaccinated i.p., but not orally, with SRB51 had increased spleen cell IFN-γ production in response to S2308 and S2308 proteins following challenge infection with S2308 at week 16 or 20 (data not shown). Nonvaccinated control mice and all groups of SRB51-vaccinated mice had spleen cells which spontaneously produced high and similar levels of TNF-α following challenge infection of the mice with S2308 at week 12, 16, or 20 (data not shown). The

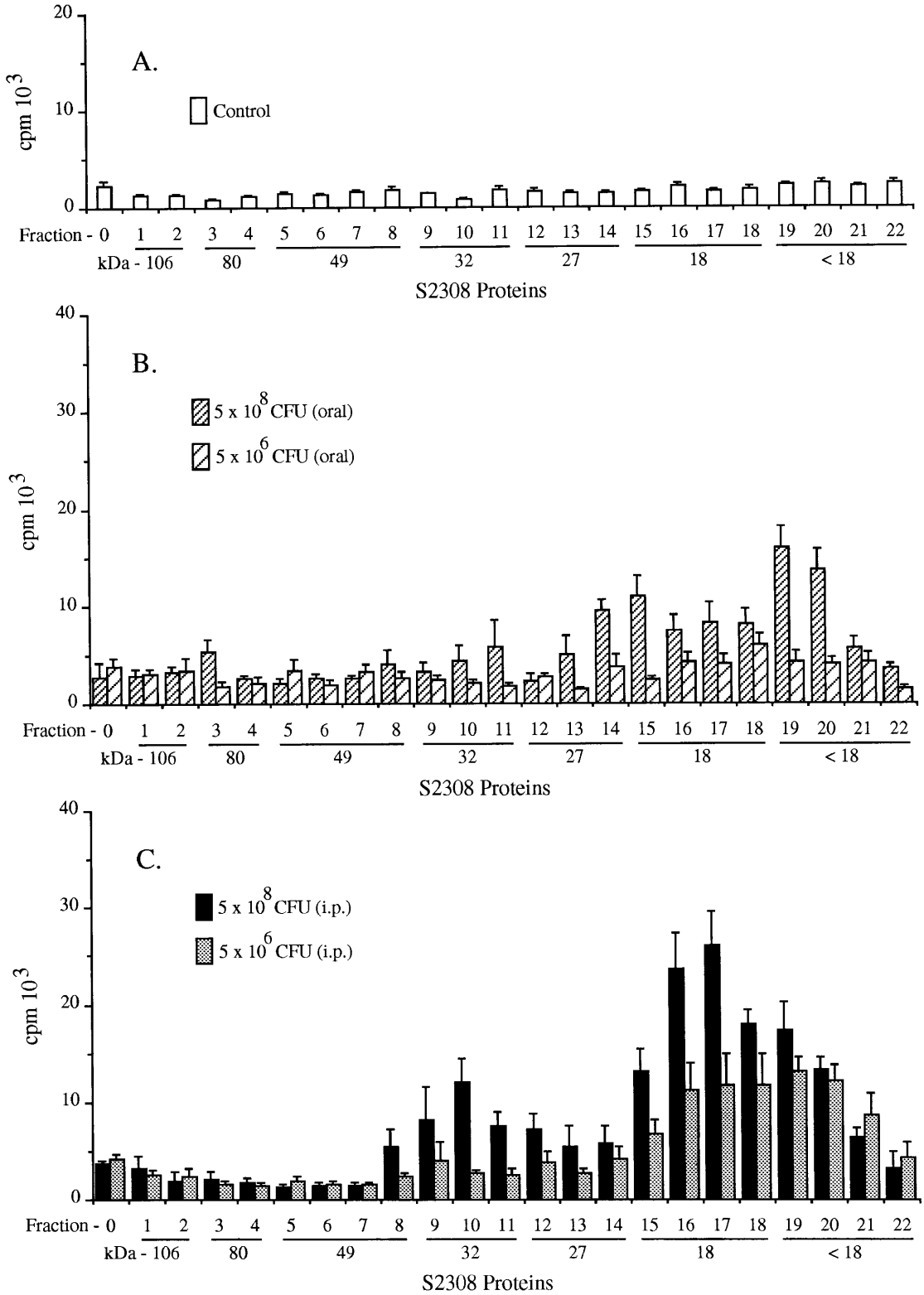

FIG. 4. Spleen cell proliferation in response to S2308 proteins in SRB51-vaccinated mice following challenge infection with S2308. Mice were challenged with S2308 at 12 weeks after i.p. or oral vaccination with SRB51 (5×10^8 or 5×10^6 CFU). Spleen cell suspensions were prepared from nonvaccinated control mice (A) and from orally (B) and i.p. (C) vaccinated mice at 2 weeks after challenge infection with S2308. Cells (3×10^5 cells per well) were incubated alone (fraction 0) or with S2308 proteins (fractions 1 to 22) for 5 days and then pulsed for 18 h with [^{3}H]thymidine. Results are expressed as mean ± SEM ($n = 6$).

TABLE 1. Serologic responses in mice vaccinated with SRB51

SRB51 vaccine ($n = 5$)[a]		Log_{10} antibody titer to SRB51 at time (wk) after vaccination[b]			
Dose (CFU)	Route given	2	4	8	12
5×10^8	i.p.	3.17 ± 0.18**	3.76 ± 0.15***	3.83 ± 0.12***	3.22 ± 0.07**
5×10^6	i.p.	1.02 ± 0.42*	2.68 ± 0.07**	2.51 ± 0.10**	2.74 ± 0.06**
5×10^8	Oral	0	1.02 ± 0.43*	1.34 ± 0.40*	1.08 ± 0.48*
5×10^6	Oral	0	0	0.90 ± 0.38	0.52 ± 0.31
None		0	0	0	0

[a] Mice were vaccinated i.p. or orally with SRB51 (5×10^8 or 5×10^6 CFU).
[b] Dot ELISA antibody titers to SRB51 were measured in vaccinated mice at 2, 4, 8, or 12 weeks. Results are expressed as mean ± SEM. Groups with one or more asterisks are different ($P \leq 0.05$) from controls and each other.

production of TNF-α was not further increased by incubation of spleen cells from these mice with S2308 or each of the 22 protein fractions (106 to <18 kDa) of S2308 (data not shown).

DISCUSSION

Previous studies have noted that i.p. or subcutaneous SRB51 vaccination effectively increases the resistance of mice to S2308 infection (15, 24, 32) and that subcutaneous vaccination is less effective than i.p. vaccination (15). Results from the current study indicate that oral vaccination is also less effective than i.p. vaccination with SRB51, since enhanced resistance to S2308 infection occurred at 12 to 20 weeks in mice vaccinated i.p. with 5×10^8 or 5×10^6 CFU of SRB51 but occurred at 12 weeks only in mice vaccinated orally with 5×10^8 CFU of SRB51. Oral vaccination of mice with 5×10^6 CFU of SRB51 failed to enhance their resistance to S2308 infection from 12 to 20 weeks after vaccination.

Neither oral or i.p. vaccination with SRB51 induced anamnestic antibody responses to the surface antigens of S2308 following challenge infection of the vaccinated mice with S2308. These results suggest that antibody responses were probably not responsible for the shorter persistence of protective immunity in mice vaccinated orally with SRB51. Other studies have also reported that anamnestic antibody responses to the surface antigens of S2308 either do not occur (24) or occur infrequently (15) in response to S2308 infection in SRB51-vaccinated mice. Results from the current and previous studies (15, 24) cannot preclude that anamnestic antibody responses to internal S2308 antigens may have occurred. However, these antibodies would probably play no role in protective immunity, since they would be incapable of fixing complement and acting as agglutinins and opsonins by binding to the surface of live S2308 bacteria in plasma or tissues of S2308-infected mice. In addition, antibody responses to SRB51 appear to play no role in protective immunity, since protective immunity cannot be passively transferred to recipient mice by injection with serum from SRB51-vaccinated mice (15).

Current evidence indicates that vaccination of mice with SRB51 induces immunity to infection with S2308 by cell-mediated immune responses but not by antibody immune responses (15, 31, 35). In addition, it has been shown that IFN-γ increases the ability of murine macrophages to kill or inhibit the replication of *B. abortus* (13, 14, 34) and that endogenous production of IFN-γ plays an important role in resistance to *B. abortus* infections in mice (42). In the present study, mice vaccinated i.p. with SRB51 and challenged with S2308 had spleen cells which proliferated better and produced higher levels of IFN-γ in response to S2308 than did orally SRB51-

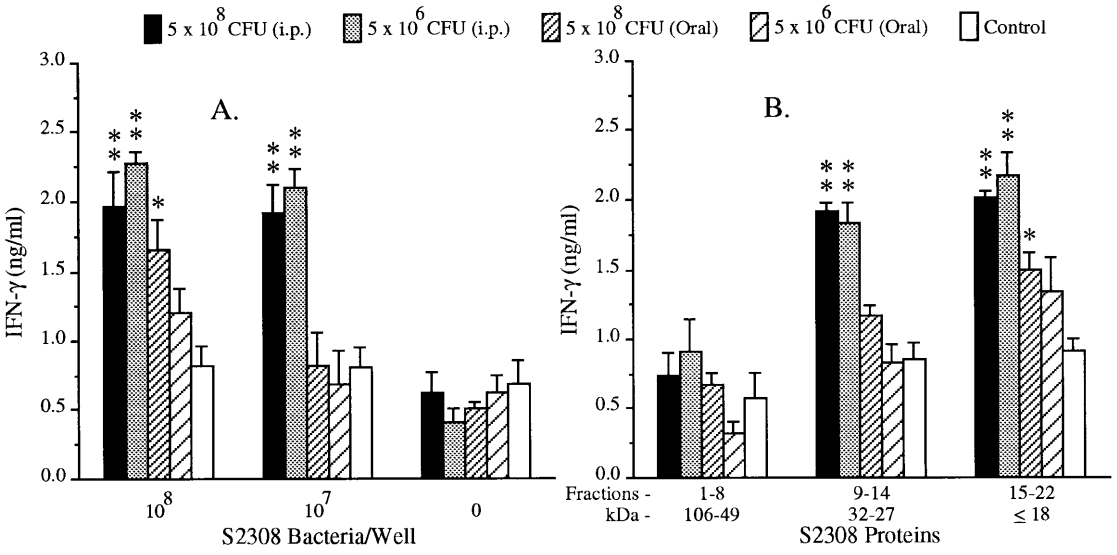

FIG. 5. Spleen cell IFN-γ production in response to S2308 or S2308 proteins in SRB51-vaccinated mice following challenge infection with S2308. Mice were challenged with S2308 at 12 weeks after i.p. or oral vaccination with SRB51 (5×10^8 or 5×10^6 CFU). Spleen cell suspensions were prepared from nonvaccinated control mice and from vaccinated mice at 2 weeks after challenge infection with S2308. Cell cultures (3×10^5 cells per well) were incubated for 5 days with 10^8 or 10^7 γ-irradiated-killed S2308 bacteria (A) or with S2308 proteins (B, fractions 1 to 22). The concentration of IFN-γ in the culture supernatants was assayed by an ELISA. Results are expressed as mean ± SEM ($n = 6$). $P \leq 0.01$ (**) or $P \leq 0.05$ (*) versus controls. For simplicity, the results for the production of IFN-γ in response to each of the 22 protein fractions were combined into three groups (fractions 1 to 8, 9 to 14, and 15 to 22).

vaccinated mice which had been challenged with S2308. In addition, i.p. vaccination with SRB51 resulted in spleen cells which produced IFN-γ in response to two groups of S2308 proteins (32 to 27 kDa and ≤18 kDa), whereas oral vaccination resulted in spleen cell IFN-γ production in response to only one group of proteins (≤18 kDa). These findings suggest that resistance to S2308 infection may have been lower and less persistent in mice vaccinated orally with SRB51, because these mice produced smaller amounts of IFN-γ in response to S2308 infection than did mice vaccinated i.p. with SRB51.

Nonvaccinated mice and mice vaccinated orally or i.p. with SRB51 had spleen cells which spontaneously produced similar and large amounts of TNF-α following infection with S2308. The results indicate that TNF-α production in S2308-infected mice was not appreciably altered by prior vaccination of the mice with SRB51. The role of TNF-α in resistance to brucellosis has not been definitively determined. However, TNF-α may be important, since macrophages from *B. abortus*-infected mice produce large amounts of TNF-α in response to the bacteria (43, 44), and even though TNF-α does not inhibit the growth of *B. abortus* in macrophages (14), it does aid the killing of *B. abortus* by IFN-γ-activated macrophages (1).

Mice vaccinated orally with SRB51 had antibody responses to SRB51 and spleen cell-mediated immune responses to S2308 which were lower than the responses which occurred in mice vaccinated i.p. with SRB51. These results probably occurred as a result of lower spleen cell stimulation, since SRB51 colonized the spleen in mice vaccinated i.p. with SRB51 but not in those vaccinated orally with SRB51. However, mice vaccinated orally with SRB51 did have bacterial antigens for 4 weeks in the parotid lymph node which drains the oral cavity. In addition, SRB51 in the lymph node induced the formation of well-developed germinal centers containing mitotic figures, tingible body macrophages, and residual bodies which are indicators of immune system stimulation and increased cell replication (39). Collectively, these results indicate that SRB51 is not highly invasive when given orally to mice and that the bacteria apparently stimulate immune responses primarily in the lymph nodes which drain the oral cavity. Similar findings have been noted in cattle vaccinated subcutaneously with SRB51, in that bacteria colonize and stimulate immune responses in the draining lymph nodes (28, 29) but are not present in the blood and spleen (5).

Results from our previous studies indicate that mice vaccinated i.p. with SRB51 have spleen cells which proliferate in response to S2308 proteins of 32 to <18 kDa but not in response to 106- to 49-kDa proteins following S2308 infection (31, 32). Data from the present study confirm and extend these findings in that 32- to <18-kDa but not 106- to 49-kDa proteins appeared to be the most important in inducing IFN-γ production when SRB51-vaccinated mice are challenge infected with S2308. Proteins with similar molecular masses from S19 (36 to 26 and <14 kDa) have also been shown to stimulate IFN-γ production following S19 vaccination of mice (45). Lymph node cells from SRB51-vaccinated cattle (28, 29) and spleen cells from SRB51-vaccinated mice (31, 32) proliferate in response to the same S2308 protein fractions. However, the S2308 protein fractions which stimulate IFN-γ production in SRB51-vaccinated cattle have not been identified.

Mice vaccinated orally or i.p. with SRB51 in the present study had antibody which reacted much better with SRB51 than with S2308 in the dot ELISA. The same findings were noted in our previous studies with SRB51-vaccinated mice and cattle (26, 33), and we speculated that the binding of SRB51 antibodies to S2308 is probably inhibited by steric hindrance of the S2308 LPS O side chain (26). The SRB51 antibodies in mice which bind to S2308 have not been fully characterized, although in cattle they are nonagglutinating antibodies, which do not react with S2308 LPS but instead react primarily with S2308 proteins of 29 to <20 kDa (26).

We previously reported that mice vaccinated i.p. with SRB51 at a dose of 5×10^8 or 5×10^6 CFU have increased resistance to infection with S2308 and that 5×10^8 CFU is close to the maximum dose which can be tolerated by mice (32). On the basis of these findings, these two doses were given as oral vaccines to mice in the present study. Only the highest oral dose of SRB51 (5×10^8 CFU) induced protective immunity to S2308 infection, and the immunity was lower and less persistent than that achieved by the same dose of SRB51 given i.p. However, it cannot precluded that a higher oral dose of SRB51 than was used in the present study may be tolerated by mice and may stimulate immune responses and resistance to infection with S2308 which are comparable to those obtained by i.p. vaccination with SRB51.

Mice were vaccinated orally in the present study by placing SRB51 on to the pharyngeal mucosa. This site was chosen because of ease of administration and because it represents a likely site of exposure in orally vaccinated ruminants when vehicles such as feed pellets or capsules containing SRB51 are initially masticated and ingested and when partially digested ruminal contents mixed with the released SRB51 are regurgitated into the oral cavity, remasticated, and reingested during rumination. Mice were challenged by an i.p. injection with S2308 in the present study, since this method (i) results in a systemic infection in which the spleen and other major lymphoid organs in nonvaccinated mice are reliably infected at a high and uniform frequency and (ii) produces an experimental infection in which the efficacy of the immunizing effect of vaccines can be accurately and quantitatively determined by measuring the number of brucella organisms contained in the spleen (3, 17, 18). Although ruminants are most probably infected by the oral route under natural field conditions (10), this route was not used to challenge mice, since they apparently have an innate resistance to oral infection (2) and because oral challenge is an inefficient method of producing a uniform and high frequency of infection in mice (4).

Nonvaccinated BALB/c AnNHsD mice in the present and other studies (32, 34, 35) exhibit approximately a 10-fold-larger number of CFU in the spleen at 2 weeks after i.p. challenge infection with S2308 than is present at 2 weeks after S2308 challenge infection of nonvaccinated BALB/c ANCrLBR mice (19–21). On the basis of these results, it may be possible to conclude that AnNHsD mice are more susceptible to infection with S2308 than are ANCrLBR mice. However, a side-by-side comparison of these two substrains of BALB/c mice inoculated with the same stock of S2308 would be needed to confirm these differences.

In summary, mice vaccinated orally with SRB51 had less persistent cell-mediated immune responses and protective immunity to S2308 infection than did mice vaccinated i.p. with SRB51. From data obtained with the mouse model, it may be feasible to predict that oral vaccination would be less effective than parenteral vaccination with SRB51 in preventing brucellosis in ruminants. Furthermore, repeated oral vaccinations with SRB51 or a dose of SRB51 given orally to ruminants at higher levels than that used for parenteral vaccination may be needed to induce durable levels of protective immunity that are comparable to those obtained by parenteral vaccination. However, it should be noted that our studies have indicated that SRB51 vaccination of ruminants (cattle) apparently provides much better protective immunity (6, 7) than would have been expected from studies evaluating SRB51 vaccine-induced

protective immunity in mice (15, 32). Ruminants vaccinated orally with SRB51 may also have greater protective immunity than mice vaccinated orally with SRB51, since (i) brucellosis in ruminants most probably results from oral infection under natural field conditions and (ii) SRB51 vaccine-induced oral mucosal immune responses may play a role in preventing brucellosis following the initial oral infection. It is not known if oral mucosal immunity plays a critical role in protecting ruminants from brucellosis. In addition, it is not known if oral SRB51 vaccination must stimulate mucosal or systemic immunity or both to be effective in preventing brucellosis in ruminants. These types of considerations will undoubtedly be important in determining the efficacy of SRB51 as an oral vaccine in ruminants under field conditions.

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