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Protection against Infection in Mice Vaccinated with a *Brucella abortus* Mutant

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This study determines whether a genetically engineered mutant of *Brucella abortus*, strain M-1, possesses differences in protective properties compared to the parental strain, vaccine S19. M-1 is a mutant unable to express BP26, a periplasmic protein with potential use in diagnosis. Mice vaccinated with S19 developed antibodies against BP26, while those vaccinated with M-1 did not. However, mice vaccinated with S19 or M-1 were similarly protected against challenge with pathogenic strain 2308, suggesting that the lack of BP26 does not affect the induction of the protective immune response exerted by S19. These and previous results showing that bacterial invasion and growth or replication in mouse spleens were indistinguishable between strains M-1 and S19 could indicate that the mutant is an attenuated strain which maintains the same protective properties as S19.

Brucellosis, a zoonotic disease, is caused by members of the genus *Brucella*. The current vaccine for cattle used in most of the world against this disease is an attenuated bacterial strain, *Brucella abortus* S19 (13). The antigenic similarity between this strain and wild-type virulent strains, mainly in the immunodominant antigen lipopolysaccharide (LPS), causes difficulty in serological differentiation of vaccinated from infected animals when common immunoassays are used (4).

In a previous work (14), we described the properties of a periplasmic antigen of *B. abortus*, a 26-kDa protein (BP26). This protein is recognized in immunoassays performed with sera from infected animals and at very low levels in few sera from vaccinated animals. By genetic engineering techniques, the gene encoding BP26 was subcloned in a pBR322 derivative plasmid (a suicide plasmid in *Brucella*), and it was inactivated by insertion of a kanamycin cassette; this plasmid was introduced in *B. abortus* S19, and the mutant was isolated (2). The mutant strain, M-1, seems to have many physiological characteristics in common with its parental strain, mainly low virulence and survival in mouse spleens (2). The main advantage of using mutant strain M-1 as a vaccine strain would be the presence of anti-BP26 antibodies in infected but not in vaccinated cattle, allowing differentiation of these animals. Thus, the protective properties of the mutant need to be evaluated. In this study, we investigated the protective capability of mutant strain M-1, compared to that of the original S19.

Several *Brucella* mutants produced by gene replacement have already been studied; the deletion of the Cu/Zn superoxide dismutase (SOD) from *B. abortus* does not significantly modify virulence in mice (11). Survival and intracellular growth were similar in two mammalian cell lines when comparing S19 or 2308 and their respective mutants containing the BCSP31 gene deletion (9). Another study (6) showed that S19-vaccinated and nonvaccinated heifers failed to produce antibodies against superoxide dismutase or the 31-kDa (BCSP31) proteins when challenged with 2308, concluding that using S19ΔSOD or S19ΔBCSP31 as a vaccine would not offer an improvement for the identification of *B. abortus* infections in vaccinated cattle over the current S19 vaccine. By in vitro assays, Drazek et al. (8) found that *Brucella melitensis* with a *purE* deletion failed to grow in human monocyte-derived macrophages, suggesting that this mutant could be attenuated in animals and humans. Recent work shows that this strain is attenuated in mice (7) and goats (5), rendering this mutant a good candidate as a vaccine strain, although no protection experiments with animals were reported.

In order to determine the kinetics of anti-BP26 antibody production in mice immunized with S19 or M-1, mouse sera, obtained at different times after injection, were tested in a Western blot against BP26 (14). For this, *Escherichia coli* DH5α whole-cell lysates carrying the plasmid pBa52, which expresses BP26 (14), were prepared. The second antibody was goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase. As seen in Fig. 1, pooled sera from five mice immunized with S19 developed antibodies against BP26 30 days postinjection (lane 12), while those vaccinated with M-1 did not (lane 4). The band of 29 kDa corresponds to BP26 preprotein that is always present in *E. coli* extracts but not in *Brucella* extracts (14), and since the antibodies against BP26 react with the preprotein, the 29-kDa band is seen in this Western blot. The same figure shows a strong reaction band in the area of BP26 in samples from S19-vaccinated mice, taken 15 days after challenge with 2308 (lane 8), but not in those from mice vaccinated with M-1 and equally challenged (lane 6). It is also shown that mice infected with *B. abortus* 2308 developed antibodies against BP26 at 30 days postinjection (lane 14) as well as those vaccinated with the vaccinal strain S19 (lane 12). We assume that the band of 34 kDa reacting with all the mouse sera corresponds to an *E. coli* antigen, since it is present with the same intensity from early stages of immunization (day 1) and it is absent in lane 15, in which rabbit serum against purified BP26 was used.

In order to determine the protective potential of *B. abortus* M-1 compared to S19, cells were grown in tryptic soy broth (TSB) agar (Difco) at 37°C for 4 days, resuspended in phosphate-buffered saline (PBS), washed in the same buffer, and

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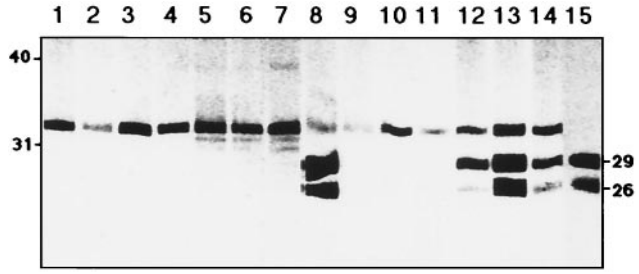

FIG. 1. Western blot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *E. coli* DH5α (pBa52) whole-cell lysate expressing *B. abortus* BP26 with sera pooled from five female mice. Lanes 1 to 5, sera from M-1-vaccinated mice at 1, 7, 15, 30, and 60 days postvaccination, respectively; lane 6, sera from mice vaccinated with M-1 and challenged 45 days postvaccination with *B. abortus* 2308; lane 7, sera from mice mock vaccinated and challenged with 2308; lane 8, sera from mice vaccinated with S19 and challenged 45 days postvaccination with 2308; lanes 9 to 13, sera from S19-vaccinated mice at 1, 7, 15, 30, and 60 days, respectively; lane 14, sera from 2308-infected mice at 30 days postinfection; lane 15, sera from rabbits immunized with purified BP26. Positions of the 26-kDa protein (BP26) and the 29-kDa protein (BP26 plus the signal peptide that allows the export to the periplasm) (14) are indicated on the right, and molecular weight (in thousands) markers are indicated on the left.

diluted to the desired concentration. Following immunization and challenge protocols previously described (3, 10, 12), 8-week-old BALB/c female mice (five per treatment group) were injected intraperitoneally with 0.2 ml of PBS (control group) or 0.2 ml of PBS containing 10^4 CFU of strain S19 or M-1 mutant. Since Araya et al. (1) showed that nonspecific resistance to infection against unrelated bacteria is very low at 6 weeks after immunization with *Brucella*, all groups were challenged 45 days postinoculation with 10^5 total CFU of virulent *B. abortus* 2308 per mouse.

Fifteen days postchallenge, mice were killed and their spleens were removed, weighed, and homogenized in 5 ml of PBS. Lysates were diluted in PBS and duplicate plated onto TSB agar and TSB agar plus 0.1% erythritol for differentiation of *B. abortus* S19 or M-1 from S2308 (12). After 4 days at 37°C, colonies were visualized and CFU per gram of tissue were determined after subtracting the number of S19 or M-1 colonies. Comparison of groups was made by analysis of variance with a significance level of 5% ($P < 0.05$). Bonferroni pairwise comparison of means was made with the same level of significance. Figure 2A shows that mice vaccinated with S19 or M-1 (10^4 CFU/mouse) had significantly ($P \leq 0.01$) enhanced resistance to infection with S2308 at 8 weeks after vaccination compared to nonvaccinated control mice. No differences were observed between S19- and M-1-vaccinated animals ($P \geq 0.5$).

On the other hand and by the same statistical analysis (Fig. 2B), spleen weights of S19- and M-1-vaccinated mice were not significantly different ($P \geq 0.5$) but were significantly lower with respect to the control group ($P \leq 0.005$).

Using *B. abortus* RB51, a rifampin-resistant mutant of *B. abortus* 2308 lacking LPS, described by Schurig et al. (15), Cheville et al. (6) found that this strain was a good vaccine candidate to be used against brucellosis in cattle. However, mice vaccinated with this strain had lower resistance to challenge with the pathogenic strain 2308 than did mice vaccinated with S19, probably due to the rapid clearance of SRB51 (17) and to the absence of antibodies against LPS (16).

We show here that *B. abortus* M-1 mutant, a bacterial strain incapable of expressing the periplasmic 26-kDa protein (BP26), possesses the same protective capability as the parental strain, and as expected, mice vaccinated with M-1 did not develop antibodies against BP26 as did mice vaccinated with S19. These results, together with previous studies (2) showing common properties between the mutant and the parental strain such as splenomegaly and bacterial survival in spleens, would indicate that inactivation of the gene encoding BP26 might also have little effect on the physiological behavior of the bacteria in the host animal. Maintaining the physiological behavior of *Brucella* is a prerequisite for the development of a modified live bru-

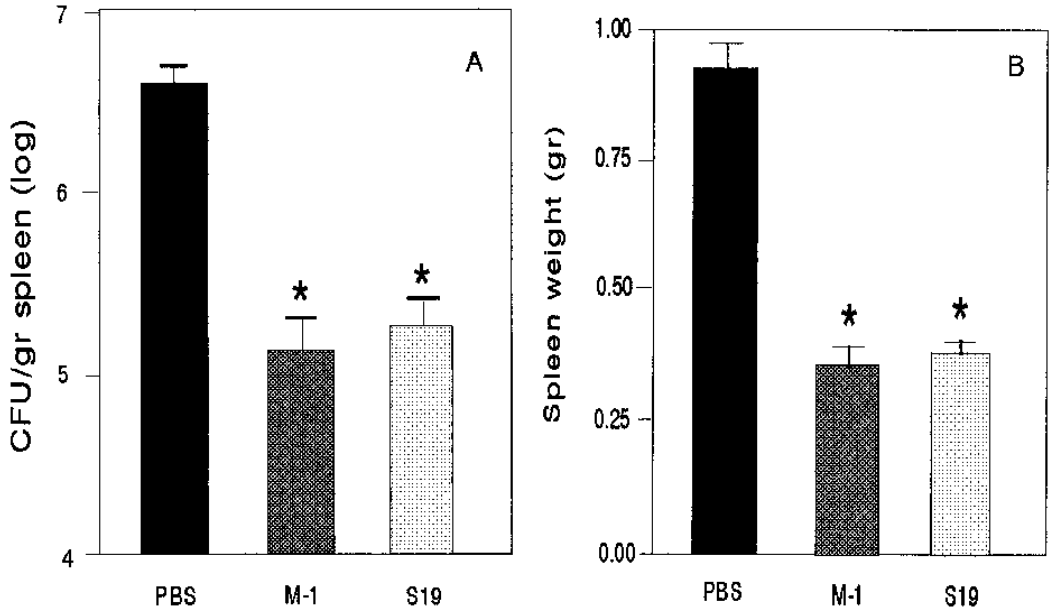

FIG. 2. Persistence of *Brucella* in spleen and spleen weight in vaccinated and control mice after challenge with *B. abortus* 2308. Mice were vaccinated with S19 or M-1 (10^4 CFU/mouse) or mock vaccinated with PBS and challenged 45 days later with 10^5 CFU of *B. abortus* 2308 per mouse. Numbers of bacteria in spleen (A) and spleen weight (B) were determined at 15 days after challenge by plating on TSB agar with or without erythritol. Differences are expressed as means ± standard errors of the means ($n = 5$). Groups with asterisks are different from controls by analysis-of-variance test analysis and Bonferroni pairwise comparisons.

cellosis vaccine with a distinctive immunological signature. We conclude that this mutant strain of *B. abortus* is a potential candidate as a vaccine strain since it protects animals to the same extent as S19 but without induction of antibodies against BP26, allowing differentiation between vaccinated and infected animals as previously postulated (14).

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