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Deletion of *purE* Attenuates *Brucella melitensis* Infection in Mice

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We previously showed that a *purE* mutant (Δ*purE201*) of *Brucella melitensis* 16M is attenuated for growth in cultured human monocytes (E. S. Drazek, H. H. Houng, R. M. Crawford, T. L. Hadfield, D. L. Hoover, and R. L. Warren, Infect. Immun. 63:3297–3301, 1995). To determine if this strain is attenuated in animals, we compared the growth of the Δ*purE201* mutant with that of strain 16M in BALB/c mice. The number of bacteria in the spleen and spleen weight peaked for both strains between 1 and 2 weeks postinfection (p.i.), though the number of Δ*purE201* cells was significantly less than the number of 16M cells recovered from the spleens of infected mice. During the next 6 weeks, Δ*purE201* was essentially eliminated from infected mice (three of five mice sterile; <100 CFU in two of five mice at 8 weeks p.i.), whereas bacteria persisted at a high level in the spleens of 16M-infected mice (about 10^6 CFU per spleen). The number of bacteria in the livers and lungs of mice infected with either strain paralleled those in the spleen. Mice infected with 16M had a strong inflammatory response, developing dramatic and prolonged splenomegaly (five to eight times normal spleen weight) and producing serum interleukin-6. In contrast, mice infected with Δ*purE201* developed only mild, transient splenomegaly at 1 week p.i. and produced no interleukin-6 in their serum. We further characterized the host response to infection by measuring changes in immune spleen cell populations by flow cytometry. CD4- and CD8-positive lymphocytes declined by 1 week in both experimental groups, while MAC-1-positive cells increased. T-cell subpopulations remained low or declined further, and MAC-1 cells increased to three times normal levels during 8 weeks of infection with 16M but returned to normal by 4 weeks after infection with Δ*purE201*. These results document infectivity and attenuation of Δ*purE201* and suggest that it should be further evaluated as a potential vaccine.

Brucellae cause human disease and significant worldwide economic loss due to infection of livestock (11). Live, attenuated vaccines reduce abortion in animals but cause bacteremic infection when administered to humans (10). The genetic basis for attenuation of current vaccine strains has not been elucidated. Recently, *Brucella abortus* 19 (S19) was shown to have a deletion in the *eri* region of the chromosome that results in sensitivity to erythritol (13). However, it seems unlikely that this defect is responsible for attenuation of S19, since erythritol-resistant revertants of S19 remain attenuated for cattle (13). Development of vaccines for humans or improvement of animal vaccines may require construction of genetically defined mutants. An effective live vaccine must induce a strong immune response but cause minimal disease. Auxotrophic mutants of *Salmonella* species which have defects in purine biosynthetic pathways have shown promise as vaccines, but finding the balance between attenuation and immunogenicity has been difficult (15). We have previously characterized the *purEK* operon of *Brucella melitensis* and have created a defined *purE* mutant (Δ*purE201*) by homologous recombination (6a). This mutant requires addition of purines for growth on minimal medium. In contrast to its parent strain, *B. melitensis* 16M, Δ*purE201* fails to replicate in human monocytes in vitro (3). In this paper, we report that Δ*purE201* is attenuated in a murine model of brucellosis. We characterize the murine infection by bacterial burden in macrophage-rich organs, by effect of infection on spleen mononuclear cell populations, and by serum levels of proinflammatory cytokines. We find that Δ*purE201* causes murine infection that qualitatively resembles that caused by 16M, but with lower intensity of infection, earlier clearance of bacteria from target organs, and less profound alteration of spleen cell phenotype and proinflammatory cytokine response. These findings indicate that Δ*purE201* should be further investigated as a live, attenuated vaccine for brucellosis.

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MATERIALS AND METHODS

Animals. Specific-pathogen-free BALB/c female mice were obtained from Harlan Sprague Dawley (Frederick, Md.) and were housed in a BSL-3 barrier facility. Mice were used at 8 to 12 weeks of age. All studies were performed in accordance with Armed Forces Institute of Pathology regulation 70-1 and all statutes, regulations, directives, and guidelines relating to the use of laboratory animals in research.

***B. melitensis* strains and cultures.** *B. melitensis* 16M and Δ*purE201* were used for infections of mice. 16M was obtained from G. Schurig (Virginia Polytechnic Institute, Blacksburg), and stock cultures were prepared in *Brucella* broth (Remel, Lenexa, Kans.) and stored at −70°C. Twenty-four hours before infection of mice, a stock vial was thawed, cultured in broth overnight (log-phase growth), washed in sterile saline, and adjusted turbidimetrically to the desired concentration. The exact count was measured retrospectively by plate count on *Brucella* agar (Remel). Δ*purE201* is a genetically defined auxotrophic mutant derived by genetic replacement from wild-type 16M (6a). Δ*purE201* contains a mutated *purE* operon of *B. melitensis* 16M in which the 3′ half of the *purE* gene was replaced by a kanamycin cassette. Complementation of Δ*purE201* with a plasmid carrying only the *purE* gene restores the capacity of Δ*purE201* to grow in minimal medium lacking purine supplements under atmospheric concentrations of CO_2. This finding suggests that *purK* is expressed under the control of the kanamycin

TABLE 1. Growth of *B. melitensis* strain 16M in BALB/c mice[a]

Dose (CFU)	Log brucellae in spleens (mean ± SD)[b]			
	1 wk	2 wk	4 wk	8 wk[c]
10^4	5.61 ± 0.63	5.77 ± 0.31	5.43 ± 0.80	>5
10^5	6.66 ± 0.45	6.00 ± 0.31	6.16 ± 0.29	>5
10^6	6.68 ± 0.32	6.06 ± 0.10	6.42 ± 0.29	>5
10^7	7.17 ± 0.23	6.12 ± 0.26	6.41 ± 0.35	>5

[a] Mice were infected intraperitoneally with various numbers of strain 16M cells. Growth of bacteria in spleens was determined at several time points.
[b] Five mice per group.
[c] Plate contamination prevented precise enumeration.

cassette. Stock cultures, inoculum, and culture plates of Δ*purE201* were prepared as described for 16M.

Infection of mice and collection of tissues. Groups of mice were inoculated intraperitoneally with 0.2 ml of saline (controls) or various doses of *B. melitensis* 16M or Δ*purE201* in 0.2 ml of saline. Blood samples, spleens, and in some instances livers and lungs were obtained from age-matched noninfected control mice and infected mice at 3 days and 1, 2, 4, 6, and 8 weeks postinfection (p.i.) (five mice per treatment group).

Preparation of tissues and bacterial counts. Spleens from individual mice were weighed, placed in 5 ml of RPMI 1640 in a petri dish, and then gently pressed between frosted ends of two sterile glass slides to release the cell contents from the capsule. Aliquots of the spleen suspension were prepared for differential processing. One milliliter was homogenized in the presence of 0.1% saponin, diluted, and plated onto *Brucella* agar (Remel), and bacterial colonies were counted after incubation for 72 h at 37°C. Debris from the remaining portion of spleen suspension was allowed to settle for 5 min. Supernatant fluid was removed, and cells were pelleted by centrifugation. The pellet was suspended in 1 ml of 0.83% NH_4Cl for 1 min to lyse erythrocytes, then diluted to 10 ml in 10% heat-inactivated fetal bovine serum in RPMI 1640 (F-RPMI), and pelleted by centrifugation. Cells were resuspended in F-RPMI, counted, and distributed for further analysis.

Spleen cell surface phenotyping. Cells were washed once in Ca^{2+}-Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA-PBS). One million cells were pelleted, blocked for 10 min with hamster anti-mouse FcRII (FC block; Pharmingen, San Diego, Calif.), and then stained for 30 min at 4°C with fluorochrome-conjugated antibodies (Pharmingen) titrated to optimally differentiate cell populations. Staining combinations were to CD3 (total T cells), CD4, CD8a, CD45R/B220 (B cells), and CD11b (MAC-1 [macrophages]). Generally, CD4-fluorescein isothiocyanate and CD8-phycoerythrin were added in a single tube, and other antibodies were used individually. Additional cells were stained with fluorochrome-conjugated isotype controls to determine nonspecific binding, which was consistently found for less than 1% of cells. Stained cells were washed once in BSA-PBS and then fixed in 0.5% formaldehyde in PBS. After sterility of cell preparations was verified for safe handling, 5,000 cells per sample were acquired on a FacSort (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), and the percentage of positively stained cells was determined by using Lysis II software (Becton Dickinson). Data are presented as the ratio of mean percent positive spleen cell populations in infected compared with uninfected mice at each time point.

Cytokine assays. Serum interleukin-6 (IL-6) activity was determined by induction of proliferation of B9 cells (a gift from Lucien Aarden) (5). Dilutions of heat-inactivated (56°C, 30 min) serum or standard recombinant human IL-6 (Boehringer Mannheim, Indianapolis, Ind.) were added to 0.2 ml of B9 cells cultured in 96-well plates at 2×10^3 cells per well in F-RPMI. After 72 h, 0.5 μCi of [^{3}H]thymidine was added per well. Sixteen hours later, cells were harvested onto glass fiber filters, and incorporation of [^{3}H]thymidine was quantitated by liquid scintillation counting. IL-6 content of supernatant fluids was determined from the standard curve and expressed as units per milliliter (1 U equals the cytokine concentration required to induce half-maximal cell proliferation). IL-1 activity was determined by induction of proliferation of D-10(N4)M cells (a gift from Stephen Hopkins) (6). Dilutions of unheated serum or recombinant human IL-1α (Boehringer Mannheim) were cultured with 10^4 cells in 0.2 ml of F-RPMI with 2×10^{-5} M 2-mercaptoethanol for 72 h before addition of [^{3}H]thymidine and harvest for scintillation counting and analysis as described above. Serum gamma interferon and tumor necrosis factor alpha were determined by commercial enzyme-linked immunosorbent assays (Endogen, Boston, Mass.) used according to the manufacturer's instructions.

Statistical analysis. Samples from each mouse (five per group) were kept separate for all treatments and analysis. Data from groups of mice were compared by using a two-tailed Student *t* test.

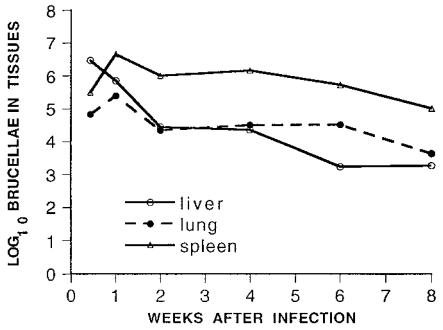

FIG. 1. Course of infection of *B. melitensis* 16M in BALB/c mice. Mice were infected intraperitoneally with 10^5 brucellae, and growth of bacteria in liver, lungs, and spleen was determined at several time points for 8 weeks. Results are expressed as the mean of five individual mice per group.

RESULTS

Growth of *B. melitensis* 16M and spleen weights in BALB/c mice. Table 1 shows the effects of different initial intraperitoneal infection doses of wild-type *B. melitensis* on the growth of bacteria in mice. Peak numbers of bacteria in the spleen occurred 2 weeks p.i. for all infectious doses. Animals infected with 10^5 to 10^7 brucellae had peak counts of 6.6 to 7.2 logs, compared with 5.6 logs for animals infected with 10^4 brucellae. The number of bacteria recovered from the spleens remained at or close to peak levels for the duration of the experiment. One should note that the 8-week p.i. point represents a conservative estimate of actual bacterial numbers, since diluted samples could not be interpreted because of contamination of the diluent. Values presented in the table are reasonable approximations of bacterial numbers based on counts of >200 colonies per plate in undiluted samples. In another experiment, constant numbers of bacteria (5.5 to 6.2 logs) were recovered between 3 days and 8 weeks p.i. from mice infected with 10^5 brucellae (data not shown). Figure 1 shows bacterial growth in the livers and lungs of mice infected with 10^5 organisms. In these mice, the numbers of bacteria in the liver and lung peaked at 3 days and 1 week, respectively. By 2 weeks, the numbers declined to about 4 logs, a level that persisted for the duration of the experiment. Although the numbers of bacteria in the spleens of mice infected with 10^5 to 10^7 bacteria were not different, spleen weight increased as the infectious dose increased (Fig. 2). Mice receiving 10^7 brucellae developed spleens weighing 930 ± 16 mg by 2 weeks p.i. In contrast, mice infected with 10^4 CFU developed peak spleen weights of only

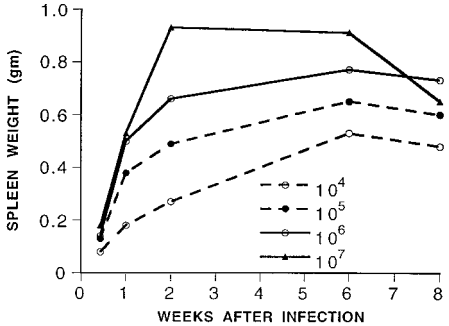

FIG. 2. Spleen weights during *B. melitensis* 16M infection in BALB/c mice. Mice were infected intraperitoneally with various numbers of 16M cells. Spleen weights were measured at several time points over 8 weeks. Results are expressed as the mean of five individual mice per group.

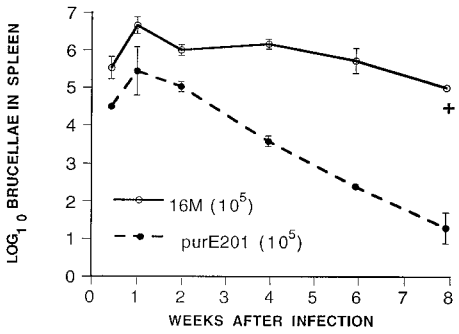

FIG. 3. Course of infection of *B. melitensis* 16M and Δ*purE201* in BALB/c mice. Mice were infected intraperitoneally with 10^5 brucellae, and growth of bacteria in the spleen was determined at several time points for 8 weeks. Results are expressed as the mean ± standard error of the mean ($n = 5$). +, minimum estimate; higher dilutions contaminated.

530 ± 27 mg by 2 weeks. The discrepancy between bacterial burden and the degree of splenomegaly in mice infected with different numbers of brucellae suggests that events early in the course of infection influence host inflammatory response and subsequent control of infection intensity.

Attenuation of Δ*purE201*. We compared the numbers of bacteria in the spleen and the inflammatory responses of mice infected with 10^5 CFU of Δ*purE201* or 16M. Growth of these two strains was parallel over the first 2 weeks, but mean numbers of bacteria in the spleens of Δ*purE201*-infected mice were always significantly lower ($P = 0.05$) than the numbers of bacteria in the spleens of 16M-infected mice (Fig. 3). In mice infected with Δ*purE201*, the number of bacteria peaked at 1 week p.i., decreased slightly at 2 weeks p.i., and then dropped steadily over the next 6 weeks. At 8 weeks p.i., no bacteria (detection limit, 10 CFU) were detected in the spleens of three of five mice infected with Δ*purE201*, and CFU counts of only 1.3 ± 0.36 logs per spleen were detected in the other two mice. In contrast, all spleens from mice infected with 16M had $>10^5$ CFU per spleen during the entire 8-week period. Δ*purE201* also colonized the lung and liver. In contrast to 100% colonization of the liver and lung by 16M, we detected Δ*purE201* in the livers of only two of five mice and in the lungs of only three of five mice. One should note that our lower limit of detection in these organs was 10^3 organisms per organ. No bacteria were detected in either of these tissues 2 weeks or more after infection with Δ*purE201*. These results established that Δ*purE201* was attenuated in the mouse.

Figure 4 shows that Δ*purE201*-infected mice had significantly reduced splenomegaly compared with mice infected with 16M. While spleen weight peaked at 6 weeks p.i. in mice infected with 16M, spleen weight peaked at 1 week and returned to normal values by 6 weeks in mice infected with Δ*purE201*. Since the increase in spleen weight observed during *Brucella* infection, particularly with 16M, probably reflected an increase in the number of spleen cells, we measured the total number of mononuclear cells in the spleens of these groups of animals. The total mononuclear cell count in the spleens of infected animals peaked at about 2 weeks p.i. (data not shown). Again, 16M-infected mice had the greatest increase (approximately twofold, or 2×10^8 cells per spleen) in spleen cells.

Quantitative changes in splenic mononuclear cell populations. Spleen cell populations were analyzed by flow cytometry to determine the percentages and total numbers of CD4, CD8, MAC-1, and B cells at various times after infection with 10^5 16M or Δ*purE201*. Compared with cells from uninfected control mice, the percentage of CD4 cells significantly decreased by 1 week p.i. in both experimental groups, though the decrease was greater in 16M-infected mice (Fig. 5). CD4 cells in Δ*purE201*-infected animals never declined below 80% of the values found in control mice. In contrast, in 16M-infected mice, CD4 cells dropped to a low of about 50% of the control value by 2 weeks p.i. Moreover, the decline in CD4 cells in Δ*purE201*-infected mice was reversed by 8 weeks p.i. Conversely, CD4 cells in 16M-infected mice remained below 60% of the control level for the duration of the experiment. CD8 cells declined like CD4 cells (Fig. 5). The percentage of CD8 cells decreased in both experimental groups by 1 week, returned to normal by 4 weeks in strain Δ*purE201*-infected mice, but continued to decline in 16M-infected mice, reaching a low of 40% of the control level by 6 weeks p.i. In contrast to these remarkable decreases in T-cell subpopulations in *Brucella*-infected animals, MAC-1-positive cells increased (Fig. 5). MAC-1-positive cells increased in 16M-infected animals by more than twofold by 2 weeks and continued to climb, reaching a threefold increase by 8 weeks p.i. MAC-1-positive cells also increased slightly at 1 and 2 weeks in Δ*purE201*-infected mice but then declined to the control level. The effect of *Brucella* infection on B cells was less clear (data not shown). In the first experiment, the percentage of B cells (CD45 receptor) in the spleens of 16M-infected mice, like the percentage of T cells, decreased by 20 and 25% at weeks 2 and 4 p.i. and then returned to normal. In the second experiment, we observed no significant differences in the B-cell population.

Serum proinflammatory cytokine levels. Sera collected from infected and noninfected mice at various times after infection were assayed for the cytokines gamma interferon, tumor necrosis factor alpha, IL-1α, and IL-6. Only IL-6 was detected in any sera from Δ*purE201*-, 16M-, or saline-injected mice. A few animals in each group had a transient increase in serum IL-6 3 days after injection, and two mice infected with Δ*purE201* had detectable levels of IL-6 in their sera at 2 weeks. In contrast, four of five mice infected with 16M had detectable levels of serum IL-6 by 1 week p.i., and all 16M-infected animals were positive by 2 weeks (Fig. 6). IL-6 levels in this group peaked at 4 weeks p.i. and then declined to lower levels until the end of the experiment.

DISCUSSION

These studies establish and begin to characterize a murine model to study *B. melitensis* infection and evaluate vaccine candidates. Using this model, we find that deletion of the *purE* gene from *B. melitensis* dramatically reduces bacterial viru-

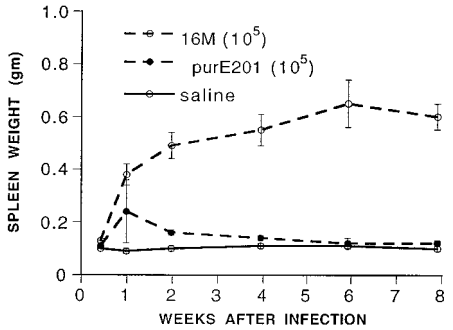

FIG. 4. Spleen weights during *B. melitensis* 16M and Δ*purE201* infection in BALB/c mice. Mice were infected intraperitoneally with 10^5 brucellae, and spleen weights were measured at several time points over 8 weeks. Results are expressed as the mean ± standard error of the mean ($n = 5$).

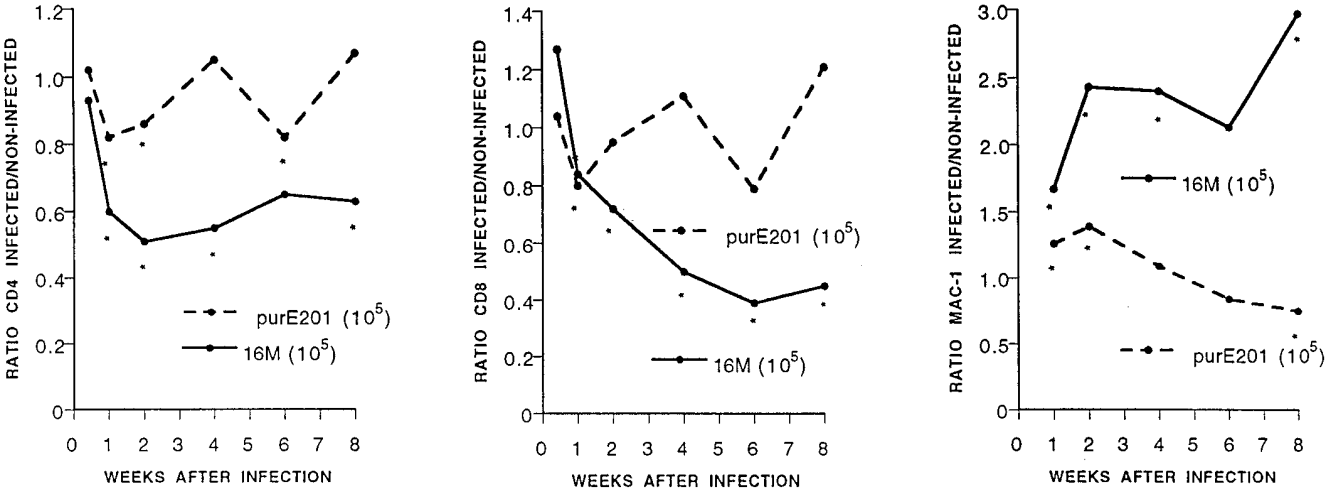

FIG. 5. CD4-, CD8-, and MAC-1-positive cells in the spleen during 16M and Δ*purE201* infection. Mice were infected intraperitoneally with 10^5 brucellae, and CD4 cells were analyzed by flow cytometry at several time points over 8 weeks. Results are presented as the ratio of mean percent positive spleen cell populations in infected compared with uninfected mice at each time point ($n = 5$). Asterisks indicate differences ($P < 0.05$) between infected and noninfected mice, using a two-tailed Student *t* test.

lence. Over the 8-week study period, mice clear the mutant Δ*purE201* more rapidly than the parent strain and display less splenomegaly, reduced spleen cell phenotypic changes, and less inflammatory cytokine response.

While the mouse has been used as a model to study *B. abortus* and *Brucella ovis* infection and to evaluate *B. abortus* vaccines, there are no comprehensive studies of *B. melitensis* infection in the mouse (1, 7, 9, 14). In addition, many studies of the immunology of *B. abortus* infection have used bovine vaccine strains rather than the virulent strain 2308 (1, 2, 15, 18). Whether the mechanisms of recovery from infection with attenuated strains are similar to those required for recovery from infection with virulent organisms is not known. Our findings indicate that *B. melitensis* 16M, like *B. abortus* 2308, produces a chronic steady-state, though not overt, infection in BALB/c mice that persists for at least 8 weeks after inoculation.

The remarkable chronicity and slow resolution of brucellosis, though well documented in both humans and animals, remains unexplained. One of the primary target organs of *Brucella* infection in mice is the spleen. Histopathologic studies of spleen in *B. abortus* infection showed increases in macrophages and disruption of normal architecture (4, 12). Severe lymphoid depletion in the spleen 3 to 6 weeks after infection of mice with *B. abortus* S19 or 2308 is associated with decreased lymphoproliferative responses (14). In the present study, examination of the mononuclear cell composition of the spleen by flow cytometry supported these findings. We showed a decreased percentage of both $CD4^+$ and $CD8^+$ T cells in the spleens of infected mice. The reduction in T cells contrasts with findings by Zhan and Cheers, who noted that CBA mice infected with S19 had increased T cells (both $CD4^+$ and $CD8^+$) compared with uninfected animals (16). Whether this difference reflects differences in mouse strains or bacterial species is unknown. Zhan and Cheers, however, did detect increases in macrophages in the spleens of infected mice by flow cytometry (16). We should emphasize that percentage measurements of the constituents of a rapidly enlarging spleen may not accurately reflect the total number of cells of each of the various mononuclear cell populations. In view of the two- to threefold increase in the percentage of macrophages (from 6% to 12 to 18%) and the decreased percentage of T cells, however, it is likely that macrophage-T cell interactions would be profoundly altered in splenic microenvironments during the course of infection.

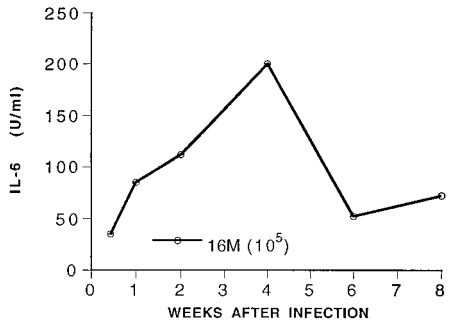

FIG. 6. Production of IL-6 in sera of 16M-infected mice. Mice were infected intraperitoneally with 10^5 brucellae; serum samples were collected at several time points for 8 weeks and assayed for IL-6. Each point represents the mean ± standard deviation for individual sera from five mice.

It is generally recognized that nonliving (subunit) vaccines are ineffective at stimulating protective immunity against intracellular bacterial pathogens like *Brucella* species (17). In this report, we address one of the limitations of current live vaccines, namely, the lack of genetic characterization. We have created a deletion in the *purE* operon of *B. melitensis* by homologous recombination (6a). The resulting smooth Δ*purE201* requires purines for growth on minimal media and fails to replicate in cultured human monocyte-derived macrophages (3). Despite this in vitro evidence of attenuation, however, the degree of attenuation was not easily predictable. Virulence in vivo of *pur* mutants varies with bacterial species, concentrations of nutrients available in challenged tissues or intracellular compartments, and the location of the mutation in the purine synthesis pathway (15). When inoculated into mice, Δ*purE201* behaved much like parental 16M for the first 2 weeks. Both infections peaked at 1 week, although the peak number of bacteria recovered for Δ*purE201* was lower than that for 16M. The increase in numbers of Δ*purE201* early after inoculation

may be attributable to use of purines by extracellular organisms, especially hypoxanthine, which may be increased in mammalian plasma by a variety of stressors (8). However, in contrast to 16M, Δ*purE201* was cleared from the tissues of BALB/c mice. Interestingly, this course of infection bears a striking resemblance to that reported for mice challenged with S19 (9). In parallel with the reduced bacterial burden in the spleen, the intensity and persistence of host responses in Δ*purE201*-infected mice were significantly reduced. Mean spleen weight and spleen cell phenotypes showed only transient changes. Even IL-6 was only minimally and transiently elevated. Nevertheless, this abbreviated course and reduced intensity of infection by Δ*purE201* met the first goal of live vaccine development: reduced virulence by the vaccine strain compared with the wild-type organism. In addition to reduced virulence, however, effective vaccines must also have the ability to induce an immune response sufficient to protect against challenge with virulent organisms. Recently, Stevens et al. showed that *B. abortus* RB51 cleared more rapidly from mice and was less immunogenic than S19 (15). Moreover, mice immunized with RB51 were less resistant than mice immunized with S19 to infection with the fully virulent 2308. This result was partially attributed to the absence of antilipopolysaccharide antibodies in RB51-immunized mice. Current efforts in our laboratory are directed toward analysis of cytokine, antibody, and macrophage activities during infection with 16M and Δ*purE201* to identify antibacterial host responses. In addition, the efficacy of Δ*purE201* as a live vaccine against *B. melitensis* is under investigation.

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