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Lymphocyte Proliferative Responses of Goats Vaccinated with *Brucella melitensis* 16M or a Δ*purE*201 Strain

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The response to a *Brucella melitensis purEK* deletion mutant, Δ*purE*201 (referred to as strain 201), was compared with the response to its parental strain, 16M, in juvenile goats. Proliferative responses to γ-irradiated bacteria were detected earlier in strain 201-infected goats. Lymphocytes from strain 16M- or 201-infected goats proliferated in response to one-dimensional polyacrylamide gel electrophoresis-separated proteins of similar mass isolated from strain 16M or *Brucella abortus* RB51. Data from this study suggest that some antigens stimulating cell-mediated responses are conserved among *Brucella* species, as 201- and 16M-infected goats recognized similar proteins expressed by RB51 and 16M.

Brucella melitensis is an intracellular bacterium that causes abortions in goats and sheep (1). It is the least species-specific *Brucella* species and is readily transmissible from sheep or goats to other species. In humans it is highly pathogenic, and brucellosis is considered one of the more serious zoonoses in the world (1). The current vaccine for sheep and goats, *B. melitensis* Rev1, is a live vaccine which retains some virulence. Disadvantages of Rev1 include abortions in pregnant animals and the inability to serologically distinguish antibody responses from wild-type *B. melitensis* infections in conventional *Brucella* tests. The purpose of this study was to characterize immune responses, clearance, and lymph node (LN) histology of a new vaccine candidate for goats and humans, a *B. melitensis* Δ*purE*201 strain (201). Strain 201 was constructed by homologous recombination to have a defined deletion in the *purEK* operon (5). This operon encodes two enzymes of the de novo purine synthesis pathway (6), PurE and PurK, which catalyze conversion of 5-aminoimidazole ribonucleotide (AIR) to 4-carboxyAIR. Deletion of *purEK* abrogates the ability of *B. melitensis* to grow in human monocytes (5) and leads to more rapid clearance of bacteria from spleens of infected BALB/c mice (4).

Data regarding serologic responses, persistence of strains 201 and 16M, and histological lesions of the goats used in this study are reported elsewhere (2). This study describes the cell-mediated responses to γ-irradiated bacteria and one-dimensional polyacrylamide gel electrophoresis (1D-PAGE)-separated 16M proteins. As protein antigens may be conserved among *Brucella* species, proliferative responses to 1D-PAGE-separated proteins of the rough *Brucella abortus* strain, RB51, were also characterized. This strain is a potential vaccine for goats, as a mouse model demonstrated that RB51 vaccination induced significant protection against challenge with strain 16M (7). As RB51 does not interfere with serologic identification of *Brucella*-infected cattle, it may also offer diagnostic advantages over Rev1 in sheep and goats (9).

***B. melitensis* and *B. abortus* cultures and proteins.** Strain 16M or 201 used for vaccination was grown on tryptose agar, whereas bacteria prepared for γ-irradiation were grown on tryptose agar containing 5% bovine serum. Strain RB51 was prepared as previously described (12). Killed suspensions of 16M, 201, and RB51 cells were prepared by γ-irradiating 8×10^{13} CFU/ml in 0.01 M phosphate-buffered saline (pH 7.2) with 1.4×10^6 rads. The killed bacteria were washed in phosphate-buffered saline three times, aliquoted, and then stored at −70°C.

Whole-cell lysates (1D-PAGE-separated protein fractions) from irradiated 16M or RB51 cells (approximately 1.5×10^{11} CFU) were separated into 106- to 18-kDa proteins by sodium dodecyl sulfate-PAGE, eluted into 22 fractions with a Bio-tetlutor (Biometra, Göttingen, Germany), and concentrated by membrane filtration (12). A 50-μl aliquot of each of the protein fractions (undiluted and 1:2 and 1:4 dilutions) was added in duplicate to wells of a 96-well microtiter plate, and plates were stored at −70°C until use. Protein assays (Bio-Rad, Richmond, Calif.) of the concentrated fractions indicated that aliquots added to individual wells contained approximately 0.25 to 1.25 μg of protein.

Vaccination of goats. Eighteen juvenile (4 months of age) female goats were obtained from brucellosis-free herds and were randomly assigned to one of two vaccine (16M or 201) treatments or to a control treatment (6 goats per treatment). All goats were given bilateral, subcutaneous injections (8×10^9 live bacterial CFU suspended in 2 ml of saline per side) in sites drained by the superficial cervical (prescapular) lymph node. Control animals received 2 ml of saline per side. Animals were housed in a biosafety level 3 facility.

Culture medium. Peripheral blood mononuclear cells (PBMC) were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 300 mg of L-glutamine per liter, 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

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streptomycin per ml, and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co, St. Louis, Mo.).

Collection of PBMC and LN cells. Blood was obtained from the jugular veins of goats and placed in an acid-citrate-dextrose solution. PBMC were isolated by density centrifugation using a Ficoll-sodium diatrizoate gradient.

The left or right superficial cervical LN was biopsied from each of two goats in each group 3 days and 1, 2, 4, 8, or 12 weeks after vaccination. Goats were sedated with xylazine, and the LNs were surgically removed as previously described (3). At 16, 17, and 18 weeks postvaccination, two animals from each group were euthanized and supramammary LNs were removed. LNs were placed on a sterile 60-mesh stainless steel screen, minced with scissors, and processed to form a single-cell suspension as described previously (12).

LN cells and PBMC were diluted in RPMI 1640 medium to 1×10^7 viable cells per ml as determined by trypan blue dye exclusion.

Lymphocyte proliferation assays. Fifty microliters of each cell suspension containing 5×10^5 LN cells or PBMC was added to each of two separate flat-bottom wells of 96-well microtiter plates that contained 100 μl of RPMI 1640 medium and either γ-irradiated strain 16M, 201, or RB51 cells (10^9 to 10^5/well) or strain 16M or RB51 1D-PAGE-separated protein fractions. Cell cultures were incubated for 7 days at 37°C under 5% CO_2. Microtiter plates were placed on a MicroShaker II (Dynatech Laboratories Inc., Alexandria, Va.) every 2 days during the incubations and mixed at an instrument setting of 3.5 for 1 min. After 7 days of incubation, cell cultures were pulsed with 1.0 μCi of [^{3}H]thymidine per well for 18 h. Cells were harvested onto glass filter mats, and radioactivity was counted in a liquid scintillation counter. Cell proliferation results were expressed as mean counts per minute (cpm) ± the standard error of the mean. Stimulation indexes (SI) were calculated by dividing the proliferative response (cpm) of cells incubated with the protein fractions by the proliferative response (cpm) of cells incubated alone.

Statistical analysis. Differences in responses between treatments at each sampling time were compared by a general linear model procedure. Protein fractions were compared at each sampling time with a two-way analysis of variance model. For statistical comparisons, [^{3}H]thymidine incorporation was also evaluated as the logarithm of the cpm value and as the SI (cpm of antigen-stimulated cells/cpm of nonstimulated cells). Means for individual treatments were separated by use of a least significant difference procedure ($P < 0.05$).

PBMC proliferative responses. As superficial cervical LN cells from all treatments failed to demonstrate proliferative responses to γ-irradiated bacteria (Fig. 1) at 8 weeks postinfection or earlier, we elected to evaluate PBMC to characterize proliferative responses of 16M- or 201-vaccinated goats. Initial evaluations of PBMC from two goats per treatment at 9 weeks postinfection also failed to demonstrate proliferative responses to γ-irradiated bacteria or 1D-PAGE-separated 16M or RB51 proteins (data not shown).

At 11 weeks postinfection, PBMC from strain 201-infected goats demonstrated significantly greater proliferation ($P < 0.05$) in response to γ-irradiated strain 16M, 201, or RB51 cells than PBMC isolated from 16M-infected or noninfected goats (Fig. 2). Proliferative responses to 16M proteins by PBMC from 16M- or 201-infected goats were confined to protein fractions 17 to 22 (≤18 kDa, 16M [Fig. 3]). In these fractions, SIs of greater than 10 were demonstrated in three of five 201-infected goats as compared with only one of five 16M-infected goats and none of the noninfected controls. SIs of 201- and 16M-infected goats to the remaining 16M fractions were less than 4. Antigens present within fractions 17 to 20 of 1D-PAGE-separated RB51 proteins also stimulated proliferative responses (RB51 [Fig. 3]). Within RB51 fractions 17 to 20, four of five 201-infected goats had SIs that exceeded 10, compared with only one of five 16M-infected goats. SIs of noninfected goats to all 1D-PAGE 16M or RB51 fractions remained less than 5.

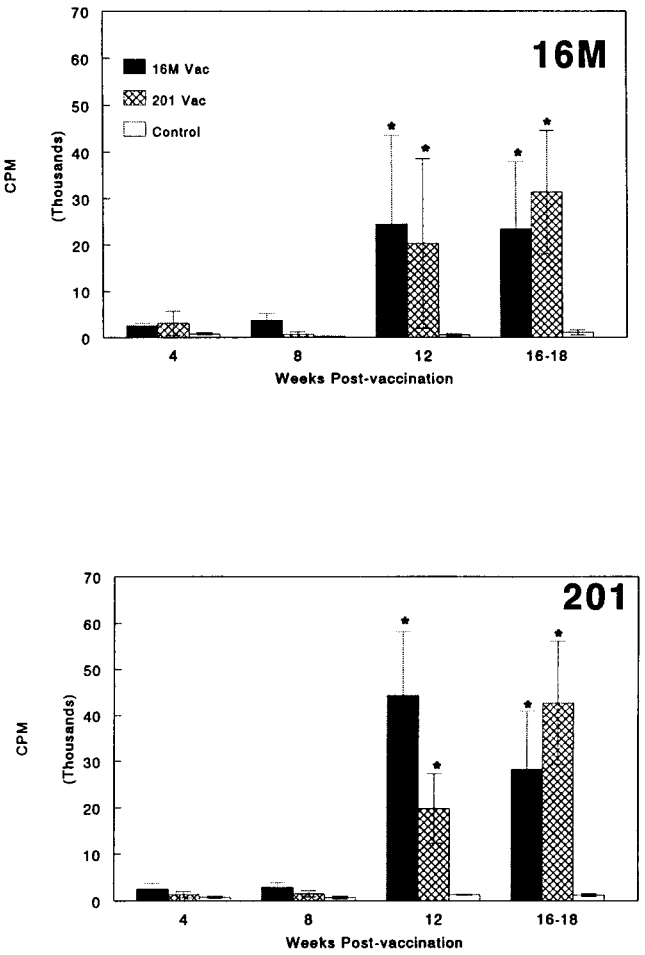

FIG. 1. Proliferation of LN cells from 16M-infected, 201-infected, or noninfected goats in response to 10^7 γ-irradiated 16M or 201 cells per well. Cells were obtained by biopsy 4, 8, and 12 weeks (prescapular, $n = 2$ goats per treatment per time) after infection or at necropsy, 16, 17, or 18 weeks after infection (supramammary, $n = 4$ goats per treatment per time). LN cells were incubated with killed bacteria in duplicate for 7 days and pulsed for 18 h with [^{3}H]thymidine. Results are presented as mean counts per minute ± the standard error of the mean. Mean responses of 201- or 16M-infected goats identified with asterisks are statistically different ($P < 0.05$) from mean responses of noninfected goats when evaluated as the logarithm of the counts per minute value.

Proliferative responses of LN cells. Superficial cervical LN cells from all treatments failed to demonstrate proliferative responses to irradiated bacteria (Fig. 1) or 1D-PAGE-separated 16M or RB51 proteins (data not shown) at 8 weeks postinfection or earlier. At 12 weeks postinfection, proliferative responses of superficial cervical LN cells from 16M- and 201-infected goats ($n = 2$) to irradiated 16M or 201 bacteria were greater ($P < 0.05$) than the responses of nonvaccinated goats (Fig. 1). Although greater in magnitude, responses by LN cells of goats from both 16M and 201 treatment groups at 12

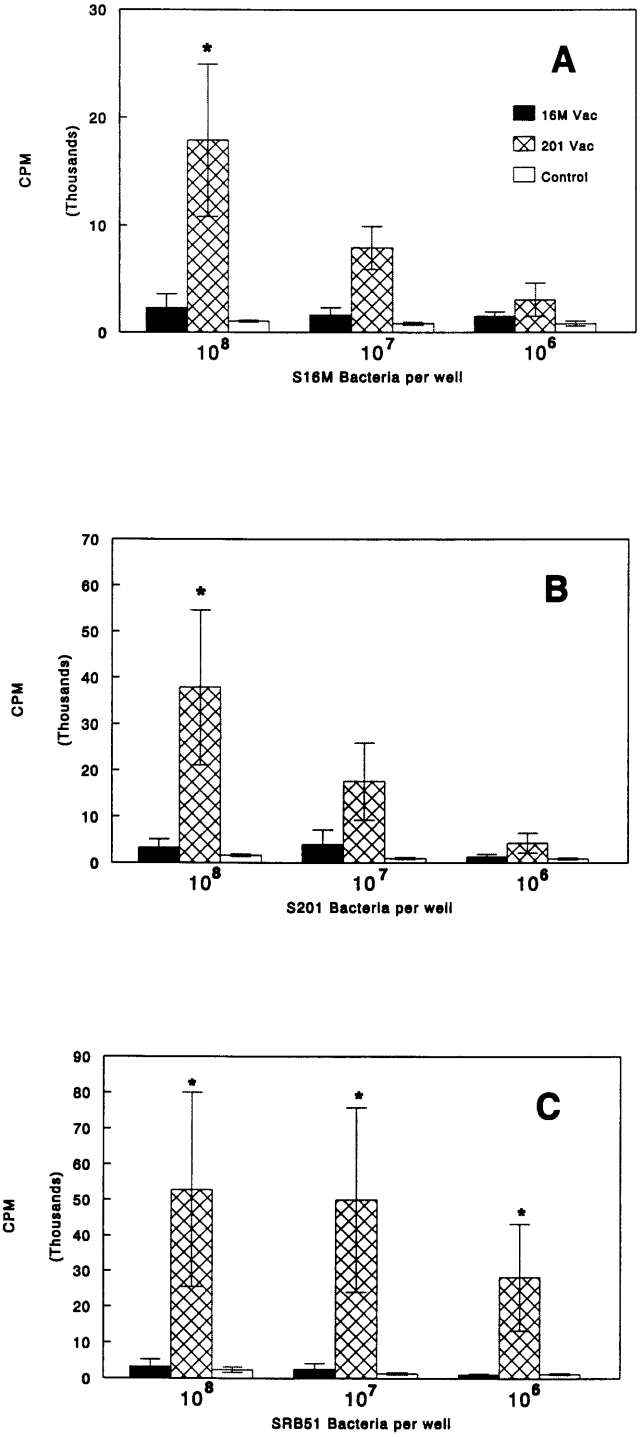

FIG. 2. Proliferation of PBMC in response to γ-irradiated strain 16M (A), 201 (B), or RB51 (C) 11 weeks after subcutaneous injection of 16M, 201, or saline (n = 5 goats per treatment). PBMC were incubated with killed bacteria in duplicate for 7 days and pulsed for 18 h with [^{3}H]thymidine. Results are presented as mean counts per minute ± the standard error of the mean. Mean responses of 201-infected goats identified with asterisks are statistically different ($P < 0.05$) from mean responses of noninfected or 16M-infected goats.

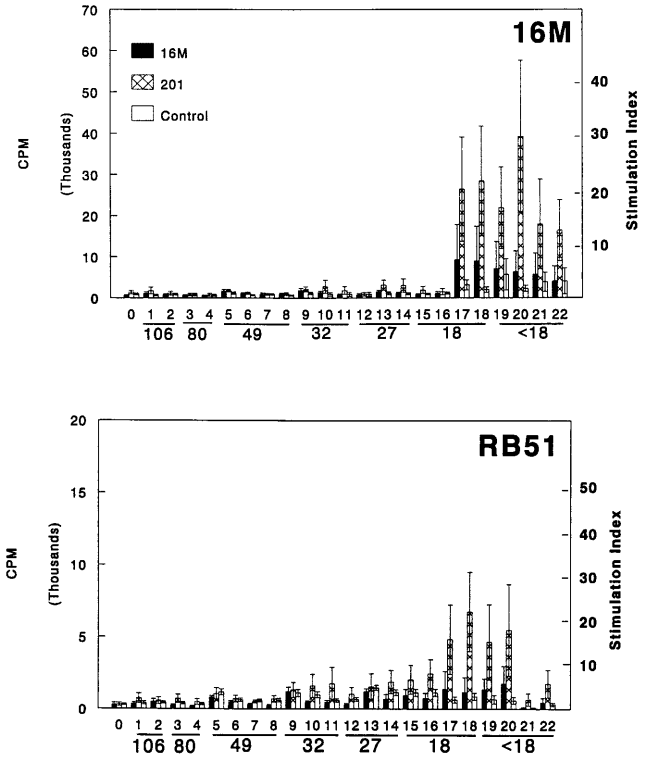

FIG. 3. Proliferation of PBMC in response to 1D-PAGE-separated protein fractions of strain 16M or RB51 11 weeks after subcutaneous injection of 16M, 201, or saline (n = 5 goats per treatment). PBMC were incubated with the protein fractions in duplicate for 7 days and pulsed for 18 h with [^{3}H]thymidine. Fraction 0 represents proliferation of PBMC when incubated alone (no proteins). Results are presented as mean counts per minute ± the standard error of the mean.

weeks postinfection were present within the same 16M protein fractions (≤18 kDa, fractions 17 to 22 [data not shown]) which had stimulated proliferation of PBMC from 201-infected goats 1 week previously.

At necropsy at 16, 17, and 18 weeks after infection, supramammary LN cells from 16M- and 201-infected goats demonstrated proliferative responses to strains 16M and 201 (Fig. 1) that were similar to the responses by prescapular LN cells at 12 weeks postinfection. Compared with cells from control animals, LN cells from 16M- or 201-infected goats showed significantly ($P < 0.05$) increased responses to 12 of 14 lower-molecular-mass (32 kDa to <18 kDa) RB51 protein fractions (Fig. 4). Responses of LN cells obtained from 201-infected goats consistently exceeded responses of cells obtained from 16M-infected goats ($P < 0.05$ for 9 of 12 active fractions). Responses of supramammary LN cells obtained at necropsy to 1D-PAGE-separated 16M proteins could not be determined because of contamination of protein fractions prepared for use at that sampling time. In a similar manner, responses to irradiated bacteria or 1D-PAGE-separated RB51 proteins could not be determined in one control goat, two 16M-vaccinated goats, and two 201-vaccinated goats at necropsy because of high background responses (>30,000 cpm) of supramammary LN cells incubated alone.

Our data indicate that PBMC isolated from goats vaccinated with strain 201 proliferated in response to *Brucella* antigens earlier than those from goats vaccinated with similar numbers of 16M cells. However, proliferative responses to irradiated bacteria of LN cells from 16M- or 201-infected goats were

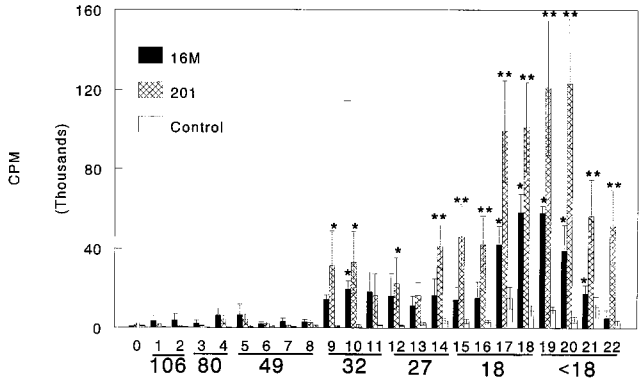

FIG. 4. Proliferation of goat supramammary LN cells in response to 1D-PAGE-separated RB51 proteins. LN cells were obtained at necropsy, 16, 17, or 18 weeks after injection with 16M, 201, or saline ($n = 4$ goats per treatment for vaccine groups, $n = 5$ goats for control group). LN cells were incubated with the protein fractions in duplicate for 7 days and pulsed for 18 h with [^{3}H]thymidine. Fraction 0 represents proliferation of LN cells when incubated alone (no proteins). Results are presented as mean counts per minute ± the standard error of the mean. Mean responses of 201-infected goats identified with two asterisks are statistically different ($P < 0.05$) from mean responses of noninfected or 16M-infected goats. Mean responses identified with one asterisk are statistically different ($P < 0.05$) from mean responses of noninfected goats.

similar at 12 and 16 weeks. The PBMC responses in this study suggest that strain 201 is slightly attenuated in vivo compared with strain 16M. This is supported by LN histology, although culture data suggest that clearance of both strains is similar (2).

Our inability to demonstrate proliferative responses in PBMC or LN cells prior to 11 weeks postinfection is similar to results of studies in which LN cells were isolated from *B. abortus* 19- or RB51-vaccinated cattle. In these cattle studies, no response, or very low responses, to *Brucella* antigens were detected 6 weeks after vaccination, whereas strong proliferative responses were elicited 10 weeks after vaccination (12). Goats infected with strain 16M or 201 had acute lymphadenitis of draining LNs that persisted histologically for approximately 4 weeks (2). The failure of PBMC to respond to stimuli while local LN inflammatory reactions are at maximal intensity may reflect depletion of circulating antigen-specific lymphocytes in LNs. This possibility is supported by histological data indicating that although lymphodepletion and inflammation occurred in both vaccine groups, they were more severe in the 16M group (2). Another possibility is that lymphocyte trafficking was altered, with preferential localization of antigen-responsive cells within local LNs. However, this hypothesis is not supported by our data, as both PBMC and LN cells failed to respond until 11 or 12 weeks postvaccination. A third hypothesis, that antigen-specific cells have impaired responsiveness because of immunosuppressive accessory cells, cannot be excluded by our data. This hypothesis is supported by a study in which reduced proliferative responses of spleen cells in *Brucella*-infected mice were attributed to the relative predominance of macrophages over lymphocytes (8). Whether a similar phenomenon occurs in blood is unknown. Regardless of the mechanism, our data suggest that lymphocyte blastogenic responses of 201-vaccinated goats can be demonstrated earlier and are greater in magnitude than responses of lymphocytes from 16M-vaccinated goats.

The 22 protein fractions of 16M and RB51 did not contain a single protein but instead contained groups of proteins with similar molecular masses. Therefore, adjustment of each fraction to the same protein concentration would not have been of benefit in characterizing fractions which stimulate proliferative responses. Because of the inherent inability to equalize the concentrations of all antigens within a fraction, the possibility that other 16M or RB51 proteins of immunological importance were not present in sufficient quantities to induce proliferative responses cannot be excluded. We attempted to address this problem by dramatically increasing the concentration of bacteria used for protein isolation per well compared with the concentration of whole bacteria which stimulated the largest proliferative responses.

Some of the *B. melitensis* proteins recognized by 16M- or 201-infected goats are expressed by *B. abortus* RB51. In our study, proliferative responses were demonstrated in fractions that contained 16M or RB51 proteins of similar molecular masses. The range of RB51 proteins inducing responses is similar to that in previous work using either LN cells from *B. abortus* 2308-infected cattle (11) or spleen cells from *B. abortus* 19-, 2308-, or RB51-infected mice (10). Data from these studies suggest that *Brucella* proteins approximately 18 kDa in size or smaller may be important in stimulating lymphocyte proliferative responses and that these proteins may be conserved between some strains of *B. abortus* and *B. melitensis*. These data are also supported by a study which demonstrated that RB51 protects BALB/c mice against challenge with 16M (7), thereby implying conservation of some antigens which induce protective responses.

Strain 201 may have potential for vaccine use in goats. Antibody responses of 201-infected goats are similar to those of 16M-infected goats in that they are detectable 2 weeks after vaccination and persist past 16 weeks (2). Proliferative responses of goats infected with strain 201 were greater in magnitude than, and encompassed the same 16M 1D-PAGE-separated protein fractions as, responses of 16M-infected goats. However, further studies that include challenge of pregnant animals with virulent strains of *B. melitensis* will be required to determine the value of 201 as a vaccine for goats.

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