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Vaccine Potential of Attenuated Mutants of *Corynebacterium pseudotuberculosis* in Sheep

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Corynebacterium pseudotuberculosis, a gram-positive facultative intracellular bacterial pathogen, is the etiological agent of the economically important disease caseous lymphadenitis (CLA) in both sheep and goats. Attenuated mutants of *C. pseudotuberculosis* have the potential to act as novel vaccines against CLA and as veterinary vaccine vectors. In this report, we have assessed the virulence of both *aroQ* and *pld* mutants of *C. pseudotuberculosis* in sheep and concurrently their capacity to act as vaccines against homologous challenge. The results suggest that *aroQ* mutants of *C. pseudotuberculosis* are attenuated with regard to both lymph node persistence and vaccination site reactogenicity. Immunologically, *aroQ* mutants failed to elicit detectable specific gamma interferon (IFN- γ)-secreting lymphocytes and induced low levels of antibodies to *C. pseudotuberculosis* culture supernatant antigens. Following subcutaneous vaccination, the immune responses induced by *aroQ* mutants did not protect sheep from infection with the wild-type strain but did appear to reduce the clinical severity of disease resulting from challenge. Conversely, an attenuated *C. pseudotuberculosis* strain expressing an enzymatically inactive phospholipase D exotoxin, when used as a vaccine, elicited a protective immune response. Protection appeared to correlate with in vivo persistence of the vaccine strain, the induction of IFN- γ -secreting lymphocytes, and relatively high levels of antibodies to culture supernatant antigens. The results suggest that *aroQ* mutants of *C. pseudotuberculosis* may be overly attenuated for use as a CLA vaccines or as vaccine vectors.

Corynebacterium pseudotuberculosis is a gram-positive, mycolic acid-containing facultative intracellular pathogen which is phylogenetically related to *Mycobacterium tuberculosis* (16). *C. pseudotuberculosis* is the etiological agent of caseous lymphadenitis (CLA) in both sheep and goats. CLA is a chronic disease characterized by the formation of necrotic lesions that in sheep are typically located in superficial lymph nodes and the lungs (1). Transmission of disease is thought to occur via contamination of shearing wounds with viable bacteria originating from the discharging lung abscesses of infected sheep (6, 19). In Australia, CLA is one of the most prevalent diseases of sheep and, as a consequence, has an economic impact due to reduced wool production by infected animals and condemnation of carcasses and skins in abattoirs (17, 18). *C. pseudotuberculosis* infection of humans has also been reported (20).

While the pathogenic process employed by *C. pseudotuberculosis* in causing CLA in sheep and goats is not well defined, at least two major virulence determinants have been identified. One of these is the toxic lipid cell wall, which may mediate the bacterium's resistance to killing by phagocytic cells (7, 8). The other identified virulence determinant is a sphingomyelin-degrading phospholipase D (PLD) exotoxin (12). PLD is thought to mediate dissemination of the pathogen within the host by increasing local vascular permeability (1). CLA vaccines formulated from concentrated, formalin-inactivated *C. pseudotuberculosis* culture supernatants containing PLD have considerable efficacy (3–5). A role for PLD in the virulence of *C. pseudotuberculosis* was confirmed when two independently

constructed *pld* mutants were shown to be attenuated in sheep (10) and goats (15), respectively. One of these mutants (Toxminus), when used as a vaccine against CLA in sheep, elicited a protective immune response (10). Such live attenuated mutants of *C. pseudotuberculosis* hold promise as veterinary vaccine vectors, since immune responses to coexpressed antigens can be elicited in vaccinated sheep (11). Importantly, the immune response to an antigen delivered by a live vector can potentially be long lasting, thus circumventing the requirement for multiple vaccinations.

There is, however, evidence from studies of attenuated *Salmonella typhimurium* mutants to suggest that the type of attenuating mutation used to construct a vaccine vector can critically affect the immunogenicity of the strain. This has been attributed to the different in vivo growth rates or levels of host persistence of the mutants and concomitant altered interaction with the host immune system (13). Toward the development of new attenuated strains of *C. pseudotuberculosis* for use as vaccine vectors, we have previously constructed and assessed the vaccine potential of an attenuated *aroQ* mutant in a mouse model (24). The *aroQ* gene encodes a type II 3-dehydroquinase enzyme likely to be involved in the biosynthesis of aromatic amino acids in the bacterium. This mutant, when used as a vaccine in mice, elicited an immune response which protected vaccinees from wild-type *C. pseudotuberculosis* challenge (24). The aim of the present study was to compare the vaccine efficacies of *aroQ* and *pld* mutants of *C. pseudotuberculosis* with regard to induction of immune responses which are protective against ovine CLA. The capacity of these mutants to elicit protective immune responses may correlate with their potential as vaccine vectors for the delivery of heterologous antigens to sheep.

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MATERIALS AND METHODS

Bacterial strains and culture. *C. pseudotuberculosis* C231 is a sheep-pathogenic wild-type strain (2). *C. pseudotuberculosis* TB521 is a *pld* mutant obtained through allelic exchange of the native *pld* gene with a cloned *pld* sequence specifically mutated at the position encoding the active site of the exotoxin. Previous studies have established that the histidine at position 20 of the mature PLD protein is part of the enzyme active site (9, 26). Through site-directed mutagenesis of the *pld* gene sequence, substitution of the histidine residue to serine at position 20 of the mature PLD protein rendered the molecule enzymatically inactive (26). By homologous recombination, this mutated *pld* sequence was recombined with the C231 chromosome so as to replace the native sequence. The resultant mutant, TB521, expressed enzymatically inactive PLD at levels approximately equivalent to that of the wild-type parent (data not shown). The technique of achieving site-specific allelic exchange at the *pld* locus and the screening of mutants has been previously described (10). To verify allelic exchange, the DNA sequence encompassing the mutation was amplified by PCR, and the PCR product was sequenced to verify replacement of the native *pld* sequence with the mutated sequence. TB521 has previously been shown to be attenuated in both mice (24). Strains CS100 and CS200 are *aroQ* mutants of C231 and TB521, respectively, and were constructed by allelic exchange (24).

TB111 is a Δ *pld* mutant of *C. pseudotuberculosis* harboring the entire transcriptional unit of the *pld* gene on the *Escherichia coli*-*C. pseudotuberculosis* shuttle vector pEP-2 and has been described previously (26). Supernatants from TB111 cultures were used to partially purify PLD for serological assays. *C. pseudotuberculosis* strains were grown at 37°C in brain heart infusion (BHI; Oxoid, Basingstoke, England) broth or agar supplemented with erythromycin (30 µg ml⁻¹) when appropriate. As previously described (10), BHI plates containing 5% sheep erythrocytes and 10% filtered *Rhodococcus equi* supernatant were used to identify strains which expressed PLD. For vaccine preparation, *C. pseudotuberculosis* strains were grown in 500 ml of BHI broth at 37°C for 20 h with shaking and then pelleted. Cells were then washed once with phosphate-buffered saline (PBS), pelleted, and resuspended in 10 ml of PBS prior to vaccination.

Serological assays. The serological responses of sheep following vaccination to *C. pseudotuberculosis* antigens were determined in an enzyme-linked immunosorbent assay (ELISA). To assess responses to secreted proteins, supernatants from TB111 were first precipitated by 45% ammonium sulfate precipitation of filter-sterilized culture supernatants. The precipitated proteins were resuspended in PBS (pH 7.4), and salt was removed by dialysis against four changes of PBS (4°C for 24 h). The composition of the precipitated proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. The results indicated PLD comprised at least 90% of the precipitated protein. Culture supernatant proteins isolated in this manner were solubilized at 10 µg/ml in bicarbonate buffer (pH 9.6) and used to coat 96-well microtiter plates (Nunc Immunoplates). For preparation of whole-cell lysate antigens, washed overnight cultures of *C. pseudotuberculosis* were passed through a French press three times at a pressure of 1,000 lb/in². Insoluble material was removed by centrifugation, and the supernatant containing soluble cellular proteins was removed and freeze dried. Freeze-dried antigens were solubilized at 10 µg/ml in carbonate buffer and used to coat 96-well microtiter plates, which were then incubated at 4°C overnight. The protein concentration in solutions of coating buffer were determined by specific protein determination assay (Bradford assay), using a dye reagent concentrate (Bio-Rad, Hercules, Calif.). Following antigen coating, ELISA plates were washed and blocked with 3% bovine serum albumin in PBS for 1 h prior to addition of serum. Serum was diluted in PBS containing 0.3% bovine serum albumin and 0.05% Tween 20 and, following serial dilution, incubated in wells for 2 h at 37°C. Wells were washed with PBS containing 0.05% Tween 20, 100 µl of the appropriate mouse anti-sheep immunoglobulin G (IgG) subclass-specific antibody (kind gifts from Ken Beh, CSIRO Division of Animal Health, Sydney, Australia) was added, and the mixture was incubated for 2 h at 37°C. Following washing, a sheep anti-mouse horseradish peroxidase-conjugated antibody (Silenus) was added for 2 h at 37°C. Wells were then washed, and bound conjugate was detected by using Immunopure *o*-phenylene diamine (Pierce) with H₂O₂ as substrate. Reactions were stopped with 20 µl of 2.5 M H₂SO₄, and optical densities (ODs) were read at 492 nm. Titers were expressed as the reciprocal of the dilution which gave an OD threefold above the OD of preimmune serum analyzed on the same plate. Titers obtained from preimmune serum of individual animals were always below 300.

Assay for cellular immune responses. Cellular responses to *C. pseudotuberculosis* antigens were quantified by detection of gamma interferon (IFN-γ) in plasma of 1-ml whole-blood cultures stimulated with 5 µg of a *C. pseudotuberculosis* soluble whole-cell lysate. Whole blood was incubated in duplicate with and without antigen for 18 h at 37°C in 5% CO₂, when plasma was collected and stored at -20°C until analyzed. Levels of IFN-γ in plasma were determined by using a commercial capture ELISA for the detection of ovine and bovine IFN-γ (CSL Ltd., Parkville, Victoria, Australia) according to the manufacturer's instructions. Levels of IFN-γ were expressed as stimulation indices. These were calculated by dividing the mean ELISA OD attained with plasma derived from antigen-stimulated blood by the mean OD obtained when blood was cultured without antigen.

Sheep vaccination and challenge. Nine-month-old merino wethers were selected from a flock with no history of vaccination or CLA. Prescreening of sheep involved analysis of serum antibody for reactivity with *C. pseudotuberculosis* whole-cell lysate antigens in an ELISA. The use of whole-cell lysates as antigens in ELISAs has previously been applied to identify sheep with CLA (25). Of 63 sheep screened, 30 with the lowest *C. pseudotuberculosis*-specific serum antibody levels were randomly divided into six groups of five. Sheep were vaccinated subcutaneously above the left hind lateral claw, a site drained by the left popliteal lymph node. Groups of sheep received either 10⁶ or 10⁸ CFU of the *aroQ* mutants CS100 and CS200; sheep given the *pld* mutant TB521 received either 10⁶ or 10⁴ CFU. Sheep in one group were vaccinated with 10⁶ CFU of the wild-type strain C231. The rationale for including in this study sheep vaccinated with wild-type strain C231 came from studies by Pepin et al. (21) and Hodgson et al. (10) which demonstrated that sheep experimentally infected with virulent *C. pseudotuberculosis* developed concomitant, acquired immunity to reinfection. Consequently, these sheep could serve as positive controls for protective immunity. All sheep were allowed to graze freely and were bled fortnightly for isolation of serum and for blood cultures. Observation of vaccine-induced reactogenicity at the vaccination site were made weekly. Thirty-eight days postvaccination, all sheep, including naive controls, were challenged subcutaneously in the right hind leg with 10⁶ CFU of C231. All sheep were sacrificed 38 days postchallenge, and subjected to a full necropsy. At necropsy, the left and right hind popliteal lymph nodes were individually collected for bacterial culture. The iliofemoral, medial iliac, superficial cervical, and superficial inguinal lymph nodes were dissected in situ for evidence of abscessation. The lungs, kidneys, and intestines were removed from the carcass and similarly analyzed for evidence of abscessation.

Bacterial culture from lymph nodes. The bacterial load in popliteal lymph nodes was determined by fine dissection of the lymph nodes with scissors followed by homogenization in 5 ml of saline, using a medical Stomacher 80 (Seward, London, England). Where abscesses were noted in other lymph nodes, pus was collected and cultured on BHI agar. Identification of *C. pseudotuberculosis* *aroQ* mutants was made on BHI plates containing erythromycin. Identification of the wild-type strain was based on culture morphology and capacity to cause hemolysis on BHI plates containing sheep erythrocytes and *R. equi* supernatant. Conversely, identification of the *pld* mutant, TB521, was based on lack of hemolysis on blood plates.

Statistical analysis. Bacterial counts in popliteal lymph nodes from vaccinees were compared to counts from unimmunized animals by using the nonparametric Mann-Whitney test. Total IgG1 and IgG2 antibody responses in vaccinated sheep were compared by the student *t* test.

RESULTS

***aroQ* mutants of *C. pseudotuberculosis* elicit less severe site reactions.** The purpose of this study was to compare the levels of virulence of *aroQ* and *pld* mutants of *C. pseudotuberculosis* and assess their efficacy as live vaccines against CLA in sheep. An ovine model of *C. pseudotuberculosis* infection, established previously (10), facilitated this comparison. This model allows determinations of a strain's virulence, based on (i) lymph node colonization/abscessation and immunization site reactogenicity and (ii) its capacity to elicit a protective immune response, based on clearance of wild-type challenge bacteria from a distal draining lymph node.

Semiquantitative observations of reactogenicity at the vaccination site on day 9 postvaccination indicated that site reactions in sheep vaccinated with 10⁶ CFU of CS100 or CS200 were less severe than those in sheep administered 10⁶ CFU of C231 or TB521, respectively (Fig. 1). Sheep administered TB521 at a comparable dose had site reactions which were marginally less severe, but resolved more quickly, than those observed in sheep administered C231. Site reactions typically resolved by day 21 postinjection in all sheep except those administered C231, which persisted till day 28 in some animals.

Humoral immune responses following primary *C. pseudotuberculosis* vaccination. The humoral immune response following vaccination with *C. pseudotuberculosis* strains was assessed at day 38 postvaccination. The results indicated that following vaccination, all sheep developed IgG1 and IgG2 antibody responses specific for *C. pseudotuberculosis* cell-associated antigens (Fig. 2A). The magnitude of the antibody response to cell-associated antigens was, however, lower than the response to antigens isolated from *C. pseudotuberculosis* culture supernatants, of which a major protein component is PLD (Fig. 2B).

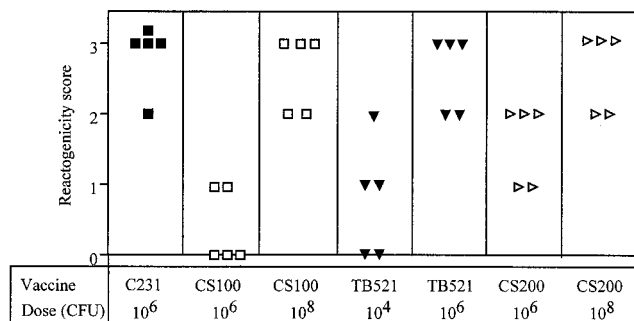


FIG. 1. Clinical scores of adverse reactions occurring 9 days postvaccination at the injection site of sheep vaccinated with *C. pseudotuberculosis* C231, the *pld* mutant TB521, or the *aroQ* mutant CS100 or CS200. Site reactions were scored semiquantitatively, using the following criteria: 0, no significant adverse reaction; 1, >0.5-cm-diameter defined nodule but <1.5 cm with no pustulence evident; 2, >1.5-cm-diameter defined nodule but <3 cm with no pustulence evident; 3, >3-cm-diameter defined nodule with surrounding swelling and/or soft pustulant head.

All vaccine strains elicited antibodies to culture supernatant antigens, with a bias toward the detection of IgG2 over IgG1 (Fig. 2B). Since the ratio of IgG2 to IgG1 did not change significantly between vaccination groups, we believe the differences observed are attributable to the different binding affinities of the IgG1 and IgG2 antibody conjugates. The magnitude of the antibody response to *C. pseudotuberculosis* antigens was vaccine dependent, however, with some vaccine strains inducing significantly higher total antibody titers. The sum of the IgG1 and IgG2 antibody titers to culture supernatant antigens was significantly lower ($P < 0.05$) in sheep vaccinated with 10⁶ CFU of CS100 than in sheep vaccinated with an equivalent number of the parental strain, C231 (Fig. 2B). Similarly, the sum of the antibody titer from sheep vaccinated with 10⁶ CFU of CS200 was significantly lower ($P < 0.05$) than that observed from sheep vaccinated with 10⁶ CFU of its parental strain, TB521 (Fig. 2B). Despite these vaccine-dependent differences in antibody levels, at the time of challenge, all vaccinated sheep had serum antibodies to *C. pseudotuberculosis* culture supernatant and cell-associated antigens.

Cellular immune responses to primary *C. pseudotuberculosis* infections. Cellular immune responses to *C. pseudotuberculosis* were assessed in groups of sheep which received the highest dose of each vaccine strain. The detection of IFN- γ in plasma of antigen-stimulated whole-blood cultures was used as an indicator of antigen-specific cellular immune responses. On day 14 postvaccination, only sheep vaccinated with 10⁶ CFU of C231 or 10⁶ CFU of TB521 had circulating lymphocytes which produced IFN- γ upon antigen stimulation in vitro (Fig. 3). IFN- γ was not detected in the plasma of stimulated blood cultures on day 7 postvaccination (data not shown). Stimulated blood cultures from sheep vaccinated with either CS100 or CS200 did not produce detectable IFN- γ at any time point postvaccination.

Clinical findings at necropsy. In situ dissection and qualitative observation of different lymph nodes and organs in each animal indicated there were vaccine-dependent differences in the degree of abscessation resulting from *C. pseudotuberculosis* challenge (Table 1). Four of five unvaccinated sheep challenged with C231 displayed clinical signs of CLA. Indeed, in three of these animals, abscesses extended to lymph nodes other than the right popliteal. Despite evidence of infection by the challenge strain in sheep vaccinated with 10⁸ CFU of CS100 or CS200, these animals displayed less severe clinical

symptoms of CLA compared to unvaccinated controls. Thus, while there was abscessation in the right popliteal lymph nodes of some vaccinated animals, lower numbers of other lymph nodes were affected than in unvaccinated sheep. Conversely, with regard to popliteal lymph node abscessation, sheep vaccinated with 10⁶ CFU of CS100 or CS200 appeared as susceptible as unimmunized animals. Without exception, sheep vaccinated with 10⁶ CFU of TB521 were free from CLA caused by the wild-type challenge strain. One animal in this group did, however, have an abscess in the left popliteal lymph node that was attributed to colonization of the vaccine strain. Two of five sheep vaccinated with 10⁴ CFU of TB521 displayed abscessation which was restricted to the right popliteal lymph node. Sheep vaccinated and challenged with C231 had more suppurative lesions in the left popliteal lymph node (three of five sheep) than the right popliteal lymph node (two of five sheep). Correspondingly, there were more sheep with abscesses in the left iliofemoral lymph node (four of five sheep) than in the right iliofemoral node (none of five sheep).

Persistence of *C. pseudotuberculosis* vaccine strains. At necropsy, the left (draining vaccination site) and right (draining

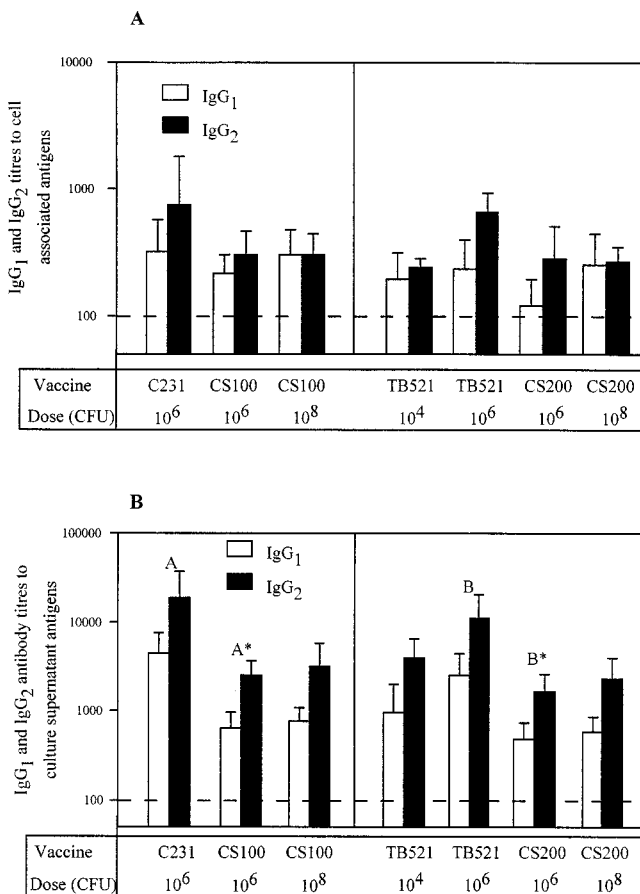


FIG. 2. Mean (plus standard deviation) IgG1 and IgG2 antibody titers specific for *C. pseudotuberculosis* soluble whole-cell lysate antigens (A) and culture supernatant antigens (B) at day 38 postvaccination. There was a significant difference ($P < 0.05$) in the sum of the antibody responses to secreted proteins in sheep vaccinated with 10⁶ CFU of C231 compared to sheep vaccinated with 10⁶ CFU of CS100 (denoted by A and A*, respectively). Similarly, there was a significant difference ($P < 0.05$) in the sum of the antibody responses in sheep vaccinated with 10⁶ CFU of TB521 compared to sheep vaccinated with 10⁶ CFU of CS200 (denoted by B and B*, respectively). The dashed line represents the limit of antibody detection.

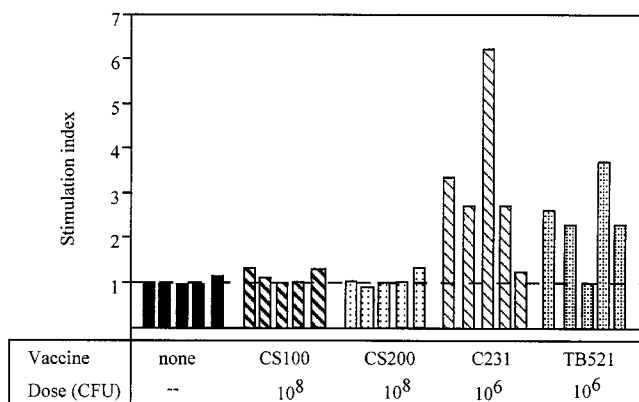


FIG. 3. Stimulation indices representing *C. pseudotuberculosis* antigen-specific IFN- γ release from stimulated whole-blood isolated from individual sheep 14 days postvaccination. Blood from uninfected control sheep did not produce IFN- γ following antigen stimulation.

challenge site) hind popliteal lymph nodes were aseptically removed from all sheep and processed for quantitative bacterial culture of the vaccine strain (left) and the challenge strain (right) (Fig. 4). Left popliteal lymph nodes from sheep infected with CS100 and CS200 were sterile and contained no abscesses. This result indicated that at the doses given, CS100 and CS200 were either unable to colonize or unable to persist in the left popliteal lymph nodes for 76 days. Conversely, four of five sheep vaccinated with 10⁶ CFU of TB521 harbored between 10² and 10⁴ bacteria in their left popliteal lymph nodes (Fig. 4). Indeed, there was evidence of abscessation in one of these lymph nodes from which TB521 was isolated (Table 1). All randomly selected colonies isolated from this node were nonhemolytic on blood plates, suggesting that abscessation resulted from TB521 colonization. Four of five left popliteal lymph nodes isolated from sheep vaccinated with C231 harbored significant numbers of bacteria (range, 10² to 10⁷ CFU) (Fig. 4). Three of these nodes contained abscesses (Table 1).

Colonization by the wild-type challenge strain. The quantitative observations made regarding the number of challenge bacteria in the right popliteal lymph node of each animal partially reflected the number of abscesses found at necropsy.

Enumeration of the number of wild-type challenge bacteria isolated from the right popliteal lymph nodes of unvaccinated sheep indicated that these animals were highly susceptible to infection (Fig. 4). Low numbers of C231 were isolated from the left popliteal lymph node of one unvaccinated animal, which also had the highest bacterial load in the right lymph node. The presence of challenge bacteria in the left popliteal lymph node may have arisen as a result of systemic spread of the organism, since the right iliofemoral lymph node of this animal was also severely abscessed. Importantly, the isolation of C231 in all unimmunized sheep confirmed the infectious nature of the challenge inoculum. Sheep vaccinated with CS100 or CS200, irrespective of the dose, also harbored high numbers of wild-type bacteria in the right popliteal lymph nodes (Fig. 4). This result indicated that CS100 and CS200, when used as vaccines, were unable to elicit an immune response that prevented infection. One animal in the group vaccinated with 10⁸ CFU of CS200 also carried C231 challenge bacteria in the left popliteal lymph node. This animal also had the highest bacterial counts in the right popliteal lymph node, which was correspondingly severely abscessed. Given that no other lymph nodes were visibly abscessed in this animal, contamination during lymph node collection cannot be excluded.

In sheep vaccinated with 10⁶ CFU of TB521, there was a significant difference ($P < 0.01$) in the median number of challenge bacteria recovered from the right popliteal lymph node compared to unimmunized control animals (Fig. 4). There was no evidence of abscessation in the right popliteal lymph nodes of TB521-vaccinated animals (Table 1). Sheep vaccinated with 10⁶ CFU of C231 and subsequently challenged with the same strain also harbored significantly fewer ($P < 0.05$) challenge bacteria (between 10 and 10⁴ CFU) in the right popliteal lymph nodes compared to unimmunized control animals (Fig. 4). Despite the significant reduction in the number of challenge bacteria in these nodes, two of five lymph nodes contained abscesses (Table 1).

DISCUSSION

We have previously reported that *aroQ* mutants of *C. pseudotuberculosis* are highly attenuated in a BALB/c mouse model of infection yet, at the appropriate dose, could elicit an immune response that protected mice from homologous challenge (24). Our results for sheep, a natural host for *C. pseudo-*

TABLE 1. Number of sheep in each vaccine group ($n = 5$) with abscesses in specific lymph nodes or organs at necropsy (day 76) from which *C. pseudotuberculosis* could be recovered^a

Lymph node or organ affected	No. of sheep in each vaccine group with abscesses (by location)						
	CS100 (10 ^{8b})	CS200 (10 ⁸)	TB521 (10 ⁶)	C231 (10 ⁶)	CS100 (10 ⁶)	CS200 (10 ⁶)	TB521 (10 ⁴)
Lymph node							
Left popliteal	0	0	1 ^c	3	0	0	0
Right popliteal	3	2	0	2	5	4	2
Left iliofemoral	0	0	0	4	0	0	0
Right iliofemoral	1	0	0	0	0	0	0
Right medial iliac	0	0	0	0	1	0	0
Left superficial cervical	0	0	0	0	0	0	0
Right superficial inguinal	0	0	0	0	0	0	0
Lung	0	0	0	0	1	0	0
Kidney	0	0	0	1	0	0	0

^a With one exception, wild-type *C. pseudotuberculosis* was recovered from all abscesses.

^b Dose (CFU).

^c Abscess from which TB521 was recovered.

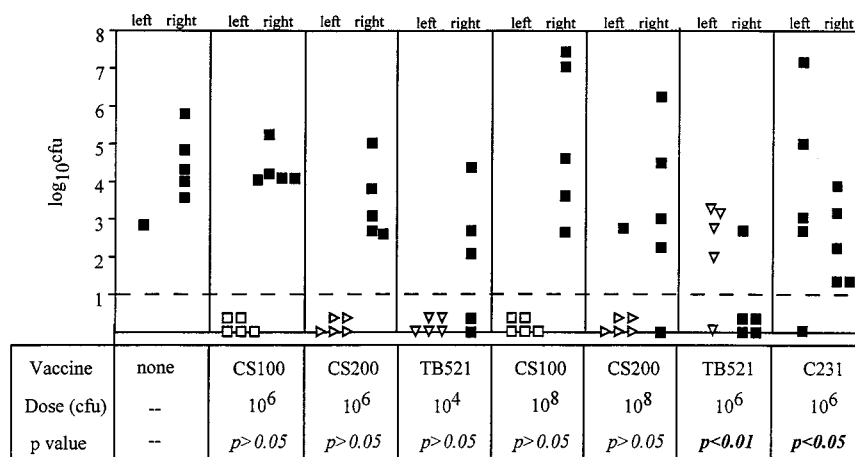


FIG. 4. Isolation of *C. pseudotuberculosis* mutants and wild-type bacteria from left (draining the vaccination site) and right (draining the challenge site) popliteal lymph nodes from individual sheep at necropsy (day 76). Bacteria isolated from lymph nodes were identified as described in Materials and Methods. Symbols for mutant and wild-type bacteria: □, CS100; ▽, CS200; ▽, TB521; ■, C231. There was a significant reduction in the median number of wild-type bacteria (■) recovered from the right popliteal lymph nodes of sheep vaccinated with 10⁶ CFU of C231 ($P < 0.05$) or TB521 ($P < 0.01$) compared to lymph nodes from naive controls. The dashed line represents the limit of detection.

tuberculosis infections, support the observation that these *aroQ* strains are indeed attenuated. However, at the doses used for vaccination in the current trial, these strains did not elicit immune responses which protected sheep from CLA.

The observation that the degree of vaccination site reactivity in sheep given 10⁶ CFU of CS100 or CS200 was less severe than that found in sheep given 10⁶ CFU of either C231 or TB521 is consistent with the hypothesis that *aroQ* mutants have a reduced capacity to multiply in vivo. This hypothesis is supported by the observation that the left popliteal lymph nodes of sheep immunized with the *aroQ* mutants were sterile at necropsy. This finding suggests that *aroQ* mutants are either unable to persist in or unable to colonize this draining lymph node. In direct contrast, in four of five sheep vaccinated with 10⁶ CFU of the *pld* mutant, TB521, nonhemolytic bacteria could be recovered from the left popliteal lymph node, which drains the vaccination site. Indeed, the left popliteal lymph node from one of these sheep displayed abscessation that was attributed to colonization by TB521. In comparison to sheep vaccinated with C231, however, animals immunized with TB521 had reduced vaccination site reactivity, lower vaccine strain colonization of the left popliteal lymph node, and a concomitant reduction in the number of vaccination-induced abscesses. These observations confirm that mutation of the gene encoding the PLD exotoxin (His20→Ser20) is sufficient to significantly attenuate the bacterium in its natural host. *C. pseudotuberculosis* mutants either with deletions in the *pld* gene (10) or with histidine-to-tyrosine substitutions at position 20 of the mature PLD protein (23) have previously been shown to be attenuated.

While all sheep infected with *C. pseudotuberculosis* strains developed IgG antibodies to both cell-associated and culture supernatant antigens, there were some significant differences in the magnitude of the responses. Despite different vaccine doses, sheep infected with 10⁶ CFU of C231 or TB521 had significantly higher antibody titers to culture supernatant antigens compared to sheep vaccinated with 10⁸ CFU of CS100 or CS200, respectively. The detection of IFN- γ in the plasma of stimulated whole blood isolated from sheep vaccinated with 10⁶ CFU of TB521 or C231 suggests that these strains also elicit cellular immune responses. The inability to detect IFN- γ from sheep vaccinated with CS100 or CS200 suggests either

that these strains are relatively poor stimulators of IFN- γ -secreting cells or that the kinetic of the acquired immune response is different. The capacity of TB521 and C231 to elicit IFN- γ -secreting lymphocytes in whole blood of vaccinees correlated with a reduction in the number of challenge bacteria in the right popliteal lymph node. Thus, the induction of an adequate Th1-type T-cell response, characterized by IFN- γ production, may be an essential component in the induction of acquired resistance to *C. pseudotuberculosis* infection by live attenuated vaccines. Previous studies of mice (24) and sheep (27) support this contention. Furthermore, the importance of Th1-type immune responses in acquired resistance to other facultative intracellular bacterial pathogens is well characterized (14).

While Th1-type cellular immune responses are likely to be an important component in acquired resistance to *C. pseudotuberculosis*, humoral immune responses, particularly to antigens found in culture supernatants, have also been suggested to mediate immunity. In Australia, current commercial vaccines against CLA consist of inactivated *C. pseudotuberculosis* culture supernatant antigens, of which PLD is a component (3–5, 11). It has been hypothesized that the presence of anti-PLD antibodies at the time of *C. pseudotuberculosis* challenge could abolish toxin induced vascular permeability and thus limit dissemination of the pathogen (12). Evidence that immune responses to PLD can mediate immunity has been shown in sheep studies using chromatographically purified PLD as a subunit vaccine (4). However, a correlation has not been established in individual sheep between the magnitude of the antibody response to PLD and protection from CLA (3). Another protein secreted by *C. pseudotuberculosis*, a 40-kDa serine protease, has also been used as a subunit vaccine to elicit protective immune responses in sheep (28, 29). The presence of antibodies to the 40-kDa protein did not correlate with protection from challenge, however, leading the authors to suggest that cellular immune responses mediated protection (28).

Despite the equivocal role of antibodies to proteins secreted by *C. pseudotuberculosis* in protective immunity, the magnitude of the mean antibody response to culture supernatant antigens by sheep immunized with C231 or TB521, in this trial, correlated with fewer challenge bacteria in the right popliteal lymph node. Clearly though, the mere presence of these antibodies did

not prevent infection of the lymph node draining the challenge site, since CS100- and CS200-vaccinated sheep, while having antibodies to culture supernatant antigens, had significant numbers of challenge bacteria in their right popliteal lymph nodes.

While most sheep immunized with the *aroQ* mutant CS100 or CS200 harbored high numbers of challenge bacteria and also some abscesses in their right popliteal lymph nodes, the total number of lymph nodes displaying clinical signs of CLA in these sheep appeared to be lower than in unvaccinated control animals. Thus, the immune responses induced by the *aroQ* mutants used as vaccines did not protect sheep from infection but did appear to reduce the clinical severity of disease resulting from wild-type challenge. We hypothesize that the presence of antibodies to *C. pseudotuberculosis* antigens at the time of challenge may help limit the systemic dissemination of the pathogen to other lymph nodes. In contrast, the immune response elicited by administration of 10^6 CFU of TB521 prevented colonization with the wild-type challenge strain. Interestingly though, despite the apparent immune status of these animals, a majority of vaccinees were unable to clear the vaccine strain from the lymph node draining the immunization site. We hypothesize that this is due to the inaccessible location of the bacteria within granulomas. Indeed, a key process in the induction of a protective immune response may be the formation of microscopic pyogenic granulomas, which, by helping to prevent bacterial dissemination, allow the host to mount an effective, T-cell-mediated immune response (22). Conversely however, these granulomas allow persistence of the pathogen by excluding immunological effectors.

In this study, we have assessed the virulence of *aroQ* and *pld* mutants of *C. pseudotuberculosis* in sheep and simultaneously their capacity to act as vaccines against homologous challenge. The *aroQ* mutants did not elicit a protective immune response against CLA. These mutants may be overly attenuated with respect to in vivo growth to elicit the required response. In contrast, at the appropriate dose, the *pld* mutant TB521 elicited a protective immune response, and this was correlated with persistence of the vaccine strain, the induction of IFN- γ -secreting lymphocytes, and relatively high levels of antibodies to culture supernatant antigens. Importantly, vaccination with TB521 did not cause overt CLA in vaccinees. As a result, TB521, like the previously constructed Δpld mutant (Toxminus) (10, 11), holds promise as a live vaccine vector for the induction of cellular and humoral immune responses to heterologous antigens expressed by the bacterium.

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