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 reactivity. 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 attenuation 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.
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 antibody titer were administered C231, which persisted till day 28 in some animals. 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 fibrinolysin, ultrafiltrable, 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 mechanical 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 mutants was made by 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, TB251, 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 aroQ 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 reactivity 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 reactivity at the vaccination site on day 9 postvaccination indicated that site reactions in sheep vaccinated with 10^6 CFU of CS100 or CS200 were less severe than those in sheep administered 10^9 CFU of C231 or TB251, respectively (Fig. 1). Sheep administered TB251 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).
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 10^6 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 10^6 CFU of CS200 was significantly lower (P < 0.05) than that observed from sheep vaccinated with 10^6 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 10^6 CFU of C231 or 10^8 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 10^6 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 10^6 CFU of CS100 or CS200 appeared as susceptible as unimmunized animals. Without exception, sheep vaccinated with 10^6 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 10^6 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
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 $10^8$ CFU of TB521 harbored between $10^3$ and $10^5$ 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, $10^2$ to $10^7$ 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 $10^8$ 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 $10^6$ 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 $10^6$ CFU of C231 and subsequently challenged with the same strain also harbored significantly fewer ($P < 0.05$) challenge bacteria (between 10 and $10^4$ 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. pseudotuberculosis* 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 $10^8$ 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.

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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 10^6 CFU of CS100 or CS200 was less severe than that found in sheep given 10^9 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 10^6 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 10^6 CFU of C231 or TB521 had significantly higher antibody titters to culture supernatant antigens compared to sheep vaccinated with 10^8 CFU of CS100 or CS200, respectively. The detection of IFN-γ in the plasma of stimulated whole blood isolated from sheep vaccinated with 10^6 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 10⁶ 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 Apld mutant (Tosmini) (10, 11), holds promise as a live vaccine vector for the homologous and heterologous antigens expressed by the bacterium.

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