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Protection of Sheep against Caseous Lymphadenitis by Use of a Single Oral Dose of Live Recombinant *Corynebacterium pseudotuberculosis*

ADRIAN L. M. HODGSON,* MARY TACHEDJIAN, LEIGH A. CORNER, AND ANTHONY J. RADFORD†

CSIRO Division of Animal Health, Animal Health Research Laboratory, Parkville, Victoria 3052, Australia

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An inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D (PLD) gene was constructed and expressed in a PLD-negative strain (designated Toxminus) of *C. pseudotuberculosis*. Antibody responses specific to Toxminus and both Toxminus and PLD proteins were detected in sheep following oral administration of Toxminus or Toxminus expressing the PLD toxoid, respectively. However, only those sheep vaccinated with Toxminus expressing PLD toxoid were protected against wild-type challenge. These results confirm the importance of PLD as a protective antigen and demonstrate both the potential for developing an oral caseous lymphadenitis vaccine and *C. pseudotuberculosis* Toxminus as a live vaccine vector.

Caseous lymphadenitis (CLA) is a chronic, granulomatous disease of sheep and goats caused by the gram-positive bacterium *Corynebacterium pseudotuberculosis*. The most common mode of entry of *C. pseudotuberculosis* into the host is believed to be via skin wounds (e.g., shearing cuts) or by aerosol infection of the lungs (21). Consequently, CLA is characterized primarily by the formation of abscesses within the superficial lymph nodes in addition to those draining the lungs (2, 21). CLA is a major disease of Australian sheep; for example, the average prevalence in Western Australian flocks is 45% (21), and the overall cost of CLA to the Australian sheep industry has been estimated to be $10 to 15 million (Australian dollars) from lost wool production and $10 million (Australian dollars) for the inspection and subsequent trimming of abscesses from carcasses, particularly in export abattoirs (21, 22).

C. pseudotuberculosis secretes a phospholipase D (PLD) exotoxin (5, 11, 26, 27) that has for some years been implicated as a major virulence factor (2). It is thought that PLD enhances the virulence of *C. pseudotuberculosis* by limiting bacterial opsonization, increasing vascular permeability and thus bacterial dissemination, enabling the bacteria to escape from neutrophils, and impairing neutrophil chemotaxis toward the site of infection (31). Vaccines formulated by use of partially or fully purified PLD toxoid offer significant protection against CLA to sheep (3, 6, 7), and deletion of the PLD gene from *C. pseudotuberculosis* (creating the strain designated Toxminus) attenuated this pathogen by at least 3 log doses (12), thus further substantiating the importance of PLD in the infection process. Although we recognize that vaccines based upon the *C. pseudotuberculosis* PLD (e.g., Glanvac 6; CSL Ltd.) protect sheep against CLA, there is general interest in developing single-dose vaccines. Here we described protection of sheep against CLA by use of a single oral dose of live *C. pseudotuberculosis* Toxminus (12) expressing a genetically inactivated version of the PLD gene. The relevance of these results with respect to our goal of developing *C. pseudotuberculosis* Toxminus as a live vaccine vector (12, 13) will also be discussed.

* Corresponding author. Mailing address: CSIRO Division of Animal Health, Animal Health Research Laboratory, Private Bag No. 1, Parkville, Victoria 3052, Australia. Phone: (03) 342 9784. Fax: (03) 347 4042.

† Present address: AMRAD Corporation, Kew, Victoria 3101, Australia.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Escherichia coli* DH5-α (Bethesda Research Laboratories) was the host for all plasmids and clones. The *C. pseudotuberculosis* PLD gene (10) cloned into the *Eco*RI-*Hin*dIII site of pTZ18U (Promega) produced pTB351. The His-20→Tyr mutant variant of the PLD gene in pTZ18U was called pTB270 and, when subcloned as a *Sal*I fragment into the *Sal*I site of pEP2 (24), produced pTB253. *E. coli* transformants were grown on Luria medium supplemented with either 50 μg of ampicillin (Sigma) per ml or 50 μg of kanamycin sulfate (Sigma) per ml. *E. coli* CJ236 (Bio-Rad) transformed with pTB351 was grown on 2× YT agar (25) supplemented with 30 μg of chloramphenicol (Sigma) per ml and 50 μg of ampicillin per ml. *C. pseudotuberculosis* Toxminus was transformed by electroporation (12) and grown at 37°C on brain heart infusion (BHI; Difco) agar supplemented with 50 μg of kanamycin sulfate per ml. To detect PLD activity from transformed *E. coli*, *C. pseudotuberculosis* Toxminus, or the wild-type strain of *C. pseudotuberculosis*, bacteria were grown on sheep blood agar plates (12). For vaccine preparation, Toxminus and Toxminus containing pTB253 were grown with vigorous shaking for 16 h in BHI broth and BHI broth supplemented with 50 μg of kanamycin sulfate per ml, respectively. Cultures were subcultured 1:5 into fresh medium and grown for an additional 16 h, and 40-ml doses (equivalent to 10^{10} CFU) were pelleted, washed in sterile phosphate-buffered saline (PBS), and then resuspended in 10 ml of sterile PBS. To ascertain the number of CFU per vaccine dose, viable counts were performed on a sample of each vaccine by using the appropriate media.

DNA techniques. Unless detailed, all DNA manipulations were conducted by use of standard protocols (25). The nucleotide sequence of the mutated PLD gene was determined from double-stranded templates by use of a T7 DNA polymerase sequencing kit (AMRAD Pharmacia Biotech) and an oligonucleotide (5′ CTATTTATCGAAACTTGG 3′) designed from the PLD sequence 138 bp upstream of His-20. DNA sequence data were stored, assembled, and analyzed by using DNASIS software (AMRAD Pharmacia Biotech).

Site-directed mutagenesis. A 2.1-kb *Eco*RI-*Hin*dIII fragment containing the entire transcriptional unit of the PLD gene was cloned into pTZ18U (pTB351) and transformed into *E. coli* CJ236. A single-stranded DNA template was prepared, and site-directed mutagenesis was performed with a Muta-Gene Phagemid in vitro mutagenesis kit (version 2) as described in the manufacturer's specifications (Bio-Rad). The complementary-strand mutagenic oligonucleotide primer was designed to convert His-20 to Tyr by the underlined two-base substitution (both C to T): 5′ AGTGGTTAAAACGCG ATA GGCAATCGCATAGAC 3′. The Tyr codon (TAT) was chosen to conform with the PLD gene codon bias. The mutagenesis mix was electroporated into *E. coli* DH5-α and transformants were patched onto selective sheep blood agar plates. Plasmid DNA was isolated from colonies showing no hemolysis, digested with *Sal*I, and compared with the wild-type PLD gene on agarose gels.

In vitro transcription and translation. Plasmid DNA from *E. coli* clones harboring the mutated PLD gene that showed the same restriction pattern as the wild-type gene was transcribed and translated in vitro by use of an S30-coupled in vitro transcription-translation system (Promega) as described in the manufacturer's protocol. To eliminate the 31.5-kDa vector-encoded β-lactamase protein, plasmid DNA was digested with *Sal*I, *Sca*I, and *Dra*I before the in vitro reactions were performed. ^{35}S-labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Amplify (Amersham)-enhanced autoradiography. Gels were dried with a Bio-Rad slab drier. For molecular weight markers, a mixture of ^{14}C-methylated proteins (Amersham) was used.

Western blot analysis. Western blot (immunoblot) analysis was performed by the method of Sambrook et al. (25). PLD-specific antiserum was raised in sheep following one subcutaneous vaccination with PLD purified as a fusion with glutathione *S*-transferase (AMRAD Pharmacia Biotech) formulated with Freund's complete adjuvant and then two booster doses, 3 weeks apart, formulated in Freund's incomplete adjuvant. The antiserum was used at a dilution of 1:50, and it was preadsorbed with 1 ml of total Toxminus protein prepared by sonicating Toxminus cells. The conjugate, used at a dilution of 1:1,000, was mouse monoclonal antibody specific for ovine immunoglobulin G (IgG) conjugated to horseradish peroxidase (Silenus) with tetramethylbenzidine (17) as the substrate. Toxminus-expressing PLD mutants were grown for 24 h in BHI agar containing kanamycin and sonicated, and the total protein concentration was measured by the BCA assay (8). Samples containing 70 μg of total protein or 20 μl of culture supernatant were analyzed by SDS-PAGE, and proteins were transferred to nitrocellulose (Bio-Rad) by using a Bio-Rad Transblot Cell as described in the manufacturer's specifications.

Sheep vaccination and challenge trial. Each of nine sheep was orally drenched with either 10 ml of PBS containing 10^{10} CFU of Toxminus or Toxminus expressing the His-20→Tyr PLD toxoid (pTB253). An additional nine unvaccinated sheep served as controls. Sheep were challenged 6 weeks postvaccination with 10^6 CFU of wild-type *C. pseudotuberculosis* suspended in 500 μl of sterile PBS. To direct the challenge toward the popliteal lymph node, the bacteria were administered subcutaneously just above the coronet of the left hind lateral claw. All sheep were necropsied 6 weeks postchallenge, and the challenge site, major lymph nodes, and organs were examined for abscesses. The left popliteal lymph nodes, in addition to all abscessed tissues, were collected for microbiological examination.

Assessment of fecal shedding. Fecal samples were collected from all sheep every day for 7 days after oral drenching. Feces (1 g) were added to 20 ml of sterile PBS containing about 20 0.5-cm-diameter glass beads. The mix was vortexed for 1 to 2 min and allowed to settle for 30 min. The supernatant (400 μl) was spread onto BHI agar supplemented with 150 μg of erythromycin (Boehringer GmbH, Mannheim, Germany) per ml. Plates were held at 37°C for 2 days and then examined for colonies characteristic of *C. pseudotuberculosis* Toxminus.

Antibody assays. All sheep were bled prior to vaccination and then at weekly intervals for the duration of the experiment. Antibody responses to *C. pseudotuberculosis* and the PLD toxoid were measured by an indirect enzyme-linked immunosorbent assay (ELISA) with 0.5 μg of sonicated *C. pseudotuberculosis* Toxminus protein preparation or purified glutathione *S*-transferase–PLD, respectively, per well. Titers were determined as the reciprocal of the dilution that gave half the maximum optical density obtained. Mean titers are expressed as geometric means. IgG1 and IgG2 specific to Toxminus protein were independently measured in an ELISA with mouse monoclonal antibodies (Ken Beh, CSIRO Division of Animal Health, Glebe, Sydney, Australia) specific for the particular isotype. Titers were calculated as described above. Five serum samples were selected at random from the 10 samples taken from sheep 2 weeks following oral vaccination with Toxminus bacteria. Five serum samples from our previous study (12), taken 2 weeks following subcutaneous vaccination with 10^7 Toxminus bacteria, were also used in the isotype analysis.

RESULTS

Analysis of PLD mutants. Following site-specific mutagenesis of the PLD gene, the nonhemolytic phenotype in *E. coli* transformants was obtained at a frequency of 50%. Two clones selected for in vitro transcription and translation analysis produced a 31-kDa protein and had the desired substitution of His-20 (CAC)→Tyr (TAT) as assessed by DNA sequencing. This result indicated that the mutagenesis had been successful and supported the previous finding (9) that substituting His-20 for Tyr inactivated the *C. pseudotuberculosis* PLD.

Expression of PLD toxoid in Toxminus. To assess expression of the PLD toxoid molecule in Toxminus, the mutated PLD gene was cloned into pEP2 (pTB253) and electroporated into Toxminus. Western blot analysis of total cell and culture supernatant proteins showed that the 31-kDa PLD toxoid was recognized by PLD-specific sheep antiserum. However, in contrast to the parent molecule, secretion of the PLD toxoid could not be detected by Coomassie blue staining (Fig. 1A). Western blot analysis revealed that secretion of the PLD toxoid was greatly reduced but not eliminated (Fig. 1B). Substituting His-20 for Tyr was therefore found to reduce secretion of the PLD molecule.

Antibody responses. To determine the antibody response to both Toxminus and the PLD toxoid expressed within Toxminus following a single oral dose of live bacteria, sheep were bled weekly and specific antibody responses were measured by an ELISA (Fig. 2). Strong antibody responses to Toxminus antigen developed rapidly and peaked 2 weeks postvaccination (Fig. 2A). Wild-type *C. pseudotuberculosis* challenge at week 6 produced an anamnestic response that peaked 2 weeks postchallenge. Unvaccinated controls responded to challenge by mounting a Toxminus-specific antibody response that peaked 2 weeks following challenge. PLD-specific responses were detected only in sheep vaccinated with Toxminus expressing PLD toxoid, and as observed with the Toxminus-specific response, an anamnestic response occurred following challenge at week

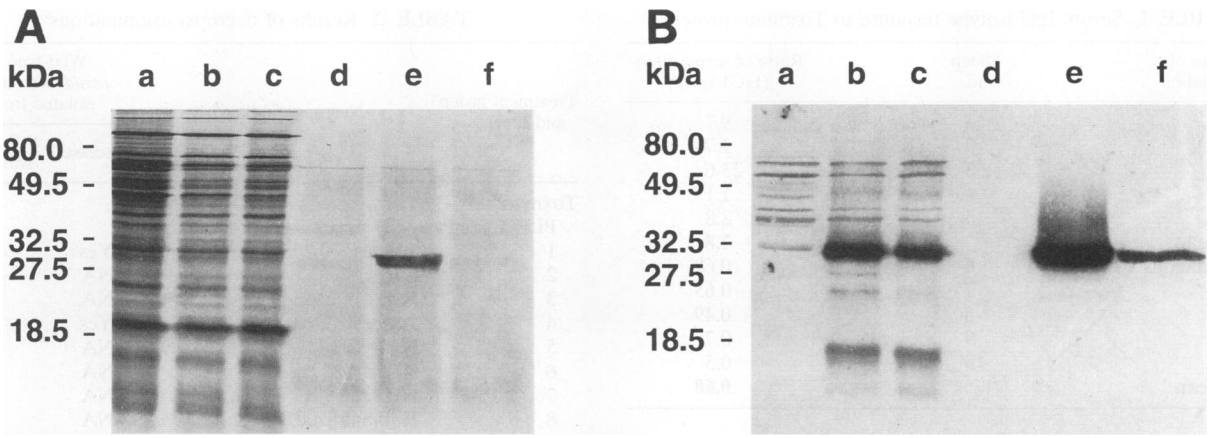

FIG. 1. Expression and secretion of PLD toxoid. (A) Coomassie blue-stained polyacrylamide gel. Lanes: a, Toxminus sonicate; b, sonicate of Toxminus expressing the PLD gene from the multicopy plasmid pEP2; c, sonicate of Toxminus expressing the His-20→Tyr variant of PLD from pEP2; d, Toxminus culture supernatant; e, culture supernatant from Toxminus expressing PLD from pEP2; f, culture supernatant from Toxminus expressing the His-20→Tyr variant of PLD. (B) Same as panel A but Western blotted with PLD-specific antiserum. Molecular mass markers are shown to the left of the gels.

6 (Fig. 2B). Unvaccinated controls and sheep vaccinated with Toxminus alone mounted PLD-specific antibody responses following challenge; however, these were weaker and occurred later than responses to Toxminus antigen, possibly reflecting differences in the relative amounts of antigen in the challenging bacteria. These results showed that antibody responses can be stimulated in sheep following oral vaccination; it should be noted that no attempts were made to protect the bacteria from the potential damaging effects of low pH within the abomasum.

The antibody response to Toxminus protein elicited following oral vaccination of sheep was predominately IgG1, whereas the IgG2 isotype was dominant in sheep vaccinated subcutaneously (Table 1). This trend was confirmed when the ELISA was repeated with the same serum samples (data not shown).

Fecal shedding. To investigate the possibility for transmission of *C. pseudotuberculosis* Toxminus from sheep to the environment, fecal samples were cultured daily for 7 days postvaccination. Only on the third day postvaccination *C. pseudotuberculosis* Toxminus was found in the feces of one of the sheep that had been dosed with Toxminus expressing the PLD toxoid. This result showed that viable Toxminus was shed only rarely from orally dosed sheep.

Necropsy examination of sheep. The disease CLA is characterized by abscess formation within the lymphatic system (and/or organs) following infection by *C. pseudotuberculosis*. The objective of this study was to determine the degree of protection to sheep against CLA provided by two different oral vaccines. To determine the effect of challenge on vaccinated and unvaccinated sheep, necropsy examinations were conducted 6 weeks postchallenge. All of the major lymph nodes were sectioned, and any abscessed tissue and the draining popliteal lymph nodes were cultured for *C. pseudotuberculosis* (Table 2). The greatly reduced incidence and degree of abscess formation clearly indicated that single-dose oral vaccination of sheep with live *C. pseudotuberculosis* expressing the PLD toxoid provided effective (100%) protection against CLA (Table 2). Overall, clinical signs in sheep vaccinated with Toxminus alone (50% of sheep had CLA) differed little from those observed in the unvaccinated controls (66% of sheep had CLA), indicating that a single oral dose of Toxminus did not provide substantial protection against challenge. Hemolytic *C. pseudotuberculosis* could be isolated from all abscessed tissue and from most of the draining popliteal lymph nodes even in the absence of abscesses (Table 2). These results confirmed

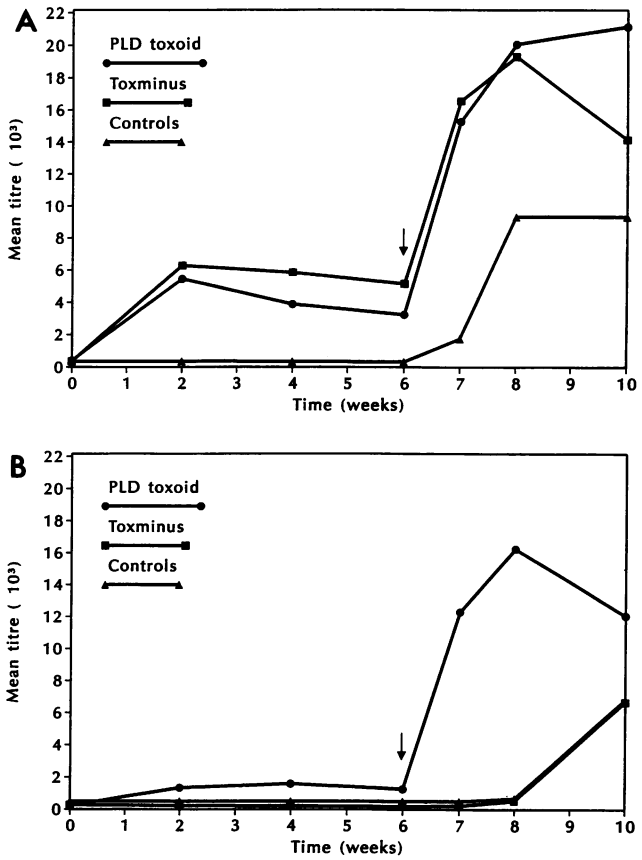

FIG. 2. Serological responses of sheep following oral vaccination with Toxminus or Toxminus expressing PLD toxoid. (A) Groups of nine sheep were vaccinated orally with 10^{10} CFU of either Toxminus or Toxminus expressing PLD toxoid. The sheep were bled at weekly intervals, and the average antibody titer raised against Toxminus protein was measured with an ELISA at the indicated times. The arrow indicates when challenge with virulent *C. pseudotuberculosis* (10^6 CFU) was performed. Control sheep remained unvaccinated. (B) Same as panel A except that the average antibody titer raised against PLD was measured.

TABLE 1. Serum IgG isotype response to Toxminus protein

Route of vaccination	Sheep no.	Ratio of serum titers (IgG1/IgG2)
Oral	1	9.7
	2	1.7
	3	23.6
	4	1.1
	5	2.8
Mean		**7.8**
Subcutaneous	6	0.68
	7	0.65
	8	0.49
	9	0.7
	10	0.5
Mean		**0.60**

that abscess formation was a result of *C. pseudotuberculosis* infection and showed that *C. pseudotuberculosis* can reside within a lymph node even in sheep with no clinical signs of disease.

DISCUSSION

With a view to developing a new vaccine against CLA, we previously deleted the gene for the secreted PLD from the *C. pseudotuberculosis* chromosome and showed that a single subcutaneous vaccination of the attenuated strain, Toxminus, protected sheep against wild-type challenge (12). However, the live attenuated vaccine produced an undesirable abscess at the site of inoculation, albeit transiently (12). In addition to providing a convenient means of administration, oral dosing is one way of avoiding the disadvantages associated with site reactions. We therefore sought to evaluate the use of Toxminus as a live oral CLA vaccine. Since PLD is a major protective antigen (3, 6, 7), a genetically inactivated analog of the PLD gene was derived and expressed in Toxminus for delivery to the ovine immune system. Toxoiding of the PLD gene was accomplished site specifically by mutating PLD His-20 to Tyr, a substitution that had been shown previously to remove enzymatic activity (9). To minimize the likelihood of reversion to the wild type, we deliberately replaced 2 bp to effect the amino acid conversion, and since our site-specific mutation inactivated PLD, this confirmed that His-20 is involved in the active site of the enzyme. In addition to rendering enzymatic activity undetectable, however, the single amino acid substitution greatly reduced the amount of PLD secreted from Toxminus (Fig. 1). This is not unprecedented; secretion of both the *Bacillus subtilis* neutral protease (28) and *Pseudomonas aeruginosa* elastase (14, 16) was removed concomitantly with catalytic activity. However, this is understandable since in both cases enzyme function is required to process the precursor proteins during secretion. In another example, amino acid substitutions within the *B. subtilis* levansucrase also reduced secretion (23). In this case, it was suggested that structural changes in the altered region affected the ability of the molecule to undergo metal-dependent refolding essential for secretion. Since we do not known the mechanisms involved in the secretion process of PLD, we can only speculate as to why the His-20→Tyr substitution within PLD affected export. One possibility is that the structural changes to the putative active site of PLD, which were sufficient to inactivate the molecule, also impacted the processing of the signal peptide, perhaps by masking the cleavage site located between alanine −1 and alanine 1 (11).

TABLE 2. Results of necropsy examinations[a]

Treatment group and sheep no.	Necropsy findings[b]	Wild-type *C. pseudotuberculosis* isolated from[c]:	
		Abscesses	Popliteal lymph node
Toxminus + PLD toxoid			
1	Abscess (0.2 cm) at SOI	Yes	No
2	NIL	NA	Yes
3	NIL	NA	No
4	Abscess (1 cm) at SOI	Yes	Yes
5	NIL	NA	Yes
6	NIL	NA	Yes
7	NIL	NA	Yes
8	Euthanatized	NA	NA
9	Euthanatized	NA	NA
Toxminus			
10	NIL	NA	Yes
11	Multiple lymph duct and popliteal abscesses	Yes	Yes
12	Multiple lymph duct and popliteal abscesses	Yes	Yes
13	Multiple lymph duct and popliteal abscesses	Yes	Yes
14	Abscess (0.5 cm) at SOI	Yes	NC
15	NIL	NA	No
16	Abscess (0.3 cm) at SOI	Yes	Yes
17	Popliteal abscess (1 mm)	Yes	Yes
18	Euthanatized	NA	NA
Unvaccinated			
19	NIL	NA	No
20	Popliteal abscess (1 cm)	Yes	Yes
21	Multiple lymph duct and popliteal abscesses	Yes	Yes
22	Multiple lymph duct and popliteal abscesses	Yes	Yes
23	Multiple lymph duct and popliteal abscesses	Yes	Yes
24	Abscess (1.0 cm) at SOI and multiple lymph duct abscesses	Yes	Yes
25	Abscess (0.1 cm) at SOI	Yes	Yes
26	Abscess (0.1 cm) at SOI	Yes	Yes
27	Abscesses as follows: SOI (1.0 cm), spleen (0.5 cm), lung (1.0 cm), lymph duct (0.5 and 1.0 cm), popliteal lymph node (multiple and 8 by 6 cm)	Yes	Yes

[a] Sheep were orally vaccinated with either Toxminus expressing PLD toxoid or Toxminus alone or they remained unvaccinated. All sheep were then challenged with wild-type *C. pseudotuberculosis*. Sheep were examined at necropsy for abscesses resulting from challenge. The draining popliteal lymph node and all abscesses were collected for microbiological culture.

[b] SOI, site of inoculation; NIL, no abscesses.

[c] Culture for wild-type challenge strain of *C. pseudotuberculosis*, colony morphology, and zones of hemolysis were diagnostic. Two sheep from the Toxminus expressing PLD toxoid group and one from the Toxminus group required euthanasia prior to challenge. Necropsy examinations failed to reveal the cause of morbidity, and there was no evidence of wild-type *C. pseudotuberculosis* infection. NA, not applicable; NC, not collected.

In contrast to subcutaneous vaccination of sheep with Toxminus (12), oral vaccination did not provide significant protection against challenge (Table 2). This cannot be attributed to immunological nonresponsiveness since orally dosed Toxminus stimulated a strong antibody response that was

boosted upon challenge (Fig. 2A). In an attempt to explain this difference, we measured the levels of the IgG1 and IgG2 components of the antibody response. Sheep vaccinated orally produced a higher proportion of IgG1, whereas in those vaccinated subcutaneously, the IgG2 isotype predominated (Table 1). These isotype profiles are consistent with current immunological dogma. Murine T-helper cell responses have recently been divided into two types, TH1 and TH2, on the basis of the cytokines that they secrete (18). TH1 cells secrete, for example, gamma interferon and tend to drive the antibody response toward the IgG2 isotype, whereas TH2 clones secrete, among other cytokines, interleukin 4 and preferentially induce the production of IgG1, IgA, and IgE (19). The introduction of live microorganisms to the mucosa primarily stimulates secretory (IgA) and humoral (IgG) responses (4, 29), whereas subcutaneously, the predominant responses are humoral (IgG) and cellular (e.g., gamma interferon) (1, 20, 30). Consistent with this, in the present experiments, subcutaneous vaccination of sheep with Toxminus bacteria stimulated mainly IgG2, and we also previously detected a gamma interferon response following subcutaneous administration (12). In orally vaccinated sheep, IgG1 predominated, but unfortunately, we have been unable to assess IgA production because of a lack of a sheep anti-IgA antibody. One explanation for the difference in protection between the two routes of vaccination, therefore, is that a TH1 response to Toxminus proteins is required to protect sheep against CLA.

Notwithstanding the above speculation, one result that is clear from this study and which confirms previous observations (6, 7) is that antibodies directed against PLD (Fig. 2B) provide strong protection against CLA (Table 2). In addition to demonstrating the potential for developing an oral single-dose CLA vaccine, an important principle shown here relates to the ability to stimulate humoral responses to a recombinant, vectored protein (PLD toxoid) following oral vaccination. Traditionally, attenuated *Salmonella* strains have been used as the vectors of choice to deliver antigens to the mucosa, and success in stimulating antibody responses specific for foreign antigens has been reported (13). Recently, oral vaccination of guinea pigs with recombinant *Mycobacterium bovis* BCG was found to stimulate IgA, IgG, and cellular responses specific for the foreign protein (15). We have now shown that *C. pseudotuberculosis* Toxminus has potential as a live vaccine vector (12) for both parenteral and oral administration. Further studies are in progress to assess foreign gene expression in this bacterium.

Inherent in the use of live bacterial vaccines and vaccine vectors for oral administration is the issue of transmission of the vaccine to the environment through fecal shedding. In the current study, the Toxminus bacterium was shed, on only 1 day, from only 1 of 18 orally dosed sheep. Since the vaccine doses were 10^{10} CFU, there is the possibility of reducing the potential for shedding even further by determining the minimum dose required to stimulate desired immune responses. Spread to secondary hosts is just one of several issues (10) that will require close consideration before new, live recombinant vaccines and vaccine vectors can be commercialized.

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