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Michel Pépin, Hf Seow, L Corner, Js Rothel, Alm Hodgson, et al.. Cytokine gene expression in sheep following experimental infection with various strains of *Corynebacterium pseudotuberculosis* differing in virulence. *Veterinary Research*, 1997, 28 (2), pp.149-163. hal-00902468

HAL Id: hal-00902468

<https://hal.science/hal-00902468>

Submitted on 11 May 2020

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Cytokine gene expression in sheep following experimental infection with various strains of *Corynebacterium pseudotuberculosis* differing in virulence

M Pépin^{1*}, HF Seow², L Corner², JS Rothel²,
ALM Hodgson², PR Wood²

¹ Laboratoire de pathologie des petits ruminants et des abeilles, CNEVA Sophia-Antipolis, BP 111, 06902 Sophia-Antipolis, France;

² CSIRO, Division of Animal Health, Private Bag 1, Parkville, Victoria 3052, Australia

(Received 23 July 1996; accepted 15 October 1996)

Summary — *Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis in sheep and goats. This disease is characterized by the development of pyogranulomas in the lymph nodes and lung tissue. To measure the cytokine gene expression in *C pseudotuberculosis* lesions, sheep were inoculated with two attenuated strains (Tox⁻ and PLD-t) and a wild-type (WT) strain of *C pseudotuberculosis* and were necropsied at 7 or 28 days post-inoculation. The Tox⁻ strain showed a strong reduction in virulence as assessed by the absence of disseminating lesions in the lymph nodes draining the inoculation site in contrast with the WT strain. The PLD-t strain showed an intermediate reduction in virulence. The two attenuated strains, however, induced the same amount of antibodies and IFN- γ production as the WT strain. Using a semi-quantitative RT-PCR technique, the expression of inflammatory cytokines was found to be higher in the inoculation site, whereas expression of T-cell associated cytokines was more intense in the draining lymph node. On the whole, the infected sheep produced high levels of cytokines in at least one organ on days 7 or 28 post-inoculation. No significant differences in cytokine gene expression were shown between sheep infected with strains differing in virulence. Higher cytokine expression was measured in sheep with pyogranulomas in the draining lymph nodes as compared to those without, especially for interleukin-1 β and interleukin-8. Overall, these results taken together confirmed the attenuation of virulence in Tox⁻ and PLD-t strains of *C pseudotuberculosis* and showed the important role of PLD in disseminating the bacteria from the inoculation site to the draining lymph nodes. The pathogenesis of ovine caseous lymphadenitis was shown to be associated with production of cytokines at the pyo-

* Correspondence and reprints

Tel: (33) 04 92 94 37 00; fax: (33) 04 92 94 37 01; e-mail: vasa20@calvacom.fr

granuloma level, but the local cytokine patterns associated with different courses of infection were not distinguished.

caseous lymphadenitis / sheep / attenuated strain / cytokine / RT-PCR

Résumé — Expression des gènes de cytokines chez des moutons infectés expérimentalement avec des souches de *Corynebacterium pseudotuberculosis* de virulence variée. *Corynebacterium pseudotuberculosis* est l'agent causal de la lymphadénite caséuse chez les ovins et les caprins. Cette maladie est caractérisée par le développement de pyogranulomes dans les nœuds lymphatiques et les poumons. Afin de mesurer l'expression des cytokines produites par les cellules des lésions induites par *C pseudotuberculosis*, des moutons ont été inoculés avec deux souches (Tox- and PLD-t) de virulence atténuée et une souche sauvage (WT) de *C pseudotuberculosis*, puis autopsiés 7 et 28 jours après l'inoculation. La souche Tox- a montré une forte réduction de virulence avec l'absence de lésions dans les ganglions drainant le site d'inoculation en comparaison avec la souche sauvage ; la souche PLD-t a montré une réduction intermédiaire de virulence. En revanche, la production d'anticorps et d'IFN- γ ont été identiques pour les trois souches. Mesurée par une technique semi-quantitative d'amplification en chaîne par polymérase après rétrotranscription (RT-PCR), l'expression des cytokines inflammatoires a été plus intense au site d'inoculation, alors que l'expression des cytokines produites préférentiellement par les lymphocytes T a été plus forte au niveau des ganglions drainants. Au total, les moutons infectés ont produit des niveaux élevés de cytokines dans au moins un organe et à au moins un jour après inoculation (j7 ou j28). Cependant, la différence de virulence des souches n'a pas permis de mettre en évidence une différence dans l'expression des cytokines étudiées. Si la comparaison porte sur la présence ou l'absence de lésion au niveau d'un organe, les animaux avec pyogranulome ont exprimé des niveaux de cytokines plus élevés par rapport aux animaux sans lésion, ceci étant particulièrement vrai pour l'interleukine-1 β et l'interleukine-8. L'ensemble de ces résultats confirme l'atténuation de la virulence des souches Tox- et PLD-t de *C pseudotuberculosis* et démontre le rôle important de l'exotoxine dans la dissémination des bactéries depuis le site d'inoculation jusqu'au ganglion drainant. Le développement des pyogranulomes est associé à la production locale de cytokines, sans qu'il soit possible de distinguer un profil de cytokines en relation avec une différence de virulence des souches de *C pseudotuberculosis*.

lymphadénite caséuse / mouton / souche de virulence atténuée / cytokine / RT-PCR

INTRODUCTION

Caseous lymphadenitis is a common disease in sheep and goats. *Corynebacterium pseudotuberculosis*, a Gram-positive rod, is the causative agent of this bacterial disease, which is characterized by the development of pyogranulomas in the lymph nodes and lungs (Batey, 1986; Pépin et al, 1994b). These pyogranulomas are characterized as focal lesions involving macrophages, lymphocytes and

other cell types. Previous reports have described the kinetics of pyogranuloma formation (Pépin et al, 1991a,b), and the phenotype of cells involved in this lesion during experimental infections of sheep with *C pseudotuberculosis* (Ellis, 1988; Guilloteau et al, 1990; Walker et al, 1991; Pépin et al, 1992, 1994a). In particular, heterogeneity in cell phenotype both for macrophages and lymphocytes has been described (Pépin et al, 1992, 1994a). One of the key functional

parameters determining the outcome of the immune responses to infectious agents is the nature of the cytokines locally produced by the immune cells. The organisation and characterization of the cells in the pyogranuloma underline the importance of the mediators released by stimulated specific T lymphocytes and macrophages as these mediators have been found in various models of chronic infections (Heinzel et al, 1989; Squires et al, 1989; Amiri et al, 1992; Murray et al, 1992; Pirmez et al, 1993). These mediators play a role in the accumulation, organisation and activation of the cells present in granulomas (Elias et al, 1990; Lukacs et al, 1994). For example, in human leprosy, which presents a clinical and immunological spectrum (Modlin et al, 1988), it has been demonstrated that two distinct patterns of cytokine production correlate with the known degree of cell-mediated immunity against the infection. High expression of interleukin-2 (IL-2) and gamma interferon (IFN- γ) mRNAs correlate with the tuberculoid lesions and the resistance to growth of *Mycobacterium leprae*, whereas high levels of expression of IL-4, IL-5, and IL-10 are characteristic of lepromatous lesions harbouring large quantities of organisms (Yamamura et al, 1991).

In the present study, the sheep were experimentally infected with three strains of *C pseudotuberculosis*, which differed in virulence. Mutants of *C pseudotuberculosis* that do not produce the exotoxin, a phospholipase D (PLD), were recently obtained. These mutants were shown to have an attenuated phenotype (Hodgson et al, 1992). The two modified strains included one that did not produce PLD (Toxminus strain; Tox⁻) (Hodgson et al, 1992) and one in which the *pld* gene was genetically modified, leading to the production of a detoxified PLD (PLD-toxoid strain; PLD-t) (Hodgson et al, 1994). The sheep were experimentally infected with the two attenuated strains and with a wild-type (WT) strain. They were necropsied 7 or 28 days following inoculation. These two time periods correspond

to, respectively, an amplification phase and a stabilisation phase of the pyogranuloma formation (Pépin et al, 1991a,b). Using a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), the cytokine gene expression was measured at the inoculation site and at the regional lymph nodes from the sheep infected with these three strains of *C pseudotuberculosis*. This protocol allowed a comparison of cytokine production according to the sample, time after infection, virulence of strain, and the presence or absence of pyogranulomas in draining lymph nodes.

MATERIALS AND METHODS

Bacterial strains

The wild type *C pseudotuberculosis* strain 231(WT strain) was obtained from D Burrell (CSIRO, Division of Animal Health, Glebe, Australia). The Tox⁻ strain was obtained as previously described by replacing the chromosomal phospholipase D gene (*pld*) with a deleted *pld*-erythromycin resistance gene cassette (Hodgson et al, 1990, 1992). The PLD-t strain was obtained by replacing the deleted *pld* gene with a *pld* gene coding for a detoxified PLD (Hodgson et al, 1994). Preparation of bacteria for inoculation was performed as previously described (Hodgson et al, 1992).

Experimental infection of lambs

Nineteen male lambs of 9 months of age were used for this experiment. They were seronegative for antibodies against the PLD of *C pseudotuberculosis* prior to the experimental infection. Five or six sheep per strain were injected subcutaneously with 2 mL of phosphate-buffered saline (PBS) containing 2×10^8 CFU (WT and Tox⁻ strains) or 2×10^7 CFU (PLD-t strain) of *C pseudotuberculosis*. The sheep were inoculated just above the coronet of the right hind limb. Two control lambs were similarly injected with 2 mL of PBS. The infected sheep were

maintained in an isolated pen during the experimental period.

Clinical examination and immunological assays

The infected and control sheep were observed regularly. Blood samples were collected regularly for isolating peripheral blood lymphocytes (PBLs) as previously described (Wood et al, 1990). Cellular immunity was measured both by determining the level of gamma interferon (IFN- γ) mRNA in isolated PBLs using a semi-quantitative RT-PCR technique, and by quantifying the release of IFN- γ by PBLs (5×10^6 /mL) following incubation for 24 h with either 5 μ g per mL of *C pseudotuberculosis* Tox⁻ sonicate or PLD.

Necropsy and enumeration of lesions

The challenged and control sheep were necropsied either on day 7 post-inoculation (pi) or on day 28 pi. The inoculation site, popliteal (right and left), prefemoral (right and left), iliac and mediastinal lymph nodes were removed for lesion examination. The lungs were also removed and examined for the presence of lesions. Representative pieces of these organs were sampled, embedded in OCT medium (Miles, Eckhart, USA) and immersed in liquid nitrogen before storage at -70°C . The presence of *C pseudotuberculosis* was confirmed when a pyogranuloma was observed, and the causative strain was identified.

Titration of IFN- γ

The amounts of IFN- γ released by PBLs following overnight incubation were assayed using a commercial capture-tag ELISA (CSL Ltd, Parkville, Australia) for the detection of bovine and ovine IFN- γ (Rothel et al, 1990). The interferon levels from antigen-stimulated PBLs were expressed in units and compared with the levels

obtained from PBLs either stimulated with concanavalin A (ConA; 5 μ L/mL; positive control), or not stimulated (negative control).

RNA extraction

For RNA extraction, cryostat sections (four to six sections 10 μ m thick) were obtained from the frozen pieces of organs. These sections were collected in 2 mL Eppendorf tubes and mixed with 1 mL of solution D (4 M guanidinium isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, 100 mM 2-mercaptoethanol), and stored at -70°C . Total RNA was extracted from cryostat sections of the popliteal lymph nodes or inoculation sites as previously described (Chomczynski and Sacchi, 1987). In brief, the cryostat sections in solution D were thawed and homogenised by passing them through 21G needles. After the different steps of extraction, the RNA was resuspended in 70% ethanol, precipitated, washed again, dried, and dissolved in 20 μ L of DEPC-treated water containing RNA-guard (Pharmacia, Biotechnology AB, Uppsala, Sweden). The RNA yield was determined and the purity of the RNA was verified. The total RNA was then adjusted to the desired concentration and stored at -70°C until use.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analysis was performed as previously described (Mohler and Butler, 1991; Kennedy et al, 1992; Seow et al, 1995). The total RNA (5 μ g) was converted to cDNA utilizing oligo dT₁₂₋₁₈ (100 μ g/mL) as a primer and avian myeloblastosis virus (AMV) reverse transcriptase (Pharmacia) according to the manufacturer's instructions. The reaction mixture (2 μ L of 10x amplification buffer, 1 μ L of oligo dT₁₂₋₁₈, 0.5 μ L of RNAGuard, 2 μ L of 10 mM deoxynucleoside triphosphates (dNTPs; Pharmacia), 1 μ L of 50 mM MgCl₂, 5 μ L of total RNA, 1 μ L of AMV reverse transcriptase, and 6.5 μ L of sterile water) was incubated at 42 $^\circ\text{C}$ for 1 h, heated to 95 $^\circ\text{C}$ for 2 min, chilled on ice, and stored at -20°C .

The cDNA pool from the reverse transcription reaction was diluted 1:10, 1:20, and 1:40 in sterile H₂O, and 5 µL of each dilution was subjected to the cycles of PCR in a 96-well format (programmable thermal controller, MJ Research, MA, USA). The sequences for each primer pair are listed in table I. The PCR reagents were used as follows: 1 µL of primer 1 (200 µg/mL), 1 µL of primer 2 (200 µg/mL), 4 µL of 10x amplification buffer, 5 µL of first strand cDNA, 1 µL of Tth polymerase (diluted 1:4; Promega, Madison, WI, USA), and 28 µL of sterile water. Each cycle consisted of 95 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 min. In addition to the experimental samples, each PCR run included three controls. The positive controls consisted of a titration of a plasmid containing the cDNA of the tested cytokine, and a

titration of cDNA obtained from either alveolar macrophages stimulated with lipopolysaccharide (LPS) (for tumor necrosis factor-α (TNF-α), IL-1β, monocyte chemoattractant protein 1 (MCP-1), IL-6, IL-8) or from peripheral blood lymphocytes cultured in the presence of ConA for 18 h (for IL-2, IFN-γ, IL-4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The negative control consisted of the PCR mixture without cDNA. For PCR using plasmid DNA, the mixture was composed as follows: 1 µL of plasmid DNA including cytokine cDNA (20 ng), 4 µL of 10x amplification buffer, 0.8 µL of 10 mM dNTPs, 1.2 µL of 50 mM MgCl₂, 2 µL of each primer (200 µg/mL), 1 µL of Tth polymerase (diluted 1:4) and 28 µL of sterile water.

A first series of experiments was designed to test the effect of various numbers of PCR

Table I. Primer sequences of sheep cytokines and control used for mRNA detection.

<i>Cytokine</i>	<i>Primer sequence (S = sense, A = antisense)</i>	<i>Fragment size (bp)</i>
IL-1β	S: CGCGGATCCATGGCAACCGTACCTGAACCC A: CCGGTCGACTAGGGAGAGAGGGTTCCATTC	801
IL-2	S: ATGTACAAGATACAACCTTTGTCTT A: GTCATTGTTGAGTAGATGCTTTGAC	468
IL-4	S: GGAATTCATGGGTCTCACCTCCCAACTGCTT A: CGGTTCGACTAGCTCGAACACTTTGAATATTT	540
IFN-γ	S: GGGCTGCAGCGTCTTATGCCAGGGC A: AAAGAATTCTCACATTGCTCTCCGGCC	498
TNF-α	S: ACACTCAGGTCATCTTCTCAAGCC A: CAGGGCGATGATCCCAAAGTAGAC	712
IL-8	S: CCGGATCCATGAGTACAGAACTTCGA A: GGAATTCCTCATGGATCTTGCTTCTC	1 100
MCP-1	S: CCGGATCCCAGCCAGATGCAATTAAC A: GGATTCCTCAAGGCTTCGGAGTTTG	310
IL-6	S: CTG GGA GAA GAT TTC AAA AAT GAC A: CTG CTT CAT CGG AAT ACG TCT CAG	504
GAPDH (control)	S: ATCACTGCCACCCAGAAGACT A: CATGCCAGTGAGCTTCCCCTT	200

cycles on the quantity of the generated PCR product. Twenty-five cycles gave a good linear relationship between the amount of input template and the amount of PCR product generated (ie, counts/band), so 25 cycles were used in all further experiments. An example of the results obtained with RT-PCR is illustrated in figure 1 concerning the relative quantitation of IFN- γ mRNA in various samples. After undergoing 25 cycles, an aliquot of each sample (10 μ L) was removed and treated with 0.4 M NaOH before application to Hybond N (Amersham, UK) filters via a dot-blot apparatus. The filters were briefly rinsed in 2x SSC (saline sodium citrate). The PCR products were fixed to the filter with UV light, and stored between two plastic films at room temperature until use. The remaining PCR products were submitted to 25 additional cycles, and stored at 4 $^{\circ}$ C.

Hybridisation was performed with specific probes corresponding to each cytokine (Radford et al, 1991; Seow et al, 1991a,b; Andrews et al, 1993; Seow et al, 1993; Haig et al, 1994; Seow et al, 1994, 1995) using Rapid-hyb buffer (Amer-

sham) according to the manufacturer's instructions. Hybridisation was carried out at 65 $^{\circ}$ C for 2 h using a hybridisation oven. The blots were washed once in 50 mL 2x SSC, 0.1% (W/V) SDS (sodium dodecyl sulfate) at room temperature for 20 min, and washed twice in 50 mL 0.2x SSC, 0.1% SDS at 65 $^{\circ}$ C for 15 min. Before exposing the blots to X-ray film, they were counted on a Trace-96 counter (Innotech, Mulgrave, Australia) to obtain the actual amount of radioactivity associated with each dot.

The procedure for determining the relative quantity of cDNA in each sample has been described previously (Kennedy et al, 1992; Seow et al, 1995). The data are expressed as relative units: a relative unit was defined as the reciprocal of the dilution of the experimental sample $\times k \times$ the equivalent dilution of control cDNA (k is a variable factor and was determined for each cytokine, according to the variable activity of each standard control). It is important to note that these measures of activity (i) did not determine the exact amount of mRNA/cDNA present in each sample, and (ii) did not allow for

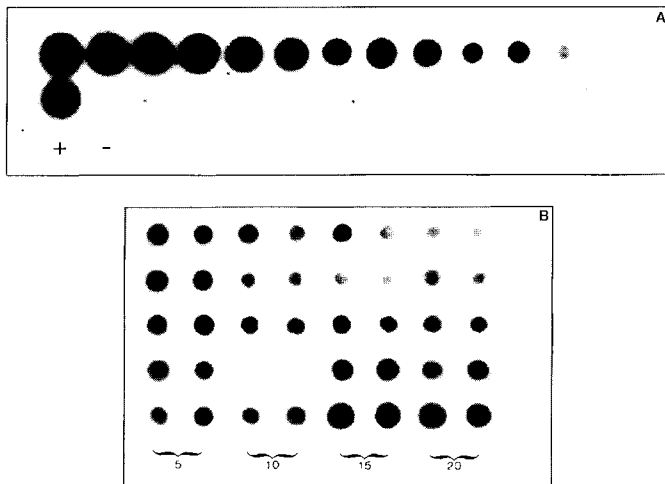


Fig 1. Representative experiment of the semi-quantitative RT-PCR protocol. Hybridization of PCR products after an amplification of 25 cycles with the IFN- γ primers; the products were transferred to a nylon membrane and hybridized with a IFN- γ probe. Part **A** represents the serial dilutions of the positive control (plasmid with IFN- γ cDNA) and two wells corresponding to the positive control (+) and the negative control (-); part **B** represents the samples in duplicate (the numbers 5, 10, 15 and 20 represent the cumulative number of samples per columns; two wells per sample).

direct comparisons of specific levels (ie, relative units) of mRNA between the different cytokines (eg, IL-1 β vs TNF- α). In addition, the number of relative units was adjusted for differences in the expression of a housekeeping/control cDNA (ie, GAPDH) when necessary.

Agarose gel electrophoresis

Following 50 PCR cycles, the products were analysed by agarose gel electrophoresis with a horizontal 1% agarose gel in 1x Tris-acetate-EDTA (TAE) buffer supplemented with 0.005% ethidium bromide for DNA staining. Undiluted PCR product (10 μ L) and 2 μ L of bromophenol blue was applied to each well. The gels were run in 1x TAE buffer at 50 V for 1.5 h. The PCR products were visualised and photographed on a UV transilluminator. This procedure was undertaken to confirm that the correct size DNA fragment was amplified (table I).

Statistical analysis

Differences for the four parameters (strain, day of necropsy, sample, and presence or absence of pyogranuloma) were tested by using analysis of variance (ANOVA; Statview Software on

Apple Macintosh). The level of significance applied to the data was $P < 0.05$.

RESULTS

Clinical findings

The sheep infected with the different strains of *C pseudotuberculosis* were regularly examined, particularly at the site of inoculation and draining lymph node (right popliteal lymph node). The evolution of inoculation site scores between the three groups of sheep were different. Beginning on day 14 pi, the sheep infected with the WT strain developed more severe reactions at the inoculation site than the sheep infected with either the PLD-t strain or Tox⁻ strain (table II). All sheep infected with the WT strain developed gross pyogranulomatous lesions at the inoculation site, whereas the other sheep developed either no visible lesions or only small lesions. No lesion was recorded in the two control sheep.

Necropsy findings

On day 7 pi, pyogranulomas were found at both the inoculation site and the draining lymph

Table II. Kinetics of lesion score at the inoculation site in sheep experimentally infected with strains of *C pseudotuberculosis* differing in virulence.

Days pi	Lesion score ^a (mean \pm SEM) in different strains		
	Tox ⁻	PLD-t	WT
0	0	0	0
4	3.33 \pm 0.33	3.33 \pm 0.33	3.00 \pm 0.37
7	3.17 \pm 0.4	3.00 \pm 0.37	3.83 \pm 0.31
14	1.00	1.33 \pm 0.33	2.00 \pm 0.58
21	1.00	2.00 \pm 0.58	2.67 \pm 0.67
28	0.67	1.83 \pm 0.73	2.33 \pm 0.88

^a The reaction at inoculation sites was examined during the experiment by determining a score: 0, no reaction; 1: slight reaction; 2: moderate reaction; 3: severe reaction and 4: very severe reaction. The parameters considered were: size, induration, presence of pus (six sheep per strain from days 0 to 7 pi and three sheep per strain from days 14 to 28 pi).

node in sheep infected with the WT strain. The sheep infected with the two attenuated strains (Tox⁻ and PLD-t strains; table III) developed only minor lesions at the inoculation site and no pyogranulomas were observed in the right draining lymph node.

On day 28 pi, the necropsy results were similar and confirmed the observations of day 7 pi. No lesions were observed in the draining lymph nodes for sheep infected with the Tox⁻ and PLD-t strains, with the exception of one animal infected with the PLD-t strain. Two

of the three animals inoculated with the Tox⁻ strain had no lesions at the inoculation site (table III). The presence of *C pseudotuberculosis* and the subsequent identification of the corresponding strain were checked in all lesions found at necropsy.

Titration of IFN- γ

To determine the role of cell-mediated immunity, the levels of IFN- γ from PBLs were mea-

Table III. Necropsy findings in lambs experimentally infected with various strains of *C pseudotuberculosis*.

Lamb	Days pi	Presence and severity of pyogranulomas (number) ^a		
		Inoculation site	Right popliteal LN	Other location
Tox ⁻ strain (2 x 10 ⁸ viable bacteria)				
1	7	++ ^b (1)	-	-
2	7	++(1)	-	-
3	7	++(1)	-	-
4	28	-	-	-
5	28	+(1)	-	-
6	28	-	-	-
PLD-t strain (2 x 10 ⁷ viable bacteria)				
7	7	++(1)	-	-
8	7	+(1)	-	-
9	7	+(1)	-	-
10	28	+++ (2)	+(1)	-
11	28	+(1)	-	-
WT strain (2 x 10 ⁸ viable bacteria)				
12	7	+++ (3)	++++ (>4)	-
13	7	++(1)	++(1)	-
14	7	++++ (1)	+++ (1)	-
15	28	-	+++ (2)	++(1) ^c
16	28	++++ (1)	++++ (4)	-
17	28	++(1)	++(1)	-
Controls				
18	7	-	-	-
19	28	-	-	-

Lambs were necropsied on days 7 or 28 pi, and checked for the presence or absence of pyogranulomas at the inoculation site and lymph nodes (LN) or organs (other locations). ^a Number of macroscopic pyogranulomas ^b The severity of gross lesions were determined according to the following scale of lesion diameter: - : no lesion; + : <0.5 cm; ++ : 0.5 to 1 cm; +++ : >1 to 2 cm and ++++ : >2 cm. ^c Pyogranuloma found in lung tissue.

sured following an overnight incubation with different stimuli or antigens. The titration of IFN- γ from the nine remaining sheep on day 14 pi did not show any significant differences between the three groups of sheep inoculated with the different strains of *C pseudotuberculosis* (fig 2A). This result appeared to be associated with the IFN- γ mRNA levels as measured by a semiquantitative RT-PCR technique (fig 2B). As observed for the antibody response (data not shown), sheep infected with the Tox⁻ strain did not produce either IFN- γ protein or IFN- γ mRNA when stimulated with the PLD antigen.

Cytokine gene expression at inoculation sites and in lymph nodes

The control sheep did not express the mRNA for the cytokines tested in this experiment, with the exception of MCP-1, for which the control sheep necropsied on day 28 pi expressed significant amounts of mRNA (fig 3). The control sheep also expressed very low levels of IL-4, IFN- γ , TNF- α or IL-8 in some circumstances (figs 3 and 4).

Cytokine gene expression during an experimental *C pseudotuberculosis* infection can be analysed by different patterns. First of all,

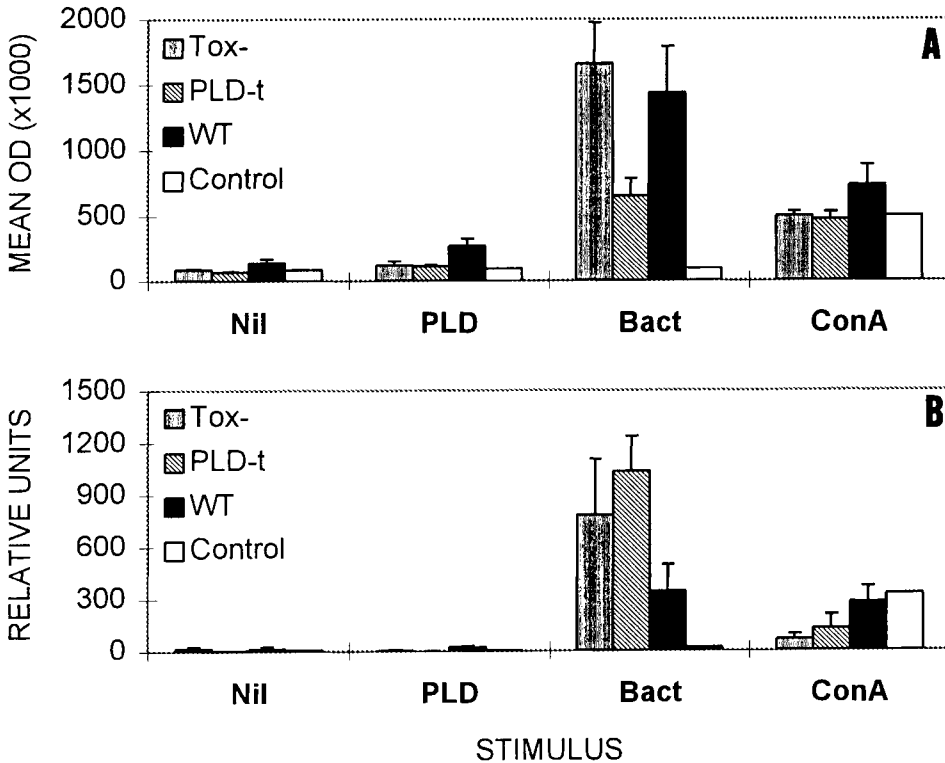


Fig 2. Production of IFN- γ by peripheral blood lymphocytes (PBLs) in lambs experimentally infected with various strains of *C pseudotuberculosis* on day 14 pi. Peripheral blood lymphocytes were incubated with different stimuli: none (nil); negative control, purified PLD (PLD), Tox⁻ sonicate (bact), and concanavalin A (ConA; positive control). (A) Measurement of IFN- γ by ELISA in supernatants of PBLs following overnight stimulation by an ELISA test; (B) measurement of IFN- γ mRNA using a semi-quantitative RT-PCR method as described in Materials and Methods. Except for the control (one lamb), each bar represents the mean (\pm se) of two or three lambs.

differences were obvious between the tissues sampled at necropsy, ie, between the inoculation site and the right popliteal lymph node, draining the inoculation site. Inflammatory cytokines (IL-1 β , $P = 0.02$, TNF- α , $P = 0.05$) were present at high levels in the inoculation site, whereas the expression of T-cell associated lymphokines (IL-2 and IL-4, $P = 0.05$) was greater in the right popliteal lymph node

than in the inoculation site. Secondly, there were also significant differences depending on the time delay from the inoculation. The levels of gene expression for TNF- α ($P = 0.03$), IL-2 ($P = 0.05$) and IFN- γ ($P = 0.05$) were higher on day 7 pi than on days 28 pi. In contrast, IL-6 ($P = 0.02$) and IL-1 β ($P = 0.03$) mRNA were expressed at higher levels on day 28 pi. Overall, the infected sheep pro-

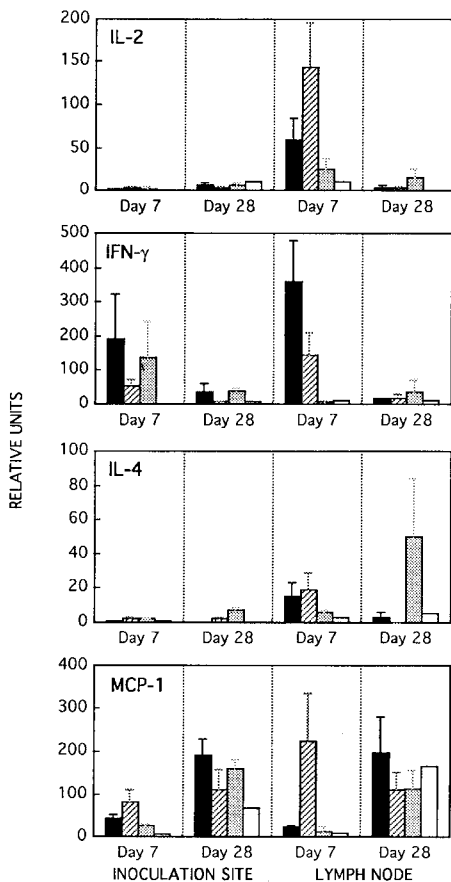


Fig 3. T-cell associated cytokines (IL-2, IFN- γ and IL-4) and MCP-1 gene expression in sheep experimentally infected with various strains of *C pseudotuberculosis* differing in virulence. The levels of mRNA are expressed in relative units by using a semi-quantitative RT-PCR technique. Each bar represents the mean (\pm SD) of two or three sheep, except for the control sheep. ■ Tox⁻, ▨ PL D-t, ▩ wt, □ control.

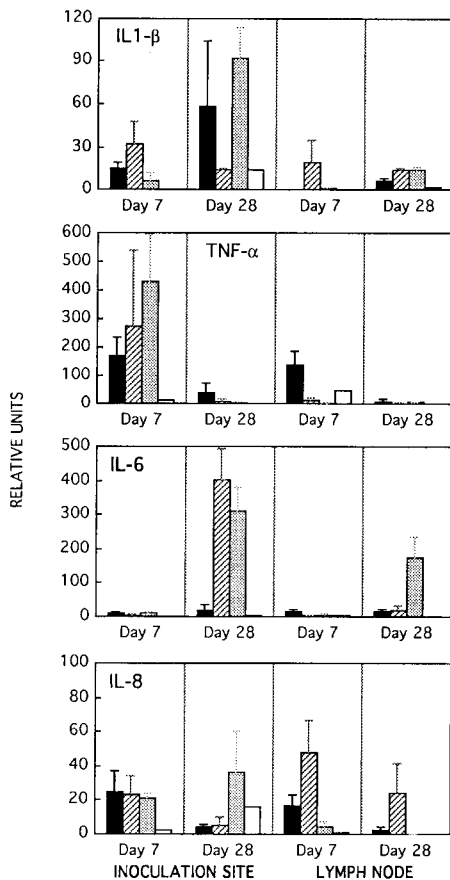


Fig 4. Inflammatory cytokines (IL-1 β , TNF- α and IL-6) and IL-8 gene expression in sheep experimentally infected with various strains of *C pseudotuberculosis* differing in virulence. The levels of mRNA are expressed in relative units by using a semi-quantitative RT-PCR technique. Each bar represents the mean (\pm SD) of two or three sheep, except for the control sheep. ■ Tox⁻, ▨ PL D-t, ▩ wt, □ control.

duced high levels of mRNA in at least one organ on days 7 or 28 pi in comparison with the controls, irrespective of the cytokine. The exception was MCP-1 where the control and infected sheep had similar levels of mRNA on day 28 pi (fig 3).

Whereas the clinical and lesional parameters were different in the sheep infected with the three strains differing in virulence, no significant differences could be detected in cytokine mRNA expression in these sheep. The sheep infected with the WT strain seemed to express relatively higher levels of cytokine mRNA in comparison with the two other groups (Tox⁻ strain and PLD-t strain). However, there was a large amount of individual variabilities within the groups. In contrast, if the presence or absence of lesions was considered, significant differences in cytokine gene expression were detected for two cytokines, IL-1 β and IL-8, between animals within the right popliteal lymph node pyogranulomas and animals without pyogranulomas (15.9 versus 4.5 relative units for IL-1 β , $P = 0.05$; 31.0 versus 6.4 relative units for IL-8, $P = 0.02$). In addition, although the differences were not significant, sheep with pyogranulomas had higher levels of mRNA for the other cytokines tested, except for TNF- α and IL-6.

DISCUSSION

The present report describes (i) the differences in virulence of two attenuated strains of *C pseudotuberculosis* in comparison with a wild-type strain, and (ii) the adaptation of a semi-quantitative RT-PCR technique to analyse the expression of eight cytokines from the inoculation site and the draining lymph nodes of sheep infected with these various strains of *C pseudotuberculosis*.

The deletion of the *pld* gene in *C pseudotuberculosis* or the replacement of this gene by a gene coding for a genetically inactivated analogue produces a decrease in virulence, particularly for the Tox⁻ strain. The results concerning the PLD-t strain were less obvious,

because the lambs were experimentally infected with a ten times lower amount of viable bacteria than the other two groups (Tox⁻ and WT strains) (Hodgson et al, 1992, 1994). The decrease in Tox⁻ virulence was characterised by a lack of bacterial dissemination from the subcutaneous inoculation site to the draining lymph node. Previous reports have shown that the dissemination of *C pseudotuberculosis* is restricted to the draining lymph nodes and associated with the development of typical pyogranulomas. This development is the major event in experimental, and probably in natural infections with *C pseudotuberculosis* (Pépin et al, 1991a,b). In this report, the Tox⁻ strain was unable to disseminate to the draining lymph node, at least in sufficient quantities to develop a subsequent lesion. Also, the Tox⁻ strain induced minimal lesion development at the inoculation site. The formation of a local pyogranuloma at the inoculation site together with little difference in lesion severity among the three bacterial strains before day 14 demonstrated (i) that PLD did not participate in the early events leading to the formation of pyogranuloma, and (ii) that other virulence factors are involved (Jolly, 1966; Hard, 1975; Muckle and Gyles, 1983). However, the rapid healing in the two lambs necropsied on day 28 pi, and together with their absence of pyogranuloma at the inoculation site, suggested that the production of PLD by bacteria inside the mature pyogranuloma may contribute to the persistence of the lesions. This observation obtained from two out of three sheep needs to be confirmed to ascertain the role of PLD in maintaining chronic lesions induced by *C pseudotuberculosis*.

The role of PLD in the dissemination of bacteria from an inoculation has been well recognised (Jolly, 1965; Carne and Onon, 1978). This report supports this role for PLD in the dissemination of *C pseudotuberculosis*, and is in agreement with a recent study that used targeted mutagenesis of the *pld* gene (McNamara et al, 1994). It also shows that Tox⁻ and PLD-t strains persisted long enough to induce

a strong immunity as demonstrated by the antibody response against bacterial antigens (data not shown) and the cellular reactivity as measured by the production of IFN- γ by PBLs, at both the mRNA and protein levels. The induction of a strong immune response, which can protect sheep following virulent challenge (Hodgson et al, 1992), is probably not solely due to the persistence of bacteria at the inoculation site. As described in other bacterial infections (Fensterbank et al, 1985; Fontaine et al, 1994), some bacteria probably reach the lymph node where immunocompetence is then established. An enumeration of viable bacteria during the days following experimental inoculation would clarify this possibility.

The semi-quantitative RT-PCR technique demonstrated that the eight cytokines analysed in this report were produced at least once following experimental infection, by days 7 or 28 pi. The low levels of mRNA for some cytokines found in the tissues of the uninfected sheep may reflect normal homeostasis. This has been described in other studies and it is likely that the presence of low levels of mRNA reflect the dynamic nature of immune regulation even in the absence of microbial invasion (Iizawa et al, 1992).

The important features emerging from this study were (i) the differences in cytokine gene expression between the inoculation site and the draining lymph node, and (ii) the differences associated with the time after experimental infection. Inflammatory and T-cell associated cytokines were more highly expressed at the inoculation site and in the popliteal lymph node, respectively. These data suggested an important inflammatory process in the first week followed by a stabilisation by day 28 pi. This is correlated with the known process of pyogranuloma development in sheep following experimental infection with *C pseudotuberculosis*, during which it is possible to distinguish three phases. The first phase (days 1–4) is the initiation process characterized by the recruitment of neutrophils both to the inoculation site and to the draining

lymph node. The second phase (amplification phase; days 5–10) is characterized by the early development of pyogranuloma involving macrophages and lymphocytes, and the third phase which corresponds to a relative stabilisation of the process and is characterized by the maturation and persistence of the pyogranuloma (Guilloteau et al, 1990; Pépin et al, 1991a,b, 1992, 1994a,b). It is consistent with these pathogenic phases that inflammatory cytokines are more involved in the first phase, whereas the T-cell associated cytokines are more involved in the draining lymph node and in the later stages of the infection process. This pattern is not clear-cut since some inflammatory cytokines such as IL-6 remained involved on day 28 pi, although the expression of this cytokine was low. Another important conclusion from this study was the fact that there were differences between animals with and without lesions. The mRNA levels for five cytokines, namely, IL-1 β , IL-8, TNF α , IL-6 and MCP-1, were higher in animals with pyogranulomas in the draining lymph node. Even if the differences were only significant for IL-1 β and IL-8, this suggested that the presence of pyogranuloma is characterized by a more active production of cytokines. This study did not allow us to determine which cells produced these cytokines, but it is likely that the macrophages and lymphocytes, which are distributed in two separate layers around the central necrosis, are a major source of cytokines. It is interesting to note that in a previous report, we demonstrated that the granuloma macrophages produced IL-1 β by using a monoclonal antibody raised against ovine IL-1 β (Rothel et al, 1997). This study also supported an important role for TNF- α in granuloma development, since the levels of TNF- α were high on day 7 pi and seemed to decrease in the later stages of disease. This is in agreement with previous reports in which TNF- α has been recognised as an important factor in *C pseudotuberculosis* infection in sheep (Ellis et al, 1991, 1994, 1995), and in granuloma persistence (Chensue et al, 1989; Kindler et al, 1989; Amiri et al, 1992).

In contrast, the study failed to detect major changes between sheep infected with strains of *C pseudotuberculosis* differing in virulence; these differences may exist, but it is likely that the large individual animal variability did not allow us to detect these differences. This accentuates the difficulty in studying large animal immunology, but it is likely that an analysis of a larger group of animals would more fully define the cytokine pattern associated with the different strains. It is also likely that an analysis performed at a later time after infection would determine if the cytokine gene expression in sheep infected with attenuated strains or with a fully virulent strain of *C pseudotuberculosis* differed. Among the cytokines examined, IFN- γ , in particular, requires further study. It is assumed that IFN- γ production is of particular value in intracellular infections through IFN- γ -mediated enhancement of cellular immunity (Squires et al, 1989; Farrar and Schreiber, 1993; Flynn et al, 1993; Zhan and Cheers, 1993; Xiong et al, 1994). The significant production of IFN- γ in all the infected sheep, whatever the bacterial strain, on day 7 pi indicates that infection with *C pseudotuberculosis* is associated with its production. It is interesting to note that the levels of IL-4 produced were relatively low. This is consistent with the reciprocal production of IFN- γ and IL-4, according to the paradigm of TH1 and TH2 cells (Scott, 1993). These differences have been reported in numerous other infectious or parasitic diseases in mice or humans (Heinzel et al, 1989; Yamamura et al, 1991; Hsieh et al, 1993; Pirmez et al, 1993). It will be worth testing for the presence of other cytokines in this model of pyogranuloma in the future, particularly those known as immunoregulatory cytokines, such as IL-10 and IL-12 (Locksley, 1993; Moore et al, 1993).

In conclusion, our results (i) showed the production of cytokines in sheep infected with *C pseudotuberculosis* with variations in relation to the type of tissue and time following infection, but failed to detect differences between sheep infected with attenuated or virulent strains and (ii) suggested that the development

of the pyogranuloma can proceed in the presence of TH1, TH2 and inflammatory cytokines, namely, IFN- γ , IL-2, IL-4, TNF- α and MCP-1 whose levels peak during early granuloma formation. These cytokines may play a role in the development of pyogranuloma, which is important in reducing the dissemination of bacteria.

ACKNOWLEDGMENTS

The authors thank M David and L Hurst for their technical help and N Collins and A Cameron for their assistance with the sheep experiment. This work was supported by grants from the McMaster Research Fellowship, CSIRO, Australia (M Pépin) and from INRA, France.

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