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Dynamics of experimentally induced *Staphylococcus epidermidis* mastitis in East Friesian milk ewes

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The responses of five lactating East Friesian milk ewes to experimental mammary infection with *Staphylococcus epidermidis* and of five control ewes were examined over a period of 10 weeks. Infection caused an influx of neutrophils into milk, the numbers of which started to rise 4 h post infection and peaked 24 h after infection. The initial response was accompanied by mild fever and mild leucopaenia in blood (8 h after infection). No other signs of systemic infection were observed. Milk appeared normal at all times, although the milk yield of infected ewes tended to decline. Staphylococci were absent in milk from four ewes at 2 d and at 3 d after infection, but re-emerged intermittently in four of five ewes at subsequent samplings. Cytokines in milk were measured by ELISA. IL-8 was elevated in infected glands at 2 h and peaked at 8 h. In the four ewes intermittently shedding bacteria, IL-8 remained elevated until the final sampling at 10 weeks. IL-1β was transiently elevated at 1 d and 2 d and showed a pronounced peak in one sheep. Milk samples from this ewe were bacteriologically negative, somatic cell count (SCC) was within the normal range and the concentrations of IL-1β, as well as IL-8, were similiar to the control group ($n=5$) from 1 week after infection until the final sampling. Histological examination revealed leucocytic infiltrates in the four glands remaining infected at the end of the experiment, and a high level of CD5+ lymphocytes in three ewes. The results suggest that the relationship between the initial neutrophil influx and the proinflammatory cytokines may be responsible for determining the course of infection. Subclinical mastitis due to coagulase-negative staphylococci leads to minor changes in milk yield and milk constituents.

Keywords: Mastitis, dairy ewes, *Staphylococcus epidermidis*, subclinical, cytokines.

Sheep milk and milk products are becoming increasingly popular, mainly because raw milk products are thought to benefit human health. Consequently, the number of dairy flocks is increasing, with East Friesian milk sheep being the predominant breed. The adoption by the industry of a quality payment system based on somatic cell count (SCC) and total bacterial count has led to substantial financial losses for dairies with a high prevalence of subclinical mastitis. In addition to a reduced unit value of milk, subclinical mastitis is associated with decreased milk yield (Saratsis et al. 1999), changed milk composition (Jelinek et al. 1996; Burriel, 1997) and lowered processing quality (Klei et al. 1998).

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Although subclinical intramammary infections due to coagulase-negative staphylococci (CNS) in sheep dairy flocks are increasing (Deutz et al. 1990; Rammelmayr, 1997; Wittek et al. 1998; Leitner et al. 2001; Moroni & Cuccuru, 2001), the pathogenic mechanisms that lead to subclinical mastitis after infection with CNS are largely unknown. An essential part of the udder's defence is the accumulation of neutrophils, which is induced by bacterial products and pro-inflammatory cytokines. In other types of bacterial mastitis, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) are released (Persson Waller et al. 1996, 1997; Boudjellab et al. 1998; Boudjellab et al. 2000; Riollet et al. 2000; Hagiwara et al. 2001; Kehrli & Harp, 2001; Winter & Colditz, 2001). The presence of these cytokines is also associated with changes in mononuclear cell populations, which are important for both inflammatory and

immunological reponses in the udder (Persson Waller & Colditz, 1998, 1999; Rivas et al. 2000). However, cytokine and cellular responses in the mammary gland following acute CNS infection, and their role in inducing resolution or progression to subclinical infection, have not been determined. In the present study, the effect of intramammary challenge of East Friesian milk sheep with *Staphylococcus epidermidis* on milk yield and several parameters of host defence were examined over a period of 10 weeks.

Materials and Methods

Experimental procedures

The study was approved by the Austrian Federal Ministry for Education, Science and Culture (GZ 68.205/40-Pr/4/2001). Ten clinically healthy multiparous East Friesian ewes in the third month of lactation were used; five receiving experimental challenge with CNS and five controls. The infused and control ewes were housed indoors and were milked with the same machines at the same time, twice a day, in a milking parlour. They were free access to hay, which was supplemented with 500 g commercial feed concentrate at each milking. Milk collected aseptically from udder halves before the experiment was bacteriologically negative. Sampling times were before infection (0), 2, 4, 8 h after infection, 1, 2, 3 d and 1, 2, 3, 4, 6, 8 and 10 weeks after infection. At these times each ewe was clinically examined, and feed intake and rectal temperature were recorded. Milk samples were collected in sterile containers by hand stripping, and blood samples were taken from the jugular vein in vacutainer tubes containing EDTA. On these days, individual milk yield was recorded by means of graduated measuring cylinders attached to individual milking units. Morning and evening milk yield were summed and expressed as total daily milk yield.

Challenge

The inoculum organism had been isolated in pure culture from the mammary secretion of a ewe with subclinical mastitis and was identified as *Staph. epidermidis* by the following characteristics: mixed aerobic and anaerobic growth; medium colony size; non-pigmented colonies; haemolysis-negative; coagulase-negative; DNAse-positive; phosphatase-positive; novobiocin susceptible; lipase-positive; urease-positive; fructose-positive; lactose-positive; maltose-positive; mannitol-negative; sucrose-positive; ribose-positive; trehalose-negative; xylose-negative; xylitol-negative. Challenge inocula were prepared by growing the *Staph. epidermidis* in 5 ml of Trypticase soy broth (Difco) for 18 h at 37 °C. The supernatant was discarded and the bacteria resuspended in 5 ml pyrogen-free saline (PFS). The number of viable bacteria in the inoculum after 18 h of incubation was $3{\cdot}3 \times 10^7$ cfu/ml. Five ewes received 1 ml inoculum by infusion into the right mammary gland and five control ewes were treated with 1 ml PFS.

Laboratory procedures

Leucocytes were counted using a haematology analyser (CA 580 A, Medonic, Stockholm, Sweden) calibrated for sheep blood. The somatic cells in each milk sample were counted by means of a fluorescence-optical method (Fossomatic) as described by Schmid-Madsen (1975). Fat, protein and lactose contents of milk were measured using an IR spectrophotometer (Milko Scan 133B; Foss Electric, Hillerød, Denmark).

A loopful (0·01 ml) of each milk sample was inoculated onto Columbia blood agar and incubated aerobically at 37 °C for 24–48 h. Bacterial growth was scored semi-quantitatively as follows: 0 cfu per plate=negative; 1–10 cfu per plate=(+); 11–50 cfu per plate=+; 51–100 cfu per plate=++; >100 cfu per plate=+++. Milk samples carrying fewer than 500 cfu/ml may have yielded false negative results on bacterial culture.

For cytokine analysis, milk samples were centrifuged (2500 ***g*** for 20 min at 4 °C) and the fat-free and cell-free milk fractions were collected. These samples were then frozen at −30 °C until assayed. The concentrations of cytokines IL-8 and IL-1β in these milk samples were determined by ELISA as described by Persson Waller et al. (1996). Monoclonal antibodies to ovine IL-8 and IL-1β were obtained from Serotec (Biomedica, Vienna, Austria), and recombinant ovine cytokine standards were a kind gift from Peter McWaters, CSIRO Livestock Industries, Geelong, Australia.

The five infected animals were killed 10 weeks after infection and promptly necropsied. Milk samples from the left (unchallenged) glands of these five ewes were examined 2 d before euthanasia and were found to be bacteriologically negative and to have SCC comparable to the control group. These unchallenged glands were subsequently used as control tissues for histology. After gross examination, three tissue samples from both mammary glands were removed for histological investigation. The samples were selected from the deep parenchyma near the dorsal surface of the gland, in the centre of the gland and at the point of transition from teat cistern to gland cistern. Tissue samples were fixed in neutral buffered formaldehyde (70 g/l). After embedding in paraffin, 4-μm sections were cut and routinely stained with haematoxylin and eosin (HE). For cell types of interest, sections were examined at ×40 magnification and were scored as follows: 0 cells=negative; a few cells=(+); mild infiltration=+; moderate infiltration=++; high infiltration=+++. For immunohistochemical investigations, tissue samples from the same locations were frozen in isopentane/liquid nitrogen and stored at −20 °C. Sections were prepared and stained as described by Jörundsson et al. (1999). Table 1 shows the monoclonal antibodies used to determine the presence

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Table 1. Monoclonal antibodies used to identify leucocyte populations in milk

Surface molecules	Antibodies	Cells marked/Function	Reference
CD 4	SBU-T4 (pool)	T-helper cells	Maddox et al. 1985
CD 8	SBU-T8 (38,65)	Cytotoxic and suppressor T cells	Maddox et al. 1985
γδ T-cells	SBU-T19 (19–29)	T19 subset of γδ T cells	Mackay et al. 1989
CD 5	SBU-T1 (25–91)	T cells and subpopulation of IgM+ B cells	Mackay et al. 1985 Chevallier et al. 1998
MHCII	SBU-II (38–27)	Major histocompatability class II antigen	Puri et al. 1987

Table 2. Somatic Cell Counts (SCC) in milk from challenged (1–5) and control ewes

Values are least square means ± SEM ($n=5$ per group)

	SCC ($\times 10^{-3}$) cells/ml								
	Challenged								
	Ewe number					Controls			
Time p.i.†	1	2	3	4	5	Mean I	SEM I	Mean C	SEM C
0 h	26	95	62	35	50	53·6	26·9	37·6	2·7
2 h	39	137	90	50	89	81·0	38·7	78·6	38·7
4 h	3131	5747	5170	4118	6001	4833·4	1196·0	94·2	35·0
8 h	7868	8253	7570	7286	7543	7704·0	369·8	97·6	44·8
1 d	8335	9160	9041	7860	5563	7991·8	1457·7	190·0	43·4
2 d	7285	6095	6976	6992	5133	6496·2	882·8	105·4	29·8
3 d	6159	6488	6239	3152	3199	5047·4	1713·2	80·6	13·7
1 w	1241	1691	2738	595	1118	1476·6	806·0	57·0	21·2
2 w	4923	905	7348	6485	634	4059·0	3127·6	74·6	14·9
3 w	6264	307	5484	3799	6011	4373·0	2467·5	87·0	31·6
4 w	2572	115	2797	848	2367	1739·8	1187·3	66·6	14·2
6 w	6626	210	6244	1584	7503	4433·4	3296·4	128·0	53·4
8 w	2444	91	8388	850	3486	3051·8	3265·2	63·8	22·3
10 w	1975	23	839	844	7441	2224·4	2997·8	47·4	62·2

† post infection

and distribution of leucocytes expressing CD4, CD5, CD8, γδ T cell receptor and MHCII surface antigens. Tissue sections from right supramammary lymph nodes of challenged ewes served as positive controls and sections from organs not normally containing lymphocytes were used as negative controls. Positive cells staining brown following the peroxidase reaction were scored as above.

Statistical analysis

Results are presented as means ± SEM. As repeated measurements were made on each animal, results were analysed as a split plot design (Crowder & Hand, 1990; Montgomery, 1991) using the following model:

$$y_{ijk} = \mu + G_i + S_{ij} + T_k + (GT)_{ik} + (GST)_{ijk} \begin{cases} i=1,\ 2 \\ j=1,\ \ldots,\ 5 \\ k=1,\ \ldots,\ 14 \end{cases}$$

where G=group, S=sheep and T=time.

Results

Control ewes

Control animals remained clinically healthy throughout, and the results of bacteriological examination of their milk were negative. Mean SCC for controls was 37·6–190·0 ($\times 10^3$) cells/ml (Table 2). Cytokines IL-1β and IL-8 were 0·00–0·38 ng/ml, and from 0·00–4·92 ng/ml, respectively (Figs. 1 and 2).

Challenged ewes

Clinical examination. Feed intake and behaviour were not affected by experimental challenge. A slight and short-lived increase of the rectal temperature (39·9±0·4 °C) and swelling of the infected glands were observed 8 h after infection. Simultaneously, leucocyte counts in blood dropped to their lowest level of 2·6±0·6 ($\times 10^9$) cells/ml. Subsequently, rectal temperatures and leucocyte counts in blood were within a normal range, 38·8±0·3 °C,

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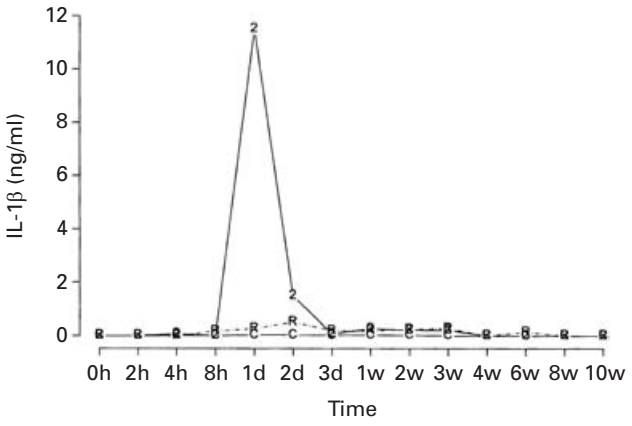

Fig. 1. IL-1β concentrations in milk of challenged and control ewes (C). For challenged ewes the symbol (2) represents ewe 2 and (R) represents ewes 1, 3, 4 and 5.

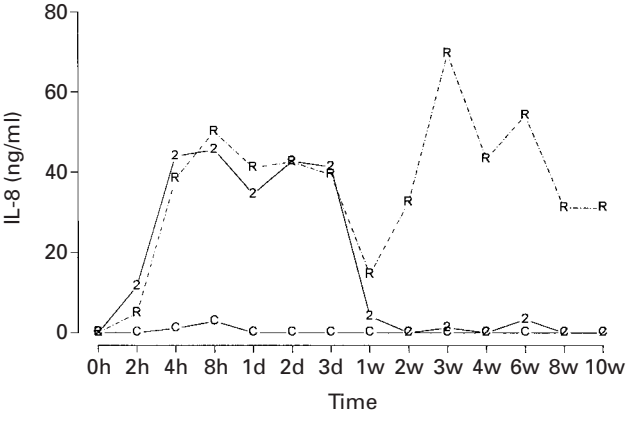

Fig. 2. IL-8 concentrations in milk of challenged and control ewes (C). For challenged ewes the symbol (2) represents ewe 2 and (R) represents ewes 1, 3, 4 and 5.

6·4±1·3 ($\times 10^9$) cells/ml, respectively. Milk appeared normal, was not discoloured and did not contain any flakes or clots.

Bacteriological examination. Immediately after infection (2, 4, 8 h) bacteria were isolated from milk samples of all infected animals. Milk from four infected ewes was bacteriologically negative at 2 d and 3 d after infection. However, bacteria re-emerged episodically in milk from four infected glands at subsequent samplings until the end of the experiment, while milk from ewe 2 remained free of bacteria after 8 h (Table 3).

SCC. Milk SCC increased 4 h after infection and peaked 1 d after infection at 7991·8±1457·7 ($\times 10^3$) cells/ml. SCC then remained elevated until the end of the experiment (Table 2). SCC differed significantly between the infected and control groups, differed with time ($P<0·01$) and there was a significant time-by-group interaction ($P<·01$, Table 4). In concordance with the bacteriological clearance of the milk from ewe 2, SCC dropped to a physiological level in this animal by the end of the experiment (Table 2).

Table 3. Results of bacteriological examination of the milk samples from challenged ewes

	Ewe				
Time p.i.†	1	2	3	4	5
0 h	–	–	–	–	–
2 h	+++	++	+++	++	+++
4 h	+++	+++	+++	+++	+++
8 h	+++	+	+++	++	++
1 d	++	–	–	++	+
2 d	(+)	–	–	–	–
3 d	–	–	(+)	–	–
1 w	++	–	+	++	+++
2 w	+	–	–	+	+++
3 w	–	–	–	–	–
4 w	(+)	–	(+)	++	–
6 w	++	–	–	–	++
8 w	–	–	++	(+)	–
10 w	–	–	+++	+	+

– = negative, (+) = 1–10 cfu per 10 µl, + = 11–50 cfu per 10 µl, ++ = 51–100 cfu per 10 µl, +++ = >101 cfu per 10 µl)
† post infection

Table 4. Statistical significance of the effect of group, time and group-by-time interaction on SCC, IL-1β, IL-8, milk yield and milk composition

	Effects (P)		
	Group	Time p.i.†	Time p.i. × group
SCC	<0·01	<0·01	<0·01
IL-1β	0·26	0·10	0·36
IL-8	<0·01	<0·01	<0·01
milk yield	0·43	<0·01	<0·01
lactose	0·46	0·08	0·24
protein	0·44	<0·01	0·18
fat	0·29	<0·01	0·25

† post infection

Cytokine production. Concentrations of IL-8 in milk were significantly elevated in the infected group from 4 h until 3 d and peaked at 46·19±8·21 ng/ml 8 h after infection (Fig. 2). Milk from ewe 2 showed high IL-8 concentrations over the period from 2 h to 3 d after infection. Subsequently the concentration subsided to the level of the control group (Fig. 2). IL-1β was elevated 24 h after infection, showing a pronounced peak (11·52 ng/ml) in milk from ewe 2 (Fig. 1). Concentrations of IL-1β did not differ significantly between the infected and control groups. The time and time-by-group interaction effects were not significant (Table 4).

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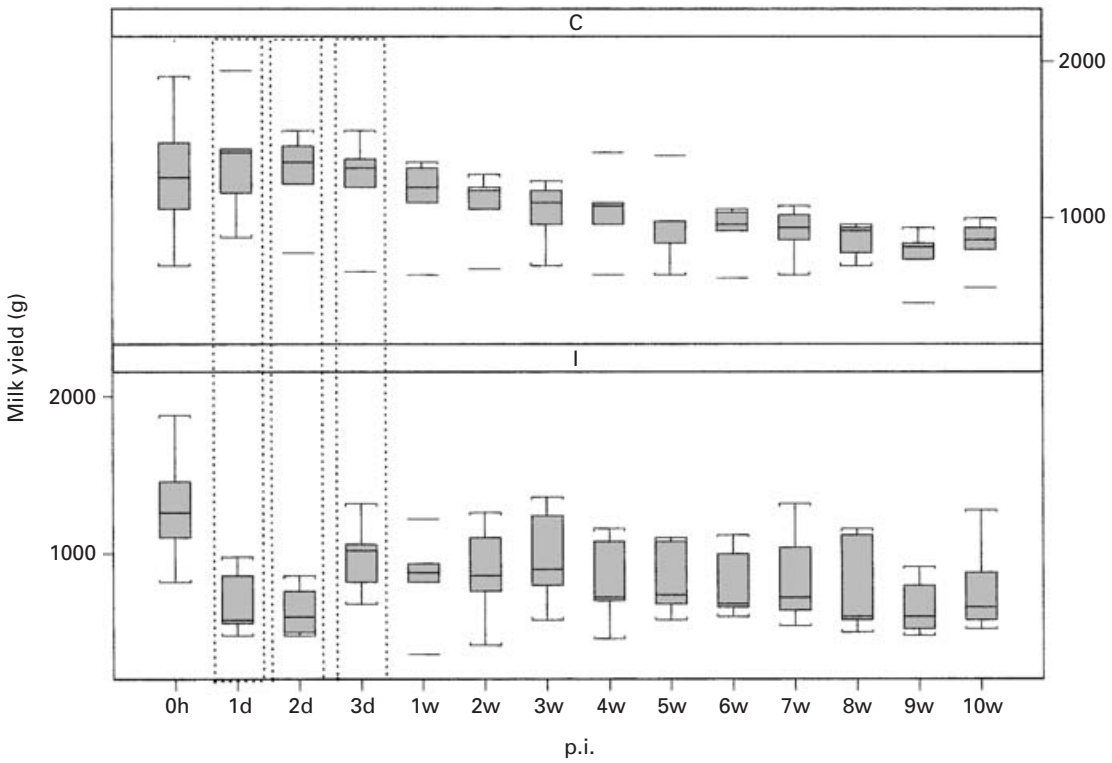

Fig. 3. Milk yield of control (C) and challenged (I) groups. Milk yields at timepoints marked by dotted lines differ significantly between groups ($P<0.01$).

Yield of milk and milk constituents

Across treatment groups, milk yield, protein and fat content varied significantly during the experiment ($P<0{\cdot}01$, Table 4), and there was a significant group-by-time interaction for milk yield ($P<0{\cdot}01$, Table 4). Milk yield of the infected ewes were significantly depressed 1, 2 and 3 d after infection (Fig. 3). A small increase of fat in milk from infected glands could be observed 1 d after infection (infected: 7·6%, control: 6·0%). Protein in milk from infected glands showed a peak 1 week after infection (infected, 5·8%; control, 5·1%). No significant differences were found for lactose.

Histopathological and immunohistochemical examination

Small numbers of lymphocytes were seen in the mammary parenchyma from control glands. However, there were no signs of neutrophilic infiltration, epithelial degradation or fibrous tissue proliferation. Both in control and in infected glands, fat globules and corpora amylaceae were present in some alveoli.

There were slight histopathological differences between the infected sheep but, in general, a mild chronic mastitis was observed. The most characteristic lesions consisted of focal intra-alveolar neutrophil infiltrates, degeneration of some epithelial cells, mononuclear cell infiltrates and proliferation of connective tissue. It is notable that the lesions were localized and not widespread in infected glands. The tissue alterations and cellular infiltrates in the three udder regions sampled are described in Table 5. Ewe 2, which was clear of infection by the time tissue samples were collected at week 10, showed only mild proliferation of fibrous tissue in one section; otherwise the mammary gland appeared normal. The strongest reaction was seen in ewe 3, where large numbers of neutrophils and moderate numbers of mononuclear cells were observed in the deep and central tissue samples.

From immunohistochemical examination, the strongest immunoreactivity was observed for CD5. These cells were particularly prevalent in ewes 1, 3 and 5 (Fig. 4), in contrast to the results of ewe 2 (Fig. 5). A moderate number of CD4+ cells were seen in the central tissue sample in ewe 3 whereas no, or only a few, cells could be determined in the tissue samples of the remaining challenged ewes. A low number of CD8+, γδ T cells and MHCII-expressing cells were observed in samples from challenged ewes.

Discussion

After experimental intramammary challenge in five ewes, mastitis with high SCC was established without gross abnormalities in milk. Thus by the criteria commonly used to identify mastitis in milking parlours (heat and swelling of the gland and gross abnormalities in volume

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Table 5. Results of the pathohistological examination of the challenged ewes (ewe 1–5)

	Ewe number														
	1			2			3			4			5		
	L	G	CT	L	G	CT	L	G	CT	L	G	CT	L	G	CT
Dorsal parenchyma	++	++	+	–	–	–	++	+++	+	+	(+)	+	+	(+)	+
Central parenchyma	+	+	–	(+)	–	++	++	+++	+	(+)	–	+	+	(+)	+
Teat-gland junction	(+)	+	(+)	–	–	–	+	+	(+)	+	(+)	+	+	(+)	+

L = lymphocytes, G = granulocytes, CT = connective tissue; – = no cells, (+) = a few cells, + = mild infiltration, ++ = moderate infiltration, +++ = high infiltration

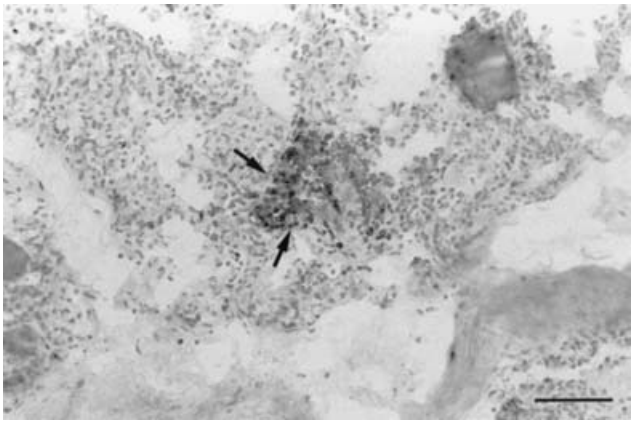

Fig. 4. Immunohistochemical staining of many CD5+ cells (arrows) in ewe 3; central tissue sample; frozen section; bar = 84 μm.

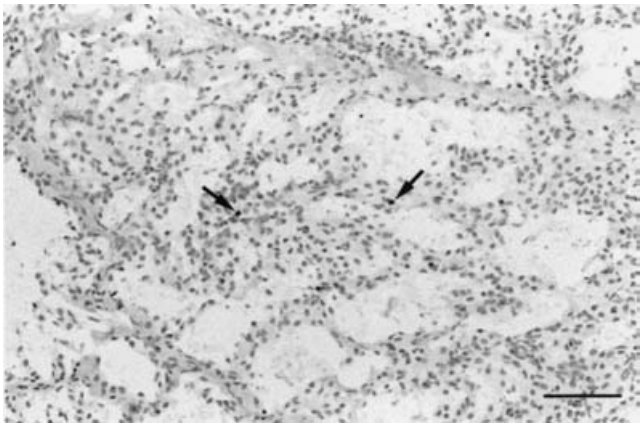

Fig. 5. Immunohistochemical staining of CD5+ cells in ewe 2; only a few positive cells (arrows) were observed in the central tissue sample; frozen section; bar = 84 μm.

and appearance of milk), the mastitis induced by the intramammary challenge was subclinical. Nonetheless, a limited acute systemic response characterized by an increase of rectal temperature and a moderate nadir of white blood cells was observed 8 h after infection, which might be attributable to the high challenge dose used in this study. As previously seen during experimental intramammary infection of Merino wool sheep with CNS (Winter & Colditz, 2001), an early recruitment of leucocytes led to a transient clearance of bacteria from milk of four ewes in the present experiment. SCC in milk of these ewes remained elevated throughout the experiment and was accompanied by elevated IL-8 concentrations and by intermittent re-emergence of bacteria in milk. In contrast, one sheep cleared bacteria from the gland by 24 h and its milk remained free of bacteria for the remainder of the experiment.

IL-8 and IL-1β were analysed for associations with leucocyte recruitment and bacterial clearance. IL-8 is thought to play a prominent role in the recruitment of neutrophils in the mammary gland, and is produced by mammary epithelial cells (Eckmann et al. 1993; Boudjellab et al. 1998; Barber et al. 1999). IL-8 appeared in milk 2 h after infection and preceded the appearance of IL-1β, which peaked at 24 h. The early appearance of IL-8, production of which can be stimulated by bacterial products, is in accord with previous studies (Cybulsky et al. 1988; Persson Waller et al. 1997; Persson Waller & Colditz, 1999; Winter & Colditz, 2001). A marked contrast in cytokine presence in milk occurred between ewe 2, which cleared infection at 24 h, and the remaining four sheep. While IL-8 was present in milk from all sheep at 2 h and subsequent time points, a pronounced peak in IL-1β seen in ewe 2 at 24 h did not occur in the other sheep. Clearance of bacteria from the milk of ewe 2 was followed by a gradual return of IL-8 concentrations in milk to levels seen in control animals within 1 week. Although it is based on observations in only one animal, this finding suggests that IL-1β may have played an important role in terminating *Staph. epidermidis* infection and that, once bacteria were removed from the gland, the stimulus for production of IL-8 decreased. It is noteworthy that transient peaks in IL-1β in other sheep may have be been missed because of the sampling schedule.

During chronic *Staph. aureus* mastitis, neutrophils are the major cell population in milk (Riollet et al. 2001). However, the sustained shedding of bacteria seen in chronic mastitis suggests that neutrophils lack the phagocytic efficacy needed to eliminate bacteria at this time (Shoshani et al. 2000). The IL-1β peak seen in ewe 2 might

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have activated neutrophils for enhanced phagocytosis and intracellular killing of bacteria. Failure to eliminate bacteria rapidly may lead to hyporesponsiveness of defence mechanisms in the mammary gland (Maas & Colditz 1987; Young et al. 2001) and survival of bacteria within leucocytes and epithelial cells (Bayles et al. 1998).

In addition to neutrophils, mononuclear cells are thought to play an important role in mammary defence. Although changes in prevalence of mononuclear cell subsets occur during mastitis, associations with resistance to infection have been hard to demonstrate. Increased expression of CD11b and CD3 markers 1 d after experimental challenge was negatively associated with presence of *Staph. aureus* in milk 9–14 d later (Rivas et al. 2001). In contrast, increased numbers of CD8+ lymphocytes are associated with persistent *Staph. aureus* infections (Shoshani et al. 2000; Riollet et al. 2001), while no changes in the percentage of γδ+ cells were observed during chronic staphylococcal infection (Riollet et al. 2001). In the present study there were no apparent differences in the presence of CD4+, CD8+, MHCII+ and γδ+ cells in infected and control animals. However, CD5+ cells were present in higher numbers in the mammary glands still suffering from subclinical mastitis. CD5 is expressed on 98% of T cells (Mackay et al. 1985) and an IgM+ subpopulation of B cells (Cheavallier et al. 1998) in sheep. CD5+ lymphocytes have previously been noted to cluster around vascular tissue in the ovine mammary gland (Lee et al. 1989) and to increase in number in milk during mastitis (Ayoub et al. 1996). In the present study, the histological and immunohistological examinations were undertaken after a longer period of infection than in the studies mentioned above, and suggest that T cell responses to chronic infection were occurring in some glands. The routine histological findings confirmed the self-cure in ewe 2, while the remaining infected glands showed similiar alterations to those described by Burriel (1998).

In accordance with previous studies, subclinical mastitis was associated with a depressed milk yield (Fthenakis & Jones, 1990; Burriel, 1997; Leitner et al. 2001; Albenzio et al. 2002). Neutrophils entering the gland may contribute to decreased milk production by damaging secretory epithelial cells (Burvenich et al. 2000). In addition, a high SCC is usually accompanied by leakage of serum components from extracellular fluid into milk (Sheldrake et al. 1983) where hydrolytic enzymes from serum can further modify milk composition by breaking down casein (Grieve & Kitchen, 1985). In addition, variation between bacterial species in production of toxins may influence milk composition during mastitis. In the current experiment, the effects of group and the group-by-time interaction on milk constituents were not significant, indicating that changes in milk composition following challenge were moderate, agreeing with the results of Albenzio et al. (2002).

Although SCC and bacteriological examinations are regarded as the most reliable indicators of chronic mastitis, they can fail to diagnose subclinical infections. An aetiological agent is not always isolated from glands with high SCC, and conversely, isolation of a mastitis pathogen is not necessarily accompanied by an increase in SCC (Albenzio et al. 2002). Further work is required to identify strategies to enhance mammary immune defence against CNS. The present study highlights the importance of the initial host response in eliminating intramammary infections with CNS. IL-1β may hold promise as a therapeutic agent to prevent subclinical infections, which can cause changes in milk yield and quality.

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