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Staphylococcus aureus leucocidin, a virulence factor in bovine mastitis

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The involvement of *Staphylococcus aureus* exosecretions in bovine udder infection (Younis et al. 2003) suggests that four different monomer protein bands appearing between 36 and 31 kDa, are associated with the severity of the cow's infection response. Three out of these four bands have been identified by means of protein sequencing. Band B, with a MW of 35 kDa was identified as Panton-Valentaine leucocidin LukF'-PV chain- *Staph. aureus*; band C, with a MW of 32 kDa was identified as leucocidin chain LukM precursor- *Staph. aureus*; and band D was found to be similar, but not identical, to phosphatidylinositol-specific phospholipase-C-X. Bands B and C were purified by gel filtration using FPLC. The ability of these proteins to induce udder inflammation *in vivo,* and proliferation response *in vitro* and cytokine secretion were tested for both the crude exosecretions and purified bands. Three cows were inoculated intracisternally, with three quarters receiving either 0·007–0·008 mg (as total proteins) of *Staph. aureus* FR2449/1 bacterial exosecretion, pooled fraction 39–41 (bands B and C), or culture broth medium. The fourth quarter was left free as a control. Quarters that received fraction 39–41 of *Staph. aureus* FR2449/1, exhibited induced inflammation, which was indicated by increased somatic cell count and enhanced NAGase activity that was significantly higher than that of the original *Staph. aureus* FR2449/1 bacterial exosecretion. Proliferation tests of bovine blood lymphocytes *in vitro* showed that the pooled fraction 39–41 stimulated bovine proliferation of mononuclear cells much more than the original *Staph. aureus* FR2449/1 bacterial exosecretion. Secretion of TNF-α, IL-1β, IL-6 and IL-8 was in accordance with the contents of LukF'-PV and LukM precursor in the exosecretions. The results suggest that LukM/LukF' induce inflammation into the udder by a mechanism similar to that of LPS or by a unique mechanism(s) which requires further investigation.

Keywords: *Staphylococcus aureus,* leucocidin, mastitis, bovine.

Staphylococcus aureus mastitis is a major cause of economic losses to the dairy industry. The bacterium can cause clinical or subclinical mastitis, which leads to chronic disease in most incidences. In a previous study (Younis et al. 2000), hundreds of *Staph. aureus* strains, isolated from bovine mastitis in Israeli dairy herds, were phenotypically characterized and correlated with the severity of the disease. The findings revealed large differences among cow inflammation responses to the various strains, as measured by somatic cell count (SCC), as well as N-acetyl-β-D-glucosaminidase (NAGase) activity. These results suggested that the inflammation response was related to the bacterial exosecretions rather than the adherence molecule. Therefore, a further attempt was made to determine the influence of bacterial exosecretions on the severity of the cow's response by inoculating several different bacterial exosecretions into bovine mammary glands (Younis et al. 2003) and by testing the ability of those exosecretions to modulate bovine mononuclear cells *in vitro*

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(Leitner et al. 2002). These previous studies revealed that bacterial exosecretions that contained four protein bands (A,B,C,D) with molecular weights between 31 and 36 kDa caused more severe responses in the cow and activated greater proliferation of bovine mononuclear cells than did exosecretions devoid of these proteins. Moreover, differences in the killing effect [killing sensitivity by polymorphonuclear neutrophils (PMN) *in vitro*] were found among the various *Staph. aureus* isolates and a negative correlation was observed between killing effect and the expression of the two protein bands: B and C (unpublished observations) i.e., *Staph. aureus* isolates that identify with at least one of these bands were more resistant to killing than the isolates with no bands. *Staph. aureus* can produce several exotoxins with haemolytic and cytotoxic activities which may impair the phagocytosis and killing of the cells by these bacteria (Adlam et al. 1977; Rogolsky, 1979; Gemmell et al. 1982; Bhakdi et al. 1988). Moreover, *Staphylococcus* is capable of producing numerous extracellular proteins and exoenzymes such as coagulase (Aarestrup et al. 1994) which are associated with its pathogenicity and resistance to phagocytosis. *Staph. aureus* isolates from bovine mastitis can produce pyrogenic toxins (Bohach et al. 1990), enterotoxins (Zhang et al.1988; Kenny et al. 1993; Munson et al. 1998; Dinges et al. 2000; Akineden et al. 2002) which exhibit superantigenic activities and are capable of causing polyclonal activation and proliferation of various subpopulations of T lymphocytes. Staphylococcal Panton-Valentaine Leucocidin (PVL) (family leucotoxin) is one of these exotoxins, which is capable of selectively killing PMN cells (Blobel et al. 1980; Prevost et al. 1998; Lina et al. 1999). It is involved in various organ infections and causes tissue and cell necrosis, especially the destruction of leucocytes. This toxin comprises two classes of components – class F (Luk-F'-PV, Luk-F-I, Luk-M) and class S (Luk-S-PV, Luk-S-R, Luk-S-I) – and its toxicity depends on the synergic action of the two components with another group of *Staph. aureus* cytotoxins, best known as gamma-haemolysins (HlgA, HlgB and HlgC) (Pedelacq et al. 1999; Szmigielski et al. 1999). Therefore, the aims of the present study were to identify and to purify these four proteins and to examine their direct influence on the severity of the cows' response, as well as their ability to modulate the proliferation of bovine mononuclear cells *in vitro*.

Materials and Methods

Bacteria and bacterial exosecretions

Four strains of S*taph. aureus* isolates, FR2449/1; AU3133; AL32/4 and FR780/1, were isolated from cases of subclinical–chronic bovine mastitis (Younis et al. 2000). The isolates were identified with 1–4 protein bands with molecular weights between 31 and 36 kDa (Younis et. al. 2003): Bands A–D (FR2449/1), A and B (FR780/1) A and C (AU3133), and A (AL32/4). The isolate FR2449/1 was used as a source for the identification of the four protein bands. Bacterial exosecretion was harvested as described previously (Younis et al. 2003). In brief, *Staph. aureus* isolates were cultured by incubation in 100 ml of non-pyrogenic Columbia broth supplemented (Difco, Detroit MI, USA) with 0·1% D-glucose, yeast extract and 0·5% NaCl (CB), at 37 °C for 12 h. The crude supernatants, which included the exosecretions, were collected by centrifugation, filtered through 0·2-µm membranes and their protein concentrations were determined with a protein assay kit (Bio-Rad, Hemel Hempstead, UK).

Band identification and sequencing

One-dimension NuPAGE 10% Bis–Tris gel for small proteins (NOVEX-Electrophoresis, San Diego CA, USA) was used. Protein concentrations in the bacterial exosecretions were adjusted to 0·5 mg/ml by dilution with phosphate-buffered saline (PBS), pH 7·5. To 62 µl of the sample were added 25 µl of 4 × NuPAGE sample buffer, 3 µl of ultrapure water and 10 µl of reducing agent. The sample mixtures were heated for 10 min at 70 °C and loaded onto the gel (30 µl per lane). Unstained markers mixture (NOVEX Mark 12, wide range standard) with a molecular weight of 14·4–200 kDa was used. The gel was stained with silver (NOVEX-Electrophoresis). The four stained protein bands in the gel of isolate FR2449/1 were cut out with a clean razor blade, reduced with 10 mM-DTT and modified with 100 mM-iodoacetamide in 10 mM-ammonium bicarbonate buffer, pH 8. The gel pieces were treated with 50% acetonitrile in 10 mM-ammonium bicarbonate buffer (pH 8) to remove the stain from the proteins and then dried. The dried pieces were rehydrated with 10 mM-ammonium bicarbonate buffer (pH 8) containing about 0·1 g trypsin per sample, and were incubated overnight at 37 °C. The resulting peptides were recovered with 60% acetonitrile containing 0·1% trifluoroacetate. The tryptic peptides were resolved by reversed-phase chromatography on a 1 × 150-mm Vydac C-18 column, eluted with an 80-min linear gradient of 5 to 95% acetonitrile containing 0·025% trifluoroacetic acid in water at a flow rate of 40 µl/min. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ, Finnigan, San Jose CA, USA). Mass spectrometry (MS) was performed in the positive ion mode, with repetitive full MS scan followed by collision induced dissociation (CID) of the most dominant ion selected from the first MS scan. Mass spectrometry data were compared with data obtained by simulated proteolysis and CID of the proteins in the 'nr' database (NCBI) by means of Sequent software (J Eng and J Yates, University of Washington and Finnigan, San Jose).

Protein purification

Bacterial exosecretions (0·5 ml at a concentration of 0·5 mg/ml) of the four isolates were applied to a Superdex

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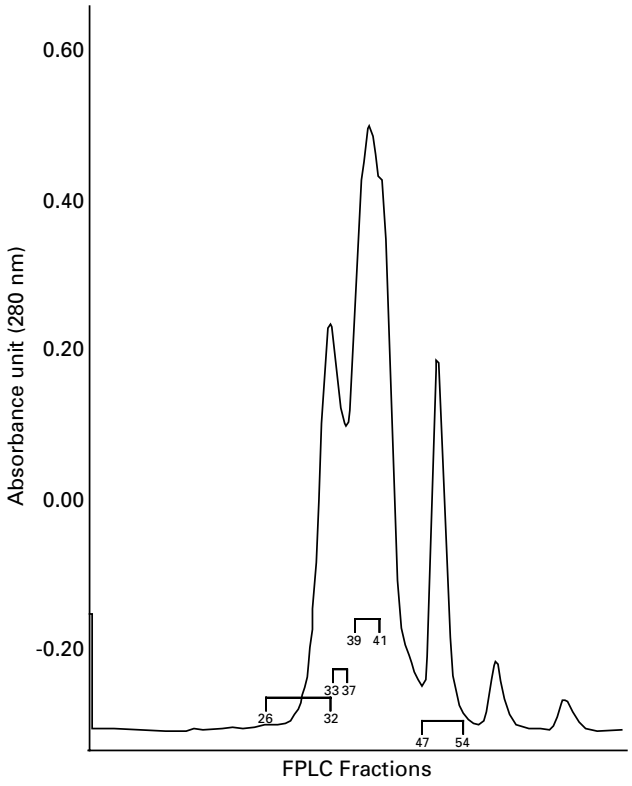


Fig. 1. Purification of *Staph. aureus* FR2449/1 bacterial exosecretions on a Superdex 200 gel filtration column using a FPLC System. The column was developed with 50% PBS at 0·5 ml/min, collecting 0·5 ml fractions. A u.v. detector monitored u.v. absorbance at 280 nm. Groups of fractions (26–32, 33–37, 39–41 and 47–54) were pooled and concentrated to protein contents of 0·5 mg/ml by a Speed-Vac concentrator.

200 (10 × 300 mm) gel filtration column using a FPLC System (Pharmacia, Uppsala, Sweden) equipped with a u.v. detector (280 nm), a conductivity meter and a fraction collector. The column was developed with 50% PBS at 0·5 ml/min, collecting 0·5 ml fractions. The results of *Staph. aureus* FR2449/1 bacterial exosecretion exhibit in Fig. 1. Groups of fractions (0–26, 26–32, 33–37, 39–41 and 47–54) were pooled and concentrated to protein contents of 0·5 mg/ml by a Speed-Vac concentrator (Savant Inc, Farmingdale NY, USA). The pooled fractions, containing 0·007–0·008 mg/ml protein, were loaded into NuPAGE 10% Bis–Tris gel (Fig. 2). Two bands (B and C) with molecular weights corresponding to those of exotoxins, Panton-Valentaine Leucocidin LukF′-PV chain-Staphylococcus aureus-35 kDa and Leucocidin chain LukM precursor- Staphylococcus aureus-32 kDa, were revealed in fractions 33–37 and 39–41; the other two bands (A and D) were not visualized. For the study *in vivo,* fraction 39–41 was chosen because it generated visually cleaner bands, with no other bands present, in contrast to the pooled fractions 33–37.

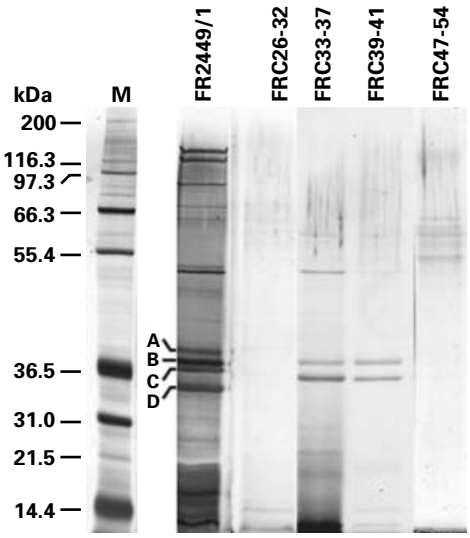


Fig. 2. NuPAGE 10% Bis–Tris gel of *Staph. aureus* FR2449/1 bacterial exosecretions and the combined fractions 26–32, 33–37, 39–41 and 47–54.

Studies in vitro

Proliferation assay. Bovine blood lymphocyte proliferation was determined *in vitro* by means of the ^{3}H-thymidine proliferation assay as previous described (Leitner et al. 2002). *Staph. aureus* bacterial exosecretion, and pooled fractions 26–32, 33–37, 39–41, and 47–54 were assayed in descending serial dilutions starting from 0·005 mg/ml total protein, with CB and Con-A used as a negative and positive controls, respectively. Results were expressed by an index, which was calculated as:

$$\frac{\text{Cpm exosecretion test} - \text{Cpm background}}{\text{Cpm Con A} - \text{Cpm background}} \times 100$$

Where Cpm is counts per min.
The assay was performed six times with blood samples from three different cows. The variations among the assays were small and insignificant (less than 1%).

Cytokine assay. IL-6 quantification was performed by ELISA according to the supplier's directions (BioSource International, Camarillo CA, USA), IL-8 and TNF-α assays were performed according to the supplier's directions (HyCult Biotechnology, Uden, The Netherlands), and IL-1β was assayed according to the manufacturer's directions (BD Bioscience, San Jose CA, USA). Whole blood from a human donor was collected in 10-ml vacutainer tubes with anticoagulant (heparin 5000 i.u./ml) and diluted 1 : 5 with RPMI 1640 (Biological Industries, Bet Haemek, Israel). To achieve maximum response, *Staph. aureus* FR2449/1 bacterial exosecretion was tested at serial dilutions: 1 : 50, 1 : 100, 1 : 1000, 1 : 10000 and incubation time of 0, 1, 3, 6, and 24 h. The highest cytokine levels were achieved with a dilution of 1 : 50 (0·001 mg/ml) at

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6 h, thus the assays were conducted at those parameters. A 40-µl sample of each of *Staph. aureus* bacterial exosecretions or pooled fractions: 33–37, 39–41, and 47–54 was added to 2 ml blood and incubated at 37 °C in 5% CO_2 for 6 h. A negative control (CB) and a positive control (lypopolysaccharide, LPS, 10 µg/ml) (*Escherichia coli* O55:B5; Difco) were tested in parallel to each assay. The assay was run in three consecutive repetitions. Plasma was separated by centrifugation at 500 ***g*** for10 min and was stored at −80 °C.

Layout of the study in vivo

Three Israeli-Holstein dairy cows in mid-lactation with milk yields >25 l/d (milked three times daily) were used. The cows that were chosen for the assay had all quarters free of bacteriology infection with SCC $<150\times10^3$ cells/ml and NAGase activity <10 units when tested in three consecutive daily examinations of quarter milk samples prior to the experiment. SCC was determined with a Coulter cell counter (Z1 model, Coulter Electronics Limited, Luton, UK) with a limit count of 5000×10^3 cells/ml and NAGase activity measured with a kit (ADC Applied Diagnostics Corporation, Helsinki, Finland). An NAGase value of 100 units corresponded to a release of about 5 µmol of product per l per min at 25 °C. On the day of the experiment, the cows were brought to the stalls 1–2 h after milking and their teats were washed with warm water, dried, disinfected with individual non-woven towelets, and moistened with chlorhexidine, cetrimide and ethanol (Medi-Wipes, AL Baad, Messuot Itzhak, Israel) after which the first three milk squirts were discarded and the teats were again disinfected. Each quarter of each cow was inoculated intracisternally with (in 5 ml pyrogen-free saline) 0·007–0·008 mg/quarter (as total proteins) of *Staph. aureus* FR2449/1 bacterial exosecretion, pooled fraction 39–41, CB, or left free as a control. The cows were monitored at 0, 4, 8, 12, 24, 48 h post inoculation (p.i.) for rectal temperature, heart and respiratory rates, alimentary tract activity (rumen contraction) and udder temperature, pain, oedema and udder size. Milk samples were collected according to the same schedule, for determination of bacteriological status, SCC and NAGase activity. NAGase activity and SCC measurements are presented as the pre-inoculation (time zero) level in each quarter subtracted from the value measured in each subsequent time p.i. and are referred to as Delta NAGase or Delta SCC, respectively.

Results

Band identification and sequencing

Of the four protein bands that were contained in bacterial exosecretions of FR2449/1 *Staph. aureus* isolates and which were associated with the increase of the cows' inflammation response in the mammary gland, only three bands (B, C and D) were identified (Fig. 2 lane 2). Band B, with a molecular weight of 35 kDa, was identified as Panton-Valentaine leucocidin LukF-PV LukF'-PV chain–*Staph. aureus*. Its position sequence (NCBI BLAST link) was identical to that of the Panton-Valentaine leucocidin amino acid sequence between 71 and 80 (SYDKDTLILK) and between 208 and 223 (DSSDSLYGNELFLGGR).

Protein sequence:

MKFKNIVKSS	VATSITLIML	SNTVDAAQHI
TPVSEKKVDD	KITLYAKTTAT	SDSDKLKISQ
ILTFNFIKDK	SYDKDTLILK	AAGNIYSGYT
QPTSDSSINS	QFYWGAKYNV	FVSSESKDSV
NIVDYAPKNQ	NEEFQVQQTL	GYSYGGDINI
INGLTGGLNG	SKSFSETINY	KQESYRTTID
RKTNHKSIGW	GVEAHKIMNN	GWGPYGRDSS
DSLYGNELFL	GGRQSSSNAN	QNFLPTHQMP
ILARGNFNPE	FISVLSHKQK	YQREMDRYEN
FWNNLHWIGY	NIKNQKRATH	TSIYEIDWEK
HTVKLVASQS	SE.	

Band C, with a molecular weight of 32 kDa, was identified as leucocidin chain LukM precursor–*Staph. aureus*. Its position sequence (NCBI BLAST link) was identical to that of the leucocidin chain LukM precursor amino acid sequence between 126 and 144 (NKIETVDVGQTLGYNIG GK) and between 247 and 260 (DSGDTSEFEITYGR).

Protein sequence:

MFKRKLLVTT	TPEQGSKATT	NAEDIGDDAE
VIKRTEDVSS	RKWGVTQNVQ	FDFVKDKKYN
KDLIIKMQG	FINSRTTFND	VKQNRANKRM
VWPFQYNIGL	TSKDQNTSLI	NYLPKNKIET
DVGQTLGYN	IGGKFQSVPS	IGGNGSFNYS
KSIKYSQKSY	VSEVEQQSSK	TIKWGVKANS
FVIAGHRWSA	YDELLFIRNT	TRGPNARDYF
VDDNELPPLI	TSGFNPSFIA	TVSHEKDSGD
TSEFEITYGR	NMDVTYATYL	PKLGLYPERK
HNEFVNRNFV	VKYEVNWKTY	EIKVKGHN.

Band **D**, with a molecular weight of 32 kDa, was identified as similar but not identical to phosphatidylinositol-specific phospholipase-C-X (PI-PLC-X).

Similarity to other bacterial PI-PLC and other similarity that were found in Domain X are conserved in different forms of PLC and are essential for catalytic activity.

Protein sequence: 311AA

MSGWHSAHA	SDSLSKSPEN	WMSKLDDGKH
LTEINIPGSH	DSGSFTLKDP	VKSVWAKTQD
KDYLTQMKSG	VRFFDIRGRA	SADNMISVHH
GMVYLHHELG	KLFDDKYYL	SAYPNETIVM
SMKKDYDSDS	KVTKTFEEIF	REYYYNNPQY
QNLFYTGSNA	NPTLKETKGK	IVLFNRMGGT
YIKSGYGADT	SGIQWADNAT	FETKINNGSL
NLKVQDEYKD	YYDKKVEAVK	NLLLKLKTDS
NKDNVYVNEL	SVASGGSDLS	TYNYSHINP
EIAKTIKANG	KARTGWLIVD	YAGYTWPGYD
DOVSEIIDSN	K.	

The 1-phosphatidylinositol phosphodiesterase chain peptides were located between 27 and 311. The domain X

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Table 1 Mean proliferation index of bovine mononuclear cells treated *in vitro* with the bacterial exosecretions and pooled fractions produced by four *Staph. aureus* strains at various dilutions starting from 0·005 mg/ml total proteins and CPM of positive (CON-A; 5 μg/ml) and negative (CB) controls

Values are means with SD for $n=18$

	Proliferation index[1] ± SD Total proteins, mg/ml		
Bacterium	0·005	0·0005	0·00005
FR2449/1	2·8±0·9	13·9±0·8	11·2±1·4
Fractions 26–32	<2	<2	<2
Fractions 33–37	7·9±0·2	13·5±0·3	9·5±0·2
Fractions 39–41	7·9±0·3	14·2±0·4	8·1±0·3
Fractions 47–54	<2	<2	<2
AU3133	11·1±1·2	8·2±0·8	3·8±0·5
Fractions 39–41	7·8±0·6	4·7±0·6	<2
FR780/1	12·9±0·3	8·3±1·0	3·7±0·5
Fractions 39–41	15·6±1·2	8·7±0·6	3·6±0·2
AL32/4	<2	<2	<2
Fractions 39–41	8·3±0·5	3·8±0·6	<2
		CPM	
Con-A (5 μg/ml)[2]		47 741±4975	
CB		3064±116	

[1] Index = [(Cpm bacterium test − Cpm background)/CpmCon A − Cpm background)] × 100
[2] Con-A and CB proliferation responses are given in counts per minute

was located between 27 and 176, the active site general base and general acid sites were located by similarity at amino acid residues 40 and 90, respectively.

Proliferation assay

Staph. aureus bacterial exosecretions of FR2449/1, AU3133 and FR780/1, but not that of AL32/4, stimulated bovine mononuclear cell proliferation (Table 1). Similarly, the pooled fractions 33–37 and 39–41 of *Staph. aureus* bacterial exosecretions FR2449/1 stimulated bovine mononuclear cell proliferation, whereas the pooled fractions 26–32, and 47–54 did not. Pooled fractions 39–41 of *Staph. aureus* FR780/1 stimulated bovine mononuclear cell proliferation in all three dilutions while that of AU3133 and AL32/4 stimulated proliferation only at the lowest dilution. Peak cell proliferation of *Staph. aureus* bacterial exosecretions and pooled fractions of FR2449/1 was achieved at a protein concentration of 0·0005 mg/ml, while with the other strains, it was achieved at a protein concentration of 0·005 mg/ml. Lower responses of FR2449/1 fractions in the concentration 0·005 mg/ml indicate a toxic effect.

Cytokine assay

No TNF-α, IL-1β, IL-6 or IL-8 cytokines were detected in plasma before (Time 0) and after 6 h of spontaneous release or stimulated with CB (Table 2). After 6 h of stimulation with LPS, high levels of all these four cytokines were measured in plasma. Secretion of TNF-α and IL-6 were detected in whole blood supernatants stimulated with bacterial exosecretions of *Staph. aureus* FR2449/1, AU3133 and FR780/1 but not with the one stimulated with the exosecretion of AL32/4 strain. IL-1β was detected only after stimulations with FR2449/1 and FR780/1 bacterial exosecretions while IL-8 was detected after stimulation with all four *Staph. aureus* bacterial exosecretions. Pooled fractions 39–41 of all four strains tested stimulated the cells to secrete TNF-α, IL-1β, or IL-8 cytokine in significantly higher ($P<0·005$) levels that that of the bacterial exosecretions. IL-6 was not tested in the supernatants stimulated with the pooled fractions. In comparing the levels measured in the pooled fractions of all *Staph. aureus* tested with that of the LPS, TNF-α was lower than that of IL-1β and IL-8 with no deferent (Table 2).

Table 2 TNF-α, IL-1β, IL-6 and IL-8 levels (pg/ml) in human whole blood supernatants stimulated for 6 h with different *Staph. aureus* bacterial exosecretions

Values are means with SD for $n=6$

Staph. aureus exosecretions	TNF-α	IL-1β	IL-6	IL-8
FR2449/1	50±11^{c}	54±8^{c}	754±98^{b}	107±21^{ab}
FR2449/1: 39–41	124±33^{b}	735±24^{a}	ND	312±10^{a}
AU3133	5±9^{d}	0	121±32^{c}	140±7^{ab}
AU3133: 39-41	39±10^{c}	474±18^{d}	ND	279±30^{a}
FR780/4	12±2^{d}	51±21^{c}	263±32^{c}	54±5^{b}
FR780/4: 39–41	66±1^{c}	535±11^{d}	ND	329±14^{a}
AL32/4	0	0	0	91±25^{b}
AL32/4: 39–41	76±9^{bc}	450±32^{d}	ND	237±36^{a}
Time 0″	0	0	0	0
Spontaneous release	0	0	0	0
CB	0	0	0	0
LPS	472±3^{a}	790±42^{a}	3031±57^{a}	292±72^{a}

[a-e] Values within a column without a common superscript differ significantly ($P<0·05$)

Study in vivo

The following systemic symptoms appeared in all three cows: tachycardia (70–80 beats/min), tachypnoea (78–96 breaths/min), elevated rectal temperature (39–41 °C) and low rumen activity (1–2 contractions/min) that appeared as early as 4 h p.i., peaked at 8–12 h and disappeared at 24 h p.i. Local symptoms in the udder, which included swelling, rigidity, pain and increased sensitivity and decreased milk yield, were recorded only in the quarters inoculated with *Staph. aureus* FR2449/1 bacterial exosecretion and with pooled fractions 39–41. The local symptoms appeared as early as 4 h p.i., peaked at 8–12 h and disappeared at 24–48 h p.i. No major changes were noticed in the control quarters that had been left free, or in those inoculated with CB, both of which maintained constant NAGase activity and SCC values throughout the experiment. In all the

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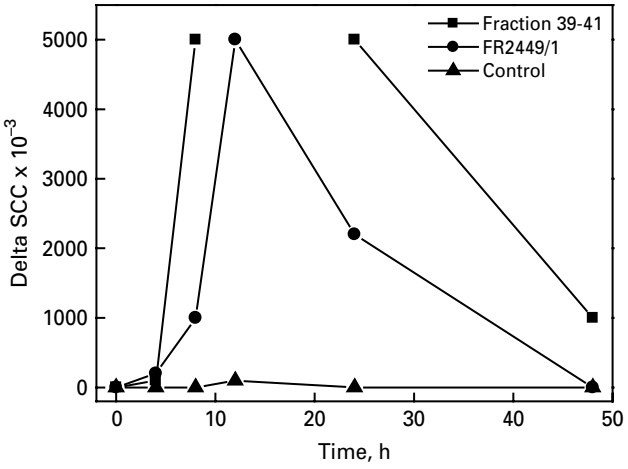


Fig. 3. Delta-SCC values: response to quarter inoculation with pooled fraction 39–41 (Band C), and *Staph. aureus* FR2449/1 bacterial exosecretions and values in non-inoculated quarter.

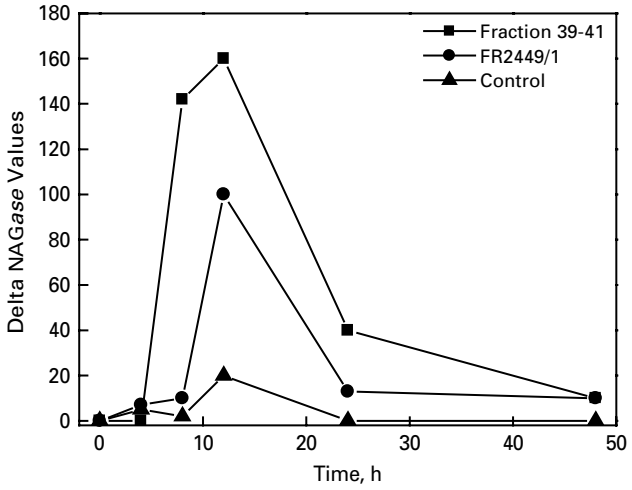


Fig. 4. Delta-NAGase values: response to quarter inoculation with pooled fraction 39–41 (bands B and C), and *Staph. aureus* FR2449/1 bacterial exosecretion, and values in non-inoculated control quarter.

quarters inoculated with bacterial exosecretions and pooled fraction 39–41, NAGase activity and SCC increased in parallel after 4 h p.i., peaked at 12 h and decreased to their original levels at 48 h p.i. However, the severity differed amongst individual cows. The increases in NAGase activity and SCC of the quarters inoculated with the fraction 39–41 in all three cows, were 2–3-times higher than those inoculated with the bacterial exosecretion, and they took longer to return to their original levels. Responses of SCC and NAGase in one cow are presented in Figs 3 and 4, respectively.

Discussion

Staphylococcal PVL produced by *Staph. aureus* as well as by *Staph. intermedius* (Prevost et al. 1998; Lina et al. 1999) are involved in infections of various organs, where they cause tissue necrosis and destruction of cells, especially leucocytes. Fewer than 5% of the *Staph. aureus* strains isolated from human infection produce SPVL (Lina et al. 1999). These toxins cooperatively lyse human and rabbit PMN and rabbit erythrocytes because of their pore-forming properties, and disrupt the ion exchange (Ca^{2+}, Na^{+}, K^{+}) via the calcium channel, finally leading to cell swelling and haemolysis (Siqueira et al. 1997; Nishiyama et al. 1998; Prevost et al. 1998; Staali et al. 1998; Sugawara et al. 1999). In an earlier study (unpublished observations) various species of staphylococcal exosecretions were tested in alteration phagocytosis by causing apoptosis and/or necrosis of bovine PMN. In that study, a positive association was found between killing sensitivity of the *Staph. aureus* strains and the bacterial exosecretion containing the protein bands B and C. Moreover, incubation *in vitro* for 60 min only with the bacterial exosecretion of those strains decreased the number and changed the morphology of remaining PMN, which expressed the annexin-V receptor. The involvement of these toxins in mastitis of cows, goats and ewes was studied based on the presence or absence of the genes encoding for LukM/LukF′-PV in 128 *Staph. aureus* isolates (Rainard et al. 2003). In that study, it was found that exosecretion of LukM-positive isolates were much more leucotoxic than LukM-negative isolates. In Israeli dairy herds, 40% of the *Staph. aureus* strains isolated from bovine mastitis (Younis et al. 2000) contain SPVL, F′ (band B) and M (band C) as inferred from protein sequences. It is possible, however, that these proteins were not detected in the exosecretions of some of the *Staph. aureus* strains used in the present study; although these strains carried the genes for SPVL, these genes did not express the leucocidins under the growth conditions used in this experiment. This would mean that the frequency of occurrence of these leucocidins might be even higher than indicated by the present results. Indeed, Bronner et al. (2000) who attempted to detect these genes encoding leucotoxins by RNA analysis, found that some of the isolates carried these genes.

The inflammation responses (SCC and NAGase activity) of the udder quarters to the purified LukM/LukF′ were clearly stronger than those to the *Staph. aureus* FR2449/1 bacterial exosecretions. Although the pooled fractions 39–41 did not represent absolutely pure LukM/LukF′, the relative amounts of these two proteins were higher than those in the crude bacterial exosecretions, indicating that the major trigger of the inflammation was LukM/LukF′. The mechanism by which endotoxin of Gram-negative bacteria, LPS induces migration of leucocytes in the presence of microorganisms is well documented (Tizard, 1996). The processes are mediated by proinflammatory agents such as LPS binding to acute phase protein and release TNF-α, (Shumann et al. 1990) or by agents produced by damaged tissue such as thrombin, histamine, IL-1, TNF-α,

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(Dinarello, 2000). The present results suggest that LukM/LukF′ induce inflammation in the udder by a mechanism similar to that of LPS or by a unique mechanism(s) which requires further investigation.

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