Mammary infection with *Staphylococcus aureus* in cows: progress from inoculation to chronic infection and its detection

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Summary. The progress of *Staphylococcus aureus* infection from inoculation to the early chronic stage was examined in 12 Israeli-Holstein cows (four primiparous and eight multiparous) for up to 48 d after inoculation. Before inoculation, the primiparous cows were free from any infection and the multiparous cows were infected by coagulase-negative staphylococci. Two quarters in each cow were inoculated intracisternally following milking with 2000 cfu of a local prevailing *Staph. aureus* strain, VL-8407. Infection was established in 21 out of 24 quarters. The control quarters remained free from infection during the study, with no significant change in function. No statistically significant differences were found between primiparous and multiparous cows in the responses examined. Somatic cell count (SCC) increased within 24 h of inoculation and remained high for the duration of the study. In the infected quarters mean ln (SCC) increased within 24 h from $9{\cdot}9 \pm 0{\cdot}5$ before inoculation to $13{\cdot}0 \pm 0{\cdot}2$ after inoculation; most of the cells were neutrophils. *N*-acetyl-β-glucosaminidase activity, expressed as ln (nmol/min per l), was increased from $0{\cdot}9 \pm 0{\cdot}6$ to $2{\cdot}4 \pm 0{\cdot}2$ by inoculation, and was highly correlated with SCC. The *Staph. aureus* count fluctuated with no particular relationship with SCC. The phagocytic activity of neutrophils was significantly lower in the inoculated than in the control quarters and this difference increased with time after inoculation. $CD8^+$ T lymphocytes were the main subpopulation of lymphocytes found in inoculated quarters. After inoculation, maximum but not minimum electrical conductivity (EC) recorded during milking increased significantly. The rises in maximum EC varied significantly among cows. The rises in SCC were associated with a persistent increase in EC in only one of the eight cows examined. No clinical signs were observed, and milk yield and composition were not affected during the study period. The results suggest that some strains of *Staph. aureus* may induce a relatively mild response in mammary glands of cows in mid lactation, and that the concomitant development of such chronic *Staph. aureus* infections in two quarters may not be detected by changes in the EC of composite milk and in the yield of the cow.

Chronic subclinical mastitis causes major economic losses in dairy cattle, due to damage to alveolar tissues, impairment of alveolar function, reduction in milk yield and deterioration in milk composition (Gudding *et al.* 1984; Nickerson, 1989).

Staphylococcus aureus is a principal pathogen causing this form of mastitis. Chronic subclinical infection may be detected by yearly quarter bacteriological examinations and monthly somatic cell counts (SCC) in composite milk, as practiced in the mastitis monitoring program in Israel. The bacteriological and SCC tests are not sufficiently frequent to detect an infection soon after the pathogens' penetration into the gland. The efficiency of antibiotic therapy in curing chronic infections during lactation or even dry periods is debatable (Soback *et al.* 1990). It is possible that the cure rate might be improved if infections could be detected before they evolve into the chronic form.

Infections may induce the appearance of uncommon compounds in milk or alter the concentrations of normal constituents. The concentration of electrolytes increases in intramammary infections, resulting in a rise in the electrical conductivity (EC) of the milk (Linzell & Peaker, 1971; Kitchen, 1981). The EC of milk could be measured at each milking using on-line milking systems, perhaps offering a way of detecting infections at an early stage. The value of on-line EC measurement during milking for the detection of mastitis has been examined in several studies in which EC has been measured in individual quarters (Fernando *et al.* 1985; Nielen *et al.* 1995). Detection of subclinical mastitis based on EC alone is of high specificity but of low sensitivity (Maatje *et al.* 1992; Lansbergen *et al.* 1994). The detection sensitivity may be improved by also taking account of changes in milk yield (Nielen *et al.* 1992; Shoshani & Berman, 1998).

The efficiency of mastitis detection might be improved if the sequence of signs from the pathogens' entry to their establishment in the gland were known. The events following inoculation with *Staph. aureus* have been frequently described. Inoculation is followed by elevated SCC, mostly polymorphonuclear cells, rises in *N*-acetyl-β-glucosaminidase (NAGase), phagocytosis and bacterial death (Newbould & Neave, 1965; Niemialtowski *et al.* 1988; Nickerson, 1989; Paape *et al.* 1991; Kehrli & Shuster, 1994). A cyclic pattern of the number of viable bacteria shed in milk is evident, irrespective of inoculum dose (Sears *et al.* 1990). This cyclic shedding of bacteria is accompanied by an inverse cycle in polymorphonuclear cell (PMN) numbers (Daley *et al.* 1991). It is noteworthy that changes in phagocytic capacity are not necessarily accompanied by parallel changes in bactericidal capacity (Daley *et al.* 1991). The survival and multiplication of *Staph. aureus* are attributed to a reduction in the phagocytic activity of PMN in milk (Craven & Williams, 1985; Niemialtowski *et al.* 1988). Inoculation with *Streptococcus uberis* or *Staph. aureus* results in increased EC of fore milk samples at one milking after challenge, but this increase is observed in composite udder milk only after four milkings (Hillerton & Walton, 1991). A computerized milking system (Afimilk, SAE, Afikim 15148, Israel), which records the highest EC measured during milking in composite milk, issues warnings on the probable occurrence of mastitis events on the basis of combined changes in both milk EC and milk yield.

This study examined the progress of infection by a local strain of *Staph. aureus* from inoculation to the early chronic stage and the capacity to detect the onset of infection in two glands by deviations in composite milk production and EC.

Materials and methods

Animals

Israeli-Holstein cows in mid lactation (four primiparous and eight multiparous) in which SCC was < 70000 cells/ml in each of the three monthly tests preceding the

inoculation date were selected from commercial herds. Quarter samples were cultured for bacteriological tests weekly for 3 weeks, and on 3 consecutive days before inoculation. Cows selected for the study were free from infection or were infected by only a minor pathogen (coagulase-negative staphylococci). From 3 weeks before inoculation the cows were kept in an open shelter providing for each cow 10 m^2 shaded slatted floor and 10 m^2 concrete surfaced yard at the experimental dairy herd of the Agricultural Research Organization Center, Bet Dagan 25250, Israel. Food was offered *ad lib*. in mangers located in the sheds.

Milking system

Cows were milked three times daily at 8 h intervals with a computerized milking system (Afimilk) in which cows are identified automatically and composite milk yield, its EC and milking times are recorded at each milking. Composite milk yield is measured volumetrically by counting the number of 200 ml ($\pm 2\%$) fractions passing through the milk meter and the hourly milk production rate (MPR) for each milking interval computed. Stainless steel electrodes located in the measuring cell sense EC ($\pm 1\%$ in mS) in each milk fraction passing through it and the highest value is recorded. Warnings of potential mastitis are issued if a combination of two deviations in MPR and in EC occur in three consecutive milkings. The deviations involved are: a fall in MPR of $> 20\%$ of the mean of nine preceding milkings at same time of day, and an EC increase of $> 10\%$ of the mean of nine preceding milkings at same time of day. Two consecutive deviations in MPR are not considered to indicate a mastitis event.

Bacteriological examination

Duplicate samples of quarter fore milk were taken aseptically according to International Dairy Federation (1981) and bacteriological tests performed according to National Mastitis Council protocols (Barnes-Pallesen *et al*. 1987). A portion (0·01 ml) from each milk sample was spread over blood agar plates (Bacto-Agar; Difco, Detroit, MI 47202, USA) containing washed sheep red blood cells (5 g/l), and over MacConkey agar plates. The minimal detection limit was 5 cfu in pure culture. *Staph. aureus* numbers after inoculation were counted by serial dilution in phosphate-buffered saline (PBS, 0·01 M-sodium phosphate–0·138 M-NaCl), and plating on solid blood agar for enumeration of viable bacteria.

Inoculation procedure

The cows were inoculated with the *Staph. aureus* strain, VL-8407, that prevailed in the experimental herd and caused mostly subclinical mastitis cases. Two diagonally opposing quarters in each cow were inoculated intracisternally at the end of milking with 2000 cfu/ml pyrogen-free saline. The teat end was cleaned and disinfected with alcohol (700 ml/l) before inoculation. The inoculation was carried out on diagonally opposite quarters to reduce the risk of cross contamination during milking. The other two quarters in each cow served as controls.

Freeze-dried *Staph. aureus* VL-8407 stock culture (1 ml) in tripticase–soya broth (TSB) was thawed at room temperature, added to 6 ml TSB and incubated overnight at 37 °C. A portion (0·1 ml) of the latter culture was added to 9·9 ml TSB and titrated serially to a 10^{-7} dilution. Samples (0·1 ml) of each of the dilutions between 10^{-5} and 10^{-7} were spread over a blood agar plate and incubated overnight at 37 °C. During this procedure the original stock culture was kept at 5 °C. On the day of inoculation, the culture was diluted in pyrogen-free saline to the specified dosage. From that

culture, 0·1 ml was plated in triplicate on a blood agar plate to determine the actual bacterial count.

Clinical examination

Clinical examinations were carried out daily from 3 d before inoculation, every 4 h on the day of inoculation and daily until 5 d after inoculation. Rectal temperature, respiratory rate, clinical mastitis score (on a scale of 7–35; Anderson *et al.* 1986), alimentary tract activity, and cows' behaviour were monitored. Jugular blood samples were taken into sterile vacutainer tubes containing heparin for determinations of haematocrit, leucocyte count, sodium, calcium, phosphate, urea, creatinine, glutamic–oxaloacetic transaminase (EC 2.6.1.1) and total plasma protein. The chemical analysis was carried out with an automated analyser (Selective Chemistry Analyzer; Kone, FIN-02100 Espoo, Finland).

Antibodies and conjugates

The monoclonal antibodies (mAb) used for the detection of different milk cell subpopulations (from VMRD Inc., Pullman, WA 99163, USA) were: anti-lymphocyte, CACT 138A anti-CD4 (Th1), CACT 80C anti-CD8 (Tc/s) and BAS9A anti-B, all diluted 1:500; anti-monocyte/macrophage, CH137 used with the primiparous cows and BAQ151A used with the multiparous cows, diluted 1:500; and anti-neutrophil, CH138A diluted 1:100. All mAb were species-reactive with bovine cells.

The polyclonal antibody used was goat F(ab′)2 anti-mouse IgG (H + L) conjugated with fluorescein isothiocyanate (1:100 dilution), which exhibited minimal cross reaction to human, bovine and horse serum proteins (Jackson Immuno Research Laboratories, West Grove, PA 19390, USA).

Laboratory procedures

Milk samples were kept on ice for ~ 2 h before processing. Concentrations of fat, protein and lactose were measured with a Milkoscan (Foss Electric, DK-3400 Hillerød, Denmark) and SCC were determined to ± 10 % with a Fossomatic 360 (Foss Electric) at the Israel Dairy Herd Laboratory (Bitan Aharon 38900, Israel). SCC were also measured by flow cytometry (Becton Dickinson, San Jose, CA 95131, USA). Differential leucocyte counts were carried out by both light microscopy and flow cytometry. Sodium and potassium concentrations were determined by flame photometer (model 400; Ciba Corning Diagnostics, Halstead CO9 2DX, UK) and chloride with a chloride analyser (model 926; Ciba Corning Diagnostics), all to ± 0·5 %. NAGase activity was determined by the method of Kitchen (Kitchen *et al.* 1980; Kitchen, 1981) as modified by Mattila & Sandholm (1985). EC of milk samples were measured at 20 °C using a portable conductivity meter (Model HI 873; Hanna Instruments, El-Rom 12466, Israel).

Differential leucocyte count

Light microscopy procedure. Differential cell counting was carried out by light microscopy as follows. The milk sample (10 ml) was centrifuged in a conical tube at 500 ***g*** and 4 °C for 10 min; the fat layer and supernatant were discarded and the pellet was dispersed in 1 ml PBS. Cell concentration and viability were determined by mixing 10 μl of this solution with 10 μl trypan blue (0·2 g/l) and counting stained cells in a haemacytometer. PBS was then added to the cell suspension to produce a final concentration of 5×10^5 cells/ml. Cell viability was > 90 % in practically all

samples. Cell suspension (0·1 ml) was mixed with 0·1 ml TSB (0·2 g/l) and 400μl of the final suspension was centrifuged in cytospin tubes at 150 ***g*** for 20 min. Differential count of cells was carried out after staining by a modified Wright Giemsa stain. Absolute numbers of PMN, macrophages, lymphocytes, eosinophils and epithelial cells were calculated from the total cell count determined by the Fossomatic counter.

Flow cytometry procedure. Milk samples were kept at room temperature and analysed not > 3 h after milking. To calculate the volume of milk needed for a cell concentration of $\sim 1 \times 10^6$ cells/ml, 1 ml whole milk was diluted 1:2, 1:4 and 1:8 in PBS and read at the following settings. Forward-angle light scatter was set at 1 with threshold controls at 200. Logarithmic transformations were used for the side-light scatter and green fluorescence. The number of events at each dilution was read at a flow rate of 60 μl/min, multiplied by 16·7 and by the dilution factor, and the mean value for the three dilutions was used to calculate the cell count.

To assess the different types of leucocytes, milk samples containing $\sim 1 \times 10^6$ cells/ml were distributed into one tube for each mAb plus a control. The samples were centrifuged (230 ***g***, 4 °C, 10 min), the fat removed by aspiration, the supernatant discarded and the pellet resuspended in 15 ml PBS and washed by centrifugation once more (230 ***g***, 4 °C, 5 min). To each tube, 50 μl of one of the mAbs or PBS (as negative control) was added, mixed gently and incubated at 4 °C for 1 h. Cells were washed in PBS and 50 μl of the conjugated antibody was added, mixed gently and incubated at 4 °C for 30 min. After incubation, the cells were washed and resuspended in PBS to a final volume of 1 ml. To calculate the proportions of the different leucocytes, 10000 events were read per sample. The cursor was set such that only 2 % of the events in the control were to the right of it. In samples labelled with mAb, the percentage of events to the right of the cursor was considered positive. Cell numbers for each leucocyte type were calculated from SCC determined by Fossomatic counter. In addition, total lymphocytes and PMN were evaluated by gating the cells on a dot–plot forward-angle light scatter and by side-light scatter map (Miller *et al*. 1993).

Phagocytic activity

After 24 h incubation in neutral broth the *Staph. aureus* culture was washed by centrifugation (1000 ***g***, 4 °C, 15 min), labelled with fluorescein isothiocyanate (0·5 mg/ml) at 4 °C for 1 h and then washed three times with PBS. The labelled *Staph. aureus* suspension was added to 1×10^6 milk leucocytes at a cell:bacteria ratio of 1:10, PBS was added to a final volume of 1 ml and the mixture incubated at 37 °C. After 1, 10, 30 and 60 min, samples (200 μl) were taken and the reaction was stopped by adding ice cold PBS. The sample was washed three times with ice cold PBS, resuspended in 2 ml PBS and divided into two. One portion was stored at 4 °C and used for estimating the number of both adhered and phagocytosed bacteria, the other for estimating the number of adhered bacteria. To quench the adhered bacteria, 1 μg ethidium bromide was added and the mixture incubated for 20 min. The excess ethidium bromide was removed by washing the cells twice in ice cold PBS and the cells were resuspended in 1 ml PBS and kept at 4 °C until analysis. The proportions of PMN with both adhered and phagocytosed bacteria and PMN containing only phagocytosed bacteria were calculated from the flow cytometry cell counts for the first and second samples respectively. Estimations were carried out at four 4 weekly intervals after inoculation.

Study layout

Quarter milk sampling was more frequent, analyses of samples more detailed and observations extended over a longer period for the primiparous than for the multiparous cows to provide a more detailed and extended view of the establishment of a chronic infection.

In the primiparous cows, quarters were milked individually by a quarter milking machine on 23 occasions from 18 d before to 48 d after inoculation: twice weekly for 3 weeks before inoculation, three times during the first week after inoculation, and twice weekly thereafter for 7 weeks. A sample (250 ml) of the composite milk from each quarter was taken on each such milking for determination of EC, NAGase, SCC, differential cell count, phagocytic activity, contents of fat, protein and lactose, and concentrations of sodium, potassium and chloride. The relative yields of inoculated and control quarters were recorded.

With the multiparous cows, quarter milk samples were taken three times in the week before and in the 2 weeks after inoculation. In these samples fat, protein, and lactose contents, NAGase activity, and total and differential cell counts were determined. In the primiparous cows both flow cytometry and light microscopy were used for differential leucocyte counts, while only flow cytometry was used for the multiparous cows.

Results obtained at observation times identical in the primiparous and the multiparous cows, i.e. on days −6, −3, 0, 1, 4, 6, and 12, were analysed using SAS general linear model procedures and type III mean squares (SAS, 1990, 1997). The distributions of SCC and NAGase values were normalized by transformation to their natural logarithms. Separate statistical analyses were carried out for results from the pre-inoculation and post-inoculation periods. The analysis concentrated on the effects of inoculation, day and inoculation × day interactions. The effects of parity on these terms was not significant, and is therefore not included in the analyses presented here. Contrasts were used to estimate significance of differences.

The model used was

$$Y_{ijkl} = \mu + C_i + I_j + I_j D_k + Q_m(I_j C_I) + e_{ijkl},$$

where Y_{ijkm} is the variable within cow, inoculation, quarter and day, C_i the cow class effect, I_j the inoculation class effect, D_k the day class effect, $I_j D_k$ the inoculation × day interaction effect, $Q_m(C_I I_j)$ the quarter within cow inoculation error term for inoculation effect and e_{ijkl} the residual error.

This statistical model yielded results for statistical significance closely similar to those using a repeated measures analysis of variance model. The same model was used for analysis of results that related only to primiparous cows, i.e. phagocytic activity, and concentrations of sodium, potassium and chloride.

The MPR for each milking interval and the maximum EC of milk recorded during each milking from 10 d before to 10 d after inoculation in multiparous cows were used to assess their potential for detecting infection. Minimum EC, maximum EC and MPR were analysed by a model similar to the one above, with day as repeated measures.

Results

MPR, milk composition and SCC of inoculated and control quarters did not vary significantly before inoculation. *Staph. aureus* was detected 24 h after inoculation in seven out of eight inoculated quarters in the primiparous cows and in 14 out of 16

Table 1. *Numbers of somatic and polymorphonuclear cells, T lymphocytes bearing CD8⁺, total lymphocytes, macrophages, eosinophils and epithelial cells in the milk from control and inoculated quarters of dairy cows before and after inoculation with* Staphylococcus aureus *VL-8407*

(Values are ln (no. of cells), least square means ± SEM)

	Control quarters		Inoculated quarters	
Cell type	Before	After	Before	After†
Somatic	9·9 ± 0·5	10·6 ± 0·3	9·9 ± 0·5	13·0 ± 0·1
Polymorphonuclear	8·3 ± 0·8	9·1 ± 0·4	8·4 ± 0·7	12·0 ± 0·2
Lymphocytes	7·8 ± 0·6	8·1 ± 0·4	7·9 ± 0·7	10·3 ± 0·2
T lymphocytes with CD8⁺	5·9 ± 0·6	7·6 ± 0·2	6·4 ± 0·7	9·4 ± 0·3
Macrophages	8·1 ± 0·4	8·1 ± 0·3	7·8 ± 0·4	10·5 ± 0·2
Eosinophils	7·0 ± 0·2	7·7 ± 0·2	6·8 ± 0·2	9·0 ± 0·2
Epithelial ‡	9·0 ± 0·1	9·6 ± 0·1	9·1 ± 0·1	11·0 ± 0·1

† Cell counts in this column were significantly different from those in other columns: $P < 0{\cdot}01$.
‡ Means are for epithelial cells found in the primiparous cows only.

in the multiparous cows, and in all subsequent milk samples. The shedding of *Staph. aureus* in milk varied significantly between quarters, as well as between days within quarters ($P < 0{\cdot}05$). The control quarters remained free from infection during the study and there were no consistent differences in SCC. During the study we observed no acute clinical signs, neither were there any effects of inoculation on body temperature, respiratory rate, haematological values, general behaviour, alimentary tract activity or daily milk yield. In the primiparous cows, the ratio of milk produced by the inoculated quarters to that produced by the control quarters was not significantly different before and after inoculation ($1{\cdot}05 \pm 0{\cdot}07$ *v.* $1{\cdot}14 \pm 0{\cdot}04$), neither was there any indication of change during 48 d after inoculation.

In the inoculated quarters ln (SCC) rose from $9{\cdot}9 \pm 0{\cdot}5$ before inoculation to $13{\cdot}0 \pm 0{\cdot}2$ at 24 h after inoculation and fluctuated moderately throughout the remainder of the study. It was significantly higher ($P < 0{\cdot}01$) than the value for control quarters by 1 d after inoculation and remained so for the duration of the study. There was a high correlation ($r = 0{\cdot}91$, $P < 0{\cdot}01$) between ln (NAGase) and ln (SCC).

The correlations between PMN and total lymphocytes determined by mAb reading with flow cytometry and by haemacytometer count were 0·93 ($P < 0{\cdot}001$) and 0·94 ($P < 0{\cdot}01$) respectively. The subpopulations of lymphocytes were determined by flow cytometry. Macrophage number was determined by haemacytometer count only, as the mAb used in these animals (CH137) was inadequate for labelling milk macrophages, though it had previously been found to label monocytes from blood successfully. The poor binding of this mAb by milk macrophages may have been due to a change in affinity during diapedesis. In multiparous cows, milk macrophages were determined by mAb BAQ151A.

The number of cells as determined by flow cytometry or haemacytometer count and calculated from the Fossomatic count are presented in Table 1. The increase in SCC was mostly due to PMN cells. At 4 d after inoculation, the PMN counts were 13- and 26-fold (primiparous and multiparous cows respectively) those before inoculation and they remained high throughout the study. PMN number fluctuated markedly between days within quarters as well as between quarters on a particular day. Most PMN had three to five lobes; the degree of lobulation did not change during the observation period.

Table 2. *Percentage of polymorphonuclear cells* (*PMN*) *from composite milk samples that internalized* Staphylococcus aureus *VL-840* in vitro *at different times after inoculation of the mammary glands with this strain*

(Values are least square means ± SEM)

Time after inoculation, d	Control	Inoculated
16	28·2 ± 4·2	24·7 ± 8·7
36	19·8 ± 2·9	12·2 ± 3·9
48	42·3 ± 8·7	10·8 ± 5·9
60	16·2 ± 13·1	9·4 ± 4·9
Mean	26·2 ± 3·1	14·7 ± 3·2*

* Significantly different from value for control quarters: $P < 0{\cdot}05$.

Inoculation increased the number of lymphocytes, owing to the elevation in SCC, but as a proportion of SCC they decreased by ~ 50 % (Table 1). The T cells bearing $CD8^+$ constituted the main subpopulation of lymphocytes in milk; the number of B lymphocytes and $CD4^+$ bearing T lymphocytes was negligible. However, not all the cells identified as lymphocytes were labelled with mAb; the proportion of unlabelled cells was not significantly different between control and inoculated quarters, neither did it change significantly during the study period. Macrophages increased only in the inoculated quarters, although their relative proportion declined. Epithelial cells, examined in the cytospin prepared slides, were present in high numbers only in primiparous cows. The high proportion of epithelial cells found is open to question, however, owing to the difficulty in distinguishing between macrophages and epithelial cells by light microscopy (Kitchen, 1981). Eosinophils increased in the inoculated quarters but their proportion remained negligible.

Phagocytic activity was measured four times, on days 16, 36, 48 and 60 after inoculation. The proportion of neutrophils with adhered and internalized bacteria after being exposed to *Staph. aureus* organisms *in vitro* was not significantly different between inoculated and control quarters. However, the proportion of neutrophils that contained internalized bacteria was significantly lower in the inoculated than in the control quarters ($P < 0{\cdot}05$). There was a trend for the difference between inoculated and the control quarters to increase with time after inoculation ($P < 0{\cdot}07$; Table 2).

Milk yield and composition

The effects of inoculation on the milk yield of control and inoculated quarters and on electrolyte concentration, NAGase activity and EC in the fore milk of primiparous cows was examined, and was significant only for Na concentration and NAGase activity ($P < 0{\cdot}01$, Table 3).

Infection and electrical conductivity

The feasibility of identifying the onset of infection in two quarters by on-line measurement of EC in composite milk and changes in MPR was examined only for multiparous cows, as the results for primiparous cows were lost owing to computer disk problems. During the 9 d preceding inoculation, minimum and maximum EC of milk were not significantly different at the three milking times (Table 4). Minimum EC varied from day to day before inoculation, though not significantly or very markedly ($P < 0{\cdot}12$ between days). In contrast, both maximum EC and MPR varied significantly between days during this period ($P < 0{\cdot}01$). There was no pattern to the

Table 3. *Concentrations of NaCl and* N*-acetyl-β-glucosaminidase activity in the milk from control and inoculated quarters of primiparous cows before and after inoculation with* Staphylococcus aureus *VL-8407*

(Values are least square means ± SEM)

	NaCl concn, mM		*N*-acetyl-*β*-glucosaminidase activity, ln (nmol/min per l)	
	Control	Inoculated	Control	Inoculated
Before	27·2 ± 0·5	26·5 ± 0·5	2·1 ± 1·4	2·6 ± 1·4
After	25·8 ± 0·3	27·7 ± 0·3*	1·1 ± 0·7	6·5 ± 0·7*

* Values were sigificantly different from corresponding control values: $P < 0·05$.

Table 4. *Minimum and maximum electrical conductivities of milk and hourly milk production rate of dairy cows at the three daily milkings during the 9 d before inoculation with* Staphylococcus aureus *VL-8407*

(Values are least square means ± SEM)

	Electrical conductivity, mS/10 mm		
Milking time	Minimum	Maximum	Milk production rate, kg/h
Morning	4·01 ± 0·06	4·64 ± 0·01	0·71 ± 0·03
Noon	4·07 ± 0·07	4·69 ± 0·01	0·71 ± 0·04
Evening	4·02 ± 0·06	4·69 ± 0·01	0·87 ± 0·03**

** Significantly higher than the values for the other two milking times: $P < 0·01$.

Table 5. *Minimum and maximum electrical conductivities of milk and hourly milk production rate of dairy cows at the three daily milkings during the 9 d before and the 9 d after inoculation with* Staphylococcus aureus *VL-8407*

(Values are least square means ± SEM)

	Electrical conductivity, mS/10 mm		
Period	Minimum	Maximum	Milk production rate, kg/h
Before	4·04 ± 0·01	4·67 ± 0·19	0·75 ± 0·03
After	4·05 ± 0·01	4·88 ± 0·18**	0·79 ± 0·04

** Significantly different from value before inoculation: $P < 0·01$.

variation in maximum EC, but there was a slight increase in MPR during the period preceding inoculation. Before inoculation there was a significant partial correlation between minimum and maximum EC ($r = 0·653$, $P < 0·01$), but not between minimum or maximum EC and MPR. It is noteworthy that MPR was higher in the interval between noon and evening milking times. However, neither minimum nor maximum EC was affected by the higher MPR during the afternoon milking interval.

The mean maximum EC for the 9 d observation period after inoculation increased by 3·2 % ($P < 0·01$), while minimum EC remained practically unchanged (Table 5). The response of minimum and maximum EC to inoculation was not affected by the time of day at which they were measured, as indicated by the non-significant time × inoculation interaction. The slight trend for increasing MPR before inoculation continued after inoculation ($P < 0·06$). We found no significant partial correlation between maximum and minimum EC and MPR in the 9 d after inoculation. The differences in mean maximum EC before and after inoculation

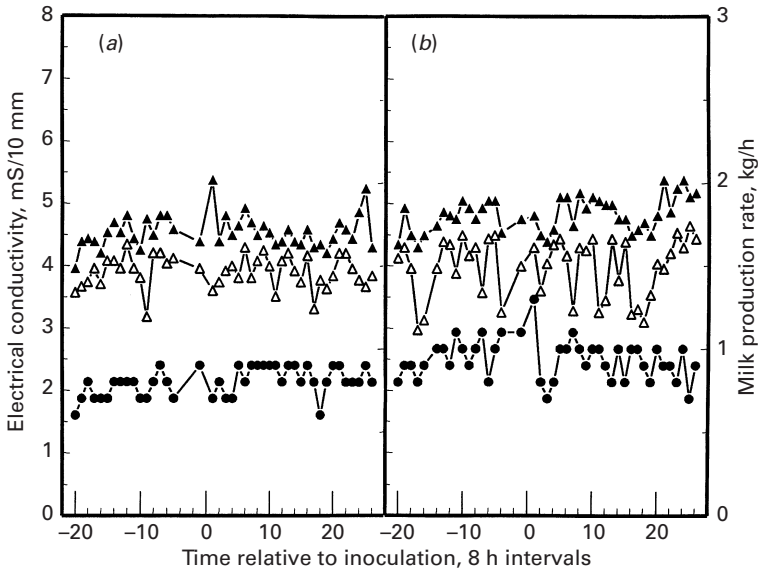

Fig. 1. ▲, Maximum and △, minimum electrical conductivity (EC) recorded by an automated procedure in composite milk during milking and ●, hourly rate of milk production at 8 h intervals of two dairy cows before and after inoculation with *Staphylococcus aureus*. (*a*) In this cow there was a persistent increase in maximum EC with no effects upon minimum EC and milk production rate; (*b*) in the other cow, there was a transient rise in maximum and minimum EC and a temporary fall in milk production after inoculation.

varied significantly between cows ($P < 0{\cdot}01$), from -4 to $+19{\cdot}6\,\%$. The rise in maximum EC after inoculation was associated with a larger variation in maximum EC, as shown by the correlation between the individual increments in mean maximum EC and their respective SD ($r = 0{\cdot}75$, $P < 0{\cdot}01$). However, this was not true for minimum EC or MPR. Inoculation did not alter the relationships among mean maximum EC, mean minimum EC and MPR at the three milking times.

In all cows increased SCC concentrations ranging from $0{\cdot}9 \times 10^6$ to 17×10^6 cells/ml were reached between days 1 and 13 after inoculation. In all but one cow, these rises were not associated with a persistent increase in the maximum EC of composite milk. In this individual the persistent increase in maximum EC and the high SCC (17×10^6/ml) were not accompanied by a fall in MPR (Fig. 1*a*). In another cow, there was a transient rise in maximum EC and a temporary reduction in MPR after inoculation (Fig. 1*b*). In these two cases, but not in the other cows, the infection that followed inoculation produced deviations in EC and milk yield that were detected by the milking system. In three other cows inoculation was followed by a small transient rise in maximum EC. In one cow no change was observed in EC and only a very transient decrease in MPR.

DISCUSSION

Challenging healthy mammary glands of cows in mid lactation with 2000 cfu *Staph. aureus* VL-8407 was followed by development of chronic subclinical mastitis, as indicated by shedding of the organisms over a 50 d period. No clinical signs were detected in the inoculated glands after inoculation. Although two glands were inoculated in each cow, this did not result in any detectable fall in milk yield or change in composition. Quarter milk composition and yields of infected quarters,

measured as the relative proportions of milk from inoculated and control quarters, remained unaffected over 50 d. These results disagree with other reports (e.g. Kitchen, 1981); perhaps our strain was less virulent, or mid-lactation cows are more resistant.

Strain and number of organisms inoculated may play a role in these different responses. Clinical mastitis was not reported after cows were inoculated with 20–600 cfu *Staph. aureus* Newbould 305 or 0053 (Heald, 1979; Nickerson & Heald, 1981; Schultze & Paape, 1984; Daley *et al.* 1991; Hillerton & Walton, 1991; Schukken *et al.* 1994). Inoculation of 40–60 cfu *Staph. aureus* Newbould 305 induces a rise in SCC only after 4–13 d (Erskine *et al.* 1990). Tissue damage in cows inoculated with 100 cfu *Staph. aureus* Newbould 305 does not increase significantly 3–8 d after inoculation (Gudding *et al.* 1984). The secretory potential of mammary tissue of cows inoculated with 240 cfu *Staph. aureus* Newbould 305 falls 2–10 d after inoculation, with large variations in the extent of secretory tissue impairment (Nickerson & Heald, 1982). Mild clinical mastitis develops following inoculation of 5000 cfu *Staph. aureus* Newbould 305 (Heald, 1979) or 1000 cfu *Staph. aureus* 0053 (Milner *et al.* 1996). Thus, it is probable that the particular strain and the number of organisms inoculated may explain the absence of clinical mastitis in the present study.

Stage of lactation also may be involved in resistance to disease. The cows inoculated in this study were in mid lactation, as with most *Staph. aureus* inoculation studies on lactating cows in which stage of lactation was reported (Postle *et al.* 1978; Gudding *et al.* 1984; Schultze & Paape, 1984; Schukken *et al.* 1994; Milner *et al.* 1996). The incidence of both subclinical infections and clinical mastitis is highest in early lactation (Daniel *et al.* 1982; Smith *et al.* 1985; Faye & Fayet, 1986; Morse *et al.* 1987; Hogan *et al.* 1989; Shpigel *et al.* 1998). Penetration of organisms into the mammary gland is more likely during early lactation (Grindal & Hillerton, 1991; Grindal *et al.* 1991), but although this is a factor in spontaneous infection, it would not affect experimental inoculation into the teat cistern. In cows inoculated at different stages of lactation with *Staph. aureus* (strains 106–6 and 107–59) the proportion of quarters infected increases with advancing lactation (Poutrel & Lerondelle, 1978). This would suggest that organisms become established less readily in the glands of animals in early lactation. However, there is also persuasive evidence to the contrary. In uninfected cows, the SCC curve has the form of an inverted lactation curve (Serieys, 1985) and the success of experimental infection is higher when SCC is low (Poutrel & Lerondelle, 1978; Schultze & Paape, 1984). Leucocyte migratory response to a low endotoxin dose (10^{-7} mg) as well as the capacity of skim milk to support phagocytosis are also lower in early than in mid lactation (Miller *et al.* 1985; Grommers *et al.* 1989). In experimental *Staph. aureus* infections PMN migrate into the luminal space mostly at damaged tissue sites (Harmon & Heald, 1982), the area of which may be expected to increase with advancing lactation owing to the cumulative effects of previous infections. Taken as a whole, the bulk of the evidence suggests that in early lactation there is greater likelihood of spontaneous cisternal infection and for them to develop into chronic mammary disease. However, to the best of our knowledge, there is no controlled experimental evidence to indicate that chronic mammary infections in early lactation are more likely to evolve into acute clinical mastitis.

There seems to be no information on the proportion of spontaneous subclinical *Staph. aureus* infections that evolve into clinical mastitis. The risk of clinical mastitis may be estimated from epidemiological records of dairy herds. The incidence of *Staph. aureus* isolates in lactating cows in Israel is $\sim 7\,\%$ (Saran, 1997). This figure

is similar to that of *Staph. aureus* isolates in clinical cases in seven Israeli herds located in the same region as the experimental herd and similarly managed, in which the mean lactational incidence of clinical mastitis is 0·2–0·3 cases/cow-year (Shpigel *et al.* 1998). Calculation on this basis suggests that for 16 animals over a 2 week period the incidence of clinical mastitis would be ~ 0·4 cases/group-month. Assuming the cows in this study and those in other farms in the region to be similar, a clinical event in this study was not very likely.

In this study on mid-lactation animals, all infections became chronic, as indicated by the persistent shedding of *Staph. aureus*, and remained subclinical. The possibility cannot be ruled out that some cases might have evolved into clinical mastitis had the animals been in earlier stages of lactation, the *Staph. aureus* strain more virulent, the observation period longer or the animals managed differently.

In the present study, SCC increased within 24 h of inoculation, but only moderately. This delayed and moderate leucocytosis contrasts with the rapid and massive leucocytosis observed after the administration of *Esch. coli* endotoxin that induces clinical mastitis (Heald, 1979; Guidry *et al.* 1983; Saad & Ostensson, 1990), but resembles the moderate leucocytosis observed in experimental infections with *Staph. aureus* 305 (Heald, 1979; Gudding *et al.* 1984; Daley *et al.* 1991). These different responses may be related to the different modes of action of the two organisms in the host tissues. *Esch. coli* has rapid systemic effects, the onset of which is only slightly different with intravenous and intramammary infusions, which suggests a rapid passage of toxin from mammary gland to blood (Jackson *et al.* 1990). The initial phase in the pathogenesis of *Staph. aureus* involves adherence to the mammary epithelium (Frost, 1975; Wanasinghe, 1981) resulting from its high affinity for components of the extracellular matrix (Cifrian *et al.* 1994). This initial adherence is apparently followed by multiplication of the organisms, their spread via the duct system (Gudding *et al.* 1984) and eventual progression to chronic infection. The infection of glands in this study became chronic, as indicated by the sustained shedding of *Staph. aureus* in milk from inoculated quarters. This occurred despite the influx of PMN, probably owing to rapid multiplication. The similar pattern of *Staph. aureus* and SCC in this study is in contrast with the findings of Daley *et al.* (1991), but the apparent differences probably stem from the less frequent observations in our study.

PMN were the main type of SCC migrating to the mammary gland (cf. Nickerson, 1989; Paape *et al.* 1991; Kehrli & Shuster, 1994). The adherence of *Staph. aureus* organisms to neutrophils did not change significantly between inoculation and 50 d later. However, the proportion of neutrophils that ingested bacteria tended to fall with time, but this can be countered by a rise in their numbers in milk, so that total phagocytosis capacity may increase (Paape *et al.* 1991). In the present study, the percentage of neutrophils ingesting bacteria fell from ~ 25 to ~ 8% between days 16 and 60, but the PMN concentration remained more or less stable between days 1 and 50, suggesting that total phagocytosis capacity fell with time.

Phagocytosis may decline with the time spent by neutrophils in milk, as they ingest milk fat and casein particles, resulting in degranulation and loss of pseudopodia, which reduces their bactericidal potential *in vitro* (Russell *et al.* 1977). This was probably not the case in the present study, as milk was extracted three times a day and the transit time for neutrophils from blood into the lacteal secretions is ~ 24 h (Persson *et al.* 1992). Since the degree of lobulation of PMN did not change, it is unlikely that infection modified the mean age of PMN in milk under these infection conditions. However, changes in phagocytosis do not completely reflect

changes in bactericidal capacity, as oxidative burst activity may increase together with a fall in phagocytic activity (Paape *et al.* 1991). Additional mechanisms may be present in chronic *Staph. aureus* infections, as indicated by a reduced phagocytic capacity of neutrophils concomitant with a reduction in the number of IgG receptors and non-immunologically mediated receptors (Niemialtowski *et al.* 1988).

The number of macrophages increased in both groups studied. The mAb used (CH137) in the first group did not label milk macrophages although it did label blood monocytes, suggesting a possible change in macrophage function. However, the mAb (BAQ151A) used with the second group labelled both blood and milk macrophages. A rise in macrophage numbers is not always found in subclinical and clinical forms of mastitis (Concha, 1986).

The mean EC of composite milk from individual quarters was not altered after inoculation, suggesting that it probably does not fully reflect the development of a *Staph. aureus* infection. In its initial stages, *Staph. aureus* infection may remain confined to parts of the gland (Heald, 1979; Nickerson & Heald, 1981; Gudding *et al.* 1984), so that an overall EC value may give no indication of infection. It is thus possible that sequential or continuous measurement of EC during milking may be better for detecting the initial stages of an infection. Although we found high SCC in all cows after inoculation, this was associated with a persistent high maximum EC in only a single cow. A high SCC of composite milk is detected by quarter maximum EC recorded during milking with a sensitivity of only 55%, as EC is not always increased during periods of high SCC that are not associated with clinical events (Nielen *et al.* 1995). In contrast, inoculation of 600 cfu *Staph. aureus* mexicana produces a subclinical infection with a $>15\%$ difference in mean quarter EC between control and infected quarters that correlates with SCC (Hillerton & Walton, 1991). A similar sequence of events also follows the infusion of either 10 or 100 μg *Esch. coli* endotoxin that results in clinical signs (Guidry *et al.* 1983). Clinical mastitis is detected with a sensitivity of 75% and a specificity of 95% by changes in EC at the milking at which clinical signs are observed (Nielen *et al.* 1995). In an experimental *Staph. aureus* 0053 infection, changes in EC of fore milk were detected concurrent with or in advance of clots in 88% of cases in which they appeared (Milner *et al.* 1996).

These results, taken collectively, suggest considerable variation among cases in the development of inflammation leading to clinical mastitis. They also suggest that the likelihood of detecting subclinical mastitis is quite low, and that inflammation is only likely to be detected by changes in EC when it is close to being clinically observable. This latter conclusion is limited to the conditions of this study, i.e. the organism inoculated, the milking system and its detection algorithms, and the general management of the herd.

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