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Influence of *Staphylococcus aureus* Exosecretions Isolated from Bovine Mastitis on Leukocyte Activity *In Vitro*

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With 2 figures and 4 tables

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Summary

The role of *Staphylococcus aureus* and coagulase-negative staphylococcal exosecretions was tested for its ability to elicit *in vitro* proliferation of bovine blood lymphocytes, which we determined by means of the ^{3}H-thymidine proliferation assay and by flow cytometry. Exosecretions of 32 field strains of *S. aureus* isolated from bovine udder infection and one of each of *S. intermedius* (M2), *S. hyicus* (M5), *S. xylosus* (M6) and *S. chromogenes* (M10) were used. Of the 32 *S. aureus* bacterial exosecretions, only 14 stimulated bovine mononuclear cells to proliferate. A high degree of association was found when the proliferation indexes were compared with the virulence as determined by intracisternal inoculation. All the six *S. aureus* strains that were categorized as highly virulent and that were tested in the proliferation assay exhibited a proliferation index > 20, whereas the five *S. aureus* strains that were categorized as low did not stimulate at all. Cells treated with media or Columbia broth supplemented with 0.1% D-glucose, yeast extract, and 0.5% NaCl (CBs) did not exceed 15% of the T-cells double positive with $CD25^+$, whereas incubation with Con A activated the T-cells to display $CD25^+$ up to 90%. Cells treated with one of the exosecretions that stimulated bovine mononuclear cells to proliferate, stimulated $CD3^+$ and $CD4^+$ T-cells to exhibit $CD25^+$ receptor significantly higher ($P < 0.05$) than that found in media and CBs treatments, but lower than those found in Con A treatments. The exosecretions that did not stimulate mononuclear cells to proliferate also did not activate T-cells to exhibit $CD25^+$ receptor. Con A activated 74% out of the total $CD8^+$ to exhibit ACT2 receptor and 50% out of the total $CD4^+$ to exhibit ACT3 receptor. A few but not all of the exosecretions that activated the CD25 receptor on T-cells also activated the ACT3 receptor on $CD4^+$ cells.

Introduction

The severity of mastitis resulting from *Staphylococcus aureus* and other staphylococci is partly associated with their bacterial exosecretions, which exhibit pyrogenic toxin (PT) (Bohach et al., 1990) and superantigen (SAg) activities. After colonization, mastitis results in severe damage to the mammary gland epithelial cell (Almeida et al., 1996), which increases the inflammation response in the gland. Staphylococci isolated from humans as well as from mastitis cows produced antigenically distinct enterotoxins, toxic shock syndrome toxin-1 (TSST-1) and pyrogenic exotoxins A and B (Barsumian et al., 1978; Bohach and Schlievert, 1987; Zhang et al., 1988; Munson et al., 1998; Akineden et al., 2001; Orwin et al., 2001). Enterotoxins and other PTs are potent T-cell activators (Peavy et al., 1970), and insights into the basis for their mitogenic activity have been gained from the important findings that enterotoxins and other PTs bind to major histocompatibility class II molecules (MHC-II) and to the $\alpha\beta$ T-cell receptor (TCR) (Fraser, 1989; White et al., 1989). Moreover, evidence has been found that suggests that *S. aureus* SAgs have a suppressive effect through the activation of lymphocytes with immunosuppressive activity (Park et al., 1992, 1993; Davis et al., 1996). Two potentially immunosuppressive T-cell subpopulations have been linked to staphylococcal mastitis: a subpopulation of activated $CD8^+$ T-lymphocytes that express a novel activation molecule, ACT2, which down-regulates the response of $CD4^+$ T-cells to heat-killed *S. aureus*; and another sub-population of $CD8^+$ lymphocytes that express an activation molecule, ACT3 (Park et al., 1992, 1993; Davis et al., 1996). Recently, our group phenotypically characterized over 400 *S. aureus* strains (Younis et al., 2000) and several dozen coagulase-negative staphylococci (CNS) isolated from bovine mastitis in Israeli dairy herds. None of the isolates were identified as producers of enterotoxins that included SEA, SEB, SEC1, SEC2, SEC3, SED, SEE and TSST-1, as tested by the microslide-immuno-diffusion-gel technique (Younis et al., submitted for publication). Moreover, in comparing the cleaved monomeric proteins of the isolates with standard enterotoxins of ATCC strains of *S. aureus*, by means of one-dimensional NuPAGE 10% Bis–Tris gel electrophoresis, no enterotoxins and no protein band could be matched with the standard which lie between 29 and 26 kDa (Younis et al., 2000). In the latest study (Younis et al., submitted for publication), the various bacterial exosecretions were inoculated into mammary glands and the gland response (exhibited as changes in somatic cells and *N*-acetyl-β-D-glucosaminidase activity) was monitored. While some of the exosecretions caused local responses other did not, and the responses to them did not differ from that to the control. When the exosecretions were tested with one-dimensional NuPAGE (7%) Tris-acetate gel electrophoresis, four different bands between 36 and 31 kDa, were detected, two of which were found to be associated with the cow's responses and could be attributed to the virulence of the bacteria. The objective of the present study was to test the ability of the representative *S. aureus* bacterial exosecretions used in the *in vivo* study, to modulate bovine mononuclear cells

in vitro, and to associate the result with those obtained by inoculation of the cows' mammary gland (Younis et al., submitted for publication).

Materials and Methods

Bacteria

Thirty-two field strains of *S. aureus* (Younis et al., submitted for publication) and one of each of *S. intermedius* (M2), *S. hyicus* (M5), *S. xylosus* (M6) and *S. chromogenes* (M10) of bovine mastitis were included (Table 1). These strains do not produce any enterotoxins or TSST-1, according to tests on microslide-immuno-diffusion-gel (Younis et al., submitted for publication). The production of haemolysins (alpha- and beta-haemolysin) was done on washed blood agar plates together with a *Streptococcus agalactiae* as well as by testing the bacterial supernatant against rabbit or sheep erythrocytes, according to Nilsson et al. (1999). Phage typing was performed in the National Staphylococcus Reference Center (Microbiology Dept., Rabin Medical Center, Petach-Tiqva, Israel) according to Blair and Williams (1961), with phages issued by the International Reference Laboratory, Colindale, UK, as modified by Samra and Gadba (1993).

Bacterial supernatant preparation

Each of the isolates was cultured in 100 ml Columbia broth supplemented with 0.1% D-glucose, yeast extract, and 0.5% NaCl (CBs), and incubated at 37 °C for 10 h. Bacteria were separated by centrifugation (1000×***g*** for 15 min). The crude supernatants, which included the exosecretions were collected and filtered through 0.2-μm pore-size membranes. The bacterial exosecretions were concentrated tenfold in 5 ml of 7500 MWCO membranes Vivapore concentrator (Vivascience, Lincoln, UK) in order to achieve protein concentrations of 0.4–0.5 mg/ml; they were then stored at –20°C. Protein concentrations were determined with the Protein Assay Kit (Bio-Rad, Munchen, Germany) after Bradford (1976). Sterility was tested by incubation of the bacterial exosecretions (1 ml) at 37°C for 6 days, followed by culture on a blood agar plate for 24 h at 37°C.

Antibodies and conjugates

The monoclonal antibodies (VMRD Inc., Pullman, WA, USA) used for the detection of the various leukocytes were mouse anti-bovine: (1) anti-lymphocyte: anti-CD3 – MM1A (IgG-1), anti-CD4 – CACT 138A (IgG-1), anti-CD8 – CACT 80C

Table 1. Virulence, as determined by intracisternal inoculation of a cows udder with 0.04–0.05 mg/quarter (total proteins) in 5 ml pyrogen-free saline (Younis et al., submitted for publication) and identification of strains used: 32 of *S. aureus*, two of *S. chromogenes*, and one of each of *S. intermedius* and *S. xylosus*

Bacteria	Coagulation	Virulence[a]	Phage type[b]	Haemolysis
AH4855	+	NT	3/A,3/C,6,29,42E,77,81,83/A,84,09,92, $D11^+/HK_2$ [55,75,94,96]	–
AH9130	+	NT	3/A,3/C,6,29,42E,77,81,83/A,84,09,92, $D11^+/HK_2$ [55,75,94,96]	$\alpha+\beta$
AL32/4	+	Low	3/A,3/C, [29,88/89	$\alpha+\beta$
AL246	+	NT	29,75,79,85,90, [42E,47,54,77,80,83/A95]	$\alpha+\beta$
AL302/1	+	Low	3/A,3/C, [29,88/89]	α
AN51/3	+	High	85,55[3/A,29,52,52A,92]	β
AU2873	+	NT	3/A,3/C,71[55]	β
AU3133	+	High	0 [0]	–
AZ/1	+	Moderate	3/A,3/C, $D11^+/HK_2$	β
BH4532	+	NT	3/A,3/C,55,71	χ
BH4751	+	NT	3/A,3/C,55,71	–
BH4792/1	+	Moderate	3/A,3/C,55,71	$\alpha+\beta$
BH5059	+	NT	3/A,3/C,55,71	$\alpha+\beta$
BS1	+	High	81	$\alpha+\beta$
CM619	+	NT	6,42E,47,53,75,83/A,85,90,$D11^+/HK_2$ [54,81,84]	–
CM1242/1	+	Low	3/A,3/C	$\alpha+\beta$
FR749	+	NT	0 [0]	–
FR780/4	+	Moderate	0 [0]	–
FR2449/1	+	High	0 [0]	$\alpha+\beta$
GL62	+	High	52/A,53,83A,90,92,$D11^+/HK_2$	–
IB4411	+	NT	6,52,79,81,42E,75,88/89,90,92,$D11^+/HK_2$	β
MH784/3	+	Low	0 [0]	χ
MH3081	+	NT	71 [3/A]	χ
MH3270	+	NT	3/A,3/C,55	β
MW3289	+	NT	52/A,53,83A,90,92,$D11^+/HK_2$	–
NR888/2	+	Moderate	3/A,3/C,71 [55]	–
NR2063/2	+	Moderate	3/A,3/C,71 [55]	–
NR2597/4	+	High	3/A,3/C,71 [55]	–
NR2602/4	+	Low	3/A,3/C,71 [55]	α
SY2794/2	+	Moderate	3/A,3/C,55,71	β
SY2846/4	+	Low	3/A,3/C,71 [55]	β
ZU452	+	NT	3/A,3/C,71 [55]	β
S. intermedius (M2)	–	Moderate	–	–
S. hycus (M5)	–	NT	–	–
S. xylosus (M6)	–	High	–	–
S. chromogenes (M10)	–	NT	–	–

[a]NT, not tested.
[b]0, no identification with the phage set used.

(IgG-1), anti-CD21 – GB 25A (IgG-1); (2) anti- ACT2 – CACT 77A (IgM) and anti-ACT3 – CACT 114A (IgG2b). All monoclonal antibodies were species-reactive with bovine cells. The secondary polyclonal antibodies used were: goat anti-mouse IgG-1 conjugated with TRI-COLOR (TC), goat anti-mouse IgG-2b PE and goat anti-mouse IgG-3 FITC conjugated affinity isolated (CALTAG Laboratories, Burlingame, CA, USA).

In vitro lymphocyte proliferation response using ^{3}H-thymidine and flow cytometry

The exosecretion of each of the isolates was tested for its ability to elicit proliferation of bovine blood lymphocytes, which we determined by means of the ^{3}H-thymidine proliferation assay and by flow cytometry (FACScan, Becton Dickinson Immunocytometry System, San Jose, CA, USA). Mononuclear cells were obtained from heparinized cows' blood by density centrifugation at $600 \times g$ for 30 min at room temperature with the aid of Lymphoprep (produced for Robbins Scientifics USA by Nycomed Pharma, Norway). The cells were collected from the plasma–Ficoll interface, diluted with an equal volume RPMI-1640 (Biological Industry, Bet HaEmek, Israel), washed twice and counted and adjusted to a concentration of 1×10^6 cells/ml in complete medium.

A. Thymidine assay

Cells (1×10^6 per ml) in complete medium (CM) (RPMI-1640 supplemented with 5% foetal calf serum, antibiotic penicillin (100 g/ml) and streptomycin (100 U/ml) (Biological Industry, Bet HaEmek, Israel) were cultured in triplicate (1×10^5 cells/well) in round-bottom 96-well plates (Nunc, Denmark). Cells were stimulated with a range of BEs (dilutions of $1:10^2$–$1:10^5$ in CBS) in an end volume of 200 μl. Con A (Sigma, USA) was used as a positive control, at a concentration 5 μg/ml. Plates were placed in a humidified incubator at 37 °C and 5% CO_2. After 52–54 h of incubation, 1 μCi ^{3}H-thymidine (10 µl) (New England Nuclear, Boston, MA) 6.7 μCi/mmol were added and 20 h later (a total of 72 h), the cells were harvested with a Skarton Harvester (Flow Lab., Rockville, MD, USA) into Glass Mico-Fibre Filter (Whatman). Wells and filters were washed with distilled water. Three millilitres of OPTI-FLUOR fluid (Packard BioScience Comp., Groningen, Netherlands) were added to the filter paper discs mini-vials in and were counted by Packard 1600 TR scintillation counter (Packard, Downers Grove, IL). Results were expressed as an index, which was calculated as:

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

where Cpm denotes counts per minute.

B. Flow cytometry analyses

Aliquots of mononuclear cell suspensions were prepared for flow cytometry analyses and for incubation with the various exosecretions. For flow cytometry, 1×10^6 cells/ml in phosphate buffered saline (PBS) were transferred into tubes. To each tube was added 50 μl of one or two of the mAbs or PBS (as a negative control); it was mixed gently and incubated for 1 h at 4°C. Cells were washed (3×) in PBS and 50 μl of one (single) or two (double) of the conjugated antibodies were added, mixed gently and incubated for 30 min at 4°C. Following incubation the cells were washed and re-suspended in PBS to a final volume of 1 ml. To calculate the percentages of the various lymphocytes, 10 000 events were read per sample. For incubation, 1×10^6 cells/ml cell suspensions in complete medium were cultured in 24-well plates (1 ml/well) (Costar, #3524, Cambridge, MA, USA). Cells were stimulated with the various exosecretions in 1 : 1000 dilutions. After 72 h, the cells were collected, live cells were counted with a light microscope and then analysed by the flow cytometry procedure.

Statistical analysis

Data were analysed with the SAS General Linear Model (GLM) procedure (SAS/STAT® User's Guide, 1990).

Results

Of the 32 *S. aureus* bacterial exosecretions tested, only 14 stimulated bovine mononuclear cells to proliferate (Table 2). The four CNS exosecretions tested did not elicit proliferation. Some of the exosecretions were toxic to the cells at a final concentration of $< 1:10^2$ (protein concentration of 0.004 mg/ml). The assay with each of the exosecretions was performed three to six times with blood from different cows. However, in the light of small variations among the assays and among the cows, all of the exosecretions were tested again with blood from cow number 2089, and the results of this experiment are summarized in Table 2. No significant differences were found between the results obtained in that assay and those obtained previously (data not shown). The proliferation response varied among the isolates and among the dilutions; however, most of the proliferation peaks were found with the $1:10^3$ dilution (protein concentrations of 0.0004 mg/ml), therefore, this dilution was used for subsequent cell stimulation assays for cell distribution. Comparison of the proliferation index with the results of the virulence as determinate by the intracisternal inoculation of the cow's udder with 0.04–0.05 mg/quarter (Younis et al., submitted for publication), revealed a high degree of association between the two (Table 2). All the six *S. aureus* strains that were categorized as highly virulent and that were tested in the proliferation assay exhibited a proliferation index > 20. Of the six *S. aureus* strains that were categorized as moderate, three had proliferation indexes > 20, one an index of 9.5 and two strains did not stimulate at all (proliferation index < 2) whereas all the five *S. aureus* strains that were categorized as low did not stimulate at all. The *S. intermedius* (M2), categorized as moderate, and the *S. xylosus* (M6), categorized as highly virulent, did not stimulate the cells to proliferate *in vitro* (Table 2). The distributions of lymphocytes in the bovine blood are displayed in Fig. 1 and Table 3. The percentages of T-cells were: $CD3^+$, $53.4 \pm 2.4\%$; $CD4^+$, $27.5 \pm 2.8\%$; and $CD8^+$, $19.3 \pm 3.0\%$; that of B-cells was $28.1 \pm 2.2\%$. None of the cells were found positive with CD25 or with ACT2 mAbs (Fig. 1 and Table 4). On the other hand, one-third of the $CD3^+$ T-cells were also positive to ACT3, and most of those cells were $CD4^+$. The mAb agents ACT2 receptors was also labeled $> 50\%$ of the B-cells (data not shown). After incubation for 72 h, no significant

Table 2. Virulence, as determined by intracisternal inoculation of cow udder with 0.04–0.05 mg/quarter (total proteins) in 5 ml pyrogen-free saline (Younis et al., submitted for publication) and proliferation index of bovine mononuclear cells treated *in vitro* with the exosecretions produce by the various *S. aureus* strains, *S. intermedius* (M2) or *S. xylosus* (M6). Results in the table are those obtained with blood from cow 2089

		Dilution (proliferation index[a])			
Bacterium	Virulence	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$
AN51/3	High	5.5	22.0	26.7	14.8
AU3133	High	28.0	21.5	8.4	3.1
BS1	High	9.2	31.2	28.2	16.0
FR2449/1	High	5.9	34.4	28.4	16.3
GL62	High	29.3	32.9	30.6	22.1
NR2597/4	High	28.8	27.6	9.1	4.3
AZ/1	Moderate	2.1	9.5	11.8	13.5
BH4792/1	Moderate	< 2	< 2	< 2	< 2
FR780/4	Moderate	27.7	29.6	13.8	2.4
NR888/2	Moderate	28.5	23.5	7.9	5.3
NR2063/2	Moderate	35.2	23.5	11.3	4.5
SY2794/2	Moderate	< 2	< 2	< 2	< 2
AL302/1	Low	< 2	< 2	< 2	< 2
CM1242/1	Low	< 2	< 2	< 2	< 2
AL32/4	Low	< 2	< 2	< 2	< 2
MH784/3	Low	< 2	< 2	< 2	< 2
SY2846/4	Low	< 2	< 2	< 2	< 2
MW3289	NT	29.3	32.6	30.2	20.8
NR2602/4	NT	3.6	18.8	7.7	4.1
AL246	NT	7.2	14.3	15.6	6.8
FR749	NT	21.8	21.1	9.1	4.1
S. intermedius (M2)	Moderate	< 2	< 2	< 2	< 2
S. xylosus (M6)	High	< 2	< 2	< 2	< 2
CBS[b]		2109			
Con A		92 459			

[a]Index = [(Cpm exosecretions test − Cpm background)/Cpm Con A − Cpm background)] × 100.
[b]CBS and Con A proliferation response are given in counts per minute.

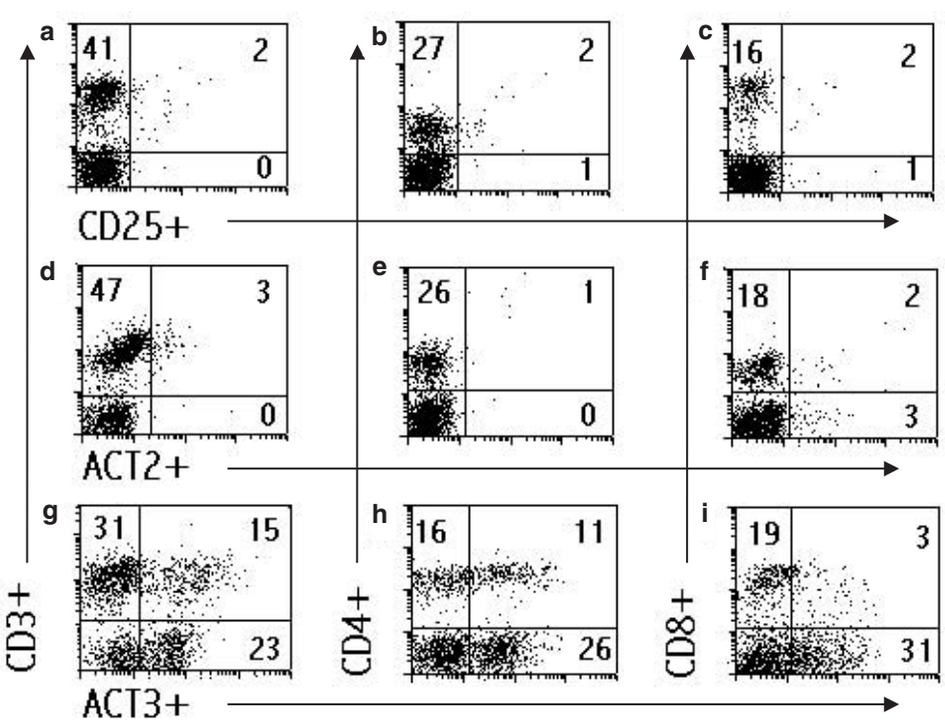

Fig. 1. Representative two-colour flow-cytometric dot plots of phenotype ($CD25^+$, $ACT2^+$ or $ACT3^+$: FL1) and ($CD3^+$, $CD4^+$ or $CD8^+$: FL2) of bovine blood lymphocytes. Percentages of cells with the subset phenotype were calculated from 10 000 cells.

Table 3. Lymphocytes distribution identified by flow cytometry before and after *in vitro* treatment with exosecretions produce by selective *S. aureus* strains. For cells identified with each mAb, results are given as: total = percentage of cells labelled by the mAb, out of 10 000 cells. $CD25^+$ = percentage of the 10 000 cells labelled with both mAbs. % = the percentage of the 10 000 cells labelled by both mAbs

		$CD3^+$			$CD4^+$			$CD8^+$			$CD21^+$		
Treatment[1]	#[2]	Total	$CD25^+$	%	Total	$CD25^+$	%	Total	$CD25^+$	%	Total	$CD25^+$	%
Pre	8	53.4 ± 2.4	0	0	27.5 ± 2.8	0	0	19.3 ± 3.0	0	0	28.1 ± 2.2	0	0
Media	8	67.3 ± 9.4	6.8 ± 1.0^b	10.1	26.8 ± 7.9	3.5 ± 0.8^b	13.1	21.5 ± 3.6	1.3 ± 0.2^b	5.8	13.1 ± 3.4	0.5 ± 0.2	3. 8
CB	3	57.1 ± 3.4	8.2 ± 1.0^b	15.0	16.1 ± 2.4	0.5 ± 0.1^b	3.1	13.4 ± 0.3	1.5 ± 0.2^b	11.5	17.1 ± 3.4	0.5 ± 0.2	2.9
Con A	8	68.1 ± 3.9	56.3 ± 5.0^a	83.6	31.3 ± 1.5	28.4 ± 1.8^a	90.8	26.8 ± 2.9	20.5 ± 2.1^a	76.6	14.6 ± 1.2	2.6 ± 0.6	18.0
AN51/3	2	69.0 ± 1.4	45.5 ± 2.5^a	65.9	39.5 ± 8.8	27.0 ± 3.5^a	68.4	20.3 ± 2.7	7.05 ± 0.5^a	34.5	10.0 ± 0.7	2.0 ± 0.3	20.0
AU3133	2	65.0 ± 9.6	36.5 ± 7.4^a	55.4	31.5 ± 8.1	14.5 ± 1.4^a	46.0	24.0 ± 1.4	6.5 ± 2.5^a	27.1	18.5 ± 1.5	2.5 ± 1.1	13.5
BS1	2	68.0 ± 2.1	41.5 ± 1.1^a	61.0	33.0 ± 5.3	19.0 ± 4.9^a	56.7	24.5 ± 4.6	12.1 ± 1.4^a	50.0	14.1 ± 1.5	3.0 ± 0.7	21.0
FR2449	3	70.7 ± 6.7	43.3 ± 3.4^a	60.8	41.3 ± 9.5	25.0 ± 5.0^a	61.0	20.3 ± 2.7	7.05 ± 0.5^a	34.5	16.7 ± 5.5	2.0 ± 0.9	12.0
GL62	2	79.0 ± 2.5	46.2 ± 4.9^a	58.2	46.5 ± 5.3	28.5 ± 1.7^a	61.3	23.5 ± 1.1	7.5 ± 1.8^a	31.9	8.5 ± 2.5	1.0 ± 0.4	11.8
AH9130	2	64.0 ± 1.5	$6.6^b \pm 3.5$	9.4	29.0 ± 0.3	$2.9^b \pm 1.5$	10.0	20.0 ± 1.3	$2.0^b \pm 1.5$	10.0	14.6 ± 2.4	2.8 ± 2.4	19.1
CM1242	1	43.5	5.6^b	12.9	19.9	2.1^b	10.6	17.4	1.5^b	8.6	17.8	0	0
BH4532	1	58.3	3.8^b	6.5	26.6	1.5^b	5.6	22.4	1.0^b	4.4	10.4	2	19.2
Pr > F		NS	< 0.0001		NS	< 0.0001		NS	< 0.0001		NS	NS	

[a,b]Means within columns with no common superscript differ significantly ($P < 0.05$).
[1]Supernatant treated cells.
[2]Number of repeats.

differences were found among the various treatments: media, CBs, Con A and the various exosecretions, in the total numbers of alive cells counted by light microscopy (data not shown). Moreover, no significant differences were found in the distributions of the cells labeled with anti-CD3, -CD4, -CD8 or -CD21 (Table 3). However, significant differences ($P < 0.0001$) were found among $CD3^+/CD25^+$, $CD4^+/CD25^+$ and $CD8^+/CD25^+$. Cells treated with media or CBs did not exceed 15% of the T-cells double positive with $CD25^+$, whereas incubation with Con A activated the T-cells to display $CD25^+$ up to 90% ($CD3^+/CD25^+$, 83.6%; $CD4^+/CD25^+$, 90.8% and $CD8^+/CD25^+$, 76.6%). The number of B-cells stimulated with Con A did not significantly differ from the numbers stimulated by media or CBs; however, after incubation with Con A, the percentage of B-cells positive to CD21 and CD25 exceeded 18%, compared with 3–4% with media or CBs. Out of the 14 *S. aureus* exosecretions that stimulated mononuclear cells to proliferate (Table 2), 11 were also tested for lymphocyte distributions after *in vitro* treatment. These 11 exosecretions stimulated $CD3^+$ and $CD4^+$ T-cells to exhibit $CD25^+$receptor (Table 3). The percentages of those double positive cells ($CD3^+/CD25^+$, $CD4^+/CD25^+$) were found to be significantly higher ($P < 0.05$) than were found in media and CBs treatments, but lower than those found in Con A treatments. Not all of those 11 exosecretions activated $CD8^+$ cells, to exhibit $CD25^+$ receptor. The three exosecretions that did not stimulate mononuclear cells to proliferate (AH9130; CM1214; BH4532) also did not activate T-cells to exhibit $CD25^+$receptor. Activation of B-cells to exhibit $CD25^+$ receptor was similar to that by Con A with all exosecretions except CM1242. Con A activated 74% out of the total $CD8^+$ to exhibit ACT2 receptor and 50% out of the total $CD4^+$ to exhibit ACT3 receptor (Fig. 2 and Table 4). A few but not all, of the exosecretions that activated the CD25 receptor on T-cells also activated the ACT3 receptor on $CD4^+$ cells. As can be seen in Fig. 2, with exosecretions of FR2449/1, 36% out of the total $CD8^+$ to exhibit ACT2 and 45% out of the total $CD4^+$ and 32% out of the total $CD8^+$were also positive with ACT3 ($CD4^+/ACT3^+$; $CD8^+/ACT3^+$). All cells positive to ACT2 or ACT3 molecules were also positive to $CD25^+$. None of the exosecretions tested activated $CD4^+$ to exhibit ACT2 receptor.

Discussion

Attention has been focused on the role of *S. aureus* exosecretions, which exhibit SAg and PTs activity (Bohach et al., 1990). Previously (Younis et al., submitted for publication), in an *in vivo* study with Israeli–Holstein dairy cows which had been inoculated intracisternally with the various *S. aureus* or CNS exosecretions, identified three categories of virulence: high, moderate and low. In an attempt to relate the virulence to some known PTs, none of the sought-for enterotoxins (A–F) or TSST-1 were detected in any of the tested *S. aureus* or CNS exosecretions. However, two protein bands, of 35 and 31 kDa (bands B and C), were identified which were strongly associated with virulence (Younis et al., submitted for publication). In the present study, the focus was put on testing the ability of the representative *S. aureus* and CNS exosecretions that were examined in the *in vivo* study, to modulate bovine mononuclear cells *in vitro*, and trying to associate the results with those

Table 4. Lymphocytes distributions identified by flow cytometry before and after *in vitro* treatment with exosecretions produce by *S. aureus* strains FR2449. For cells identified with both mAbs, results are given as: total = percentage of cells labelled by the mAb, out of 10 000 cells

	$CD3^+$		$CD4^+$		$CD8^+$	
Treatment[a]	$ACT2^+$	$ACT3^+$	$ACT2^+$	$ACT3^+$	$ACT2^+$	$ACT3^+$
Pre	2.2 ± 2.0	20.3 ± 4.4	1.3 ± 2.7	13.3 ± 3.1	2.2 ± 0.7	3.5 ± 0.7
Con A	12.6 ± 2.8	24.2 ± 4.0	2.2 ± 1.1	16.3 ± 4.6	12.7 ± 4.5	7.8 ± 3.5
FR2449	9.0 ± 2.7	23.2 ± 3.7	3.0 ± 2.2	19.2 ± 4.8	7.0 ± 5.8	7.6 ± 1.6

[a]Supernatant treated cells.

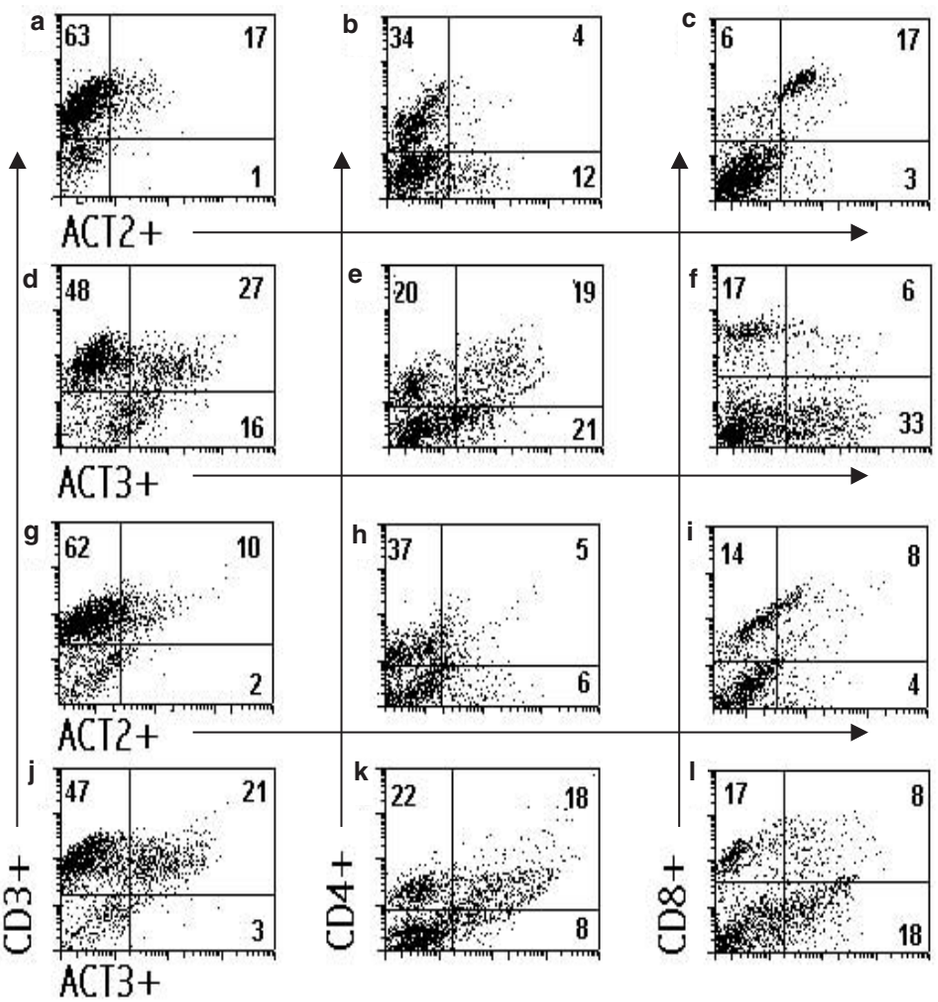

Fig. 2. Representative two-colour flow-cytometric dot plots of phenotype ($CD25^+$, $ACT2^+$ or $ACT3^+$: FL1) and ($CD3^+$, $CD4^+$ or $CD8^+$: FL2) of bovine blood lymphocytes after incubation for 72 h with Con A (A–F) or with exosecretions of FR2449/1 (G–L). Percentages of cells with the subset phenotype were calculated from percentages of cells with the subset phenotype were calculated from 10 000 cells.

obtained in the cows' mammary glands. The strong association between the strain virulence (*in vivo*) and proliferation of T-cells, mainly $CD4^+$ (*in vitro*), which was found in this study, suggests that the inflammation response in the mammary gland results, at least in part, from the activity of stimulated immune cells. Although the inflammation in the mammary gland and transfer of immune cells from the blood into the infected gland is important in the elimination of the intruder (successfully in the case of Gram-negative bacteria), the signals which directed the specific immunity, are crucial for the longer-term response, in case the intruder survived and/or in case of new penetration by the some intruder. Two potentially immunosuppressive T-cell sub-populations – $CD8^+ACT2^+$ (Park et al., 1993; Davis et al., 1996) and $CD8^+ACT3^+$ (Ferens et al., 1998a,b) – were identified and were found to down-regulate the response of $CD4^+$ T-cells *in vitro*. As in the case of SEC, the predominant SEC associated with the disease in ruminants (Deringer et al., 1997; Ferens et al., 1998b), stimulation of bovine mononuclear cells by the exosecretions expressed in the bands B and C in the present study, also

partially activated $CD4^+$ and $CD8^+$ to exhibit the ATC3, as well as the CD25 molecules. These results, as in the case of the one previously reported with the SEC, demonstrated the strong association of the exosecretions with virulence in the mammary gland and its roles in the suppression of the immune response to *S. aureus*. Interestingly, the two CNSs tested in the present study – exosecretions of *S. intermedius* (M2) or *S. xylosus* (M6) – which ranked as moderately or highly virulent, respectively, in the *in vivo* study, did not stimulate bovine mononuclear cells to proliferate. These results suggest that, although the M6 isolates were found with the protein band marked as B and those of M2 with band C, those bands represented different proteins and may be involved in the mammary gland response, but they did not activate lymphocytes as the *S. aureus* did. In conclusion, these findings could indicate the mechanisms by which *S. aureus* manipulates the immune system to reduce its activity against the bacteria and by this means to remain in the udder. Further studies are in progress, aiming to identify and purify these to proteins in order specifically to study their mechanisms and, more generally, to determine how *S. aureus* modulates the immune system.

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