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Development of a *Staphylococcus aureus* vaccine against mastitis in dairy cows I. Challenge trials

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Abstract

A vaccine composed of three field isolates of *Staphylococcus aureus* (*S. aureus*) derived from cases of mastitis in cows was developed. The vaccine was administered to nine uninfected cows while 10 other cows were used as controls. All cows were challenged with a highly virulent *S. aureus* strain administered into two quarters of each cow. Quarters were tested for clinical signs, secretion of *S. aureus*, and somatic cell count (SCC). No systemic effects were observed in any of the cows, vaccinated or control. Vaccinated cows had 70% protection from infection compared with fewer than 10% in the controls. Moreover, all quarters challenged in the vaccinated cows, regardless of whether they were successfully infected or not with *S. aureus*, exhibited very mild inflammatory reactions, identified by their low SCCs (<100,000).

Keywords: Cattle; Mastitis; Vaccine; *S. aureus*

1. Introduction

Staphylococcus aureus (*S. aureus*) is the main causative agent of bovine mastitis and is difficult to eliminate. Vaccination is a logical approach to the control of infectious diseases in food production animals. The article ''*Staphylococcus aureus* vaccine against mastitis in dairy cows, composition and evaluation in mouse model'' (Leitner et al., companion paper—this issue) describes a new vaccine, designated ''MASTIVACS I''. This vaccine, upon administration to mice elicited high levels of IgG-specific antibodies and exhibited highly significant protection against challenge with *S. aureus* strains, either homologous or heterologous, in a mouse model. In the present paper we describe the efficacy of the new vaccine in controlled experiments in cows which were exposed to intrammamary challenge with a

Abbreviations: *S. aureus*, *Staphylococcus aureus*; SCC, somatic cell count; CNS, coagulase-negative staphylococci; cfu, colony forming units; mAbs, monoclonal antibodies; FITC, fluorescein isothiocyanate; IDF, international dairy federation; CC, coulter® counter; NAGase, *N*-acetyl-β-D-glucosaminidase; FACS, flow cytometry; PMN, polymorphonuclear; FLS, forward-angle light scatter; SLS, side-light scatter; LM, light microscopy

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virulent field strain of *S. aureus* after vaccination with "MASTIVACS I".

2. Materials and methods

2.1. Animals

Nineteen non-pregnant Israeli–Holstein cows in their first to second mid-lactation period were included in two consecutive trials. The cows were located in several different farms and were chosen for the experiment according to their udder condition as confirmed by three consecutive bacteriological and somatic cell count (SCC) examinations of quarter milk samples. The cows were free of udder infection or contamination with coagulase-negative staphylococci (CNS), and had SCCs lower than 300×10^3 cells/ml; they had no specific antibodies against *S. aureus*. The cows were milked three times daily and had milk yields of 25–35 kg per day. Food was offered in mangers located in free-stall barns.

2.2. Vaccine, vaccination and challenge

The vaccine is described comprehensively under *Patent* # 122829 and *Patent PCT/IL* # 98/00627 and in Leitner et al., companion paper—this issue. Briefly: bacterial strains (three different strains: VLVL8407—non-hemolytic; ZO3984—β-haemolytic; and BS449 α + β-hemolytic) were grown in Columbia broth (Difco, Detroit, MI, USA). Before vaccination the separate strain preparations: bacterial homogenates of BS449 and ZO3984, and exosecretion of VLVL8407 were mixed (1:1:1 by protein concentrations) and then was mixed 1:1 with incomplete Freund's adjuvant (IFA) (Difco). Each cow was immunized subcutaneously with 1 ml of the adjuvant vaccine under the tail root and with 1 ml without the adjuvant in the area of the supra-mammary lymph node. Second and third (boost) doses of the vaccine were administered that were similar to the first administration but without adjuvant. In trial 1, only the second dose was administered, 36 days after the primary immunization; in trial 2, the second and third doses were administered 36 and 56 days, respectively, after the primary immunization. Control cows were vaccinated according to the same schedule, with a vehicle only.

One of the bacterial strains that make up the vaccine (VLVL8407) was used for challenge. This strain was grown in Nutrient Broth (Difco, Detroit, MI) for 24 h at 37 °C and harvested by centrifuging at $3000 \times g$ for 15 min at 4 °C. The pellet was resuspended in non-pyrogenic PBS (pH 7.6; 0.01 M) and washed, and the bacterial concentration determined by serial dilution on blood–agar plates. The original pellet was stored on ice for 16 h. Each cow was challenged by injection of 1 ml of the suspension, containing 1000 colony-forming units (cfu), into two quarters (except for one cow that received it in one quarter and one that received it in three quarters) through the streak canal into the gland cistern.

2.3. Antibodies and conjugates

The monoclonal antibodies (mAbs) (VMRD Inc., Pullman, WA) used for the detection of the various leukocytes were: (1) anti-lymphocyte [anti-$CD4^+$: CACT 138A, anti-$CD8^+$: CACT 80C and anti-B: BAS9A]; (2) anti-monocyte/macrophage [BAQ151A]; and (3) anti-granulocyte [CH138A (G1) and MM20A (G3)]. All mAbs were species-reactive with bovine cells. The polyclonal antibody used was goat $F(ab')_2$ anti-mouse IgG (H + L) conjugated with fluorescein isothiocyanate (FITC) that exhibited minimal cross-reaction to human-, bovine- and horse-serum proteins, (Jackson Immuno Research Laboratory, West Grove, PA, USA).

2.4. Bacteriological examinations

Duplicate quarter foremilk samples were taken aseptically according to the International Dairy Federation (IDF) (1985) procedures and submitted to the laboratory within 1 h. Bacteriological analysis was performed according to accepted standards (Hogan et al., 1999). A 0.01 ml aliquot from each milk sample was spread over blood–agar plates (Bacto-Agar; Difco Laboratory) containing 5% sheep red blood cells. All plates were incubated at 37 °C and examined for growth at 18 and 42 h. Colonies suspected to be staphylococci were tested for coagulase production ((tube test) Anilab, Rehovot, *Israel*) and strain identification was performed with the ID 32 Staph Kit (Bio Merieux S.A., 69280 Marcy-l'Etoile, France).

2.5. Somatic cell count

SCC was performed with a Coulter® Counter (CC) Model Z1 (Coulter Electronics Ltd., Beds., England) according to the revised protocol of the A2B Sub-Group of Mastitis Experts from the IDF (1991).

2.6. NAGase test

N-Acetyl-β-D-glucosaminidase (*NAGase*), the lyzosomal enzyme concentrate in milk, was determined fluorometrically by means of the ADL MILK *NAGase* test (Applied Diagnostics Corporation (ADC), Helsinki, Finland).

2.7. Immunological assay

2.7.1. Differential leukocyte count by flow cytometry (FACS)

The assay was performed as previously described (Leitner et al., 2000a,c). Milk samples were kept at room temperature, and were analyzed not more than 3 h after collection. The number of somatic cells in each sample was determined by CC and a volume of milk containing approximately 1×10^6 cells was divided among 15 ml test tubes, one tube for each mAb and one for the control. After washing, 50 μl of each mAb or PBS (as a negative control) was added to cell pellets in each tube, mixed gently and incubated for 1 h at 4 °C. After further washing (three times), 50 μl of conjugated antibodies were added, mixed gently, incubated for 30 min at 4 °C, washed again, and resuspended in PBS to a final volume of 1 ml. To calculate the percentages of the various leukocytes, 10,000 events were read per sample. The absolute cell number for each cell type was calculated from the SCC, as determined by CC. The results are given as the percentage of positive cells labeled by each relevant mAb. The percentage of polymorphonuclear (PMN) cells, gated by forward-angle light scatter (FLS) and by side-light scatter (SLC) of the flow cytometry map are also presented.

2.7.2. Differential leukocyte count by light microscopy (LM)

Differential cell counts were carried out by LM according to Jensen and Eberhart (1981); they were performed on samples of approximately 100–150 cells.

2.7.3. Antibody detection

2.7.3.1. ELISA. The assay was performed as previously described (Leitner et al., 2000b). Each serum (blood or milk) was tested twice, once on *S. aureus* VLVL8407 and once on BS449 as antigen. In brief, 96-well immunoplates (Nunc, Kamstrup, Denmark) were coated with killed agitated *S. aureus*. After blocking with 3% casein, tested blood serum (1:2000) or milk (1:50) was added in duplicate, incubated for 1 h at 37 °C and affinity-purified goat anti-Bovine IgG(γ) chain peroxidase conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. The bound antibodies were detected by adding 100 μl of 2.2′-azino-di (3-ethyl-benzthiazolin sulfonate) (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Plates were read in a microplate autoreader (Dynatech MR5000, Guernsey, UK) at 410 nm. To each plate, positive and negative serum standards were added. The optical density of each tested sample was modified by linear regression and the end point dilution was then calculated, to compensate for the differing dilutions of blood sera and skim milk that were used.

2.8. Study layout

In each trial, cows were divided into two groups according to farm and udder condition. In the first trial, five cows were immunized and five were used as controls; in the second trial four cows were immunized and five were used as controls. The schedules of the two trials are summarized in Table 1. On specified days (Table 1), bacteriological status, SCC, differentiated count; *NAGase* activity and antibodies to *S. aureus* in blood and milk were determined. In the first trial, cows were revaccinated once following their initial vaccination, whereas in the second trial they were revaccinated twice. Cows were initially vaccinated at their farm of origin, and were transferred to the Volcani Center research farm 5–7 days before challenge. In the 48 h post-challenge period, cows were closely observed for symptoms of illness. At the end of each trial, tissue from the area of vaccine administration and epithelial tissue from challenged and unchallenged teats were submitted for histological examination.

Table 1
Schedule of the two vaccination regimes and challenge trials

Trial 1																
−60	−25	−13	0	13	22	37	43	57	63	65	66	72	73	78		84
			*			*		*		*				*		
			Vaccine I			Vaccine II		"Volcani"		Challenge				Histology		
Trial 2																
−30	−5	0	9	21	36	45	56	69	72	77	78	79	84	90		98
		*			*		*		*	*				*		
		Vaccine I			Vaccine II		Vaccine III		"Volcani"	Challenge				Histology		

On each of the days marked, bacteriological status, SCC, differentiated count, *NAGase* activity and antibodies to *S. aureus* in blood and milk were determined. In addition, on the days marked with (∗), the activity written below the mark was performed. "Volcani" stand for the Volcani center research farm.

2.9. Statistical analysis

Data were analyzed by means of the SAS general linear model (GLM) procedure (SAS, 1990).

3. Results

During the entire period of the experiment, none of the cows showed any abnormal signs of sensitivity to the vaccine except for a local swelling for up to 10 days after vaccination. Prior to vaccination, the cows did not have antibodies to *S. aureus*, whereas following vaccination; antibodies were detected in the blood sera of all vaccinated cows, reaching the highest-level 7–20 days after the revaccination. In the second trial no change in the antibody titer was observed following the second vaccination (Table 2). Specific IgG antibodies to both strains of *S. aureus* tested were detected in milk of all the quarters in parallel to those detected in the blood. The end point dilutions of the antibodies in the milk derived from the vaccinated cows were in the same range in all quarters, with no significant differences, and the titers were only 10–30% of that of the blood. Following vaccination, observations of bacteriological status, SCC, differential counts, *NAGase* and milk yield showed no significant changes until the challenge was presented (data not shown). At the time of challenge, all vaccinated cows had antibodies specific to the two strains tested (VLVL8407 and BS449). The end points dilutions were somewhat lower than those at the peak, but the differences were not significant (Table 2). Antibody to *S. aureus* was not detected in any of the control cows.

There were no significant differences between the results obtained in trials 1 and 2 in any of the parameters tested, therefore, in the subsequent calculations data collected over both trials were combined. *S. aureus* was isolated in 19 out of the 21 quarters (90.5%) of the unvaccinated control cows 1 day after challenge. Those 19 quarters continued to secrete *S. aureus* in their milk over the entire period of the

Table 2
Levels of antibodies to *S. aureus* vaccine in blood and milk of vaccinated and control cows

Treatment		Antibody (end point)[a]		
		Time pre-vaccination	Peak[b]	Challenge time
Vaccinate	Blood	0	1:16000–1:64000	1:16000–1:32000
	Milk	0	1:800–1:2400	1:400–1:1200
Control	Blood	0	0	0
	Milk	0	0	0

Antibody levels were determined by ELISA, with VLVL8407 as antigen.
[a] Range of end point dilution.
[b] Peak: highest value (7–20 days after the cows were revaccinated).

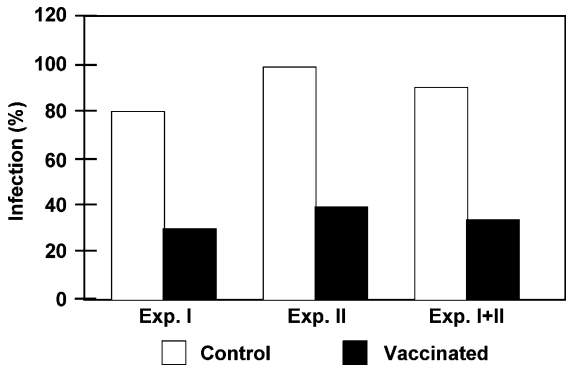

Fig. 1. Percentages of infected quarters, 2 weeks after experimental challenge with *S. aureus* of cows previously vaccinated with experimental *S. aureus* vaccine or with the vehicle only (control).

experiment (19–21 days). In two challenged quarters, *S. aureus* was not detected at any time. In the vaccinated cows, nine of the 17 challenged quarters secreted *S. aureus* after the first day. In successive attempts to isolate it, on days 7 and 13, *S. aureus* was found in only 6 of the 17 challenged quarters (35.3%). Fig. 1 summarizes the status of the challenged quarters that shed *S. aureus* in their milk after the first days post-challenge, according to trial (1 or 2) and treatment (vaccinated or control). The difference in the rate of infection between quarters of vaccinated and control cows (35.3 and 90.5% respectively) were found significant ($P < 0.01$). Moreover, the number of cows for which at least one of their challenged quarters shed *S. aureus*, was significantly lower ($P < 0.05$) among the vaccinated cows (4/9) than among the controls (9/10).

One day post-challenge, SCC increased significantly ($P < 0.01$) in all quarters challenged with *S. aureus* in vaccinated and control cows, including those in whose milk no *S. aureus* could be detected at that time. In parallel, no changes in SCC were recorded in the unchallenged control quarters. In the challenged quarters of the vaccinated cows (17 quarters), mean SCC values increased from 109×10^3 cells/ml before challenge to 1644×10^3 cells/ml 1 day after challenge; they decreased to values not significantly different from those before challenge by days 7 (380×10^3 cells/ml) and 13 (60×10^3 cells/ml) post-challenge (Table 3). In the challenged quarters of the control cows (21 quarters), the mean SCC increased from 148×10^3 cells/ml before challenge to 1420×10^3 cells/ml 1 day after challenge; it remained relatively high until the end of the experiment (879×10^3 cells/ml on day 7 and 525×10^3 cells/ml on day 13) (Table 3). Thus, the difference between the vaccinated and control cows, in the SCCs of their challenged quarters, was significant ($P < 0.05$). Moreover, when the SCCs in the milk of all the quarters that secreted *S. aureus* at day 13 post-challenge were

Table 3
Mean ± S.E. of proportions (%) of cells and numbers of neutrophils counted in light microscopy, proportion of PMNs gated or labeled by mAb (G1), macrophage (Mo), lymphocytes bearing $CD4^+$ or $CD8^+$ labeled by the different mAbs and epithelial cell counts by light microscopy, in milk from infected quarter with *S. aureus* of vaccinated and control cows

Treatment	Time[a]	SCC × 10^3	Neutrophil (LM)[b]		PMN (FACS)		Mo (FACS) (%)	$CD4^+$ (FACS) (%)	$CD8^+$ (FACS) (%)	Epithel (LM) (%)
			%	Number	(Gate) %	%				
Vaccinate (17)	0	109 ± 25A	38 ± 5	41	38 ± 4	36 ± 3	16 ± 2	1.4 ± 1	7.6 ± 1	27 ± 4
	1	1644 ± 289B	63 ± 4	1044	62 ± 5	56 ± 4	13 ± 2	0 ± 0	3.6 ± 1	17 ± 3
	7	380 ± 155A	59 ± 4	223	61 ± 5	52 ± 3	16 ± 1	1.8 ± 1	5.3 ± 1	15 ± 4
	13	60 ± 28A	52 ± 4	90	55 ± 3	53 ± 3	15 ± 1	3.7 ± 1	7.4 ± 1	20 ± 3
Control (21)	0	148 ± 48C	45 ± 5	72	48 ± 5	45 ± 4	19 ± 2	3.8 ± 2	6.8 ± 1	22 ± 3
	1	1420 ± 302D	73 ± 4	1073	74 ± 5a	36 ± 6b	14 ± 2	2.4 ± 1	2.9 ± 1	5 ± 3
	7	879 ± 244D	72 ± 3	633	75 ± 6a	41 ± 6b	17 ± 2	2.3 ± 1	5.1 ± 1	2 ± 1
	13	523 ± 145D	68 ± 4	551	71 ± 4a	46 ± 5b	18 ± 2	2.4 ± 1	5.8 ± 1	4 ± 2

A and B or C and D means within a column and treatment with different letters differ significantly ($P < 0.05$). The a and b means within a row and PMN with different letters differ significantly ($P < 0.05$).

[a] Time post-challenge (days).

[b] Neutrophils or epithelial cells counted by light microscopy; absolute numbers of neutrophils were calculated from the SCCs determined by CC.

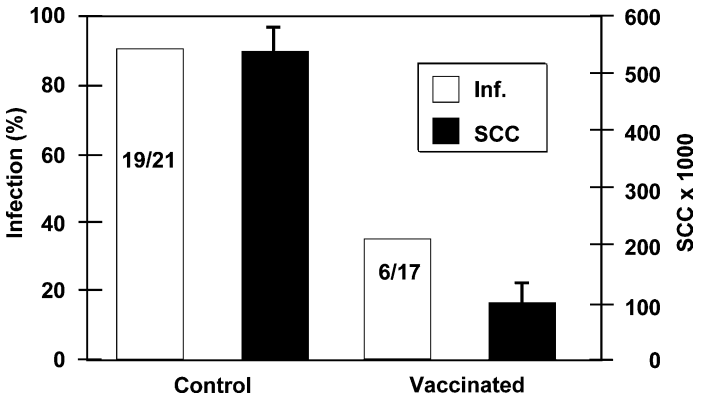

Fig. 2. Percentages and numbers of infected quarters and the SCCs of those quarters 2 weeks after experimental challenge with *S. aureus* of cows previously vaccinated with the said vaccine or with the vehicle only (control).

compared between control and vaccinated cows (i.e. 19 out of 21 control quarters and 6 out of 17 vaccinated quarters), a significant difference ($P < 0.05$) was again found: the mean SCC in the control cows was 540×10^3 cells/ml whereas that in the vaccinated ones was only 100×10^3 cells/ml (Fig. 2). Similar results were observed with *NAGase* concentrations (data not shown).

The main change in the distribution and number of cell types of the SCC in the challenged quarters (vaccinated and control) was expressed in a sharp increase in numbers of PMN cells, mainly neutrophils, as recorded by LM (Table 3). This change in the distribution of the neutrophils remained high in all the challenged quarters. However, the absolute numbers in the vaccinated animals decreased because of the general decrease in the SCC. No differences were found in the percentages of neutrophils and PMN cells gated (FLS by SLC. However, in the control cows the percentage of PMNs (FACS and LM) labeled by the mAb G1 was significantly lower (Table 3). This phenomenon was not observed in the vaccinated cows. The proportions of macrophages and lymphocytes bearing $CD4^+$ or $CD8^+$ changed slightly, following the challenge, but not significantly. However, the absolute numbers of lymphocytes increased in those samples in which SCC was also significantly higher (Table 3). The proportion of epithelial cells decreased significantly ($P < 0.05$) in the control cows only.

Histopathological examination of the inductive areas showed normal structures with no pathological findings. In the control cows, epithelial tissue of all challenged teats (including the two quarters from which no *S. aureus* was isolated) exhibited a mild suppurative inflammatory reaction and/or lymphocytic infiltration. In the vaccinated cows a slight lymphocytic infiltration was found only in the quarters that shed *S. aureus* (6 out of 17 quarters). Epithelial tissue from unchallenged teats of both vaccinated and control cows exhibited normal structure.

4. Discussion

Upon administration of the vaccine to cows, nogeneral cytotoxic or local tissue damage was observed. Thus the vaccine appears to be safe. The pathogenicity of one bacterial strain could be evaluated according to the degree of infection, as indicated by secretion of the bacteria and by the SCC. The level of infection established in the control cows clearly demonstrated a transient and mildnature, similar to that observed in a previous study demonstrating characteristic chronic infections (Shoshani et al., 2000). Highly significant differences were found between the vaccinated and the unvaccinated (control) cows: whereas 19 out of 21 challenged quarters of the control group were infected and developed continuous infection, only 6 out of 17 challenged quarters of the immunized cows were infected. Moreover, a comparison between the SCC in the infected quarters of the immunized cows and that of the control cows revealed that the immunized cows had normal values (100×10^3) whereas the

control cows showed elevated counts (540×10^3), indicating a state of inflammation. These results are consisted with the findings of a previous challenge trial (Pankey et al., 1985) in which the vaccinated cows had significantly lower SCCs than the control animals.

At the time of challenge all quarters were healthy. Moreover, the distribution of the SCCs was similar to that found in our previous study, with regard to milk leukocyte population in heifers free of udder infection (Leitner et al., 2000a). It is important to note that epithelial cells rather than macrophages were the main cell type found, and that the proportion of macrophages did not exceed 17%. These findings are in agreement with those of Giesecke and van den Heever (1967) but differ from those of other studies, that found that macrophages were the predominant cell type in milk (60%) and tissues (Lee et al., 1980; Concha, 1986; Sordillo et al., 1988). In our present experiments, neutrophils increased in number and proportion soon after the inoculation and were the main cell type in all challenged quarters. These findings are consistent with typical acute mastitis (Burvenich et al., 1995). However, leukocytes that were identified by LM as neutrophils and were gated as PMN by the FACS, received significantly less labeling by the mAb G1 in the control cows than in the vaccinated ones. These results are in agreement with those previously reported for acute mammary infection by *E. coli* and *S. aureus* (Leitner et al., 2000c). This phenomenon was not observed in the vaccinated cows. The remaining of the receptor in the vaccinated animals could suggest that the neutrophils in the vaccinated cows may have a higher function although the activity of the receptor for this mAb (G1) is unknown. All vaccinated cows exhibited antibodies specific to *S. aureus* in their sera and milk, and these persisted throughout the whole period of the experiment. These antibodies may play a significant role in the acquisition of immunity against subsequent *S. aureus* infection.

The cows were challenged with one of the three *S. aureus* strains from which the vaccine was prepared. It is important to note that the whole bacteria of two strains were included in the vaccine, whereas only the supernatant of the challenge strain, secreted in the first 24 h of incubation, was used as a component of the vaccine. Upon immunization trials in a mouse model (data not shown) the antigens composed of only the supernatant could not elicit significant protection against the challenge of the homologous strain, while the whole vaccine did so successfully. Thus, the challenge in this experiment can be considered as a semi-homologous one. In the mouse model experiments, a significant protection could be obtained, when challenge was presented by different strains of *S. aureus* from those used in the vaccine. We can hypothesize that the vaccine might protect not only against the strains it comprises, but also against a large variety of other pathogenic *S. aureus* strains. However, this hypothesis needs to be evaluated in a large-scale field trial.

In conclusion, the vaccine used in this experiment proved to be safe: it provided about 70% protection from *S. aureus* infection and complete protection from inflammatory reactions as expressed by the SCC.

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