ABSTRACT

Staphylococcal enterotoxin C (SEC), a superantigen, is the most frequently expressed enterotoxin by bovine strains of Staphylococcus aureus causing mastitis. To examine the possible impact of SEC on the immune response of the bovine mammary gland, we monitored changes in lymphocyte subpopulations in mammary glands of four lactating cows after intramammary instillation of S. aureus strain Rn4220 transformed with a plasmid containing a gene coding for SEC1. Four other lactating cows received the same strain transformed with the plasmid without the SEC1 gene (positive control), and four cows were untreated (negative control). Mammary quarter milk samples for somatic cell count (SCC) analysis and determination of N-acetyl-β-D-glucosaminidase (NAGase) activity levels were collected daily for 21 d postinstillation. Flow cytometry utilizing three-color analysis was used to phenotype lymphocyte subpopulations isolated from milk samples collected on d 0, 4, 7, 11, 14, 18, and 21 postinstillation from all the cows. Milk from mammary gland halves (positive control and experimental) or all mammary quarters (negative control) was collected for flow cytometric analysis. Increased NAGase activity, SCC, and isolated S. aureus demonstrated that infection was established in mammary quarters intramammary instilled with bacteria. There were no significant differences (P > 0.05) in the proportions of BoCD4 helper T lymphocytes or BoCD8 cytotoxic T lymphocytes between the two infected treatment groups. There was a significant day × treatment difference of the proportion of a γδ T cell subpopulation that did not express BoCD2, but did express the ACT2 activation molecule and a significant treatment difference of a γδ T cell subpopulation that expressed BoCD2, but not the ACT2 activation molecule (P < 0.05). Results do not support the hypothesis that the presence of the gene for SEC1 alters the mammary BoCD4 or BoCD8 T lymphocyte response to infection.

(Key words: Staphylococcus aureus, mastitis, staphylococcal enterotoxin C, superantigen, γδ T lymphocytes, lymphocytes)

Abbreviation key: E = experimental, EC = E noninfected mammary quarter, EI = E infected mammary quarter, NAGase = N-acetyl-β-D-glucosaminidase, NC = negative control, PC = positive control, PCI = PC infected mammary quarters, PCC = PC noninfected mammary quarter, SEC = staphylococcal enterotoxin C.

INTRODUCTION

Mastitis is one of the costliest diseases in animal agriculture. Contagious mastitis, transmitted from cow to cow during milk removal, is mainly caused by Staphylococcus aureus (Fox and Gay, 1993). Unlike other major mastitis pathogens that are associated with transient infections, S. aureus causes infections of longer duration. Bovine S. aureus strains express many virulence factors (Sutra and Poutrel, 1994), which may enable this organism to cause chronic infections. One class of virulence factors is the staphylococcal enterotoxins, which function as superantigens (Marrack and Kappler, 1990).

Depending on the geographical area studied, a varying proportion (0 to 50%) of bovine mastitis S. aureus isolates have one or more genes coding for a staphylo-
MAMMARY IMMUNE RESPONSE TO SEC 2045
coccal enterotoxin (Lee et al., 1998). Staphylococcal enterotoxin C (SEC) is the most common enterotoxin secreted by bovine mastitis isolates (Kenny et al., 1993). Similar to other superantigens, SEC is able to stimulate an aberrant immune response by bovine peripheral T lymphocytes as demonstrated in vitro by Ferens et al. (1998). Analysis of the composition of lymphocytes in milk from healthy cows has shown the proportions of \( \alpha \beta \), \( \gamma \delta \) T, and B lymphocytes are similar throughout cycles of lactation. The \( \alpha \beta \) T lymphocytes are the predominant population. Very few B lymphocytes have been found at any stage of lactation (Park et al., 1993; Taylor et al., 1994, 1997). In contrast to peripheral blood, the ratio of CD4 to CD8 is less than one. The inverse ratio appears to be attributable to the presence of a subset of CD8\(^+\) \( \alpha \beta \) T lymphocytes that constitutively express ACT2 (Park et al., 1993). Two subsets of \( \gamma \delta \) T cells are present that are distinguished by a difference in the expression of CD2. As noted here and in previous studies, the majority of \( \alpha \beta \) T lymphocytes have a memory phenotype CD45R0 low (Taylor et al., 1994) or CD45R0 high or CD45R CD45R0 high. The latter two populations present in peripheral blood of cattle have been shown to contain memory T lymphocytes (Bembridge et al., 1993). Although the relative proportions of lymphocyte subpopulations appear not to change at different stages of lactation, analysis of cytokine mRNA profiles from enriched preparations of CD4\(^+\) T lymphocytes from milk have shown that the message for IL-2 and IFN-\( \gamma \) is present in cells obtained at mid- and late lactation, and that for IL-4 and IL-10 is present in cells obtained postpartum. Expression of these cytokines is associated with T helper 1 and T helper 2 effector activity respectively (Shafer-Weaver et al., 1999). Functional studies have provided data that suggest that the proliferative response of CD4\(^+\) T lymphocytes to antigen is down regulated in the presence of CD8\(^+\) ACT2\(^+\) T lymphocytes (Park et al., 1993). Studies in cows with staphylococcal mastitis suggest this population could impair CD4 T cell responses to the pathogen.

The recent finding that SEC elicits aberrant activation of CD8\(^+\) T lymphocytes in vitro suggested that SEC might have a similar activity in vivo and alter the composition of lymphocyte subpopulations and their state of activation (Ferens et al., 1998). Studies by Park and coworkers (1992, 1993, 1994) did not determine whether the S. aureus causing infection produced enterotoxins. Thus, in vivo studies examining intrammary bovine T cell responses to superantigens are lacking. To this end, we examined lymphocyte subpopulations isolated from a mammary gland in the course of a S. aureus IMI. A recent report highlights the possible link between BoCD4 T lymphocytes responses and resistance to mastitis (Shafer-Weaver et al., 1999). The hypothesis tested in this study was that S. aureus possessing a gene coding for SEC would affect the immune response of the bovine mammary gland, specifically by aberrant interaction of the SEC protein with BoCD4 and BoCD8, causing abnormal activation of T lymphocytes. Specifically, we investigated whether SEC positive bacteria altered the proportion of memory and naive T lymphocytes in milk during the course of infection and also whether there was an associated aberrant alteration in expression of activation molecules ACT2 and the interleukin 2 receptor (CD25).

MATERIALS AND METHODS

Twelve lactating Holstein cows from the Washington State University dairy herd were assigned to three treatment groups (Table 1). Animals were split into three main groups, a negative control (NC), a positive control (PC), and an experimental (E). Positive-control and experimental cows received intramammary instillations of S. aureus to both quarters of one udder half. The EI cows were instilled with a strain of S. aureus RN4220, which was transformed and contained a plasmid containing a gene coding for SEC1. The PC cows received the same strain of S. aureus, which lacked the gene coding for SEC1. Negative control cows were untreated. The animals were maintained according to the Association for the Assessment and Accreditation of Laboratory Animal Care, and guidelines established by the Animal Care and Use Committee at Washington State University.

Staphylococcus aureus strain RN4220 was transformed with isogenic plasmids, either pMIN121 (SEC+) (Hovde et al., 1990) or pMIN164 (SEC−) (Marr et al., 1993), both of which encoded erythromycin resistance. The bacteria and plasmids were maintained as described (Hovde et al., 1990). Intramammary instillations of bacteria were prepared as by Postle et al. (1978). Bacterial suspensions instilled into the mammary quarters contained 300 cfu of bacteria. To prevent growth of S. aureus that was void of the plasmid, 6 ml of erythromycin (Agri Laboratories, St. Joseph, MO) was instilled into each of the four mammary quarters of positive control and experimental cows on d 0, 4, 7, 11, 14, and 18.

Milk was collected by the gallon for lymphocyte isolation on d 0, 4, 7, 11, 14, and 18, and 21 postbacterial postinfection. Lymphocytes were isolated from milk as described by Park et al. (1992). Expression of cell surface molecules on subpopulations of milk lymphocytes was monitored by three-color flow cytometry as described by Davis et al. (1995). As outlined in Table 2, combinations of three mAb were used to identify the respective subpopulations T helper (CD4), cytotoxic T lymphocytes...
Table 1. Intramammary treatments received by the various cow groups.

<table>
<thead>
<tr>
<th>Main group</th>
<th>Intramammary instillation</th>
<th>Instilled mammary gland quarters</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (NC)</td>
<td>None</td>
<td>All quarters</td>
<td>NC</td>
</tr>
<tr>
<td>Positive control (PC)</td>
<td>SEC1-bacteria</td>
<td>Either the left or right quarters</td>
<td>PCC</td>
</tr>
<tr>
<td>Experimental (E)</td>
<td>Sterile saline</td>
<td>Either the left or right quarters</td>
<td>EI</td>
</tr>
</tbody>
</table>

1Staphylococcus aureus strain RN4220 with pMIN164 was intramammarily instilled to establish an experimental infection. SEC = Staphylococcal enterotoxin.

2Staphylococcus aureus strain RN4220 with pMIN121 was intramammarily instilled to establish an experimental infection.

3Milk samples for cellular isolation were collected from all mammary gland quarters as a composite sample.

4Treatments were randomly assigned to either the left or right mammary gland half.

(CD8), and γδ T lymphocytes; determine whether they expressed isoforms of CD45 expressed on memory (CD45R0) or naive (CD45R) T lymphocytes; and whether they expressed molecules associated with activation ACT2 and IL-2Rα. The lymphocyte subpopulations were measured on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). For analysis of data we initially, selectively gated on the main subpopulations of T lymphocytes (BoCD4, BoCD8, γδ T lymphocytes) to obtain the primary dataset for analysis. After selecting for one of these populations, we determined whether subsets of the selected subpopulation of lymphocytes 1) expressed molecules associated with a memory or naïve phenotype on αβ T lymphocytes (CD45R and CD45R, respectively) or a molecule (CD2) that distinguishes the two subpopulations of γδ T lymphocytes, and 2) whether the identified subpopulation subsets expressed activation molecules (ACT2 or CD25). These subsets were categorized as either a positive/positive, positive/negative, negative/positive, or negative/negative phenotype for the specified secondary and tertiary cell surface molecules. With the combinations of cell surface molecules, 32 subsets of lymphocytes were measured (12 subsets of BoCD4 lymphocytes, 12 subsets of BoCD8 lymphocytes, and 8 subsets of γδ T lymphocytes).

Table 2. Combinations of mAb used to identify lymphocyte subsets and determine their proportions in milk.

<table>
<thead>
<tr>
<th>Gated population</th>
<th>Secondary cell molecule</th>
<th>Tertiary cell molecule</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoCD4(+)+</td>
<td>CD45R (+)</td>
<td>CD45R0 (+)</td>
<td></td>
</tr>
<tr>
<td>BoCD8(+)+</td>
<td>CD25 (+)</td>
<td>ACT2 (+)</td>
<td></td>
</tr>
<tr>
<td>BoCD2(+)</td>
<td>BoCD2 (+)</td>
<td>CD25 (+)</td>
<td></td>
</tr>
</tbody>
</table>

1IL-A11A, IgG2a, VMRD, Pullman, WA (Baldwin et al., 1986).
2T2B, IgG2a, Dr. W. C. Davis, Washington State University, Pullman, WA (Unpublished).
3TCR1-N24,GB21A, IgG2b, (δ chain specific) VMRD, Pullman, WA (Davis et al., 1996).
4GC44A, IgG1, VMRD, Pullman, WA (Baldwin et al., 1995).
5BAQ95A, IgG1, VMRD, Pullman, WA (Davis and Splitter, 1991).
6GS5A, IgG1, VMRD, Pullman, WA (Baldwin et al., 1995).
7ACT146A, IgG1, VMRD, Pullman, WA (Naessens et al., 1992).
8ACT26A, IgG1, VMRD, Pullman, WA (Park et al., 1993).
9ACT77A, IgM, VMRD, Pullman, WA (Park et al., 1993).
10ACT77B, IgM, VMRD, Pullman, WA (Park et al., 1993).

RESULTS

The average daily arithmetic mean, NAGase activity (nmole/min per milliliter), over the 21-d trial of the

Journal of Dairy Science Vol. 84, No. 9, 2001
infected mammary quarters (PCI and EI) was significantly higher than the contralateral control mammary quarters (PCC and EC), which were all significantly higher than the NC mammary glands (Table 3). The NAGase activity of the infected mammary quarters were not significantly different from each other (Table 3). Similarly, the average daily geometric mean SCC (Table 3) over the 21-d trial of the PCI and EI quarters were significantly greater than PCC and EC quarters, which were all significantly higher than NC quarters (Table 3). The SCC of the infected mammary quarters were not significantly different from each other.

Using different combinations of the mAb listed previously (Table 2), 32 T lymphocyte subpopulation proportions were analyzed. The major subpopulations included subsets of αβ helper and cytotoxic T lymphocyte and γδ T lymphocyte that expressed different combinations of cell surface accessory molecules (BoCD2, CD45R0, or CD45R) and cell surface activation molecules (CD25 and ACT2).

In general, there were very few significant treatment group differences in the proportion of lymphocytes with the BoCD4 and BoCD8 surface molecules (Tables 4 and 5). However, where differences were found, it was generally between the mammary quarters infected with S. aureus (Tables 4 and 5). The general linear model analysis indicated that the overall proportions of EI and PCI lymphocyte populations with these molecules were similar and significantly different from each other. Treatment group populations.

There were major differences in the proportions of certain subpopulations of γδ T lymphocytes over time. On d 4 postinstillation, 47% of the γδ T lymphocytes of the PCI treatment group and 16% of the EI treatment group expressed the ACT2 activation molecule, but not BoCD2; data not shown. The general linear models indicated a significant day × treatment basis for those γδ BoCD2+, ACT2+ lymphocytes. Data also demonstrated that 24.8% of the γδ T lymphocytes of the PCI treatment group and 18.8% of the EI treatment group expressed BoCD2, but did not express ACT2 over the 21-d treatment period (Table 4). Save for the γδ+, BoCD2+, CD25+, the S. aureus infected quarters (EI and PCI) had similar proportions of lymphocytes that expressed these molecules. Expression differed from that of lymphocytes from control quarters of the same cow (Table 6).

**DISCUSSION**

The aim of this research was to test the hypothesis that a S. aureus strain possessing a gene coding for SEC would alter an immune response of the mammary gland. This hypothesis was tested by comparing the proportions of selected mAb-defined subsets of BoCD4, BoCD8, and γδ T lymphocytes during an experimental IMI with S. aureus strain RN4220 transformants with and without an SEC1 gene. These were the populations of T lymphocytes present in the bovine mammary gland that were likely to change in response to a S. aureus IMI (Park et al., 1992, 1993, 1994). We also examined in detail subpopulations of lymphocytes that appeared to be aberrantly stimulated in vitro by SEC1 (Ferens et al., 1998). To this end, the current study was undertaken to test the hypothesis and to refine hypotheses that would evolve into future research on particular subpopulations of T lymphocytes that were atypically stimulated in vivo by an experimental S. aureus IMI when a SEC1 gene was present.

The elevation of milk SCC and NAGase activities in infected mammary quarters indicates that IMI induced an inflammatory response. The IMI in EI and PCI glands appeared to have an affect on adjacent noninfected quarters, as the SCC and NAGase activities were significantly lower in NC glands (Table 3). However, such changes in milk composition in a mammary quarters adjacent to a quarter with a noxious agent has been reported and may be related to glandular changes in the blood-milk barrier (Fox et al., 1981).

There were no statistically significant (P < 0.05) differences in the 24 subset proportions of BoCD4 and BoCD8 T lymphocytes analyzed from either the PCI or EI mammary quarters. This finding indicates that the presence of the SEC1 gene did not affect the major parameters of the mammary BoCD4 and BoCD8 T lymphocyte response to intramammary S. aureus infection. Park et al. (1992) found that the ACT2 activation molecule was upregulated on mammary BoCD8 T lymphocytes compared with BoCD8 peripheral blood T lymphocytes and was more upregulated on BoCD8 T lymphocytes from naturally infected mammary glands. A subpopulation of BoCD8 T lymphocytes that expressed ACT2 suppressed BoCD4 T cells in a dose-dependent manner (Park et al., 1993). In our study, BoCD8 γδ

---

**Table 3.** Mean (±SD) N-acetyl-β-D-glucosaminidase activity (NAGase) and SCC from milk collected from statistical treatment group quarters during the 21-d trial.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>NAGase activity</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCI</td>
<td>82.8 ± 51.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.9 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PCC</td>
<td>40.9 ± 20.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EI</td>
<td>95.7 ± 101.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>EC</td>
<td>41.5 ± 34.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NC</td>
<td>19.0 ± 8.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.1 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>nMeans within a column not sharing a superscript are significantly different, (P < 0.05).

<sup>b</sup>SCC, log<sub>10</sub> cells/ml.

<sup>c</sup>Treatment groups described in Table 1.
intramammary instillation, mammary quarter adjacent to PCI quarter. Mean percentage of lymphocytes expressing cell surface molecules by treatment group for BoCD8.2

Table 4. Mean1 percentage of lymphocytes expressing cell surface molecules by treatment group for BoCD4.2

<table>
<thead>
<tr>
<th>Cell surface markers</th>
<th>NC</th>
<th>EI</th>
<th>EC</th>
<th>PCI</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45R+</td>
<td>69.2 ± 19.8bc</td>
<td>69.4 ± 21.3bc</td>
<td>82.1 ± 16.9a</td>
<td>64.8 ± 21.3a</td>
<td>77.5 ± 16.5ab</td>
</tr>
<tr>
<td>CD45R–</td>
<td>15.8 ± 11.5a</td>
<td>13.3 ± 15.8a</td>
<td>8.0 ± 5.4a</td>
<td>13.3 ± 8.5a</td>
<td>9.8 ± 8.4ab</td>
</tr>
<tr>
<td>CD45R+</td>
<td>4.6 ± 6.3a</td>
<td>6.2 ± 8.4a</td>
<td>4.9 ± 8.7a</td>
<td>8.7 ± 11.3a</td>
<td>4.6 ± 4.9a</td>
</tr>
<tr>
<td>CD45R–</td>
<td>9.0 ± 8.8ab</td>
<td>10.8 ± 11.3a</td>
<td>4.0 ± 6.3a</td>
<td>13.2 ± 13.8a</td>
<td>7.8 ± 7.8ab</td>
</tr>
<tr>
<td>CD45R+</td>
<td>66.1 ± 21.4b</td>
<td>63.4 ± 16.5b</td>
<td>75.2 ± 14.7a</td>
<td>65.0 ± 15.0b</td>
<td>74.8 ± 15.7a</td>
</tr>
<tr>
<td>CD45R–</td>
<td>20.6 ± 16.6ab</td>
<td>21.0 ± 9.9a</td>
<td>16.5 ± 9.1ab</td>
<td>19.3 ± 7.6ab</td>
<td>14.8 ± 10.2b</td>
</tr>
<tr>
<td>CD45R+</td>
<td>8.7 ± 8.5ab</td>
<td>12.2 ± 13.6a</td>
<td>6.5 ± 8.2b</td>
<td>9.8 ± 7.4ab</td>
<td>8.6 ± 8.8ab</td>
</tr>
<tr>
<td>CD45R–</td>
<td>4.7 ± 7.7ab</td>
<td>3.4 ± 4.0ab</td>
<td>1.8 ± 3.4b</td>
<td>6.0 ± 11.2b</td>
<td>1.9 ± 1.6b</td>
</tr>
<tr>
<td>CD45R+</td>
<td>71.8 ± 16.8b</td>
<td>76.3 ± 15.5b</td>
<td>81.0 ± 16.0b</td>
<td>75.5 ± 12.5b</td>
<td>78.0 ± 17.3ab</td>
</tr>
<tr>
<td>CD45R–</td>
<td>14.3 ± 10.1a</td>
<td>10.7 ± 6.2a</td>
<td>10.6 ± 8.3a</td>
<td>12.1 ± 10.2a</td>
<td>10.5 ± 8.3a</td>
</tr>
<tr>
<td>CD45R+</td>
<td>10.1 ± 9.3a</td>
<td>10.9 ± 10.1a</td>
<td>6.7 ± 11.2a</td>
<td>8.7 ± 5.1a</td>
<td>9.1 ± 10.1a</td>
</tr>
<tr>
<td>CD45R–</td>
<td>3.8 ± 3.9a</td>
<td>2.8 ± 4.0a</td>
<td>1.7 ± 2.9a</td>
<td>3.8 ± 4.8a</td>
<td>2.4 ± 4.1a</td>
</tr>
</tbody>
</table>

1Means in the same row not sharing a common superscript were significantly different (P < 0.05).

2NC = Negative control, no intramammary instillation; EI = intramammary instillation with *Staphylococcus aureus* strain RN 4220 with pMIN 121; EC = no intramammary instillation, mammary quarter adjacent to EI quarter; PCI = intramammary instillation with *S. aureus* strain RN 4220 with pMIN 164; PCC = no intramammary instillation, mammary quarter adjacent to PCI quarter.

Table 5. Mean1 percentage of lymphocytes expressing cell surface molecules by treatment group for BoCD8.2

<table>
<thead>
<tr>
<th>Cell surface markers</th>
<th>NC</th>
<th>EI</th>
<th>EC</th>
<th>PCI</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45R+</td>
<td>59.2 ± 18.3b</td>
<td>60.6 ± 19.0b</td>
<td>62.2 ± 16.3a</td>
<td>56.3 ± 19.0a</td>
<td>65.4 ± 21.6ab</td>
</tr>
<tr>
<td>CD45R–</td>
<td>18.9 ± 10.2a</td>
<td>15.9 ± 7.8ab</td>
<td>14.0 ± 7.1b</td>
<td>19.2 ± 12.1a</td>
<td>14.7 ± 7.0ab</td>
</tr>
<tr>
<td>CD45R+</td>
<td>7.2 ± 7.5a</td>
<td>7.7 ± 9.7a</td>
<td>5.4 ± 12.8a</td>
<td>7.9 ± 10.5a</td>
<td>6.1 ± 7.6a</td>
</tr>
<tr>
<td>CD45R–</td>
<td>14.0 ± 5.8ab</td>
<td>15.8 ± 13.5a</td>
<td>9.2 ± 7.5a</td>
<td>16.6 ± 13.1a</td>
<td>13.9 ± 13.3ab</td>
</tr>
<tr>
<td>CD45R+</td>
<td>67.7 ± 18.9b</td>
<td>69.8 ± 17.9ab</td>
<td>78.4 ± 15.2a</td>
<td>67.3 ± 15.1a</td>
<td>75.3 ± 18.3b</td>
</tr>
<tr>
<td>CD45R–</td>
<td>14.0 ± 15.2a</td>
<td>11.4 ± 8.6ab</td>
<td>8.5 ± 6.6a</td>
<td>13.9 ± 11.4a</td>
<td>7.9 ± 7.7b</td>
</tr>
<tr>
<td>CD45R+</td>
<td>15.5 ± 7.5b</td>
<td>15.7 ± 11.5a</td>
<td>11.1 ± 8.9b</td>
<td>15.7 ± 5.1a</td>
<td>15.4 ± 13.3a</td>
</tr>
<tr>
<td>CD45R–</td>
<td>2.8 ± 3.5a</td>
<td>3.0 ± 3.7a</td>
<td>2.1 ± 5.2a</td>
<td>3.1 ± 2.9a</td>
<td>1.5 ± 1.9a</td>
</tr>
<tr>
<td>CD45R+</td>
<td>48.6 ± 17.5b</td>
<td>58.6 ± 16.1a</td>
<td>62.3 ± 17.3a</td>
<td>59.2 ± 12.1a</td>
<td>56.7 ± 16.0ab</td>
</tr>
<tr>
<td>CD45R–</td>
<td>34.9 ± 18.3a</td>
<td>22.8 ± 11.9b</td>
<td>26.1 ± 13.6b</td>
<td>22.5 ± 11.3b</td>
<td>26.0 ± 15.7b</td>
</tr>
<tr>
<td>CD45R+</td>
<td>12.5 ± 6.6ab</td>
<td>14.7 ± 12.2a</td>
<td>7.8 ± 6.3a</td>
<td>14.8 ± 8.7a</td>
<td>14.1 ± 14.4a</td>
</tr>
<tr>
<td>CD45R–</td>
<td>4.0 ± 2.8a</td>
<td>3.9 ± 3.3a</td>
<td>3.8 ± 5.5a</td>
<td>3.6 ± 3.1a</td>
<td>3.2 ± 3.1a</td>
</tr>
</tbody>
</table>

1Means in the same row not sharing a common superscript were significantly different (P < 0.05).

2NC = Negative control, no intramammary instillation; EI = intramammary instillation with *Staphylococcus aureus* strain RN 4220 with pMIN 121; EC = no intramammary instillation, mammary quarter adjacent to EI quarter; PCI = intramammary instillation with *S. aureus* strain RN 4220 with pMIN 164; PCC = no intramammary instillation, mammary quarter adjacent to PCI quarter.

Table 6. Mean1 percentage of lymphocytes expressing cell surface molecules by treatment group for γδ T cell.2

<table>
<thead>
<tr>
<th>Cell surface markers</th>
<th>NC</th>
<th>EI</th>
<th>EC</th>
<th>PCI</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BoCD2–</td>
<td>13.7 ± 7.6ab</td>
<td>12.0 ± 8.3b</td>
<td>7.4 ± 5.4a</td>
<td>15.6 ± 7.9a</td>
<td>10.8 ± 4.7b</td>
</tr>
<tr>
<td>BoCD2+</td>
<td>12.8 ± 7.6a</td>
<td>15.4 ± 15.3b</td>
<td>12.6 ± 8.4a</td>
<td>14.6 ± 9.9a</td>
<td>13.3 ± 12.5a</td>
</tr>
<tr>
<td>BoCD2–</td>
<td>33.5 ± 13.7b</td>
<td>46.2 ± 18.5b</td>
<td>36.0 ± 21.5bc</td>
<td>42.0 ± 12.5ab</td>
<td>32.7 ± 14.0b</td>
</tr>
<tr>
<td>BoCD2+</td>
<td>40.8 ± 17.6b</td>
<td>26.5 ± 16.8b</td>
<td>43.5 ± 21.9a</td>
<td>27.8 ± 14.5a</td>
<td>43.1 ± 15.4a</td>
</tr>
<tr>
<td>BoCD2–</td>
<td>19.9 ± 12.0a</td>
<td>11.2 ± 5.4a</td>
<td>8.6 ± 7.3a</td>
<td>15.6 ± 13.4a</td>
<td>11.5 ± 7.7a</td>
</tr>
<tr>
<td>BoCD2+</td>
<td>29.6 ± 15.9a</td>
<td>24.6 ± 14.3ab</td>
<td>28.3 ± 14.6ab</td>
<td>20.6 ± 12.5a</td>
<td>20.9 ± 14.0b</td>
</tr>
<tr>
<td>BoCD2–</td>
<td>29.7 ± 15.4a</td>
<td>45.4 ± 19.0a</td>
<td>31.1 ± 17.9a</td>
<td>39.1 ± 14.3b</td>
<td>34.7 ± 17.6a</td>
</tr>
<tr>
<td>BoCD2+</td>
<td>20.9 ± 9.4b</td>
<td>18.8 ± 12.3b</td>
<td>32.2 ± 14.0b</td>
<td>24.8 ± 13.0a</td>
<td>33.0 ± 15.5a</td>
</tr>
</tbody>
</table>

1Means in the same row not sharing a common superscript were significantly different (P < 0.05).

2NC = Negative control, no intramammary instillation; EI = intramammary instillation with *Staphylococcus aureus* strain RN 4220 with pMIN 121; EC = no intramammary instillation, mammary quarter adjacent to EI quarter; PCI = intramammary instillation with *S. aureus* strain RN 4220 with pMIN 164; PCC = no intramammary instillation, mammary quarter adjacent to PCI quarter.
T lymphocytes from mammary quarters instilled with bacteria containing the SEC gene did not exhibit any differences in ACT2 expression compared with BoCD8 γδ T lymphocytes isolated from mammary quarters instilled with bacteria without the SEC gene.

Major differences between experimental groups that could be ascribed to the presence of the SEC1 gene were statistically significant differences in two populations of γδ T lymphocytes. A population of γδ T lymphocytes that expressed the ACT2 activation molecule, but did not express the BoCD2 accessory cell surface molecule, was increased nearly threefold on d 4 in PCI cows compared with EI cows. A population of γδ T lymphocytes that did not express the ACT2 activation molecule but expressed the BoCD2 accessory molecule was also increased in PCI animals compared with EI animals.

The γδ T lymphocytes did not appear to be strongly activated in vitro by SEC1 in bovine lymphocyte studies (Ferens et al., 1998). In a study by Park et al. (1994), γδ T lymphocytes expressing BoCD8, the ACT2 activation molecule, and presumably, the BoCD2 cell molecule in the mammary gland were suppressive for BoCD4 T lymphocytes and were upregulated during a natural S. aureus IMI. It is not clear at this time if the subpopulation of γδ T lymphocytes that expressed ACT2 in this study is the subpopulation observed in previous studies because the BoCD2 cell molecule was not directly measured in previous studies. However, it is known that a subpopulation of γδ T lymphocytes that expresses BoCD8 also coexpress the BoCD2 cell molecule. It is not known whether S. aureus strains isolated from natural IMI contained a gene for SEC1 (Park et al., 1992).

The hypothesis of this study was based on results of previous work completed by collaborating laboratories. There are several possible explanations of differences between the previous in vitro studies and the current in vivo work. First, the effects of a S. aureus IMI may overshadow or mask the effects of SEC1. We saw statistically significant differences of some BoCD4 and BoCD8 subpopulation proportions between infected mammary quarters and uninfected mammary quarters, indicating that experimental methods were sufficient to detect differences attributable to an experimental intramammary S. aureus infection. However, differences in lymphocyte proportions attributable to SEC during an IMI may not be detectable by these same methods. Second, the bovine mammary gland may be too voluminous for SEC1 to affect the immune response. Lymphocytes isolated from the mammary secretions were from the whole mammary gland. Staphylococcus aureus may have colonized specific sites within the gland, which could allow the SEC1 to act at the specific site without affecting the remainder of the mammary gland. The amount of milk present in the mammary gland may dilute any SEC present to ineffective concentrations to affect the response of all mammary lymphocytes. Moreover, varying levels of SEC production may affect a local immune response in either an up-regulatory, neutral, or down-regulatory manner. Third, lymphocytes used in vitro studies were isolated from the peripheral blood. Lymphocytes isolated from peripheral blood exhibit different phenotypes than lymphocytes isolated from mammary secretions (Park et al., 1992). Lymphocytes present in mammary secretions express certain cell surface molecules that are not expressed, or expressed in low amounts in peripheral blood. These cell surface molecules may not be altered by SEC.

Fourth, the impact of SEC may not be readily detectable by the analysis of the expression of activation molecules used in this study. Fifth, this research included a 21-d experimental period. In vivo effects of SEC1 may be cumulative and need more time to manifest. Finally, 32 subsets of T lymphocytes were analyzed in this study. With a $P$ value of 0.05, it can be expected that one subset would be statistically significant by chance. In two different analyses, day $\times$ treatment and treatment, only one subset exhibited statistically significant differences in each analysis. Statistical significance does not necessarily equate to biological significance.

This study looked at some of the many cell surface molecules present on T lymphocytes. Future research in this area should focus on some of the other known molecules. The ACT3 activation molecule, an activation molecule normally found on BoCD4 T helper lymphocytes, is upregulated on BoCD8 T cytotoxic lymphocytes in cell cultures stimulated with SEC1 (Ferens et al., 1998). To increase our knowledge of γδ T lymphocytes, it would be informative to observe any suppressive effects of γδ T lymphocytes that express the ACT2 activation molecule, but not BoCD2, on other lymphocytes, especially BoCD4 T helper lymphocytes.

CONCLUSIONS

The current study did not demonstrate that presence of an SEC1 gene alters the response of BoCD4 or BoCD8 T lymphocytes in the bovine mammary gland during an experimental intramammary S. aureus infection. However, two subpopulations of γδ T lymphocytes displayed significantly different responses in the presence of the SEC1 gene. Results of this study did not support the hypothesis that the presence of the SEC1 gene contributes to the chronicity of an experimental S. aureus intramammary infection.

ACKNOWLEDGMENTS

The authors would like to thank Dorothy Newkirk, Mary Jo Hamilton, Claudia Deobald, John Swain, and
C. Gaskins for their advice and technical assistance. This research was funded in part by the Washington State Dairy Products Commission, Grant #0187, USDA Grant 99-35201-8581, NIH Grant P20RR15587-01, and the Washington State University Monoclonal Antibody Center.

REFERENCES


