Opsonization of \textit{Staphylococcus aureus} by Bovine Immunoglobulin Isotypes

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\section*{ABSTRACT}

The ability of specific bovine Ig isotypes to enhance phagocytosis of \textit{Staphylococcus aureus} by polymorphonuclear neutrophils was studied. Polymorphonuclear neutrophils were isolated from the blood of 14 lactating Holstein cows. Antibodies against \textit{S. aureus} M10 were produced by two Holstein cows immunized via intramuscular injections and injections in the area of the supramammary lymph node with M10 emulsified in dextran sulfate. The IgG1, IgG2, and IgM were prepared from immune sera. Fluorescein-labeled, formalin-killed \textit{S. aureus} M10 were opsonized with the respective isotypes prior to incubation with polymorphonuclear neutrophils. Percentage of polymorphonuclear neutrophils phagocytosing averaged 37.4, 1.1, 15.9, and 9.4\% for immune sera, IgG1, IgG2, and IgM, using a M10:polymorphonuclear neutrophils ratio of 10:1; and 77.1, 1.8, 32.1, and 57.9 using a 40:1 ratio. When IgG1 was incubated with either IgG2 or IgM, phagocytosis was reduced to 10.0 and 5.0\%, respectively, using the 10:1 ratio and 24.2 and 44.7\%, respectively, using the 40:1 ratio. Significant variation occurred among cows in the ability of polymorphonuclear neutrophils to undergo phagocytosis independent of isotype and \textit{S. aureus} M10:polymorphonuclear neutrophil ratio. These data show that IgG2 and IgM are opsonic for bovine polymorphonuclear neutrophils and that IgG1 inhibits the activity of both. These results will be helpful to determine immunization protocols to solicit synthesis of bovine IgM and IgG2 specific for \textit{S. aureus}.

(Key words: immunoglobulin, opsonization, isotype)

\small{Abbreviation key: PMN = polymorphonuclear neutrophil.}

\section*{INTRODUCTION}

\textit{Staphylococcus aureus} is a major cause of mastitis in cows (5, 25, 26). Polymorphonuclear neutrophil (PMN) phagocytosis is the most effective defense against \textit{S. aureus} infection of the mammary gland (4, 19). However, because of the ingestion of milk fat globules by milk PMN and a deficiency in phagocytosis-promoting antibodies (opsonins) in milk, milk PMN are less phagocytic than blood PMN. Therefore, high concentrations of milk PMN \((9 \times 10^5 \text{ of PMN/ml})\) are required to prevent infection (22). This concentration far exceeds the number of PMN in the uninfected gland \((<1 \times 10^5 \text{ of PMN/ml})\) (4). Increased phagocytic efficiency of PMN via increased specific antibodies decreases the number of PMN needed to protect the mammary gland against \textit{S. aureus} infection.

The four major classes (isotypes) of bovine antibodies are IgA, IgG1, IgG2, and IgM. Guidry et al. (7, 8, 9) showed a significant correlation between IgA and phagocytosis of \textit{S. aureus} by bovine PMN. Mackie et al. (14) later found that IgA was opsonic for \textit{Streptococcus agalactiae}, but their data were based on whey from acutely infected glands. Specific IgA from an immunized cow showed no opsonic effect when tested using the IgA concentration normally found in milk (2). Therefore, IgA is not considered to be a major opsonin in the bovine mammary gland.
Conversely, IgG1 exists in large quantities in lacteal secretions, particularly in colostrum, but IgG1 is not opsonic for bovine PMN, nor have receptors for IgG1 been identified on bovine (12, 16) or ovine (23) PMN. Williams and Hill (27) hypothesized that IgG1 may even block the effectiveness of other opsonins, but this hypothesis has not been tested. Receptors for IgG2 have been identified on PMN and were opsonic for bovine (11, 12, 15, 16) and ovine PMN (23, 24).

Grewal et al. (6) demonstrated Fc receptors for IgM on bovine PMN. Williams and Hill (27) showed that S. aureus and Escherichia coli were more readily phagocytosed when they were opsonized with IgM than with IgG2. Other reports (1, 7, 17) have also shown that IgM was correlated with blood PMN phagocytosis of S. aureus.

Those studies also suggest that IgG2 and IgM are the opsonins for bovine PMN, but none of those studies investigated either the additive or the inhibitory effects of these isotypes. Hambleton (10) showed that cows immunized with S. aureus produce specific IgG1, IgG2, and IgM isotypes, but the timing of the response varied. She (10) also showed that the peak IgG1 response was associated with decreased phagocytosis, which suggested an inhibition of opsonization and confirmed a previous observation (9). The current study was conducted to determine the ability of isolated bovine Ig isotypes from immunized cows to promote or to inhibit phagocytosis of S. aureus by bovine PMN.

MATERIALS AND METHODS

Preparation of Specific Isotypes

Because Ig bind nonspecifically to S. aureus protein A, S. aureus M10 (gift from P. Oeding, University of Bergen, Norway), a protein A-free isolate obtained from a case of bovine mastitis, was chosen for this study. A vaccine was prepared by emulsification of 1 ml of S. aureus M10 (5.0 × 10^9 formalin-killed S. aureus M10/ml in .01 M PBS, pH 7.4) with 1 ml of dextran sulfate (500 mg/ml of PBS, molecular weight 500,000; Sigma Chemical Co., St. Louis, MO). Two Holstein cows were immunized via injection of 1 ml of vaccine in the semitendinosus muscle and 1 ml in the area of the supramammary lymph node 14 d prior to drying off, at drying off, and at 28 and 56 d into the dry period. Jugular blood was collected on d 41 of the dry period and 23 d postpartum.

Fifty milliliters of serum, diluted 1:5 in starting buffer (.01 M PBS with .25 M NaCl, pH 7.0), were added to mouse monoclonal anti-bovine IgM conjugated to Sepharose 4b (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and rotated for 2 h at 23°C. The IgM-Sepharose 4b conjugate was filtered and washed three times with .85% saline. Bound IgM was eluted with .1 M Na2HPO4 (pH 2.5). Eluted IgM was adjusted to pH 7.2 and quantitated by absorbance at an optical density of 280 nm. Similar absorptions were performed using mouse monoclonal anti-bovine IgG2, IgG1, and IgA bound to Sepharose 4b.

Isotype purity of the antibodies was determined using ELISA. Eluted isotypes (200 ILl; 10 μg of Ig/ml of .1 M Na2CO3-NaHCO3, pH 9.6, with .02% NaN3) were placed in Immulon 2™ microtiter plate wells (Dynatech Laboratories, Inc., Chantilly, VA) and were incubated for 2 h at 23°C. The plates were washed four times with .85% NaCl, .05% Tween 20™ (Sigma). Each monoclonal ascites was diluted 1:500 (vol:vol) in diluent (.01 M PBS, .85% NaCl, .05% Tween 20), 200 ILl were added to each microtiter well, and the plates were incubated for 2 h. After the plates were washed four times, goat anti-mouse IgG, IgA, IgM (heavy and light chain) alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) was added, and the plates were washed four times, substrate (p-nitrophenyl phosphate disodium; Sigma) was added, and the plates were read on a Bio-Tek EL312 reader (Bio-Tek, Winooski, VT) at optical densities of 405 and 620 nm. The isotypes were reabsorbed until no crossreactivity was observed.

Agglutination Titers

Serum and purified isotypes (50 μl) were placed in Linbro™ conical microtiter plate wells (Dynatech Laboratories Inc.) and serially diluted 1:1 (vol:vol) in diluent. Formalin-killed M10 (25 μl, 1 × 10^9 organisms/ml) in diluent were added to microtiter wells and incubated overnight in a humidity chamber at 23°C. The
highest dilution showing a positive reaction was recorded as positive. Purified isotypes were diluted to equal agglutination titers for use in the phagocytosis assay (18).

**PMN Isolation**

Blood samples were collected from the jugular or coccygeal vein of healthy Holstein cows. Acid-citrate dextrose (10%) was used as an anticoagulant. Siliconized glassware was used for all cell isolations.

A granulocyte-enriched population was obtained using the method of Carlson and Kaneko (3). Whole blood was centrifuged (1500 x g, 5 min, 4°C), and plasma, buffy coat, and one-third of the red blood cell pellet were aspirated. The cell suspension was then mixed with a double volume of distilled water and mixed for 45 s to lyse the red blood cells. Isotonicity was restored by addition of a single volume of 2.7% PBS. The suspension was centrifuged (500 x g, 3 min, 4°C), and the pellet was washed in PBS three times (500 x g, 3 min, 4°C). Total cell counts were determined using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL). Differential cell counts were determined microscopically using Wright’s stain. Cell viability was determined using trypan blue exclusion (Sigma) (13).

**Phagocytosis**

Phagocytosis was determined using flow cytometry according to Saad and Hagelton (21) with minor modifications. Modifications were 1) the bacteria:PMN ratios were 10:1 and 40:1, 2) the bacteria plus PMN were incubated for 30 min, and 3) the extracellular fluorescence was quenched with 1% methylene blue.

Fluorescein-labeled M10 were incubated with purified isotypes from immune sera, purified isotypes from nonimmune sera, and Hanks balanced salt solution for 1 h at 23°C with gentle rocking. Polymorphonuclear neutrophils were added and incubated for 30 min at 37°C with gentle rocking. The reaction was stopped with 1.5 ml of ice-cold 85% NaCl with 0.02% EDTA. Extracellular bacteria were quenched with 1% methylene blue, and the samples were analyzed by flow cytometry, using an EPICS Profile flow cytometer (Coulter Electronics) equipped with a 488-nm argon ion laser. After gating on PMN, the percentage of PMN phagocytosing was determined after quenching, based on forward light scatter and log side scatter: percentage of PMN phagocytosing = percentage of PMN fluorescing after quenching noninternalized bacteria.

**Statistical Analysis**

Statistical analysis was performed using PROC GLM (SAS Institute, Cary, NC). Cow and Ig isotype were the independent variables. All parameters were checked for homogeneity of variance.

**RESULTS AND DISCUSSION**

Neither control nor immune IgG1, using either the S. aureus M10:PMN ratio of 10:1 or 40:1, showed an effect on percentage of PMN phagocytosing (Table I), which confirms previous studies (16, 18, 27). No difference existed between control IgG2 (4.6%) and control IgM (4.1%) at the 10:1 ratio. At the 40:1 ratio, the percentage of PMN phagocytosing was significantly greater in the presence of control IgM (46.4%) than in the presence of control IgG2 (6.1%), which agrees with the greater bactericidal activity of normal serum IgM in the report by Williams and Hill (27). The percentage of PMN phagocytosing varied among cows (P < .01) and was independent of isotype and S. aureus M10:PMN ratio. Similar variation among cows in the ability of bovine PMN to phagocytose S. aureus has been

<table>
<thead>
<tr>
<th>Sera</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgM</th>
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<tbody>
<tr>
<td>10:1 Bacteria:PMN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.6a,x</td>
<td>4.6a,x</td>
<td>4.1a,x</td>
</tr>
<tr>
<td>Immune</td>
<td>37.4</td>
<td>15.9b,y</td>
<td>9.4b,x</td>
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<td>40:1 Bacteria:PMN</td>
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<td></td>
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<tr>
<td>Control</td>
<td>0a,x</td>
<td>6.1b,y</td>
<td>46.4b,x</td>
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<tr>
<td>Immune</td>
<td>77.1</td>
<td>32.1b,y</td>
<td>57.9b,x</td>
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a,b,Means in a column without common superscripts differ significantly (P < .05).

x,y,Means in a row without common superscripts differ significantly (P < .05).

1,immune sera, from which the immune isotypes were isolated, were added to the incubation mixture.

Figure 1. Combined effect of IgG2 and IgM on polymorphonuclear neutrophil phagocytosis of Staphylococcus aureus M10. Means without common letters (a,b) differ significantly ($P < .05$).

reported (7, 8, 9). In the present study, the increase in phagocytosis with immune IgM (57.9%) compared with that occurring with immune IgG2 (32.1%) further demonstrates the importance of IgM as an opsonin in the bovine. These results agree with reports of others (1, 17, 18, 27) who have found association between IgM and phagocytosis. However, the transient nature of IgM following immunization compared with the more prolonged IgG2 response (20) could limit the effectiveness of IgM as a protective opsonin.

These data clearly show the importance of the bacteria:PMN ratio in phagocytosis assays. Immunization significantly increased the percentage of phagocytosis for both IgG2 and IgM at the low and high bacterial ratios. At the lower ratio, the effect of IgG2 and IgM on the percentage of PMN phagocytosis was additive (IgG2 + IgM = 21.1%) (Figure 1), suggesting separate binding sites on S. aureus, or PMN, or both. At the higher ratio, the immune IgM (57.9%) and the immune IgG2 plus IgM (57.8%) were essentially the same, suggesting that IgG2 played no active role. However, because immune IgG2 significantly increased percentage of phagocytosis from 6.1 to 32.1%, at the higher S. aureus M10:PMN ratio, the PMN were more likely to have reached maximum capacity to phagocytose.

Conversely, IgG1 inhibited phagocytosis when it was combined with IgG2 at the S. aureus M10:PMN ratio of 10:1 and with both IgG2 and IgM at the 40:1 ratio (Table 2). This inhibition suggests either competition of IgG1 for the same antigens as IgG2 and IgM or stearic hinderance of closely associated antigens on the bacteria. Competition for binding sites on the PMN was ruled out, because no binding sites for IgG1 exist on bovine PMN (12, 16).

The inhibition of IgG2 and IgM opsonization by IgG1 that was observed in this study supports previous observations of lower phagocytosis concomitant with elevated concentrations of specific IgG1 (10). This inhibition of IgG2 and IgM could be a crucial factor in decisions to determine the optimal mode of immunization, such as adjuvant, site of injection, and timing, which affects the specific isotype formed. For example, because the greatest incidence of bacterial infection of the mammary gland occurs shortly after drying off and around parturition, timing of immunization to effect an IgM response during either of these periods may prove to be protective.

Because IgG1 is the predominant isotype in lacteal secretions, particularly at calving, and readily responds to immunization, additional studies are needed to determine the role of IgG1 in defense of the mammary gland. Also, studies are needed to determine the opsonic ability of each isotype using organisms positive for protein A.

### Table 2. Ability of IgG1 to inhibit opsonization by IgG2 and IgM ($n = 7$).

<table>
<thead>
<tr>
<th>IgG1</th>
<th>IgG2</th>
<th>IgM</th>
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<tbody>
<tr>
<td>10:1</td>
<td>Bacteria:PMN&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>40:1</td>
<td>Bacteria:PMN</td>
<td></td>
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<tr>
<td>0x</td>
<td>32.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1x</td>
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<td>43.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>4x</td>
<td>24.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Means in a column without common superscripts differ significantly ($P < .05$).

<sup>1</sup>No IgG1 (0x) and IgG1 preincubated with bacteria at equal concentration (1x) and at four times (4x) the concentration of IgG2 and IgM.

<sup>2</sup>Polymorphonuclear neutrophils.
OPSONIZATION BY ISOTYPE

REFERENCES


