Immunoechemical Specificities of the Combining Sites of Bovine Immunoglobulins Reactive with Sepharose 4B

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(Received 29 August 1990/accepted 1 November 1990)

ABSTRACT. Binding specificities of calcium-dependent and -independent bovine IgM and IgG reactive with unsubstituted Sepharose 4B were determined by competitive binding assays. The binding of 125I-labeled calcium-dependent bovine IgM to unsubstituted Sepharose 4B was most effectively inhibited by lactose which was about 100 times more reactive than galactose and melibiose. Other inhibitors were much less potent. With 125I-labeled calcium-independent bovine IgM and IgG, on the other hand, lactose was as potent as galactose. Melibiose was about 10 times less potent than lactose and galactose, whereas other mono- and disaccharides were much less potent. From these findings, the combining site of calcium-dependent bovine IgM reactive with unsubstituted Sepharose 4B may be specific for lactose, whereas those of calcium-independent bovine IgM and IgG specific for galactose. — Key words: binding specificity, bovine immunoglobulin, Sepharose 4B.

Materials and Methods

Reagents: Bovine serum was isolated from whole blood of a cow which had been kindly provided by a local slaughter house, Atsugi, Kanagawa, Japan. Sugar inhibitors were purchased from Nakarai Chemical Ltd. Co., Kyoto, Japan. Peanut agglutinin (PNA) was the product of E. Y. Laboratories, San Mateo, CA, U.S.A.

Isolation of bovine immunoglobulins reactive with Sepharose 4B: Calcium-dependent and -independent bovine IgM and IgG reactive with unsubstituted Sepharose 4B were isolated by the methods described previously [22].

Hemagglutination and hemagglutination inhibition: Hemagglutination and hemagglutination inhibition were performed using saline with or without 50 mM CaCl2 as a diluent according to the methods described previously [22]. To determine anti-T (Thomsen-Freidenreich) antibody activities in bovine IgM and IgG, hemagglutination inhibition was carried out with PNA and neuraminidase-treated human type O erythrocytes since PNA is reactive with the T antigen [15].

Competitive binding assays: Bovine IgM and IgG were labeled with the Bolton-Hunter reagent (Amersham International, Amersham, UK) by the methods described previously [1, 23]. Competitive binding assays were carried out in a total volume of 500 μl using 50 mM Tris-HCl, pH 8.0, containing 0.2
M NaCl and 0.5% bovine serum albumin with or without 50 mM CaCl₂ as a diluent. In competitive binding assays with 125I-labeled bovine IgM (or \(-\)-labeled IgG) and unsubstituted Sepharose 4B, 50 \(\mu l\) of 20% Sepharose 4B was found to be sufficient to bind to 50-60% of approx. 4,000 cpm of 125I-labeled IgM (or \(-\)-labeled IgG). A mixture of labeled bovine IgM (or IgG) and inhibitor was added to the suspension of unsubstituted Sepharose 4B. The tubes containing 125I-labeled IgM (or \(-\)-labeled IgG) and Sepharose 4B were mixed by end-over-end rotation at 4°C for 3 hr. Separation of bound labeled bovine IgM (or IgG) from unbound labeled ones was done by centrifugation. An aliquot (450 \(\mu l\)) of the supernatant was assayed for unbound 125I-labeled IgM (or IgG). All determinations were performed in triplicate.

The data are expressed graphically as percentage inhibition of the binding of labeled bovine IgM or IgG against micromoles of different substances used as inhibitors. The formula used to calculate percentage inhibition is:

\[
\text{Percent Inhibition} = \left(1 - \frac{\text{total cpm - cpm in supernatant with inhibitor}}{\text{total cpm - cpm in supernatant without inhibitor}}\right) \times 100
\]

RESULTS

Determination of anti-T antibody activities in bovine IgM and IgG reactive with Sepharose 4B: As reported previously [22], bovine IgM reactive with Sepharose 4B showed weak hemagglutinating activities to human erythrocytes. Since natural anti-T antibodies and PNA are demonstrated to bind to Sepharose beads [6, 25], anti-T antibody activities in bovine IgM and IgG were determined by hemagglutination inhibition. No hemagglutination of intact human type O erythrocytes was found by calcium-dependent and \(-\)-independent bovine IgM and IgG reactive with Sepharose 4B at the highest concentrations used. Hemagglutination of neuraminidase-treated human type O erythrocytes was observed by calcium-independent bovine IgM reactive with Sepharose 4B, whereas no hemagglutination was found by other calcium-dependent and \(-\)-independent bovine IgM and IgG. However, hemagglutination of neuraminidase-treated human type O erythrocytes by PNA was not effectively inhibited by any of these bovine IgM and IgG at the highest concentrations used.

Competitive binding assays: To study specificities of bovine IgM and IgG reactive with unsubstituted Sepharose 4B, competitive binding assays were performed using different inhibitors. The inhibitory activities of these substances are shown in Figs. 1-3 and their minimum amounts to give 50% inhibition are summarized in Table 1. In competitive binding assays with Sepharose 4B and 125I-labeled calcium-independent IgM and IgG reactive with Sepharose 4B, similar results were obtained (Figs. 1 and 2, and Table 1). Of inhibitors tested, lactose (Lac) and Gal were the most potent. They were more reactive than

![Graph](image-url)

Fig. 1. Competitive binding assays by different substances of the binding of 125I-labeled calcium-independent IgM reactive with Sepharose 4B to Sepharose 4B. Inhibitors used were galactose (○), lactose (○), methyl a-D-galactopyranoside (◇), melibiose (⊙), mannose (△), glucose (×), N-acetylglucosamine (■), N-acetylgalactosamine (▲), L-fucose (◇), Na-lactate (○), Na-propionate (⊙), and Na-pyruvate (□).
Fig. 2. Competitive binding assays by different substances of the binding of 
$^{125}$I-labeled calcium-independent IgG reactive with Sepharose 4B to Sepharose 
4B. Inhibitors used were the same as those in Fig. 1.

Table 1. Inhibitory activities of different substances to the binding reaction between $^{125}$I-labeled bovine IgM or -labeled bovine IgG and Sepharose 4B

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Minimum amount ($\mu$ moles) to give 50% inhibition</th>
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<tbody>
<tr>
<td></td>
<td>Ca$^{2+}$-dependent IgM</td>
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<tr>
<td>----------------------------</td>
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</tr>
<tr>
<td>Galactose</td>
<td>$&gt;$17.8</td>
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<tr>
<td>Methyl $\alpha$-D-galactopyranoside (MeaGal)</td>
<td>ND*</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.2</td>
</tr>
<tr>
<td>Melibiose</td>
<td>$&gt;$31.0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>$&gt;$17.0</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>$&gt;$34.0</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>$&gt;$19.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>$&gt;$19.9</td>
</tr>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Na-lactate</td>
<td>$&gt;$26.0</td>
</tr>
<tr>
<td>Na-propionate</td>
<td>$&gt;$34.0</td>
</tr>
<tr>
<td>Na-pyruvate</td>
<td>$&gt;$51.0</td>
</tr>
</tbody>
</table>

*ND: not determined.

Fig. 3. Competitive binding assays by different substances of the binding of 
$^{125}$I-labeled calcium-dependent IgM reactive with Sepharose 4B to Sepharose 
4B. Inhibitors used were the same as those in Fig. 1.

methyl $\alpha$-D-galactopyranoside (MeaGal), whereas melibiose (Meli) was less potent than MeaGal. Less than 15–20% inhibition was obtained by other inhibitors such as N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), L-fucose (LFuc), mannose (Man), glucose (Glc), Na-lactate, Na-
propionate, and Na-pyruvate at the highest concentrations used. In competitive binding assays with unsubstituted Sepharose 4B and $^{125}$I-labeled calcium-dependent IgM reactive with Sepharose 4B, on the other hand, Lac was a most potent inhibitor which was about 100 times more reactive than Gal and Meli (Fig. 3 and Table 1). Forty-five% inhibition was obtained by Gal and Meli at the highest concentrations used, whereas less than 10% inhibition was found by other inhibitors (Fig. 3). The specificity of calcium-dependent bovine IgG reactive with Sepharose 4B was not tested since $^{125}$I-labeled calcium-dependent bovine IgG did not show good binding to Sepharose 4B.

**DISCUSSION**

Natural antibodies reactive with mannan, bacterial polysaccharides, or glycoproteins are found in human and animal sera [3, 7, 12, 16, 17, 19, 21] since human and animals are always exposed to normal gastrointestinal or pulmonary flora. Thus, their production seems to continue throughout life by constant antigen stimulation as found for anti-blood group antibodies. Although natural antibodies against carbohydrates have been reported to usually possess calcium-independent binding properties [3, 5, 8, 18, 20, 24], bovine serum contains calcium-dependent and -independent antibodies against polysaccharides [27]. The present study gave a suggestion that there may be some difference in the specificities of calcium-dependent and -independent bovine IgM and IgG reactive with polysaccharides such as Sepharose 4B.

Among immunoglobulins reactive with unsubstituted Sepharose 4B, the specificity of some human monoclonal IgM against *Klebsiella* K polysaccharides containing pyruvylated Gal has been most extensively studied [8]. Since Sepharose 4B is a purified form of agar which was known to have 4, 6 pyruvic acetylated Gal in its repeating unit, the specificities of bovine antibodies reactive with unsubstituted Sepharose 4B were determined by competitive binding assays to see whether or not they are also reactive with pyruvylated Gal. With $^{125}$I-labeled calcium-dependent bovine IgM reactive with Sepharose 4B, Lac was found to be a most potent inhibitor which was about 100 times more reactive than Gal. Meli was a less potent inhibitor than Gal. With $^{125}$I-labeled calcium-independent bovine IgM and IgG reactive with Sepharose 4B, on the other hand, both Lac and Gal were similarly potent. Mear Gal was a less potent inhibitor but was more reactive than Meli. Much less inhibition was obtained by other substances at the highest concentrations used in the binding of $^{125}$I-labeled bovine immunoglobulins reactive with Sepharose 4B. No inhibition was obtained by Na-pyruvate, Na-lactate, and Na-propionate at the highest concentrations used. These results were quite different from those with human monoclonal IgM with specificities for *Klebsiella* K polysaccharides containing pyruvylated Gal [8] since the precipitation of these human monoclonal IgM has been effectively inhibited by Na-pyruvate, Na-lactate, or Na-propionate [8]. Furthermore, these three inhibitors have been more reactive with the combining sites of human monoclonal IgM than Gal [8], whereas they were much less specific for those of bovine IgM (or IgG) reactive with Sepharose 4B than Gal.

On the other hands, guinea pig natural antibodies to the T antigen and PNA have been also demonstrated to bind to Sepharose 6B [6, 25]. However, bovine IgM and IgG reactive with Sepharose 4B may not contain anti-T antibody activities since hemagglutination of neuraminidase-treated human type O erythrocytes exposing the T antigen by PNA was not effectively inhibited by any of bovine IgM and IgG reactive with Sepharose 4B at the highest concentrations used. From these findings, the specificity of the combining site of bovine IgM (or IgG) reactive with Sepharose 4B may be different from those of human monoclonal IgM with a specificity for *Klebsiella* K polysaccharides and of anti-T antibodies although all of them commonly bind to unsubstituted Sepharose 4B or Sepharose 6B [8, 25]. Thus, the combining site of calcium-dependent bovine IgM to Sepharose 4B may be specific for Lac, whereas that of calcium-independent bovine IgM (or IgG) specific for Gal.

**ACKNOWLEDGEMENT.** This study was supported in part by the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan.

**REFERENCES**