Immunochemical Studies on Blood Groups

THE COMBINING SITE SPECIFICITIES OF MOUSE MONOCLONAL HYBRIDOMA ANTI-A AND ANTI-B*

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Mouse monoclonal hybridomas, five anti-blood group A, three anti-B, and one anti-AB, produced by various methods of immunization, have been characterized by quantitative precipitin tests and the fine structures of their combining sites have been mapped by oligosaccharide inhibition assays. The combining sites of antibodies of each specificity differed among themselves. Three of the five monoclonals were specific for difucosyl and two for monofucosyl A determinants. All but the anti-AB were strictly specific for blood group A or blood group B erythrocytes; all of the anti-A monoclonals gave essentially equivalent titers in hemagglutination tests with A1 and AB erythrocytes, except for a monoclonal anti-A prepared by immunization with a human gastric cancer cell line. The data provide additional evidence for the heterogeneity of the antibody response to the different antigenic determinants present on blood A and B substances and emphasize the importance of difucosyl determinants which comprise most of the determinants on the water-soluble blood group substances.

Since the discovery of the four human blood groups, A, B, O, and AB (1, 2), human sera containing anti-A and anti-B have been used in blood grouping. Such anti-A and anti-B sera contain heterogeneous populations of antibody combining sites. Initially, sera containing high titers of anti-A and anti-B were selected for blood grouping, but when it was found that injection of hog or human blood group A substance into individuals of groups B and O and of horse or human blood group B substances into individuals of groups A and O yielded high titer antisera (3, 4), anti-A from immunized B individuals and anti-B from immunized A individuals came into widespread use. Although these antisera contained heterogeneous populations of antibody molecules, their combining sites were studied by assays of the capacities of various isolated blood group oligosaccharides to inhibit the precipitin reaction of anti-A by blood group A substances and of anti-B by blood group B substances (5-8), the site specificities being considered most complementary to the oligosaccharides giving inhibition at the lowest concentrations. Thus many anti-A sera were found (7, 9, 10) to be most complementary to the following:

\[
\text{L-Fuc} \alpha_1^1 \text{L-Fuc} \text{Gal} \alpha_1^1 \text{D-GalNAc} \text{O-} \text{R} \text{1} \text{Type 1}
\]

or

\[
\text{L-Fuc} \alpha_1^1 \text{D-GalNAc} \text{O-} \text{R} \text{2} \text{Type 2}
\]

and anti-B sera to

\[
\text{L-Fuc} \alpha_1^1 \text{D-Gal} \text{O-} \text{R} \text{1} \text{Type 1}
\]

and

\[
\text{L-Fuc} \alpha_1^1 \text{D-Gal} \text{O-} \text{R} \text{2} \text{Type 2}
\]

R₁ is 3-linked α-galactosyl formed by alkaline borohydride and R₂ is 6-linked β-hexametetrol formed by alkaline β-elimination (peeling) and reduction by borohydride of a 3,6-linked galactose. In both of these structures, the nonreducing trisaccharides were immunodominant and contributed a large part of the binding energy of the intact determinant. Since the trisaccharide portions of both the A and B structures are identical, specificity differences, except for the terminal non-reducing GalNAc⁺ and Gal, among various anti-A and among anti-B sera involve the fourth sugar and the linkage of the trisaccharide to it. Any differences among antibody molecules with sites larger than the trisaccharide might not be detectable in view of the heterogeneity and the predominant

* This work was aided by National Science Foundation Grant NSF-PCM-81-02322, National Institute of Allergy and Infectious Diseases Grant 1RO1 AI-19042 (to E. A. K.), and by Cancer Support Grant CA13696 to Columbia University. This is paper LXXIII in the series. The abbreviations used are: GalNAc, N-acetyl-D-galactosamine; ELISA, enzyme-linked immunosorbent assays; Ap, alkaline phosphatase; BSA, bovine serum albumin; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Fuc, L-fucose; AbN, antibody nitrogen. When not given, all sugars are in D except for fucose which is L. 1 The abbreviations used are: GalNAc, N-acetyl-D-galactosamine; ELISA, enzyme-linked immunosorbent assay; Ap, alkaline phosphatase; BSA, bovine serum albumin; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; Fuc, L-fucose; AbN, antibody nitrogen. When not given, all sugars are in D except for fucose which is L.
were prepared in this laboratory from human ovarian cyst fluid or precisely mapped total binding energy. Blood group A and B substances contain a second fucose—provided by Drs. A. Lundblad and L. Messeter, Department of Clinical Chemistry and University Hospital, University of Lund, Lund, Sweden (21). AH16 was from Dr. Sen-Ihiro Hakomori, University of Washington, Seattle (22). Anti-AB A15/1A4.7.3 was shown to be monoclonal (20) since absorption with A or with B erythrocytes involved one or two fucoses. All of these gave satisfactory and comparable results as blood grouping reagents.

**Materials and Methods**

**Mouse Monoclonal Hybridoma Antibodies—**Anti-A AC-1001 and anti-B BC-1004 were obtained from Dr. Don Baker, University of Alberta, Edmonton, Alberta, Canada (17). A15/3D3.92.1, MH2/6D4.12.1, NH40/5B2.6.5 were provided by Dr. A. D. Lowe, and Z. Lennox, Medical Research Council Laboratory of Molecular Biology, Cambridge, England (18-20). Anti-A A003=40/5G7 and anti-B B003=46/2D7 were provided by Drs. A. Lundblad and L. Messeter, Department of Chemical Chemistry and University Hospital, University of Lund, Lund, Sweden (21). AH16 was from Dr. Sen-Ihiro Hakomori, University of Washington, Seattle (22). Anti-B A15/1A4.7.3 was shown to be monoclonal (30) since absorption with A or with B erythrocytes reduced both anti-A and anti-B levels proportionally. Table I gives the combining sites and that determinants may be type 1 or type 2 and involve one or two fucoses. All of these give satisfactory and comparable results as blood grouping reagents.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Antigen used for immunization</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Anti-A AC-1001</td>
<td>A1 erythrocytes and 01 coupled to BSA or to O erythrocytes</td>
<td>12</td>
</tr>
<tr>
<td>A15/3D3.92.1</td>
<td>A1 erythrocytes and A substance</td>
<td>18</td>
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<tr>
<td>MH2/6D4</td>
<td>Tissue culture cells (HT-29) from human group A colon carcinoma case</td>
<td>19</td>
</tr>
<tr>
<td>A003=40/5G7</td>
<td>A2 substance phenol-insoluble cyst 14</td>
<td>20</td>
</tr>
<tr>
<td>AH16</td>
<td>Human gastric cancer cell line MKN 45</td>
<td>21</td>
</tr>
<tr>
<td>Anti-B BC-1004</td>
<td>02 coupled to BSA or to O erythrocytes</td>
<td>17</td>
</tr>
<tr>
<td>NB10/5B2.45</td>
<td>B erythrocytes and B substance</td>
<td>17</td>
</tr>
<tr>
<td>B005=46/2D7</td>
<td>B substance</td>
<td>20</td>
</tr>
<tr>
<td>Anti-AB A15/1A4.7.3</td>
<td>A1 erythrocytes and A substance</td>
<td>21</td>
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</table>

<table>
<thead>
<tr>
<th>MONOCLONAL ANTIGEN USED FOR IMMUNIZATION</th>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>1</td>
</tr>
<tr>
<td>Acetate and Glucose</td>
<td>2</td>
</tr>
<tr>
<td>Acetate, Glucose, and D-Galactose</td>
<td>3</td>
</tr>
<tr>
<td>Acetate, Glucose, D-Galactose, and L-Fucose (Sigma); methyl-α-D-galactoside (Sigma); melibiose (Eastman Kodak Company); galactinol; Qu3.4; A511; Beach P100D62; GalNAc(α1-3) (Fucα1-2) Gal; Galα(1-3) (Fucα1-2) Gal; and Glucose, D-galactose, D-glucose, and L-Fucose (Sigma)</td>
<td>4</td>
</tr>
</tbody>
</table>

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**Monoclonal Antibodies**—The following were available: d-galactose, N-acetyl-d-galactosamine, N-acetyl-d-glucosamine, and L-fucose (Sigma); methyl-α-D-galactoside (Sigma); melibiose (Eastman Kodak Company); galactinol; Qu3.4; A511; Beach P100D62; GalNAc(α1-3) (Fucα1-2) Gal; Galα(1-3) (Fucα1-2) Gal; and Glucose, D-galactose, D-glucose, and L-Fucose (Sigma) | 4     |

**Inhibition Assays by ELISA—**Coupling of alkaline phosphatase to goat anti-mouse IgM was performed by the one-step glutaraldehyde procedure (58, 39). The required quantities of enzyme and antibody were mixed in the given volume and dialyzed overnight against 0.05 M phosphate-buffered saline, pH 7.2, at 4 °C. The 25% glutaraldehyde was added to a final concentration of 0.2% while the solution was gently stirred and the reaction mixture was kept at room temperature for 2 h. After dialysis against the same buffer overnight at 4 °C the mixture was diluted with 5% BSA, 0.05 M Tris/MgCl₂/Na azide buffer to the appropriate concentration and stored in the refrigerator at 4 °C.

**Hemagglutination Assays—** These were carried out using a Takatsy microrotator with 0.025 ml loops and a 2% suspension of human A₁, A₂, or B erythrocytes. A constant amount of mouse monoclonal hybridomas antibody containing 5 to 8 µg of antibody nitrogen was added to varying quantities of blood group substance (0-70 µg), the total volume was adjusted to 200 µl with saline (0.15 M NaCl), and the contents of each tube were mixed and incubated at 37 °C for 1 h and kept at 4 °C for 5 days with daily mixing. The tubes were centrifuged at 4 °C at 2000 rpm, the supernatants were decanted, and the precipitates were washed twice with 0.5 ml of chilled saline. Total nitrogen in the washed precipitates was determined by ninhydrin assay (37).
RESULTS

Hemagglutination Assays—Table II gives the hemagglutination titers of the various anti-A, anti-B, and anti-AB monoclonals. The titers of four anti-A monoclonals toward a, B, and anti-AB erythrocytes only varied over a 2-fold range. With AH16, produced to human gastric cancer cells, the titer with A, B, and anti-AB monoclonals was completely A- or B-specific with the undiluted material. With the anti-AB monoclonal the titers toward A, B, and B erythrocytes were 2048, 1024, and 1024, respectively.

Quantitative Precipitin Studies—Fig. 1 shows the quantitative precipitin curves obtained with various blood group substances and the five monoclonal anti-A samples studied. All five monoclonals showed blood group A specificity and did not react with B, H, Le^a, and Le^b substances. The blood group A substance from hog gastric mucosa and the A, B substances from human ovarian cyst fluids reacted strongly but tended to show relatively small differences in reactivity per microgram of A substance added. With AC-1001 and A15/3D3.92.1, the substances tend to fall into three groups but the members of each group differ. The most potent (group 1) in precipitating were cyst 9, MSM 10%, MSS 10% 2X with both; Hog 4 10% was in group 1 with the former and Hog 75 10% in group 1 with latter monoclonal. The differences between groups 1 and 2 were about 25%. With A003=40/5G7, the distinction between the groups was not as clear but MSM 10% and Hog 4 10% reacted slightly better than the others, and with MH2/6D4 and AH16 the two groups were not seen.

The most striking finding was the reaction of the monoclonals with human blood group A substances. With AC-1001, A003=40/5G7, MH2/6D4, and AH16, A, B substances phenol-insoluble cyst 14 was considerably more potent than saliva A, B substances. With A003=40/5G7, AH16, and A-hexa-, the A-tetrasaccharide, and MSMA3RL0.56 which was 0.3 as active as the A-heptasaccharide with AC-1001 but was considerably less active. However, anti-A A15/3D3.92.1 reacted considerably less well with A, B than with A, B substances, and both A, B substances, phenol-insoluble cyst 14 and phenol-insoluble W. G., gave indistinguishable quantitative precipitin curves and showed about 30% and 40% of the activity of the group 1 and group 2 substances, respectively.

With the anti-AB monoclonal, A15/1A4.7.3, the precipitin curves were completely different (Fig. 2). In order of decreasing activity, the A substances were Hog 4 10%, MSM 10%, MSS 10%, 2X, Hog 75 10%, phenol-insoluble Mc Donan 15%, and phenol-insoluble cyst 9; among B substances Horse 4 25% was the only B substance tested which gave any precipitation; phenol-insoluble Tij II, phenol-insoluble PM, and phenol-insoluble Beach did not react. A15/1A4.7.3 did not precipitate with A, B substances, phenol-insoluble cyst 14 and phenol-insoluble W. G. Each of the blood group substances precipitated a different amount of AbN at the maximum ranging from 5.5 to 3.0 µg of total N precipitated.

Quantitative precipitin studies could only be carried out on two of the three anti-B monoclonals since NB10/5B2.4.5 did not precipitate with B substances. The two other anti-B monoclonals, BC-1004 and B003=46/2D7, were B-specific (Fig. 2) in that they did not react with A, H, Le^a, and Le^b substances. Typical precipitin curves were obtained with human ovarian cyst B substances, phenol-insoluble Tij II and phenol-insoluble Beach, with human saliva B, phenol-insoluble PM and Horse 4 25%. Precipitin curves of all four substances fell into a narrow range with BC-1004 but differed considerably with B003=46/2D7, the most active being about 3-fold better than the least potent. All B substances precipitated comparable amounts of total N at the maximum within experimental error.

Water-soluble Cow 35 of 10% ppt did not precipitate with anti-AB A15/1A4.7.3, nor with the two anti-B ascites; it reacted as well as the A, B substances with A15/3D3.92.1 and A003=40/5G7 and less strongly than A, B substances with the other anti-A ascites.

Quantitative Inhibition Assays—Quantitative inhibition assays by ELISA showed differences in fine structure among anti-A combining sites (Fig. 3). Inhibition assays with AH16 were carried out with alkaline phosphatase-labeled protein A (22). The five anti-A sera showed different patterns; AC-1001 and MFI2/6D4 were identical in order of reactivity with the A-heptasaccharide being best followed by the A-penta-, the A-hexa-, the A-tetrasaccharide, and MSMA2R0.56 which were equal, and by 01 and the A-trisaccharide. The relative inhibiting powers of the other oligosaccharides to the A-heptasaccharide differed; with AC-1001 the hepta-, penta-, and hexasaccharides were very close, the three differing over less than a 2-fold range, whereas with MH2/6D4 the A-heptasaccharide was three times more potent per nanomole than the A-pentasaccharide and six times better than the A-hexasaccharide. MSMA2R0.56 and the A-tetrasaccharide were 0.3 as active as the A-heptasaccharide with AC-1001 but only 1/10 as potent in MH2/6D4. The ratios of nanomoles required for 50% inhibition of MSMA2R0.56 and the A-

### Table II

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Titors with human erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A, B</td>
</tr>
<tr>
<td>Anti A</td>
<td>2.048 1.024 0</td>
</tr>
<tr>
<td>BC-1004</td>
<td>0 0 16.384 1.024 8.192</td>
</tr>
<tr>
<td>A15/1A4.7.3</td>
<td>2.048 1.024 0</td>
</tr>
<tr>
<td>A003=40/5G7</td>
<td>16.384 16.384 0</td>
</tr>
<tr>
<td>MH2/6D4</td>
<td>8.192 0 0</td>
</tr>
<tr>
<td>AH16</td>
<td>256 16 0</td>
</tr>
<tr>
<td>A003=40/5G7</td>
<td>16.384 16.384 0</td>
</tr>
<tr>
<td>MH2/6D4</td>
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</tr>
<tr>
<td>A15/1A4.7.3</td>
<td>2.048 1.024 0</td>
</tr>
</tbody>
</table>

*Highest dilution giving definite agglutination.
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Anti-A AH-16 was inhibited by the A-hexa- and A-trisaccharides (Table III).

The anti-B samples showed substantial differences. With NB10/5B2.45, Beach BR0.44 was the best inhibitor and three times as potent as 02, with 50% inhibition requiring 10.5 and 31 nmol, respectively. The B-trisaccharide was much less potent, only 9% inhibition being obtained at 55 nmol. Anti-B B003-46/2D7, Beach BR0.44, and another fraction, Beach BR0.39, with the same structure showed identical inhibition and were about six times more potent than 02, with 21 and 120 nmol being required for 50% inhibition; the B-trisaccharide gave no inhibition at 55 nmol, the largest quantity available. With anti-B BC-1004, however, 02 was much more potent than the B-trisaccharide followed by Beach R0.44 in a ratio at 50% inhibition of 1.8:18.29, respectively.

With anti-AB A15/1A4.7.3, only two oligosaccharides, tetrasaccharide to 01 and the A-trisaccharide were 1:2.4:10 for AC-1001 and 1:1.4:5.4 for MH2/6D4. Dr. Don Baker of Chembiomed independently determined the relative inhibiting power of A-pentasaccharide:A-tetrasaccharide:01 as 1.0:0.29:0.12 in close agreement with our value of 1:0.34:0.14.

Anti-A A003=40/5G7 differed from the other two in that the order of decreasing ratio of nanomoles for 50% inhibition was: A-hepta-, 1.0; A-hexa-, 1.4; A-penta-, 3.2; MSMA;R0.56 and 01, 4.8; and A-trisaccharides, 26.

Anti-A A15/3D3.92.1 gave a completely different pattern: MSMA;R0.56 and the A-hexa- and A-tetrasaccharide being equal and most active, with A-pentasaccharide and 01 being about 50%, and the A-hepta- and A-trisaccharides about 29% as active (cf. Ref. 49).

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**FIG. 1.** Quantitative precipitin curves of mouse monoclonal anti-A by various blood group substances. Symbols used are: A, phenol-insoluble cyst 3 (A1); A, MSM 10% (A2); A, MSS 10% XX (A3); A, McDon 15% (A4); A, Hog 4 10% (A5); A, Hog 75 10% (A6); A, phenol-insoluble cyst 14 (A7); A, phenol-insoluble W. G. (Saliva A1): V, water-soluble Cow 35 of 10% ppt (A8); O, phenol-insoluble Tij 2 (B1); O, phenol-insoluble Beach cyst (B2); O, Horse 4 25% (B3); O, phenol-insoluble PM (saliva B4); O, phenol-insoluble N-1 (Lea); O, phenol-insoluble JS (HLea).

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2 D. Baker, personal communication.
The monoclonal anti-A, anti-B, and anti-AB were produced using different methods of immunization (Table I); some involved erythrocytes or tissue culture cells from carcinoma cell lines; others, soluble blood group A\textsubscript{1} or A\textsubscript{2} substances; and others, the synthetic A or B blood group nonreducing trisaccharides glycosidically linked to $-\text{O}(\text{CH}_2)_n\text{COOH}$ and coupled to BSA or to O erythrocytes; in some instances, mixtures of two of these were used. The structures of the antigens are so different, the antibody response so heterogeneous, and the selection of the hybridomas from so large a population of spleen cells, that one cannot ascribe the differences among the hybridomas to the method of immunization except anti-B hybridoma BC-1004, the best inhibitor (02 antigen, Table III), indicated specificity not only for the B-trisaccharide but included the $-\text{O}(\text{CH}_2)_n\text{COO}^-$ portion of the artificial antigen. With the corresponding A synthetic antigen, A\textsubscript{1} erythrocytes were also used for immunization and this anti-A clone was evidently specific for the A\textsubscript{1} erythrocytes since the 01 antigen was poorer than all other oligosaccharides except the A-trisaccharide. The number of monoclonals available was too small to obtain any idea of the size of the repertoire, but the anti-A and anti-B monoclonals were of high titer and strictly specific for A or B erythrocytes. Except for AH16, the anti-A titers were at most one tube less with A\textsubscript{2} than the A\textsubscript{1} erythrocytes.

Quantitative precipitin curves showed A\textsubscript{1} substances to fall into two groups, with some members of each group not being the same with the different monoclonals. Although with AC-1001, MH2/6D4, and A003=40/5G7, A\textsubscript{2} substance phenol-insoluble cyst 14 was about as active as the group 2 A\textsubscript{1} substances, and saliva A\textsubscript{2} substance phenol-insoluble W. G. was distinctly less reactive; with anti-A A15/3D3.92.1, both A\textsubscript{2} samples were equal but considerably poorer than all A\textsubscript{1} substances and, with AH16, the A\textsubscript{2} substances were both substantially less potent than all A\textsubscript{1} substances with A\textsubscript{2} phenol-insoluble cyst 14 being much more active than A\textsubscript{2} phenol-insoluble W. G. B, Le\textsuperscript{a} and Le\textsuperscript{b}, substances did not precipitate.

With the anti-B monoclonal BC-1004, all four B substances fell into one group, whereas with B003=46/2D7 phenol-insoluble PM was most active, followed by phenol-insoluble Tij II and then by Horse 4 25% and phenol-insoluble Beach, the last two being of equal potency.

The differences among A and among B substances are ascribable to differences in the specificities of the different monoclonals and the heterogeneity of the different blood group substances with respect to numbers of mono- and difucosyl type 1 and type 2 chains as well as to additional determinants in which the type 1 and type 2 A- and B-tetrasaccharides are linked either to galactose or to N-acetylgalactosamine (42, 43). The predominant determinants in saliva blood group substances are difucosyl type 1 and type 2 chains (44).

Monoclonal anti-B NB10/5B2.4.5 did not precipitate with the same four B substances which precipitated with the other two monoclonals. This has not previously been encountered. The most plausible suggestion is that the antibody sites on each molecule are lacking flexibility so that most of the interactions take place with individual molecules of B substance and that sufficient cross-linking and lattice formation for precipitation does not occur.

Monoclonal anti-B NB10/5B2.4.5 did not precipitate with the same four B substances which precipitated with the other two monoclonals. This has not previously been encountered. The most plausible suggestion is that the antibody sites on each molecule are lacking flexibility so that most of the interactions take place with individual molecules of B substance and that sufficient cross-linking and lattice formation for precipitation does not occur. Two monoclonal myeloma proteins, DOB (IgG1\textsubscript{c}) and LBC, both have 15 amino acid deletions in the hinge region (see Ref. 45) and so could not express effector functions. Data on IgM monoclonals are not available; perhaps some IgM monoclonals might also have
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...deletions which might reduce flexibility of their Fab regions.

If increasing amounts of these B substances were bound to plastic and an amount of Nb10/5B2.4.5 equal to that used in the inhibition studies was added, the curves obtained resembled quantitative precipitin curves (Fig. 6). Thus, although no precipitation was seen in agarose gels or by quantitative precipitin assays, binding of this monoclonal anti-B to B substance took place.

The anti-AB monoclonal was also unusual in that each A, substance tested gave an individual curve, the efficacy of precipitation being A1 Hog 4 10% > A1, MSM 10% = A1, MSS 10% 2X > A1, Hog 75 10% > A1 phenol-insoluble McDon 15% > A1 phenol-insoluble cyst 9 > B Horse 4 25%. B phenol-insoluble PM, B phenol-insoluble Beach cyst, B phenol-insoluble Tij II, A2 phenol-insoluble cyst 14, phenol-insoluble W. G. and Cow 35 water-soluble of 10% did not precipitate. It is of interest that A phenol-insoluble cyst 9 and B Horse 4 25% precipitated the same amount of total N.

The most insight into the differences among the monoclonals was gained by the quantitative inhibition assays by ELISA (Figs. 3 to 5 and Table III). Anti-A monoclonals, AC-1001 and MH2/6D4, gave identical inhibition patterns in terms of the order of activity of the various A-active oligosaccharides used: hepta- > penta- > hexa- > MSMAR0.56 = tetra- > 01 > trisaccharide. The relative activities of each oligosaccharide to the heptasaccharide differed substantially (Fig. 3, a and c), the hepta- being very much better than the pentasaccharide in MH2/6D4 than in AC-1001; also in AC-1001 the hepta-, penta-, and hexasaccharide were very close whereas, with MH2/6D4, there was a 6-fold range between the hepta- and the hexasaccharide. Monoclonal anti-A, A003=40/5G7, was also similar except that the hexasaccharide was somewhat more active than the pentasaccharide. The differences between these two oligosaccharides are negligible; they could be identical within experimental error. MSMAR0.56 and the tetrasaccharide are essentially identical with three anti-A monoclonals, whereas MSMAR0.56 and 01 are essentially identical with A003=40/5G7.
The following mono- and oligosaccharides gave no significant inhibition with the anti-A monoclonals: GalNAc, GlcNAc, L-Fuc, Rl.34 [GalNAc] and Gal. The anti-B monoclonals were not inhibited by: D-GalNAc, D-GlcNAc, L-Fuc, galactinol, melibiose, methyl-α-D-galactoside, Beach P.R.0.62, Beach P.R.0.62, Beach P.R.0.62, Beach P.R.0.62, Beach P.R.0.62, Beach P.R.0.62, Beach P.R.0.62.

### Table III

**Oligosaccharides active in inhibition assays by ELISA** (see Figs. 3-5)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Anti-A</th>
<th>50 Percent Inhibition (nanomoles)</th>
<th>kcat (Kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalNAc1Gal</td>
<td>A-Tri</td>
<td>132</td>
<td>125</td>
</tr>
<tr>
<td>D-GalNAc</td>
<td></td>
<td>33</td>
<td>82.5</td>
</tr>
<tr>
<td>D-GlcNAc</td>
<td></td>
<td>13.5</td>
<td>36</td>
</tr>
<tr>
<td>L-Fuc</td>
<td></td>
<td>4.6</td>
<td>82.5</td>
</tr>
<tr>
<td>GalNAc1Gal1Gal1Gal</td>
<td>A-Penta</td>
<td>5.9</td>
<td>36</td>
</tr>
<tr>
<td>GalNAc1Gal1Gal1Gal1Gal1Gal</td>
<td>A-Hexa</td>
<td>3.7</td>
<td>2%</td>
</tr>
<tr>
<td>GalNAc1Gal</td>
<td>A-Hepta</td>
<td>4.6</td>
<td>82.5</td>
</tr>
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</table>

The fourth anti-A monoclonal, A15/3D3.92.1, was quite different from three others, MSMA3R.0.56, A-hexa-, and A-tetrasaccharide, which were more potent and equal as inhibitors followed by the A-pentasaccharide and 01, which were also identical, with the A-trisaccharide being somewhat less active; the A-heptasaccharide was about as active as the A-trisaccharide. These data are not too different from the monoclonal anti-A, TL5, studied by Gooi et al. (46), which reacts with the receptor for epidermal growth factor of human epidermoid carcinoma cell line A431. AH16 differed from the others in that it was inhibited only by the two oligosaccharides satisfying the monofucosyl type 1 structure (22) of the A glycolipid from the cancer cells used for immunization. Thus, one may conclude that all of the six monoclonal hybridoma...
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13215

50μl Anti-B BC-1004 diluted 1:43.2 (1.12μg IgM)
0.25μg Tij II phenol insoluble
100μl AP-coupled goat anti-mouse IgM 1:1500

NANOMOLES INHIBITOR ADDED

FIG. 4. Inhibition by various oligosaccharides of binding of mouse monoclonal anti-B by blood group substance B phenol-insoluble Tij II. In Fig. 4c, ⊥ represents Beach BR0.39.

anti-As studied differ in fine structure of their combining sites. The immunodominant portion of the site is the A-

trisaccharide D-GalNAcα1→3p-Gal, which accounts for the efficacy in all except AH16, reacting with A2 as well as with A, erythrocytes. This may be due to the relatively small difference in ΔΔG° between the A-trisaccharide and the A-hexasaccharide (−0.3 kcal/mol) as compared to all the other anti-A monoclonals (ΔΔG° = −0.62 to −1.68 kcal/mol).

However, the rest of the sites differ from one monoclonal to another in terms of their relative contribution to the total binding energy. This is best seen as a ΔΔG° = 273Rln(x/y) (15, 40, 41); y was arbitrarily set at 0 kcal/mol except for BC-1001 for which 02 was set at 0 kcal/mol since the trisaccharide did not reach 50% inhibition. This permits ready comparisons of all compounds to one another relative to an A- or B-active trisaccharide which was actually isolated from a blood group glycoprotein. Table III summarizes the results. The precision of the assays is such that differences of about 0.1 kcal/mol are significant but, from the energetic considerations, differences in ΔΔG° of less than 0.3 kcal/mol are probably of limited significance (15, 41). With the A monoclonals, the contribution of the \(^\text{1p}A\)-linked GlcNAc (MSMA3R40.56) over \(^{\text{4}}\alpha l^+4\)-linked Glc (A-tetrasaccharide) is negligible for all four anti-A monoclonals. If the trisaccharide is linked \(\text{p}A^+3\) to GlcNAc, there is substantially tighter binding with AC-1001 and A003=40/5G7; with MH2/6D4 there is a slight

Fig. 5. Inhibition by various oligosaccharides of binding of mouse monoclonal anti-AB by blood group substance A Hog 4 10% or blood group substance B Horse 4 25%.

50μl Anti-AB A15/1A4.7.3 diluted 1:124 (0.67μg IgM)
0.7μg Hog 4 10%
100μl AP-coupled goat anti-mouse IgM 1:1000

50μl Anti-AB A15/1A4.7.3 diluted 1:56.3 (0.675μg IgM)
50μl Anti-AB A15/1A4.7.3 diluted 1:124 (0.67μg IgM)
0.7μg Horse 4 25%
100μl AP-coupled goat anti-mouse IgM 1:1000

NANOMOLES INHIBITOR ADDED

Fig. 6. Binding curves of the mouse monoclonal anti-B NB10/5B2.4.5 by various blood group substances using ELISA. Symbols are the same as in Fig. 1.
increase in binding, and with A15/3D3.92.1 there is no
difference.

The contribution of the second fucose, if linked α-1→3 to
the type 2 chain, was substantial by about 0.5 kcal/mol with
AC-1001, MH2/6D4, and A005=40/5G7, but in A15/3D3.92.1
it actually reduced binding affinity by about 0.45 kcal/mol. If
the second fucose was linked α-1→4 to the type 1 chain, with
MH2/6D4 the increase in binding was about 1 kcal/mol, with
AC-1001 and it was only about 0.2 kcal/mol and, with A15/
3D3.92.1, binding affinity was essentially minimal, 50% bind-
ing not being reached. The ΔΔG° values of the A-hepta-, A-
hepta-, and A-hexasaccharide for AC-1001 are very close.

The inhibition data on A15/3D3.92.1 clearly indicate that
the site is most specific for at least a monofucosyl tetrascar-
daride GalNAcα1→3Galβ1→3GlcNAcβ1-2 structure with the
Fucα-1

difucosyl compound blocking entry into the site, whereas the
other three anti-A monoclonals are most specific for a difu-
cosyl oligosaccharide. MH2/6D4 and A005=40/5G7 clearly
involve the type 1 difucosyl pentasaccharide; with AC-1001 it
is not clear whether the specificity is for the difucosyl type 1
(A-pentasaccharide) or type 2 (A-heptasaccharide) oligosac-
charides. Thus, although there has been a tendency to accept
the monofucosyl type 1 and type 2 oligosaccharides as the A
determinants (9, 10) and essentially to ignore the difucosyl
determinants, three of the four monoclonals turn out to have
a combining site most complementary to difucosyl moieties.

Two of the three anti-B monoclonals, NB10/5B2.4.5 and
B003=46/2D7, clearly do not have difucosyl type 2 specificity
since these compounds were inactive compared to the mono-
cosyl type 2 oligosaccharides; in both instances maximum
amounts of the B-trisaccharide used did not give 50% inhibi-
tion; this level was attained by oligosaccharide 02 and is
perhaps ascribable to the additional β-linkage to the
-O(CH2)6COOCH3 aglycone with or without the aglycone
making an additional hydrophobic contribution to binding.
The monofucosyl type 2 oligosaccharide, Beach BR0.44, was
considerably more active. BC-1004 was unusual in that the
02 was -1.2 kcal/mol more negative than the B-trisaccharide,
establishing that a substantial portion of the binding energy
was directed toward the aglycone and that its antibody
binding site was larger than the trisaccharide as would be
expected since O2, with its carboxyl coupled to ε erythrocytes
or to bovine albumin, had been used for immunization. That
ΔΔG° of the mono- and difucosyl type 2 oligosaccharides were
much less potent suggests that the combining site of BC-1004
was most specific for the B-trisaccharide plus the β-linkage
and that the aglycone-specific part of its site could not accom-
mmodate B determinants larger than the trisaccharide. Despite
this, it is a highly satisfactory blood group reagent. Lemieux
et al.3 have studied two IgM affinity purified hybridoma anti-
B monoclonals from clones 3E-4 and A1-2, produced by
immunization with the same B-trisaccharide antigen as
BC-1004. As with BC-1004, attaching the aglycone
-(CH2)6COOCH3 made ΔΔG° substantially more negative,
but if the same aglycone was linked β to the B-tetrasaccharide
in which the B-trisaccharide was linked β-1→3 to a D-GlcNAc,
the ΔΔG° became more than 1 kcal/mol more positive, indi-
cating that the combining site could not accommodate a D-
GlcNAc in the position occupied by the aglycone in the
trisaccharide aglycone, a finding in complete agreement with

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