SPECIFICITY OF SERUM ANTI-A\(_{di}\) IgG ANTIBODIES FROM PATIENTS WITH GASTROINTESTINAL CANCER

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Changes in the glycosylation in cancer may lead to an aberrant expression of A, B incompatible or xenogeneic blood group related antigens. To characterize the specificity of IgG antibodies to A, B, and related glycans in sera of gastrointestinal cancer patients, serum probes and affinity-isolated antibodies were analyzed in the indirect and competitive ELISA using a set of homogenous polyacrylamide (PAA) glycoconjugates. Monoreactive antibodies recognizing Adi (I) and cross-reactive antibodies to Adi = Bdi = Btri (II) or Adi = Atri = Fsdi = Core5 (III) were affinity-isolated on Adi-PAA-Sepharose. The population I showed a higher affinity to Adi-PAA than cross-reactive antibodies. The antibodies II were more specific to Bdi and may belong to the core alpha-Gal reactive antibodies but are also capable of recognizing Adi. The antibodies III were more specific to Atri; they agglutinated A-erythrocytes and belong to anti-A isoantibodies reactive to xenogeneic oligosaccharides. The purified antibody samples were non- or faintly reactive to Tn. The IC50 values of PAA glycoconjugates ranged from $6 \times 10^{-8}$ to $7 \times 10^{-6}$ M. No or weak binding of antibodies to the unrelated antigens used in the detection of polyreactivity (ferritin, casein, and DNA) was observed.

**Keywords** A, B isoantibodies, alpha-Gal, Core5, disaccharide, Forssman, glycolipids, polyacrylamide glycoconjugates, Tn, trisaccharide

**INTRODUCTION**

Various types of human malignant tumors are characterized by changes in the glycosylation involving histo-blood group antigens. A and B epitopes may be aberrantly expressed or deleted with an accumulation of their precursors. In addition, the expression of an incompatible A epitope in tumors of individuals with H or B phenotypes may occur. The Adi, Forssman
(Fs) antigen, and αGal are xenogeneic A and B blood group related determinants, and they may also be expressed in malignant tumors.\textsuperscript{[4–8]} The expression of \( \text{A}_{\text{dii}} \) antigen on cervical cancer had a statistically significant correlation with the five-year survival rate of the patients.\textsuperscript{[4]}

Numerous studies have been carried out on the expression of carbohydrate antigens in tumors, but data about spontaneously occurring antibodies to tumor-associated carbohydrate antigens in cancer patients are scanty. Antibodies to A and B blood group related determinants deserve a thorough study in specificity and cross-reactivity to clarify the role of antibodies in cancer progression. Being inherent in the innate and adaptive immunity, anti-carbohydrate antibodies are constantly produced in humans. Many natural IgM antibodies are polyreactive and responsible for the innate immunity.\textsuperscript{[9]} In the case of the adaptive immune response, IgG antibodies may demonstrate the specificity that is restricted to cognate, related, and some mimetic antigens.

In humans, the natural αGal antibodies are abundantly produced presumably in response to antigenic stimulation by gastrointestinal bacteria.\textsuperscript{[10,11]} The αGal antibodies comprise a very high proportion of anti-A and anti-B activities. Reactivities exclusive to αGal and the core αGal epitope of B antigen or within A and B antigens have been revealed in healthy individuals or recipients of ABO-incompatible grafts.\textsuperscript{[12–14]} The αGal antibodies and their minimal antigen (Galz1-3Galβ, αGal disaccharide, B\textsubscript{dii}) have been relatively well studied thanks to their role in rejecting of xenografts in transplantation to humans.\textsuperscript{[14]} However, the analogous \( \text{A}_{\text{dii}} \) antigen (GalNAcz1-3Galβ, the epitope of afuco-A antigen) and antibodies have received little attention. Being also a foreign determinant for humans, \( \text{A}_{\text{dii}} \) exhibits the highest affinity to the C-type lectin of human dendritic cells in the glycan microarray screening.\textsuperscript{[15]} The α- and βGalNAc residues are exclusive ligands of the C-type lectin of human macrophages, which is involved in the uptake and presentation of glycosylated antigens.\textsuperscript{[15,16]} The prevalence of \( \text{A}_{\text{dii}} \)-reactive antibodies in human serum has been established using a microchip glycan array.\textsuperscript{[17]}

The authors have undertaken a long-term follow-up of cancer patients to determine changes in the level of \( \text{A}_{\text{dii}} \) antibodies, as well as to elucidate their association with the progression of cancer and rates of survival. The study of \( \text{A}_{\text{dii}} \) antibodies is part of a vast investigation of differences and dynamic changes in the level of anti-carbohydrate antibodies in cancer patients.\textsuperscript{[18–22]} Being xenoreactive, \( \text{A}_{\text{dii}} \) antibodies may be cross-reactive to A, B, and related antigens expressed in some tumors thereby to influence the tumor growth and spread. The cross-reactivity of \( \text{A}_{\text{dii}} \) antibodies to incompatible A, B and related tumor-associated glycans may resemble the cross-reactivity of B\textsubscript{dii} (αGal) antibodies in recipients after transplantation of ABO-incompatible grafts.\textsuperscript{[12–14]} To purify carbohydrate antibodies and
to characterize their specificity, the synthetic homogenous PAA glycoconjugates\cite{23} and affinity sorbents were used.\cite{24,25} The aim of the present study was to characterize the specificity and cross-reactivity to A, B and related glycans of $\text{A}_{\text{di}}$ antibodies of cancer patients.

**EXPERIMENTAL**

**Patients**

The study was carried out in accordance with the ICH GCP Standards and approved by Tallinn Medical Ethics Committee. The informed consent was obtained from patients under study. Serum of the blood collected during the follow-up was frozen to create the bank of sera. Gastric ($n=32$) and colorectal cancer patients ($n=44$) with different blood groups (A–25, B–19, O–26, AB–6 cases) were investigated. Diagnosis in stages I–III was verified by the pTNM system. Patients who received blood or plasma transfusion or chemo- and X-ray therapy were excluded from the study. The median age was 65 years (the age ranging from 36 to 78). Data about patients selected for isolation of antibodies are presented in Table 1.

**Glycoconjugates**

The regular PAA glycoconjugates (30 kDa) are the soluble derivatives of N-substituted poly(N-hydroxyethylacrylamide), with a glycan density of 20\%mol. The conjugates and affinity adsorbents were obtained from Lectinity (Russia). The following saccharide-PAA conjugates were used: $\text{A}_{\text{di}}$, GalNAc$\text{z}$1-3Gal$\beta$; Tn, GalNAcz; $\text{F}_{\text{s}}$ (the terminal disaccharide of the Forssman glycolipid), GalNAc$\text{z}$1-3GalNAc$\beta$; Core 5, GalNAc$\text{z}$1-3GalNAc$\beta$; $\text{A}_{\text{tri}}$, GalNAc$\text{z}$1-3(Fuc$\text{z}$1-2)Gal$\beta$; $\text{B}_{\text{di}}$ (zGal disaccharide), Galz1-3Gal$\beta$; $\text{B}_{\text{tri}}$, Galz1-3(Fuc$\text{z}$1-2)Gal$\beta$; $\text{X}_{\text{2}}$ (the terminal disaccharide of the $\text{X}_{\text{2}}$ glycolipid), GalNAc$\beta$1-3Gal$\beta$; GalNAc$\beta$. Tris-PAA, tris(hydroxymethyl)aminomethane-PAA, was used as a negative control. The affinity adsorbents were designed as PAA conjugates covalently attached to the Sepharose FF or Macroporous glass (MPG) and contained 0.6\%mol saccharide per mL.

### Table 1 Data About Patients From Whose Sera Antibodies Were Isolated

<table>
<thead>
<tr>
<th>Code</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Blood Group</th>
<th>Antibody Sample</th>
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</thead>
<tbody>
<tr>
<td>GE</td>
<td>Colon cancer, II, pT$_3$N$_0$M$_0$G$_2$</td>
<td>65</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>LH</td>
<td>Colon cancer, II, pT$_3$N$_0$M$<em>0$G$</em>{2,3}$</td>
<td>74</td>
<td>O</td>
<td>1</td>
</tr>
<tr>
<td>KA</td>
<td>Rectal cancer, II, pT$_3$N$_0$M$<em>0$G$</em>{2,3}$</td>
<td>60</td>
<td>A</td>
<td>2</td>
</tr>
<tr>
<td>JV</td>
<td>Gastric cancer, II, pT$_3$N$_0$M$_0$G$_3$</td>
<td>62</td>
<td>A</td>
<td>3</td>
</tr>
<tr>
<td>VN</td>
<td>Gastric cancer, II, pT$_3$N$_0$M$_0$G$_1$</td>
<td>61</td>
<td>O</td>
<td>4</td>
</tr>
</tbody>
</table>
Indirect ELISA

The sera were collected by centrifugation of clotted venous blood after incubation for 2 h at 37°C. The sera were kept at 4°C for no longer than three weeks or were frozen (−50°C) and thawed once before use. The glycoconjugates with PAA (5 μg/mL) in 0.05 M carbonate buffer, pH 9.2, were applied to the Nunc-Immuno plate (MaxiSorp) and held overnight at 4°C. After washing with 0.05 M Tris HCl/0.2 M NaCl/0.02% sodium azide, pH 7.5 (TBS)/0.05% Tween 20, the plate was coated with human sera diluted (1:25–1:200) in TBS/0.05% Tween 20/5 mM EDTA/0.2% bovine serum albumin (BSA) or coated with antibodies diluted in TBS/0.05% Tween 20/5 mM EDTA and incubated for 2 h at 26°C. The plate was washed with TBS/0.05% Tween 20 and goat anti-human IgG-alkaline phosphatase conjugate (Flow Labs., USA) in TBS/0.05% Tween 20 was added. The plate was kept for 1.5 h at 26°C and washed. The absorbance (A) at 405 nm was measured using a Labsystem Multiscan MCC/340 (Finland) after incubation for 1 h at 26°C with a p-nitrophenylphosphate disodium salt (1 mg/mL in 0.1 M glycine-buffer, pH 10.3). Each sample was analyzed in duplicate, including analysis with Tris-PAA (control). The antibody level was estimated as a ratio of $A_{test}/A_{control}$, where $A_{test}$ is the absorbance with the PAA-glycoconjugate and $A_{control}$ with the Tris-PAA. The variation coefficient was 3%. The analogous determination of the glycopeptide-reactive IgM and IgA was performed using alkaline phosphatase labeled secondary antibodies, rabbit anti-human IgM, or anti-human IgA (DAKO).

The concentration of IgG in preparations was determined by ELISA using the calibration curve. Briefly, the immunoplate was coated with an affinity-purified rabbit-anti-human IgG (DAKO) and incubated overnight at 4°C. The plate was washed and blocked with a 10% fetal calf serum (1 h, 26°C). Suitable dilutions of human IgG (Sigma) or samples of antibodies were added to the wells, and the plate was incubated (3 h, 26°C). The goat-anti-human IgG antibodies labeled with an alkaline phosphatase were used as secondary antibodies, which were diluted in TBS/0.05% Tween-20 containing 2% of the rabbit serum.

Competitive ELISA

In the competitive assay, one of the glycoconjugates was adsorbed onto plates, the other or the same glycoconjugate was incubated with the serum or purified antibodies in the buffer. The human sera diluted (1:25–1:200) with TBS/0.05% Tween-20/5 mM EDTA/0.2% BSA or purified antibodies diluted in the same buffer without BSA were incubated with the solution of glycoconjugates at different concentrations for 2 h at 26°C. The mixture was applied to the wells with coated glycoconjugates, and the plate was
incubated (2 h, 26°C). The sera or antibodies incubated with the buffer at an appropriate dilution were used as control. The plate was washed, and the goat anti-human IgG-phosphatase conjugate was added as described above in the indirect ELISA. The negative control Tris-PAA was included as well. The inhibition of IgG binding to the adsorbed ligand by the soluble ligand was evaluated in percentages using the subtracted value of absorbance.

**Purification of IgG Antibodies**

The serum probes with high IgG antibody levels ($A_{\text{test}}/A_{\text{control}} > 2$, dilution 1:50) were used to isolate antibodies on the Adi-PAA-Sepharose FF. The once freeze-thawed serum or the serum mixture was treated to remove the complement on the silica gel-adsorbed human IgG (silica gel-IgG). The mixture of silica gel “Chemapol” (30 mg, washed with 1 M HCl, deionized water and dried) and 0.6 mL of the solution of human IgG (5 mg/mL) in 0.05 M Tris HCl buffer (pH 7.5) was rotated for 1 h at room temperature. The silica gel-IgG was washed with TBS and centrifuged. The mixture of the serum (3 mL) with the silica gel-IgG was rotated for 1 h at room temperature and centrifuged. The supernatant was shaken intensively with 0.5 mL of hexane to remove the lipids. The emulsion was then centrifuged for 20 min at 10000 g. The serum was diluted to 1:1 with TBS/10 mM EDTA and put through the column with 1–3 mL of the sorbent in a closed cycle (4 h, room temperature). The column was washed with TBS/5 mM EDTA to remove unbound proteins. The elution of antibodies was performed at 4–8°C by using 2–6 mL of the eluent: 8 M urea/0.1 M glycine/0.05 M Tris HCl/10 mM EDTA/0.2 M NaCl/0.02% sodium azide, pH 7.3 (the denser eluent moved upwards, in the opposite direction to the flow in the antibody adsorption). The treatment with urea causes a reversible denaturation of IgG and after removal of the denaturant, the immunoglobulin regains the functional properties of the native conformation.\[^{[26]}\] The dialysis of the product was started during elution without delay. The product was concentrated by ultrafiltration. The admixture of anti-Adi IgA and anti-Adi IgM was separated from the affinity-purified anti-Adi IgG by immunoadsorption on the goat-anti-human IgA (α-chain specific)- or goat-anti-human IgM (μ-chain specific)-agarose (Sigma-Aldrich). The term “key ligand” designates the saccharide used in affinity chromatography for the isolation of the corresponding antibodies.

**High-Performance Thin-Layer Chromatography (HPTLC)**

HPTLC and immunostaining with antibodies were performed as described by Smorodin et al.\[^{[25]}\] Briefly, the probes of the chloroform/methanol
extract of rabbit erythrocytes were separated by HPTLC in the solvent of chloroform/methanol/water 60:35:8 (v/v). The sheet was dried in vacuum (2h, 50°C) and impregnated in hexane by dipping on one side, then soaked for 1 min with 0.1% isopropyl myristate in hexane. The sheet was dried in vacuum, sprayed with TBS, and blocked in TBS/1%BSA/0.1% normal goat serum (2h, room temperature). All further operations were performed at 4°C. The sheet was incubated overnight with \(A_{di}\) or \(B_{di}\) antibodies in TBS/0.2%BSA. After washing with TBS, the sheet was incubated for 1.5 h with the goat-anti-human IgG-alkaline phosphatase conjugate in TBS/0.1%BSA/0.1% normal goat serum. The sheet was washed and incubated with the buffer containing a 5-bromo-4-chloro-indolylphosphate disodium salt (0.2 mg/mL) and nitroblue tetrazolium (0.4 mg/mL, Sigma). The stained sheet was washed with TBS, then impregnated with the distilled water to remove TBS and dried. The dip in the distilled water causes the detachment of the silica gel from the aluminum base.

**Hemagglutination Assay**

The agglutination of human A or B red blood cells was performed with a different antibody titer as described in reference.[18]

**Statistical Analysis**

A Sigma Plot (version 10), Curve Expert (version 1.34), Difference test (Statistics 6), and the Mann–Whitney U-test were used. The linear regression analysis was conducted by using a Statgraphics Plus 5.0.

**RESULTS**

**The Cross-Reactivity of Antibodies to Oligosaccharides with an External GalNAc\(\beta\) Fragment in Serum Probes**

In the competitive ELISA, the soluble PAA-glycoconjugate (used as an inhibitor) at a concentration of 20 μg/mL inhibited the binding of serum antibodies to the same adsorbed PAA-glycoconjugate more than 60% (hereafter, PAA-glycoconjugate is referred to as a ligand). The tenfold concentration of the soluble ligand (200 μg/mL) was used in the competitive immunoassay to saturate serum antibodies in the determination of the antibody reactivity to the related ligand with a similar structure. The antibody-positive sera with the IgG binding to either both A, B ligands (H-phenotype) or one of them (A or B, antibody-negative phenotypes, respectively) were examined. The results were compared against...
affinity-isolated antibodies. The values of inhibition of IgG antibodies by cross-reactive ligands at a concentration of 200 μg/mL in serum probes and in samples of the antibodies isolated from these probes were found to be close.

A partial cross-reactivity of serum antibodies to Tn, A<sub>di</sub>, A<sub>tri</sub>, F<sub>si</sub><sub>di</sub>, and Core 5 was observed. In pairs of Tn with A<sub>di</sub>, F<sub>si</sub><sub>di</sub>, or Core 5, the interval of inhibition was from low to moderate (Figure 1). In the pair Tn/A<sub>di</sub>, the soluble Tn ligand almost did not inhibit the binding of IgG to the adsorbed A<sub>di</sub> (Figure 1, the strip A<sub>di</sub>), but the soluble A<sub>di</sub> moderately inhibited the binding of IgG to the adsorbed Tn (the strip Tn). This may be interpreted as a moderate one-sided reactivity of Tn antibodies to A<sub>di</sub>, i.e., antibodies with the higher affinity to Tn may demonstrate some reactivity to A<sub>di</sub>. No or weak reactivity of A<sub>di</sub> antibodies to Tn was also confirmed by using the affinity-purified anti-A<sub>di</sub> IgG (see Samples 1–4). No inhibition of IgG binding was observed in the pair A<sub>tri</sub>/Tn and vice versa (Figure 1). In the pair Tn/F<sub>si</sub><sub>di</sub>, the soluble Tn ligand inhibited the IgG binding to the adsorbed F<sub>si</sub><sub>di</sub> in a bit broader interval (Figure 1, strip F<sub>si</sub><sub>di</sub>), but F<sub>si</sub><sub>di</sub> was a weak inhibitor of IgG binding to Tn (strip Tn). In the pair Tn/Core 5, in which the GalNAC<sub>z</sub> residue in Core 5 is an external and internal fragment, the Tn ligand moderately inhibited the IgG binding to Core 5, but

![Figure 1](image.png)

**Figure 1** The cross-reactivity of serum IgG antibodies to structurally related ligands as established in the competitive ELISA. The ligands adsorbed onto the plates are shown under the horizontal zero axis. The ligands-inhibitors are written over intervals of inhibition. The interval designates the extreme values of inhibition for the three to four sera used. The reactivity of antibodies was calculated as (A<sub>test</sub> − A<sub>control</sub>). A<sub>test</sub> is the absorbance with PAA glycoconjugate; A<sub>control</sub> is the absorbance with Tris-PAA. The blood group phenotype is shown in brackets.
Core 5 was a weak inhibitor of IgG binding to Tn (Figure 1, strips Core 5 and Tn).

In the pair Adi = Fsdi, both the ligands inhibited the antibody binding mutually (Figure 1). The cross-reactivity between these ligands was observed in purified anti-Adi IgG: Antibodies contained either a small amount of the antibody population reactive to Adi and Fsdi or demonstrated an entire cross-reactivity (sample 1 or 4, respectively).

A total inhibition of IgG binding to the adsorbed Core 5 by the soluble Fsdi was observed, while the inhibition of IgG binding to Fsdi by Core 5 was partial (Figure 1). This may be explained by a significantly lower affinity of antibodies to Core 5 than to Fsdi. This explanation may be extended to the pairs Adi/Core 5 and Tn/Core 5. In fact, the soluble ligands differed in potency to inhibit the IgG binding to the adsorbed Core 5: Fsdi > Adi > Tn. But the soluble Core 5 was a less potent inhibitor of IgG binding to the corresponding ligands. As a rule, Fsdi- and Adi-reactive sera demonstrated a comparatively lower reactivity to the Core 5 ligand as tested by the serum dilution in the indirect ELISA. Also, the purified Adi antibodies showed a weak reactivity to Core 5 in the indirect ELISA, and Core 5 inhibited antibodies at higher concentrations (see Sample 4).

The level of antibodies to Adi vs. Atri, Adi vs. Fsdi, and Atri vs. Fsdi correlated in serum probes (Table 2), which may be due to their partial cross-reactivity to the ligands.

The cross-reactivity of antibodies in some serum probes was observed in pairs Adi/Bdi, Adi/Btri, and Adi/Atri (Figure 1). This was verified in the assay of reactivity of isolated antibodies (see Samples 2–4).

**The Characterization of Purified Adi Antibodies**

The serum probes demonstrating a low or high cross-reactivity to related ligands were selected for the isolation of antibodies on Adi-PAA-Sepharose. The IgG antibody level in sera is shown in Table 3. High levels of IgM and IgA antibodies were not observed, and in some samples they were insignificant. For sample 1, the Adi antibodies were isolated from the mixed sera collected during the follow-up of two patients with a similar diagnosis (Table 1). The cross-reactivity of antibodies in both serum probes

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>R</th>
<th>P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adi vs. Atri</td>
<td>0.43</td>
<td>0.005</td>
<td>42</td>
</tr>
<tr>
<td>Adi vs. Fsdi</td>
<td>0.60</td>
<td>&lt;0.001</td>
<td>37</td>
</tr>
<tr>
<td>Atri vs. Fsdi</td>
<td>0.40</td>
<td>0.046</td>
<td>25</td>
</tr>
</tbody>
</table>
was low. For other samples, the antibodies were isolated from the sera of individual patients. An excess of serum probes was applied for isolation of samples 1–3 because the column did not totally deplete antibodies. The anti-Tn and anti-Bdi IgG isolated earlier\cite{24,25} were compared with Adi antibodies. The optimal concentration of purified antibodies used in ELISA varied appreciably (Table 4).

**Sample 1**

The reactivity of purified anti-Adi IgG to the related ligands is shown in Table 5. The reactivity of antibodies to the other ligands, viz. Tn, Core 5, Bdi, Btri, and X2di, was negligible. The Bdi and Atri ligands (both at 200 µg/mL) inhibited the binding of antibodies to Adi by 5–7%. The reactivity of antibodies to Fsdi was 10% of that to Adi. The Fsdi ligand inhibited the binding of antibodies by up to 21%, while its higher concentrations did not augment the inhibition (Figure 2). In vice versa conditions, where

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>The Characterization of Sera Used for Purification of Anti-Adi IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.</td>
<td>Phenotype</td>
</tr>
<tr>
<td>1</td>
<td>O, B</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>O</td>
</tr>
</tbody>
</table>

*The ratio of A_{test}/A_{control}, where A_{test} is the absorbance with PAA-glycoconjugate and A_{control}, with Tris-PAA.

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>The Total Yield of IgG (µg) and its Optimal Final Concentration (µg/mL) Used in Indirect ELISA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>1, Adi</td>
</tr>
<tr>
<td>Yield</td>
<td>74</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*The samples were diluted to provide the increment of absorbance of 0.6–0.9 units/h.

| TABLE 5 | The Reactivity of Purified Antibodies to Ligands (A_{test} – A_{control} in %, Indirect ELISA) |
|---|---|---|---|---|---|---|---|
| Sample, Key Ligand | Adi | Atri | Bdi | Btri | Fsdi | Core 5 | Tn | X2di |
| 1, Adi | 100 | 5 | 3 | 0 | 10 | 0 | 0 | 0 |
| 2, Adi | 100 | 0 | 58 | 8 | 8 | 0 | 4 | 0 |
| 3, Adi | 100 | 0 | 354 | 10 | 1 | 0 | 0 | 0 |
| 4, Adi | 100 | 105 | 3 | 0 | 73 | 9 | 14 | 0 |
| Bdi | 1 | 0 | 100 | 5 | 0 | 0 | 0 | 0 |

The reactivity to the key ligand used in affinity chromatography was taken as 100%.
Fsdi-PAA was adsorbed onto immunoplates, both Adi and Fsdi inhibited the binding of purified antibodies to the Fsdi ligand in a dose-dependent manner, but Fsdi inhibited the binding of antibodies to a higher extent and in lower doses (not shown). The data obtained are indicative of the presence of a low amount of the IgG population reactive to both Adi and Fsdi. But the purified antibodies mostly demonstrated monoreactivity to Adi in the indirect and competitive ELISA. The affinity of Adi to the antibodies of sample 1 was significantly higher than that of Adi to the cross-reactive antibodies of samples 2, 3, and 4. Actually, the IC\textsubscript{50} value of the Adi of sample 1 was twice lower than that of sample 3 (Table 6, 0.23 vs. 0.47). Moreover, the IgG concentration of sample 1 used in ELISA was 15 times lower than that of sample 3 (Table 4).

**Sample 2**

The isolated anti-Adi IgG showed a similar pattern of reactivity to the related ligands as in sample 1, including a slightly higher reactivity to Fsdi, which was 8% of that to Adi. However, unlike the first sample, the antibody reactivity to the Bdi and Btri ligands was observed (Table 5). Moreover, Bdi inhibited the IgG binding to Adi (Table 6, IC\textsubscript{50} = 1.37 μM), while Btri was a weaker inhibitor (44% of the inhibition at a Btri-PAA concentration of 200 μg/mL). These results confirm the data obtained using the serum probes of some individuals with an A phenotype, in the case of which an apparent cross-reactivity of antibodies to pairs Adi/Bdi and Adi/Btri was observed (Figure 1).
The purified IgG antibodies did not show any reactivity to Tn, Atri, Fsi, Core 5, and X2di. However, their reactivity to Bdi was very high, being 354% of that to Adi. The Bdi ligand itself was a more potent inhibitor of antibodies than Adi, as its IC50 value was six times lower (Table 6, 0.08 vs. 0.47; Figure 3). In vice versa conditions, where Bdi-PAA was adsorbed to the solid phase, both the ligands also inhibited antibodies efficiently but in higher concentrations (Figure 4, light rings and dark squares). Under these conditions, Bdi was nine times as strong an inhibitor of antibodies as the Adi ligand (Table 6, 0.75 vs. 6.7). Both the Adi-reactive and Adi-nonreactive

**Sample 3**

The purified IgG antibodies did not show any reactivity to Tn, Atri, Fsi, Core 5, and X2di. However, their reactivity to Bdi was very high, being 354% of that to Adi. The Bdi ligand itself was a more potent inhibitor of antibodies than Adi, as its IC50 value was six times lower (Table 6, 0.08 vs. 0.47; Figure 3). In vice versa conditions, where Bdi-PAA was adsorbed to the solid phase, both the ligands also inhibited antibodies efficiently but in higher concentrations (Figure 4, light rings and dark squares). Under these conditions, Bdi was nine times as strong an inhibitor of antibodies as the Adi ligand (Table 6, 0.75 vs. 6.7). Both the Adi-reactive and Adi-nonreactive

![FIGURE 3](image)

**FIGURE 3** Sample 3. The inhibition of the binding of purified anti-Adi IgG to the adsorbed Adi-PAA by the soluble conjugates: Adi-PAA (△, IC50 = 0.47 μM), Bdi-PAA (○, IC50 = 0.08 μM), and Btri-PAA (▲, IC50 = 1.19 μM).
populations of B_{di} antibodies were present in the serum used because A_{di}-nonreactive anti-B_{di} IgG remained in the fraction after a complete depletion of A_{di} antibodies. The purified antibodies were reactive to B_{tri} (Table 5). The B_{tri} ligand also showed a dose-dependent inhibition of antibodies but in higher concentrations than A_{di} (Figure 3, and Table 6, 1.19 vs. 0.47). This was observed in different combinations of ligands (Table 6, Figures 3 and 4). Thus, the ligands showed a total cross-reactivity to antibodies and differed in affinity: B_{di} > A_{di} > B_{tri}.

**Sample 4**

To isolate different A_{di}-reactive antibodies, a lower volume of the serum probe was applied to the column (Table 3). The sorbent exhausted serum A_{di} antibodies completely. These antibodies differed from those of other samples in reactivity to related ligands. The purified antibodies demonstrated a strong binding to the A_{tri} and F_{si} ligands but bound weakly to Core 5 (Table 5). Antibodies reacted to neither B_{di}, B_{tri}, nor X2_{di}. In the serum used, Tn antibodies were present, but the antibody reactivity to Tn in the isolated sample was weak (Tables 3 and 5). Furthermore, Tn (200 μg/mL) inhibited the purified A_{di} antibodies only by 7%. This remaining reactivity to Tn is rather due to the presence of Tn antibodies, which are less reactive to A_{di} because the A_{di} ligand inhibited IgG binding to Tn in the serum used in isolation but not vice versa (Figure 1, strips A_{di} and Tn). The ligand A_{tri} was 10 times as potent an inhibitor of antibodies
as \( \text{Adi} \) (Table 6, 0.06 vs. 0.6; Figure 5), while Core 5 had the lowest potency. Also, \( \text{Atri} \) was 19 times as strong an inhibitor of IgG binding to the adsorbed \( \text{Adi} \) ligand as \( \text{Adi} \) itself (Table 6, 0.13 vs 2.44; Figure 6). The \( \text{Adi} \) and \( \text{Fsdi} \) ligands showed mutual cross-reactivity in different combinations of ligands

**Figure 5** Sample 4. The inhibition of the binding of purified anti-\( \text{Adi} \) IgG to the adsorbed \( \text{Adi-PAA} \) by the soluble conjugates: \( \text{Adi-PAA} \) (○, \( \text{IC}_{50} = 0.60 \mu \text{M} \)), \( \text{Atri-PAA} \) (△, \( \text{IC}_{50} = 0.06 \mu \text{M} \)), \( \text{Fsdi-PAA} \) (▲, \( \text{IC}_{50} = 1.32 \mu \text{M} \)), and Core 5-PAA (■, \( \text{IC}_{50} = 3.37 \mu \text{M} \)).

**Figure 6** Sample 4. The inhibition of the binding of purified anti-\( \text{Adi} \) IgG to the adsorbed \( \text{Atri-PAA} \) by the soluble conjugates: \( \text{Atri-PAA} \) (○, \( \text{IC}_{50} = 0.13 \mu \text{M} \)) and \( \text{Adi-PAA} \) (△, \( \text{IC}_{50} = 2.44 \mu \text{M} \)). The inhibition of the binding of antibodies to the adsorbed \( \text{Fsdi-PAA} \) by the soluble conjugates: \( \text{Fsdi-PAA} \) (○, \( \text{IC}_{50} = 0.40 \mu \text{M} \)) and \( \text{Adi-PAA} \) (▲, \( \text{IC}_{50} = 0.18 \mu \text{M} \)).
as well but the affinity of $A_{dii}$ was higher (Figures 5 and 6, and Table 6). Thus, the ligands demonstrated cross-reactivity and inhibited antibodies totally (until saturation): $A_{tri} > A_{dii} > Fs_{dii} > Core 5$.

All the purified anti-$A_{dii}$, anti-$Tn$, and anti-$B_{dii}$ IgG did not bind to unrelated antigens used in the detection of antibody polyreactivity, namely ferritin and casein, and weakly bound to DNA ($E. coli$, plasmid). Unlike anti-$B_{dii}$ IgG, $B_{dii}$-reactive anti-$A_{dii}$ IgG did not bind the $z$Gal glycolipids of rabbit erythrocytes (Figure 7). The negative immunostaining on HPTLC may be due to the lower affinity of $A_{dii}$ antibodies to the $z$Gal epitope, particularly to its whole structure in glycosphingolipids. In fact, in the inhibition assay using $B_{dii}$-PAA, the $A_{dii}$ antibodies showed 19 times as low an affinity as $B_{dii}$ antibodies (Table 6, 0.75 vs. 0.04). This was observed at similar concentrations of IgG used (Table 4, 0.45 vs. 0.54 $\mu$g/mL).

The $A_{dii}$ antibodies of sample 4 agglutinated A erythrocytes effectively at concentrations of 10–20 $\mu$g/mL but did not agglutinate B erythrocytes. The antibodies of samples 1, 2, and 3, as well as Tn and Bdi antibodies, agglutinated neither A nor B erythrocytes.

**FIGURE 7** The HPTLC of the glycolipids extracted from rabbit erythrocytes: (A) immunostaining with anti-$A_{dii}$ IgG (sample 3, dilution 1:50) and (B) anti-$B_{dii}$ IgG (dilution 1:250).
DISCUSSION

The Monoreactivity of $A_{\text{di}}$ Antibodies

In human serum, antibodies reactive to cognate and some structurally related antigens, as well as polyreactive antibodies are present. Most polyreactive antibodies belong to the IgM class, but some are of IgG and IgA isotype and can bind to several randomly selected antigens: proteins, carbohydrates, lipids and nucleic acids. The anti-$\alpha$Gal antibody is not a polyreactive antibody. The isolated anti-$A_{\text{di}}$ IgG as well as anti-Tn and anti-$B_{\text{di}}$ IgG also belong to mono- or oligoreactive antibodies. No or faint binding of antibodies to the unrelated antigens was observed. We examined a limited number of oligosaccharides selected on the basis of structures with an external GalNAc$\alpha$ fragment. Therefore, the antibodies of sample 1 classified as “monoreactive” can bind certain saccharides or unrelated antigens if they would be screened in the panel with a number of antigens. But the affinity of such saccharides would be lower instead.

In the serum probes used for the isolation of sample 1, the reactivity of antibodies to other related ligands was observed (Table 3). But monoreactive $A_{\text{di}}$ antibodies were isolated due to their higher affinity. Only the minor population of antibodies reactive to $A_{\text{di}}$ and $F_{\text{di}}$ was present in sample 1. Also, the isolated $A_{\text{di}}$ antibodies of sample 3 did not react to $F_{\text{di}}$. These results are indicative of the presence of $A_{\text{di}}$-monoreactive antibodies in human serum.

The antibodies of sample 1 were more specific to $A_{\text{di}}$. Both the IgG concentration of sample 1 and the IC$_{50}$ value of the key ligand were lower than those of samples 2, 3, and 4 (Tables 4 and 6). The higher specificity of purified $B_{\text{di}}$ antibodies to $B_{\text{di}}$ was observed as well. The concentrations of IgG in $B_{\text{di}}$ antibodies and the $B_{\text{di}}$-reactive sample 3 were similar (Table 4), but the affinity of the $B_{\text{di}}$ ligand to $B_{\text{di}}$ antibodies was significantly higher (Table 6, 0.04 vs. 0.75). The anti-$B_{\text{di}}$ IgG was isolated from the sera of non-B individuals with a low antibody reactivity to $B_{\text{tri}}$ using the $B_{\text{di}}$-sorbent. The reactivity of anti-$B_{\text{di}}$ IgG is shown in Table 5. Unlike the $A_{\text{di}}$ antibodies that did not bind $A_{\text{tri}}$ and $B_{\text{tri}}$ (sample 1 isolated from the sera of non-A individuals), the $B_{\text{di}}$ antibodies showed a dose-dependent inhibition by the $B_{\text{tri}}$ ligand. The monoreactive $A_{\text{di}}$ antibodies did not agglutinate human A or B erythrocytes as expected.

The Similarity and Difference Between $A_{\text{di}}$ and $B_{\text{di}}$ Antibodies and Their Reactivity to $A_{\text{tri}}$ and $B_{\text{tri}}$

The cross-reactivity of antibodies to $A_{\text{di}}/B_{\text{di}}$, $A_{\text{di}}/B_{\text{tri}}$, and $A_{\text{di}}/A_{\text{tri}}$ was not associated with tumor stage and localization. The pronounced
cross-reactivity of antibodies to pairs Adi/Bdi and Adi/Btri was not revealed in non-A phenotypes, but it was found in A-phenotype (Figure 1, strips Adi, Bdi, and Btri). Either high or low values of inhibition in the sera of A-phenotype were demonstrated for the pair Adi/Bdi (n = 6), while high values were not observed in O and B individuals (n = 12, P = 0.03, U-test). Also, intervals of the inhibition were significantly higher in A individuals than in O or B individuals for the pair Adi/Btri (P = 0.03, U-test). The IgG antibodies were isolated on Adi-PAA-Sepharose from two selected sera of blood group A individuals. The antibodies of samples 2 and 3 were cross-reactive to the Adi, Bdi, and Btri ligands. The affinity of Bdi to the antibodies of sample 3 was six to nine times as high as that of Adi, and both the ligands showed a total cross-reactivity. This may reflect the molecular mimicry in the recognition of zGal-terminated and defucosylated blood group A (Adi-terminated) glycosphingolipids by human natural anti-zGal IgG.\[27]\n
We named the antibodies after the key ligand used in chromatography. But taking into consideration their higher specificity to the Bdi ligand, antibodies should be named as “anti-Bdi IgG” cross-reactive to Adi.

The reactivity of the anti-Adi IgG of samples 2 and 3 to Btri but not to Atri was demonstrated. The results may be interpreted as the recognition of Bdi and the core Bdi epitope in Btri, i.e., antibodies resembled the zGal antibodies in reactivity to the B antigen\[12–14]\ but were furthermore capable of recognizing the Adi epitope with the terminal GalNAc residue.

Bdi is a minimal ligand reactive to zGal antibodies.\[28]\ The disaccharide structure of Adi also appears to be minimal for the binding of Adi antibodies, as the antibody reactivity to the Tn monosaccharide was not observed or was faint in all the samples tested.

The Bdi antibodies isolated on the Bdi-sorbent were found to be reactive to the zGal-glycosphingolipids of rabbit erythrocytes. Like monoclonal antibodies,\[29]\ they detected a similar pattern of two bands.\[25]\ However, the Bdi-reactive anti-Adi IgG (sample 3) did not detect the bands, probably because of their lower affinity to zGal. In the inhibition assay, the Bdi antibodies and antibodies of sample 3 bound to Btri, but both were unable to agglutinate B erythrocytes, possibly owing to their weak affinity to Btri and a weaker interaction of IgG with erythrocytes. As it is known, the IgM antibodies are stronger agglutinins.

Serum antibodies were cross-reactive to pairs Adi/Atri and Bdi/Btri (Figure 1). In the inhibition assay, as a rule, the cross-reactivity was more pronounced when antibodies to both the related ligands were present. This was observed for both the pairs Adi/Atri and Bdi/Btri. In Atri- and Btri-antibody negative serum samples (A and B, respectively, or AB blood groups), the interval of inhibition was lower than in positive serum probes in which antibodies to both Adi and Atri or Bdi and Btri were present (Figure 1, strips Adi and Bdi). Nevertheless, in the anti-Btri-negative B, AB serum probes, the
self-antigen $B_{tri}$ inhibited the binding of IgG to $B_{di}$ (Figure 1, the strip $B_{di}$). This may be interpreted as an “autoreactivity” of the serum $B_{di}$ antibodies to $B_{tri}$. A similar pattern of the inhibition of IgG binding to $A_{di}$ by the $A_{tri}$ ligand was also demonstrated in A blood group persons, not only in B and 0 persons (Figure 1, the strip $A_{di}$). These results are in conformity with findings of other researchers, showing that the IgG antibodies reactive to the self A and B antigens are present in the serum of A and B individuals, but their autoreactivity is masked by an interaction with autologous complementary molecules.\[30\]

The $\alpha$Gal antibody level in the serum is associated with the blood group phenotype. A significantly higher level in non-B antigen-expressing individuals than in B and AB individuals was observed.\[31\] We also showed analogous phenotype-dependent differences in serum $A_{di}$ antibody levels. The level of IgG antibodies reactive to $A_{di}$ was significantly higher in group O, B (non-A) than in group A, AB ($P = 0.0004$, $n = 38$). This may be explained by the presence of an additional $A_{di}/A_{tri}$-reactive population of IgG in O and B individuals. The population of antibodies was isolated from the serum, showing the cross-reactivity to $A_{di}/A_{tri}$ (O blood group, sample 4).

**$A_{di}/A_{tri}/F_{sdi}/Core 5$ Reactive Antibodies**

The cross-reactivity of antibodies was observed in combined pairs, but in pairs with Tn, it was lower or was missing at all (Figure 1). The purified antibody samples 1, 2, and 3 did not show any significant reactivity to Tn but the level of Tn antibodies in serum probes used for isolation was also insignificant (Table 3). The faint reactivity of antibodies of sample 4 to Tn was observed when they were isolated from Tn-reactive serum. Also, the affinity-purified anti-Tn IgG did not show any reactivity to $A_{di}$, $F_{sdi}$, and Core 5 as well as to GalNAc$\beta$ ligands, despite the heterogeneous mixture of the serum probes used for purification, in which the antibody populations reactive to these ligands were present.\[24\] The Tn-monoreactive antibodies were isolated because of the high affinity of the Tn ligand (Table 6) and an excess amount of the serum applied.

The levels of antibodies to $A_{di}$, $A_{tri}$, and $F_{sdi}$ in serum probes correlated, which may be indicative of the cross-reactivity of ligands (Table 2). Indeed, a potent mutual inhibition of antibodies by $A_{di}$, $A_{tri}$, and $F_{sdi}$ was demonstrated for antibodies isolated from the serum of blood group O individual (sample 4). Core 5 was a less potent inhibitor, and this is in conformity with the low potency of Core 5 to inhibit $A_{di}$ antibodies in serum probes. The $A_{tri}$ ligand was the most effective inhibitor of antibodies. Because of the higher specificity to the A determinant ($A_{tri}$) and capability to agglutinate human A erythrocytes, these antibodies belong to anti-A isoantibodies.
reactive to xenogeneic oligosaccharides. As known, human antibodies to A antigen cross-react partially with the xenogeneic Forssman glycolipids.[32]

Trisaccharides Atri or Btri are minimal external determinants of A or B blood group antigens that are produced by the transfer of the GalNAc or Gal residue to the galactosyl residue of the H antigen. The alpha-fucosyl residue of the H-determinant is essential because either the A- or B-enzyme cannot transfer the substrate sugar to the carbohydrate chain lacking an alpha-fucosyl residue.[33] In this respect, A\textsubscript{di} (afuco-A) and B\textsubscript{di} (afuco-B) are cryptic determinants and should be generated in human cells only as a core epitope within A and B antigens. Similarly to the αGal antibodies, the isolated A\textsubscript{di} antibodies can be either monoreactive or recognize A and B determinants.

Thus, the cross-reactivity shown in Figure 1 was not associated with the stage and localization of tumor. A significant difference between A and non-A phenotypes in cross-reactivity to pairs A\textsubscript{di}/B\textsubscript{di} and A\textsubscript{di}/B\textsubscript{tri} was observed. Cross-reactive serum probes (A individuals) and those having low cross-reactivity (O, B individuals) were selected to isolate and characterize antibodies. In addition, antibodies demonstrating cross-reactivity to A\textsubscript{tri} and xenogenic glycans (sample 4, O individual) were isolated and characterized. These isoantibodies showed a high specificity to A\textsubscript{tri} and ability to agglutinate A erythrocytes. Isoantibodies might be involved in targeting the A antigen in tumor cells and thereby share in “cancer immunoediting.”[34] As is known, the occurrence of tumor in A blood group individuals compared to O individuals is higher. Incompatible A antigen and A-cross-reacting antigens may be expressed in tumors of O or B individuals.[3] Also, the deletion of A and B antigens in carcinomas or inversely their presence (which depends on the tumor localization) is an indicative of an unfavorable outcome.[35] But if tumor localization is not taken into account, these results are contradictory. These dissimilarities may be due to the missing link of the host immune response to antigens studied. The cross-reactive antibodies to A and B blood group related glycans might belong to antibodies autoreactive to tumor-associated antigens.[36] One can hypothesize that immune response to incompatible tumor antigens may be similar to described different types of the response to ABO antigens after transplantation of the incompatible grafts, i.e., rejection, accommodation, and tolerance.[14] Whether a high level of A\textsubscript{di} antibodies, its dynamics and antibody cross-reactivity are related to survival and have prognostic significance, which will be shown by further investigations.

**CONCLUSIONS**

Three human IgG antibody populations, namely A\textsubscript{di} monoreactive, A\textsubscript{di}/B\textsubscript{di}/B\textsubscript{tri}, and A\textsubscript{di}/A\textsubscript{tri}/F\textsubscript{5di}/Core 5 cross-reactive antibodies, were isolated
using Aβ₁-PAA-Sepharose. The reactivity of antibodies to Tn was faint or was missing. The first population belongs to the Aα₁-xenoreactive antibodies. The second population showed a higher specificity to Bβ₁ and the third one to Aα₁. The second population isolated from A individuals may belong to the αGal-antibodies with anti-B reactivity, but they are also capable of recognizing Aβ₁. The third population belongs to anti-A isoantibodies reactive to xenogeneic oligosaccharides. It is noteworthy that the total mutual inhibition of cross-reactive antibodies by the corresponding ligands took place.

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