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THE CHARACTERIZATION OF IgG ANTIBODIES TO GalNAc BETA-TERMINATED GLYCANS OF GASTRIC CANCER SURVIVORS

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The elevated anti-GalNAcβ IgG level of serum was shown to be associated with the significantly better survival of patients with gastrointestinal cancer. *Aim:* To characterize the specificity of IgG antibodies to GalNAcβ-terminated glycans of long-term gastric cancer survivors. *Methods:* Serum antibodies and affinity-isolated antibodies were analysed by the indirect and competitive ELISA using glycan-polyacrylamide (PAA) conjugates as well as by isoelectric focusing and Western blotting. *Results:* In the serum probes, a partial cross-reactivity of antibodies to GalNAcβ, GalNAcβ1–3Galβ (X2$_{di}$), GalNAcβ1–3GalNAcβ (PF$_{di}$) and GlcNAcβ was observed. The isolated anti-GalNAcβ IgGs demonstrated the cross-reactivity to the X2$_{di}$ glycan mainly. The affinity of the X2$_{di}$-PAA to anti-GalNAcβ IgGs was 11–21 times lower than that of the GalNAcβ-PAA. Anti-X2$_{di}$ and anti-PF$_{di}$ IgGs demonstrated monoreactivity to their key glycans-PAA used in isolation. The IC$_{50}$ values of key glycoconjugates ranged from 1 to 5 · 10^{-7} M. No polyreactivity of antibodies to the unrelated antigens (ferritin, casein and DNA) was found. The polyclonal or oligoclonal distribution of IgG bands was established and the monoreactivity of antibodies was not associated with the clonal distribution of bands. *Conclusion:* The cross-reactivity of anti-GalNAcβ antibodies to X2$_{di}$ and related glycans deserves attention in the clarification of the role of antibodies in cancer progression and enhancement of the prognostic potential in the combined determination of antibody markers.
Key Words: gastric cancer, glycan-polyacrylamide, anti-GalNAc-beta, para-Forssman, X2, oligoclonal IgG, microbiota.

Many anti-glycan antibodies in humans are constantly stimulated by enteric microorganisms of the alimentary tract. In different pathologies, including cancer, mucosal barrier dysfunction occurs that frequently causes the altered bacterial colonization and translocation [1, 2]. This may change the pattern and level of circulating anti-glycan antibodies and predispose to an adaptive IgG immune response. We observed a high serum level of IgG antibodies to glycans, including tumor-associated carbohydrate antigens, as well as the respective changes in patients with gastrointestinal cancer during follow-up [3–5]. The determination of the level of some circulating anti-glycan antibodies in cancer patients was found to be of diagnostic and prognostic significance [3, 6–9]. The study of the antibody specificity recognizing immunogens may be of clinical value but is complex because of the antibody heterogeneity and the diversity of natural immunogens. Synthetic antigens like polyacrylamide (PAA) glycoconjugates may help to solve this problem. The PAA-glycoconjugates are homogenous antigens with a single reiterative glycotope [10], enabling a precise detection and isolation of glycotope-specific antibodies. Using appropriate glycoconjugates and their optimal combination in the determination of antibodies level could improve diagnostic and prognostic parameters. The specificity of affinity-isolated anti-glycan antibodies from the serum of patients with cancer was investigated using a set of PAA-glycoconjugates [11–13]. Earlier we had found two populations of IgG antibodies reactive

to GalNAcβ and GalNAcβ1–3GalNAcβ glycans (the latter is the terminal disaccharide of the para-Forssman glycolipid, PF$_{di}$) [11]. The elevated serum level of anti-GalNAcβ IgG was associated with the significantly better survival of patients with gastrointestinal cancer while the anti-PF$_{di}$ IgG level was higher in the serum of cancer patients than in donors [4, 8].

The aim of the present study was to characterize the specificity of serum IgG antibodies to terminated GalNAcβ glycans in long-term gastric cancer survivors having a high level of antibodies.

MATERIALS AND METHODS

Patients. The study was carried out in accordance with the ICH GCP Standards and approved by Tallinn Medical Research Ethics Committee. The informed consent was obtained from patients under study. The diagnosis of patients with gastric cancer (n = 78) was verified by the pTNM system [14]. Patients who received blood or plasma transfusion or chemo- and X-ray therapy were excluded from the study. Long-term survivors were selected for isolation and characterization of antibodies (Table 1).

Table 1. The characterization of gastric cancer patients

Code	Diagnosis	Age, sex	Blood group	Antibody sample	Postoperative survival in months
UA	Stage II, pT3N0M0G1	63, m	A	1	154
PK	Stage II, pT3N0M0G2	46, f	O	2	135
GV	Stage I, pT1N0M0G2–3	72, f	A	3	159
JL	Stage III, pT4N0M0G2	74, f	A	4	82
SK	Stage II, pT3N0M0G3	41, f	B	5	162
GL	Stage III, pT3N1M0G2–3	67, f	O	6	140

Glycoconjugates. The synthetic glycoconjugates and affinity sorbents were obtained from Lectinity, Russia. The soluble glycoconjugates with the N-substituted poly[N-(2-hydroxyethyl)acrylamide] (the ethanolamine-type conjugate, 30 kDa, a glycan density of 10–20%mol) were designated as glycan-PAA. The following glycan-PAA conjugates were used:

GalNAcβ; X2$_{di}$ (the terminal disaccharide of the X2 glycolipid), GalNAcβ1–3Galβ; PF$_{di}$ (the terminal disaccharide of the para-Forssman glycolipid), GalNAcβ1–3GalNAcβ; GlcNAcβ; Tn, GalNAcα; TF, Galβ1–3GalNAcα; T$_{ββ}$ (TFβ), Galβ1–3GalNAcβ; GA2 (asialo-GM2), GalNAcβ1–4Galβ1–4Glcβ; Lac-di-NAc, GalNAcβ1–4GlcNAcβ; Fs$_{di}$ (the terminal disaccharide of the Forssman glycolipid), GalNAcα1–3GalNAcβ; A$_{di}$ (A-disaccharide), GalNAcα1–3Galβ; B$_{di}$ (αGal disaccharide), Galα1–3Galβ. 20% Tris-PAA (Tris(hydroxymethyl)aminomethane-PAA) was used as a negative control.

Indirect and competitive ELISA. Assays were performed as described earlier [12]. In the indirect ELISA, the level of antibodies was assessed as a ratio of A$_{test}$/A$_{control}$, where A$_{test}$ is the absorbance with the glycan-PAA and A$_{control}$, with the Tris-PAA. To evaluate the cross-reactivity of serum antibodies, an excess of soluble glycans-PAA (200 µg/ml) was used in the competitive immunoassay. The competitive ELISA of isolated antibodies was performed at different concentrations of glycans-PAA. Briefly, one of the glycoconjugates was adsorbed onto plates, the other or the same glycoconjugate was mixed with the serum or purified antibodies in the buffer. The mixture was applied to the wells with adsorbed glycoconjugates after incubation. The sera or antibodies incubated with the buffer at an appropriate dilution were used as control. The inhibition of IgG binding to the adsorbed ligand by the soluble ligand was evaluated in percentages of subtracted A-values. The goat anti-human IgG-phosphatase conjugate was used as secondary antibodies. The concentration of IgG in antibody samples was determined by ELISA as well [12].

Purification of human IgG antibodies. Antibodies were purified as described in reference [12]. Briefly, serum probes with a high level of antibodies were applied to the glycan-PAA-Sepharose FF. The elution of antibodies was performed at 4–8 °C by using 8 M urea in the buffer. The product was dialyzed and concentrated by ultrafiltration. The admixture of IgM or IgA was separated from the affinity-purified IgG antibodies by immunoadsorbtion on the goat-antihuman IgM or IgA-agarose. The term "the key glycan-PAA" designates the ligand used in affinity chromatography for the isolation of the corresponding antibodies. In some cases, the reactivity of isolated antibodies to the key glycan-PAA recovered gradually after storing the solution for one month at 4 °C. A little of precipitate may be formed, which should be removed by centrifugation.

Isoelectric focusing and Western blotting. IEF was performed in a 1% agarose IEF (GE Healthcare Bio-Sciences AB, Sweden) containing 10% glycerol. A pH gradient was formed in the gel with 5% Ampholytes high resolution 3–10 (Sigma-Aldrich) or in the gel with 4% Ampholites and 1% Pharmalyte 5–8 (GE Healthcare Bio-Sciences AB, Sweden). The antibody samples were focused in the electric field of 10–50 V/cm during 8 h at 3 °C. After focusing, the fractions were transferred for 1 h to the nitrocellulose sheet (pore size 0.45 µm, Trans-blot, Bio-Rad, Hercules, CA, capillary transfer under pressure). The sheet was kept

in TBS overnight at 4 °C and then blocked in TBS/0.1% Tween-20/0.5% BSA/0.1% normal goat serum (NGS) for 2 h at 20 °C. The sheet was washed once with TBS/0.05% Tween-20 and incubated with the goat anti-human IgG-alkaline phosphatase conjugate in TBS/0.05% Tween-20/0.1% BSA/0.1% NGS for 1.5 h at 20 °C. After that, the sheet was washed and incubated at 20 °C with the solution of a 5-bromo-4-chloro-indolylphosphate disodium salt (0.2 mg/ml) and nitroblue tetrazolium (0.4 mg/ml) (Sigma). After the development of a colour reaction, the sheet was washed with TBS and distilled water and dried.

Statistical analysis. A Sigma Plot (version 10) and Curve Expert (version 1.34) were used. The linear regression analysis was conducted by using a Statgraphics Plus 5.0.

RESULTS AND DISCUSSION

The cross-reactivity of IgG antibodies to glycans-PAA in serum probes. A partial cross-reactivity of serum antibodies to GalNAcβ, X2$_{di}$, PF$_{di}$ and GlcNAcβ was observed while the cross-reactivity to other related glycoconjugates was lower or insignificant (Fig. 1). Serum antibodies were cross-reactive to the glycoconjugates with an external GalNAcβ residue but not to T$_{ββ}$ having an internal GalNAcβ. The cross-reactivity of antibodies to terminal GalNAcβ1–4 linked glycans (GA2, Lac-di-NAc) as well as to the glycans with the terminal GalNAcα anomer (Tn, Fs$_{di}$) was lower or insignificant.
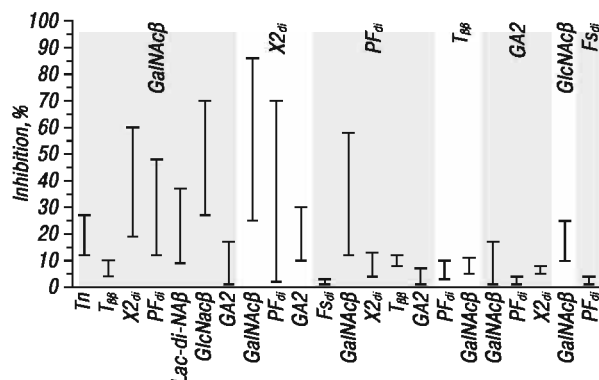
Fig. 1. The cross-reactivity of serum IgG antibodies to structurally related ligands as established in the competitive ELISA. The ligands-inhibitors are shown under the horizontal zero axis. The ligands adsorbed onto the plates are written in the figure. The interval designates the extreme values of inhibition for the four sera used. The reactivity of antibodies was calculated as (A$_{test}$ minus A$_{control}$). A$_{test}$ is the absorbance with PAA glycoconjugate; A$_{control}$ is the absorbance with Tris-PAA

The correlation between the levels of some serum antibodies was observed that may be due to their partial cross-reactivity to the corresponding glycans (Table 2).

Table 2. The correlation between the levels of serum IgG antibodies to structurally related glycans by indirect ELISA

Antibodies	r	P	n
GalNAcβ vs GA2	0.38	0.034	41
GalNAcβ vs X2$_{di}$	0.36	0.075	37
GalNAcβ vs PF$_{di}$	0.41	0.006	52
GalNAcβ vs GlcNAcβ	0.33	0.14	34
X2$_{di}$ vs PF$_{di}$	0.33	0.102	37

When both the glycans inhibit the antibody binding to each other, their mutual cross-reactivity could con-

tribute to better correlation between the antibodies levels. Correlation was usually weaker when one-sided cross-reactive and non-cross-reactive antibodies were presented in serum probes [12, 13].

The reactivity of isolated anti-GalNAcβ IgG (samples 1 and 2). The antibodies of sample 1 were isolated on the column with a GalNAcβ-PAA-Sepharose (2.8 ml). The serum probes (12 ml) with the level of anti-GalNAcβ IgG in the range of 4.2–9.9 (dilution 1:25) were applied. Sample 2 was isolated from the serum probe of another patient with the level of antibodies of 2.6. In serum probes, the reactivity of IgG antibodies to Tn, $X2_{di}$, PF_{di}, GlcNAcβ and GA2 as well as their partial cross-reactivity to these ligands was demonstrated (see Fig. 1). The column depleted IgG reactivity to GalNAcβ-PAA in sera totally. The isolated IgG antibodies showed the cross-reactivity to $X2_{di}$-PAA (Tables 3, 4; Fig. 2, 3). The anti-GalNAcβ IgG isolated from pooled serum probes obtained from six patients was also cross-reactive to $X2_{di}$-PAA and, similarly to samples 1 and 2 (Table 3), bound faintly or not at all other related glycoconjugates (not shown). The antibody samples demonstrated close values of IC_{50} for key glycans. The affinity of $X2_{di}$ to antibodies was 11–21 times lower than that of GalNAcβ (Table 4). The inhibition of antibodies of both samples by PF_{di}-PAA (200 µg/ml) was in the range of 14–18%. The reactivity of anti-GalNAcβ IgG samples to Tn (GalNAcα) did not exceed 5%. The human monoclonal antibody (6D4), which recognized the GalNAcβ ligand but did not bind Tn, has been described by other authors [15]. As found earlier, Tn-monoreactive antibodies were isolated on GalNAcα-PAA sorbent from the heterogeneous mixture of serum probes obtained from eight patients [11]. The results are indicative of the sufficient selectivity of GalNAc-PAA-sorbents for isolation of IgG antibodies.

Table 3. The reactivity of isolated IgG antibodies to PAA glycoconjugates (A_{test} minus $A_{control}$ in%, indirect ELISA)

Conjugate	Sample and key glycan-PAA*					
	1, GalNAcβ	2, GalNAcβ	3, $X2_{di}$	4, $X2_{di}$	5, PF_{di}	6, PF_{di}
GalNAcβ	100	100	10	2	2	0
$X2_{di}$	48	24	100	100	4	0
PF_{di}	1	10	6	3	100	100
GlcNAcβ	0	7	2	0	0	0
Tn	0	5	0	0	0	0
TF	0	0	0	0	0	0
$T_{ββ}$	0	0	0	1	9	2
GA2	5	3	6	1	0	1
Lac-di-NAc	1	0	0	0	0	0
Fs_{di}	0	0	0	0	0	0
A_{di}	0	0	0	0	0	0
B_{di}	0	0	0	0	0	0

Note: *The key glycans-PAA is a ligand used for the isolation of antibodies. The reactivity of antibodies to the key glycan-PAA was taken as 100%.

Table 4. The IC_{50} values (µM) of glycans-PAA to the isolated antibodies

Sample, antibodies	Conditions				
	GalNAcβ	GalNAcβ	$X2_{di}$	$X2_{di}$	PF_{di}
	GalNAcβ	$X2_{di}$	GalNAcβ	$X2_{di}$	PF_{di}
1, GalNAcβ	0.30	6.39	0.121		
2, GalNAcβ	0.24	2.62	NT		
3, $X2_{di}$				0.30	
4, $X2_{di}$				0.49	
5, PF_{di}					0.34
6, PF_{di}					0.14

Note: The conditions of the assay: the glycan above is an adsorbed (solid phase) glycan-PAA and the below one is an inhibitor. NT – not tested.
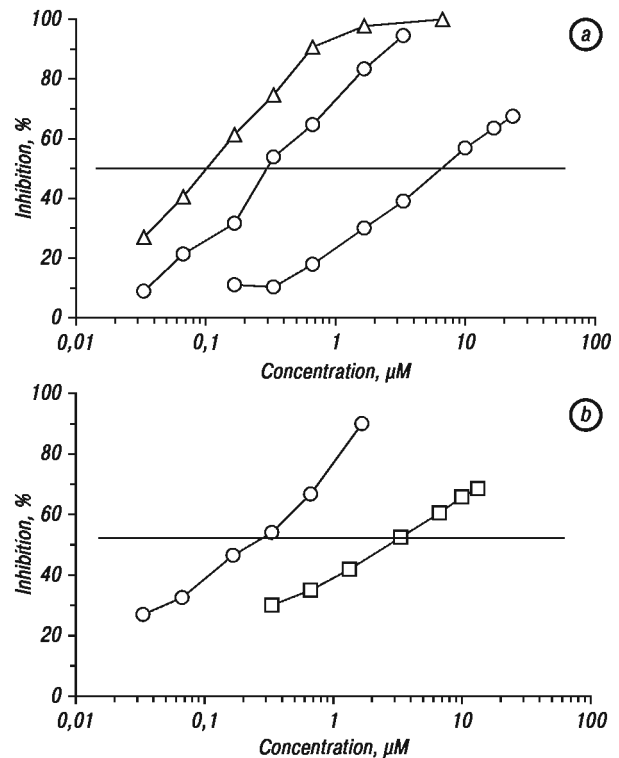
Fig. 2. *a:* Sample 1. The inhibition of the binding of purified anti-GalNAcβ IgG to the adsorbed GalNAcβ-PAA conjugate by the soluble GalNAcβ-PAA (O) and the soluble $X2_{di}$-PAA (O). The inhibition of the binding of anti-GalNAcβ IgG to the adsorbed $X2_{di}$-PAA by the soluble GalNAcβ-PAA (△). *b:* Sample 2. The inhibition of the binding of purified anti-GalNAcβ IgG to the adsorbed GalNAcβ-PAA conjugate by the soluble GalNAcβ-PAA (O) and the soluble $X2_{di}$-PAA (□)
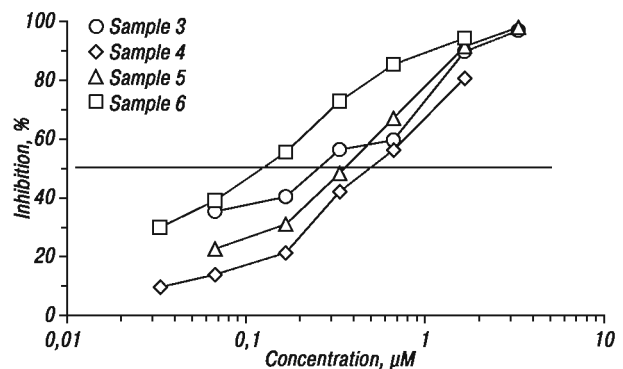

Fig. 3. Sample 3 and sample 4. The inhibition of the binding of purified anti-$X2_{di}$ IgG to the adsorbed $X2_{di}$-PAA by the soluble $X2_{di}$-PAA. Sample 5 and sample 6. The inhibition of the binding of purified anti-PF_{di} IgG to the adsorbed PF_{di}-PAA by the soluble PF_{di}-PAA

The reactivity of isolated anti-$X2_{di}$ IgG (samples 3 and 4). The antibodies of sample 3 were isolated on the column with a $X2_{di}$-PAA-Sepharose (2.1 ml). The serum probes (7 ml) with the level of anti-$X2_{di}$ IgG in the range of 5.9–14.9 (dilution 1:25) were applied. In the same way, sample 4 was isolated from the serum probe of another patient with the level of antibodies of 2.4. In one case, probes with a low IgG reactivity to the GalNAcβ and GA2 glycans and a high reactivity to PF_{di} were used. In the other case, the serum with a high IgG reactivity to GalNAcβ, GA2 and PF_{di} was employed. The column depleted the serum anti-$X2_{di}$ IgG incompletely in the first case but completely

in the second case. In both cases the purified antibodies showed a high reactivity to $X2_{di}$-PAA. The reactivity of antibodies to related glycans was low or absent (Table 3). In the competitive assay, related glycans-PAA at a concentration of 200 µg/ml inhibited antibodies in the range of 2–13%. The antibody samples demonstrated close IC_{50}-values of $X2_{di}$-PAA (Table 4; Fig. 4).
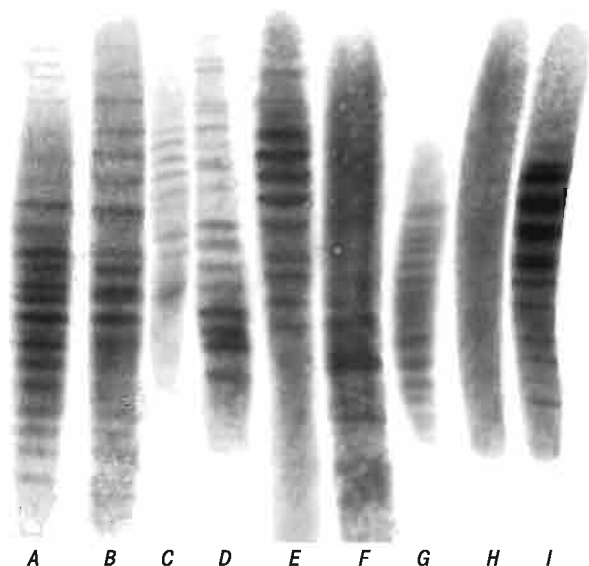
Fig. 4. Isoelectric focusing in a 1% agarose and Western blotting of antibody samples. A–G — focusing in the gel with Ampholytes 3–10. A — anti-GalNAcβ IgG (sample 1). B, C — monoreactive anti-PF_{di} IgG (samples 5 and 6, respectively). D, E — monoreactive anti-$X2_{di}$ IgG (samples 4 and 3, respectively). F — monoreactive anti-Tn IgG [11], G — monoreactive anti-A_{di} IgG [12]. H, I — focusing in the gel with Ampholites 3–10/ Pharmalyte 5–8. H — Human IgG (total, Sigma). I — anti-$X2_{di}$ IgG (sample 3)

The reactivity of isolated anti-PF_{di} IgG (samples 5 and 6). The antibodies of sample 5 were isolated on the column with a PF_{di}-PAA-Sepharose (2.8 ml). The serum probe (6 ml) with the level of anti-PF_{di} IgG in the range of 6.8–14.3 (dilution 1:25) were applied. Sample 6 was isolated from the serum probe with the antibody level equal to 11.2. The IgG reactivity to GalNAcβ was observed in the serum of the first patient (sample 5) but the reactivity of antibodies to $X2_{di}$ and $T_{ββ}$ was insignificant. The serum of the second patient (sample 6) showed the IgG reactivity to $X2_{di}$ but not to GalNAcβ and $T_{ββ}$. The column depleted the serum anti-PF_{di} IgG totally in both cases. In both cases, the purified anti-PF_{di} IgG was specific to PF_{di}-PAA. Only a slight binding to $T_{ββ}$ (9%) was observed for sample 5 (Table 3). In the competitive assay $T_{ββ}$-PAA (200 µg/ml) did not inhibit antibody binding to PF_{di}-PAA. The IC_{50}-value of PF_{di}-PAA to the antibodies of sample 6 was lower than that of sample 5 (Table 4; Fig. 4).

The isolated IgG antibodies did not bind ferritin and casein, and only weakly bound DNA (*E. coli*, plasmid) that are antigens used for the determination of antibody polyreactivity. The optimal concentration of IgG used in ELISA had similar values (0.02–0.03 µg/ml).

The results of study of the specificity of isolated antibodies obtained in the present paper and earlier may be summarized as follows:

- Antibodies recognized well β and α anomers of external GalNAc. Anti-GalNAcβ IgG did not bind GalNAcα-PAA and *vice versa*, the earlier isolated anti-GalNAcα IgG did not bind GalNAcβ-PAA [11]. Anti-$X2_{di}$ IgG did not bind A_{di}-PAA and *vice versa* [12]. Anti-PF_{di} IgG did not bind Fs_{di}-PAA.
- Antibodies recognized the external GalNAcβ and Galβ residues. The anti-PF_{di} IgG bound weakly or did not bind $T_{ββ}$ (TFβ)-PAA (indirect or competitive ELISA, respectively) and *vice versa,* TFβ-reactive antibodies bound weakly or did not bind PF_{di}-PAA [13].
- Antibodies distinguished GalNAcβ(α)-terminated disaccharides from monosaccharides. Anti-$X2_{di}$ and anti-PF_{di} IgG bound weakly or did not bind GalNAcβ-PAA. Similarly, anti-A_{di} IgG bound weakly GalNAcα-PAA [12].
- Anti-GalNAcβ IgG recognized the GalNAcβ residue in $X2_{di}$ but bound weakly or did not bind PF_{di} and $T_{ββ}$ in PAA-conjugates.
- Anti-GalNAcβ IgG did not bind or bound weakly GlcNAcβ.
- Anti-GalNAcβ and anti-$X2_{di}$ IgG recognized GalNAcβ1–3Galβ linked glycans but bound weakly or did not bind GA2 and Lac-di-NAc having the GalNAcβ1–4 linkage.
- Anti-GalNAcβ IgG and anti-GalNAcα IgG differed in reactivity: the former bound the $X2_{di}$ (GalNAcβ1–3Galβ), whereas the latter did not bind A_{di} (GalNAcα1–3Galβ) [12].

Noteworthy is that the reactivity and specificity of pair samples were similar (Tables 3, 4), despite some differences in the reactivity between the pairs of serum probes taken for isolation. This may point to some selectivity of GalNAcβ-terminated conjugates and affinity sorbents. On the other hand, heterogeneous populations of IgG antibodies that differ in specificity to di- and trisaccharides with external GalNAcα or Galα are present in the serum of different blood groups, and were isolated using only GalNAcα1–3Galβ-PAA-Sepharose for all probes [12]. The limited number of observations was due to that the number of long-term survivors having a high level of serum antibodies was low. In donors, the level of antibodies to GalNAcβ-terminated glycans was lower. The anti-GalNAcβ IgG isolated from the serum of four subjects with the elevated level of antibodies demonstrated a similar cross-reactivity to $X2_{di}$. These findings are in agreement with those of the study on anti-glycan immunoglobulins in donors, which demonstrated either polyspecificity or narrow epitope specificity [16].

Earlier we characterized anti-TF/TFβ cross-reactive IgG populations in long-term cancer survivors and found a significantly better survival of gastrointestinal cancer patients with an increased serum level of antibodies [9, 13]. The elevated serum level of anti-GalNAcβ IgGs cross-reactive to $X2_{di}$ was associated with the significantly better survival of patients with gastrointestinal cancer as well [8]. Cross-reactive antibodies deserve attention in the clarification of their function in cancer progression.

Analysis of antibodies by IEF. The isolated IgG samples were analysed by IEF and the Western blotting. IgG samples showed either the oligoclonal or polyclonal distribution of bands (Fig. 4). The monoreactivity of antibodies was not associated with the clonal distribution of bands. The antibody response in humans to many common pathogens is oligoclonal, with restricted usage of Ig V-genes [17]. Bacterial polysaccharides with a repeated carbohydrate unit have relatively few determinants and complementary combinations. Therefore, despite the diversity of anti-glycan antibodies, the oligoclonal pattern of affinity-isolated anti-glycan IgG was expected.

Putative natural immunogens. The immunopathological role of antibodies remains poorly understood unless the immunogen or relevant autoantigen is known. Enteric microbiota in human is most likely immunogens for antibodies. GalNAcβ is one of the exclusive ligands of the C-type lectin of human macrophages, which is involved in the uptake and presentation of glycosylated antigens [18, 19]. The GalNAcβ-terminated glycans are present in antigens of bacteria and protozoa [20, 21]. Neoplasia can disrupt the intact mucosal barrier to enteric microbiota. The elevated level of some anti-glycan IgGs in sera of patients with gastrointestinal cancer may reflect the adaptive immune response to microorganisms and may be indirectly associated with better or worse survival *via* beneficial or detrimental interrelation between microorganisms and tumor.

The X2 and PF glycolipids have been isolated from human erythrocytes and X2 has been found in various human tissues including carcinomas. The above antigens are extremely minor components; therefore, they cannot be detected on the cell surface [22–25]. It seems to be improbable that the antibodies described could be autoreactive, binding the minor glycolipids of cellular membranes. Moreover, the presumably "auto" antibodies were found to bind short fragments of larger glycans whereas recognition of the same fragment in the context of the whole natural chain was completely abolished [16].

Thus, the anti-GalNAcβ IgGs isolated from the serum of gastric cancer survivors demonstrated the cross-reactivity to the $X2_{di}$ glycan mainly. The isolated anti-$X2_{di}$ and -PF_{di} IgGs demonstrated monoreactivity to the corresponding key glycan-PAA used for isolation of antibodies. The findings provide additional information for elucidation of the immunopathological role of cross-reactive antibodies to GalNAcβ-terminated glycans in cancer and enhancement of the prognostic potential in the combined determination of antibody markers.

ACKNOWLEDGMENTS

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