THE RELATION OF SERUM ANTI-(GalNAc BETA) AND -PARA — FORSSMAN DISACCHARIDE IgG LEVELS TO THE PROGRESSION AND HISTOLOGICAL GRADING OF GASTROINTESTINAL CANCER

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Earlier we found two unusual IgG-antibody specificities to GalNAcβ and GalNAcβ1-3GalNAcβ (Para — Forssman disaccharide, PFdi) carbohydrate ligands in human serum. The aim of the study was to evaluate whether elevated antibody levels are related to the progression of gastrointestinal cancer and the histopathological grading. Methods: Specific IgG levels were tested in 159 patients with gastric cancer, 88 patients with colorectal cancer and 96 blood donors by the ELISA using synthetic polyacrylamide (PAA) conjugates, GalNAcβ-PAA and PFdi-PAA. Biochemical and haematological analyses were performed using automatic equipment. Results: The anti-PFdi IgG levels were significantly higher in patients with gastric and colorectal cancer than in donors: in stages II—IV, \( P = 0.0002 – 0.04 \) (U-test). The elevated anti-PFdi IgG level was associated with the advanced gastric cancer: in stages II, III, IV vs stage I \( (P = 0.004 – 0.06) \) and in case of the tumor size T2 + T3 vs T1 (stages I, II; \( P = 0.03 \)). Differences in anti-GalNAcβ IgG level were insignificant. No relation between antibody levels and the regional and distant metastases of gastric or colorectal cancer was found. The lower anti-GalNAcβ IgG level was associated with lower-differentiated carcinomas \( (P = 0.01 – 0.04) \). Prolonged postoperative changes in the levels of both antibodies during the follow-up were established. An elevation of both antibody levels in patients with gastrointestinal cancer was revealed after a surgical removal of G3-tumors \( (P = 0.003 – 0.01) \). The anti-PFdi IgG levels correlated with the levels of the C-reactive protein: \( r = 0.50, P = 0.003 \). The anti-GalNAcβ IgG levels correlated with the percentage of peripheral blood monocytes: \( r = 0.42, P = 0.002 \). Conclusion: The association of the anti-PFdi IgG level with cancer progression suggests the implication of antibodies in the pathogenesis of gastrointestinal cancer. Further studies are required to identify natural targets of antibodies, their relation to other diseases, prognostic significance in cancer.

Key Words: GalNAc beta1-3GalNAc beta, Para — Forssman, polyacrylamide-glycoconjugates, IgG antibodies, gastric cancer, colorectal cancer.

The immunopathological role of antibodies remains poorly understood until the relevant autoantigen and the exogenous immunogen are unknown. Normal individuals as well as individuals with autoimmune diseases and cancer produce antibodies reacting with a variety of carbohydrate determinants [1]. Human natural anticalbohydrate antibodies belong mostly to the IgM-class and their individual physiological level is sustained by immune homeostasis. In some patients with cancer, the unusually high levels of the IgG antibody to the mucin-type carbohydrate epitopes were found [2]. This may be explained by an adaptive immune response indicating an antibody class switching to IgG and undergoing affinity maturation. As a rule, the serum IgG antibodies affinity-purified on synthetic sorbents exhibited a low specificity to tumor-associated mucins, possibly due to the presence of a clustered form of carbohydrates in mucins [3, 4]. As in immunoassays or the purification of antibodies polyacrylamide (PAA)-glycoconjugates with a low epitope density \( (10–20 \text{ mol.} \%) \) were used, the specificity of the antibodies examined may be directed to glycolipids (natural targets with non-clustered saccharides).

PAA-glycoconjugates are homogenous antigens with a single reiterative epitope that enables the detection of epitope-specific antibodies [5]. In immunoassays, synthetic glycoconjugate-models have certain advantages over natural antigens containing usually different determinants. The high reproducibility of antibody detection by the ELISA and the low background in the control make PAA-conjugates a promising tool for comparative investigations [2, 6]. The measurement of antibodies to glycolipids is technically demanding and different laboratories have demonstrated that the immunoassays varied widely in sensitivity and the criteria employed for a positive test [7].

Taking into consideration the high frequency of human antibodies to glycolipids and their possible role in the pathogenesis of cancer [8], we tested the serum of patients by the ELISA using a set of PAA-β-glycoconjugates, and found two populations of IgG antibodies that were specific to GalNAcβ and GalNAcβ1-3GalNAcβ ligands [3] (the latter is an outer disaccharide of Para — Forssman glycolipid, PFdi). The aim of the present study was to evaluate whether serum antibody levels are related to the progression of gastrointestinal cancer and the histopathological grading. The level of anti-PFdi IgG was found to be associated with the advanced cancer and that of anti-GalNAcβ IgG was associated with the histopathological grading.

MATERIALS AND METHODS

Subjects. The investigation was carried out in accordance with the ICH GCP Standards and approved

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Abbreviations used: A – absorbance; CRP – C-reactive protein; PAA – polyacrylamide; PFdi – Para — Forssman disaccharide; TF – Thomsen-Friedenreich antigen; αGal – Galα1-3Galβ; Tn – GalNAco; TBS – Tris-buffer/0.05% Tween-20.
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by Tallinn Medical Research Ethics Committee. The informed consent from the subjects under study was obtained. Blood transfusion donors and patients with gastric, colon or rectal cancer with a verified diagnosis (histology and tumor staging by the pTNM system) were examined [9]. The median age of donors was 52 years (range 39 to 65 years); that of gastric cancer patients, 59 years (range 28 to 74 years); and of colorectal cancer patients, 60 years (range 41 to 75 years). In all cases, except special investigations (the effect of a surgical removal of the tumor or the follow-up study), blood samples were taken before the surgical operation. The gastrectomy and extended D2 limphadenectomy, but not spleectomy, for gastric cancer and the resection of local lesions for colorectal cancer were performed. The patients who received chemo- or X-ray-therapy were excluded from the study. Concomitant diseases in patients were examined based on the history of the disease and a personal conversation.

Glycoconjugates. The glycoconjugates with polyacrylethanolamide (PAA) — GalNAcβ-PAA and GalNAcβ1-3GalNAcβ-PAA were received from Lectinity, Russia. The epitope density was 20 mol. %. Tris(hydroxymethyl)aminomethane-PAA (Tris-PAA) was used as a negative control.

The determination of epitope-specific antibody levels in sera. The venous blood serum was kept at −70°C. In the ELISA, the serum frozen/thawed once was used. The method was performed as described in [2]. Briefly, PAA-glycoconjugates (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 Tris-buffer/0.05% Tween-20 (TBS), the serum diluted to 1:25–1:400 in TBS/0.05% casein/5 mM EDTA-disodium salt was added. The dilution of sera with a buffer containing 5 mM EDTA reduces the background significantly, but influences weakly the specific antibody binding [2]. After incubation for 3 h at 26°C, the plate was washed and incubated with the goat anti-human IgG-alkaline phosphatase conjugate for 1.5 h at 26°C. The absorbance (A) at 405 nm of the reaction with a p-nitrophenylphosphate disodium salt (1 mg/ml in the glycine-buffer, pH 10.3, 26°C, 2 h) was measured using a Labsystem Multispec MCC/340 (Finland). The ratio of the mean A_{\text{test}}/A_{\text{control}} was calculated, where A_{\text{test}} is the absorbance with the PAA-glycoconjugate and A_{\text{control}} with the Tris-PAA. The variation coefficient was 3%.

Clinical analysis of blood samples. The biochemical and haematological analyses were performed in North-Estonian Regional Hospital Oncological Centre using automatic equipment: a Hitachi 912 and Elescys 2010, Roche Diagnostics; Sysmex XE-2100, Sysmex Corporation. Blood samples were taken from patients before and/or after a surgical operation during the planned visits to the physician for health control. The antibody levels were correlated with the levels of the C-reactive protein (CRP), tumor markers (CA19-9, CEA), alanine aminotransferase, glucose, haemoglobin, circulating red blood cells (count), leucocytes (count), neutrophils (%), monocytes (%), lymphocytes (%), platelets (count) and eosinophils (%). The CRP concentration was determined by using a turbidimetric method and tumor markers by an electrochemiluminescence immunoassay.

Statistical methods. The Mann — Whitney U-test, 2D Cartesian box plots graph and regression analysis were used in the study. The differences were considered significant when P < 0.05. The graphs were plotted by means of a SigmaPlot 2000 program and Statgraphics Plus 5.1.

RESULTS

The relation of antibody levels to cancer. The subjects were analysed by age, gender and ABO(H) blood group phenotype to assess the possible influence of these parameters in the comparative study. In donors as well as in patients with cancer the levels of anti-GalNAcβ and -PFdi IgG antibodies were not related to age and gender. The relation between antibody levels and the blood group phenotype in donors was not observed either. The higher anti-PFdi IgG levels for the A-blood group as compared to those for B or O groups were revealed in cancer patients (P = 0.03). Therefore, the influence of the blood group was taken into account: an approximately equal ratio of A/B and A/O was chosen for the comparison of cancer patients with donors as well as for analysis by stage of cancer.

High levels of antibodies, particularly anti-PFdi IgG, were found in the serum of patients with cancer (Fig. 1). The anti-PFdi antibody level was significantly higher in patients with gastrointestinal cancer, especially in stages II—IV, than in donors, whereas the difference in the level of anti-GalNAcβ IgG was insignificant (Table 1). An asymmetrical distribution of anti-PFdi IgG values was characteristic of more advanced cancer (Fig. 2, boxes 2, 3 and 4). The median values in stage I of gastric cancer were very close to those in donors (Table 1, 2), but the median in stages II and IV was significantly higher (Table 2). Taken together, stages I and II were analyzed for tumor size: the differences in T1 vs T2 + T3 remained significant. A similar stage-dependent change of median values was observed for colorectal cancer but differences were significant only between stages I and II: P = 0.047 (tumor size status in stage I was T1 + T2 vs T3 + T4 in stage II). The level of anti-GalNAcβ IgG was not related to the stage of gastric or colorectal cancer. These results and our previous data [10] show that the levels of serum anti-carbohydrate antibodies in patients with cancer are either directly or inversely associated with tumor progression (PFdi, TF, cGal) or do not depend on it (GalNAcβ, Tn), (Table 3, summarized data).

Table 1. Analysis of serum IgG antibody levels in donors and cancer patients

<table>
<thead>
<tr>
<th>Serum</th>
<th>Anti-GalNAcβ</th>
<th>Anti-PFdi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Median</td>
</tr>
<tr>
<td>Donors</td>
<td>96</td>
<td>1.34</td>
</tr>
<tr>
<td>Gastric cancer, all stages</td>
<td>159</td>
<td>1.57</td>
</tr>
<tr>
<td>Stages II, III, IV</td>
<td>121</td>
<td>1.88</td>
</tr>
<tr>
<td>Colorectal cancer, all</td>
<td>88</td>
<td>1.38</td>
</tr>
<tr>
<td>Stages II, III, IV</td>
<td>69</td>
<td>1.72</td>
</tr>
<tr>
<td>Total cancer, all stages</td>
<td>247</td>
<td>1.37</td>
</tr>
</tbody>
</table>
The effect of the surgical removal of tumors on antibody levels was investigated: blood samples from each patient were taken before and after surgery at intervals of three to sixteen months. The postoperative level of antibodies was increased in 80–90% of patients having G3-tumors, differences were significant. Differences in pre- vs postoperative levels of anti-GalNAcβ or anti-PFdi IgG were not significant for G1 + G2-tumors (Table 5, Fig. 3).

The follow-up study of sixty-eight patients with gastrointestinal cancer carried out since 1994 has shown both the stimulation and suppression of the immune response to take place. A long-term high anti-PFdi IgG level was also observed but a common trend towards the decline of antibody levels occurred during the last period of observation (Fig. 4).

Table 2. The relation between the anti-PFdi IgG level and the stage and size status of the tumor in patients with gastric cancer

<table>
<thead>
<tr>
<th>Stage, size</th>
<th>n</th>
<th>Median</th>
<th>Comparison, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>39</td>
<td>1.38</td>
<td>I vs II, 0.029</td>
</tr>
<tr>
<td>II</td>
<td>42</td>
<td>1.76</td>
<td>I vs III, 0.056</td>
</tr>
<tr>
<td>III</td>
<td>44</td>
<td>1.49</td>
<td>I vs IV, 0.004</td>
</tr>
<tr>
<td>IV</td>
<td>35</td>
<td>2.26</td>
<td>III vs IV, 0.065</td>
</tr>
<tr>
<td>T1 (I)</td>
<td>31</td>
<td>1.24</td>
<td>T1 vs T2 + T3,</td>
</tr>
<tr>
<td>T2 + T3 (I, II)</td>
<td>50</td>
<td>1.68</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Table 3. The association of the serum anticarbohydrate IgG level with the progression and histopathological grading of cancer

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Tumor progression</th>
<th>Histopathological grading*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn [10]</td>
<td>Gastric cancer</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>GalNAcβ</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>TF [10]</td>
<td>No</td>
<td>Direct</td>
</tr>
<tr>
<td>αGal [10]</td>
<td>Inverse</td>
<td>No</td>
</tr>
<tr>
<td>PFdi</td>
<td>Direct</td>
<td>No</td>
</tr>
</tbody>
</table>

*Direct association: lower levels are associated with lower-differentiated carcinomas (the comparison is shown in Table 4).
ND: not determined.

Fig. 1. Binding of the serum IgG-antibody with PFdi-PAA (●) and GalNAcβ-PAA (■) adsorbed onto immunoplates in patients with gastric cancer. Light symbols show the control binding with Tris-PAA, respectively.

Fig. 2. The distribution of anti-PFdi IgG levels in donors and patients. Donors: box No 1; gastrointestinal cancer in stage I: box No 2; gastric cancer in stages II–IV: No 3; colorectal cancer in stages II–IV: No 4. The lower boundary of the box indicates the 25th percentile data, a line within the box marks the median, and the upper boundary of the box indicates the 75th percentile. Bars above and below the box indicate the 90th and 10th percentiles.

No relation between the levels of anti-GalNAcβ or anti-PFdi antibodies and regional lymph node metastases (stages II and III) and distant metastases (stages III vs IV) in patients with gastric or colorectal cancer was observed. The relation between antibody levels and the histopathological grading was found only for anti-GalNAcβ IgG: the median for lower-differentiated carcinomas was significantly lower (Table 4).
The relation of antibody levels to blood parameters and other diseases. The anti-PFdi IgG level was found to correlate with CRP (Fig. 5). In a follow-up study, anti-GalNAcβ IgG levels correlated with the monocytes percentage ($r = 0.42, P = 0.002$, $n = 50$). The correlation with tumor markers and other parameters (see Materials and Methods) was not established.

The autoimmunity is an immunodominant. Probably, the Para — Forssman glycolipid is a natural cross-reactive ligand to antibodies, whose postoperative level was increased in a majority of patients with gastrointestinal low-differentiated carcinomas (a manuscript in preparation). The levels of Forssman antibodies in the sera of patients have been reported to be also elevated significantly after a radical resection of the tumor [12, 15]. The total levels of IgG and other isotype antibodies remained unchanged after gastrectomy [16]. Taken together, these results may be interpreted as a common suppressive influence of carcinomas (low-differentiated mainly) on the production of anti-carbohydrate antibodies. Although the postoperative elevation of anti-PFdi IgG levels (as well as anti-αGal IgG, unpublished data) was observed, the relation between their levels and histopathological grading was not registered for gastric and colorectal cancer (Table 3). Perhaps, the direct association of the level of these antibodies with tumor progression does not allow a statistical evaluation of their relation to grading.

The terminal β-linked GalNAc residues are specific ligands of the C-type lectin of human macrophages, whose recognition and targeting modulates immune response [17]. We observed the correlation of anti-GalNAcβ IgG levels with the percentage of peripheral blood monocytes that might reflect the adaptive immune response and the production of specific IgG antibodies.

**The putative natural antigens.** The specificity of the antibodies examined in the ELISA using PAA-conjugates with a low epitope density may be directed to glycolipids. Usually, an external oligosaccharide in carbohydrate moieties is an immunodominant. Probably, the Para — Forssman glycolipid is a natural cross-reactive ligand to antibodies, which we named “anti-PFdi”, because it contains a terminated GalNAcβ1-3GalNAcβ disaccharide, the same as in PFDi-PAA [18, 19]. The serum anti-PFdi IgG did not react with the Forssman disaccharide (GalNAcβ1-3GalNAcβ) and other ligands [3]. It is in agreement with vice versa investigations: the lack of the cross-reactivity between Forssman and Para — Forssman glycolipids was observed earlier for anti-Forssman antibodies [18]. We did not find any literature data about the expression of the Para — Forssman antigen in human tissues, except in erythrocytes, where it is present in low amounts [18].
Human natural anti-Para — Forssman antibodies appeared to have been described earlier neither. Whether the Para — Forssman antigen is expressed in human gastric carcinoma similarly to the Forssman antigen remains to be clarified [20].

The natural ligand for anti-GalNAcβ antibodies may be glycolipid with a terminated GalNAcβ residue, for example GA2, an x, glycolipid or a P antigen. The serum anti-GalNAcβ IgG reacted weakly with Tn (GalNAcα) and other ligands [3]. The IgG antibodies affinity-purified on GalNAcβ-sepharose from human serum were specific and did not show any reactivity to Tn and the other GalNAc-ligands tested earlier [3], but reacted weakly with synthetic GA2 (GalNAcβ1-4Galβ1-4Glcβ) (unpublished observations). The specificity of purified antibodies to PAA-glycoconjugates and their reactivity to tumor-derived glycolipids, as well as the characterization of carbohydrate moiety with monoclonal antibodies will be subjected to further study.

The exogenous origin of an antigenic stimulus should not be neglected either. A widespread human parasite Giardia intestinalis might be related to the production of anti-PF antibodies because its cyst wall antigen contains the β(1-3)-GalNAc-homopolymer [21]. Although the polymer is highly insoluble, its degraded products (oligomers), if they form, could be potential immunogens.

The Helicobacter pylori infection is one of the main reasons for gastric disorders and its relation to cancer is well documented [22]. There is evidence that the cancer-related TF-epitope is expressed in surface membrane glycoconjugates of H. pylori and is associated with a modulation of the natural immune response to a TF-antigen in infected subjects [23]. No correlation or only a tendency to correlation between the levels of anti-GalNAcβ or anti-PFdi IgG and serum IgG antibodies against H. pylori cell surface antigens was observed in donors and cancer patients. The treatment of H. pylori-infected patients with gastric ulcer (a standard one-week triple therapy) did not influence the level of antibodies, irrespective of the efficacy of therapy. The origin of immunogens remains unclear and needs further exploration.

Thus, significant differences in antibody levels were found owing to the application of the highly reproducible immunoassay with synthetic homogeneous neoantigens. The high anti-PFdi antibody level in patients and its association with advanced cancer as well as prolonged postoperative changes during the follow-up suggest the presence of adaptive antibodies that are involved in tumor pathogenesis directly or indirectly. A further monitoring of patients and the survival analysis are now underway to evaluate the prognostic significance of antibodies.

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**REFERENCES**


СООТНОШЕНИЕ МЕЖДУ УРОВНЕМ IgG АНТИТЕЛ К GalNAc БЕТА И ДИСАХАРИДУ ПАРА — ФОРСМАННА В СЫВОРОТКЕ КРОВИ И ПРОГРЕССИЕЙ, А ТАКЖЕ СТЕПЕНЬЮ ДИФФЕРЕНЦИРОВКИ ОПУХОЛЕЙ ЖЕЛУДОЧНО-КИШЕЧНОГО ТРАКТА

В сыворотке крови человека ранее выявлены IgG-антитела к углеводородным липидац GalNAcβ и GalNAcβ1-3GalNAcβ (дисахарид Пара — Форсмания, PFdi). Цель исследования — выяснение связи повышенного уровня антител с прогрессией и гистологическими особенностями опухолей желудочно-кишечного тракта. Методы: обследованы 159 больных раком желудка, 88 — раком кишечника и 96 здоровых людей — доноров крови. Уровень специфических IgG-антител определяли методом ИФА с использованием синтетических конъюгатов полиакрилат (ПАА), GalNAcβ-ПАА и PFdi-ПАА. Определение биохимических и гематологических показателей проводили с использованием автоматических анализаторов. Результаты: уровень анти-PFdi IgG у пациентов со злокачественными новообразованиями в стадиях II–IV (P = 0,0002 — 0,04) достоверно выше, чем у доноров. Повышенный уровень анти-PFdi IgG ассоциировался с прогрессией рака желудка (в стадиях II–IV по сравнению со стадиями I (P = 0,04 — 0,06) и увеличением размера опухоли (T2 + T3 по сравнению с T1, стадии I, II; P = 0,03). Различия в уровнях анти-GalNAcβ IgG незначительные. Не выявлено взаимосвязи между уровнем антител и метастазированием опухолей желудка и кишечника. Более низкий уровень анти-GalNAcβ IgG определяли у больных с менее дифференцированными опухолями (P = 0,01 — 0,04). В период послеоперационного наблюдения отмечали изменения уровня обоих типов антител. После хирургического удаления опухолей органов желудочно-кишечного тракта (стадия дифференцировки G3) повысился уровень как анти-PFdi IgG, так и анти-GalNAcβ IgG (P = 0,003 — 0,01). Уровень анти-PFdi IgG коррелировал с уровнем C-реактивного белка (r = 0,50, P = 0,003), а уровень анти-GalNAcβ IgG — с относительным количеством моноцитов в периферической крови (r = 0,42, P = 0,002). Выводы: установлена зависимость уровня анти-PFdi IgG от опухолевой прогрессии, что подтверждает их участие в патогенезе злокачественных новообразований органов желудочно-кишечного тракта. Необходимы дальнейшие исследования по определению приоритетных мишений для антител, роли в развитии других заболеваний и прогностическом значении при онкологической патологии.

Ключевые слова: GalNAc бета1-3GalNAc бета, Пара — Форсмания, полиакрилат-гликоконъюгаты, IgG антитела, рак желудка, рак кишечника.