Natural IgM and IgG Antibodies to Thomsen-Friedenreich (T) Antigen in Serum of Patients with Gastric Cancer and Blood Donors

Relation to Lewis (a,b) Histo-blood Group Phenotype

Oleg Kurtenkov, Ljudmila Miljukhina, Jevgeni Smorodin, Kersti Klaamas, Nikolai Bovin, Malle Ellamaa and Valentin Chuzmarov

From the Department of Oncology, Institute of Experimental and Clinical Medicine (O. Kurtenkov, L. Miljukhina, J. Smorodin, K. Klaamas), Tallinn, Estonia, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (N. Bovin), Moscow, Russia, the Estonian Blood Bank (M. Ellamaa), and Estonian Oncological Center (V. Chuzmarov), Tallinn, Estonia

Correspondence to: Oleg Kurtenkov, MD, ScD, Institute of Experimental and Clinical Medicine, Hiiu 42, 11619 Tallinn, Estonia. Tel: +372 2 514 501. Fax: +372 6 706 814. E-mail: oleg@ekmi.online.ee

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A possible association of serum anti-T IgM and IgG antibody levels with Lewis blood-group phenotype was investigated in 168 blood donors and 132 gastric cancer patients using ELISA with synthetic T-disaccharide-polyacrylamide conjugate as antigen. The donors of Le(a − b +) phenotype showed the highest anti-T IgM level irrespective of ABO(H) blood group. A significant decrease in anti-T IgM in serum was observed among cancer patients of Le(a − b +) phenotype: 95% of weak responders versus 17.5% for related groups of donors ($p < 10^{-6}$). In contrast, no significant difference between patients and donors was found for Le(b −) individuals. Thus, a level of natural anti-T antibodies in serum of blood donors and its decrease in patients with gastric cancer are related to Le(a,b) phenotype. This should be taken into account where anti-T antibody level in the serum is used as a tumour marker or for monitoring patients during cancer immunotherapy with mucin-type vaccines.

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The Thomsen-Friedenreich (T) antigen (Galβ1-3GalNAcα/β-O-Ser/Thr) is one of the core structures of malignant-related carbohydrate antigens (1–4). An expression of T epitope in the majority of human carcinomas is usually accompanied by a decrease in natural anti-T specific antibodies in the serum (5–7). A low level of these antibodies is also associated with some premalignant conditions (5, 8, 9). This phenomenon was shown to be of diagnostic value for cancer (5, 6), but the clinical use of this approach is in large part limited because of the absence of a uniform and practical assay (10).

A level of natural anti-T antibodies in the serum is fairly stable for a given individual (7) but the reason for variations between individuals in health and disease remains unclear. Recently, we developed an enzyme-linked immunosorbent assay (ELISA) for the determination of serum anti-T antibodies using a fully synthetic T disaccharide-polyacrylamide conjugate as antigen (9). A significant decrease in anti-T antibody has been demonstrated already at the very early stages of gastric and breast cancer compared with age-matched healthy blood donors (11). However, discrimination between patients with cancer and related benign disorders was insufficient, particularly for the stomach diseases, suggesting that the differences observed might be altered by factors unrelated to cancer. In the present study we report that the serum level of anti-T antibodies in healthy blood donors and its decrease in patients with gastric cancer are related to the Lewis (a,b) blood group phenotype of the individual.

MATERIAL AND METHODS

Subjects and samples

Blood samples were obtained from 168 blood transfusion donors and 132 consecutive patients admitted to an Estonian Cancer Centre with histologically verified gastric carcinoma. The numbers of patients and controls tested for IgM and IgG T-antibodies including the individuals within subgroups of different Lewis(a,b) phenotype are presented in Table 1. Tumour staging was based on the histopatho-

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logic (pTNM) classification system (12). Individuals who received treatment with antibiotics within a month before testing were excluded from the study.

The sera were separated after blood clotting (2 h incubation at 37°C), stored at −20°C and tested within 1–2 months.

Lewis and ABO(H) phenotyping

Lewis and ABO(H) phenotyping of erythrocytes was carried out using anti-Le^a and -Le^b monoclonal antibody gel system (DiaMed, Switzerland) and anti-A and anti-B monoclonal antibody (Monocarb AB, Sweden) according to the manufacturers' instructions.

Determination of serum anti-T IgM and IgG antibodies by ELISA

Plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with synthetic T-hapten-polyacrylamide (PAA) conjugate (Synthesom, Munich, Germany; 10 mol% of carbohydrates) 2 μg/ml in carbonate buffer 50 mM Na_2CO_3/$NaHCO_3$ and 0.02% NaN_3, pH 9.6 or 1% BSA in PBS (control wells) at 4°C overnight. After washing three times with PBS – (0.13 M NaCl, 8 mM NaH_2PO_4/KH_2PO_4, 3 mM KCl, 0.02% sodium azide, pH 7.2) – 0.05%Tween 20, the plates were blocked with 0.15 ml 1% BSA in PBS for 1 h at room temperature (RT) and washed in PBS-Tween. Serum (100 μl) diluted 1 : 500 in PBS-Tween was added and incubated overnight at RT. The plates were then

Table 1

Blood donors and the patients with gastric cancer tested for serum IgM and IgG anti-T antibody level and Le(a,b) blood group phenotype

	Blood donors	Cancer patients
N	168	132
Median age (range)	49.4 (26–65 yrs)	61.3 (28–87 yrs)
Men	68	89
Woman	100	43
Tested for IgM T-antibodies	168	132*
Tested for Le(a,b) phenotype and IgM T-antibodies	168	76
Le(a+b−)	25	4
Le(a−b+)	120	60
Le(a−b−)	23	12
Tested for IgG T-antibodies	78	132
Tested for Le(a,b) phenotype and IgG T-antibodies	57	69
Le(a+b−)	16	5
Le(a−b+)	36	52
Le(a−b−)	5	12

* The distribution of patients with gastric cancer by disease stage: stage I—18, stage II—27, stage III—36 and stage IV—51 patients.

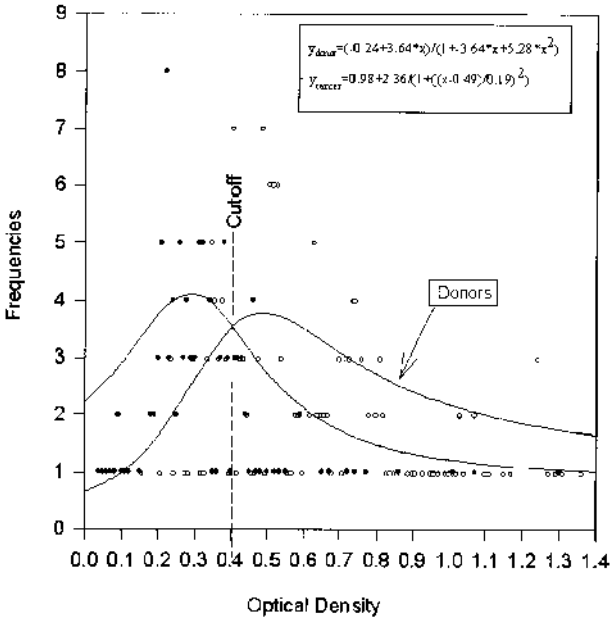

Fig. 1. The dot-plot and the distribution curves of optical density values for IgM anti-T-antibody level in serum of patients and controls. Donors (open bars, n = 168); cancer patients (closed bars, n = 132). Dotted line—the cut-off limit. The distribution curves are optimized and described using the CurveExpert v.1.2 program.

washed and bound IgM was detected with 100 μl alkaline phosphatase-conjugated rabbit anti-human IgM (Dako, Denmark) diluted 1 : 500 in PBS-Tween. Following an incubation of 90 min at RT and washing, the plates were developed with p-nitrophenylphosphate (Sigma, St. Louis, MO), 1 mg/ml in 1 M diethanolamine buffer, pH 9.8 for 30 min and absorbance values at 405 nm were registered with Labsystem Multiscan MCC/340 (Finland). Anti-T IgG antibodies were determined by a similar procedure, except that the serum diluted 1 : 100 was incubated for 2 h at RT and alkaline phosphatase conjugated goat anti-human IgG (Gibco, BRL, Life Technologies, Gaithersburg, MD, USA) 1 : 1000 was used. Optical density (O.D.) values of control wells were subtracted from the values of the wells coated with the T conjugate and net O.D. values of more than 0.4 and 0.3 were considered as strong IgM and IgG responses, respectively. These cut-off limits were calculated as shown in Fig. 1. More than 80% of preliminary tested blood donors (n = 50) were above these cut-off limits. Each serum was analysed in duplicate. Two reference sera from weak and strong responders were run in every plate as an internal standard in order to control the experimental conditions and to correct the interassay variations in the consecutive assays. The intra-assay variations did not exceed 7%.

Statistical analysis

The distribution of the O.D. values in patients and controls was evaluated using the SigmaPlot, version 1.01 and

CurveExpert, version 1.2 statistical programs to select proper cut-off limits. Statistical comparisons between the groups were done by χ^2 test. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were used to measure an association between the parameters studied. The difference was considered to be significant when $p < 0.05$.

RESULTS

The distribution of all individual O.D. values for IgM anti-T antibodies in the group of cancer patients and blood donors (irrespective of ABO(H), Lewis phenotype or stage of cancer) is presented in Fig. 1. An abnormal distribution was found for both groups, suggesting that non-parametric statistics should be applied. According to the statistical calculations, the cut-off limit which allowed the best discrimination between patients and controls was found to be equal to O.D. = 0.4 and 0.3 for IgM and IgG T-antibodies (not shown), respectively. These cut-off limits were used for further comparisons.

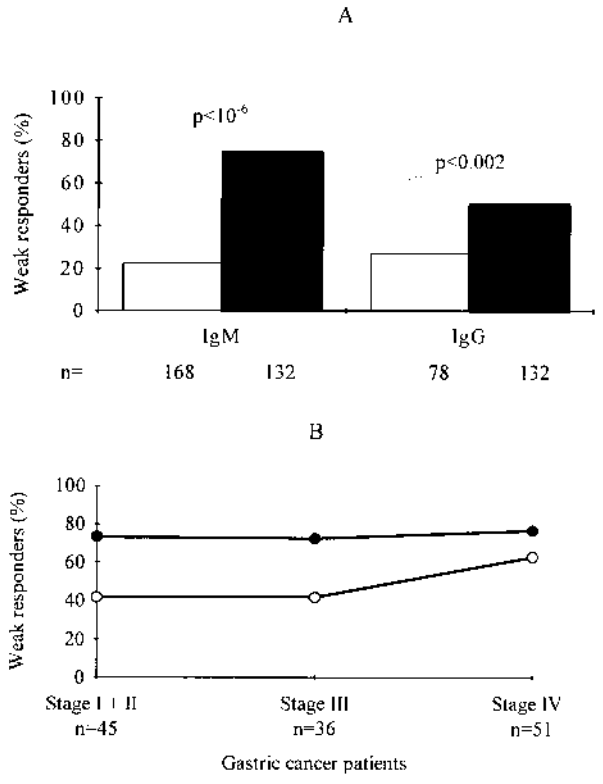

Fig. 2. The proportion of IgM and IgG weak responders to T-antigen in blood donors and patients with gastric cancer (A); relation to the disease stage (B). A: Donors (open bars, n = 168) and gastric cancer patients (closed bars, n = 132) were tested for serum IgM anti-T antibody. IgG anti-T antibody level was further determined in serum of 78 donors and of 132 cancer patients. Statistical comparisons between the groups were carried out by χ^2 test. B: The patients with gastric cancer (n = 132) were tested in parallel for serum IgM and IgG anti-T antibody. On the ordinate axis—the proportion of weak responders.

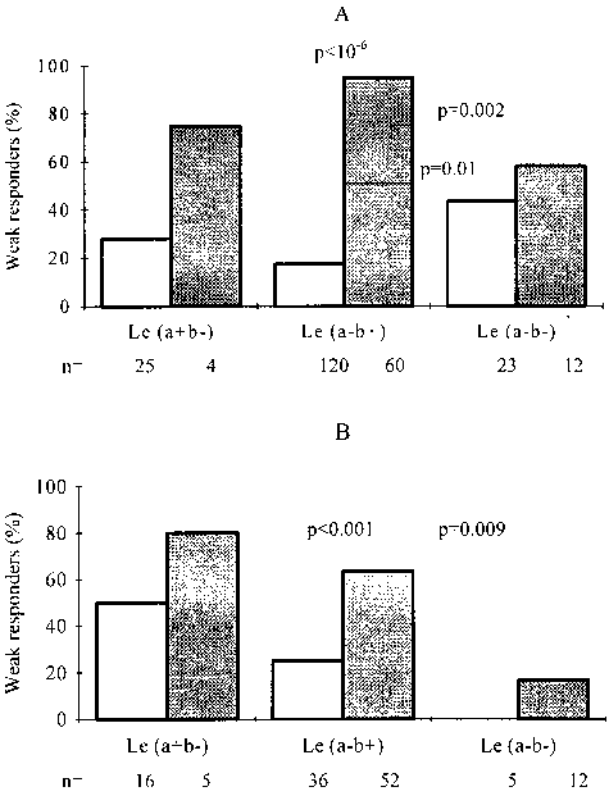

Fig. 3. The proportion of IgM (A) and IgG (B) weak responders to T-antigen among blood donors and gastric cancer patients in relation to the Lewis blood group phenotype. A: Lewis phenotyped donors (open bars, n = 168) and gastric cancer patients (closed bars, n = 76) were tested for serum IgM anti-T antibody. B: Donors (open bars, n = 57) and gastric cancer patients (closed bars, n = 69) were tested for serum IgG anti-T antibody. Statistical comparisons were carried out by χ^2 test.

A highly significant difference ($\chi^2 = 77.4$, K = 1, n = 300, $p < 10^{-6}$; OR = 9.9, 95% CI 5.6–17.4) in the proportion of anti-T IgM weak responders was found between cancer patients and donors when the data were analysed irrespective of Lewis phenotype (Fig. 2). A less pronounced but significant difference was noted for IgG response ($\chi^2 = 9.8$, K = 1, n = 126, p = 0.017; OR = 2.7, 95% CI 1.4–5.2). A proportion of weak IgM responders in cancer patients was not dependent on the disease stage (73–76%), whereas IgG response was lower in the patients with advanced gastric cancer.

No significant association with ABO(H) blood group phenotype was found for either IgM or IgG immune response of patients with cancer and donors (data not shown). The patients possessing blood group A antigen (groups A and AB) showed a slightly lower proportion of IgM weak responders compared with those of blood groups O and B: 68% and 80%, respectively, p = 0.1). Males and females also showed very similar patterns in both groups of individuals. The only difference for IgG response was an insignificantly higher proportion of weak

responders among individuals of blood group O (42.3%) compared with other blood groups (13–21%).

The proportion of weak and strong responders varied significantly among the individuals of different Lewis phenotype in both donors and patients (Fig. 3). Blood donors of Le(a − b +)/secretor type showed the highest anti-T IgM level regardless of ABO(H) blood group (Fig. 3A). Twenty-one of 120 donors with this phenotype were weak IgM responders. Conversely, the related group of cancer patients revealed the most pronounced decrease in IgM anti-T antibody: 95% of them (57 out of 60) were found to be weak responders compared with 17.5% for donors ($\chi^2 < 94.7$, $K = 1$, $p < 10^{-6}$; OR = 89.6, 95% CI 23.0–221.5). In contrast, the difference between patients and controls of Le(a + b −) or Le(a − b −) phenotype was insignificant.

Similarly, only the patients of Le(a − b +) showed a significantly lower anti-T IgG response (Fig. 3B) compared with the related group of donors ($\chi^2 = 11.1$, $K = 1$, $p = 0.0008$; OR = 5.2, 95% CI 1.8–15.0).

Thus the best discrimination between patients with gastric cancer and healthy blood donors was noted in Le(a − b +)/secretors for both IgM and IgG anti-T immune response. The differences were more pronounced for IgM than IgG anti-T antibodies. In contrast, the donors of Le(a + b −) and Le (a − b −) phenotype showed a high proportion of weak responders and did not differ significantly from the patients with cancer. Similarly, no significant differences between donors and patients were found in the combined Le(b −) groups for both IgM ($\chi^2 = 2.5$; $p = 0.1$) and IgG ($\chi^2 = 0.02$; $p = 0.8$) anti-T antibodies. A decrease of anti-T antibody level was minimal in cancer patients of Lewis negative Le(a − b −) phenotype.

DISCUSSION

The data show that the patients with gastric cancer showed a significant decrease in both IgM and IgG anti-T antibody levels in the serum compared with blood donors. Unlike many tumour markers that are produced by tumour cells and detected in the circulation, a decrease in anti-T IgM antibodies is observed already at very early stages of tumour growth. We found that a natural immune response of healthy blood donors to T-antigen is dependent on Le(a,b) blood group phenotype and is stronger in the subjects of Le(a − b +)/secretor phenotype. Interestingly, the most significant decrease in T-antibody in serum of patients with gastric cancer was also observed among the Le(a − b +) individuals, thus giving the best discrimination between patients and controls. Since the level of natural antibody is a rather stable individual characteristic, it seems that the higher the level of anti-T antibody before neoplasia, the more appreciable the suppression of the response expected after tumour development.

Evidence has been previously presented suggesting the involvement of natural antibody to tumour-associated carbohydrate epitopes in natural resistance mechanisms against cancer (5, 13). Though this hypothesis is still not well established, the successful attempts of active immunotherapy of cancer using simple mucin-type epitopes (T, Tn, sialyl-Tn) as haptens further support this idea. An increase of vaccination-induced serum antibodies against T and sialyl-Tn antigens was associated with a more favourable prognosis (4, 14, 15) suggesting that these blood group-related carbohydrate epitopes may be effective targets for cancer immunotherapy.

It should be noted that the T-epitope is normally expressed on the type 3 chain of gastric glycoconjugates in non-secretors exclusively but it is further fucosylated in secretors (16, 17). Therefore, a lower response to this epitope should be expected in non-secretors. Our data showing that Le(a + b −) donors and patients had a higher proportion of weak responders are in line with these findings. In this respect, it would be interesting to determine whether the prognosis of patients with cancer depends on the Lewis phenotype and/or secretor/non-secretor status.

A lower level of anti-T antibodies in serum of cancer patients could not be explained by an increased expression of T-epitope on cancer cells. If this were the case, a more pronounced decrease of T-antibody level would be observed in patients with advanced cancer. We did not find any such association. Since further fucosylation of T-disaccharide on simple mucin type-3 chain is related to the *Se*/*H* gene encoded for α1–2F-T but not to the *Le* gene encoded for α 1–3/4 fucosyltransferases (18, 19), the differences found might be expected to be associated more with *Se*/*se* status.

In the present study we did not determine the *Se*/*se* status. The main comparisons were made between different *Le* types including the Le(a + b −) and Le(a − b +) individuals, which are known to be *se* and *Se*, respectively, at least in healthy individuals. The main differences for both patients and controls were observed between individuals of Le(a − b +)/secretors and those of Le(a − b −) phenotype. However, the last Lewis negative group is known to be mostly (about 90%) the secretors (20), suggesting that an expression or non-expression of Le antigens, in particular Le(b), might be related to the differences observed.

Blood group-related carbohydrate structures play an important part in host-microbe interplay and in determining susceptibility to infectious agents (21). It could be speculated that a spectrum of the T-antigen positive resident intestinal flora, which is thought to be an antigenic stimulus for natural T-antibody synthesis (22), may differ quantitatively or quanlitatively in individuals of various Le phenotype.

It has been shown that the proportion of Le(b −) individuals, as defined by Lewis phenotype testing on erythrocytes, is significantly raised in patients with gastrointestinal cancers (up to 43–55% for pancreatic carcinoma patients) including those with gastric cancer (28.2%) (23). This must be taken into account when the assay is used as a tool for cancer diagnostics because a

rather high percentage of false negative results with low IgM T-antibody serum level may occur in Le(b −) individuals. Along with the use of fully synthetic T hapten-polyacrylamide conjugate as antigen in ELISA, this approach could be of value for further improving the assay for cancer diagnostics as well as for monitoring the immune responses of cancer patients during active immunotherapy with mucin-type vaccines and for the selection of the patients for such therapy.

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