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An Immunochemical Assay for Natural IgM Antibodies with an Affinity to Galactose and Whose Titer is Reduced in the Sera of Cancer Patients*

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Abstract—*Normal sera contain an IgM fraction which binds to free galactosyl groups of agarose. This fraction causes complement-dependent lysis of Ehrlich ascites tumor cells (EATC). Cancer sera show a much reduced titer of this lytic activity. In this paper we describe an immunochemical assay for these galactophilins (GALF). Agarose columns were immunoabsorbent and bound proteins were detected by labeled antibody to human IgM. All* 157 *normal sera contained GALF. The serum GALF concentrations of normal sera were linearly correlated to the serum cytolytic titers. However, EATC absorbed only* 15% *of normal serum GALF. The titers in normal females are higher than in males. The titers in* 147 *nonmalignant chronic disease patients were lower than the normals only in women over* 40 *years of age. The sera of* 138 *cancer patients, taken before any therapy, were significantly lower than the corresponding normal or chronic disease controls. Lymphoproliferative neoplasms were associated with the lowest titers while melanoma sera showed normal titers. It is likely that GALF is bound to tumor associated antigens with free galactosyl groups. These are on the cell surface and/or are shed into the circulation. GALF titration may prove useful in following treatment efficacy in individual cancer patients.*

INTRODUCTION

We have shown previously that normal human sera contain natural IgM antibodies which lyse Ehrlich ascites tumor cells (EATC) in the presence of complement. Some of these antibodies are directed against antigenic determinants containing D-galactosyl moieties. Thus agarose, a galactose polymer, absorbed about 30% of the cytolytic activity [1–3]. Furthermore we found that this cytolytic activity is significantly lower in the sera of cancer patients [4]. In this paper we describe an immunochemical method for the assay of agarose-absorbed IgM natural antibodies. We used this assay to find the titers in normal human sera, in the sera of cancer patients and in the sera of patients from various nonmalignant diseases. For convenience, we call this group of antibodies galactophilins (GALF).

MATERIALS AND METHODS

Galactophilin titers were assayed in the sera of 157 healthy blood donors, 138 patients with suspected cancer before therapy, and from 147 patients with nonmalignant chronic diseases. Only data from patients with histologically proven malignancy were included in the cancer group. Patients under treatment for cancer were omitted from this study.

Venous blood was withdrawn from donors and left at room temperature for coagulation. We separated the sera by centrifugation and stored them at −20°C.

The assay consisted of two steps. In the first step agarose columns absorbed the galactophilin from the serum. In the second step we added to the columns ^{125}I-labelled anti-human IgM. The labelled antibody bound to the galactophilic IgM

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fraction absorbed on the agarose.

The columns were prepared by autoclaving a suspension of 2% agarose in phosphate-buffered saline, pH 7.4 (PBS) for 30 min at 120°C. We poured the solution into large Petri dishes and allowed it to cool. We cut the resultant gel into small pieces. Then it was homogenized in a Sorvall homogenizer for 50 s at 100 V, with several volumes of PBS. The PBS contained 0.2% sodium azide. The column was a 6 ml polyethylene syringe barrel closed at the bottom with a plastic cap. A porous polyethylene disc, previously treated with the detergent Brij was placed in the bottom of the column. The detergent treatment consisted of placing the disks in a boiling 5% solution of polyoxyethylene–lauryl ether (Brij SP) for 30 min. We filled the columns with enough homogenate to provide 1.5 ml of agarose gel. They were then closed on top with a plastic cap and stored at room temperature.

We labelled rabbit antiserum to human IgM mu chain (Dako) with ^{125}I using iodogen (Pierce Chemical Co.). We dissolved the iodogen in chloroform and added 0.3 ml containing 500 mcg of iodogen to an empty column with a bottom disk. The solvent evaporated overnight in a fume hood, leaving a coating of fine iodogen crystals on the disk and the walls of column. About half a millicurie of iodination grade $Na^{125}I$ (Amersham) together with 500 mcg of antibody in 0.2 ml of PBS were added to the iodogen column and allowed to react for 1.5 min. We stopped the reaction by washing the mixture from the column with 2 ml of PBS. We dialysed the protein solution against PBS to remove free iodide. We then diluted the iodinated protein stock solution with PBS, containing 0.1% Tween 20 (J.B. Baker).

After removing the excess buffer, the columns were washed twice with 6 ml of PBS containing 0.1% Tween 20. For each serum, five aliquots ranging from 0.5 to 5 µl were added to triplicate columns. They were incubated for 1 h at room temperature. The bottom stoppers were removed and the unbound plasma proteins were washed out with two 6 ml volumes of PBS. We then added 0.3 ml of 10% bovine serum albumin in PBS to the columns. This was followed by 10 mcg of labelled anti-IgM in 0.2 ml and the columns were left at room temperature for 24 h. Then, 23 gauge hypodermic needles were fitted to the bottom tips of the columns. This slowed the rate of passage of the washing buffer. We washed the columns with a total of 80 ml of a Tween-containing PBS.

The columns were then counted in a well-type gamma counter (Packard or LKB). The radioactivity remaining on the column was proportional and linear to the volume of serum added to the column. The slope of the curve of radioactivity

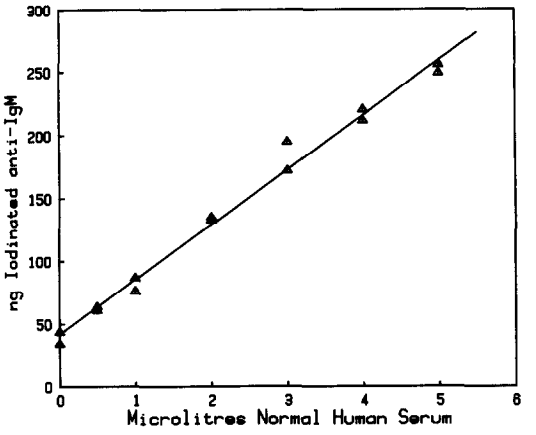

Fig. 1. This graph shows a typical standard curve of the fraction of normal human serum that bound to agarose and was detectable with a ^{125}I-labelled antibody to human IgM. The specific activity of the labelled protein was about 2500 cpm/100 ng. *The load was* 10,000 ng *for each sample. The solid line was calculated by regression analysis* (r = 0.995) *and was highly linear.*

versus serum volume was a measure of the galactophilin titer in the serum. A typical assay curve is shown in Fig. 1. We calculated the slopes using regression analyses. These gave us galactophilin titers expressed as nanograms anti-IgM per ml of serum. In each assay, 10–12 unknown sera were titrated together with a normal control serum pool, consisting of 20 normal blood bank sera. This enabled us to correct for variations in the potency of the anti-IgM reagent. The potency slopes of the unknown sera were normalized to the slope of the control serum and the data expressed as units/ml (A/ml) according to the following formula:

$$\text{titer (U/ml)} = \frac{\text{unknown serum (ng/ml)}}{\text{control serum (ng/ml)}} \times 100.$$

The interassay variance for 70 titrations of the control serum was 17%. The interassay variance of normalized titers calculated from five assays on the same serum, averaged 10%. All calculations and statistical analyses were done on a PDP-11/34 digital computer.

RESULTS

We wanted to see if there was any relationship between the cytolytic [4] and the immunochemical titration of the galactophilic fraction of normal human sera. Each of 16 different sera were titrated by the two assays. By regression analysis there was a significant correlation ($P < 0.01$) between the two titers. In our previous work, we found that pretreatment of serum with Ehrlich ascites tumor cells removed all cytolytic activity. When normal sera were similarly treated and then assayed immunochemically for galactophilin, ascites cells absorbed approx. 15% of the galactophilic activity. Thus the immunochemical assay described in this paper reflects the levels of serum cytolytic galacto-

Table 1. Serum galactophilin titers in normal subjects and patients with non-malignant chronic diseases

Sex	*n*	Units/ml Mean	Range
Females			
Normals	87	95	35–302
Chronic disease	91	76	17–321
Males			
Normals	80	69	9–206
Chronic disease	56	62	7–308

The significant differences by the Mann–Whitney test are as follows: female normals vs. male normals $P < 0.002$; female normals vs. female chronic disease $P < 0.01$.

Table 2. Serum galactophilin titers in females divided into two age groups

	n	Units/ml Mean	Range
Age 40 years or younger			
1. Normals	62	91	35–250
2. Chronic disease	30	101	41–321
3. Cancer	13	67	23–158
Age 41 years or older			
4. Normals	25	106	52–302
5. Chronic disease	60	69	17–174
6. Cancer	70	53	0–132

The significant differences by the Mann–Whitney test are

Group	vs. Group	*P*
3	1	0.03
3	2	<0.02
6	4	<0.001
6	5	<0.005.

philin, but also detects additional serum IgM proteins that bind to agarose.

A total of 167 sera from healthy individuals were assayed for galactophilin. The values were not normally distributed and so groups were compared using the nonparametric statistical Mann–Whitney test. As an additional control group, 147 sera from patients with nonmalignant chronic diseases were assayed. Table 1 shows the data. The titers for the normal female group were significantly higher than those of the normal males. This difference was much smaller when the corresponding chronic disease groups were compared. Since the average age of the normal female controls was significantly younger than the female chronic disease group, we examined the effect of age on the titers. The data are shown in Table 2. The populations were divided in those aged 40 years or less (average 28 years), and those older than 40 years of age (average 57 years). The young chronic disease group did not differ from the normal controls. Apparently chronic nonmalignant disease causes a lowering of galactophilin titers around or after the age of menopause.

Table 3. Serum galactophilin titers in males more than 40 years old

	n	Units/ml Mean	Range
1. Normals	21	66	9–144
2. Chronic disease	60	69	17–144
3. Cancer	55	48	0–153

The significant differences by the Mann–Whitney test are

Group	vs. Group	*P*
3	1	<0.05
3	2	<0.001.

In Table 2 are also shown the titers for the corresponding cancer groups. In both age groups the titers of the cancer patients are significantly lower than either the normal controls or the chronic disease controls. We analyzed the titers of the male groups similarly. The data in Table 3 are only for the older group, since there were too few cancer cases in the younger group. There is no difference between the normal and the chronic disease group. However the titers of the cancer group are significantly less than either of the two other groups. Thus the presence of early cancer causes a depression of circulating galactophilin.

We examined the effect of cancers at specific sites on the GALF titers. In women (Table 4), the lowest titers were found in cases of malignancy of the lymphatic system. Titers were not lowered in cases of cancer of the reproductive tract, except the ovary. The other groups studied showed 40–50% lowering of the titers. In men (Table 5), the presence of cancer of the pancreas and lung and malignant melanoma had no significant lowering effect. The other groups were associated with about a 50% decrease in galactophilin levels.

DISCUSSION

We previously showed [4] that all normal sera tested contain an IgM fraction which lyses Ehrlich ascites tumor cells (EATC) in the presence of complement (C′). Part of this fraction binds to the galactosyl groups of agarose. The lytic activity of this fraction is low in the sera of cancer patients. In this paper we describe the development of an immunochemical assay for these galactophilins (GALF). We used agarose columns as the immunoabsorbent and detected the bound proteins with an ^{125}I-labeled antibody to human IgM. The test quantifies the serum level of IgM antibodies that bind to free galactosyl groups. All normal sera tested contained GALF. The GALF concentrations in a test group of normal sera were linearly correlated to the cytolytic titers assayed with EATC and C′. However, while exposure to EATC removed all cytolytic activity

Table 4. Serum galactophilin titers in female patients according to cancer site

	n	Mean	Percentage	*P* vs. normal
Normal	87	95	100	
Carcinoma of				
Colon and rectum	15	61	64	<0.001
Other GI*	8	49	52	<0.01
Ovary	17	63	66	<0.001
Reproductive tract†	19	96	101	ns
Breast	31	59	62	<0.001
Lymphoma	8	28	29	<0.001
Melanoma and cancer of unknown origin	8	58	62	ns

*Cancer of esophagus, stomach, jejunum, hepatoma and pancreas.
†Cancer of cervix, vulva and uterus.
ns = not significant.

Table 5. Serum galactophilin titers in male patients according to cancer site

	n	Mean	Percentage	*P* vs. normal
Normal	81	69	100	
Carcinoma of				
Stomach	13	39	57	<0.01
Colon and rectum	13	47	68	<0.05
Pancreas	5	44	64	ns
Other GI*	5	40	58	<0.05
Lung	6	57	83	ns
Cancer of urinary bladder	9	46	67	<0.05
Melanoma	3	93	135	ns

*Cancer of esophagus, hepatoma and jejunum.
ns = not significant.

from serum, only 15% of the serum GALF is removed by this treatment. Thus 85% of GALF does not bind to EATC and therefore would not contribute to the cytolytic titer. This could explain the differences found between the two assays, which we discuss in the following paragraph.

The mean GALF value for normal women is significantly higher by about 40% than the mean male value. The mean cytolytic titers were the same in both sexes. This sexual dimorphism of GALF is consistent for that found for the whole immune system, including the observations that the IgM antibody class occurs in higher concentration in females [5]. In addition, serum levels of some natural antibodies are higher in women [6]. There is another discrepancy between the two assays, when we compare the two sets of data for the nonmalignant chronic disease group. The mean GALF titer for the female group is significantly lower than the mean normal titer by about 20%. No such differences were seen with the cytolytic assay. Hospitalized patients with chronic diseases, which were very similar to our group, showed a reduced natural antibody level when compared with healthy controls [6]. The mechanism is not known. It would appear to be a reduced rate of antibody production.

The GALF titers of the cancer groups were significantly lower than the corresponding normal or chronic disease groups. We can postulate two possible mechanisms. The first could be that the presence of cancer in the body lowers the production of natural antibodies. This is not likely since natural humoral immunity is normal in cancer patients [7]. The second possibility is that GALF is bound by galactosyl groups on the tumor cell membrane and/or circulates as immune complexes with galactose containing antigens, shed from cells into the circulation. The findings of Sakamoto *et al.* [8] provide strong support for this mechanism. They found that a wide variety of human tumor cell lines express Lewis blood group antigens which have free galactosyl groups. The frequency of this expression was

100% for the lines from lymphoproliferative neoplasms and zero for melanoma lines. This parallels our findings that GALF values were most depressed in lymphatic malignancy and were not significantly lowered in melanoma (Tables 4 and 5). These authors also found that over 90% of biopsies for colon cancer showed this surface antigenicity. This indicates that tumors *in situ* express Lewis antigenicity. Tumor cells shed these antigens, since they were found in the pleural and ascitic fluids of patients with various types of cancer [9]. It is likely that the GALF titer in a cancer patient reflects the net effect of three factors. These are (a) the degree of expression of galactosyl antigens on the tumor cells, (b) the mass of the tumor and (c) the degree of antigen shedding into the circulation. In a preliminary study of cancer patients under treatment, the GALF levels seem to reflect the degree of control of the disease process in each individual case.

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