Eur J Cancer Clin Oncol, Vol. 22, No. 1, pp. 13–19, 1986.
Printed in Great Britain.

0277-5379/86$3.00+0.00

Complement-dependent Lysis of Erhlich Ascites Tumor Cells by Human Serum (Ascitolysin) is Lowered in Cancer Patients and Raised in Pregnant Women*

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Abstract—*Normal human serum has been shown to contain IgM antibodies that are lytic to Ehrlich ascites tumor cells in the presence of complement. We call this activity ascitolysin (AL). Using a* ^{51}Cr *release assay, the AL titers of* 164 *normal sera,* 211 *sera from patients with non-malignant disease,* 62 *sera from* 25 *pregnant women and* 170 *sera from cancer patients were estimated. The patient sera were taken preoperatively, and were assigned to the cancer or non-cancer group after histological diagnosis. In normals, higher titers were found in sera of A and O blood groups but not B and AB. The difference was due to a fraction absorbed by B red blood cells. Sera from untreated cancer patients showed significantly reduced titers when compared to normal, benign tumor, chronic and other non-malignant disease groups. In pregnancy, titers were positively correlated with duration and remained elevated for at least* 6 *weeks post partum. About* 30% *of AL activity was absorbed by the free galactose groups of agarose. This fraction was significantly lower in untreated cancer patients. Anti-T antigen antibodies do not contribute to AL levels. Galactophilic blocking factors were also found; eight times more frequently in cancer sera than in normals. The non-absorbed fraction was also lower in cancer sera. The data suggest that AL antibodies may be directed against human oncofetal antigens.*

INTRODUCTION

NATURAL IgM antibodies are detectable in the sera of humans and animals some months after birth. In previous work [1–3] we have shown that normal human serum is cytolytic to Ehrlich ascites tumor cells (EATC) in the presence of complement. This activity was in the IgM fraction and was absorbed from serum by EATC. We call this activity ascitolysin (AL).

The aim of this study was to see if in patients with early untreated cancer the circulating titers of AL were changed. A sensitive assay for this group of antibodies was developed. Serum AL titers were assayed in normals and in patients with malignant and non-malignant diseases.

MATERIALS AND METHODS

Sera were prepared from the blood of fasting individuals and stored at −20°C. Four groups of sera were examined: (a) from healthy donors (n = 164); (b) from 25 normal pregnant women (n = 62); (c) from hospitalized patients with non-malignant diseases (Table 4); and (d) sera obtained 24 hr preoperatively from patients admitted for the diagnosis of possible cancer. The sera from the last group were assigned to the cancer group only after a histologically confirmed diagnosis (n = 170).

Assay methods

Ascitolysin activity was measured by a two-step assay. AL was first absorbed from human sera by 6- to 8-day-old Ehrlich ascites tumor cells (EATC), previously labeled with radioactive chromium (^{51}Cr) [4]. Complement (C′), contained in normal human umbilical cord serum (NCS), was then added to cause lysis. The buffer used was phosphate-buffered saline (PBS, pH 7.4), free of Ca^{2+} and Mg^{2+} [5]. The divalent cations were added to the buffer only for the activation of complement. To remove non-specific toxicity to EATC, pooled cord sera were absorbed either with agar (125 mg/ml serum) [6] or with EATC (50 × 10^6 cells/ml serum). When control cell mortality due to C′ alone exceeded 30%, resistant cells were

Accepted 1 July 1985.
*This work was supported in its initial stage by an Israeli Government grant. The later work was supported generously by anonymous friends.
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selected by exposing the whole cell population to the complement of NCS.

In each assay a control pooled standard sera and 8–10 unknown sera were titrated. To measure the non-specific lysis due to C′, two serum-free control tubes were used. Ascitolysin from human sera was absorbed on EATC by adding increasing amounts of human sera to tubes containing 5×10^6 cells. The final concentrations of serum were 0.15, 0.25, 0.45, 0.7, 1.0 and 1.5%. To measure maximum cell lysis, tubes containing 2.5–5.0% serum were included. To inhibit the endogenous C′ activity of the sera, D-glucosamine HCl was added in a final concentration of 0.075 M [7]. The total volume was adjusted to 1 ml with buffer. The tubes were incubated for 30 min at 4°C, centrifuged at 1000 ***g*** for 1 min, washed twice with PBS and resuspended in 0.5 ml PBS containing Ca^{2+} and Mg^{2+}, using a Vortex mixer. Two hundred microliters of C′ (NCS) were added to the cells and the volume adjusted to 1 ml. The tubes were then incubated for 30 min in a shaking water bath at 37°C. The reaction was stopped by putting the tubes into ice water for 5 min. They were then centrifuged for 1 min at 1000 ***g***. A 0.5 ml aliquot was taken from the supernatant of each tube. Both sediments and aliquots were counted in a gamma counter to a total count of at least 2000.

Calculation of ascitolysin titers

The total ^{51}Cr in each tube was determined by adding the counts for the supernatant aliquot to the cpm for the cell residue and remaining supernatant. The ^{51}Cr released was obtained by multiplying the cpm of the supernatant aliquot by 2. This was expressed as the percentage of total radioactive chromium less the percentage in the tubes with non-specific cell death. The percentages were plotted as probits against the natural logarithm of the serum concentration. Straight lines were obtained which were computed by a least-squares linear regression program. The natural logarithm of the serum concentration causing 50% release was determined from the equation of the regression line. Latterly these data were obtained using the four-parameter model of Rodbard and Hutt [8]. The antilog of this value gave the amount of serum equivalent to 1 LD_{50} unit. The reciprocal of this value gave the titer in terms of LD_{50} units/ml. The unknown titers were expressed as a percentage of the normal serum control.

Absorption of sera by lyophilized RBC ghosts

RBCs of groups A and B were washed by centrifugation at 850 ***g***, once with normal saline and then three times with distilled water. The ghosts were resuspended in 20 ml distilled water and stored at −20°C overnight. After thawing, the ghosts were centrifuged and washed again with distilled water. The sediment was transferred with a minimum amount of water to a small flask and lyophilized. Sera were absorbed with stroma in a ratio of 1:1 at 4°C for 30 min. The ghosts were removed by centrifugation and the sera were titrated for ascitolysin.

Absorption of sera by Sepharose

Sepharose 4–B (Pharmacia) was washed with PBS to pH 7.4 and diluted 1:1. Barrels from 5 ml syringes were used as columns after the insertion of a Brij-treated porous polyethylene disc. The columns were filled with Sepharose to a height of 2 cm and the upper gel surface was covered with a second porous disc. Serum in a volume of 0.3 ml was added to the column and left at 4°C from 45 mins. PBS in a volume of 2.7 ml was added and allowed to penetrate the Sepharose. The eluate from the column was collected in graduated tubes. Non-absorbed sera were diluted similarly with PBS and kept under the same experimental conditions as controls. Absorbed and non-absorbed sera were assayed for ascitolysin titers.

RESULTS

The cytolytic activity against Ehrlich ascites tumor cells was detected in all normal sera assayed. The titers were log-normally distributed. The data were therefore calculated as the means and deviations of the natural logarithms of the titers.

The influence of sex, age and blood group on normal ascitolysin titers was examined. There was no difference between the mean serum titers of females and males. Previously higher titers were found in the sera from A and O blood groups than those from the B and AB groups [1]. This finding was confirmed in this study (Table 1). In A and O sera there was a significant negative correlation between log titer and age (ln (titer) $5.964 - 0.01813 \times$ yr, $n = 113$, $P < 0.01$). Thus the differences between the blood groups disappeared after age 40 yr (Table 1). This difference was also abolished by absorbing A and O normal sera with cell blood group B stroma (Table 2). This anti-beta hemolysin activity decreases with age, following the pattern described by Grundbacher [9].

Serum samples from patients suffering from various pathological conditions were titrated. The results in the B and AB blood group samples (Table 3) show a marked significant reduction in serum titers from untreated cancer patients. The titers of the non-malignant disease group are not significantly lower than those of the normal control. The data of the A and O blood group samples are given in Table 4. The cancer sera again show markedly reduced titers when compared to the control or non-malignant disease groups ($P < 0.001$).

Table 1. The effect of blood group and age on ascitolysin titers in sera from normal individuals

Blood group	Mean ln (titers) ± S.E.M. Donors <40 yr old	Donors >40 yr old
A & O	5.51±0.11* (n = 61)	5.02±0.14** (n = 54)
LD_{50} U/ml	247	151
B & AB	5.00±0.19 (n = 19)	4.85±0.22 (n = 29)
LD_{50} U/ml	148	128

The numbers in parentheses are the number of tests in each group.
*A & O vs B & AB significantly different in the < 40 yr group ($P < 0.01$).
**The difference between the two A & O age groups is significant ($P < 0.01$).

Table 2. Absorption of 32 A or O normal sera with B red cell stroma on ascitolysin titers

Group	Mean ln (titers) ± S.E.M.	Mean titer LD_{50} U/ml
Control	5.54 ± 0.16	255
Absorbed	5.17 ± 0.17*	176

*Using the paired t-test, t = 6.7 for d.f. 31, i.e. $P \ll 0.001$.

Table 3. Ascitolysin titers in sera from patients with cancer non-malignant disease and normal controls with blood groups B or AB

Sera from:	Mean ln (titers) ± S.E.M.	Mean titer LD_{50} U/ml
Normal Controls	4.91 ± 0.15 (48)	136
Non-malignant disease	4.56 ± 0.33 (50)	96
Cancer	3.17 ± 0.29 (53)	24

The numbers in parentheses represent the number of sera tested.
Cancer vs normal, $P < 0.001$.
Cancer vs non-malignant, $P < 0.001$.

Table 4. Comparison of titers in sera from patients with cancer and non-malignant disease with the expected value from age vs titer regression line for normal controls with blood group A or O*

Serum group	Mean age (yr)	Mean ± S.E.M. ln (titers)	Significance (paired t)
Normals	40	5.29 ± 0.09 (111)	—
Non-malignant	44	4.80 ± 0.13 (156)	N.S.
Chronic diseases	55	4.43 ± 0.30 (51)	$P < 0.05$
Benign tumor	37	5.01 ± 0.17 (49)	N.S.
Mastopathy	41	5.03 ± 0.17 (29)	N.S.
Male sterility	31	5.32 ± 0.16 (16)	N.S.
Surgical problems	53	4.14 ± 0.65 (11)	N.S.
Cancer	59	3.78 ± 0.19 (115)	$P < 0.01$
Intestinal tract	63	3.74 ± 0.26 (66)	$P < 0.001$
Breast	55	3.97 ± 0.40 (24)	$P < 0.02$
Various other	57	3.72 ± 0.37 (25)	$P < 0.05$

N.S. not significant.
*The normal population with A or O blood groups showed a negative correlation of ln (titer) with age ($0.02 > P > 0.01$). The equation for the regression line was ln (titer) = 5.96–0.01813 × yr. The expected normal titer was estimated by solving the equation with the age of the individual in the groups shown above. The expected titer was compared with the found titer using the paired t test to determine significance.

The differences in the mean ages of the groups could introduce an artifactual bias (Table 4). This was tested in the following analysis of A and O blood groups data. Using the regression equation for age vs ln (titer) given above, the expected normal titer for donor age was determined for the non-malignant and cancer groups. The expected and found titers were compared by a paired *t* test (Table 4). The cancer group data as a whole and for each of the three subgroups were significantly different from the titers expected for the age of the donors. The data for the total non-malignant group do not differ significantly from the expected values. However the sera from the chronic disease subgroup had significantly lower titers than the normals. In subsequent analyses the chronic disease group was examined separately. The non-malignant group now includes only the sera from patients with benign tumors, mastopathy, sterility or surgical problems.

To determine if the antibody is directed to an embryonic antigen, the AL activity was assayed in serum throughout human pregnancy (Fig. 1). It increases during the course of pregnancy ($P < 0.005$) and remains high for at least 6 weeks *post partum*. Thus the placenta and/or the embryo stimulate AL production, an effect opposite to that caused by cancer.

The possibility that AL included 'natural' cytotoxins to the T- antigen [10] was tested. Group O RBCs were treated with neuraminidase as described by Banai *et al.* [11] to reveal the T antigen groups on the cell surface. Normal sera were absorbed with the treated RBCs. In five sera tested there was no significant change in ascitolysin levels after absorption. Antibodies to T antigen are not a significant component of AL.

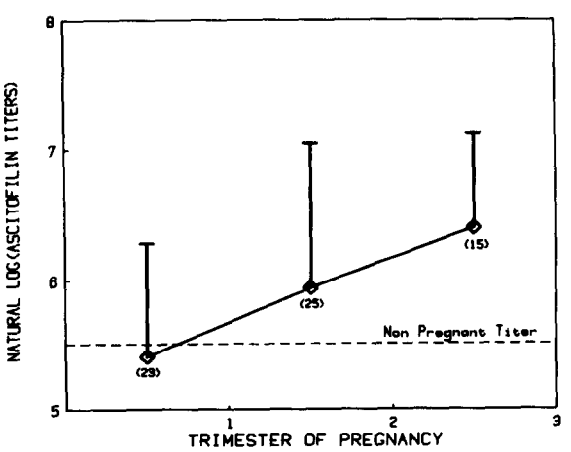

Fig. 1. The curve shown is the regression line for ln (AL titer) vs months of pregnancy. The correlation was linear and significant at $P < 0.005$. *The diamond symbols represent the means of pooled data for each trimester plus one standard deviation. The 6 weeks post partum group showed a mean titer of* 6.31 ± 0.78 *for eight samples. This mean was not significantly different from the value for the third trimester* (6.4 ± 0.73) *but was significantly greater than the first trimester titer* ($P < 0.02$).

Previously, the AL activity was shown to be partially inhibited by D-galactose [3]. Thus antigenic sites for AL on EATC have free galactosyl groups. Sera were assayed before and after absorption by the galactosyl groups of Sepharose. The difference between the two titers was taken as the galactophilic fraction titer, and constituted about 30% of normal AL activity. This fraction, in the normal population, was not influenced by blood group or age. The data did not follow a normal distribution, therefore non-parametric methods were used for statistical analyses. The titer of the galactophilic fraction is lowered very significantly in cancer patients (Table 5). There were no significant differences between the non-cancer groups. The unbound AL titers in cancer sera were also significantly lower than in the control or chronic disease groups. There was no significant difference between the cancer and the non-malignant disease groups.

Some sera showed a higher cytolytic titer after absorption than before. This suggested that absorbed serum factors were blocking the cytolytic reaction. The numbers of such sera in the three experimental groups are shown in Table 6. While the two non-cancer groups showed an incidence of blocking antibodies of about 5%, about 40% of cancer sera tested showed this phenomenon.

DISCUSSION

In this paper we show that all normal human sera tested contain a complement-dependent cytolytic activity to Ehrlich ascites tumor cells, which we have called ascitolysin (AL). These IgM antibodies can be considered to be part of the large group of 'natural antibodies' found in normal human serum [12–18]. Many of these are absorbed by or are lytic to tumor cells.

Sera from untreated cancer patients showed significantly lower AL titers than sera from healthy individuals or patients with non-malignant diseases or benign tumors. On the other hand, the sera of pregnant women showed increasing titers with the duration of pregnancy. In a similar study [19] the effect of human sera on the growth of EATC cultures was tested. These authors found increased toxicity in pregnancy sera, analogous to our results. However, they found that the sera from cancer patients were more toxic than healthy controls, contrary to our results with AL. It is reasonable to hypothesize that AL or some of its constituent molecules are natural antibodies to human oncofetal/differentiation antigens.

We now turn to a discussion of the rise of serum ascitolysin during the course of human pregnancy. The stimulus for this rise could be the entry into

Table 5. *Titers of the fractions of ascitolysin separated on Sepharose from sera of normals and patients with non-malignant disease or cancer*

Sera from:	Mean LD_{50} titers (range) Sepharose-bound fraction	Unbound fraction	No. of sera
Normal controls	79** (0–403)	248* (10–1057)	70
Non-malignant disease†	86** (0–717)	236 (13–849)	25
Chronic disease	96** (0–517)	307* (10–1086)	25
Cancer	19 (0–135)	185 (0–719)	40

Statistical significance was tested by using the Mann–Whitney *u* test; cancer vs the other groups: ** $P < 0.001$; * $P < 0.05$.
†This group includes sera from patients with benign tumors, mastopathy, and sterility or surgical problems.

Table 6. *The number of sera showing 'blocking factors' to ascitolysin absorbed on Sepharose in the normal, non-malignant disease and cancer groups*

Sera from:	No. of sera	%
Normal controls	4/70	5.7
Non-malignant disease	2/50	4.0
Cancer	16/39*	41.0

A serum was considered to contain blocking factors when the cytolytic titer of the effluent from the Sepharose column was significantly greater than the titer of the unabsorbed serum (see Materials and Methods).
*Cancer vs the two other groups: $P < 0.01$ (χ^2 test).

the maternal circulation of either fetal cells or fetoplacental products as antigens. In fact fetal cells have been found in maternal plasma as early as at 15 weeks of gestation [20]. Equally, there are pregnancy-specific glycoproteins that are secreted into the mother's blood. These proteins have also been demonstrated in the blood and tumors of patients with non-trophoblastic cancers [21]. The fact that female sex hormones, which are high in the blood in pregnancy, also enhance division and maturation of maternal immunocompetent cells [22] may also contribute to the rise in AL activity. This is supported by the finding that IgM-bearing lymphocytes are increased in the blood during pregnancy [23] and that serum IgM may be raised in some cases [24]. In animals, maternal antibodies are produced in response to embryonic antigens expressed *in utero* [25]. One such antigen binds to *Ricinus communis* lectin [26], which is a lectin most avidly bound to EATC (see discussion below). There is a maintained increased production of AL in the *post partum* period when pregnancy-associated hormones and proteins have returned to virtually normal levels. This was shown by the fact that at 6 weeks *post partum* the blood AL levels were not lower than the third trimester mean, despite the 5-day half-life of IgM in the circulation.

Ascitolysin activity could be divided into at least two separate fractions. This was based on differing affinities of the antibodies to the free D-galactosyl groups of Sepharose. The galactophilic fraction of AL constituted about 30% of the total AL activity. This fraction was lowered or absent in the sera of cancer patients but was not affected by the presence of chronic disease or non-malignant tumors. This fraction also showed the presence of blocking factors to AL activity. The frequency of occurrence of these blockers was some four-fold greater in cancer sera than in the control sera. The presence of cancer affects the titers of galactophilic antibodies in human serum to the Thomsen–Freidenreich (T) antigen [10]. However, no AL activity was absorbed by neuraminidase-treated RBCs, and so there is no detectable anti-T antibodies in AL. The non-galactophilic fraction was also depressed in cancer patients when compared to the normal and chronic disease groups. However, the

data from the non-malignant group, which included sera from patients with benign tumors, mastopathy, sterility and surgical problems, was not significantly different from the cancer group. This may be due to the selection of cancer sera with reasonably measureable AL titers for the absorption assays.

There are data on the surface antigens of EATC that may be involved in binding ascitolysin antibodies. Thus Eckhardt *et al.* [27] showed that a B blood group-specific lectin is bound to EATC. This would explain the anti-B activity to EATC found in sera of A and O blood groups. The EATC membrane has free terminal D-galactosyl groups [28–30]. The binding of *Ricinus communis* to EATC membrane is inhibited by lactose. This indicates the presence of D-Gal (–)D-glucosyl groups, which are probably responsible for binding the galactophilic fraction. This grouping attached to ceramide has been found in the glycolipid fraction of human rectum adenocarcinoma cells [31]. *Ricinus* also binds to Gal–GlcNac groups [32], indicating the possibility that the human embryonic antigens described by Feizi [33] may also be present on the surface membrane of EATC. This might account for AL activity in sera from pregnant women. This further supports the hypothesis that AL may contain natural antibodies against oncofetal/differentiation antigens. The membrane antigen responsible for the non-galactophilic fraction of AL activity is as yet undetermined.

The reduction of serum AL titers in cancer patients may be due to several mechanisms. There is increasing evidence of the presence of common oncofetal antigens on both animal and human tumour cells [34]. Some of these antigenic groups are present on EATC. An increasing tumor mass could bind AL. An IgM component of normal human serum has been shown histochemically to bind to sections of breast cancer, and in some cases endogenous IgM was found on the cells [16]. Reduced titers of AL in cancer sera could be due to the formation of immune complexes. This mechanism is operative in melanoma patients to reduce the levels of autologous antibody to melanoma and a number of other cancer and normal cells [35].

The blocking of cytolysis, found more frequently in cancer sera, may be due to blocking factors that bind to Sepharose and EATC. Another mechanism could involve the anti-immunoglobulins to cytotoxic antibodies to fetal and tumor cells described by Morgan *et al.* [36]. These anti-immunoglobulins blocked the ability of natural anitbodies to react with and lyse their target cells.

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