Isolation of a glycoprotein responsible for the enhanced concanavalin A agglutinability of erythrocytes in Yoshida-ascites-sarcoma-bearing rats: the mechanism of paraneoplastic syndromes

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As a model for the development of paraneoplastic syndromes, we have studied the mechanism by which erythrocytes in the circulation of rats bearing intraperitoneal Yoshida ascites sarcoma acquire higher agglutinability with concanavalin A (Con A). The *in vitro* incubation of erythrocytes from normal animals with the cell-free ascites fluid or the plasma of tumour-bearing animals is able to confer an enhanced agglutinability on the cells. Fractionation of the ascites fluid has yielded three subfractions that are active *in vitro*. Two of these, occurring in small amounts, are a particulate fraction rich in plasma-membrane markers and a soluble fraction containing protein of molecular mass equal to or less than 50 kDa. These two are, however, unable to affect the agglutinability of erythrocytes *in vivo*, i.e. when injected intraperitoneally into normal rats. The third, and major, fraction consists of proteins of molecular mass equal to or greater than

680 kDa, and is able to modify the erythrocyte agglutinability *in vivo*. From this fraction, by using a combination of Con A affinity chromatography, gel filtration, $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-Sephadex chromatography, an active protein has been purified to apparent homogeneity. It yields a subunit of 310 kDa in the presence of SDS and further breaks down into a polypeptide of 170 kDa when reduced with 2-mercaptoethanol. It has a pI of 5.35. The protein is rich in Glx, and appears to contain hybrid-type *N*-linked oligosaccharides. The protein is also present in the blood plasma of tumour-bearing, but not normal, rats. The radioiodinated protein binds to the erythrocyte surface adding about 7400 molecules/cell. The study unequivocally demonstrates that a protein from the tumour fluid can appear in the circulation, interact with host cells that are not in contact with the tumour and modify their properties.

INTRODUCTION

The effects of the tumour on distantly located host cells are known as paraneoplastic syndromes or tumour–host relations [1,2]. Some of these effects are responsible for consequences such as cachexia, anorexia, hypercalcaemia and anaemia which are associated with considerable morbidity or even mortality [1]. Experiments [3,4] with parabiotic pairs of animals have suggested that circulatory tumour products may be responsible for these effects. We have earlier reported [5] that in rats bearing Yoshida ascites sarcoma the erythrocytes in the circulation acquire a surface modification that shows up in their higher agglutinability with concanavalin A (Con A). It was found that (i) the higher Con A agglutinability of erythrocytes arises 2 days after tumour transplantation; (ii) the cell-free ascites fluid and the plasma of tumour-bearing animals (2 days after transplantation, but not earlier) are able to confer higher Con A agglutinability on normal erythrocytes after *in vitro* incubation; (iii) the putative activity-conferring substance(s) can diffuse from the ascites fluid into the circulation; and (iv) the modification of the erythrocyte surface is not the result of a host reaction or the action of proteinases. Since the tumour and the erythrocytes reside in separate anatomical compartments, the system offers an excellent model for the study of the mechanism of paraneoplastic syndromes. It has three additional advantages: (i) being an ascites tumour, the tumour products appear first in the ascites fluid offering a convenient source of purification of the active substance(s); (ii) agglutinability of erythrocytes is a rapidly assessable property; (iii) there is already considerable information available

on the lectin-mediated agglutination [6], especially the Con A-mediated agglutination, of erythrocytes [7–9]. The latter information can help in evaluating the mechanism by which the tumour products can enhance the agglutinability of erythrocytes.

Here we report the purification and partial characterization of a protein from the Yoshida ascites fluid that is responsible for the modification of the erythrocyte surface.

MATERIALS AND METHODS

Materials

All fine chemicals, except Con A, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Con A, the gel-filtration and ion-exchange media and ampholines were obtained from Pharmacia LKB, Uppsala, Sweden. Na^{125}I was from The Radiochemical Centre, Amersham, Bucks., U.K. Dibutyl phthalate was obtained from E. Merck, Darmstadt, Germany. The laboratory reagents were purchased locally.

Con A–Sephrose was prepared as described by March *et al.* [10]. About 10 mg/ml Con A was bound per ml of the gel.

Tumour cells, ascites fluid and erythrocytes

Yoshida ascites tumour was propagated intraperitoneally in Wistar rats. The tumour fluid was collected over 1 mg/ml EDTA, and 0.1 mM phenylmethanesulphonyl fluoride (PMSF) and 0.1 mM *N*-ethylmaleimide were added. Thereafter, the temperature was maintained at 4 °C or below unless otherwise noted. After 30 min, the tumour cells were isolated at 100 *g* as previously

Abbreviations used: Con A, concanavalin A; DAB, 3,3'-diaminobenzidine; MeMan, methyl α -D-mannopyranoside; PMSF, phenylmethanesulphonyl fluoride; TBS, Tris-buffered saline (0.01 M Tris/HCl, pH 7.4, containing 0.15 M NaCl).

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Scheme 1 Scheme for purification of the protein responsible for the modification of erythrocyte agglutinability

The yield of each protein fraction, in terms of percentage of the starting protein, is given in parentheses. The values are averages of three preparations. Because of averaging and losses occurring during fractionation, the yields do not add up to the value of the parent fraction. The protein fractions showing *in vitro* erythrocyte modification activity are indicated by asterisks.

described [5]. The fluid was then centrifuged at 1000 *g* for 5 min. The supernatant was re-centrifuged, and is referred to as cell-free ascites fluid. This was further centrifuged at 100000 *g* for 1 h. The pellet was washed once by suspension in the original volume of Tris-buffered saline (TBS) and centrifugation. The supernatant was re-centrifuged and carefully removed discarding about 1 ml from the bottom. This is called the 'soluble fraction'.

Rat blood was obtained by cardiac puncture after anaesthesia and anticoagulated with EDTA. It was centrifuged in the cold at 1000 *g* for 5 min. Plasma and buffy coat were removed, and erythrocytes were washed four times in 10 vol. of TBS in the cold.

Protein purification

The purification is described in Scheme 1. All operations were carried out at 25 °C unless indicated otherwise.

Figure 1 Enhancement of Con A agglutinability of erythrocytes by ascites fluid fractions

Washed erythrocytes from a normal rat were incubated in the particulate fraction (0.04 mg/ml) (■), the soluble fraction (50 mg/ml) (●) and TBS (---). After the erythrocytes had been washed, the Con A agglutinability of the cells was determined as described in the Materials and methods section.

The soluble ascites fluid fraction containing 2 g of protein in 40 ml was passed through a Con A-Sepharose column (1.2 cm × 40 cm), and the bound protein was eluted with 0.04 M glucose in TBS, followed by 0.2 M methyl α -D-mannopyranoside (MeMan) in TBS. The latter was concentrated by vacuum dialysis in the cold, and fractionated on a column (2.5 cm × 132 cm) of Sephadex G-200. The eluate was monitored at A_{280} . Of the five peaks, the protein eluted in the void volume (peak I) and peak V were found to be active. Peak I was concentrated and applied to a Sepharose 4B column (2.15 cm × 95 cm). The protein in the second peak was fractionated with $(\text{NH}_4)_2\text{SO}_4$ in cuts of 0–40% and 40–90% saturation. The former, after dialysis in the cold in 0.01 M Tris/HCl, pH 8.0, and 0.1 M NaCl, was applied to a DEAE-Sephadex column (1 cm × 10 cm) in chloride form and eluted with 0.1–0.4 M NaCl in Tris/HCl, pH 8.0.

Activity determination

The activity of protein fractions was tested by incubation of washed erythrocytes in 10 vol. of the fraction (500 μg of protein/ml, except when otherwise indicated) for 2 h at 37 °C. The cells were washed four times in TBS in the cold after the incubation, and used for assessment of their agglutinability at various concentrations of Con A [7]. The control consisted of erythrocytes incubated and washed in TBS.

The *in vivo* activity of fractions was determined by intra-peritoneal injection in normal rats. The soluble fractions were passed through nitrocellulose filters of 0.45 μm porosity before injection. For the pellet, the entire procedure of isolation was carried out aseptically. In earlier experiments, intraperitoneal

Figure 2 Fractionation of the Con A-binding protein on Sephadex G-200

(a) The MeMan eluate of the Con A-Sepharose-bound protein was separated on a column of Sephadex G-200 as described in the Materials and methods section. Five fractions were obtained as shown. (b) The Con A agglutinability of erythrocytes treated with each of the five fractions *in vitro*. —, TBS; ●, I; ◻, II; ▼, III; ◆, IV; ▲, V. (c) SDS/PAGE of (A) ascites fluid soluble fraction, (B) Con A-non-binding protein, (C) Con A-bound protein, (D–H) peaks I to V respectively; 70 μg of protein was applied in lanes (A)–(C), and 30 μg each in lanes (D)–(H) on a 6–12% gradient gel under reducing conditions. The standard proteins were: 240 kDa, erythrocyte spectrin 1; 220 kDa, spectrin 2; 116 kDa, β -galactosidase; 97 kDa, phosphorylase b; 80 kDa, erythrocyte band 4.1 protein; 68 kDa, BSA; 50 kDa, immunoglobulin heavy chain.

injection of the soluble ascites fluid was found to modify the erythrocyte agglutinability in 24 h [5]. Therefore the agglutinability of erythrocytes was tested 24 h after the injections. The effect of the pellet was also examined 48 h after the injection.

Electrophoresis

PAGE was carried out as described by Laemmli [11], in the presence or absence of SDS and mercaptoethanol. Homogeneous or gradient gels were used as described in the Figure legends. Limit-pore electrophoresis was performed using a 4–18% gradient gel for 24 h at 100 V at 25 °C. The standard proteins used

were thyroglobulin (669 kDa), apoferritin (443 kDa) and urease (hexamer, 545 kDa; trimer 272 kDa). The gels were stained with 0.2% Coomassie Brilliant Blue or silver [12].

For two-dimensional electrophoresis [13], isoelectrofocusing was performed in a 4% acrylamide tube gel using ampholines in the range pH 3.5–10. SDS/PAGE was carried out in the second dimension with a 6–12% gradient slab gel.

Immunization, Immunohistochemistry and Immunoblotting

About 100 μg of protein in complete Freund's adjuvant was injected intradermally into a rabbit at several sites, followed 2

weeks later by 50 μ g of protein in incomplete adjuvant. Blood was collected a week later and the immunoglobulin fraction was prepared [14] and reconstituted in the original volume of the antiserum. The antiserum titre was 1:64 by immunodiffusion, and it gave a strong staining of 1.0 μ g of 170 kDa-subunit protein at a dilution of 1:10000 by immunoblotting (see below). The preimmune serum did not react with the protein. The rabbit was intermittently boosted by injection of 50 μ g of protein in incomplete Freund's adjuvant.

The presence of the 170 kDa-subunit protein was probed in methanol/acetone-treated smears of 10^6 washed Yoshida sarcoma cells using the undiluted and variously diluted antiserum, peroxidase/antiperoxidase, and 3,3'-diaminobenzidine (DAB) as the substrate. The preimmune serum was used in parallel. For detection of the protein by immunoblotting, approximately 2×10^8 cells were lysed in 1 ml of a solution containing 1% Triton X-100, 0.25 M NaCl, 25 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1 mM PMSF, 10 μ g/ml each aprotinin, leupeptin and pepstatin A in the cold. After centrifugation, the supernatant was used for protein estimation and immunoblotting.

The proteins from polyacrylamide gel were electrophoretically transferred to nitrocellulose [15]. The blot was blocked with 1.75% gelatin and washed with 0.5 M NaCl in 20 mM Tris/HCl, pH 7.5, containing 0.05% Tween 20. It was then sequentially treated for 1 h with the anti-(170 kDa protein) antibody, anti-rabbit antibodies raised in goat and horseradish peroxidase-conjugated antiperoxidase (in rabbit), with washing after each treatment. Finally, the colour was developed by addition of the substrate DAB. All operations were carried out at room temperature.

Amino acid and carbohydrate compositions

The purified protein was hydrolysed in 6 M HCl at 100 °C for 24, 48 and 72 h. The amino acid composition was determined using an LKB amino acid analyser. The concentrations of serine, threonine and tyrosine were obtained by extrapolation to time zero, and those of valine, isoleucine and leucine were calculated from the 72 h sample. Cysteine and methionine were oxidized by treatment of the protein with performic acid before hydrolysis, and were estimated as cysteic acid and methionine sulphone respectively. Tryptophan was not determined.

For N-terminal amino acid analysis, manual Edman degradation and t.l.c. were employed [16].

For amino sugar analysis, the protein was hydrolysed in 6 M HCl for 6 h at 100 °C. *N*-Acetylglucosamine and *N*-acetylgalactosamine were quantified on the amino acid analyser according to the program suggested in the instrument manual. The neutral sugars were liberated from the protein in 4 M trifluoroacetic acid at 100 °C for 4 h. The sugars were converted into alditol acetates [17] and analysed by gas chromatography. Sialic acid was hydrolysed at 80 °C in 0.05 M H_2SO_4 for 1 h, and quantified by the thiobarbituric acid method [18].

Radiolodination of the protein and binding to cells

The purified protein was radioiodinated using Iodogen [19]. The specific radioactivity of the protein was 57000 c.p.m./ μ g.

Rat erythrocytes (5.95×10^7 in 0.1 ml of TBS) were incubated with various concentrations of the ^{125}I -labelled protein at 37 °C for 2 h. The cell-bound and free radioactivities were separated by transferring a portion of the labelled cell suspension in 0.3 ml of dibutyl phthalate and centrifugation at 15000 *g* for 2 min. The radioactivity in the pellet was counted.

Other methods

Purified 170 kDa-subunit protein (200 μ g) was extracted twice in 1 ml of chloroform/methanol (2:1, v/v), and then twice in chloroform/methanol (1:2, v/v) containing 5% water [20]. The combined extracts were evaporated to dryness, and the 'total' sugar content was estimated by the phenol/ H_2SO_4 method [21]. Yoshida sarcoma cells were homogenized as described by Maeda et al. [22]. The activities of ($Na^+ + K^+$)-activated ATPase [23], 5'-nucleotidase [24], glucose-6-phosphatase [25] and succinate dehydrogenase [26] were estimated in the homogenate and the pellet suspension. P_i and protein were estimated by the methods of Ames [27] and Peterson [28] respectively.

Statistical analysis

The agglutination values of TBS-incubated normal cells and those treated with the protein fraction were analysed by the two-tailed paired *t* test. *P* values of 0.05 or less were considered significant, and the fractions were regarded as active.

RESULTS

Purification of the active protein

The cell-free ascites fluid (Scheme 1) yielded a particulate and a soluble fraction, both of which could enhance the Con A agglutinability of erythrocytes *in vitro* (Figure 1). The particulate fraction was enriched in the plasma-membrane markers 5'-

Figure 3 *In vivo* activity of Sephadex G-200 peaks I and V and the pellet

The aseptically isolated pellet (200 μ g of protein equivalent) was suspended in 5 ml of TBS. Sephadex G-200 peaks I and V were isolated from the soluble fraction as described in Scheme 1, and sterilized by filtration. Five ml of each containing 4.37 mg and 0.137 mg of protein respectively and the pellet suspension were injected intraperitoneally into normal rats. A control rat received 5 ml of sterile TBS. The amounts of the three fractions were chosen on the basis of their yields from several experiments. The volume represents the average volume (approx. 5 ml) of the cell-free ascites fluid usually obtained at the time of tumour harvest (3 days after transplantation). Con A agglutinability of erythrocytes from animals injected with pellet (●), Sephadex G-200 peak I (▲) and V (△) and TBS (—) is shown.

Figure 4 Fractionation by DEAE-Sephadex chromatography

(a) The elution pattern of the 0–40% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate from DEAE-Sephadex. The elution was carried out using a NaCl gradient of 0.1–0.4 M in 0.01 M Tris/HCl, pH 8.0. No significant amount of protein was eluted subsequently by 0.4–1.0 M NaCl gradient. (b) The Con A agglutinability of erythrocytes incubated with TBS (— — —), fraction I (●) and fraction II (◆). (c) SDS/PAGE of fractions I and II under reducing conditions on a 7% gel; 4 μg of protein was applied per lane and stained with silver. The standard protein markers were the same as in Figure 2 (c). (d) SDS/PAGE of fraction I under non-reducing conditions on a 5% gel; 4 μg of protein was applied. The gel was stained with silver. The markers were: 330 kDa, thyroglobulin; 240 kDa, spectrin 1; 220 kDa, spectrin 2; 150 kDa, IgG whole molecule; 116 kDa, β -galactosidase.

nucleotidase and $(\text{Na}^+, \text{K}^+)\text{-ATPase}$, 35- and 7.4-fold respectively, and depleted in glucose-6-phosphatase and succinate dehydrogenase 0.74- and 0.31-fold respectively, relative to the tumour cell homogenate. It possibly represents the plasma-membrane vesicles shed by the tumour cells. The soluble protein was subfractionated on Con A-Sepharose into bound and unbound fractions. The active Con A-bound protein (MeMan eluate) yielded five peaks on Sephadex G-200 (Figure 2a), of which, I, the void peak, and V were found to be active (Figure 2b). Figure 2(c) shows the SDS/PAGE patterns, under reducing conditions, of proteins present in the ascites fluid, Con A-binding fraction and the five peaks eluted from the Sephadex G-200 column. When the active gel-filtered peaks I and V and the pellet were

individually injected into normal rats, only the Sephadex G-200 peak I was found to be active (Figure 3).

The *in vivo* active Sephadex G-200 peak I was further fractionated on Sepharose 4B and by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Scheme 1). The 0–40% saturation cut was subjected to DEAE-Sephadex chromatography where two fractions were obtained (Figure 4a). The fraction eluted at 0.25 M NaCl possessed high activity (Figure 4b), and showed a single polypeptide of 170 kDa on SDS/PAGE under reducing conditions (Figure 4c). The second fraction eluted slightly before 0.3 M NaCl also showed some activity (Figure 4b), and contained some 170 kDa polypeptide (Figure 4c). The polypeptide of 155 kDa, and its degradation products of 90, 70, 64 and 36 kDa (evidence not presented), were

Table 1 Amino acid and sugar composition of the 170 kDa-subunit protein

Amino acid	Content (mol/100 mol)
Aspartic acid	7.08
Threonine	5.98
Serine	6.08
Glutamic acid	17.74
Proline	6.29
Glycine	7.15
Alanine	6.22
Half-cystine	3.47
Valine	7.65
Methionine	1.00
Isoleucine	3.76
Leucine	7.32
Tyrosine	3.42
Phenylalanine	3.82
Histidine	2.08
Lysine	6.82
Arginine	4.12
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Sugar	Content (mol/mol of 170 kDa polypeptide)
N-Acetylglucosamine	37.60
N-Acetylgalactosamine	0.00
Mannose	65.26
Galactose	17.75
Glucose	0.00
Fucose	0.00
Sialic acid	3.60

the major constituents of this fraction. The 155 kDa protein was also purified (not shown) and found to be inactive. Thus the activity of the *in vivo* active Sephadex G-200 peak I resides solely in the 170 kDa polypeptide. The other fractions that possessed some activity (Scheme 1), namely Sepharose 4B peak I and 40–90%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction, were also found to contain the 170 kDa polypeptide (not shown).

Incubation of cells with various concentrations of the purified protein, up to 1 mg/ml, showed that 500 μg of the protein/ml was sufficient to confer maximal agglutinability on erythrocytes.

Characterization

The purified active protein had a molecular mass of 170 kDa by SDS/PAGE under reducing conditions (Figure 4c) and of 310 kDa when unreduced (Figure 4d). By gel filtration on a column of Sepharose 4B [calibrated with thyroglobulin, apo-ferritin and catalase (232 kDa)], a molecular mass of 680 kDa was obtained for the native molecule (results not shown). Non-denaturing limit-pore electrophoresis also yielded the same value (results not shown). The results indicate that the protein is homotetrameric, made up of two disulphide-bonded dimers, which in turn are made of non-covalently linked monomers of 170 kDa.

Two-dimensional electrophoresis of the protein showed a single spot (not shown) with pI of 5.35.

The amino acid composition of the purified protein does not show any distinctive features except a high content of Glx (Table 1). The sugar composition (Table 1) is suggestive of N-linked hybrid oligosaccharides. In view of the absence of N-acetylgalactosamine, the presence of O-linked sugars is ruled out.

Figure 5 Binding of radiolabelled 170 kDa-subunit protein to rat erythrocytes

The purified protein was radioiodinated. Various concentrations of the ^{125}I -protein were incubated with 5.95×10^7 erythrocytes in 0.1 ml of TBS at 37 °C for 2 h. The cell-bound and free radioactivities were separated by centrifugation on dibutyl phthalate, and the cell-bound radioactivity was counted. The results are expressed as ng of protein bound to 5.95×10^7 cells.

No N-terminal amino acid could be detected. It is possible that it is blocked.

The possible presence of glycolipid in the purified protein was examined; however, no lipid-linked sugar was extracted.

Binding of the protein to erythrocytes

The radioiodinated protein consisting of the 170 kDa subunit was found to bind to normal rat erythrocytes in a non-saturable manner (Figure 5). At 512 $\mu\text{g}/\text{ml}$, the highest concentration used, 7400 molecules were bound per cell. The Scatchard plot (results not shown) of the data appeared quite complicated, initially showing a straight line with negative slope followed by an upward curvature, possibly indicating positive co-operativity.

Presence in blood plasma

The presence of the 170 kDa-subunit protein in plasma of normal and tumour-bearing rats was probed using the antibody generated against the isolated protein (Figure 6). The protein was found to be present in the plasma of tumour-bearing but not normal rats. The plasma of the rat bearing the tumour for 1 day showed a small amount of the protein. The concentration increased after 2 and 3 days of tumour transplantation. On the third day, on the basis of immunostaining, the protein appears to be present in greater amounts in the plasma of the tumour-bearing rat than in ascites fluid. At this time, the plasma possesses greater potency to enhance the Con A agglutinability of erythrocytes *in vitro* [5].

Sephadex G-200 peak V and the pellet failed to show immunological cross-reactivity with the 170 kDa-subunit protein.

Figure 6 Detection of the 170 kDa-subunit protein in the pellet, fraction V and the plasma of normal and tumour-bearing rats, and in tumour cells

(a) 80 μ g each of (A) soluble ascites fluid, (B) Sephadex G-200 peak V, (C) pellet (D), normal rat plasma (E, F, G) plasma of rats bearing the tumour for 1, 2 and 3 days respectively were electrophoresed on 6–12% gel, and transblotted onto nitrocellulose. The blot was probed with anti-(170 kDa-subunit protein) antibody (1:5000 dilution) as described in the Materials and methods section. (b) The detergent extract of tumour cells was prepared as described in the Materials and methods section. The blot, probed with 1:1000 dilution of the antiserum, shows the 170 kDa polypeptide in about 2 mg of the extract. The additional band seen is most probably a degradation product since the purified protein on storage also shows this band.

Presence of the 170 kDa-subunit protein in tumour cells

Immunohistochemistry using even the undiluted antiserum failed to detect the 170 kDa-subunit protein in the cells. The detergent extract of the cells showed a barely discernible band of the protein when the amount on the immunoblot was 135 μ g, and the antiserum dilution was 1:1000. Figure 6(b) shows the 170 kDa polypeptide in approx. 2 mg of the extract protein at 1:1000 dilution of the antiserum.

DISCUSSION

Three fractions possessing *in vitro* erythrocyte surface modification activity are present in the Yoshida ascites fluid, the particulate fraction, enriched in plasma-membrane markers, being one of them. It may represent vesicles shed by the tumour cell membrane [29]. The second fraction consists of soluble proteins present in Sephadex G-200 peak V. This fraction, comprising several polypeptides, forms about 0.08% of the starting protein. The third, and major, fraction that is active in the modification of erythrocyte surface is Sephadex G-200 peak I. This fraction, of the three, was the only one that was active *in vivo*. Its further fractionation yielded an active protein that was homogeneous by SDS/PAGE.

The molecular mass of the purified protein in non-denaturing conditions is 680 kDa. SDS splits it into 310 kDa molecules, which on disulphide bond reduction yields a polypeptide of 170 kDa. The native molecule is evidently a homotetramer consisting of two S-S bonded dimers. The protein has a pI of 5.35. The amino acid composition shows a high content of Glx, and the sugar composition is indicative of hybrid N-linked oligosaccharides with the absence of O-linked structures.

The protein binds to erythrocytes in about 7400 copies/cell at 520 μ g/ml. The binding up to this concentration shows no evidence of saturation. At roughly this concentration, the protein brings about maximal agglutinability in erythrocytes. Since coupling of only 6000 molecules of mannose to the surface of an erythrocyte can make the Con A-non-agglutinable human erythrocytes highly agglutinable [30], it is possible that the added number of the purified protein molecules can make the cells more agglutinable.

The antibody produced against the purified active component reveals the presence of the protein in the plasma of tumour-bearing animals, but not in normal rat plasma. The plasma of the animal bearing the tumour for 1 day shows a barely detectable amount which increases on subsequent days. The enhancement

in Con A agglutinability of erythrocytes occurs only on the third day after transplantation [5]. This suggests that a sufficient concentration of the protein in plasma is necessary to modify the cell agglutinability.

Thus at least one specific protein is found to exist in the ascites fluid and blood plasma of Yoshida sarcoma-bearing rats that is absent from the plasma of normal animals. Since neither a non-specific stress-induced reaction [5] nor the native or (lipopolysaccharide-) activated peritoneal macrophages are responsible for the synthesis of the active protein (R. D. Kalraiya, A. Sanjay and N. G. Mehta, unpublished work), the latter must be a tumour product. Although immunohistochemistry of the tumour cells failed to reveal the protein, the presence of the protein in the detergent extract of tumour cells was demonstrated by immunoblotting. In the latter case a minimum of 135 μ g of the tumour protein had to be applied for the band to be just detectable by the antiserum at a dilution of 1:1000. It is known that immunoblotting is about 10 times as sensitive as immunohistochemistry [31]. This emphasizes that the amount of the protein synthesized by the tumour cells is extremely small, and also that it is not stored to any significant extent in the cell before secretion. The very low yield (0.027%) of the protein from the ascites fluid containing over 1×10^8 cells/ml is consistent with this result. The isolated protein is able to bind to normal erythrocytes and alter their agglutinability in a similar way to that occurring in the tumour-bearing animal. The passage of the active protein from the peritoneal cavity, the site of tumour growth, into the circulation has been demonstrated. This evidence is consistent with the long-suspected and indirectly supported mechanism of paraneoplastic syndromes [2], namely that tumour products can reach distantly located host cells by entering the circulation and can alter their properties by non-specifically interacting with them.

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