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Enhanced Concanavalin A-agglutinability of Erythrocytes in Rats Bearing Yoshida Ascites Sarcoma: a Study of the Mechanism*

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Abstract—*Erythrocytes of normal Wistar rats are maximally agglutinated at* 250 μg/ml *concanavalin A (Con A). In rats bearing Yoshida ascites tumor (i. p.), however, the red cells in circulation and those contaminating the tumor fluid agglutinate the most at* 50 μg/ml *of the lectin. The enhanced agglutinability of circulatory RBCs arises* 3 days *after tumor transplantation, but the erythrocytes in the ascites fluid show the alteration within a day. Incubation of normal erythrocytes in the cell-free ascites fluid or its soluble or membranous component modifies their agglutinability. Intraperitoneal injection of the cell-free ascites fluid in a normal rat also leads to modification of its erythrocytes in* 24 hr. *Thus the modification activity resides in the fluid, from which it can enter into circulation. The blood plasma of the animal, collected on the third day after tumor transplantation but not earlier, is also able to modify red cells* in vitro. *The erythrocyte modification activity of the fluid is insensitive to the temperature of incubation, and prior boiling enhances its activity. Comparison of the sugar content, and protein and glycoprotein profiles of the normal and modified circulatory erythrocytes shows no differences. Thus proteolytic enzymes are not responsible for the enhanced agglutinability of red cells from tumor-bearing animals. The membranes of erythrocytes from the ascites fluid, however, have somewhat reduced contents of sugars and diminished amounts of glycoproteins, indicating that they are probably acted upon by proteases in the ascites fluid. Approximately* 5% *of the protein in the ascites fluid binds to Con A-Sepharose. This glycoprotein fraction, consisting of* 14 *polypeptides, possesses* in vitro *modification activity, while the protein devoid of affinity for Con A is inactive. It is proposed that the tumor cells secrete or shed Con A-binding glycoproteins in the ascites fluid, which bind to the surface of erythrocytes present in the fluid and modify their agglutinability with the lectin. The glycoproteins enter circulation and, after reaching a sufficient concentration in plasma, alter the membranes of red blood cells.*

INTRODUCTION

SEVERAL reports in the literature indicate modification of the erythrocyte membrane in cancer patients and tumor-bearing animals. The modification often manifests itself in altered chemical or immunochemical properties of the cells. Many blood group A_1 patients with carcinoma at any one of various sites exhibit *B* group antigens on their red cells [1]. In chronic myeloid leukemia, increased sialic acid content and altered glycoprotein profile of red cell membranes is reported [2]. The agglutinability of erythrocytes from normal and leukemic individuals is found to be qualitatively different with many seed extracts [3]. In addition to chemical properties, modifications cause alterations in physiological properties of the cells. Decreased red cell survival and/or their increased osmotic fragility have been reported in cancers of various sites [4–6]. Changes have also been observed in various properties of red cells in animals bearing experimental tumors. The deformability of erythrocytes is decreased in Lewis lung carcinoma-bearing rats [7], and the morphology as well as the membrane carbohydrate com-

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ponents of erythrocytes are altered in mice bearing Ehrlich ascites carcinoma [8]. The erythrocytes in the latter case, unlike the cells from normal animals, agglutinate with concanavalin A (Con A) [9]. Thus the tumor is able to influence surface properties of host cells not directly in contact with it. The mechanism by which the tumor causes such effects is not known.

We have found that the Con A-agglutinability of erythrocytes in rats bearing Yoshida ascites sarcoma is enhanced. We have used this system to investigate the mechanism whereby the erythrocyte property is altered in the tumor-bearing animals.

MATERIALS AND METHODS

Chemicals

Concanavalin A (Grade IV), crystalline bovine serum albumin, α-methylmannoside (α-MM), Tris(hydroxymethyl)aminomethane (Tris) and *N*-acetylneuraminic acid were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Galactose was from E. Merck, Darmstadt, W. Germany, and was recrystallized from ethanol. Cyanogen bromide-activated Sepharose 4B was from Pharmacia, Uppsala, Sweden. Sodium dodecylsulfate (SDS) was a product of Fluka AG, Buchs SG, Switzerland. Acrylamide was purchased from Koch Light & Co., Coinbrook, Bucks, England, and *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were obtained from Eastman Organic Chemicals, Rochester, NY, U.S.A. EDTA was a product of British Drug House, Poole, England. Other chemicals were of reagent or better grade and were purchased either from British Drug House, Bombay or Sarabhai M. Chemicals, Baroda, India.

Cells

Yoshida ascites sarcoma was maintained by transplantation of 100×10^6 cells in inbred Wistar female rats, $2\frac{1}{2}$–3 months of age. Ascites fluid was collected using 1 mg/ml EDTA. The fluid, which usually contains a variable amount of blood depending on the day of harvest, was immediately centrifuged in the cold. The cells were resuspended in the original volume of 0.15 M NaCl in 0.01 M Tris–HCl (TBS), and the suspension was centrifuged in an International PR-2 centrifuge at 4°C at $\sim 100\,g$ for 1 min. Under these conditions most of the sarcoma cells sediment with a few erythrocytes. The erythrocyte-rich supernatant was recentrifuged under identical conditions three more times, after which the red cells were pelleted and washed four times in TBS. These cells were termed peritoneal-RBCs (pRBC), and were found to be free of other cell types.

Blood from normal as well as tumor-bearing rats was collected by cardiac puncture using EDTA as the anticoagulant. The cells were centrifuged and washed four times with TBS in the cold, each time discarding a portion of the pellet from the top to ensure maximal removal of leukocytes. The red cells from circulation of normal and tumor-bearing animals were called $cRBC_N$ and $cRBC_T$, respectively.

Ascites fluid fractionation

Cell-free ascites fluid was obtained by centrifugation of the tumor fluid at $1000\,g$ for 5 min. The supernatant was centrifuged once more and called *crude ascites supernatant.* It was left at 4°C if used within an hour, otherwise it was stored at −20°C. The crude ascites supernatant was centrifuged at $100{,}000\,g$ in a Kontron ultracentrifuge for 1 hr. The pellet was washed by resuspension in TBS and centrifugation. The clear supernatant from the first centrifugation and the membranous pellet were called the *soluble supernatant fraction* and the *pellet* respectively. The latter was suspended in the original volume of TBS and both fractions were stored at −20°C. Their activity is retained for at least three months under this storage condition.

In vitro *incubation of erythrocytes*

Erythrocytes were incubated with crude ascites supernatant, the soluble fraction or the pellet suspension in the ratio of 1:9 (v/v) in a total of 0.2 ml in stoppered tubes at 37°C with shaking, usually for 2 hr. (The ratio of 1:9 was chosen since this is roughly the ratio of erythrocytes and crude ascites supernatant on the third day of tumor transplantation. Much of the work was done with the ascites fluid obtained at this time after the transplantation.) RBCs were incubated in blood plasma under the same conditions, except that the ratio was 1:1.5 (v/v). In all experiments cells incubated in TBS were used as control. After incubation, the cells were washed four times in TBS in the cold.

Agglutination

For agglutination, 0.1 ml of a 0.2% (v/v) suspension of erythrocytes in TBS was mixed with 0.1 ml of freshly prepared solution of Con A in TBS and incubated at 37°C for 1 hr. Several Con A concentrations were used, and one tube, usually with 250 μg/ml Con A, also contained α-MM at 5×10^{-2} M. After incubation each tube

was gently tapped several times and the free cells were counted in a hemocytometer. Aggregates of three or more cells were considered as clumps. It was repeatedly observed that rat erythrocytes incubated in the absence of Con A adhered strongly to the glass tube. Hence for calculating percentage agglutination the tube with Con A plus α-MM was taken as control. No clumps were ever observed in the presence of the saccharide inhibitor.

Con A-Sepharose chromatography

Con A-Sepharose was prepared according to Allan and Crumpton [10] using cyanogen bromide-activated Sepharose 4B. Approximately 9 mg of Con A was coupled to 1 ml of the sedimented gel. A 3-ml column was used for separation of Con A-binding material at 25°C. Two ml of the soluble supernatant fraction containing about 80 mg protein was applied to the column previously equilibrated with TBS. After loading the sample the column was washed with TBS to remove the non-Con A-binding protein. After the absorption of the wash fluid at 280 nm had returned to the base line, the column was further washed with 100 ml TBS and the Con A-bound material was eluted with α-MM.

Ghost preparation and SDS-polyacrylamide gel electrophoresis

Erythrocyte ghosts were prepared according to the procedure of Dodge *et al.* [11], except that Tris buffer was used in place of phosphate buffer for lysis and washing of ghosts.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to Laemmli [12] in cylindrical tubes.

Analytical methods

Protein was estimated according to the method of Lowry *et al.* [13], including 0.1% SDS in final concentration. Bovine serum albumin was used as the standard. Neutral sugars were estimated by the phenol–sulfuric acid procedure of Dubois *et al.* [14], with galactose as the standard. For the release of amino sugars the ghost protein was hydrolyzed in 3 N HCl at 100°C for 4 hr [15]. The amino sugars were estimated by the Elson–Morgan procedure [16], using glucosamine hydrochloride as the standard. Sialic acid was estimated by the Warren procedure [17] following hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 hr. *N*-Acetylneuraminic acid served as the standard.

RESULTS

Con A-mediated agglutination of erythrocytes from normal and Yoshida ascites tumor-bearing rats

Normal rat erythrocytes were found to be agglutinated with Con A, but the agglutination was not linear with increasing concentrations of Con A. Maximal agglutination occurred at 250 μg/ml of the lectin, beyond which there was partial reversal of agglutination (Fig. 1). On a few occasions the maximal response was observed at 100 μg/ml of Con A (e.g., Fig. 2A). The agglutination was totally abolished in the presence of 5×10^{-2} M α-MM. In contrast to normal erythrocytes, both types of red cells from tumor-bearing animals, i.e., the cells in circulation and those isolated from the ascites fluid, showed maximal agglutination at 50 μg/ml Con A. In these cases also, there was partial reversal of agglutination above 50 μg/ml of the lectin. α-MM prevented the agglutination, indicating that the reactions were Con A-specific.

Agglutination of pRBC and $cRBC_T$ following tumor transplantation

The Con A-induced agglutinability of pRBC and $cRBC_T$ samples, obtained on successive days following tumor transplantation, was examined. The response of $cRBC_T$ was found to be similar to that of normal erythrocytes for 2 days after the transplantation. Thereafter, the $cRBC_T$ agglutinated maximally at 50 μg/ml of Con A (not shown). Samples of pRBC, however, showed enhanced agglutinability from the first day onwards. Thus a direct con-

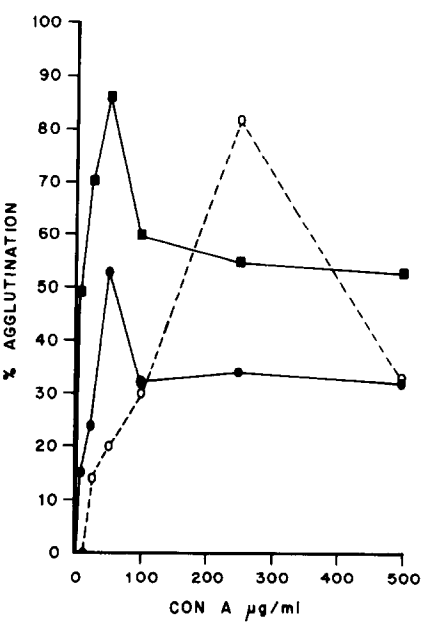

Fig. 1. Con A-agglutinability of erythrocytes from normal and Yoshida ascites tumor-bearing rats. $cRBC_N$ (O---O); $cRBC_T$ (●——●); pRBC(■——■).

tact with the ascites fluid for a period ≤24 hr was enough to modify the agglutinability of cells, whereas the RBCs in circulation showed a similar modification only after more than 48 hr.

Incubation of cells in crude ascites supernatant

When erythrocytes from normal rats were incubated with crude ascites supernatent, their agglutinability was enhanced similar to that of pRBC, and $cRBC_T$ after 3 days of transplantation (Fig. 2A). Human red cells, which are normally not agglutinated by Con A even at 500 μg/ml, became agglutinable at 50 μg/ml after incubation with the crude supernatant (Fig. 2B). The surface modification was complete in 2 hr of incubation with the crude supernatant (Fig. 2B; results with only human cells are shown).

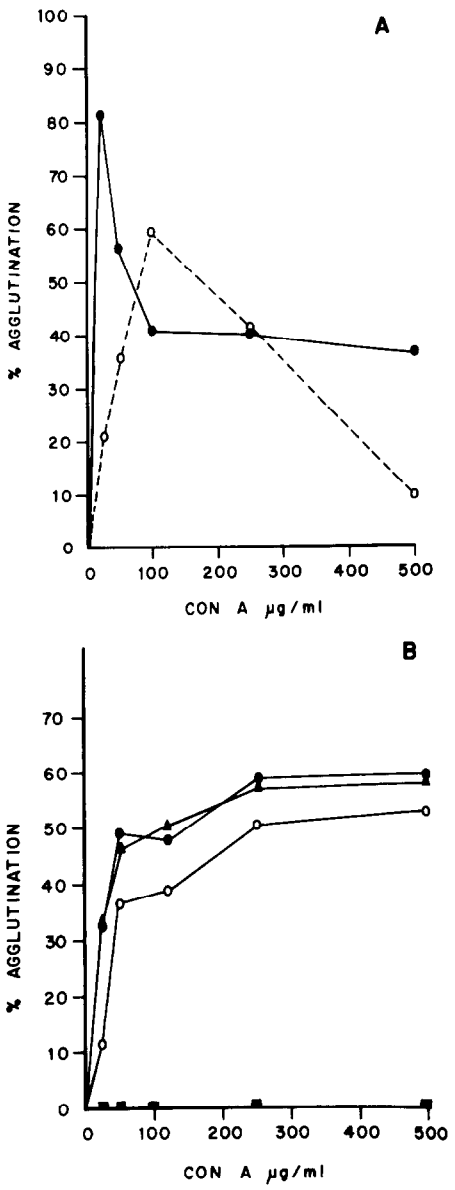

Fig. 2. Modification of erythrocyte agglutinability by in vitro *incubation of cells in crude ascites supernatant. (A) Rat erythrocytes: TBS-incubated cells (O---O); cells incubated in the crude ascites supernatant (●——●). (B) Human erythrocytes: TBS-incubated cells (■——■); cells incubated in the crude ascites supernatant for* 1 hr (O——O); 2 hr (●——●) *and* 4 hr (▲——▲).

Modification of cells by the soluble supernatant fraction and the pellet suspension

The factor(s) in the crude supernatant responsible for altering the agglutinability of erythrocytes could either be a soluble substance(s) elicited by the tumor cells, or membrane fragments shed from the tumor cell surface. When rat erythrocytes were incubated with the soluble supernatant fraction and the pellet suspension, both the fractions were active in modification (Fig. 3). Qualitatively the soluble fraction showed somewhat greater activity, with maximal agglutination of the modified cells occurring at 25 μg/ml Con A. The pellet-treated erythrocytes, similar to those treated with the crude supernatant, showed highest agglutination at 50 μg/ml of the lectin.

Modification of erythrocytes of a normal animal following an injection of soluble supernatant fraction

Although both the soluble supernatant fraction and the pellet may contribute to the activity, we have currently worked with only the soluble fraction. A normal rat was injected with 3.0 ml of Millipore membrane-filtered soluble supernatant fraction. After 24 hr the

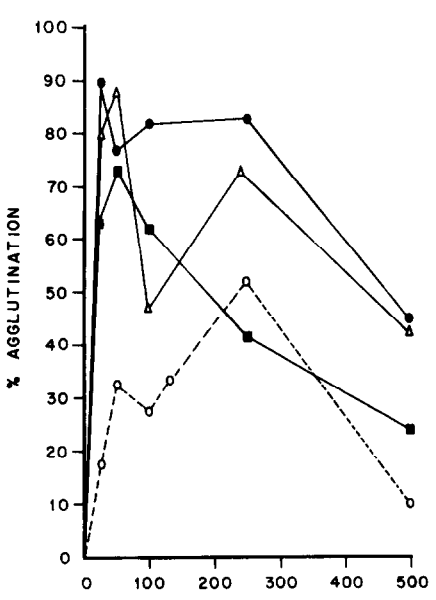

Fig. 3. Agglutination of erythrocytes incubated with crude ascites supernatant, the soluble supernatant fraction and the pellet suspension. TBS-incubated cells (O---O); cells incubated with soluble supernatant (●——●); RBCs treated with crude supernatant (△——△); cells incubated with membranous pellet (■——■).

animal was killed and the agglutinability of its erythrocytes was tested. Cells from another normal animal were incubated *in vitro* with an aliquot of the fluid used for injection. In both cases the erythrocytes showed enhanced agglutinability with Con A (Fig. 4). This result indicates that the modification activity present in the ascites fluid in the peritoneal cavity can appear in the circulation.

Activity in blood plasma of tumor-bearing animals

The circulating erythrocytes in tumor-bearing rats showed enhanced agglutinability on the third day following transplantation, while pRBC in direct contact with the ascites fluid always exhibited higher agglutinability (see above). Since the surface modification activity present in the soluble supernatant fraction is able to diffuse into the circulation (Fig. 4), the three-day lag seen in the modification of $cRBC_T$ may be due to the time required to achieve a sufficient concentration in blood plasma. If such is the case, the blood plasma of tumor-bearing animals 3 days after transplantation, but not earlier, should be able to modify normal erythrocytes *in vitro*. When normal rat erythrocytes were incubated in plasma from animals bearing the tumor for 0, 1, 2 and 3 days, only the plasma from the 3-day-old tumor-bearing rat was found to enhance the Con A-agglutinability of cells (Fig. 5; results with 0, 1 and 2-day plasma from the 3-day-old tumor-bearing rat was found to enhance the Con A-agglutinability of cells (Fig. 5; results with 0, 1 and 2-day plasmas are not shown since they were similar to the TBS-incubated cells).

Effect of cold incubation and of boiling on the activity of soluble supernatant fraction

Secretion of proteolytic enzymes is a well-known property of tumor cells [18]. Treatment of normal cells by proteolytic enzymes is also known to make them agglutinable at low concentrations of lectins [19, 20]. It is, therefore, possible that the factor(s) in the supernatant causing alteration of erythrocyte agglutinability is a tumor-secreted protease(s). Thus, if the modification observed in the experiments is an enzymatic process, it would be expected to slow down at low temperatures; and the enzyme may be heat-labile. The *in vitro* modification of cells by ascites fluid (Fig. 2B) and the soluble supernatant (not shown) is complete in 2 hr at 37°C. Incubation of cells at 0°C for 2 hr was found to be equally effective. Thus low temperature does not affect modification. Heating the soluble supernatant fraction for 5 min at 100°C and incubation of normal erythrocytes in the clarified extract at 37°C revealed a much higher activity compared to the original supernatant. Also, there was no reversal of agglutination at higher concentrations of Con A. The agglutination patterns of normal and the boiled extract-treated erythrocytes looked qualitatively similar (Fig. 6).

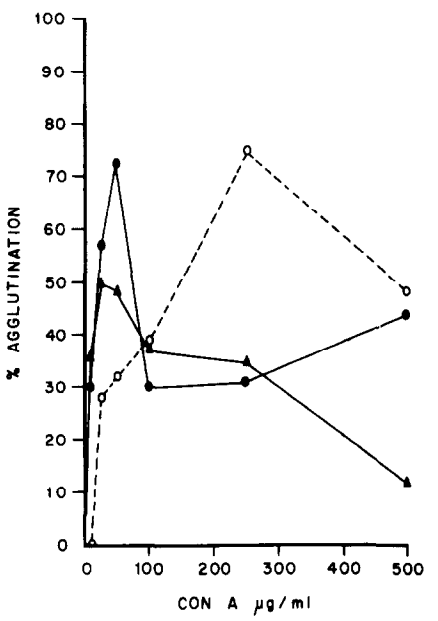

Fig. 4. Enhanced agglutinability of erythrocytes from a rat given an i.p. injection of the soluble supernatant fraction. Normal TBS-incubated erythrocytes (O---O); *normal erythrocytes treated* in vitro *with the soluble supernatant* (▲——▲); *erythrocytes from the supernatant-injected rat* (●——●).

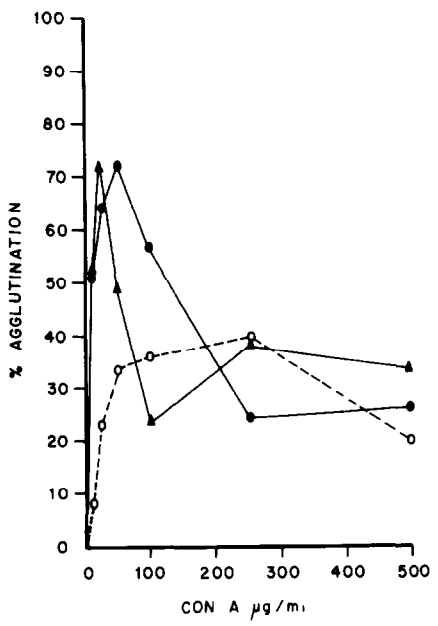

Fig. 5. Modification of erythrocyte agglutinability by plasma from tumor-bearing animals. TBS-incubated normal cells (O---O); *erythrocytes treated with ascites fluid supernatant* (●——●); *cells treated with plasma of an animal bearing the tumor for three days* (▲——▲).

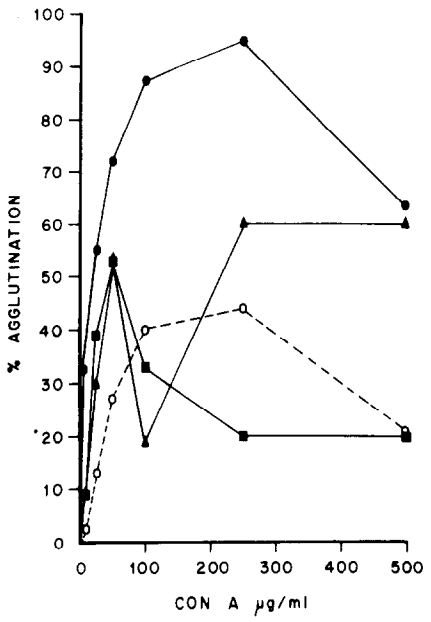

Fig. 6. Effect of cold-incubation and of boiling on the in vitro *modification activity of the soluble supernatant fraction. TBS-incubated cells* (O---O); *erythrocytes incubated in the soluble supernatant at* 37°C (■——■); *cells incubated in the soluble supernatant at* 0°C (▲——▲); *cells treated with the boiled supernatant at* 37°C (●——●).

Sugar composition and electrophoretic patterns of normal and modified erythrocyte membrane proteins

Protease treatment of erythrocytes would remove glycopeptides from the cell surface, leading to a decrease in the sugar content of their ghost protein and also to a reduction in the staining intensity or absence of some of the protein bands, with simultaneous appearance of lower molecular weight species on SDS-PAGE. The content of neutral sugars, amino sugars and sialic acid of $cRBC_N$ and $cRBC_T$ were similar. The values for pRBC were, however, somewhat reduced (Table 1). The SDS-PAGE patterns of the Coomassie blue-stained components were almost identical for the three ghost species, with only slight quantitative variations in the lower molecular weight proteins. The periodic acid Schiff-staining revealed two bands (plus one at the dye front) in each case, the intensities of which were reproducibility reduced in pRBC but not in $cRBC_T$ (Fig. 8).

Con A-binding proteins in the soluble supernatant fraction and the modification of erythrocytes

It was conceivable that the soluble supernatant fraction might contain Con A-specific glycoprotein(s) which could bind to the surface of cells and modify their agglutinability. These molecules could either be secreted by the tumor cells or could arise from the Con A-receptors on the cell surface. In such a case, the modification activity could be neutralized by addition of Con A to the fluid. When the lectin was added to the soluble supernatant a heavy precipitate developed. This was washed several times in TBS to remove soluble components. On addition of 0.1 M α-MM it went into solution, indicating that it consisted of Con A and glycoprotein. The Con A-binding protein from the supernatant fraction was purified by Con A-Sepharose chromatography. In three experiments the yield of the binding protein was 4.85, 6.25 and 3.75% of total protein in the ascites fluid, the average being 4.95%.

The Con-A-binding protein and the non-binding material were tested for their modification activity. Erythrocytes treated with the non-lectin-binding protein showed similar agglutination to normal cells but less agglutination at higher concentrations of Con A. The Con A-binding material, on the other hand, was highly active (Fig. 7). The erythrocytes incubated with it showed substantial agglutination even at 10 μg/ml of Con A. Their agglutination pattern was similar to that of erythrocytes treated with the boiled soluble supernatant (Fig. 6). The cells inoculated with a mixture of the Con A-binding and non-binding fractions yielded a pattern similar to the one obtained by treatment with the unfractionated fluid (Fig. 7).

The material purified by Con A-Sepharose chromatography showed a complex composition on SDS-PAGE, revealing 14 bands by Coomassie blue staining and 11 bands by the PAS reaction (Fig. 9). The precipate obtained

Table 1. Sugar composition of erythrocyte ghost protein

Ghost	Neutral sugars	Amino sugars	Sialic acid
$cRBC_N$	112.2 ± 12.8*	37.5 ± 6.0	20.0 ± 3.4
$cRBC_T$	110.2 ± 3.3	35.1 ± 4.0	19.2 ± 5.4
pRBC	94.9 ± 6.3	25.4 ± 5.2	16.2 ± 4.4

*The values are in μg/mg protein and indicate mean ± S. E. The results are from three ghost preparations.

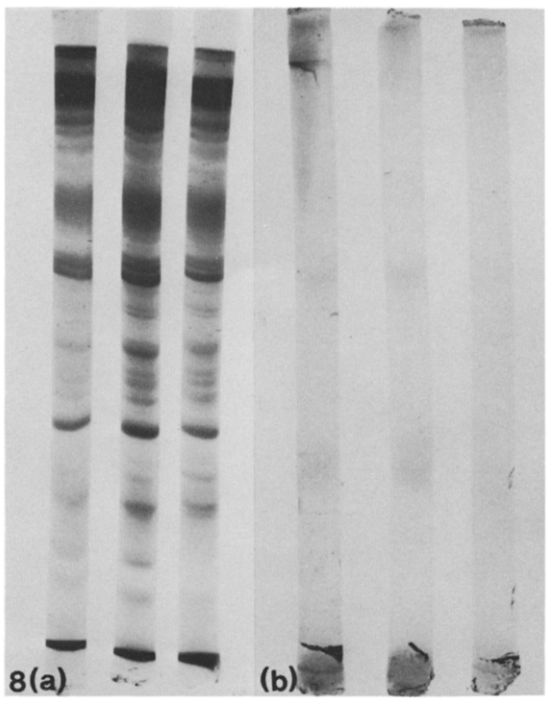

Fig. 8. SDS-polyacrylamide gel electrophoresis of erythrocyte ghost proteins and glycorproteins. Left: $cRBC_N$, 113 μg; middle: $cRBC_T$, 110 μg; right: pRBC, 116 μg protein. (a): Coomassie blue staining; (b) periodic acid-Schiff staining.

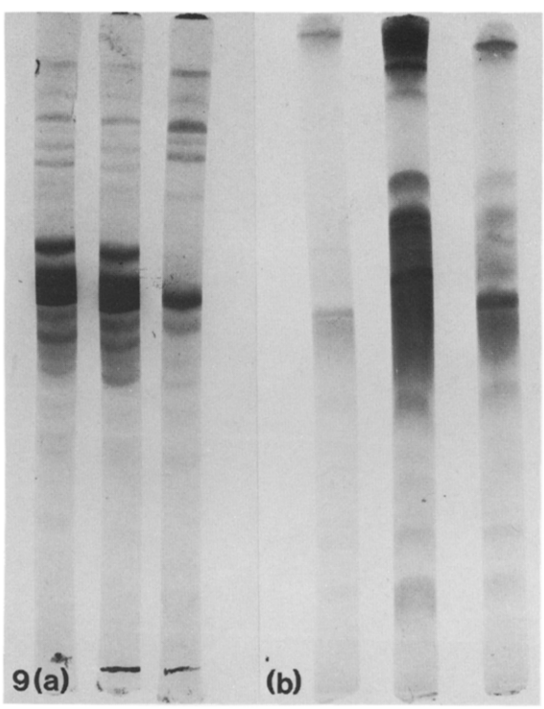

Fig. 9. SDS-polyacrylamide gel electrophoresis of soluble supernatant fraction, and its Con A-binding and non-binding components. (a) Coomassie blue staining. Left non-Con A-binding protein, 70 μg; middle: unfractionated soluble supernatant, 80 μg protein; right Con A-binding protein, 70 μg. (b) Periodic acid-Schiff staining. Left: non-Con A-binding protein, 140 μg; middle: unfractionated soluble supernatant, 160 μg protein; right: Con A-binding protein, 140 μg.

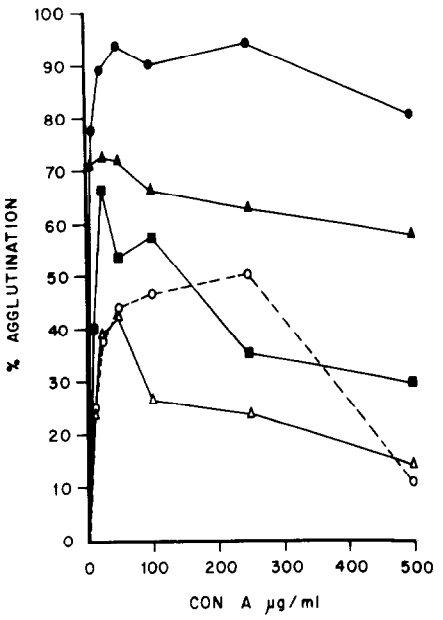

Fig. 7. Modification of erythrocyte agglutinability by the Con A-binding fraction of the soluble supernatant. Normal red cells incubated with TBS (O---O); *erythrocytes treated with unfractionated soluble supernatant* (■——■); *cells incubated with Con A-binding protein* (●——●); *cells treated with* 1:1 (*v/v*) *mixture of Con A-binding and non-binding fractions* (▲——▲); *erythrocytes treated with the non-Con A-binding fraction* (△——△).

by addition of Con A to the soluble supernatant also showed the same polypeptide composition (not shown). The unfractionated supernatant showed 21 polypeptides by Coomassie blue staining, while the non-Con A-binding protein contained 17 polypeptides (Fig. 9). Eleven polypeptides were common on the three gels, indicating the heterogeneous nature of many bands.

DISCUSSION

The results presented above show that erythrocytes from rats bearing Yoshida ascites sarcoma exhibit enhanced Con A-agglutinability. As mentioned earlier, the Con A-agglutinability of erythrocytes in mice bearing Ehrlich ascites carcinoma is altered [9]. Chaudhury *et al.* [21] reported that fibroblasts from a histologically normal region neighboring a uterine cervical carcinoma were agglutinated with Con A, unlike the cells obtained from the tissue of a healthy donor.

In vitro incubation of normal red cells in the cell-free ascites fluid modifies their surface property. It is therefore reasonable to conclude that a substance(s) present in the fluid is responsible for the modification, and that contact *per se* with tumor cells is not necessary. Since *in vitro* modification occurs in a matter of 1–2 hr of incubation, the enhanced agglutinability of pRBC observed in 24 hr of tumor transplantation is not surprising. The appearance of the surface modification in $cRBC_T$ on the third day post-transplantation and the simultaneous presence of the modification activity in plasma of the animals suggest that the latter activity is responsible for the modification of cells in circulation. The lack of increased agglutinability of these cells on the first and second days after tumor inoculation and the absence of activity in the respective blood plasmas support this conclusion. The source of modification activity in plasma must obviously be the ascites fluid, from which the active substances do diffuse into circulation. The delay of over 48 hr in manifestation of the activity in blood perhaps reflects the time required to build up a sufficient concentration of the modifying substance(s).

The soluble supernatant fraction and the membranous component of the crude ascites supernatant both possess the modification activity. The soluble fraction seems to be more active since the cells treated with it are maximally agglutinated at 25 μg/ml Con A, whereas the pellet-treated cells require 50 μg/ml for maximal agglutination. The membranes, in all probability, represent fragments (vesicles) shed from the tumor cell surface. Some of them could also arise from dead cells. While these could perhaps enter circulation and modify red cells, at present we have studied only the soluble agents occurring in the 100,000 *g* supernatant.

What is the nature of the modifying agent elicited by the tumor cells and present in the soluble supernatant fraction? Several lines of evidence indicate that it is not a protease: the lack of difference in the polypeptide and sugar components of $cBRC_N$ and $cRBC_T$ membranes, the lack of temperature effect on *in vitro* modification kinetics and the enhancement of modification activity of the ascites fluid supernatant upon boiling attest to this. The staining intensity of pRBC glycoproteins on SDS-PAGE and the sugar content of their membrane proteins are decreased relative to $cRBC_N$, and thus it is distinctly possible that the red cells in ascites fluid are subjected to proteolysis. However, since both $cBRC_T$ and pRBC show similar modification with respect to Con A agglutinability, and the former show no evidence of proteolytic action, surface modification by proteases does not seem to be the determining factor for enhanced agglutinability of erythrocytes in tumor-bearing

animals. The apparent protease action on pRBC seems to be an additional, but not crucial, factor, besides the one that determines the modification of $cRBC_T$. Moreover, proteolytic enzymes cannot be expected to remain active in blood plasma: the plasma contains several proteolytic inhibitors against different classes of proteases. At least one inhibitor, α_2-macroglobulin, is known to inhibit all classes of proteolytic enzymes and occurs in large concentrations in plasma [22]. Many of these inhibitors form a part of acute phase plasma proteins, and their concentrations rise in many diseases including cancer [16, 23]. These should inactivate any proteases entering circulation. In some experiments (results not shown) the level of acute phase proteins was boosted up a day prior to tumor transplantation by s.c. injection of turpentine in rats [24]. This treatment should have delayed the appearance of surface modification in $cRBC_T$ if proteolytic enzymes were the cause of modification of erythrocytes in circulation. However, the circulating red cells of these animals became agglutinable at low Con A concentrations on the second day, a day earlier than in the non-boosted, tumor-bearing animals. (The early modification may be due to the appearance in enormous quantities of a recently discovered Con A-binding glycoprotein in the plasma of turpentine-injected rats [25].)

Precipitation of glycoproteins on addition of Con A to the soluble supernatant fraction indicated the presence of a relatively large amount of Con A-binding material in the fluid. This comprises approximately 5% of total protein in the soluble supernatant fraction, and it possesses *in vitro* modification activity. The agglutination pattern of cells treated with this fraction is found to be similar to the one shown by cells treated with the boiled supernatant (cf. Figs 6 and 7). The activity of the Con A-binding fraction is also heat-stable (not shown). The bulk of the supernatant protein, devoid of affinity for Con A, possesses no modification activity. In fact, the cells treated with it agglutinate even less than normal cells at lectin concentrations greater than 50 μg/ml. The reversal of agglutination observed with pRBC and $cRBC_T$ at higher concentrations of Con A is evidently due to the influence of the non-Con A-binding protein. It does not, however, affect human cells (Fig. 2B). The erythrocyte surface modification activity of the soluble supernatant fraction thus resides entirely in the Con A-binding glycoproteins. A comparison of Coomassie blue-stained components on SDS-PAGE of the soluble supernatant fraction and the Con A-binding material shows that 14 out of 21 polypeptides in the supernatant bind to Con A-Sepharose. Many of the components seen on the electrophoretic gels are obviously heterogeneous since 11 of the 14 components in the lectin-binding fraction have mobilities identical to components present in the non-binding protein. How many of the 14 Con A-specific glycoproteins are involved in erythrocyte modification remains to be determined.

The Con-A-binding proteins possessing erythrocyte modification activity may be secretory products of the tumor cells. Yoshida sarcoma is a hepatoma and, like its parent liver cell, may secrete glycoproteins. However, the active glycoprotein(s) would have to be a protein specifically synthesized and secreted by the tumor cells. It also cannot be a plasma glycoprotein synthesized by the liver or any other organ in response to the tumor. In such an event the modification activity would first appear in plasma, affecting $cRBC_T$ earlier than pRBC. Another possibility, which we currently favor, is that some of the cell surface glycoproteins of the tumor cells may be digested by proteolytic enzymes present in the ascites fluid. Yoshida sarcoma cells, like other tumor cells, are highly agglutinated by Con A. The Con A-receptors on the tumor cell surface in addition to other proteins/glycoproteins could be cleaved by proteases. Such a possibility is supported by the results of Baumann and Doyle [26] and Howard *et al.* [27]. The released Con A-binding glycoproteins would accumulate in the ascites fluid and eventually appear in the circulation. At present, of course, there is no evidence to decide between these possibilities.

Since the modification activity resides in soluble glycoprotein(s) it is unlikely that the glycoprotein gets inserted into the erythrocyte lipid bilayer. It probably binds to peripheral components of the outer cell surface by ionic forces involving, for example, sialic acid or lysine residues of membrane glycoproteins/proteins.

We have studied the Con A-agglutinability of five chronic myeloid leukemia patients' erythrocytes. In contrast to normal human erythrocytes, which do not agglutinate with Con A, the erythrocytes of one patient were found to agglutinate at as low as 10 μg/ml of the lectin.

Apart from the mechanism described above, two other mechanisms are conceivable: one is that in response to the tumor, the bone marrow may release abnormal cells in circulation which may have different agglutinability than the cells in the peripheral circulation. However, in such

an event, two populations of red cells with agglutination peaks at 50 and 250 μg/ml should be detectable. This is not the case; the second possibility is that red cells are modified only in the ascites fluid and are subsequently absorbed into the circulation. Red cells can be taken up into circulation from the peritoneal cavity [28]. However, in such a case the properties of $cRBC_T$ and pRBC should be identical. Also, there should be a gradual increase in the appearance of modified cells in the circulation rather than a sudden conversion of the entire population on the third day of transplantation. The existence of the modification activity in blood plasma is also inconsistent with this mechanism. In one experiment (results not shown) the ascites tumor was inoculated subcutaneously in a rat, which produced a solid tumor at the site of injection. The red cells in the circulation of this animal examined a week later showed maximal agglutination at 50 μg/ml Con A.

It is possible that the mechanism modifying agglutinability of normal cells adjacent to uterine cervical carcinoma [21] and of erythrocytes in Ehrlich ascites tumor-bearing mice [9] is similar to the one found in the present study. The modifications occurring in other properties such as survival, deformability, osmotic fragility, etc., may or may not be similar. Recently it has been reported that the drug-metabolizing ability of normal tissue regions neighboring a hepatoma in rats and in humans is enhanced [29]. Thus, apart from surface modification, other functions of host cells are affected by the tumor [30].

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