Difference of Lectin Receptors Between the Free Cell Type and Island Forming Cell Type of Rat Ascites Hepatoma Cells

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Summary

The cell surface glycoconjugates of various rat ascites hepatoma cell lines with different degrees of adhesiveness were compared, by binding assays, using ^{125}I-labelled lectins. Effects of neuraminidase, TPCK-trypsin and chondroitinase ABC treatment of the cells, on the number of lectin receptor sites were also studied. The TPCK-trypsin treatment caused a marked decrease in the number of peanut agglutinin receptor sites on the island forming and mixed cell types. The decrease of wheat germ agglutinin receptor sites and the increase of castor bean agglutinin receptor sites, after neuraminidase treatment, were larger on the free cell type. It is therefore possible that α-sialyl-β-D-galactosyl residues are abundant on the cell surface of this type, and that its low cell adhesiveness may be due to a electrostatic repulsion of negative charges of the sialic acid.

Introduction

A decrease or loss of cell adhesiveness has been reported to be one of the causes of the malignancy of tumor cells (Hirono, 1958, Abercrombie et al., 1962). Since cells are in contact with each other, the substances of the cell surface, especially glycoconjugates, are likely to be involved in cell adhesiveness. Rat ascites tumor cells are particularly useful for the study of cell adhesiveness, since there are a number of cell lines with differing degrees of adhesiveness. These cell lines can be classified into three types; an island forming cell type, a free cell type and a mixed cell type. The island forming cell type maintains cell adhesiveness and forms cell aggregates called 'islands' in ascitic fluid. The free cell type has completely lost cell adhesiveness, and exists as free cells and has a tendency to kill the host rat more quickly than the island forming cell type. The mixed cell type has both small cell aggregates and free cells. Saito (1973), has studied the mucopolysaccharide contents of various rat hepatoma

cells and reported that the island forming cell type has heparan sulfate as a major mucopolysaccharide, while the free cell type has chondroitin sulfate as a major component. Yamashina et al. (Funakoshi et al., 1974, Nakada et al., 1975, and Mutoh et al., 1976), have purified various glycoproteins from the cell lines; AH66, AH130 and AH130FN and Taki et al. (1978), have reported on the glycolipid contents of the cell lines; AH130, AH130FN, AH7974 and AH7974F. The elucidation of the particular glycoconjugate components responsible for cell adhesiveness by classical methods would require prohibitively large amounts of material and time consuming analyses. However, a survey of the cell surface glycoconjugates by lectin binding assays can be performed with only small amounts of tumor cells and can give a quantitative and comprehensive characterization of sugar chains on the intact cell. This is because lectins have affinities for specific sugar structures of glycoconjugates, although lectin binding assays do not provide information regarding the actual identity of the glycoconjugates.

In this study, lectin binding assays were performed on intact tumor cells and also on tumor cells modified with various enzymes to yield information on the nature of the lectin receptors. The possible relations between the lectin receptors and cell adhesiveness are discussed.

Materials and Methods

Materials

$Na^{125}I$ (393 mCi/m*l*) was obtained from New England Nuclear, Boston, Mass., and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin of bovine pancreas from Worthington Biochemicals Ltd., Freehold, New Jersey, chondroitinase ABC of *Proteus vulgaris* from Seikagaku Kogyo Co., Ltd., Tokyo, Japan, and neuraminidase of *Arthrobacter ureafaciens* from Nakarai Chemicals Ltd., Kyoto, Japan.

Lectins

Crude lectin preparations were obtained as previously reported, RCA (castor bean, *Ricinus communis* agglutinin) and SBA (soybean agglutinin) by the method of Allen and Johnson (1976), WGA (wheat germ agglutinin) by LeVine et al. (1972), AHA (peanut, *Arachis hypogaea* agglutinin) by Terao et al. (1975), and Con A (Concanavalin A) by Agrawal and Goldstein (1972). Crude lectins were purified by affinity chromatography using new affinity adsorbents (Matsumoto et al., 1981). Iodination of purified lectins was performed by the chloramine-T method (Matsumoto et al., 1974) using $Na^{125}I$. The specific activities of the iodinated lectins were $0.1\text{-}1.7\times10^{15}$ cpm/mole.

Preparation of Tumor Cells

Four free cell lines of rat ascites hepatoma, Yoshida sarcoma (YS), AH7974F, AH108AF and AH13, one mixed cell line, AH7974, and two island forming cell lines, AH60C and AH108A, were used. Each cell was transplanted intraperi-

toneally into male Donryu rats weighing 250-300 g. The tumor cells were harvested at pure culture state, and were washed several times with phosphate buffered saline (15 mM Na_2HPO_4-KH_2PO_4-0.15 M NaCl-0.05% NaN_3 pH 7.2, PBS), to remove blood components.

Trypsin Treatment

Cells were washed three times with 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and suspended at a final concentration of 3×10^8 cells in 5 m*l* of the same buffer. The cell suspension was incubated with 20 units of TPCK-trypsin for 10 min at 37° with gentle shaking (Codington et al., 1975). The treated cells were washed three times with PBS containing 0.25% bovine serum albumin (PBS-BSA) by centrifugation and finally resuspended in the same buffer for binding assay.

Chondroitinase Treatment

Cells were washed three times with Veronal buffered saline (0.05 M CH_3COONa-HCl-0.05 M sodium barbiturate-0.14 M NaCl, pH 8.0) containing 0.1% BSA. To a suspension of 4×10^8 cells in 5 m*l* of this buffer was added 5 units of chondroitinase ABC followed by incubation for 30 min at 37° with gentle shaking (Kojima et al., 1971). The treated cells were washed three times with PBS-BSA before use.

Neuraminidase Treatment

Cells were washed two times with PBS (pH 6.8) and suspended at a final concentration of 3×10^8 cells in 5 m*l* of PBS. Then the suspension was incubated with one unit of neuraminidase for 60 min at 37° with gentle shaking (Kawaguchi et al., 1976). The treated cells were washed three times with PBS-BSA before use.

Binding Studies

Binding assays were performed essentially according to the method previously described (Matsumoto et al., 1974). After the incubation for the indicated times, at the temperature indicated, cells were washed three times with cold PBS-BSA by centrifugation at $1500\times g$ for 2 min and then the amount of bound ^{125}I-labelled lectin was determined by the Aloka autowell gamma system ARC-500. Corrections were made for the nonspecific binding of lectin and inactivated lectin. The data for binding assays were plotted by the method of Steck and Wallach (1965).

Results and Discussion

Optimal Conditions for Lectin Binding Assay

To find the optimal conditions, the lectin binding assay using ^{125}I-RCA was performed on two kinds of cells, the AH60C line and the human erythrocyte, at different temperatures with different incubation times. The amount of RCA

bound to erythrocytes reached a plateau in 20 min at 19°, whereas that bound to AH60C cells increased gradually even after 20 min as shown in Figure 1. The effects of temperature on lectin binding were studied for the incubation time of 60 min as shown in Figure 2. An almost linear decrease of lectin binding was observed with erythrocytes as the temperature increased. These results are in good agreement with the report that the affinity constant of the lectin to cell surface saccharides is maximal at 0° (Noonan et al., 1973). On the other hand, the RCA binding to AH60C cells, around room temperature, increased with increasing times of incubation, giving a convex curve as shown in Figure 2. The difference between the RCA binding patterns of erythrocytes and AH60C cells is explained by the endocytosis of RCA by AH60C cells, as was reported for other tumor cells (Aubery et al., 1972, Nicolson et al., 1975, and Noonan et al., 1973). To obtain reproducible data, all of the lectin binding studies thereafter were performed at 0° for 30 min, for which the binding reached a plateau and no endocytosis occurred.

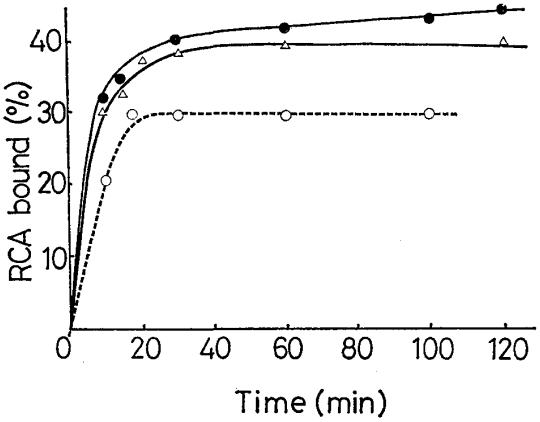

Fig. 1. Time course of RCA binding to AH60C cells (○ or ●) and human erythrocytes (△). After different times of incubation at 0° (-----) or at 19° (——), the amount of bound RCA to the cells was determined as described in 'Materials and Methods'.

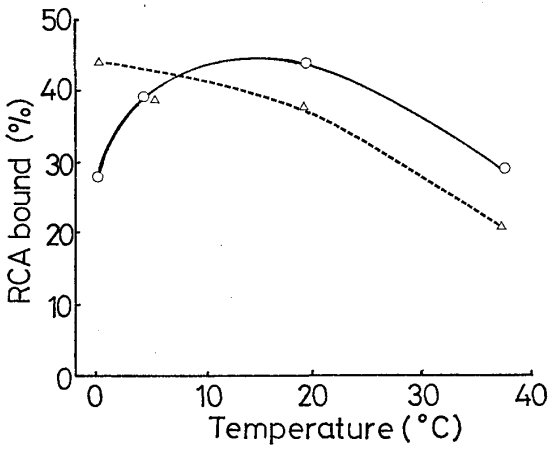

Fig. 2. Effect of temperature on RCA binding to AH60C cells (○) and human erythrocytes (△). The incubation was performed for 60 min at various temperatures (0°-37°).

Lectin Receptor Sites on Intact Tumor Cells

The results of lectin binding assays on intact tumor cells are shown as 'untreated' in Figure 3 and Table I. For the number of receptor sites for the lectin, the following order was observed for each cell line; WGA>RCA>Con A≧AHA=SBA, while no characteristic differences were observed between the free cell type and island forming cell type. Since WGA is known to bind specifically with either α-sialyl or *N*-acetyl-β-D-glucosaminyl residues of the cell surface (Greenaway et al., 1973, and LeVine et al., 1972), these sugar chains must have been the most abundant on the cell surface of each cell line. The number of receptor sites for RCA was markedly larger than that of AHA or

SBA. The specificities of these lectins have been studied mainly on the sugar chains of glycoproteins. Irimura et al. (1975), have reported that RCA has a high affinity for Gal$\overset{\beta}{-}$GlcNAc structures usually found in *N*-glycosidically linked sugar chains of glycoproteins, whereas AHA has high affinity for Gal$\overset{\beta}{-}$GalNAc structures usually found in *O*-glycosidically linked sugar chain of mucin type glycoprotein. Therefore, each cell line may have more *N*-glycosidically linked sugar chains than *O*-glycosidically linked sugar chains on the cell surface.

Con A is known to be specific for either the non-reducing terminal region of a mannose rich sugar chain or the mannose core region of *N*-glycosidically linked sugar chains of glycoproteins. Since the number of receptor sites for Con A was smaller than that of RCA, it is assumed that the amount of mannose rich type *N*-glycosidic sugar chains, of the glycoproteins, was small. Furthermore, if most of the RCA receptors were *N*-glycosidically linked sugar chains of glycoproteins, there must have been a large amount of the *N*-glycosidic sugars with more than four branches, to which Con A would be unable to bind (Ogata et al., 1975), alternatively the non-reducing terminal of some galactose residues might have been substituted with sialic acids (Debray et al., 1978).

Effect of Enzyme Treatment of Tumor Cells on the Lectin Receptors

The effects of enzyme treatments and of washing or incubation of cells without enzyme treatments on the lectin receptor sites of tumor cells are shown in Table I. One of the most significant differences between the cell types examined was a modification of the number of AHA receptor sites by trypsin treatment. In the case of the two island forming cell types, AH60C and AH108A, and a mixed cell type, AH7974, the number of AHA receptor sites was markedly reduced by trypsin treatment, whereas it was not changed in the three free cell type cell lines, YS, AH7974F and AH108AF. Therefore, it was assumed that there are large amounts of trypsin sensitive glycoproteins, as AHA receptors on the cell surface of the island forming cell type and mixed cell type. Although the free cell type was not changed by tryptic treatment, as observed microscopically, cells of the island forming and mixed cell types were found to be disaggregated into either free cells or small cell aggregates. These changes were not observed in the control experiments without enzymes. Since the decrease of the number of AHA receptors was the only common phenomenon concomitant with the dissociation of cell aggregates, upon tryptic treatment of island forming and mixed cell types, it was assumed that the trypsin sensitive AHA receptors of glycoproteins contribute to the cell adhesiveness of island forming and mixed cell types. Though Kudo et al. (1974), reported that the glycoprotein isolated from the cell surface of AH136B could aggregate free cells of AH109A, the relation between their aggregating factor and our trypsin sensitive AHA receptor is unknown and left for future studies.

Some of the AHA receptors on the free cell type, that were also trypsin

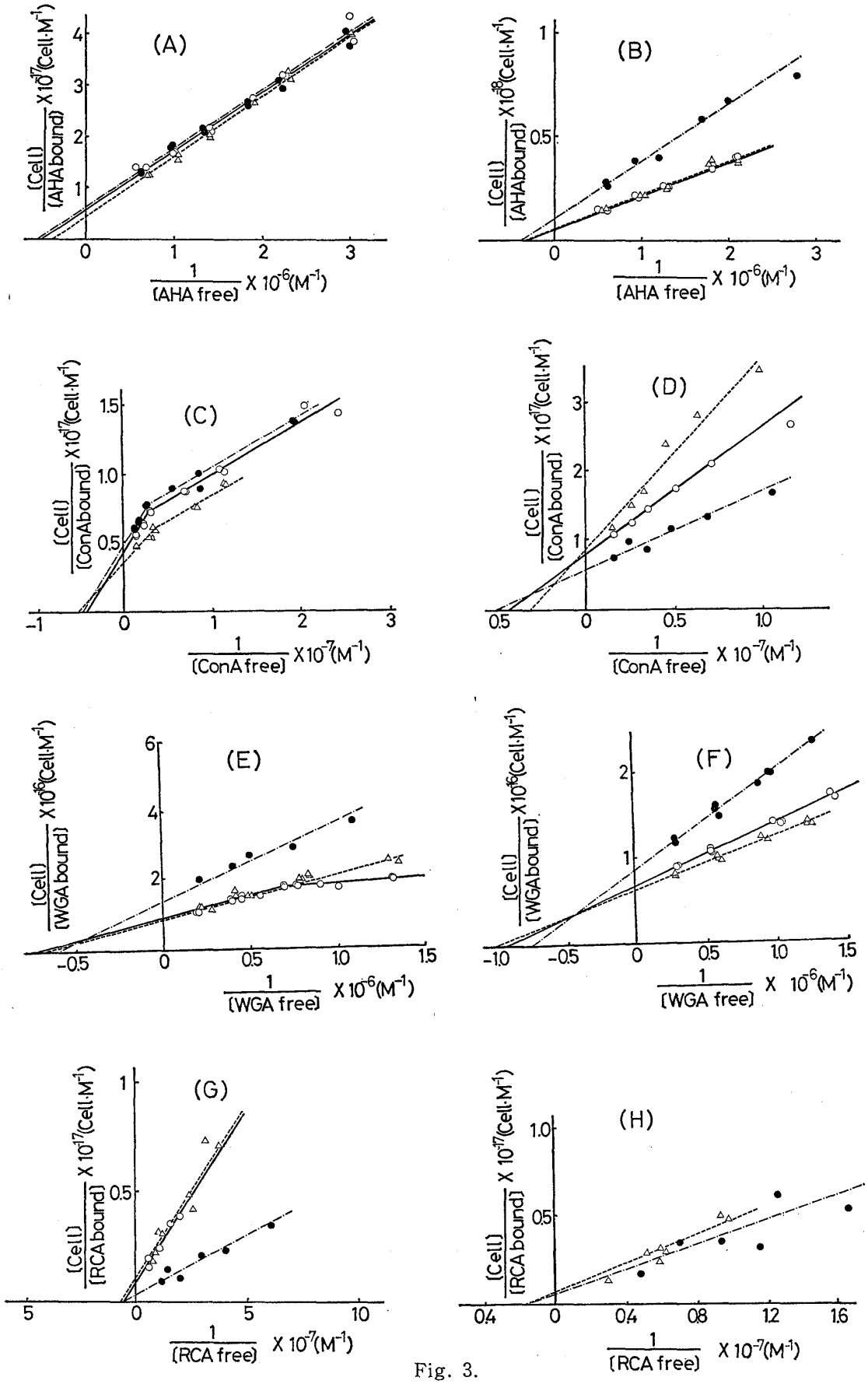
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
{Cell} / {AHA bound} X 10^17 (Cell·M^-1)
{Cell} / {AHA bound} X 10^18 (Cell·M^-1)
{Cell} / {ConA bound} X 10^17 (Cell·M^-1)
{Cell} / {WGA bound} X 10^16 (Cell·M^-1)
{Cell} / {RCA bound} X 10^17 (Cell·M^-1)
1 / {AHA free} X 10^-6 (M^-1)
1 / {ConA free} X 10^-7 (M^-1)
1 / {WGA free} X 10^-6 (M^-1)
1 / {RCA free} X 10^-7 (M^-1)

Fig. 3.

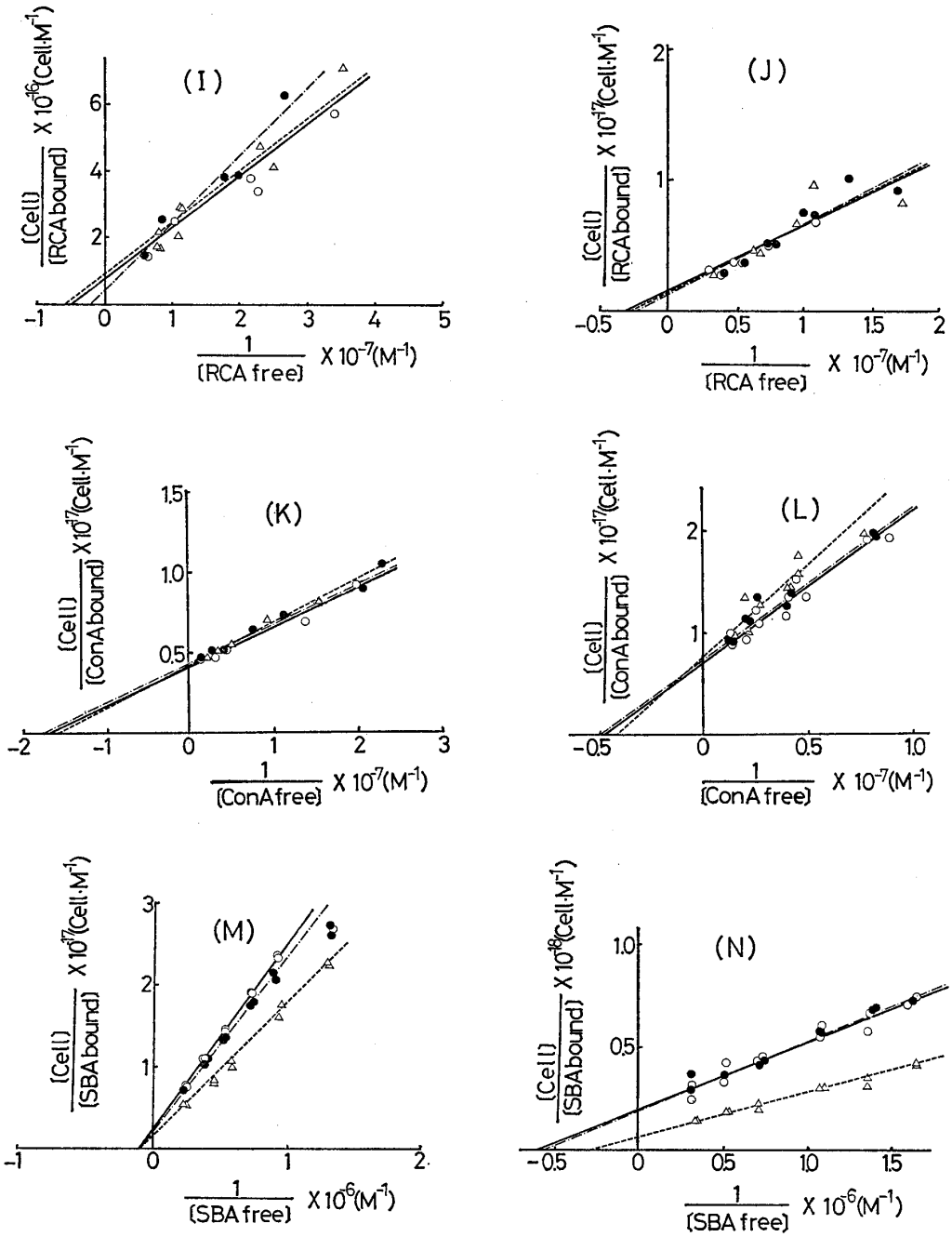

Fig. 3. Binding of ^{125}I-labelled lectins to YS cells and AH60C cells. The data of binding studies are plotted according to Steck et al. (1965). The symbols are for cells, untreated (- -△- -), enzyme-treated (-·-●-·-) and control (—○—). [Lectin free]=the molar concentration of unbound lectin. $\frac{[\text{Cell}]}{[\text{Lectin bound}]}$=the ratio of the cell concentration to the bound lectin concentration.

Effect of TPCK-trypsin treatment on AHA binding to YS cells (A), to AH60C cells (B).
Effect of TPCK-trypsin treatment on Con A binding to YS cells (C), to AH60C cells (D).
Effect of neuraminidase treatment on WGA binding to YS cells (E), to AH60C cells (F).
Effect of neuraminidase treatment on RCA binding to YS cells (G), to AH60C cells (H).
Effect of chondroitinase treatment on RCA binding to YS cells (I), to AH60C cells (J).
Effect of chondroitinase treatment on Con A binding to YS cells (K), to AH60C cells (L).
Effect of chondroitinase treatment on SBA binding to YS cells (M), to AH60C cells (N).

Table I. Lectin binding assays for enzyme-treated and untreated cells

Cell	lectin	AHA	Con A	WGA	RCA	RCA	Con A	SBA
	enzyme	trypsin	trypsin	NANAse	NANAse	CHase	CHase	CHase
YS (free cell type)	untreated	1.3[1](0.4)[2]	1.5 (5.4)	7.1 (0.7)	7.1 (6.3)	7.1 (6.3)	1.4 (5.4)	2.7 (0.2)
	control	1.0 (0.6)	1.4 (4.4)	6.8 (0.7)	7.2 (5.1)	7.6 (5.6)	1.4 (5.7)	2.2 (0.2)
	treated	0.9 (0.6)	1.3 (4.6)	3.9 (0.6)	16 (3.8)	11 (2.7)	1.4 (5.7)	2.2 (0.2)
AH7974F (free cell type)	untreated	0.80(0.5)		15 (3.4)	1.3 (2.4)	1.3 (2.4)		0.77(1.0)
	control	0.58(1.6)	—	—	—	1.9 (1.6)	—	0.92(1.1)
	treated	0.46(0.7)		5.3 (6.3)	4.4 (1.5)	1.6 (1.7)		0.84(1.2)
AH108AF (free cell type)	untreated	0.28(1.5)	0.26(17)	6.2 (1.4)	2.6 (1.4)	2.6 (1.4)	0.26(17)	1.0 (1.2)
	control	0.34(1.1)	—	—	2.5 (1.4)	3.3 (1.1)	0.36(9.8)	1.1 (1.3)
	treated	0.34(1.0)	0.28(14)	2.3 (3.6)	3.3 (6.3)	3.9 (1.0)	0.40(9.5)	1.3 (1.0)
AH13 (free cell type)	untreated	0.40(0.1)	1.7 (3.3)	2.9 (1.8)	2.8 (0.3)	2.8 (0.3)	1.7 (3.3)	0.42(2.7)
	control	—	1.7 (4.5)	2.9 (2.4)	2.1 (0.6)	1.5 (1.4)	1.9 (3.8)	0.51(2.5)
	treated	0.34(0.2)	3.6 (7.1)	0.29(13)	5.4 (0.7)	1.9 (0.6)	1.8 (4.3)	0.51(1.8)
AH7974 (mixed cell type)	untreated	2.6 (0.8)	2.7 (1.6)	3.1 (3.7)	5.5 (1.3)	5.5 (1.3)	2.7 (1.6)	0.76(2.8)
	control	2.9 (0.9)	3.2 (1.5)	1.7 (6.4)	—	3.2 (1.4)	1.9 (2.1)	0.57(1.9)
	treated	0.5 (1.9)	1.7 (1.4)	1.3 (9.4)	6.5 (2.4)	3.6 (1.6)	2.5 (1.5)	0.61(1.8)
AH60C (island-forming cell type)	untreated	1.2 (0.3)	0.71(3.1)	9.7 (1.0)	6.9 (4.8)	4.1 (3.0)	0.80(4.1)	0.79(0.3)
	control	1.2 (0.3)	0.74(4.4)	8.7 (1.0)	—	3.6 (3.4)	0.81(5.1)	0.29(0.6)
	treated	0.61(0.4)	1.1 (5.1)	6.7 (0.8)	9.4 (4.8)	4.9 (2.1)	0.78(5.4)	0.29(0.6)
AH108A (island-forming cell type)	untreated	3.9 (2.6)	7.4 (0.1)				7.4 (0.1)	2.4 (0.9)
	control	—	6.6 (0.1)	—	—	—	—	1.2 (1.9)
	treated	1.5 (1.1)	3.6 (0.2)				3.6 (0.2)	1.3 (1.8)

Various amounts of ^{125}I-labelled lectin were added to the suspension of intact cells, or cells treated with TPCK-trypsin, neuraminidase (NANAse) or chondroitinase ABC (CHase) as described in the Methods section. For the control, cells were incubated without enzyme and washed by same procedure. After incubation for 30 min at 0°, the amount of lectin bound to the cells was determined, and the binding data were analyzed by Steck and Wallach's method (1965). The number of lectin receptor sites per cell were calculated from the reciprocal of the ordinate (Fig. 3). The affinity constants shown in parentheses were obtained from the intercept of the abscissa. Each value is the average of duplicate or triplicate experiments.

1) $=10^{-7}\times$number of lectin receptor sites per cell. 2) $=10^{-6}\times$affinity constant (M^{-1}).

resistant, appeared to be other glycoconjugates apart from glycoproteins. Studies on the inhibitory activities of glycolipids to the hemagglutination of lectins revealed that ceramide trihexoside and paragloboside bind with AHA, RCA and SBA, while ceramide dihexoside having a shorter sugar chain could bind with AHA but not with RCA and SBA. Taki et al. (1978), reported that the content of glycolipids was relatively high in the free cell membrane. Therefore, it was assumed that various glycolipids are also among AHA receptors.

After treatment with trypsin, the number of Con A receptor sites of the mixed cell type AH7974 and island forming cell type AH108A were decreased, while those of the free cell type AH13 and island forming cell type AH60C were increased. This modification of the number of receptor sites for Con A showed no common effect amongst the cell lines in contrast to the case with AHA receptors of the island forming and mixed cell types. Nakada et al. (1978) has reported that the Con A binding capacity of the free cell type, AH130FN, was decreased by trypsin treatment. This was not found with the free cell type as shown in Table I.

The neuraminidase treatment induced a decrease of the number of receptor sites for WGA as well as an increase of that for RCA, on all cell lines, as shown in Table I. These changes were greater on the free cell type than on the island forming and mixed cell types. Some of the WGA receptors may have been α-sialyl residues since they were removed by neuraminidase treatment. Sialyl residues have already been reported to be WGA receptors on various cells (Aubery et al., 1978, Greenaway et al., 1973, Monsigny et al., 1980, Nicolson et al., 1975, and Rosen et al., 1977). Since more WGA receptor sites on the free cell type were removed by the neuraminidase treatment, more sialyl residues seemed to be located on the cell surface of the free cell type than on that of other types. This is in good agreement with the results of cell electrophoresis by Kojima and Maekawa (1972), showing that the free cell type moved to the anode faster than either the island forming cell type or neuraminidase treated free cell type. Regarding the specificities of WGA, it was also reported that sialyl residues of both glycolipids (Monsigny et al., 1980) and glycoprotein can bind with WGA. Taki et al. (1978), reported that glycolipids of the free cell type have a higher content of sialic acid than the corresponding island forming cell type. Therefore, sialyl WGA receptors on the free cell type may be not only glycoproteins but also glycolipids.

Kojima et al. (1972), suggested that a high content of sialic acids on the free cell type contributes to the dissociation of cell clamps by the electrostatic repulsion of carboxyl groups of sialyl residues. The observations obtained in this study, that the free cell types AH108AF and AH7974F were aggregated to form island after neuraminidase treatment, may support their finding.

The increase of the number of RCA receptor sites by neuraminidase treatment seemed to be due to the unmasking of β-galactosyl residues by the removal of terminal sialyl residues, suggesting the presence of $SA\overset{\alpha}{-}Gal\overset{\beta}{-}$ structures on

the cell surface (Nicolson et al., 1975). The increase of the number of RCA receptor sites was greater on the free cell type than on the island forming and mixed cell types. Despite setting optimal conditions for the lectin binding assay, some experimental errors were inevitable and this seemed to be one of the reasons why the increase in the amount of RCA receptors did not always correspond to the decrease in the amount of WGA receptors.

The number of receptor sites for SBA was not changed on any of the cell types by chondroitinase treatment. Therefore, chondroitin sulfate is probably not the SBA receptor on the cells, although *Phaseolus vulgaris* agglutinin with similar specificities to SBA was reported to bind with chondroitin sulfate (Buonassisi et al., 1977). Interestingly, some of the receptor sites for SBA on the island forming cell type were found to be removed by only washing and incubation procedures without chondroitinase, but their structures are unknown.

The treatment with chondroitinase induced a high increase of RCA receptor sites on the YS cells, but not on the AH60C cells. This is in good agreement with the report by Nakada et al. (1977), that chondroitinase treatment induced an increase of RCA receptor sites on the free cell type, AH130FN, but not on the island forming cell type, AH130, and did not change the number of Con A receptor sites on either cell type. It was reported in a previous paper (Saito, 1973) that YS cells have chondroitin sulfate as a main mucopolysaccharide component and that AH60C cells have heparan sulfate. Therefore, it was assumed that the RCA receptor sites were exposed by the removal of chondroitin sulfate on the YS cells, but not on the AH60C cells. However, such an increase of RCA receptor sites was not observed on other free cell types; AH7974F, AH108AF and AH13, and was assumed not to be a common property of the free cell type.

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