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Surface Properties of Cell Membrane Tested by Lectin Induced Cytoagglutination (I)

In Kyung Lim and Yoon Soo Kim

Department of Biochemistry, Yonsei University College of Medicine, Seoul, Korea

This report describes surface properties of several cell membranes tested by lectin-induced agglutination reactions which were quantitated using the microquantitative particle counter agglutination assay of Davis *et al.* (1976). The quantitative assays of concanavalin A (con A) induced agglutination were performed for rabbit erythrocyte, rat erythrocyte, human erythrocyte, and sarcoma 180 mouse ascites cells.

The percent agglutination versus the con A concentration revealed a sigmoid curve in all cases, but the steepness of the sigmoid curve is variable depending on the cell types. It varies even with the same cell but in different species.

Optimum cell concentration was (0.92-0.95) $\times 10^7$ cells/ml final concentration in the hanging drop, for rabbit erythrocytes, (0.77-1.64) $\times 10^7$ cells/ml for rat erythrocytes, (1.59-2.7) $\times 10^7$ cells/ml for human erythrocytes and (0.23-0.39) $\times 10^7$ cells/ml for sarcoma 180 mouse ascites cells.

When minimal and maximal agglutination percentages were defined as the concentration of con A/ml/1 $\times 10^6$ cells corresponding to 10% and 95% agglutination, minimal and maximal agglutination occured at 0.56 ug, 19.98 ug for human erythrocytes at 0.56 ug, 2.24 ug for rat erythrocytes at 0.08 ug, 1.43 ug for rabbit erythrocytes at 0.12 ug, 14.8 ug for sarcoma 180-mouse ascites cells respectively.

The order of inhibitory activity of α-methyl-D-mannopyranoside (αMM) for each corresponding cells from the highest inhibition was human erythrocytes, rat erythrocytes, sarcoma 180 mouse ascites cells and rabbit erythrocytes. The concentrations of αMM required for 50% inhibition per ml of the final concentration in the hanging drop per 1×10^7 cells were 0.565 umoles for rabbit erythrocytes, 0.072 umoles for rat erythrocytes, 0.018 umoles for human erythrocytes and 3.677 umoles for sarcoma 180 mouse ascites cells, respectively.

From our experimental results we conclude that the cytoagglutination activity was increased with con A, the inhibitory activity with αMM in the presence of con A was decreased, however the sarcoma 180 mouse ascites cells revealed a contradictory result, and might be due to the topological distribution of agglutination site changes to a distribution more favorable for agglutination.

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In recent years, much experimental data suggested major differences in membrane structure between normal and malignant cells ——— plasma membrane alteration (Wallach, 1968; Emmelot, 1973), glycolysis difference (Warburg, 1930), increased sugar transport (Hatanaka, 1974) due to lack of proteins in the cell membrane that function in the active transport, the decreased intracellular communications (Loewenstein and Kanno, 1967 ; Haeysman, 1973 ; Pollack *et al.*, 1975 ; Goldman *et al.*, 1975) by loss of normal cellular affinity, loss of contact inhibition of growth (Coman, 1953 ; Abercrombie and Ambrose, 1962) indicating that the plasma membrane of cancer cells is very different from that of normal cells. The selective precipitation properties by lectin seem to cause a molecular change at the cell surface during transformation (Sharon and Lis, 1972, 1975 ; Nicolson, 1974 ; Annette *et al.*, 1974 ; Ribinson and Bellett, 1975; Sharon, 1977), ——however, we are far from understanding exactly how the surface of the cell changes in the course of malignant transformation.

Several laboratories have reported that the lectins are multivalent plant proteins which bind to a specific cell surface saccharide of the membrane and induce cytoagglutination by cross-linking receptors on different cells (Burger, 1969 ; Inbar and Sachs, 1969 ; Nicolson, 1972 ; Rosenblith *et al.*, 1973). As a result of these properties, lectins have become useful molecular probes to investigate the structure and biological function of plasma membrane saccharide-bearing components (Nicolson, 1974).

Because the molecular mechanisms operative in lectin-induced cytoagglutination are not fully understood, and because so many factors influence lectin induced cytoagglutination (Inbar *et al.*, 1971 ; Rosenblitz *et al.*, 1973 ; Noonan and Burger, 1973 ; Nicolson, 1973 ; Inbar *et al.*, 1973 ; Inbar and Sachs, 1973 ; Gunther *et al.*, 1973 ; Vlodavsky *et al.*, 1973 ; Schnebli and Bächi, 1975 ; Willingham and Pastan, 1975 ; Bächi and Schnebli, 1975 ; Schnebli *et al.*, 1976 , Singer and Morrison, 1976 ; Grieg and Brooks, 1979 ; Gordon and Young, 1979) the assay system used to assess lectin induced cell agglutination seems to be very important, since many laboratories studying the same agglutination reaction have reported dissimiliar, or contradictory results (Vlodavsky *et al.*, 1972 ; Schnebli and Bächi, 1975 ; Singer and Morrison, 1976 , Grieg and Brooks, 1979), however, recently, Davis *et al.* (1976) have reported that a microquantitative method, which utilizes an electronic particle counter, is simple to perform, requires only ug quantities of lectin and receptor, and eliminates the subjectivity inherent in some other assay systems (Oppenheimer and Odencrantz, 1972 ; Deman and Bruyneel, 1973 ; Hwang *et al.*, 1974 ; Rottmann *et al.*, 1974).

The studies with lectin have convinced many workers that the cell membrane plays a central role in modulating control of growth, but it is not known how the control is effected. Much more investigation of surface properties of normal cells with lectin induced cell agglutination are required before it is clear whether the cell surface alterations detected with lectin are merely correlated with transformatin or have a causal role in early malignant processes. We hope to settle these problems by progress in this field in our laboratory.

The report describes the surface properties of several normal cell plasma membranes tested by concanavalin A (con A) induced agglutination reactions and cytoagglutination inhibition reactions by α-methyl-D-mannopyranoside and these were quantified using the microquantitative particle counter agglutination assay of Davis *et al.* (1976) and compared with those of normal cell agglutination properties of lectin with that of a transformed cell induced by a chemical

carcinogen.

MATERIALS

Human erythrocytes were prepared from blood (group O.Rh^+) freshly drawn in a heparin tube (1,000μ/ml, Riker Lab. Inc.,). Rabbit erythrocytes were prepared from blood freshly obtained by cardiac puncture in the heparin tube. Rat erythrocytes were prepared from the blood freshly obtained by puncture of orbital fossa of rat after ether anesthesis. Sarcoma 180 mouse ascites cells were collected by syringe using a 17 gauge needle from the ascites of mice inoculated 2 weeks previously by sarcoma 180 mouse ascites cells.

The cells were washed 3 times in phosphate buffered saline (PBS), pH 7.4, at 2000rpm for 10 minutes (International centrifuge size 2, Model V), discarding the upper 5% of the cell mass in each of three successive washings, and used for experiments within 2 hours of preparation.

A stock solutins of con A (type III, Sigma Chem. Co.,) containing 0.2 mg/ml, 2.0 mg/ml in PBS, pH 7.4 were prepared and frozen. Serial dilutions (1:2, 1:4, 1:8, ..., 1:128) of this stock solution were made in PBS, pH 7.4 and allowed to stand over night at 4°C prior to use.

25 μmole/ml α-methyl-D-mannopyranoside (Grade III, Sigma Chem. Co.,) stock solution was prepared with PBS, pH 7.4 for cytoagglutination inhibition studies. Serial dilutions (1:2, 1:4, ..., 1:128) of stock solution were made in cold PBS, pH 7.4.

A 1% bovine serum albumin (BSA) solution in PBS, pH 7.4 was made freshly on the day of the assay. BSA, 1 time crystallized and lyophilized, was obtained from Sigma Chem. Co., St. Louis MO.

Agglutination and inhibition assays were performed using the quantitative particle counter agglutination assay of Davis *et al.* (1976) as described for Novikoff hepatoma cells, on a Model ZBI Coulter counter (Coulter Electronics Hialeah, FL.).

The counter was equipped with a 100 μ apperture and a 0.5 ml manometer. Erythrocytes were counted at settings of 1/amplification = 1/2, and 1/aperture current = 1.0 with a window 14-100. While sarcoma 180 mouse ascites cells were counted at a setting of 1/amplification = 32, 1/aperture current = 1/8 with a window 24-100.

METHODS

Agglutination of con A for single type cell and inhibition studies were carried out at room temperature (23±1°C) to avoid the temperature dependent depolymerization of con A (Gordon and Marquardt, 1974).

Cytoagglutination properties of rabbit, rat, human erythrocytes, and sarcoma 180 mouse ascites cells were determined using the method of Davis *et al.* (1976). It was performed as follows; to a 20ml DiLu-Vial® (Elkay products Inc., Mass, 01545) was added 10 μl of 1% BSA in PBS, pH 7.4 and 50 μl of a serial dilution of con A in PBS, pH 7.4 and 50 μl of cell suspension containing $(2.03\text{-}2.1) \times 10^7$ cells/ml of rabbit erythrocytes, 2.54×10^7 cells/ml of rat erythrocytes, $(3.5\text{-}6.23) \times 10^7$ cells/ml of human erythrocytes, $(0.5\text{-}0.85) \times 10^7$ cells/ml of the mouse ascites cells in PBS, pH 7.4. The vial was capped, inverted and allowed to stand at 37°C in a water bath for 1 hour. After 1 hour, 10ml of cold PBS, pH 7.4 were pipetted down the side of the vial, the vial was gently inverted three times and the single cells counted. The percent agglutination was defined as $100 \times [1.00\text{-}(a\text{-}b)/(c\text{-}b)]$: where a, is the number of single cells present at a serial dilution of lectin; b, the number of single cells present at maximal agglutination; and c, the number of single cells present in absence of lectin.

Assay for inhibition of con A induced cytoagglutination was accomplished in the following manner; to a 20 ml DiLu-Vial® was added 10 μl of 1% BSA in PBS, pH 7.4, 25 ul of a serial dilution of inhibitor in PBS, pH 7.4, 25 μl of dilution of con A which agglutinated 95% of the cells as determined by a prior agglutination assay. The vials were capped and allowed to stand for 30 minutes at 37°C water bath. After this incubation time, 50 μl of cell suspensions were added into the con A-inhibitor solution. The vials were capped, inverted and allowed to stand at 37°C in a water bath for 1 hour.

All counting was performed identically to that of the agglutination assay. The percent inhibition of agglutination was defined as 100 x [(d-e)/(f-e)]: where d, is the number of single cells present at a serial dilution of inhibitor; e, the number of single cells present in absence of inhibitor; and f, the number of single cells present in the absence of lectin and inhibitor.

RESULTS

Agglutination of rabbit, rat, human erythrocytes and sarcoma 180 mouse ascites cells

Plots of % agglutination versus the con A concentration revealed a sigmoid curve in all cases, but the steepness of the sigmoid curve and the optimal concentration are variable depending on the cell types, and it varies even with the same cell but in different species. Optimal cell concentrations used in the assay were (0.92-0.95) x 10^7 cells/ml for rabbit, (0.77-1.64) x 10^7 cells/ml for rat erythrocytes, (1.59-2.73) x 10^7 cells/ml for human erythrocytes and (0.23-0.39) x 10^7 cells/ml for sarcoma 180 mouse ascites cells expressed as the final concentration in the hanging drop.

Optimal con A concentrations required to give maximal agglutination were 90 μg/ml of the final concentration in the hanging drop technique employed in this assay except for the human erythrocytes which required 10 times higher than that of con A concentration and optimal minimal con. A concentrations to give 10% agglutination were variable depending on the cell types (Fig. 1-4, Table 1). When minimal and maximal agglutination percentages were defined as the concentration of con A/ml/l x 10^6 cells corresponding to 10% and 95% agglutination, minimal and maximal agglutination occurred at 0.08 μg, 1.43 μg, for rabbit erythrocyte, 0.56 μg, 2.24 μg for rat erythrocyte, 0.56 μg, 19.98 μg for human erythrocyte and 0.12 μg, 14.81 μg for sarcoma 180 mouse ascites cell, respectively. These results indicate another difficulty in optimizing conditions for the agglutination assay.

Fig. 4 demonstrates that the degree of cytoagglutination was dependent on the cell concentration for sarcoma 180 mouse ascites cells, in the presence of a low concentration range below 11.36 μg of con A/ml final concentration in the hanging drop, but it was evident that the agglutination in the presence of high

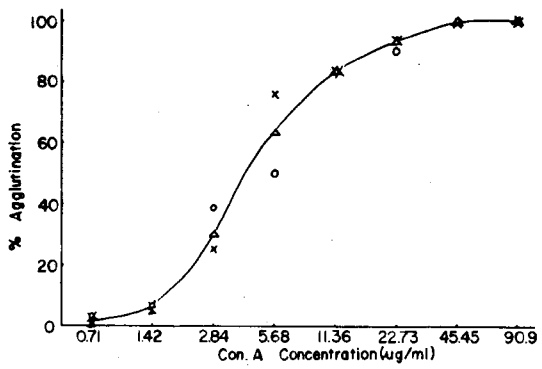

Fig. 1. Con A induced agglutination of rabbit erythrocytes
Abscissa: con A concentration is expressed as ug/ml final concentration in the hanging drop.
Ordinate: % agglutination
Cell concentration range used in the assay was (0.92-0.95) x 10^7 cells/ml final concentration in the hanging drop. The data points (○ x △) shown on the Graph are the value of % agglutination at specific con A concentrations in each assay.

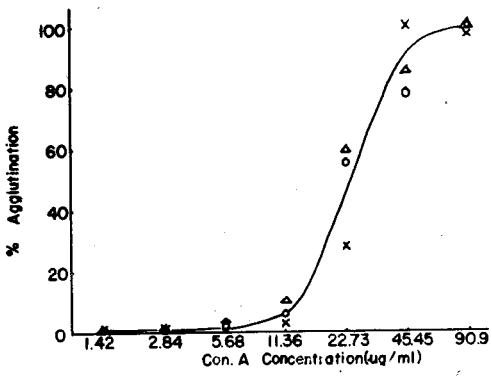

Fig. 2. Con A induced agglutination of rat erythrocytes

Abscissa: con A concentration is expressed as ug/ml final concentration in the hanging drop

Ordinate: % agglutination

Cell concentration range used in the assay was $(0.77\text{-}1.64) \times 10^7$ cells/ml final concentration in the hanging drop. The data points (○ × △) shown on the graph are the value of % agglutination at specific con A concentrations in each assay.

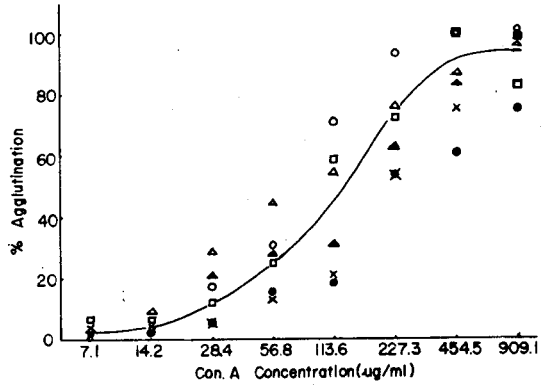

Fig. 3. Con A induced human erythrocyte ($O.Rh^+$) agglutination

Abscissa: con A concentration is expressed as ug/ml final concentration in the hanging drop.

Ordinate: % agglutination

Cell concentration range used in the assay was $(1.59\text{-}2.73) \times 10^7$ cells/ml final concentration in the hanging drop. The data points (○ × △ □ ● ▲ shown on the graph are the value of % agglutination at specific con A concentrations in each assay.

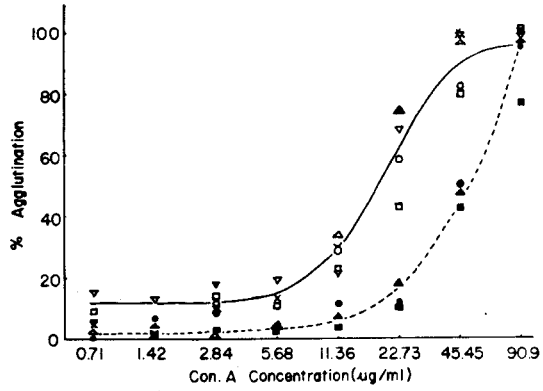

Fig. 4. Con A induced agglutination of sarcoma 180 mouse ascites cells

Abscissa: con A concentration is expressed as ug/ml final concentration in the hanging drop.

Ordinate: % agglutination

The line (———) shown on the graph was derived from the data of $(0.23\text{-}0.39) \times 10^7$ cells/ml final cell concentation in the hanging drop, the line (- - - -) shown on the graph was dervied from the data $3 \times (0.23\text{-}0.39) \times 10^7$ cells/ml final cell concentration in the hanging drop. The data points (○ × ▽ □ △ ● ■) shown on the graph are the value of % agglutination at specific con A concentrations in each assay.

concentration range above 45.45 μg of con A/ml final concentration in the hanging drop becomes progressivly independent of the con A concentration, and it seemed to behave differently and resulted in cell aggregation by formation of clusters rather than cell agglutination.

Inhibition of con A-induced cytoagglutination

The inhibitory activities of α-methyl-D-mannopyranoside (αMM) were quantitated as shown in Fig. 5 and Table 2. The order of % inhibition in the presence of specific αMM concentration for each cell type a from the highest inhibition was human erythrocyte, rat erythrocyte, sarcoma 180 mouse ascites cell and rabbit erythrocyte. The concentrations of αMM required for 50% inhibition per ml of final concentration in the hanging drop per

Table 1. Optimal con A concentration required to give maximal and minimal cytoagglutination

Cell	*Optimal cell concentration ($\times 10^{-7}$ cells/ml)	*Con A concentration (ug/ml) required for cytoagglutination of each cell per 1×10^6 cells	
		95% agglutination	10% agglutination
Rabbit erythrocyte	0.92-0.95	1.43 (100)	0.08
Rat erythrocyte	0.77-1.64	2.24 (170)	0.56
Human erythrocyte	1.59-2.73	19.98 (1390)	0.56
Sarcoma 180 mouse ascites cell	0.23-0.39	14.81 (1040)	< 0.12

* means final concentration in the hanging drop.

Parenthesis represents agglutination susceptibility expressed as percentage of the value for the rabbit erythrocyte.

Table 2. Inhibitory activities of α-methyl-D-mannopyranoside

Cell	Inhibitory unit
Rabbit erythrocyte	0.565* (100)
Rat erythrocyte	0.072 (13)
Human erythrocyte	0.018 (3)
Sarcoma 180 mouse ascites cell	3.677 (651)

* Means αMM final concentration in the hanging drop (umoles/ml) required to yield 50% inhibition in the presence of the amount of con A required to agglutinate 95% of the cells per 1×10^7 cells.

Parenthesis represents inhibitory susceptibility expressed as percentage of the value for the rabbit erythrocyte.

1×10^7 cells were 0.565 umoles for rabbit erythrocytes, 0.072 umoles for rat erythrocytes, 0.018 umoles for human erythrocytes and 3.677 umoles for sarcoma 180 mouse ascites cells, respectively (Table 2).

The cytoagglutination properties with con A (Table 1) and the inhibitory properties on methyl α-D-mannopyanoside suggest that the difference of agglutination with con A by cell type and cell species might be due to the total number of binding site (receptor) on the cell membrane surface: The increment of agglutin-

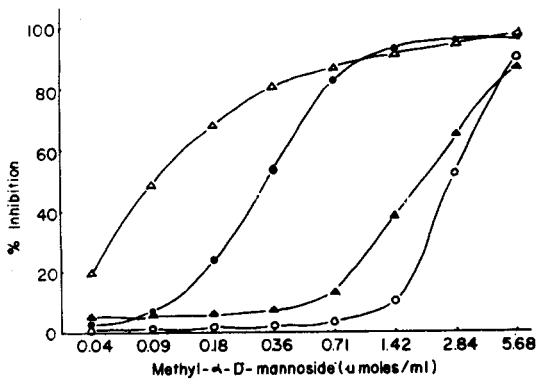

Fig. 5. Effect of inhibitor on con A induced cytoagglutination

Abscissa: concentration of methyl α-D-mannoside (umoles/ml) final concentration in the hanging drop.

Ordinate: % inhibition

The con A concentration used was the concentration required to give 95% agglutination as determined by a prior agglutination assay (Table I). Cell concentration used in this assay was 2.2×10^7 cells/ml for rabbit erythrocytes (○), 2.1×10^7 cells/ml for rat erythrocytes (●), 2.3, $\times 10^7$ cell/ml for human erythrocytes (△) and 0.3×10^7 cells/ml for sarcoma 180 mouse ascites cells (▲). The each line shown on the graph was derived from 5 data points. *expressed as final concentration in the hanging drop.

ability with con A and per unit cells indicates the cell contains more receptors on the cell membrane surface.

DISCUSSION

The research reported in this paper describes the surface properties of several normal and sarcoma 180 mouse ascites cell plasma membranes tested by con A induced agglutination reactions and cytoagglutination inhibition reactions by α-methyl-D-mannopyranoside and these were quantitated by measuring the disappearance of single cells from a cell suspension containing varying amount of con A and its inhibitor.

Because of the complexity and difficulty of lectin induced agglutination reaction and the limitations of the instrumentation, the optimal condition could not always be attained. It varies even with the same cell but from different species. Several parameters which had an influence on lectin induced cytoagglutination reaction were studied to optimize the assay condition for normal rabbit erythrocyte, rat erythrocyte, human erythrocyte and sarcoma 180 mouse ascites cell except other factors, which have been reported to be involved on BSA concentration (Davis *et al.*, 1976), incubation time (Davis *et al.*, 1976), temperature (Vlodavsky *et al.*, 1972 ; Schnebli and Bächi, 1975 ; Davis *et al.*, 1976), metabolic activity (Yahara and Edelman, 1972 ; Gunther *et al.*, 1973 ; Schnebli and Bächi, 1975), receptor mobility (Yahara and Edelman, 1972 ; Vlodavsky et al., 1972 ; Gunther *et al.*, 1973 ; Schnebli and Bächi 1975), mechanical shear (Schnebli and Bächi, 1975 ; Davis *et al.*, 1976) in the lectin induced cytoagglutination reaction.

The data depicting optimal cell concentrations used in the assay were (0.92-0.95) x 10^7 cells/ml final concentration for rabbit erythrocytes, (0.77-1.64) x 10^7 cells/ml for rat erythrocytes, (1.59-2.7) x 10^7 cells/ml for human erythrocytes and (0.23-0.39) x 10^7 cells/ml for sarcoma 180 mouse ascites cells and the optimal cell concentration for each cell was measured at the plateau in the presence of a unique number of cells by a plot of % agglutination versus the con A concentration. Fig. 4 illustrates the effect of cell concentration on cytoagglutination. For sarcoma 180 ascites cells, the optimal cell concentration was (0.23-0.39) x 10^7 cells/ml, however, when 3 times the optimal cell concentration was used, the shape of % agglutination versus the con A concentration seemed to behave quite differently and it was hard to reach a plateau at 45.45 μg and 90.9μg con A/ml but the agglutination revealed progressive independence on the con A concentration and resulted in cell aggregation by formation of clusters rather than cell agglutination and it also revealed less than 10% agglutination in the low concentration of con A (less than 11.36 μg/ml), this indicated that the use of high concentrations of the cell would decrease the sensitivity of the assays and may be especially important in that optimal concentration which exhibits high sensitivity for determination of con A induced cytoagglutination for a single cell type can be determined. Con A concentration required to give maximal and minimal cytoagglutination of cell per 1 x 10^6 cells for a single cell type by using corresponding optimal cell concentration was varied (Table 1). Our experiment has shown that the agglutination activity, which is defined as the con A concentration required to give maximal cytoagglutination per 1x10^6 cells was 1.43 μg for rabbit erythrocyte, 2.24 μg for rat erythrocyte, 19.98 μg for human erythrocyte, 14.81 μg for sarcoma 180 mouse ascites cell, respectively. This indicates that the susceptibility to agglutination is much higher in the rabbit erhtyrocyte than other cells. Previously it has been shown that the agglutination activity with soybean agglutinin as determined by the spectrophotometric me-

thod (Liener, 1955 ; Lis *et al.*, 1970) for rabbit and human erythrocyte is 40 units and 2 units respectively, that is, in the rabbit erythrocyte, the susceptibility to agglutination is higher by at least 20 fold over that of the human (Gordon *et al.*, 1972), these results are very similar to our experimental finding on the rabbit and human erythrocyte agglutination of 14:1 tested by the con A, and it may be a good comparison of the susceptibility difference to agglutination by a different assay system.

Inhibition of con A induced agglutination of rabbit, rat, human erythrocytes and sarcoma 180 mouse acites cell was quantitated in a similar manner by using optimal cell concentrations for corresponding cells in the agglutination. Inhibitory activities of α-methyl-D-mannopyranoside on con A induced cytoagglutination for a single cell type can be comparable by our agglutination inhibitory unit difined as the amount of inhibitor required to yield 50% inhibition in the presence of con A concentration required to agglutinate 95% of the cells (Table 2), and this inhibitory activity was campared to the values of corresponding cell obtained by agglutination susceptibility studies. Agglutination activities for the rabbit erythrocyte determined in this assay was 1.43 μg con A/ml, which was the most sensitive, and 19.98 μg con A/ml, for human erythrocyte, was the least sensitive. Our experimental results indicate that the reciprocal relation between agglutination activities and inhibitory activities, the higher the agglutination activity, of a single cell type revealed the less inhibitory activity was observed in same assay system.

Our experimental result leads us to conclude that the cytoagglutination activity was increased with con A, the inhibitory activity with α-MM in the presence of con A was decreased however. Sarcoma 180 mouse ascites cell revealed contradictory results, which might be due to the topological distribution of agglutination site changes without change of the total number of surface receptor sites and to a distribution more favorable for agglutination which was demonstrated in transformed cells by other investigators (Cline and Livingston, 1971 ; Ozanne and Sambrook, 1971 ; Sela *et al.*, 1971 ; Singer and Nicolson, 1972).

Experiments are now under way to extend studies of the agglutination properties of lectin on transformed liver cells which induced to chemical carcinogen for comparison to those of normal liver cells and of the molecular change of topological distribution of agglutination sites on the surface receptor sites of the cell membrane in the neoplastic stage.

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