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SMALL AMOUNT OF CONCANAVALIN A MODIFIES RADIATION-INDUCED ALTERATION IN CELL-SURFACE CHARGE DEPENDING ON ITS BINDING CONDITION

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SUMMARY

Cell electrophoretic mobility of cultured melanoma cells or rat erythrocytes decreased with time after X-irradiation. Addition of tetravalent concanavalin A or divalent succinyl-concanavalin A before (not after) irradiation, completely blocked the mobility reduction in greater concentrations than 5 μg/l. At 5 μg/l only $3.7 \cdot 10^3$ concanavalin A molecules bound to receptors per cell, while $4.18 \cdot 10^7$ molecules/cell bound at saturating concentrations. Preincubation with concanavalin A at 37 °C was effective even when the cells were treated with α-methylmannoside immediately after irradiation. At low temperature, however, concanavalin A was not effective despite a sufficient amount of bound ^{125}I-labelled concanavalin A. Treatment with α-methyl-mannoside following the binding of concanavalin A at 37 °C before irradiation inhibited the concanavalin A effect depending on temperature. The residual amount of bound lectin could not account for the temperature dependence. The amount of sialic acid (the main charged substance) was not altered by X-irradiation with or without the lectin. Divalent succinyl-concanavalin A was also effective in blocking the radiation effect on electrophoretic mobility. These results seem to suggest that binding of a very small amount of concanavalin A without causing cell agglutination or clustering of its receptors, induces some alteration in the conformation of receptor glycoprotein, which blocks the internalization of acidic sugar residues by subsequent irradiation.

INTRODUCTION

In our previous papers [1–4], we reported changes in electrophoretic mobility of cells and isolated nuclei after X-irradiation in cultured mammalian cells and lymphocytes. The electrophoretic mobility decreased with time and reached a minimum, depending on the dose, 4 h after exposure. As the mechanism of the X-ray induced mobility reduction, we tentatively proposed a change in the conformation of

the cell surface [5], based upon the following findings. (1) Electrophoretic mobility was mainly dependent on the negative charge of sialic acid, chondroitin sulfates, and hyaluronic acid of the cell surface. Radioactive labels incorporated into these acidic sugars were not affected by X-irradiation at the time when the electrophoretic mobility showed minimum value. (2) Sulfhydryl-blocking agents (*p*-chloromercuribenzoic acid, *N*-ethylmaleimide and iodoacetamide) in a concentration range from $1 \cdot 10^{-8}$ to 10^{-6} M completely prevented the reduction in electrophoretic mobility after irradiation. (3) Addition of phytohemagglutinin-M to cell cultures before or after irradiation also stopped the decrease in mobility.

In this experiment, we used concanavalin A as a modifier of X-ray effect to investigate further the mechanism of mobility change because the lectin is well known to bind its specific receptor sites on cell surface and induce a topographic change of receptor sites depending on incubation temperature [6, 7]. Existence of its hapten inhibitor, α-methylmannoside, facilitated the regulation of binding conditions. By utilizing ^{125}I-labelled concanavalin A, the amount of bound concanavalin A on the cell surface was measured to analyse its relation with the blocking effect of the lectin.

MATERIALS AND METHODS

A cultured melanoma cell line B16-C2W [8] and erythrocytes obtained from Wistar-King A rats by heart puncture were employed in this experiment. The culture media used were Ham's F12 medium [9] +10 % fetal calf serum for C2W cells, and Dulbecco's phosphate buffered saline (pH 7.2) for erythrocytes. C2W cells in logarithmic growing phase were used in the whole experiment ($2–4 \cdot 10^6$ cells/9 cm diameter dish).

Cells were washed twice with phosphate buffered saline, and then incubated with concanavalin A or α-methylmannoside at different temperatures immediately before or after X-irradiation. 2 or 4 h after irradiation, C2W cells were mechanically stripped off from culture dish with a rubber policeman for the measurement of cell electrophoretic mobility.

Three times crystallized concanavalin A (carbohydrate free) was obtained from Miles-Yeda Ltd., U.S.A. The lectin had no proteolytic enzyme and glycosidase activities as far as tested. Concanavalin A was iodinated with Na^{125}I (carrier free) by slight modification of Sonoda's method [10]. This labelled concanavalin A was applied on Sephadex G-200 column, eluted with phosphate buffered saline containing α-methyl-D-mannoside, and then dialized. The iodinated lectin newly prepared was used in the experiment.

For the measurement of ^{125}I-labelled concanavalin A bound to cell membrane, C2W cells were washed 4 times with phosphate buffered saline, and then mechanically stripped off from a Falcon plastic dish with a rubber policeman. Radioactivity of gathered cells was measured with Beckman's γ-counter. The cell number was counted with Fuchs-Rosenthal hemocytometer.

Succinyl-concanavalin A was prepared by the procedure of Gunther et al. [11] with a slight modification introducing affinity chromatography on Sephadex G-75 after the final succinylation to remove inactive form. The preparation contained 11 mol of succinyl residues/mol of concanavalin A subunit.

Cell electrophoresis was carried out in 1/15 M phosphate buffered saline

supplemented with 5.4% glucose (pH 7.3, ionic strength 0.167), as previously reported [12]. Measurements of cellular electrophoretic mobility were made at 25±0.5 °C with a Zeiss cytopherometer. Each value of the mobility was obtained by timing the movements of at least 40 cells with a reversal of polarity after each measurement. In the given buffer, the mean mobility of rat erythrocytes was -1.100 ± 0.040 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$.

The amount of sialic acid in erythrocyte membrane was determined by Aminoff's method [13].

Irradiation of the cells in a culture dish was carried out with X-rays at room temperature (15–20 °C). The physical factors of exposure were: 200 kVp, 20 mA, 0.5 mm Al+0.5 mm Cu filter added, HVL 1.13 mm Cu, 40 cm target-sample distance, and dose rate 105 R/min.

RESULTS

Change in electrophoretic mobility

The time-course change of electrophoretic mobility of C2W cells after irradiation is indicated in Fig. 1. With 500 R-irradiation only, the mobility decreased progressively with time and reached a minimum 4 h after exposure. When concanavalin A (0.1 μg/ml) was added before irradiation and remained for the subsequent incubation at 37 °C, the mobility reduction was completely blocked. Addition of concanavalin A immediately after irradiation, or administration of α-methylmannoside with concanavalin A before irradiation did not show the blocking effect.

Fig. 2 exhibits the relationship between the concentration of concanavalin A in phosphate buffered saline and electrophoretic mobility 4 h after irradiation with 500 R in C2W cells. Concanavalin A was added before irradiation and remained during

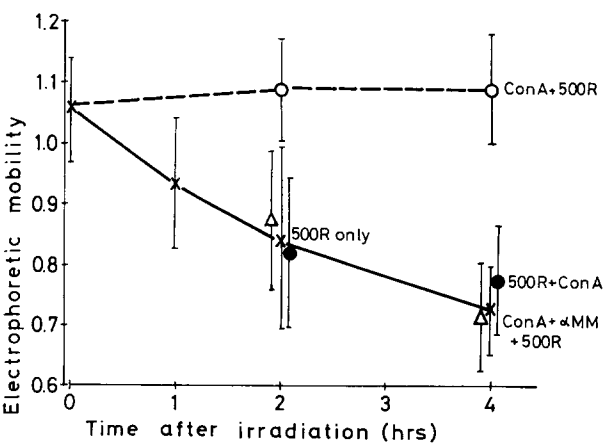

Fig. 1. Change in the electrophoretic mobility ($-\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) with time after X-irradiation with 500 R in C2W cells. Addition of concanavalin A (Con A) (0.1 μg/ml) or α–methylmannoside (αMM) (0.05 N) before irradiation (Con A+500 R, – – –) or (Con A+αMM+500 R, △). Addition of concanavalin A immediately after irradiation (500 R+Con A, ●). Concanavalin A and α-methylmannoside persisted during the subsequent incubation. The vertical lines represent one standard deviation for 30–100 measurements.

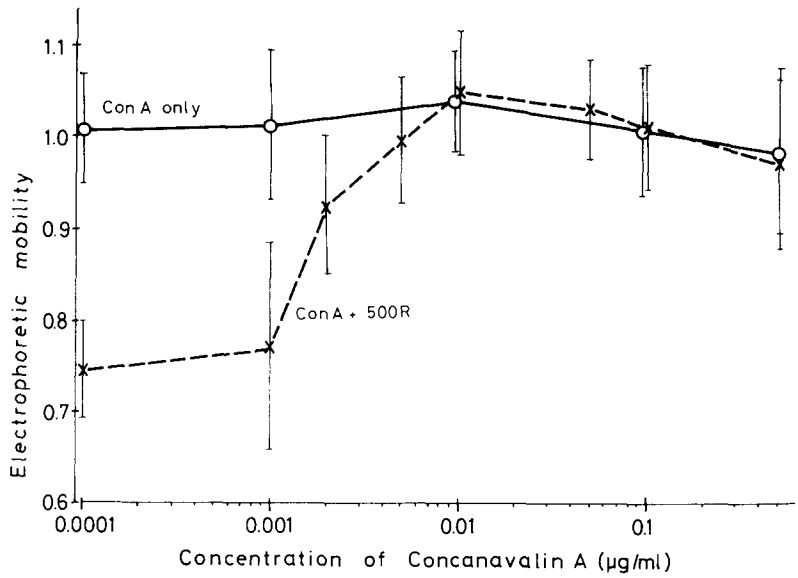

Fig. 2. Relationship between the concentration of tetravalent concanavalin A in phosphate buffered saline (pH 7.2) and electrophoretic mobility ($-\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) 4 h after irradiation with 500 R in C2W cells. Concanavalin A was added before irradiation and remained during subsequent incubation for 4 h. The vertical lines represent standard deviations for 30–100 measurements.

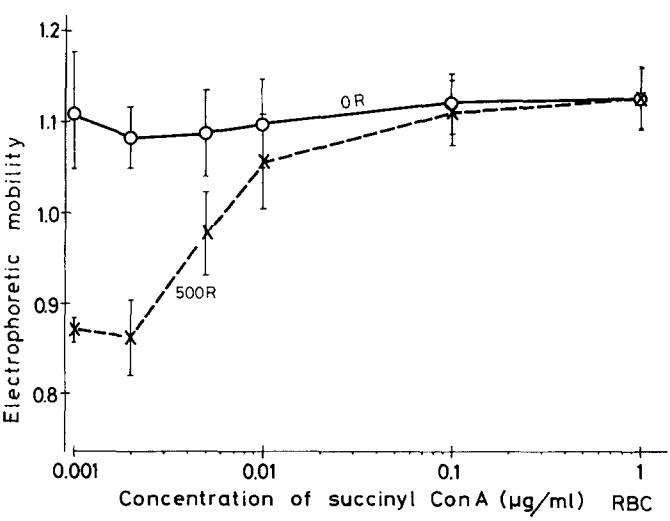

Fig. 3. Relationship between the concentration of succinyl-concanavalin A in phosphate buffered saline and electrophoretic mobility ($-\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$) 4 h after irradiation with 500 R in rat erythrocytes. The vertical lines represent standard deviations for 20–50 measurements.

the next 4 h incubation. Concanavalin A in concentrations higher than 10^{-2} μg/ml exhibited a complete blocking of mobility change, and in concentrations less than 10^{-3} μg/ml showed no effects. Treatment with concanavalin A per se had no substantial effect on electrophoretic mobility at a concentration range from 10^{-4} to 10^{-1} μg/ml.

Fig. 3 demonstrates that divalent succinyl-concanavalin A could block the mobility decrease by irradiation in erythrocytes. The effect of succinyl-concanavalin A varied markedly at concentrations between $2 \cdot 10^{-3}$ and 10^{-2} μg/ml, as demonstrated with tetravalent concanavalin A in Fig. 2 in C2W cells. Agglutination of C2W cells was evident by tetravalent concanavalin A in concentrations above 5 μg/ml, but not apparent by succinyl-concanavalin A up to 10 μg/ml.

Table I indicates the relationship between the sequence of treatments and electrophoretic mobility 4 h after irradiation with 500 R in C2W cells. Treatment with concanavalin A (0.1 μg/ml), α-methylmannoside (0.05 N), X-irradiation and subsequent incubation were carried out in phosphate buffered saline. Incubation of cells with concanavalin A for 5, 10 or 30 min at 37 °C or 20 °C before irradiation, completely blocked the decrease in electrophoretic mobility by X-irradiation even when concanavalin A was removed by α-methylmannoside immediately after irradiation.

Administration of concanavalin A at 3 °C resulted in a slight effect. Treatment with α-methylmannoside at 37 °C (but not at 3 °C) before irradiation negated the concanavalin A effect. Addition of concanavalin A immediately after irradiation exhibited no effect. The effect of the lectin changed drastically with an incubation temperature between 20 and 3 °C. Strict caution was required to precool all of the cells, buffer solution for washing and concanavalin A solution. This experiment was repeated five times, but standard deviation was still large.

TABLE I

RELATIONSHIP BETWEEN THE SEQUENCES OF TREATMENT AND THE CELL ELECTROPHORETIC MOBILITY 4 H AFTER IRRADIATION IN C2W CELLS

Concanavalin A (Con A) 0.1 μg/ml, α-methylmannoside (αMM) 0.05 M in phosphate buffered saline. Results are the mean ±S.D.

Treatments	Electrophoretic mobility ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$)
No treatment	−1.059±0.087
500 R only	−0.727±0.076
3 °C 10 min	−1.064±0.086
αMM 37 °C 10 min	−1.057±0.084
Con A 37 °C 30 min; 500 R; αMM 37 °C 10 min	−1.096±0.089
Con A 37 °C 5 min; 500 R; αMM 37 °C 10 min	−1.093±0.087
Con A 20 °C 10 min; 500 R; αMM 37 °C 10 min	−1.057±0.090
Con A 3 °C 10 min; 500 R; αMM 37 °C 10 min	−0.856±0.113
Con A 37 °C 30 min; αMM 37 °C 10 min; 500 R	−0.770±0.077
Con A 37 °C 2 h; αMM 37 °C 10 min; 500 R	−0.814±0.089
Con A 37 °C 2 h; αMM 3 °C 10 min; 500 R	−1.104±0.093
500 R; Con A	−0.775±0.090
Con A, αMM; 500 R	−0.715±0.090

Amount of bound concanavalin A on cell surface

The specific activity of the original ^{125}I-labelled concanavalin A obtained by iodination and purification was $7.18 \cdot 10^5$ cpm/μg. For use in experiments, specific activity was adjusted to give from $5 \cdot 10^2$ to $5 \cdot 10^4$ cpm for each cell sample after incubation for 30 min with a wide range of concanavalin A concentrations. Fig. 4 shows the relationship between a concentration of concanavalin A in phosphate buffered saline and the amount of bound lectin to 10^5 C2W cells after incubation for 30 min at 37 °C. The bound concanavalin A increased proportionally to concentrations up to 10 μg/ml and saturated at higher concentrations. The binding amount after incubation with concanavalin A (100 μg/ml) for 60 min at 37 °C was 0.710 μg/10^5 cells. The time course experiment indicated that this was the maximum binding quantity. Since molecular weight of tetrameric concanavalin A is reported as 102 000 [14], 0.710 μg/10^5 cells is calculated as $4.18 \cdot 10^7$ molecules/cell. At a concentration of 5 μg/l which showed almost complete blocking of mobility reduction after X-irradiation, only $3.7 \cdot 10^3$ concanavalin A molecules bound to receptors per cell (about 1 in 10^4 receptors). Incubation with concanavalin A (0.1 μg/ml) at 3 °C for 30 min resulted in binding of the lectin of $8.7 \cdot 10^{-4}$ μg/10^5 cells. This amount of

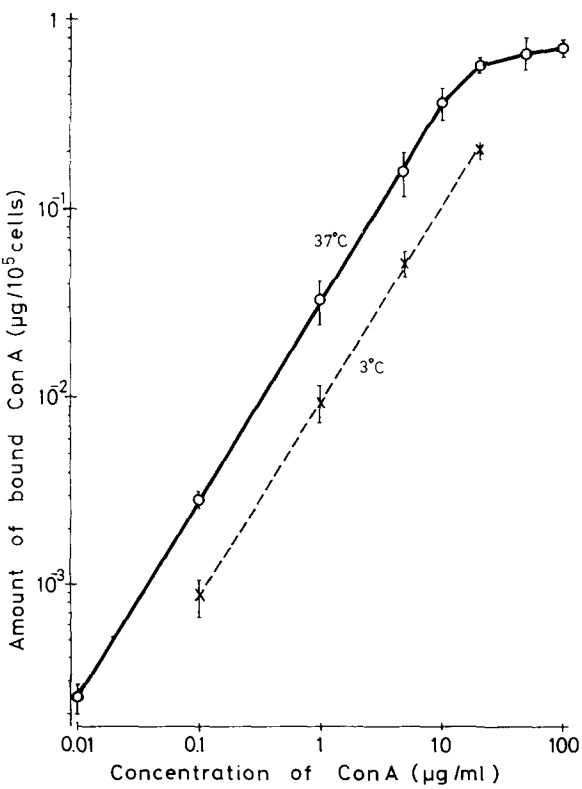

Fig. 4. Relationship between the concentration of concanavalin A in phosphate buffered saline and the amount of bound concanavalin A /10^5C2W cells after the incubation for 30 min. The vertical lines represent standard deviations for 6–18 measurements in 3 separate experiments.

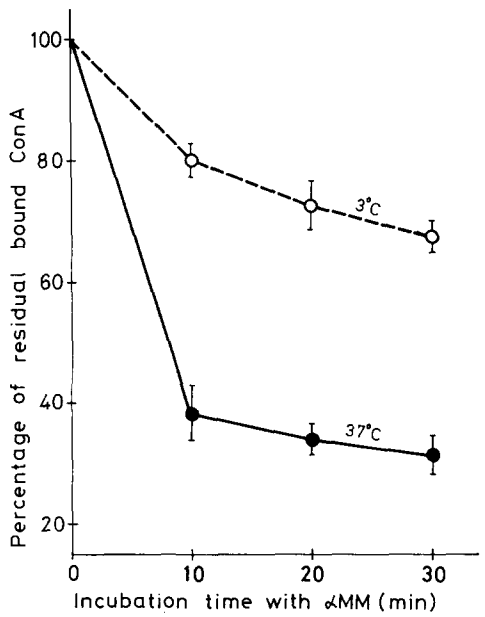

Fig. 5. Change in the amount of residual bound concanavalin A on cell membrane with time of incubation with α-methylmannoside (αMM) (0.05 N). C2W cells were preincubated with ^{125}I-labelled concanavalin A (1 μg/ml) for 30 min at 37 °C, washed 3 times with phosphate buffered saline, and then incubated with α-methylmannoside at 3 or 37 °C. The vertical lines represent standard deviations for 6–12 measurements in 3 separate experiments.

concanavalin A was sufficient to block mobility reduction, if such cells were shifted up to 37 °C before irradiation without change in the amount of bound concanavalin A.

Fig. 5 shows the time course of the release of ^{125}I-labelled concanavalin A from cell surface by incubation with α-methylmannoside. C2W cells were preincubated with ^{125}I-labelled concanavalin A (1 μg/ml) for 30 min at 37 °C, washed with phosphate buffered saline, and then incubated with α-methylmannoside (0.05 N) at 3 or 37 °C. About twice as much concanavalin A was released from the cell surface at 37 °C as compared to 3 °C. The residual amount of concanavalin A ($1.2 \cdot 10^{-2}$ μg/ml) after the treatment with α-methylmannoside at 37 °C, however, should have been a sufficient quantity to block the mobility reduction by X-irradiation.

Amount of sialic acid

Rat erythrocytes were used in this experiment, because in erythrocytes, sialic acid is the single main negatively charged substance, and cytoplasmic membrane can be easily obtained free from cytoplasm and deoxyribose which sometimes interfere in the estimation of sialic acid. Removal of sialic acid from erythrocyte surface by the treatment of neuraminidase (1 unit/ml at 37 °C for 30 min) resulted in a 60 % reduction in electrophoretic mobility.

The amounts of sialic acid in erythrocyte ghosts (prepared by hypotonic hemolysis) were 87.2 μmol/g protein in unirradiated control cells, and 90.1 in irradiated cells with 500 R, 4 h previously, 86.4 in irradiated cells with 0.1 μg/ml concanavalin A, and 82.7 μmol/g protein in cells of concanavalin A treatment only. This result indicates

that the amount of sialic acid in erythrocyte membrane was not altered by X-irradiation with or without concanavalin A unrelated with electrophoretic mobility.

DISCUSSION

Cell agglutination [6, 7, 15], resistance to trypsinization [16], clustering of agglutinin-binding sites [17–19, 6], and change in cell electrophoretic mobility (ref. 20, and Kojima et al., unpublished data) have been reported as concanavalin A effects at 37 °C but not at 3 °C. Blocking of radiation-induced electrophoretic mobility reduction by concanavalin A also exhibited temperature dependence. A noteworthy difference of this newly found effect is the requirement of a very low concentration of concanavalin A. Cell agglutination and resistance to trypsinization appeared at a concentration of 5 μg/ml and became almost complete at 20 μg/ml [16]. Blocking of mobility reduction, however, appeared at 0.002 μg/ml and became complete at 0.01 μg/ml. Incubation with concanavalin A in concentrations 5 μg/ml or 20 μg/ml for 30 min at 37 °C resulted in the binding of concanavalin A molecule to 21 or 80 % saturation. At 0.002 μg/ml or 0.01 μg/ml, however, the fraction of receptors bound with concanavalin A were $6 \cdot 10^{-5}$ or $3 \cdot 10^{-4}$ saturation, respectively. Preincubation with the lectin was required. Added concanavalin A after X-irradiation was not effective. The effectiveness varied substantially with incubation temperature despite the amount of bound lectin at 3 °C. Low fluidity of the membrane lipids is thought to be responsible for the lack of movement of the membrane components at low temperatures in the fluid mosaic model of the membrane [21]. A low concentration of tetravalent concanavalin A or succinyl-concanavalin A did not induce the agglutination of C2W cells or erythrocytes, nor did succinyl-concanavalin A induce capping of the lectin receptors [11]. The effect of a very small amount of bound concanavalin A or succinyl-concanavalin A (1 molecule in 10^4 receptors) which we reported in this experiment, seems not necessarily related to the lateral movement of receptor proteins by cross-linkage with multi-valent lectins, as proposed in mitogenic effect [11]. The use of monovalent concanavalin A would clarify the relation. A configuration change of receptor glycoprotein itself by the binding of concanavalin A before irradiation might have blocked a further conformational change by X-irradiation. Ineffectiveness of binding at low temperature or inhibition of the concanavalin A-effect by treatment with α-methylmannoside at 37 °C despite a sufficient amount of bound concanavalin A at irradiation suggest that a membrane alteration is not induced under these binding conditions. Low fluidity of membrane lipid might restrict not only a lateral movement of receptor proteins, but also restrict a intramolecular rearrangement of receptor proteins by concanavalin A and its reversion by α-methylmannoside through protein-lipid interaction.

Cell electrophoretic mobility is determined by the net negative charge of the cell surface within 10 Å of the hydrodynamic shear plane under the present measuring conditions [22]. Since the amount of sialic acid in membrane was not altered by X-irradiation with or without concanavalin A irrespective of electrophoretic mobility, change in the localization of sialic acid such as to be withdrawn under the surface layer of 10 Å depth is suggested as the mechanism of mobility reduction after irradiation.

Although there are some conflicting literatures [23–25] on cell surface charge

after X-irradiation, the discrepancy seems attributable to a difference in the cell conditions and in the measuring conditions. We found that the cells in saturated cell density had lower electrophoretic mobility than the cells in logarithmic growth, and did not exhibit the mobility change after irradiation. Measurement of the mobility in the buffer solution of low ionic strength [25] did not reveal the mobility reduction by irradiation.

Possible involvement of ATP and contractile proteins of membrane was suggested in mobility reduction after irradiation [26]. Binding of concanavalin A is also reported to inhibit the mobility of immunoglobulin receptors on the lymphoid cell surface [27], and phagocytosis by polymorphonuclear leukocytes [28]. Further investigation is required to clarify the existence and the character of conformational change in glycoprotein induced by concanavalin A or X-irradiation using physico-chemical methods.

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