ROLE OF CELL SURFACE RECEPTOR
MOBILITY IN CONCANAVALIN A-INDUCED
AGGLUTINATION OF NOVIKOFF HEPATOMA
AND NORMAL RAT LIVER CELLS

PAOLA MASTROMARINO, GIOVANNI NERI,* ANGELO SERRA
AND EARL F. WALBORG, Jr†
Department of Human Genetics, Catholic University School of Medicine,  
00168 Rome, Italy, and †The University of Texas System Cancer Center,  
Science Park-Research Division, Smithville, Texas 78957, U.S.A.

SUMMARY
The relationship between Con A-receptor mobility and Con A-induced agglutination of Novikoff hepatoma and normal rat liver cells was investigated. Novikoff cells, incubated with fluorescein-labelled Con A at 3 °C displayed uniform, ring-like surface fluorescence. Increasing the temperature of the cells to 37 °C caused capping of Con A receptors in approximately 65% of the cells, a phenomenon that could be prevented by prefixing the cells with glutaraldehyde. In spite of these variations in Con A-receptor distribution, Con A-induced agglutination was remarkably constant over a temperature range from 3 to 37 °C. In contrast to Novikoff cells, normal hepatocytes displayed a uniform, ringlike surface fluorescence at both 3 and 37 °C. No capping was observed. However, hepatocytes, similar to Novikoff cells, were agglutinable by low concentrations of Con A. These findings indicate that, in this model system, Con A-induced cytoagglutination is not dependent upon long-range lateral mobility of Con A receptors. The qualitative differences in the lateral mobility of cell-surface Con A receptors of normal and malignant rat liver cells may represent a marker for neoplastic transformation during hepatocarcinogenesis, adaptation to growth in ascitic form, or progression of a tumour to a more malignant state.

INTRODUCTION
Since the first report by Aub, Sanford & Cote (1965) and subsequently by others (Burger & Goldberg, 1967; Inbar & Sachs, 1969; Sela, Lis, Sharon & Sachs, 1970; Nicolson & Blaustein, 1972) that malignantly transformed cells are more readily agglutinated by plant lectins than their normal counterparts, a large effort has been directed toward elucidation of the factors responsible for preferential lectin-induced cytoagglutination of malignant cells (reviewed by Nicolson, 1974). In a very simplified way, lectins can be viewed as acting like molecular bridges extending between neighbouring cells and causing agglutination by a mechanism analogous to that responsible for antibody-induced cell agglutination.

To determine whether alterations of cell-surface lectin receptors are responsible for the increased agglutinability of tumour cells, investigations have focused mainly on

* To whom correspondence and reprint requests should be addressed at: Istituto di Genetica Umana, Università Cattolica, Via Pineta Sacchetti, 644-00168 Rome, Italy.
cell-surface saccharide-containing components, particularly glycoproteins. Several differences have been reported between normal and malignant cells with respect to the number, structure, topography and dynamics of cell-surface lectin receptors (reviewed by Smith & Walborg, 1977). Several hypotheses have been advanced to explain the increased lectin-induced agglutination of tumour cells (Rapin & Burger, 1974; Smith & Walborg, 1977). To date, none of these hypotheses has proved of general applicability, possibly because different mechanisms are operating in different cell types. Moreover, the concept that increased agglutinability is a distinctive property of cancer cells is no longer tenable. A number of studies on various mouse tumours failed to show positive correlation between malignancy and lectin-induced agglutinability (Gantt, Martin & Evans, 1969; Friberg, 1972; Dent & Hillcoat, 1972). More recently Starling, Capetillo, Neri & Walborg (1977) showed that Novikoff hepatoma cells and normal rat hepatocytes are both agglutinatable by low concentrations of concanavalin A (Con A) and wheat germ agglutinin. In this same model system, it was found that glutaraldehyde-fixed Novikoff cells were still agglutinatable by Con A, suggesting that Con A-induced agglutination of these cells is independent of cell-surface receptor mobility (Walborg et al. 1975). Moreover, microfilament-active drugs, such as cytochalasins b and d, proved capable of inhibiting Con A-induced agglutination of Novikoff cells, but did not have any apparent effect on the topography of Con A receptors (Glenney, Hixson & Walborg, 1979). The present study was undertaken to clarify the relationship between Con A-induced cytotoxicity and the mobility of Con A receptors on Novikoff hepatoma and normal rat liver cells.

MATERIALS AND METHODS

Cells
Novikoff hepatoma cells (Novikoff, 1957) were maintained in 6- to 10-week-old female Sprague-Dawley rats (Charles River Italia, Calco, Italy) by weekly intraperitoneal transplantation. Normal hepatocytes were obtained as a suspension of cells (largely single cells) from the liver of 6- to 10-week-old female Sprague-Dawley rats by the collagenase perfusion method of Starling et al. (1977). Papain digestion and control incubation of Novikoff hepatoma cells were performed as previously described (Neri, Smith, Gilliam & Walborg, 1974). Cell viability was assessed on the basis of vital dye exclusion test, using nigrosine (Kaltenbach, Kaltenbach & Lyons, 1958).

Labelling of Con A with fluorescein
Con A (Grade III Sigma Chemical Co., St Louis, Missouri) was conjugated with fluorescein isothiocyanate component A (Serva Feinbiochimica, Heidelberg, West Germany) by direct mixing at pH 9.0, as described by Mallucci (1976). The conjugate was purified by affinity chromatography on a column of Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). Material that did not bind to the column or that could be removed by elution with 0.01 M glucose was discarded. The active fraction, eluted from the column with 0.1 M glucose, was concentrated by ultrafiltration on Amicon PM 10 membrane (Amicon B.V., Costerhout, Holland) to a volume of approximately 3.0 ml, and dialysed exhaustively against standard salt solution, pH 7.4 (Mallucci, 1976) to remove glucose. The ratio of the optical densities at 495 and 280 nm, a measure of the degree of conjugation, was 0.5. The concentration of Con A in the conjugate (53 mg/ml) was calculated from the extinction coefficient of the lectin.
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(E_{280} = 13), corrected for the absorption of fluorescein at 280 nm. A virtually identical estimate of the concentration of Con A was obtained by an independent method, i.e. by measuring the haemagglutinating activity of fluorescein-labelled Con A (Fl-Con A) using the haemagglutination assay of Smith, Neri & Walborg (1973). This assay also showed that Fl-Con A had the same specific activity as freshly prepared, unlabelled Con A.

Assay of Con A receptor mobility

Untreated, control-incubated and papain-treated Novikoff cells or untreated normal hepatocytes were washed three times in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS) prepared according to Dulbecco & Vogt (1954), resuspended in the same buffer at a concentration of 5 x 10^6 cells/ml and submitted to the following procedure. To a 4-ml aliquot of cell suspension a specified amount of Fl-Con A was added, and the mixture allowed to incubate for 30 min (unless otherwise specified) at either 3 °C, over crushed ice, at 23 °C, in a temperature-regulated waterbath, or at 37 °C in a metabolic water incubator. Cell suspensions were then chilled to 3 °C and washed 3 times with cold CMF-PBS in a refrigerated centrifuge. The cells were resuspended in 4 ml of buffer and fixed for 30 min at 3 °C by addition of glutaraldehyde to a final concentration of 2 %. The cells were subsequently washed 3 times in CMF-PBS, resuspended in 0.5 ml of the same buffer and observed under a coverslip with a Leitz Orthoplan ultraviolet photomicroscope equipped with epi-illumination and specific filters for fluorescein. In a few experiments the cells were fixed with glutaraldehyde prior to incubation with Fl-Con A. The specificity of binding of Fl-Con A to the cell surface was assessed by incubating the cells with Fl-Con A in presence of 0.5 M methyl-α-D-mannopyranoside (α-MM), a specific inhibitor of Con A (Poretz & Goldstein, 1970). Alternatively, cells incubated with Fl-Con A were washed 3 times in CMF-PBS and post-incubated twice for 15 min at 37 °C in CMF-PBS containing of 0.2 M α-MM. Visual scoring of Fl-Con A-labelled cells allowed their assignment to 1 of 3 classes of cells with different patterns of fluorescence: ringlike, caplike and patchlike. The proportions of cells belonging to the 3 classes were then estimated for the various experimental conditions, and the trend of these proportions was analysed.

Cytoagglutination assays

Untreated, control-incubated or papain-treated Novikoff cells were washed 3 times in CMF-PBS and assayed for Con A-induced agglutination following the procedure of Neri et al. (1974). Con A, purchased from Pharmacia Fine Chemicals, was dissolved in CMF-PBS. Protein concentration was determined spectrophotometrically using the extinction coefficient, E_{280} = 13. The cytoagglutination assays were performed at 3, 23 and 37 °C, with different concentrations of Con A. For the assay at 3 °C, the cytoagglutination plates were set on a thin metal sheet lying on a pan filled with crushed ice. After pipetting the cells and the lectin into the wells, and mixing with a toothpick, the plates were transferred to the cold room, and removed 30 min later for microscopic scoring. For the assay at 23 °C, all operations were performed at room temperature. For the assay at 37 °C, pipetting of the lectin and cells and mixing were performed at room temperature. The plates were then immediately transferred to an incubator at 37 °C, covered with Petri dishes to minimize evaporation, and removed 30 min later for scoring. All agglutination assays were repeated on different days using fresh reagents. The degree of agglutination was scored visually on a serological scale from 0 to 4 (Wray & Walborg, 1971). Then, the least square regression lines of agglutination on the log of the Con A concentration at the 3 temperatures were fitted.

RESULTS

Con A receptor mobility of Novikoff cells

Incubation of Novikoff cells with Fl-Con A resulted in labelling of the cell surface in a ringlike or in a caplike pattern. Caps could take the form of either a protruding fluorescent mass at the uropod of the cell, or a condensation of fluorescence at one
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pole of the cell. Cell-surface labelling was specific, and could be completely inhibited by the presence of 0.5 M α-MM. Discrete patches of fluorescence were also observed in some of the cells scored. The location of these patches at the surface of the cells seems unlikely since they could not be removed by postincubation of the cells with α-MM. Examples of these fluorescence patterns are illustrated in Fig. 1 (panels A–E). The cells shown in the plate were incubated with Fl-Con A (66 μg/ml) for 30 min at 37 °C (except for panel A, showing a cell incubated at 3 °C). These experimental conditions yielded maximal capping as shown in Figs. 2 and 3. Fig. 2, where the percentage of cells with cap is plotted against the concentration of Con A, shows that at 66 μg of Con A/ml the proportion of cells with cap was maximal. This concentration of Con A is sufficient for maximal cell agglutination (Neri et al. 1974). In receptor-mobility experiments, however, cytoagglutination was largely prevented by using conditions, such as low cell concentration and repeated pipetting of the cell suspension, that would minimize cell–cell contacts. The incubation time (30 min) was chosen to render receptor mobility data directly comparable to cytoagglutination data, previously standardized on a 30-min incubation (Neri et al. 1974), and was optimal in terms of allowing maximal capping. This is shown in Fig. 3, where the percentage of cells with cap is plotted against time.

![Graph](image)

**Fig. 2.** Cap formation in Novikoff cells incubated with various concentrations of Fl-Con A for 30 min at 37 °C.

**Fig. 1.** Patterns of fluorescence in Novikoff cells (A–E) and normal hepatocytes (F) treated with Fl-Con A (66 μg Con A/ml) for 30 min at 37 °C (except for A, showing a cell incubated with Fl-Con A at 3 °C). × 540. A, ringlike pattern; B, cap; C, uropodal cap; D, patchlike pattern; E, absence of cell-surface fluorescence in Novikoff cells treated with Fl-Con A in presence of 0.5 M α-MM; F, ringlike fluorescence in normal hepatocyte.
The proportions of Novikoff cells showing ring, cap or patch fluorescence at the 3 temperatures tested, and at a Con A concentration of 66 µg/ml, are reported in Table 1. In untreated cells, incubation with Fl-Con A at 3 °C resulted in the appearance of ringlike fluorescence in 95% of the cells, and of caps (all non-uropodal) in 5%. Increase in temperature was accompanied by a significant shift in the pattern of surface fluorescence from ringlike to caplike. The increase in the percentage of capped cells from 3 to 37 °C was about 13-fold. The trend analysis of proportions of capped cells showed that the observed increase with the temperature is not linear.
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The appropriate statistics (Armitage, 1971; Snedecor & Cochran, 1971) indicated that the deviation of proportions from their linear regression on the temperature is highly significant ($\chi^2 = 23.485; P < 0.001$), suggesting that a sudden change probably occurs at 37 °C. Patchlike fluorescence was absent at 3 or 23 °C, and present in only 6% of the cells incubated at 37 °C. Control-incubated cells behaved in an apparently identical way. Here also, the deviation from linearity was highly significant ($\chi^2 = 164.470; P < 0.001$). Treatment of Novikoff cells with papain resulted in the appearance of a relevant proportion of cells with caplike fluorescence at 3 °C. The percentage of capped cells increased further upon raising the temperature to 37 °C, at which, however, their proportion was only 31%, significantly lower than that observed in the untreated or control-incubated cells. This effect is clearly correlated with a significant increase in the number of cells displaying patchlike fluorescence. Nevertheless, the deviation from linearity, suggesting a sudden change at 37 °C, was evident also in this experimental condition ($\chi^2 = 44.112; P < 0.001$). Fixation of Novikoff cells with glutaraldehyde prior to incubation with Fl-Con A under any of the experimental conditions employed, resulted in a pattern of surface fluorescence comparable to that observed at 3 °C under those same conditions.

Con A-receptor mobility of normal hepatocytes

Isolated normal rat hepatocytes were incubated with Fl-Con A under experimental conditions identical to those described for Novikoff cells. Only untreated cells, i.e. cells that had not undergone papain treatment or control incubation, were tested. Surface fluorescence had a ringlike form in 100% of the viable cells at all 3 temperatures tested, and at Con A concentrations ranging from 1.3 to 66 µg/ml. The latter concentration was routinely used, because it gave more distinct and visible rings. A normal hepatocyte is illustrated in Fig. 1F.

Effect of temperature upon cytoagglutination of Novikoff cells

The fitted least-square lines of regression of Con A-induced agglutination of Novikoff cells upon Con A concentration at 3, 23 and 37 °C are reported in Fig. 4. It clearly appears, on inspection, that agglutination is dependent on the concentration of Con A. The intercept of each agglutination line with the abscissa represents the concentration of Con A required for threshold agglutination at that particular temperature. The point on the abscissa corresponding to the score value of 2 on the ordinate represents the concentration of Con A necessary for half-maximal agglutination. Threshold and half-maximal values are reported in Table 2. The untreated cells show at 37 °C a slight increase in agglutinability, since a smaller concentration of Con A is required for threshold and half-maximal agglutination. Although a constant enhancement of agglutinability at 37 °C is also suggested by the parallelism of the regression lines (Fig. 4A) the approximate standard error of the estimated line ($s_{y|x} = 0.485$) indicates, however, that such increase cannot be considered significant. The behaviour of the control-incubated cells was virtually superimposable on that of the untreated cells. Treatment of the cells with papain caused an increase in agglutinability by Con A, an effect that had been previously observed (Neri et al. 1974). At
the same mean concentration of Con A (log $\bar{x} = 1.32$) the mean agglutinability at $37^\circ$C was $3.13 \pm 0.27$ for papain-treated cells against $2.48 \pm 0.12$ for incubated cells or $2.27 \pm 0.10$ for untreated cells. A test for the difference of means shows that this increase is significant both with respect to untreated cells ($t = 2.938; P < 0.001$) and to incubated cells ($t = 2.667; P < 0.001$).

**DISCUSSION**

Interest in lectin-induced cell agglutination was greatly stimulated by the observation that neoplastic cells are usually agglutinated by lower concentrations of lectins than their normal counterpart. The molecular mechanisms responsible for the differential lectin-induced cytoagglutination of normal and malignant cells has been the subject of numerous investigations (Nicolson, 1974; Smith & Walborg, 1977). Much of this research focused on the role of lectin receptor mobility as a determinant of lectin-induced cytoagglutination. From work of Rosenblith, Ukena, Berlin & Karnowski (1973), of Nicolson (1973) and of Guerin et al. (1974) it might be con-
cluded that clustering of lectin receptors in discrete areas of the cell surface is a prerequisite for the increased agglutination of transformed cells. The evidence, however, is only circumstantial, since, as pointed out by Smith & Walborg in their comprehensive review (1977), these authors did not investigate the agglutinability of cells carrying clustered sites (e.g. transformed cells incubated with Con A at 37 °C) as compared with the same cells carrying uniformly distributed sites (transformed cells incubated with Con A at 0 °C). More direct evidence was provided by Inbar et al. (1973), who showed that aldehyde fixation of mouse lymphoma cells inhibited agglutination by Con A. Similarly, Noonan & Burger (1973) reported that Py3T3 or SV3T3 cells, that were not agglutinable by Con A at 0 °C, became agglutinable when the temperature was raised to 22 °C. On the other hand, Schnebli et al. (1977), in a series of detailed studies utilizing human erythrocytes as well as cells from normal and malignant tissues, came to the conclusion that cell agglutination by Con A was not blocked at 0 °C, a temperature at which mobility of receptors in the plane of the membrane was inhibited. Rutishauser & Sachs (1974), in a study utilizing normal...
mouse lymphocytes and lymphoma cells attached to nylon fibres, provided evidence that cell-cell binding induced by Con A required short-range lateral movements of cell-surface lectin receptors, and that clustering of the receptors was not necessary and seemed to hinder cell-cell binding.

The purpose of the present report was to clarify the relationship between the topography and dynamics of Con A receptors at the cell surface and Con A-induced cytoagglutination of normal and malignant rat liver cells. These studies utilized Novikoff hepatoma cells and normal rat hepatocytes, cells that are agglutinable by low concentrations of Con A (Neri et al. 1974; Starling et al. 1977). We took advantage of the fact that lectin-induced receptor movement is a temperature-dependent phenomenon and can be blocked at 0-4 °C, when the membrane lipid bilayer is

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**Fig. 4.** Estimated regression lines of the degree of agglutination upon Con A concentration at temperatures of 3, 23 and 37 °C respectively. A, untreated cells; B, control-incubated cells; C, papain-treated cells. Abscissa: Con A concentration on a logarithmic scale. Ordinate: agglutination scores. The dotted lines represent the approximate standard error of the estimated regression line at 37 °C.
'frozen' (Reinert & Steim, 1970). Con A receptor movements were monitored at various temperatures utilizing fluorescein-conjugated Con A, and were compared with Con A-induced agglutination, scored under the same experimental conditions, using a modification of a previously described method (Neri et al. 1974). We found that temperature variations had profound effects on the distribution pattern of Con A receptors on Novikoff cells. At 3 °C the distribution was uniform over the entire cell surface as indicated by the ringlike shape of the membrane fluorescence in 95% of the cells examined. This uniform distribution probably represents the inherent topographical pattern of the Con A receptors, since an identical pattern was observed when the cells were fixed with glutaraldehyde, prior to incubation with Fl-Con A. Upon shifting the temperature to 37 °C, the Con A receptors underwent a redistribution, as indicated by the appearance of caps in 65% of the cells scored. In a few cells the fluorescence appeared clustered in discrete patches over the cell. However appropriate controls in presence of α-MM, indicated that the fluorescence could not be removed by post-incubating the cells with α-MM, suggesting that the fluorescent patches were intracellular and probably represented pynocytosed material. The situation at 23 °C was similar to that observed at 3 °C. This accounts for the non-linearity of the increase of the proportion of capped cells with temperature, but seems rather surprising, since the transition temperature of the membrane lipids is below 23 °C (Reinert & Steim, 1970). At the latter temperature the lipid bilayer should be in a fluid state and should allow lateral diffusion of membrane macromolecules. If this does not happen, the most likely explanation is that the process of cap formation requires energy and is therefore partially inhibited at 23 °C. The previous observation of Glenney et al. (1979) that at 22 °C ferritin-labelled Con A was uniformly distributed over the surface of Novikoff cells, is in agreement with the present finding.

In spite of these variations in Con A-receptor distribution, Con A-induced cell agglutination was remarkably stable over the temperature range from 3 to 37 °C. This observation suggests that Con A-induced agglutination of Novikoff cells does not depend upon diffusion and capping of the lectin receptors. It was previously

### Table 2. Con A concentration (μg/ml) for threshold and half-maximal agglutination of Novikoff cells at different temperatures

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Treatment of cells</th>
<th>Threshold, μg/ml</th>
<th>Half-maximal agglutination, μg/ml</th>
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<td>Untreated</td>
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<td>Incubated</td>
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*a* Threshold agglutination.

*b* Half-maximal agglutination.

¢ As in Table 1.
demonstrated that Novikoff cells become more agglutinable by Con A after papain treatment (Neri et al. 1974). This finding was quantitatively confirmed in the present report, and additional evidence was provided, showing that the papain effect was of the same magnitude at each of the 3 temperatures tested. On the other hand, temperature-dependent Con A-receptor diffusion could still be observed in the papain-treated cells, indicating once more that capping of receptors and cytoagglutination varied independently. The only significant effect of papain treatment upon Con A-receptor dynamics, especially apparent at 37 °C, was an enhanced internalization of Fl-Con A, as indicated by the increased percentage of cells with patchlike fluorescence, that could not be removed by post-treatment with α-MM. A less marked effect was the appearance of caps in a small percentage of cells incubated with Fl-Con A at 3 °C.

Additional evidence that cytoagglutination is not dependent upon receptor clustering was provided by a study of normal rat hepatocytes. These cells are also agglutinable by low concentrations of Con A (Starling et al. 1977). Yet, when assayed for receptor mobility using Fl-Con A, all of the viable cells scored invariably displayed a ring of surface fluorescence with no evidence of receptor diffusion and redistribution into caps or patches, even at 37 °C. The molecular basis for the observed difference in Con A-receptor mobility between normal hepatocytes and hepatoma cells is presently unclear. At any rate, it may be concluded that Con A-induced cytoagglutination and long-range Con A-receptor mobility vary independently in normal hepatocytes and Novikoff hepatoma cells. Furthermore Con A-receptor mobility may represent another marker of neoplastic transformation during hepatocarcinogenesis, adaptation to growth in the ascitic form, or progression of a tumour to a more malignant state.

This work was supported in part by grant NCT 75 00699.04 from the Consiglio Nazionale delle Ricerche of Italy.

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Con A receptor mobility and cyt-Toagglutination


*(Received 17 July 1979)*