J. Membrane Biol. 13, 129–142 (1973)


# Alterations in the Surface Glycoproteins of Chicken Erythrocytes Following Transformation with Erythroblastosis Strain R Virus

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Received 8 March 1973

*Summary.* Erythroblasts from marrows of chicks infected with RNA-virus (strain R *erythroblastosis* virus) were found to possess a small but consistent increase in the number of concanavalin A binding sites per cell compared to erythroblasts derived from the marrows of phenylhydrazine-treated birds. Both types of erythroblast possessed more surface glycoproteins per cell accessible to concanavalin A (Con A) than marrow and peripheral blood erythrocytes. Employment of concanavalin A conjugated to ferritin showed marked differences in the spatial arrangement of the Con A receptors between phenylhydrazine and virus-induced erythroblasts but little difference was observed in the surface density of the Con A sites between erythrocytes and erythroblasts, a result which agrees with the amount of bound labeled Con A when this data is expressed in terms of the cell surface.

The amount of labeled Con A bound to erythrocytes derived from the marrow was greater than that derived from the peripheral circulation, a result which is substantiated by the ferritin Con A studies which show an increase in the density of Con A sites on the marrow blood cells. Trypsinization increases the number of sites and the agglutininability of the marrow cells.

The increase in the susceptibility of the cells to agglutinate with concanavalin A paralleled the observed increase in the number of binding sites per cell.

The erythroblastosis strain R virus which has been shown to belong to avian tumor virus subgroup B [9] was chosen as an experimental model to study the surface changes following transformation of the host erythrocytes because of the possibility of obtaining: (a) high cell yields *in vivo* and (b) a rapid preparation of plasma membranes from dividing cells. The study was therefore commenced by employing the plant lectin-concanavalin A(Con A) to determine whether the number and arrangement of the surface glycoproteins are altered by the viral induction of the erythroleukemia. The degree of binding of this carbohydrate-binding protein labeled with $^{63}$Ni apparently

showed a difference between SV 40 transformed and nontransformed 3T3 cells [7], a result which has been disputed by Cline and Livingston [5], who reported difficulty in preparing active Con A labeled with $^{63}$Ni and who therefore utilized an $^{3}$H-acetylated derivative of Con A to show no apparent differences between virally transformed and normal 3T3 cells. Similar results were obtained with $^{125}$I-labeled Con A by Ozanne and Sambrook [19]. Correlation between the agglutinability by Con A and its binding to various transformed cells is also a subject of considerable discussion, Inbar and Sachs [8] having shown that transformation of 3T3 cells with SV 40 renders them available to agglutination by Con A, a property which can also be realized by trypsinization. Ozanne and Sambrook [19] and Cline and Livingston [5], however, have shown with a variety of transforming agents that no correlation exists between the sensitivity of the cells to agglutinate and the amount of Con A bound per cell. Recent work [20] has also shown that normal cells become more susceptible to agglutination following infection with nononcogenic viruses, and fetal cells are apparently more susceptible to agglutination by Con A than adult [24]. Nicolson [16] employing Con A coupled to the electron-dense ferritin molecule showed that the surface density of concanavalin A binding sites is greater with SV 40 transformed 3T3 cells and that there were more glycoprotein clusters on the transformed cell's surface. Shoham and Sachs [22] employing Con A coupled to a fluorescent dye showed differences in the surface properties of transformed and trypsinized cells when compared to normal 3T3 cells in interphase and in mitosis.

## Materials and Methods

The avian erythroblastosis strain R (AEV-R) virus was initially obtained from Dr. J. W. Beard (Duke University, Durham, N.C.) and was passed into 1- to 2-month-old White Leghorn chickens by I. V. inoculation in the wing vein. Plasma from injected chickens was sterilized and stored at $-70$ °C for use as virus stock. (The influence of host age and the dose response of this line of chickens has been well characterized [2].) The chickens were sacrificed on the ninth day, the wing bones dissected and the marrow collected using compressed air. Phenylhydrazine-induced erythroblastosis was produced by I. V. injection of phenylhydrazine (50 mg of phenylhydrazine/kg animal weight) in sterile water adjusted to pH 7.2 with 1 mM NaOH and these birds were sacrified on the third day. It was found, in accordance with other workers [23] that phenylhydrazine induced 60 to 80% erythroblastosis and the virus, 50 to 60% erythroblastosis. Marrow erythroblasts were dispersed in $Ca^{2+}$-free Hanks solution by passage through an 18 gauge needle. The cell suspension was filtered through hospital gauze, washed three times in $Ca^{2+}$-free Hanks solution and then suspended in four volumes of Hanks solution containing 10% (v/v) sterile chicken serum. The cells were then placed on a barrier [23] of 5% (w/v) Dextran T 70 (Pharmacia) in $Ca^{2+}$-free Hanks solution containing 10% (v/v) chicken serum and centrifuged at $120 \times g$ for 3 min. Erythroblasts passed through the barrier while

erythrocytes remained on the surface. Samples of the purified cell suspensions were stained with Giemsa or New Methylene Blue and examined under the microscope. It was found, following purification, that the percentage of erythroblasts had increased to 90 to 100%. Frequently, low magnification electron-microscopy was employed as a check on cell purity. The normal chick marrow contained 80% erythrocytes [14]. Marrow erythrocytes were therefore prepared in a manner similar to that used with the erythroblasts except that: (a) the dextran barrier was omitted and (b) the cell suspensions were washed (3 times) and the buffy coat of immature cells removed by aspiration. Erythrocytes from the peripheral blood were obtained by cardiac puncture and collected in heparin (1,000 units/ml blood). The cells were centrifuged at $1{,}000 \times g$ for 5 min and suspended in 0.14 M NaCl, 10 mM phosphate buffer, pH 7.4.

Cell counts were made by employing a hemacytometer; hemoglobin was determined by lysis of the cells in water followed by determination of the absorbance at 541 nm ($E_{541}^{0.1\%} = 0.88$ [12]). The cell surface areas were measured according to Williams [25]. Trypsinization was carried out by reacting trypsin (Sigma) 0.001% (w/v) in 0.14 M NaCl, 10 mM phosphate buffer, pH 7.4 (CMP buffer) with $2 \times 10^7$ cells for a maximum time of 1 min at 25 °C. More drastic hydrolysis caused lysis. The treatment was stopped by cooling the mixture to 4 °C and rapid washing of the cells in the CMP buffer. Agglutination was measured by the method described by Burger and Goldberg [3]. Cells ($2 \times 10^7$) in 0.1 ml of CMP buffer were mixed with 0.1 ml of Con A in 0.1 ml of chicken serum, and incubated at 25 °C for varying times from 0 to 60 min. The differences seen in agglutination patterns at 15 min were enlarged at 60 min but remained qualitatively similar.

$^{63}$Ni-Con A was demetallized [10] and then reacted with $^{63}NiCl_3$ (Amersham Searle, Toronto) and $CaCl_2$ at final concentrations of 0.5 mM and 1.6 mM, respectively. Nonbound metal ions were removed by dialysis against 0.4 M NaCl, 10% (w/v) Tris, pH 7.4, the buffer being changed every 30 min for 2.5 hr. The $^{63}$Ni-Con A was prepared within 2 hr of the binding study. Acetylation of Con A was performed by scaling down the procedure described by Agrawal *et al.* [1]. Con A (80 mg) in 0.2 M NaCl 0.05 M acetate buffer, pH 5.2 (10 ml) was acetylated with 25 mC of $^3$H-acetic anhydride (4.1 C/mM, Amersham Searle) diluted with 95 µliters of unlabeled acetic anhydride for 1 hr at 0 °C. Excess acetic anhydride was removed by dialysis against water, the protein was freeze-dried and redissolved in the 0.2 M NaCl 0.5 M acetate buffer, pH 5.2. The acetylated Con A was stored at −20 °C and used within two weeks of preparation.

Binding was performed with $2 \times 10^7$ cells/ml in CMP buffer with varying concentrations of Con A for 30 min at 25 °C. Duplicate tubes were run with 0.1 M α-methyl-D-glucoside as an inhibitor, the difference in radioactivity between the two tubes being considered as the specific binding of the lectin [7]. Inhibition was 50 to 60%, independent of the method used to prepare the labeled Con A. The cell suspension was washed three times in CMP buffer and then resuspended in 0.25 ml of CMP buffer and 0.25 ml of 2 N NaOH and left overnight at 25 °C. The hemoglobin was bleached by addition of 5 drops of 30% (w/v) $H_2O_2$; finally 0.5 ml of formic acid was added and the mixture counted with a scintillation liquid — 4 g Omnifluor, 80 g naphthalene, 300 ml cellosolve, 300 ml dioxane, made up to 1 liter with toluene. Cell suspensions containing fewer than $2 \times 10^7$ cells gave count rates which were too low. With suspensions of higher concentration the quenching due to hemoglobin became a problem.

Low magnification micrographs were obtained with cells fixed for 1 hr at 4 °C in 4% (w/v) glutaraldehyde, in 0.1 M cacodylate buffer, postfixed in 1% (w/v) $OsO_4$, dehydrated by an ethanol series and embedded in Epon. The sections were then stained with saturated uranyl acetate and lead citrate as described by Reynolds [21]. Ferritin conjugated to Con A was prepared as outlined by Nicolson and Singer [18]. Grids were prepared by adding a 20% (w/v) cell suspension to a water-filled Erlenmeyer flask over which parafilm with small

holes had been stretched. A Formvar-coated grid was touched to the water surface to remove the lysed membranes, which were then conditioned by the addition of 1 drop of a 5% (w/v) BSA solution in 0.2 M NaCl 50 mM phosphate buffer, pH 6.8, followed by 1 drop of the ferritin Con A conjugate. After 3 min the drop was washed off by touching the grid consecutively to the surface of 6 drops of 0.2 M NaCl 50 mM phosphate buffer followed by 2 drops of water and then dried. All micrographs were obtained with a Hitachi 12 microscope.

## Results

Binding curves of $^{63}$Ni-Con A with marrow erythrocytes, with phenylhydrazine- and virus-induced erythroblasts are presented in Fig. 1. (The number of cells employed in all experiments was $2 \times 10^7$/ml, which corresponded to equal amounts of hemoglobin (0.8 mg) and the time of incubation was fixed at 30 min because saturation of all cells was obtained after this time period.) The erythroblasts obviously bind more Con A than the erythrocytes and there were differences between virus- and chemical induced erythroblasts at low concanavalin A concentrations. To determine whether these

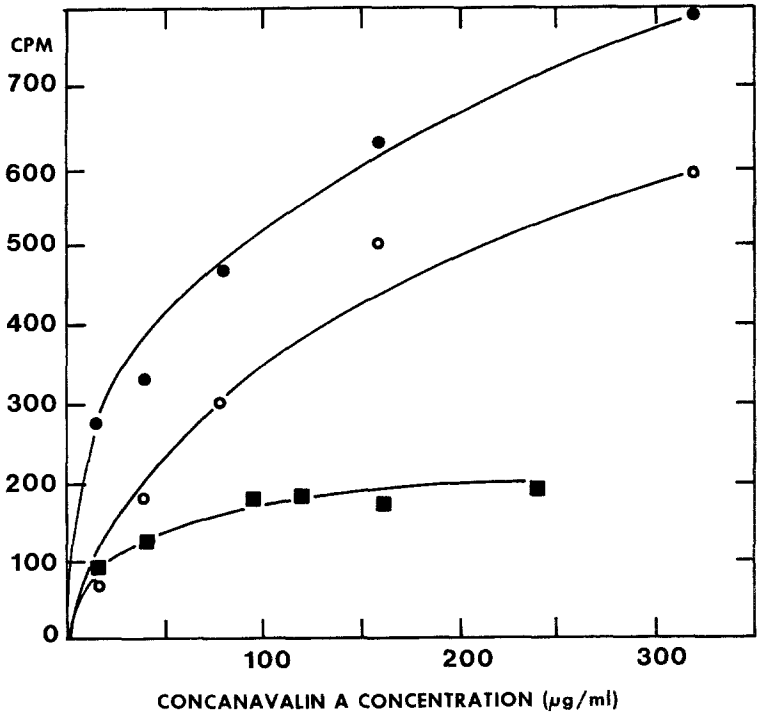


Fig. 1. The binding of $^{63}$Ni-labeled Con A to virus-induced (●— ●), phenylhydrazine-induced (○—○) erythroblasts and marrow erythrocytes (■— ■). The counts per minute refers to the difference in radioactivity (per $2 \times 10^7$ cells) observed in the presence and absence of the Con A inhibitor, 0.1 M methyl-D-glucoside. (Each point represents 5 determinations.)

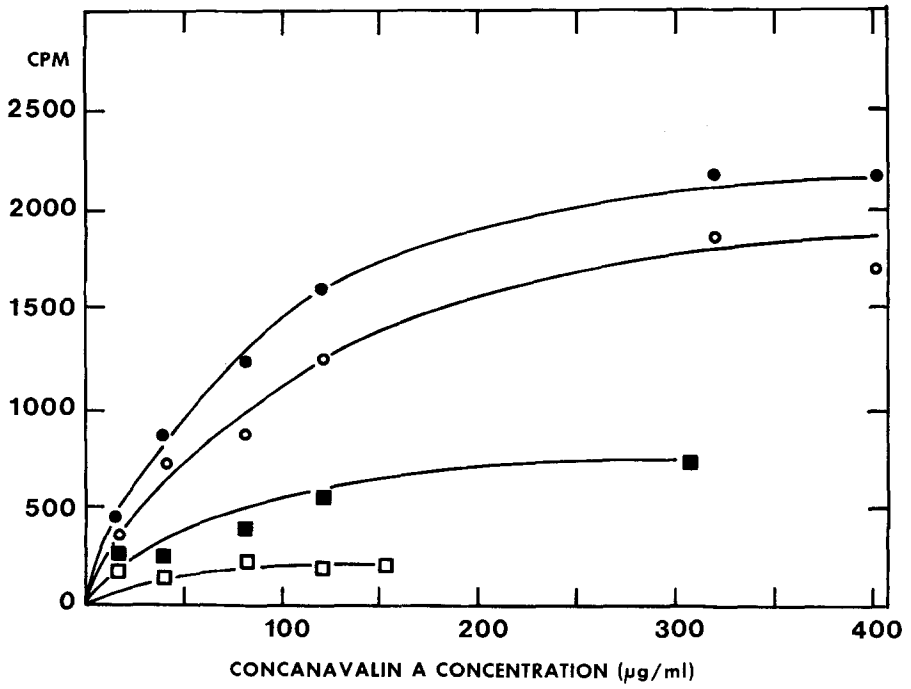


Fig. 2. The binding of $^{3}$H-acetyl-Con A to virus-induced (●— ●), phenylhydrazine-induced (○—○) erythroblasts and marrow (■ — ■) and peripheral blood erythrocytes (□ — □). The counts per minute refers to the difference in radioactivity (per $2 \times 10^{7}$ cells) observed in the presence and absence of the Con-A inhibitor, 0.1 M methyl-D-glucoside. (3 determinations)

differences were only detectable with $^{63}$Ni-labeled Con A, the $^{3}$H-acetyl-labeled derivative was prepared and binding curves obtained (Fig. 2). Similar but smaller differences were observed between the erythrocytes and the erythroblasts and between the virus- and phenylhydrazine-induced erythroblast. Differences were also apparent between the marrow and peripheral blood erythrocytes, the marrow cells binding more Con A than those from the circulation.

The curves obtained with the $^{63}$Ni-Con A binding to erythroblasts appear not to reach a plateau whereas saturation was observed with the acetyl derivative. The total binding and nonspecific binding curves for $^{63}$Ni-Con A are presented for both classes of erythroblast in Fig. 3 (*a* and *b*). It can be seen that both total and nonspecific binding increases with both the virus- and phenylhydrazine-induced erythroblast, the effect being more noticeable with the former. The error in both curves is of the order of 10%; obviously the error in the subtracted curve is even greater. The errors involved in calculating

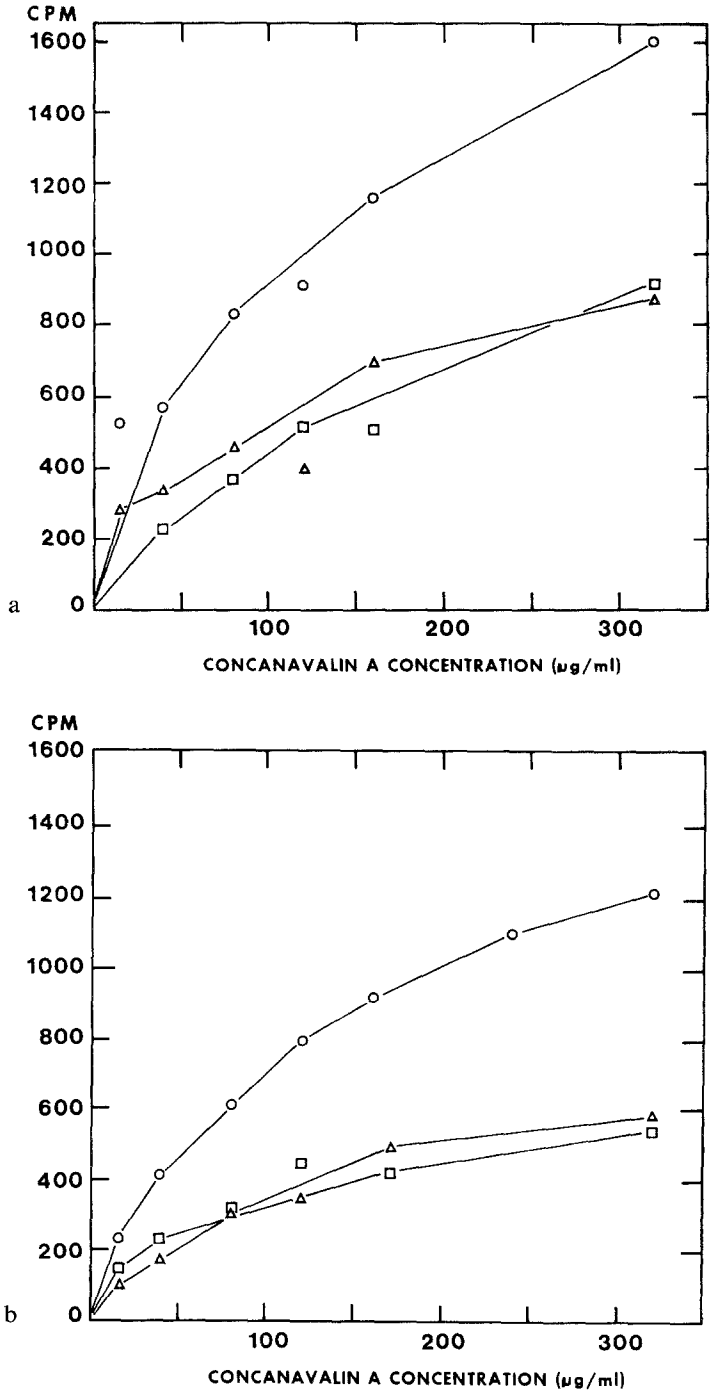


Fig. 3. (*a*) The total binding (○—○), nonspecific binding (□—□) and specific binding (△—△) of $^{63}$Ni-labeled Con A to virus-induced erythroblasts. (*b*) The total binding (○—○), nonspecific binding (□—□) and specific binding (△—△) of $^{63}$Ni-labeled Con A to phenylhydrazine-induced erythroblasts. In both experiments the number of cells employed was $2 \times 10^7$ cells, time of incubation 30 min at 37 °C, and the nonspecific binding being that obtained in the presence of 0.1 M methyl-D-glucoside

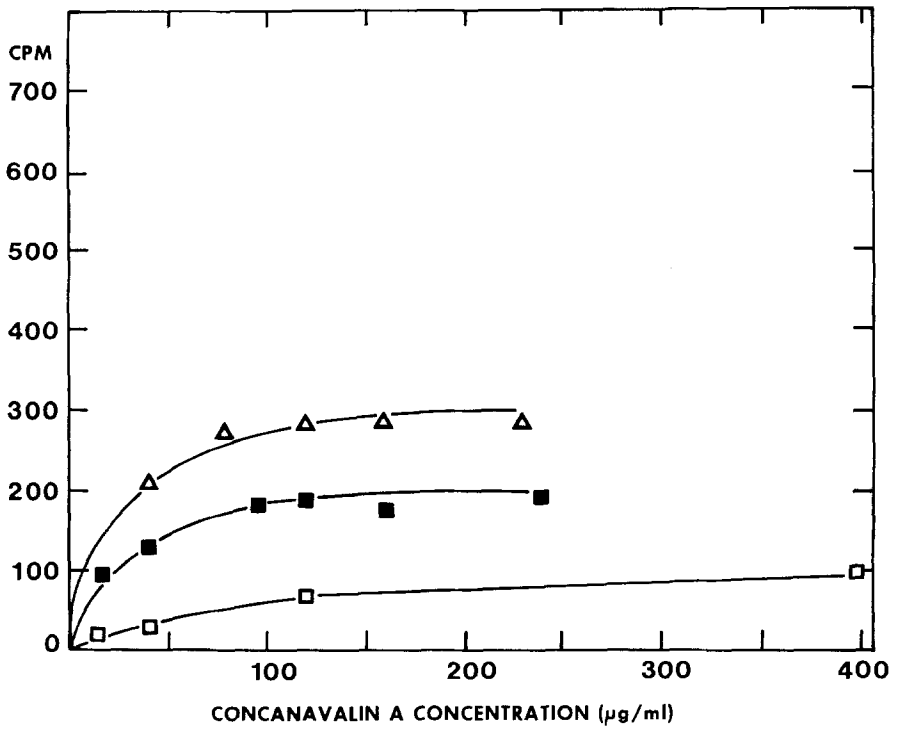


Fig. 4. The binding of $^{63}$Ni-Con A to trypsinized (△—△), normal (■—■) marrow erythrocytes and peripheral blood (□—□) erythrocytes. The counts per minute refers to the difference in radioactivity (per $2 \times 10^7$ cells) observed in the presence and absence of the Con A inhibitor, 0.1 M methyl-D-glucoside. (5 determinations)

the acetyl-derivative curves are lower because the specific activity of the Con A was greater. The lack of a plateau in the virus-induced erythroblast (the phenylhydrazine erythroblast curve can be drawn to a plateau; *see* Fig. 1) may be explained by experimental error and/or by dissociation of a small amount of the $^{63}$Ni-Con A complex and the binding of $^{63}$Ni to the cell surface. This latter hypothesis would explain the marked increase in nonspecific binding as the Con A concentration was increased with the virus-induced erythroblast.

Trypsinization (Fig. 4) of the marrow erythrocytes increased significantly the amount of $^{63}$Ni-Con A binding to the cell. Unfortunately, due to the erythrocyte's fragility only minimal proteolysis was possible.

The greater difference observed between marrow and peripheral cells with the metal-labeled agglutinin than with the acetyl derivative may be due

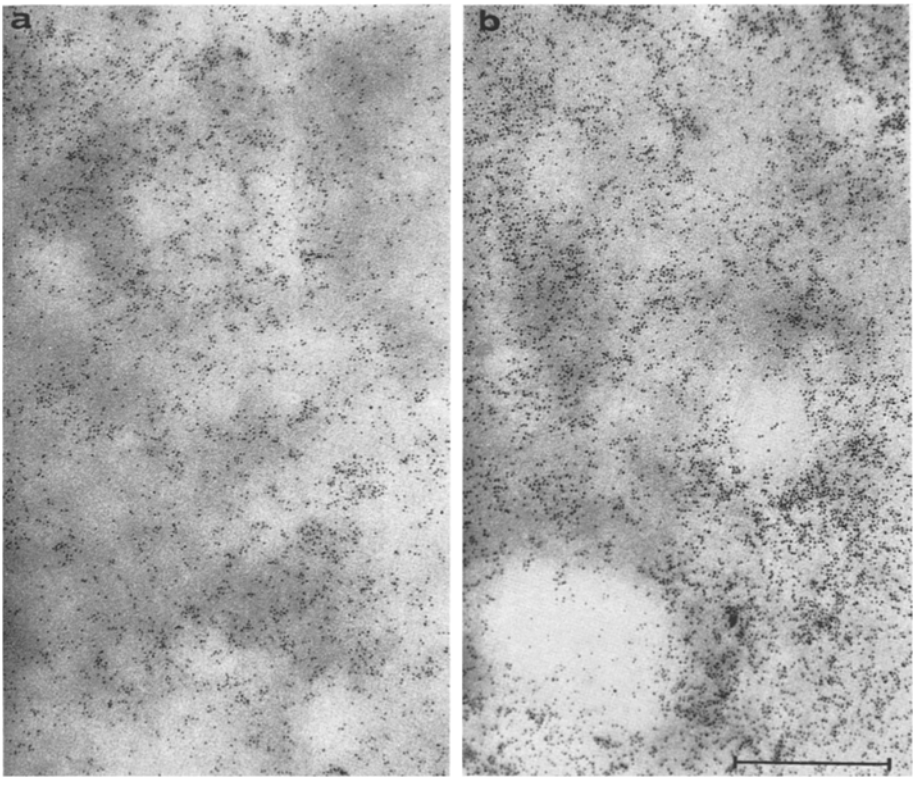


Fig. 5. Electron-micrographs of membranes of peripheral blood (*a*) and marrow (*b*) erythrocytes treated with Con A-ferritin conjugates. The bar refers to 500 nm and the final magnification is $\times 75{,}000$

to acetylation resulting in a modified Con A molecule unable to bind to all of the membrane glycoprotein sites, a hypothesis which is strengthened by the finding that acetylated Con A [1] does not: (a) possess a similar electrophoretic mobility to native Con A and (b) bind carbohydrate so avidly, although its carbohydrate specificity remains unaltered. The absolute amount of acetylated Con A apparently bound per cell is also lower than with the metal-labeled derivative also showing that acetylation has altered the binding capacity of the lectin. This effect does not negate the binding data since the relative amount of nonspecific binding is no greater than that found with $^{63}$Ni-Con A.

The differences in binding behavior observed between erythrocytes in the circulation and those in the marrow were also observed when concanavalin A conjugated to ferritin was employed (Fig. 5, *a* and *b*), the density of ferritin

being greater on the surface of the marrow cell. The shapes of both types of cell appeared microscopically to be identical and only small differences were found to exist in the respective cell volumes (the mean cell volume of marrow and peripheral erythrocytes being $160 \pm 20$ and $120 \pm 20$ $\mu m^3$, respectively). Therefore, similar surface areas are predicted for either cell. The amount of hemoglobin per cell was also identical for both types of cell. Thus, the two techniques show that there is an increase in the number of Con A binding sites situated on marrow as opposed to peripheral erythrocytes.

The most startling changes seen with the electron-microscope were differences observed between the phenylhydrazine-induced (Fig. 6 *b*) and the virus-induced erythroblasts (Fig. 6 *c*) and the apparent similarity between the phenylhydrazine-induced erythroblast and the marrow erythrocyte (Fig. 6 *a*). (N. B., Fig. 6 is not comparable with Fig. 5 since a higher concentration of ferritin-Con A was employed in the latter.) The increased density on the surface of the virus-induced erythroblast is partly due to a contraction of the membrane when it is lysed, since the membrane area of the virus-induced erythroblast is approximately 0.5 to 0.8 that of the phenylhydrazine-induced erythroblast but this decrease in surface area cannot explain the three- to fourfold increase in the density of the ferritin-Con A observed in the "cluster regions" of the micrographs. It is also possible but not very likely that the dense clusters observed on the transformed cell's surface are *only* detected because of the membrane contraction. The relatively small increase in ferritin-Con A staining between the marrow erythrocyte and chemically-induced erythroblast, as compared to the large increase observed in the binding of Con-A to the two classes of cell can be partially explained by the greater surface area of the erythroblast (mean cell volume of both classes of erythroblast is $330 \pm 30$ $\mu m^3$), but it is difficult to compare the cell surfaces with precision because the cellular shapes are different. It is however apparent that the density of ferritin-con A receptor sites is still greater on the erythroblast than on the erythrocyte (*cf.* Fig. 6, *a*, *b*). Fig. 6 (*d*) presents the virus-induced erythroblast which had been treated with ferritin-Con A in the presence of α-methyl-glucoside, a Con A inhibitor [16]. It is obvious that the density of staining is markedly reduced. Sucrose was also employed but gave ambiguous results. Dense bodies were seen which are not due to ferritin-Con A conjugates, virus or sucrose treatment of the membrane.

Agglutination studies (Table 1) were performed with both erythrocytes and erythroblasts and it is seen that as the total concanavalin A binding increases so does the agglutinability of the cells. The experiment showed small repeatable differences between chemical-induced and virus-induced erythroblasts at low concentrations of concanavalin A.

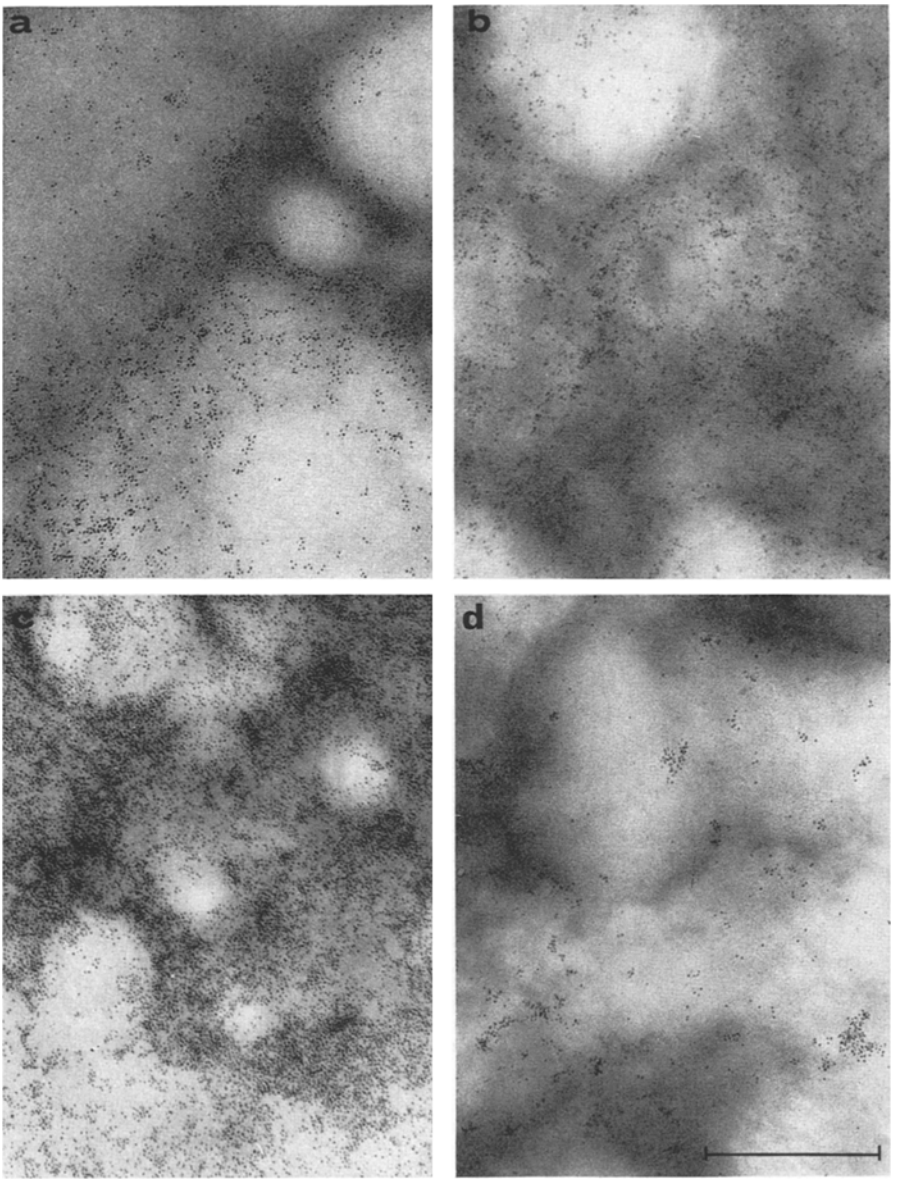


Fig. 6. Electron-micrographs of membranes from marrow erythrocytes (*a*), phenylhydrazine-induced erythroblasts (*b*) and virus-induced erythroblasts (*c*) treated with Con A-ferritin conjugates. The final micrograph (*d*) refers to membranes from virus-induced erythroblasts treated with the conjugates in the presence of an inhibitor (0.1 M α-methyl-glucoside). The bar refers to 500 nm and the total magnification is ×75,000. (N. B., a lower concentration of ferritin-Con A was used than in Fig. 4)

Table 1. Agglutination of virus and phenylhydrazine-induced erythroblasts, marrow and peripheral blood erythrocytes, with varying concentrations of concanavalin A

| Cell sample | Concentration of concanavalin A (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 16 | 32 | 64 | 120 | 240 | 480 |
| Erythrocytes (peripheral blood) | − | − | − | − | − | |
| Erythrocytes (marrow) | − | − | ± | ± | + | + |
| Erythrocytes (marrow) subsequent to trypsinization | − | − | ± | ± | + | ++ |
| Erythroblasts (phenylhydrazine-induced) | ± | ± | ± | + | ++ | +++ |
| Erythroblasts (virus-induced) | + | + | + | ++ | ++ | +++ |

[a] Trypsin treatment was performed as described in Materials and Methods. The following method of scoring was employed: − = 0 to 25%; ± = 50%; + + = 75%; + + + = 75 to 100%. The cells and concanavalin A were incubated for 1 hr at 25 °C.

## Discussion

Our first tentative conclusion is that an increase in the age of the cell (even if apparently nondividing, e. g. erythrocyte) apparently decreases the number of surface glycoproteins (containing terminal α-D-glucose or α-D-mannose residues) since a decrease was observed in the amount of Con A binding to peripheral as compared to marrow erythrocytes. Such a change is difficult to explain by alterations in the cell size or shape since none are obvious by microscopic examination [14] and also the density of Con A sites is seen to increase by employing ferritin-Con A conjugates.

The startling difference in the amount of Con A binding to a chemical-induced (control) erythroblast and an erythrocyte is due at least partially to an expansion in the surface area of the cell, although this is difficult to quantitate. The relatively small increase in the density of ferritin-Con A bound to the erythroblast as compared to the erythrocyte supports this idea. If the results are expressed in terms of hemoglobin content these results are the same, since the intracellular hemoglobin concentrations are similar for all four cell types. The other point that should be made is whether a phenylhydrazine-induced erythroblast provides a reasonable control. Unfortunately attempts to produce erythroblastosis by other methods (i. e., severe bleeding) were not satisfactory. However, there appears to be little difference between control erythroblasts and normal 3T3 cells in the arrangement of the ferritin-Con A conjugates on the cell surface suggesting that phenylhydrazine has not produced any marked effects on the arrangement of the surface glycoproteins.

Agglutination experiments showed a correlation between the "age" of the cell with the total amount of Con A bound per cell, but any correlation between the latter and agglutinability must be interpreted with caution. Gordon *et al.* [6] have shown that rabbit erythrocytes following trypsin treatment possess a 100- to 200-fold increase in agglutinability *without* any detectable increase in the amount of soybean agglutinin binding per cell. However, agglutination experiments did show a small but significant difference between virus-induced and chemical-induced erythroblasts; a result which can be correlated with the small increase in the number of Con A sites per cell. The apparent size of both types of erythroblast is the same, although a relatively small and perhaps undetectable change in mean cell shape could explain the binding results. Concanavalin A conjugated to ferritin shows perhaps the most distinct change between the virus-induced and control erythroblast as the glycoproteins in the former appear arranged in clusters somewhat similar to that shown by Nicolson with 3T3 cells transformed with SV 40 virus [16] or following treatment with trypsin [17]. However, the possibility exists that the "clustering" is induced by the multivalent binding of the Con A.

We therefore conclude that virus-induced erythroblasts may possess surface glycoproteins arranged spatially in a manner different from chemically-induced erythroblasts or erythrocytes, and that there is a small but discrete increase in the amount of agglutinin bound per cell and sensitivity to agglutination of a leukemic as compared to control erythroblast. Our results showing little change in the agglutination properties of these cells transformed by an RNA-virus are similar to those of Moore and Temin [15] and Burger and Martin [4] who showed little change following RNA virus transformation if no pretreatment was performed with hyaluronidase; results which were contrary to those of Lehman and Sheppard [13] and Kapeller and Doljanski [11] who showed large changes in the agglutination properties of chick embryo cells following infection with RSV virus. Marrow erythrocytes also possess more Con A binding sites, a greater susceptibility to agglutination and a greater density of binding sites on their surface than the circulating cell, a property perhaps related to the age of the cell.

It should be pointed out that another added advantage of employing this *in vivo* system is that prior treatment of the cells by such agents as EDTA or trypsin is unnecessary. Treatment by such agents could remove, to differing degrees, surface glycoproteins involved in the binding process.

This work was supported by the Cancer Research Society Inc., Montreal and the Canadian National Cancer Institute. H. S. is a Canadian Medical Research Council Scholar and E. B. is a fellow of the Cancer Research Society Inc., Montreal.

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