CHANGES IN NUMBER, MOBILITY, AND TOPOGRAPHICAL DISTRIBUTION OF LECTIN RECEPTORS DURING MATURATION OF CHICK ERYTHROID CELLS

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ABSTRACT

Plant lectins have been used to probe changes in cell surface characteristics that accompany differentiation in a complete series of chick erythroid cells. Dramatic differences in lectin receptor mobility were observed between the most immature cells of the series, the proerythroblasts, and cells at the next stage of maturation, the erythroblasts. Both concanavalin A and *Ricinus communis* agglutinin form caps on proerythroblasts, whereas they develop a patchy distribution on erythroblasts. Erythroid cells at later developmental stages show a homogeneous distribution of surface-bound *R. communis* agglutinin. Concanavalin A also shows a uniform distribution on the cell periphery, but appears to be concentrated in a ring above the perinuclear region of the cell. In addition to changes in mobility of lectin receptors, a large reduction (50–70%) in the number of lectin receptors per cell accompanies maturation of proerythroblasts to erythroblasts. Pretreatment of the cells with neuraminidase results in enhanced binding of *R. communis* agglutinin to proerythroblasts. The number of additional *R. communis* agglutinin receptors exposed by enzyme treatment remains relatively constant during subsequent cell maturation.

The migratory or invasive behavior of embryonic and malignant cells has been attributed to similarities in cell surface properties. For example, both embryonic and malignant cells from various sources tend to be more agglutinable by plant lectins than their more mature or nontransformed counterparts (8–10, 19, 20, 23). In the case of at least one malignant cell line, the SV40-transformed 3T3 fibroblast (SV3T3), the high agglutinability may be explained by increased mobility of cell surface glycoprotein receptors for lectins. Lectin-receptor complexes aggregate in patches on SV3T3 cells, permitting multiple lectin crossbridges at points of cell-cell contact. By contrast, the parental 3T3 cells maintain a homogeneous or dispersed distribution of lectin-receptor complexes and hence show less cell-cell agglutination even though the total amount of lectin bound per cell is not different from the amount bound to transformed cells (22). In the case of embryonic cells changes both in receptor mobility (8, 20) and in the number of available receptors (9) have been suggested to explain changes in agglutinability during cell differentiation and maturation. However, very little direct evidence for either hypothesis has been presented.

We considered that the relationship of changes in the density and mobility of lectin receptors to

differentiation could be approached by systematic examination of these surface properties through a well-defined developmental cell series. A complete series of developing erythroid cells derived from chick embryos was selected for study.

MATERIALS AND METHODS

Materials

Purified RCA I (mol wt 120,000) was prepared in this laboratory by Dr. H. H. Yin according to Nicolson et al. (16). Concanavalin A (Con A) (3 × crystalline) was obtained from Miles-Yeda Laboratories, Kankakee, Ill. and neuraminidase from *Vibrio cholerae* from Calbiochem, Inc., San Diego, Calif.

Fertilized eggs of the White Leghorn breed were obtained from Spafas Co., Norwich, Conn. Nutrient Mixture F12 (HAM) with glutamine, heat-inactivated fetal calf serum and antibiotic-antimycotic solution were obtained from Grand Island Biological Co., Grand Island, N. Y.

Cells

The eggs were incubated in a Humidaire incubator (model no. 50) at 100°F (dry bulb) and 85°F (wet bulb) for various times. Homogeneous populations of proerythroblasts were obtained from cultures of dissociated blastoderm embryos by the technique of Chan and Ingram (6). Cells were harvested from the medium after 44–48 h of culture at 37°C, washed with isotonic saline, and resuspended in phosphate-buffered saline (PBS). Cell number was determined with a hemocytometer counting chamber.

Erythroblasts and more mature erythroid cells were collected from the circulation of embryos into cold saline solution after various times of incubation, washed until free of yolk, and resuspended in PBS. Erythroblasts could also be obtained from 70–74-h cultures.

Nucleated ghosts were prepared by hypotonic lysis in a buffer containing 10 mM Tris HCl (pH 7.5), 10 mM KCl, and 1.5 mM $MgCl_2$. Maximal swelling without lysis was achieved by treating cells with diluted PBS (43% vol/vol). Swollen cells were restored to their normal shapes by resuspending in PBS or in Nutrient Mixture F12 containing 10% chicken serum.

Surface sialic acid residues were cleaved by incubation of 5×10^5 cells in 1 ml of 0.9% NaCl containing 5 mM $CaCl_2$ and 10 U neuraminidase. Excepted as noted, the incubation with enzyme was carried out for 30 min at 37°C followed by washing in PBS.

Light Microscopy

Light microscopy was performed with a Zeiss Photomicroscope III. The stages of maturation of the erythroid cells were determined by bright-field examination of slides prepared in the Cytospin (Shandon Southern Instruments, Inc., Sewickley, Pa.). The cell monolayers were fixed in methanol, stained with benzidine reagent for hemoglobin, and counterstained with mixed Wright-Giemsa's.

Fluorescence Microscopy and Photometry

Fluorescein isothiocyanate (FITC)-conjugated lectins were prepared by a modification of the procedure of Nicolson (13). FITC-Con A was purified on Sephadex-G50 columns and FITC-RCA I on Bio-gel agarose, 0.5 M (Bio-Rad Laboratories, Richmond, Calif.). A single preparation of each FITC-lectin conjugate was used for all the experiments described. The dye to protein ratios were different for the two conjugates.

Unless otherwise noted, cells (4×10^5) were suspended in 0.5 ml PBS containing FITC-Con A (22 μg) or FITC-RCA I (37 μg) and incubated at room temperature for 5 min. An equal volume of freshly prepared 2% paraformaldehyde was added to fix the cells during a further 10-min incubation. The cells were then collected by centrifugation, washed once with PBS, and suspended in 0.1 ml PBS.

To elute surface-bound lectin, the cells were diluted with an equal volume of cold (4°C) PBS containing 0.1 M concentrations of α-methyl-D-mannoside (for Con A) or D-galactose (for RCA I) after the 5-min labeling period. The suspensions were immediately centrifuged and the cell pellet was resuspended in PBS-sugar (2 ml) and incubated at 37°C for 30 min. The cells were washed once in PBS-sugar and then fixed.

The distribution of fluorescence was observed by epi-illumination, as previously described by Oliver et al. (18), using a Zeiss Universal fluorescence microscope. Fluorescence intensity per cell was quantified with a Zeiss photometer in series with the microscope. The photometer was set to give an arbitrary high reading for proerythroblasts labeled with either lectin, and the same settings were used for all cells of the erythroid series. A series of cell measurements was always completed in a single day, without adjustment to the optical system of the microscope. A duplicate of the first slide was repeated at the end of the series to ensure that no fluctuations in the system had occurred. Fluorescence intensity was routinely measured on 50 cells from a given cell suspension, and the mean and standard error of the mean were calculated. No attempt was made to relate fluorescence intensity to the absolute amounts of lectin bound per cell.

The photometric studies were all done with cells labeled to saturation with lectin. The results of preliminary experiments established that the routine lectin concentrations (22 μg/0.5 ml for FITC-Con A, 37 μg/0.5 ml for RCA I) are at least twice those needed for maximal binding to proerythroblasts during a 5-min incubation. Furthermore, increasing the labeling time beyond 5 min at room temperature does not increase the amount of bound lectin per cell on either immature (3 day [3d]) or mature (16 day [16d]) erythroid cells.

The results of other preliminary experiments estab-

lished that the measured intensity was directly proportional to the amount of fluorescent lectin bound. For these experiments cells were labeled at the same total concentration of lectin but with various dilutions of the fluorescent lectin. The correlation between relative intensities and labeled lectin concentrations was highly significant, with a correlation coefficient of 0.997 for Con A and 0.999 for RCA I.

RESULTS

Morphology of Erythroid Cells

Fig. 1 shows the morphology of erythroid cells at progressive stages of maturation after fixation and staining with benzidine-Wright-Giemsa's. The most immature erythroid precursors (Fig. 1 *a*) are cells harvested from cultures incubated for 44–48 h at 37°C. These cells have been identified to be typical proerythroblasts according to morphologic criteria at both the light and electron microscope levels (5, 27). Cells at this stage of culture often occur in large clusters, reminiscent of the blood islands observed in the area pellucida of 2d chick embryos (21). These grapelike clusters can be mechanically dissociated into single cells. If left undisturbed, they spontaneously dissociate, after 72 h of culture, into single cells which are, by morphological criteria, erythroblasts. Erythroid cells at later stages of maturation (Fig. 1 *b*–1 *e*) were obtained from the embryonic circulation at different days of incubation.

The most striking morphologic differences exist between proerythroblasts and more mature erythroid cells. Proerythroblasts are large and amorphous in shape. They frequently show vacuolation,

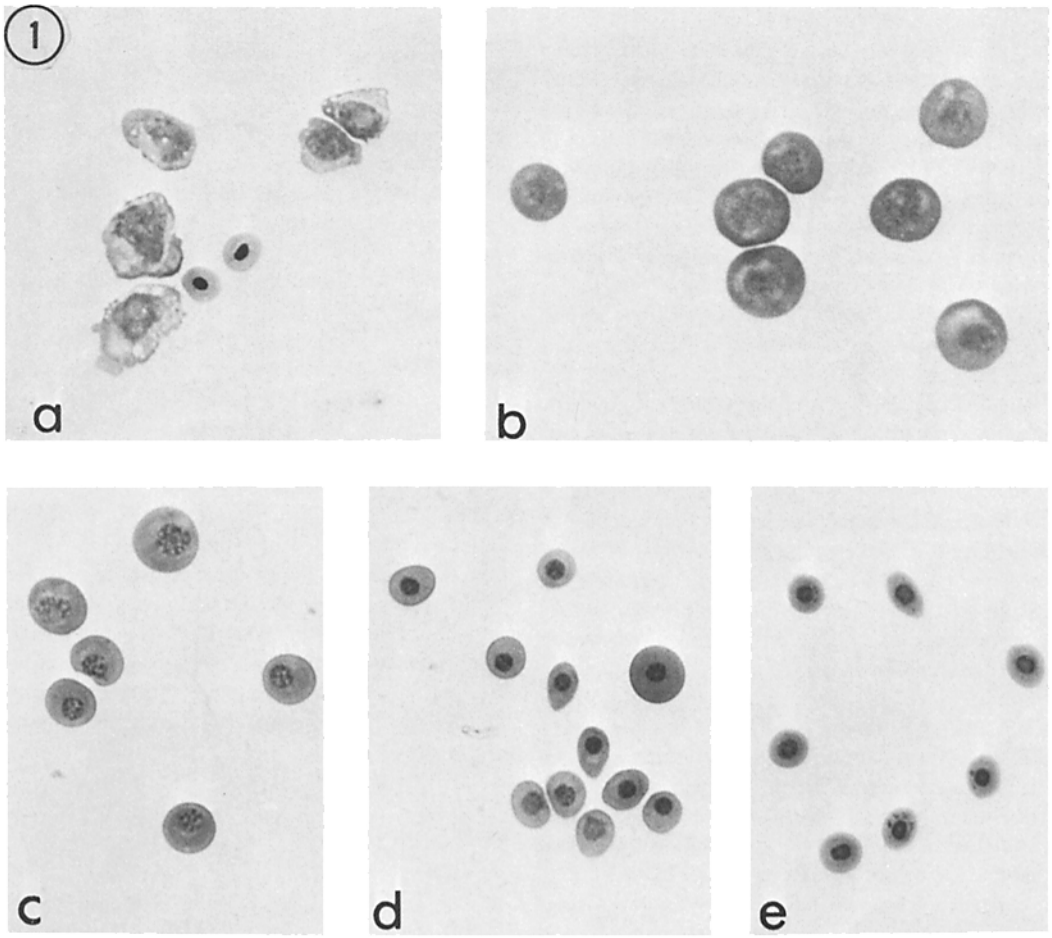

FIGURE 1 Light micrographs of chick erythroid cells at different stages of maturation stained with benzidine-Wright-Giemsa's. (*a*) Proerythroblasts from 2d cultures with two mature erythrocytes present for comparison. (*b*)–(*e*) Erythroid cells from the circulation of 3d, 5d, 7d, and 16d embryos, respectively.

and the cytoplasm is strongly basophilic and does not contain detectable amounts of hemoglobin. Multiple cytoplasmic projections are seen on these cells by Nomarski or dark-field microscope examination of unfixed cells.[1] In contrast, the erythroblasts from the circulation of 3d embryos already have a defined cell shape, and 90% of these cells contain hemoglobin. Other features which characterize the progressive maturation of the erythroid cells include a decrease in cell size, a decrease in cytoplasmic basophilia, a decrease in the nuclear to cytoplasm ratio, an increase in chromatin clumping and nuclear pycnosis, and increased hemoglobinization. These morphologic changes are characteristic of erythroid cells of both the primitive series and the definitive series (5).

Distribution of FITC-Lectins

RCA I: The distribution of RCA I on chick erythroid cells at various stages is illustrated in Fig. 2 and summarized in Table I, column 1. When cells at any stage in the series are prefixed with 1% paraformaldehyde before labeling, then the distribution of FITC-RCA I is uniform over the whole cell surface (Fig. 2 *a*, *c*, *e*). RCA I bound to prefixed cells can be quantitatively removed by postincubating with the competing sugar, D-galactose, confirming its surface location.

Cells that are labeled with RCA I for 5 min at room temperature before fixation show characteristic changes in the topographical distribution of bound lectin. RCA I is concentrated into a single polar aggregate or cap on the surface of a high proportion of proerythroblasts (Table I, Fig. 2 *b*). These polar caps are slightly different from those seen in lectin-treated mammalian lymphocytes or leukocytes (17, 18); whereas the surface of mammalian cells is usually cleared of bound lectin except in the capped region, some lectin always remains distributed over the entire cell surface of chick proerythroblasts. When longer incubation periods at room temperature are employed or when proerythroblasts are labeled at 37°C for 5 min, the caps are internalized and fluorescence can no longer be removed with D-galactose. Erythroblasts from 3d and 4d embryos do not form surface caps when labeled with RCA I (Table I). However, the RCA I is not completely immobilized; some dispersed patching is seen after the 5-min labeling period (Fig. 2 *d*), and with longer incubation periods (greater than 15 min) surface-bound lectin is internalized and appears as vesicles near the cell periphery (not shown: illustrated in Fig. 3 for Con A). In more mature cells, neither capping nor redistribution of RCA I is seen during short incubation periods (Fig. 2 *f*) and very little internalization is seen even after prolonged incubation.

CON A: The distribution of FITC-Con A on prefixed proerythroblasts is uniform as described for FITC-RCA I (Table I, Fig. 3 *a*). Similarly, Con A forms caps on proerythroblasts labeled for 5 min before fixation (Table I, Fig. 3 *b*). These caps can be eluted with α-methyl-D-mannoside after a short labeling period but they become internalized and inaccessible to the competing sugar when incubation is continued for 15 min or longer. 3d erythroblasts do not cap with Con A (Table I, Fig. 3 *d*) but surface patching occurs and bound lectin appears in vesicles inside the cells after prolonged incubation (Fig. 3 *f*). More mature cells show little internalization with long incubations.

There is one striking difference between the distribution of Con A and that of RCA I on erythroblasts and older cells. Both prefixed (10 min—12 h) and postfixed cells show a nonuniform distribution of surface-bound Con A; the lectin is concentrated in a ring in the perinuclear area of the cell (Fig. 3 *c–g*) The frequency of cells with perinuclear Con A rings is somewhat higher in prefixed cells that in their postfixed counterparts; in 4d cells, 87% of prefixed cells and 70% of postfixed cells show rings, and in 15d cells, the proporation of cells with rings is 99% and 93% for prefixed and postfixed cells, respectively.

This ring is readily removed by incubating the cells with the competing sugar, α-methyl-D-mannoside, suggesting a surface location. It is dependent on the nucleus, as shown by the double ring on a binucleate erythroblast (Fig. 3 *e*). The proportion of cells with surface rings after a 5-min labeling period increases from 0–6% on proerythroblasts to 50–70% on 3d–4d erythroid cells, and to greater than 90% on mature cells, including cells isolated from adult chicken. The intensity of the ring with Con A on 5d and older erythroid cells varies somewhat from cell to cell, ranging from a relatively low intensity on about 10% of cells, through cells with an intermediate intensity to bright and well-defined rings on about 60% of cells.

Prolonged incubation of cells with FITC-Con A results in a decrease in the proportion of cells with

[1] J. M. Oliver and L.-N. L. Chan. Unpublished observations.

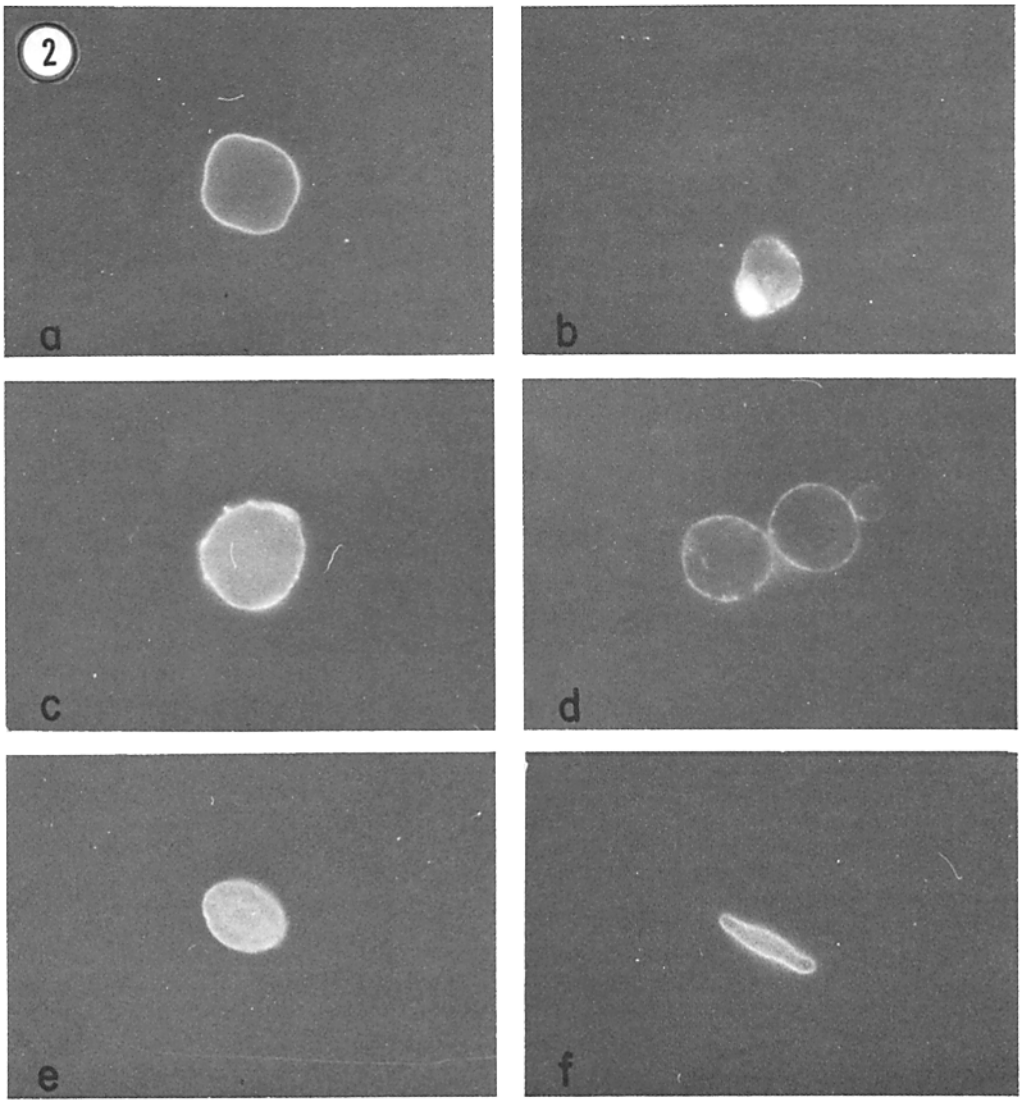

FIGURE 2 The distribution of FITC-RCA I on erythroid cells. Prefixed (left column) or unfixed (right column) cells were labeled as described in Materials and Methods. (*a*) Prefixed proerythroblast, with irregular shape and random labeling. (*b*) Proerythroblast fixed after labeling, showing a surface cap. (*c*) Prefixed 3d erythroblast, labeled randomly. (*d*) 3d erythroblast fixed after 5 min of labeling showing some surface patching. (*e*) Prefixed 15d erythroid cell. (*f*) 15d erythroid cell fixed after labeling, with no apparent redistribution of bound lectin. Photographed with 30-s exposures. Film developing times were modified to give micrographs of similar intensity for all developmental stages.

surface rings, most probably as a result of internalization of lectin. In 4d cells which show rapid endocytosis of bound Con A, the percent of cells with perinuclear rings decreases in a linear fashion with time from 70% after 5 min of labeling to 36% after 90 min of labeling. The decrease is less rapid in more mature cells where endocytosis is also slow. Thus, the percent of 15d cell with perinuclear rings decreases from 93% after 5 min of labeling to 87% after 90 min of labeling.

Con A ringing is also readily abolished by hypotonic lysis of cells to yield nucleated ghosts, or by swelling of the cells followed by labeling (Table II). Cells that are swollen and then returned to isotonic medium do not regain the ring even after extensive postincubation at 37°C in nutrient me-

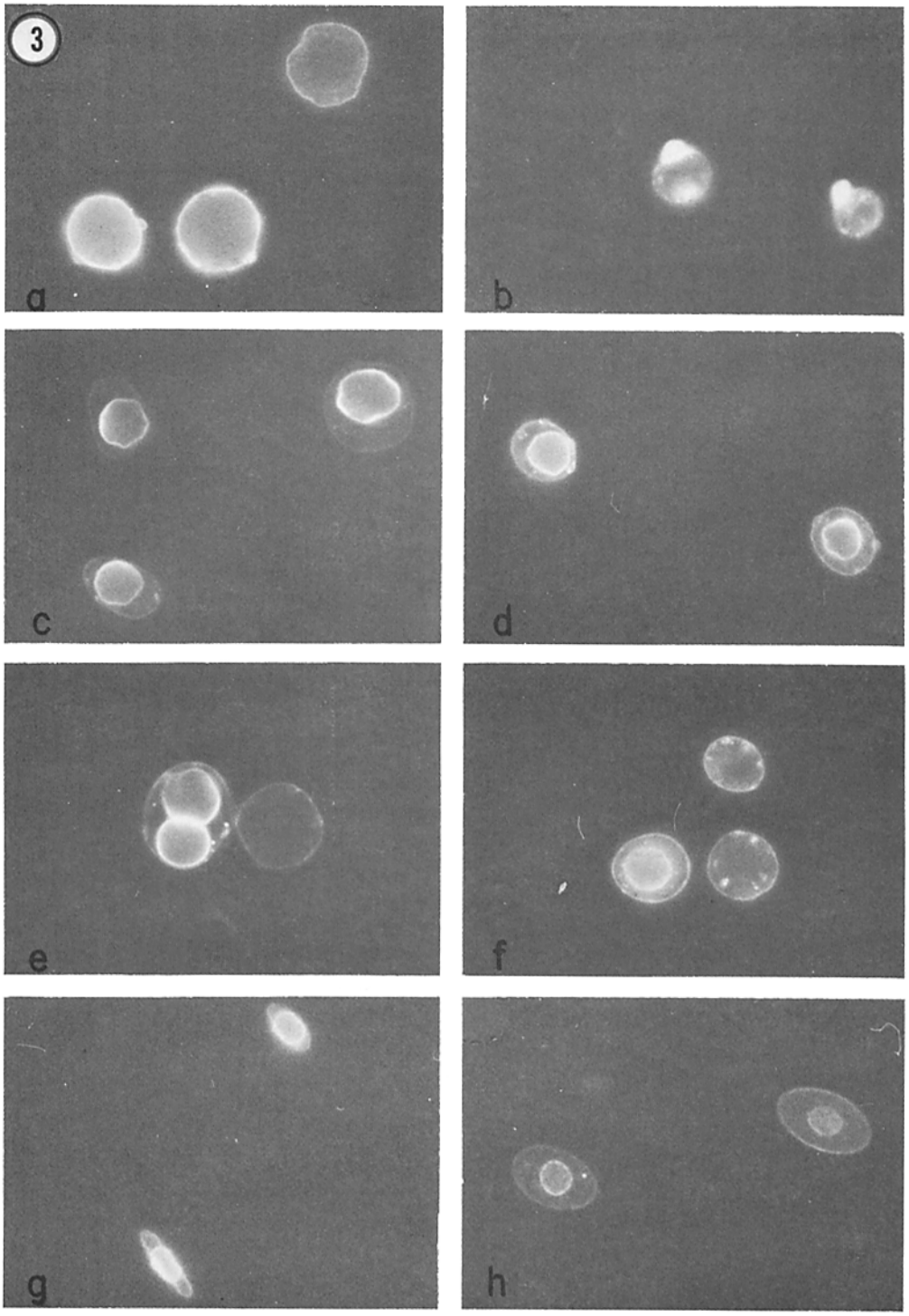

FIGURE 3 The distribution of FITC-Con A or erythroid cells. (*a*) Prefixed proerythroblast, showing uniform surface labeling. One cell shows less intense labeling and is beginning to develop a perinuclear ring (early erythroblast). (*b*) Proerythroblasts fixed after labeling, showing surface cap formation. (*c*) Prefixed 3d erythroblasts, showing Con A concentrated in perinuclear rings and peripheral patching. (*d*) 3d erythroblasts fixed after 5 min of labeling, showing perinuclear rings and limited surface patching but no surface caps. (*e*) Binucleate 3d erythroblast fixed after 5 min of labeling, showing a double ring. (*f*) 3d erythroblasts fixed after 15 min of labeling. Internalization of fluorescence has occurred from the cell periphery and from the ring. (*g*) Prefixed 15d erythroid cells. The perinuclear ring is seen on cells oriented at 90° from the usual plane of viewing. (*h*) 15d erythroid cells fixed after 5 min of labeling, with perinuclear rings and uniform peripheral distribution of fluorescence. 30-s exposure. Increasing times for film development with progressively older cells.

TABLE I

Distribution of FITC-Lectins on Erythroid Cells

Cells	Condition	Percent cells with surface caps	
		RCA I	Con A
Proerythroblasts	Fixed, labeled 5 min	0	0
Proerythroblasts	Labeled 5 min, fixed	57	64
Proerythroblasts	Neuraminidase-treated, labeled 5 min, fixed	60	61
3d erythroid cells	Labeled 5 min, fixed	2	1
3d erythroid cells	Neuraminidase-treated, labeled 5 min, fixed	2	1
10d erythroid cells	Labeled 5 min, fixed	0	0

100 cells on duplicate slides were chosen at random and scored for the presence or absence of surface caps.

TABLE II

Con A Ring Formation on 16d Erythroid Cells

Treatment	Cells with perinuclear rings
	%
Label, fix	80
Lyse, label, fix	0
Fix, lyse, label	63
Label, lyse, fix	9
Swell, label, fix	0
Swell, fix, label	0
Swell, shrink, label, fix	1
Swell, shrink, incubate 30 min, label, fix	3
Swell, shrink, incubate 60 min, label, fix	2
Swell, shrink, incubate 90 min, label, fix	6

100 cells from duplicate slides were chosen at random and scored for the presence or absence of perinuclear rings.

dium containing adult chicken serum. However, if the cells are lightly fixed before swelling or lysis, the ring is retained.

The Amount of Lectin Binding during Cell Maturation

Fluorescence intensity was measured on cells labeled with FITC-lectins for 5 min. The fluorescence intensity per erythroid cell from increasingly older embryos is plotted (as percentage of fluorescence intensity on proerythroblasts) in Figs. 4 and 5.

A very striking decrease in binding of both Con A and RCA I occurs between proerythroblasts and 3d erythroblasts. Con A binding falls by approximately 70% during this period and RCA binding by greater than 50%. Binding is identical whether erythroblasts are obtained from the culture system or from the embryonic circulation, indicating that cultured cells do not show elevated lectin binding as a result of adsorption of glycoprotein secreted into the culture medium. Thereafter, binding per cell remains relatively constant on 3d–5d cells and then continues to gradually decrease to about 12d. Fluorescence intensity is the same for nucleated ghosts which have lost their cytoplasm as for intact cells at the same stage of maturation, indicating that no significant quenching of fluorescence by cytoplasmic constituents occurs.

The Effect of Neuraminidase on Lectin Binding

Sialoproteins, a major component of erythrocyte membranes, almost invariably have a terminal sialic acid-galactose sequence (3). Nicolson (14) has previously established that treatment of erythrocytes and other mammalian cells with neuraminidase results in increased binding of RCA I, presumably due to removal of sialic acid and exposure of new galactose receptors for this lectin. Con A binding was unchanged after enzyme treatment in mammalian cells. A similar response to neuraminidase occurs in chick erythroid cells. Cells (11d) which were pretreated with 10 U/ml of neuraminidase for various times before labeling with either Con A or RCA I show a large (sixfold) increase in RCA binding, which is maximal after 30 min of enzyme treatment, whereas Con A binding to these cells is unchanged after enzyme

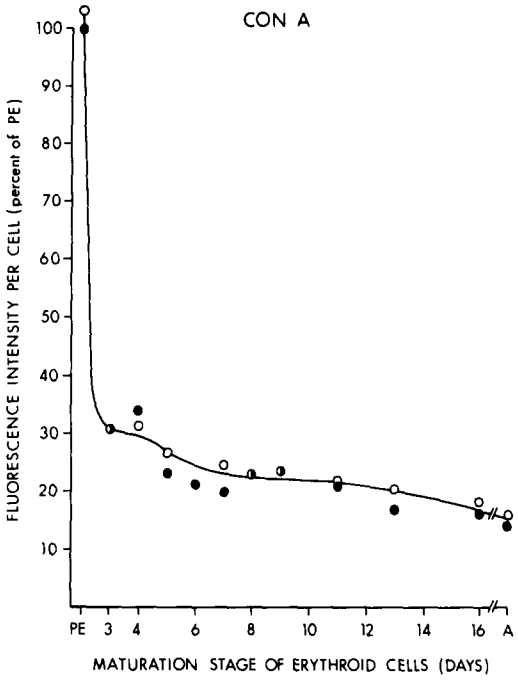

FIGURE 4 Changes in FITC-Con A binding during erythroid cell maturation. Mean fluorescence intensity per cell was calculated from measurements of fluorescence intensity of 50 cells at each developmental stage from proerythroblasts (PE) through later embryonic cells (3d–16d) to adult (*A*) erythrocytes. The maximum standard error of the mean for determinations at any stage was ±5%. Closed circles, fluorescence intensity of control erythroid cells plotted as percentage of intensity measured on control proerythroblasts. Open circles, fluorescence intensity of neuraminidase-treated erythroid cells plotted as percentage of intensity measured on control proerythroblasts. Absolute values of mean fluorescence intensity per proerythroblasts were 101.57 ± 3.99 (control cells) and 104.57 ± 3.61 (neuraminidase-treated cells).

treatment. Neuraminidase does not inhibit proerythroblast capping with either lectin, nor does it promote capping on 3d erythroblasts (Table I).

We considered that elevation of RCA I binding to cells treated with neuraminidase for 30 min at 37°C should provide an index of changes in membrane sialoproteins during maturation. Con A binding through the series of neuraminidase-treated cells was also measured to control against possible contamination of the neuraminidase by proteases. As shown in Fig. 4, neuraminidase pretreatment does not affect Con A binding to cells at any stage in the maturation process. However, RCA I binding is greatly increased on proerythroblasts and is maintained at high levels throughout the cell series (Fig. 5). In fact, when the difference between RCA I binding with and without enzyme treatment is plotted (Fig. 5) it can be seen that RCA I binding to the newly exposed receptors decreases only gradually through the cell series, with the reduction being evident only after the cells are greatly reduced in size and hence in surface area.

DISCUSSION

Our data show that the most striking changes in surface characteristics during chick erythroid cell differentiation occur between proerythroblasts and immature erythroblasts. The surface properties of proerythroblasts have not previously been described, presumably due to the difficulty in obtaining these cells from embryos. The culture system we use to obtain homogeneous populations of proerythroblasts permits morphological and bio-

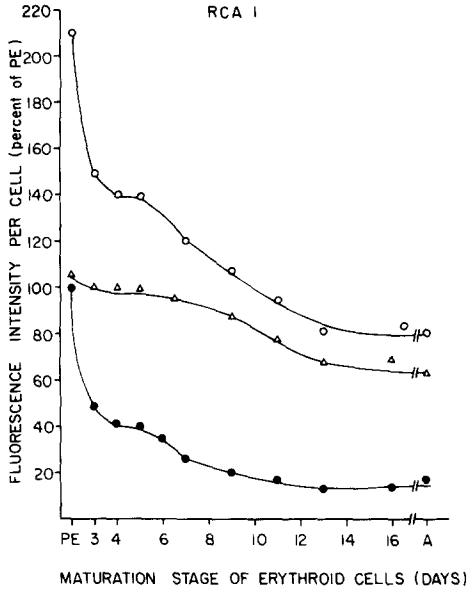

FIGURE 5 Changes in FITC-RCA I binding during erythroid cell maturation. See legend to Fig. 8. Closed circles, intensity measured on control erythroid cells; open circles, intensity measured on neuraminidase-treated proerythroblasts; open triangles, fluorescence intensity due to RCA I binding only to new receptors exposed by neuraminidase (intensity on neuraminidase-treated cells minus intensity on control cells); all plotted as percentage of intensity on control proerythroblasts. Absolute values of mean fluorescence intensity per proerythroblast were 63.76 ± 2.34 (control cells), and 133.75 ± 7.29 (neuraminidase-treated cells).

chemical study of these most immature cells of the erythroid series.

It has been established that marked changes in the mobility of lectin receptors occur during maturation of chick erythroid cells. Proerythroblasts are characterized by a very high mobility of surface receptors for Con A and RCA I that results in surface cap formation. By contrast, 3d erythroblasts show a greatly reduced mobility of receptors, as judged from the development of dispersed surface patches but not caps. The receptors on more mature erythroid cells are almost completely immobilized. These changes in lectin-receptor mobility are similar to changes reported by D'Amelio (7) for chick erythroid cell receptors for fluorescein-conjugated antiadult blood cell serum: extensive surface patching of fluorescence was observed after a 2-h incubation with antiserum on basophilic erythroblasts, the most immature developmental stage that was examined, and the distribution of fluorescence was uniform on cells from 5d and older embryos.

The loss of mobility is accompanied by a reduction in the number of lectin receptors per cell. However, it seems unlikely that reduced binding contributes significantly to the loss in mobility First, neuraminidase-treated erythroblasts bind 50% more RCA I than untreated proerythroblasts (Fig. 5). Nevertheless, these erythroblasts still form surface patches and are not induced to cap as a result of the increased amount of surface-bound lectin (Table I). As a control for this experiment, it was shown that the extent of RCA I capping on proerythroblasts is unaffected by enzyme treatment (Table I). In addition, the amount of bound lectin per cell is not greatly reduced between erythroblasts from 3d–4d embryos and erythroid cells from 5d–6d embryos, yet surface patching occurs only on the former.

The mechanism by which the highly mobile lectin receptors on proerythroblasts become immobilized on more mature cells is not yet known. It is possible that a membrane protein or membrane-associated protein that inhibits the movement of other proteins is absent from proerythroblasts but develops in erythroblasts and is present in all older cells. Preliminary studies have established that the proteins of isolated proerythroblast membranes analyzed by SDS-acrylamide gel electrophoresis are significantly different from the protein of erythroblast membranes.[2] Changes in the lipid composition of the membrane, for example, an increased proportion of saturated fatty acids or changes in cholesterol content in more mature cells, could also reduce lectin receptor mobility by reducing the fluidity of the lipid matrix of the membrane.

The apparent nonuniformity in the distribution of fluorescence due to FITC-Con A is of particular interest. In 3d erythroblasts and more mature cells, fluorescence is concentrated in a ring in the perinuclear region of the cell. These rings are observed at similar frequencies on cells whether they were prefixed (up to 12 h) before labeling or postfixed, indicating that the asymmetric distribution of Con A receptors is intrinsic to the cells and not caused by Con A binding. By contrast, all other cells so far studied (fibroblasts, leukocytes, lymphocytes, mammalian erythrocytes, etc.) show a homogeneous distribution of surface-bound Con A when examined by both fluorescence and electron microscope techniques, provided the cells are fixed before labeling or labeled in the cold to prevent lectin-induced rearrangements of receptors (17). The possibility that the rings of fluorescence seen with Con A are simply an artifact due to the curvature of the membrane over the protruding nuclei of avian red cells seems highly unlikely since the distribution of FITC-RCA I is homogeneous on prefixed cells at any stage of maturation.

There are only two possible locations for the perinuclear rings: they are either on the outer surface of the cells or in the perinuclear zone inside the cells. Several lines of evidence indicate a surface location. First, the rings are readily removed by incubation with the competing hapten, α-methyl-D-mannoside, whereas internalized fluorescence caps or patches are not. Second, the frequency of rings is observed to increase with age of the cells, whereas the pinocytic acitivities of the cells decrease with age, so that 5d and older cells no longer show internalization of fluorescence even after prolonged labeling. The third line of evidence comes from studies with the lysed cells which retain their nuclei. It was observed that these nucleated ghosts, when labeled with Con A, did not show any perinuclear rings unless the cells were lightly prefixed before lysis. If the fluorescence rings are due to the accumulation of FITC-Con A inside the cells, one would expect this accumulation to occur, if not be enhanced, in nucleated ghosts since the label now has free access to the inside of the cells. Last, and perhaps most convincingly, prefixation (up to 12 h) of the cells

[2] L.-N. L. Chan. Unpublished observations.

before labeling preserves the rings rather than abolishes them. It is not only extremely unlikely that cells after such extensive fixation can still take in significant amounts of a large molecule such as FITC-Con A, but it is even more unlikely that prefixed cells can selectively incorporate Con A but not RCA I. Thus, although we have as yet no direct proof that the perinuclear rings represent an asymmetry in the distribution of surface-bound Con A, we conclude that this is the most likely explanation.

The basis of this apparent heterogeneity is not yet understood. However, the absence of rings from cells that were swollen or lysed and then returned to a shape that is indistinguishable from that of control cells suggests that the structural integrity of the cells is intimately involved with the maintenance of the rings.

Published studies indicate that this apparent segregation of membrane components during erythroid cell maturation may not be restricted to avian systems. It has been reported that the nuclei extruded from rabbit erythroblasts are surrounded by plasma membrane that is significantly enriched for Con A receptors and surface antigens and depleted in sialic acid receptors for charged ferric oxide as compared to the density of these same receptors on the residual plasma membrane of enucleate cells (24, 26). These data suggest that a segregation of membrane determinants may precede nuclear extrusion. Similarly, Ackerman (1) found by direct observation that cationic ferritin shows increased binding over the nucleus of human reticulocytes before nuclear expulsion and that the membrane surrounding extruded nuclei is correspondingly enriched for this membrane marker.

The very marked decrease in the binding of both Con A and RCA I to their respective receptors (glucosides and mannoside for Con A, galactosides for RCA I) between proerythroblasts and erythroblasts is of significant interest. The number of receptors per cell is reduced by 50% for RCA I and 70% for Con A during this early period of cell maturation. After this rapid drop, a slow continuous decline in the number of receptors for both lectins per cell occurs through to cells from about 12d embryos. Binding thereafter remains relatively constant.

These data suggest an extensive and rapid remodeling of membrane glycoproteins, at least during early cell differentiation. A number of possible explanations exist. In dividing cells (proerythroblasts and erythroblasts), synthesis of specific glycoproteins may cease at an early stage so that, for example, a finite number of glycoproteins on proerythroblasts are divided among several daughter erythroblasts. In addition, in both dividing and non-dividing (7d and older) cells, removal of glycoproteins from the membrane, loss of the appropriate sugars perhaps by addition of sialic acid or other groups that could render the sugar inaccessible to the lectin could account for the reduction in number of receptors per cell.

In agreement with Nicolson's (15) observations with mammalian cells, it has been established that treatment of chick erythroid cells with neuraminidase removes sialic acid residues from sialoproteins and exposes subterminal galactose residues that now become available for RCA I binding. Additional RCA I binding after neuraminidase treatment is maintained at a high level on early cells and decreases only slowly at later stages, implying that the number of sialated receptors remains relatively unchanged as the number of nonsialated receptors decreases. This may reflect either continued synthesis of cell surface sialoproteins as erythroid cells divide and mature or it may reflect the kind of remodeling of glycoproteins suggested above. For example, oligosaccharide chains with accessible sugars for RCA I binding on proerythroblasts could become modified by addition of sialic acid on more mature cells. This would permit a reduction in total receptors but an increase in the proportion of receptors that are sialated.

We cannot conclude with certainty that the reduction in numbers of receptors per cell corresponds to a reduction in receptor density as cells mature, since differentiation of erythroid cells is accompanied by a progressive reduction in cell size and hence in surface area. Accurate determinations of surface area are not available. However, it seems unlikely that a reduction in surface area as large as 50–70% (the magnitude of reduction of RCA I and Con A binding) occurs between proerythroblasts and 3d erythroblasts. First, air-dried stained cells do not show a significant reduction in size by visual examination between the proerythroblasts (Fig. 1 *a*) and 3d erythroblasts (Fig. 1 *b*). Similarly, when unfixed proerythroblasts and 3d erythroblasts are viewed by dark-field or Nomarski illumination, they are similar in size and extent of surface projections.[1] Thus, at least for the immature cells, we consider that the decrease in number of receptors per cell, which parallels the reduction in cellular adhesiveness between proerythroblasts and erythroblasts, most

probably involves a decrease in receptor density. We have insufficient data to decide whether the relatively constant number of additional RCA I receptors per cell after neuraminidase treatment represents no change or an increase in the density of sialic acid moieties during maturation.

Reduction in the density of lectin receptors during maturation has previously been reported in mammalian systems. Ackerman and Waksal (2), using the Con A-peroxidase-diaminobenzidine reaction to visualize receptors electron microscopically, found that maximal binding of Con A to erythroid cells in human bone marrow occurred on erythroblasts while mature erythrocytes showed minimal surface reactivity. Electron microscope studies of Con A-ferritin binding to rabbit bone marrow erythroid cells by Skutelsky (24) provide further information about the sequence in mammalian cells. A progressive increase in Con A binding was observed during successive divisions of erythroblasts followed by an abrupt reduction in surface-bound Con A upon expulsion of the nucleus from reticulocytes. These data differ from our observations in chick cells where the number of receptors per cell and most probably receptor density decrease during early maturation. Skutelsky (24) also reported a significant increase in Con A binding after neuraminidase treatment. This result is contrary to previous studies with human erythrocytes (4) and other mammalian cells (14) where Con A binding was unaffected by neuraminidase. It is also contrary to our observations in chick cells where only RCA I and not Con A binding is enhanced after exposure to neuraminidase.

Published information about changes in sialoprotein density during development is very limited. Ackerman (1) observed that binding of colloidal iron to human erythroid cell surfaces is similar at all developmental stages. Since this positively charged particle reacts primarily with sialic acid (11, 15) these results suggest that the density of surface sialic acid is relatively constant during human erythroid cell maturation. Similarly, our data suggest that the density of sialoproteins is either unchanged or possibly increased during maturation of chick erythroid cells. By contrast, Skutelsky and Danon (25), and Skutelsky (24) found a large reduction in binding of charged colloidal ferric oxide during rabbit erythroblast maturation, followed by a small increase during reticulocyte maturation.

Finally, one purpose of this study was to explore previous suggestions of similarities between the surface properties of embryonic and malignant cells as compared to their corresponding mature or nonmalignant counterparts. We have established at least one similarity and one difference. The mobility of lectin receptors is high on both the earliest chick erythroid cell type (this study) and certain malignant cells (22). This increased mobility, if it is a general phenomenon, may account, at least in part, for the high agglutinability by lectins characteristic of various embryonic and malignant cells. However, in SV3T3-transformed fibroblast membranes, the density of lectin receptors is the same as in normal 3T3 fibroblast membranes, whereas mature erythroid cells show a lower number and probably a lower density of surface receptors than the proerythroblasts. This reduction in surface receptors could be a feature unique to maturing proerythroblasts since a decrease in cell to cell adhesiveness, which may be determined by cell surface carbohydrates (12), is a prerequisite for their release as single cells into the ciruclation.

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