Solubilization and Developmental Distribution of Embryonic Antigen of the Chick Red Blood Cell Plasma Membrane

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ABSTRACT

An antigen(s) on the surface of embryonic and newly hatched chick red blood cells was studied with antiseraum absorbed with adult red blood cells. Because of the reappearance of the antigen or some cross-reactive antigen during myeloblastosis, attempts at solubilization and characterization of the antigen were pursued. Antigen was solubilized from whole red blood cell lysates or from red blood cell ghosts with 0.01 M Tris:0.1% Nonidet P40, pH 8.0. Antigen was assayed with an enhancement of agglutination assay. Enhancement apparently occurs because of available specific receptors for the antigen on newly hatched chick red blood cells. Antigen was also found to be present in plasma, and both the membrane-derived antigen and the plasma antigen were excluded from Sephadex G-100. Isoelectric focusing of the antigen extract indicated the presence of more than one molecular species with antigenic activity.

INTRODUCTION

An embryonic antigen present on the surface of developing chick RBC disappears or becomes cryptic about 120 days posthatching (2, 8). It is, therefore, of interest to determine the nature and the biological function of this antigen and its role to the development of the chick RBC or other cells. This antigen (or antigens) reappears on the RBC surface of chickens when avian myeloblastosis occurs, resulting in the former designation of chicken fetal leukemic antigen, more properly, chicken embryonic leukemic antigen (10, 11). Evidence for the possible multiplicity of antigens comes from the observations of Dietert and Sanders (3) specific for embryonic chick RBC which are augmented in this report. The presence of a "family" of antigens detectable by a single antiseraum may mean that each of the constituent molecules plays a phase-specific role in development of, for example, the erythropoietic system. The association of some or all of these antigens with the leukemogenic process also tends to implicate them with the development of the hematopoietic system. Insight into both normal and neoplastic development might be provided by an understanding of the biological significance of these surfaces antigens. As a step toward this goal, isolation and characterization of the relevant molecule(s) were attempted. This paper deals with the solubilization of such an antigen(s) from the surface of 1-day chick RBC, the use of an enhancement of agglutination assay to detect the solubilized antigen(s), the phase-specific nature of the antigen(s), and the localization of the antigen(s) in both RBC membranes and plasma during early development of the chick.

MATERIALS AND METHODS

Preparation of RBC

Blood was collected from 1-day-old White Leghorn chicks in 50% Alsever's solution and washed 7 times in 0.85% NaCl solution. Theuffy coat was removed after each wash by aspiration. RBC obtained in this way were used either for direct lysis or for preparation of ghosts as described below.

Preparation of Solubilized Antigen

Total Cell Lysate Preparation. RBC were lysed, and antigens were solubilized by the addition of 30 volumes of 0.01 M Tris:0.1% NP 40* (Shell Chemical Company, Carson, Calif.), pH 8.0, with shaking for 10 min. The residual fraction was removed by centrifugation at 10,000 rpm at 4°, and the supernatant was collected. The pellet was reextracted as before with 10 volumes of lysing and solubilizing buffer. The supernatants of both extractions were pooled and dialyzed overnight against distilled water at 4°. The nondialyzable substances were then lyophilized to dryness, and the powder was stored at -20° until further use.

RBC Ghost and Antigen Preparation. The procedure of Hanahan and Ekholm (4) was used for the preparation of RBC ghosts as outlined below. RBC were collected and washed with 0.85% NaCl solution as before, and the buffy coat was removed by aspiration between each wash. The RBC were then washed once in 0.172 M Tris, pH 7.6 (310 mosM). The pelleted cells were cooled on ice, and isotonic Tris medium was added 1:1 (v/v). Care was taken to suspend all cells in the medium. Five-ml aliquots of the 50% cell suspension were transferred to 50 ml polyethylene centrifuge tubes. Thirty ml of 20 mosM Tris, pH 7.6 (1:15.5 dilution of 0.172 M Tris) were forcefully added into the cell suspension with a syringe. The tubes were allowed to stand for 5 min before centrifugation at 20,000 × g for 40 min at 4°. The supernatant was carefully decanted, and the membranes were well mixed on the Vortex mixer before adding another 30 ml of buffer (20 mosM). The membranes were centrifuged as above, and the wash procedure was repeated 4 times to remove all the trapped hemoglobin. Even

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after 4 washes the membranes did retain a slight pink color, indicating that some small amount of hemoglobin was still trapped.

The washed membranes were then extracted with 20 ml of 0.01 M Tris:0.1% NP 40, pH 8.0, at 20° for 1 hr. The extract was centrifuged at 20,000 x g for 40 min and the supernatant was collected. The membranes were reextracted as before, and the supernatants from each extraction were pooled. The NP 40 extracts were dialyzed against distilled H$_2$O overnight, and the dialysate was collected and lyophilized to dryness. Extracts were stored at −20° either as lyophilized powders or solubilized in 0.01 M Tris:0.1% NP 40, pH 8.0.

**Preparation of Plasma**

Plasma was collected from whole blood in 50% Alsever’s solution after removal of blood cells by centrifugation. The plasma:Alsever’s mixture was then dialyzed overnight against distilled water at 4°. The dialysate was lyophilized to dryness and stored at −20°.

**Preparation of Antisera**

Antiserum was prepared from female New Zealand White rabbits by injecting 0.5 ml (40% suspension) 1-day-old chicken RBC i.v. once weekly for 3 weeks followed by 1-ml injection i.m. (0.5 ml 40% RBC suspension plus 0.5 ml mineral oil) weekly. Rabbits were tested weekly for 5 weeks. Sera with titters of greater than 640 hemagglutination units were saved for absorption with adult RBC. Sera were heat inactivated at 56° for 30 min and absorbed 3 times with an equal volume of packed adult RBC as previously described (10, 11).

**Assay for Soluble Antigens**

An enhancement of hemagglutination assay was developed to detect solubilized antigens. The assay was done in microtiter plates (Cooke, Santa Monica, Calif.) with the use of 5 µl of antiserum and the addition of either 0, 5, 10, or 20 µl of a 20-µg/ml solution (0.01 M Tris:0.85% NaCl solution:0.1% NP 40, pH 7.6) of the antigen preparation to be tested. The antiserum:sample mixture was diluted to 100 µl by the addition of 0.85% NaCl solution. Control assays containing 0, 5, 10, or 20 µl of assay buffer were also done. The antiserum:sample mixture was then serially diluted in 2-fold steps. Agglutination was calibrated by adding 50 µl of 0.8% solution of 1-day-old chick RBC prewashed and diluted in 0.01 M Tris:0.85% NaCl solution. Enhancement of agglutination was determined as increased hemagglutination units (antigen added) above the normal agglutination units (control level) achieved by antibody alone (no antigen added). The hemagglutination assay showed a S.D. of ±0.3 unit.

**Iodination of Cell Surface Components**

RBC were washed as described before (preparation of RBC) and were washed once more in isotonic Tris, pH 7.6 (310 mosm). One ml of packed cells was then suspended in 3 ml of isotonic Tris, and 1 ml of this suspension was used for iodination (approximately 3 x 10$^9$ cells). One hundred µl of Na$^{125}$I (100 µCi; Schwarz/Mann, Orangeburg, N. Y.) and 50 µl of lactoperoxidase (2 mg/ml; Calbiochem, La Jolla, Calif.) were added to the reaction mixture, and the iodination was initiated by the addition of 10 µl of H$_2$O$_2$ (8.8 x 10$^{-4}$ M) essentially as described by Phillips and Morrison (7). Ten-µl aliquots of H$_2$O$_2$ were added every 2 min for a total of 30 min. The cells were then diluted by the addition of 40 ml of isotonic Tris, pH 7.6, and centrifuged at 800 x g for 10 min. The pelleted cells were washed twice more as above and were suspended in 2.5 ml of isotonic medium before forcefully adding 40 ml of lysis buffer (20 mosm Tris, pH 7.6). The membranes were removed by centrifugation after a 5-min lysing period, and the RBC ghosts were washed as previously described. The $^{125}$I ghosts were extracted twice in 20 ml of 0.01 M Tris:0.1% NP 40, pH 8.0, and the extracts were dialyzed and lyophilized as described.

**Preparation of Immobilized Antigen on Sepharose Beads**

CNBr-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, N. J.) were prepared according to the procedures outlined in the Pharmacia handbook. For linkage of extracted antigens to the beads, either 10 mg of lyophilized extract from 1-day RBC ghosts or 20 mg of lyophilized total lysate extract were dissolved in 1 ml of coupling buffer (0.1 M NaHCO$_3$:0.5 M NaCl:0.1% NP 40, pH 8.3). These antigen preparations were added to a 0.75-ml aliquot of swollen prewashed CNBr-activated Sepharose 4B beads and tumbled for 2 hr at room temperature. After the coupling period, 3 ml of 1 µl Tris, pH 8.0, were added to each mixture, and the samples were tumbled for 16 hr at 4°. The antigen:bead preparations were then washed 5 times with 0.1 M NaHCO$_3$:0.5 M NaCl, pH 8.3, followed with alternate washings for 5 cycles each with pH 4.0 (0.1 M acetate:1.0 M NaCl) and pH 8.0 (0.1 M borate:1.0 M NaCl) buffer. The antigen:bead preparations were then washed 3 times with 0.01 M Tris:0.85% NaCl, pH 8.0, and stored in the same solution.

**RESULTS**

The capacity of antigens to enhance a standard agglutination assay was utilized as an assay method for solubilized antigens. In this assay, the agglutination of 1-day chick RBC by RA-CELA can be enhanced by the addition of RBC surface extract (antigen) to the mixture. As is noted in Table 1, enhancement is several hemagglutination units and is easily detected. When RBC from 9-week-old chickens (instead of 1-day-old) are used for agglutination, no enhancement results from the addition of membrane antigens. RBC from adult chickens (older than 6 months) cannot be agglutinated with RA-CELA nor can they be made agglutinable by the extracts or lysates of 1-day RBC. This enhancement phenomenon can be explained by suggesting the presence of antigen-binding sites on the surface of newly hatched RBC. The antigen, therefore, would possess 2 binding sites, phenomenon can be explained by suggesting the presence of a factor in the membrane extracts that causes
localization or patching of antigenic sites of the 1-day-old chick RBC thereby enhancing their capacity for agglutination. As 9-week-old chick RBC cannot be enhanced, this proposal would therefore suggest a restriction in this mobilization phenomenon with development. Since the agglutination assay has been used throughout this investigation, the antigen-binding site and the mobilization factor proposals were further evaluated.

Evidence in favor of both the free binding-site model and the mobilization factor model is obtained from absorption experiments with solubilized antigen(s). When aliquots of solubilized antigens are absorbed several times with 1-day chick RBC, the enhancing activity is completely removed from solution (Chart 1). RBC from adult chickens have no capacity to remove this activity. These data support the thesis that (a) the soluble antigens are capable of binding to available sites on the surface of 1-day-old RBC and (b) a mobilization factor may bind to the same sites. Furthermore, the data in Table 1 indicated that 9-week-old RBC cannot be enhanced by added antigens, presumably because free binding sites were no longer available at this stage of development or 9-week-old RBC are not capable of mobilization.

For further determination of which of these proposals might be a more suitable explanation of the enhancement phenomenon, extracts from other whole cells or from RBC ghosts were coupled to CNBr-activated beads. The solubilized extracts were then used to absorb the rabbit antisera. In 2 experiments, beads coupled with the total lysate extract were capable of absorbing more, but not all, of the agglutinating activity of the antisera (Table 2). Beads coupled with antigen extract from RBC ghosts absorbed the antisera even more effectively than did the lysate. Not all of the agglutinating activity was absorbed, even in a second experiment in which additional quantities of coupled beads and extended absorption times were utilized.

These data support the antigen-receptor proposal, since the remaining agglutination after absorption and the coupled beads should be enhanced if a mobilization factor was involved. The data also suggest the possibility of 3 categories of antigen(s): (a) the antigen(s) that are not extractable by the NP 40 procedure or are not capable of being coupled to beads and account for the residual agglutination after absorption, (b) antigen(s) extractable with NP 40 that account for the loss in agglutination capacity of absorbed antiserum (e.g., 160 → 44), (c) antigen(s) extractable with NP 40 that account for the enhancement of agglutination. The fact that the enhancement activity of the antiserum is so readily absorbed would suggest that this might be different from the agglutination activity.

**Table 1**
Enhancement of agglutination of 1-day-old chick RBC by added antigen

<table>
<thead>
<tr>
<th>Agglutination units (reciprocal titer)</th>
<th>Membrane antigen only</th>
<th>RA-CELA + membrane antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-day RBC</td>
<td>6.5 (480)</td>
<td>9.0 (2560)</td>
</tr>
<tr>
<td>9-wk RBC</td>
<td>7.0 (640)</td>
<td>7.0 (640)</td>
</tr>
<tr>
<td>Adult RBC</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Chart 1. Twenty μl of lysate (20 mg/ml) were diluted to 100 μl with 0.01 M Tris:0.85% NaCl solution, pH 8.0, and adsorbed with 100 μl of packed 1-day RBC for 30 min at room temperature for 4 successive times. After each adsorption, 20 μl of lysate were removed and tested for enhancing activity by adding 5 μl of RA-CELA, allowing the mixture to stand for 15 min, diluting out, and adding 50 μl of 0.8% RBC as before. First column, agglutination with the rabbit antiserum; second column, enhancement following addition of lysate; third and fourth columns, the loss of enhancement following adsorption of lysate with 1-day-old chick cells.

**Table 2**
Absorption of RA-CELA with membrane antigens immobilized on Sepharose beads

<table>
<thead>
<tr>
<th>Reciprocal titer</th>
<th>RA-CELA and solubilized membrane antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong>: 200 μl beads-antigen</td>
<td>Unadsorbed 160 704</td>
</tr>
<tr>
<td>Adsorbed, beads-lysate antigen</td>
<td>96 88</td>
</tr>
<tr>
<td>Adsorbed, beads-membrane antigen</td>
<td>44 48</td>
</tr>
<tr>
<td><strong>Experiment 2</strong>: 300 μl beads-antigen</td>
<td>Unadsorbed 160 896</td>
</tr>
<tr>
<td>Adsorbed, beads-lysate antigen</td>
<td>104 120</td>
</tr>
<tr>
<td>Adsorbed, beads-membrane antigen</td>
<td>44 52</td>
</tr>
</tbody>
</table>

Developmental Occurrence of Antigen(s) and Available Antigenic Binding Sites. Hemagglutination of embryonic RBC can be detected readily by the seventh day of incubation (Chart 2). The agglutinability of the RBC with antiserum...
Localization of Antigen(s). For determination of whether antigen was a membrane component, RBC ghosts were prepared by the method of Hanahan and Ekholm (4). After the soluble contents of the RBC were dialyzed, lyophilized, and assayed for enhancing activity, only a slight enhancing capacity could be detected (Table 4). However, when the RBC ghosts were extracted with Tris:NP 40 and the extracts were dialyzed, lyophilized, and then assayed for enhancing activity, this fraction contained considerable enhancing activity. The association of the antigen with the plasma membrane is, therefore, hydrophobic, and the small amount of activity found in the polar fraction of the RBC extract is probably derived from the membrane during lysis of the RBC.

Some Characteristics of Chick RBC Membrane Antigens. Sephadex fractionation of 1-day RBC lysates allows a 10-fold purification of the enhancing activity. Chromatography of soluble antigen lysates over Sephadex G-100 (Chart 3) results in a fractionation of all the enhancing activity in the void volume fractions. No activity is detected in any other fraction from the column, and the activity is completely separated from the hemoglobin that is the main component in the lysates. Antigen can also be detected in the plasma of 1-day-old chicks suggesting that RBC might shed the antigen. Fractionation of the plasma over Sephadex G-100 (Chart 4) shows that the plasma antigen also elutes in the void volume fractions similar to the RBC lysate-derived antigen.

Isoelectric focusing of the total lysate extract from 1-day chick RBC shows some heterogeneity of antigenic species when these fractions are assayed for enhancing activity in the hemagglutination assay (Chart 5). At least 2 major isoforms of antigen can be detected.

Localization of antigen in RBC membrane

<table>
<thead>
<tr>
<th>Source of antigen</th>
<th>Control agglutination</th>
<th>Cytoplasm (+ Tris soluble antigen)</th>
<th>Membrane (+ NP 40-soluble membrane antigen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-day-old RBC</td>
<td>192</td>
<td>304</td>
<td>2944</td>
</tr>
<tr>
<td>7-week-old RBC</td>
<td>208</td>
<td>320</td>
<td>1472</td>
</tr>
</tbody>
</table>

*Reciprocal titer of antiserum in agglutination assay with no antigen extract added.*
were carried out by adding 30 /¿I of the sample in 10 /¿I of RA-CELA, dryness. Pooled samples were redissolved in 1 ml of 1.5 M NaCI:0.1% NP 40:0.05 M Tris, pH 8.0, and chromatographed over a 2.5 × 30-cm Sephadex G-100 column. Fractions were collected and pooled as designated and dialyzed against distilled water overnight and lyophilized to dryness. Pooled samples were redissolved in 1 ml of 1.5 M NaCl:0.1% NP 40:0.05 M Tris, pH 8.0, except Pool 1, which was taken up to 2 ml. Assays were carried out by adding 30 µl of the sample in 10 µl of RA-CELA, incubating at room temperature for 15 min and assaying by enhancement of agglutination of 1-day RBC as before.

Chart 3. Thirty mg of lyophilized lysate were dissolved in 5 ml of 1.5 M NaCl:0.1% NP 40:0.05 M Tris, pH 8.0, and chromatographed over a 2.5 × 30-cm Sephadex G-100 column. Designated fractions were pooled, dialyzed overnight against distilled water, lyophilized to dryness, and assayed as in Chart 3.

enhancing activities are observed eluting at pH 5.6 and 6.2. Some minor activity is also evident eluting after pH 7.0. The main protein contained in the lysate preparation is hemoglobin, and it focuses at an elution pH of 7.4.

When washed, RBC are labeled with 125I, and ghosts are prepared and extracted as before, the 125I-extracted proteins show a distribution from pH 4.9 to approximately pH 7.7 after isoelectric focusing (Chart 6). The 125I surface proteins show a multiplicity of peaks in a similar pH range to that of the enhancing activity (Chart 5). Immunoprecipitation of focused fractions with RA-CELA, followed by coprecipitation with goat anti-rabbit IgG, shows 1 major molecular species with 125I label to be precipitable with an elution pH of 5.5 (Chart 6). This 125I-immunoprecipitable species is remarkably close in elution pH to one of the enhancing activities previously observed (Chart 5).

The iodination data suggest that the enhancing species eluting at pH 5.4 to 5.6 is readily accessible on the cell surface while presumably other major enhancing species (eluting at pH 6.2) are not so easily iodinated. It is also possible that the process of iodination has modified the isoelectric point of the 125I species, and its similarity of elution to the enhancing activity is merely a coincidence. However, the 125I species does interact with the specific antiserum and is immunoprecipitable. Iodination may have caused a shift in pI of the antigen but has not affected the binding site to RA-CELA.
It has already been established that membrane preparations from chick erythrocytes contain both nuclear membranes and plasma membranes (1, 9, 12). It is probable that CELA is a plasma membrane molecule because of the methods of antisera preparation, the methods of 125I surface labeling, and the fact that these antigens interact with the RBC surface.

**DISCUSSION**

Some antigenic determinants unique for the embryonic chick RBC have been shown to be dissociable in a nonionic detergent NP 40. These antigens have binding sites on the surface of developing chick RBC, and these binding sites are only partially saturated in early developmental stages. The display of antigens, which are dissociable at the 1-day stage, has been shown to have at least 2 major components and probably some minor components. Analyses of developmental displays of antigens are being conducted at this time. The fact that more than 1 species of antigen are noted suggests that there are multiple antigenic determinants, an observation that is supported by the absorption and the focusing data. Interestingly, 1-day RBC from other avian species that can be agglutinated by RA-CELA cannot be enhanced by added soluble antigen (Table 3). These data indicate that all the available binding sites for antigen are saturated at the stage tested or that the binding sites on other avian species do not have the same binding specificity as chick, even though some antigenic determinants are shared. These data indicate that either the antigens from other species are not solubilized by NP 40 and therefore have a different association with the membrane than do the chick antigens or that these antigens cannot bind to the available binding sites on the surface of the chick RBC.

Direct identification of the enhancing factor as antigen by absorption of 125I-labeled IgG derived from RA-CELA with the NP 40 extract (antigen) has been attempted but not achieved. Various methods of iodination of the IgG have been used, (chloramine T and lactoperoxidase with and without the glucose oxidase modification) but without evidence of binding. Since the enhancing factor is precipitated with specific antisera (Chart 6), there seems to be little doubt that the factor and antigen(s) are identical or equivalent. Lack of binding in the experiment described above is attributed to denaturation of the IgG by the iodination process, at least with respect to the binding site.

The presence of antigenic activity in the plasma of 1-day chicks and its ability to bind to RBC suggest the possibility of secretion (shedding) of the antigen by some tissue or cell other than the RBC. It has been previously shown by indirect fluorescence (7, 11) that some cells of liver, spleen, kidney, bone marrow, bursa, and thymus in 1-day chick have antigenic activities on their surface, suggesting that a stem cell population common to those tissues may shed the antigen. Some cells of liver, spleen, kidney, and bone marrow in adult chickens also carried the antigen(s), indicating a continued synthesis and possible secretion, even in adult chickens (11).

The restriction of antigenic species (detectable by RA-CELA) in the adult RBC population can be interpreted in several ways: (a) it is possible that the gradual sequential disappearance of individual antigenic species may occur with development, resulting in a relatively small group of antigen(s) in the adult stage; (b) alternatively, it could be postulated that all the antigenic species diminish or disappear simultaneously; (c) it is also possible that none of the antigens disappear with development but become masked by a topographical modification of the cell surface. The cryptic nature of antigens remaining in the adult RBC is suggested by the agglutination enhancement capacity of membrane extracts from adult RBC, even though these cells fail to agglutinate in the presence of RA-CELA. Should the restriction of antigenic species during development occur with the disappearance of individual antigens in a phase-specific manner, then the identification of the RBC surface antigens that reappear during leukemogenesis might be of particular diagnostic significance.

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