Chicken Fetal and Adult Antigen Expression on Erythroleukemia Cells before and after Induced Differentiation

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ABSTRACT

Avian erythroblastosis virus strain R (AEV)-transformed, cloned erythroleukemia cells from three different ages of SC strain chickens were analyzed before and after differentiation induced by 1.0 mm butyric acid for expression of chicken fetal antigens (CFAs) and chicken adult antigens (CAAs) for hemoglobin expression. Immunofluorescent analyses show the loss of individual CFA determinants from erythroleukemia cells with induced differentiation, although there appeared to be no correlation between CFA loss and onset of hemoglobin production. Erythroleukemia cells were examined by cell surface labeling followed by immunoprecipitation with antisera specific to CFAs and CAAs. Erythroleukemia cells expressed CFAs and CAAs on their membranes that are not reported to be expressed by the target cell of AEV. The expression of CAAs and the enhanced expression of CFAs by erythroleukemia cells may be due to limited cellular differentiation, alterations in regulatory controls of genes coding for CFAs and CAAs, or increased levels of production of previously undetected CFAs and CAAs following AEV transformation. Control and induced erythroleukemia cells expressed CFAs and CAAs that differed both quantitatively and qualitatively from normal erythroid cells. Molecular weight variations of CFAs and CAAs observed in the erythroleukemia cells may represent glycosylation differences between AEV-transformed cells and normal erythroid cells.

INTRODUCTION

During chicken development, definitive-series RBC display phenotypic variation in the expression of 2 membrane antigens, CFAs and CAAs (2, 36, 37).

CFA is an antigenically complex system of hematopoietic-lymphoid cell surface markers (5, 7, 8, 28). Originally described as embryonic peripheral RBC antigens (36), CFA determinants have since been detected on all immature cells of the erythroid maturation series in adult bone marrow (31), on peripheral and splenic lymphocytes, thymus cells, bursa cells, and on certain lymphoblastoid cell lines (3, 30, 37). CFA determinants are gradually lost from the membranes of primitive and definitive peripheral RBC during chicken development (6, 8, 32). CFA was established as an oncodevelopmental antigen when studies showed that peripheral RBC from avian myeloblastosis virus-infected adult chickens expressed CFAs (38, 39). CAA, another complex system of cell surface markers, appears on a small number of chicken peripheral RBC at hatching, gradually increases in expression, and persists throughout adult life.

Three distinct populations of definitive RBC have been demonstrated in the circulation of the developing chicken (2, 24): RBC expressing predominately CFAs, present from before hatching to 60 days after hatching; RBC expressing both CFAs and CAAs, present from hatching to 130 days after hatching; and RBC expressing predominately CAAs, present from shortly after hatching through adult life (24). Equivalent embryonic and adult RBC membrane antigens have been reported by Blanchet (2).

AEV is a replication-defective acute leukemia virus that causes specific transformation of avian erythroid precursors (11, 14, 15, 17). A blockage in cellular differentiation has been proposed as a mechanism of leukemogenesis (13, 37). Murine and avian virally transformed erythroleukemia cells differentiate to a more mature state following chemical induction (9, 14). A variety of agents are effective inducers of in vitro cellular differentiation (9, 18, 27, 34). One effective inducer of AEV-transformed erythroleukemia cells in chickens is butyric acid (16, 21). In vitro differentiation can be monitored by various parameters, among them hemoglobin production (9, 14, 16, 21), the production of RBC-specific antigens (1, 14, 16), and alterations in RBC-specific antigenic determinants (10, 19) that parallel those associated with normal in vivo erythropoiesis (4, 29).

Objectives of this study were to evaluate CFAs and CAAs as erythroid differentiation markers. Cloned AEV-transformed erythroid cells were utilized to test this hypothesis. Specifically, studies examined (a) the expression of CFAs and CAAs on cloned erythroleukemia cells before and after differentiation induced by butyric acid and (b) clones derived from different ages of chickens for phenotypic variation in the expression of CFAs and CAAs.

This paper shows that (a) erythroleukemia cells express both CFAs and CAAs; (b) CFA determinant expression decreases and the percentage of cells expressing hemoglobin increases in erythroleukemia cell cultures induced to undergo differentiation by butyric acid; and (c) chemically induced differentiation of erythroleukemia cells results in changes in both CFA and CAA expression; and (d) erythroleukemia cell clones derived from chickens of different ages expressed CFA and CAA molecules of identical molecular weights that were altered in a similar manner after differentiation induced by butyric acid.

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**MATERIALS AND METHODS**

**Experimental Animals.** All chickens used in this study were of the inbred SC strain purchased as fertile eggs from Hyline International (Johnston, Iowa), hatched, and raised to various developmental ages.

**Preparation of Antiserum.** Anti-CFA sera were prepared as described by Sanders (36). Anti-CFA determinants 1 to 6 sera were prepared according to the procedures outlined by Sanders (36). Anti-CAA sera were prepared in rabbits using washed peripheral RBC (buffy coat removed) from chickens older than 180 days following procedures described by Sanders for anti-CFA sera production (36). Antiserum to adult RBC were made adult specific by exhaustive adsorptions with washed peripheral RBC from 18-day-old embryos. The resulting absorbed antiserum are referred to as anti-CAA sera.

**Virus Stocks.** Viruses used in this study were AEV strain R with the associated natural helper virus (20). Viral stocks were obtained from spent culture medium of producer erythroleukemia cells. (Original stock courtesy of Dr. Jacalyn Hoelzer, Department of Microbiology, The University of Texas, Austin, Texas).

In Vivo Transformation. H14 were given i.v. and i.p. injections every other day for 6 days of 1.0 ml of undiluted virus stock (untittered). Progress of the erythroleukemia was monitored by examination of wing webs for petechiae or examination of smears of peripheral blood stained with Wright's stain for the presence of neoplastic cells. Chickens were sacrificed when neoplastic cells appeared or when there was evidence of peripheral hemorhaging (usually within 10 to 14 days after the primary injection). Bone marrow cells were harvested aseptically from femurs with ringing with RPMI 1640 (Irvine Scientific, Santa Ana, Calif.). Single-cell suspensions were achieved by gentle teasing of the marrow plug. Then cells were either cultured in 25-ml flasks for 3 days before cloning or cloned directly into 0.35% Noble agar (Difco Laboratories, Inc., Detroit, Mich.) as described in the section on soft-agar cloning.

In Vitro Transformation. H2 and H14 bone marrow were harvested by aseptic removal of femurs which were rinsed with RPMI 1640. Single-cell suspensions were obtained by the same procedures as described above. Yolk sacs from E8 were dissected into culture medium, and the cells were gently teased apart. All cells were washed twice in RPMI 1640. Then 1.0 ml of undiluted virus stock was added to 1 x 10^6 cells. After 1 hr of incubation at 37°C, the virus-transformed cells were cloned directly into 0.35% Noble agar as described in the section on soft-agar cloning. Separate cultures were maintained for cells obtained from each of the 3 different-aged chickens.

**Cell Culture.** All erythroleukemia cells were maintained in serum-free RPMI 1640 with 5% glutamine (Sigma Chemical Co., St. Louis, Mo.) prepared from a 200 mm stock solution, 15% fetal calf serum (M. A. Bioproducts, Walkersville, Md.), 1% penicillin (Grand Island Biological Co., Grand Island, N. Y.) prepared from a stock solution of 10,000 IU/ml, 1% streptomycin (Grand Island Biological Co.) prepared from a stock solution of 10,000 µg/ml, and 0.5% fungizone (Flow Laboratories, Inc., McLean, Va.) prepared from a stock solution of 250 µg/ml. Serum-free RPMI 1640 containing these supplements is hereafter referred to as supplemented medium. All cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°.

**Soft-Agar Cloning.** Serial dilutions (1.0 x 10⁶ to 0.03 x 10⁶/0.01 ml) of E8 yolk sac or H2 or H14 bone marrow cells were cloned into 0.36% Noble agar prepared with supplemented medium with the addition of 3% chicken serum (KC Biologicals, Lenexa, Kans.) and 4% beef embryo extract (Grand Island Biological Co.) and the omission of glutamine. Erythroleukemia cell clones of approximately 1-mm diameter were picked and placed into 24-well microtiter plates containing 0.5 ml of conditioned medium (supplemented medium in which erythroleukemia cells had been cultured previously). Over a period of 3 to 5 days, the culture volume was gradually increased to 1.5 ml with conditioned medium. Next, the 1.5-ml cultures were transferred to 25-ml culture flasks and 1.5 ml of conditioned medium were added. Over a period of approximately 5 days, the cultures were gradually increased to 5.0 ml with conditioned medium. At this time, the cultures were split into 25-ml culture flasks containing 2.5 ml of conditioned medium. When the cells were growing well, they were centrifuged at 1000 x g and 5.0 ml fresh supplemented medium were added. The cultures were maintained in supplemented medium until sufficient numbers of cells were obtained for study.

**Butyric Acid Induction of Erythroleukemia Cells.** Preliminary experiments using different inducing agents at different final concentrations revealed that butyric acid at a final concentration of 1.0 mM was the best inducer. Only low levels of induced cells (i.e., hemoglobin-positive cells) were obtained with dimethyl sulfoxide treatment while no induction was observed with hexamethylene bisacetamide treatment. Prior to induction, clones were analyzed for viability by trypan blue dye exclusion and for percentage of cells "spontaneously" expressing hemoglobin by a modification of the benzidine technique of Ralph (33). During log growth, approximately 1 x 10⁶ cells from a single culture flask were centrifuged at 1000 x g, the supernatant was removed, and the cells were split into 2 flasks. A full volume of supplemented medium was added to the preinduced (control) flask. One-half volume of supplemented medium and and one-half volume of a solution of 2.0 mM n-butyric acid (M, 88.1; Sigma) in supplemented medium was added to the induced flask accompanied by gentle swirling to ensure mixing [final concentration, 1.0 mM butyric acid, according to Leder and Leder (27)]. After 72 hr of incubation, cells were harvested from the preinduced flask and the butyric acid-induced flask and washed prior to use. Cell viability of control and induced cells was evaluated by trypan blue dye exclusion, and percentage of hemoglobin-containing cells was determined by benzidine staining. In all cases, cell viability was greater than 70%, with an average viability of 83%.

**Cell Surface Labeling.** In vitro AEV-transformed erythroleukemia cells from E8, H2, and H14 were labeled following the procedures of Kesski-Oja et al. (22). Briefly, cells were washed 3 times in DPBS followed by a single wash in DPBS containing 1 µM potassium iodide. Approximately 2.1 x 10⁶ cells were brought up to a total volume of 0.5 ml in DPBS. Next, 10 µl of lactoperoxidase (1 µg/µl in DPBS; Sigma) and 0.2 IU glucose oxidase (Sigma) were added to each sample, followed by the addition of 250 µCi ¹²⁵I in 0.5 ml of 10 mM glucose. Samples were mixed well and incubated at room temperature for 15 min with frequent gentle mixing. The reaction was quenched by the addition of 10 µl of sodium azide in DPBS and washing the cells 3 times in ice-cold DPBS containing 5 mM potassium iodide. Cells were resuspended in 0.5 ml DPBS and lysed by adding 0.5 ml of 1.0% Nonidet P-40 in 10 mM Tris, pH 7.3. After 30 min on ice, the lysates were spun at 36,000 x g for 20 min to remove insoluble material.

**Immunoprecipitation and Analysis of Cell Surface Proteins.** Immunoprecipitation was carried out by using heat-killed, formalin-fixed Staph A, prepared as described by Kessler (23). Cell extracts (1.0 ml) were incubated for 1 hr with 0.25 ml 10% Staph A in wash buffer (0.01 M Tris-HCl, pH 8.0, containing 0.15 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). After removal of the bacterial by centrifugation (7000 x g, 1 min), 100 /µl of the cleared extracts were incubated (4°, 16 hr) with anti-CFA sera or anti-CAA sera. The anti-CFA and anti-CAA sera had been blocked previously by incubation with 5% Nonidet P-40 extracts of unlabeled adult or unlabeled 1-day-old RBC, respectively. Immune complexes were then collected by incubation with 100 µl of 10% Staph A for 1 hr. The Staph A were harvested by centrifugation (7000 x g, 1 min) and washed 3 times with the wash buffer. Bound antigens were then eluted by resuspension of the Staph A in 50 µl of SDS sample buffer (0.6 M Tris-HCl, pH 6.7, containing 2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol, and 0.002% bromphenol blue) and incubated at 100° for 5 min. The antigens were then analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (10% acrylamide, 0.26% bisacrylamide) as described previously (26). Slab gels were stained with Coomassie Brilliant Blue R250, dried on a slab drier, and exposed on SRP-1 Kodak film. The following proteins were used as molecular weight markers: bovine serum albumin (M, 68,000); ovalbumin (M, 43,000); and carbonic anhydrase (M, 29,000) (Sigma).
Approximations of protein quantities were made by scanning autoradiographs on an EC Apparatus Corp. densitometer and calculating the area under the peaks indicating individual proteins.

**Analysis of Hemoglobin Production.** The production of hemoglobin in control and induced erythroleukemia cells was evaluated using a modification of the benzidine technique of Ralph (33). Cells were harvested, washed twice in 0.75% NaCl solution, and resuspended in 1% bovine serum albumin in 0.75% NaCl solution. Slides were prepared, fixed in absolute methanol, and stained with a 1% solution of 3,3′-dimethoxybenzidine (Eastman Chemical Co., Rochester, N. Y.) in methanol with 25% hydrogen peroxide in 70% ethanol. Two hundred cells/clones were scored, and the percentage of benzidine-reactive cells was calculated.

**Indirect Immunofluorescence.** Erythroleukemia cells from H14 in vivo-transformed clones (control and induced) were harvested, washed 4 times in phosphate-buffered saline solution [0.07 M NaCl, 0.75 M KH₂PO₄, and 0.075 M Na₂HPO₄ (pH 7.2)], and assayed with anti-CFA sera (Determinants 1 to 6) by indirect immunofluorescence using previously described techniques (31). Two hundred cells/clone were scored for positive or negative fluorescence on a modified Leitz epifluorescence microscope.

### RESULTS

**CFA Determinant Expression and Hemoglobin Production by Cloned AEV-transformed Erythroleukemia Cells before and after Butyric Acid Induction.** Four clones of erythroleukemia cells derived from in vivo AEV-transformed H14 bone marrow cells were analyzed by indirect immunofluorescence for the expression of CFA determinants 1 to 6 before and after butyric acid induction (Table 1). The production of hemoglobin, as determined by benzidine staining, was used as an established marker of erythroid differentiation (Table 1).

Preinduced erythroleukemia cells exhibited heterogeneity for the expression of CFA Determinants 1 to 6. The number of erythroleukemia cells reactive with the CFA determinant specific antisera significantly decreased after butyric acid induction for all CFA determinants with the exception of CFA Determinant 3. Although there was a 34% decrease in the percentage of induced erythroleukemia cells expressing CFA Determinant 3, there was considerable variation among the 4 clones. The induced erythroleukemia cells exhibited a significant increase in the number of benzidine-positive cells after induction, although the number of benzidine-positive cells per clone was variable. Preinduction numbers of cells per clone producing hemoglobin were from 0 to 30% while postinduction levels ranged from 21 to 67%.

**CAA and CFA Expression on Cloned AEV-transformed Erythroleukemia Cells before and after Butyric Acid Induction.** Immunochemical analyses of CAA and CFA expression were conducted on clones of erythroleukemia cells derived from in vitro-transformed E8 yolk sac and H2 and H14 bone marrow cells. Clones were selected for immunochemical analyses that exhibited high viability (assayed by trypan blue dye exclusion analyses) and low numbers of cells which spontaneously expressed hemoglobin (assayed by benzidine staining). Prior to induction, the E8, H2, and H14 clones exhibited 2, 2, and 4% hemoglobin-containing cells, respectively; and all 3 clones had greater than 87% viability (87, 88, and 90%, respectively). Cells from the 3 clones were divided into 2 groups: control and induced. The control and induced groups were incubated for 3 days and received identical treatment with the exception that the induced groups were cultured in the presence of butyric acid. The control clones did not change in either percentage of hemoglobin-containing cells or viability during the 72-hr culture period. The butyric acid-induced clones exhibited increased numbers of hemoglobin-positive cells (63, 52, and 67%, respectively) and were greater than 84% viable (86, 84, and 88%, respectively) after 72 hr of culture with butyric acid.

CAAs were isolated from extracts of radiiodinated cells of E8, H2, and H14 clones by immunoprecipitation with anti-CAA sera, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Before induction with butyric acid, all clones expressed a major molecule with an apparent molecular weight of 84,000 and a minor protein with a molecular weight of 92,000. After induction, all clones expressed an additional protein with an average molecular weight of 45,000 (Fig. 1A). Quantitative relationships of CAA expression were determined by densitometric analyses of autoradiograms (Chart 1A). In all clones examined, the M, 84,000 molecule showed a decrease of 44% after induction while the induced M, 45,000 molecules showed a marked increase, representing 22 to 64% of total major proteins immunoprecipitated after induction.

CFAs reactive with anti-CFA sera were analyzed by SDS-polyacrylamide gel electrophoresis as described above for CAAs. Before and after induction, all 3 clones expressed molecules with apparent molecular weights of 94,000 and 87,000 and a third major component with an average molecular weight of 59,000. With the possible exception of E8 preinduced cells, all clones expressed a fourth protein with an apparent molecular weight of 50,000 (Fig. 1B). Quantitative analyses of autoradiograms (Chart 1B) showed that all 3 clones exhibited

### Table 1

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<thead>
<tr>
<th>Erythroleukemia cells positive for CFA determinants (%)</th>
<th>% of hemoglobin-positive cells</th>
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<tbody>
<tr>
<td>Expression of CFA determinants 1 to 6 and hemoglobin production by pre-induced and butyric acid-induced cloned erythroleukemia cells</td>
<td></td>
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<tr>
<td>Erythroleukemia cells</td>
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</tr>
<tr>
<td>Preinduced 85 ± 5</td>
<td>74 ± 18</td>
</tr>
<tr>
<td>Induced 58 ± 25</td>
<td>31 ± 30</td>
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* Four lines of cloned erythroleukemia cells (obtained from AEV-infected H14) were assayed for CAAs and hemoglobin before (preinduced) and after (induced) butyric acid-induced differentiation.

* A minimum of 200 cells in each clone (800 total) were scored and percentages were calculated. The expression of CFA determinants 1 to 6 on control and induced erythroleukemia cells was assayed by indirect immunofluorescence using rabbit antibody rendered monospecific for the individual CFA determinants. Anti-CFA sera, adsorbed with RBC from avian species possessing differing combinations of CFA determinants, yield monospecific antibodies that identify different CFA determinants (6, 7, 37).

* Average ± S.D., determined by combining data from 4 different clones of erythroleukemia cells.
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Fig. 1. Erythroleukemia cells transformed from E8, H2, and H14 were examined by cell surface labeling followed by immunoprecipitation with anti-CAA sera (A) and anti-CFA sera (B). Induced erythroleukemia cell clones were harvested after 72 hr in culture with 1.0 mM butyric acid. Control (C) and induced (I) cells were labeled, immunoprecipitated, and analyzed by SDS-polyacrylamide gel electrophoresis as described in “Materials and Methods.” The exposure times for the autoradiograms depicted in A and B were 5 and 2 hr, respectively. Apparent molecular weights indicated in kilodaltons (Kd) were determined using the following molecular weight standards: bovine serum albumin (M, 68,000); ovalbumin (M, 43,000); and carbonic anhydrase (M, 29,000); which were electrophoresed with each assay.

Chart 1. Densitometer tracings of autoradiographs shown in Fig. 1. See legend to Fig. 1. Autoradiographs were scanned on an EC Apparatus Corp. densitometer. Overlays of tracings from uninduced control erythroleukemia cell clones (-----) with induced erythroleukemia cell clones (-----) clearly illustrate alterations in the quantities of proteins expressed before and after butyric acid-induced differentiation. A corresponds to the autoradiogram depicted in Fig. 1A and B corresponds to the autoradiogram depicted in Fig. 1B. Tracings of autoradiograms of normal rabbit serum (NRS) controls are also depicted. Apparent molecular weights of proteins designated in kilodaltons (Kd) are shown.
weight shifts were observed in the heterogeneous M, 59,000 molecules after induced differentiation. The heterogeneity of the M, 59,000 molecules exhibited by the erythroleukemia cells may be due to glycosylation patterns unique to each specific clone. Induced erythroleukemia cells showed a 70 to 100% reduction in levels of the M, 87,000 molecule.

Finding CFAs and especially CAAs on both control and induced AEV-transformed cells from clones derived from all 3 of the different ages of chickens tested was not expected and is of interest. It has been reported that the AEV target cell is a committed BFU-E which does not express CAAs but does exhibit a low level of a subset of CFA determinants (12). Assuming that BFU-Es are the targets of AEV infection, the expression of CAAs and the enhanced expression of CFAs by uninduced erythroleukemia cells must occur after infection with AEV and may represent: (a) limited or altered cellular differentiation after viral transformation which has been suggested by Graf and Beug (14); (b) alterations in regulatory controls of genes coding for CFAs and CAAs due to viral infection; or (c) increased levels of production of CFAs and previously undetected CAAs following AEV transformation.

In chickens, erythroid differentiation proceeds from stem cells to erythrocytes through several intermediate steps which have been summarized by Gazzolo et al. (12) as: colony-forming units in marrow → BFU-Es → CFU-Es → erythroblasts → erythrocytes. Samarat et al. (35) proposed the presence of 4 age-related CFU-E stem cell populations in the developing chicken that express different CFA and CAA phenotypes: CFA only; CFA and CAA; CAA only; and neither CFA nor CAA. Although definitive stem cell analyses have not been performed in our laboratory, immunofluorescence analyses show immature erythrocytes undergoing cellular maturation in the bone marrow to express both CFAs and CAAs, regardless of the age of the chicken. Furthermore, in this study, over 40 erythroleukemia cell clones (23 of which were transformed from bone marrow derived from H14) have been examined thus far and all expressed both CFAs and CAAs. Butyric acid induced the cells in all clones irrespective of the age of the chicken from which the cells were taken for in vitro AEV transformation to express an adult-related M, 45,000 molecule. Assuming that 4 distinct CFU-E age-related stem cells exist, these data support limited or altered cellular differentiation following AEV transformation on BFU-E precursors and suggest that AEV transforms BFU-E target cells which give rise only to the CFU-E stem cell population that expresses both CFAs and CAAs. Alternatively, there may be only one type of CFU-E stem cell, regardless of the age of the chicken, where CFA and CAA expression is cell maturation dependent. If this is the case, erythroleukemia cells might be viewed as cells that are blocked at a specific maturation stage where both CFAs and CAAs are expressed.

REFERENCES


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