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Changes in the Expression of Membrane Antigens During the Differentiation of Chicken Erythroblasts

Michael J. Hayman, Hartmut Beug, and Keith W. Savin

Imperial Cancer Research Fund, London WC2A 3PX, England (M.J.H., K.W.S.) and Institut für Virusforschung, Deutsches Krebsforschungzentrum, Im Neuenheimer Feld 280,6900 Heidelberg 1, Federal Republic of Germany (H.B.)

Chicken erythroblasts can be transformed by the avian retrovirus, avian erythroblastosis virus (AEV). Earlier studies have shown that the mechanism of transformation appears to involve a "block" in differentiation, in that when erythroblasts are transformed by a temperature-sensitive mutant of *ts*34 AEV and incubated at the nonpermissive temperature, the cells start to differentiate and produce hemoglobin. We have decided to use this system to isolate pure populations of chicken erythroblasts and raise monoclonal antibodies against their cell surface proteins. Three monoclonal antibodies were isolated and tested for their ability to bind to various hematopoietic cell types; two were shown to be erythroid-specific, whereas the other antibody bound to proliferating cells but not to erythrocytes or granulocytes. Of the erythroid-specific antibodies, one precipitated a 94,000 molecular weight protein, whereas the other precipitated a 11,000 molecular weight protein that was tentatively identified as hemoglobin. The use of this system and approach to identify and evaluate changes that occur during the differentiation is discussed.

Key words: monoclonal antibodies, erythroid differentiation

Avian erythroblastosis virus (AEV) is a replication-defective leukosis virus causing acute erythroid leukemia and slowly developing sarcomas in chickens [1]. In vitro infection of bone marrow cells gives rise to colonies of rapidly dividing erythroblasts, and cloned virus is also able to transform fibroblasts [2 (review)]. Characterization of the in vitro transformed erythroblasts with regard to their antigenic phenotype showed that it was identical to that of the erythroblasts generated in vivo [3]. The mechanism of transformation by AEV has been postulated to involve a "block" in differentiation of the erythroblasts [4]. This postulate is based on experiments with a temperature-sensitive mutant of AEV (designated *ts*34 AEV). This mutant can transform erythroblasts at the permissive temperature of

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35°C, but when the cells are shifted to the nonpermissive temperature, 41.5°C, the viral transforming functions are inactivated and the cells appear to differentiate [4]. This differentiation is most markedly manifested by the increased synthesis of hemoglobin.

We have decided to exploit this virus-induced transformation system to look at the changes that occur during the differentiation of chicken erythroblasts. The advantages of this system are basically twofold. Firstly, the ability to transform chicken bone marrow cells in an in vitro colony assay means that clonal populations of erythroblasts can be isolated in large numbers, up to 10^8 cells, and therefore, pure populations of a cell type that normally represents less than 1% of bone marrow cells can be isolated. Secondly, the availability of the temperature-sensitive mutant, *ts*34 AEV, enables one to study the differentiation of pure populations of cells in vitro in a system where differentiation is induced by merely growing the cells at 41.5°C, a temperature that is essentially the normal blood temperature of a chicken. The approach taken has been to inject virally transformed erythroblasts into mice with the aim of producing monoclonal antibodies against the cell surface molecules of the erythroblasts. These antibodies will be used to characterize the molecules on the cell surface of the erythroblasts and to check whether they change during differentiation by seeing whether they are expressed on chicken erythrocytes. When differentiation specific molecules are identified, we intend to use the *ts*34-AEV transformed erythroblast system to study the control of the expression of these molecules and try to identify their role, if any, in the differentiation process.

In this paper, we report the preliminary isolation and characterization of monoclonal antibodies against molecules that change during differentiation and point out certain pitfalls of the production of such antibodies.

METHODS

Cells

Normal bone marrow cells were prepared from 1–4-week old SPAFAS chickens as described previously [5].

Erythroblasts transformed by AEV were obtained from infected bone marrow cultures as previously described [5]. Cell lines derived from colonies transformed by wt AEV or *ts*34 AEV were used throughout [Beug et al, in preparation]. These were designated LSCC-HD1 (AE/EB), or AEV-Ebl, 6C2, and LSCC-HD3 (*ts*AE/EB/np) or C1, respectively. Cells were cultured at 35°C or 41°C as described [3]. Erythroblasts and peripheral blood erythrocytes were washed twice in phosphate-buffered (10 mM sodium phosphate, pH 7.2) saline before use.

The following virally transformed cells were used to test the binding of the hybridoma supernatants: LSCC HD11, a line of avian myelocytomatosis virus strain MC29 transformed macrophages [3]; LSCC HD12, a line of avian myeloblastosis virus; AMV, transformed myeloblasts [Beug et al, in preparation]; LSCC TLT, an avian leukosis virus (ALV) transformed B-lymphoid line [6]; and MDCC-MSB-1, a Marek's disease virus (MDV) T-lymphoid line [7]. Avian reticuloendotheliosis virus (REV) transformed cells were prepared from in vitro transformation of chick bone marrow and are thought to represent immature lymphoid cells [8].

Hybridomas

A Balb/c mouse was injected intravenously with 2×10^6 erythroblasts of the cell line C1 that had been growing for five days at 41°C, the restrictive temperature of *ts*34 AEV. Four days later the spleen was removed and immune splenic leukocytes fused to cells of the HAT-sensitive mouse myeloma line P3/NSI/I-Ag-4 [9] according to [10]. The resulting cell suspension was divided between 144 two-ml cell culture wells and hybrids propagated in RPMI-1640 culture medium containing 20% fetal calf serum. HAT was added to each well 24 h after fusion, and its presence was maintained until after final cloning of hybridomas by either limiting dilution or single-cell micromanipulation.

Hybridoma supernatants were screened initially by cell surface immunofluorescence on C1 cells grown at 41°C, using FITC-labeled rabbit anti-mouse IgG (H + L)(Miles Laboratories, Slough, UK), basically according to [11]. Supernatants positive by fluorescence were then screened for their ability to immunoprecipitate a protein from the detergent lysate of ^{35}S-methionine-labeled cells of the above line [12]. Finally, cell surface binding of supernatants from cloned hybridomas was assessed by a quantitative ^{125}I-rabbit anti-mouse IgG (H+L) binding assay [13].

Antisera

The preparation and characterization of the anti-erythrocyte, anti-erythroblast, and anti-macrophage sera has been described previously [3].

RESULTS

Isolation of Monoclonal Antibodies

Using the schedule described in Methods, mice were injected with *ts*34-AEV transformed erythroblasts that had been grown at 41.5°C for five days. This cell population was chosen since after this time approximately 50% of the cells had started to differentiate by the criteria that they had accumulated sufficient hemoglobin to be stained with benzidine. Therefore, the cell population could be expected to contain both immature erythroblasts as well as more mature erythroid cells, and consequently, it was theoretically possible that antibodies would be produced against a whole spectrum of antigens. The mice were test bled, and when they could be shown to produce antibody against erythroblasts as detected by immunofluorescence, they were boosted intravenously with erythroblasts. Four days later the spleen was removed from an individual mouse and used for fusion. After approximately 14 days, the individual wells were tested for antibody production and following this initial screen 21 of the 144 wells were analyzed further. The supernatant fluids from these wells were then tested in an indirect ^{125}I-antibody binding assay for their ability to bind to both erythroblasts transformed by wild-type AEV and peripheral blood chicken erythrocytes. The results are shown in Figure 1. There was a varied reaction with the two cell types, the antibodies from the wells either reacting well with both cell types, for example samples 12, 18, and 21 reacting better with red blood cells than erythroblasts, eg, samples 1; and four or more commonly reacting with erythroblasts and not with red blood cells, eg, samples 2, 15, 16.

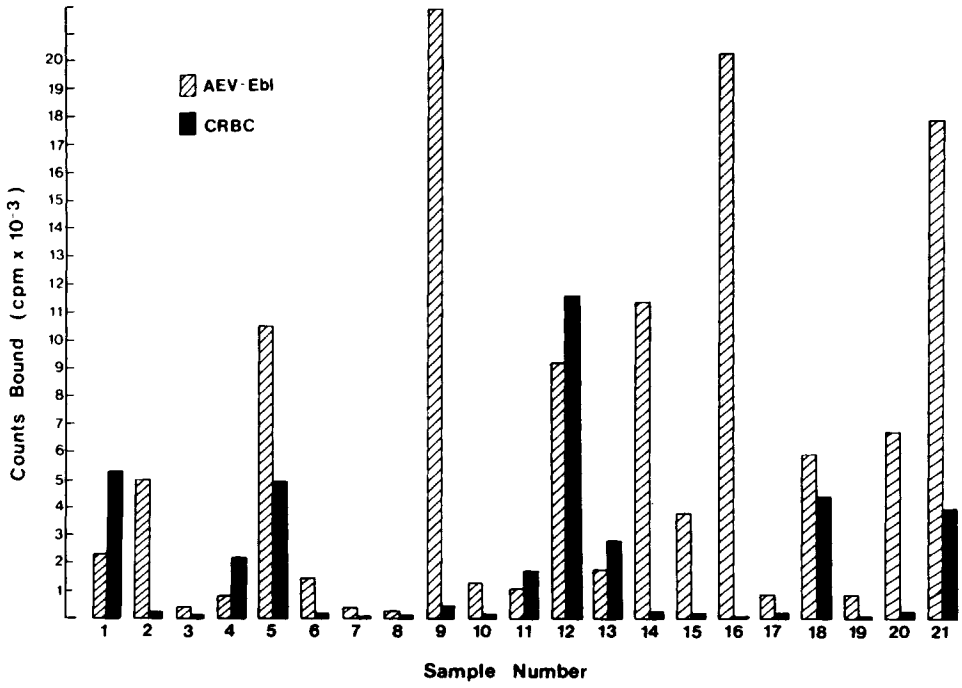

Fig. 1. Analysis of tissue culture supernatant for binding to RBC and erythroblasts. Two hundred microliters of supernatant was tested for its ability to bind to 1 × 10^6 chicken RBC or to 1 × 10^6 6C2 erythroblasts as described in Methods.

At the same time, the culture supernatants were tested for their ability to immunoprecipitate proteins from ^{35}S-methionine-labeled cell extracts of *ts*34-AEV infected erythroblasts that had been grown at 41.5°C for three days. The results of the experiments are summarized in Table I. A wide variety of molecules were precipitated with apparent molecular weights ranging from 11,000 to 94,000 daltons. In this initial screen, three of the supernatants failed to precipitate any proteins. At the present time the reasons for this are unknown. It is interesting to note that supernatants with essentially identical binding characteristics react with different proteins; for example, samples 2 and 15. From these data, we decided to reclone cells from wells 4.2A5 (sample 4 in Fig. 1), 4.5A5 (sample 16), and 4.6Cl (sample 21) as examples of all three types of binding reaction, and the cells from the other wells were frozen down for future analysis.

Analysis of Monoclonal Antibody 4.2A5

The cells from this well were recloned three times, the antibody-producing cells grown up, and the secreted antibody used for further characterization. We initially tested the binding of this antibody to various hematopoietic cell types, mainly virally transformed cells representative of the different lineages, to get large numbers of pure cell types. As can be seen in Table II, in the indirect binding assay using live cells, 4.2A5 only reacted with cells of the erythroid lineage and in addition reacted more strongly with erythrocytes than with erythroblasts as

TABLE I. Immunoprecipitation Studies of the Hybridoma Supernatants

Sample no[a]	Hybridoma designation	Apparent molecular weight of protein precipitated ($\times 10^{-3}$)[b]
1	4.1B3	11
2	4.1C2	38
3	4.1D4	NP
4	4.2A5	11
5	4.2B4	68
6	4.2C3	90
7	4.2C4	NP
8	4.2D5	60
9	4.3A2	38
10	4.3B2	50
11	4.3B6	11
12	4.4A3	28
13	4.4B5	40
14	4.4C4	40
15	4.4D1	90
16	4.5A5	NP
17	4.6A5	11
18	4.6C2	50
19	4.6D2	28
20	4.6D5	28
21	4.6C1	94

[a]The same number as shown in Figure 1.

[b]NP indicates nothing precipitated.

already shown in Figure 1. Since most of the cell types tested above were virally transformed, it was considered possible, although unlikely, that there was something strange about their membrane antigens. Therefore we also tested the binding of 4.2A5 on smears of fixed normal bone marrow cells using indirect double immunofluorescence (Fig.2). Much to our surprise 4.2A5 reacted much more strongly with the cytoplasms of the cells than with the membrane (Fig 2,A,B left panels); indeed in experiments using live cells, no membrane fluorescence could be seen, and binding was only seen in cells that were slightly damaged during the handling procedures (data not shown). Also in this test, 4.2A5 not only reacted with erythroid cells, which were identified by counter staining for either hemoglobin (Fig. 2A right panel) or for the erythroid specific histone H5 (Fig. 2B right panel), but also reacted in a granular fashion with granulocytes. In preliminary experiments, 4.2A5 could be shown to precipitate a hemoglobin-like molecule from erythroblasts. This molecule comigrated on SDS-polyacrylamide gels with hemoglobin and the precipitation could be blocked by adding partially purified hemoglobin (data not shown). Whether or not this molecule is hemoglobin obviously requires further analysis. Whatever the result, the binding to the granulocytes will have to be explained since antibody to authentic hemoglobin does not exhibit this property (Fig. 2A right panel).

Characterization of Monoclonal Antibody 4.5A5

Following two single-cell clonings, the antibody produced by these cells still retains the characteristic shown in Figure 1, that is, they bind strongly to

TABLE II. Reactivity of the Monoclonal Antibodies 4.2A5 and 4.5A5 With Various Virus-Transformed and Normal Chicken Hematopoietic Cells*

	Cell types							
Monoclonal	AMV-myeloblasts	MC29-macrophages	REV-"lymphocytes"	ALV-B lymphocytes	MDV-T lymphocytes	AEV-erythroblasts	Bone marrow erythrocytes	Peripheral erythrocytes
4.2A5	–	–	–	–	–	+	ND[a]	+ + +
4.5A5	+ + +	+	+ +	+ + +	+ + +	+ + +	–	–

*Reactivity of the monoclonal antibody was assessed by the binding of ^{125}I-labeled rabbit anti-mouse immunoglobulin as described in Methods. – = background binding approximately 800 cpm. + = 3,000–5,000 cpm bound. + + = 5,000–10,000 cpm bound. + + + = 10,000–25,000 cpm bound. All values represent plateau binding values with approximately 1 × 10^6 cells.

[a]ND: not done.

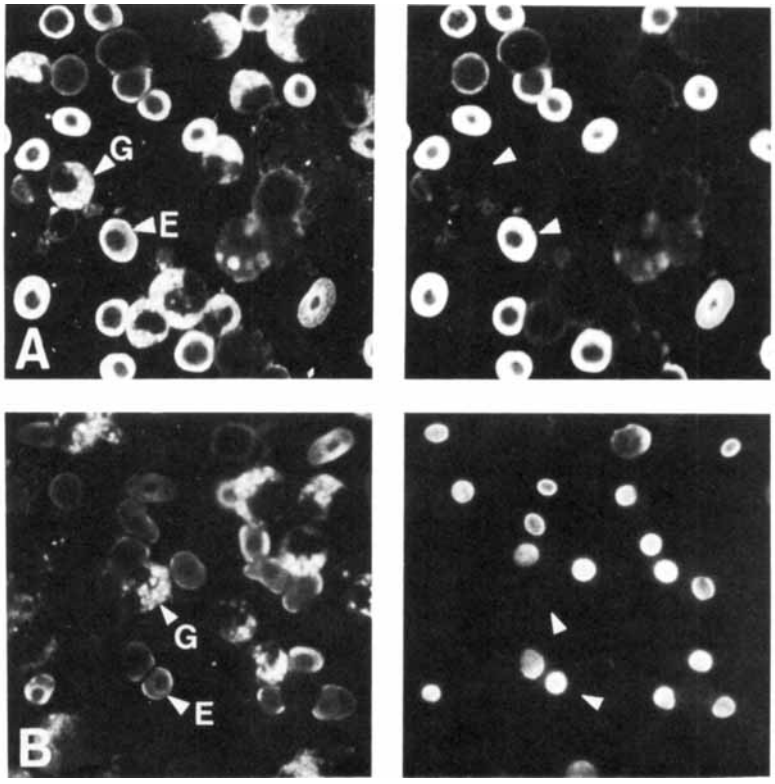

Fig. 2. Indirect immunofluorescence analysis of 4.2A5 on chicken bone marrow. Fluorescence was performed as described in Methods using in both the left-hand panels 4.2A5 counterstained with FITC-rabbit anti-mouse immunoglobulin and in the right-hand panels either rabbit anti-chicken hemoglobin (A) or rabbit anti-Histone H5 (B), counterstained with TRITC-goat anti-rabbit immunoglobulin.

erythroblasts but not at all to red blood cells (Fig. 3). The antibody was then tested for its ability to bind to the various virally transformed cell lines that represent cells from different stages of the various lineages. Table II shows that this antibody binds to all the cell types tested except red blood cells. However, it should be noted that the binding to the MC29-transformed macrophage-like cells was significantly lower than the binding to other cell types.

The antibody was then tested by indirect immunofluorescence for its binding to viable normal bone marrow cells (Fig. 4). The bone marrow cells were counterstained as shown in the right-hand panels with rabbit antisera specific for erythrocytes (A), erythroblasts (B), or macrophages (C). The fluorescence with the 4.5A5 antibody is shown in the middle panels in every case with the left-hand panels showing the same field under bright field illumination. In this case, the fluorescence is membrane fluorescence, and there was very little cytoplasmic fluorescence. The binding of the antibody in this assay reflected the binding seen using the ^{125}I-antibody binding technique in that 4.5A5 bound to all blast-like cells

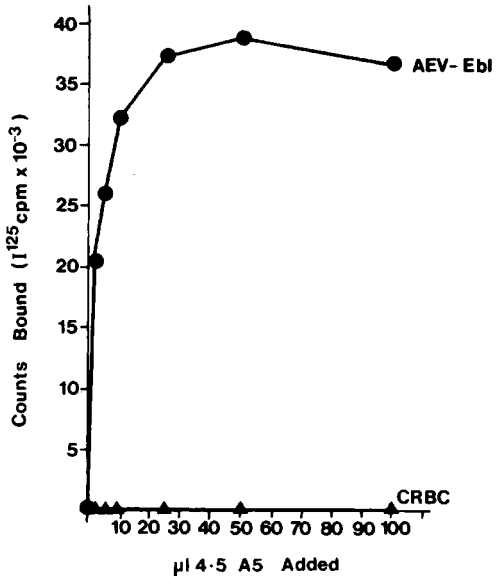

Fig. 3. RIA-binding analysis of 4.5A5. Supernatants were tested as described in Figure 1.

in the preparation, be they immature erythroid cells (marked Eb), or immature myeloid cells (M). However, it did not bind to either erythrocytes (E) or to mature granulocytes (G). Therefore this antibody seems to recognize an antigen that is present on all actively dividing hematopoietic cells but is not expressed when the cells have reached some kind of end-cell stage. Immunoprecipitation studies on ^{35}S-methionine-labeled cell extracts of erythroblasts have so far failed to identify the antigen that this antibody reacts with, as mentioned in Table I.

Analysis of Antibody 4.6Cl

We were interested in characterizing this antibody since as shown in Table I, it was capable of immunoprecipitating a protein of molecular weight 94,000. This was of interest to us since earlier studies using rabbit antisera against erythrocytes and erythroblasts had demonstrated the increased synthesis of an apparently erythroid-lineage-specific 94,000 dalton protein following the temperature induced differentiation of *ts*34 AEV-infected erythroblasts [14]. Immunofluorescence studies on normal bone marrow cells demonstrated that this antibody was also apparently specific for the erythroid lineage (data not shown). Therefore, we tested this antibody for its ability to bind to erythroblasts infected with *ts*34 AEV and grown at 35°C and 41°C, and we included erythroblasts infected with wt AEV as a control. As can be seen in Figure 5, this antibody bound to erythroblasts equally well whether they were grown at 35°C or 41°C. Thus, it appears that either the 4.6Cl antibody is directed against a different molecule from the one detected by the rabbit antiserum or the binding assay does not pick up the increased synthesis of the 94,000 dalton protein. At present, experiments are under way to resolve these possibilities.

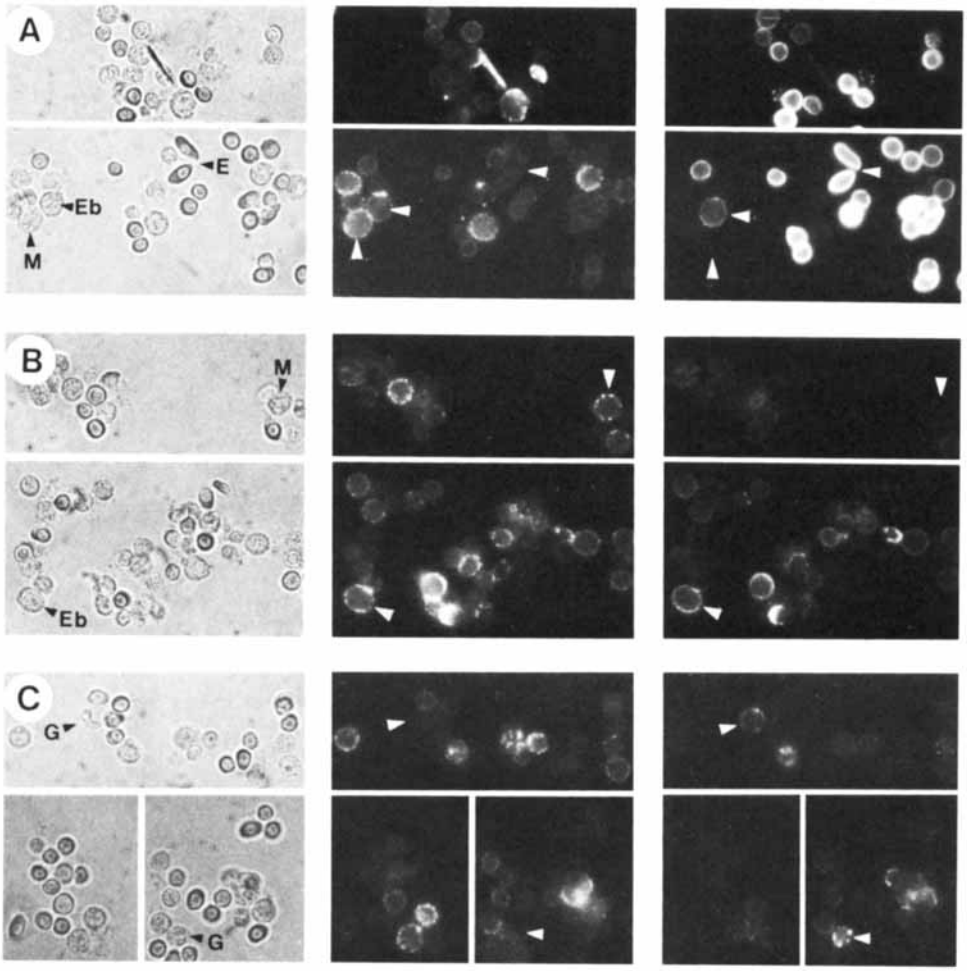

Fig. 4. Immunofluorescence analysis of 4.5A5 on bone marrow cells. The three horizontal rows show the same field viewed with bright field illumination (left panel), with epifluorescence for FITC (middle panel), or TRITC (right panel). Cells were incubated with mixtures of monoclonal antibody 4.5A5 and rabbit anti-erythrocyte serum (A), anti-erythroblasts serum (B), or anti-macrophage serum (C), then reacted with FITC goat anti-mouse IgG plus TRITC goat anti-rabbit IgG. The cells marked with arrowheads were identified as; E = erythrocytes; Eb = immature erythroid cells; M = immature myeloid cells; and G = mature granulocytes or macrophages.

DISCUSSION

Our major interests lie in understanding the changes that occur during the differentiation of chicken erythroblasts. The approach we have used has been to take advantage of the clonally pure populations of chicken erythroblasts that can be obtained following infection of bone marrow cells with AEV and use these cells to raise monoclonal antibodies against their cell surface antigens. Our intention

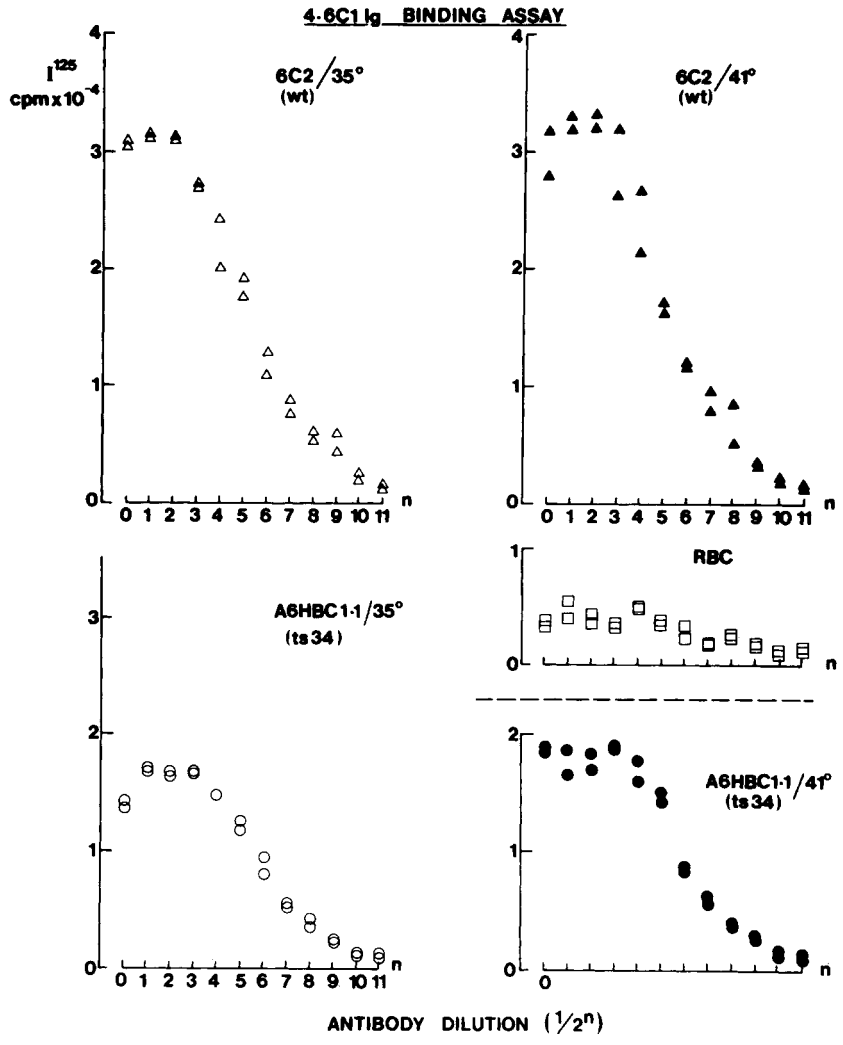

Fig. 5. Binding of 4.6CI antibody to erythroblasts and erythrocytes. A crude immunoglobulin (Ig) fraction was made from concentrated culture supernatant of 4.6Cl hybridoma cells and its binding to cell surfaces assayed by the ^{125}I-binding assay referred to in Methods. Doubling dilutions of the Ig fraction (50 μl) were incubated with 10^5 cells of the 6C2 cell line (wt AEV), the A6HBCl-1 cell line (*ts*34 AEV), or fresh, peripheral blood erythrocytes (RBC). The cell lines were grown at either 35°C or 41°C.

was to use such reagents in order to characterize in detail the surface phenotype of the erythroblasts and then analyze changes that occur in the expression of these molecules as the cells differentiate. As mentioned earlier, we intend to use an in vitro differentiation system for this analysis that takes advantage of the availability of a temperature-sensitive mutant of AEV. Although this in vitro system will greatly facilitate the biochemical characterization of the differentiation process, it is obviously necessary to confirm that the changes we observe also occur in normal hemopoiesis and do not represent in vitro artifacts, and where possible, this will be checked.

The cell membrane proteins were chosen for analysis since it is already known that the cell surface of the erythrocyte is a very specialized and fascinating structure, as is amply documented in this volume and is therefore well worthy of investigation. In addition, preliminary experiments have shown that our in vitro system requires erythropoietin for maximum differentiation to take place [Beug et al, unpublished observations] and, therefore, almost certainly is controlled via cell surface receptors.

In the preliminary observations reported here, it is already apparent that one of the major changes that occurs is the loss of certain cell surface antigens. This conclusion is based on the fact that 13 of the 21 hybridoma supernatants tested failed to react with mature erythrocytes but were positive on erythroblasts. Immunoprecipitation studies showed that these represented at least eight different antigens. (It should be noted that the viral antigens of AEV are well-defined, being proteins of 75,000 and 40,000 daltons [15], only one of which could be the same as these eight antigens detected by the hybridomas.) The monoclonal antibody 4.5A5 defines such an antigen, which is lost as the erythroblasts differentiate and become erythrocytes. This antigen, however, was also strongly expressed on all actively dividing cells we have examined and therefore potentially represents a molecule that is required for cell proliferation. Since erythrocytes are of course nonproliferating whereas erythroblasts divide very rapidly, we expect to find many such proliferation-linked antigens that will be lost upon differentiation. The eventual identification of these antigens as, for example, transport proteins or receptors, will allow interesting experiments to be performed to assess the role these proteins play in cell growth control.

The reaction of the antibody 4.2A5 with essentially cytoplasmic proteins and not with membrane proteins, we found a surprise. We had assumed that the antibody binding assay on live cells would only detect membrane antigens, an assumption that is apparently incorrect. The apparent reaction of this antibody with hemoglobin by the criteria of immunoprecipitation of a protein with the same mobility on SDS gels as hemoglobin and the blocking of such precipitation with hemoglobin would make perfect sense except for the immunofluorescence with granulocytes. However, since it is known that monoclonal antibodies can identify rare antigenic cross-reactions [16], perhaps this is not surprising but it obviously requires further work before we will understand exactly what is happening. Unfortunately, this antibody probably will not be very useful to understand membrane antigen changes.

The other antibody, 4.6C1, seems to detect a protein of 94,000 daltons that does not change as the erythroblasts partially differentiate in that it is also present on mature red blood cells (Fig. 1) and does not change in the *ts*34-AEV differentiation system. This too was a disappointment since we had hoped that it was directed against the 94,000 daltons protein that we had identified in earlier studies as appearing upon differentiation [14]. However, it will undoubtably prove useful as a control reagent for future studies, and it will be interesting to elucidate what the function of this erythroid-lineage-specific membrane protein is.

It is worth mentioning that a large series of monoclonal antibodies against chicken erythrocytes have been isolated by Longenecker and co-workers and that there is a preference for mice to respond to the polymorphic antigens of the chicken M.H.C. region [17]. Furthermore, some of these alloantigens are

erythroid specific, being expressed only on mature erythrocytes and erythrocyte progenitors [18]. Unfortunately, we cannot test these existing hybridoma antibodies in our system since the cells we use express antigens of a different B locus than those used by Longenecker et al, and consequently, their antibodies do not react with our cells [Longenecker, personal communication]. However, it will obviously be useful to see if any of our monoclonal antibodies react with molecules encoded by the M.H.C. region.

These are obviously very early days in our analysis of the changes in the surface proteins during the differentiation of erythroblasts. We have to isolate many more monoclonal antibodies and characterize them in great detail. Only then will we be able to build up a picture of the changing surface phenotype of the cells and to pick out the changes that are crucial for the differentiation to occur.

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