External Surface Membrane Proteins in Normal and Neoplastic Murine Erythroid Cells

J. Glass,* S. Fischer, L. M. Lavidor, and T. Nunez

Department of Medicine and Thrombolytic Laboratory, Beth Israel Hospital and Harvard Medical School, Boston 02215 [J. G., L. M. L.], and Department of Biology, M.I.T. Harvard-M.I.T. Programs in Health Sciences and Technology Cambridge, Massachusetts 02138 [S. F., T. N.]

SUMMARY

The developmental pattern of one class of plasma membrane proteins, the external surface proteins, was examined in neoplastic and nonneoplastic differentiating murine erythroid cells. Neoplastic erythroid precursor cells were obtained from spleens of CD-1 mice after infection with Friend erythroleukemia virus while the nonneoplastic cells were obtained from spleens of mice with phenylhydrazine-stimulated erythroid hyperplasia. Erythroid precursors at different stages of development were isolated from these erythroid cell populations by sedimentation at unit gravity. The surface proteins were labeled by lactoperoxidase-catalyzed iodination, solubilized in sodium dodecyl sulfate, and separated by sodium dodecyl sulfate gradient gel electrophoresis. Multiple labeled bands were found at all stages of neoplastic and nonneoplastic erythroid differentiation examined. The pattern of external membrane proteins labeled in neoplastic erythrophils and in reticulocytes from peripheral blood were qualitatively similar and not altered by infection with Friend virus.

The nucleated precursor cells from noninfected mice exhibited distinct differences from erythrocytes, and with increasing differentiation an evolutionary pattern of several minor proteins was seen. Clear-cut differences in lactoperoxidase-reactive proteins were also observed between neoplastic and nonneoplastic precursors. The most marked differences were observed between the most immature cells. The youngest neoplastic cells from CD-1 mice possessed a protein with a molecular weight of 80,000 not seen in normal erythroid cells. Additionally, there was an absence of normally occurring protein with a molecular weight of 13,000 and increased amounts of a protein with a molecular weight of 140,000. With increasing maturation of the neoplastic cells, labeling of the protein with a molecular weight of 8,000 decreased while the protein with a molecular weight of 13,000 became apparent, so that a labeling pattern similar to that of nonneoplastic cells was obtained.

These studies demonstrate both distinct alterations of lactoperoxidase-reactive surface membrane proteins in neoplastic erythroid cells during cell maturation and in nonneoplastic erythroid cells as compared with nonneoplastic erythroid cells at similar stages of development.

INTRODUCTION

Although erythrocyte plasma membrane proteins are of obvious importance in mechanical and metabolic properties of the mature erythrocyte, little information is available as to the development of these proteins during RBC differentiation. Fischer et al. (5) have noted changes in the amount of membrane glycoproteins and glycolipids in mature RBC as compared with reticulocytes. During maturation, membrane protein synthesis has been observed to become restricted to lower-molecular-weight proteins (9). Rabbit reticulocytes are capable of synthesizing a limited number of plasma membrane proteins, and loss of membrane proteins has been described during the maturation of rabbit reticulocytes (5, 9, 10). Knowledge of the developmental changes of plasma membrane proteins of nonneoplastic erythroid cells is essential to the interpretation of alterations in plasma membrane proteins in malignant erythroid cells.

We have undertaken an examination of external surface membrane proteins in nonneoplastic murine erythroid cells at different stages of maturation. It was then possible to perform a similar study in Friend virus-infected erythroid cells at similar stages of maturation, allowing correlation of modification of surface proteins either with stage of differentiation or with transformation to the neoplastic state.

Friend virus infection stimulated marked erythroid hyperplasia accompanied by differentiation of the infected erythroblasts (12, 14). Techniques of cell separation previously utilized by this laboratory (7) permitted isolation of infected and noninfected erythroid precursor cells at different stages of maturation. The external surface proteins in these erythroid cell populations were specifically iodinated by a modification of the lactoperoxidase technique (13) and then were separated by SDS* slab gel electrophoresis. The results demonstrate that, during erythroid differentiation, definite qualitative and quantitative changes occur in this class of proteins and that the virus-infected cells exhibited distinctive changes in surface proteins compared with normal erythroid cells at similar stages of differentiation.

MATERIALS AND METHODS

Cell Preparation. Noninfected erythroid precursors were obtained from the spleens of CD-1 mice (Charles River

* The abbreviations used are: TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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MAY 1977

1497
Breeding Laboratories, Wilmington, Mass.) after treatment with phenylhydrazine as described previously (7). Neoplastic erythropoiesis was induced by the i.p. injection of 0.1 to 0.2 ml of polycythemic Friend virus stock previously passaged in CD-1 mice. (Virus was kindly supplied by Dr. Stuart Levy, Tufts University School of Medicine.) Spleens were excised about 14 days after injection of virus, at which time the mice had developed polycythemia with hematocrits greater than 60%. Cell suspensions of normal and malignant erythroid cells were prepared (7). Nonviable cells, as estimated by trypan blue dye exclusion, were removed by bovine serum albumin centrifugation (15) so that cell viability was always greater than 90%. Cells were then separated by velocity sedimentation at unit gravity. Preliminary immunolysis of mature erythroid cells was eliminated in order to obtain a larger spectrum of maturing erythroid cells and to minimize the percentage of nonerythroid cells. Cell counts, Wright-Giemsa benzidine staining, and differential counts were performed as described (7). Peripheral blood was obtained from the same animals after decapsulation by collection into heparinized PBS. Cells were washed 3 times in large volumes of PBS prior to iodination.

Iodination. The iodination procedure was a modification of the method of Phillips and Morrison (13). Viable cells (1 × 10^7) were iodinated in 0.1 ml of PBS at 30° with 13 μg of lactoperoxidase (kindly supplied by P. J. Durda, Center for Cancer Research, Massachusetts Institute of Technology) and 20 μCi of ^125I (New England Nuclear, Boston, Mass.). One ml of 0.003% H2O2 was added with a Hamilton syringe every min for a total of 10 additions, and the reaction was allowed to continue for an additional 20 min. The reaction was stopped by addition of a large excess of cold PBS containing 10^-4 M KI, and the cells were washed 3 times in the same buffer. Aliquots of iodinated cells were precipitated with cold 10% TCA, collected either on glass fiber filters of Whatman No. 3MM filter paper, and washed twice with cold 10% TCA. Total cellular radioactivity was measured in a Nuclear Chicago Isocap scintillation counter or in a Packard well-type gamma counter.

Distribution of Radioactivity. The amount of ^125I incorporated into cytoplasmic protein was determined by lysing the washed cells with 20 volumes of 5M sodium phosphate (pH 8.0). After centrifugation at 40,000 × g, aliquots of the soluble fraction were precipitated by 10% TCA on filter paper, the radioactivity was measured, and the percentage of cytoplasmic label was calculated from the total radioactivity determined as described above.

SDS-Polyacrylamide Gel Electrophoresis. Iodinated cells were solubilized in 100 to 200 μl of SDS sample buffer, heated to 100° for 1 min or 85° for 15 min, and samples applied to 1.5-mm slab gel with an acrylamide gradient of 7.5 to 30% as described by Maizel (11). Gels were run for 16 hr at 50 volts and dried, radioautographs were made with Kodak X-ray film (RP/R54), and densitometry was performed at 550 nm. The protein standards used for molecular weight determinations were β-galactosidase (Boehringer Mannheim Corp., New York, N. Y.), bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), ovalbumin (Sigma), and cytochrome c (Sigma). Peaks of radioactivity were numbered in sequence and not by reference to any previous enumeration scheme (3).

RESULTS

Cell Separation. The erythroid precursor cells from spleens of mice infected with Friend virus were morphologically similar to erythroid cells in mice developed in response to phenylhydrazine. With the neoplastic cells, however, the percentage of nonviable cells (about 25%) was greater than with the normal cells (less than 10%). The nonviable cells were removed prior to velocity sedimentation by a simple bovine serum albumin density gradient centrifugation (15) to minimize the possible effects of nonviable cells on subsequent iodination (8). Table 1 shows the differential cell counts of erythroid cells for a typical velocity sedimentation separation of infected, neoplastic cells from CD-1 mice compared with that obtained from phenylhydrazine-stimulated mice. Fraction I, containing the most rapidly sedimenting cells, is composed primarily of pronormoblasts, the largest and most immature erythroid precursors. As the rate of sedimentation decreases the fractions contain smaller, more mature cells. For example, Fraction VIII is composed primarily of orthochromatophilic normoblasts as polychromatophilic erythrocytes. Contamination with nonerythroid cells was always less than 10%.

Iodination. Initial experiments with nonneoplastic erythroid cells demonstrated that iodination as measured by TCA-precipitable radioactivity was dependent upon the ratio of cell number to peroxide concentration. Table 2 illustrates that more than twice the iodination per cell was obtained when 10^7 mature erythrocytes were iodinated in the presence of 0.003% H2O2 as when 10^6 cells were iodinated at the same peroxide concentration. A higher concentration of peroxide, 0.03%, did not increase the iodination of 10^7 but did increase the iodination of 10^6 cells. Under all of these conditions, no more than 5% of the radioactivity was found in the cytoplasm (Table 2). A similar degree of cytoplasmic labeling was found with early erythroid cells from splenic cell suspensions. When peroxide was generated by glucose oxidase and glucose, the label was similarly partitioned with less than 5% of the radioactivity being cytoplasmic. Furthermore, upon SDS gel electrophoresis of iodinated erythrocytes, reticulocytes, or spleen cells with subsequent radioautography, no label was found corresponding to globin.

The extent of iodination was also dependent on the stage of erythroid maturation. Erythrocytes were iodinated 2.26 ± 0.35-fold more and nucleated erythroid cells 3.89 ± 0.06-fold more intensely than reticulocytes (the mean ± S.D. for 6 experiments).

Analysis of Iodinated Membrane Proteins of Infected and Noninfected Erythrocytes and Reticulocytes from Peripheral Blood. Chart 1 shows the lactoperoxidase-catalyzed ^125I labeling pattern of noninfected erythrocytes (Chart 1a) and reticulocytes (Chart 1b) and infected erythrocytes (Chart 1c). In both noninfected cell types, despite the differences in extent of labeling, the predominant labeled proteins had molecular weights of 28,000 and 13,000 (Bands 8 and 9) with other proteins of molecular weights of 100,000, 65,000, 50,000, and 41,000 (Bands 3 to 6). Similar labeling patterns were obtained when extensively washed membranes were prepared from iodinated cells, as opposed to the usual assays performed with whole cells, or after eryth-
Table 1
Differential counts of erythroid cells of velocity sedimentation fractions from phenylhydrazine-treated or Friend virus-infected CD-1 mice

As detailed in "Materials and Methods," erythroid precursors were obtained from spleens either after phenylhydrazine treatment or about 14 days after infection with Friend virus, when hemato-
crits were greater than 60%. The precursor cells were fractionated by velocity sedimentation. Differential counts were performed on at least 400 cells; enucleated cells were counted simultane-
ously. The results presented are for a representative experiment; the iodination labeling pattern of the external membrane proteins for these fractions is presented in Figs. 3 and 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction</th>
<th>Pronormoblasts (%)</th>
<th>Basophilic normoblasts (%)</th>
<th>Polychromatophilic normoblasts (%)</th>
<th>Orthochromatophilic normoblasts (%)</th>
<th>Enucleated RBC (%)</th>
</tr>
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<tbody>
<tr>
<td>Friend virus</td>
<td>I</td>
<td>81.3</td>
<td>17.5</td>
<td>1.2</td>
<td>0.6</td>
<td>0.0</td>
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<tr>
<td>infection</td>
<td>III</td>
<td>62.2</td>
<td>24.7</td>
<td>13.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>VIII</td>
<td>0.0</td>
<td>0.0</td>
<td>8.5</td>
<td>40.4</td>
<td>51.1</td>
</tr>
<tr>
<td>Phenylhydra-</td>
<td>I</td>
<td>79.1</td>
<td>13.1</td>
<td>6.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>zine</td>
<td>III</td>
<td>47.2</td>
<td>31.2</td>
<td>1.6</td>
<td>10.1</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>VIII</td>
<td>0.4</td>
<td>4.0</td>
<td>4.0</td>
<td>41.3</td>
<td>54.1</td>
</tr>
</tbody>
</table>

Table 2
Membrane protein iodination—effect of cell number and peroxide concentration

Erythrocytes were iodinated as described in "Materials and Methods," except cell concentration and H2O2 were varied as shown. Total radioactivity was determined on an aliquot of the extensively washed cells and cytoplasmic radioactivity on the super-

<table>
<thead>
<tr>
<th>Cell concentration (cells/0.1 ml)</th>
<th>Concentration of added H2O2 (µl)</th>
<th>Total radioactivity (cpm/10^7 cells)</th>
<th>% radioactivity in cytoplasmand</th>
<th>A. 550</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^7</td>
<td>0.003</td>
<td>255,600</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>10^6</td>
<td>0.003</td>
<td>96,200</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>10^7</td>
<td>0.03</td>
<td>242,350</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>10^8</td>
<td>0.03</td>
<td>220,000</td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>

Erythrocytes were subjected to the conditions of sedimentation used in the studies of nucleated spleen cells. The band with a molecular weight of 13,000 was not altered by chloroform-
methanol extraction of labeled cells.

The lactoperoxidase-reactive proteins of erythrocytes from infected animals (Chart 1c) were qualitatively similar to those from noninfected mice. In another mouse strain, DBA/2J, slight quantitative differences were observed, with the infected erythrocytes demonstrating a consistent decrease in labeling of the moiety with a molecular weight of 63,000 (Band 4).

Analysis of Iodinated Membrane Proteins of Infected and Noninfected Nucleated Erythroid Precursors from Spleens. The pattern of labeling consistently observed with cells from normal CD-1 mouse is shown in the upper panels of Chart 2. For simplicity only the patterns for velocity sedimentation Fractions I, III, and VIII are shown; the other fractions displayed patterns consistent with a transition from Fractions I to VIII. The predominant reactive proteins had molecular weights of 100,000 (Band 3) and 13,000 (Band 9). As erythroid maturation progressed from the least mature cells of Fraction I to the most mature of Fraction VIII, the following changes in minor proteins were observed: proteins with molecular weights of 140,000 (Band 2.2) and 35,000 (Band 7) diminished, and new proteins with molecu-
becomes quite similar to that of normal erythocytes (Chart 1).

Similar alterations in the profiles of the lactoperoxidase-reactive proteins were observed in phenylhydrazine-treated and virus-infected DBA/2J mice. In the DBA strain the changes were most marked in the earliest cells, where again the protein with a molecular weight of 13,000 was absent, a new protein with a molecular weight of 8,000 appeared, and a protein with a molecular weight of 140,000 was accentuated.

DISCUSSION

This study was designed to examine a particular class of plasma membrane proteins, the external surface proteins, in Friend virus-infected erythroid cells. Infection of mice with the polycythemic strain of Friend virus stimulates proliferation of erythroid cells, which are neoplastic yet differentiated to produce markedly increased numbers of apparently normal erythocytes (12, 14). Techniques previously utilized for separation of normal erythroid precursor cells into populations enriched for different stages of maturation (7) were found to be applicable to the erythroid cells produced in response to Friend virus infection. Hence, it was possible to compare the external surface proteins of cells that shared similar morphological features and sedimentation profiles.

Specificity of labeling of the cell surface relies on the relatively large molecular weight of lactoperoxidase which excluded the iodination reaction from the interior of the cell. In addition, since nonviable cells are unable to exclude the enzyme probe and permit labeling of internal proteins (8) in the experiments reported, great care was taken to remove nonviable cells to always less than 10%. In this study not only was cytoplasmic labeling always less than 5%, but on electrophoresis of labeled whole cells no iodine was detected on the major cytoplasmic protein, globin, or on a plasma membrane, internal membrane protein, spectrin. The absence of globin labeling was true in the well-homogenized cells in which hemoglobin binding to cell membrane occurs (4). Hence, cytoplasmic structures were not labeled.

The mouse reticulocyte plasma membrane was iodinated to less than one-half the extent of the mature erythrocytes. However, the profile of iodinated proteins was minimally altered. Since the iodination reaction is specific for tyrosine groups (13), changes in the extent of labeling may reflect rearrangements of the cell surface altering accessibility of the labeling probe to tyrosine groups in surface proteins. Differences in the extent of labeling have not been previously described in comparisons of reticulocytes and erythrocyte surface proteins.

The iodination profiles of reticulocytes and erythrocytes compared with cells from the velocity sedimentation fractions were strikingly different. These changes were present when erythrocytes and reticulocytes were subjected to sedimentation in bovine serum albumin and hence were not due to alterations in pH, ionic strength, etc. These changes cannot be ascribed to the enucleation process since Fraction VIII was comprised primarily of polychromatophilic, nonnucleated erythrocytes. Possibly, surface remodeling of reticulocytes (2) after their release from the erythropoietic compartment is associated with specific loss (inaccessibility) of the protein with a molecular weight of 180,000 and appearance (accessibility) of the proteins with molecular weights of 50,000 and 41,000. The infected erythrocytes must undergo a similar process, since in the transition from Fraction VIII to erythrocyte a normal appearing surface protein was also achieved.

The changes in iodination profiles of noninfected cells from the velocity sedimentation fractions occurred primarily in minor surface proteins during maturation of nucleated erythroid cells. Perhaps greater differences would have been observed if purer cell populations had been obtained. Infection with Friend virus produced erythroid hyperplasia with striking alterations of the lactoperoxidase-reactive membrane proteins of the sedimentation fractions. The most marked changes, both qualitative (Band 10) and quantitative (Band 2.2) occurred in the most immature cells. With increasing maturation the lactoperoxidase-reactive proteins approached the pattern observed in noninfected cells.

The observed differences in lactoperoxidase-reactive proteins in the neoplastic compared with the noninfected cells and in the noninfected nucleated cells compared with cells in the peripheral blood have several possible explanations. Since the iodination reaction is specific for exposed tyrosine groups (8), a protein might appear to be absent if tyrosine residues were inaccessible to the labeling reagent or if the protein itself were not synthesized. Quantitative
differences may also be explained by accessibility to labeling reagents or differences in the rates of synthesis of particular proteins. After infection the appearance of a new protein (Band 10) could result from the proteolysis of a normal surface constituent, e.g., Band 9. Newly expressed proteins in infected cells could also represent viral proteins either integrated into the host membrane or on budding virions. The latter is an unlikely explanation for the appearance of Band 10 in view of the known molecular weights of the Friend virus coat proteins (1).

The ability to monitor a particular class of membrane proteins throughout erythroid differentiation serves as an important model for investigating alterations of structural and functional characteristics of the RBC surface in malignant disorders of erythropoiesis. This study has demonstrated clear-cut differences in membrane properties of differentiating normal erythroid cells and between neoplastic and nonneoplastic erythroid cells. For the time being, these remain simply descriptive observations awaiting explanation in physiological or metabolic terms. As the functions of erythroid plasma membrane proteins are elucidated it should become possible to correlate these virus-induced surface modifications with specific functional changes and, in particular, with the neoplastic properties of the transformed cells.

REFERENCES


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