Immunofluorescent Detection of Erythrocyte Sialoglycoprotein Antigens on Murine Erythroid Cells

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ABSTRACT
A sialoglycoprotein fraction isolated from murine (DBA/2) erythrocytic ghosts (see companion article, Sarris and Palade, 1982, J. Cell. Biol. 93:583-590) was used to raise antibodies in rabbits. By immune-IgG (serum)-[125I] protein A overlays, the antibodies were found to react positively with the four sialoglycoprotein monomers (gp-2.1, gp-2.2, gp-3.1, and gp-3.2) of the original fraction, with the sialoglycoproteins detected in erythrocytic ghosts (gp-2.1 and gp-3.1), with a diffuse component (probably a macroglycolipid) trailing around gp-3.1 in SDS polyacrylamide gel electrophoretograms of solubilized ghosts, and with a minor sialoglycoprotein hidden under this trail.

IgG's isolated from immune and nonimmune rabbit sera were conjugated to tetramethylrhodamine isothiocyanate and used to survey, by fluorescence microscopy, the distribution of the cognate antigens on the three different erythroid lines known to succeed each other during the life span of the mouse. In the peripheral blood of the adult, the antibodies recognized only mature erythrocytes; they did not crossreact with either platelets, monocytes, or different types of granulocytes. In the spleen of adult anemic mice, the antibodies reacted weakly with proerythroblasts and strongly with all types of erythroblasts. In enucleating erythroblasts, antigens were preferentially segregated on the cell membrane of the nascent reticulocyte. In the 10-day-old embryo, antigens were already present on the primitive nucleated erythrocytes (produced by the blood islets of the yolk sac), and in the 14-d fetus they were found on all hepatic erythroblasts and derived non-nucleated erythrocytes. A positive immunoreaction was also obtained on Friend erythroleukemic cells, before or after induction by dimethyl sulfoxide. Nonimmune serum, or nonimmune IgGs gave negative reactions in all cases. The antibodies were species-specific: they did not crossreact with either human or rat erythrocytes.

We recently purified and partially characterized the sialoglycoprotein fraction from the erythrocyte ghosts of DBA/2 mice and demonstrated that it is free of detectable contamination by any of the major ghost proteins (spectrins, bands 3, 4.1, 4.2, and actin) (see companion paper, reference 56). We also presented evidence that this fraction contains all the major—as well as some minor—sialoglycoproteins of the murine erythrocyte ghosts, as well as a diffuse component which we assume to be a macroglycolipid or a heterogeneous sialoglycoprotein (56).

We raised in rabbits a polyclonal antiserum that is capable of recognizing all the components of the sialoglycoprotein fraction and is free of major detectable contamination by antibodies against the major ghost proteins (spectrins, bands 3, 4.1, 4.2, actin, and hemoglobin). We determined that this serum recognizes antigens which are restricted to the erythrocytes, but are not present on any other cells (leukocytes or platelets) of the murine peripheral blood. Crossreacting antigens are not present on any cell of the peripheral blood of either man or rat.

In this paper we demonstrate the presence and time of appearance of crossreacting antigens in three erythroid lines present during the life span of the mouse. First, on erythroblasts which arise in the blood islands of the yolk sac, and develop into large, nucleated erythrocytes. Second, on proerythroblasts of the fetal liver which yield on intermediate
population of large, non-nucleated erythrocytes. And finally, on the erythroblasts of the adult hematopoietic spleen which generate adult-size, non-nucleated erythrocytes.

MATERIALS AND METHODS

Materials

All chemicals were reagent or analytical grade. Tetramethylrhodamine isothiocyanate, (isomer R, lot F6EHRT) was from BBL Microbiology Systems, Becton, Dickinson & Co. (Cockeysville, MD); DE-52 cellulose from Whatman Inc. (Chemical Separation Div., Clifton, NJ); complete Freund’s adjuvant from Difco Laboratories (Detroit, MI); agars from Bio-Rad Laboratories (Richmond, CA); rabbit transferrin from Miles Laboratories (Research Products Div. Elkhart, IN); glucose oxidase and phenylenediamine from Sigma Chemical Co. (St. Louis, MO); and lpectoperoxidase from Calbiochem-Behring Corp., American Hoechst Corp. (La Jolla, CA). Immunoplates for double immunodiffusion were from Hyland Diagnostics Div., Travenol Laboratories, Inc. (Costa Mesa, CA). Formalin-fixed and heat-killed Staphylococcus aureus (Cowan I strain) was a generous gift from Dr. Doris Wall (Yale University School of Medicine) or was prepared by us according to Kessler (43) and stored at -70°C. DBA/2 and CD-1 mice were obtained either from the Jackson Laboratories (Bar Harbor, ME) or from the Charles River Breeding Laboratory (Wilmington, VT). Pregnant CD-1 or NIH Swiss females were obtained from Charles River Breeding Laboratories or the Yale University Division of Animal Care.

Antigen and Its Purity

We used as antigen the sialoglycoprotein fraction of mouse erythrocyte ghosts, purified as previously described (56), and tested it for possible contamination with major ghost proteins by the following procedures: (a) We have previously shown that the sialoglycoproteins of murine erythrocyte ghosts can be stained by Coomassie Brilliant Blue R-250 (CB) only in the absence of isopropanol (whereas all other ghost proteins are stained in its presence, as well as in its absence). In the presence of isopropanol, CB staining of SDS polyacrylamide gel electrophoretograms of up to 200 μg sialoglycoproteins revealed no stained bands, and thus excluded detectable contamination by any of the major ghost proteins. The same tube gel stained in the absence of isopropanol revealed several CB bands (a, b, c, gp-1, gp-2 and gp-3 with its diffuse tail) which coincided with periodic acid-Schiff reagent (staining procedure) (PAS) positive bands; some minor sialoglycoproteins were also observed (Fig. 3). (b) Iodination of the sialoglycoprotein fraction by the lactoperoxidase-glucose oxidase (LPO-GP) technique, followed by dissociation into monomeric units, SDS PAGE and prolonged autoradiography, revealed only gp-2.1, gp-2.2, gp-3.1 and gp-3.2 without detectable amounts of globin or any other ghost proteins (56). These results were confirmed on both Laemmli and Steck-Yu gels using several concentrations of acrylamide which resolved bands are designated as in the Laemmli system. The only exception is 5% Steck and Yu gel (64) was used. The gp symbols are omitted in all figures (e.g., 3.2), but they are always used in the text (e.g., gp-3.2).

Iodination of Reagents

The isolated sialoglycoproteins and protein A (from Staphylococcus aureus) were labeled with K125I (37, 56). Murine erythrocytes and their ghosts were radioiodinated (35, 36).

SDS PAGE

SDS PAGE was performed according to Laemmli (44) in the presence of 0.2% (wt/vol) SDS as in (56). Gel composition is given in each figure legend; the resolved bands are designated as in the Laemmli system. The only exception is Fig. 4 for which a 5% Steck and Yu gel (64) was used. The gp symbols are omitted in all figures (e.g., 3.2), but they are always used in the text (e.g., gp-3.2).

Immunoprecipitations

The mixture of 125I-labeled sialoglycoproteins was immunoprecipitated as described by Kessler (43). The polypeptides of individual bands were obtained by electro-
Fetal Erythrocytes and Erythroblasts from Fetal Liver at Day 14

Pregnant mice (CD-1 or NIH Swiss) were obtained from sources given under Materials. The day a vaginal plug was found was designated as day 0 of gestation. Fetal blood was obtained by the following modification of the Brotherton et al. (9) procedure. On days 10–14 of gestation, pregnant mice were anesthetized lightly with ether, their uterine horns were removed and placed in phosphate-buffered saline (PBS), at room temperature. Each placenta and the attached amniotic sac were dissected intact from the uterine decidua and, after washing away the maternal blood, the amniotic sac was opened. The umbilical cord was cut, the intact placenta and the amnion were removed, and the embryos were exsanguinated in a small plastic petri dish filled with PBS. The cells were collected by centrifugation at 4°C and cytocentrifuge smears were prepared as already described. To visualize erythroblasts from day 14 fetal liver, the exsanguinated embryos were partially dissected, and their livers removed and minced with scissors in a PBS-containing petri dish. Fetal erythroblasts, hepatocytes, and erythrocytes were recovered together by centrifugation as above.

Friend Erythroleukemia Cells

Friend erythroleukemia cells, clone 745, were cultured in DME supplemented with 10% FCS in a humid atmosphere at 37°C (27). They were induced to differentiate partially, along the erythroid line, by treatment with 2% (vol/vol) dimethyl sulfoxide (DMSO), and were used at day 5 post induction when they were 95% positive for hemoglobin by the benzidine test (27). Smears of induced or noninduced Friend erythroleukemia cells were prepared and fixed as indicated for buffy coat cells.

Direct Immunofluorescent Staining of Smears

After methanol fixation and air-drying, the slides were incubated for 30 min at room temperature with 180 μg/ml of immune-TMR-IgG, or 170 μg/ml nonimmune-TMR-IgG. They were then rinsed with PBS, washed with the same buffer (with gentle shaking) for 4 h at room temperature, mounted in 90% (vol/vol) glycerol-10 mM sodium phosphate, pH 7.5 (28), and sealed with nail polish.

Fluorescence Microscopy and Light Microphotography

We used a Zeiss Photomicroscope II equipped with a Zeiss epi-illumination system, a mercury HBO light source, a BP546 exciter filter, a FT580 beam splitter, and a LP590 barrier filter for rhodamine fluorescence. All micrographs were obtained with a ×100 oil immersion Zeiss camera; for fluorescent microscopy, the exposures varied from 30 to 60 s according to the intensity of the fluorescence. Images were recorded on Kodak Ektachrome film for color slides (ASA 400) or on black and white Kodak Tri-X Pan film (ASA 400). Color slides were developed by a commercial laboratory to ASA400, and were duplicated using black and white Kodak Plus-X Pan film (ASA 125) for use in this paper. Black and white films were developed to their respective ASA speeds using Kodak Microdol-X developer following the instructions supplied by Kodak. In all plates, each phase-contrast micrograph on the left is accompanied on the right by a fluorescent micrograph of the same field.

RESULTS

Characterization of the Serum

After the fourth antigen injection, a double immunodiffusion test in agar (52) revealed the presence of precipitating antibodies in the serum of all three rabbits used. By the same criterion, there were no detectable antibodies against hemoglobin or an EDTA extract from erythrocyte ghosts, both from DBA/2 mice. The EDTA extract contained mainly spectrin and actin as assessed by SDS PAGE (data not shown). These results cannot exclude, however, the presence of antibodies to the proteins mentioned, because the pertinent antigens and antibodies might be far from equivalence (13). To circumvent this objection, we used the immunoprecipitation of 125I-labeled antigens by their antibodies taking advantage of the interaction of the latter with a staphylococcal bacterial adsorbent (43); this reaction does not depend on the formation of precipitating antigen-antibody complexes. Serum from one of the immunized rabbits (G-3) recognized all four monomers present in the sialoglycoprotein fraction (Fig. 1; notice that gp-2.1 and gp-2.2 were tested together), whereas nonimmune serum did not recognize any. To detect the possible presence of antibodies against other ghost proteins, we labeled all proteins of unsealed ghosts with 125I (Fig. 2, lane 4) and used them for immunoprecipitation as above. Nonimmune serum did not recognize any ghost protein (Fig. 2, lane C), and immune serum precipitated gp-2.1 and gp-3.1 but not any other ghost component, globin included (Fig. 2, lane D).

By the immune overlay method, we detected in the same serum antibodies against the diffuse component that trails behind (and partly overlaps) gp-3 on Steck and Yu gels (Fig. 3, lane B, immune serum). This antigen was present in much smaller amounts in the sialoglycoprotein fraction since similar exposure did not stain to the same extent the corresponding region of the sialoglycoproteins (Fig. 3, lane C, immune serum). Immune serum did not recognize any of the major ghost proteins (spectrins, band 3, bands 4) which did not comigrate with the sialoglycoproteins in this system, nor any molecular weight markers (Fig. 3, immune serum lanes B and A). Nonimmune serum recognized neither molecular weight markers, nor any ghost proteins, nor any of the sialoglycoproteins (Fig. 3, lanes A, B, and C, respectively). The immune serum might recognize, however, some of the minor components which migrated between gp-2 and gp-3, a finding in apparent agreement with data obtained in preliminary pulse-chase experi-
FIGURE 2. Immunoprecipitation of sialoglycoproteins from radioiodinated ghosts. Autoradiogram of a SDS polyacrylamide gel electrophoretogram performed on 10–15% gradient Laemmli gel. Lanes B, C, and D are the autoradiograms of the input ghosts and their nonimmune and immune precipitates respectively. In lane E are $[^{125}]$sialoglycoproteins heated at low concentration so that they dissociate almost entirely to their monomers. Lanes B–E were exposed for the same length of time. Lane A is a shorter exposure of lane B.

FIGURE 3. Detection of antigens in a Steck-Yu SDS polyacrylamide gel electrophoretogram. Molecular weight markers (A), 50 μg of DBA/2 ghosts (B), and 150 μg of sialoglycoproteins (C) were separated on a 5% gel, and the antigens were detected using serum from rabbit #3 and $[^{125}]$protein A. The last three lanes are controls reacted with nonimmune serum. Note that the sialoglycoproteins were not stained with the CB method (10) used in this experiment. The lines to the left of the figure give the position of the main molecular markers used; their Mr were 130, 94, 68, 43, and 25.7 X 10^3 (from top to bottom). The arrow marks the position of Pyronin-Y that comigrated with lipid.

FIGURE 4. Quality control of nonimmune-TMR-IgG and immune-TMR-IgG. These experiments establish the absence of nonspecific staining by the conjugated IgG fractions we have used. Smears were prepared with the cytocentrifuge from human (a, b, e, and f) or mouse blood (c, d, g, and h) and stained with nonimmune-TMR-IgG (a–d) or with immune-TMP-IgG (e–h). B, basophil granulocyte; G, polymorphonuclear granulocyte; L, lymphocyte; M, monocyte; P, platelet. Note that immune TMR-IgG stains all murine erythrocytes, but the intensity of the staining varies from one cell to another (h). Immune-TMR-IgG does not stain murine lymphocytes, granulocytes or platelets; it does not stain either human erythrocytes or leukocytes. All nonimmune controls are negative.

Specificity of Conjugated IgG

The specificity of the conjugated IgG fractions was determined by immunofluorescent staining of human and mouse blood cells. Nonimmune-TMR-IgG stained no cells from either human or murine blood (Fig. 4b and d). Immune-TMR-IgG stained erythrocytes but not platelets, lymphocytes or granulocytes from murine blood (Fig. 4h); the staining was species-specific, since the antibody did not stain any cell from either human (Fig. 4f) or rat blood (micrographs not shown). This quality control was repeated periodically without any detectable decrease in specific staining or appearance of any nonspecific staining even after 6 mo of storage at 4°C.

Splenic Cells from Anemic Mice

The spleen is a minor hematopoietic organ in the adult mouse, but after severe hemolysis, induced in our case by intraperitoneal injections of phenylhydrazine, the spleen enlarges as it becomes filled with proliferating erythroid precursor cells (21, 63).

Nonimmune-TMR-IgG did not stain any splenic cells at any time after the beginning of the phenylhydrazine treatment (Fig. 5b). Immune-TMR-IgG stained all recognizable erythroid precursors at all times examined—after the induction of anemia (66, 88, 120, and 160 h). Proerythroblasts which, in phase-contrast microscopy, appeared as large cells with euchromatic nuclei and a small rim of cytoplasm, stained very faintly (Fig. 5f and h). The presence in the same field (Fig. 5e vs. f) of large cells (identified as splenic macrophages) which did not stain at all was taken as an internal check for the absence of nonspecific staining, and as a further indication that even the faint staining detected in some cells was specific.

In the phase-contrast microscope, basophilic and polychromatophilic erythroblasts were easily recognized because they were smaller than proerythroblasts, had characteristic heterochromatin masses in their nuclei, had a smaller nucleus/cytoplasm ratio, and a cytoplasm that appeared grey under phase-
contrast optics (5), on account of hemoglobin accumulation. All erythroblasts were stained intensely by the immune-TMR-IgG (Fig. 5 d, f, and h). Enucleating erythroblasts were stained intensely over the cytoplasmic part on its way to become a reticulocyte. There was much less, yet detectable staining of the plasma membrane which wrapped the nucleus as it was extruded (Figs. 5 f and h and 6 b and d). Lymphocytes (Fig. 5 d), macrophages (Fig. 5 f), basophilic (Fig. 5 h), and other types of granulocytes (micrographs not shown) were always negative.

**Primitive Nucleated Erythrocytes of the Fetal Circulation**

In the mouse embryo, the first primitive erythroblasts appear during the eighth day of gestation in the blood islands of the yolk sac. During the ninth day, they are released into the circulation where they divide and differentiate synchronously. In these cells the nucleus condenses progressively, and by day 14 it becomes heterochromatic but is not extruded (4, 17, 20). These primitive erythroid cells synthesize exclusively fetal hemoglobins until day 11 of gestation (9, 24), when they switch to adult hemoglobin, and by day 12 they accumulate enough of it to give a positive reaction with an antibody against adult hemoglobin.

Immune-TMR-IgG stained all primitive erythrocytes at the tenth day of gestation and all contaminating maternal adult erythrocytes, but not any nonerythroid cells (Fig. 7); nonimmune-TMR-IgG did not stain any cell (micrographs not shown). On the day 14 of gestation the antigens were still present on all fetal erythrocytes, which by this time had orthochromatic cytoplasm and pyknotic nuclei (Fig. 8). Occasionally, a bright fluorescent halo was visible around the nucleus (Fig. 7 b and d). This halo was not due to the staining of the nuclear envelope, but to that of the plasmalemma which had become wrapped over the protruding nucleus as the cell flattened during the cytocentrifuge run. The fluorescent rim was visible when the plane of focus was close to the top of the nucleus, but disappeared when the microscope was focused at the level of the flattened cell body (where the phase-contrast ring of the nuclear periphery was still visible [micrographs not shown]).

**Erythroblasts from Day 14 Fetal Liver**

Stem cells originating in the yolk sac seed the fetal liver (51, 69) which becomes an erythropoietic organ during the tenth day of gestation and produces non-nucleated erythrocytes (4, 17, 24, 51). By day 16 of gestation, these cells—which are larger than adult mouse erythrocytes—become the only erythrocytes in the circulation.

Hepatic proerythroblasts, with large euchromatic nuclei and scant cytoplasm were faintly stained by immune-TMR-IgG (Fig. 9 b and f). Next to them, negative hepatocytes served as an additional internal control for the specificity of the staining (Fig. 9 e vs. f). All recognizable erythroblasts from fetal liver were stained intensely by the immune-TMR-IgG (Fig. 9 b, d, and f). The plasma membrane of enucleating erythroblasts stained more intensely over the cytoplasm than over the nucleus being extruded, as described previously for the splenic erythroblasts of anemic mice (micrographs not shown). The intermediate size erythrocytes derived from hepatic erythroblasts were also stained (Fig. 9 b, d, and f) through variably and less intensely than erythroblasts. Hence, crossreacting antigens are present on all murine, hepatic erythroblasts.

**Friend Erythroleukemia Cells**

Friend erythroleukemia cells were originally derived from splenic implants of erythroleukemic mice (26, 54), and permanent cultured lines consist predominantly of blast cells and proerythroblasts, with only a few differentiated erythroblasts (54) whose numbers can be greatly increased by exposure to DMSO or to several other agents (25, 27, 49).

Immune-TMR-IgG stained all Friend erythroleukemia cells before or after exposure to DMSO. In the absence of DMSO, most cells were hemoglobin-free, but they all stained. The diffuse staining without a pronounced rim effect was probably due to the flattening of the cells during the cytocentrifuge run (Fig. 10 b). After a 5-d exposure to DMSO, 95% of the cells contained hemoglobin and all were stained by immune-TMR-IgG more intensely than noninduced cells. There was diffuse staining over the flattened cytoplasm and intense staining over (or within) intracytoplasmic vacuoles in areas where phase-contrast microscopy demonstrated refractile bodies, and where Golgi complexes were found by transmission electron microscopy (Sarris and Palade, unpublished observations).
and f). No cells were stained by nonimmune-TMR-IgG before or after exposure to DMSO (micrographs not shown).

DISCUSSION

During the fetal development of all mammals, erythropoiesis is carried out in succession by three different cell lines. In mice, erythroid cells appear first in the yolk sac—on the eighth day of gestation; then, in the fetal liver—during the tenth day; and finally in the spleen and bone marrow—during adult life (for review, see references 47, 48). However, it was not known whether the erythroblasts and erythrocytes derived from these cell lines express the sialoglycoprotein antigens of adult erythrocytes. Incomplete data on this point exist for bone marrow erythroblasts from mouse, rabbit, and man.

Polyspecific sera against mouse and rabbit intact erythrocytes (50, 62) were used to demonstrate that the cognate antigens accumulate progressively on the surface of differentiating erythroid cells in the bone marrow. The specificity of the antisera towards individual surface antigens (glycoproteins, glycolipids, band 3, and others) was not established, and consequently the relative contribution of each of these chemically diverse components to the total antigen density could not be determined. This is particularly relevant to rabbit erythrocytes since they do not have discrete major sialoglycoproteins (55, 68); consequently, the observed antigenic sites probably represented glycolipids or band 3. Gahmberg et al. (30, 31) raised an antiserum against the purified sialoglycoprotein fraction of human erythrocytes and used a staphylococcal rosetting technique (53) to detect the presence of extracellularly located crossreacting antigens on intact human bone marrow cells. However, they did not show either that their antigen was free of contaminating ghost proteins or that their antiserum did not recognize any other ghost proteins (as, for instance, the externally located segments of band 3). With this technique they demonstrated the existence of sialoglycoprotein antigens on erythrocytes and all erythroblasts, but not on proerythroblasts. The strongest antigenic determinant on human glycophorin A seems to be on a peptide derived from the protein’s endodomain (16; and V. Marchesi, personal communication), and the apparent absence of the antigen from proerythroblasts could be due to inaccessibility to this determinant, as well as to the sensitivity of the technique, which was not determined.

We have isolated the sialoglycoprotein fraction of mouse erythrocyte ghosts without contamination by other ghost proteins that can be either stained with CB or radioiodinated enzymatically. We have raised an antiserum to this fraction and we have proven—by double immunodiffusion and immunoprecipitation of radioiodinated ghosts—that it does not recognize hemoglobin or any of the ghost proteins that can be labeled with $^{125}$I using the LPO-GO procedure (35). In addition we have evidence from immunoprecipitation of solubilized ghosts labeled in vivo with $[^{35}S]$methionine that this antiserum does not recognize other $[^{35}S]$methionine labeled proteins, such as...
as band 3 and spectrins (Sarris and Palade, unpublished observations). The serum recognizes all major components of the purified sialoglycoprotein fraction (gp-2.1, gp-2.2, gp-3.1, and gp-3.2), and immunoprecipitates gp-2.1 and gp-3.1 from $^{125}$I-labeled ghosts. As suggested by evidence we have already presented (56), gp-2.2 and gp-3.2 probably arise from gp-2.1 and gp-3.2, respectively, by proteolytic degradation during the preparation of the sialoglycoprotein fraction. The antiserum also recognizes in ghosts a diffuse component which cannot be labeled by the LPO-GO technique. This component is present in large amounts in the ghosts, but is recovered only to a limited extent in the sialoglycoprotein fraction. Finally, the antiserum appears to bind to minor bands hidden under the diffuse component. We realize, however, that at present we cannot strictly rule out the presence in our antiserum of non-precipitating antibodies to other antigens, or to antigens which are not radioiodinated by the LPO-GO procedure, or not labeled by $[^{35}S]$methionine, and which migrate between gp-2 and gp-3 on Fairbanks gels.

We have isolated IgG fractions from nonimmune and immune rabbit sera, have prepared TMR-IgG conjugates, and have used them to demonstrate by direct immunofluorescence the existence of crossreacting antigens on all recognizable erythroblasts and erythrocytes encountered during the life span of the mouse. We have also shown that crossreacting antigens are not present on lymphocytes, polymorphonuclear leukocytes, platelets, monocytes, and fetal hepatocytes.

The antigens can be localized on the nucleated erythrocytes of the 10-d-old fetus even before these cells start synthesizing adult hemoglobin (during the day 11 of gestation); they are present in the fetal liver erythroblasts that give rise to an intermediate population of non-nucleated erythrocytes, larger than the erythrocytes of the adult mouse; and they are detected on proerythroblasts and on all erythroblasts present in the erythropoietic spleen of adult, anemic mice.

On the continuous plasmalemma of enucleating erythroblasts, most of the antigens segregate on the plasmalemmal domain that will go with the reticulocyte; few remain on the domain that surrounds the expelled nucleus. This sialoglycoprotein segregation parallels the segregation of spectrin in the enucleating erythroblasts (34), and the differential distribution of negative charges, presumed to be sialyl residues, on the enucleating erythroblast of the rat (60, 61).

In fetal liver and in splenic cells, the intensity of immunofluorescent staining increases from proerythroblasts to more mature erythroblasts, probably reflecting continuous synthesis and accumulation of antigens. The pattern of staining of all erythroblasts suggests the existence of a cytoplasmic pool (pre-

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**Figure 8** Day 14 mouse embryos; blood cells stained with nonimmune-TMR-IgG (a and b) or with immune-TMR-IgG (c and d). Adult non-nucleated erythrocytes (AE) serve as standard of staining and size. LE, large liver erythrocyte; PLE, polychromatophilic erythroblast (probably from fetal liver); and PNE, primitive nucleated erythrocyte. Most of the patches of intense staining on some of the primitive nucleated erythrocytes in d are probably folds of the plasmalemma.
sumably present in the rough endoplasmic reticulum, Golgi elements, and small vesicles) draining into a plasmalemmal "sink."

Our findings agree with those of Gahmberg et al. (30) for erythroblasts; but not for proerythroblasts: glycophorin A was not found on human bone marrow proerythroblasts (30), but we have detected sialoglycoprotein antigens on mouse proerythroblasts. The discrepancy may be due to species differences in the appearance of major and minor sialoglycoproteins during the course of normal erythroid differentiation or to differences in the sensitivities of the experimental techniques applied in the two studies. Since our antiserum recognizes all major components of the sialoglycoprotein fraction—and perhaps some minor ones—we can specify neither the nature of the individual antigens present at each developmental stage nor the order of their accumulation during differentiation, should they appear asynchronously.

We have also detected crossreacting antigens on cultured Friend erythroleukemia cells, a population of erythroid precursor cells at various morphological stages of differentiation (25, 49, 54, 59). The antigens are present before as well as after partial differentiation induced by DMSO. However, after induction the staining becomes more pronounced, and intense fluorescence is seen over globular structures in the Golgi complex region. At present, the nature of these crossreacting antigens and the reason for their accumulation in Golgi com-

**Figure 9** Erythroblasts from the liver of day 14 mouse embryos. All micrographs were obtained after staining with immune-TMR-IgG. Nonimmune-TMR-IgG did not stain any cells (not shown). **BE**, basophilic erythroblast; **H**, hepatocyte; **LC**, large unidentified cell; **LE**, large erythrocytes of fetal liver origin; **PE**, proerythroblast; and **PLE**, polychromatophilic erythroblast.
plex regions is unclear. More work, needed to answer this question, is currently in progress.

It is already established that there is progressive accumulation of surface anionic groups during the development of rat erythroblasts and that these groups (presumably sialyl residues) segregate preferentially on the surface of reticulocytes but not on the membrane enclosing extruded nuclei (60, 61). It is also known that the mature erythrocytes of the adult mammal lose progressively their surface sialyl residues as they age (14; see also reference 46). Yet these cells retain practically the same surface charge density throughout their aging process, as shown by a variety of electrophoretic procedures (45). To reconcile these apparently contradictory sets of data, net membrane loss—compensating for loss of charges—has been postulated (45). At present, the functional role of sialyl residue—and charge variations in controlling the differentiation process and the life span of erythrocytes—is still an object of controversy. In vitro removal of sialyl residues by neuraminidase or by trypsin digestion enhances the rate at which the treated erythrocytes are removed by splenic and hepatic macrophages upon reintroduction in the systemic circulation (23, 33, 39, 40). This enhancement is not due to the exposure of terminal galactosyl residues (39), and this it does not seem to depend on a mammalian lectin similar to the hepatic receptor which clears asialoglycoproteins from the circulation (3, 37, 41). IgG-induced opsonization of erythrocytes promotes their phagocytosis by mononuclear cells in vitro (1) and reduction of surface sialyl residues on old erythrocytes results in attachment of IgG to unspecified surface receptors and to their subsequent in vitro phagocytosis by mononuclear cells (42). A similar process presumably leads to removal of old erythrocytes in vivo (42). The sialoglycoproteins of erythroid cells account for a significant fraction of the total sialyl residues on these cells' surface. These residues may inhibit the attachment of IgG to unspecified surface receptors and thus prevent the phagocytosis of erythroid cells in liver, spleen, and bone marrow. The relative paucity of sialoglycoproteins on the plasmalemmal domain that segregates with the extruded erythroid nuclei could explain (by reduced negative charge and prompt antibody attachment) the rapid phagocytosis of nucleated remnants by mononuclear cells in the bone marrow (58, 60, 61).

Yet, since surface charge density does not change during

**FIGURE 10** Friend erythroleukemia cells stained with immune-TMR-IgG (a–f). a and b are noninduced cells grown in DME. c–f are cells induced by growth for 5 d in DME containing 2% (vol/vol) DMSO. They were 95% positive for hemoglobin by the benzidine test. Note prominent staining in the Golgi complex regions of some of the cells in d and f.
erythrocyte aging (45), more complex interactions than those so far envisaged are probably involved in the control of the differentiation and life span of erythrocytes. A similar conclusion is suggested by the existence of a rare recessive human allele, En(a-), associated with the absence of the major sialoglycoproteins and a significant reduction of surface sialyl residues in human erythrocytes (18, 19, 29, 36). En(a-) homozygotes do not have a much shorter erythrocyte survival than normal individuals, and do not present symptoms usually associated with a chronic hemolytic condition. The increased glycosylation of band 3 in En(a-) homozygotes (32, 67) suggests that the latter have other mutations that compensate for the lack of glycoporphins, but clearly more work is limited to establish the actual function of erythroid sialoglycoproteins, and to explain the apparent hemolytic normality of En(a-) homozygotes.

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