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Expression of Red Cell Membrane Proteins in Erythroid Precursor Cells

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Specific antibodies to human glycophorin A and spectrin were used to study the expression of these membrane proteins in normal and pathologic human bone marrow. In immunofluorescence experiments spectrin and glycophorin A are found in 50–60% of the nucleated cells in normal bone marrow. These two proteins are expressed at all stages of red cell differentiation and can be traced at least to the earliest morphologically recognizable nucleated red cell precursor, the proerythroblast; the two proteins are specific for cells of the red cell series and are not found to be expressed in lymphocytic, granulocytic cells or platelets. These conclusions were drawn from studies on bone marrow in patients with a temporary block in erythropoiesis at the level of stem cells or of the pronormoblast. Bone marrow from these individuals either lacked all nucleated cells stainable for glycophorin A and spectrin or contained only pronormoblasts. Similar findings were obtained on spleen cells from mice which were made severely anemic by multiple injections with N-acetyl-phenylhydrazine. Antibodies to a sialoglycoprotein isolated from mouse red cell membranes stain 70–80% of all cells in the spleen of anemic animals, while only 1–2% of such cells are seen in the spleen of normal animals. Spectrin and glycophorin A could be labeled metabolically and isolated using specific antibodies. The human tumor cell line K562 expresses both membrane proteins, but induction experiments with various agents thus far have failed to change their expression.

Key words: erythroid precursors, glycophorin A, spectrin, bone marrow, anemic mouse spleen, plasma membrane

The human erythrocyte membrane proteins spectrin and glycophorin A have been studied with regard to structure and function. Spectrin, a large cytoskeletal fibrillar-like membrane protein which probably exists as a tetramer or polymer of even higher molecular weight in the intact cell [1], is thought to influence shape and provide structural rigidity to the red cell membrane by binding to an assembly of other cytoskeletal and membrane-associated proteins [2]. Glycophorin A, a much smaller membrane-spanning sialoglycoprotein which carries the MN blood group antigen [3] has an unknown function, but its structure and organization within the membrane are quite consistent with a role as a receptor.

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The expression of these proteins during mammalian erythroid differentiation has been studied by several investigators in both normal marrow and blood cells [4–7] and in erythroleukemia cell lines [8–10]. It has been reported for human bone marrow that glycophorin is expressed in basophilic normoblasts and later stages of development, while pronormoblasts do not seem to contain it [4]. In mouse marrow cells spectrin is expressed in nucleated red cell precursors [5].

Using a combination of fluorescent antibody microscopy and immunoassay of biosynthetically labeled cell proteins, we have investigated the early expression of spectrin and glycophorin in human marrow, anemic mouse spleen cells, and human K562 erythroleukemia cells. We present evidence that (a) spectrin and glycorphorin A are expressed solely in erythroid cells and (b) that they are present in the earliest morphologically recognizable erythroid precursor cells. These proteins are also expressed in the human cell line K562 and, unlike hemoglobin, do not appear to increase in amount after treatment of these cells with various agents known to induce differentiated erythroid cell functions.

MATERIALS AND METHODS

Reagents

Formalin-treated Staph aureus (Pansorbin) was purchased from Calbiochem-Behring Corp, LaJolla, CA. Fetal calf serum (heat inactivated) and RPMI-1640 medium were purchased from Grand Island Biological Co., NY. L-[^{35}S]-methionine (900–1300 Ci/mmole) and D-[^{3}H]-galactose (2.1 Ci/mmole) were purchased from New England Nuclear, Boston, MA.

Isolation of Sialoglycoproteins from Mouse Erythrocyte Membranes

Blood from mice of different strains was obtained from the retroorbital plexus or by cardiac puncture and was drawn into a solution of 3.8% sodium citrate (approximately 1 part per 3 parts of blood). The red cells were washed 5 times with PBS with careful removal of the buffy coat after each centrifugation and were lysed at 4°C with a volume of 5 mM sodium phosphate, pH 8.0, 20–30 fold in excess over the volume of packed cells. The membranes were resuspended and centrifuged 3 times with large volumes of the same buffer. After final suspension in 10 mM Tris-HCl, pH 8.0, and centrifugation the resulting ghosts were hemoglobin-free and were lyophilized. A sialoglycoprotein fraction was isolated as described previously [11] by the lithium diiodosalicylate phenol method [12]. After extraction of the dialyzed and lyophilized fraction with 100% ethanol and relyophilization, the preparation gave an electrophoretic peptide pattern on SDS-polyacrylamide gels which was identical to data obtained by Sarris and Palade [13]. Since several bands are observed which are stainable with the periodic acid–Schiff's reagent, individual peptide bands were isolated from unfixed slab gels (16 × 14 × 0.5 cm) prepared according to the method of Laemmli [14]. A portion of the sialoglycoprotein fraction was dansylated as described previously [15] and added to a ninefold excess of unlabeled protein. Portions (20 mg) of protein were dissolved in 2 ml SDS-buffer and loaded onto individual slab gels. After electrophoretic separation the peptide bands were visualized under UV light and the gels were cut with a razor blade. The gel strips were homogenized with a Tekmar homogenizer (Tekmar, Cincinnati, OH) in 0.05% SDS in 50 mM sodium bicarbonate containing 0.02% sodium azide, and after shaking overnight at 37°C the gel particles were removed by centrifugation. The supernate of a second distilled water extract was combined with the first supernate and was dialyzed at 4°C against distilled water. After lyophilization the

peptides were dissolved in 0.05% SDS in 50 mM sodium phosphate, pH 8.0, and chromatographed on a column (95 X 2.5 cm) of ultrogel AcA 54 (LKB) equilibrated with the same buffer to remove glycine and acrylamide. The sialic acid containing protein peak was dialyzed extensively against distilled water and lyophilized. To remove remaining SDS the peptides were finally extracted twice with 100% ethanol at 0°C and relyophilized. The chemical characterization of the mouse sialoglycoproteins will be described elsewhere (Furthmayr, in preparation).

Preparation of Antibody Reagents

Antisera to the carboxyterminal fragment of human erythrocyte glycophorin A were raised in rabbits, and specific antibodies were purified by affinity chromatography as described [16]. Antisera to human erythrocyte spectrin were obtained by immunization of white albino rabbits with three to four biweekly injections each of 2 mg spectrin purified by methods described [17] and mixed with complete Freund's adjuvant. Specific antibodies were isolated by affinity chromatography on a column of spectrin coupled to cyanogen bromide-activated Sepharose 4B using procedures as detailed elsewhere [16]. Antibody IgG was eluted from the column with 1N acetic acid containing 0.15M NaCl. The purified antibodies reacted only with the two polypeptide chains of spectrin when analyzed by two-dimensional immunoelectrophoresis of human erythrocyte membranes [15].

Six rabbits were immunized with individual mouse sialoglycopeptides after separation on polyacrylamide gels. For this purpose 20 mg of the mouse red cell membrane sialoglycoprotein fraction was electrophoresed on slab gels, and the gel strips containing protein were cut with razor blades and were homogenized. After mixing with an equal volume of complete Freund's adjuvant, sufficient material was injected to contain 1–2 mg of protein. Injections were repeated at biweekly intervals three more times. The antisera exhibited the zone phenomenon when tested for hemagglutination with mouse erythrocytes and had titers of 1:32. In immunodiffusion experiments (Hyland plates, pattern D) the precipitation titers ranged from 1:1 to 1:4 at sialoglycoprotein concentrations of 1 mg/ml or 0.3 mg/ml. No difference was observed for glycoprotein preparations isolated from red cell membranes of different strains of mice. The antisera were also tested by immunofluorescence as described below. After heat inactivation at 56°C for 30 minutes, the antisera showed positive fluorescence at dilutions of 1:50 with intact cells or with dried and fixed membranes after absorption with intact mouse erythrocytes (1 ml of washed and packed red blood cells incubated at room temperature with 1 ml of antiserum). Rabbit anti-mouse sialoglycopeptide sera did not react with human glycophorin A in these tests. The antibodies also reacted specifically with the sialoglycopeptides when mouse erythrocyte membrane proteins or isolated sialoglycopeptides were separated by SDS-polyacrylamide electrophoresis and the gels were incubated with dilutions of the antisera by procedures described elsewhere [15].

Immunofluorescence Assays

Blood smears from peripheral blood were prepared on glass slides according to standard procedures. Various lymphoblastic cell lines were kindly provided by J. Strominger, Harvard University; K562-4 cells by B. Lozzio, University of Tennessee; a K562 clone by C. Gahmberg, Helsinki; and HL-60 cells by R. Hoffman, Yale University. Cells in 10% fetal calf serum containing medium were centrifuged onto Ovalbumin-coated glass slides with a cytocentrifuge and air dried. After fixation for 10 minutes in acetone and drying, a circle one cm in diameter was engraved with a diamond. The preparation was rehydrated with

PBS for 10 minutes followed by incubation with sufficient amounts of appropriate diluted specific antibodies (3 μg/100 μl), antiserum, or rabbit normal serum (1:25–1:50) for 30 minutes in a humid chamber. Care was taken to prevent drying of the cells. After three washes with PBS the cells were overlaid with a 1:20 dilution (30 μg/μl) of affinity purified, fluorescein isothiocyanate conjugated, sheep anti-rabbit IgG and incubated for one hour. The slides were then washed again three times with PBS and cover slipped with a polyvinyl alcohol-glycerol medium (Gelvatol, Monsanto, Indian Orchard, MA). Controls included incubation without primary antibodies and with antibodies which had been preincubated with the specific antigen. A Zeiss standard binocular 14 fluorescence microscope equipped with a Mercury lamp and a vertical illuminator with 450–490 nm excitation and 520 nm barrier filters was used to view the slides. Photographs were taken with high-speed Ektachrome (ASA 400) film using 4-minute exposure times. The fluorescein-conjugated affinity purified sheep anti-rabbit IgG was prepared using standard techniques [32]. The F/P ratio was 4:1, the labeled antibody solution was stored at −70°C at a concentration of 100 μg/ml.

Preparation of Mouse Spleen Cells

Mice (C57/bl) were injected intraperitoneally at 0, 16, and 24 hours with 1 mg (in 100 μl) of neutralized N-acetyl phenylhydrazine (Sigma) in 0.15 M NaCl [18]. Spleens were removed from untreated animals and from injected animals at various time points after the initial injection. The cells were teased into RPMI-1640 medium containing 10% fetal calf serum and the nucleated cells were isolated by one-step Ficoll-Isopaque density gradient centrifugation LSM-solution, Litton Bionetics, Kensington, MD. Boyum [19] slides for immunofluorescence staining were prepared as described above.

Maintenance and Labeling of K562 Cells and Mouse Spleen Cells

K562 cells were maintained in suspension in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in 5% CO_2 at a cell density of 5×10^4/ml to 8×10^5/ml.

These cells were labeled biosynthetically with either [^{35}S]-methionine or [^{3}H]-galactose. For long-term (18–48 hours) labeling with methionine, the cells were first resuspended at 10^5/ml in RPMI-1640 without methionine in the presence of serum; for short-term (less than 2 hours) labeling cells were resuspended in RPMI-1640 without methionine but supplemented with 0.2% bovine serum albumin. For [^{3}H]-galactose labeling, cells were resuspended in their maintenance medium.

The spleen cells from anemic mice were washed by centrifugation and resuspended in methionine-free medium (for methionine labeling) or in medium with serum (for galactose labeling). The cells were usually labeled at a concentration of about 5×10^7 nucleated cells/ml.

Cell Fractionation

Both K562 and spleen cells were suspended in 10 mM Tris-HCl, 3 mM $MgCl_2$, pH 8, with 0.03 mM PMSF and 1 mM DFP for five minutes on ice ($1–5 \times 10^7$ cells/ml). The hypotonically swelled cells were lysed with a tight-fitting glass Dounce homogenizer (20 strokes) and then centrifuged at 1,900g for 20 seconds to pellet the nuclei. The post-nuclear supernatant, which consisted of suspended membrane vesicles, was again centrifuged and the supernatant was then made up to 10 mM EDTA. For further fractionation into a cytosol and membrane preparation, the post-nuclear supernatant was centrifuged at 100,000g for one hour.

Immune Precipitation of Cell Fractions

Prior to immune precipitation, methionine-labeled fractions were mixed with 3% SDS, heated to 80–100°C for one minute, and then mixed with a five- to sevenfold excess of Triton X100 before diluting sixfold with 10 mM Tris HCl, pH 8. Galactose-labeled fractions were mixed with Triton X100 to a final concentration of 1% (10 mg Triton per 10^7 cells). Detergent-treated samples were then mixed with about 20 μg of affinity purified antibody or 20 μl of antiserum per 10^7 cells, incubated on ice overnight, and then mixed with a 10% detergent suspension of washed formalin-fixed Staph aureus (10 μg/μl of antibody or 10 μl/μl of antisera). After incubation on ice for one hour, the Staph aureus was pelleted at 2,400 g for five minutes and washed three times by centrifugation, first with detergent and finally with 10 mM Tris HCl, pH 8. The washed pellet was incubated with a tenfold excess of SDS solubilizing buffer at 80–100°C, and aliquots were analyzed by SDS polyacrylamide gel electrophoresis according to the methods of Fairbanks et al [20] or Laemmli [14].

Authentic standards used were the membrane proteins of red blood cell ghosts prepared by the method of Fairbanks et al [20].

Slab gels were stained with Coomassie Blue and then prepared for fluorography by impregnation with DMSO and PPO [21]. Tube gels were separated into 1-mm fractions with a Gilson automated gel slicer. These fractions were eluted with 0.2% SDS and radioactivity was determined in a Beckman scintillation counter after suspension in an aqueous scintillation cocktail (New England Nuclear formula 963).

RESULTS

Fluorescent Antibody Studies

When normal human bone marrow cells were incubated with rabbit antibodies raised against either glycophorin A (Fig. 1a) or spectrin and then fluorescein-labeled with sheep anti-rabbit IgG, 50–60% of the nucleated cells showed positive fluorescence in addition to mature erythrocytes. No staining was seen when these cells were first incubated with non-immune rabbit IgG or when the antibodies were preadsorbed with the specific antigen.

Due to the poor preservation of cellular morphology and loss of characteristic staining properties for histochemical stains, it was not possible to unambiguously assign nucleated cells to the erythroid lineage or to determine their exact stage of differentiation.

In order to make these distinctions we took advantage of two patients with blocks in erythroid cell development at different stages. The first patient, S.M., was a 24-year-old white female who was found to have an anemia (initial hematocrit of 12.9%) due to an erythroid hypoplasia of unknown etiology. Examination of her bone marrow cells showed a great predominance of pronormoblasts with few early and almost no late normoblasts. Marrow cells from this patient were tested with glycophorin A and spectrin antibodies (Fig. 1b). A few large nucleated cells showed positive fluorescence. The percentage of nucleated cells which fluoresced using either antibody was similar to or slightly larger than the percentage of cells which could be identified as blasts on cell smears treated with Wrights stain. In addition, mature erythrocytes were visualized as fluorescent positive with both antibodies.

The second patient, J.A., was a 46-year-old white male with tuberculosis who developed anemia (hematocrit of 15.5%) from a pure red cell aplasia following treatment with isoniazid and ethambutol. Examination of his bone marrow cells by conventional staining

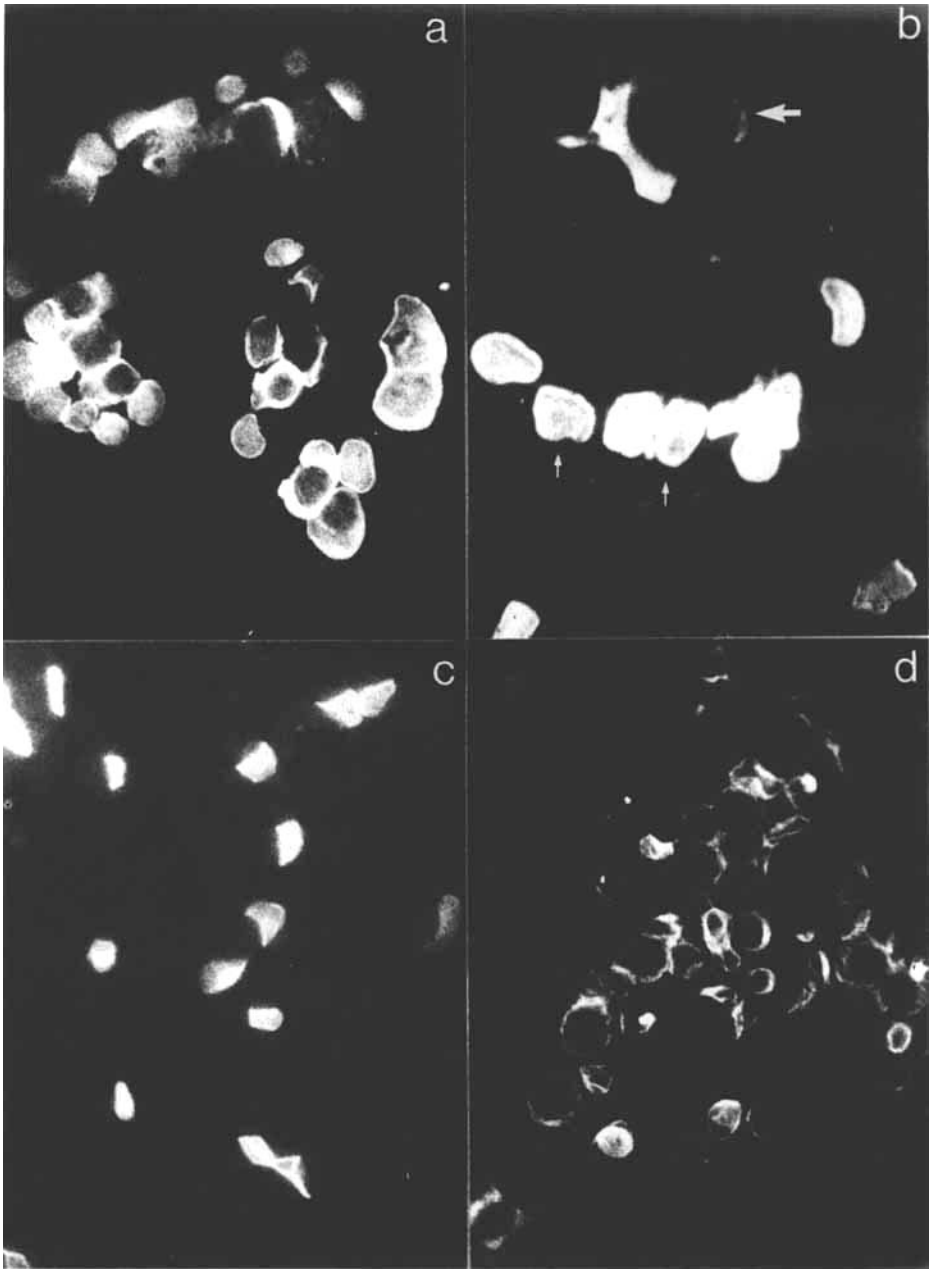

Fig. 1. Fluorescent antibody staining of human bone marrow cells. Air-dried and fixed cells were stained with antibodies to glycophorin A. Identical results obtained with antibodies to spectrin are not shown. a) Normal human bone marrow cells. b) Marrow cells from a patient (S.M.) with a block in erythropoiesis at the pronormoblast stage. Pronormoblast (large arrow), mature red cells (small arrows). c) Marrow cells from a patient (J.A.) with a drug-induced pure red cell aplasia. d) Cells from the same patient (J.A.) following withdrawal of drugs and with restoration of erythroid population.

techniques revealed a marked reduction in all erythroid cells with little effect on other marrow elements. Following withdrawal of the patient's anti-tuberculosis drugs, the marrow was repopulated with erythroid cells. When the aplastic marrow cells were treated with glycophorin or spectrin antibodies (Fig. 1c), virtually no nucleated cells were fluorescent positive; however, when marrow cells were analyzed one month after withdrawal of drugs, the percentage of fluorescent-positive nucleated cells dramatically increased (Fig. 1d). Since no cells other than erythroid elements were affected in the bone marrow of this patient, it is quite apparent that both proteins are specific products of cells belonging to the red cell series. In support of this conclusion are studies on various lymphoblastic cell lines (SLT, IM-1, RAMS, RAJI, SB) and the granulocytic precursor cell line HL-60, which do not react in fluorescence assays with either antibodies to glycophorin A or spectrin (data not shown).

The human erythroluekemia cell line K562 and one clone derived from it were analyzed by the fluorescent antibody technique. A large proportion of these cells were stainable when cells were incubated with glycophorin and spectrin antibodies (Fig. 2). When these cells were grown for several days to a week in the presence of either 1% DMSO, 5 mM hexamethylene-bis-acetamide, 0.2 mM hemin, or 1 mM sodium butyrate, no apparent increase in fluorescence intensity nor in number of fluorescence positive cells was seen when treated with antiglycophorin or anti-spectrin (data not shown). These diverse compounds

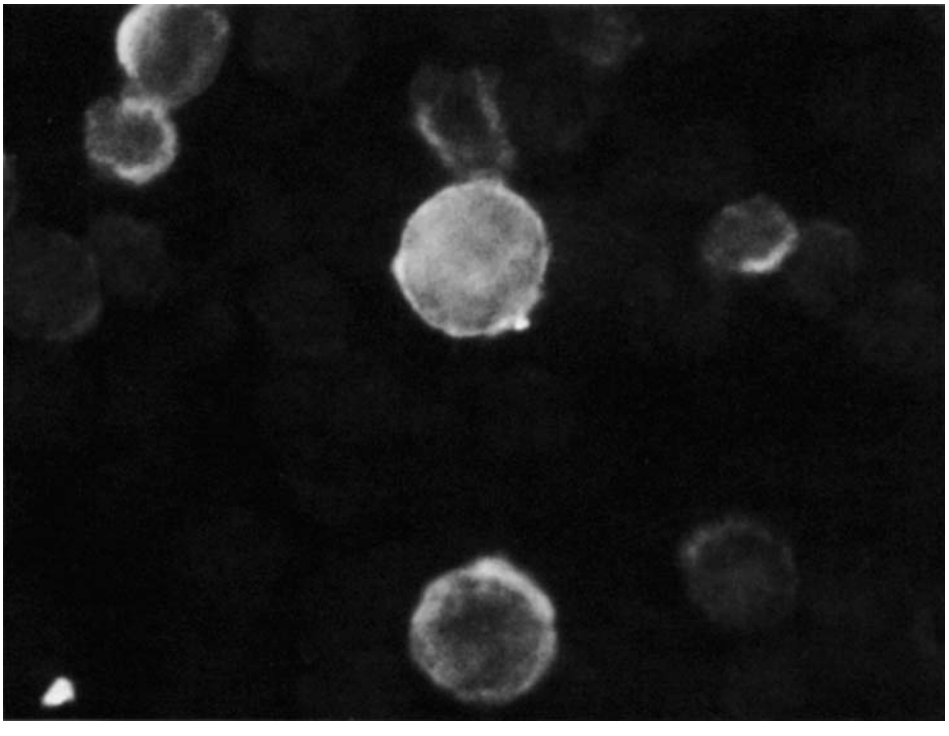

Fig. 2. Fluorescent antibody staining of air-dried and fixed human K562 cells. Staining of human K562 cells in log phase growth with antibody to human glycophorin A. Notice the difference in size and fluorescence intensity in different cells. Cells stained with antibodies to human spectrin had the same appearance.

are known to induce hemoglobin synthesis in either K562 cells [10] or hemoglobin and spectrin synthesis in mouse erythroleukemia Friend cells [8, 22], although the effect of sodium butyrate on K562 cells has been disputed [23]. In contrast to K562 cells, several other cell lines such as HL60 and various B-cell lymphoblastic lines mentioned above were evaluated. When these cells were grown with or without 1% DMSO for up to nine days, no fluorescence could be detected after incubating the cells with either glycophorin A or spectrin antibodies.

In support of the conclusions drawn above for the human system, namely that glycophorin A and spectrin are specific red cell proteins, further studies were done in mice. Mice were made anemic by injections of N-acetyl-phenylhydrazine, and up to 90% of the nucleated spleen cells isolated 120 hours after the first injection were recognizable as erythroid by Wright's staining, while only 2–4% of the untreated mouse spleen cells were erythroid. When splenic cells isolated 48, 72, 96, 120, and 140 hours after initial injection were stained with antibodies to a unique sialoglycoprotein isolated from mouse erythrocyte membranes or with anti-human spectrin (which readily cross reacts with mouse spectrin), an increasing number at 48 hours and high proportion of nucleated cells at later times showed positive fluorescence (Fig. 3). After adsorption of the anti-mouse sialoglycoprotein with intact red cells, immune fluorescence was lost from live cells but not from dried and fixed cells, indicating that the antibodies reacted also with a cytoplasmic portion of the molecule similar to human erythrocyte glycophorin A [16].

Characterization of Cellular Antigens

In order to confirm the data presented above which indicate that nucleated erythroid cells express glycophorin A and spectrin, human K562 cells were labeled biosynthetically with [^{35}S]-methionine for one hour at 37°C. After washing, the cells were lysed and the postnuclear supernatant fraction was mixed with SDS and Triton X100. After addition of affinity-purified spectrin antibodies, labeled material was bound to formalin-treated Staph aureus, and the washed precipitate was analyzed by polyacrylamide gel electrophoresis in the presence of SDS. The major proteins migrated as two bands (230,000 and 250,000 daltons) in positions which were identical to authentic human spectrin (Fig. 4). These bands were not seen if nonimmune IgG was used instead of antispectrin (Fig. 4). When the postnuclear supernatant was further separated by ultracentrifugation (100,000 g for 1 hour) into cytosol (supernatant) and membrane (pellet) enriched fractions and immune precipitated with anti-spectrin, only about one third of the labeled spectrin was found associated with the membrane pellet. Of total cellular proteins synthesized, 0.15% (4.5 μg/10^7 cells) is represented by spectrin (determined by radioimmunoassay).

In the mature red cell spectrin has been reported to be exclusively associated with the membrane [24], although some spectrin apparently fractionates as soluble material after hypotonic lysis of red cells [25]. If one assumes that spectrin binds to the K562 membrane with the same affinity with which it binds to the red blood cell membrane (K_D of 10^{-7} M; [26]), then the apparent discrepancy may simply be due to the comparatively low concentration of spectrin in the erythroleukemia cells. Although it cannot be excluded that 10 mM Tris buffer lysis in itself induces some spectrin dissociation from the membrane, it may be that spectrin binds differently to the membranes in the tumor cell line.

K562 cells were also labeled with [^{3}H]-galactose overnight at 37°C and cytosol and membrane-enriched fractions were prepared and, after solubilization with Triton X-100, were immunoprecipitated with anti-glycophorin A antibodies. The precipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The membrane fraction gave two major

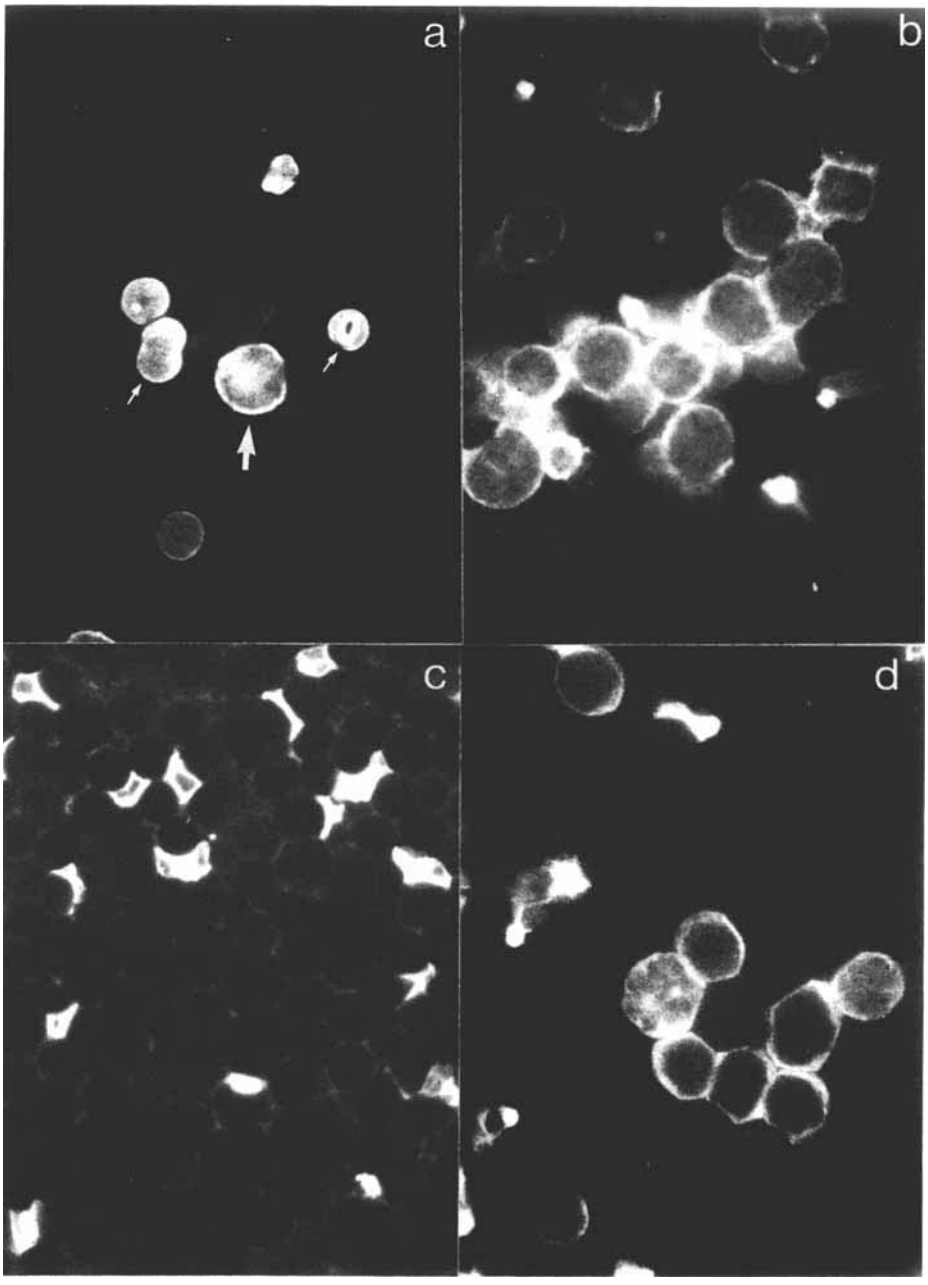

Fig. 3. Fluorescent antibody staining of air-dried and fixed erythropoietic mouse spleen cells. a) Normal mouse spleen cells stained with antiserum to the major mouse erythrocyte membrane sialoglycoprotein ("mouse glycophorin"). Mature red cells are indicated by small arrows and an occasional nucleated red cell by large arrow. b) Erythroid mouse spleen cells (140 hours after initial injection of mice with N-acetyl phenylhydrazine) stained with antiserum to sialoglycoprotein. c) Normal mouse spleen cells stained with antibodies to human spectrin. d) Erythroid mouse spleen cells (120 hours after initial injection of mice with N-acetyl-phenylhydrazine) stained with antibodies to human spectrin.

peaks with apparent mobilities identical to the two bands of human red cell glycophorin A, PAS-1 and PAS-2 (Fig. 5). No labeled glycophorin could be immune precipitated from the cytosol fraction.

The K562 cells were evaluated for increased synthesis of spectrin and glycophorin after growth for several days in the presence of either 1% DMSO, 1 mM sodium butyrate, or 0.2 mM hemin. No increase in the rate of incorporation of [^{35}S]-methionine or [^{3}H]-galactose into immunoprecipitated spectrin or glycophorin was observed (data not shown).

The HL-60 human leukemia cell, either before or after treatment with 1% DMSO, could not be shown to synthesize spectrin when analyzed by immune precipitation, although the induced cells did contain two polypeptides of about the same molecular weight as spectrin (data not shown).

Spleen cells harvested from mice 120 hours after initial treatment with N-acetyl-phenylhydrazine were incubated in the presence of [^{35}S]-methionine in methionine-free medium for several hours. A postnuclear supernatant was prepared and after solubilization

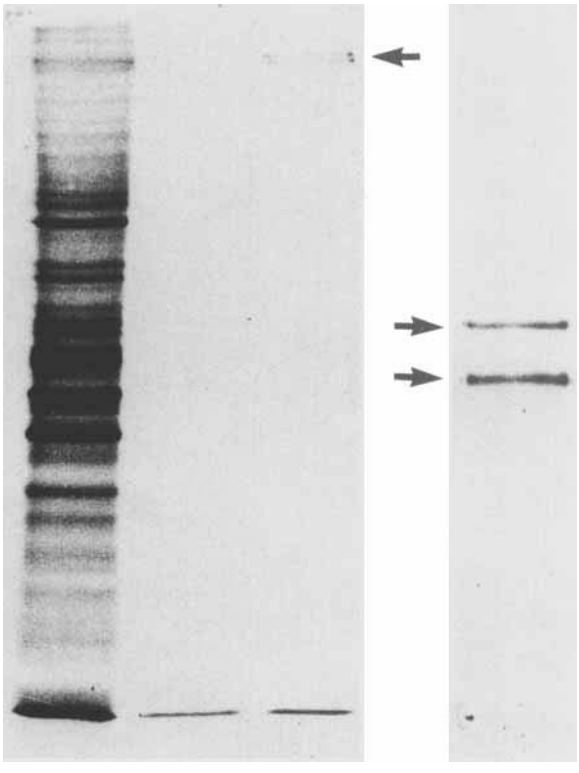

Fig. 4. Fluorograms of labeled spectrin isolated from K562 cells by immune precipitation and analyzed by SDS-polyacrylamide gel electrophoresis. K562 cells were labeled with 1 mCi/ml of [^{35}S]-methionine at 37°C for one hour. A post-nuclear supernatant was mixed with SDS and Triton X100 and immune precipitated with antispectrin and formalin-treated Staph aureus. The bound protein was analyzed by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels (a, b, c) and 5% gels (d) according to the method of Laemmli [14]. a) Post-nuclear supernatant before immune precipitation. b) Incubation with nonimmune IgG. c) Incubation with affinity purified rabbit anti-spectrin. d) Specific immune precipitate electrophoresed for extended time period.

with SDS/Triton X100, labeled material was immune precipitated with anti-human spectrin and was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 6). Two major bands were observed which migrate identically to authentic human spectrin.

Spleen cells from N-acetylphenylhydrazine-treated mice were also labeled with [^{3}H]-galactose for three hours. The material which could be precipitated with rabbit anti-mouse sialoglycoprotein serum gave three radioactive peaks on SDS-polyacrylamide gels with apparent molecular weights of 56,000, 116,000, and 230,000 daltons, one of which co-migrated with the mouse sialoglycopeptide isolated from mature red cells (Fig. 7). The two species with higher molecular weight represent SDS-resistant multimeric forms of presumably the same glycopeptide. Such aggregates are known to occur with human [11] and mouse [27] sialoglycoproteins.

DISCUSSION

Using immunofluorescence and metabolic labeling techniques we have found that both spectrin and glycophorin A are synthesized and expressed by human erythroid precursor cells at least as early as the pronormoblast stage. Similarly in mouse spleen, spectrin and sialoglycoprotein are expressed early in erythroid development. Furthermore,

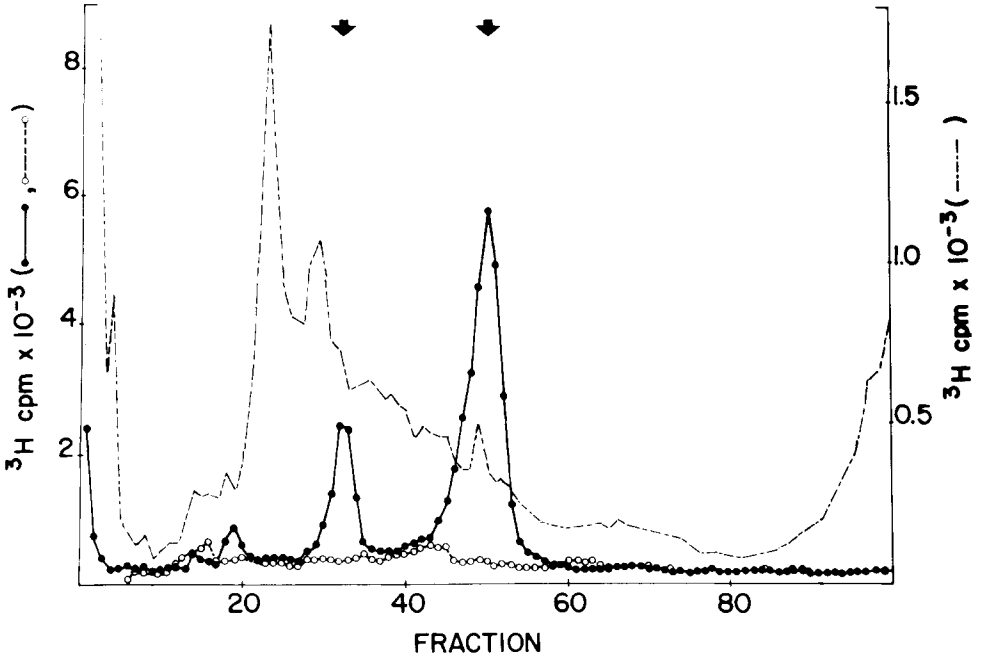

Fig. 5. SDS-polyacrylamide gel electrophoresis of labeled glycophorin A isolated from K562 cells by immune precipitation. K562 cells (2.7×10^7) were labeled with [^{3}H]-galactose at 10 μCi/ml for 22 hours and fractionated into cytosol and membrane material. Aliquots of cytosol and a 1% Triton X100 solution of membranes were incubated with either rabbit anti-glycophorin A or rabbit nonimmune IgG, and the immune precipitates were analyzed on 5.6% SDS-polyacrylamide tube gels [20]. The gels were separated into 1-mm fractions. Arrows indicate the position of PAS stained human red cell ghost proteins. Anti-glycophorin precipitate, ●—●; normal rabbit IgG precipitate, ○---○; total membranes before immune precipitation, ----.

by using marrow cells with blocks in erythropoiesis and mouse spleen cells before and after drug-induced erythropoiesis, we have established that spectrin and glycophorin are unique to the erythroid lineage and are not found in myeloid cells, lymphocytes, or megakaryocytes and platelets. These two proteins are, therefore, specific erythroid antigenic markers which are expressed at early stages of red cell development. Our findings are supported by earlier studies. Gahmberg et al [4], by analyzing labeled cell surface glycoproteins with glycophorin antiserum, found that glycophorin is expressed in the basophilic normoblast and later cell stages of human marrow erythropoiesis, and Geiduschek and Singer [5] detected spectrin by immune fluorescence in nucleated and enucleating mouse marrow normoblasts. They noted an apparent increase in the concentration of spectrin in the more differentiated cells. Furthermore, peripheral T- and B-lymphocytes, monocytes, granulocytes, and platelets do not carry these proteins in their plasma membrane (reviewed in Furthmayr [28]) as determined by cell-surface labeling of radioimmunoassay techniques.

To test the validity of our results obtained with the immunofluorescence technique on dried and fixed cells, the recently discovered human erythroleukemia cell line K562 proved to be of great value. Since these cells can be maintained in tissue culture, cellular

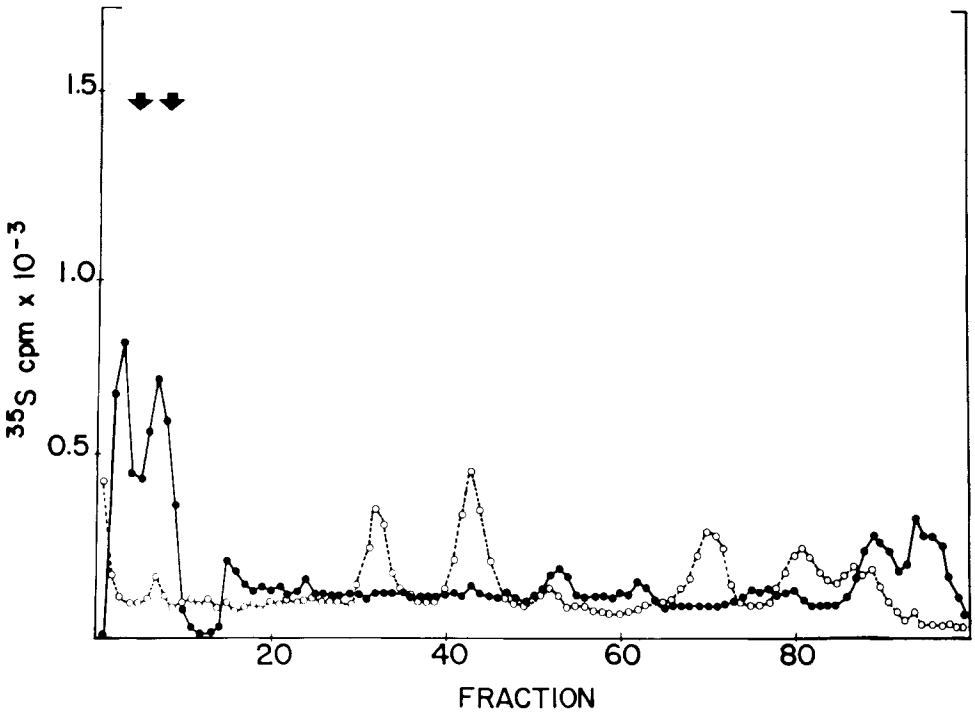

Fig. 6. SDS-polyacrylamide gel electrophoresis of labeled spectrin isolated from erythroid mouse spleen cells by immune precipitation. Mouse spleen cells (4×10^8) harvested 120 hours after initial injection with N-acetyl phenylhydrazine were incubated with 100 μCi/ml of [^{35}S]-methionine for one hour. A post-nuclear supernatant was prepared and solubilized with SDS and Triton X100. Aliquots were then incubated with either rabbit anti-human spectrin affinity purified antibodies or normal rabbit IgG, and the Staph aureus adsorbed protein was analyzed on 5.6% SDS-polyacrylamide gels [20]. The gels were sliced into 1-mm fractions. Arrows indicate the migration position of authentic spectrin. Anti-spectrin precipitate, •—•; rabbit normal IgG precipitate, ○– – –○.

components can be labeled metabolically and the products analyzed with specific antibodies. The K562 cells have the morphological appearance of blast cells and have been shown to express both glycophorin and spectrin as well as trace amounts of hemoglobin [9, 10]. We have confirmed the presence and synthesis of glycophorin A and spectrin in this cell line after labeling with [^{3}H]-galactose and [^{35}S]-methionine. While glycophorin was found to be associated exclusively with the membrane fraction, spectrin, which represents 0.15% of total protein synthesis, was present in both cytoplasmic and membrane fractions. The cell line obtained from Dr. Lozzio appeared, however, quite heterogeneous with respect to size of the cells and the expression of these membrane proteins with only 30–50% of all cells labeled in the immunofluorescence procedure. Cloning of the cells apparently does not remedy this situation, because a clone obtained from Dr. Gahmberg did not yield a more uniform cell population. Since in Friend leukemia cells grown in DMSO and treated with spectrin antibodies the percent of fluorescent positive cells increased from 0.3% (0 time) to 38% at 48 hours and 92% at 92 hours induction time [8], it is possible that the K562 cells are analogous to the 48-hour stage of induced differentiation of these cells.

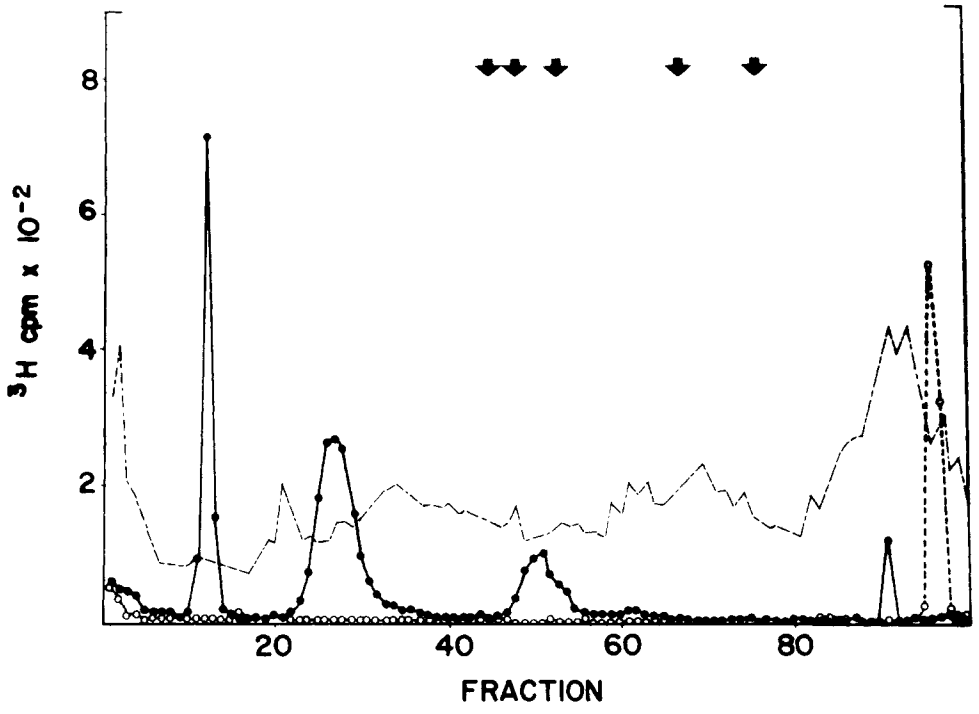

Fig. 7. SDS-polyacrylamide gel electrophoresis of labeled sialoglycoprotein isolated from erythroid mouse spleen cells by immune precipitation. Mouse spleen cells (2×10^8) harvested 120 hours after initial injection with N-acetyl phenylhydrazine were labeled with 0.8 mCi/ml of [^{3}H]-galactose for three hours. A post-nuclear supernatant was prepared, solubilized with SDS and Triton X100, and aliquots were incubated with rabbit anti-mouse sialoglycoprotein antiserum or normal rabbit serum. The formalin-treated Staph aureus adsorbed protein was analyzed on 10% SDS-polyacrylamide gels [14]. Gels were sliced into 1-mm fractions. Anti-sialoglycoprotein precipitate, •—•; normal rabbit serum precipitate, ○– – –○; post-nuclear supernatant before immune precipitation, ————.

It has been reported [10] that hemoglobin synthesis can be induced in K562 cells with sodium butyrate from a measured globin cellular concentration of 20 μg/gram to 150 μg/gram total protein. Preliminary studies confirm this hemoglobin induction phenomenon in one of the clones we studied [29]. However, we have been unable to demonstrate an increase in the amount of either spectrin or glycophorin in these cells with a series of inducing agents. This is consistent with the idea that globin synthesis is not tightly coupled to spectrin and glycophorin synthesis and that both are regulated separately during erythropoiesis. In support of this concept spectrin-inducible, globin non-inducible Friend leukemia cell variants [30] have been described. Furthermore, Eisen, Bach, and Emery [8] in their evaluation of a spectrin- and hemoglobin-inducible Friend cell variant found that the rate of spectrin synthesis was maximal 48 hours after induction began, while hemoglobin synthesis was maximal after 96 hours. In addition, while hemoglobin is expressed late during marrow erythropoiesis [31], it is clear from our observations that spectrin and glycophorin are expressed in the earliest morphologically recognizable erythroid cell, the pronormoblast, and possibly in a cell at an even earlier stage in development.

It is of interest to speculate why the erythroid precursor cell synthesizes spectrin and glycophorin long before hemoglobin, and this leads one to search for possible early functions for these proteins. The molecular topography of glycophorin A suggests that it may be well suited to serve as a cell surface receptor important for differentiation. This role could include a receptor function for growth hormones or to mediate cell interactions in the bone marrow. Given that rare individuals who lack glycophorin in their peripheral mature red blood cells [3] exhibit no abnormal symptomatology, it may be that glycophorin is important *only* during early erythropoiesis. Spectrin, on the other hand, which in the mature erythrocyte apparently stabilizes the membrane, could be involved in the organization of plasma membrane proteins at this early stage in red cell development and could maintain a particular composition of the plasma membrane during the extrusion of the nucleus of the late erythroblast. It has been observed by fluorescent labeling [5] that spectrin segregates with the incipient reticulocyte during enucleation while actin, on the other hand, does not.

Regardless of the role these plasma membrane proteins play in development, they do provide specific markers for immature cells of the red cell series. They can be used to define other less mature cell intermediates which are characterized at present only by functional criteria, or they can be used as diagnostic tools for human leukemias.

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REFERENCES

1. Marchesi VT: J Memb Biol 51:101, 1979.
2. Lux SE: Nature 281:426, 1979.
3. Furthmayr H: Nature 271:519, 1978.

4. Gahmberg CG, Jokinen M, Andersson LC: Blood 52:379, 1978.
5. Geiduschek JB, Singer SJ: Cell 16:149, 1979.
6. Koch PA, Gardner FH, Gartrell JE, Carter JR: Biochim Biophys Acta 389:177, 1975.
7. Light ND, Tanner MJA: Biochim Biophys Acta 508:571, 1978.
8. Eisen H, Bach R, Emery R: Proc Natl Acad Sci USA 74:3898, 1977.
9. Andersson LC, Nilsson K, Gahmberg CG: Int J Cancer 24:143, 1979.
10. Andersson LC, Jokinen M, Gahmberg CG: Nature 278:364, 1979.
11. Furthmayr H, Marchesi VT: Biochemistry 15:1137, 1976.
12. Marchesi VT, Andrews EP: Science 174:1247, 1971.
13. Sarris A, Palade GE: J Biol Chem 254:6724, 1979.
14. Laemmli UK: Nature 227:680, 1970.
15. Furthmayr H: J Supramol Struct 9:79, 1978.
16. Cotmore SF, Furthmayr H, Marchesi VT: J Mol Biol 113:539, 1977.
17. Ralston G, Dunbar J, White M: Biochim Biophys Acta 491:345, 1977.
18. Dickerman HW, Cheng JC, Kazazian HH, Spivak JL: Arch Biochim Biophys 177:1, 1976.
19. Böyum A: Scand J Clin Lab Invest 21:1(Suppl 97), 1968.
20. Fairbanks G, Steck TL, Wallach DFH: Biochemistry 10:2606, 1971.
21. Bonner WM, Laskey RA: Eur J Biochem 46:83, 1974.
22. Marks PA, Rifkind RA: Ann Rev Biochem 47:419, 1978.
23. Lozzio CB, Lozzio BB, Machado EA, Fuhr JE, Lair SV, Bamberger EG: Nature 281:709, 1979.
24. Ziparo E, Lemay A, Marchesi VT: J Cell Sci 34:91, 1978.
25. Kansu E, Krasnow SH, Ballas SK: Biochim Biophys Acta 526:18, 1980.
26. Bennett V, Branton D: J Biol Chem 252:275, 1977.
27. Sarris A: PhD Thesis, Yale University School of Medicine, 1980.
28. Furthmayr H: In Ginsburg V (ed): "Biology of Complex Carbohydrates," Vol I, New York: J. Wiley & Sons, in press.
29. Hoffman R, Murnane MH, Benz EJ, Prohaska R, Floyd V, Dainiak N, Forget BG, Furthmayr H: Blood 54:1182, 1979.
30. Harrison PR, Rutherford T, Conkie D, Affara N, Sommerville J, Hissey P, Paul J: Cell 14:61, 1978.
31. Chang H, Lanzer PJ, Lodish HF: Proc Natl Acad Sci USA 73:3206, 1976.
32. Wells AF, Miller CE, Nadel MK: Appl Microbiol 14:271, 1966.