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BIOSYNTHESIS OF MOUSE ERYTHROCYTE MEMBRANE PROTEINS BY FRIEND ERYTHROLEUKEMIA CELLS

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Summary

The synthesis of mouse erythrocyte membrane proteins by Friend erythroleukemia cells during dimethyl sulfoxide-induced differentiation was studied. Untreated and dimethyl sulfoxide-treated cells were incubated with L-[^{3}H]leucine and the incorporation of radioactivity into total trichloroacetic acid-insoluble proteins and into proteins immunoprecipitated with a multivalent rabbit antibody to mouse erythrocyte membranes was determined. The immunoprecipitated membrane proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and radioactivity was detected by fluorography. The incorporation of L-[^{3}H]leucine into total cell proteins was linear for 20 min in both untreated and treated cells. Exposure of the cells to dimethyl sulfoxide had an inhibitory effect on protein synthesis, with a significant decrease noted on the fourth day of treatment and a continued decline occurring until the seventh day when protein synthesis was 42% that of untreated cells. The synthesis of erythrocyte membrane proteins was 0.49% that of total cell proteins in untreated cells, was increased to 1.27% by the third day of treatment and remained at about 1% of total protein synthesis from the fourth to the seventh day. Untreated cells synthesized low levels of spectrin, bands 5 and 6 proteins. Treatment with dimethyl sulfoxide caused a staggered increase in synthesis of a number of erythrocyte membrane proteins. Spectrin synthesis increased 4-fold by the third day of treatment and declined thereafter. The synthesis of membrane proteins with electrophoretic mobilities similar to bands 3 and 4 was increased 2—3-fold by the fourth day, while bands 6 and 5 proteins attained maximal synthesis (4-fold) on the fifth and sixth days of treatment.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

Introduction

The Friend erythroleukemia cell offers several advantages for the study of membrane biogenesis. The untreated cell shows few erythroid characteristics but upon treatment with chemical inducers such as dimethyl sulfoxide, it undergoes partial differentiation in the erythroid pathway from proerythroblast to normoblast [1—4]. During this process the cell acquires hemoglobin, some erythrocyte membrane proteins [5,6] and surface antigens [7,8] and undergoes cell surface charge alterations [9]. Studies of spectrin synthesis during dimethyl sulfoxide-induced differentiation showed that this protein is present in uninduced cells and that treatment results in a 10—20-fold increase in spectrin content. The intracellular concentration of spectrin reaches a peak on the third day of treatment, after which it falls to levels found in mouse erythrocytes [5]. These studies imply that Friend cells synthesize some erythrocyte membrane proteins during dimethyl sulfoxide-induced differentiation.

Much is known about the chemical composition and structure of the erythrocyte membrane, but at present there are few useful experimental models with which to study membrane biogenesis, since the mature red blood cell has no protein-synthesizing ability. Weise and Chan [10] have investigated membrane protein synthesis in embryonic chick erythroid cells. They found that immature chick red cells at the early, middle and late polychromatophilic erythroblast stages synthesized all the major classes of membrane proteins, although the rate of labeled leucine incorporation into membrane as well as total polypeptides was found to decrease as the cells matured. Pulse-chase experiments using excess cold leucine or cycloheximide revealed two classes of membrane proteins: those of which the synthesis is followed immediately by association with the plasma membrane and those apparently requiring post-translational processing before appearing in the membrane.

In a different experimental system, studies of erythrocyte membrane protein synthesis in phenylhydrazine-treated anemic rabbits suggested that red cell proteins are synthesized asynchronously during the life cycle of the cell. The larger polypeptides are presumably made predominantly in the earlier marrow stages of development, while certain of the smaller polypeptides are still being synthesized at the reticulocyte stage [11].

Studies of virus coat proteins have provided most of the detailed information on how integral membrane proteins are synthesized and are inserted into the membrane [12—15]. Recently, however, studies by Jokinen et al. [16] using a human continuous leukemia cell line indicate that the erythrocyte membrane protein, glycophorin, may be synthesized in a fashion similar to the glycoproteins of vesicular stomatitis virus. In both of these instances, the proteins are thought to be inserted directly into the endoplasmic reticulum membrane during synthesis on membrane-attached polysomes and the nascent proteins are thought to span the membrane with the N-terminal portion of the protein on the luminal face of the endoplasmic reticulum and the carboxyterminal portion on the cytoplasmic surface. Proximal glycosylation of the nascent protein occurs at this early stage, and further processing of the protein occurs at a later stage during its passage to the plasma membrane.

Recent studies with spleen cells from anemic mice by Sabban et al. [17]

indicate that band 3 protein is quickly incorporated into membranes and that it is synthesized by membrane-attached polysomes. This suggests that band 3 protein follows an intracellular route similar to that taken by integral viral membrane proteins. Studies of the biosynthesis of other erythrocyte membrane proteins have not been performed.

Because Friend erythroleukemia cells may be a useful model to study the biosynthesis of erythrocyte membrane proteins, we have examined protein synthesis in untreated and dimethyl sulfoxide-treated cells and have determined, using a multivalent antibody to mouse erythrocyte membrane proteins, which erythrocyte membrane proteins undergo increased biosynthesis during differentiation and at what times after treament this occurs.

Materials and Methods

Culture conditions. Friend erythroleukemia cells (Line 745) were obtained from Dr. Charlotte Friend (through Dr. O. Pogo). Cells were grown in suspension in Dulbecco's modified Eagle's medium containing 10 mM Hepes and supplemented with 10% dialyzed heat-inactivated fetal calf serum. Under these conditions, the doubling time was 12 h until a density of $1—2 \cdot 10^6$ cells/ml was reached. To induce differentiation, cells were seeded at a density of $5 \cdot 10^4$ cells/ml and grown in the presence of 1.8% (v/v) dimethyl sulfoxide for up to 7 days. Treatment with dimethyl sulfoxide for 7 days resulted in 80—90% differentiation. Cell differentiation was measured using the benzidine stain method [18] for hemoglobin.

Antibody preparation. Membranes for antibody production were prepared from erythrocytes of female DBA/2J mice by using the method of Adachi and Furusawa [19]. Antibodies were produced in rabbits by injecting, subcutaneously, 2 mg of membrane protein in complete Freund's adjuvant following the schedule of MacDonald et al. [20]. Sera were heat inactivated at 56°C for 30 min and stored at —20°C.

Incubation of Friend erythroleukemia cells with L-[^{3}H]leucine. Cell proteins were labeled by a modification of the method described by Weise and Chan [10]. Cells were washed with F-12K media (amino-acid free) supplemented with 10% fetal calf serum, diluted to a density of $2.5 \cdot 10^7$ cell/ml in prewarmed leucine-free F-12K medium and the incubation was started by the addition of L-[^{3}H]leucine (10 μCi/ml). Cells were incubated at 37°C with gentle shaking and the incorporation of radioactivity into protein was measured at various times up to 2 h.

Immunoprecipitation of membrane proteins. Friend cells were incubated with L-[^{3}H]leucine for 20 min, collected by centrifugation and washed twice with cold isotonic Tris-HCl buffer, pH 7.4. The washed cells were lysed and homogenized in 10 mM Tris-HCl buffer, pH 7.5, with 10 mM KCl, 1.5 mM $MgCl_2$. The homogenate was treated with 1% sodium deoxycholate, centrifuged for 1 h at 166 500 $\times g$ in a 50 Ti Beckman rotor. Phosphate-buffered saline (0.75 ml) was added to 1 ml of the detergent-soluble fraction and the radioactive membrane proteins were immunoprecipitated by the addition of 0.25 ml of rabbit antibody to mouse erythrocyte membrane proteins. Preimmune rabbit serum was added to the control samples. Incubations were performed for 1 h at 37°C

and overnight at 4°C. Immunoprecipitation was aided by the addition of 0.1 ml of 10% formaldehyde-fixed *Staphylococcus aureus* for 30 min at room temperature [21]. The Staphylococcus-immune complex was collected by centrifugation, washed with 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.05% Triton X-100, resuspended in lysing buffer [22] containing 8 M urea and heated at 100°C for 2 min. An aliquot of the supernatant fraction, which contains the radioactive erythrocyte membrane proteins which were part of the immune complex, was counted for total erythrocyte membrane protein radioactivity and the remainder was then subjected to SDS-polyacrylamide gel electrophoresis and fluorography to determine the incorporation of radioactivity into individual erythrocyte membrane proteins.

SDS-polyacrylamide gel electrophoresis and fluorography. Membrane proteins were analyzed by electrophoresis on SDS-polyacrylamide slab gels using a modification of the method described by Fairbanks et al. [22]. The gels contained 0.1% SDS and 7.5% acrylamide with 4% acrylamide in the stacking portion of the gel. The separated proteins were detected by staining the gels with Coomassie blue, followed by fluorography [23]. The radioactivity in the separated proteins was determined by cutting the gels, digesting them with $HClO_4$ and H_2O_2 at 60°C for 3 h and counting in a liquid scintillation counter.

Materials. Dulbecco's modified Eagle's medium, amino acid-free F-12K medium, and fetal calf serum (dialyzed) were purchased from Grand Island Biological Company, Grand Island, NY; L-[4,5-^{3}H]leucine from Amersham Corp., Arlington Heights, IL; dimethyl sulfoxide from Fisher Scientific Co., Fair Lawn, NJ; heat-inactivated formaldehyde-treated *Staphylococcus aureus* (Pansorbin®) from Calbiochem-Behring Corp., La Jolla, CA.

Results

Protein synthesis during dimethyl sulfoxide treatment

Preliminary to the study of the biosynthesis of erythrocyte membrane proteins, the synthesis of total proteins in untreated and dimethyl sulfoxide-treated Friend cells was examined. Cells were incubated with L-[^{3}H]leucine (as described in Materials and Methods) for varying periods of time up to 2 h and the incorporation of radioactivity into trichloroacetic acid-precipitable proteins was measured. The results are shown in Fig. 1. In both the untreated and treated Friend erythroleukemia cells the incorporation of L-[^{3}H]leucine into protein was linear for 20 min. Treatment of cells with dimethyl sulfoxide for up to 3 days had little effect on the incorporation rates, but after 4 days in dimethyl sulfoxide, cells showed significantly lower rates of protein synthesis. At 4 days of treatment about 30% of the cells contain hemoglobin, as determined by benzidine staining. The inhibitory effect of dimethyl sulfoxide on protein synthesis was more pronounced on the following days of treatment when the number of benzidine-positive cells increased to about 80%. On the seventh day of treatment protein synthesis was 60% less than that of untreated cells.

Immunoprecipitation

Friend erythroleukemia cells were incubated with L-[^{3}H]leucine for 20 min and the washed cells were homogenized and prepared for immunoprecipitation

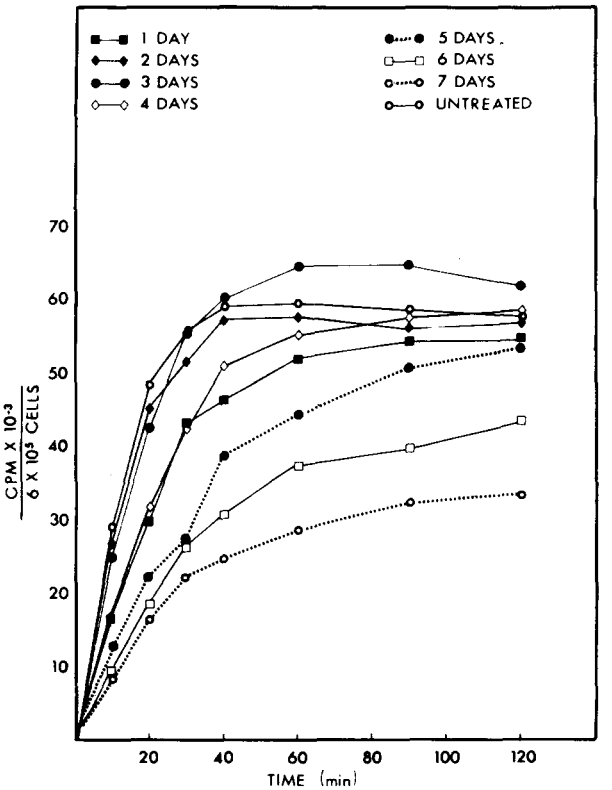

Fig. 1. Effect of dimethyl sulfoxide on total protein synthesis by Friend erythroleukemia cells. Protein synthesis in untreated Friend cells and cells grown in the presence of dimethyl sulfoxide for up to 7 days was measured. Cells were incubated with L-[^{3}H]leucine at 37°C for 2 h. Duplicate aliquots were taken at the indicated times and the incorporation of radioactivity into trichloroacetic acid-precipitable proteins was determined.

as described in Materials and Methods. The proteins precipitated by preimmune and immune sera were compared by SDS-polyacrylamide gel electrophoresis followed by fluorography. Both the immune and the preimmune sera caused the precipitation of several proteins by formaldehyde-treated *S. aureus*. The Coomassie blue-stained patterns of these proteins are shown in Fig. 2, lanes A and B. Fluorography revealed, however, that only the immune serum was capable of precipitating nascent radioactive proteins from Friend erythroleukemia cells (Fig. 2, lane C). The proteins precipitated by the preimmune serum were not radioactive (Fig. 2, lane D) and presumably represent non-specific adsorbtion of proteins not synthesized by Friend erythroleukemia cells.

Incorporation of L-[^{3}H]leucine into erythrocyte membrane proteins

Using the multivalent antibody described above, we have studied the synthesis of erythrocyte-like membrane proteins by Friend erythroleukemia cells in order to determine which proteins are produced during dimethyl sulfoxide-induced differentiation. Friend cells, at various times after dimethyl sulfoxide treatment, were incubated with L-[^{3}H]leucine for 20 min and the radioactivity in total trichloroacetic acid-precipitable proteins and in erythrocyte membrane

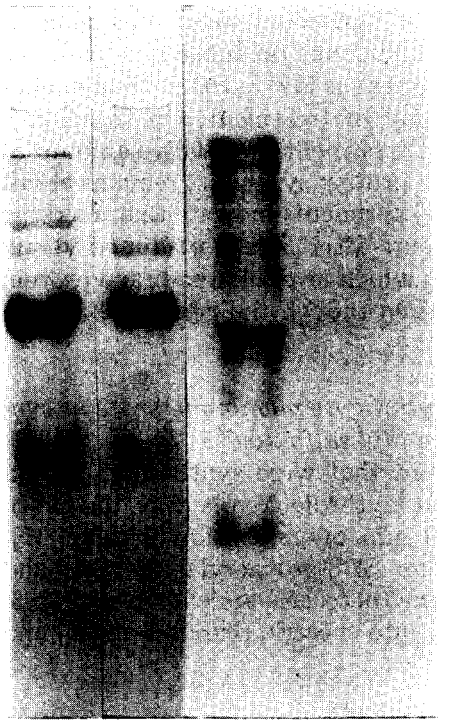

Fig. 2. Immunoprecipitation of Friend erythroleukemia cell proteins by pre-immune and immune sera. Rabbit multivalent antibodies against mouse erythrocyte membrane proteins and preimmune sera from the same rabbits were used to precipitate L-[^{3}H]leucine-labeled Friend erythroleukemia cell proteins with the aid of formaldehyde-fixed *Staphylococcus aureus* as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane A shows the Coomassie blue-stained patterns of proteins precipitated by the immune serum and lane B that by the preimmune serum. Lane C shows the fluorogram of radioactive proteins precipitated by immune serum and lane D shows that by preimmune serum.

TABLE I

INCORPORATION OF L-[^{3}H]LEUCINE INTO TOTAL AND ERYTHROCYTE MEMBRANE PROTEINS

Untreated and dimethyl sulfoxide-treated cells were incubated with L-[^{3}H]leucine for 20 min and membrane proteins were immunoprecipitated. Radioactivity in total trichloroacetic acid-precipitable proteins and in aliquots of immunoprecipitates was determined.

Days in dimethyl sulfoxide	Protein radioactivity (cpm/4.5 · 10^7 cells)		%
	Total protein	Erythrocyte membrane	
0	6 836 600	33 350	0.49
1	5 659 600	32 950	0.58
2	6 535 800	43 500	0.67
3	5 338 800	68 000	1.27
4	4 274 500	46 900	1.10
5	2 992 000	29 600	0.99
6	3 072 800	35 280	1.15
7	2 845 400	26 600	0.93

proteins was determined. Protein biosynthesis is high in untreated cells, remains relatively unaltered for the first three days and drops on subsequent days of treatment. In contrast, membrane protein synthesis is low in the untreated cells and reaches maximal activity on the third day of treatment. On the fourth through the seventh day of dimethyl sulfoxide treatment the relative amount of membrane protein synthesis (percent of total protein synthesis) remains about twice that in untreated cells (Table I). These experiments indicate that maximal erythrocyte membrane protein synthesis occurs after 3 days of dimethyl sulfoxide treatment and that Friend cells sustain a high level of membrane protein synthesis on subsequent days of treatment even though the general ability to synthesize proteins has declined.

SDS-polyacrylamide gel electrophoresis of erythrocyte membrane proteins synthesized following treatment of cells with dimethyl sulfoxide

In order to identify the membrane proteins that were synthesized by untreated and dimethyl sulfoxide-treated Friend erythroleukemia cells, the radioactive immunoprecipitates obtained after 20 min of incubation with L-[^{3}H]-leucine were subjected to electrophoresis on SDS-polyacrylamide gels and fluorography. The fluorograms obtained from these gels are shown in Fig. 3. The untreated cells incorporated radioactivity into spectrin (bands 1 and 2) and

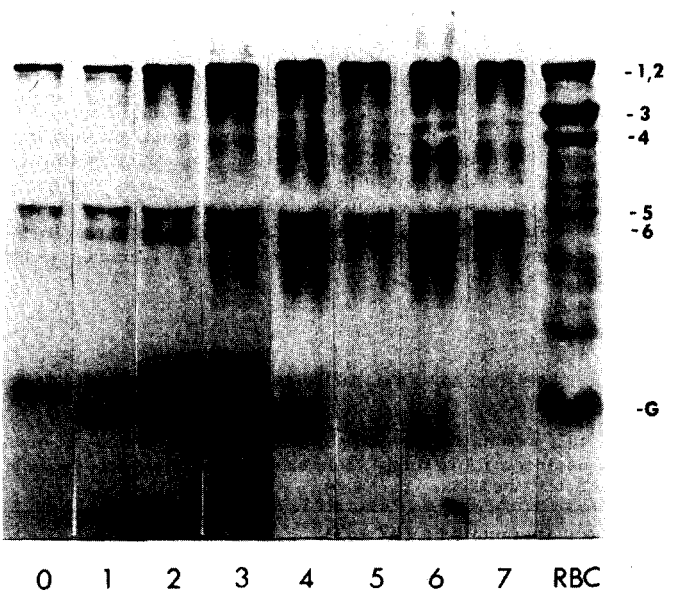

Fig. 3. Fluorograms of membrane proteins in untreated and dimethyl sulfoxide-treated Friend erythroleukemia cells. Cells were incubated with L-[^{3}H]leucine for 20 min and membrane proteins were isolated from detergent-treated homogenates by immunoprecipitation with antibodies directed against mouse erythrocyte membrane proteins. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and radioactivity was determined by fluorography [23]. Polyacrylamide gel electrophoresis was performed on different days under identical conditions. The figure shown is a composite of several gels. For orientation, the right-hand lane shows mouse red blood cell (RBC) membrane proteins. The nomenclature of Fairbanks et al. [22] is used in describing the relative electrophoretic mobilities of the proteins. The numbers at the bottom (0—7) indicate length of exposure of cells to dimethyl sulfoxide (days).

into proteins with electrophoretic mobilities similar to bands 5, 6 and globin. On the first day of treatment more radioactivity was incorporated into these proteins but the pattern of radioactive proteins remained similar to that of the untreated cell. On the second day of treatment there was again a marked increase in the amount of radioactivity in spectrin and in proteins with mobilities similar to bands 5, 6 and globin. On the third day of treatment the biosynthesis of band 4 protein became apparent. On days 5, 6 and 7 of dimethyl sulfoxide treatment the cells produced the same proteins as on the fourth day of treatment, but there was a marked decline in the biosynthesis of spectrin and globin.

Biosynthesis of specific membrane proteins

Untreated cells and cells treated with dimethyl sulfoxide were pulse-labeled for 20 min with L-[^{3}H]leucine. This is a time at which protein synthesis is linear in both untreated and treated cells (Fig. 1). The radioactive membrane proteins synthesized during this period were isolated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The radioactivity in spectrin, globin, and proteins with electrophoretic mobilities similar to bands 3, 4, 5 and 6 was determined. In these experiments the radioactivity in spectrin comprises that in both bands 1 and 2, and the radioactive area with mobility near that to bands 4.1 and 4.2 is termed band 4. Radioactive bands 5 and 6 (shown in

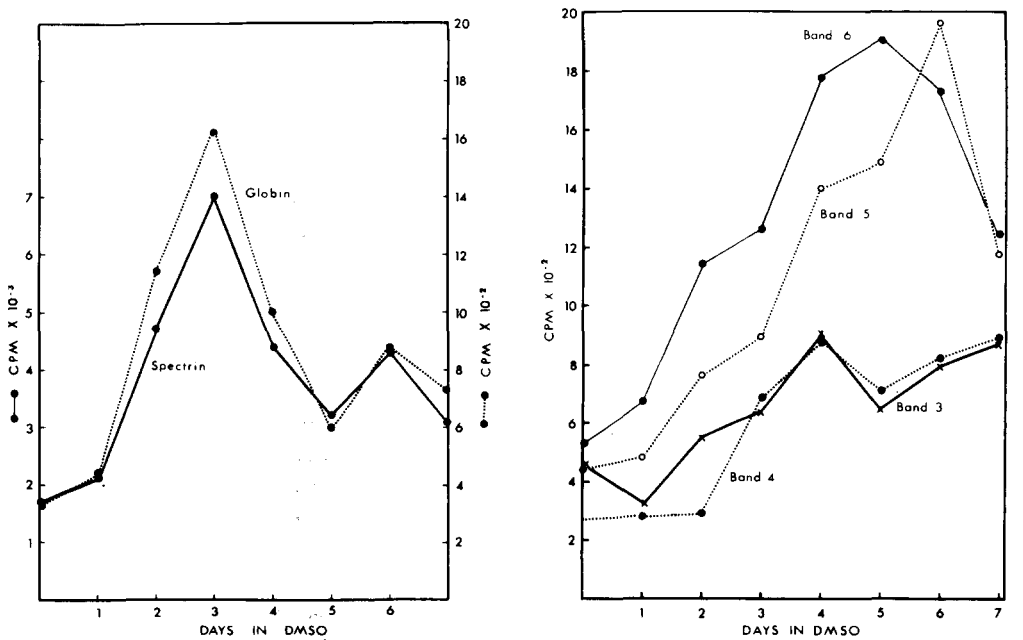

Fig. 4. Spectrin and globin synthesis during dimethyl sulfoxide-induced differentiation. The bands corresponding to spectrin and globin in the gel shown in Fig. 3 were excised and their radioactivity determined. DMSO, dimethyl sulfoxide.

Fig. 5. Synthesis of membrane proteins during dimethyl sulfoxide-induced differentiation. Areas in the gel shown in Fig. 3 corresponding to bands 3, 4, 5 and 6 were excised and the radioactivity present in each band was determined.

Fig. 3) have mobilities similar, but not identical, to the corresponding mouse erythrocyte membrane proteins.

The synthesis of both spectrin and globin increased dramatically on the second day of treatment with dimethyl sulfoxide, reaching a peak on the third day and declining rapidly on following days, but remaining twice as high on the seventh day of treatment when compared with untreated cells (Fig. 4).

The biosynthesis of other membrane proteins (bands 3, 4, 5 and 6) showed different responses to dimethyl sulfoxide treatment. The radioactive proteins with electrophoretic mobilities similar to bands 3 and 4 reached maximal synthetic rates on the fourth day of treatment, while bands 5 and 6 attained maximal biosynthesis on the fifth day of treatment (Fig. 5). The rate of synthesis of bands 3 and 4 on the fourth day of treatment was 2—3-times greater than that in undifferentiated cells, while the synthesis of bands 5 and 6 was stimulated 4-fold on the sixth and fifth day of treatment. The biosynthetic rate of proteins in bands 3 and 4, which increased from the second to the fourth day of treatment, did not decline on subsequent days of treatment, while the rates of synthesis of bands 5 and 6 proteins declined on the seventh day of dimethyl sulfoxide treatment (Fig. 5).

Discussion

Much has been written about the process of plasma membrane biogenesis but at present there is little detailed knowledge of how it is accomplished. This is due mostly to the lack of a suitable experimental system. Most investigators interested in membrane biogenesis have chosen to study specialized membranes, which are produced at a rapid rate under certain conditions. However, this advantage of rapid intensive biogenesis is not available in the membrane about which we have the most information on the structure and composition, namely the erythrocyte membrane. The mature red blood cell does not have a protein-synthesizing system and thus new membrane proteins cannot be made while the cells are in the peripheral circulation.

Our studies show that the Friend erythroleukemia cell system offers several advantages for the study of membrane biogenesis. The uninduced cells and the cells treated with dimethyl sulfoxide, even after 7 days of treatment, a time at which 80—90% of the cells contain hemoglobin, are capable of protein synthesis (Fig. 1). The cells are able to synthesize several mouse erythrocyte membrane proteins and some of these membrane proteins (spectrin, and proteins with similar electrophoretic mobilties to bands 3, 4, 5 and 6) undergo marked increases in biosynthesis on treatment with dimethyl sulfoxide. For example, the rate of spectrin biosynthesis on the third day of treatment is 4-times that of the untreated cell, production of bands 5 and 6 is increased 4-times on the sixth and fifth day of treatment and synthesis of bands 3 and 4 is increased 2—3-fold by the fourth day of treatment. The increased rates of biosynthesis make these proteins useful candidates for further study to determine their intracellular routes and how they are processed and inserted into the plasma membrane. It should be noted, however, that at present these radioactive proteins have not been well characterized and identification is based on the fact that they react immunologically with antibodies to mouse erythrocyte mem-

brane proteins and that they have similar electrophoretic mobilities on SDS-polyacrylamide gels. Nascent radioactive membrane proteins may occur in precursor forms and thus, because they have not been fully processed, may behave differently on SDS-polyacrylamide gel electrophoresis from the mature plasma membrane proteins.

These studies also indicate that even the undifferentiated Friend cells are capable of producing some mouse erythrocyte membrane proteins. The untreated cells synthesize spectrin and proteins with similar mobilities to bands 5 and 6 proteins. On treatment with dimethyl sulfoxide there is a staggered increased production of several erythrocyte membrane proteins. The synthesis of spectrin and bands 5 and 6 proteins, which are being produced by the untreated cell, increases first after dimethyl sulfoxide treatment. In contrast, the synthesis of band 4 protein is not noticed for the first 2 days of treatment and its synthesis is seen only on the third and subsequent days of treatment. This staggered synthesis of membrane proteins, and the decline in biosynthetic rates of some membrane proteins during dimethyl sulfoxide treatment may reflect the order in which erythrocyte membrane proteins are assembled into the plasma membrane of Friend erythroleukemia cells as these cells become erythroid-like.

Acknowledgments

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