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# Different Sequences of Expression of Band 3, Spectrin, and Ankyrin During Normal Erythropoiesis and Erythroleukemia

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***Expression of the erythrocyte anion exchanger band 3, and ankyrin and spectrin, two cytoskeletal proteins of the red blood cell membrane, was studied by immunofluorescence using 1) smears of human bone marrow from healthy donors and from a patient with erythroleukemia, 2) human red blood cell precursors grown in cell culture, and 3) murine erythroleukemia cells grown in cell culture. Double immunostaining with antibodies to band 3 in combination with spectrin or ankyrin revealed that these proteins become expressed synchronously during normal human erythropoiesis. In contrast, both murine erythroleukemia cells (induced by fibronectin and dimethyl sulfoxide to differentiate in vitro) and erythroblasts from a patient suffering from erythroleukemia displayed distinct asynchronicity in expression of these proteins, ie, ankyrin and spectrin were synthesized first, followed by band 3 at a later stage of erythroid development. After the onset of band 3 expression in human erythroleukemia cells, an increase of membrane-associated fluorescence was detectable for both ankyrin and spectrin, supporting the general view that band 3 promotes assembly of the membrane cytoskeleton. These findings indicate that the current concept of a sequential expression of spectrin/ankyrin and band 3 is valid only for erythroleukemia cells or transformed erythropoietic cell lines but does not occur in normal erythropoiesis, during which these proteins become expressed simultaneously. (Am J Pathol 1993, 142:1565–1573)***

The erythrocyte membrane skeleton is composed mainly of spectrin filaments, which are linked by ankyrin molecules to the anion exchanger (band 3) of the plasma membrane.[1,2] It is generally accepted that this spectrin scaffold helps red blood cells to resist mechanical stress in the circulation. Studies on murine erythroleukemia (MEL) cell lines indicated that during erythropoiesis band 3 and components of the membrane skeleton do not become synthesized and assembled synchronously.[3–12] Expression of spectrin and ankyrin has been demonstrated to start first in development, probably between the colony-forming unit and proerythroblastic stage of differentiation. On the other hand, band 3 was suggested to become expressed much later, at about the normoblast stage of development. Based on these observations it was assumed that band 3 binds to a preassembled cytoskeletal network.[7,13]

Linkage of the cytoskeleton to band 3 is widely believed to stabilize the cytoskeleton and to protect it from disassembly and degradation.[7,8] However, as outlined above, these observations of an asynchronous expression of erythrocyte membrane components were obtained by experiments on transformed erythroid cell lines, such as, for example, chicken red blood cell precursors infected with the avian erythroblastosis virus[12] or mouse erythroblasts infected with the anemia-inducing strain of Friend virus[3–6,8–11] (MEL cells). These transfected cells become arrested in development at a point well before terminal differentiation. By chemical induction with dimethyl sulfoxide (DMSO) or, more physiologically, with erythropoietin these cells can be induced to synchronously pass through terminal differentiation. This includes expression of hemoglobin and results finally in the enucleation of normoblasts. It appeared as a major advantage of these cell lines that homogeneous populations of both nondifferentiated cells and cells progressing in terminal differentiation were available.

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Thus, for the first time, biochemical investigations were possible on homogeneous populations of precursor cells at different stages of maturation. The major outcome of these studies was that uninduced erythroid cells did not contain detectable amounts of band 3 but were distinctly positive for spectrin and ankyrin. Following induction, band 3 became expressed, and, concomitantly, the total amounts of cytoskeletal proteins increased significantly.

However, these investigations have suffered from several important limitations. First of all, transformed erythroid cell lines cannot be considered to be in all points representative for normal erythropoiesis. This was indicated by growth factor-independent proliferation, which is a common feature of these cell models.[4,12] Second, some of these data have been obtained by studies on transformed chicken precursor cells, which in many aspects differ profoundly from mammalian erythroblasts.[7] Third, the whole concept of sequential expression of spectrin, ankyrin, and band 3 was based solely on biochemical analysis of either induced or noninduced cell fractions. To our knowledge, no report has been published so far that demonstrates an asynchronous expression of cytoskeletal proteins at the cellular level, eg, by immunolabeling techniques.

To address this problem we have undertaken a double immunostaining approach and found that in normal human erythropoiesis *in situ* and *in vitro* band 3 is expressed synchronously with ankyrin and spectrin. However, in MEL cells and in a case of human erythroleukemia the situation was similar to that described by biochemical methods for avian and murine erythroleukemia cells, ie, asynchronous expression of band 3 and spectrin/ankyrin, indicating that it is difficult to extrapolate from studies with erythroleukemia cells on normal erythropoiesis.

## Materials and Methods

### Antibodies and Materials

Mouse and rabbit antibodies directed to human erythroid ankyrin, spectrin, and the cytoplasmic domain of band 3 were prepared and characterized as described previously.[14,16] By immunoblotting, the antibodies used in this study were strongly reacting with their respective antigens without showing cross-reactivity to other proteins (Figure 1). Tetramethylrhodamine isothiocyanate (TRITC)-labeled goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG were from Bayer Diagnostic (Munich, Germany). Human recombinant erythropoietin and interleukin-3 were generous gifts from the Behringwerke (Marburg, Germany). Iscove's medium, Dulbecco's modified Eagle's medium, and fetal calf serum (FCS) were from Gibco (Deisenhofen, Germany). Alpha-thioglycerol, hydrocortisone-hemisuccinate, DMSO, bovine plasma fibronectin, diisopropylfluorophosphate, and leupeptin were obtained from Sigma Chemical Co. (Deisenhofen, Germany).

### Human Bone Marrow Cells

Air-dried smears of human bone marrow of healthy individuals and of bone marrow from a patient with malignant erythroleukemia were kindly provided by the hematology units of the university hospitals of Marburg and Würzburg, Germany.

### Human Bone Marrow Cell Culture

Human erythroblasts were grown in cell culture as described previously.[16] Briefly, pieces of human bone marrow, obtained from patients undergoing hip surgery, were placed in phosphate-buffered saline (10 mmol/L sodium phosphate; 140 mmol/L NaCl, pH 7.4; PBS) anticoagulated with heparin (10 USP U/ml). The spongiosa pieces were dissected mechanically, and larger tissue pieces were removed by filtration through a 100-µm nylon mesh. The filtrate was loaded on a Ficoll Cushion (density

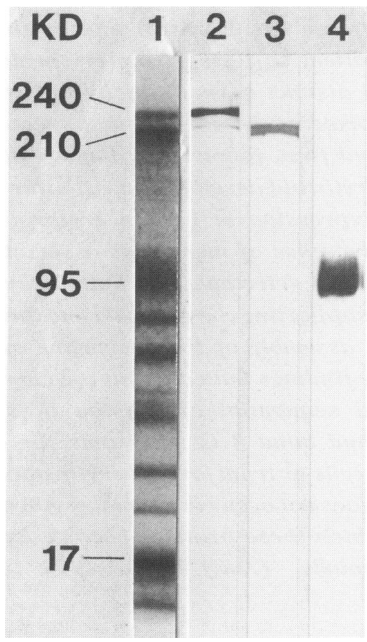



**Figure 1.** *Immunoblot analysis of human bone marrow membranes documenting the specificity of antibodies used in this study. Lane 2, anti-α-spectrin; lane 3, anti-ankyrin; lane 4, anti-band 3. A corresponding Coomassie blue-stained lane is also shown (lane 1).*

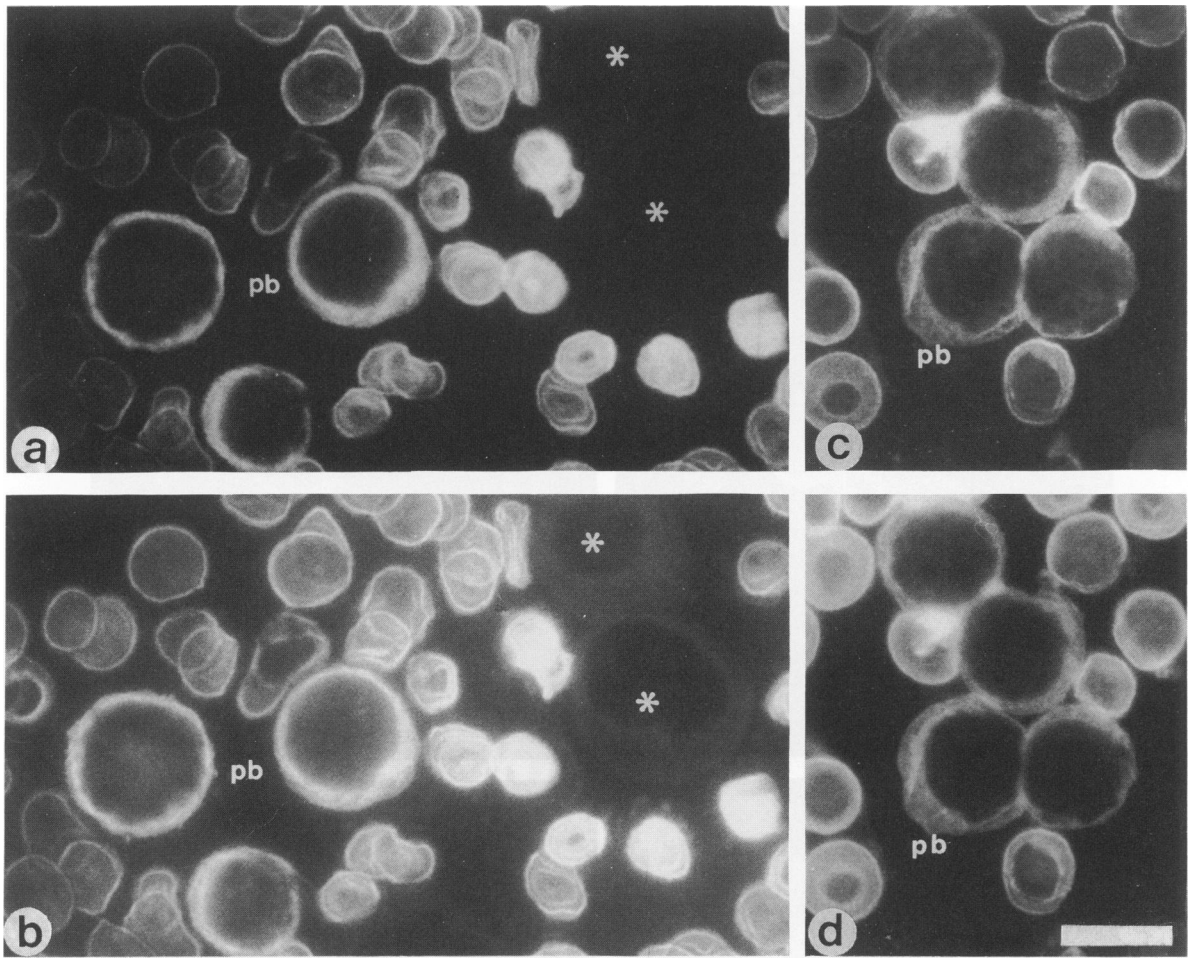



Figure 2. *Double immunostaining of human bone marrow erythroblasts with antibodies to erythrocyte spectrin* (a), *band 3* (b *and* d), *and ankyrin* (c). *Red blood cell precursors of different stages of maturation are simultaneously labeled by antibodies to band 3, ankyrin, and spectrin, indicating coexpression of these proteins. Proerythroblasts (pb) are already positive for band 3. The antibodies are nonreactive with nonerythroid cells (eg, myelocytes) (*), which were visualized by phase contrast optics (not shown). Bar, 10 μm.*

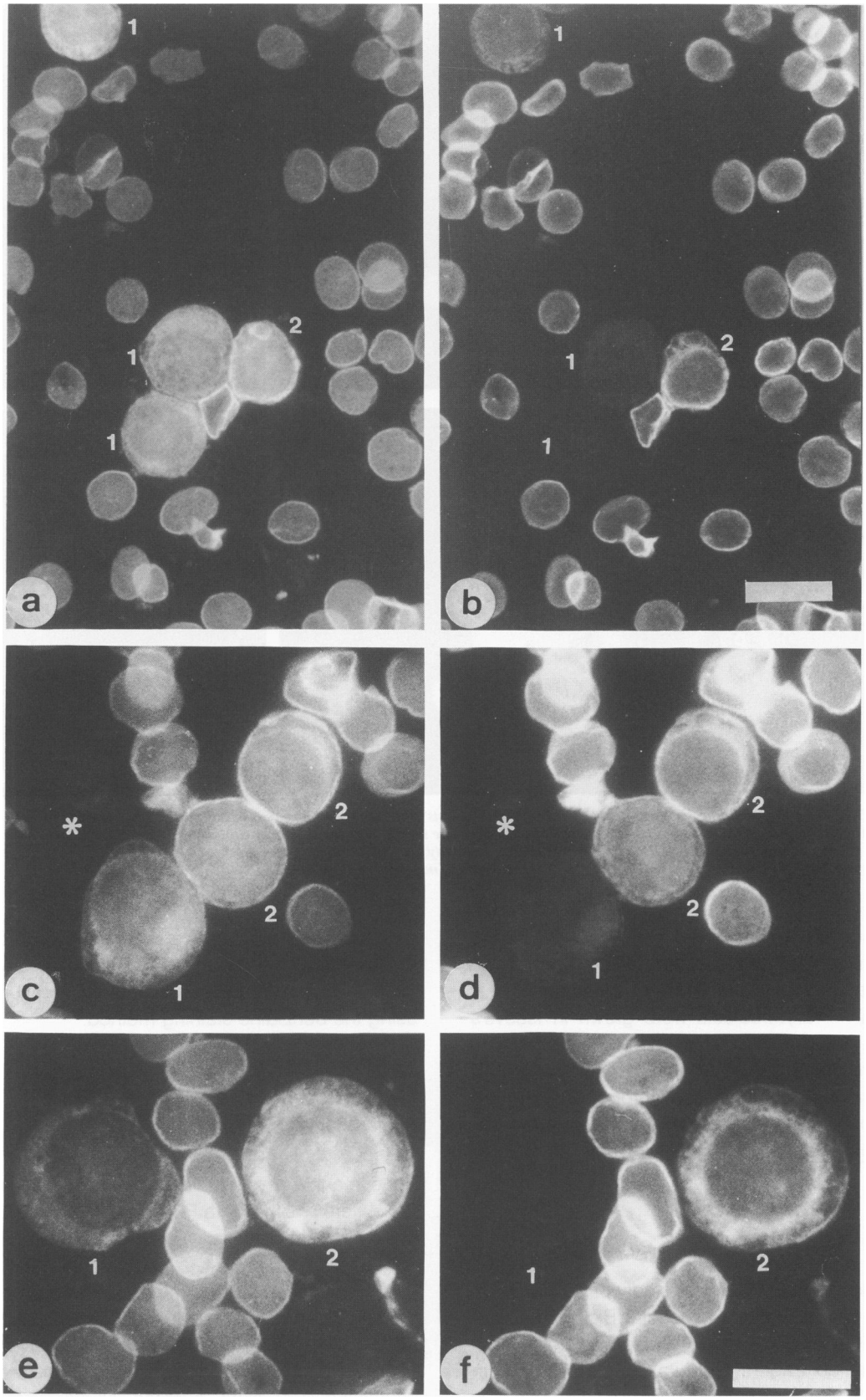
1.09) and centrifuged for 20 min at 800 × *g*. The cells on top of the cushion were seeded on fibronectin-coated glass slides at a density of 5 × $10^6$ cells/ml. Erythroblasts were grown in Iscove's medium with 25% FCS supplemented with 5 × $10^{-6}$ mol/L-hydrocortisone, 0.1 mmol/L α-thioglycerol, 3 U/ml human recombinant erythropoietin, and 50 ng/ml human recombinant interleukin-3 in a humidified atmosphere with 12.5% $O_2$ and 5% $CO_2$ at 37°C.

## *Murine Erythroleukemia Cell Culture*

MEL cells, clone FN 46, were kindly provided by Dr. Thomas Graf, European Molecular Biology Laboratory, Heidelberg, Germany, and grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. To induce MEL cells to differentiate *in vitro*, cells were plated on fibronectin-coated dishes as described[9] and stimulated in 1.8% (v/v) DMSO in Dulbecco's modified Eagle's medium (10% FCS) for 2–6 days. Cell differentiation was measured by using the benzidine staining method.

## *Immunoblotting of Bone Marrow Membranes*

Bone marrow was obtained by sternal aspiration. Cells were washed with PBS (3 × 10 min, 300 × *g*, 4°C) and then lysed for 10 min in 5 mmol/L sodium phosphate (pH 7.4, 4°C) containing 2% β-mercaptoethanol, 0.5 mmol/L diisopropylfluorophosphate, and 10 μg/ml leupeptin. Lysed cells were sedimented at 20,000 × *g* (for 30 min), suspended, and homogenized in the same solution containing 0.32 mol/L sucrose. Nuclei were spun down at 1000 × *g* (20 min), and the membranes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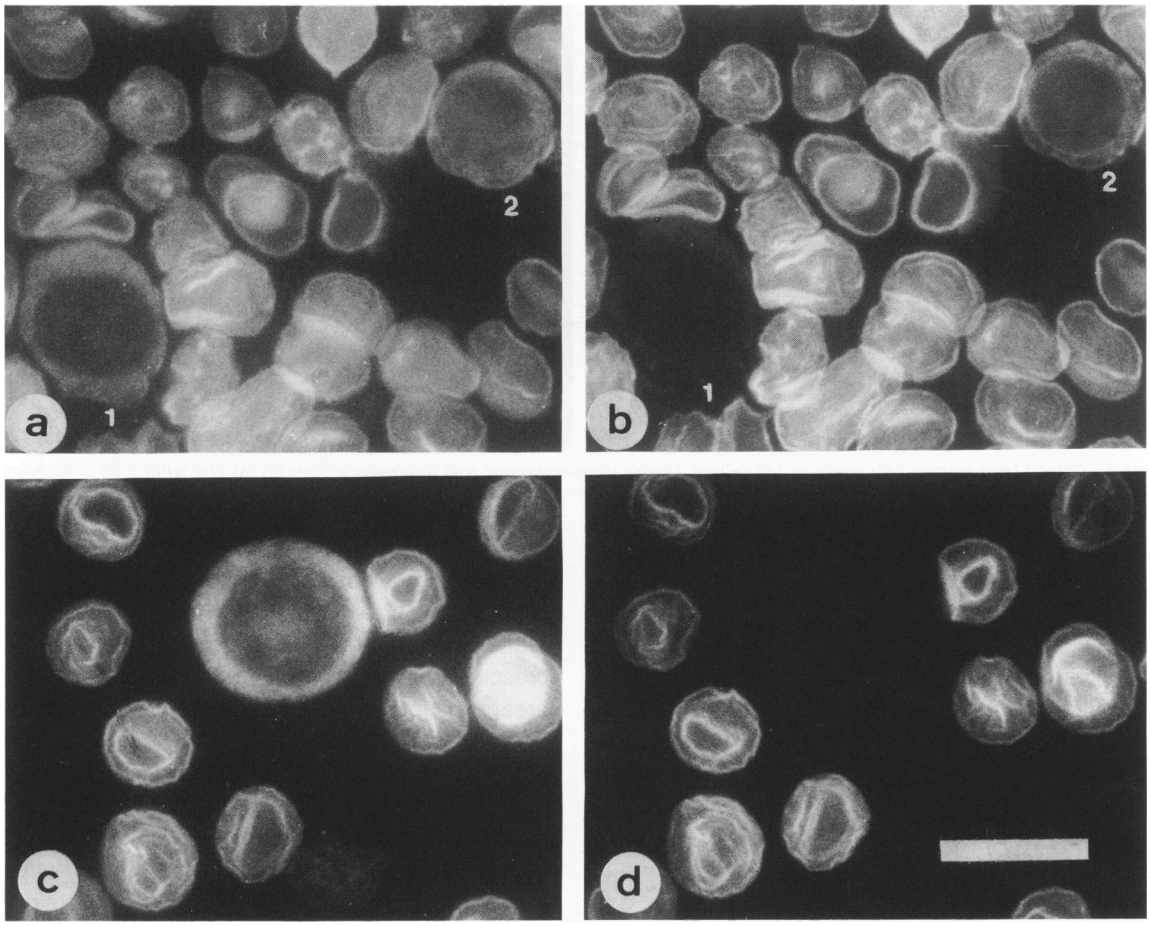


Figure 4. *Double immunostaining of human erythroleukemia cells with anti-erythroid spectrin* (a, c) *and anti-band 3* (b, d). a *and* b, *two erythroid precursors. One cell (1) is positive for spectrin but not for band 3, whereas the other, smaller cell (2) is labeled for both proteins.* c *and* d, *a proerythroblast-like precursor cell that reacts only with anti-spectrin but not with anti-band 3. Bar, 10 μm.*

and cell ghosts of the supernatant were then pelleted at 40,000 × *g* (30 min). The membrane pellet was directly dissolved in sample buffer and subjected to sodium dodecyl sulfate polyacrylamide gel (10–15%) electrophoresis. The separated proteins were electroblotted on nitrocellulose filters and incubated with antibodies (1:500) and $^{125}$I-Staphylococcal protein A as described elsewhere.[14]

## *Immunostaining*

Air-dried bone marrow smears or coverslips with cells attached to fibronectin were fixed in 2% formaldehyde in PBS (pH 7.4) and permeabilized thereafter in 1% Triton X-100 (Sigma) in PBS for 2 min. Alternatively, cells were fixed in 100% methanol for 3 min at −20°C. These fixation procedures did not result in different patterns of immunostaining. Primary antibodies were diluted 1:30 in PBS, and the incubation of cell smears was conducted for 60 min at 20°C. For double immunostaining primary antibodies directed to band 3 (mouse) were mixed with antibodies directed to either ankyrin or spectrin (rabbit). After incubation with primary antibodies samples were rinsed three times for 5 min each with PBS and then incubated for 30 min at 20°C with TRITC- or FITC-labeled second antibodies (diluted 1:50 in PBS). For double immunostaining, a mixture

Figure 3. *Double immunostaining of human erythroleukemia cells with anti-ankyrin* (a, c, e) *and anti-band 3* (b, d, f). *All frames show proerythroblast-like cells (indicated by 1) that are positive for ankyrin but have not yet expressed band 3. All cells of erythroblast morphology are coexpressing band 3 and ankyrin (indicated by 2).* e *and* f, *two proerythroblast-like cells that express ankyrin. One of these cells (2) is already coexpressing band 3. Note the association of the band 3 stain with reticular cytoplasmic structures that may belong to the endoplasmic reticulum and Golgi apparatus.* c *and* d, *asterisks, a nonreactive lymphocyte-like cell. Bars, 10 μm.*

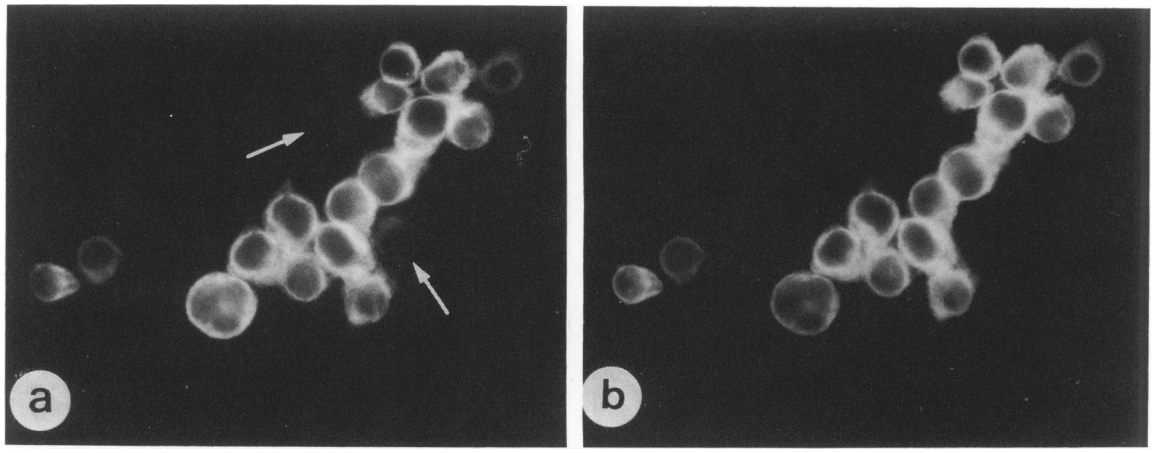


**Figure 5.** *Human bone marrow cells grown in cell culture. Double immunostaining with antibodies to ankyrin* (a) *and band 3* (b). *Cells belonging to the erythroid lineage of development show simultaneous staining for both ankyrin and band 3. Arrows, two cells (erythroid progenitor cells or myeloblasts) unlabeled by both antibodies. Bar, 15 μm.*

of TRITC-labeled goat anti-mouse IgG and FITC-labeled goat anti-rabbit IgG was used. After repeated rinsing with PBS, smears were mounted with coverslips in 60% glycerol/PBS to which 1.5% *n*-propyl-gallate has been added as an antifading compound. Smears were examined with an Olympus $BH_2$ fluorescence microscope equipped with Zeiss optics. Appropriate barrier filter combinations were used to separate TRITC from FITC fluorescence.

## Results

### Immunoblotting

Immunoblot analysis of human bone marrow membranes with antibodies to human erythrocyte α-spectrin, ankyrin, and band 3 revealed specific labeling of the corresponding protein bands (Figure 1). Anti-α spectrin cross-reacted weakly with β-spectrin. No cross-reaction of anti–α-spectrin was seen with purified brain spectrin (fodrin; not shown).

### Immunofluorescence

Marrow smears from healthy donors incubated with antibodies to band 3, erythroid ankyrin, or spectrin revealed that nonerythroid cells, eg, myelocytes, remained completely unstained by these antibodies (Figure 2). On the other hand, the vast majority of cells of the erythroid lineage were found to stain simultaneously for band 3, ankyrin, and spectrin. Proerythroblasts that could be readily discriminated from other erythroblast stages by their size and rather large nucleus were the first cells in erythropoiesis to be labeled by band 3 antibodies. As a rule, the label specific for band 3, ankyrin, and spectrin was distinctly confined to the plasma membrane of erythroblasts. Occasionally, band 3-like fluorescence could be detected in the perinuclear area, probably reflecting pathways of synthesis and intracellular transport of the anion transporter from the endoplasmic reticulum to the Golgi apparatus and the plasma membrane (Figure 3). Whereas normal human erythroblasts showed coexpression of band 3, ankyrin, and spectrin, transformed erythroblasts from a patient suffering from erythroleukemia displayed asynchronous expression of band 3 and ankyrin/spectrin (Figures 3 and 4). Transformed erythroblasts could be identified by periodic acid-Schiff staining and appeared to differentiate in a normal way, as indicated by appearance of periodic acid-Schiff-labeled erythrocytes in peripheral blood smears (not shown).

Immature erythroleukemia cells that stained for ankyrin/spectrin but not yet for band 3 always exhibited a diffuse cytoplasmic distribution of the ankyrin/spectrin label throughout the cell body. The onset of band 3 expression was regularly paralleled by a shift of the ankyrin/spectrin stain from the cytoplasm toward the plasma membrane (Figures 3 and 4). Concomitantly, an increase of membrane-associated fluorescence was observed for both ankyrin and spectrin. The assembly process seemed to be completed at about the stage of the late erythroblast, since no further increase of membrane signals could be observed beyond this point.

To exclude the possibility that apparent synchronous expression of erythroid proteins in normal red blood cell precursors was due to an absence of sufficient numbers of more immature stem cells in bone marrow smears (eg, colony-forming unit–

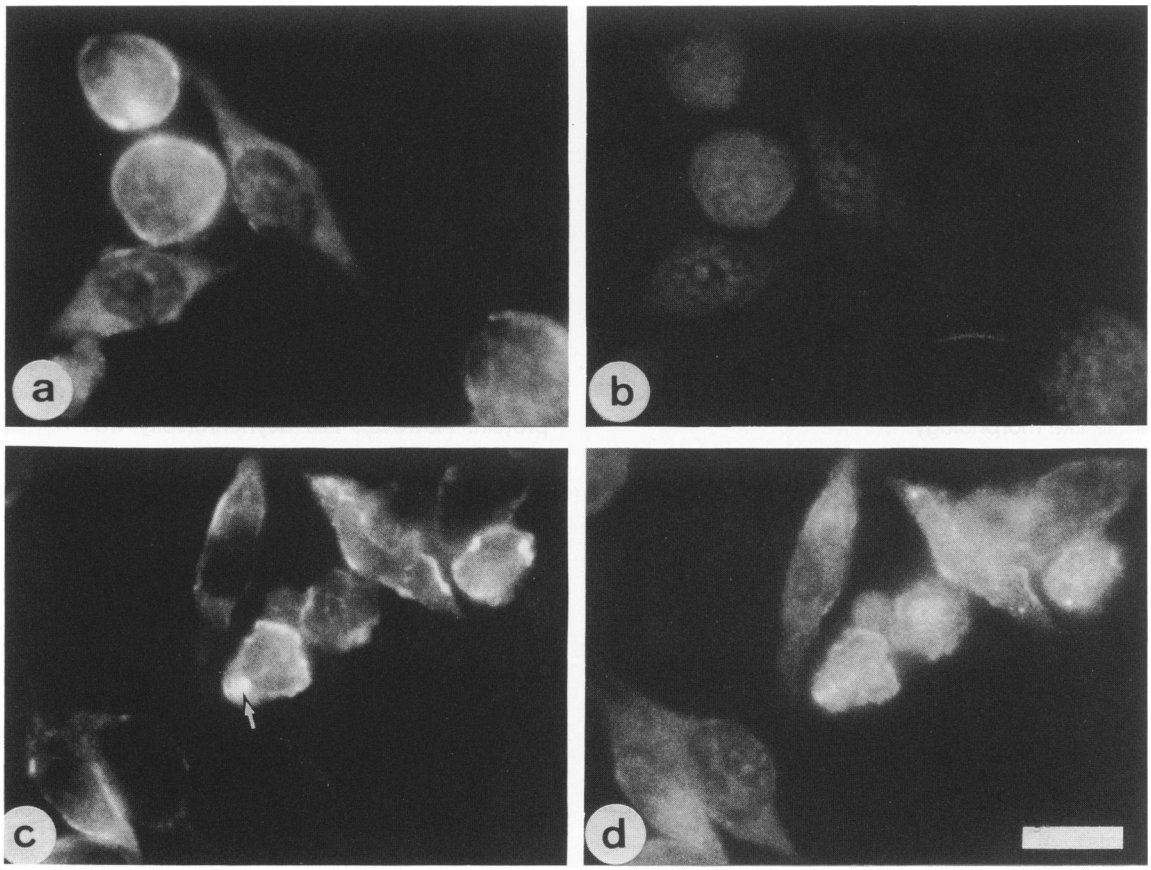


**Figure 6.** *Mouse erythroleukemia cells grown in cell culture. MEL cells were grown on fibronectin and induced by DMSO for 3 days* (a, b) *and 5 days* (c, d). *Double immunostaining with antibodies to spectrin* (a, c) *and band 3* (b, d). *At day 3 of differentiation, cells are clearly reactive for erythroid spectrin* (a) *but are nonreactive for band 3* (b). *At day 5, some spectrin-positive MEL cells* (c) *show diffuse staining for band 3* (d). *The spectrin label in MEL cells is partially concentrated in cytoplasmic inclusions (arrow in* c). *Note that some MEL cells have acquired a spindle-shaped morphology. Bar, 10 μm.*

erythroid) we have established a human bone marrow cell culture system in which red blood cell precursors differentiated through all stages of erythropoiesis. The cell fraction used for plating was obtained from the Ficoll interphase, which consisted predominantly of small lymphocyte-sized cells and appeared to be essentially free of erythrocytes and of erythroblasts of more advanced stages of erythropoiesis. Since erythropoiesis in this culture system could be maintained for up to 6 weeks, numerous immature erythroid stem cells must have been included in the cell population. Double immunostaining of bone marrow cells grown *in vitro* again revealed coexpression of ankyrin, spectrin, and band 3 in all cells belonging to the erythroid lineage of development (Figure 5). Conversely, we have not been able to detect cells that stained solely for ankyrin or spectrin but not for band 3.

Asynchronous expression of erythroid membrane proteins has been demonstrated previously only for transformed erythroid cell lines, in particular for MEL cells. MEL cells have been analyzed in these investigations by biochemical methods rather than by immunostaining. To test whether our experimental approach would give comparable results with this cell line, we have examined terminally differentiated MEL cells by double immunostaining. At day 3 after initiation of induction, 30% of the cells showed clearly recognizable staining for spectrin. Band 3, in contrast, was not detectable at this developmental stage (Figure 6, **a** and **b**). At day 5, increasing amounts of cells were labeled for both spectrin and band 3 (Figure 6, **c** and **d**). The distribution of spectrin and band 3 in MEL cells differed considerably from that in normal erythroblasts. Only a fraction of the cellular spectrin in MEL cells was associated with the plasma membrane. In many cells a diffuse cytoplasmic stain as well as intensely stained cytoplasmic inclusions were seen. Such inclusions were never observed in any type of erythroblasts of normal bone marrow. Band 3 was mainly localized in the cytoplasmic space (probably bound

to internal membranes) with only a small amount of label associated with the plasma membrane of MEL cells.

In summary, immunofluorescence analysis of MEL cell differentiation supports the general view according to which MEL cells exhibit an asynchronous expression of erythroid membrane proteins. In addition, immunostaining revealed that DMSO-treated MEL cells behave rather differently from normal cells with regard to the cellular distribution of spectrin and band 3. As a further sign of abnormality, a large proportion of MEL cells which had attached to fibronectin-coated dishes acquired a spindle-like morphology.

## Discussion

We have shown at the cellular level that during normal human erythropoiesis the major cytoskeletal proteins ankyrin and spectrin are expressed synchronously with band 3. As judged by cell size, proerythroblasts were found to be the most immature precursors to react with antibodies to these proteins. However, the early developmental stages (between colony-forming units–erythroid and proerythroblasts) of erythroid differentiation, which are characterized by smaller diameters than those of proerythroblasts, could not be safely discriminated from basophilic erythroblasts by immunofluorescence. Therefore, we are not able to decide whether the expression of ankyrin, spectrin, and band 3 starts at a point prior to proerythroblast differentiation.

Previous estimations of the onset of band 3 synthesis during erythropoiesis were based on studies on virally transformed cells that were analyzed by biochemical methods but not classified by cytological criteria.[4–13] The general conclusion drawn from these studies was that the synthesis of band 3 in virally transformed cells occurs only after the induction of terminal differentiation by DMSO or erythropoietin. Prior to induction, expression of band 3 was absent or negligible in these cell lines.[3–6,8–11] Low amounts of ankyrin and spectrin, however, were detectable in murine erythroleukemia cells even before terminal differentiation was started. The apparent discrepancy with our own findings is most readily explained by the different types of cells used for these studies. It is tempting to speculate that immortalization of erythroid cells either by viral transformation or by genomic mutations is accompanied by changes in the expression of genes encoding for band 3 and components of the membrane cytoskeleton. This may occur either by a premature onset of the expression of ankyrin and spectrin or by a suppressive effect on transcription or translation of the band 3 gene. Our findings do not allow us to safely exclude the possibility that a certain degree of asynchronous expression of these proteins might also occur in normal erythropoiesis. In this case, however, we would expect the intervals between the onset of ankyrin/spectrin expression and expression of band 3 to be extremely short.

In conclusion, there is no doubt that both murine and human erythroleukemia cells display the asynchronous expression of ankyrin/spectrin and band 3 protein. However, this asynchronicity was not observed in normal human erythropoiesis. Human erythroleukemia cells have allowed us for the first time to observe by immunostaining that delivery of band 3 to the plasma membrane leads to an increase of membrane-associated fluorescence for ankyrin and spectrin. Thus, these observations lend further support to the general concept that band 3 has a role in the assembly and stabilization of the erythroid membrane cytoskeleton.

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