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ERYTHROPHAGOCYTOSIS AND ITS RELATION TO BAND 3 CLUSTERING IN CHRONIC MYELOGENOUS LEUKEMIA

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Summary : Band 3, a major erythrocyte membrane glycoprotein, undergoes topographic redistribution leading to enhanced clustering, in chronic myelogenous leukemia (CML). This is probably due to the binding of heme compounds to the CML erythrocyte membrane resulting from depletion of cellular levels of reduced glutathione (GSH). Band 3 clustering appears to be one of the factors associated with increased erythrophagocytosis in CML.

Keywords : Chronic myelogenous leukemia, erythrophagocytosis, band 3.

Introduction

Over the years, several approaches have been developed in different laboratories to identify the mechanisms involved in removal of senescent, or otherwise damaged erythrocytes, from the circulation. Naturally occurring autoantibodies bind to the surface of senescent erythrocyte (1-3) and promote their phagocytosis by macrophages. In the case of senescent erythrocytes, several mechanisms have been proposed to explain how these erythrocytes are removed from the circulation. These include clustering of the transmembrane protein band 3 (4-6), sialylation of surface carbohydrate residues of band 3 (7), generation of band 3-derived fragments (8) and changes of phospholipid asymmetry (9-11). Such changes are recognized by macrophages leading to erythrophagocytosis (12, 13). Recent studies have also proposed that macrophages recognize sialyl residues associated with glycophorin A in erythrocytes with oxidized protein sulfhydryl groups (14).

Chronic myelogenous leukemia (CML) is a hematologic malignancy resulting from the development of an abnormal stem cell (15). Our previous studies have identified several defects in associations of membrane proteins (16, 17) and increased binding of ^{125}I-protein A to CML erythrocytes (18), leading to the speculation that the rearrangement of band 3 into clusters provides recognition sites for autologus antibodies, ultimately leading to premature

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removal of these erythrocytes from the circulation. The present report provides evidence in support of the view that the clustering of band 3 is associated with increased erythrophagocytosis in CML. This is probably due to the binding of heme compounds to the CML erythrocyte membrane.

Materials and Methods

Collection of samples. Blood was collected from CML patients from the Society for Research on Haematology and Blood Transfusion, Calcutta, and from normal healthy volunteers, in heparin. Erythrocytes were separated from whole blood on Ficoll by centrifugation at 3,000 rpm for 10 min in a table-top centrifuge, and washed in phosphate-buffered saline (PBS). All procedures were carried out at $4^{\circ}C$.

Phagocytosis of erythrocytes by macrophages. Macrophages were obtained from the peritoneal cavity of 7-10 week old Balb/C male mice 4 days after an intraperitoneal injection of 2% starch solution. Macrophages were collected by injecting chilled PBS into the intraperitoneal cavity after sacrificing the mice. Cells were centrifuged down at 500 g for 10 min and resuspended in RPMI1640 medium supplemented with 20 mM Hepes, pH 7.2, 50 units/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf serum (FCS) at $2x10^6$ cells/ml. From this, 0.2 ml of the cell suspension was layered over square glass coverslips and incubated at $37^{\circ}C$ in 5% CO_2 for 3 h. Non-adherent cells were removed by washing with PBS three times. Washed erythrocytes were suspended in RPMI/FCS medium at a concentration of $5x10^6$ cells/ml. From this 100 µl cell suspension was layered over each coverslip and incubated at $37^{\circ}C$ for 45 min. Non-adherent cells were removed by gentle washing with PBS and surface-bound erythrocytes were removed by treatment with 0.83% NH_4Cl/17 mM Tris-HCl, pH 7.6 for 5 min. Coverslips were dried overnight at room temperature. Cells were fixed in methanol for 10 min and stained with diaminobenzidine for staining erythrocytes, and counterstained with Giemsa stain for staining macrophages. Counting was done at 400x magnification. For each experiment the number of macrophages that ingested one or more erythrocytes was determined. Phagocytosis was calculated in terms of the percentage of macrophages that ingested one or more erythrocytes (12).

Lateral distribution of band 3. The distribution of band 3 was examined by immunofluorescence microscopy using the procedure of Waugh et al. (20). Briefly, blood smears were prepared, allowed to dry at room temperature, permeabilized and fixed with acetone, and then washed with PBS. Small areas of the smears were overlaid with antibodies against the cytoplasmic domain of band 3. After incubation, smears were washed with PBS and then incubated with rhodamine-conjugated anti-rabbit IgG. After repeated washing, the stained cells were overlaid with buffer or glycerol, sealed under a coverslip and viewed under a fluorescence microscope (Reichert-Jung, Austria).

Binding of heme compounds to the erythrocyte membrane. Ghosts were prepared by lysing 120 µl packed erythrocytes in 5 mM phosphate buffer, pH 8 (Buffer A) at $4^{\circ}C$ and washed thrice with the same buffer. For estimation of membrane-bound hemoglobin, the volume of the ghosts was made upto 15 µl with Buffer A and over that, 6 volumes of Buffer A containing 1% SDS was added to solubilize the membranes. A 50 µl aliquot was removed for protein estimation. The reaction mixture was incubated at $37^{\circ}C$ for 15 min and at room temperature for 15 min. Absorbance was read at 406 nm. A calibration curve for bound hemoglobin was obtained from standard solutions of hemoglobin, containing SDS at the same concentration as that used for solubilization (21).

Estimation of peroxidation of membrane lipids. Lipid peroxidation was estimated in terms of thiobarbituric acid-reactive substances (TBARS). Malondialdehyde (MDA) was taken to represent the TBARS. Briefly, 50 µl of packed erythrocytes was suspended in 12 volumes of PBS. An aliquot of 10 µl was removed and the volume was made upto 1 ml by adding PBS. 0.1 ml of 50% trichloroacetic acid (TCA) was then added and centrifuged. To 1 ml of the resultant supernatant, 0.27 ml of thiobarbituric acid (TBA) was added and incubated at 95°C for 20 min. The absorbance was read at 532 nm. A calibration curve was obtained by generating MDA, freshly prepared from 1,1',3,3'-tetramethoxypropane (19).

Measurement of cellular GSH content. Reduced glutathione (GSH) levels in normal and CML erythrocytes were determined as described in reference 22. Briefly, 0.2 ml of whole blood was centrifuged down and the cells were lysed by addition of 2 ml of distilled water. 0.2 ml of the hemolysate was removed for measurement of hemoglobin. To the rest of the lysate, 3 ml of precipitating solution (consisting of 1.67 gm of glacial metaphosphoric acid, 0.2 gm of Na_2EDTA and 30 gm NaCl, per 100 ml) was added. The contents were mixed by inversion and filtered through Whatman no. 1 filter paper. The clear filtrate was used for GSH estimation. A blank was prepared by adding 3 ml of precipitating solution to 2 ml of distilled water. 0.8 ml of 0.3 M Na_2HPO_4 was added to 0.2 ml of the clear filtrate and the absorbance was read at 412 nm (A_1). In the same mixture, 0.1 ml of a 0.02% solution of 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB] in 1% sodium citrate was added and the absorbance was read at 412 nm (A_2). The amount of GSH was calculated as follows:

GSH(µmoles per gm hemoglobin) = $[(A_2-A_1)x1.01]$/ gm hemoglobin per ml hemolysate.

Determination of Vitamin E (α-tocopherol) content in erythrocytes. Vitamin E content in erythrocytes was measured according to Simoes et al. (23). Erythrocytes were washed with buffer containing 15 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 5 mM glucose, 20 mM Tris-HCl, pH 7.4. Cells were packed and 100 µl packed cell was made upto 1 ml with the same buffer. 50 µl of ethanolic butylated hydroxytoluene (20 mg/ml) was added. To 1 ml of the cell suspension, 2 ml of methanol was added while vortexing. Thereafter, the mixture was extracted twice with 2 ml of hexane and the hexane layer was taken for analysis. It was evaporated under nitrogen and the extract was dissolved in 100 µl of a mixture of methanol:ethanol (4:1, v/v). 20 µl of this extract was injected on a Lichrosorb RP-18 column (10 µ; 4x250 mm). Analysis was done by isocratic elution with methanol containing 0.1% (w/v) ammonium acetate at a flow rate of 1 ml/min and detection at 287 nm.

RESULTS

Phagocytosis of erythrocytes by macrophages. Macrophages recognize and phagocytose foreign cells as well as senescent self cells. In a survey of 6 normal and 6 CML patients it was found that CML erythrocytes were phagocytosed 12±1% more than normal ones.

Lateral distribution of band 3. Normal erythrocytes, permeabilized and immunostained with anti-band 3 antibody, showed a diffuse staining over the entire cell surface (Fig. 1A). In contrast, CML erythrocytes showed distinct patches of intense fluorescence confirming the presence of band 3 clusters (Fig. 1B).

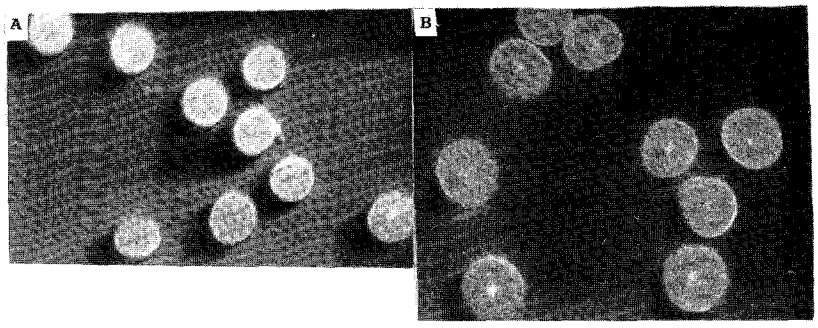

FIG. 1. Lateral distribution of band 3 in normal and CML erythrocytes. Erythrocytes were immunostained with anti-band 3 antibody as described under "Materials and Methods, and viewed by immunofluorescence microscopy. A : normal; B : CML

Binding of heme compounds to the erythrocyte membrane. Attachment of heme compounds to the CML erythrocyte membrane was higher than in the case of normal erythrocytes (Table I).

Lipid peroxidation in normal and CML erythrocytes. Peroxidation of membrane lipids is believed to be one of the causes leading to sulfhydryl oxidation of membrane proteins and possible clustering of band 3. However, in this case, no significant difference was found in malondialdehyde production between normal and CML erythrocytes (Table II).

Glutathione (GSH) and Vitamin E levels in normal and CML erythrocytes. Glutathione levels in CML erythrocytes were significantly lower than in the case of normal erythrocytes (Table III). On the other hand, no significant difference was observed in Vitamin E levels of normal and CML erythrocytes.

DISCUSSION

Previous studies undertaken by us had identified reduced proportions of spectrin tetramers and defective binding of ankyrin to band 3 (16,17) among several abnormalities of the erythrocyte membrane which may account for the premature removal of CML erythrocytes from the circulation. We had also demonstrated increased binding of ^{125}I-Protein A to CML compared to normal erythrocytes, and speculated that the clustering of band 3 probably generates autologous antibody binding sites in CML erythrocytes and provides the signal for their recognition by macrophages and removal from the circulation (18). This speculation has been tested and corroborated in the present study which has shown that CML eythrocytes are phagocytosed 12±1% more than normal erythrocytes. Clusters of band 3 were convincingly demonstrated in CML

TABLE I

Attachment of heme compounds to the erythrocyte membrane

	Number	mg heme protein/mg non-heme protein
Normal	4	0.031 ± 0.005
CML	4	0.056 ± 0.01

Results represent the mean ± S.D.

TABLE II

Lipid peroxidation in normal and CML erythrocytes

	Number	Malondialdehyde produced (nmoles/ml RBC)
Normal	4	0.73 ± 0.2
CML	4	0.79 ± 0.13

Results represent the mean ± S.D.

erythrocytes by immunofluorescence with anti-band 3 antibody. Furthermore, the increased attachment of heme compounds to the CML erythrocyte membrane suggested that this may trigger clustering of band 3. The deposition of heme compounds to the CML erythrocyte membrane was probably due to greater susceptibility of CML erythrocytes to oxidative stress. No significant difference in the level of lipid peroxidation was observed between normal and CML erythrocytes.

Aerobic cells are equipped with a battery of oxidant defense mechanisms. Among the chemical antioxidants providing protection against the deleterious effects of lipid peroxidation, α-tocopherol plays a major role. GSH is required for the detoxification of several organic hydroperoxides (24). GSH is also required for maintaining the sulfhydryl status of erythrocyte membrane proteins. The α-tocopherol content of CML erythrocytes, which was similar to that of normal erythrocytes, was probably adequate to protect the cells against peroxidative damage of lipids. The elevated oxidant sensitivity of CML erythrocytes reflected in the binding of heme compounds to the membrane appears to be at least partly a consequence of reduced cellular GSH concentration. The reduction of spectrin dimer self-association observed by

TABLE III

Vitamin E and GSH levels in normal and CML erythrocytes

	Number	GSH (μmoles/gm hemoglobin)	Vitamin E (pmoles/100μl packed RBC)
Normal	5	7.8 ± 0.26	164 ± 7.8
CML	5	5.7 ± 0.19	149 ± 5.2

us previously (16), may also be a consequence of its complexation with hemoglobin, since hemoglobin-spectrin complexes interfere with spectrin tetramer formation (25). Globin-bound uncoupled spectrin dimers probably lead to the increased lateral mobility of band 3 molecules in CML erythrocytes and their clustering into the senescence antigen (4).

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