

# Alterations in band 3 protein and anion exchange in red blood cells of renal failure patients

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## Abstract

The precise nature of band 3 protein and its involvement in oxalate exchange in the red blood cells (RBCs) of renal failure patients has not been studied in detail. Therefore, here we studied the oxalate exchange and binding by band 3 protein in RBCs of humans with conditions of acute and chronic renal failure (ARF and CRF). The RBCs of ARF and CRF patients exhibited abnormal red cell morphology and an increased resistance to osmotic hemolysis. Further, an increase in the cholesterol content and decrease in the activities of Na<sup>+</sup>-K<sup>+</sup>-, Ca<sup>2+</sup>-, and Mg<sup>2+</sup>-ATPases of membranes were observed in the RBCs of ARF and CRF patients. A decrease in the oxalate flux was observed in the RBCs of ARF and CRF patients. The oxalate-binding activities of the RBC membranes were significantly lower in ARF (20 pmoles/mg protein) and CRF (5.3 pmoles/mg protein) patients as compared to that in the normal subjects (36 pmoles/mg protein). DEAE-cellulose and Sephadex G-200 column chromatography purification profiles revealed a distinctive shift in oxalate-binding activity of band 3 protein of RBCs of ARF and CRF patients as compared to that of the normal subjects. It was also observed from the binding studies with a fluorescent dye, eosin-5-maleimide, which specifically binds to band 3 protein, that the RBCs of ARF and CRF patients exhibited only 53 and 32% of abundance of band 3 protein, respectively, as compared to that in the RBCs of the normal subjects, thus revealing a decrease in the band 3 protein content in ARF and CRF patients. These results for the first time showed a decrease in the oxalate exchange in RBCs of patients with ARF and CRF, which was also concomitant with the low levels of abundance of band 3 protein. (*Mol Cell Biochem* 273: 11–24, 2005)

*Key words:* band 3 protein, red blood cell, acute and chronic renal failure, anion exchange, erythrocyte oxalate transport

## Introduction

The anion exchange of the red blood cell (RBC), mediated by the anion transport protein, namely band 3 protein (96 kDa), has been the subject of intensive investigation for the past decade [1, 2]. Human band 3 protein, a member of the anion exchanger (AE1) family of proteins, is a 911-amino

acid glycoprotein of the RBCs accounts for ~25% of the total membrane protein [3]. It is also detected in the nuclear, Golgi, and mitochondrial membranes [4–6]. Band 3 protein comprises two distinct structural and functional domains, of which the 52 kDa C-terminal membrane domain carries out the anion exchange and the 43 kDa N-terminal cytoplasmic domain anchors the protein to the peripheral membrane

proteins, forming an anchorage site for the RBC cytoskeleton [7]. It is involved in maintaining the acid–base balance and respiration [8]. Under normal conditions, the high rate of  $\text{Cl}^-/\text{HCO}_3^-$  exchange across the human RBC membrane is mediated by the high abundance of band 3 protein [9]. As a multi-functional integral membrane protein, it is expressed in the RBCs and the intercalating cells of the distal tubule of kidney [10, 11].

The rates of exchange of chloride and bicarbonate across the RBC membrane are very high when compared with those of other cells [12]. The function of  $\text{Cl}^-/\text{HCO}_3^-$  exchange is to increase the  $\text{CO}_2$  carrying capacity of blood [13]. Recently, it has been reported that nitric oxide is translocated from hemoglobin (SNO-Hb) *via* band 3 protein [14]. In addition, many other anions including oxalate, are also transported by band 3 protein [15]. Our earlier studies on the nucleated RBCs showed an increase in oxalate flux [16]. Moreover, we also established the presence of oxalate-binding proteins in the mitochondria, nuclei, nuclear pore and histones of tissues of rat and human under both the normal and renal stone forming conditions [17]. The association between the abnormality of RBCs and renal stone formation has been established and the patients with primary calcium oxalate nephrolithiasis have a significantly elevated RBC oxalate exchange [18]. However, little is known about the involvement of band 3 protein in the transport and binding of oxalate in the RBCs of humans during other renal failure conditions, including ARF and CRF. Hence, in the current study, we investigated the exchange and binding of oxalate by the band 3 protein of the RBCs of humans with conditions of ARF and CRF. The present study revealed (1) an alteration of band 3 protein, (2) a decreased binding of oxalate by band 3 protein, (3) a reduction in the abundance of number of copies of band 3 protein, and (4) a decreased rate of anion transport in RBCs of ARF and CRF patients. Our results, for the first time, revealed a significant decrease in the oxalate transport/binding in RBCs of the ARF and CRF patients.

## Materials and methods

### *Selection of patients*

ARF and CRF patients were identified, categorized and selected according to their daily urinary volume output and the levels of serum creatinine. CRF patients undergoing hemodialysis, twice per week at Royapettah Government Hospital, Chennai, India, were selected. A total number of 60 patients (38 males and 22 females) with mean age  $49 \pm 11$  were selected for the study. All the CRF patients studied had a minimal urine excretion volume of  $<100$  ml with proteinuria. These patients had elevated levels of blood urea, serum creatinine, and low levels of serum albumin. The blood was

obtained before dialysis. ARF patients, a total number of 55 (32 males and 23 females) of an average age  $47 \pm 8$  years, with mild renal failure, were included in the study. Nearly 115 healthy individuals with normal renal function (62 males and 53 females) of an average age of  $40 \pm 15$  years were recruited as control subjects. Studies on human subjects were conducted in compliance with the ethical standards of human experimentation.

### *Materials*

$^{14}\text{C}$ -Oxalate (specific activity of 5.86 mCi/mmmole) was obtained from the Board of Radiation and Isotope Technology, Bhaba Atomic Research Center (Mumbai, India). Eosin-5-maleimide (E5M) was purchased from Molecular Probes Inc. (Eugene, OR). Band 3 monoclonal antibody (Clone B-III 136), sodium dodecyl sulfate (SDS), Coomassie brilliant blue-R 250, phenylmethyl sulfonyl fluoride (PMSF), DEAE-cellulose and Sephadex G-200 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), Triton X-100, PPO and POPOP were purchased from Sisco Research Laboratories (Mumbai, India). Pre-stained molecular weight marker (Benchmark ladder JJC15) was obtained from Life Technologies (Bethesda, USA). Rabbit anti-mouse IgG-HRP was obtained from the National Institute of Immunology (New Delhi, India). All other reagents were of analytical grade and purity.

### *Biochemical analysis*

Blood was drawn by slow venepuncture through a 21 gauge disposable needle and one portion of it was carefully transferred to a sterile screw-capped tube containing heparin (2 mg/10 ml blood) and the other portion was used for the separation of plasma. After centrifugation at  $500 \times g$  for 15 min at  $4^\circ\text{C}$ , the plasma and the buffy coat were aspirated carefully. RBCs were washed thoroughly with physiological saline and the RBC suspensions were used for the oxalate transport studies, membrane preparation and isolation of band 3 protein. RBC membranes were isolated according to Dodge *et al.* [19]. The activities of  $\text{Na}^+/\text{K}^+$ -,  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -ATPases in the RBC membranes were determined according to the established procedures [20–22]. Lipids from the membranes were extracted by Folch extraction method [23]. Cholesterol and phospholipid contents of the RBC membranes were determined according to published methods [24, 25]. Erythrocyte osmotic fragility was assayed according to the widely followed method of Dacie *et al.* [26]. Protein concentrations were determined by the Lowry method [27].

### Examination of RBC morphology

Smears of blood from normal subjects and ARF and CRF patients were prepared on clean glass slides, fixed in absolute methanol for 5 min, dried in air, and stained with Giemsa stain for 30 min [28]. The slides were then washed with distilled water, dried and pictures were captured on a light microscope with 200× magnification.

### Isolation of band 3 protein

Band 3 protein was isolated from RBCs according to Wolosin *et al.* [29]. For this purpose, white or slightly pink erythrocyte ghosts were prepared according to Dodge *et al.* [19]. All procedures were carried out at 4 °C. All the reagents used in band 3 protein isolation contained 0.2 mM dithiothreitol, protease inhibitor cocktail (Roche Diagnostics, IN) and PMSF (1 mM). RBC membranes were resuspended in 5 volumes of phosphate buffered saline (PBS, pH 8) for 20 min and then centrifuged at 5000 × *g* for 10 min at 4 °C. In order to remove sialoglycoproteins, the pellet was incubated for 20 min in 6 volumes of 1:5 diluted PBS containing 0.45% Triton X-100 and then centrifuged at 15,000 × *g* for 30 min at 4 °C. Removal of Triton X-100 was accomplished by washing the pellet several times with 1:15 PBS. The pellet thus obtained was subsequently incubated for 10 min with 5 volumes of 2 mM EDTA at pH 12. The suspension was centrifuged at 50,000 × *g* for 60 min at 4 °C and the pellet was resuspended in 5 mM potassium phosphate buffer (pH 7.4) containing 200 mM sucrose at a final concentration of 1 mg of protein/ml. This suspension was snap frozen by liquid nitrogen and stored at –80 °C for further processing.

The partially purified band 3 protein was loaded onto the DEAE-cellulose column and eluted with 50 mM potassium phosphate buffer (pH 7.4) with increasing concentrations of NaCl from 0.1 to 0.2 M in the buffer (pH 7.4). Fractions of 1 ml volume, in each eluting buffer, were collected. Presence of proteins in each fraction was monitored by measuring the absorbance of each fraction at 280 nm in a UV spectrophotometer. Oxalate-binding activity of the protein in each fraction was determined by measuring the extent of <sup>14</sup>C-oxalate binding by the protein aided by liquid scintillation counting. The <sup>14</sup>C-oxalate-bound protein fraction obtained from the DEAE-cellulose column purification was then loaded onto the Sephadex G-200 column and eluted with 50 mM potassium phosphate buffer (pH 7.4) and 20, 1 ml fractions were collected in succession. The absorbance of protein at 280 nm and radioactivity in each fraction were measured as described earlier. The eluted fractions were also tested for their immunoreactivity to the monoclonal antibody against band 3 protein.

### <sup>14</sup>C-Oxalate transport

Oxalate exchange measurements in RBCs were performed according to the methods of Baggio *et al.* and Selvam and Saradhadevi [16, 30]. The data were extrapolated and the flux rate (*k*) was calculated according to the formula  $\ln(a_t - a_\infty) = \ln(a_0 - a_\infty) - Kt$ , where '*t*' is the time, '*K*' the flux constant and '*a*' the labeled oxalate at times 0, *t*, and  $\infty$ .

### Determination of <sup>14</sup>C-oxalate-binding assay

The oxalate-binding activity was determined according to Laxmanan *et al.* [31]. An aliquot of RBC membranes or band 3 protein (100 μg) was incubated with 100 nM <sup>14</sup>C-oxalate (10,000 cpm/100 μl) in a total volume of 1 ml of 0.05 M potassium phosphate buffer (pH 7.4) at room temperature for 20 min. Following incubation, the mixture was filtered through 0.45 μm Millipore membrane filter under vacuum. The filters were washed with 0.05 M potassium phosphate buffer (pH 7.4) and dried under vacuum. Each filter was then placed in a mini scintillation vial followed by addition of 4 ml scintillation cocktail. The contents of the vials were vortexed and stored overnight at 4 °C to minimize quenching. The radioactivity of <sup>14</sup>C-oxalate was measured in a liquid scintillation counter (Kontron). Specific binding was calculated by subtracting non-specific binding from the total binding. Oxalate-binding activity was expressed as picomoles per milligram protein.

### Electrophoresis of membrane proteins

The proteins in isolated RBC membranes and purified band 3 protein were resolved on 8% SDS-polyacrylamide gel electrophoresis (PAGE) in a discontinuous buffer system of Laemmli [32]. Protein ladder was included along with the samples and the gels were stained with Coomassie brilliant blue. Each sample containing 100 μg protein was mixed with the sample-solubilization buffer, boiled for 5 min, cooled and aliquots of equal volumes were loaded into the wells. The electrophoresis was carried out at 4 °C with a constant voltage set at 100.

### Determination of E5M binding by band 3 protein

RBCs were labeled with E5M according to Jennings *et al.* [33] and suspended in PBS containing 0.5% BSA. Aliquot of 10 μl cells were transferred onto a glass slide and viewed under the epifluorescence microscope (Nikon EC-400, Japan) at 520 nm of excitation and 540 nm of emission.

### Preparation of fluorescent labeled RBC membrane

RBCs labeled with E5M were lysed by treating them with 40–50 volumes of hypotonic buffer containing 5 mM  $\text{NaH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  (pH 7.4). The membranes were obtained by centrifugation at  $27,000 \times g$  for 10 min at 4 °C. E5M-labeled membrane pellet was washed four to five times, until the preparation was essentially free of hemoglobin [33]. Fluorescence of E5M was measured at 520 nm of excitation and 545 nm of emission. The content of band 3 protein in the RBC membrane was measured fluorimetrically and number of molecules of band 3 protein was calculated from the number of molecules of E5M bound to the membrane.

### Statistical analysis

All values obtained from the biochemical analyses were averages of eight independent determinations and statistical difference between two groups was determined by Student's *t*-test with significance set at  $p < 0.05$ .

## Results

### RBC morphology

The abnormal concentrations of creatinine, urea and albumin in serum of the renal failure patients [34] prompted us to further conduct studies on the structural alterations of RBC in ARF and CRF patients. Light microscopy revealed the abnormal morphology of RBCs with increased number of eichinocytes or burr cells. About 5 and 9% of burr cells were seen in ARF and CRF patients, respectively, which accounted for the erythrocyte abnormality when compared with the RBCs of normal subjects. (Figs. 1A–1C). Thus, these observations revealed morphological abnormalities of RBCs associated with the conditions of ARF and CRF in patients.

### Osmotic fragility and RBC membrane composition

As we demonstrated earlier in the current study that the morphological alterations of RBCs in patients were closely associated with the ARF and CRF conditions, we further investigated the biochemical composition of the membranes, ATPase activity and osmotic fragility of those RBCs. Table 1 shows the results of biochemical analysis performed on the RBCs obtained from the ARF and CRF patients, as well as from the normal subjects. The amount of cholesterol significantly increased in the ARF (28%) and CRF (52%) patients as compared to that of the normal subjects. The ratio of cholesterol to protein in the RBC membranes of ARF

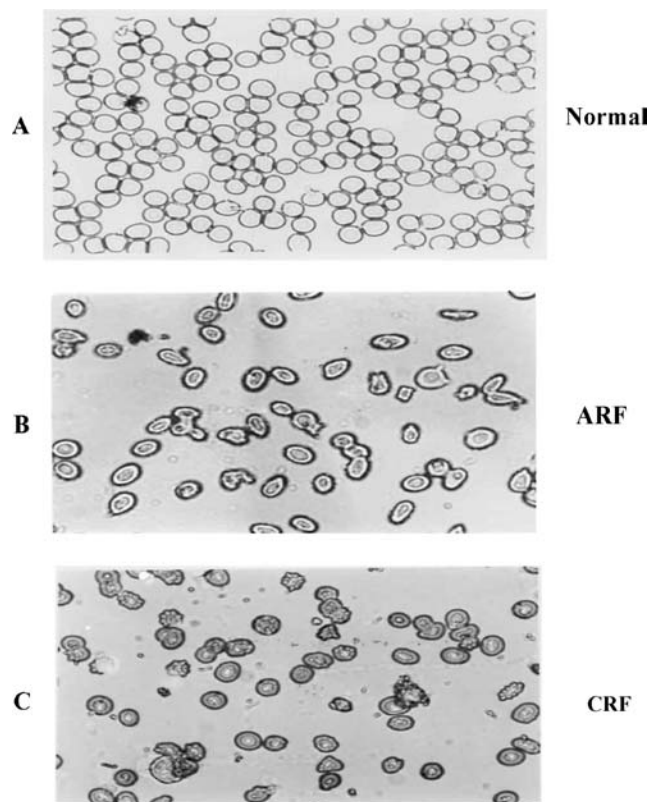


Fig. 1. Morphological alterations of RBCs of ARF and CRF patients. RBCs were isolated from blood of normal subjects and ARF and CRF patients. Their morphology was examined as described in the “Materials and methods” section. RBCs were stained with Giemsa, viewed under light microscope at  $200 \times$  magnification, and photographed.

and CRF patients was significantly higher as compared to that of the normal subjects. Among the two different disease states studied, the RBC membranes of CRF patients showed significantly higher levels of cholesterol and cholesterol to protein ratios. Although the total phospholipid content in the RBC membranes of ARF and CRF patients did not change significantly as compared to that in the normal subjects, the total protein content of the RBC membranes of ARF and CRF patients significantly declined, wherein the latter showed the greatest change as compared with the normal subjects (Table 1). The activities of  $\text{Na}^+$ - $\text{K}^+$ -,  $\text{Mg}^{2+}$ -, and  $\text{Ca}^{2+}$ -ATPases were significantly lower in the membranes of RBCs of only the CRF patients, whereas that in membranes of RBCs of ARF patients it was close to that of the normal subjects (Table 1). As shown in Fig. 2, RBCs of ARF and CRF patients were resistant to hypotonic hemolysis (osmotic fragility) as compared to the RBCs from normal subjects. These results clearly indicated a relationship between (1) elevated cholesterol levels, (2) reduced protein content and (3) decreased activities of ATPases and the resistance of RBCs to osmotic fragility in ARF and CRF patients.

Table 1. RBC membrane lipid composition and membrane-bound ATPases in CRF, ARF and control subjects

Index	Normal	ARF	CRF
Cholesterol (mmol/l RBC)	2.1 ± 0.10	2.7 ± 0.13**	3.2 ± 0.2***
Phospholipids (mmol/l RBC)	3.09 ± 0.23	3.2 ± 0.19	3.4 ± 0.20
Membrane protein (mg/ml)	2.7 ± 0.20	1.48 ± 0.1***	1.06 ± 0.9***
Chol:membrane protein ratio	0.50 ± 0.08	0.93 ± 0.12**	1.57 ± 0.29**
Chol:PLmolar ratio	0.79 ± 0.02	0.82 ± 0.04	0.85 ± 0.05
# Na <sup>+</sup> /K <sup>+</sup> ATPases	0.56 ± 0.06	0.50 ± 0.03	0.39 ± 0.03***
# Ca <sup>2+</sup> ATPases	0.33 ± 0.02	0.25 ± 0.03	0.20 ± 0.02***
# Mg <sup>2+</sup> ATPases	0.29 ± 0.03	0.21 ± 0.02	0.20 ± 0.02***
# Osmotic fragility	0.45 ± 0.06	0.33 ± 0.02*	0.28 ± 0.05***

RBC membranes were isolated from RBCs of control subjects, ARF and CRF as described in 'Materials and methods' section. (#) Enzyme protein activity is expressed as nanomoles of Pi liberated per 10<sup>10</sup> cells/h; (##) Concentration of NaCl in percentage at 50% hemolysis. Comparisons were made between ARF, CRF and control. Values are expressed as mean ± S.D. Values are statistically significant when \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001 compared with control.

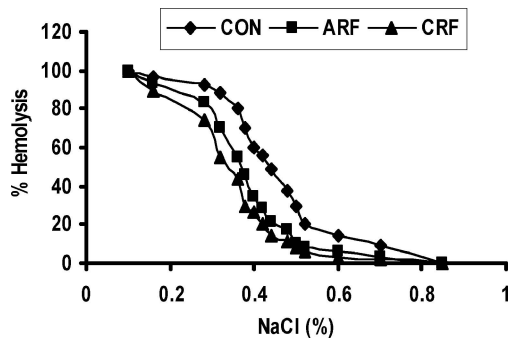


Fig. 2. Osmotic fragility of RBCs of normal subjects and ARF and CRF patients. RBCs were isolated and incubated in medium containing increasing concentrations of NaCl ranging from 0.1 to 0.9%. Hemolysis (%), as an index of osmotic fragility, was measured as described in 'Materials and methods' section.

### RBC oxalate flux

Oxalate flux studies revealed that <sup>14</sup>C-oxalate transport rate in the RBCs of normal subjects was higher with concomitant decrease in <sup>14</sup>C-oxalate in the medium, whereas the oxalate exchange rate was slow in the RBCs of ARF and CRF patients. The rates of oxalate flux were (*k* = 0.35/min) and (*k* = 0.11/min) for RBCs from ARF and CRF patients, respectively, when compared to the normal subjects (0.51/min) (Fig. 3). These results clearly indicated lower oxalate flux rates in the RBCs of ARF and CRF patients as compared to that in the normal subjects.

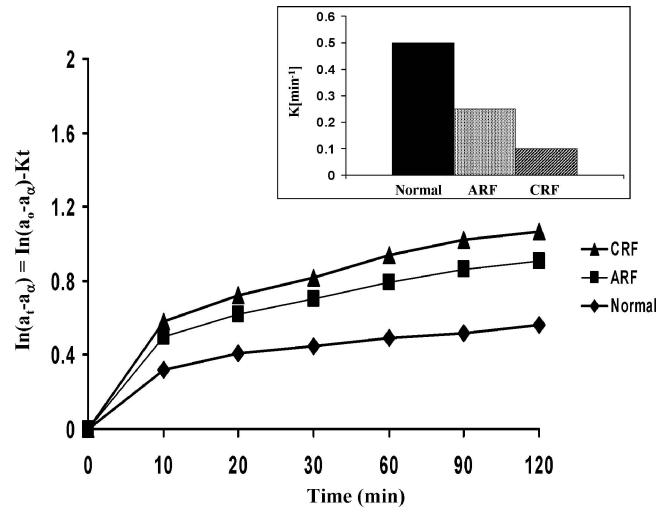


Fig. 3. Oxalate flux rate in RBCs of normal subjects and ARF and CRF patients. <sup>14</sup>C-oxalate flux was determined and calculated as described in 'Materials and methods' section. Each individual point is an average of eight independent determinations.

### Oxalate-binding activity

Oxalate-binding activity in the RBC membranes of ARF and CRF Patients significantly decreased by 45 and 85%, respectively, when compared to that in the normal subjects (Fig. 4). These results showed that the oxalate-binding activity in RBC membranes of ARF and CRF patients was lower as compared to that in the normal subjects. Further, the oxalate-binding activity in the partially purified band 3 protein was dramatically reduced by 60 and 80% in ARF and CRF patients, respectively (Fig. 4).

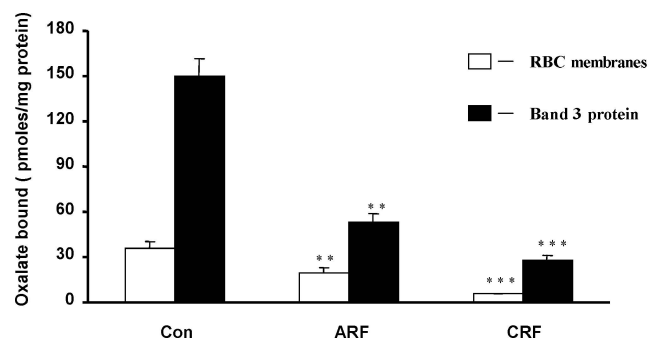


Fig. 4. Oxalate-binding activity in the membranes and isolated band 3 protein of RBCs of normal subjects and ARF and CRF patients. <sup>14</sup>C-oxalate-binding activity was measured as described in 'Materials and methods' section. Each individual point is an average of eight independent determinations. \*\*Significantly different from the normal subjects at *p* < 0.05. \*\*\*Significantly different from the ARF subjects at *p* < 0.05.

Table 2. Purification profile of RBC membrane band 3 proteins from RBCs of healthy controls, ARF and CRF

Details	Total volume (ml)			Total protein (mg)			Total radioactivity (pmoles) <sup>a</sup>			Specific activity (pmoles/mg protein)			Fold purification			Yield (%)		
	Normal	ARF	CRF	Con	ARF	CRF	Normal	ARF	CRF	Normal	ARF	CRF	Normal	ARF	CRF	Normal	ARF	CRF
RBC membrane	1	1	1	8.0	8.0	8.0	1700 ± 163	530 ± 43	170 ± 6.5	125	71.6	32	1	1	1	100	100	100
Crude band 3 protein	1	1	1	3.0	1.2	0.44	800 ± 71	250 ± 21	53 ± 4.3	266	208	132	2.1	2.9	1.5	22.2	16.5	8.3
DEAE-cellulose column	2	2	2	1.5	0.65	0.12	500 ± 44	170 ± 15	29 ± 2.0	333	161.5	142	2.6	3.4	3.1	11	8.7	2.2
SG-200 column	2	2	2	0.8	0.25	0.05	385 ± 31	75 ± 8	17 ± 2	481	243	214	3.8	3.9	3.6	6	4	1

A total volume of 1 ml RBC membranes obtained from 0.5 ml suspension (4.30 ml/mm<sup>3</sup>) cells were incubated with 50,000 cpm <sup>14</sup>C-oxalate for 30 min. The total radioactivity was measured and the oxalate-binding activity was expressed as specific activity. The membranes were washed twice with PBS, pH 7.4 and extracted with 0.45% Triton X-100 to remove the peripheral proteins. Crude band 3 protein was loaded onto the DEAE-Cellulose column and 1 ml fractions were collected. The fractions with protein bound radioactive peak were pooled and loaded onto SG-200 column, 1ml fractions were collected. Protein and radioactivity were measured as described in 'Materials and methods' section.

<sup>a</sup>Total radioactivity measured in an aliquot that was filtered through 0.45 μM Millipore and retained in the membrane filter. Values are expressed as mean ± S.D. for eight independent determinations and are statistically significant when compared with normal (\*\* *p* < 0.01; \*\*\* *p* < 0.001).

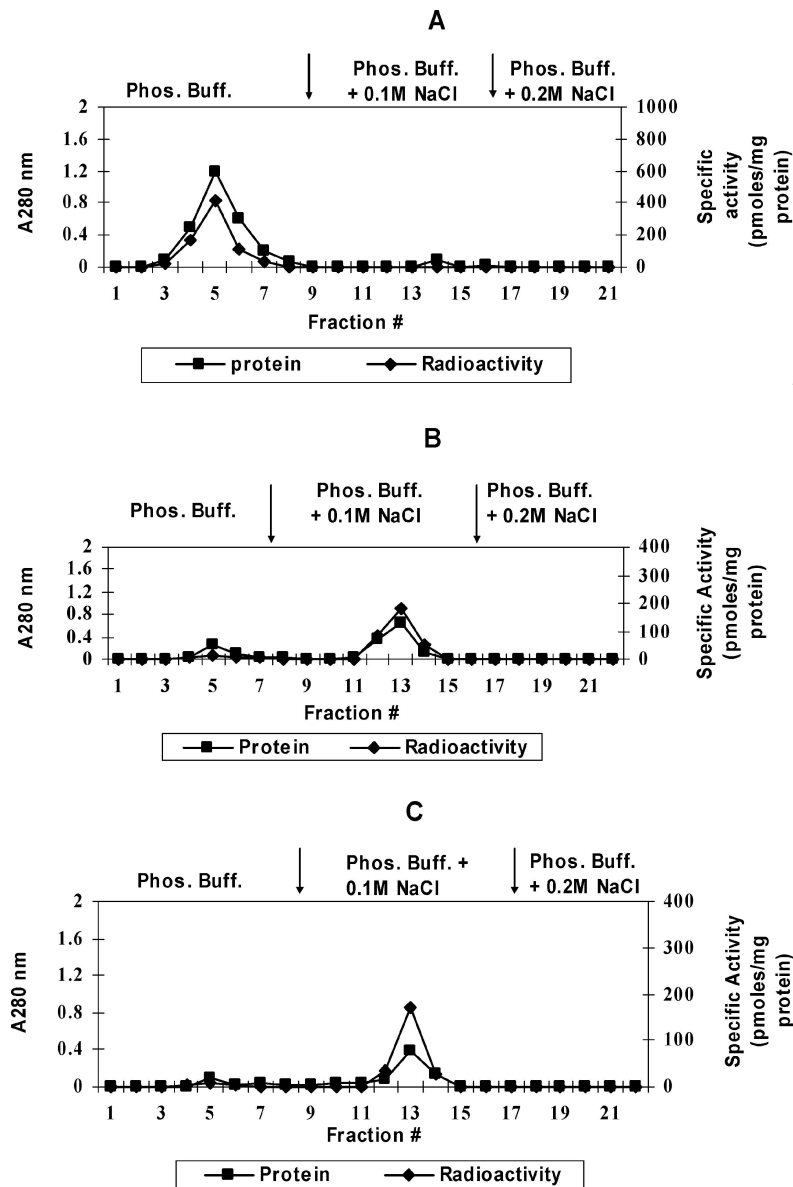
#### *Alteration of band 3 protein as revealed from the DEAE-cellulose and Sephadex G-200 column chromatography purification*

Band 3 protein from the RBCs of normal subjects as well as the renal failure patients was isolated and purified and the results are shown in Table 2. The partially purified band 3 protein (3 mg) from the RBCs of normal subjects was loaded onto the DEAE-cellulose column and eluted with 50 mM phosphate buffer (pH 7.4) and the fraction containing the oxalate-binding protein (1.5 mg) was collected. The fraction 5 with the total <sup>14</sup>C-oxalate-binding activity of 500 and 333 pmoles of specific activity gave a yield of 11% with 2.6-fold purification (Table 2). There was no further elution of protein peaks when the column was subjected to increasing concentration of NaCl (0.2 M) in the buffer (Fig. 5A). The crude anionic band 3 protein isolated from the RBCs of ARF or CRF patients was loaded onto the DEAE-cellulose column and eluted with 50 mM phosphate buffer (pH 7.4). The protein fraction obtained from the elution with phosphate buffer (50 mM, pH 7.4) did not bind to <sup>14</sup>C-oxalate, whereas the fractions (12 and 13) obtained from elution with 50 mM phosphate +0.1 M NaCl (pH 7.4) buffer showed <sup>14</sup>C-oxalate binding (Figs. 5B–5C). Band 3 protein, with 3.9-fold purity, obtained from RBCs of ARF patients showed a specific activity of 261 pmoles with 8.7% yield (Table 2). However, the band 3 protein, obtained from the RBCs of CRF patients with 3.1-fold purity, showed a specific activity of 241 pmoles with 2.2% yield (Table 2). These results clearly indicated that the yield (%), specific activity and the <sup>14</sup>C-oxalate-binding activity of band 3 protein of RBCs of ARF and CRF patients were markedly lower as compared to that of the normal subjects.

Further purification of the band 3 protein from the RBCs of normal subjects on Sephadex G-200 column chromatography

yielded the first peak (fraction 5) which showed a very high radioactive (<sup>14</sup>C-oxalate-binding) protein peak. Among the 40, 1 ml fractions collected, the oxalate-binding protein (0.8 mg) was eluted in fraction 5 had a total radioactivity of 385 pmoles and specific activity of 248.1 pmoles/mg protein with 6% yield and 3.8-fold purity (Table 2 and Fig. 6a). The protein fractions purified by DEAE-cellulose column chromatography were pooled and further purified by Sephadex G-200 column chromatography. This yielded two protein fractions, out of which the later fractions possessed <sup>14</sup>C-oxalate-binding activity.

When the pooled protein fractions (5 and 13) of ARF and CRF band 3 protein obtained from DEAE-cellulose column was loaded onto Sephadex G-200 column and eluted with buffer, two protein fractions (5 and 12–13) were obtained (Figs. 6B and 6C). However, protein peaks at fractions 12–13 of RBCs of ARF and CRF patients contained <sup>14</sup>C-oxalate-binding activity, while the band 3 protein of RBCs of normal subjects was eluted at fraction 5 and had a very high oxalate-binding activity (Fig. 6A). Band 3 protein purified from the RBCs of ARF patients showed a 3.9-fold purity with the specific activity of 210 pmoles and 4% yield, whereas the same purified from the CRF patients showed a 4.4-fold purity with a specific activity of 152 pmoles and 10% yield (Table 2). However, the control band 3 protein had a specific activity of 481 pmoles with 3.8-fold purification and 6% yield (Table 2). Protein fractions obtained from the Sephadex G-200 purification of RBC membranes from ARF and CRF patients showed positive immunoreactivity to band 3 protein as determined by ELISA. Fractions 5, 12 and 13 from RBCs of ARF and CRF patients exhibited band 3 protein-positive immunoreactivity, whereas only the fraction 5 from the RBCs of normal subjects was positive to band 3 protein immunoreactivity (Figs. 6A–6C). This suggested a probable proteolytic



*Fig. 5.* Partial purification of band 3 protein of RBCs by DEAE-cellulose column chromatography. Band 3 protein of RBC membranes of (A) normal subjects, (B) ARF patients and (C) CRF patients was purified by DEAE-cellulose column chromatography as described in 'Materials and methods' section. RBC membrane protein (8 mg) was treated with  $^{14}\text{C}$ -oxalate (10,000 cpm), loaded onto the DEAE-cellulose column and eluted with phosphate buffer (pH 7.4) without or with 0.1 and 0.2 M NaCl. Eluted fractions were collected and analyzed for presence (1) of protein at 280 nm and (2)  $^{14}\text{C}$ -oxalate radioactivity (index of oxalate-binding activity) as described in 'Materials and methods' section.

degradation of band 3 protein in the renal failure patients. SDS-PAGE analysis confirmed the purity of band 3 protein of RBCs of normal subjects (Fig. 7).

#### *Properties of band 3 protein*

##### *Fluorescent binding studies*

The fluorescence intensity as observed under the fluorescence microscope following the binding of E5M is directly proportional to the abundance of cellular band 3 protein. We

followed this approach and observed that RBCs from the CRF patients, pre-loaded with fluorescent E5M, did not exhibit any noticeable fluorescence, thus indicating either deficiency or defective band 3 protein in those RBCs (Fig. 8). Studies on E5M binding with RBC membranes also revealed that the extent of E5M binding by the RBC membranes of ARF and CRF patients was significantly lower as compared to that of the normal subjects (Table 3). Among the RBCs of ARF and CRF patients, the membranes of the latter exhibited lower E5M binding (39%) as compared to the same

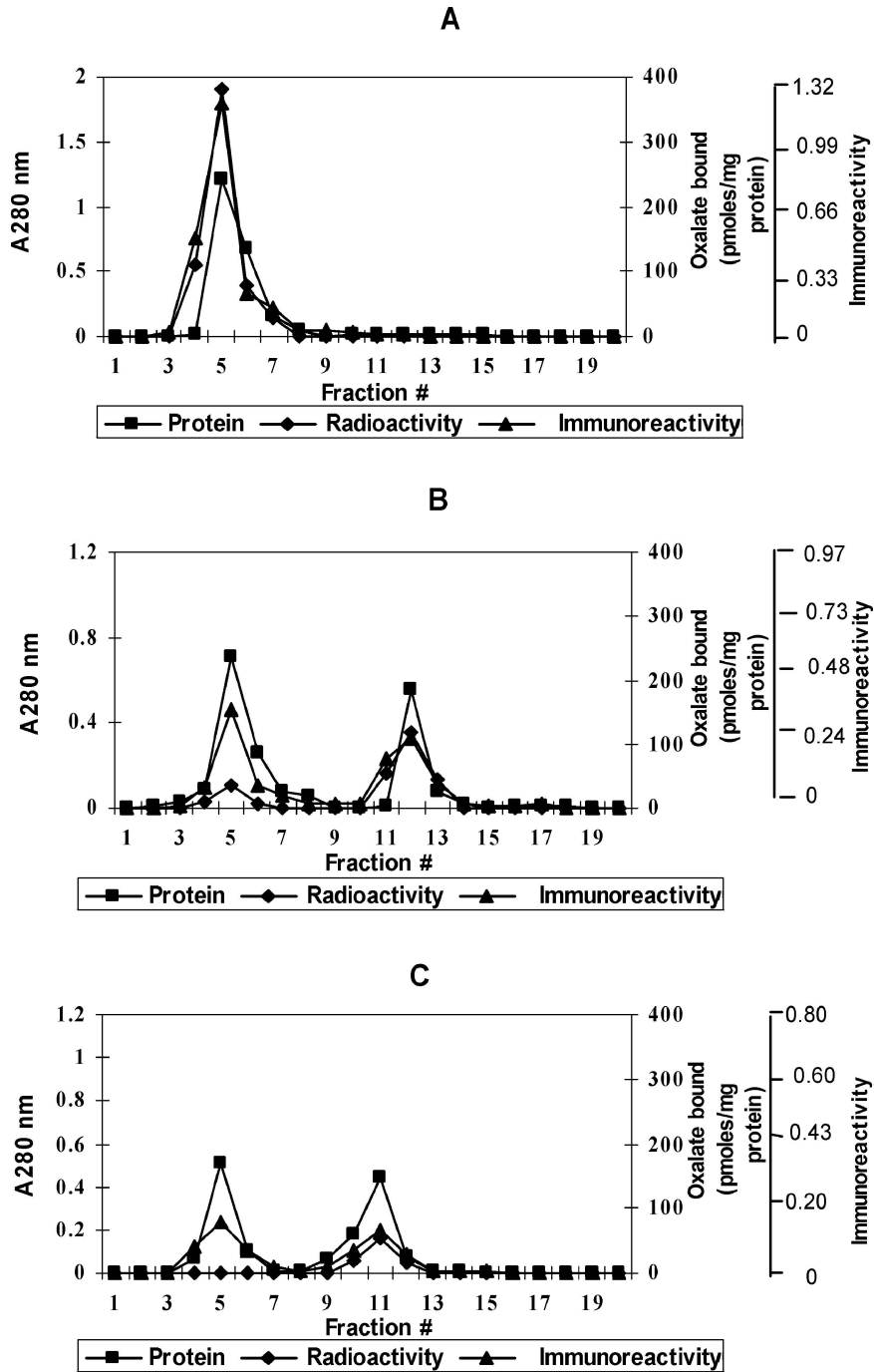


Fig. 6. Sephadex G-200 column chromatographic purification of band 3 protein partially purified by DEAE-cellulose column chromatography. Proteins of RBC membranes of normal subjects and ARF and CRF patients were subjected to DEAE-cellulose column chromatography as described in 'Materials and methods' section. Eluted fractions 4–6, 12–14, and 5–6 and 12–14 of RBCs of normal subjects, ARF patients, and CRF patients, respectively, were collected, pooled, concentrated and subjected to Sephadex G-200 column chromatographic separation as described in 'Materials and methods' section. Proteins were eluted with phosphate buffer (pH 7.4). Eluted fractions were collected and analyzed for presence (1) of protein at 280 nm and (2) <sup>14</sup>C-oxalate radioactivity (index of oxalate-binding activity) as described in 'Materials and methods' section.

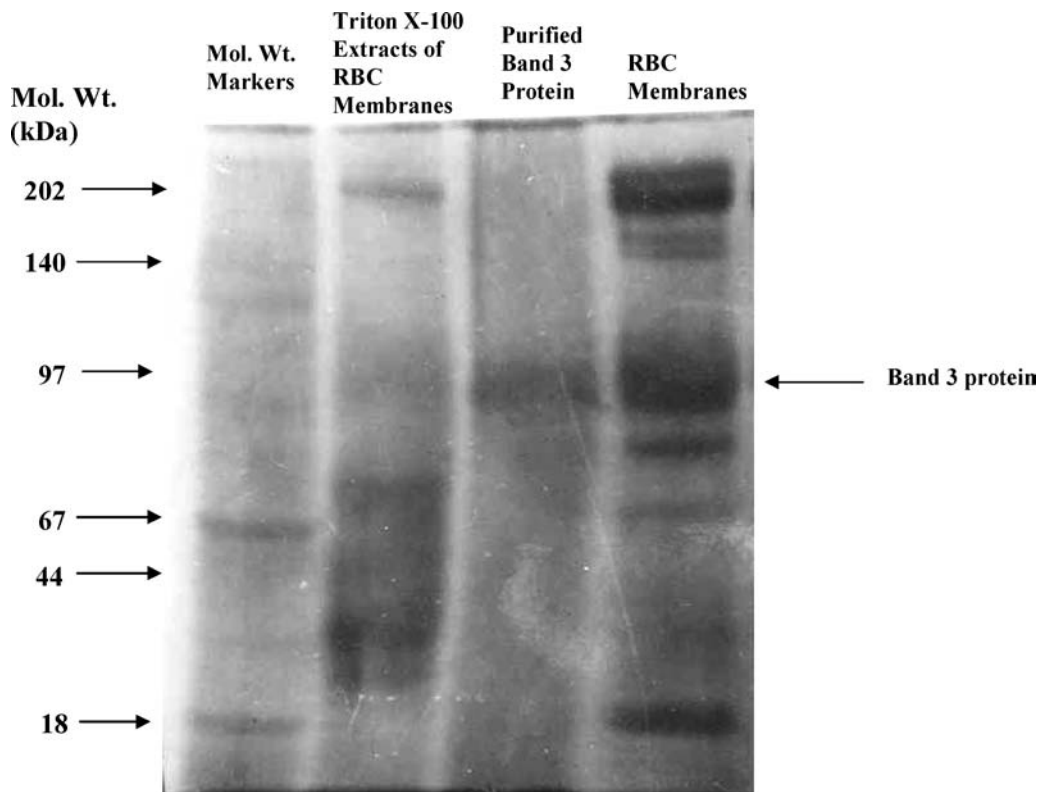


Fig. 7. SDS-PAGE detection of band 3 protein. Total proteins, Triton X-100 extractable proteins, and purified band 3 protein of RBC membranes of normal subjects were separated on SDS-PAGE as described in 'Materials and methods' section.

Table 3. Determination of band 3 protein abundance by E5M binding in RBC membranes of normal, ARF and CRF subjects

Subject	Fluorescence (arbitrary units)	Relative band 3 content	No. of band 3 copies per cell
Normal	1470 ± 135	100%	1.2 × 10 <sup>6</sup>
ARF	830 ± 79**	56.46%	0.63 × 10 <sup>6</sup>
CRF	576 ± 44***	39.18%	0.38 × 10 <sup>6</sup>

E5M-labeled RBC membranes (100 µg protein) obtained from 0.5 ml cell suspension ( $2 \times 10^6$  cells/mm<sup>3</sup>) of control, ARF and CRF subjects were subjected to fluorescence determination at 520 nm of excitation and 545 nm of emission as described in the 'Materials and methods' section. Based on the 1:1 binding of E5M to the band 3 protein at the level of  $1.2 \times 10^6$  copies per cell in normal subjects, the number of E5M molecules bound to RBCs of ARF and CRF subjects was calculated. Values are expressed as mean ± S.D. of 10 independent determinations.

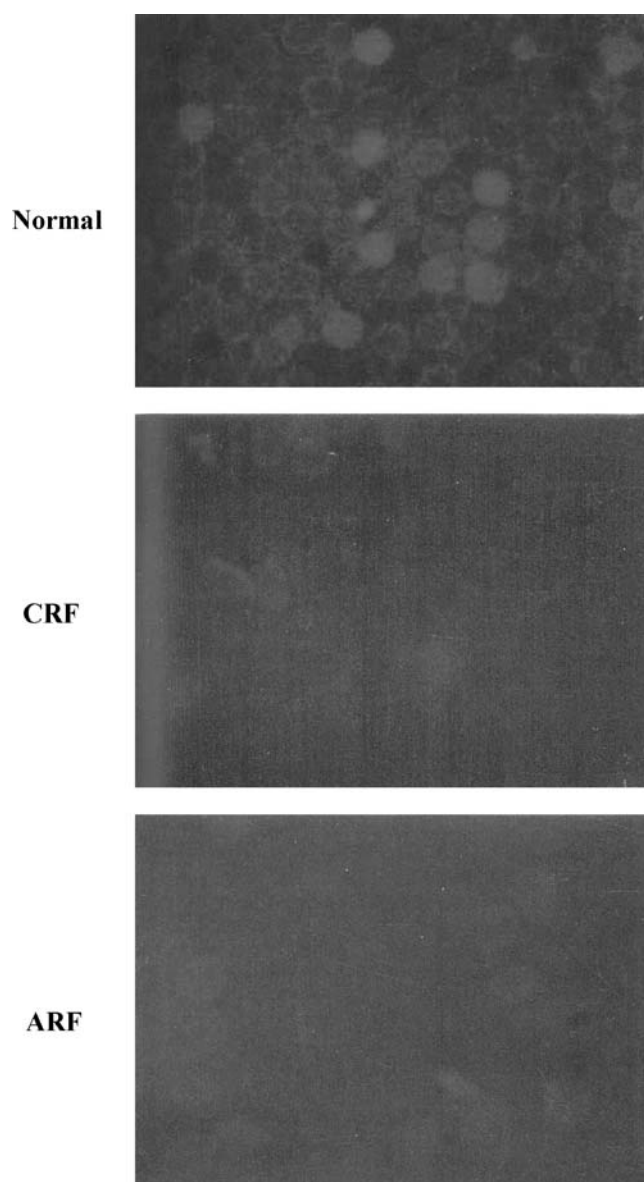
\*\*Significantly different at  $p < 0.01$  and  $p < 0.001$  from the normal.

in the ARF patients (56%). These results also confirmed that the membranes of RBCs of ARF and CRF patients had 52.5 and 32% of abundance of (number of copies per cell) of band 3 protein, respectively, as compared to that in the RBCs of normal subjects (Table 3, Fig. 9). Based on a 1:1 binding between E5M and band 3 protein ratio, the number of E5M

molecules required to cause 100% inhibition of oxalate binding was calculated to be  $1.2 \times 10^6$  per cell, that was directly proportional to  $1.2 \times 10^6$  copies of band 3 protein. However, in the RBCs of ARF and CRF patients, the number of E5M molecules required for 100% inhibition of oxalate-exchange was less, suggesting a decreased number of band 3 molecules in those RBCs (Fig. 10).

## Discussion

Several studies have reported alterations among a number of biochemical parameters in the renal failure patients. An increase in the levels of blood urea, plasma creatinine, cholesterol, uric acid and a decrease in the plasma albumin were noticed in patients with ARF and CRF [34]. In renal failure condition, the amount of urea is elevated proportionally more than creatinine due its reabsorption by the tubular cells [35]. Hypercholesterolemia may contribute to the pathogenesis of atherosclerosis associated with CRF [36]. A decrease in MCV, MCH and MCHC is observed in the renal failure condition [37]. Hence, it is envisioned that certain cellular or molecular damage in RBCs apparently is associated with this decrease. Anemia occurs with relatively high incidence



*Fig. 8.* Loss of band 3 protein in ARF and CRF patients. Total proteins of RBC membranes of normal subjects and ARF and CRF patients were subjected to SDS-PAGE separation as described in 'Materials and methods' section.

in CRF patients as indicated by a decrease in the hematocrit, RBC count and hemoglobin concentration [38], which may be due to the decreased production of RBCs and shortened life span of RBCs. Down-regulation of AE1 immunoreactivity has been reported in the liver of patients with chronic liver diseases and in the kidney tubular tissue of patients with renal tubular acidosis. The derangement of band 3 function disturbs acid-base balance and favors metabolic acidosis [39]. Also disorders in NO respiratory cycle may be due to the alterations of the band 3 protein complex in addition to certain hemoglobinopathies [14].

Reduction of the amount of band 3 protein in human RBC membrane results in membrane defects and the complete absence of the protein in human is life threatening and requires intervention [40]. It has been previously reported that mutations in band 3 gene resulted in band 3 protein deficiency, decreased anion exchange across the erythrocyte membrane, and increased osmotic fragility of RBCs [41]. Oxalate has been used as a substrate to study the function of band 3 protein in RBCs under different disease states [30]. Therefore, in the present study, we used  $^{14}\text{C}$ -oxalate as a substrate to study the anion transport in RBCs. Our study revealed an abnormal reduction in the oxalate exchange in the RBCs of CRF and ARF patients. This reduction in oxalate exchange may be associated with the observed defects in the cell membrane such as the abnormal cell morphology (shape) and biochemical composition of the membranes of RBCs of ARF and CRF patients. Alterations in cell morphology have been observed in patients with hereditary spherocytosis [42] and Southeast Asian ovalocytosis (SAO) [43]. Similar morphological changes have been reported in RBCs when the suspension medium is changed [44]. Anion transport across the RBC membrane that is mediated by band 3 protein is strongly influenced by the lipid composition of the membrane [45]. The observed decrease in the density of protein with concomitant increase in the cholesterol content may be responsible for the increase in resistance of RBCs of ARF and CRF patients to osmotic lysis and the decreased anion transport. The current study also revealed a higher number of morphologically abnormal cells (burr cells) which may be due to the elevated levels of urea and abnormal membrane lipid composition of RBCs. This may facilitate the increased susceptibility of RBCs to degradation resulting in anemia. Our study revealed an increase in the cholesterol content of the RBC membranes, while their protein content decreased in ARF and CRF patients. Further, our study also showed that the activities of the membrane-bound ATPases were also lower, suggesting alterations in the functions of membranes of ARF and CRF patients. Similar reports have been made on the decrease in the activities of ATPases and increase in the levels of cholesterol in CRF patients [46]. Thus, the changes in membrane composition apparently have contributed to the enhanced resistance of RBCs to hypotonic hemolysis in ARF and CRF patients.

Abnormal red cell oxalate transport is a risk factor for idiopathic calcium nephrolithiasis [47]. In the current study, we also demonstrated that the oxalate flux and oxalate-binding activity was significantly lower in the RBC membranes of ARF and CRF patients. This decrease was apparently associated with the changes in cell morphology and membrane lipid changes, thus leading to the defective anion transport in renal failure conditions. The observed decrease in oxalate transport of the RBCs in renal failure patients may be due to the alteration in their structure as well as in the content of the band 3

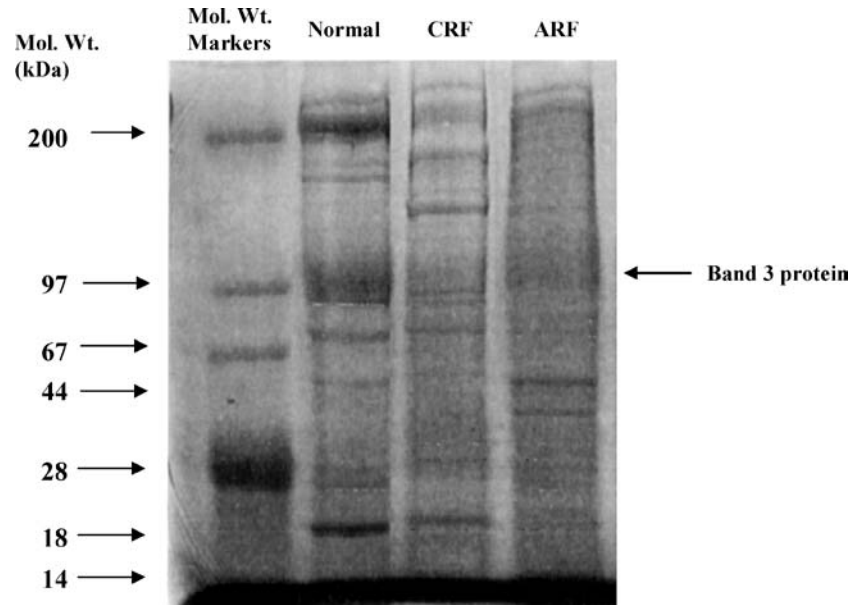


Fig. 9. Decreased binding of E5M to band 3 protein of intact RBCs of ARF and CRF patients. Binding of E5M to band 3 protein of intact RBCs of normal subjects and ARF and CRF patients was examined under fluorescence microscope as described in 'Materials and methods' section.

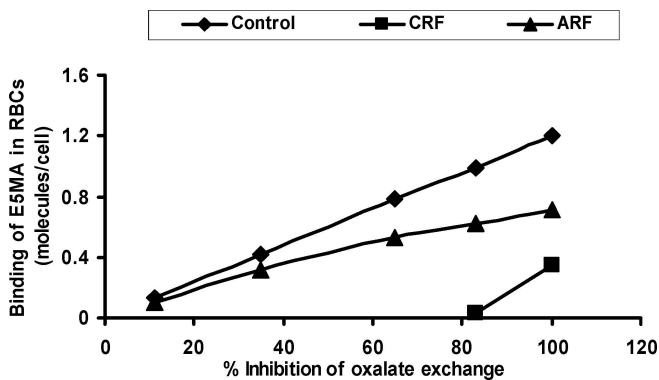


Fig. 10. Inhibition of oxalate exchange by E5M in RBCs of ARF and CRF patients. RBCs, preloaded for 30 min with 1 mM E5M, were incubated with  $^{14}\text{C}$ -oxalate and the rates of oxalate flux were determined as outlined in 'Materials and methods' section. Determination of inhibition (%) is based on the number of E5M molecules bound to band 3 protein. The number of molecules of E5M required to cause 100% inhibition of oxalate exchange in RBC was  $\sim 0.3 \times 10^6$ , thus reflecting the decrease in number of band 3 protein copies present in ARF and CRF patients.

protein as observed by our cell morphology and fluorescent binding studies. Mutations in the cytoplasmic domain of band 3 rendered alteration in the cell shape and anion transport [48]. In the current study, we also showed that the band 3 protein derived from the RBCs of CRF patients showed a shift in SG-200 elution profile as compared to that of RBCs of normal subjects. The current study also demonstrated an abnormal elution pattern, suggested the fragmentation of the band 3 protein. The oxalate-bound fraction appeared to be

a low molecular weight protein. The fragmentation of the band 3 protein was also confirmed by SDS-PAGE analysis (data not shown).

Thus, the observed decrease in oxalate-binding activity in RBCs of ARF and CRF patients was apparently associated with the fragmentation of the band 3 protein. This was further supported by the evidence from our current investigation that the antibody against band 3 protein reacted with band 3 protein fragments of RBCs of CRF patients eluted at fractions 5 and 12–13, although only fractions 12–13 exhibited the oxalate-binding activity. Degradation of the band 3 protein has been shown in a variety of pathophysiological conditions [49]. The cytoplasmic  $\text{Ca}^{2+}$ -activated thiol endopeptidase, calpain, is also known to be responsible for limited protein degradation of cytoskeletal proteins and plays a role in the membrane-associated events [50]. The enhanced sensitivity of band 3 protein in aged individuals to degradation by calpain appears to be quantitative and may play a role in the events leading to the shortened life of the erythrocytes in aged individuals [51]. The degradation of the N-terminal cytoplasmic domain of band 3 protein would affect its association with cytoskeletal and cytoplasmic components. As a result, the cell structure stability may be altered [52]. The decreased anionic activity in the RBCs of ARF and CRF patients may be due to the limited degradation of a fraction of the N-terminal cytoplasmic domain of the band 3 protein thus leading to the perturbation of the outer surface of the membrane.

E5M binding to the band 3 protein and Rh-related proteins forms the basis of a screening test for hereditary spherocytosis [53]. Hence, we determined the content and function of

the band 3 protein based on its property to bind with E5M, a dye that specifically binds to band 3 protein at the external anion transport site of the 17 kDa membrane-bound fragment [33]. The observed decrease in oxalate transport in RBCs of ARF and CRF patients might be due to the loss of membrane band 3 protein as the fluorescence of E5M bound to band 3 was dramatically low in the RBCs of ARF and CRF patients. It has been suggested that Lys-430 of band 3 protein which binds with E5M is located in the external transport site and the resultant blockade of this channel inhibits anion exchange [54]. E5M binds, one per subunit, at the transport site of the protein [55]. Using this concept, the number of band 3 copies per cell was calculated. In the RBCs of CRF patients, the number of copies of band 3 protein was reduced by 60%.

Reduction in the abundance of copies of band 3 protein of RBCs has been reported in sickle cell anemia [56]. The membrane domain of the abnormal ovalocytic band 3 protein has a substantially altered structure and the protein is defective in anion transport activity [57]. The heritable frameshift insertion of 10 nucleotides into the band 3 protein associated with spherocytosis leads to a partial deficiency of the band 3 protein and the deficiency of band 3 protein has been detected in hereditary spherocytosis kindered [58]. Band 3 protein deficiency affects primarily the laterally and rotationally mobile dimeric fraction of band 3 protein that is not associated with the underlying membrane skeleton. A decrease in the amount of band 3 protein leads to the formation of protein-free areas of the lipid bilayer, leading to the lack of stabilization of protein–lipid and protein–protein interactions and this renders the membranes unstable and susceptible to undergo detachment as membrane fragments, suggesting a mechanism of surface area deficiency and spherocytosis in the hereditary spherocytosis-deficient subjects [59]. The membrane domain of the abnormal ovalocyte band 3 protein has a substantially altered structure and that the protein is defective in anion transport activity [60] and a decrease in the content of band 3 protein has been reported in uremic patients [61], suggesting an alteration in the  $\text{Cl}^-/\text{HCO}_3^-$  anion exchanger in RBCs of CRF patients. These studies further support our current findings that the decrease in the number of copies of band 3 protein in CRF patients could very well be the reason for the decreased oxalate transport and oxalate-binding activity in RBCs. Furthermore, degradation of band 3 protein could be one of the causes for the decreased content of band 3 protein in the RBC membranes of ARF and CRF patients. Though the exact mechanism of degradation of band 3 protein is not known in the renal failure condition, further studies are warranted on the mechanism of degradation of band 3 protein. Overall, this study revealed the alteration of RBC band 3 protein and its possible involvement in the alteration of acid–base exchange in the renal failure condition which leads to acidosis. As the extent of alteration of band 3 protein parallels

the progression of the disease towards a chronic stage, such alteration in band 3 protein can be used as a marker in the diagnosis of severity of the disease.

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