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Oxidation as a possible mechanism of cellular aging: Vitamin E deficiency causes premature aging and IgG binding to erythrocytes

(senescent-cell antigen/band 3/anion transport/macrophage phagocytosis)

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ABSTRACT **Senescent-cell antigen is a "neo-antigen" that appears on the surface of senescent cells and initiates IgG binding and cellular removal. As an approach to evaluating oxidation as a possible mechanism for generation of senescent-cell antigen, we studied erythrocytes from vitamin E-deficient rats. Vitamin E is localized primarily in cellular membranes. Its major role is the termination of free-radical chain reactions propagated by the polyunsaturated fatty acids of membrane phospholipids. Results of our studies indicate that erythrocytes of all ages from vitamin E-deficient rats behave like old erythrocytes from normal rats, as determined by their susceptibility to phagocytosis, IgG binding, anion transport ability, and glyceraldehyde-3-phosphate dehydrogenase activity. Increased breakdown products of band 3 were observed with immunoblotting in membranes of erythrocytes from vitamin E-deficient rats. Breakdown products of band 3 are known to increase as cells age in normal individuals. The data suggest that oxidation may be a possible mechanism for erythrocyte aging and generation of senescent-cell antigen *in vivo*.**

One hypothesis for the removal of senescent erythrocytes is that a "neo-antigen" appears on the surface of senescent cells leading to IgG binding and cellular removal (1–10). The "neo-antigen" is recognized by the antigen binding, Fab, region (3, 10, 11) of a specific IgG autoantibody in serum that attaches to it and initiates the removal of cells by macrophages (1–4). A number of studies performed by us (1–11) and by others have demonstrated the presence of IgG on senescent, damaged, and stored erythrocytes (12–20, ¶). In addition, workers in several laboratories have recently presented evidence that IgG binding is also involved in the removal of erythrocytes in diseases such as thalassemia (21) and sickle cell anemia (22, 23). IgG has also been implicated in the removal of aging platelets (24).

We have named the neo-antigen on senescent erythrocytes "senescent-cell antigen" (5). Senescent-cell antigen is a glycosylated 4.5 region polypeptide that appears to be derived from band 3 (5, 8, 10, 25–27). Senescent-cell antigen is located on an extracellular portion of band 3 that includes most of the 38-kDa carboxyl-terminal segment and 30% of the 17-kDa anion-transport region (27).

Although the senescent-cell antigen was first demonstrated on the surface of senescent human erythrocytes (1, 2), it has since been demonstrated on the surface of lymphocytes, polymorphonuclear leukocytes, platelets, embryonic kidney cells, and adult liver cells (5). A molecule immunologically related to band 3, the molecule from which senescent-cell antigen is derived, has been observed on all cells examined (28, 29).

Workers in several different laboratories agree that appearance of senescent-cell antigen initiates IgG binding and destruction of erythrocytes (1–20, ¶). However, they do not agree on the molecular changes responsible for generating the antigen. Our evidence suggests that senescent-cell antigen is derived from band 3, probably by degradation (10, 25–31); Lutz and his colleagues have suggested that senescent-cell antigen is a dimer of band 3 (32); Sayare *et al.* (33) have shown that band 3 can cross-link with hemoglobin under oxidative conditions; and Low *et al.* (34) have presented evidence that clustered band 3 can generate senescent-cell antigen. These membrane events are not mutually exclusive. Indeed, we have postulated that generation of senescent-cell antigen may result from oxidation-induced cross-linking followed by proteolysis (26).

As an approach to evaluating oxidation as a possible mechanism responsible for generation of senescent-cell antigen, we studied erythrocytes from vitamin E-deficient rats. The importance of vitamin E as an antioxidant, providing protection against free radical-induced membrane damage, has been well documented (35–38). Vitamin E is primarily localized in cellular membranes, and a major role of vitamin E is the termination of free-radical chain reactions propagated by the polyunsaturated fatty acids of membrane phospholipids. Vitamin E-deficient erythrocytes are defective in their ability to scavenge free radicals (38, 39).

The erythrocyte has many potential sources for generating free radicals. Hemoglobin is known to catalyze lipid peroxidation as well as enhance the decomposition of lipid hydroperoxides to the corresponding free radicals (40). Autooxidation of oxyhemoglobin to methemoglobin results in the generation of a superoxide radical (41, 42). The reaction of a superoxide radical with peroxides in the erythrocytes produces highly reactive intermediates, such as the hydroxyl radical ('OH). These radicals in turn react with the lipid and protein components of the membrane, damaging its integrity and leading to eventual hemolysis of the cell (40). Lipid peroxidation in the erythrocyte membrane can result in accumulation of an aldehyde, which can cause a reduction in deformability (43, 44) and formation of irreversibly sickled cells (45).

In humans, vitamin E deficiency shortens erythrocyte life span, causing a compensated hemolytic anemia in patients with cystic fibrosis (45). In newborns, vitamin E deficiency causes a hemolytic anemia that develops by 4 to 6 weeks of age (46).

Specific biochemical alterations in the membrane of erythrocytes from vitamin E-deficient rhesus monkeys have been described (47, 48). Furthermore, vitamin E deficiency represents a "physiological" method for rendering cells suscep-

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tible to free-radical damage and may simulate conditions encountered *in situ*. In contrast, methods that have been used to induce oxidative damage *in vitro*, such as exposing cells to malondialdehyde, peroxide, etc., result in numerous pronounced membrane changes that are not observed in cells aged *in situ* (unpublished observations).

The role of free-radical damage in aging has received a great deal of attention. It is interesting that there is a correlation between life span and natural antioxidant levels in a variety of species and that the level of such antioxidants appears to correlate with metabolic activity of individual species (49). Evidence for free-radical damage associated with aging is the presence of lipofuscin and ceroid, so called aging pigments, which represent accumulated breakdown products of polyunsaturated fatty acids and proteins. It has been suggested that free radicals may be mediators of aging and specific pathologies, such as inflammation, arthritis, adult respiratory distress syndrome, and other conditions (50, 51). Free radicals have also been implicated as causative agents in mutagenesis and carcinogenesis as well as causing cross-linking of macromolecules and the formation of age pigments (51).

Results of the experiments presented here indicate that erythrocytes from vitamin E-deficient rats age prematurely. The results suggest that oxidation may accelerate cellular aging and may be a mechanism for generation of senescent-cell antigen.

MATERIALS AND METHODS

Diet of Vitamin E-Deficient Rats. Weanling male Wistar–Kyoto (W/K) rats (Charles River Breeding Laboratories) were randomly separated into three groups of 12 rats each. One group received a vitamin E-deficient diet (Dyets, Bethlehem, PA). The second and third groups received the identical diet supplemented with *dl*-α-tocopheryl acetate, hereafter referred to as vitamin E, at 50 and 200 mg/kg, respectively. Rats were tested at 11, 16, 20, and 30 wk after being placed on their assigned diet. Rats fed a diet containing no vitamin E are referred to as "deficient" rats, those receiving vitamin E at 50 mg/kg are referred to as "normal," and those receiving 200 mg/kg are referred to as "high."

All animals were given food and water *ad libitum*, housed in stainless steel wire floored cages, and kept on a 12-hr light/dark cycle. Animals from each group were anesthetized with metofane (Pittman–Moore, Washington Crossing, NJ) and blood was collected by cardiac puncture. Blood was collected in sterile tubes with $CPDA_1$ as an anticoagulant. Blood was shipped on ice from New Jersey to Texas. Analysis was performed within 24 hr of collection. Since samples from all groups were handled the same way, samples from rats given normal amounts of vitamin E served as controls for the effects of shipping as well as normal vitamin E levels.

Addition of Vitamin E *in Vitro*. Vitamin E was added *in vitro* to blood samples obtained from vitamin E-deficient rats in an amount (1.6 mg/dl) necessary to bring the serum vitamin E level to that of the level of rats fed diets containing vitamin E at 200 mg/kg. D-α-tocopherol (1 mg) was dissolved in absolute ethanol, and an aliquot was taken and diluted to a concentration of 1.6 mg per dl of vitamin E in plasma. The vitamin E was then added to whole blood, which was incubated for 30 min at room temperature and then shipped on ice. From previous experiments, we know that vitamin E will equilibrate between plasma and erythrocytes within this time.

Serum Analysis. Vitamin E levels were determined as described using high-pressure liquid chromatography (52). Haptoglobin and transferrin were determined on an automated Beckman Immunochemistry Analyzer II; iron and lactate dehydrogenase (LDH) were determined with a DuPont Automatic Clinical Analyzer II; LDH isoenzymes were determined with a Helena Auto Scanner with Quick Quant II; bilirubin was determined with a Beckman Automated Stat/Routine Analyzer.

Cell Separation. Cells were separated into populations of different ages on Percoll gradients as described (4).

Phagocytosis Assay. Rat erythrocytes were washed three times with RPMI 1640 medium with 5% fetal calf serum. Ten erythrocytes per macrophage were added to tubes containing either human or mouse macrophages (1×10^6) as indicated in the text. The human cell line used was U937; the mouse cell line was WEHI-3. The phagocytosis assay was performed as described (2–4, 53).

Immunoblotting. Polyacrylamide gel electrophoresis was performed using 6–24% or 2–16% polyacrylamide gradient gels and the discontinuous gel system of Laemmli (54). Immunoblotting was performed as described (26, 53, 55).

Glycoprotein and Protein Stains. Polyacrylamide gels were stained with silver stain followed by Coomassie blue (Bio-Rad kit). This double-staining technique permits visualization of both glycoproteins and proteins. Gels were scanned with a laser scanning densitometer (LKB).

Glyceraldehyde-3-Phosphate Dehydrogenase Assay. Cells were washed four times in 50–60 vol of phosphate-buffered saline (pH 7.4) and lysed in "lysis buffer" (5 mM Na_2HPO_4/1 mM EDTA/1 mM EGTA/1 mM DFP, pH 7.4) at a concentration of 2×10^8 cells per 0.1 ml. The hemolysates were aliquotted and either assayed for glyceraldehyde-3-phosphate dehydrogenase or frozen at −80°C. Freezing and thawing once did not affect the activity of the enzyme under the conditions used. Glyceraldehyde-3-phosphate dehydrogenase was measured at 37°C in the forward reaction using the method of Schrier (56) except that the volume of the reaction mixture was reduced from 3 ml to 1 ml. D-Glyceraldehyde 3-phosphate was obtained from Sigma as the diethyl acetal barium salt, which was deionized on Dowex 50W (Sigma) and heated for 3 min at 100°C to obtain the free aldehyde.

Anion Transport Measurements. The "self-exchange flux" of sulfate was determined from the sulfate exchange at Donnan equilibrium, essentially following the method of Lepke and Passow (57) and Schnell *et al.* (58). Erythrocytes were assayed either the same day or 1 day after the blood was collected. Density separation was performed as described above, and the cells were washed 4 times in 40 vol of saline.

"Influx" experiments were performed according to Schnell *et al.* (58) with the following modifications (P. Hallaway and J. W. Eaton, personal communication). Washed cells were suspended at $3–4 \times 10^8$ cells per ml in Hanks' balanced salt solution, containing various concentrations of sulfate (pH 7.4) and equilibrated at 37°C for 1 hr.

RESULTS

Serum Vitamin E Levels. Serum vitamin E levels were evaluated at the end of the experiment as an independent means of determining whether the rats had been maintained on the assigned diet. The serum vitamin E measurements indicated that the assigned diets had been maintained (serum vitamin E levels: high, 1.52, 1.54, and 1.56 mg/dl; normal, 0.88 and 1.1 mg/dl; deficient, <0.05, <0.05, and 0.18 mg/dl (limits of detection, 0.05 mg/dl).

Hematologic and Serum Studies. Clinical studies indicated accelerated destruction of erythrocytes and were consistent with the vitamin E-deficient rats having a compensated hemolytic anemia (Table 1) as is observed in vitamin E-deficient humans. Serum haptoglobin was significantly reduced in vitamin E-deficient rats. Haptoglobin binds specifically and tightly to the protein (globin) in hemoglobin. The hemoglobin–haptoglobin complex is cleared within minutes

Table 1. Hematologic and serum indices of vitamin E-deficient and control rats

Index	Vitamin E diet			Deficient + vitamin E
	High	Normal	Deficient	
RBC, $\times 10^{-6}$	10.31 ± 0.08	10.39 ± 0.08	10.35 ± 0.14	
Hb, g/dl	16.65 ± 0.12	16.76 ± 0.12*	16.26 ± 0.18	
Hct, %	50.53 ± 0.38	51.26 ± 0.40*	49.6 ± 0.55	
MCV, μm^3	48.99 ± 0.21†	49.28 ± 0.14†	47.84 ± 0.16	
	(n = 9)	(n = 10)	(n = 11)	(n = 9)
Haptoglobin	5.5 ± 0.2	5.9 ± 0.4		BDL
Fe	177 ± 59	224 ± 122	229 ± 175	149 ± 61
TIBC, %	28.9 ± 0.4	29.7 ± 1.3	31.1 ± 0.1	30.8 ± 2.2

Four experiments were performed. Serum data are from the fourth experiment. The number of rats (n) from which the serum data were obtained is shown above the serum data. Haptoglobin results on pooled samples from the third experiment were as follows: high, 5.23 mg/dl; normal, 5.13 mg/dl; deficient, <0.83 mg/dl. The reticulocytes in the same experiment were as follows: high, 0.4%; normal, 0.2%; deficient, 3.38%. Results presented are the mean ± 1 SD. *$P \leq 0.05$; †$P \leq 0.001$ as compared to deficient. There was no significant difference in unconjugated bilirubin or lactate dehydrogenase. RBC, erythrocytes; Hb, hemoglobin; Hct, hematocrit; MCV, mean cell volume; TIBC, total iron-binding capacity; BDL, below the limits of detection. Detection limit is 0.83 mg/dl.

by the mononuclear–phagocyte system, while free haptoglobin has a prolonged circulation time. Reticulocytes were increased in vitamin E-deficient rats.

Phagocytosis of Old Erythrocytes from Normal Rats and Erythrocytes of All Ages from Vitamin E-Deficient Rats. The phagocytosis assay was performed on both age-separated and unseparated erythrocytes from rats fed a diet containing normal amounts of vitamin E or a diet deficient in vitamin E (Table 2). Old erythrocytes obtained from rats fed a diet containing normal amounts of vitamin E were phagocytized; whereas young and middle-aged erythrocytes were not. In contrast, young and middle-aged as well as old erythrocytes were phagocytized when obtained from vitamin E-deficient rats. There was a significant difference in phagocytosis between erythrocytes obtained from normal rats and vitamin E-deficient rats, even when unfractionated erythrocytes were used for the assay (Table 2).

To determine whether the observed defects in the erythrocytes obtained from vitamin E-deficient rats were reversible and/or were the result of oxidative events occurring *in vitro* during shipping, vitamin E was added to aliquots of blood prior to shipping, 24 hr before the samples were tested. It should be noted that the hematologic and serum studies indicate that erythrocyte destruction is occurring *in situ* (e.g., reticulocyte count and haptoglobin). Thus, it is unlikely that erythrocyte destruction is a phenomenon occurring only *in vitro*, secondary to lack of vitamin E.

Addition of vitamin E *in vitro* to erythrocytes from vitamin E-deficient rats did not alter the membrane defect that rendered these erythrocytes susceptible to phagocytosis (Table 3). However, the erythrocytes that were "reconstituted" with vitamin E *in vitro* were less susceptible to hemolysis during Coombs testing and storage than were cells from vitamin E-deficient rats to which vitamin E had not been added.

Erythrocytes from vitamin E-deficient rats both with and without vitamin E added *in vitro* had a positive direct antiglobulin test with antibodies to rat IgG.

Effect of Age and Vitamin E Deficiency on Anion Transport by Erythrocytes. We suspected that anion transport might be altered with cellular aging because our previous studies had indicated that senescent-cell antigen is derived from band 3 by cleavage in the transmembrane anion transport region (27, 30, 31). If this suspicion proved to be correct, then we would have a functional assay for aging of band 3, the major anion transport protein of the erythrocyte membrane.

Transport studies on age-separated rat erythrocytes indicated that anion transport decreased with age (Table 4). The kinetic Michaelis constant (K_m) increased and the maximal velocity (V_{max}) decreased in old erythrocytes as compared to middle-aged erythrocytes.

These data provided us with an assay of cellular function to use to determine whether erythrocytes from vitamin E-deficient rats exhibited characteristics of old erythrocytes prematurely. Results of the anion transport studies on erythrocytes from vitamin E-deficient rats revealed that their

Table 2. Phagocytosis of unfractionated and age-separated erythrocytes from vitamin E-deficient and normal rats

Vitamin E diet	Erythrocyte fraction	% phagocytosis
Normal (50 mg/kg)	Young	2 ± 3
	Middle-aged	1 ± 1
	Old	72 ± 3
	Unfractionated	13 ± 3
Deficient (0 mg/kg)	Young	89 ± 1
	Middle-aged	88 ± 1
	Old	99 ± 0
	Unfractionated	87 ± 1

The phagocytosis assay was performed with U937 cells. Erythrocytes were incubated with macrophages overnight at 37°C in a humidified atmosphere containing 5% CO_2. Data are presented as the mean ± 1 SD; n = 3. Diet is expressed as mg of vitamin E per kg of diet.

Table 3. Phagocytosis of unseparated erythrocytes from vitamin E-deficient and normal rats

Vitamin E diet	Vitamin E added *in vitro*, mg/dl	% phagocytosis
Macrophage source: WEHI-3 (mouse)		
High (200 mg/kg)	0	5 ± 10
Normal (50 mg/kg)	0	24 ± 7
Deficient (0 mg/kg)	0	85 ± 3
Deficient (0 mg/kg)	1.6	92 ± 1
Macrophage source: U937 (human)		
High (200 mg/kg)	0	5 ± 4
Normal (50 mg/kg)	0	25 ± 2
Deficient (0 mg/kg)	0	77 ± 4
Deficient (0 mg/kg)	1.6	64 ± 2

Data are presented as the mean ± 1 SD (n = 3).

Table 4. Effect of cellular aging and vitamin E deficiency on anion transport system of rat erythrocytes

	K_m, mM	V_{max}, mol × 10^{-8} per 10^8 cells per min
Cell age		
Middle-aged	0.5 ± 0.2	41.8 ± 1.9
Old	1.6 ± 0.4*	17.3 ± 3.8*
Vitamin E diet		
Normal	0.7 ± 0.1	41.2 ± 3.7
Deficient	2.0 ± 0.3*	16.2 ± 2.0*
Deficient + vitamin E	2.0 ± 0.3*	12.1 ± 1.7*

Results presented as the mean ± 1 SD. There is no statistical difference between deficient, deficient + vitamin E, and old cells. K_m, concentration at half-maximal exchange, corresponding to an apparent Michaelis–Menten constant (in mM); V_{max}, maximal flux, determined at 37°C and pH 7.2.
*$P \leq 0.01$.

anion transport was impaired, as was transport in old erythrocytes (Table 4).

Effect of Age and Vitamin E Deficiency on Glyceraldehyde-3-Phosphate Dehydrogenase Activity. We examined the erythrocyte glyceraldehyde-3-phosphate dehydrogenase activity for two reasons. First, glyceraldehyde-3-phosphate dehydrogenase is one of the enzymes that attaches to the cytoplasmic segment of band 3 (59). Second, we know from previous studies on vitamin E-deficient rhesus monkeys that glyceraldehyde-3-phosphate dehydrogenase activity is reduced in erythrocytes from these monkeys because of modifications in band 3 and not glyceraldehyde-3-phosphate dehydrogenase (48).

Our initial studies indicated that glyceraldehyde-3-phosphate dehydrogenase did not bind to rat erythrocyte membranes. All of the glyceraldehyde-3-phosphate dehydrogenase activity was present in the erythrocyte cytoplasm as soluble enzyme. Thus, glyceraldehyde-3-phosphate dehydrogenase in rats differs from that of humans, in which a fraction of the enzyme is membrane bound. Therefore, we examined glyceraldehyde-3-phosphate dehydrogenase activity to compare erythrocytes from vitamin E-deficient rats to old rat erythrocytes rather than as a "probe" for band 3.

Our studies indicate that glyceraldehyde-3-phosphate dehydrogenase activity is reduced in old erythrocytes and in erythrocytes from vitamin E-deficient rats (Table 5). Thus, cells from vitamin E-deficient rats behave like old cells with respect to glyceraldehyde-3-phosphate dehydrogenase activity.

Examination of Erythrocyte Membranes from Vitamin E-Deficient Rats Using Glycoprotein and Protein Stains, and Immunoblotting with Antisera to Band 3. Differences were not detected in protein or glycoprotein composition of erythrocyte membranes from control and vitamin E-deficient rats. Although high molecular weight polypeptides or polymers were detected with Coomassie blue staining of 2–16% polyacrylamide gels, there were no differences in the number or amount of these polypeptides between control and experimental samples. Immunoblotting studies revealed increased breakdown products of band 3 in cells from vitamin E-deficient rats (Fig. 1).

DISCUSSION

As a mechanism for cellular aging and generation of senescent-cell antigen, free-radical reactions and oxidation are considered probable candidates (25). Most free-radical reactions involve the reduction of molecular oxygen, leading to the formation of highly reactive oxygen, species such as superoxide anion ($O_2^{\cdot}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). The production of

Table 5. Effect of cellular aging and vitamin E deficiency on glyceraldehyde-3-phosphate dehydrogenase activity in rat erythrocytes

	Enzyme activity, IU per 10^{10} cells
Cell age	
Young	0.209 ± 0.045 (3)
Middle-aged	0.200 ± 0.022 (4)
Old	0.091 ± 0.030 (6)
Vitamin E diet	
High	0.201 ± 0.038 (4)
Normal	0.199 ± 0.023 (7)
Deficient	0.103 ± 0.015 (7)
Deficient + vitamin E	0.116 ± 0.15 (3)

Enzyme activities are expressed as international units (IU) (mean ± 1 SD). The number of determinations is indicated in parentheses. Glyceraldehyde-3-phosphate dehydrogenase values are significantly lower for old than for middle-aged cells ($P \leq 0.01$). Deficient and deficient + vitamin E glyceraldehyde-3-phosphate dehydrogenase values are significantly lower than normal and high ($P \leq 0.01$).

these highly reactive oxygen species as metabolic intermediates appears to be an evolutionary consequence of aerobic existence because the spin state of oxygen favors univalent pathways of reduction (60).

We used vitamin E deficiency as a model for studying oxidation because studies show that, in mammals, vitamin E functions as an antioxidant, and because vitamin E deficiency simulates conditions encountered *in situ* more closely than does chemical treatment of cells *in vitro*.

The results presented here indicate that vitamin E deficiency causes premature aging of erythrocytes and IgG binding. Erythrocytes from vitamin E-deficient rats behave like old erythrocytes in the phagocytosis assay, and in anion transport and glyceraldehyde-3-phosphate dehydrogenase activity. In addition, increased breakdown products of band 3 were observed in erythrocyte membranes from vitamin E-deficient rats. We have not observed high molecular weight complexes containing band 3 in membranes from vitamin E-deficient rats or old cells aged *in situ*, except under conditions that precipitate IgG (unpublished observations).

Other investigators have reported oligomers and polymers of band 3 in cells treated with ionophore A23187 plus calcium (61) and in processed Triton extracts of old cells (32). Although we have not been able to demonstrate high molec-

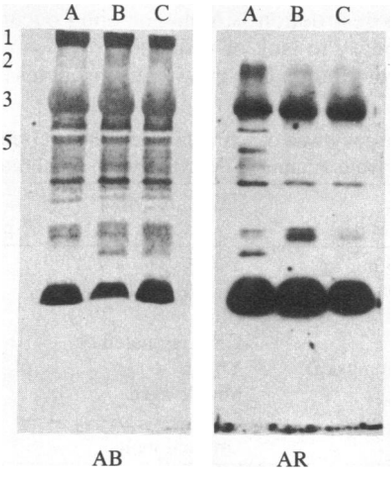

FIG. 1. Immunoblots of erythrocyte membrane proteins from vitamin E-deficient and normal rats incubated with antibodies to band 3. AB, amido black; AR, autoradiograph. Lanes: A, vitamin E deficient; B, normal; C, high. Band designations are at left.

ular weight band 3 complexes on cells aged *in situ*, we speculate that they exist, but that they are short-lived presumably because their presence is "life-threatening" to the cell. We postulate that proteolytic cleavage of "cross-linked" band 3 occurs to preserve membrane fluidity. We think it unlikely that dimerization of band 3 generates senescent-cell antigen as suggested by others (32). Data from many laboratories indicate that band 3 normally exists as a dimer or tetramer in the erythrocyte membrane (62, 63). Senescent-cell antigen does not appear to be an exposed galactose moiety as suggested by some investigators (64). Recent evidence indicates that senescent-cell IgG does not have an α- or β-galactose specificity (22, 65) and that the protein component of band 3 is required for binding of senescent-cell IgG (65).

Anion transport across the membrane, which is the best-defined physiological function of band 3, is reduced by both cellular aging and vitamin E deficiency. Differences between normal unseparated rat and human erythrocytes are observed in the position of the concentration maximum [e.g., K_m = 0.5–0.8 mM for rat cells and 3–5 mM for human cells (unpublished observations)]. Also, the maximal sulfate transport activity of rat erythrocytes was found to be 2–3 times as high as that of human erythrocytes (unpublished observations), in agreement with the data reported by Gruber and Deuticke (66) for phosphate ions.

The decrease in transport activity observed with vitamin E deficiency is not reversed by addition of vitamin E *in vitro*. This latter observation may indicate that the observed effects are not caused by a direct effect of vitamin E on membrane fluidity.

A decrease in band 3 functioning with erythrocyte age supports the data indicating that alteration of band 3 occurs during erythrocyte aging and generation of senescent-cell antigen (27, 30, 31). Impairment of anion transport was anticipated based on our previously published data indicating that senescent-cell antigen is generated by cleavage of band 3 in the anion transport region (27).

Results of the experiments reported here suggest that oxidation can cause aging of band 3. We suspect that this may be one of the mechanisms of cellular aging *in situ*. At this time, it appears that general cellular damage such as lysis (unpublished observations), cross-linking of band 3 by hemoglobin (34), and oxidation can result in the generation of senescent-cell antigen. This suggests to us that many different cellular insults have a final common pathway that results in generation of senescent-cell antigen.

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