Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5834–5838, August 1989
Cell Biology

Alteration in membrane protein band 3 associated with accelerated erythrocyte aging

(autoantibodies/anion and glucose transport/proteolysis/ankyrin binding/senescent cell antigen)

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Communicated by Herbert E. Carter, March 31, 1989 (received for review February 19, 1989)

ABSTRACT **We report a human band 3 alteration that is associated with anemia as determined by a reticulocyte count of 20%. Erythrocyte defects included increased IgG binding, increased breakdown products of band 3, and altered anion- and glucose-transport activity in middle-aged cells. These changes were observed during normal erythrocyte aging *in situ*. Binding of ankyrin to band 3 was normal. Serum/cell crossover studies indicated that a neoantigen appears on the propositus' erythrocytes to which IgG from both propositus and control serum binds as measured with a protein A binding assay. IgG eluted from the propositus' erythrocytes appeared to have a specificity for senescent cell antigen as determined by a phagocytosis inhibition assay. Immunoelectron microscopy showed that antibodies to band 3, which do not normally bind to intact erythrocytes, bound to the propositus' erythrocytes. Antibody 980 binds to normal old cells but not young or middle-aged cells. It also binds to a distinct region of band 3 in immunoblots of membranes from the propositus' middle-aged cells. Cells from both of the propositus' parents exhibited increased IgG binding and altered anion and glucose transport. The results of these studies suggest that (*i*) band 3 is aging prematurely in erythrocytes from the propositus, (*ii*) senescent cell antigen appears on the propositus' middle-aged red cells, and (*iii*) band 3 alterations observed in the propositus may have a genetic component.**

As part of our ongoing studies on mechanisms of cellular aging, we searched for models of accelerated and decelerated cellular aging. We anticipated that such models would allow us to dissect molecular aging and provide insight into mechanisms. Initially, we investigated models for aging *in vitro* (1).

We subjected intact human erythrocytes to treatments that have been reported to result in changes in band 3 (the major anion-transport polypeptide) and/or to mimic aging *in vitro*. The validity of these treatments as model systems for erythrocyte aging was evaluated by using a "red cell aging panel" that provides a biochemical profile of a senescent red cell (1, 2). Neither incubation with the free radical-generating xanthine oxidase/xanthine system nor treatment with malondialdehyde, an end product of free radical-initiated lipid (per)-oxidation, resulted in age-specific changes (1). Loading of the cells with calcium and oxidation with iodate resulted in increased breakdown of band 3 but did not lead to increased binding of autologous IgG. Only erythrocytes that had been stored for 3–4 weeks showed the same structural and functional changes as observed during aging *in vivo*.

We then began a search for "experiments of nature" that might provide insights into the process of normal cellular aging (2). Initially, we studied glucose-6-phosphate dehydrogenase (G6PD) deficiency and hemoglobin Köln as potential models (2). Membranes from both the G6PD-deficient and the hemoglobin Köln cells that we studied have been reported to contain high molecular weight polymers (3). In addition, hemoglobin Köln cells contain hemoglobin precipitates. However, accelerated cellular aging was not present as determined by a red cell aging panel including lack of phagocytosis and IgG binding to young and middle-aged erythrocytes, normal ankyrin binding, normal anion transport, normal glyceraldehyde-3-phosphate dehydrogenase activity, and no increase in band 3 breakdown products (2).

Therefore, we began a search for mutations and/or clinical alterations of erythrocyte band 3. Our search for band 3 protein alterations resulted in the discovery of two different ones (4, 5). One alteration, high molecular weight band 3, results from an addition of tyrosine-containing peptides in the transmembrane, anion-transport region of band 3 (4). It appears to result from an autosomal recessive mutation. This mutation is associated with acanthocyte ("thorny" cell) formation. However, erythrocyte survival is normal *in situ* as determined by the reticulocyte count, and the erythrocytes do not exhibit accelerated aging as determined by the red cell aging panel (4).

A second band 3 alteration also exhibits acanthocytosis (5). This alteration is associated with ion- and glucose-transport abnormalities and neurologic disease. The neurologic disease, choreoacanthocytosis, is an autosomal recessive.

In this paper, we describe a band 3 alteration that is characterized by accelerated cellular aging, as determined by a red cell aging panel, and cellular removal. The propositus' reticulocyte count is ≈20%, indicating the destruction and replacement *in situ* of 20% of circulating erythrocytes daily. Both peripheral blood findings (e.g., the presence of nucleated erythrocytes and precursors of monocytes and lymphocytes) and bone marrow biopsy are consistent with a hemolytic anemia. We gave this band 3 alteration the descriptive name "fast-aging" band 3 because the propositus' young and middle-aged cells exhibit all the characteristics of old erythrocytes (increased IgG binding, decreased anion and altered glucose transport, and increased breakdown products of band 3 as observed on immunoblots). Immunoelectron microscopy showed that antibodies to band 3, which do not bind to normal intact erythrocytes, bind to intact middle-aged erythrocytes from the propositus. Antibodies to aged band 3, which do not bind to normal young or middle-aged red cells, bind to a distinct region of band 3 in immunoblots of membranes of the propositus' middle-aged red cells. We suspect that fast-aging band 3 is more susceptible to proteolysis than is normal band 3.

MATERIALS AND METHODS

General Cellular Methods. Erythrocytes from the propositus were studied in 10 different experiments over 27 months. Family members were studied in three different experiments. Red cells were separated into populations of different ages on Percoll gradients (6). The amount of IgG on cells was

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quantitated with ^{125}I-labeled protein A (7). Autoantibody was eluted from erythrocyte membranes prepared by digitonin lysis (8, 9) and the phagocytosis inhibition assay was performed (8–11).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis and Immunostaining Methods. Proteins were analyzed in NaDodSO$_4$/polyacrylamide 6–25% linear gradient gels with the discontinuous buffer system of Laemmli (12). To remove carbohydrates, washed erythrocytes were treated with 9 units of endoglycosidase F (New England Nuclear) in 10 volumes of buffer for 2 hr at 37°C (4). Enzymatic fragments of erythrocytes were generated by standard methods (4, 13, 14). Rabbit antibody to band 3 and antibody 980, a rabbit antibody to aged band 3 (precursor to senescent cell antigen), were prepared as described (15–17). Monoclonal antibodies were a gift from Michael Jennings (University of Texas at Galveston). Immunoautoradiography was performed by the immunoblotting technique of Towbin *et al.* (18) with the modifications described previously (10, 13, 15, 16).

Transport Measurements. The "self-exchange flux" of sulfate was determined at Donnan equilibrium, essentially by the methods of Lepke and Passow (19) and Schnell *et al.* (20). Measurements were made at six concentrations of sulfate between 2 and 30 mM and at three time points within the first 8 min after the start of the assay. K_m, the sulfate concentration at which half the maximal velocity, V_{max}, is reached, was determined by extrapolation from the linear part of the velocity vs. concentration graph. Characteristics of the glucose transport system (zero trans-exit and infinite trans-entry) were determined by the methods of Naftalin *et al.* (21). V_{max} and K_m of zero trans-efflux (22) and V_{max} of infinite trans-entry were calculated from an integrated rate equation (19).

Ankyrin Binding Studies. Ankyrin binding was studied by the procedure of Hargreaves *et al.* (23) as described by Goodman *et al.* (24). Data were corrected for nonspecific binding (24) and plotted by using the Scatchard equation (25). Protein was determined according to Lowry *et al.* (26).

Immunoelectron Microscopy. Immunoelectron microscopy was performed as described (27, 28). Control and experimental cells were incubated with rabbit anti-human band 3 or preimmune serum, washed, and incubated with F(ab')$_2$ goat anti-rabbit IgG conjugated to gold colloid.

RESULTS

IgG Binding to Erythrocytes. IgG binding to unseparated and middle-aged cells from individuals with fast-aging band 3 was higher than that of normal controls (Table 1).

Antibodies Bound to Fast-Aging Erythrocytes Are Directed Against Altered Band 3. The propositus had IgG on middle-aged as well as old red cells, in contrast to normal individuals, who have significant amounts of IgG only on old erythrocytes (Table 1; refs, 1, 2, 4, 8–11). The presence in the propositus' serum of an "abnormal" autoantibody against normal erythrocytes was excluded on the basis of crossover experiments in which control cells were incubated in either autologous or propositus serum, and propositus cells were incubated in autologous or control serum. As determined by binding of ^{125}I-labeled protein A, significantly more IgG bound to the propositus' cells regardless of whether incubations were performed in autologous or control serum (Table 2). This indicates that IgG in normal serum recognizes the antigen appearing on the propositus' erythrocytes and that the defect resides in those erythrocytes. Incubation of the propositus' erythrocytes in control serum resulted in increased IgG binding as compared to incubations in autologous serum ($P \leq 0.001$), suggesting that the propositus' serum is depleted of a normally occurring antibody, presumably physiologic IgG autoantibody against senescent cell antigen.

Table 1. IgG binding to erythrocytes from individuals with normal or fast-aging band 3

Individual	Cell age	Bound IgG, no. of molecules per cell
	Experiment 1	
Control	Unseparated	8 ± 2
Propositus	Unseparated	106 ± 10†‡
Control	Middle	9 ± 2
Propositus	Middle	99 ± 4‡
Control	Old*	102 ± 11‡
Propositus	Old*	119 ± 12‡
	Experiment 2	
Control	Unseparated	10 ± 4
Propositus	Unseparated	198 ± 11†
Control	Young/middle	11 ± 5
Propositus	Young/middle	92 ± 25†
	Experiment 3	
Control	Middle	10 ± 3
Propositus	Middle	68 ± 10‡
Father	Middle	57 ± 8‡
Mother	Middle	33 ± 6‡
Sibling 1	Middle	6 ± 1
Sibling 2	Middle	15 ± 3§
Control	Old*	89 ± 19‡
Propositus	Old*	229 ± 20‡

IgG binding was determined by using ^{125}I-labeled protein A. Data are presented as mean ± SD of 8–12 determinations.
*Second band of four bands of old cells on Percoll gradient.
†$P \leq 0.001$ compared to control cells.
‡$P \leq 0.001$ compared to control middle-aged cells.
§$P \leq 0.05$ compared to control middle-aged cells.

These data suggested that the autoantibody might be against senescent cell antigen, which is derived from band 3. To test this hypothesis, a phagocytosis inhibition assay was performed. Stored erythrocytes were phagocytosed when they were incubated with IgG eluted from fast-aging cells (28 ± 11% phagocytosis; $P \leq 0.01$ compared to controls) but not when they were incubated with IgG eluted from an equal volume of control cells (0% phagocytosis) or with IgG eluted from fast-aging cells and absorbed with purified senescent cell antigen (0% phagocytosis).

Table 2. Characterization of antibody binding to fast-aging erythrocytes by crossover studies

Days in serum	Cell source	Serum source	Bound IgG, no. of molecules per cell
1	Control	Propositus	22 ± 4†
	Propositus	Control	131 ± 5‡
	Control	Control	16 ± 3
	Propositus	Propositus	41 ± 3
6	Control	Control	51 ± 9
	Propositus	Control	386 ± 88‡
	Control	Propositus	29 ± 1§
	Propositus	Propositus	103 ± 11‡
10	Control	Control	58 ± 5
	Propositus	Control	1092 ± 32‡
	Control	Propositus	44 ± 4
	Propositus	Propositus	261 ± 13‡

Results of Scatchard analysis of IgG binding to erythrocytes are expressed as mean ± SD of four concentrations of protein A (n = 4–8). Cells were received on day 1.
*IgG bound was calculated by using a standard curve (7) for day 1.
†$P \leq 0.05$ for control cells in control serum compared to propositus cells in control serum.
‡$P \leq 0.001$ for propositus cells in propositus serum compared to propositus cells in control serum.
§$P \leq 0.01$ for control cells in control serum compared to propositus cells in control serum.

Antibody 980, which was raised against aged band 3, bound to a distinct region of band 3 in immunoblots of membranes prepared from the propositus' cells and from normal old cells but not to band 3 in immunoblots of membranes from normal young and middle-aged cells (Fig. 1). Antibody 980 did not bind to band 3 of membranes of erythrocytes with a different band 3 alteration, high molecular weight band 3 (Fig. 1*B*), which does not result in shortened erythrocyte life-span (4).

These results suggest that senescent cell antigen and/or its precursor molecule appears prematurely on the propositus' erythrocytes. Thus, they appear to be aging prematurely.

Immunoelectron microscopy studies further suggested an abnormal band 3 in the propositus' erythrocyte membranes. The propositus' cells were labeled with rabbit antibody to band 3, whereas normal control cells were not (Fig. 2). Preimmune rabbit serum labeled neither propositus cells nor control cells. We previously showed that neither human nor rabbit antibodies to band 3 bind to normal erythrocytes (28). Even enzymatic digestion of normal erythrocytes with trypsin or chymotrypsin did not result in the level of binding observed with the propositus' cells (28). The number of antibody binding sites is sufficient to cause aggregation following incubation with antibodies.

Increased Breakdown of Band 3 from Fast-Aging Cells. Increased band 3 breakdown products were observed in immunoblotting studies (Fig. 3). Changes in membrane proteins other than band 3 were not detected. The electrophoretic mobility of band 3 from both control and propositus cells was increased to the same extent following treatment with endoglycosidase F, as would be expected following removal of glycosyl groups.

Anion Transport. Unseparated and middle-aged red cells from the propositus had abnormally decreased anion transport (increased K_m, decreased V_{max}; Table 3). The mother exhibited an increased K_m. The parents' V_{max} of sulfate transport was normal in two experiments and decreased in the other. The father, two siblings, and two daughters exhibited normal K_m of anion transport. Anion transport was inhibited by 4,4′-diisothiocyanato-2,2′-stilbenedisulfonate (DIDS). [^{3}H]DIDS binding was the same for propositus (350 ± 9 cpm) and control cells (330 ± 25 cpm). Increased K_m and decreased V_{max} are observed in normal old human and rat erythrocytes (10).

Glucose Transport. Erythrocytes from the propositus exhibited slower glucose uptake (influx V_{max}) and faster release (zero trans-efflux V_{max}) than normal cells (Table 4). The same changes were observed in normal old erythrocytes. Cells from both parents showed decreased glucose influx and increased glucose efflux.

Ankyrin Binding. The number of high-affinity ankyrin binding sites and the dissociation constant (K_d) for fast-aging cells were the same as for control cells (data not shown).

Analysis of Band 3 Domains for Structural Alterations. The anion transport studies suggested that the individual with fast-aging band 3 had an alteration in the anion-transport region of the protein. As a more definitive approach to localizing and defining alterations in band 3, we performed immunoblotting studies with monoclonal antibodies II E 1 (to the M_r 17,000 transmembrane segment of band 3) and IV F 12 [to the 150-residue region that is involved in ion transport (29) on the M_r 35,000 carboxyl-terminal segment of band 3]. Antibodies were used to probe immunoblots of both intact and chymotrypsin-treated membranes (see legend to Fig. 4 for details). Results with monoclonal antibody II E 1 showed that band 3 in inside-out vesicles prepared from fast-aging red cell membranes was more susceptible to enzymatic digestion because it was degraded to smaller fragments. Band 3 degradation produced a M_r 17,000 fragment and a fragment migrating just below band 7 that was labeled with monoclonal antibody II E 1 (Fig. 4). For molecular weight comparisons, band 7 has M_r 29,000 and band 8 has M_r 24,000. Monoclonal antibody IV F 12, directed against the M_r 35,000 carboxyl-terminal segment, revealed only a faint band at M_r 60,000 in autoradiographs of immunoblots of fast-aging "60R" membranes (data not shown), corresponding to the band at M_r 60,000 in the amido black stain (Fig. 4, AB, lane f), suggesting degradation to smaller fragments. A much larger band was observed at M_r 60,000 in control "60R" membranes. In addition, there was a band 3 breakdown product migrating in the high 4.5 region in undigested membranes from fast-aging but not control cells.

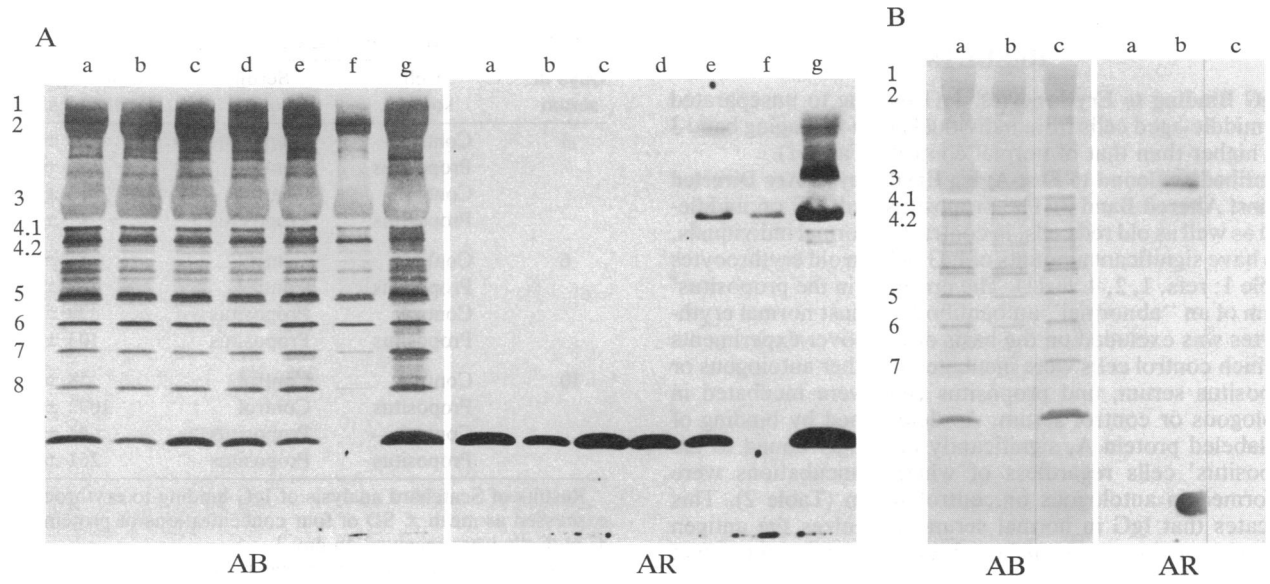

FIG. 1. Binding of antibody 980 (anti-aged band 3) to immunoblots of erythrocyte membranes. Erythrocyte protein bands are numbered at left. AB, amido black stain for proteins; AR, autoradiograph. (*A*) Lanes: a, normal young cells; b, normal upper middle-aged; c, normal middle middle-aged; d, normal lower middle-aged; e, normal old (first band); f, normal old (second band, oldest erythrocytes); g, fast-aging band 3 (middle-aged erythrocytes from the propositus). (*B*) Lanes: a, normal membranes; b, from individual with fast-aging band 3; c, from individual with high molecular weight band 3 mutation.

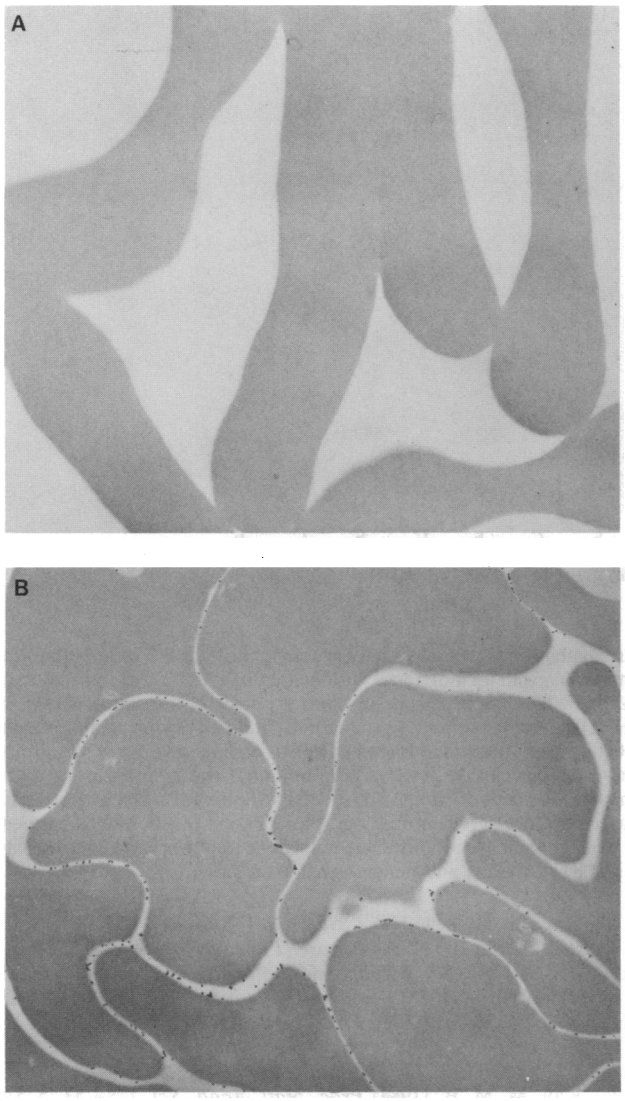

FIG. 2. Electron micrographs of normal (*A*) and fast-aging (*B*) erythrocytes incubated with antibodies to band 3 followed by gold colloid conjugated to anti-rabbit IgG.

DISCUSSION

Middle-aged red cells with fast-aging band 3 exhibit the following characteristics of senescent erythrocytes (1): increased IgG binding, decreased sulfate transport (increased K_m, decreased V_{max}), decreased glucose influx and increased glucose efflux (V_{max}), and increased breakdown of band 3. In addition, antibodies to aged band 3 bind to band 3 in fast-aging cells, but not in normal control cells or in cells with a band 3 mutation that does not result in accelerated aging or shortened erythrocyte life-span. This latter mutation results in additional peptides in the anion-transport region of band 3 (9). Immunoelectron microscopy with antibodies to band 3 also suggests an altered band 3 in fast-aging cells. Furthermore, band 3 in inverted vesicles from fast-aging cells is more sensitive to chymotrypsin digestion than that from control cells.

Our interpretation of the data is that fast-aging band 3 results from a cellular alteration that renders the band 3 molecule more susceptible to proteolysis than normal band 3 under "stress" conditions. We think that the basic, underlying band 3 alteration (which we suspect is genetic) exhibited by erythrocytes from the propositus and family members is independent of the propositus' disease process but is a contributing, perhaps predisposing, factor. Erythrocytes from the propositus showed accelerated aging based on the

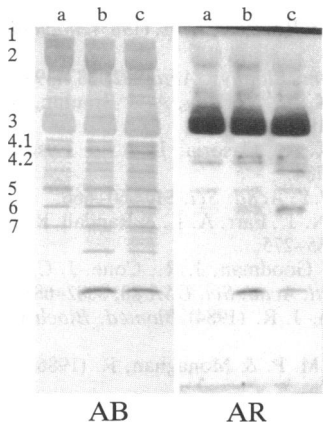

FIG. 3. Immunoblotting studies of membranes from normal and fast-aging band 3 erythrocytes. AB, amido black stain for proteins: AR, autoradiograph. Lanes: a, membranes prepared from erythrocytes of normal individuals maintained as donors by our laboratory and prepared immediately; b, control membranes drawn and prepared with those of the experimental subjects; c, propositus membranes.

Table 3. Sulfate transport by erythrocytes from individuals with normal or fast-aging band 3

Individual	Cell age	K_m, mM	V_{max}, nmol per 10^8 cells per min
		Experiment 1	
Control	Middle	0.9 ± 0.1	132 ± 14
Control	Old	1.6 ± 0.1*	77 ± 11*
		Experiment 2	
Control	Middle	0.8 ± 0.1	112 ± 8
Propositus	Middle	1.1 ± 0.1*	68 ± 8†
Mother	Middle	1.0 ± 0.1‡	83 ± 5*
Father	Middle	0.8 ± 0.1	93 ± 6‡
		Experiment 3	
Control	Middle	1.0 ± 0.1	125 ± 7
Propositus	Middle	1.4 ± 0.1*	98 ± 5*
Father	Middle	0.9 ± 0.1	125 ± 9
Mother	Middle	1.9 ± 0.1*	127 ± 10
Sibling 1	Middle	1.2 ± 0.1	111 ± 7
Sibling 2	Middle	0.8 ± 0.1	117 ± 10

Data are presented as mean ± SD. Anion transport by cells from both daughters tested was normal (data not shown).
*$P \leq 0.01$ compared to control middle-aged cells.
†$P \leq 0.001$ compared to control middle-aged cells.
‡$P \leq 0.05$ compared to control middle-aged cells.

Table 4. Glucose transport by erythrocytes from individuals with normal or fast-aging band 3

Individual	Cell age	V_{max}, μmol per ml of cells per min: Influx	Zero trans-efflux
		Experiment 1	
Control	Middle	10.4 ± 1.2	15.8 ± 0.6
Control	Old	8.6 ± 1.4*	29.1 ± 5.8†
		Experiment 2	
Control	Middle	13.2 ± 1.0	17.1 ± 1.1
Propositus	Middle	8.1 ± 1.1‡	21.2 ± 0.7‡
		Experiment 3	
Control	Middle	12.1 ± 1.2	17.1 ± 0.7
Propositus	Middle	9.2 ± 0.7*	20.1 ± 0.9‡
		Experiment 4	
Control	Middle	10.0 ± 1.3	17.2 ± 1.0
Propositus	Middle	5.7 ± 0.4‡	30.2 ± 1.6§
Father	Middle	7.3 ± 0.1*	22.8 ± 1.0§
Mother	Middle	6.5 ± 0.1‡	20.4 ± 0.4‡

Data are presented as mean ± SD.
*$P \leq 0.05$ compared to control middle-aged cells.
†$P \leq 0.01$ compared to control middle-aged cells.
‡$P < 0.01$ compared to control middle-aged cells.
§$P < 0.001$ compared to control middle-aged cells.

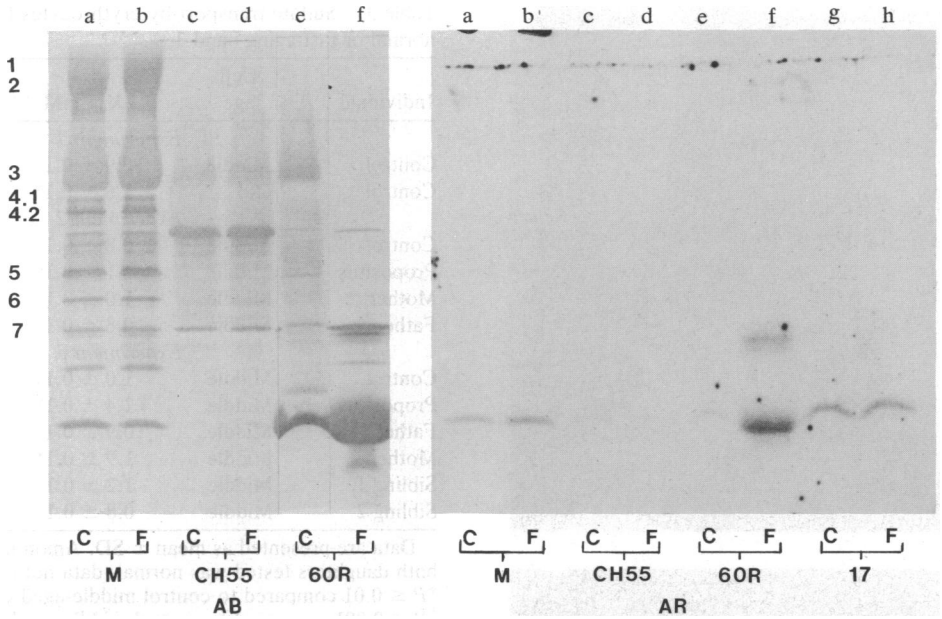

FIG. 4. Binding of monoclonal antibody (II E 1) against the M_r 17,000 transmembrane, anion-transport segment of band 3 to immunoblots of defined proteolytic fragments of band 3 from control membranes and membranes from an individual with fast-aging band 3. AB, amido black stain for proteins; AR, autoradiograph; M, untreated membranes; C, control; F, fast-aging. Lanes: a, control untreated membranes (M); b, membranes from individual with fast-aging band 3; c, control CH55 and CH38 fragments of band 3 produced by mild chymotrypsin treatment of intact red cells; d, fast-aging CH55 and CH38 fragments; e, control M_r 60,000 carboxyl-terminal fragment (60R) remaining with the membrane after removal of CH40 cytoplasmic segment by chymotrypsin treatment of inverted vesicles depleted of peripheral membrane proteins by alkali treatment; f, fast-aging 60R; g, control M_r 17,000 transmembrane, anion-transport fragment of band 3 produced by treatment with chymotrypsin at both sides of the membrane; h, fast-aging M_r 17,000 fragment. The bands at the bottom of the autoradiograph represent the M_r 17,000 segment of band 3. During the blot transfer process, band 3, CH55, and 60R migrated through the nitrocellulose sheet because they were in lower-density areas of the 6–25% gradient gel. The M_r 17,000 fragment was in the densest part of the gel and hence transferred slowly.

red cell aging panel. Although other family members (mother and father) show subtle band 3 changes, their cells do not show accelerated removal *in situ*. This suggests that they may each have a subtle, perhaps different, alteration that combines and contributes to the propositus' band 3 alteration and/or susceptibility to damage. If this is genetic, it is probably an autosomal recessive mutation or a change at different alleles in each parent that combines in the propositus.

The immunologic band 3 defects revealed by binding of antibodies to aged band 3, antibodies to band 3, and IgG *in situ* and *in vitro* to the propositus' cells may be responsible for the anemia.

We are grateful to Dr. G. Johnson for the patient samples, to Dr. T. Wajima for reading the peripheral blood and bone marrow slides, and to Dr. M. Jennings for monoclonal antibodies to band 3 fragments. We thank J. Hughes, R. Pierre, R. Poff, and G. Purser for their assistance. This research was supported by the Veteran's Administration Research Service and National Institutes of Health Grant AG08574.

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