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Brain membrane protein band 3 performs the same functions as erythrocyte band 3

(senescent cell antigen/synthetic peptides/aging/anion transport/cerebellum)

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ABSTRACT **We report the presence of band 3 protein(s) in mammalian brain that performs the same functions as those of erythroid band 3. These functions are anion transport, ankyrin binding, and generation of senescent cell antigen, an aging antigen that terminates the life of cells. Structural similarity of brain and erythroid band 3 is suggested by the reaction of antibodies to synthetic peptides of erythroid band 3 with brain band 3, the inhibition of anion transport by the same inhibitors, and an equal degree of inhibition of brain and erythrocyte anion transport by synthetic peptides of erythroid band 3 (pep-ANION 2, residues 588–602; pep-COOH, residues 812–827; pep-COOH-N6, residues 813–818). One of these segments, pep-COOH, contains antigenic determinants of senescent cell antigen. These findings suggest that the transport domains of erythroid and neural band 3 are similar functionally and structurally and support the hypothesis that the immunological mechanism of maintaining homeostasis is a general physiologic process for removing senescent and damaged cells in mammals and other vertebrates.**

The presence of band 3-related molecules in nonerythroid tissues was first demonstrated in 1983 (1). A protein immunologically related to band 3 was demonstrated in such diverse cell types as isolated neurons, hepatocytes, squamous epithelial cells, alveolar (lung) cells, lymphocytes, neurons *in vivo*, and fibroblasts by using an antibody to band 3 that reacts with the transmembrane, anion transport domain of band 3 (1). The band 3-like protein in many of these cell types appeared to be a truncated version of the erythroid protein based on its molecular mass of ≈60 kDa estimated from its migration in polyacrylamide gels. We suggested that part of the cytoplasmic amino terminus segment was missing from the band 3-like protein in these cell types and that band 3 protein was modified to perform functions in different environments (1). Since then, band 3 has been described in numerous cell types and tissues, including fibroblasts, hepatoma cells, and lymphoid cells (2–6). Band 3 is also present in nuclear (1), Golgi (7), and mitochondrial membranes (8) as well as in cell membranes. Band 3-like proteins in nucleated cells participate in band 3 antibody-induced cell surface patching and capping (1). A truncated version of band 3, which lacks the amino terminus, has also been described in kidney (4, 5). Band 3 is present in the central nervous system, and differences have been described in band 3 between young and aging brain tissue (9). One autosomal recessive neurological disease, choreoacanthocytosis, is associated with band 3 abnormalities (10, 11). The 150 residues of the carboxyl terminus segment of band 3 appear to be altered (11). In brains from Alzheimer disease patients, antibodies to aged band 3 label the amyloid core of classical plaques and the microglial cells located in the middle of the plaque in tissue sections and an abnormal band 3 in immunoblots (9, 12). However, the question of whether band 3 in nonerythroid tissues performs the same functions as it does in erythrocytes has not been addressed.

In erythrocytes, band 3 constitutes >30% of the total membrane protein (13, 14). It maintains acid–base balance by mediating the exchange of anions (e.g., chloride, bicarbonate) (for review, see ref. 15) and contributes to respiration by catalyzing CO_2 exchange.

Degradation of band 3 generates senescent cell antigen, an antigen of aging cells (16–26). Senescent cell antigen is generated on the transport domain. Senescent cell antigen is a protein that appears on old cells and acts as a specific signal for the termination of that cell by initiating the binding of IgG autoantibody and subsequent removal by phagocytes (16–26). This appears to be a general physiologic process for removing senescent and damaged cells in mammals and other vertebrates (21). Although the initial studies were done with human erythrocytes as a model, senescent cell antigen has been found on all cells examined (21). Besides its role in the removal of senescent and damaged cells, senescent cell antigen also appears to be involved in the removal of erythrocytes in clinical hemolytic anemias (24, 27) and the removal of malaria-infected erythrocytes (28). Oxidation generates senescent cell antigen *in situ* (23).

Since band 3 is such a crucial structural and functional protein and is intimately involved in cellular aging, we investigated its location, structure, and function in mammalian brain. We then determined whether senescent cell antigen, a terminal differentiation antigen, is generated in brain.

MATERIALS AND METHODS

Tissue Preparation and Immunohistochemical Analysis. Tissue preparation and immunohistochemical analysis were performed as described (29). Perfused brains from young Sprague–Dawley rats were used for immunoblotting and biochemical studies.

Brain and Erythrocyte Anion Transport. "Influx" and peptide inhibition of sulfate influx experiments were performed as described (12, 29, 30, 31).

Ankyrin Binding Studies. Binding of ^{125}I-radiolabeled ankyrin (2–20 μg/ml) to inside-out vesicles (40–60 μg/ml) from erythrocytes or brain membranes depleted of cytoskeletal proteins by 0.1 M NaOH treatment was performed as described (12, 32, 33). As an additional control, we performed competitive inhibition studies with the 40-kDa cytoplasmic segment of erythroid band 3.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis and Immunostaining of Membrane Proteins. Proteins were analyzed on

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Abbreviation: DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid.

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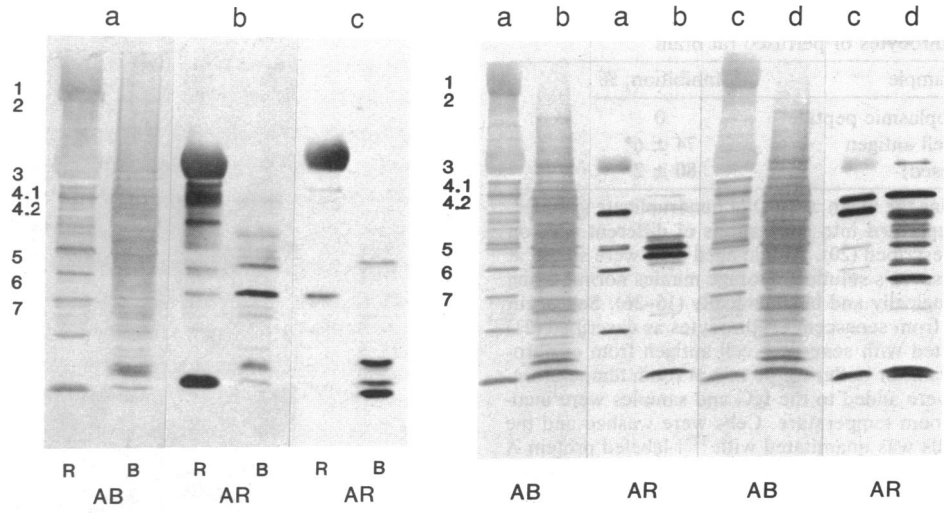

FIG. 1. Antibodies to synthetic peptides of human erythroid band 3 bind to band 3 from perfused rat brains in immunoblots. AB, amido black stain for proteins; AR, autoradiograph. (*Left*) Lanes: R, erythrocyte membranes; B, brain membranes; a, amido black; b, antibodies to pep-COOH (residues 812–827, Leu-Phe-Lys-Pro-Pro-Lys-Tyr-His-Pro-Asp-Val-Pro-Tyr-Val-Lys-Arg); c, antibodies to pep-CYTO (residues 122–144, Ala-Gly-Val-Ala-Asn-Gln-Leu-Leu-Asp-Arg-Phe-Ile-Phe-Glu-Asp-Gln). (*Right*) Lanes: a and c, erythrocyte membranes; b and d, brain membranes; a and b, antibodies to pep-ANION 1, an external loop of band 3 containing a 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS) binding site (residues 538–554, Ser-Lys-Leu-Ile-Lys-Ile-Phe-Gln-Asp-His-Pro-Leu-Gln-Lys-Thr-Tyr-Asn); c and d, antibodies prepared against a mixture of the peptides COOH, CYTO, ANION 1, and ANION 2 (residues 588–602, Leu-Arg-Lys-Phe-Lys-Asn-Ser-Ser-Tyr-Phe-Pro-Gly-Lys-Leu-Arg), an anion transport region.

6–25% or 12–25% linear $NaDodSO_4$/polyacrylamide gradient gels with a discontinuous buffer (34). Immunoautoradiography was performed by the immunoblotting technique (1, 24, 34). The rabbit antibodies to whole human erythroid band 3 used (1, 24, 34) react with peptides from all segments of the band 3 molecule including the carboxyl terminus.

IgG Binding and Inhibition Assay. Senescent cell IgG (3 μg) was absorbed with brain membranes or purified senescent cell antigen as described (19, 21) and incubated with an indicator erythrocyte. The number of IgG molecules bound to the indicator erythrocyte was quantitated before and after absorption by equilibrium binding kinetics (16, 17, 24, 34, 35).

RESULTS

Detection of Polypeptides Immunologically Related to Erythroid Band 3 and Senescent Cell Antigen in Immunoblots with Antibodies to Human Erythroid Band 3 and Synthetic Peptides of Band 3. Antibodies to synthetic peptides of human erythroid band 3 reacted with band 3 and breakdown products of band 3 in erythrocyte membranes and multiple polypeptides immunologically related to erythrocyte band 3 in membranes from perfused rat brains (Fig. 1). Band 3 breakdown products have been found to migrate in ranges between ≈5 and ≈85 kDa in erythrocytes (21–26). Some of the band 3 antibody reactive bands migrating at different molecular masses in brain may be

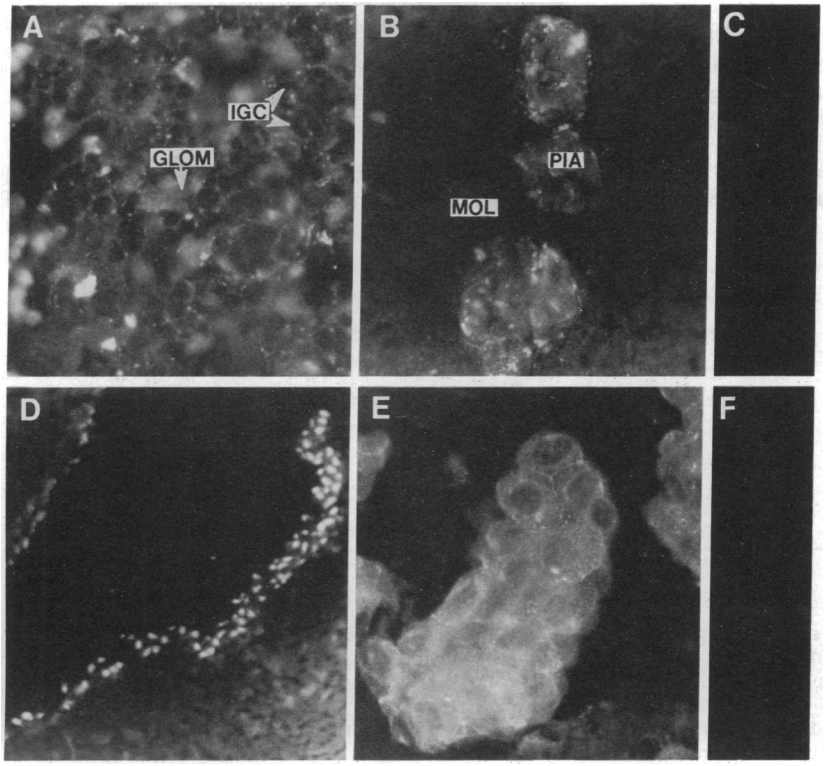

FIG. 2. Antibodies to synthetic peptides of human erythroid band 3 react with frozen sections of mouse and human brain as detected with rhodamine staining. (*A–C*) Human cerebellum stained with antibodies to pep-CYTO. (*A*) Internal granular layer showing staining of glomeruli (GLOM) and granule cells (IGC). (*B*) Pia and subjacent molecular layer (MOL). (*C*) Control consisting of antisera absorbed with the peptide. (*D–F*) Mouse brain stained with antibodies to synthetic peptides from two external loops of band 3 (16, 17). (*D*) Ependyma of the fourth ventricle stained with antibodies to pep-ANION 1. (*E*) Choroid plexus of the fourth ventricle stained with antibodies to pep-COOH. (*F*) Control consisting of antisera absorbed with pep-COOH peptide.

Table 1. Inhibition of senescent cell IgG binding by senescent cell antigen from erythrocytes or perfused rat brain

Sample	Inhibition, %
Control cytoplasmic peptide	0
Senescent cell antigen	74 ± 6*
Brain (perfused)	80 ± 2*

Data are presented as the mean ± 1 SD of quadruplicate samples. Erythrocytes were separated into populations of different ages on Percoll gradients as described (20). Middle-aged cells were stored at 4°C for 3 weeks in Alsever's solution. Storage mimics normal aging *in situ* both immunologically and biochemically (16–26). Senescent cell IgG was isolated from senescent erythrocytes as described (21) and 3 μg was incubated with senescent cell antigen from erythrocytes, perfused rat brain, or buffer for 90 min at room temperature. Intact erythrocytes were added to the IgG and samples were incubated for 90 min at room temperature. Cells were washed and the amount of IgG on cells was quantitated with ^{125}I-labeled protein A (16, 17, 24, 35). Control cytoplasmic peptide is the band 3 peptide pep-CYTO.
*$P \leq 0.001$.

brain band 3 breakdown products. However, some of these bands probably represent different forms of band 3 in brain membranes because the brains were snap frozen and homogenized in a buffer containing the following protease inhibiting agents: EGTA, leupeptin, pepstatin, aprotinin, KF, 2-mercaptoethanol, phenylmethylsulfonyl fluoride, and diisopropylfluorophosphate (12, 29). We suspect that there are multiple forms of band 3 protein that are adapted to various functions in neural and other tissue. There may be more than one type of band 3 in a cell type or different cell types may have different band 3 molecules. This requires further study.

Binding of antibodies to synthetic peptides of erythroid band 3 to brain membranes suggests that the primary structure of these segments that is recognized by the antibodies is similar in brain and erythrocytes. Senescent cell antigen has been mapped to erythroid band 3 residues 538–554 and 812–827 (16, 17).

IgG eluted from senescent erythrocytes binds to band 3 in erythrocytes and band 3-related polypeptides in membranes of saline-perfused rat brains as determined by immunoblotting (data not shown). Absorption of senescent cell IgG with brain membranes resulted in inhibition of binding in the IgG binding/inhibition assay (80% ± 2%; Table 1). For comparison, absorption with purified erythroid senescent cell antigen itself resulted in a 74% ± 6% inhibition of binding. This suggests that senescent cell antigen is generated on brain band 3.

Immunohistochemical Localization of Band 3-like Proteins in Brain with Antibodies to Erythroid Band 3 and Synthetic Peptides of Human Erythroid Band 3. Antibodies to the whole band 3 molecule and to synthetic peptides stained Purkinje

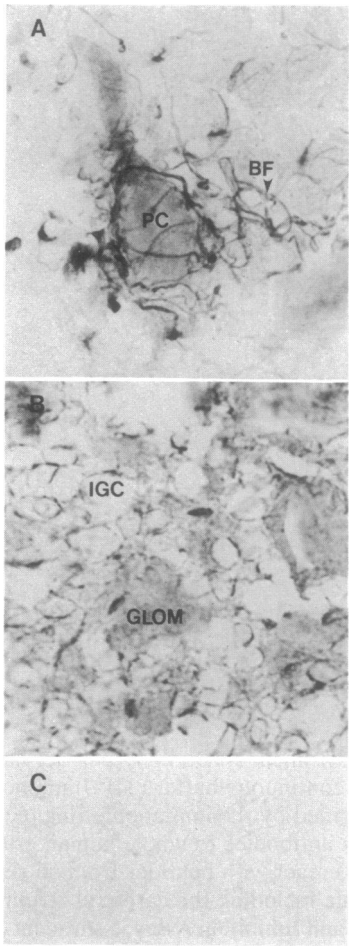

FIG. 3. Antibodies to human erythroid band 3 bind to human and mouse brain band 3 in frozen sections visualized with peroxidase staining. (*A*) Purkinje cell (PC) in human cerebellum. Basket fibers (BF), which are located in the molecular layer, are wrapped around the soma and primary dendrite of the Purkinje cell. The internal granule layer is subadjacent to the Purkinje cell at the bottom of the photograph. (*B*) Neural cells in mouse cerebellar cortex. Granule cells (IGC) and glomeruli (GLOM) (areas of synaptic contact) stain with antibodies to band 3. (*C*) Control section of human brain incubated with preimmune serum. The same results as those shown in *C* were obtained when antisera was absorbed with erythrocyte band 3. (×80.)

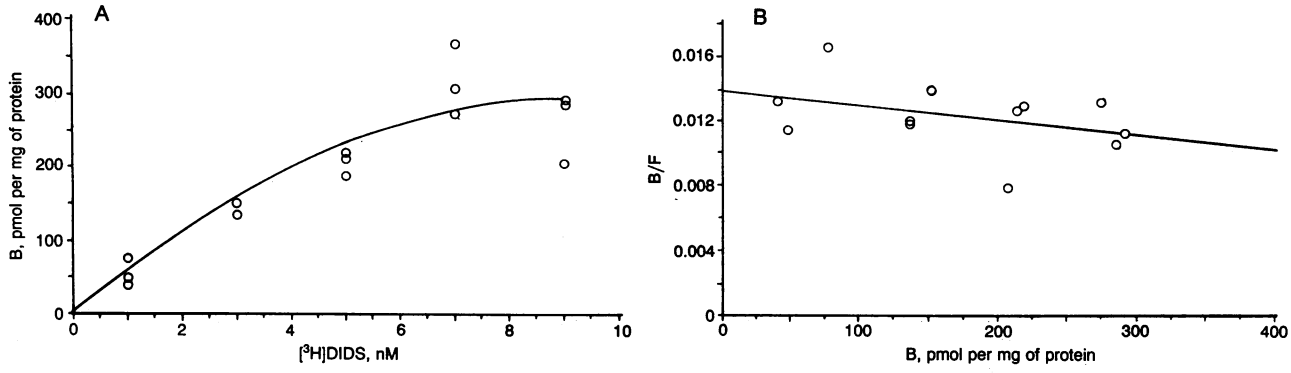

FIG. 4. Quantitation of DIDS binding sites on brain membranes. Tritiated DIDS was used to quantitate the number of band 3 molecules per mg of protein as described (12). B, bound; F, free. B_{max}, 1500 ± 255 pmol per mg of protein; K_d, 10.7 ± 1 mM. This suggests that DIDS binding sites constitute ≈14% of total brain membrane protein based on a molecular mass of 95 kDa for band 3. The calculation is based on the assumption that all band 3 molecules in brain bind DIDS and each band 3 binds a single DIDS molecule. This assumption is correct for erythrocytes. However, the proportion of band 3 in brain needs to be examined further because our data suggest that band 3-like proteins in brain have different molecular masses, and DIDS may label all neural anion transporters, not just band 3.

cell soma, axons, glomeruli (areas of synaptic contact) in the pia, internal granular layer, ependymal cells lining the ventricles, and the choroid plexus as determined by rhodamine and peroxidase staining (Figs. 2 and 3). The presence of band 3 in the choroid plexus is not surprising given the transport properties of this epithelium, which maintains the chemical stability of the cerebrospinal fluid.

Ankyrin Binding. Erythrocyte band 3 binds to ankyrin, which anchors it to spectrin. Therefore, we examined the interaction between brain band 3 and erythrocyte ankyrin. Ankyrin binding studies gave the following results: n = 16 ± 3 μg of ankyrin per mg of protein after removal of cytoskeletal proteins; K_d = 33 ± 5 nM. The 40-kDa cytoplasmic segment of erythroid band 3 competed with brain band 3 and reduced ankyrin binding to brain membranes by 58%. This indicates that ≈58% of the ankyrin binding is to band 3. Thus, brain band 3 appears to perform the same structural function of stabilizing the plasma membrane and linking it to the internal cytoskeleton as does erythrocyte band 3.

Quantitation of Band 3 Molecules. Band 3 molecules were quantitated on brain membranes with radioactive DIDS (Fig. 4).

Anion Transport Studies. Anion transport studies were performed on cerebral cortex by both the tube and the chamber techniques (Fig. 5). The presence of DIDS inhibitable anion transport systems in brain was detected by both methods.

We compared the effect of the anion transport inhibitors DIDS, phenylglyoxal, and furosemide on brain and erythrocyte anion transport (Fig. 6). Sulfate exchange is inhibited in erythrocytes by DIDS, phenylglyoxal, and furosemide. Stilbenedisulfonate derivatives such as DIDS inhibit anion transport by binding to at least two lysine residues in the membrane-spanning region of band 3 (18, 19). DIDS is a specific, irreversible inhibitor of anion transport. It binds covalently. Phenylglyoxal modifies an arginine involved in anion transport that is located on the 35-kDa carboxyl segment of band 3 (see ref. 23). The diuretic furosemide inhibits NaCl cotransport by acting at a chloride transport site. The compounds that inhibit anion transport in erythrocytes also inhibit transport in brain. Transport by brain vesicles was reduced to a level ≈74% of normal by DIDS, to ≈65% by furosemide, and to ≈45% by phenylglyoxal (Fig. 6). In contrast, transport in erythrocytes was reduced 100% by DIDS and ≈95% by furosemide. Inhibition by phenylgloxal was the same for brain and erythrocytes. Since brain anion transport was significantly inhibited but not abolished by DIDS, the data suggest the presence in brain of more than one anion transport system [i.e., a transport system(s) that is DIDS inhibitable and another or others that are not] and/or that ≈20% of the sulfate influx is due to leakage. There may be

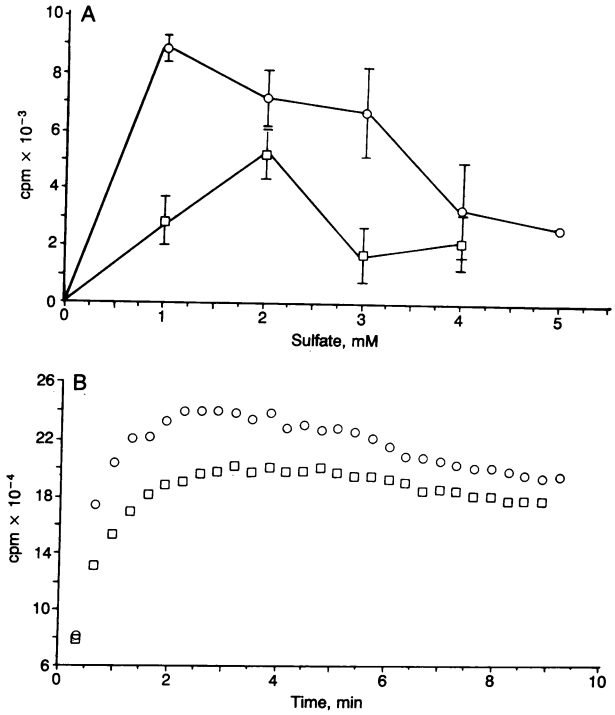

FIG. 5. Anion transport by brain membrane vesicles measured by the tube method (*A*) and the chamber method (*B*). Transport: tube, 385 ± 20 nmol per min per mg of protein without DIDS; 199 ± 34 nmol per min per mg with 25 mM DIDS; chamber, 17.7 ± 4.8 fmol per min per mg of protein without DIDS; 10.4 ± 0.9 fmol per min per mg of protein with 25 mM DIDS. Inhibition is 48% ± 9% by the tube method and 41% ± 5% by the chamber method. In the chamber method, radioactive substrate is added and buffer is pumped through the lower chamber at a constant rate with a Pharmacia pump (model P-1) connected to a LKB fraction collector. Fractions of 0.55 ml are collected directly into scintillation vials. At the beginning of fraction 20, 20 μl of either buffer or membrane vesicles (0.5–7.5 mg per ml of protein) is added to the upper chamber and 20 more fractions are collected. The experiment is carried out at 23°C. ○, Control; □, DIDS.

many transporters in each cell type present in the brain or there may be different transporters in different cell types. We suspect that the former is the case. However, the next obvious step is to examine pure cell types from brain tissue.

Peptide Inhibition of Anion Transport. Peptides from putative human erythroid transport regions were used to inhibit sulfate transport into erythrocytes and brain vesicles (F7). Peptides used were pep-ANION 1, pep-ANION 2, pep-COOH, and pep-COOH-N6. pep-COOH-6 is on the amino

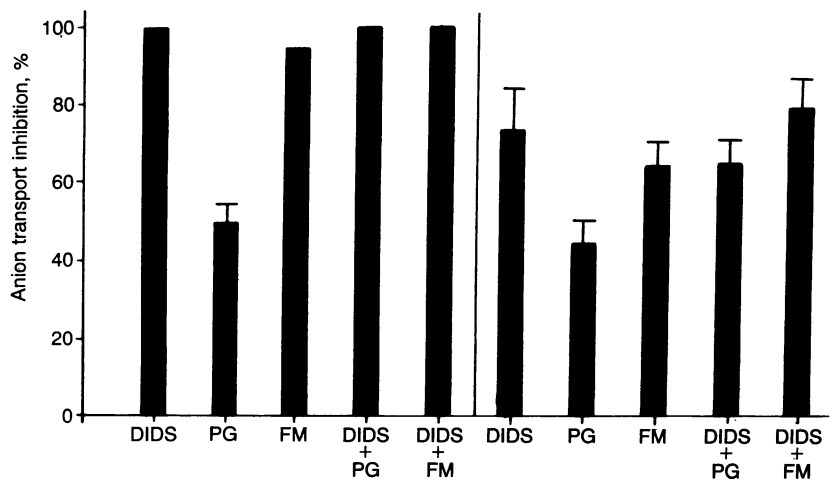

FIG. 6. Anion transport inhibition of erythrocyte (*Left*) or brain (*Right*) anion transport by the anion transport inhibitors DIDS, phenylglyoxal (PG), furosemide (FM), DIDS and PG, or DIDS and FM. Results are plotted as the mean + SD.

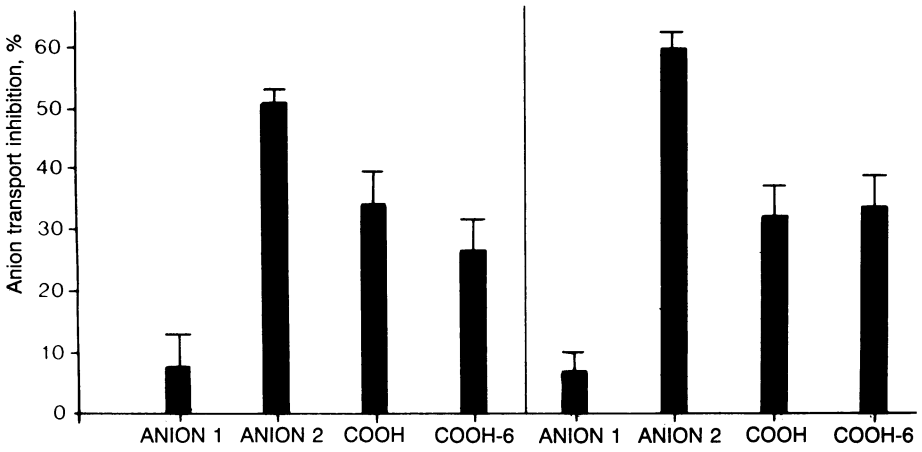

FIG. 7. Inhibition of erythrocyte (*Left*) or brain (*Right*) anion transport by synthetic peptides of human erythroid band 3. Peptides and sulfate were used in equimolar amounts. Results are plotted as mean + SD.

end of pep-COOH and contributes a significant amount of the antigenicity of senescent cell antigen (16). In previous experiments, we have shown that pep-ANION 1, a putative transport region, does not inhibit anion transport in erythrocytes (17, 36). Thus, it serves as a negative control for these experiments. Results of these experiments showed that the same peptides that inhibited anion transport in erythrocytes inhibit transport in brain (Fig. 7). This suggests that the transport sites in erythroid and neural tissue are similar.

DISCUSSION

Analogues to erythrocyte membrane cytoskeletal proteins have been found in most if not all tissues throughout the body (33, 37, 38). Thus, the proteins with which band 3 interacts in erythrocytes to provide structural stability to the membrane are present in neural tissue.

The results of these studies indicate that one or more band 3 proteins are present in mammalian brain that perform the same functions as that of erythroid band 3. These functions are anion transport, ankyrin binding, and generation of senescent cell antigen, an aging antigen that terminates the life of cells. The anion transport segments of erythroid and brain band 3 must be similar since synthetic peptides from transport regions of erythroid band 3 inhibit anion transport by brain vesicles as well as erythrocytes. In addition, the inhibitors of anion transport in erythrocytes (DIDS, phenylgloxal, and furosemide) also inhibit anion transport by brain membranes. Since senescent cell antigen is derived from band 3 and (*i*) IgG specific for this antigen binds to brain membranes in immunoblots and a senescent cell IgG binding-inhibition assay, and (*ii*) antibodies to the segments of band 3 on which senescent cell antigen resides react with brain in immunoblots and tissue sections, the data suggest that senescent cell antigen may be involved in the removal of neurons in aging and disease. This supports the hypothesis that the immunological mechanism of maintaining homeostasis is a general physiologic process for removing senescent and damaged cells in mammals and other vertebrates (21).

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