

Influence of aging and neurodegenerative disease on changes in band 3-like proteins in white blood cells

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Abstract

Fluorescent microspheres were used to measure antibody-induced capping of leukocyte membrane proteins that are immunologically related to band 3, the anion exchanger of the erythrocyte. The degree of capping was found to increase with donor age. Surface labeling and capping characteristics of cells from healthy, age-matched controls were not different from those from patients with Alzheimer's disease, multi-infarct dementia, and Down's syndrome. Immunoblots, however, indicated increased expression and/or breakdown of band 3-like proteins in leukocytes from patients when compared with young and old control donors. These findings emphasize the possible involvement of band 3-like proteins of nucleated cells in aging and disease.

Keywords: Aging; Alzheimer; Anion exchange; Capping; Cytoskeleton; Down; Lymphocyte; Membrane

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1. Introduction

During the last few years, it has become clear that band 3, the anion transporter of the human erythrocyte, belongs to the gene family of anion exchanger proteins [1,2]. The various members of this family presently known (AE1 or band 3, AE2, AE3) have been described to be expressed in many different cells and tissues, including white blood cells [1,2]. The AE proteins share extensive structural homology in their membrane domain and, in agreement with the exclusive involvement of the membrane domain of AE1 in chloride/bicarbonate exchange, they all seem to play a role in anion exchange-based regulation of intracellular pH [3,4]. The cytoplasmic domains of the AE members show considerable diversity, possibly reflecting their tissue-specific interaction with intracellular elements such as the cytoskeleton [1,2].

In addition to anion transport and providing an anchor site for the cytoskeleton, AE1 plays a pivotal role in erythrocyte aging (for a recent review, see [5]). Age-dependent changes in AE1 conformation result in the appearance of a neo-antigen, leading to recognition and removal of old cells by the immune system. The mechanism that underlies these age-related changes in AE1, and that may also be active in other cell types, seems to be altered in various diseases, including Alzheimer's disease [5–8]. Aging- and disease-related changes in AE1 structure affect anion transport as well as interaction of AE1 with the cytoskeleton [5]. The capping behaviour of surface receptors, i.e. their aggregation within the plasma membrane upon binding of an external agent such as an antibody, may indicate, among other things, the strength of their association with the cytoskeleton [9].

It occurred to us that aging- and disease-related changes in capping behaviour of AE proteins might identify aging- and/or disease-related, structural alterations. Therefore, in the course of our studies on the involvement of AE proteins in normal and pathological aging [6,7], we investigated the effect of normal aging on capping of band 3-like (AE) molecules in white blood cells. We compared the capping characteristics of band 3-like proteins in white blood cells from young and old control donors with those of patients with Alzheimer-like dementia (AD), of patients with multi-infarct dementia (MID), and of individuals with Down's syndrome (DS). For this purpose we adapted a method to visualize antigens with fluorescent microspheres [10] in order to visualize antibody-induced capping.

In this report we describe an age-dependent increase in band 3 capping in leukocytes, as well as an apparent increase in breakdown of band 3-like proteins in the leukocyte fraction of the patient donor groups.

2. Materials and methods

2.1. Patients

Patients with the diagnosis dementia of the Alzheimer-type (AD, 81 ± 5 years, $n = 8$) and multi-infarct dementia (MID, 82 ± 4 years, $n = 8$) were from a local psychogeriatric institution. Young controls (YC, 29 ± 5 years, $n = 8$) were laboratory coworkers, and old, healthy controls (OC, 82 ± 5 years, $n = 8$) were

from two local homes for the elderly. Down's syndrome patients (DS, non-demented 26 ± 3 years, $n = 3$; demented, 60 years, $n = 2$) were from an institution for the mentally handicapped. The diagnostic criteria used for selection of the various patient and control groups, as well as their clinical data have been described before [6,11–13]. None of the donors had any immunological disorders, or used medications known to affect the immune system [6].

2.2. Nucleated blood cell isolation

Blood was collected with EDTA as anticoagulant, and nucleated blood cells were isolated using a Ficoll-Paque gradient (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) as described before [6]. The viability of the various preparations was at least 95% as indicated by the ability of the cells to exclude trypan blue.

2.3. Anti-band 3 antibodies

Anti-band 3 antibodies (PM1B3) were raised in rabbits against the membrane domain of human erythrocyte band 3. Their immunoreactivity pattern with erythrocyte band 3 has been described before [13].

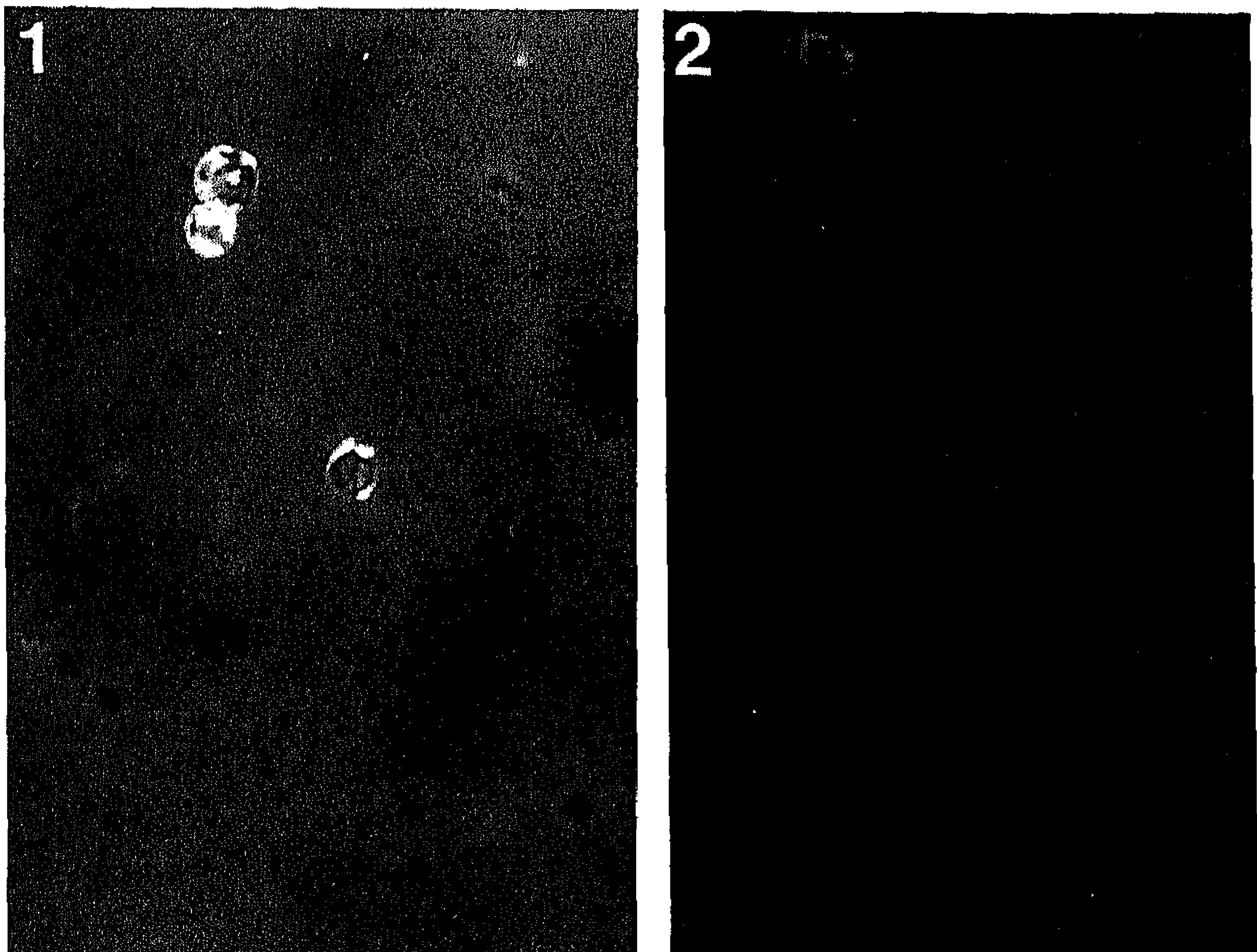


Fig. 1. Labeling and capping of band 3-like proteins in white blood cells, visualized with fluorescent microspheres. Cells were incubated with an antiserum against the membrane domain of band 3 as described in the Materials and methods section. Shown are examples of three possible results. Panel 1, from top to bottom: labeling without capping, completely covered with microspheres, and labeled with capping; Panel 2, from top to bottom: capping, labeled without capping, capping.

2.4. *Microsphere capping assay*

Lymphocytes (4×10^5 cells) were incubated with rabbit anti-human band 3 antiserum PM1B3, diluted (1:4000) with phosphate-buffered saline/1% bovine serum albumin (PBS/BSA) for 15 min at 37°C. After this period, cells were washed four times with ice-cold PBS/BSA by centrifugation ($100 \times g \times 5$ min), and fixed with formaldehyde (pH 7.0; 0.025% in PBS) for 30 min at 18°C. Labeling of fluorescent microspheres (Covaspheres MX, Duke Scientific Co., Palo Alto, CA) with swine-anti rabbit IgG, and labeling of the cells with the coated microspheres were performed exactly as described before [10]. The final cell pellet was resuspended in 15 μ l PBS/BSA, and 100–200 cells were evaluated at $400 \times$ magnification under a fluorescence microscope as in [10]. Cells were grouped into four categories: lacking any microspheres, completely covered with microspheres, non-capping (the — individually visible — microspheres were evenly distributed on the cell surface), and capping (the fluorescence was restricted to less than one half of the cell surface [14,15] (Fig. 1). Capping characteristics were determined by one of us (B.D.S.), who had no previous information of the clinical data of the donors. Blanks consisting of preserum resulted in less than 2% labeled cells.

2.5. *Immunoblot analysis*

Homogenates of nucleated blood cells were prepared following the procedure of [16], and analyzed by immunoblotting as described before [7], using the same antiserum that was used for the capping studies (PM1B3, 1:500, [13]). All lanes contained the same amount of protein (10 μ g).

3. Results

3.1. *Capping assay conditions*

Antibody dilutions resulting in labeling of the highest percentage of cells ('maximal labeling'), and incubation time and temperature resulting in the highest number of labeled cells showing capping ('maximal capping'), were determined using white blood cell preparations from young control donors. Optimal antibody dilutions were determined by combinations of serial dilutions (1:100–1:20 000 final dilution) of the rabbit anti-band 3 antiserum PM1B3 and swine-anti rabbit IgG (SAR), and the highest dilutions still yielding maximal labeling were used. These dilutions were 1:4500 for PM1B3 and 1:4000 for SAR. The mean maximal percentage of labeled cells was $64 \pm 11\%$ (range, 50–85%; $n = 7$). Variation of incubation time and temperature showed that capping was temperature-dependent; the highest percentage of capping cells ($32 \pm 3\%$; range 26–37%; $n = 7$) was reached after 15 min at 37°C.

3.2. *Ageing-related capping*

The number of cells that were not labeled with the anti-band 3 antiserum did not differ between the various donor groups (30–40%; Table 1). There also was no significant difference between the donor groups in the fraction of cells that were labeled with the antiserum, but that displayed no capping, with the exception of the

Table 1
Labeling and capping of band 3-like molecules in white blood cells of various donor groups^a

Donor	Unlabeled	Labeled without capping	Capping
YC (7)	36 ± 7	33 ± 5	32 ± 3
OC (8)	35 ± 6	23 ± 4**	42 ± 5*
MID (8)	36 ± 5	24 ± 5**	40 ± 1*
AD (8)	32 ± 9	26 ± 4**	42 ± 7*
DS (5)	33 ± 7	38 ± 4	29 ± 7

YC, young controls; OC, old, age-matched controls; MID, patients with multi-infarct dementia; AD, patients with senile dementia of the Alzheimer type; DS, donors with Down's syndrome.

^a White blood cells were labeled with anti-erythrocyte band 3 antiserum, and the degree of capping was determined with fluorescent microspheres as described in the Materials and methods section. Values represent percentages of total cells. The number of donors that was measured in one group is given in parentheses. The fraction 'Labeled without capping' also contains the cells fully covered with microspheres (see also the Materials and methods section).

* Significantly different ($P < 0.01$) from the value of YC.

** Significantly different ($P < 0.05$) from the value of DS.

DS group (Table 1). In the DS group, the fraction of capping refractory cells was significantly higher than those of the Alzheimer and MID groups ($P < 0.05$), but especially higher than that of the old control group ($P < 0.001$). There were no notable differences between the young (26 years) and old (60 years) DS donors.

The most striking finding of the capping studies is that in the white blood cells that were labeled with the anti-band 3 antiserum, capping of band 3-like molecules in the plasma membrane increases with donor age. The fraction of cells that showed capping was significantly increased in the older donors when compared with the young controls (Table 1). There was a significant positive correlation between donor age and percentage of cells with capping ($r = 0.87$; $P < 0.01$). We did not observe any Alzheimer-specific changes in capping of band 3-like molecules in white blood cells from Alzheimer patients; there were no significant differences between the AD patients, the MID patients, and the age-matched (old) control group (Table 1). It is noteworthy that the fraction of capping cells in the DS group was similar to that of the young control group, and also significantly smaller than that found in the AD, MID, and OC groups (Table 1).

3.3. Immunoblots

The white blood cell proteins from the same donor groups used in the capping analysis, were analyzed on immunoblots with the same antiserum against the membrane domain of erythrocyte band 3 that was used for the capping studies. In addition to the expected reaction of this antiserum with proteins in the 95- to 110-kDa molecular weight region of intact band 3, all fractions contained immunoreactive proteins of smaller apparent molecular weights (Fig. 2). These smaller

polypeptides arise either by breakdown of the native proteins, or may represent incomplete molecules [1,8]. Initial analysis indicated the presence of an anti-band 3 immunoreactive polypeptide with an apparent molecular weight of ~ 40 kDa in Alzheimer patients, but not in young and age-matched control donors. More extensive analysis, however, showed the presence of this polypeptide in the cells from all donors with pathology, but its almost complete absence in the white blood cells of young and old controls (Fig. 2). Also, the fractions of the patient groups seem to contain more proteins in the higher molecular weight regions. These differences were only observed with antibodies raised against the membrane domain of AE1, and not with antibodies specific for AE2 (data not shown).

4. Discussion

Detection of surface molecules on intact cells using fluorescent microspheres has several advantages compared with classical methods: a high sensitivity, a high signal/noise ratio, and (from a practical viewpoint) resistance against bleaching, and no need for dark room conditions for evaluation and quantification. One of us already demonstrated the potential of fluorescent microspheres for detection of very early erythrocyte chimaerism in patients with bone marrow transplants [10]. Here we have successfully applied fluorescent microspheres to the measurement of antibody-induced capping in nucleated blood cells.

Polypeptides of the AE family related to band 3 and senescent cell antigen are present in white blood cells [17–19]. At present the exact identity of these proteins and their distribution over the various cell types is not clear, but our preliminary immunochemical and RNA typing indicate that both AE1 and AE2 are expressed in white blood cells [19]. Capping of band 3-like molecules in white blood cells has been described before [17,18], and we show here that the extent of capping increases with increasing donor age. This is in sharp contrast with the age-related decrease of capping of other membrane receptors in white blood cells [14]. The increase in anti-band 3-induced capping seems to be related only to age, and showed no pathological component; we observed no differences in capping between the old controls and the (old) patient groups. This indicates that the process(es) responsible for this increase, through changes either in band 3-like molecules or in cytoskeleton proteins, is (are) not visibly affected in Alzheimer's disease. Changes in actin molecules of white blood cells, that play an important role in receptor capping [9], or in other components of the cytoskeleton in neurons, have been reported for Alzheimer patients [8,20]. It cannot be excluded that various Alzheimer-related changes have counteractive effects on the capping behaviour of AE proteins, resulting in overall identical capping behaviour in aged controls and AD patients, but at present we must conclude that capping of band 3-like molecules in the plasma membrane of white blood cells is not affected by Alzheimer's disease.

One explanation for increased mobility of band 3-like molecules within the membrane may be proteolytic breakdown, resulting in a decreased interaction with the cytoskeleton. Age- and disease-related breakdown of band 3 probably plays a pivotal role in the mechanism that leads to recognition and removal of old

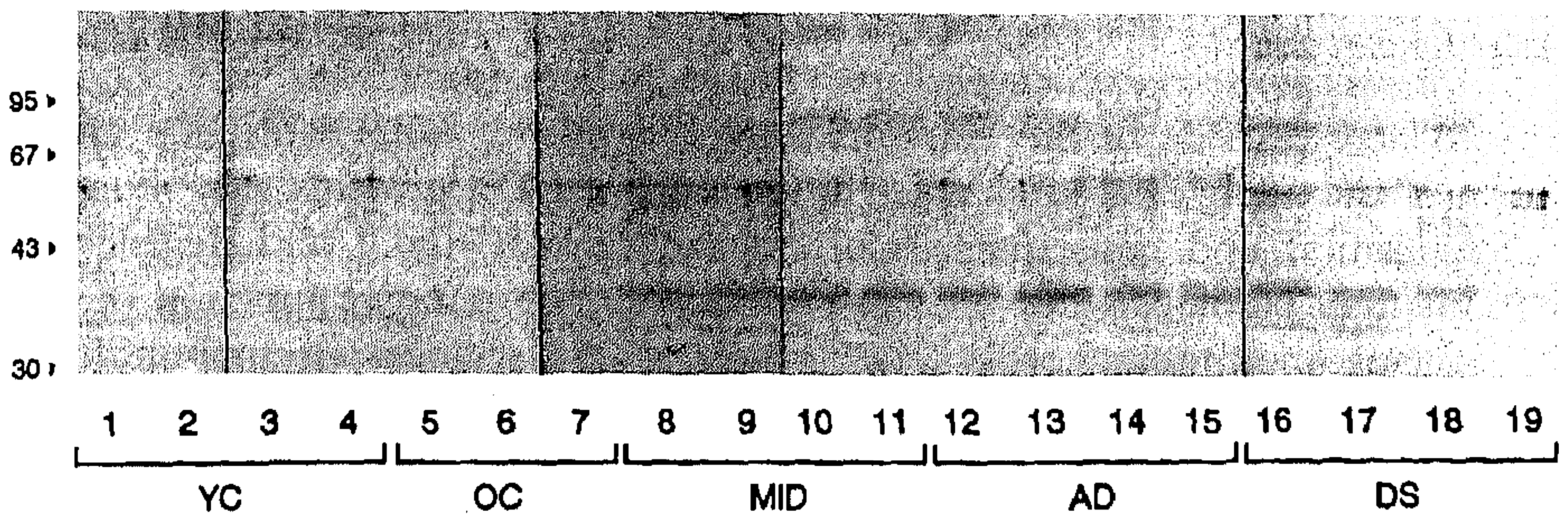


Fig. 2. Immunoblot analysis of band 3-like molecules in white blood cells of various donor groups. Cell homogenates were prepared and analyzed by immunoblotting using an antiserum raised against erythrocyte band 3 as described in the Materials and methods section. All lanes contained 10 μ g of protein, and the various blots were incubated and developed under identical conditions. For proper comparison, care was taken that the original blots contained the maximum number possible (3–4) of samples from the various donor groups (each group consisted of eight donors). YC, young controls; OC, old, age-matched controls; MID, patients with multi-infarct dementia; AD, patients with Alzheimer's disease; DS, patients with Down's syndrome.

erythrocytes by the immune system [5]. Immunoblot analysis, however, does not show any evidence for increased band 3 breakdown in lymphocytes from old control donors. Also, increased breakdown of band 3-like molecules, as especially apparent from the presence of a new immunoreactive band of 40 kDa, occurs in all the patient groups, including the DS group that has normal 'young' capping characteristics. Thus, it is unlikely that the age-related increase in capping of band 3-like molecules is directly related to increased synthesis or breakdown. However, one could speculate that the aging-related changes in capping of band 3-like membrane proteins may be related to aging-related shifts in cell populations with different capping characteristics, such as a changed composition of the T cell compartment [21]. Such aging- and/or disease-related shifts may also be responsible for the differences in band 3-like proteins in the 95- to 110-kDa region between the various donor groups (Fig. 2). Thorough investigation of the age-related changes in capping, in relation to those occurring between and within leukocyte populations, however, requires analysis of the capping properties and AE expression in individual lymphocyte populations of a large number of donors of various ages. Such studies have been initiated.

If the 40-kDa, band 3-related, protein is specific for a certain cell type, the apparently disease-related presence of the 40-kDa immunoreactive protein could be a reflection of a derangement of the major white blood cell subsets reported in DS patients [22], or of the changes in T cells in AD and MID patients [23]. Alternatively, structural changes in band 3-like proteins could be related to perturbation of intracellular ion fluxes, e.g. in the secondary messenger compartment, considering the decrease in intracellular calcium response of CD4⁺ cells in Alzheimer's disease and Down's syndrome patients recently described [24]. These possibilities are currently under investigation.

Age- and Alzheimer-related changes in structure and function of AE proteins have recently been described in erythrocytes, as well as in neurons [6,7]. The data we present here provide further support for the hypothesis that molecular characterization of the aging-related changes in band 3-like (AE) proteins may illuminate the mechanism(s) and consequences of normal cellular aging and degeneration [8,25]. This may be of broader relevance in view of the putative function of band 3-like proteins in immune cells [26,27], and of the changes in immunological parameters, which occur during normal aging and in patients with Alzheimer's disease [21,23,28,29].

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