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Activation of early components of complement targets myelin and oligodendrocytes in the aged rhesus monkey brain

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Abstract

The disruption and loss of myelin in the white matter are some of the major changes that occur in the brain with age. In vitro studies suggest a role of the complement system in the catabolic breakdown of myelin membranes. This study presents findings on activation of the early components of complement cascade in the brains of both young and aged rhesus monkeys with evidence of increased complement activation in aged animals. Complement containing oligodendrocytes (CAOs) containing C3d and C4d complement activation products bound to oligodendrocytes and myelinated fibers were found in the brain of normal young and old animals. The CAOs, which also contained activated microglia, were distributed throughout the whole brain and in significantly greater numbers in the aged monkeys. These findings, together with the demonstration of covalent binding of the C3 fragments to myelin, suggest the initiation of the complement cascade by myelin and oligodendrocytes, which are known classical complement activators. Activation of terminal complement components was not demonstrable in the CAOs.

Taken together the findings support the concept that activation of early components of complement in the brain may be a normal biological process that involves the metabolism of myelin and oligodendrocytes and up-regulates with age.

Keywords: Aging; Myelin; Oligodendrocyte; Complement activation

1. Introduction

The white matter of the brain is the site of a number of the major changes that occur in the central nervous system with age. Imaging studies have shown age-related decreases in white matter volume [2,19] while structural and biochemical studies have demonstrated an age dependent disruption of the myelin membrane with an overall loss of myelin [10,27,45,57]. Myelin loss has been found to be associated with an increase in degraded myelin proteins as revealed by the fractionation of myelin membranes isolated from brain white matter [57]. Calpain-1, a calcium dependent cysteine protease, whose known substrates include myelin proteins [3,57], was detected in an active form in degraded myelin and white matter in young and old animals [57]. Increases in the active enzyme accompanied age-related increases in degraded myelin, suggesting the participation of calpain in the observed catabolic breakdown and loss of myelin. These changes in myelin and calpain could contribute to the reported decline in cognitive function in aged subjects by impairing axonal conduction [20,25].

In vitro studies suggest that the complement system may play a role in the activation of calpain and the degradation of myelin. These studies have shown that the terminal complement complex, C5b-9, generated by complement activation induced by myelin, is capable of activating calcium dependent cysteine proteases in the myelin membrane with resulting proteolysis of myelin proteins [62,63].

In addition to C5b-9, the activation of early components of the complement system may have an affect on myelin and the myelin producing oligodendrocytes. These products include

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the anaphylatoxins, C3a, C4a and C5a and the opsonins, C3b, C3bi and C4b. They have been reported to induce the cellular release of mediators which could alter myelin metabolism [14,15,50]. These mediators include cytokines, reactive oxygen species and eicosanoids [7,16,24,28,34,37,39,46,54]. The opsonins also have been shown to enhance myelin phagocytosis and to be essential for the phagocytic removal of damaged myelin from injured myelinated nerves [6,9,47,61,65].

The activation of early and terminal components of the complement pathways is controlled by regulatory proteins, which can promote or inhibit the complement cascade. The action of these proteins have been described in a comprehensive review [32]. The activation of early complement components are derived from the degradation of C3 and C4 with C3d and C4d being the final catabolic products of these respective parent proteins. The terminal complement complex, C5b-9, results from the assembly of its protein components on the target membranes. This process is controlled by both fluid phase and membrane protein regulators.

The three major pathways of complement activation are the classical, alternate and lectin pathways. The classical cascade is initiated by the activation of the C1q subcomponent of C1 while the alternate system is induced by cleavage of C3. The lectin pathway is initiated by the cleavage of C2 and C4 by serine proteases associated with mannose binding lectin and ficolins [22,33]. Both lectin and classical complement activation lead to the formation of the classical form of C3 and C5 convertases. In addition to immune complexes and injured or apoptotic cells, myelin has been shown to be an important activator of the classical system [8,62].

The aim of the present study was to determine whether complement activation might play a role in age-related changes in brain myelin. The findings demonstrate a widespread distribution of complement containing oligodendrocytes (CAOs) containing C3d and C4d activation products bound to oligodendrocytes and myelinated nerve fibers, occurring in brain white and gray matter of both young and aged rhesus monkeys. Activation of terminal complement products was not demonstrable in these CAOs, which were present in larger numbers in aged monkeys than in young animals. Previous studies by other workers have described similar findings of CAOs but only within diseased brains; including Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease [66]. Recent studies have reported C4d containing plaques in multiple sclerosis and have implicated these CAOs in demyelination [51]. There are no published reports on the comparison of CAOs in normal young and aged humans. The monkey CAOs appear morphologically indistinguishable from the human CAOs. The findings in the present study suggest a role of myelin in initiating the activation of the complement system and the generation of C3 and C4 activation products. They, furthermore, suggest that activation of early components of complement is a normal biological function of the brain which involves the metabolism of myelin and oligodendrocytes and which upregulates with age.

2. Materials and methods

2.1. Non-human primates

Sixteen rhesus monkeys (*Macaca mulatta*) acquired from the colony of the Yerkes Regional Primate Research Center were behaviorally tested and their brains processed to evaluate age-related changes. All monkeys spent several years free ranging in social groups maintained at the Yerkes Regional Primate Research Center before being housed individually for 1–3 years during behavioral testing at either the Yerkes Regional Primate Research Center or the Laboratory Animal Science Center at Boston University School of Medicine. Both facilities are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All animal protocols were approved by the Institutional Animal Care and Use Committees of both institutions and complied with the guidelines of the National Institutes of Health and the Institute of Laboratory Animal Resources Commission on Life Sciences Guide for the Care and Use of Laboratory Animals. Animal care and medical testing was performed as previously described [21]. All monkeys had brain MRI scans and underwent behavioral testing to assess cognitive function as described [20]. For the purposes of this study animals were classified as young if under 13 years of age and old if over 20 years of age at the time of sacrifice.

2.2. Tissue preparation

At the time of sacrifice, animals were sedated with ketamine and then deeply anaesthetized with sodium pentobarbital (35 mg/kg to effect). Animals were killed by exsanguination during transcardial perfusion with 2–4 L of Krebs-Heinseleit buffer, pH 7.4 (6.41 mM Na_2HPO_4, 1.67 mM NaH_2CO_3, 137 mM NaCl, 2.68 mM KCl, 5.55 mM glucose, 0.34 mM $CaCl_2$, 2.14 mM $MgCl_2$) at 4 °C. One hemisphere was blocked, in situ, in the coronal stereotactic plane and stored at −80 °C (Rosene and Rhodes [48]). Coronal sections were cut on a cryostat into interrupted series of 15 μm thick sections spaced at 750 μm intervals. These sections were mounted onto poly-L-lysine subbed slides, rapidly dried, and stored at −20 °C until processed for immunocytochemistry. The monkey cryostat sections were coronal sections including frontal, parietal, and temporal subfields. For biochemical assays, the remaining hemisphere was dissected into neuroanatomically relevant blocks, flash frozen on dry ice, and stored at −80 °C. Triton-soluble homogenates were prepared by homogenization in five volume of phosphate-buffered saline with 1% Triton X-100 and protease inhibitors using a hand-held Teflon-coated pestle. After 30 min on ice, the homogenate was centrifuged at 16,000 × *g* for 10 min and the supernatant was assayed for protein content using the BCA assay (Pierce, Rockford, IL, USA).

2.3. Immunohistochemistry

Thirty micrometers Krebs-perfused cryostat sections of whole hemisphere Rhesus monkey brains were cut on a freezing microtome and were then dried and briefly fixed in 4% paraformaldehyde for 5–6 min. The fixed sections that were immunostained for C4d and HLA-DR were treated for 30 min with 0.3% H_2O_2 solution in 0.01 M phosphate-buffered saline, pH 7.4, containing 0.1% Triton X-100 (PBST), while sections that were immunostained for C3d and myelin oligodendrocyte-specific protein (MOSP) were treated for 30 min with 0.3% H_2O_2 solution in 0.3% Triton X-100 PBST. Sections were then blocked in 10% normal serum for 1 h before incubating overnight at 4 °C in primary antibody diluted in 1% normal serum and PBST. Monoclonal antibodies directed against C4d neo-antigen and C3d neo-antigen (Quidel) were used at a dilution of 1:500. Monoclonal antibody to HLA-DR (ICN) was used at 1:100. An IgM monoclonal antibody to myelin oligodendrocytes-specific protein (MOSP) (Chemicon), an oligodendrocyte-specific protein, was used at 1:500. Similar immunostaining procedures were performed using polyclonal antibodies to C5 (1:500, Quidel) and C9 (1:500, Quidel), and monoclonal antibodies to C1q (1:250, Quidel), and to sC5b-9 (1:500, Quidel), which recognizes a neoepitope of C5b-9.

After thorough washing, the sections were treated with appropriate biotinylated secondary antibodies for 1 h at room temperature, followed by incubation in avidin-biotinylated horseradish peroxidase complex (ABC Elite, Vector Lab) for 30 min at room temperature. Peroxidase labeling was visualized by incubation in 0.01% DAB for brown staining and with the addition of 0.6% nickel ammonium sulphate for blue/black staining. Sections were then washed, dehydrated and mounted in DPX before coverslipping. The single stained sections were counterstained with Haematoxylin. For double immunostaining, sections were treated at the end of the first incubation cycle with 0.3% H_2O_2 for 30 min and the second staining was carried out as with the first by blocking with 10% normal serum, but with a different substrate color for labeling.

2.4. Western blot analysis

Triton-soluble homogenates and purified myelin fractions were treated identically during immunoblotting. Soluble protein fractions of particular brain areas were taken from eight old and eight young Rhesus monkeys, evenly matched for sex. Prior to western blot analysis with C3 antibodies homogenates required pre-treatment with alkaline hydrolysis to release C3 fragments that covalently bind to membranes. Twenty micrograms of total protein homogenate were aliquoted to a total volume of 30 μl and the pH of the solution was altered to pH 11 with the addition of 0.01N NaOH. Samples were incubated at room temperature for 1 h and then neutralized to pH 7.4 before 2× SDS sample buffer [29,30] was added. Samples were then processed by western blot analysis under reducing conditions using β-mercaptoethanol. Dilutions (1:20,000) of both activated and non-activated normal human serum (NHS) were prepared in parallel and used as a control. NHS was activated by the addition of cobra venom before sample buffer was added. Samples were separated on a 4–20% SDS–polyacrylamide gels, following which, polypeptides were transferred to polyvinylidine difluoride (PVDF) membranes (Immobilon P, Millipore Corp., MA) using a wet blotter (BioRad) at 35 V for 16 h.

Membranes were blocked in Tris–buffered saline-0.05% Tween 20 (TBST) containing 10% skim milk for 30 min and then incubated with the appropriate primary antibody diluted in TBST plus 10% skim milk for 2 h. Antibodies to C3 (Quidel) (recognizing C3 and degradation products) and C3d (Dako) (recognizing only C3 components containing the C3d peptide) were both polyclonal and used at a dilution of 1:500. Membranes were washed in two 10 min washes of TBST and then incubated for 1 h with horseradish peroxidase-linked rabbit anti-goat IgG antibody for detection of C3d epitopes or goat anti-rabbit IgG antibody for detection of C3 epitopes (1:10,000) where appropriate. Following 3× 10 min washes in TBST, membranes were incubated with Supersignal CL-HRP chemiluminescent substrate (Pierce Chemical Co, Rockford, IL) for 5 min. Membranes were drained and exposed to X-ray film (Kodak X-MAR). Densitometric measurements were normalized for equal protein loading using immunoblotted tubulin for gray matter and post transfer coomassie-stained gels for white matter.

2.5. Intact myelin preparation

Transverse sections of medulla from four old and four young monkeys, approximately 5 mm in thickness and ranging from 0.1–0.3 g, were dissected from brainstem blocks. Samples were immediately processed after dissection and weighing. Myelin was isolated from white matter by disruption with a Dounce homogenizer and separation on discontinuous sucrose gradients as previously described [43] to yield intact myelin at the interface between 0.85 and 0.32 M sucrose [31,44]. The intact myelin fractions were assayed for total protein content using the BCA assay (Pierce, USA). Samples of the intact myelin fraction representing 200 μg total protein were delipidated by adding 1 ml of 3:2 ether: ethanol, vortexing briefly, and remaining protein was pelleted by centrifugation at 16,000 × *g* for 5 min at 4 °C. Protein pellets were lyophilized in a speed vacuum centrifuge for 5 min and resuspended in 200 μl 2× reducing sample buffer. Preparations were again alkaline hydrolyzed as above and 20 μg of total protein was loaded onto 4–20% SDS–polyacrylamide gels for analysis.

2.6. Statistical analysis

In order to determine if a difference in the number of CAOs occurred with age, hemispheres from four old and

four young monkeys were quantitatively analyzed. To take the complete brain into consideration four sections were selected from each animal at an even distribution throughout the brain hemisphere. A CAO's localization to white or gray matter was determined using Haematoxylin staining. CAOs were then counted throughout each hemisphere section. In order to eliminate bias caused by differences in section size, the area of each hemisphere was standardized between monkeys. Area of sections was determined by scanning whole stained sections on an Hewlett-Packard ScanJet 4C scanner, and computing area in pixels using Scion Image v4.02 (Scion Corp.). Counts were then divided by a ratio of section area to the area of the smallest slice counted. This was performed separately for CAOs located in white and gray, and the total corrected count was determined by adding the corrected white matter count to the corrected gray matter count. To determine if a significant difference was evident between old and young brain sections Poisson regression analyses were performed. The Poisson analysis is appropriate given that the dependent variable is measured as a count and is not typically normally distributed. Moreover, the analysis explicitly accounts for the size of the area being examined and produces estimates of effect that can be interpreted as rates, a natural measure for density.

Quantitation of all western blots was determined after scanning autoradiographs (HP ScanJet 4C). Resulting TIFF files were imported into Scion Image v4.02 and pixel density of bands of interest was measured using the Gelplot2 macro function. Resulting raw pixel density values were imported into Microsoft Excel for statistical analysis. In order to compare results at each molecular weight across the immunoblot, it was necessary to standardize raw pixel densities. This was done using a standard z-score transformation for each brain region whereby the average pixel density for all animals in that region was used as the mean. Resulting z-scores were then averaged across brain regions and the cumulative difference between young and old animals established. Statistical significance was determined using a two-tailed Student's t-test assuming unequal variance with significance determined by $P < 0.05$. When comparison of intact myelin with whole homogenate samples was made, additional standardization was done with identical concentrations of activated serum loaded onto each SDS–PAGE gel.

3. Results

3.1. C3d and C4d localize to oligodendrocytes and myelinated fibers

The immunohistochemical distribution of complement proteins in white and gray matter was investigated to assess if complement activation occurred and whether it changed with age. Cryostat whole hemisphere brain sections of both aged and young monkeys were immunohistochemically stained using monoclonal antibodies to the neo-epitopes of C3d (C3d-neo) and C4d (C4d-neo) (Quidel). The distribution of C3d and C4d appeared to be the same with both fragments localizing in the same areas of the white and gray matter of the brain of both aged and young animals (Fig. 1A and B). Staining for C3d-neo and C4d-neo was qualitatively identical and predominantly co-localized in white and gray matter. With both antibodies, immunoreactive areas contained similar process-like structures in the white matter (Fig. 1D and G) and gray matter (Fig. 1C and F). To determine the cellular composition of the CAO, antibodies to HLA-DR and MOSP were used to identify activated microglia and oligodendrocytes, respectively. Both C3d-neo and C4d-neo closely associated with MOSP positive oligodendrocytes (Fig. 2C–E) while HLA-DR positive microglia were found in close proximity but distinctly separated from the complement fragments (Fig. 2A and B, arrows). Since the relationship of C3d and C4d fragments with astrocytes has not been determined, we cannot conclude that the fragments are found exclusively on oligodendrocytes. The complement fragments also appeared to be bound to myelinated fibers (Fig. 2A arrow head). None of the CAO components stained for C1q, C5, C9 or C5b-9. Morphologically distinct areas of compact staining for C3d-neo (Fig. 1E) and Cd4-neo (Fig. 1H) were also found. These areas stained with congo red and were identified as amyloid plaques (data not shown). These plaques have been described in the aged monkey brain in previous studies [1]. However, it was noted that the number of amyloid plaques staining for C3d-neo and/or C4d-neo only represented less than 1% of the total number of plaques in the same section. Moreover, the amyloid plaques were found only in the gray matter (Fig. 1E and H). The process-like stained areas containing C3d, C4d, and oligodendrocytes are the subject of this study, and were referred to simply as CAO's. Plaques containing amyloid β peptides are referred to specifically as amyloid plaques.

3.2. Quantitation of C3d and C4d complement activated oligodendrocytes

Four sections were selected from each animal at an even distribution throughout the brain hemisphere. CAO counts between slices from each individual animal were first compared to determine if the CAO distribution varied across the hemisphere. After compensating for area differences between sections no significant differences were observed in distribution throughout the brain. Counts from the four sections per animal were then averaged, resulting in a mean CAO count for both white and gray matter for each animal. Mean total CAO counts were obtained for each animal by adding mean white matter and mean gray matter counts. Analysis revealed that the number of CAOs containing C4d and C3d was greater in both the white and gray matter of aged monkeys than of young (Fig. 3A and B). The recorded increase in CAOs with age was tested for significance by poisson regression analyses and found to be highly significant in both white and gray matter ($P < 0.0001$). The most significant change with age was the increase of C4d-containing

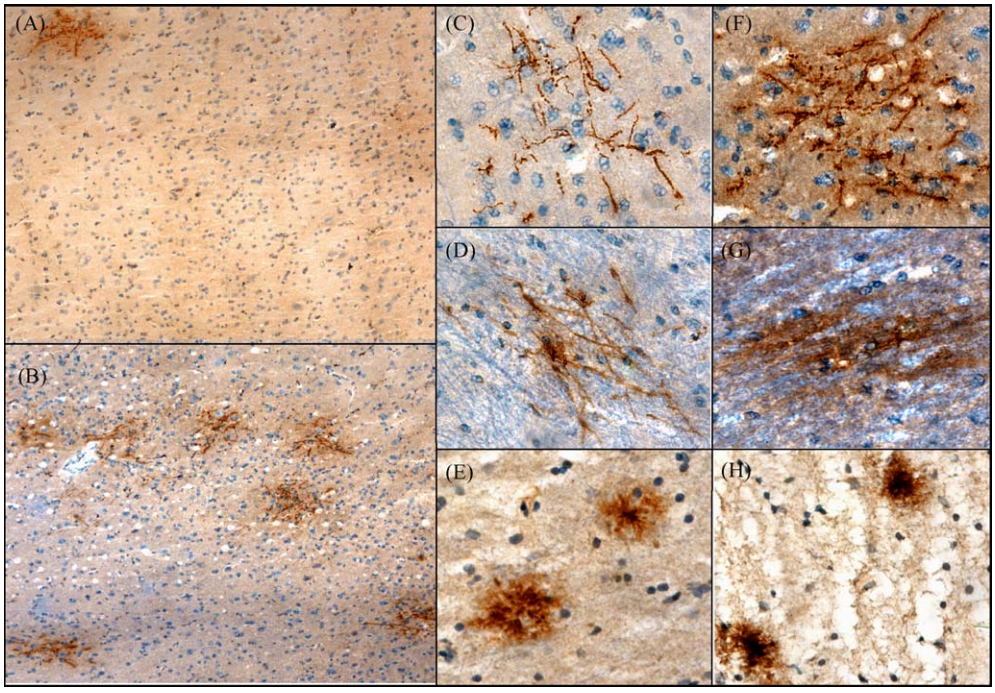

Fig. 1. Immunohistochemical distribution of C3d and C4d in brain white matter and gray matter regions. Immunohistochemical analysis of coronal temporal lobe sections from both young and old monkeys reveals localized complement immunoreactive areas, the numbers of which were increased in old animals (B) as compared to young (A). In A and B, sections are stained with a C3d antibody. Higher magnification of sections stained for C3d demonstrated the predominant immunoreactivity to be process-like in morphology in both gray (C) and white matter (D), but some compact areas of staining were also observed restricted to the gray matter (E). The same results were evident for C4d immunoreactivity; process-like C4d staining in gray (F) and white matter (G) as well as compact staining in gray matter (H). Note that the amyloid plaques seen in 1E and 1H are only found in the gray matter and the staining is of C3d and C4d associated with Aß in the plaques. (A and B) 100× magnification; (C–H) 400× magnification.

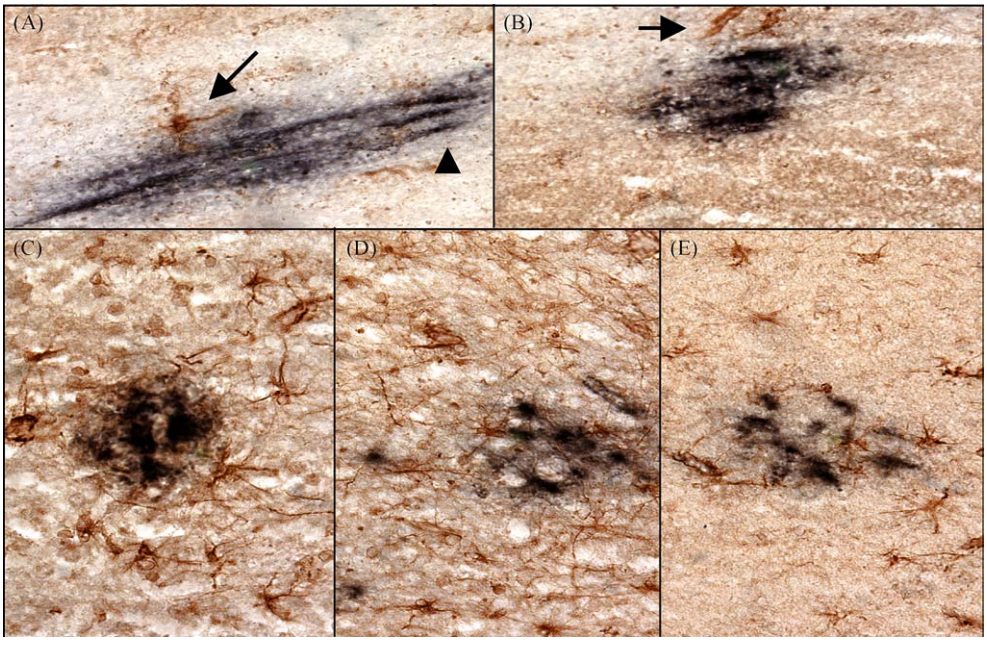

Fig. 2. Immunohistochemical double staining reveals that C4d and C3d closely associate with oligodendrocytes and myelinated fibers but not to microglia. Immunohistochemical analysis of C3d and C4d deposition revealed close association of the fragments to myelinated fibers (A, arrowhead). Regions of coronal sections of monkey brain were examined for co-localization of C3d or C4d with different glial cell types using known markers. Double staining of C4d (black/blue) and HLA-DR-positive microglia (brown) showed proximity but not colocalization (A and B, arrows). Cells immunoreactive for C4d (black/blue) were identified as MOSP-positive oligodendrocytes (brown) (C and D). C3d (black/blue) immunoreactive cells were also identified as oligodendrocytes (brown) using the same marker (E). (A–E) 400× magnification.

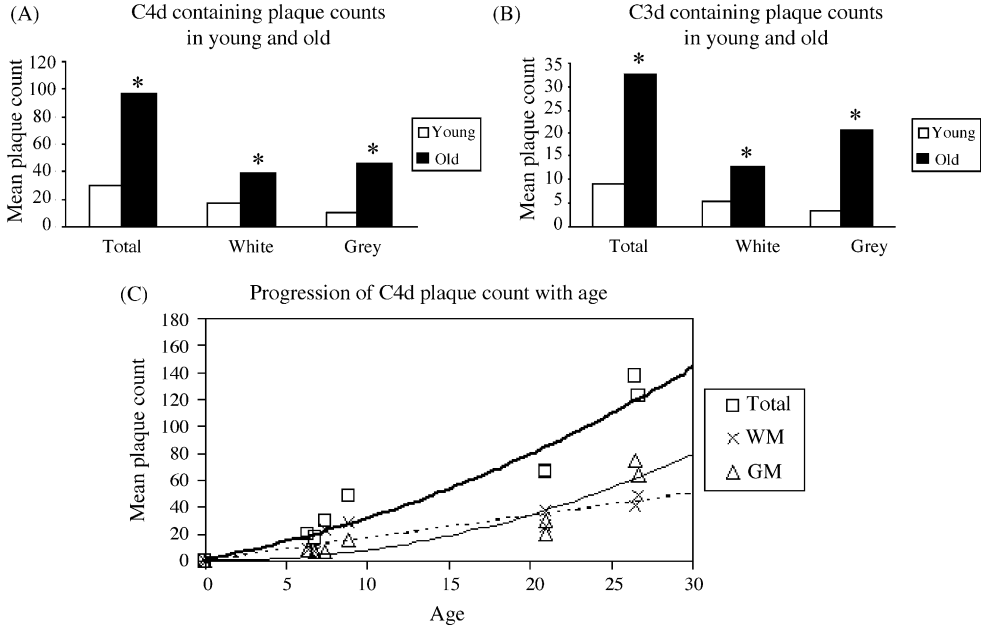

Fig. 3. Quantitation of C3d and C4d oligodendrocyte plaque frequency in cerebral whole hemisphere. Four coronal sections were stained from each of four young ($n = 16$) and four aged ($n = 16$) monkeys using anti-C3d neo. The same was repeated using anti-C4d neo. Plaque counts were obtained, and H&E staining was used to determine whether individual plaques were located in white or gray matter. Counts were then normalized by section area, in order to account for differences in slice size. Mean C4d plaque counts from old and young animals were compared (A), as were C3d plaque counts (B). A significant increase with age in the number of both C4d and C3d immunoreactive plaques was observed in white and gray matter, as well as in the total number of plaques per slice. Asterisks indicates statistical significance at the $P < 0.0001$ level using poisson regression analyses. When mean total, white matter (WM), and gray matter (GM) C4d plaque counts for each of the eight animals was plotted linearly with age (C), the trend indicated a steady increase with age until around 20 years, where a sharper increase in plaque count was observed. Total plaque count—bold solid line; gray matter plaques—solid line; white matter plaques—dashed line).

CAOs located in white matter. In both age groups, C4d-neo staining CAOs outnumbered C3d-neo staining CAOs (Fig. 3A and B).

White, gray and total brain C4d CAO counts were plotted independently with age in order to determine if age directly affected CAO count (Fig. 3C), results indicated that CAO counts rose at a steady rate until an age of about 20 years, where upon they increased notably faster. C3d CAO counts were plotted and found to follow a similar trend as seen in Fig. 3C (data not shown).

3.3. Alkaline hydrolysis enhances detection of membrane bound complement fragments

Prior to immunoblot analyses for C3 fragments, alkaline hydrolysis was used to break the covalent bonds between these fragments and cellular membranes. Immunostained bands with molecular weights characteristic of the polypeptide chains of C3 and its degradation products were detectable after alkaline treatment (Fig. 4B and C), whereas without treatment the bands were faint or absent (Fig. 4A). The detected fragments using a polyclonal anti-C3 antibody (Quidel), included C3b, C3bi, C3dg, and C3d weakly (Fig. 4B). C3d was detected at a higher expression using a polyclonal antibody to C3d (Dako) (Fig. 4C). Without alkaline hydrolysis the high molecular weight protein aggregates do not enter the gel and likely account for the weaker bands in 4A compared to 4B and C. Also, without alkaline treatment the 70 kDa beta chain of C3 was inconsistently seen whereas with alkaline treatment the band was clearly demonstrable.

3.4. C3 activation products are detected in isolated myelin

Immunoblot studies of myelin membranes isolated from the medulla of young and old monkeys were undertaken to clarify the immunohistochemical localization of C3 fragments on oligodendrocytes and myelin. Alkaline hydrolysis of intact myelin was again carried out to release covalently bound C3 fragments from myelin prior to western blot analyses. Using a polyclonal antibody to C3 or C3d (Quidel), it was shown that C3 fragments were present in the isolated myelin fractions prepared from both young and old animals. These fragments included C3b, C3bi, C3dg, and C3d (Fig. 5 inset). C1q, the membrane attack complex C5b-9, C9, and C5 were not detectable using specific monoclonal and polyclonal antibodies (data not shown).

C3 fragment levels were quantitatively compared by densitometric scanning of the immunoblots (Fig. 5). After standardization of raw densitometry against the mean value for all animals, z-score conversion revealed that higher molecular weight fragmentation products (C3b at 100 kDa and

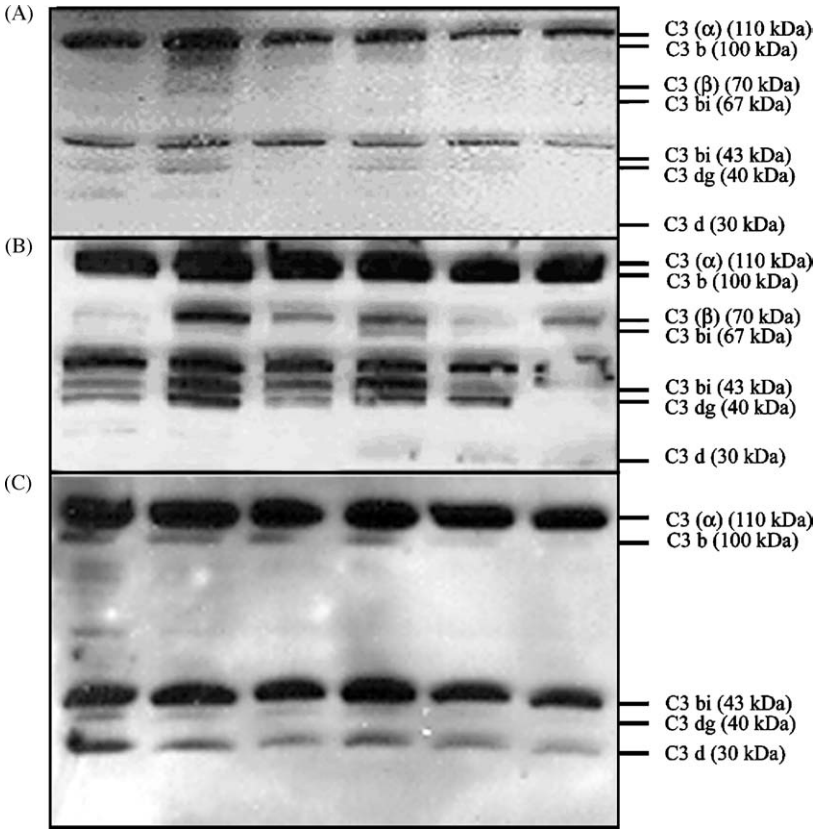

Fig. 4. Alkaline hydrolysis enhances immunoblot detection of C3 fragments. Prior to immunoblotting, homogenate samples were alkaline hydrolyzed in order to release covalently bound C3 fragments from membranes. To illustrate the increased detection of C3 and its fragments after this treatment, samples of homogenized medulla from the same animals and at the same concentrations are shown without (A: lanes 1–3, old; lanes 4–6, young) and with (B and C) alkaline hydrolysis. Western blots were performed under reducing conditions using polyclonal antibodies against C3 (A and B) and C3d (C). The polypeptide chains of C3 and its fragments, C3b, C3bi, C3dg and C3d, represented by the immunostained bands are designated according to apparent molecular weight. The membrane bound C3 fragments C3b, C3bi, C3dg and C3d were demonstrable clearly in alkaline-treated samples (B and C). Immunostaining of C3d was more pronounced using a polyclonal antibody to C3d than to C3 as shown in C and B, respectively. The degradation products of C3, as previously reported [12,41], are listed in Table 1.

C3b/C3bi at 70 kDa) were reduced with age and smaller molecular fragmentation products (C3bi at 67 kDa, C3bi at 43 kDa, C3dg at 40 kDa and C3d at 30 kDa) were increased with age. However, these changes do not reach significance at the 5% level.

Table 1
Molecular weights of C3 degradation products

C3 and its fragments	α-Chain (kDa)	ß-Chain (kDa)
C3	110–120	70–75
C3a	9	
C3b[a]	100–110	70–75
C3bi[a,b]	67/43	70–75
C3c	27/43	70–75
C3dg[a]	40	
C3d[a]	30–35	
C3g	5–10	

C3 fragments are formed as a result of the degradation of the alpha chain. The beta chain of C3 remains bound to the alpha chain in C3b, C3bi and C3c by a disulfide bond. The molecular weights in Table 1 have been reported by Frank [12] and Muller-Eberhard [41].

[a] Binds covalently to target membranes.

[b] Average molecular weight of each alpha subunit.

3.5. C3 activation products are detected in brain homogenates

Immunoblot studies of brain homogenates were undertaken to confirm the presence of C3d and other C3 fragments throughout the cerebral hemispheres. Using a polyclonal anti-C3d antibody (Dako), C3 degradation products containing C3d epitopes were demonstrated in the gray (Fig. 6A) and white matter (Fig. 7A) of the frontal, temporal, parietal, and occipital lobes of the brain in both young and old animals. The degradation products included C3d, as well as C3b, C3bi, and C3dg. Immunostained bands of these C3 fragments, except for C3d, were also detectable in all brain regions using a polyclonal antibody to C3 (Quidel) (data not shown). Major bands were quantitated using raw densitometry data standardized against the mean value for all animals from each brain area. Following this *z*-score conversion of C3 immunoreactivity in all regions of white or gray matter was done and the cumulative average *z*-scores for young and old animals were calculated (Figs. 6B and 7B). Within individual animals, no significant difference in C3 fragments was detected between

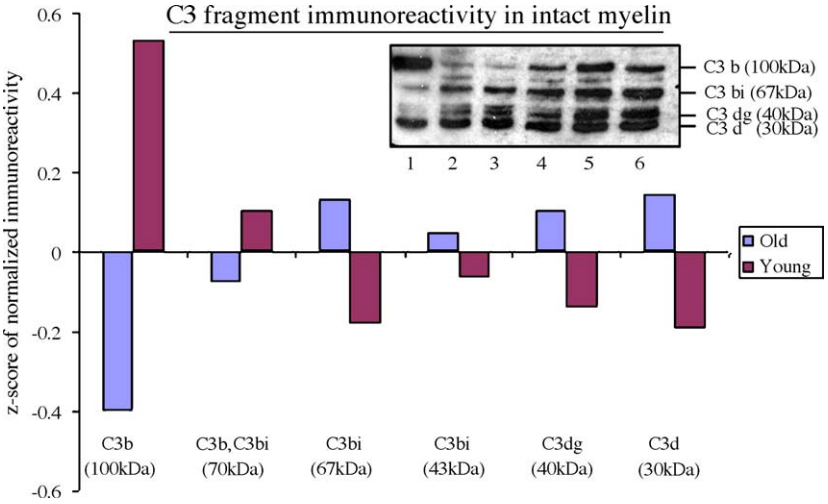

Fig. 5. Western blot detection of C3 fragments in isolated myelin. Myelin was isolated using sucrose density centrifugation from samples of medulla taken from three young and three old animals. This procedure results in an intact myelin fraction at the sucrose interface. Myelin fractions were first alkaline hydrolyzed, and then immunoblotted with a polyclonal antibody to C3 or C3d. C3 fragments were detectable in fractions from both young (lanes 1–3) and old animals (lanes 4–6). These included C3b, C3bi, C3dg and C3d. Densitometric measurements of immunoreactivity for each fragment were normalized based on the average immunoreactivity for that brain area, independent of age, and the resulting *z*-scores are represented in the graph. Quantitative differences in the C3 fragment immunoreactivity were not significant with age at the $P < 0.05$ level using a Student's *t*-test.

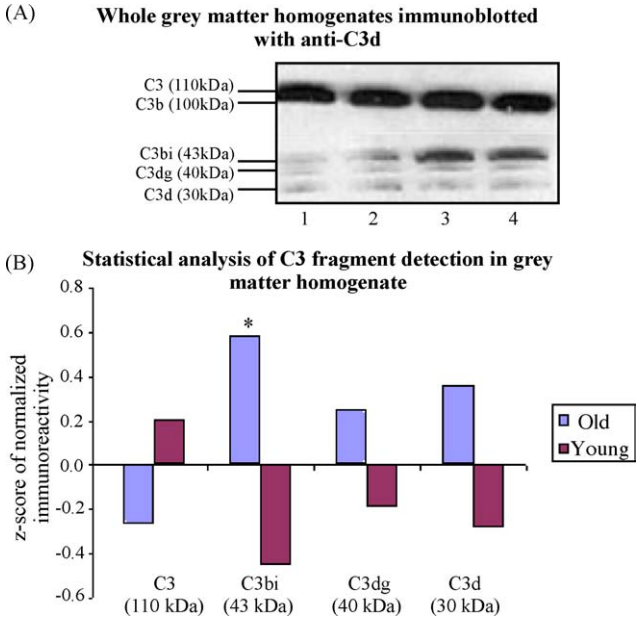

Fig. 6. Quantitation of C3 fragments in cortical gray matter. Western blots using a polyclonal anti-C3d antibody revealed comparable immunostaining of the full-length C3 protein and its degradation products in the brain gray matter (A) of each cortical area (1, temporal; 2, frontal; 3, occipital; 4, parietal of a young animal). C3 and its fragments, C3b, C3bi, C3dg and C3d, were detected in the brains of all eight young and eight old animals used in the study (data not shown). Densitometric measurements of immunoreactivity for C3 and its major fragments were normalized to tubulin. The resulting *z*-scores for each age group were averaged, and then the *z*-scores for each cortical area were averaged, resulting in one mean value for each C3 fragment in both young and aged (B). Mixed linear model analyses of these measurements showed a significant increase in the level of C3bi with age ($P < 0.05$). Increases in the other C3 fragments, along with a decrease in the full-length protein, were also seen with age but lacked statistical significance at the 5% level.

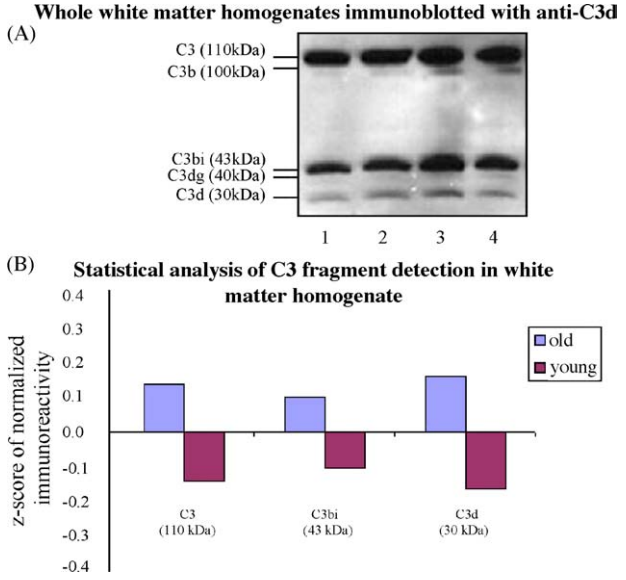

Fig. 7. Quantitation of C3 fragments in cortical white matter. Immunoblotting using a polyclonal anti-C3d antibody revealed comparable levels of the full-length C3 protein and its fragments in the white matter of each cortical area (A: 1, temporal; 2, frontal; 3, occipital; 4, parietal of a young animal), of the eight young and eight old animals (data not shown). Densitometric measurements of immunoreactivity for C3 and its fragments were normalized for equal protein loading using post transfer coomassie-stained gels. Statistical analysis of these measurements showed a trend towards an increase in C3 and its degradation products with age, but the changes were not statistically significant. The immunoblot findings in cortical white matter were comparable to those in cortical gray matter described in Fig. 6.

lobes. Age-related increases were evident in cumulative data but did not reach significance at the 5% level, except for C3bi in the gray matter (Fig. 6B, asterisk).

4. Discussion

The present study demonstrated the wide spread distribution of complement containing CAOs in the brains of both young and aged monkeys, with the number of CAOs being significantly increased with age. Immunohistochemical staining of the CAOs revealed that the C3d and C4d complement fragments closely associated to oligodendrocytes and myelinated fibers and distinctly separated from microglia of the plaque, although localization to astrocytes was not excluded. These findings were supported by immunoblot studies of isolated myelin membranes, which showed the binding of C3d and other C3 fragments to myelin. The identification of the fragments, which included C3b, C3bi, C3dg, and C3d, required alkaline hydrolysis of myelin samples prior to the immunoblot analyses suggesting covalent binding of the C3 degradation products to myelin. The binding of C3 fragments to myelin and the widespread detection of C3d in CAOs were also supported by the detection of these fragments in brain white and gray matter homogenates from both young and aged animals. Although increases in the C3 fragment concentrations were found in brain homogenates of aged monkeys, they lacked statistical significance except for C3bi. The Western blot method may not be sensitive enough to detect significant differences in the levels of C3 fragments in the brain of young and aged animals because the complement derived from the CAOs constitutes only a very small percentage of the total protein in the sample. Alternatively, the absence of complement increases in aged animals could be due to an accelerated turnover of myelin-bound complement proteins as a result of the increased myelin degradation and loss that has been found to occur with age [10,27,45,57].

C3d and C4d, which were detected in the brain and isolated myelin, are the terminal degradation products of C3 and C4, respectively. C3 plays a central role in complement activation and contains an internal thioester bond which is activated when C3 is cleaved to C3b by C3 convertase [30,64]. C3 activation results in covalent binding of C3b to target membranes. The bound C3b either is used to form C5 convertase and/or undergoes catabolic breakdown to C3bi, C3c, C3dg and finally C3d by the complement regulatory proteins factor 1 and its cofactors. With the exception of fluid phase C3c, these C3 fragments contain the membrane binding sites originally exposed with the cleavage of C3 to C3b, and remain covalently bound to the target membrane via thioester linkage which can be disrupted by alkaline hydrolysis [29,30]. The C4 fragments C4b and C4d also contain thioester bonds and bind covalently to target membranes during complement activation [26].

C4d is an activation product of the classical complement system, which has been shown to be initiated by myelin following the binding of C1 and the activation of its subcomponent, C1q [8,62]. The immunohistochemical detection of C4d and C3d on myelinated fibers, together with findings suggesting covalent binding of C3 fragments to myelin, is consistent with activation of the early components of the classical complement pathways by myelin. Oligodendrocytes, which were found to bind C3d and C4d, also have been reported to be capable of initiating classical activation [49,52]. An inability to detect C1q in CAOs and myelin could be due to the rapid turnover of the non-covalently bound protein as opposed to the slower turnover of the covalently bound C3 and C4 fragments. Lectin complement activation of the pathway is also a possible explanation for the lack of detection of C1q in the CAOs. C1q also has been reported to be undetectable in C4d containing CAOs in multiple sclerosis [51].

Although activation of early components of the complement system were demonstrated in the CAOs and in isolated myelin, evidence for activation of terminal complement components was not found. The inability to detect C5, C9, and C5b-9 in these tissues using specific monoclonal and polyclonal antibodies suggests that the proteins required for the formation of C5b-9 were not available or not synthesized in the CAO. The inhibition of C5b-9 complex formation by the complement regulatory proteins could also provide an explanation for the absence of the complex in the CAOs and myelin but would not explain the absence of C5 and C9 in these tissues. Human oligodendrocytes and myelin have been shown to express protectin (CD59), a membrane inhibitor of C5b-9 formation. CD55 and CD46, membrane inhibitors of C3 and C5 convertases, are also reported to be expressed by oligodendrocytes [29,53,67].

Most cells in the brain have been shown to synthesize complement proteins and their regulators [4,35,40]. These cells include microglia, astrocytes, neurons and endothelial cells. Recent transcription and translation studies of complement have shown that oligodendrocytes are additionally capable of synthesizing all the major proteins of the complement system [23]. Thus, the C3 and C4 fragments contained in CAOs could be derived from the synthesis of their parent proteins by oligodendrocytes as well as activated microglia in the CAOs. An increased cellular synthesis with age of C2 and C4 is supported by recent gene microarray studies comparing the brain white matter of young and old monkeys (unpublished observations).

The binding of C3b and C3bi to myelin raises the possibility that these proteins may act as opsonins and interact with the activated microglia in the CAOs with resulting phagocytosis of myelin by these cells. In vivo and in vitro studies of degenerating myelinated nerves indicate that phagocytosis of degraded myelin by macrophages requires opsonization of the myelin with C3bi [6]. In vitro studies of brain myelin have demonstrated that microglia have the capacity to phagocytose normal myelin, a process that is augmented by opsonization of the myelin [47,61,65]. Further studies are required for the assessment of myelin phagocytosis in the removal and turnover of myelin in the brain and they are

the subject of future investigations. Recent reports indicate that the opsonization of apoptotic cells may be a normal biological mechanism for the phagocytic removal of these cells [11,13,38,60]. In the present study, the C3d and C4d binding oligodendrocytes showed no morphological evidence of apoptosis although more specific studies are needed to evaluate cell death.

Activation of early components of complement may have effects on the activity and function of targeted cells. The binding of C3 and C4 fragments to myelin and oligodendrocytes of young monkeys, as well as aged animals, suggests a physiological role of these early activation products on the metabolism and function of these structures. The cleavage products of early complement components, including C3b, C3bi, C3a and C5a, have been demonstrated to induce receptor mediated formation and release of arachidonic acid and its prostaglandin and leukotriene metabolites from a number of different cell types including macrophages, neutrophils and platelets [17,18,50]. There also is evidence that arachidonic acid metabolism in oligodendrocytes, which contain C5a receptors, is stimulated by active complement fragments [42,56]. The effects on arachidonic acid metabolism appear to result from the activation of calcium dependent phospholipases induced by calcium entry into the cells [5,36,59]. Myelin membranes also have been shown to contain calcium dependent thiol-proteases which, when activated by calcium influx, cause myelin protein hydrolysis [63]. These studies suggest an important role of myelin proteases in the degradation and turnover of myelin.

The activation products of early complement components may have additional effects that are receptor mediated. Microglia have been reported to express C3a and C5a receptors as well as C3b and C3bi receptors [4]. Activation of these complement receptors has been shown to induce the release of cytokines and reactive oxygen and nitrogen species which could affect the metabolism and function of myelin and oligodendrocytes [7,16,24,28,34,37,39,46,54,55,58]. The cytokines, IL-1β and TNFα, have been reported to have both anabolic and catabolic effects on myelin [7,24,34]. Along with the oxygen free radicals, these cytokines have also been implicated in demyelination and oligodendrocyte injury [28,37,39,46,54,58]. In addition to these mediators, complement activated microglia may release calcium dependent proteases, namely calpain, whose substrates include myelin proteins [21,55,57,58]. Other studies with co-cultured microglia and oligodendrocytes report that microglial cells can release mediators that enhance myelin synthesis by oligodendrocytes [16].

In summary, the current studies present findings indicating that activation of early components of complement occurs in the brains of both young and old monkeys. C3d and C4d complement activation products are expressed in CAOs containing oligodendrocytes and activated microglia. These CAOs are distributed throughout the brain in both young and aged animals, with the number of CAOs significantly increased with age. The demonstration that C3d and C4d were bound to oligodendrocytes and myelinated fibers and that C3 fragments were covalently bound to myelin is consistent with the initiation of complement activation by these structures. Taken together, the findings support the concept that activation of early complement components in the brain may be a normal physiological process, which up-regulates with age and involves the metabolism and turnover of myelin.

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