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Induction of MC-1 immunoreactivity in axons after injection of the Fc fragment of human immunoglobulins in macaque monkeys

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Abstract Although previous studies have suggested an increased activation of humoral immunity in neurodegenerative diseases, it remains unclear whether this phenomenon is secondary to lesion formation or contributes directly to their development. Using stereotaxic injections in macaque monkey cerebral cortex, we studied the effects of human immunoglobulins on the neuronal cytoskeleton. Under these conditions, several MC-1-immunoreactive axons were observed in the vicinity of injection site. No MC-1 or TG-3 staining was detected in neuronal soma. Ultrastructurally, several axons in the same area displayed curly formations and accumulation of twisted tubules but not paired helical filaments. These data suggest that Fc fragment induce conformational changes of tau and subtle structural alterations in axons in this model. Immunocytochemical analyses in human autopsy materials revealed the presence of human Fc fragments as well as Fc receptors only in large pyramidal neurons known to be vulnerable in brain aging and Alzheimer's disease, further supporting a possible role of immunoglobulins in neurodegeneration.

Keywords Neurodegeneration · Immunoglobulin · Fc fragments · Immunocytochemistry · Macaque monkeys

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Introduction

There is evidence that increased activation of humoral immunity takes place in Alzheimer's disease (AD). For example, increased levels of immunoglobulin (Ig) G have been reported in the brains of cognitively impaired elderly individuals [4], and AD patients have high titers of anti-brain autoantibodies, mainly IgG3 [15, 19]. Importantly, human autoantibodies to neurofibrillary tangles (NFT) and astrocytes in AD brains have been produced by cell lines from AD patients and, to a lesser degree, from normal elderly individuals [6, 13]. To date, the relationship between the presence of IgG in the central nervous system and neurodegeneration remains undetermined. Increased Ig production has long been considered secondary to the accumulation of amyloid deposits and NFT, leading subsequently to the initiation of reactive changes in microglia, and release of potentially neurotoxic products [1]. Using immunocytochemical methods and electron microscopy, we have investigated the effect of stereotaxic injections of Ig on the neuronal cytoskeleton in macaque monkeys. We also examined the cellular distribution of human Ig and their Fc fragments as well as Fc receptors in the cerebral cortex in both AD and elderly control cases.

Materials and methods

Four adult male long-tailed macaque monkeys (*Macaca fascicularis*) were used in the present study. Animals were tranquilized with ketamine hydrochloride (25 mg/kg i.m.), intubated, and maintained under isoflurane general anesthesia (0.5–1.5% as necessary in air), and strict sterile surgical conditions [9]. They were placed for surgery in a Kopf stereotaxic large animal head holder. Up to 12 injections (400 nl each, 1 μg) of an aqueous solution of either

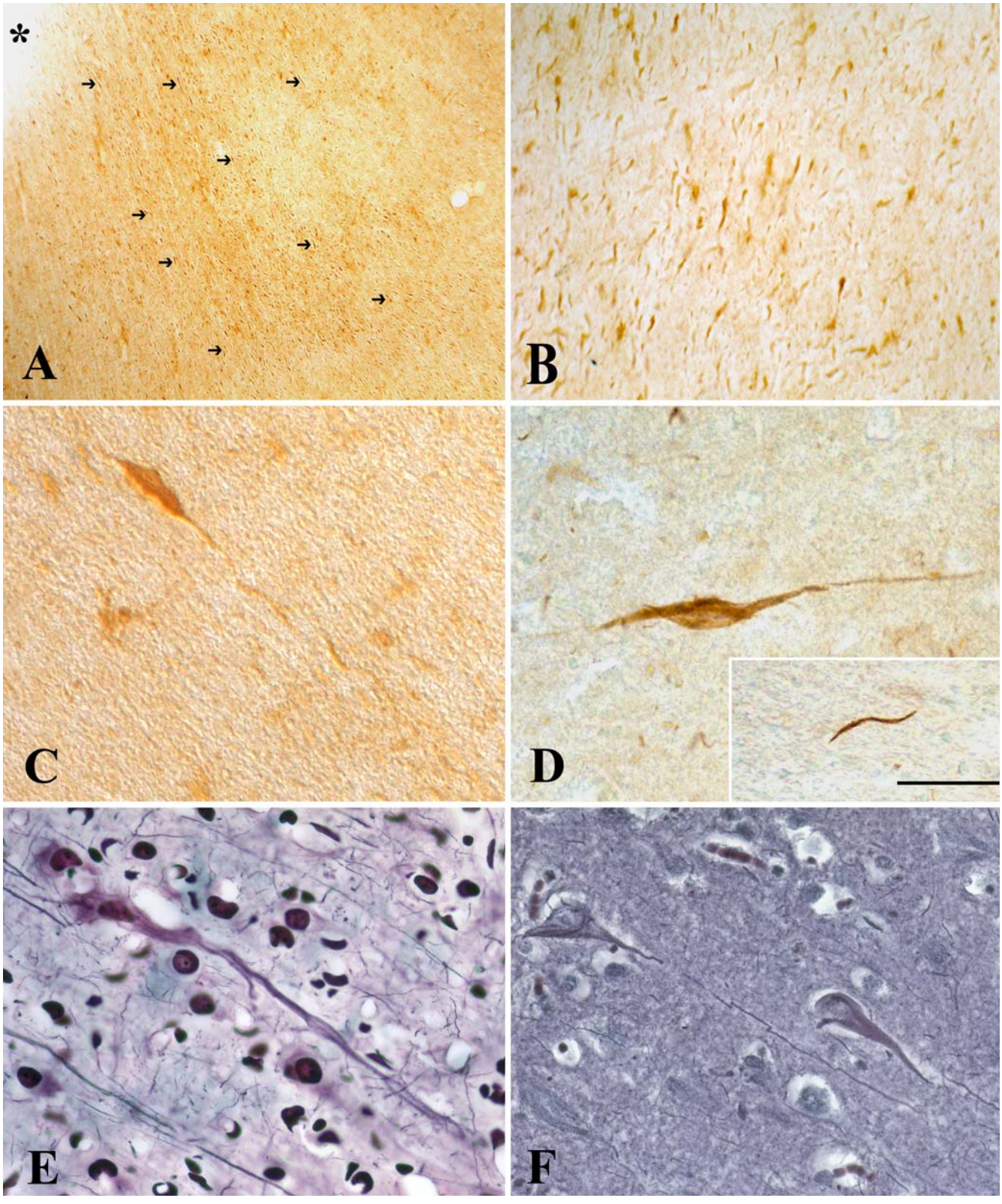


Fig. 1A–D Immunocytochemistry in macaque monkey and AD cerebral cortex. A Numerous MC-1-immunoreactive structures (*arrows*) are visible at low magnification (×5) in the vicinity of the injection site (*arrowheads*) after Ig injection (*asterisk*). B At higher magnification (×10), MC-1-immunoreactive curly axons are depicted 1 cm away from the site of Ig injection. C–F Comparison of MC-1 thread-like fibers (C, D) and Bodian-stained axons (E, F) in macaque monkey after Ig injection (C, E, ×40) and human brain tissue from a 80-year-old patient with early AD pathology in the hippocampal formation (D, F, ×40). Note the similarities between C, D and E, F (*AD* Alzheimer's disease). *Bars* A 340 μm, B 170 μm, C–F 40 μm

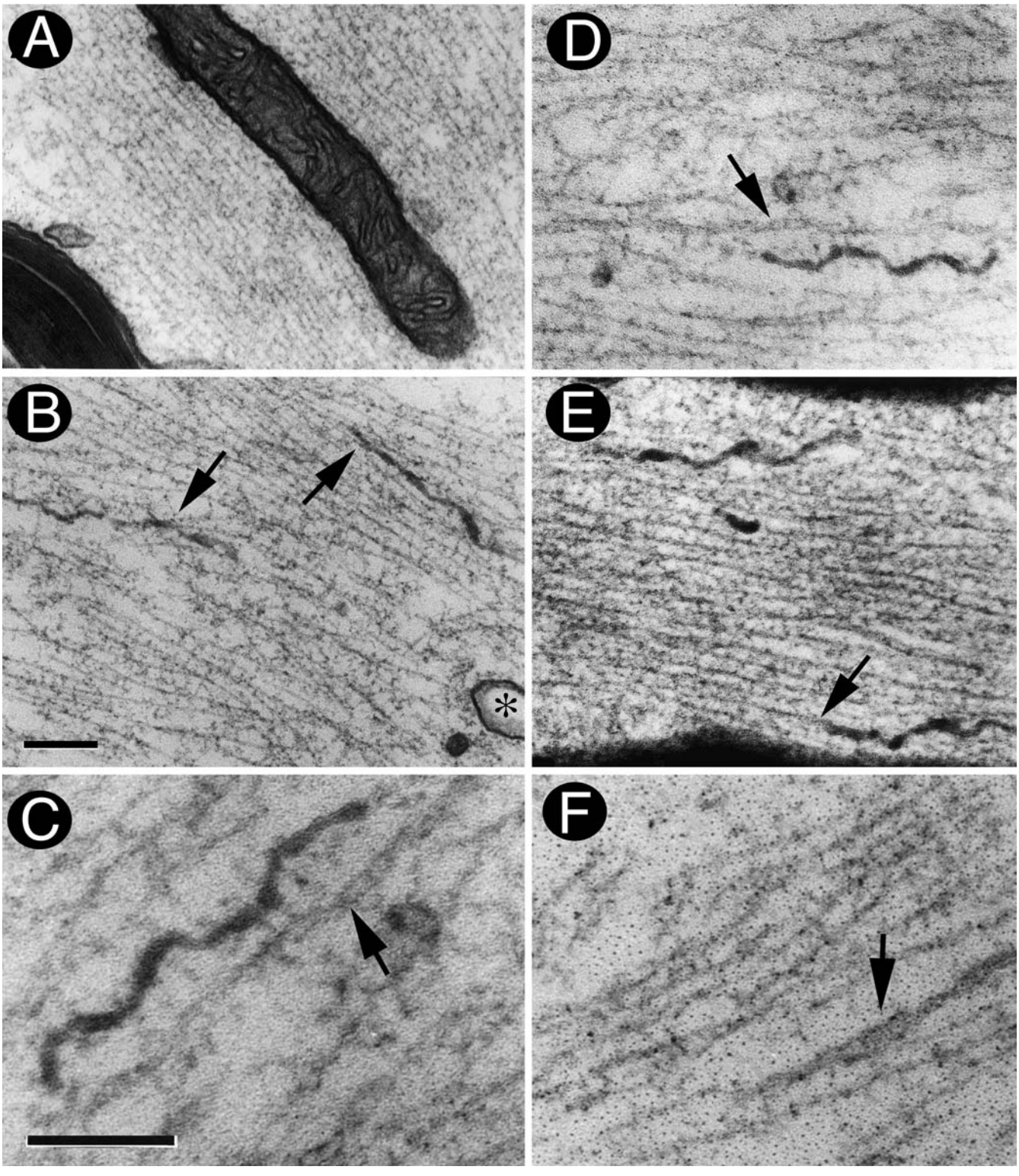


Fig. 2 Electron microscopy of axons after injection of albumin solution (A) and 12 µg Ig (B–F). A Axons were identified by the presence of the myelin sheaths. Tubules appear normal after albumin injection. B–F Examples of curly formations and accumulation of twisted tubules in axons near the injection site. *Arrows* point to regions where tubules merge and form thicker and interlinked filaments. The *asterisk* in B indicates smooth endoplasmic reticulum. B–D Axonal cytoskeleton 1 cm away from an injection site. *Bar* A 250 nm; B, E 140 nm; D 180 nm; C, F 90 nm

Ig, Fc or Fab fragments (4 injections for each of them) obtained from the serum of three elderly control volunteers (one woman, 83 years old; two men, 78 and 79 years old) were placed within the superior parietal and inferior temporal cortex using a 5-µl Hamilton microsyringe with a 24-gauge needle. Ig fractions were prepared by ammonium sulfate precipitation (50% w/v) followed by dialysis against phosphate-buffered saline (PBS, 5 mM sodium phosphate, pH 7.4, 0.9% NaCl). Fc and Fab fragments were produced by digestion of human Ig fractions with papain and pepsin,

respectively [7]. An isotonic saline solution (n=2) or albumin solution (n=2) was placed in the contralateral hemisphere. Following a survival time of 22 days, the animals were deeply anesthetized and transcardially perfused with cold 1% paraformaldehyde in phosphate buffer for 1 min followed by cold 4% paraformaldehyde for 14 min [9]. These experimental protocols were conducted within NIH guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the Mount Sinai School of Medicine.

Brains were cut into 4- to 10-mm-thick coronal blocks, postfixed for 6–7 h in 4% paraformaldehyde with 1% glutaraldehyde at 4°C, and stored in PBS until processed. Macaque monkey brain tissue was immunostained with antibody MC-1, which reacts with conformational epitopes of tau protein [10]. Adjacent sections were stained with antibody TG-3 against phosphorylated threonine 231 of tau protein, which labels early stages of NFT [11], a highly specific and fully characterized antibody to the microtubule-associated protein tau which detects both intracellular and extracellular NFT, and Bodian silver stain [24]. For electron microscopy, 1-mm^3 blocks of tissue near to (5–10 mm) and distant from (20–30 mm) the injection sites were postfixed in a solution containing equal amounts of 2% (v/v) osmium in water, and 3% potassium ferrocyanide in phosphate buffer for 30 min. After dehydration in alcohol and propylene oxide, pieces were embedded in Epon. Thin sections (70 nm thick) were cut with an ultramicrotome (Ultracut E, Reichert Jung, Wien, Austria) and placed on a copper grid. Sections were contrasted with lead citrate for 3 min and observed under a Zeiss EM 10C electron microscope.

To examine the affinity of Ig and their fragments in the human cerebral cortex, 12-µm-thick sections were prepared from left and right hippocampus, superior frontal cortex, and nucleus basalis of Meynert from six cases (three young controls, all men, mean age: 27.5±3.2 years, three aged controls, all men, mean age 82.5±3.5 years, Braak stages I or II; and three neuropathologically confirmed AD; all men, mean age 81.5±2.0 years, Braak stages V or VI) after fixation in 4% paraformaldehyde. Visualization was made with peroxidase-conjugated anti-human Ig, IgG1, IgG2, IgG3 (Dako, dilution 1:100) and 3,3'-diaminobenzidine, and counterstaining with cresyl violet (0.1%). Antibodies against human fragments Fc and Fab were obtained from Sigma (Buchs, St Gall, Switzerland). Albumin detection was performed in adjacent sections as previously described [18]. In addition, a rabbit anti-human IgG Fc receptor I (CD64) obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.) was used to visualize gamma Fc receptor-immunoreactive neurons in adjacent sections as well as in the neocortex of a 6-month-old infant who died accidentally. Double labeling was also performed in adjacent sections to examine colocalization of Ig (Fab and Fc fragments) and Fcγ receptors as well as Ig (Fab and Fc fragments) and NFT in AD cases [24]. These experiments were also performed separately for IgG1, IgG2 and IgG3.

Results

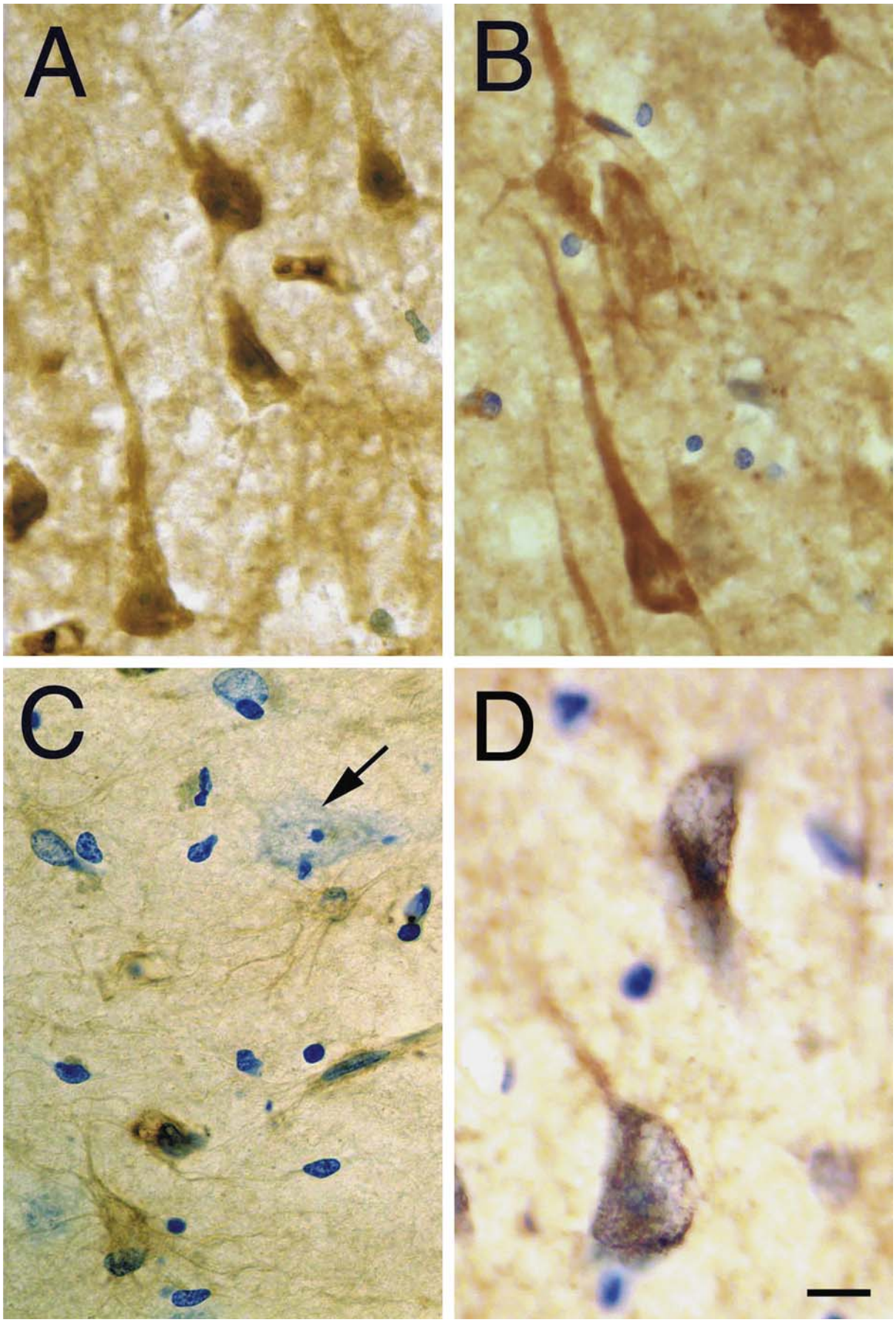
Three weeks following the injection of Ig or Fc fragments a high number of axons immunoreactive for MC-1 was detected in the vicinity (<1 cm) of the corresponding injection areas (Fig. 1A, B). In addition, numerous axons were stained with the Bodian silver impregnation. The patterns of immunostaining and Bodian staining were comparable to those observed in early phases of neurodegeneration in brain aging (Fig. 1C–F). In contrast, following ipsilateral injection of Fab fragments and injection of isotonic saline, or albumin solution in the contralateral hemisphere, no morphological changes were observed (data not shown). Neither TG3-immunoreactive pre-NFT nor intraneuronal and extraneuronal NFT were observed in any of macaque brains. At the electron microscopy level, no paired helical filament (PHF) formation or other major structural abnormality was seen. However, several axons showed curly formations and accumulation of tubules displaying filamentous structures at the beginning of the tubes (Fig. 2E, arrow). These subtle structural alterations were completely absent in control experiments.

The patterns of Ig immunoreactivity in the human hippocampus, superior frontal cortex and nucleus basalis of Meynert did not differ between control and AD cases. Immunoreactivity for the intact human Ig and Fc fragments was observed in the somatodendritic compartment of large pyramidal cells, some axons and the surrounding neuropil (Fig. 3A, B) but was absent in other neuronal types. Ig-containing neurons were consistently free of NFT. A weak immunostaining of Fab fragments was observed in astrocytes but was absent in pyramidal cells (Fig. 3C, arrow), suggesting binding selectivity of Fc fragments for neuronal elements. This binding may be partly mediated by Fcγ receptors which were identified on the somatodendritic part of Fc-immunoreactive pyramidal cells in all three areas studied (Fig. 3D). There was no difference in neuronal distribution between IgG1 and IgG2. The most intense immunostaining of large pyramidal neurons was obtained with anti-IgG3 antibody. In control experiments, no immunoreactivity was detected using antibodies against albumin in adjacent sections. Importantly, Fcγ receptors in neurons were also identified in Fc-immunoreactive pyramidal cells in a normal 6-month-old infant, excluding a cross-reaction between the antibody used and lipofuscin (Fig. 3D).

Discussion

To date, the few in vivo reports addressing the role of immune responses in neurodegenerative processes have shown that local induction of inflammatory conditions may lead to degeneration of hippocampal CA3 neurons and to spatial memory impairment [8], or initiate cholinergic and dopaminergic neuron loss in the medial septal area and substantia nigra in rats [3, 5]. The microscopic pathology and ultrastructural tubule changes reported here does not represent a nonspecific tissue damage reaction since it does not occur following isotonic saline solution, Fab fragment or albumin injection. Moreover, it has been shown that following acute physical trauma in rat neocortex, reactive changes in axon morphology are absent 14 days post-injury [12]. It is also important to note that the induction of these changes does not depend on the specificity of Ig since the injection of Fab fragment had no effect on neuronal integrity. This suggests that a possibly deleterious effect of Ig in neurons is not related to any given inflammatory disease, but is rather associated with the activation of humoral immunity. Although we cannot exclude that molecules sharing the same molecular size and charge with Fc could induce comparable morphological changes, this is unlikely since similar results were obtained after injection of the entire Ig molecule.

The induction of MC-1-immunoreactive axons by the Fc fragment prepared from the sera of elderly healthy


A
B
C
D

controls indicates that humoral immunity reactions could participate in the early phases of axonal degeneration in the absence of cognitive deterioration [14], and could induce conformational changes of tau preceding PHF formation [10]. Importantly, the present experiments did not reveal the punctuate staining characteristic of pre-NFT or full development of NFT in neuronal soma, implying that a possible effect of Ig or Fc fragments on the neuronal cytoskeleton is confined to axons and remains still insufficient to induce the full range of AD pathology [2]. Our ultrastructural data further corroborate this hypothesis. In agreement with previous lines of evidence supporting the presence of pathological phosphorylation sites of tau in the absence of structural changes [20], we did not identify either PHF in axons or other major structural abnormalities in macaque monkey neocortex following Ig and Fc fragment injection. In fact, delay between injection and tissue preparation for histological detection may be too short to develop PHF-like structures in this paradigm. However, there was a development of curly formations and twisted tubules in macaque axons which parallels the microtubule pathology reported in AD and Pick disease [19, 22] and may represent the first indication of structural alterations in axonal cytoskeleton following Fc fragment injection. It should kept in mind that it is not possible to establish a causal relationship between these ultrastructural changes and the presence of MC-1-immunoreactive axons from our data. Future studies including immunoelectron microscopy data are clearly needed to elucidate this point. Although we cannot exclude that these changes reflect collapsed tubes of endoplasmic reticulum, this is an unlikely scenario since they were absent in control experiments.

Our morphological data in the human brain showed that Ig and Fc fragment, but neither Fab fragment nor albumin, were present in NFT-free large pyramidal neurons in the hippocampus, superior frontal cortex and nucleus basalis of Meynert. Serum protein leakage has been previously reported in both non-demented aged individuals and AD patients as an evidence of blood-brain barrier damage [16, 18]. Although this phenomenon may partly reflect post-mortem changes in blood-brain barrier permeability, it is noteworthy that Ig and Fc fragments were found only in this subset of neurons. Moreover, albumin and Fab fragment immunostaining were absent in neurons, rendering it unlikely that the accumulation of Ig and Fc fragments reflects a nonspecific susceptibility to pre- and post-mortem injury. These observations suggest that Ig and Fc fragment uptake after leakage into the brain parenchyma may be confined to cells prone to degenerate in cognitively intact elderly individuals (for review see [17]). The identification by immunocytochemistry of Fcγ receptors in the same neuron subtypes that are Ig immunoreactive suggests a role of these receptors in intraneuronal penetration of Ig. Although Fc receptors have been localized mainly on microglial cells [23], one possible scenario is that when microglial defense mechanisms are not immediately activated, with subsequent phagocytosis of Ig-coated cells, some of the Ig may still penetrate into neurons and modify the neuronal cytoskeleton. Interestingly, despite their different affinity for Fcγ receptors in lymphocytes, IgG1 and IgG2 display the same pattern of neuronal distribution in the aged human brain. Moreover, IgG3 immunostaining was particularly intense in large pyramidal neurons. This suggest that Fcγ receptors are not the only possible way for intraneuronal penetration of Ig. Alternatively, the affinity of different Ig subtypes for neuronal Fc receptors may be different from that reported in lymphocytes. Further biochemical studies are warranted to elucidate the mechanisms of Ig penetration into the central nervous system, specify the affinity and binding site of Fc fragment to tau protein, examine whether phosphorylation of tau protein changes Fc-binding properties, and test the effect of chronic Ig administration on vulnerable subsets of cortical neurons.

◀ **Fig. 3A–D** Immunocytochemistry in human hippocampus from a 80-year-old patient with early AD pathology in the hippocampal formation (also reported in Fig. 1). Sections were incubated with antibodies against human Ig, Fc and Fab fragments. Both soma and dendrites of large pyramidal neurons are immunoreactive for Ig (A) and Fc fragment (B), whereas Fab immunoreactivity is detected in astrocytes (C). D Identification of Fc receptors by immunocytochemistry in the somatodendritic part of Fc-immunoreactive neurons in a 6-month-old infant who died accidentally. *Bar* 10 μm

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