Stereotaxic Injection of IgG From Patients With Alzheimer Disease Initiates Injury of Cholinergic Neurons of the Basal Forebrain

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**Context:** The participation of an immune/inflammatory process in the pathomechanism of sporadic Alzheimer disease (AD) has been suggested by evidence for activated microglia and the potential therapeutic benefit of anti-inflammatory medication.

**Objective:** To define a possible role for IgG in the immune/inflammatory process of AD in humans, we assayed the ability of IgG samples from patients with AD to target the injury to cholinergic neurons in rat basal forebrain in vivo.

**Design:** IgG purified from the serum or plasma from patients with AD and patients with other neurological disease who were used as control (DC) patients was injected stereotaxically into the medial septum of adult rats. Four weeks later coronal sections of the whole medial septum–diagonal bands of Broca region were immunostained for choline acetyltransferase (ChAT) to identify cholinergic neuronal cells.

**Setting:** University medical centers.

**Patients:** Blood samples were collected from 8 patients with probable and definite AD and from 6 age-matched DC patients.

**Main Outcome Measure:** Detection of changes in the number of ChAT immunopositive cell profiles in sections and statistical evaluation.

**Results:** Four weeks after the injections, IgG samples from patients with AD significantly reduced the number of ChAT-immunostained cell profiles in the whole medial septum–diagonal bands of Broca region compared with IgGs from DC patients. Neither DC IgGs nor saline solution significantly decreased the number of ChAT-immunopositive neuronal cell profiles.

**Conclusion:** Data document that IgG from patients with AD can target a stereotaxically induced immune/inflammatory injury to cholinergic neurons in the rat basal forebrain in vivo.

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**Alzheimer Disease (AD)** is a progressive human neurodegenerative disorder causing dementia and characterized by neurofibrillary tangles, amyloid plaques, and severe neuronal degeneration in certain regions of the brain. The loss of cholinergic neurons in the basal forebrain and the marked cholinergic deficit in the cerebral cortex are also the hallmarks of the disease. The cholinergic fiber loss displays a significant correlation with the density of neurofibrillary tangles in the cerebral cortex.

The cause of sporadic AD is unknown. However, the potential participation of an immune/inflammatory process has been suggested by evidence of activated microglia and the potential therapeutic benefit of anti-inflammatory medication. The reports of antibodies in the serum and cerebrospinal fluid samples of patients with AD directed to cholinergic neurons, and to neurofilament proteins, prompted us to test the hypothesis that IgG could help target this inflammatory response to cholinergic cells in the basal forebrain. In support of this hypothesis, we developed an animal model of an immune-mediated damage of septal cholinergic neurons. Our experiments were carried out to provide evidence that stereotaxic injection of IgG from patients with AD into the medial septum of rats could target the inflammatory response and result in cholinergic neuron injury in the basal forebrain.

**RESULTS**

**Changes in the Number of Neuronal Profiles Immunostained for ChAT in the Whole Medial Septum–Diagonal Bands of Broca Region Following IgG Injection**

A significant decrease (F7,410, df1 = 1, df2 = 12, P = .02) was demonstrated in cell profiles in the whole medial septum after
SUBJECTS, MATERIALS, AND METHODS

PATIENTS AND PREPARATION OF IgG

Serum or plasma samples were collected with informed consent from 8 patients with probable and definite AD12 and also from age-matched patients with other neurologic diseases who were used as control (DC) patients (Figure 1). Immunoglobulins were purified with a combination of precipitation with 45% ammonium-sulfate and ion exchange chromatography with a high flow rate according to Hou and Mandaro.13 Samples were equilibrated to pH 7.4, concentrated by pressure dialysis, filtered through a mesh of 0.2-µm pore size, and stored at –80°C until needed.

STEREOTAXIC INJECTION OF IgG INTO THE MEDIAL SEPTUM OF RATS

Sprague-Dawley adult female rats (Harlan Sprague-Dawley Inc, Indianapolis, Ind) in deep anesthesia were injected stereotaxically in the brain with 100-µg AD IgG, DC IgG, or saline solution (3 µL). The injection coordinates were as follows: anterior (A), 0.6 mm; lateral (L), 0.6 mm; ventral (V), ~8.0 mm from the dura. Each rat was given a single injection of IgG obtained from a particular patient. IgG from every patient was injected into 3 or 4 animals. As a control, the most effective AD IgG (sample 3) was heat-inactivated prior to injection into the medial septal of 3 rats.

HISTOLOGICAL PROCESSING

Four weeks after receiving the injections, the anesthetized animals were perfused through the heart with 100-mmol/L phosphate-buffered saline followed by fixative. Brains were postfixed with immersion in the same fixative, and the whole medial septal–diagonal bands of Broca region kept in 30% sucrose containing phosphate-buffered saline solution. Specimens were frozen in 2-methyl-butane and the septal region cut in 20-µm-thick coronal sections.

IMMUNOHISTOCHEMICAL STAINING FOR ChAT AND PARVALBUMIN

After rinsing and saturation of nonspecific antibody binding sites, normal rat brain sections were incubated with human IgG samples diluted to 30 µg/ml with phosphate-buffered saline solution containing 3% normal goat serum on a rocker for 24 hours at 4°C, followed by overnight incubation at 4°C with 1:200 dilution of FITC-labeled goat anti-human IgG (Fab specific).

DIRECT IMMUNOHISTOCHEMICAL STAINING FOR HUMAN AND RAT IgG

After blocking the nonspecific antibody binding sites, sections of the brains containing the whole medial septal–diagonal band region were incubated overnight on a rocker at 4°C with 1:200 dilution of goat anti-human IgG (Fab specific) conjugated with fluorescein-isothiocyanate (FITC) (Sigma Chemical Co). As controls, sections were preincubated with unlabeled anti-human IgG prior to incubation with FITC-labeled antibody, the FITC-labeled anti-human IgG was replaced with 1:200 dilution of FITC conjugated antirat IgG (Sigma Chemical Co), or with unlabeled antirat IgG prior to incubation with FITC-labeled antibody.

INDIRECT IMMUNOHISTOCHEMICAL ASSAY FOR DETECTION OF BINDING OF HUMAN IgG TO RAT BRAIN

After blocking nonspecific antibody binding sites, normal rat brain sections were incubated with human IgG samples diluted to 50 µg/ml with phosphate-buffered saline solution containing 5% normal goat serum on a rocker for 24 hours at 4°C, followed by overnight incubation at 4°C with 1:200 dilution of FITC-labeled goat anti-human IgG (Fab specific).
Figure 1. Distribution of the means of the numbers of choline acetyltransferase (ChAT)–immunopositive neuronal profiles in coronal sections sorted in 4 (A-D) zones (see “Numerical and Statistical Evaluation of the Effect of IgG Injections on Cholinergic Neurons in the Whole Medial Septum–Diagonal Bands of Broca Region” subsection of the “Subjects, Materials, and Methods” section for further explanation) of the whole medial septum–diagonal bands of Broca area from rats after stereotaxic injection of Alzheimer disease (AD) IgG and other neurological disease control (DC) IgG samples. Each point represents the mean of the numbers of neurons per section (in the injected and uninjected sides) immunostained for ChAT in the zone of a single animal’s brain. The left column identifies the animals (untreated, injected with saline solution, injected with 8 different AD IgG and with 6 different DC IgG). The counted cholinergic cells are located in the dark-shaded area in the middle of the sections. Especially in zones B and C the means of ChAT-immunopositive cells per section are much more scattered and less in the group of AD IgG–injected animals than in the untreated animals, in saline solution–injected, or DC IgG–injected ones. Injections of AD IgG1 and IgG3 were most effective in decreasing the number of ChAT-immunostained neuronal profiles per section. GB indicates Guillain-Barré syndrome; ALS, amyotrophic lateral sclerosis; PD, Parkinson disease; CVA, cerebrovascular accident; and MG, myasthenia gravis.
stereotaxic injection of AD IgG (mean [SEM], 118.78 [8.24]) compared with the injection of DC IgG (153.04 [9.51]).

Regardless of the type of IgG employed (AD IgG or DC IgG), fewer ChAT-positive profiles were observed on the injected side compared with the contralateral side (F10.940, df1 = 1, df2 = 12, P < .001). A similar effect might explain the interaction in the zone 3 group analysis, which was the only significant interaction obtained (F3.485, df1 = 3, df2 = 10, P = .058). The difference between the AD IgG and DC IgG groups also related to the distance from the stereotaxic injection. Pairwise comparison of groups in different zones resulted in significant differences only in zones B and C, with the following P values at the different zones: A, .24; B, .04; C, .02; and D, .09. The actual mean values in the different zones are shown in Figure 2 which also demonstrates that the ChAT-positive profile numbers after DC IgG injections were similar to those after saline solution injections in all zones on both sides. Although not analyzed quantitatively, the individual AD IgGs seemed to reduce the number of ChAT-positive cell profiles to differing extents (Figure 1).

Heat inactivation of the most effective AD IgG (sample 3) destroyed its ability to decrease the number of ChAT-immunostained profiles after local inoculation in the septum (data not shown).

REGIONAL CHANGES IN ChAT STAINING IN THE WHOLE MEDIAL SEPTUM–DIAGONAL BANDS OF BROCA REGION FOLLOWING AD IgG INJECTION

There were also regional cell losses observed mainly in the vicinity of the injection site. They appeared as circumscribed areas with a lack of cells immunostained for ChAT. This focal loss of neurons was characteristic for AD IgG injections but not for DC IgG injections (Figure 3). The focal losses were restricted to ChAT-immunopositive cells, while neurons stained for parvalbumin did not show any regional changes.

No striking lymphocytic inflammatory reaction was noted in the area after the injection of either AD IgGs or DC IgGs. Following the injection of AD IgG, some ChAT-positive cells (about 5%) in the whole medial septum–diagonal band of Broca demonstrated...
shrinkage and fragmentation, and were surrounded by glial cells (satellitosis by microglia) or underwent neuronophagia (Figure 4, A through C). Similar tissue changes were observed in the cerebral cortex after septal injection of AD IgG (patients 1, 3, and 7), and were restricted to about 5% of large pyramidal cells in the same sections (Figure 4, D).

Figure 4. Degeneration of neurons with satellitosis and neuronophagia in the whole medial septum–diagonal bands of Broca of a rat injected with IgG from patients with Alzheimer disease (AD) 4 weeks earlier (A-C, neurons have lost their structural integrity, and are surrounded by glial cells [arrows]); (cresyl violet, ×600). D, Destroyed cortical pyramidal cells (arrows) in the frontal cortex with neuronophagia in a rat 4 weeks after the septal injection of AD IgG (cresyl violet, original magnification ×300).

ACCUMULATION OF IgG IN THE WHOLE MEDIAL SEPTUM–DIAGONAL BANDS OF BROCA REGION OF INJECTED RATS

IgG from 6 of 8 patients with AD were localized to neurons of the septal region. This immunohistochemical reactivity was noted with decreasing intensity 3 days and 1 and 2 weeks after the stereotaxic injection and diminished after pretreatment of the sections with unlabeled antihuman IgG. Even 4 weeks after injection, traces of AD IgG could be detected in neurons of the horizontal diagonal band. This was in contrast with the indirect immunohistochemical binding assay of AD IgG that revealed nonselective staining of neurons in the medial septum and also in other brain regions as well in fixed tissue sections. Two AD IgG samples that had no effect on the number of cholinergic neurons immunostained vessel walls and glial cells but not neurons. IgG from DC patients could be detected with direct immunostaining within and outside neurons only up to 7 days after receiving the injection and just in close proximity to the needle track.

Direct immunostaining for rat IgG revealed that endogenous IgG also accumulated in neurons of the medial septum–diagonal bands of Broca region in the brains of rats injected with AD IgG and remained present for weeks (Figure 5). Two weeks after injection, the rats’ own IgG was also detected around large pyramidal cells in the cerebral cortex.

Figure 5. Accumulation of the endogenous (the rat’s own) IgG in neurons of the medial septum 2 weeks after the stereotaxic injection of IgG from patients with Alzheimer disease. Direct immunofluorescence reaction with fluorescein-isothiocyanate–labeled antirat IgG (original magnification ×80).

COMMENT

In this study we have demonstrated that AD IgG but not DC IgG initiated selective injury of cholinergic neurons as assayed 4 weeks after stereotaxic injection into rat septum. Maximal loss of ChAT immunostaining was found in the vicinity of the needle track, but the effect was also noted to a slightly lesser extent on the contralateral side. The decreased immunostaining was unaccompanied by any significant lymphocytic infiltrate, except for occasional microglial nodules and neuronophagia. Alzheimer disease IgG was localized to septal neurons in highest concentration immediately following injection, and decreased in intensity over the subsequent several weeks.

The goal of this study was not to define an effect on the absolute number of septal cholinergic cells injured after stereotaxic injection of IgG into the medial septum, but to contrast the relative differential effects on the septal cholinergic neuron population of AD IgG compared with DC IgG. We have previously employed a similar protocol to document that Parkinson disease IgG could target an immune/inflammatory injury to substantia nigra tyrosine hydroxylase–positive neurons.

To reduce the bias in profile counting we serially sectioned the whole medial septum–diagonal band of Broca region, and selected sections for analysis in a systematic random manner, ie, the sampling was started at a randomly selected rostrocaudal position, then sections were selected for counting at regular distances from this point. We also sorted the sections into 4 different groups according to their rostrocaudal position (zones A-D, Figure 1) to compensate for the different probabil-
ity of hitting a small cell (most anterior and medial parts) and large cell (posterior lateral parts) within a section. Within a zone, cells have an approximately similar chance to be sampled by sectioning due to their size and/or preferred orientation. Intrazone comparisons of the effect of different IgG preparations were then performed, zone by zone.

With these counting techniques, we demonstrated that the AD IgG initiated a decreased ChAT immunoreactivity of medial septal neurons compared with the group composed of DC IgG. However, it was impossible to determine whether ChAT-positive cells were actually lost or injured with resulting down-regulation of their ChAT immunohistochemical reactivity. The presence of satellitosis and neuronophagia, albeit sparse, would suggest some cell loss. Parvalbumin-positive neurons, however, were clearly spared.

The mechanism of IgG-initiated injury to septal cholinergic neurons is unclear but we do not consider the effect to be due to direct killing of neurons by the AD IgG. Our prior stereotaxic injection studies of substantia nigra injury following Parkinson disease IgG18 and septal cholinergic injury following cholinergic cell-immunized guinea pig IgG19 were associated with an enhanced microglial inflammatory reaction. We suggest that in the presence of AD IgG, a similar inflammatory response may be targeted to septal cholinergic neurons. As in the previous models IgG was demonstrated in the neuronal target, namely, whole medial septum– diagonal band of Broca region cholinergic neurons. Further, cholinergic cell injury was noted only in those animals whose septal cholinergic neurons had taken up and accumulated the AD IgG in vivo. Probably only a subgroup of IgG from a given patient with AD that binds to and accumulates in the cholinergic cells of the basal forebrain is responsible for targeting the inflammatory response to these cholinergic cells. Although the mechanism of IgG targeting the inflammatory response is unclear, it is possible that IgG bound to antigens on cholinergic neurons could activate a site-specific response in microglia through their Fc receptors,19 which in turn could amplify cholinergic cell injury.20

There is no evidence implicating a role for IgG in human AD. However, our data document that stereotoxic injection of IgG into the basal forebrain of the rat can target the inflammatory response to cholinergic neurons in vivo, and provide further evidence for the potential participation of an immune/inflammatory process in the pathogenesis of AD.

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