Experimental Destruction of Substantia Nigra Initiated by Parkinson Disease Immunoglobulins

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Background: Increased levels of free radicals and oxidative stress may contribute to the pathogenesis of substantia nigra (SN) injury in Parkinson disease (PD), but the initiating etiologic factors remain undefined in most cases.

Objective: To determine the potential importance of immune mechanisms in triggering or amplifying neuronal injury, we assayed serum samples from patients with PD to determine the ability of IgG to initiate relatively specific SN injury in vivo.

Methods: IgG purified from the serum of 5 patients with PD and 10 disease control (DC) patients was injected into the right side of the SN in adult rats. Coronal sections were cut from the whole brain at the level of the stereotaxic injections, stained for tyrosine hydroxylase and with cresyl violet, and cellular profiles were counted in identical brain regions at the injection and contralateral sides. The ratio of cell profile counts of the corresponding injected and un.injected regions was used as an internal standard.

Results: Four weeks following injection of IgG, a 50% decrease in tyrosine hydroxylase–positive cellular profiles was noted on the injected sides compared with the contralateral sides of the same animals. Similarly, applied DC IgG caused only an 18% decrease. Cresyl violet staining revealed a 35% decrease in neuronal profiles of PD IgG injected into the SN pars compacta compared with the contralateral un.injected side, whereas DC IgG caused a minimal 10% decrease. Even at 4 weeks after the PD IgG injections, perivascular inflammation and significant microglial infiltration were present near injured SN pars compacta neurons. No cytotoxic effects of PD IgG were noted in choline acetyltransferase–positive neurons after stereotaxic injections into the medial septal region. Absorption of PD IgG with mesencephalic membranes and protein A agarose gel beads removed cytotoxicity, while absorption with liver membranes did not change the cytotoxicity.

Conclusions: Our data suggest that PD IgG can initiate a relatively specific inflammatory destruction of SN pars compacta neurons in vivo and demonstrate the potential relevance of immune mechanisms in PD.

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PARKINSON DISEASE (PD) is a neurodegenerative disorder characterized by a relatively selective loss of neurons of the nigrostriatal pathway. Decreased levels of reduced glutathione, increased levels of iron, altered mitochondrial function, as well as increased lipid peroxidation suggest the involvement of excessive free radical formation and oxidative stress in substantia nigra (SN) cell injury. However, the initiating or triggering events in most patients with PD are unclear. In rare cases, the ingestion of N-methyl-D-phenyl-1,2,3,6-tetrahydropyridine has been reported to trigger injury in SN cells by its metabolite, 1-methyl-4-phenylpyridinium, which inhibits mitochondrial complex I and leads to oxidative stress.

Inflammation and immune mechanisms have been proposed as etiologic factors, but convincing evidence of their primary importance is lacking. Activated microglia have been described in the SN and antibodies to dopaminergic neurons have been found in the cerebrospinal fluid of patients with PD. In addition, cytotoxic factors have been reported in the cerebrospinal fluid of these patients. The SN can be a target of an immune-mediated injury since immunization of guinea pigs with bovine mesencephalic homogenates or hybrid dopaminergic cell line (MES 23.5) homogenates can damage the SN and decrease tyrosine hydroxylase (TH) activity as well as dopamine content in the nigrostriatal system. Herein, we provide evidence that immune mechanisms initiate or amplify a cascade of neuronal injury by demonstrating that IgG isolated from the serum of patients with PD can damage dopaminergic cells of the SN in rats in vivo.
PATIENTS AND METHODS

PATIENTS

Serum samples were collected with informed consent from 3 patients with idiopathic PD (3 men aged 56, 64, and 70 years, and 2 women aged 56 and 67 years) with moderate to severe disease of 3 to 7 years' duration; all patients were receiving dopaminergic medications. Serum samples were also collected from patients (2 samples each) with chronic inflammatory demyelinating polyneuropathy (1 woman aged 69 years and 1 man aged 61 years), Alzheimer disease (1 woman aged 70 years and 1 man aged 69 years), and amyotrophic lateral sclerosis (1 woman aged 57 years and 1 man aged 53 years), and from patients (1 sample each) with multiple sclerosis (1 man aged 66 years), Guillain-Barre syndrome (1 man aged 37 years), eosinophilia-myalgia syndrome (1 woman aged 34 years), and ischemic stroke (1 man aged 74 years). For each patient, the clinical diagnosis had been established on the basis of medical history, physical examinations, and tests specific for each disease. Immunoglobulins were purified from serum samples of patients with PD and controls using ferric ammonium sulfate precipitation, ion exchange chromatography, and filtration dialysis, as previously described. The IgG preparations were at least 80% pure. The protein concentration was adjusted to 19 to 20 mg/mL with phosphate-buffered saline (PBS) (10 mmol/L; pH, 7.4) before use.

SN INJECTIONS

Sprague-Dawley rats weighing 250 to 350 g were anesthetized with intramuscular injections of 0.6-mL/kg combination anesthetics (ketamine hydrochloride, 42.8 mg/mL; xylazine hydrochloride, 8.6 mg/mL; and acepromazine maleate, 1.4 mg/mL), while their heads were fixed in a stereotaxic device (David Kopf Instruments, Tugunga, Calif). After a small hole was drilled at the appropriate site of the right side of the skull, a total of 160 μg of IgG was administered into the SN regions of rats by 2 separate unilateral stereotaxic microinjections. Three animals were treated with each of the IgGs from 5 PD and 10 disease control (DC) patients. Six rats that were similarly injected with 0.9% sodium chloride served as healthy controls. These stereotaxic injections of the IgG samples into the right medial forebrain bundle and SN pars compacta (SNpc) were made with a 10-μL Hamilton syringe (Foxboro Company, North Haven, Conn) and a 26-gauge needle at the following coordinates: (1) medial forebrain bundle: 4.4 mm from the bregma, 1.1 mm from the midline, and 7.8 mm from the dura mater, and (2) SNpc: 3.0 mm from the bregma, 1.8 mm from the midline, and 7.6 mm from the dura mater. The injection rate was 1 μL/min, and at the conclusion of the injection, the needle was left for an additional 5 minutes to allow the solution to diffuse undisturbed. Finally, after the needle was removed, the skin was closed with metal clips over the skull. Until the rats were killed, they were kept on a 12-hour light/dark cycle, with food and water ad libitum.

TISSUE PREPARATION

Four weeks after the stereotaxic injections, the rats were anesthetized intraperitoneally with sodium pentobarbital and then transcardially perfused with 200 mL of ice-cold 0.1-mol/L PBS (pH, 7.4). The brains were quickly removed from the skulls and fixed for 3 days in 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid, followed by immersion in 30% sucrose for 3 additional days (4°C). Blocks were frozen in 2-methylbutane, chilled with liquid nitrogen, and whole brain coronal sections were cut around the position of IgG injections in a cryostat (−18°C) at a thickness of 32 μm (necessary thickness for free-floating TH immunostaining) or 16 μm (for cresyl violet staining). Immunostaining of dopaminergic neurons was undertaken with a TH polyclonal antibody (Eugene Tech.

RESULTS

EFFECTS OF PD IgG ON TH-POSITIVE SNpc NEURONS

IgG obtained from patients with PD and DCs were injected stereotactically into the SN region of adult female Sprague-Dawley rats under deep anesthesia. Four weeks after the injections, decreases in TH-positive cells were apparent in the sides injected with PD IgG (Figure 1). At a slightly higher magnification of the right side of the SN, the loss of TH-positive cells can be documented (Figure 2). Statistically, we analyzed the ratio of TH-positive cell numbers on the injected side to similar numbers on the uninjected side (Table 1). This technique was used to compensate for variations in cell counts due to different positions of sectioning in relation to the overall SN structure, the use of alternate sections for TH and cresyl violet staining, and the variability in staining. This analysis was possible because there was no statistically significant loss of neurons in the uninjected left side after the right side was injected with PD IgG, DC IgG (Table 1), or saline (the ratio ± SD of cell counts in the injected side to those in the uninjected side, as estimated on 8 sections from a single animal, was 0.92 ± 0.04). In Table 1, the PD IgG caused a 50% decrease in TH-positive cells, while DC IgG caused an 18% decrease in TH-positive cells when compared with the uninjected side. Qualitative estimations of IgG-induced changes were also made by pooling the ratios of cell counts in the injected side to those in the uninjected side in all sections from a single rat injected with a particular PD or DC IgG (Figure 3). With this analysis, differences among individual preparations were noted. However, PD IgG was even more toxic than the DC preparations in this analysis.

EFFECTS OF PD IgG ON CRESYL VIOLET-POSITIVE SNpc NEURONS

By a similar analysis of cresyl violet–stained SN neurons on injected and uninjected sides, the PD IgG injections were noted to cause a 35% decrease in SNpc neurons, while DC IgG injections caused a minimal 10% decrease (Table 1). Thus, the effects on TH activity appeared to be greater than the effects on the number of cresyl violet–stained SNpc neurons. No significant changes were found with either TH-positive staining or cresyl violet staining following saline
BEHAVIORAL EFFECTS OF PD IgG INJECTIONS

At 2, 3, and 4 weeks after injection, 2 of 3 rats injected with PD IgG developed asymmetrical (contralateral to the injection side) rotational behavior in response to apomorphine hydrochloride, 0.3 mg/kg, administered subcutaneously. These 2 animals subsequently demonstrated a loss in TH immunoreactivity in at least 50% of TH-positive SNpc neurons, respectively, while the third animal, which did not manifest rotational behavior, demonstrated a loss of TH immunoreactivity in only 44% of TH-positive neurons. The requirement for significant cell loss to document rotational behavior is in concert with other reports.19,20 However, no rats injected with other PD IgGs, DC IgGs, or rats injected with saline showed asymmetrical rotational responses at any time after injection, even though 5 other rats injected with 4 different PD IgGs demonstrated a loss in TH immunoreactivity in at least 50% of TH-positive SNpc neurons.

TIME AND DOSE RESPONSE OF PD IgG INJECTIONS

To further define the specific deleterious effects of PD IgG on SNpc neurons, we carried out a time-dependent and dose-dependent analysis of IgG effects on TH-positive cells of the SNpc (Table 2). No significant difference in the destruction of the SNpc was demonstrated at 4 weeks compared with 2 weeks. However, a definite dose dependence was demonstrated, since an injection of 160 µg was more destructive than an injection of 80 µg. Preabsorption of the IgG preparation with mesencephalic membrane preparations completely removed the toxic effect of PD IgG at ratios of membrane protein to IgG of 10:1 (Table 3). Similar pretreatment with varying doses of liver membrane proteins had no effect on the toxicity of PD IgG. Absorption of the IgG from PD preparations with protein A agarose beads completely removed toxicity11 (Table 3).

SPECIFICITY OF PD IgG INJECTIONS

The effects of PD IgG were relatively specific for SNpc neurons since stereotaxic injections of PD IgG into the medial
The septal region produced no decrease in the number of choline acetyltransferase–positive neurons in the medial septal region. Two rats each were injected with 3 different PD IgGs, and after 4 weeks, the mean ratio ± SD of the number of medial septal choline acetyltransferase–stained neurons on injected sides to the number on uninjected sides was 0.99 ± 0.14 (findings from 73 sections of 6 rats). Thus, the effects of the PD IgG were specific for SNpc dopaminergic neurons with no demonstrable effects on choline acetyltransferase–positive medial septal neurons.

**INFLAMMATORY RESPONSE TRIGGERED BY PD IgG**

Further microscopic examination of the lesions demonstrated the presence of perivascular edema and inflammation (few lymphocytes, mainly macrophages and microglial cells [Figure 4]) together with mononuclear cell infiltration near injured SNpc neurons at 4 weeks after injection. The response was similar to the many HLA-DR–positive microglia phagocytosing dopamine neurons in PD brains.12 This cellular response was absent in SNpc injected with DC IgG. The perivascular inflammation also suggests that PD IgG together with needle trauma elicited the participation of macrophage elements, while DC IgG together with trauma did not elicit a chronic inflammatory response.

**COMMENT**

Our study demonstrates that dopaminergic lesions can be transferred to experimental animals relatively selectively by immunoglobulins from the serum of patients with PD. Unilateral injections of PD IgG into the SN of rats significantly reduced both the TH reactivity and the number of SNpc neurons, while neurons of the contralateral uninjected SNpc were spared.

In this study, we attempted to characterize the cytotoxic potency of a particular IgG preparation on SN cells in the vicinity of the injections. We expected that stereotoxic injections of IgG would result in differential injury to SN neurons, depending on the distance from the site of injection, and we had no a priori data as to the size of the lesion. Thus, we did not intend to determine the absolute number of residual noninjured cells in the whole SN, which would have required the combined application of the dissector method and the Cavalieri principle.21 On the other hand, the dissector method alone would have resulted in relative numbers only (volume density of SN cells), with the additional risk of a “reference trap,”22 and would have required an exact determination of section thickness, which was unavailable in our study. Instead of attempting to determine absolute cell numbers from single sections with different correction procedures22 and with the prerequisite of constant section thickness, we used an internal standard (namely, the ratio of SNpc cells on the injected side to those on the uninjected side) to systematically characterize the relative cytotoxic potency of different IgG preparations.

Inflammation was still present 4 weeks after stereotoxic injections with perivascular edema and mononuclear cell infiltration. This inflammation appeared to be enhanced in...
In the present study, it was not possible to determine the specific titer of cytotoxic immunoglobulins or to correlate the cytotoxic potency of the IgG with the rate of the patient’s disease progression. Consequently, it was not possible to determine whether trauma was a factor in the SNpc injury mediated by PD IgG. However, without careful monitoring of the onset of the cellular infiltration, the inflammatory response could be the result of neuronal toxic effects rather than its source. Activity of microglia is one of the factors that initiate injury mediated by free radicals, and microglia are extremely high in the SN. Microglia are involved in inducing SN injury. Furthermore, activated microglia can initiate injury mediated by free radicals, and microglia are clearly present in SN lesions in PD.

Figure 3. Ratio of tyrosine hydroxylase–positive cell counts in the injected side compared with the uninjected side. Each point on the scattergram represents a single animal. The dashed line represents the mean of observations in the Parkinson disease (PD) group. PD indicates Parkinson disease; SNpc, substantia nigra pars compacta; AD, Alzheimer disease; GBS, Guillain-Barré syndrome; ALS, amyotrophic lateral sclerosis; MS, multiple sclerosis; and CIDP, chronic inflammatory demyelinating polyneuropathy.

Table 1. Quantification of SNpc Neurons After IgG Injection*

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<th>TH-Staining</th>
<th>CV-Staining</th>
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<td></td>
<td>Mean ± Variance (n)</td>
<td>Mean ± Variance (n)</td>
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<tr>
<td>PD</td>
<td>0.499 ± 0.027 (15)</td>
<td>0.651 ± 0.022 (13)</td>
</tr>
<tr>
<td>DC</td>
<td>0.818 ± 0.010 (28)</td>
<td>0.899 ± 0.014 (28)</td>
</tr>
<tr>
<td>Significance</td>
<td>F, sub, TH = 4.96</td>
<td>F, sub, CV = 4.96</td>
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Table 2. Dose and Time Dependence of PD IgG Effect on TH-Positive SNpc Neurons*

<table>
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<th>Dose of PD IgG</th>
<th>80 µg</th>
<th>160 µg</th>
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<tr>
<td>2 wk</td>
<td>0.64 ± 0.17 (14)</td>
<td>0.47 ± 0.12 (15)</td>
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<tr>
<td>4 wk</td>
<td>0.58 ± 0.18 (12)</td>
<td>0.42 ± 0.19 (14)</td>
</tr>
<tr>
<td>Significance</td>
<td>t = 0.84 (P &gt; 0.40)</td>
<td>t = 0.82 (P &gt; 0.40)</td>
</tr>
<tr>
<td>Pooled</td>
<td>0.61 ± 0.18 (26)</td>
<td>0.45 ± 0.16 (29)</td>
</tr>
<tr>
<td>Significance</td>
<td>t = 3.42 (P &lt; 0.005)</td>
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*In each time and dose group, the ratio of tyrosine hydroxylase (TH)–positive cell counts in the injected side to the uninjected side was determined on the same section, and then the data were pooled (3 animals for each time point and/or dose value; 5-6 sections per animal). Values are given as mean ± SD; number of sections analyzed are given in parentheses.

The structure of the substantia nigra pars compacta (SNpc) and the density of neurons (arrows) are preserved in the vicinity of the site of local injection with IgG from a patient with chronic inflammatory demyelinating polyneuropathy. The structure of the SNpc is destroyed, and perivascular edema (arrowheads) and mononuclear cell infiltration (mainly macrophages and few lymphocytes) are present 4 weeks after the injection of Parkinson disease IgG. Sections are 2 µm thick, embedded in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) (toluidine blue, original magnification ×220).

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not possible to suggest whether the cytotoxic IgG in the patients’ blood was a consequence or cause of SNpc injury. However, the fact that cytotoxicity could be passively transferred suggests that PD IgG may either initiate or amplify cell injury, just as the passive transfer of impaired neuromuscular transmission with serum IgG provided evidence for the importance of immune mechanisms in the pathogenesis of myasthenia gravis.25

In the present experiments, the cytotoxic effects on SNpc were relatively specific for PD IgG compared with DC IgG and were not seen in medial septal cholinergic neurons. The reasons for the selective vulnerability are not clear. Since increased levels of free radicals and oxidative stress have also been described in other neurodegenerative disorders such as amyotrophic lateral sclerosis and Alzheimer disease,26,27 the explanation may not be related to the unique sensitivity of SN neurons to oxidative stress. Anterior horn cells also may succumb to increased levels of free radicals in amyotrophic lateral sclerosis,28 but the selective vulnerability of motor neurons may be better explained by their relative lack of calcium-binding proteins.29 It is of interest that in PD, injured SNpc neurons are also relatively deficient in calcium-binding proteins.30 Other presently undefined neuronal properties that may convey selective vulnerability include constituents of the apoptotic as well as neuroprotective pathways.31 Alternatively, specific reaction-activating microglia in the SNpc with the release of cytokines such as tumor necrosis factor α or TNF-α could be triggered by the PD IgG. A definition of the initial antigenic target of this in vivo cytotoxic response could help clarify this issue.

Parkinson disease IgG can initiate the relatively specific destruction of SNpc neurons in vivo. Cellular reaction 4 weeks after stereotactic injections suggests the importance of an inflammatory response triggered by PD IgG in the subsequent destruction of SNpc neurons. Further research is necessary to define the antigenic target of the PD IgG, the mechanism of recruitment of the destructive inflammatory response, and whether the loss of SNpc neurons is mediated by free radicals and oxidative stress.

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REFERENCES


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