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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PATIENTS AND METHODS

PATIENTS

Serum samples were collected with informed consent from 5 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 55 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 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 injections.
injections (with cresyl violet staining, the ratio ± SD of cell counts in injected to uninjected sides was 0.95 ± 0.05 [findings from 10 sections of a single animal]).

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 of TH immunoreactivity in at least 50% of TH-positive SNpc neurons. However, the third animal, which did not manifest rotational behavior, demonstrated a loss of TH immunoreactivity in 52% and 77% 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 SNpc neurons.

For statistical analysis of material stained with both cresyl violet and TH, 2 groups were identified according to the type of IgG applied, ie, a PD IgG group and a DC IgG group. Within these main groups, further subgroups were created according to the source of a particular IgG: 5 subgroups were identified according to the patients in the PD group and 7 subgroups were identified in the DC group according to the different neurologic diseases. Each subgroup was created according to data from 3 to 6 experimental animals. To determine the significance of changes from different IgG injections, nested analysis of variance was used.

IgG ABSORPTION

The SNpcs containing mesencephalon were dissected from adult rat brains, homogenized in 10% sucrose in 0.1 mol/L PBS and 10-mmol/L EDTA, and centrifuged at 1000 g for 10 minutes. The supernatants were spun down at 100 000g for 1 hour, washed, and 10 mg of membrane protein was incubated with 1 mg of PD IgG for 12 hours at 4°C, followed by centrifugation at 100 000g for 1 hour. Liver membranes were isolated and incubated in a similar fashion. Protein A agarose beads (Pierce, Rockford, Ill) were incubated with PD IgG for 2 hours at 4°C, followed by centrifugation at 8000 rpm for 10 minutes and filtration to reconstitute the original volume.

MEDIAL SEPTAL INJECTIONS

For injections into the medial septal region, the stereotaxic coordinates were as follows: 0.5 mm from the bregma, 0.5 mm from the midline, and 8.0 mm from the dura mater. Immunostaining assays for cholinergic neurons were performed with a choline acetyltransferase polyclonal antibody (Chemicon, Temecula, Calif) at 1:150 dilution. Medial septal cholinergic–positive neurons of injected and uninjected sides were counted blindly, and the results were expressed as the ratio of injected to uninjected sides.

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...
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
could be the result of neuronal toxic effects rather than its cause. The fact that the microglial density is normally ex-

onset of the cellular infiltration, the inflammatory response was initiated 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 cause. The fact that the microglial density is normally extremely high in the SN and could be related to its special role in inducing SN injury. Furthermore, activated microglia can initiate injury mediated by free radicals, and microglia are clearly present in SN lesions in PD.

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 correlate the cytotoxic potency of the IgG with the rate of the disease progression.

The table below shows the quantification of SNpc neurons after IgG injection:

<table>
<thead>
<tr>
<th>TH-Staining</th>
<th>CV-Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± Variance (n)</td>
<td>Mean ± Variance (n)</td>
</tr>
<tr>
<td>PD</td>
<td>0.499 ± 0.027 (15)</td>
</tr>
<tr>
<td>DC</td>
<td>0.818 ± 0.010 (28)</td>
</tr>
<tr>
<td>Significance</td>
<td>F = 4.96</td>
</tr>
</tbody>
</table>

The significance of differences was checked by the Student t test. PD indicates Parkinson disease; SNpc, substantia nigra pars compacta.

The dose and time dependence of PD IgG effect on TH-positive SNpc neurons is shown in the table below:

<table>
<thead>
<tr>
<th>Dose of PD IgG</th>
<th>TH-Staining</th>
<th>CV-Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 µg</td>
<td>0.64 ± 0.17 (14)</td>
<td>0.47 ± 0.12 (15)</td>
</tr>
<tr>
<td>160 µg</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; .40)</td>
<td>t = 0.82 (P &gt; .40)</td>
</tr>
</tbody>
</table>

The Parkinson disease (PD) IgG was exposed to liver and mesencephalic membranes at 10 mg of membrane protein per milligram of IgG. Ratios of cell counts in the injected side compared with the uninjected side were determined on the same individual sections. Data were pooled for animals in each group (4-6 sections from each animal; 2-3 animals in each group). Values are given as mean ± SD; number of sections analyzed are given in parentheses. Isolation of membranes and incubation was performed as described by McNaught et al. TH indicates tyrosine hydroxylase; CV, cresyl violet; and SNpc, substantia nigra pars compacta.

The effects of absorption of PD IgG on TH-positive SNpc neurons are shown in the table below:

<table>
<thead>
<tr>
<th>Absorption</th>
<th>TH Staining</th>
<th>CV Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD IgG + liver membranes</td>
<td>0.42 ± 0.18 (9)</td>
<td>0.56 ± 0.23 (9)</td>
</tr>
<tr>
<td>PD IgG + mesencephalic membranes</td>
<td>0.91 ± 0.10 (10)†</td>
<td>0.98 ± 0.06 (15)†</td>
</tr>
<tr>
<td>PD IgG + protein A</td>
<td>0.93 ± 0.19 (9)‡</td>
<td>0.95 ± 0.10 (9)‡</td>
</tr>
</tbody>
</table>

The Parkinson disease (PD) IgG was exposed to liver and mesencephalic membranes at 10 mg of membrane protein per milligram of IgG. Ratios of cell counts in the injected side compared with the uninjected side were determined on the same individual sections. Data were pooled for animals in each group (4-6 sections from each animal; 2-3 animals in each group). Values are given as mean ± SD; number of sections analyzed are given in parentheses. Isolation of membranes and incubation was performed as described by McNaught et al. TH indicates tyrosine hydroxylase; CV, cresyl violet; and SNpc, substantia nigra pars compacta.

*Significant difference from PD IgG and liver membranes: P < .001 by the Student t test using the Bonferroni adjustment for multiple comparisons.
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.

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, 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, but the selective vulnerability of motor neurons may be better explained by their relative lack of calcium-binding proteins. It is of interest that in PD, injured SNpc neurons are also relatively deficient in calcium-binding proteins.

Other presently undefined neuronal properties that may convey selective vulnerability include constituents of the apoptotic as well as neuroprotective pathways. Alternatively, specific reaction-activating microglia in the SNpc with or without small neurotoxins 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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