SHORT REPORT

Antibody responses to peptides of peripheral nerve myelin proteins P0 and P2 in patients with inflammatory demyelinating neuropathy

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Background: Antibodies with reactivity to peripheral nerve myelin have previously been found in the serum, and bound to peripheral nerves of patients with Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).

Aim: To investigate the presence of antibodies reactive to specific peptide sequences within the myelin proteins P0 and P2 in patients with GBS, in patients with CIDP, in healthy controls and in patients with other neuropathies (ON).

Methods: Blood was obtained from 48 patients with GBS, 36 with CIDP, 48 with ON and 38 controls. ELISA was used to detect antibody responses to peptides of the human peripheral myelin proteins P0 and P2. Blood samples were collected from patients with GBS in early, peak and recovery stages of GBS to analyse antibody levels throughout the course of the disease.

Results: Significantly increased total IgG levels were found in patients with GBS compared with other groups. A higher percentage of patients with GBS at the peak of disease had antibody reactivity to P2_{14–25} compared with patients with CIDP and control groups. In patients with GBS and CIDP, the percentages of patients with antibody reactivity to P2_{61–70}, and peptides derived from P0, were comparable to the control groups. Although some individual patients with GBS had high titres of reactivity to the peptide antigens tested, most patients with GBS and CIDP had levels of antibody similar to controls.

Conclusion: Our data suggest that increased IgG levels and increased antibody reactivity to P2_{14–25} in patients with GBS at the peak of disease may play a contributory role in the disease process in some patients with demyelinating forms of GBS.

The most common form of Guillain-Barré syndrome (GBS) in Australia is acquired inflammatory demyelinating polyradiculoneuropathy, characterised by primary demyelination and lymphocytic infiltration of the peripheral nerve by macrophages and T cells.¹ Acute motor axonal neuropathy² and acute motor and sensory axonal neuropathy³ are variants of GBS, where axonal damage is the main finding. Clinically and pathologically similar to GBS, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) follows a protracted or relapsing course.⁴ Both GBS and CIDP are considered to be autoimmune diseases involving humoral and cell-mediated immune reactions.¹ Activation of T and B cells in the peripheral lymphoid organs is thought to be triggered by molecular mimicry between infectious agent antigens and peripheral nerve components.¹ Previous studies have found antibodies to the peripheral myelin proteins P2, P0 and PMP22, α and β tubulin, connexin-32, gangliosides and glycolipids in the sera of some, but not all, patients with GBS and CIDP.¹

We have tested for antibody reactivity to two peripheral nerve myelin proteins using purified peptide antigens from the extracellular domains P0_{56–71} and P0_{70–85}, the cytoplasmic/transmembrane segment P0_{180–199} of the glycoprotein P0 as well as P2_{14–25} and P2_{61–70} of the cytoplasmic basic protein P2. These peptides were also used in our study of T cell reactivity in GBS and CIDP.⁵ Both P2 and P0 have been reported to induce experimental autoimmune neuritis; an animal model of GBS^{6–7} and the peptides chosen have previously been found to induce experimental autoimmune neuritis.

MATERIALS AND METHODS

Patients and controls

Blood samples from patients with GBS, CIDP and other neuropathies (ON) were obtained from hospitals in south-east Queensland. Healthy controls had no symptoms of any illness. Patients with GBS and CIDP met standard diagnostic criteria.^{8–9} GBS samples were grouped into early, peak and late stage of disease (GB1, GB2 and GB3). Early (GB1) samples were collected within 10 days of the onset of neurological symptoms and before the administration of any treatment. GB2 samples were collected approximately at the time of maximum weakness, and usually after patients had been treated for some days with intravenous immunoglobulin. Follow-up (GB3) samples were taken approximately 3 months after recovery. Patients with ON included those with hereditary motor sensory, toxic and diabetic neuropathies. From some patients there was no early sample (GB1), and from some patients no follow-up sample (GB3) was collected.

Collection and preparation of samples

All blood samples were collected with written consent. Approximately 6 ml of peripheral blood was diluted with 50 ml of heparinised RPMI-1640 for extraction of lymphocytes. The plasma supernatant was stored at -70°C before assay. IgG concentrations were measured by radial immunodiffusion¹⁰ using BINDARID RID Kits (RN004.3, The Binding Site, UK).

Peptide antigens

Peptides corresponding to amino acids 56–71, 70–85 and 180–199 of the human peripheral nervous system myelin protein P0 protein and amino acids 14–25 and 61–70 of human P2 used in the study were synthesised by Auspep (Melbourne, Australia) and were purified by high performance liquid chromatography to >95% purity. These peptides were selected because they have been previously characterised as targets of autoimmunity in animal models^{6–11–13} and human disease,^{14–15} and were used in

Abbreviations: CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; GBS, Guillain-Barré syndrome; ON, other neuropathies; PBST, phosphate-buffered saline containing 0.05% Tween 20

Table 1 Percentages of patients and controls with antibody reactivity to the peptides tested

Patient group	Controls	GB1 (early)	GB2 (peak)	GB3 (3 months)	CIDP	ON
n	38	28	30	24	36	48
Male:female	19:19	31:17			24:12	24:22
Mean age (years)	42	50			56	57
Age range (years)	21–91	18–82			25–85	27–80
Median age (years)	40	52			59	58
Mean (SE) IgG concentration ($\mu\text{g}/\text{ml}$)	10 022 (435)	11 502 (1350)	18 274 (1979)	11 208 (879)	10 324 (628)	9569 (626)
Peptide P2 (14–25)						
Positive (%)	5.3	21.4	43.3	25.0	8.3	16.7
p Value		0.138	0.001*	0.366	0.67	0.174
% titre >200†	0	7.1	16.6	8.3	0	0
Peptide P2 (61–70)						
Positive (%)	13.2	17.9	26.7	25.0	11.1	17.9
p Value		0.732	0.217	0.311	1	0.765
% titre >200†	5.3	7.1	13.3	20.8	5.5	4.1
Peptide P0 (56–71)						
Positive (%)	21.1	25.0	16.7	25.0	11.1	8.5
p Value		0.561	1	0.75	0.515	0.201
% titre >200†	2.6	7.1	10	4.1	0	2
Peptide P0 (70–85)						
Positive (%)	18.4	21.4	26.7	16.7	16.7	6.4
p Value		0.765	0.557	1	1	0.103
% titre >200†	10	7.1	6.6	4.1	5.5	2
Peptide P0 (180–199)						
Positive (%)	2.6	7.1	6.7	4.2	0	8.5
p Value		0.57	0.579	1	1	0.375
% titre >200†	0	7.1	2.6	4.1	0	2

CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; GB, Guillain-Barré; ON, other neuropathies.

Samples were considered to have a positive antibody reaction to the peptide tested if the mean $\text{OD}_{485\text{nm}}$ reading in ELISA for wells containing 5 μg of total IgG was >0.2 . The percentage of patients with antibody titre >200 is shown. Patient groups were compared individually with the controls by Fisher's exact test.

*Two-tailed probability values.

†These results were non-significant.

our previous study of T cell reactivity in GBS and CIDP, using the same patients as in this study.⁵

Enzyme-linked immunosorbent assay

An indirect peptide ELISA method was optimised for detection of antibody to the peptides used. Nunc "Maxisorb" immunoplates (NUNC, Denmark) were coated with 100 μl of 10 $\mu\text{g}/\text{ml}$ peptide in coating buffer (0.08 M carbonate/0.17 M bicarbonate (pH 9.6)) overnight at 4°C. Plates were blocked with 0.5% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 (PBST) for 2 h at room temperature. Plasma samples were serially diluted in triplicate from concentrations of 100 to 1.56 $\mu\text{g}/\text{ml}$ IgG and incubated on previously coated and blocked plates for 2 h. Plates were washed four times with PBST and 100 μl of a 1/5000 dilution of goat anti-human IgG-peroxidase conjugate (Sigma Aldrich, St Louis, Missouri, USA) was added before incubation for 1 h at room temperature. After three washes with PBST, and one with phosphate-buffered saline, the plates were developed using O-phenylenediamine dihydrochloride peroxidase substrate tablets (Sigma Aldrich) for 45 min at room temperature. The colour reaction was stopped using 50 μl of 3 M hydrochloric acid and absorbances were read at 485 nm. Absorbance values >0.2 , approximately twice the mean plasma blank, were considered positive. The titre was defined at the highest dilution at which there was a positive reading.

Statistical analysis

The proportions of patients with GBS and CIDP who had a positive antibody response to P0 and P2 peptides were compared individually with the control group and patients

with ON by Fisher's exact test. A comparison was deemed to show significance at $p \leq 0.05$.

The mean IgG concentration for each patient group was compared using analysis of variance, followed by Bartlett's test for equal variances. Where p was <0.05 by analysis of variance, the Tukey-Kramer modification of the t test was used to compare each pair of groups individually.

All statistical tests were performed using GraphPad Prism V.4.00 for Windows.

RESULTS

Immunoglobulin concentration

Table 1 shows the mean IgG concentration in the various groups. The mean IgG concentrations were significantly higher in the G-B2 group than in controls (18 274 (1979), $p < 0.001$). There was no significant difference among the other groups.

Reactivity to P2 peptides

A total of 6/28 GB1, 13/30 GB2 and 6/24 GB3 patient samples had increased levels of IgG to P2_{14–25} compared with 3/36 CIDP, 2/38 HC and 8/48 patients with ON. However, of these, only the GB2 samples were significantly increased ($p = 0.0002$).

The patients with the highest titres of antibody to the P2 peptides were in the GBS and CIDP groups, where 3 patients had a titre of 1600. The percentage of patients with titre >200 was greatest in the patients at the peak of GBS, but this was not statistically significant. For P2_{61–70}, the highest percentage of positive patients and the highest percentage of patients with titre >200 was found in the patients at the peak of GBS and at 3 months after GBS, but these were not statistically significant.

Four patients with GBS and CIDP had anti-P2_{61–70} titres of 1600, whereas no control had such high titres.

Reactivity to P0 peptides

No significant difference was observed between the proportions of patients with increased IgG reactivity to the peptides derived from P0 in any of the groups (table 1) and no significant increase in the percentage of patients with titre >200. However, the patients with the highest titres of antibody reactivity to the P0 peptides were in the patients with GBS compared with controls and patients with CIDP and ON.

DISCUSSION

Both antibody and activated T cells are thought to play a role in the pathogenesis of GBS and CIDP.¹ We investigated the IgG antibody response to the myelin proteins P0 and P2 using peptides that have been previously characterised as targets of autoimmunity in animal models^{6 11–13} and human disease.^{14–16} We found a statistically significant increase in the proportion of patients with GBS at the peak of disease with an increased IgG response to P2_{14–25}, compared with the other groups. Because we used plasma rather than undiluted serum, we standardised our test to a fixed amount of IgG. As the total IgG level was significantly greater in GB2 (peak) subjects than in other groups, we underestimated the antibody reactivity at the peak of disease. The increased IgG level in these subjects is most likely due to treatment with intravenous immunoglobulin. This would also have the effect of diluting and underestimating the level of antibody produced by the patients.

Previous reports of antibody reactivity to P2 protein in GBS and CIDP have varied, with some authors finding no reactivity to P2 in either patient group,^{16 17} and others reporting low levels of antibody in patient groups and in controls.^{18 19} Many of these early studies used bovine P2 protein. More recent studies have shown increases in the level of antibodies to human P2 protein and peptide epitopes in some patients with GBS and CIDP.¹⁵ Khalili-Shiraz *et al*¹⁵ reported an anti-P2 IgM response in >30% of patients with both GBS and CIDP, but IgG responses in only 18.4% of patients with GBS and in 12.5% of patients with CIDP. We found that the proportion of subjects with IgG reactivity to P2 was greater in GBS than in other patient groups, although most patients had titres in a range similar to the control groups. Thus, this reactivity was not a general finding in GBS and may represent an abnormality found in a subgroup of patients. We do not have sufficient data to analyse this further.

In our study, anti-P0 peptide IgG levels were not significantly different in patients with GBS or CIDP compared with controls, although individual patients with GBS had high titres. Khalili-Shiraz *et al* reported increased anti-P0 IgG titres in some patients with GBS, but no IgG response to P0 in any of their patients with CIDP.¹⁵ Previous studies have found little evidence of P0 antibody in patients with GBS and CIDP.^{20 21} However, using western blotting, Yan *et al*²² and Allen *et al*²³ found significantly increased antibody to P0 in patients with CIDP compared with controls. Western blots, although less sensitive than ELISA, potentially detect a greater range of target antigens than peptide ELISAs that test only a single epitope.

We have previously reported frequencies of T cell reactivity to the peptides used in this study for this group of subjects.⁵ In that study, although no statistically significant increases in antigen-specific T cell proliferation were found in patient groups, there was some increase in T cell reactivity to P0 and P2 in some patients with GBS and CIDP.

In general, our studies of antibody and T cell responses agree with previous work that has shown that immune responses to protein antigens are not widely found in patients with GBS and CIDP. It is possible that myelin proteins are not the main target

of the immune response in GBS and CIDP. A number of studies, including our own, have shown that immunity to glycolipid antigens is important in these diseases.²⁴ Alternatively, the immune response to protein antigens may be predominantly other antigens, to a conformational epitope that is not present in peptides or to epitopes that have some modification (eg, glycosylation), which makes them unrecognisable in the peptide ELISA.

The classic definition of autoimmune disease is one in which there is a single target that elicits a response in all, or most, affected individuals. In GBS and CIDP there may be a number of different targets that are important.

CONCLUSION

We found a significant increase in patients with GBS sampled at the peak of disease, with increased levels of anti-P2_{14–25} antibody compared with control groups, which may indicate that this response contributes to the pro-inflammatory disease process in these patients. However, as has been the case in most previous studies of this type, most patients with GBS and CIDP antibody responses were within the range of the control groups.

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