Autoimmunoreactivity to Schwann cells in patients with inflammatory neuropathies

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Summary

Inflammatory demyelinating neuropathies are characterized by a loss of peripheral nerve myelin. Myelin breakdown is thought to result from an autoimmune reaction towards nerve components. Schwann cells play a crucial role in the synthesis and maintenance of peripheral nerve myelin. An immune attack targeting Schwann cells could therefore affect myelin integrity, leading to disease. We studied the reactivity of sera from patients with Guillain–Barre syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) towards Schwann cells using immunofluorescence microscopy. We found 24% of the GBS (56 out of 233) and 26% of the CIDP (12 out of 46) patients to have circulating immunoglobulin G autoantibodies against proliferating, non-myelinating human Schwann cells. In contrast, healthy donors showed positive staining in only two out of 34 sera. No reaction was found with sera from patients with non-inflammatory neurological disorders. Immunofluorescence was localized at the distal tips (leading lamella) of the Schwann cell processes. Distal tips of neurites (nerve-growth-cones) of in vitro differentiated non-myelinated hNT2 neurons also stained strongly. GBS and CIDP serum immunoreactivity was also observed in teased nerve fibre preparations. These data suggest that, at least part of the immunoreactivity is not directed against myelin, but towards non-myelin proteins and epitopes possibly involved in Schwann cell–axon interaction.

Keywords: Schwann; nerve; Guillain–Barre; neuropathy; autoantibody

Abbreviations: CIDP = chronic inflammatory demyelinating polyneuropathy; GBS = Guillain–Barre syndrome; GFAP = glial fibrillary acidic protein; HMSN = hereditary sensory and motor neuropathy; IF = immunofluorescence; IgG = immunoglobulin G; IVIg = intravenous immunoglobulins; MAb = monoclonal antibody; NDRG = N-myc downstream-regulated gene; PAb = polyclonal antibody; PBS = phosphate-buffered saline; PFA = paraformaldehyde; SMA = smooth muscle actin

Introduction

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a chronic progressive and/or relapsing immune-mediated disorder that can cause severe disability. Guillain–Barré syndrome (GBS) is an acute monophasic inflammatory neuropathy that leads in two-thirds of the patients to near complete recovery, but with a mortality of about 2–5%. Breakdown of myelin, a characteristic of these neuropathies, is thought to result from an autoimmune reaction towards nerve components (Ho et al., 1998; Steck et al., 1998). Since most patients respond well to treatment with high dose intravenous immunoglobulin G or plasma exchange (Plasma Exchange/Sandoglobulin GBS Trial Group, 1997; Archelos and Hartung, 2000), circulating autoantibodies are presumably involved in these disorders. Yan and colleagues have shown that passive transfer of demyelination is possible by serum or immunoglobulin G (IgG) from CIDP patients (Yan et al., 2000), supporting a role for autoantibodies in the pathogenesis of CIDP. However, the exact pathogenic nature of the autoimmune response, the identity of the autoantigens or the mode of action of these treatments remain elusive (Brand et al., 1996; van Schaik et al., 1997). Thus far, no in vitro assays are available to monitor disease activity, allowing adjustment of treatment and prognosis after therapy. Characterization of the autoreactive target epitopes or factors...
eliciting the autoimmune response is therefore needed. Investigation of inflammatory neuropathy disease mechanisms has focussed mainly on the immune response directed against myelin constituents. Serum antibodies against whole nerve, purified myelin proteins, nerve/myelin-associated integrins and gangliosides have been reported (Ho et al., 1998; Previtali et al., 1998; Steck et al., 1998; Archelos et al., 1999; Yan et al., 2001), but whether they cause pathology or arise in response to nerve damage is not clear. In the rat experimental autoimmune neuritis model, disease symptoms similar to GBS and CIDP can be induced by immunization with purified peripheral myelin protein zero (P0), basic protein P2 and peripheral myelin protein 22 kDa (PMP22) (Kadlubowski and Hughes, 1979; Milner et al., 1987; Gabriel et al., 1998). However, the role of antibodies against PMP22 in GBS and CIDP remains a matter of debate since our group (Kadlubowski and Hughes, 1979; Milner et al., 1987; Gabriel et al., 1998) described demyelinating anti-P0 antibodies in a subgroup of CIDP patients. Mutational analysis of heritable demyelinating neuropathies showed, that aside from myelin proteins, correct expression of non-myelin genes is also crucial for myelin synthesis and maintenance. These include mutations in the early growth response gene (EGR2/Krox-20) encoding a transcription factor (Warner et al., 1998) and N-myc downstream-regulated gene 1 (NDRG1), whose function is largely unknown (Belzen et al., 1997; Kalaydjieva et al., 2000).

Schwann cells play a crucial role in the peripheral nervous system, since they are responsible for production and maintenance of myelin (Mirsy and Jessen, 1996; Scherer, 1997). An immune attack targeting Schwann cells could therefore affect myelin integrity severely. Immuno-histochemical data have suggested involvement of terminal-complement (C5b-9) complex deposition as a disease mechanism (Hafer-Macko et al., 1996; Koski, 1997; Putzu et al., 2000; Yan et al., 2000), but the close glial-neural association in the whole nerve or even teased fibre preparations has hampered the identification of Schwann cell or axonal target epitopes in vivo. We have used in vitro cultured primary human Schwann cells (Rutkowski et al., 1995; Hanemann et al., 1998) as well as in vitro differentiated human hNT2 neurons (Pleasure and Lee, 1993; De Jonge et al., 2001) to search for autoreactive epitopes in GBS and CIDP patients. In our present study, we set out to investigate whether Schwann cell and neuronal epitopes serve as autoimmune targets in inflammatory neuropathies.

Material and methods
Patient and control sera
Serum from 193 GBS patients was collected. Multiple sera were collected at various stages of the disease from 29 of these patients. A total of 233 GBS sera were tested. Serum from 46 CIDP, 47 hereditary sensory and motor neuropathy (HSMN) type 1 and four Alzheimer’s disease patients [all fulfilling accepted diagnostic criteria (Asbury and Cornblath, 1990; Ad hoc Subcommittee of the American Academy of Neurology AIDS Task Force, 1991)], as well as 34 healthy donors was obtained after informed consent and stored at −20°C. Serum IgG concentrations were determined turbidimetrically. Patient and donor serum IgG concentrations ranged from 4 to 40 mg/ml.

Antibodies
Polyclonal antibody (PAb) anti-rat-ninjurin1 (nerve injury induced protein) and PAb anti-human-ninjurin2 was diluted 1:200 (Araki and Milbrandt, 1996, 2000). Monoclonal antibody (MAb) anti-p75 low-affinity neurotrophin receptor (p75LNTR) was diluted 1:1000 (Morrison et al., 1999). MAb anti-L1/NgCAM (neural-glial cell adhesion molecule) clone 5G3 (1:100) was a gift from A.M. Montgomery ( Scripps Institute, La Jolla, CA, USA). MAb anti-TAG-1 (transiently expressed axonal glycoprotein) clones 1C12 (IgG) and 4D7 (IgM) were diluted 1:100 (Furley et al., 1990). MAb anti-c-erbB2 clone CB11 (Biogenex Laboratories, San Ramon, CA, USA) was diluted 1:50 and clone 3B5 (Onc. Sci. OP15) was diluted 1:100. MAb anti-B-50/GAP-43 (growth-associated protein) clone NM2 (1:400) and NM6 (1:1000), PAb clones 9527 (1:1000) and 8921 (1:500) were a gift from L. Schrama (Rudolf Magnus Institute for Neuroscience, Utrecht, The Netherlands). MAb anti-ICAM (CD54), NCAM (CD56) and HNK-1 (CD57) were purchased from Becton–Dickinson Biosciences, San Jose, CA, USA and diluted 1:100. The NCAM antibody recognizes two major isoforms of human NCAM (140 and 180 kDa). MAb anti-chicken-ACAM/N-cadherin clone NCD-2 (Zymed Laboratories, San Francisco, CA, USA) was diluted 1:200. MAb anti-integrin subunits ß1 (CD29, 1:50), ß3 (CD49/C/ VLA-3, 1:5), ß4 (CD49D/VLA-4, 1:25), ß5 (CD49E/VLA-5, 1:25) and ß6 (CD49F/VLA-6, 1:25) were a gift from R. Kene (Department of Pathology, Academic Medical Centre, Amsterdam, The Netherlands). Two anti-N-myc downstream-regulated gene (NDRG1) protein polyclonal antisera were used: one serum (Drg1) was a gift from W.N.M. Dinjens (Department of Pathology, EUMC, Rotterdam, The Netherlands) and an affinity-purified polyclonal antisera was provided by L. Kalaydjieva. MAb P07 (clone 18) anti-P0-ED was raised against the extracellular domain of P0 and was a gift from J.J. Archelos (Department of Neurology, Karl-Franzens-University, Graz, Austria). Cy3 conjugated MAb anti-alpha-smooth muscle actin clone 1A4 (1:200), MAb anti-GFAP (glial fibrillary acidic protein) clone G-A-5 (1:50–100), MAb anti-S-100 (ß-subunit) clone SH-B1 (1:10 000), MAb anti-Thy-1.1 (IgM) clone TN-26 (1:2500), and fluoroisothiocyanate (FITC) and Cy3-conjugated secondary antibodies (1:100–200) were all from Sigma (St Louis, MO, USA). Anti-rabbit-IgG-Alexa Fluor 546 conjugated secondary antibody (1:500) was from Molecular Probes.
Cell culture

Primary human Schwann cell lines were established from sural nerve biopsies and nerves dissected out of amputation material as described previously (Rutkowski et al., 1995; Hanemann et al., 1998) with a few modifications (see below). We used several different passage numbers throughout different immunofluorescence (IF) experiments. Most of the experiments were performed with the same cell line. Cells with passage numbers higher than 10 were not included in the study. Biopsies were taken from patients with suspected vasculitic neuropathies or morbus Hansen, and without known mutations in myelin genes. All patients’ biopsies that were ultimately used proved to be normal after pathological examination. Alternatively, Schwann cell lines were generated from nerves isolated from patients with diabetes mellitus macroangiopathy or melanoma (amputations), respectively. No obvious differences in Schwann cell morphology or immunoreactivity between the different cell lines were observed. One cell line was established from an HSMN-Lom patient which has a mutation (Arg148Stop) in NDRG1 (Kalaydjieva et al., 2000). Schwann cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco BRL, Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) (Gibco, BRL, Life Technologies Inc., Gaithersburg, MD, USA) and 10 nM recombinant human β1-heregulin (a gift from Genentech Inc., South San Francisco, CA, USA), 2.5 μg/ml insulin (Sigma-Aldrich, St Louis, MO, USA), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (ICN, Costa Mesa, CA, USA), 0.5 μM forskolin (ICN), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were routinely subcultured every 3–4 days and maintained at 37°C with 5% CO₂.

Teased nerve fibre preparation

Sciatic nerves were dissected directly after sacrifice from 8–15 month-old wild-type (FVB) mice. Nerves were fixed immediately for 30–60 min in 4% paraformaldehyde. Teased single nerve fibres were prepared as described, plated on (adhesive) glass microscope slides (Starfrost, Knittel, Germany) and air-dried overnight at room temperature. Slides were either used immediately or stored frozen at −20°C. Prior to immunofluorescence (see below), teased nerve fibre preparations were fixed for 10 min in ice-cold acetone and rehydrated twice in phosphate-buffered saline (PBS) for 5 min.

IF microscopy

Cells were grown on either 18 × 18 mm glass cover slides in six-well plates (7 × 10⁴ cells/well) or in 9 × 9 mm eight well LabTekII (Nunc A/S, Roskilde, Denmark) chamber slides (2 × 10⁴ cells/well). For Schwann cells, 5 μg/ml laminin (from mouse EHS-sarcoma, Roche) was added to the culture medium.

For extracellular staining, cells were fixed briefly for 5 min in 4% paraformaldehyde (in PBS). In case of intracellular staining (S100β, GFAP, SMA), cells were permeabilized additionally with ice-cold 100% methanol for 10 min at −20°C. After rinsing with PBS, the cells were pre-incubated for 30 min with PBS containing 10% FCS, followed by an incubation with 100–200 μl of the diluted primary antiserum for 2 h at 37°C in a humidified chamber. After primary antibody incubation, cells on cover slips were washed three times in PBS/FCS and incubated with 1:100 diluted secondary anti-human, anti-rabbit, anti-rat or anti-mouse immunoglobulins labelled (FITC, Cy3, Alexa Fluor 546 or Biotin) conjugate. Unbound secondary antibody was removed by washing three times with 10% FCS in PBS. Biotin-conjugated secondary antibody was detected with a third incubation step using avidin-FITC or Cy3 conjugate.

For nuclear counterstaining, cells were permeabilized for 10 min at −20°C with ice-cold 100% methanol and incubated...
for 5 min in PBS/FCS containing either 5 μg/ml propidium iodide (red) or 1 μg/ml Hoechst Dye 33258 (blue) (both from Sigma). After rinsing with PBS and distilled water, the cover slips were allowed to dry and cells were embedded in Vectashield™ mounting medium (Vector Labs, Burlingame, CA, USA).

Microscope slides were analysed using an Olympus Vanox (PAES Nederland BV, Zoeterwoude, The Netherlands) immunofluorescence microscope. For comparison of patient and healthy control sera, cells were photographed at identical magnification, film sensitivity and exposure time. In our descriptions, 'strongly positive' patient sera immunolabelled ~60–90% of the Schwann cell population, while 'weak' staining refers to ~10–50% of the Schwann cell population being positive and 'negative' refers to no staining above background level (secondary antibody).

Double labelling/co-localization experiments were performed using a Leica (Leica Microsystems BV, Rijswijk, The Netherlands) confocal laser scanning microscope. Images were generated with double excitation (488 nm and 563 nm of an argon/krypton laser) and double detection (BP 530 for FITC and LP610 for Cy3/Texas Red). A pinhole setting was used giving a z resolution of ~0.7 μm. Images were adapted to the full dynamic range (8 bit) of the system. For co-localization, images were corrected for cross-talk and subsequently merged using Multi Color Analysis software (Leica).

**Recombinant protein expression in Escherichia coli**

NDRG1, two, three and four cDNA fragments encoding the entire open reading frames were obtained by reverse transcriptase–polymerase chain reaction (RT–PCR) amplification on Schwann cell cDNA. Oligonucleotide primers (sequences available upon request) were designed to introduce a BamHI restriction site at the 5'-end and a HindIII site at the 3'-end. Fragments were cloned unidirectionally into the expression vector pQE9 (Qiagen) using the BamHI and HindIII sites. N-terminal 6xHis:NDRG fusion proteins were isolated 5 h after isopropyl β-D-thiogalactoside (IPTG) induction and purified on an NTA-Nickel spin column according to the protocol of the manufacturer (Qiagen, Westburg BV, Leusden, The Netherlands).

**Recombinant protein expression in mammalian cells**

Full-length expression clones (in pcDNA3 vector) of B-50/GAP-43 and NDRG1 were kindly provided by Dr L. Schrama (Rudolf Magnus Institute for Neuroscience, Utrecht, The Netherlands) and Dr N. van Belzen (DMV International, Wageningen, The Netherlands), respectively. A stable transfected Rat1 fibroblast cell line was used for B-50/GAP-43 experiments. NDRG expression constructs were introduced into CHO-K1 cells using an electroporation apparatus with RF (radio frequency)-unit following the instructions of the manufacturer (Bio-Rad, Hercules, CA, USA). For NDRG1, two stable transfected Chinese hamster ovary (CHO-K1) cell lines were generated by culturing transfected cells in selective medium [DMEM/10% FCS/penicillin/streptomycin containing 1 mg/ml neomycin G418 (Roche)] starting 24 h post-transfection.

**Western blot analysis**

Recombinant expression in CHO-K1 cells and E.coli was verified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blot analysis (Kwa et al., 2001). For screening patient sera, a single slot comb was used to load a single sample (100 μg total protein, ~5 μg per lane) on the entire width of the gel. The membrane was mounted in a multi-well apparatus (Bio-Rad) allowing multiple serum incubations. Non-specific antibody binding was blocked with 5% non-fat milk in TBS/Tween (10 mM Tris pH 8.0, 100 mM NaCl, 0.05% Tween-20). Subsequently, the filter was incubated for 2 h with either positive control polyclonal antibodies or with the sera from healthy donors, GBS or CIDP patients. Donor and patient serum incubations were performed at normalized serum IgG concentrations of 10 μg IgG/ml (400 to 4000-fold dilution). Lower serum dilutions often resulted in the detection of non-specific protein bands. Filters were washed three times with TBS/Tween for 5 min. Subsequently, filters were incubated for 30–60 min with peroxidase conjugated secondary antiserum (1:3000). After washing with TBS/Tween (3×) and distilled water, serum immunoreactivity was detected with ECL chemiluminescence substrate (Amersham Pharmacia Biotech, Bucks, UK). Only those protein bands present in the recombinant cells and absent in the mock-transfected controls (after prolonged exposure) were scored as positive.

**Statistical analysis**

Patient variables such as age at disease onset, time between onset and serum withdrawal, mode of treatment, time between treatment and serum withdrawal, gender, pre-existing infection, spontaneous recovery, post-recovery effects were related to anti-Schwann cell IgG titres by χ² (nominal items) or Mann–Whitney test (random scales) and by multi-variant analysis of variance (ANOVA) using the statistical program SSPS for Windows (SSPS Inc Headquarters, Chicago, IL, USA).

**Results**

**Patient characteristics**

Serum was collected from a group of 46 CIDP patients with a mean age of 50 years and ranging from 6 to 83 years old. The group consisted of 33 males and 13 females. Forty-two of
these patients received intravenous immunoglobulins (IVIg), one patient was treated with plasma exchange, two patients received only corticosteroids and one patient was not treated. Serum withdrawal was performed before IVIg treatment in 12 patients, on the same day of treatment in 10 patients, within 30 days post-treatment in six patients and up to 10 years after start of treatment in 16 patients. A total of 233 GBS sera were collected from 193 patients with a mean age of 42 years (ranging from 5 to 88 years old). From 29 patients within this group, multiple sera were collected (2–4) at various stages of the disease (acute, pre-/post-treatment and post-recovery). The mean disease duration was 9.4 days (±5.8). Sixty-six patients received IVIg treatment and 26 patients were treated by plasma exchange. No treatment was given to 101 of the GBS patients; 28 patients showed spontaneous recovery. In the treated patient group, 20 sera were collected 1–12 days before treatment and 24 sera were taken on the same day of treatment. The rest of the sera in the

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**Fig. 1 In vitro culture of human Schwann cells established from sural nerve.** Cells grown on glass cover slides were fixed for 5 min in 4% paraformaldehyde (PFA) and analysed by (A) phase contrast and (B–H) IF microscopy. In addition, cells were permeabilized with methanol before intracellular staining (S100 and nuclear counterstaining). (B) The majority (90–95%) of the cells were positive to Schwann cell marker S100β. (C–H) Human serum reactivity was detected with anti-human-IgG-FITC (green) conjugate. Nuclei were counterstained with propidium iodide (red). Sera from GBS (D and E) and CIDP (F, G and H) patients reacted strongly with the distal tips of Schwann cell processes (leading lamellae). (C) This immunoreaction was not present or very weakly in healthy donor controls. Scale bars are 50 μm (A–D, F and G) and 10 μm (E and H), respectively.
treated patient group were collected at various times following treatment (ranging from a few days up to a few years).

### Cultured human Schwann cells express Schwann cell markers

We derived primary cell cultures from sural nerve biopsies and nerves dissected from amputation material (Fig. 1A). These cultures consisted primarily of Schwann cells, as shown by the expression of several Schwann cell-specific markers. About 90–95% of the cells showed reactivity with antibodies raised against $\text{S100}^\beta$ (Fig. 1B), GFAP and $\text{p75}^\text{LNTR}$ (Fig. 4A); using similar dilutions, human fibroblasts did not show staining (not shown). Fibroblast markers SMA and Thy-1.1 were negative in the majority (90–95%) of the cells. At most, 5–10% of the cells were SMA positive. Schwann cell cultures were not affected by the Thy-1.1/ complement treatment (see Material and methods). In contrast, control human fibroblast cultures (>95% SMA+, S100$^\beta+$, GFAP+, $\text{p75}^\text{LNTR}$-) showed complete complement-mediated cell lysis within 10 min (data not shown). From these data, we conclude that our selective culture method yields about 90–95% pure Schwann cells.

### Sera from CIDP and GBS patients recognize distal tips of Schwann cell processes

Reactivity of patient sera to Schwann cells was investigated by IF microscopy. A very characteristic localization of anti-Schwann cell IgG immunofluorescence was observed (Fig. 1). Distal tips of the Schwann cell processes, the leading lamellae, were stained strongly by sera from GBS patients (Fig. 1D and E) and CIDP patients (Fig. 1F, G and H).

This immunofluorescence was not detected or very weakly detected in healthy donors (Fig. 1C), HMSN type 1 patients and Alzheimer’s disease patients (Table 1). The staining was absent in cells derived from non-neuronal tissue: pancreas (MiaPaca II), lung (SW1537 S1), bladder (T24) and kidney (HEK293) carcinoma (data not shown). Schwann cell immunofluorescence is probably membrane associated since immunolabelling of the leading lamellae was eliminated by methanol/chloroform treatment (data not shown).

### GBS and CIDP sera recognize nerve-growth-cones in hNT2 neurons

GBS and CIDP serum immunoreactivity against neuronal cells was investigated using (retinoic acid-induced) in vitro differentiated human teratocarcinoma hNT2 neurons (Fig. 2). These in vitro differentiated cells grow in aggregates that are connected by long thin (unmyelinated) axon-like processes or neurites (Fig. 2A). Partially undifferentiated cells (which

### Table 1 Anti-Schwann cell IgG immunofluorescence

<table>
<thead>
<tr>
<th>Serum group</th>
<th>Positive</th>
<th>Weak</th>
<th>Negative</th>
</tr>
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<tbody>
<tr>
<td>GBS</td>
<td>24% (56 out of 233)</td>
<td>46% (108 out of 233)</td>
<td>30% (69 out of 233)</td>
</tr>
<tr>
<td>CIDP</td>
<td>26% (12 out of 46)</td>
<td>33% (15 out of 46)</td>
<td>41% (19 out of 46)</td>
</tr>
<tr>
<td>Healthy donor pool</td>
<td>all</td>
<td>all</td>
<td>all</td>
</tr>
<tr>
<td>Single healthy donors</td>
<td>6% (2 out of 34)</td>
<td>50% (17 out of 34)</td>
<td>44% (15 out of 34)</td>
</tr>
<tr>
<td>Purified IVIg</td>
<td>0% (0 out of 3)</td>
<td>33% (1 out of 3)</td>
<td>67% (2 out of 3)</td>
</tr>
<tr>
<td>HMSN type 1</td>
<td>0% (0 out of 47)</td>
<td>11% (5 out of 47)</td>
<td>89% (42 out of 47)</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>0% (0 out of 4)</td>
<td>0% (0 out of 4)</td>
<td>100% (4 out of 4)</td>
</tr>
</tbody>
</table>

Disease specific anti-Schwann cell IgG immunoreactivity is present in 24–26% of the sera from patients with an inflammatory neuropathy. Human serum immunoreactivity was determined by IF microscopy. Serum IgG concentration was normalized at 50 μg/ml. Positive staining refers to approximately 60–90% of the cell population being positive. Weak staining refers to approximately 10–50% of the cell population being positive. Negative refers to no staining above background level (secondary antibody).
were negative to anti-neurofilament antibodies (De Jonge et al., 2001) were also present in the cultures as large flattened cells. IF microscopy showed that GBS sera (Fig. 2C and D) and CIDP sera (Fig. 2E–H) strongly stained the growing ends of the neurites (the nerve-growth-cones) in differentiated hNT2 neurons. A small selection of sera from five GBS and five CIDP patients (which were positive on Schwann cells) as well as five healthy donor controls was tested on hNT2 neurons. All GBS and CIDP patient sera strongly stained the hNT2 neurons. This immunofluorescence was characteristic of the nerve-growth-cones (Fig. 2C–H). One of the CIDP sera (Fig. 2G and H) also displayed strongly stained patches on some of the undifferentiated hNT2 cells, as can be seen clearly at low magnification (50×) in Fig. 2G. The immunoreactivity seen in patient sera (Fig. 2C–H) was present in none of the five healthy donor sera (Fig. 2B), suggesting in addition that the immunofluorescence found in hNT2 neuronal cells is disease specific.

**GBS and CIDP sera show immunoreactivity in teased nerve fibre preparations**

To validate whether disease-related immunofluorescence was present not only in vitro (cell culture), we also studied serum reactivity in mouse teased nerve fibre preparations which represent a more in vivo situation. A selection of five GBS and five CIDP sera (which were positive on Schwann cells), as well as five healthy donor sera and purified IVIg was tested. None of the healthy donor sera (Fig. 3, hd) and IVIg preparations showed immunofluorescence above background level (secondary antibody only). In contrast, all GBS and CIDP patient sera strongly stained the teased nerve fibres (Fig. 3). Although immunofluorescence appeared to co-localize with P0, due to the low resolution we were not able to discriminate which cellular component of the nerve is actually stained (myelin, Schwann cell membrane, extracellular matrix, basal lamina or axon surface). From these findings, we conclude that the epitopes recognized by CIDP and GBS patient sera are present not only in cultured Schwann cells, but also in teased nerve fibres which represent a more in vivo situation.

**Screening of antisera directed against known nerve-growth-cone associated proteins and epitopes involved in Schwann cell–axon interaction**

To identify the GBS and CIDP target epitopes, we focused on known nerve-growth-cone associated proteins and proteins...
The results are listed in Table 2. Some antisera (L1/NgCAM, TAG-1, integrins) stained Schwann cell bodies or the entire cell membrane. Only three defined antisera (p75LNTR, B-50/GAP-43, NDRG1) showed an immunofluorescence staining pattern similar to GBS and CIDP sera, staining the distal tips of the Schwann cell processes (leading lamellae). Two of these epitopes were investigated further by double labelling and co-localization studies using confocal laser scanning microscopy. Double labelling of human Schwann cells with anti-p75LNTR monoclonal antibody (red fluorescence) and human serum (green fluorescence) is shown in Fig. 4A. The images were merged (overlay) yielding a white colour in case of co-localization. In Fig 4A, the upper three images are incubations with serum from a healthy donor and the lower three images represent incubations with serum from a CIDP patient, p75LNTR and the epitope recognized by the CIDP and GBS (not shown) did not co-localize. p75LNTR was therefore excluded as a target epitope in inflammatory neuropathies.

Partial co-localization (white fluorescence, overlap) was seen for CIDP (Fig. 4B, upper three images,) and GBS (Fig. 4B, lower three images) patient sera (green fluorescence) with B-50/GAP-43 antibody (red fluorescence). This finding suggests that the epitope recognized by the CIDP/GBS serum is localized on the same cellular compartment as B-50/GAP-43. Note that two monoclonal antisera (NM2 and NM6, Table 2) raised against B-50/GAP-43 peptide epitopes (residues 39–43 and 132–213, respectively) did not stain the Schwann cell leading lamella.

**Table 2** Schwann cell immuno characterization with antibodies to nerve-growth-cone associated proteins and epitopes involved in Schwann cell–axon interaction

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Extent of staining</th>
<th>Cellular location of IF</th>
</tr>
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<tbody>
<tr>
<td>p75LNTR</td>
<td>++</td>
<td>Distal tips of processes, &gt;95% of cells</td>
</tr>
<tr>
<td>ICAM (CD54)</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>NCAM (CD56)</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>HNK-1 (CD57)</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>ACAM (N-cadherin)</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>L1/NgCAM MAb 5G5</td>
<td>+</td>
<td>Distal tips of processes</td>
</tr>
<tr>
<td>B-50/GAP-43 PAb 9527</td>
<td>+/-</td>
<td>Distal tips of processes</td>
</tr>
<tr>
<td>B-50/GAP-43 PAb 8921</td>
<td>+/-</td>
<td>Distal tips of processes</td>
</tr>
<tr>
<td>B-50/GAP-43 MAb NM2</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>TAG-1 MAb 1C12</td>
<td>+/-</td>
<td>Distal tips of processes</td>
</tr>
<tr>
<td>TAG-1 MAb 4D7</td>
<td>+</td>
<td>Entire cell surface, live cells only</td>
</tr>
<tr>
<td>Ninjurin-1 PAb</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>Ninjurin-2 PAb</td>
<td>–</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>c-erbB2 MAb CB11</td>
<td>+/-</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>c-erbB2 MAb 3B5</td>
<td>+/-</td>
<td>Entire cell surface, no processes</td>
</tr>
<tr>
<td>NDRG1 PAb Drg1</td>
<td>+</td>
<td>Distal tips of processes</td>
</tr>
<tr>
<td>NDRG1 PAb affinity pure</td>
<td>+</td>
<td>Distal tips of processes, also recognizes NDRG3</td>
</tr>
<tr>
<td>Integrin β1</td>
<td>+/-</td>
<td>Entire cell surface, no processes</td>
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<tr>
<td>Integrin α3</td>
<td>+/-</td>
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<td>Integrin α6</td>
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Immunoreactivity of Schwann cells to antibodies directed against nerve-growth-cone associated proteins and epitopes involved in Schwann cell–axon interaction determined by IF microscopy. ++ = strong positive staining of the majority of the cells (90–95%); + = positive staining of 60–90% of the cells; +/- = weakly positive staining of 10–50% of the cells; – = no detectable staining above background level (secondary antibody control). Source and dilution of the antisera are given in Material and Methods.

GBS/CIDP sera do not recognize recombinant B-50/GAP-43

We next investigated whether B-50/GAP-43 is the sole epitope determining the recognition by the CIDP and GBS sera. Fig. 5 shows Rat1 fibroblasts stably transfected with a B-50/GAP-43 CMV expression construct (Aarts et al., 1999) labelled with polyclonal anti-B-50/GAP43 antibody (left panels). Since the transfected cell line was not clonal (~50% of the cells carried the expression construct), cells highly expressing recombinant B-50/GAP-43 (strong fluorescence) could easily be distinguished from untransfected cells (background fluorescence). Double staining the same cells with CIDP (upper right panel) or GBS patient serum (lower right panel) revealed that even the cells highly expressing B-50/GAP-43 did not display patient serum fluorescence above the background level. We therefore concluded that GBS and CIDP sera do not recognize recombinant B-50/GAP-43 by itself in transfected Rat1 fibroblasts.
GBS/CIDP sera show co-localization with NDRG antiserum, but do not react with NDRG1

Another epitope of potential interest is the protein encoded by N-myc downstream-regulated gene (NDRG1) (Kalaydjieva et al., 2000). This gene is mutated (Arg148Stop) in HMSN-Lom patients and our previous unpublished results have shown that two polyclonal NDRG1 antisera detect leading lamellae in Schwann cells (Table 2). GBS and CIDP patient sera showed co-localization with the two polyclonal antisera raised against NDRG1 (not shown). However, these sera showed similar reactivity to a Schwann cell line—generated from a HMSN-Lom patient—in which full-length NDRG1 mRNA was not detectable by RT-PCR analysis (data not shown). Thus, GBS/CIDP patient serum reactivity is not directed towards NDRG1. This was confirmed in NDRG1 transfected CHO-K1 cells (Fig. 6). These findings show that, in the NDRG1−/− Schwann cells, proteins highly similar to NDRG1 are present. These proteins are localized at the distal tips of the processes, the same cellular compartment that showed reactivity with GBS/CIDP sera.

Fig. 4 (A) Co-localization studies of human Schwann cells double stained with monoclonal anti-p75LNTR antibody and healthy donor or CIDP patient serum by confocal laser scanning microscopy (CLSM). Human serum reactivity was detected with anti-human-IgG-FITC conjugate (green fluorescence). p75LNTR immunoreactivity was detected with anti-rat-Ig-biotin in combination with avidin-Cy3 (red). Images were merged (overlay) yielding a white colour in the case of co-localization. No co-localization was detected between p75LNTR and the epitope(s) recognized by the CIDP patient serum. Scale bar, 20 µm. (B) Co-localization studies of human Schwann cells double stained with polyclonal anti-B-50/GAP-43 antibody and CIDP or GBS patient serum by CLSM. Human serum reactivity was detected with anti-human-IgG-FITC (green). B-50/GAP-43 immunoreactivity was detected with anti-rabbit-Ig-biotin in combination with avidin-Cy3 (red). In the overlay, human patient sera show partial co-localization (white) with anti-B-50/GAP-43 polyclonal antibody located on the Schwann cell leading lamellae and cell body. Scale bar, 20 µm.
**Polyclonal anti-NDRG1 antibody cross-reacts with NDRG3 protein**

The question arises as to which protein is responsible for the immunoreactivity in the NDRG1−/− Schwann cells. Since NDRG1 is a member of a family of four highly homologous proteins (NDRG1, 2, 3 and 4) (Zhou et al., 2001)—which all are expressed in Schwann cells—we constructed a set of four NDRG-His fusion proteins in E.coli to assess the specificity of the NDRG antiserum. Western analysis of Nickel-NTA column (partially) purified recombinant NDRGs (Fig. 7, right panel) revealed that the polyclonal antiserum raised against NDRG1 (peptides 1−19 and 21−39), aside from NDRG1 (lane 1), also recognizes NDRG3 (lane 3) but not NDRG2 and NDRG4 (lanes 2 and 4, respectively). Multiple sequence alignment confirmed that NDRG1 and NDRG3 show the highest degree of similarity relative to NDRG2 and NDRG4. Equal loading of the four different fusion proteins was assured by total protein staining (with Coomassie Brilliant Blue) of duplicate gels and re-staining of the western blot with anti-His-tag monoclonal antibody (not shown). These results strongly suggest that the affinity purified anti-NDRG1 polyclonal antibody still cross-reacts with the highly homologous NDRG3 protein. Therefore, we conclude that NDRG3 is a putative target epitope for CIDP/GBS patient sera.

**GBS and CIDP sera do not recognize NDRG3 and Schwann cell epitopes on western blot**

IF-positive serum from six CIDP and seven GBS patients, as well as a healthy donor control, was screened for reactivity to NDRG3. We performed western blot analysis using the partially purified NDRG3-His fusion protein, as well as total protein preparations from both normal and HMSN-Lom Schwann cells (not shown). Although the positive control incubations with NDRG polyclonal antibody showed strong NDRG-reactive bands of the expected size (50 kDa), none of the patient sera showed reactivity to either recombinant (NDRG3-His) or endogenous (NDRG1 and NDRG3 in normal Schwann cells, NDRG3 in HMSN-Lom Schwann cells) proteins. From these data, we conclude that under denaturing conditions (western blotting), the positive GBS and CIDP patient sera show no immunoreactivity to NDRG3 or any other Schwann cell epitope.

![Fig. 6](https://example.com/fig6) **Fig. 6 (A)** GBS and CIDP serum reactivity is not directed towards NDRG1 in stably transfected Chinese hamster ovary (CHO-K1) cells. Recombinant cells highly expressing NDRG1 (left panels, ~30% of the total) displayed no significant immunofluorescence (above background level) when double stained with CIDP (upper right panel) and GBS sera (lower right panel). Scale bar, 20 μm. **Fig. 6 (B)** Recombinant NDRG1 expression was confirmed by western blot analysis (B, arrow) of two stable lines (3 and 5). Mock-transfected cells (0) did not show detectable levels of NDRG1 expression.
**Discussion**

Here we report, for the first time, that IgG fractions of patients with inflammatory neuropathies stain non-myelinating Schwann cells. In particular, distal tips of Schwann cell processes, the leading lamellae, were stained by 24% of the GBS (36 out of 233) and 26% of the CIDP (12 out of 46) patient sera (Table 1). Moreover, these antisera also reacted in all 10 patient sera tested with nerve-growth-cones of *in vitro* differentiated hNT2 neurons. In addition, single myelinated teased nerve fibres reacted with all 10 patient sera tested. It was not possible to discriminate which cellular component of the nerve fibre (myelin, Schwann cell membrane, extracellular matrix, basal lamina or axon surface) is actually stained as was the case in *in vitro* cultured cells. In contrast, these reactivities were markedly lower in incidence and strength among the control sera from healthy donors (2 out of 34 on Schwann cells, 0 out of 5 on hNT2 cells and 0 out of 5 teased nerve fibres) and patients with non-inflammatory neurological disorders (HMSN type 1 0 out of 47, Alzheimer’s disease 0 out of 4). Immunoreactivity was independent of high dose IVIg treatment since several sera taken from non-treated patients as well as sera taken before treatment were positive. Moreover, the majority of sera taken during or immediately after IVIg treatment (within 4 weeks) showed no positivity. Cell lines derived from non-neural tissue did not show detectable staining. We conclude that the immunoreactivity with Schwann cells and hNT2 neurons is nerve tissue-specific and could be disease-related.

In view of the localization of immunofluorescence at the Schwann cell leading lamella and nerve-growth-cone, antigens such as nerve-growth-cone associated proteins (B-50/GAP-43) (Oestreicher *et al.*, 1997), cell adhesion molecules (CAMs) involved in Schwann cell–axon interaction (L1) (Haney *et al.*, 1999), Schwann cell–axon signalling (p185*erbB2*, p75LNTR) (Morrissey *et al.*, 1995; Lemke and Chao, 1988) or Schwann cell–extracellular matrix interaction (integrins) (Previtali *et al.*, 1998; Archelos *et al.*, 1999) could be involved. The nerve-growth-cone has been closely related to these processes have been well characterized (Oestreicher *et al.*, 1997; Suter and Forsher, 1998), as well as cell surface molecules expressed on the nerve-growth-cone (Walsh and Doherty, 1997). For instance, integrin expression on Schwann cells has been reported to be distinctly regulated in experimental autoimmune neuritis and GBS (Previtali *et al.*, 1998; Archelos *et al.*, 1999). We tested monoclonal and polyclonal antibodies raised against this defined group of proteins expected to be present in these structures in order to identify the epitopes recognized by the sera from patients with inflammatory neuropathies (Table 2). In our primary Schwann cell cultures, immunoreactivity was present against L1/NGCAM, TAG-1, c-erbB2 and integrin subunits. This immunofluorescence was, however, unlike disease-related immunofluorescence, not restricted to the Schwann cell leading lamella and was present diffusely on the entire cell surface. Such distribution of immunoreactivity for TAG-1 has been observed previously (D. Karagogeos, personal communication).

We identified three candidate epitopes that were almost exclusively located on Schwann cell leading lamellae (Fig. 4). First, the p75 low-affinity neurotrophin receptor (p75LNTR) or nerve growth factor (NGF) receptor (Fig. 4A) is a cell surface receptor that belongs to the tumor necrosis receptor family (Johnson *et al.*, 1986). It is upregulated after axotomy (Lemke and Chao, 1988) and is involved in the NGF-induced apoptotic-signalling pathway in Schwann cells (Soilu-Hänninen *et al.*, 1999). Double labelling studies and confocal laser scanning microscopy (Fig. 4A) showed, however, that the Schwann cell epitopes recognized by GBS/CIDP sera did not co-localize with p75LNTR. p75LNTR therefore excluded as target epitope in inflammatory neuropathies. Secondly, the pre-synaptic protein B-50/GAP-43 is highly expressed during axon sprouting and nerve regeneration after nerve injury (Plantinga *et al.*, 1993; Oestreicher *et al.*, 1997). B-50/GAP-43 has also been found in non-myelinating Schwann cells, but its function in these cells is not exactly known (Curtis *et al.*, 1992). Our studies (Fig. 4B) have demonstrated that the epitope recognized by GBS/CIDP patient sera co-localized partially with B-50/GAP-43. Double labelling of B-50/GAP-43 transfected Rat1 fibroblasts (Fig. 5) showed, however, that B-50/GAP-43 by itself is not responsible for autoreactivity in GBS and CIDP. Cellular components known to be closely associated (forming hetero-multimers) with B-50/GAP-43 (Stewart *et al.*, 1995; Suter and Forsher, 1998) are currently under investigation.

Immunofluorescence staining using polyclonal antiserum raised against a third candidate protein, NDRG1, co-localized with the GBS and CIDP sera on the Schwann cell leading lamella. Surprisingly, Schwann cells generated from a NDRG1–/– patient still showed staining localized at the
leading lamella with both NDRG1 antisemur and GBS and CIDP patient sera. Using recombinant NDRG-His fusion proteins, we demonstrated that the polyclonal anti-NDRG1 antisemur cross-reacts with NDRG3, but not with the other members of the NDRG protein family (2 and 4). Our data suggest therefore that, in HMSN-Lom Schwann cells, NDRG3 is present in the Schwann cell leading lamella and not NDRG1. However, under denaturing conditions (western blot analysis), we were not able to detect GBS or CIDP patient serum immunoreactivity to NDRG3 (expressed in E.coli) or total Schwann cell protein preparations.

The fact that the transfection studies were not able to confirm the IF microscopy data might have several explanations. First, the polyclonal antiseras raised against B-50/GAP-43 and NDRG1 may actually also recognize different proteins with regions homologous to (parts of) B-50/GAP-43 and NDRG1, respectively. The finding that monoclonal antibody clones NM2 and NM6 (which were raised against B-50/GAP-43 peptides residues 39–43 and 132–213, respectively) do not recognize the Schwann cell leading lamella supports this contention.

Secondly, post-translational modifications like (cell and/or cell-cycle-specific) glycosylation or phosphorylation may be required for recognition by the patient sera. The recombinant proteins were produced in fibroblasts (GAP-43/B-50, Fig. 4) and CHO cells (NDRG1, Fig. 6) respectively, which do not necessarily have similar post-translational modifications as Schwann cells. Recombinant NDRG3 was produced in E.coli (Fig. 7) and therefore lacks glycosylated epitopes. To rule out the possibility that the immune reaction recognizes cell-specific glycosylated epitopes exclusively, we also stained Schwann cells (which do glycosylate) with both patient sera and anti-NDRG1 polyclonal antibody (not shown). Although the endogenous (Sc) NDRG3 migrated at a marked higher position on SDS–PAGE (suggesting post-translational modifications), serum reactivity to the endogenous (Sc) protein on Western blot was absent as well (as it was to recombinant E.coli protein).

Thirdly, if a hetero-multimer consisting of more than one protein is required for recognition by the patient sera, testing separate proteins expressed in recombinant cells might not be sufficient to resolve serum reactivity. Our difficulty in demonstrating immunoreactive epitopes under denaturing conditions (western blotting) supports this hypothesis and suggests that conformational epitopes could be involved.

Our findings are novel in the fact that immune-mediated neuropathies are generally considered to arise from an immune response towards myelin constituents. The in vitro cultured Schwann cells used in this study are in a proliferative state and do not produce myelin. In addition, no myelin sheaths are present around the axons or dendrites in neural hNT2 cultures. Still, we have found for the first time characteristically localized immunoreactivity against these cells in sera of patients with inflammatory neuropathies. The GBS/CIDP serum reactivity was also present in single myelinated nerve fibre preparations, which more closely resemble the in vivo situation. GBS/CIDP related immunofluorescence appeared to co-localize with P0, suggesting that it is caused by a reaction towards myelin constituents. However, the close association between Schwann cells and axon in these fibres makes it impossible to discriminate which cell component is actually stained. Previously, the role of an immune response towards known myelin components P0 and Cx32 has been found not to play a major role in GBS and CIDP (Kwa et al., 2001). Only in a subgroup (4 out of 21) of CIDP patients demyelinating anti-P0 IgGs have been demonstrated (Yan et al., 2001). The role of anti-PMP22 antibodies in GBS and CIDP remains controversial since serum reactivity to PMP22 (Gabriel et al., 2000) was not confirmed in other laboratories (Ritz et al., 2000; Kwa et al., 2001). This non-reproducibility is probably caused by differences in assay conditions and detection methods. An immune reaction towards non-myelin components on both Schwann and neurons is apparently present, accounting for a serological response in 24–26% of the inflammatory neuropathy patients. Whether these circulating IgG antibodies are the cause of pathogenesis or arise from initial nerve damage requires further investigation.

We did not find anti-Schwann cell antibodies in all inflammatory neuropathy patient sera. This might be caused by several factors. Firstly, we made no attempt to restrict inclusion criteria such as age, date of onset, IVIg/phase exchange treatment and acute or sub-acute disease phase. However, we did not find a statistically significant (P < 0.05) correlation between anti-Schwann cell IgG and the above-mentioned clinical parameters. Although it must be noted that the serum subgroups used for our retrospective study might be too small to observe statistically significant correlations, neither treatment nor disease phase was of influence on the anti-Schwann cell antibody titre in our serum panel. This may argue against an active role of these antibodies by themselves in the disease process and in favour of them being a (closely linked) risk factor for autoimmune neuropathies. Secondly, it is well established that the clinical manifestation of both CIDP and GBS is very heterogeneous. The group of 24–26% patients with positive sera may represent a distinct subgroup displaying auto-reactivity to a group of Schwann cell epitope(s), whereas in the remainder of the patients other mechanisms or target antigens could be involved. Yan and colleagues recently demonstrated (Yan et al., 2001) that, in a subgroup of (4 out of 21) CIDP patients, IgG antibodies to P0 can cause demyelination and conduction block after intraneural injection in rats. Similarly, several antibodies to particular gangliosides have been implicated in axonal forms of GBS (Ho et al., 1999), but their role in demyelinating forms remains elusive (van Schaik et al., 1995).

Recent reports have suggested particular IgG Fc γ receptor (Fc γR) alleles as putative risk factors for GBS (Vedeler et al., 2000; van der Pol et al., 2000). For instance, Fc gamma RIILa-H131 homozygous GBS patients had an increased risk for severe disease than did patients with other genotypes. Interestingly, recent studies in a mouse autoimmune disease
model (trombocytopenia) showed that the anti-inflammatory activity of IVIg is mediated through the inhibitory Fc receptor, Fc γ RIIB, since disruption by genetic knock-out or blocking monoclonal antibody alleviated the therapeutic effect of IVIg (Samuelsson et al., 2001).

Our present data combined with recent findings suggest that, at least part of the autoimmune reactivity in inflammatory demyelinating neuropathies is not directed against myelin exclusively, but towards non-myelin proteins and epitopes possibly involved in Schwann cell–axon interaction as well. Ongoing research integrating further clinical evaluation of prospectively defined patient/serum groups, identification and molecular characterization will be necessary to assess the significance of the Schwann cell and hNT2 neuronal autoreactive epitopes in pathogenesis and clinical course of inflammatory demyelinating neuropathies.

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