Expression of the co-stimulatory molecule BB-1, the ligands CTLA-4 and CD28 and their mRNAs in chronic inflammatory demyelinating polyneuropathy

Ken-ya Murata and Marinos C. Dalakas

Neuromuscular Diseases Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA

Correspondence to: Marinos C. Dalakas, MD, Neuromuscular Diseases Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 4N248, 10 Center DR MSC 1382, Bethesda, MD 20892-1382, USA
E-mail: dalakasm@ninds.nih.gov

Summary
To examine whether the Schwann cells in patients with autoimmune neuropathies have the potential to behave as professional antigen-presenting cells, we investigated the expression of the co-stimulatory molecules BB-1, B7-1 (CD80) B7-2 (CD86) and their counter-receptors CD28 or CTLA-4 (CD152) at the protein and mRNA levels in sural nerve biopsies of patients with chronic inflammatory demyelinating polyneuropathy (CIDP), CIDP associated with human immunodeficiency virus infection (HIV–CIDP), IgM paraproteinaemic neuropathy and normal or non-immune axonal neuropathy. In single- and double-labelling experiments, we used the S-100 antigen as a pan-Schwann cell marker, myelin-associated glycoprotein as a marker for myelinating Schwann cells and the fibrillary acidic protein as a marker for unmyelinating Schwann cells. The expression of the B7 family of molecules was limited to BB-1 and was observed only on the Schwann cells. There was constitutive expression of BB-1 on unmyelinating Schwann cells in all nerves studied. However, in CIDP and HIV–CIDP, but not the other diseases, there was prominent upregulation of BB-1 on the myelinating Schwann cells. The endoneurial T cells in the proximity of BB-1-positive Schwann cells expressed the CD28 or CTLA-4 counter-receptors. Reverse transcription–polymerase chain reaction confirmed that these ligands were upregulated only in CIDP. Because the myelinating BB-1-positive Schwann cells expressed HLA-DR antigen, the findings indicate that, in CIDP, Schwann cells possess the necessary markers to function as antigen-presenting cells.

Keywords: co-stimulatory molecules; immunohistological study; RT–PCR; chronic inflammatory demyelinating polyneuropathy; antigen-presenting cells

Abbreviations: CIDP = chronic inflammatory demyelinating polyneuropathy; HIV = human immunodeficiency virus; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GFAP = glial fibrillary acidic protein; MAG = myelin-associated glycoprotein; RT–PCR = reverse transcription–polymerase chain reaction

Introduction
Chronic inflammatory demyelinating polyneuropathy (CIDP) is an acquired neuropathy caused by immune-mediated damage to peripheral nerves (Dyck et al., 1993; Hartung et al., 1994; Pollard, 1994; Dalakas, 1999; Gold et al., 1999). Although macrophage-mediated demyelination and antemyelin antibodies have been implicated (Ilyas et al., 1992; Dyck et al., 1993; Pollard, 1994; Dalakas, 1999; Gold et al., 1999), the target antigen is unknown and the type of endoneurial cell that behaves as an antigen-presenting cell remains undefined. The HLA-DR antigen, which is present on antigen-presenting cells, is not a helpful marker because it is expressed on both Schwann cells and macrophages, not only in CIDP nerves but also in non-immune-mediated neuropathies, such as hereditary motor-sensory or metabolic neuropathies (Cadoni et al., 1986; Mancardi et al., 1988; Scarpini et al., 1990).

A prerequisite for the Schwann cell to present an antigen and behave as an antigen-presenting cell is the expression of
the B7 family of co-stimulatory molecules [BB-1, B7-1 (CD80, B7) or B7-2 (CD86, B70)] on their surface and the expression of their counter-receptors CD28 and CTLA-4 (CD152) on the activated T cells. These co-stimulatory molecules are hallmark molecules of the professional antigen-presenting cell because they are essential in the maintenance of self-tolerance and the regulation of T-cell immunity (Reiser and Stadecker, 1996). B7-1 is strongly expressed on activated microglia and infiltrating macrophages within the active lesions of multiple sclerosis (De Simone et al., 1995). In addition, B7-1 and B7-2 have been shown in cardiac myocytes (Seko et al., 1998) and the B1 in human muscle fibres of patients with polymyositis (Behrens et al., 1998; Murata and Dalakas, 1999). To examine whether Schwann cells possess the properties of antigen-presenting cells in CIDP, we investigated the co-expression of co-stimulatory molecules and their ligands at the protein and mRNA levels.

**Material and methods**

**Immunocytochemical study**

Fresh-frozen sections of sural nerve biopsy specimens from three patients with CIDP and one patient with CIDP associated with HIV infection (HIV–CIDP) were processed for immunocytochemistry. All patients had the typical clinical, histological and electrophysiological features of these disorders (Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force, 1991; Dyck et al., 1993). Control specimens were obtained from three patients with mixed demyelinating/axonal IgM paraproteinaemic neuropathy and from three normal old people with mild axonal changes investigated during a study of normal ageing. All specimens had been stored in liquid nitrogen before the study.

To separate the two types of Schwann cells, we used anti-CD86 (clone BU63; Ancell, Bayport, Minn., USA), 5 µg/ml in 1% BSA/TBS for 1.5 h; (ii) IgG anti-human CD80 (clone L307.4; PharMingen), 12.5 µg/ml in 1% BSA/TBS for 1.5 h; (iii) IgG anti-human CD86 (clone BU63; Ancell, Bayport, Minn., USA), 5 µg/ml in 1% BSA/TBS for 1.5 h; (iv) IgG anti-MAG (clone 513, Roche Molecular Biochemicals, Indianapolis, Ind., USA), 5.0 µg/ml in 1% BSA/TBS for 1.5 h; (v) IgG anti-human CD28 (clone L293; Becton Dickinson, San Jose, Calif., USA), 7.5 µg/ml in 1% BSA/TBS overnight at 4°C; (vi) IgG anti-human CD152 (CTLA-4) (clone BN13; PharMingen), 5 µg/ml in 1% BSA/TBS for 1.5 h; (vii) IgG anti-human CD8 (clone SFCI21Thy2D3 (T8); Coulter, Miami, Fla., USA), 5 µg/ml in 1% BSA/TBS for 1.5 h; (viii) IgG anti-human macrophage (clone Ber-MAC3; DAKO, Carpinteria, Calif., USA), 0.35 µg/ml in 1% BSA/TBS for 1.5 h; and (x) IgG anti-human HLA-DR (clone L243; Becton Dickinson), 7.5 µg/ml in 1% BSA/TBS for 1.5 h. All sections were also incubated with rabbit polyclonal antibodies against cow S-100 (DAKO), 10.0 µg/ml in 1% BSA/TBS for 1.5 h and against cow GFAP (DAKO), 1 : 300 dilution in 1% BSA/TBS for 1.5 h. After washing for 60 min in TBS, the sections were incubated with biotinylated goat anti-mouse IgM (for the CD80 clone BB1), with goat anti-rabbit IgG (for S-100 and GFAP) or with goat anti-mouse IgG for the other proteins, and then with FITC (fluorescein isothiocyanate) avidin D. Serial sections, consecutive to those processed as described, were stained with modified Gomori trichrome. The following control, irrelevant IgG or IgM antibodies of the same isotype were also used at the same concentrations as the primary antibodies: mouse IgG1 (clone MOPC-21), mouse IgG2a (clone G155–178), mouse IgG2b (clone 27–35) and mouse IgM (clone G155–228), all from PharMingen.

We also performed double immunostaining using mouse monoclonal antibodies against CD80 (BB-1) and antibodies against S-100 or CD4+ T cells on the same sections, as described previously (Murata and Dalakas, 1999). In brief, 5 µm serial sections were fixed in acetone for 10 min at 4°C and incubated for 30 min with a blocking solution containing 2% BSA and 5% normal goat serum. The sections were incubated with the primary mouse monoclonal antibody against CD80 (BB-1), followed by biotinylated goat anti-mouse IgM and rhodamine avidin D. For double immunolocalization of BB-1 and S-100, the sections were incubated with polyclonal anti-S-100 antibody followed by FITC-conjugated goat anti-rabbit IgG. For double staining with BB-1 and CD4, the sections were incubated with anti-human CD4 followed by FITC-conjugated goat anti-mouse IgG. The slides were mounted and examined with a Zeiss microscope equipped with epifluorescence optics.

**Preparation of RNA**

Total RNA was extracted using Trizol (Gibco BRL, Grand Island, NY, USA). Frozen sural nerve biopsy sections (10 sections, 20 µm thick) were homogenized in a total volume of 0.2 ml of Trizol and the extracted RNA was precipitated according to the manufacturer’s instructions. RNA was
suspended in 8 µl of RNAase-free water, heated for 10 min at 56°C and stored at –80°C until use.

**Reverse transcription (RT)–polymerase chain reaction (PCR)**
The cDNA was prepared using a GeneAmp RNA PCR kit (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ, USA) with 1.0 µg of total RNA in a final volume of 20 µl, according to the manufacturer’s instructions. Before synthesis of the cDNA with random primers, 10 units of RNase-free DNase I (Boehringer Mannheim, Indianapolis, Ind., USA) was added per microgram of RNA, and the reaction mixture was incubated for 30 min at 37°C and 5 min at 95°C. For amplification of the cDNA, 2.5 µl of the RT reaction mixture was used for each 50 µl of the PCR reaction. Each PCR mixture contained the standard components of 1.5 mM MgCl₂ and 50 pmol CTLA-4 or CD28 primers. As an internal control for RNA quantification and standardization of the amplified RNA products, we used the primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutive and moderately expressed enzyme.

The CTLA-4, CD28 and GAPDH primers were designed using published cDNA sequences (Dariavach *et al*., 1988; Ercolani *et al*., 1988; Lee *et al*., 1990) (Genosys Biotechnologies, The Woodlands, Tex., USA), as follows: CTLA-4 forwards, 5′-AGTATGCATCTCCAGGCAAAGC-3′ (exon 1); CTLA-4 reverse, 5′-CCAGAGGAGGAAGTCAGAATCTG-3′ (exon 2); CD28 forwards, 5′-GTTGGATGTCCTTGATCATGTGC-3′ (exon 1); CD 28 reverse, 5′-GGCGATCTGCCTTCACAAAAATC-3′ (exon 2); GAPDH forwards, 5′-TGAAGGTGGTGGTGATCAACGGATTTGG-3′ (exon 2–3); and GAPDH reverse, 5′-GTTCACACCCACGACCATG-3′ (exon 6). Both sets of primers spanned at least one intron in their respective genomic sequences, resulting in amplified products of 316 bp for CTLA-4, 238 bp for CD28 and 398 bp for GAPDH. After an initial 3 min period at 95°C, the PCR mixtures were incubated for 45 cycles, unless otherwise stated, using a Perkin Elmer Model 480 thermocycler, at 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 7 min. The amplified products (12.5 µl) were analysed by 3% gel electrophoresis and the bands were visualized with ethidium bromide (0.5 µg/ml). Fluorescent bands were recorded on Polaroid Type 55 film (Polaroid, Cambridge, Mass., USA). RT–PCR analysis was done at least twice for every patient. Polaroid negatives of ethidium bromide-stained gels were digitized into 512 × 512-pixel grey-scale images.

**Fig. 1** Transverse serial frozen sections of a sural nerve biopsy from a patient with CIDP stained with monoclonal antibodies against BB-1, B7-1, B7-2 and polyclonal antibody against S-100. Note that only BB-1, but not B7-1 and B7-2 is expressed on both myelinating and unmyelinating S-100-positive Schwann cells. Scale bar = 10 µm.
**Results**

**Immunohistological study of B7 family of molecules**

Among the B7 family of molecules, only BB-1 was strongly and specifically expressed on almost all the Schwann cells that were positive for the S-100 antigen (Fig. 1). None of the inflammatory cells were positive for BB-1. In contrast, B7-1 (CD80) and B7-2 (CD86) were undetectable on the endoneurial S-100-positive Schwann cells (Fig. 1) but were present on the surface of occasional T cells (not shown). To ascertain whether BB-1 was specifically present or upregulated on myelinating or unmyelinating Schwann cells in CIDP nerves, we compared the pattern of BB-1 immunoreactivity on S-100-positive Schwann cells with the immunoreactivity pattern obtained with anti-MAG antibodies (for the myelinating Schwann cells) and with anti-GFAP antibodies (for the unmyelinating Schwann cells). There were two patterns of BB-1 expression. One consisted of diffuse, stippled staining, observed on the S-100-positive Schwann cells not only in CIDP but also in the other disease controls, as confirmed by double staining (Fig. 2). Most importantly, this pattern was identical to that obtained with anti-GFAP antibodies in serial sections (Fig. 2), indicating constitutive expression of BB-1 on the unmyelinating Schwann cells. The second and most specific pattern, noted only in CIDP and not in the controls, was the upregulation of BB-1 on the outer rings of the compact myelin, as confirmed by double immunostaining using anti-S-100 antibodies (Fig. 2B). In serial sections, the number of BB-1-positive myelinated axons corresponded with the number of MAG-positive axons (Fig. 2). Staining for HLA-DR showed expression on endothelial and perivascular cells of the endoneurial capillaries as well as on the myelinating and unmyelinating Schwann cells in all nerves that were studied (not shown). No staining was noted with the irrelevant IgG or IgM antibodies of the same isotype.

The counter-receptors of BB-1, CTLA-4 and CD28, were observed on the rare CD8+ and CD4+ T cells present in the endoneurium of CIDP nerves but not in nerves from controls (Fig. 3). With double immunostaining, these cells were in proximity to the BB-1-positive Schwann cells (Fig. 3, inset). On serial sections, most of these cells were CD4+ (not shown). The macrophages, which constitute the majority of mononuclear cells in the endoneurium, were negative not only for BB-1 but also for CTLA-4 and CD28 (not shown).

**RT–PCR**

The amount of CTLA-4, CD28 and GAPDH mRNA extracted was studied with increasing PCR cycles as described (Murata...
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Transverse serial sections of a sural nerve biopsy specimen from a patient with CIDP and a control immunostained with monoclonal antibodies against CD28 or CTLA-4. Note that CD28-positive or CTLA-4 positive T cells are observed only in CIDP. Scale bar = 10 µm. One CD4⁺ lymphocyte (green) is shown in proximity to the BB-1-positive Schwann cells (red) (inset).

Fig. 3

Transverse serial sections of a sural nerve biopsy specimen from a patient with CIDP and a control immunostained with monoclonal antibodies against CD28 or CTLA-4. Note that CD28-positive or CTLA-4 positive T cells are observed only in CIDP. Scale bar = 10 µm. One CD4⁺ lymphocyte (green) is shown in proximity to the BB-1-positive Schwann cells (red) (inset).

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Fig. 4 RT–PCR analysis of mRNA is shown for CTLA-4 (316 bp), CD28 (238 bp) and the control enzyme GAPDH (398 bp) from sural nerve biopsies of three representative patients, one with HIV–CIDP (lanes 1–3) and two with CIDP (one in lanes 4–6 and the other in lanes 7–9). Controls are represented in lanes 10–12. M indicates the size marker. mRNA expression for both ligands (CTLA-4 and CD28) was strongly amplified in HIV–CIDP (lanes 2–3). In CIDP one of the ligands was amplified, either CD28 (lane 6) or (weakly) CTLA-4 (lane 8). Neither CTLA-4 nor CD28 mRNA was amplified in the control specimens. GAPDH mRNA expression was strong in all specimens.

Discussion

We have demonstrated that in patients with CIDP and HIV–CIDP, but not in disease controls, the main co-stimulatory molecule BB-1, but not B7-1 or B7-2, is upregulated on the S-100-positive myelinating Schwann cells. Furthermore, in CIDP patients but not in controls, there is upregulation of the counter-receptors CD28 and CTLA-4 on the few endoneurial T cells found in the proximity of the BB-1-positive Schwann cells. Macrophages, which seem to be the primary effector cells invading myelin lamellae, did not express the co-stimulatory molecules associated with antigen-presenting cells. Because the co-stimulatory molecules and their ligands are required for antigen presentation, the findings indicate that in CIDP the myelinating Schwann cells express the phenotypic markers of antigen-presenting cells.

Recent data indicate that the B7 family of co-stimulatory molecules on the antigen-presenting cells include three distinct forms, B7-1 (CD80), B7-2 (CD86 or B70) and BB-1 (Boussiotis et al., 1993; Behrens et al., 1998). Although the cDNA for BB-1 has not been isolated, the BB-1 antigen is believed to be distinct from B7-1 because it is expressed on B7-1-negative cells (Nickoloff et al., 1993). It has been thought that BB-1 also reacts with CD74, an MHC class II-associated invariant chain (Freeman et al., 1998) which theoretically might be present on HLA-DR-positive Schwann cells. However, in tissue sections the anti-CD74 antibodies stain only T cells (Murata and Dalakas, 1999).

In addition to the upregulation of BB-1 on myelinating Schwann cells in patients with CIDP, BB-1 was constitutively expressed on the S-100-positive unmyelinating Schwann cells in all specimens studied. It remains to be determined whether the expression of BB-1 is also upregulated on unmyelinating Schwann cells in certain autoimmune neuropathies. It seems that such upregulation may be related to the autoimmune process, i.e. cytokines rather than inflammatory cells, because we noted mild upregulation in Schwann cells of the nerve biopsies from three patients with Sjögren syndrome who did not have inflammation (data not shown).

The finding that in CIDP, Schwann cells express the markers of antigen-presenting cells, complements various indirect in vivo and in vitro observations which support the
functional properties of these cells in antigen presentation. Upon stimulation by interferon γ or incubation with activated T cells, Schwann cells upregulate MHC class II molecules (Gold et al., 1995). Furthermore, Schwann cells containing phagocytosed myelin are able to present endogenous myelin basic protein to myelin basic protein-reactive CD4+ T cells (Wekerle et al., 1986) and to phagocyte exogenous myelin for processing to constituent proteins (Bigbee et al., 1987). In vitro, Schwann cells can present *Mycobacterium leprae* hsp70 (heat shock protein 70) antigen to hsp-specific CD4+ T cells (Ford et al., 1993), and activated P2-specific CD4+ T cells proliferate in the presence of Schwann cells (Argall et al., 1992). Accordingly, the Schwann cells in CIDP and other autoimmune neuropathies may contain myelin antigens which could be presented to T cells.

Macrophages, which in CIDP constitute the majority of the endoneurial inflammatory cells and may participate as primary effector cells in the demyelinating process, did not express BB-1 or its ligands CTLA-4 and CD28. In contrast, CTLA-4 and CD28 were upregulated only on the few T cells found in the proximity of the BB-1-positive Schwann cells. Consequently, Schwann cells may act as antigen-presenting cells by presenting antigens to the CD4+ T cells found in their proximity in association with MHC class II antigen, which is co-expressed on the Schwann cell surface. Pro-inflammatory cytokines, such as TNF-α (tumour necrosis factor α), interferon γ and IL-2 (interleukin-2), are upregulated within the microenvironment of the CIDP nerves (Mathey et al., 1999) and offer the necessary co-stimulatory signals associated with antigen presentation. Whether cytokines are also produced by Schwann cells, as shown in culture, is unclear.

In summary, our findings provide evidence that, in CIDP and HIV–CIDP, Schwann cells express the co-stimulatory molecules seen in cells with the antigen-presenting cell phenotype, and suggest that these cells should have the capacity to function as professional antigen-presenting cells.

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**References**


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