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## Detection and quantification of antibodies to the extracellular domain of P0 during experimental allergic neuritis

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### Summary

Quantification of the peripheral nerve myelin glycoprotein P0 and antibodies to P0 is difficult due to insolubility of P0 in physiological solutions. We have overcome this problem by using the water-soluble recombinant form of the extracellular domain of P0 (P0-ED) and describe newly developed assays which allow detection and quantitation of P0 and antibodies to P0, in serum and cerebrospinal fluid (CSF). These sensitive and specific assays based on the ELISA technique were used to study humoral immune responses to P0 during experimental autoimmune ("allergic") neuritis (EAN). In order to establish these tests, monoclonal antibodies to different epitopes of rodent and human P0-ED were produced. A two-antibody sandwich-ELISA allowing quantitation of P0 (lower detection limit of 0.5 ng/ml or 30 fmol/ml) and an antibody-capture ELISA (lower detection limit 1 ng specific antibody/ml) to detect antibodies to P0 in serum and CSF were developed. EAN was induced in rats by active immunization with bovine myelin or the neuritogenic protein P2 or by adoptive transfer using P2 specific CD4 positive T cells. Serum and CSF were assayed for the presence of P0-ED and antibodies to P0-ED or P2. Antibodies to P0-ED were detected during active myelin-induced EAN, but not during P2-induced or adoptive transfer EAN. The anti-P0-ED antibodies in the CSF showed a correlation with disease activity. In contrast, in the same model antibodies to P2 persisted long after the disease ceased. No soluble P0-like fragments could be found in serum or CSF during any of the three types of EAN. We conclude that P0 may be a B-cell epitope in EAN. These findings warrant a screen for antibodies to P0-ED in human immune neuropathies.

### Introduction

Experimental autoimmune ("allergic") neuritis (EAN) is an acute inflammatory demyelinating disease of the peripheral nervous system (PNS) (Hartung et al. 1993a). The close clinical, histopathological and electrophysiological similarities between EAN and the human Guillain-Barré syndrome (GBS) make this disorder an especially suitable model to offer insights into the pathophysiology of acute demyelinating disease in man (Hartung and Toyka 1990).

Recent interest has focused on P0 as a putative autoantigen involved in the etiology of GBS (Khalili-Shirazi et al. 1992). P0 with an apparent molecular weight of 28–30 kDa, is the major protein of the

peripheral nervous system accounting for over 50% of the myelin proteins of the peripheral nervous system (PNS) (Ishaque et al. 1980). P0 exhibits structural similarity to immunoglobulin-related cell adhesion proteins (Williams and Barclay 1989). The amino acid sequence of P0 has been determined in different species including rat (Lemke et al. 1985), mouse (You et al. 1991), and man (Ilyasaka et al. 1991) and is highly conserved showing few amino acid variations.

It has been shown that the protein backbone of the extracellular domain of P0 (P0-ED) acts as a homotypic adhesion molecule, has neurite-outgrowth promoting properties (Filbin et al. 1990, Schneider-Schaulies et al. 1990; D'Urso et al. 1990) and contains one of the two neuritogenic T-cell epitopes of P0 within the amino acids 56–71 (Linington et al., 1992). P0, like other myelin proteins, induces EAN in the rat (Milner et al. 1987).

Because of these properties of P0, and especially of P0-ED, it is pertinent to study immune responses to

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P0-ED during EAN and GBS. We have established a sensitive and specific antibody-capture ELISA to detect and quantitate antibodies to P0-ED in sera and cerebrospinal fluid during active and passive EAN and to compare our findings with the immune response to P2. It was also studied whether P0-like immunoreactive material is released into serum or CSF during acute inflammatory demyelination. This seemed reasonable, because it has been described that proteolytic fragments of myelin basic protein are found in the CSF during demyelinating diseases of the CNS, like multiple sclerosis (Whitaker et al. 1977). Therefore we established a two-antibody sandwich ELISA to measure P0-ED-like immunoreactive material during EAN.

## Materials and methods

### Induction of EAN

#### Active EAN

Six-week-old female Lewis rats with a body weight of 160–180 g (obtained from Zentralinstitut für Versuchstierkunde, Hannover, Germany), were inoculated into one hindleg footpad with 100  $\mu$ l of an emulsion containing an equal volume of saline and complete Freund's adjuvant with either 4 mg myelin or 500  $\mu$ g P2 from bovine spinal roots and 4 mg/ml *Mycobacterium tuberculosis* (strain H37 RA from Difco, Detroit, MI, USA)

#### Adoptive transfer EAN

Adoptive transfer EAN was induced by injection of  $4.5 \times 10^6$  P2-specific CD4-positive T cells into a rat tail vein. Generation and maintenance of this neuritogenic cells (line P2.48) have recently been described in detail (Jung et al. 1992).

### Scoring

Disease severity was assessed clinically employing the modified King-Thomas scale ranging from 0 to 10 (Hartung et al. 1988). 0 = normal, 1 = less lively, 2 = impaired righting/limp tail, 3 = absent righting, 4 = ataxic gait, 5 = mild paraparesis, 6 = moderate paraparesis, 7 = severe paraparesis, 8 = tetraparesis, 9 = moribund, 10 = death.

### Collection of serum and cerebrospinal fluid

Serum samples were collected every other day by bleeding the animal at the tail tip under anesthesia with ether and were stored at  $-30^\circ\text{C}$ .

To collect CSF, the animals were sacrificed by terminal anesthesia with ether at different stages of their disease. The atlantooccipital membrane was exposed, a

small incisure was placed under a dissecting microscope and approximately 50–100  $\mu$ l of CSF were removed with a micropipette. The CSF was centrifuged and the supernatant stored at  $-30^\circ\text{C}$  until assayed. The cellular pellet was resuspended in the same volume of PBS and the red blood cells were counted. This allowed detection and quantification of contamination with blood. Only the CSF samples showing a calculated contamination with blood of 1:1000 of the CSF volume or less were used for the assays.

### Purification of antigens

Recombinant rat P0-ED was purified as described in a separate paper (Archelos et al. 1993). In brief, bacterial inclusion bodies containing P0-ED were solubilized in 4 M urea, size-fractionated twice on a Sephadex G-50 (Pharmacia, Freiburg, Germany) column and finally reoxidized with oxidized glutathione (Calbiochem, Bad Soden, Germany) to allow refolding of the molecule. After this renaturation procedure, P0-ED remained soluble in physiological buffers. SDS-PAGE using 15% acrylamide slab gels revealed one major band at 17 kDa.

Myelin from rat or bovine PNS was prepared by density gradient centrifugation. Whole P0 was purified from rat myelin by SDS-PAGE and electroelution (Agrawal et al. 1990). P2 protein was isolated from myelin of bovine spinal roots by acid extraction of myelin followed by gel filtration on Sephadex G-50 (Pharmacia, Freiburg, Germany).

### Enzyme-linked immunosorbent assay (ELISA)

#### Antibody capture ELISA: detection of antibodies against P0-ED and P2

96-well microtiter plates (Nunc, Wiesbaden, Germany) were coated with either 5  $\mu$ g/ml recombinant P0-ED or bovine P2. To block any remaining binding sites the plates were then incubated with blocking buffer consisting of 0.5% bovine serum albumin (BSA), 1% porcine serum (Sigma, Munich, Germany) in phosphate-buffered saline (PBS), pH 7.4, overnight at  $4^\circ\text{C}$ . Plates were washed twice with PBS, 0.1% Tween 20 and incubated with serum or cerebrospinal fluid (CSF) diluted in blocking buffer overnight at  $4^\circ\text{C}$ . The subsequent steps were performed at room temperature. The plates were washed four times and horseradish peroxidase-coupled goat-anti-rat antibody (Dianova, Hamburg, Germany) diluted 1:7500 in blocking buffer was added and incubated for 30 min on a rotatory shaker (300 rounds per min). Finally, antibody binding was visualized by using 2 mM ABTS (2,2'-azino-bis-(ethylbenzthiazoline-6-sulfonic acid)) (Boehringer, Mannheim, Germany) in 0.1 M acetate buffer, pH 4.2, and 2.5 mM hydrogen peroxide. Optical density (OD) was

read at wavelengths 405 and 450 nm with an ELISA reader.

A strongly positive rat serum diluted to its end point titer was used as positive control, and normal rat sera ( $n = 9$ ) served as negative controls.

*Two-antibody sandwich ELISA: detection of P0-ED-like immunoreactivity*

This sandwich ELISA assay was established to measure water-soluble degradation products from the extracellular domain and to look whether their concentration in sera and CSF would correlate with disease activity.

Polyclonal and monoclonal antibodies P05 and P07 to P0-ED were used. These antibodies have recently been characterized (Archelos et al. 1993). Polyclonal antibodies against P0-ED obtained from rabbits were purified by gel filtration. P07, a monoclonal antibody of the IgG 1 class, was purified by affinity chromatography on protein-A-sepharose, and P05, a monoclonal antibody of the IgM class, by precipitation with polyethylene glycol 6000 and gel filtration (Neoh et al. 1986).

Microtiter plates were coated with polyclonal antibodies from rabbit acting as "capture antibodies" at a concentration of 20  $\mu\text{g/ml}$ . Plates were blocked, washed and then test samples and P0-ED standards (from 100 to 0.1 ng/ml) were added and incubated. In order to detect P0-ED-like immunoreactivity monoclonal antibodies P07 or P05 were added at a final concentration of 1 and 0.2  $\mu\text{g/ml}$ , respectively, and binding was visualised as described above. The resulting OD values were compared with the standard values obtained with defined amounts of P0-ED.

In the antibody detecting ELISA the sera were usually diluted 1:100 for anti-P0-ED- and 1:1000 for anti-P2-antibody detection. CSF was diluted 1:20 or 1:50. For the sandwich ELISA test sera were diluted 1:100, and CSF 1:1 in blocking buffer. Each sample was tested in duplicate. In order to define sensitivity and specificity of the described ELISAs, standards of P2 or P0-ED antigen and of monoclonal (P07) and polyclonal antibodies to P0-ED from different species were tested. The used standard antibodies and antigens were dissolved in normal rat serum diluted 1:100.

*Calculation of titer*

Initially, in order to titrate the antibodies to P0-ED or P2 in the test sera the samples were subsequently diluted 1:1 until the OD differed by no more than 2 SD from the value obtained with BSA or a corresponding dilution of control serum. The dilution above this value was taken as the end point titer. The standard dilution curves obtained with positive sera each showed a very similar configuration with a wide linear range

between OD values of 0.50 and 0.15 when plotted on a logarithmic scale. This behaviour and the fact that most OD values of positive samples plotted to that linear range of the standard curve at the used dilutions allowed us to calculate the end point titer from the OD value as has recently been described in detail for human samples (Pestronk et al. 1990). Sera showing values above that range were diluted and calculated in the same manner. Samples showing no significant absorbance above control at the dilutions used were plotted arbitrarily with an attributed titer corresponding to the used dilution or with their absolute absorbance value.

*SDS-PAGE and Western blot analysis*

SDS-PAGE was carried out on 15% acrylamide gels. For Western blot analysis a total of 5  $\mu\text{g}$  of P0 and P2 were loaded onto each lane. After SDS-PAGE, proteins were transferred electrophoretically to nitrocellulose membranes at 80 mA for 2 h. Unreactive binding sites were saturated for 2 h with 30% FCS (Biochrom, Berlin, Germany), 3% BSA (blocking buffer). The nitrocellulose strips were then washed with PBS, pH 8.0, 0.1% Tween and incubated, in sequence, with test serum diluted 1:100 in blocking buffer overnight at 4°C and, after washing, with alkaline-phosphatase coupled goat-anti-rat Ig (Dianova, Hamburg, Germany) diluted 1:5000 in blocking buffer for another 90 min. Nitrocellulose membranes were stained with NBT (*p*-nitrotetrazolium blue, Sigma, Munich, Germany) and BCIP (5-bromo-4-chloro-3-indolylphosphate, Sigma) diluted in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl, pH 9.4).

Monoclonal antibody P07 (mouse-anti-P0-ED), at a final dilution of 1:1000, was used as positive control, normal rat serum diluted 1:100 served as a negative control.

**Results**

*Sensitivity and specificity of the ELISAs*

The antibody capture ELISA allowed detection of P0-ED-specific antibody at concentrations as low as 1 ng per ml. Antibodies to P2 did not react with the P0-ED coated ELISA plate indicating that specifically antibodies to P0-ED were detected. Standard curves obtained with positive sera from animals with EAN showed a wide log linear range between absorbance values (405 nm) of 0.15–0.50 (Fig. 1). The two-antibody sandwich ELISA established for these experiments allowed detection of as little as 0.5 ng P0-ED/ml (or 30 fmol/ml). The standard curve obtained by addition of

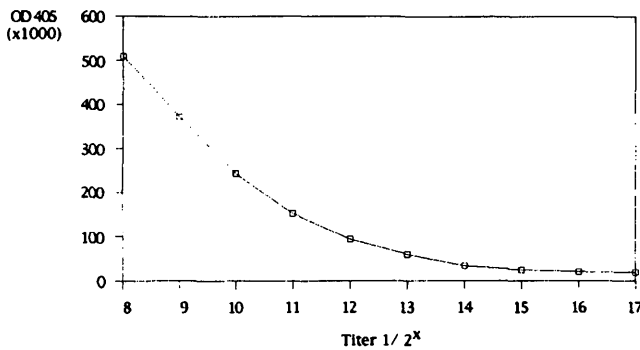


Fig. 1. Standard curve obtained with a positive serum during myelin-induced EAN using the antibody capture assay. The serum sample was diluted subsequently 1:1 and tested for antibodies to P0-ED. The dilutions used are plotted on a logarithmic scale against the optical density (OD) measured at 405 nm. Typically, the standard curve showed a linear range of OD values between 0.5 and 0.15.

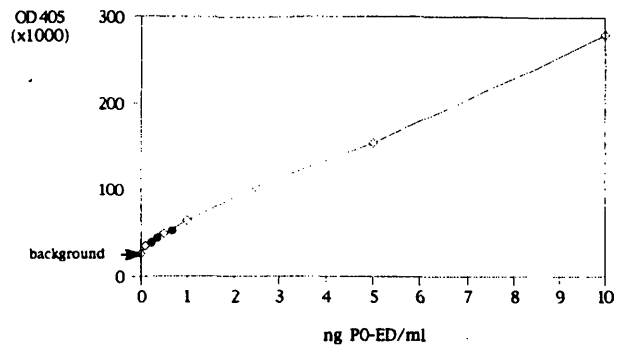


Fig. 2. Standard curve obtained with defined quantities of P0-ED ranging from 0.1 to 10 ng/ml using the two-antibody sandwich ELISA. The concentrations of P0-ED ( $\diamond$ ) are plotted against the absorbance values obtained at 405 nm. This assay allowed detection of 0.5 ng/ml or 30 fmol/ml P0-ED-like immunoreactivity. The closed circles indicate values of circulating P0-ED obtained in CSF during myelin-induced EAN.

defined amounts of P0-ED from 10-0.1 ng/ml showed a wide linear range (Fig. 2). Antigens other than P0-ED including P2 or antigens in normal rat serum were not detected with this assay.

Using P0-ED as antigen these two assays showed an approximately 200-fold higher sensitivity compared to the same assays using solubilized, detergent-containing whole human or rat P0 as antigen (data not shown).

#### Antibody titers during myelin-induced EAN

The immunized animals developed initial neurological signs after 11-12 days starting with limp tails, then developing hindleg paresis, paraplegia, sometimes tetraparesis and infrequently death (Fig. 3a). In parallel the animals experienced the weight loss symptomatic of EAN (Hartung et al. 1988)

#### Serum

Antibodies against P0-ED were first detected on day 13 just one or two days after the appearance of clinical

signs. Most animals achieved titers around 1:8000 but sometimes end-point titers up to 1:32000 were measured during maximal neurological disease. These levels were maintained on this plateau for approximately 2 weeks and then started to fall slowly. Three months after disease induction with myelin, the antibody titer to P0-ED had declined to values below 1:1000 (Fig. 3a).

The antibody response to P2 (Fig. 3b) differed from that to P0-ED. The reactivity against P2 in the test sera started 1-3 days later than the response to P0-ED and increased more slowly over the first days of disease reaching titers of 1:1-2000 when EAN was at its maximal severity. Furthermore, the antibody titer rose continuously over the next weeks despite complete clinical recovery of the animals. Titers approached values of 1:64000 during this phase being considerable higher than those obtained against P0-ED. After three months the antibody titers to P2 still maintained a mean value of 1:22000.

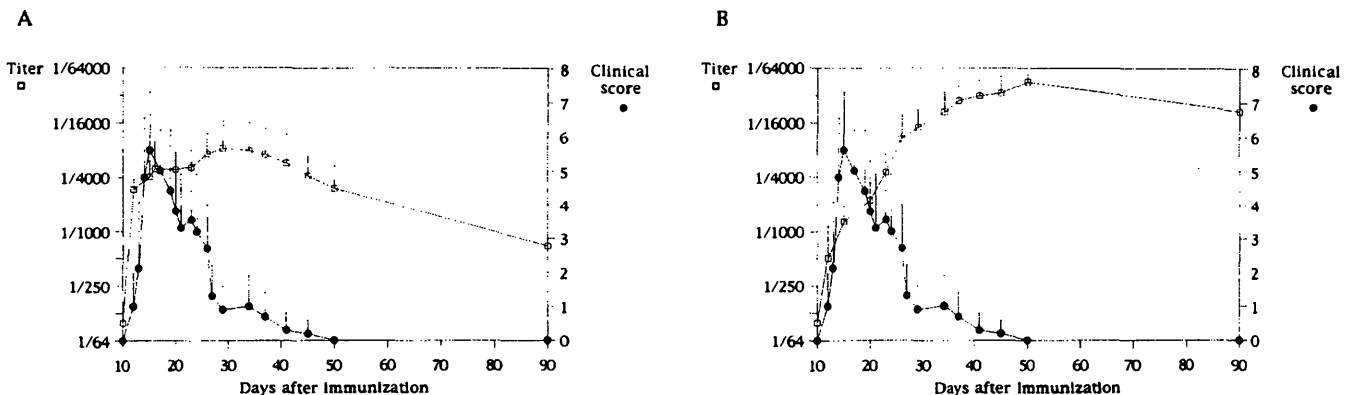


Fig. 3. Anti-P0-ED (Fig. 3a) and anti-P2 (Fig. 3b) antibody titers in serum during myelin-induced EAN. Each end point titer ( $\square$ ) represents the geometric mean + SD factor from at least 10 animals. The closed circles represent the mean clinical score + SD. Note the early and steep increase of the anti-P0-ED and the persistently high anti-P2 antibody titers.

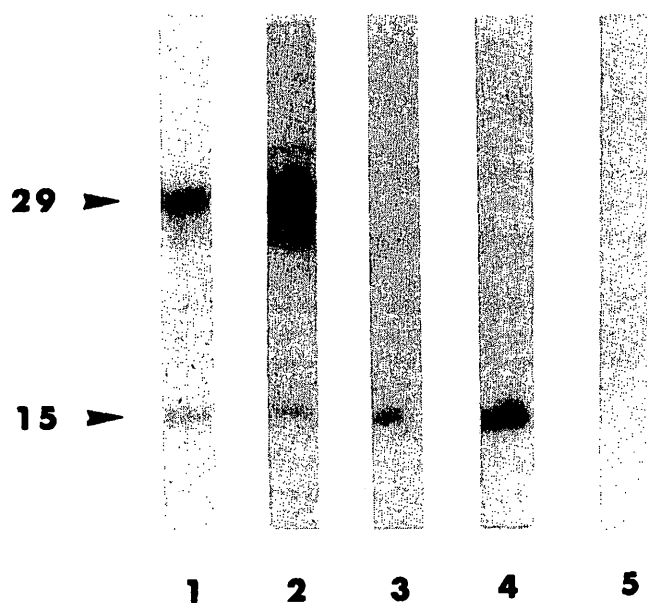


Fig. 4. Western blot analysis of serum during EAN. Total P0 and P2 were loaded on each lane and incubated with serum diluted 1:100. Lane 1 and 2 show the reaction pattern of an early (day 17) and later (day 30) stage of myelin-induced EAN, lane 3 and 4 show the staining of an early and late serum from P2-induced EAN, lane 5 shows the negative result obtained with serum from T-cell-induced EAN.

Surprisingly, two out of 12 rats developed only mild disease reaching maximal scores of 2–3. The sera of these animals showed a similar antibody response against P2 as in the severely affected animals but contained only low amounts of antibodies to P0-ED (data not shown).

These results in the ELISA were confirmed by Western blot analysis. We found that the antibody responses to whole native P0 (Fig. 4) or P0-ED (data not shown) were stronger than that against P2.

#### CSF

Antibodies against P0-ED were first detected on day 18 in CSF, several days later than in serum (Fig. 5a).

#### A

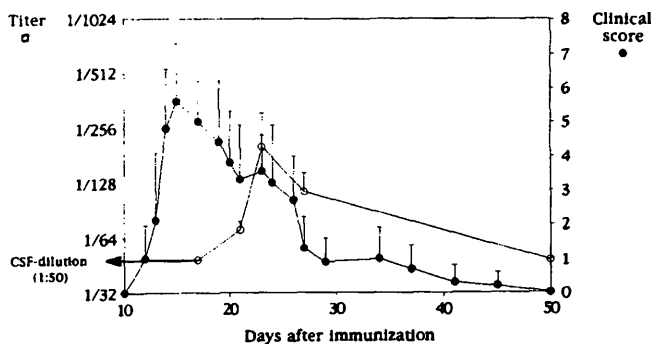


Fig. 5. Anti-P0-ED (a) and anti-P2 (b) antibody titers in cerebrospinal fluid during myelin-induced EAN. Note the closer correlation of the anti-P0-ED reactivity in CSF with clinical disease. The closed circles depict the mean clinical score + SD, the open circles the corresponding end point titer. Each point represents results obtained in at least 6 animals. Contamination with serum was calculated to be less than 1:1000.

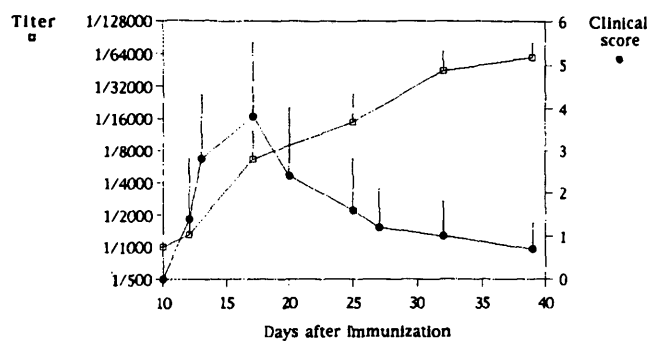


Fig. 6. Anti-P2 antibody titers during P2-induced EAN. The measured antibody titers exhibited no correlation to disease activity. Disease was less severe than in myelin-induced EAN, but serum levels of anti-P2 antibodies were higher. Each point represents the geometric mean (+SD factor) of at least 6 animals.

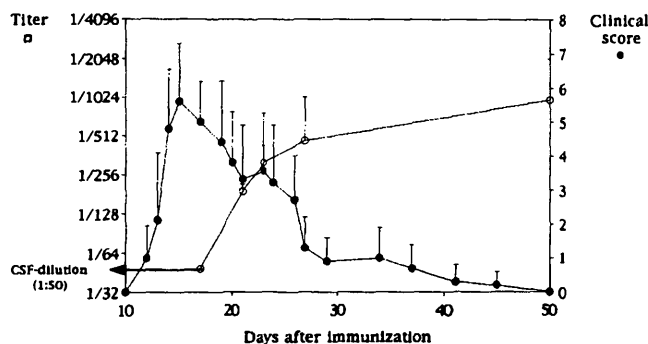
Peak titers with values of about 1:200 were detected one week after the height of the disease. After complete clinical recovery significant reactivity against P0-ED was no longer detectable in CSF.

The P2-titers started to increase at the same time as the P0-ED-titers (Fig. 5b) but persisted, similar to what was found in serum, remaining high even after neurological recovery. Here the titers usually reached levels of 1:1000.

#### Antibody titers during P2-induced EAN

The neurological signs of disease produced by P2 at the dose given were less severe than those produced by whole peripheral nerve myelin. Reactivity against P2 in the sera of these animals was first detected on day 13 and increased continuously over the following days similarly to myelin-induced EAN (Fig. 6), except that the titers increased faster and reached maximal end point titers of approximately 1:64000. In some animals titers were as high as 1:128000. No significant reactivity

#### B



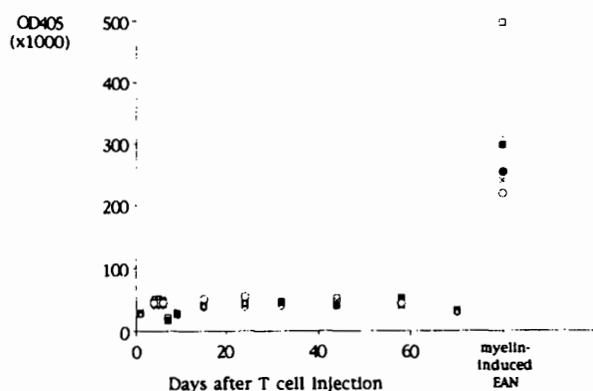


Fig. 7. Anti-P0-ED reactivity in serum during T-cell-mediated EAN. The OD 405 is plotted against the time after T cell injection (in days). No significant antibody reactivity to P0-ED was found during 60 days. Each symbol represents one value from one animal ( $n = 6$  for each day). In contrast, during myelin-induced EAN values of 0.2–0.5 were usually recorded.

against P0-ED was observed during P2-induced EAN. In order to examine late secondary formation of antibodies to P0-ED we assayed sera up to 50 days after immunization but were not able to detect these antibodies. Western blot analysis of these sera showed that only antibodies against P2 but not against the glycosylated native form of P0 (Fig. 4) or P0-ED or other myelin proteins (data not shown) were detectable.

#### *Antibody titers during adoptive transfer EAN*

First clinical signs became manifest on day 3–4 after i.v. injection of T cells. Maximal disease activity was reached by day 7–8. In comparison to active EAN, severity of the disease was less variable and residual symptoms persisted longer in the adoptive transfer model of EAN. Attempts to measure significant anti-P0-ED- or anti-P2-reactivity in the sera or CSF of these animals failed. The absorbance values found in ELISA were usually above the control values of untreated animals but much below the absorbance found in sera from animals with active EAN (Fig. 7). Furthermore the absorbance values showed no fluctuation over the course of the disease and were similar at different dilution thus indicating that although being higher than controls they were not specific. No reaction with whole native P0, P2 (Fig. 4), P0-ED or other myelin proteins (data not shown) was found in Western blots. This showed that antibodies to the cytoplasmic domain of P0 or its carbohydrates were not formed in detectable amounts during adoptive transfer EAN.

#### *Soluble P0-ED-like immunoreactivity in EAN*

Serum and CSF from animals with active myelin- or P2-induced and adoptive transfer EAN were screened

with the sandwich ELISA for the presence of P0-ED or proteolytic fragments of P0-ED. Samples were usually drawn before disease started, during maximal neurological impairment and then weekly up to 6 weeks after disease peak. With this test we were unable to detect any soluble P0-ED-like-immunoreactivity or related fragments in the serum or CSF during different stages of the three EAN models used. Some CSF samples of myelin-induced EAN showed absorbance values corresponding to calculated 0.5 ng P0-ED-like-immunoreactivity per ml during the peak of active EAN (Fig. 2). Since these values were close to the lower detection limit of these assay they were not regarded as positive.

#### **Discussion**

In this study we describe newly developed sensitive assays that allow detection of antibodies to the extracellular domain of P0 (P0-ED) and of soluble P0-ED or its fragments. The presence of antibodies to P0-ED and to P2 and the kinetics of their formation in the two animal models of the Guillain-Barré syndrome (GBS), actively induced and adoptively transferred EAN, were examined.

The etiology and pathogenesis of the GBS remain unknown (Hartung et al. 1993b). It is generally accepted that immunological mechanisms contribute to the pathogenesis of this disease (Winer et al. 1988). A number of studies have attempted to identify target antigens including P0, P2, galactocerebroside and myelin-associated glycoprotein MAG (Hughes et al. 1984; Quarles et al. 1990; reviewed by Toyka and Hartung 1992). Recent data suggest that cell-mediated immune responses against P0 may be involved in the pathogenesis of some cases of GBS (Khalili-Shirazi et al. 1992). The biophysical properties of P0 have until now prevented extensive studies on the humoral immune response during the course of EAN and GBS or allowed the use of immunoassays with relative low sensitivity. Hence, we have focused on the humoral immune response to the water-soluble extracellular domain of P0.

Immunization of Lewis rats with whole bovine spinal root myelin stimulated production of antibodies to several myelin components including P0-ED and P2. The antibody response to P0-ED in serum although of lower titer exhibited a closer relationship to the disease activity than the response to P2. The failure to correlate clinical disease with antibody titers to P2 has been previously described (Hughes et al. 1981; Milek et al. 1983; Geczy et al. 1985). The reason for the different kinetics of the humoral immune response to P0 and P2 is unclear. It parallels recent data on T cell-mediated

immune responses to both proteins in GBS (Khalili-Shirazi et al. 1992). Developmentally, it seems reasonable to limit an autoimmune response against an abundant transmembrane protein like P0, in contrast to a minor intracellular myelin protein like P2.

Little is known about the antibody levels in CSF during EAN. We found that immunoreaction to P0-ED was only detectable in CSF during overt clinical disease. This is most likely due to transient opening of the blood-CSF barrier during EAN (Hahn et al. 1985; Powell et al. 1991). The reason why the antibody titers to P2 remain high in CSF even after supposed closure of the blood-CSF barrier is unknown, but parallels our results obtained in serum. Contamination with serum could be excluded by red cell counts in CSF. Other explanations for these findings in the CSF include passive diffusion of antibodies from serum, which seems unlikely with the observed anti-P0- and anti-P2 antibody titer course, or the persistent presence of P2-specific B cells at the nerve roots in proximity to the CSF. During P2-induced active EAN, only antibodies to P2 were detected. These results demonstrate that antibodies were formed exclusively to antigens used for immunization and that no antibodies against unrelated myelin proteins were induced in response to acute inflammatory myelin destruction. This was also confirmed in adoptive transfer EAN, where later in the disease course neither a reaction to P0-ED, total P0 nor to P2 could be detected in ELISA or Western blot even at a time when an antibody response could have been mounted. Based on the data obtained in these animal models, we have no evidence that auto-antibodies against myelin proteins like P2 or P0 are formed as "epiphenomenon" after acute immune-mediated demyelination with disruption of the Schwann cells and myelin membrane and ensuing release of these antigens. Although it may be possible that small amounts of antibodies to P2 or P0 escape detection because they are absorbed to tissue antigens, this is unlikely because antibody titers did not vary and were not significantly elevated over control values during the whole observation period of two months. Antibodies against myelin proteins such as P2 or P0 found during acute inflammatory demyelination of the PNS (e.g. in GBS), may be generated in response to exogenous antigen or antigen-like structures (Hartung et al. 1993a,b). This may reflect molecular mimicry between some putative infective agents and myelin components (Fujinami and Oldstone 1985) or incidental cross-reactivity of antibodies induced by other non-related antigens. Several groups have reported cases of cross-reactivity of putative disease-related infectious agents and myelin components like P0 or gangliosides, during GBS (Fujimoto and Amako 1990; Höjeberg et al. 1991; Walsh et al. 1991; Enders et al. 1993). One interesting finding is the cross-reactivity of antibodies to herpes

simplex virus type 1 with P0, which may be relevant to the pathogenesis of Marek's disease, an avian analog of GBS induced by a herpes virus. Such cross-reactivity could play a role in the development of GBS in some cases.

Our experiments do not exclude the possibility that auto-antibodies against self myelin proteins are produced in response to chronic demyelinating neuropathy. Indeed, it is known that antibodies to myelin glycoproteins like MAG or P0 are detectable in chronic demyelinating polyneuropathies associated with monoclonal gammopathies (Bollensen et al. 1987; Burger et al. 1990), but these may be primary and causative.

Our studies suggest that antibodies to P0 may contribute to the development of demyelination. This view gains support from previous observations that antibodies to P0 when injected intraneurally to rats are capable of inducing demyelination per se (Hughes et al. 1985). Furthermore, it has been reported that antibodies to myelin-oligodendrocyte glycoprotein (MOG) if present contribute substantially to the degree of demyelination in experimental autoimmune encephalomyelitis (EAE) in the Lewis rat (Linnington et al. 1988). In fact, these authors have been able to produce a chronic relapsing model of EAE by repeated co-transfer of MBP-specific T cells and a demyelinating antibody to MOG (Linnington et al. 1992).

We were unable to detect P0-ED-like immunoreactive fragments in serum or CSF. Nevertheless, the possibility remains that these are produced in amounts below the detection limit of our assay, which is 30 pM. Furthermore, although we used monoclonal antibodies against two different epitopes it is possible that fragments if released do not contain these epitopes. Recently, an intracellular cleavage site used by a myelin-restricted protease has been identified in P0 (Agrawal et al. 1990). The presence of other cleavage sites yielding water soluble products cannot be excluded. Finally, it is conceivable that P0 is phagocytosed within myelin membranes by macrophages in areas undergoing demyelination.

In conclusion, the results of this study suggest that humoral immune responses to P0-ED may have a contributory role in inflammatory demyelination and that P0 may be a B-cell epitope in EAN and in some cases of the Guillain-Barré syndrome (unpublished observation). These findings indicate the need to screen for antibodies to P0-ED in human immune neuropathies.

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