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Peripheral Nerve P2 Basic Protein and the Guillain–Barré Syndrome

In Vitro Demonstration of P2-Specific Antibody-Secreting Cells

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SUMMARY

An immune response to the peripheral nerve basic protein P2 may be operative in the pathogenesis of the Guillain–Barré syndrome (GBS). A method is described for the purification of P2 of human origin. Purified P2 was used to investigate whether lymphocytes derived from peripheral blood of GBS patients are capable of producing P2-specific antibodies after stimulation with the antigen in vitro. Peripheral blood lymphocytes (PBL) from 5 GBS patients, from 3 patients with chronic idiopathic polyradiculoneuropathy (CIP) and from 3 normal controls were cultured in vitro in the presence of P2. PBL from the 5 GBS patients were shown to generate an antigen (P2)-specific antibody response. Contrariwise, PBL from the 3 CIP patients as well as from the 3 normal controls did not show this specific response.

Key words: *Guillain–Barré syndrome – P2 protein – P2-specific antibody – Plaque forming cell assay*

INTRODUCTION

Guillain–Barré syndrome (GBS) is an acute idiopathic polyradiculoneuropathy, frequently triggered off by a preceding infection. In many instances this infection is

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caused by one of the enveloped viruses such as cytomegaly or Epstein–Barr virus. The chronic idiopathic polyradiculoneuropathy (CIP) takes a different course; it is not preceded by an infection. Immunological mechanisms are considered to play an important part in the pathogenesis of GBS. Both humoral and cellular effector mechanisms are supposed to lead to the demyelination of the peripheral nerves characteristic of the syndrome. An argument in favour of an underlying immune dysregulation is based on the observation that GBS is identical with experimental allergic neuritis (EAN) in nearly every respect. This experimental neuritis can be induced in various kinds of laboratory animals by the injection (in Freund's complete adjuvant) of a homogenate of peripheral nerve (Waksman and Adams 1955), of peripheral nerve myelin, or of basic protein P2 (Brostoff et al. 1974). In the pathogenesis of CIP, on the other hand, immunological mechanisms do not seem to play an important role.

In a previous study (Luijten and Baart de la Faille-Kuyper 1972) we described the occurrence of IgM and complement factors along myelin sheaths of peripheral nerves in GBS. Since then, methods for the purification of the basic protein P2 have become available (Kadlubowski et al. 1980). This protein may be the putative antigen against which antibodies of the IgM class are produced in GBS.

In recent years, considerable progress has been made in the techniques of cell culture, including the induction and measuring of antigen-specific antibody (IgM) synthesis by human peripheral blood lymphocytes (PBL) in vitro (Ballieux et al. 1979). These developments enabled us to investigate the antigen (P2)-specific antibody synthesis by PBL derived from GBS patients.

MATERIALS AND METHODS

Purification of P2 basic protein

Human cauda equina was obtained, within 12 h of death, from patients who died from non-neurological diseases. The tissue was stored at −80 °C until use. All isolation steps were carried out at 4 °C.

A. Myelin isolation The procedure comprised five successive steps.

Step I. Cauda equina was disrupted in 0.88 M sucrose (5% homogenate) in a Sorvall Omni-mixer and subsequently homogenized in a Teflon-glass homogenizer.

Step II. In a number of centrifuge tubes 16 ml of homogenate in 0.88 M sucrose were overlaid by 22 ml of 0.32 M sucrose. This discontinuous gradient was centrifuged in a Beckman L5-65 preparative ultracentrifuge fitted with a SW 27 rotor for 30 min at 75000 × *g*. The crude myelin layers at the interface were collected and resuspended in 0.88 M sucrose. The same gradient was prepared and recentrifuged.

Step III. The crude myelin thus obtained was suspended in distilled water and stirred for 20 min in order to release axoplasma by means of osmotic shock.

Step IV. The suspension was then centrifuged in the Beckman ultracentrifuge for 15 min at 75000 × *g*. The pellets were dispersed in distilled water and subsequently centrifuged in a Sorvall RC 2-B centrifuge, fitted with a SS-34 rotor, for 20 min at 29000 × *g*.

Step V. The myelin pellets were collected and lyophilized.

B. Isolation of P2 protein from purified myelin

Step I. The purified myelin was homogenized in a Teflon-glass homogenizer. Acid extraction of the myelin was performed in 30 mM HCl (pH 2.1), to which 2-mercaptoethanol (1 mM) had been added. The solution was stirred for 15 h.

Step II. The suspension was centrifuged (Beckman ultracentrifuge, rotor 30) for 1 h at 78 000 × *g*. The supernatant was concentrated to a volume of 5 ml with a Diaflo ultrafiltration apparatus from Amicon Corp.; UM2 membranes were used.

Step III. The concentrate was applied to a Sephadex G-75 column (100 × 2.5 cm), equilibrated with 30 mM HCl, 1 mM 2-mercaptoethanol. The column was washed with 30 mM HCl, 1 mM 2-mercaptoethanol at a flow rate of 30 ml/h. The elution profile is shown in Fig. 1. Fractions (7 ml) were pooled as indicated, concentrated by ultrafiltration, dialyzed against water and lyophilized. The composition of the material thus obtained was investigated by means of a SDS-polyacrylamide gel electrophoresis. A discontinuous slab gel system was used (Laemmli 1970). The slab gel (146 × 134 × 2 mm) contained 3% stacking gel and 12.5% running gel.

Lymphocyte isolation

Mononuclear cells were isolated from heparinized blood by density gradient centrifugation on Ficoll–Isopaque (d = 1.077 g/cm^3) at 1 000 × *g* for 20 min and then washed twice with MEM–Tris (Tris buffered minimal essential medium) supplemented with penicillin and streptomycin (100 IU, 100 μg/ml, respectively).

Cell cultures

A total of 5 × 10^6 cells in a volume of 10 ml RPMI-1640 supplemented with 2 mM L-glutamine, 10% AB serum and antibiotics was cultured with various doses of P2 in polystyrene Falcon tubes (No. 2057) (17 × 100 mm) at 37 °C, in a humidified atmosphere of 5% CO_2 in air. The AB serum was extensively absorbed with sheep erythrocytes (SE) to prevent pseudo-plaque formation. After 6 days of culture the cells were washed twice and the plaque-forming cell assay (PFC) was performed to identify antibody-secreting cells. Details of the culture conditions have been described elsewhere (Heijnen et al. 1979).

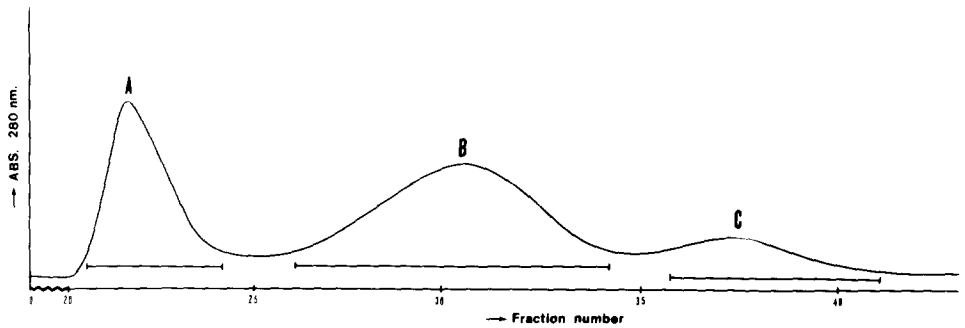

Fig. 1. Sephadex G-75 gel chromatography of acid-soluble proteins of human PNS myelin. Acid-soluble proteins (60 mg) were applied to a column (100 × 2.5 cm) and eluted with 30 mM HCl, 1 mM 2-mercaptoethanol. Flow rate: 30 ml/h.

Plaque-forming cell assay

Sheep erythrocytes, coated by means of $CrCl_3 \cdot 6H_2O$ (Goding 1976) with P2, were centrifuged for 5 min at 1000 × *g* on the bottom of Falcon microtiter plates (No. 3040) coated with poly-L-lysine (Sigma M_r = 100000) in a concentration of 100 μg/ml distilled water. Viable cultured cells in various dilutions were incubated for 60 min in the presence of SE absorbed guinea pig complement. Details of these methods have been described elsewhere (Heijnen et al. 1979).

Patients

Five patients with a typical GBS, 3 with a CIP syndrome and 3 normal controls, were investigated.

RESULTS

Characterization of human basic protein P2

SDS-polyacrylamide electrophoresis (see Fig. 2) revealed that the molecular weight of peak C material was approximately 14000D. The result suggests that peak

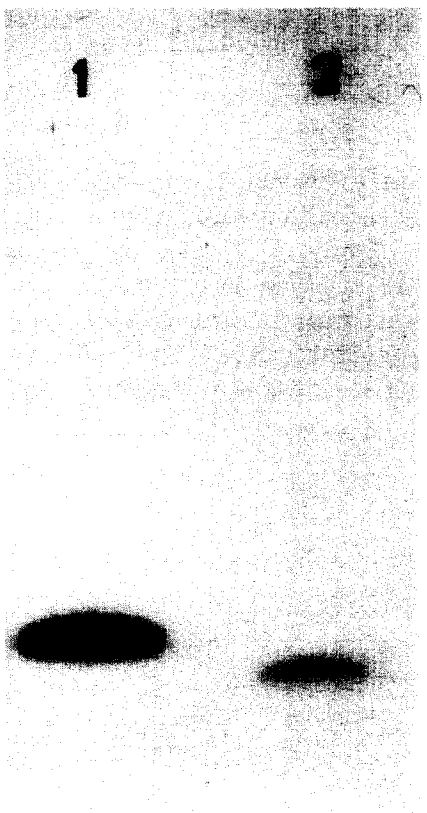

Fig. 2. SDS-polyacrylamide electrophoresis of peak C material (5 μg, lane 1) in comparison with cytochrome *c* (5 μg, lane 2). M_r = 12384.

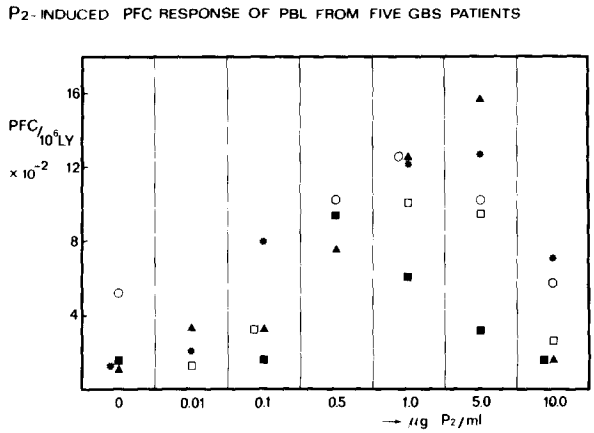

Fig. 3. Number of plaque-forming cells in relation to various doses of P2 in the culture.

C material consists of P2 protein. The isoelectric point as determined by isoelectric focussing, was 9.5. The amino acid composition was determined and found to be in accordance with the findings described for P2 (Suzuki et al. 1982).

Specific antibody response by plaque-forming cells of GBS patients

The capacity of PBL of 5 GBS patients (soon after the onset of the syndrome) for generating an antigen (P2)-specific PFC response was examined as follows. PBL of these 5 GBS patients were cultured with various doses of P2 and tested for specific antibody activity after 6 days of culture. After the culture period we determined the number of antibody-secreting cells by counting the number of plaques generated by complement-induced lysis of P2 coated erythrocytes. Figure 3 demonstrates that PBL of all of the five GBS patients were able to differentiate into PFC in the presence of low doses of the antigen; in the presence of high doses of the antigen, the PFC assay showed

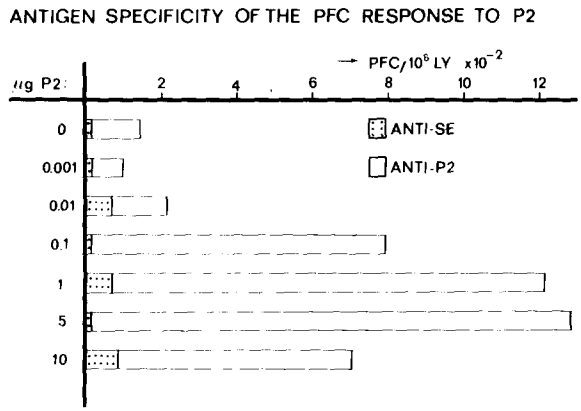

Fig. 4. Number of plaque-forming cells of PBL from 1 GBS patient cultured in the presence of various doses of P2; PFC-assay performed with P2-coated SE and with SE as a control.

TABLE 1

P2-INDUCED PFC RESPONSE OF PBL FROM 3 NORMAL CONTROLS (NC) AND 3 CHRONIC IDIOPATHIC POLYNEUROPATHIES (CIP) (PFC/10^6 ly)

μg P2/ml	NC	NC	NC	CIP	CIP	CIP
0	–	102	71	63	161	92
0.05	–	–	–	130	–	–
0.1	0	83	52	58	–	63
0.5	–	102	58	71	152	63
1.0	333	91	54	113	134	95
5.0	91	91	32	56	150	95
10.0	250	102	60	–	–	82

a decrease of the response (normal background value of the PFC test: 300 PFC/10^6 ly). When PBL of the GBS patients were reinvestigated within a few days, the same results were obtained (data not shown). The PFC assay was also performed with non-coated SE (results for 1 patient shown in Fig. 4). The number of plaques in these control experiments did not exceed the background value. Therefore one may assume that P2 does not induce a polyclonal antibody response.

Antibody response by plaque-forming cells of normal controls and CIP patients (see Table 1)

PBL obtained from 3 normal healthy control persons and from 3 CIP patients were cultured with various doses of the antigen P2; a significant PFC response was not found in any instance.

DISCUSSION

Conflicting reports are found in the literature concerning the presence of an immune response to P2 in GBS. By means of the macrophage migration inhibition assay it was found that peripheral blood lymphocytes are sensitized to P2 protein in GBS (Sheremata et al. 1975). The presence of peripheral blood lymphocytes sensitized to P2 was demonstrated in GBS patients by means of [2-^{14}C]thymidine incorporation (Abramsky et al. 1975). A recent study (Zweiman et al. 1983) could not confirm these findings; neither did this study show an unequivocally elevated level of anti-P2 antibodies in serum of GBS patients by means of an ELISA technique with P2 of bovine origin.

The results of the present study lead to the conclusion that lymphocytes capable of producing antibodies directed against the autoantigen P2 (of human origin) can be detected by means of a direct PFC assay in the peripheral blood of GBS patients, The PFC response is suppressed by a high dose of antigen in the culture medium. This finding suggests that the antibody synthesis directed against the autoantigen P2 can be negatively regulated by antigen-specific T suppressor cells. This assumption is based on

the finding that, in the case of exogenous antigens, the activation of T suppressor cells requires the antigen to be present in a high dose. (UytdeHaag et al. 1978). The activation of autoantigen-specific suppressor cells may play a part in the recovery of GBS.

As no antigen (P2)-specific PFC were found in the CIP patients (Table 1), it may be postulated that demyelination itself – with its possible release of the autoantigen P2 – does not lead to the induction of the specific autoantibody response. This finding may be an indication that the specific response in GBS is not an epi-phenomenon, but part of the aetiological mechanism.

The antibodies demonstrated by the direct PFC assay are known to be of the IgM class. The finding that PBL of GBS patients are capable of producing anti-P2-specific antibodies seems to be in accordance with the histological findings that antibodies of the IgM class (and complement) can be detected in peripheral nerves along the myelin sheaths in GBS patients (Luijten and Baart de la Faille-Kuyper 1972). It may be postulated that specific anti-P2 antibodies are produced locally by B cells which invade the peripheral nerves. Consequently, the complement activation caused by the linear deposition of (P2-specific?) antibodies of the IgM class (produced by these B cells) along myelin sheaths, may lead to complement-induced lysis of the Schwann cell membrane, resulting in demyelination. The possibility should, however, be taken into account that the demyelination is not caused exclusively by complement-induced cytolysis. The destruction of the peripheral myelin sheaths may also be caused by a (local) expansion of P2-specific T cells, which give rise to a delayed type hypersensitivity (DTH) reaction. Recently the mechanisms of disease induction in experimental allergic encephalomyelitis have been described (Ben-Nun and Cohen 1982); the findings seem to indicate that such DTH–T cells may be T helper cells. The demonstrated synthesis and secretion by primary B cells of P2-specific antibodies may be a reflection of the activation of P2-specific T helper cells that have been sensitized in the peripheral nerve lesion.

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