Immunoassay of P2 protein in cerebrospinal fluid in neurological disorders

J Colover

Abstract

Cerebrospinal fluid samples were obtained at lumbar puncture from 53 patients with a wide variety of neurological disorders. Cerebrospinal fluid samples were tested for the presence of P2 protein, a constituent of myelin, with an enzyme linked immunosorbent assay technique using a specific polyclonal antibody. High concentrations of P2 in the cerebrospinal fluid paralleled a raised IgG index (clearance ratio), the presence of oligoclonal bands, as well as raised white cell counts or depressed albumin:IgG ratios. Twenty one patients had been diagnosed as having definite or probable multiple sclerosis and the remaining 32 had other conditions. Of the 13 patients with high positive P2, 12 (92%) were in the multiple sclerosis category; of the 40 patients with low (12) or undetectable (28) P2 concentrations, only nine (23%) were diagnosed as having multiple sclerosis.

In this patient population the presence of high immunoreactive P2 concentrations in cerebrospinal fluid was closely associated with evidence of intrathecal immunoglobulin synthesis and with the clinical diagnosis of multiple sclerosis. On this basis it is suggested that immunoassay of P2 concentration in the cerebrospinal fluid may be of potential value in the investigation of patients with demyelinating disorders.

P2 protein is one of several recognised basic proteins present in human myelin that are known to be generated by oligodendrocytes.¹⁻³ The protein has a molecular weight variously stated as 13.8-14.8 kilodaltons^{4,5}; its maximum concentration is in peripheral nerve, then in the spinal cord; very little is found in the cerebral cortex.⁵ There is evidence that its localisation and provenance in nervous tissue are related to the distribution of fatty acids where it may have a physiological role in fatty acid transport.⁵ In acute demyelinating experimental allergic encephalomyelitis in guinea pigs^{6,7} myelin debris in central nervous system lesions stained strongly for P2 protein by immunohistochemical reactivity with a specific monoclonal antibody.⁸ It was therefore decided to investigate whether P2 protein could be detected in the cerebrospinal fluid of patients with multiple sclerosis and other neurological disorders by immunoassay. This paper describes the use of an enzyme

linked immunoadsorbent technique (ELISA) for detecting P2 protein in small samples of cerebrospinal fluid, from 53 such patients, and the extent to which immunoreactive P2 was associated with other pathological changes in the cerebrospinal fluid samples.

Methods

Samples of cerebrospinal fluid were derived from 53 patients with a variety of neurological disorders, mostly admitted to St Thomas's Hospital with a recent episode of neurological illness. Three of the patients had been attending the Action for Research into Multiple Sclerosis (ARMS) unit at the Central Middlesex Hospital; and a total number of 21 were assigned the diagnosis of definite or probable multiple sclerosis. The remaining 32 patients had a variety of other conditions (table 1). In some patients the diagnosis was not clear until several weeks after admission or after recurrence of neurological episodes, when both clinical and laboratory (cerebrospinal fluid) abnormalities were considered together. The diagnoses assigned by the clinicians to these 53 patients are given in table 1.

Samples of cerebrospinal fluid from these patients were obtained from the Clinical Immunology and Microbiology Departments at St Thomas's Hospital where some samples had been stored in plastic tubes at -20°C for periods up to one month. In most cases immunoassays for the presence of P2 were done without knowledge of the clinical diagnoses or of other laboratory results on the cerebrospinal fluid samples.

For the detection of P2 activity in the cerebrospinal fluid an indirect ELISA technique was set up using a rabbit polyclonal antibody to bovine spinal cord P2 protein (provided by Professor C F C MacPherson, London, Ontario) and an alkaline phosphatase conjugated goat anti-rabbit immunoglobulin reagent (Sigma). Volumes (100 µl) of cerebrospinal fluid, diluted serially twofold in buffered saline from 1 in 20 to 1 in 40 960, were incubated in 96-well plastic plates (Sterilin) overnight at 4°C and after washing, non-specific absorbance was blocked with 0.1% gelatin (200 µl). A 1 in 320 dilution of the rabbit antibody to P2 protein was then added to the wells in 100 µl volumes; plates were incubated at room temperature for three hours and then washed. The enzyme conjugated anti-immunoglobulin reagent was then added in 100 µl volumes for two hours at room temperature, followed by overnight incubation at 4°C. After washing, 100 µl of the

Department of Immunology, The Rayne Institute, United Medical and Dental Schools, St Thomas's Campus, London SE1 7EH
J Colover

Correspondence to:
Dr J Colover

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Table 1 Clinical features of 53 patients studied

Initials	Sex	Age	Diagnosis/clinical features	P2 ELISA Category*
CC	F	46	MS(d) bilateral vision loss; both legs weak	High
MG	F	45	MS(d) final diagnosis; acute pontine lesion	
VH	F	37	MS(d); vision loss; ataxia, vertigo	
BS	F	42	MS(d)	
MO	F	63	MS(d)	Low
JA	F	38	MS	
YC	M	37	MS	
JC	M	44	MS; previous Guillain-Barré; now extensor plantar reflexes	
MC	F	35	MS	High
AK	F	32	MS; transverse myelitis; optic neuritis; ?Devic's disease	
JM	M	39	MS	
SM	F	18	MS; ophthalmoplegia	
NM	M	26	MS; two episodes	Low
SH	F	34	MS	
DP	F	44	MS; bilateral loss of vision	
AW	F	27	MS; optic neuritis	
MD	F	29	MS	Undetectable
HF	M	30	MS; bilateral optic neuritis	
GL	M	51	MS; bilateral optic neuritis	
JS	F	19	MS; pale optic discs	
JW	F	49	MS; facial weakness; right leg weak; dizziness	High
PH	M	48	Central nervous system lymphoma; non-Hodgkin's disease	
KB	F	27	?Myeloma; blood/central nervous system barrier defect	
IB	F	63	Breast cancer; collapsed vertebra	
JC	M	55	Cervical spondylosis; myelogram	Low
AKa	M	48	Central nervous system lymphoma; immunoblastic high grade	
AKb	M	50	Sciatica; radiculogram	
JL	F	29	Vertebral collapse	
DP	F	44	Bilateral visual loss	Undetectable
GR	M	10	Progressive visual failure due to hereditary optic atrophy	
HB	M	27	Headache; ?subarachnoid haemorrhage	
WB	F	43	Myelogram	
WC	M	75	Motor neuropathy; ?Guillain-Barré syndrome	Undetectable
CC	M	58	Stroke	
DD	M	75	Cerebrovascular disease; confused	
ED	F	92	Neck stiffness; ?meningitis	
RD	M	33	Sciatica	Undetectable
ED	F	46	Syphilis; ?residual central nervous system pathology	
JE	M	14	Pinealoma	
MF	F	40	Cerebral systemic lupus erythematosus	
DG	M	8	Acute myeloid leukaemia; precytotoxic	Undetectable
SK	F	38	Meningococcal septicaemia	
GL	F	6m	Meningitis	
SM	M	35	Blackouts	
EM	F	49	Benign intracranial hypertension	Undetectable
MR	F	48	Lumbar disc prolapse	
BR	M	24	Neuropathy; weight loss; ?malignancy	
DS	M	63	Central nervous system lymphoma	
DS	M	5m	Sepsis; jaundice; ?meningitis	Undetectable
MT	F	64	Hydrocephalus	
MW	F	61	Bilateral retinal necrosis	
LW	M	6m	Hydrocephalus	
AZ	M	2w	Premature twin; on ventilator; ?meningitis	Undetectable

*P2 ELISA category (figure).
MS(d) definite multiple sclerosis.

substrate p-nitrophenyl phosphate was added to each well for 30 minutes in the dark, the reaction stopped by adding 50 μ l 3M-NaOH, and absorbance of the reaction product was read at 405 nm using a Titertek Multiscan spectrophotometer. Each plate was calibrated with standard quantities of bovine spinal cord P2 protein (from 1000 ng to 0.5 ng per well). The presence of P2-like activity in the cerebrospinal fluid samples was classified as high, low, or undetectable according to the characteristics of the assay curves (fig 1) and by comparing them visually with the standard curves. Coefficients of variation (CV) were obtained for intra-assay and interassay results on individual CSF samples and on the standard preparation of P2 protein. When the results of P2 assays had been obtained these were then related to the clinical diagnosis and to the results of other laboratory tests (protein content; white cell numbers; IgG index) that had been done on the original cerebrospinal fluid samples. Associations between P2 activity, clinical diagnoses, and other available

Immunoassay of P2 protein in 40 samples of cerebrospinal fluid showing three ELISA curves A, B, and C. Curve A is the average of 10 spinal fluids ("high positive"). Curve B is the average of 10 other spinal fluids ("low positive"). C is the average of 20 further cerebrospinal fluid samples categorised as "undetectable" for P2 activity. Assay points are the arithmetic means for each cerebrospinal fluid dilution and the vertical bars represent standard deviations about the mean.

cerebrospinal fluid laboratory results were evaluated non-parametrically by the χ^2 test after constructing 2×2 contingency tables.

Results

IMMUNOASSAY FOR THE PRESENCE OF P2 PROTEIN IN CSF

The figure shows cumulative results obtained with the first 40 cerebrospinal fluid samples examined. These showed that P2-like activity could be classified into three categories: high positive, low positive, or undetectable (curves A, B, and C). As judged by the mean ELISA readings at corresponding cerebrospinal fluid dilutions these distinct curves were widely separated from one another with virtually no overlap of the corresponding standard deviations. The remaining 13 samples assayed fell into one or other of these three categories.

Individual and averaged curves in these three categories were compared with dilution curves obtained with the standard sample of P2 protein (from Professor MacPherson) assayed in doubling dilutions from 1000 ng to 0.5 ng per well (100 μ l sample). By visual comparison with the standard P2 dilution assays, curve A seemed to correspond to a P2-like immuno-reactivity of 125 μ g/l, curve B to a reactivity of 16 μ g/l, and curve C to background readings only—that is, then the P2 was omitted from the reaction well. As the slopes of curves A and B were different from each other, and from the standard curves obtained with doubling dilutions of P2 in buffered saline, the terms "high-positive," "low positive," and "undetectable" were retained in classifying P2 activity in the cerebrospinal fluid for purposes of subsequent clinical and laboratory correlation (tables 2 and 3).

Coefficients of variation (CV) were calculated by averaging individual CV values

Table 2 Associations between P2 activity of cerebrospinal fluid samples and other laboratory data

Criteria compared with P2 state* of cerebrospinal fluid	No of paired comparisons (n)	χ^2 test	p Value
IgG > 0.03 g/l	24	0.06	> 0.5
Total protein > 0.42 g/l	24	0.80	0.37
Albumin > 0.23 g/l	25	2.33	0.13
IgG total protein content: > 13.5%	23	3.46	0.062
Albumin: IgG ratio of $\leq 5:1$	23	4.79	0.029
White cell content in cerebrospinal fluid of $\geq 3 \times 10^6/l$	34	5.04	0.025
Presence of oligoclonal bands on PAGE analysis	25	7.70	0.007
Raised IgG index† (clearance ratio):	24	19.70	< 0.001

*P2 state: high-positive or low/undetectable (see text).

†IgG index: $\frac{\text{IgG (cerebrospinal fluid)}}{\text{IgG (serum)}} \div \frac{\text{Albumin (cerebrospinal fluid)}}{\text{Albumin (serum)}}$

obtained at all dilution points. Within individual cerebrospinal fluid assays the arithmetic mean CV was 7.9% (range 3.6–12.0%); between CSF assays (done at eight month intervals on aliquots of corresponding cerebrospinal fluid samples) the mean CV for two patients was 14.5% (range 3.6 to 37%); while the mean interassay CV for P2 alone was 21.8% (range 9.3–29%; eight assays). It was concluded that categorisation of P2-like activity in cerebrospinal fluid into “high positive”, “low positive,” and “undetectable” was quite compatible with these coefficients of variation.

RELATION BETWEEN P2 REACTIVITY OF CEREBROSPINAL FLUID SAMPLES AND OTHER LABORATORY DATA

Table 2 displays the extent to which P2-like activity of the cerebrospinal fluid samples paralleled other laboratory results of cerebrospinal fluid analysis that were available. For these correlations P2 activity in the cerebrospinal fluid samples was classified simply into two categories: “high positive” and “low or undetectable.” As judged by this analysis P2 activities tended to run parallel with raised cerebrospinal fluid IgG indices ($p < 0.001$), the presence of oligoclonal bands ($p = 0.007$), raised cerebrospinal fluid white cell content ($p = 0.025$) and depressed albumin: IgG ratios ($p = 0.029$), rather than with raised IgG/protein content ($p = 0.062$), or just with concentrations of albumin, IgG, or total protein ($p = 0.13$ to > 0.5). It was concluded that there was a close association between high P2 activity in the cerebrospinal fluid and evidence of intrathecal immunoglobulin synthesis.

RELATION BETWEEN P2 REACTIVITY OF CEREBROSPINAL FLUID SAMPLES AND CLINICAL DIAGNOSIS

Table 3 displays the relation between P2-like

activity of cerebrospinal fluid and the clinical diagnoses assigned with particular reference to definite or probable multiple sclerosis.

Of the 21 patients with definite or probable multiple sclerosis, 16 (76%) had high or low positive P2-like reactivity in their CSF; of the 32 patients with other neurological conditions, only nine (28%) were ELISA positive ($\chi^2 = 5.38$; $p < 0.01$). If the cerebrospinal fluid samples that gave low positive ELISA reactivity were omitted from the analysis, 12 of 13 (92%) patients with high positive ELISA readings had definite or probable multiple sclerosis; on the other hand, only five of 28 (18%) patients with undetectable P2 in their cerebrospinal fluid fell into this diagnostic category ($\chi^2 = 20.3$; $p < 0.001$). If the 12 cerebrospinal fluid samples that gave “low positive” ELISA reactivity were put together with the “undetectable” samples, only nine of 40 (23%) of these patients fell into the definite or probable multiple sclerosis category in contrast to 12 of 13 (92%) patients exhibiting high positive ELISA reactivity for P2 antigen ($\chi^2 = 11.8$; $p < 0.001$).

The population of 53 patients was then divided into two clinical categories (definite and probable multiple sclerosis $n = 21$; other conditions 32 patients), and the presence of high positive P2 reactivity (compared with low or undetectable P2 reactivity) was evaluated for its predictive value in relation to the two clinical categories. By these criteria,⁹ 31 of the 32 patients with conditions other than multiple sclerosis and 12 of the 21 patients with definite or probable multiple sclerosis would have been correctly classified. On this basis, the “true positives” (TP) were 12; the “true negatives” (TN) were 31; the “false negatives” (FN) were nine; and only one patient gave a “false positive” (FP) result in either clinical category. A high positive P2 ELISA value therefore had a specificity $[TN/(TN+FP)]$ of 97% and a sensitivity $[TP/(TP+FN)]$ of 57%. In this population the predictive value of a high positive P2 ELISA for “suspicion” of multiple sclerosis $[(TP/(TP+FP))]$ would have been 92%; that of a low or undetectable value for “exclusion” of multiple sclerosis $[TN/(TN+FN)]$ would have been 78%. It was concluded that the presence of high P2 activity in the cerebrospinal fluid could be of potential value in relation to the diagnosis of multiple sclerosis.

Discussion

This study set out to determine whether immunoreactive P2 protein could be shown in cerebrospinal fluid samples from a group of patients with multiple sclerosis and other neurological disorders (table 1), whether the presence of P2 reactivity was associated with other laboratory abnormalities in the cerebrospinal fluid, and whether this might be particularly relevant to multiple sclerosis. Although samples of normal cerebrospinal fluid were not available, the ELISA technique detected P2 reactivity in 25 of 53 patient samples, and from dilution assays with a stan-

Table 3 Classification of ELISA reactivity for P2-like protein in cerebrospinal fluid samples of 53 patients in this study

Clinical diagnoses* (case Nos)	Category of ELISA reactivity (figure)		
	High positive	Low positive	Undetectable
Definite multiple sclerosis (5)	4	0	1
Probable multiple sclerosis (16)	8	4	4
Other conditions (32)	1	8	23
Totals (53)	13	12	28

*See Table 1.

dard P2 preparation the threshold of detectability was considered to be about 6 µg/l. The observation that P2 activity in the "positive" samples fell into two categories ("high" and "low"), distinct from each other by dose-response criteria (figure), suggests that certain cerebrospinal fluid samples may contain factors which regulate the release or recognition of P2 protein. Within this patient population, a high positive cerebrospinal fluid ELISA test for P2 had an overall efficiency of 75% (or 40/53) in predicting the correct assignment of patients into multiple sclerosis or non-multiple sclerosis categories (table 3).⁹

It was of particular interest that P2 immunoreactivity of the cerebrospinal fluid samples bore a close relation to the cerebrospinal fluid IgG index ("clearance ratio") as well as to other cerebrospinal fluid abnormalities consistent with intrathecal immunoglobulin synthesis rather than with blood cerebrospinal fluid barrier leakage (table 2). As the protein P2 can induce an experimental autoimmune neuritis,⁴ associations between cerebrospinal fluid P2 reactivity, intrathecal immunoglobulin synthesis, and multiple sclerosis corroborate immunological events in the central nervous system as having a role in the process of demyelination. Multifocal central nervous system demyelination is associated with chronic demyelinating peripheral neuropathy,¹⁰ which suggests that immunological recognition of P2 released during central demyelination may well occur.

The availability of standardised assays for myelin breakdown products in the cerebrospinal fluid could be of considerable diagnostic and pathogenetic interest. The present study of immunoreactive P2 is viewed as contributing towards this area.¹¹

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