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Serum antibodies against central nervous system proteins in human demyelinating disease

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SUMMARY

An immunoblotting technique has been used to screen serum samples from patients with demyelinating disease for antibody directed against central nervous system proteins. Antibodies of the IgM, IgG and IgA class directed against one or more of the particulate fraction proteins tubulin, myelin basic protein, 69 K neurofilament protein, glial fibrillary acidic protein, myelin associated glycoprotein or Wolfgram protein were present in 94, 54 and 47%, respectively, of multiple sclerosis sera examined. IgM antibodies against tubulin and myelin basic protein predominated. A similar antibody spectrum was seen in a significant proportion of sera from patients with optic neuritis, subacute sclerosing panencephalitis and motor neurone disease, in which primary or secondary demyelination occurs. Antibodies of all three classes directed against the 169 K and 220 K neurofilament proteins and against some unidentified proteins of human peripheral nerve, kidney, liver, spleen and skeletal muscle were detected in sera from healthy subjects and patients with neurological disease.

Keywords multiple sclerosis immunoblotting tubulin myelin basic protein neurofilament proteins

INTRODUCTION

Using complement fixation assays and immunofluorescent techniques, antibodies of the IgG and IgM classes directed against unidentified antigens in the particulate fraction of white matter or myelin have been reported in a proportion of sera from patients with multiple sclerosis (MS), motor neurone disease and the Guillain-Barré syndrome (see Leibowitz & Hughes, 1983; Walsh & Tourtellotte, 1983). Demyelinating factors and IgG antibodies against myelin basic protein (MBP) and oligodendrocyte antigens have been reported to occur in sera from MS patients, but their presence in sera from many healthy subjects and patients with other neurological diseases suggests that they are unlikely to be directly associated with the pathogenesis of MS.

Immunoblotting is a sensitive technique which has the advantage of enabling individual antigens to be identified. When applied to cerebrospinal fluid or brain extracts from cases of MS or subacute sclerosing panencephalitis (SSPE), no disease related antibodies against central nervous system (CNS) proteins were detected (Newcombe, Glynn & Cuzner, 1982a). However, in the present study we have demonstrated that IgM, IgG and IgA antibodies directed against several CNS proteins are present in the serum of patients with MS and other neurological diseases in which primary or secondary CNS demyelination occurs.

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MATERIALS AND METHODS

Serum samples. Serum samples were obtained from 46 MS patients (average age 39 years, range 21–64 years), eight demyelinating optic neuritis patients (average age 39 years, range 24–53 years), seven SSPE patients (average age 12 years, range 7–18 years), six motor neurone disease patients (average age 55 years, range 50–60 years), 21 patients with other neurological diseases and 16 healthy subjects (average age 31 years, range 23–52 years). None of the patients were receiving immunosuppressive therapy. $F(ab')_2$ fragments of IgG were prepared from sera from two healthy subjects and one MS patient by the method of Poulsen & Hjort (1980). Serum IgM, IgG, IgA and the $F(ab')_2$ fragment preparations were assayed by the method of Mancini, Carbonara & Heremans (1965).

Preparation of samples for electrophoresis. Plaques, plaque borders, macroscopically normal white or grey matter dissected from the brain, spinal cord or optic nerve of MS patients and CNS tissue samples from cases of SSPE and normal control subjects were fractionated by centrifugation into supernatant and particulate fractions which were prepared for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis as described previously (Newcombe, Glynn & Cuzner, 1982b). Particulate fractions of human sciatic nerve, kidney, liver, spleen and skeletal muscle from control subjects were heated with SDS-mercaptoethanol and centrifuged at 10,000 ***g*** for 15 min at room temperature to remove insoluble material. MBP was extracted from myelin (Banik & Davison, 1973) isolated from normal human white matter. CNS cytoskeletal proteins were prepared from normal spinal cord (Chiu, Norton & Fields, 1981) and tubulin was isolated from normal brain (Williams & Lee, 1982). The $F(ab')_2$ fragments were prepared for electrophoresis in order to monitor their purity.

Detection of serum antibodies. The supernatant and particulate fraction proteins were separated by SDS electrophoresis in 9·3% polyacrylamide slab gels and electrophoretically transferred for 2 h onto cellulose nitrate sheets as described previously (Newcombe, Glynn & Cuzner, 1982b). After transfer each cellulose nitrate sheet of four sample lanes was incubated (3 h, 20°C) in 25 ml of 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The sheets were then rinsed in PBS, placed in a heat sealed polythene bag with an appropriate volume of human serum containing 0·5 mg of IgM, 0·5 mg of IgA or 1·0 mg of IgG, made up to a final volume of 4 ml with 3% BSA-PBS and incubated (12 h, 4°C) on a rotator. To achieve these concentrations, serum samples were diluted 4–18, 9–24 and 32–52 times, respectively.

Each sheet was washed for 75 min on a shaking platform in a total volume of 1·25 l of PBS with six changes of PBS, followed by incubation (2 h, 20°C) in 5 ml of 3% BSA-PBS containing 50 μg of rabbit antiserum (Miles) directed against the μ chain of human IgM, the γ chain of IgG or the α chain of IgA. After further washing in PBS each sheet was incubated (2 h, 20°C) with horseradish peroxidase conjugated goat anti-rabbit IgG (Miles) at a dilution of 1 in 125 in 5 ml of 3% BSA-PBS, washed again and stained with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co) and *p*-cresol (Aldrich Chemical Co.) at pH 5·0 (Glynn *et al.*, 1982). Cellulose nitrate sheets were incubated only with second and third antibodies as a control for non-specific staining.

In adsorption experiments, human MBP or tubulin were pre-incubated (3 h, 4°C) on a rotator with selected sera to block specific IgM or IgG antibody, using 250 μg or 500 μg, respectively, of purified protein. In order to determine whether IgM rheumatoid factor was masking IgG antibody directed against CNS proteins, selected sera showing IgM but no IgG reactivity with MBP, tubulin, the 69 K neurofilament protein or glial fibrillary acidic protein were preincubated (1 h, 4°C) on a rotator with 25 mM β-mercaptoethanol.

RESULTS

Antibodies in serum from healthy humans

Serum antibodies directed against CNS proteins were assessed by visual examination of the immunoperoxidase-stained cellulose nitrate sheets. With the exception of very weak staining of

MBP, no CNS protein bands were observed on the sheets which had been incubated only with the second and third antibodies. IgM, IgG and IgA antibody activities directed against a number of CNS particulate fraction proteins were present in all human serum samples (Figs 1 & 2). Two bands of molecular weight 16 K and 22 K were probably histones and three bands of 28 K, 32 K and 33 K were not identified. Two other bands were identified as the 169 K and 220 K neurofilament proteins by incubation of human sera with cellulose nitrate bound cytoskeletal proteins prepared from human spinal cord (Fig. 2). The intensity of staining varied widely between individual serum samples. Purified $F(ab')_2$ preparations isolated from two normal control sera and one MS serum were found to retain antibody activity against the 169 K and 220 K neurofilament proteins.

A small number of unidentified protein bands, some of which may have been histones, were stained in particulate and supernatant fractions of human sciatic nerve, kidney, liver, spleen or skeletal muscle samples after incubation with sera from healthy subjects and patients with neurological diseases. This observation is in agreement with the studies of Daar & Fabre (1981) and Guilbert, Dighiero & Avrameas (1982).

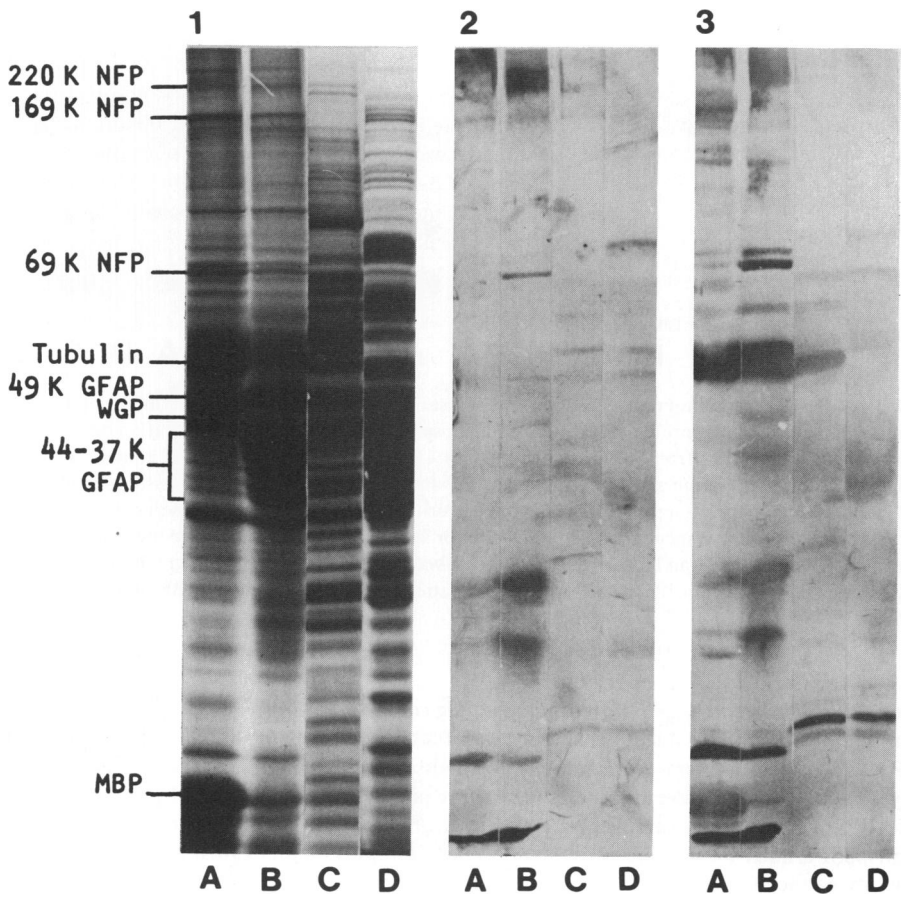

Fig. 1. Block 1: SDS-polyacrylamide (9·3%) slab gel stained with Coomassie brilliant blue. Blocks 2 & 3: cellulose nitrate sheets immunoperoxidase stained for the μ chain of human IgM after incubation with (2) normal control serum and (3) serum from a MS patient, which showed reactivity with tubulin and myelin basic protein (MBP). Lanes A & B: particulate fractions of (A) normal control white matter and (B) a MS plaque. Lanes C & D: supernatant fractions of (C) normal control white matter and (D) a MS plaque. NFP: neurofilament protein; GFAP: glial fibrillary acidic protein; WGP: Wolfgram protein.

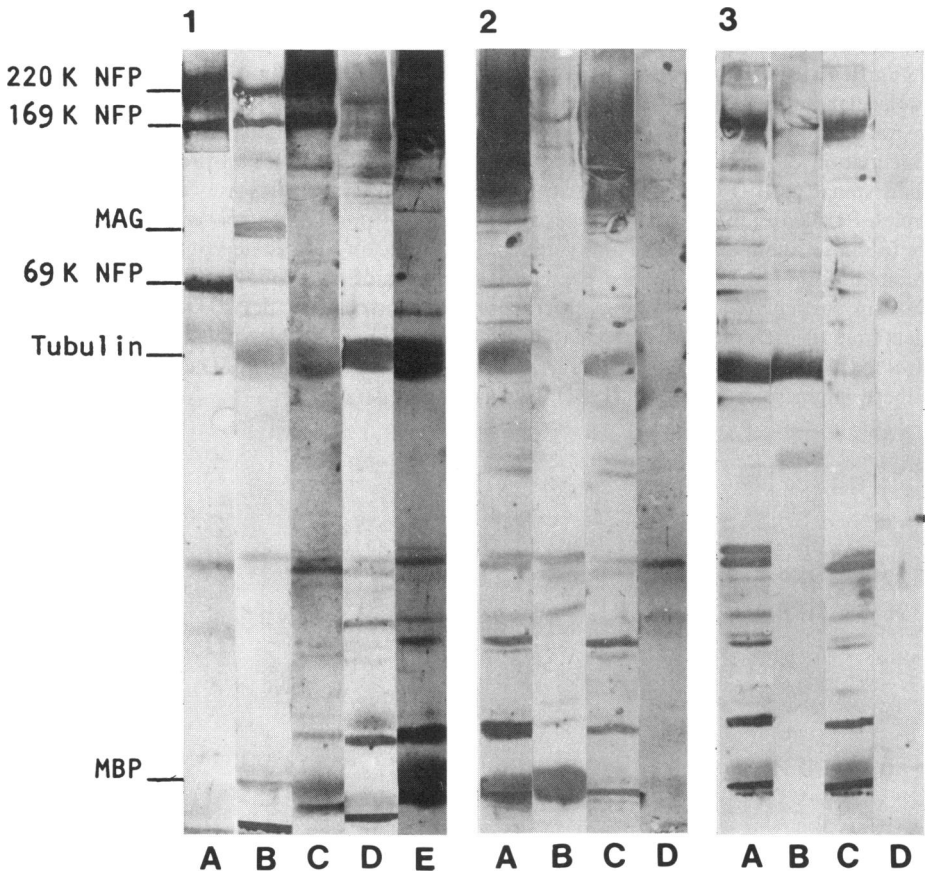

Fig. 2. Blocks 1, 2 & 3: immunoperoxidase stained cellulose nitrate sheets. Lane 1A: IgG staining of the 69 K, 169 K and 220 K neurofilament proteins (NFP) in a human spinal cord cytoskeletal preparation on a sheet which was incubated with serum from a MS patient. Lanes 1B–E: particulate fraction of control white matter incubated with (1B) MS serum and stained to show IgG antibodies against the myelin-associated glycoprotein (MAG) and tubulin; (1C) MS serum (IgA); (1D) optic neuritis serum (IgM); (1E) SSPE serum (IgM). Blocks 2 & 3 were stained for IgM after incubation with MS sera; for 2C and D, MS serum was pre-incubated with human myelin basic protein (MBP), and for 3C & D, MS serum was pre-incubated with human tubulin. Lanes 2A, 2C, 3A, & 3C: particulate fraction of normal human white matter; lanes 2B & D: human MBP; lanes 3B & D: human tubulin.

Serum antibodies against CNS proteins in neurological disease

Examination of cellulose nitrate sheets showed that IgM antibodies directed against tubulin or MBP were present in sera from patients with diseases in which primary or secondary CNS demyelination occurs. These antibodies were more prevelant in sera from MS patients (Figs 1 & 2, Table 1). Although fewer samples were examined, the majority of sera from patients with optic neuritis, SSPE and motor neurone disease also had IgM antibodies against tubulin and MBP. The specificity of the antibody binding to tubulin or MBP was confirmed by adsorption of a selected number of positive sera with the purified proteins (Fig. 2). IgM antibodies against tubulin and MBP were also seen in a small proportion of sera from patients with other neurological diseases (14 and 24%, respectively) and from normal controls (19%).

However, with the exception of four cases of MS, IgG antibodies against tubulin were not detected in any sera (Table 2). With respect to IgG antibodies against MBP, there was no distinction between patients with CNS demyelination or other neurological diseases as an average of 28% of

Table 1. Serum IgM antibodies

Subject	Number of sera	Percentage of positive sera			
		Tubulin	MBP	69K NFP	GFAP
Multiple sclerosis					
Stable	13	77	46	—	8
Acute	18	78	67	28	28
Progressive	15	80	87	53	13
Optic neuritis	8	63	63	—	13
SSPE	7	71	57	—	—
Motor neurone disease	6	83	50	—	—
Cerebrovascular accident	5	—	20	—	20
Alzheimer's disease	4	—	—	—	—
CNS tumours	4	25	25	—	—
Encephalitis	3	—	33	—	—
Peripheral neuropathy	2	50	50	—	—
Myasthenia gravis	3	33	33	—	33
Normal	16	19	19	—	—

MBP: myelin basic protein; NFP: neurofilament protein; GFAP: glial fibrillary acidic protein; SSPE: subacute sclerosing panencephalitis.

Table 2. Serum IgG antibodies

Subject	Number of sera	Percentage of positive sera			
		Tubulin	MBP	69K NFP	GFAP
Multiple sclerosis					
Stable	13	—	8	8	15
Acute	18	6	39	17	6
Progressive	15	20	33	27	13
Optic neuritis	8	—	25	—	—
SSPE	7	—	29	—	—
Motor neurone disease	6	—	33	17	—
Cerebrovascular accident	5	—	—	20	—
Alzheimer's disease	4	—	50	—	—
Encephalitis	3	—	33	—	—
CNS tumours	4	—	25	—	—
Peripheral neuropathy	2	—	50	—	—
Myasthenia gravis	3	—	33	—	33
Normal	16	—	6	—	6

sera in both groups were positive. IgM and IgG antibodies against the 69 K neurofilament protein and glial fibrillary acidic protein were found in 12–29% of MS patients. Antibodies directed against the myelin associated glycoprotein and Wolfgram proteins were detected only in 7 and 3%, respectively, of the MS group; no IgM or IgA antibodies against these proteins were observed. These antigens were identified by incubating sera with purified tubulin, myelin proteins, CNS cytoskeletal proteins and sclerotic MS plaque samples on cellulose nitrate sheets.

IgA antibody activity was examined in sera from 19 cases of MS, five cases of optic neuritis,

three cases of SSPE and three cases of motor neurone disease. Antibodies against tubulin, MBP and the 69 K neurofilament protein were seen only in 16, 21 and 21%, respectively, of the MS group. IgA antibodies against GFAP were seen in two MS sera, one optic neuritis serum and one SSPE serum. No IgA activity with any of these four antigens were seen with sera from three cerebrovascular accident patients, three CNS tumour patients, two peripheral neuropathy patients or with eight healthy subjects. No correlation between serum IgM, IgG or IgA levels and antibody reactivity with CNS proteins was observed. With the exception of tubulin, no antibody activity specifically directed against any CNS supernatant fraction proteins was detected.

DISCUSSION

Antibodies of the IgM isotype directed against tubulin and MBP were found to occur with highest frequency in sera from patients with the inflammatory primary demyelinating diseases MS and optic neuritis. These antibodies were also present in a high proportion of sera from patients with SSPE and motor neurone disease, in which secondary demyelination occurs. Antibodies against the 69 K neurofilament, glial fibrillary acidic, Wolfgram proteins and the myelin associated glycoprotein were found almost exclusively in sera from MS patients. All of these six proteins have been shown to undergo quantitative changes in demyelinated and macroscopically normal white matter in MS and SSPE (Newcombe *et al.*, 1980; Newcombe, Glynn & Cuzner, 1982b). In all human sera examined in the present study, antibodies against the two high molecular weight neurofilament proteins and a number of other CNS proteins were detected; the intensity of staining varied widely between individual serum samples. The specificity of IgG antibody binding to the 169 K and 220 K neurofilament proteins was validated by purification of $F(ab')_2$ fragments. IgG antibodies against neurofilaments have been reported in sera from 10% of control subjects in an immunofluorescence study in which high titres were detected in sera from patients with CNS degenerative diseases (Sotelo, Gibbs & Gajdusek, 1980).

In MS the specificity of intrathecally synthesized immunoglobulin is largely unknown. In a previous study employing immunoblotting, we were unable to detect any IgG or IgM antibodies againt CNS proteins in the cerebrospinal fluid in MS or SSPE (Newcombe, Glynn & Cuzner, 1982a). In both MS and SSPE, serum antibodies entering the cerebrospinal fluid would be diluted by intrathecally synthesized IgG. Screening for IgG antibodies in cerebrospinal fluid was carried out with only 200 μg of IgG, in contrast to 1 mg of serum IgG used in the present study, at which level cerebrospinal fluid antibody reactivity might have escaped detection. However, both anti-MBP and anti-tubulin activity were still clearly apparent in positive MS serum samples tested at 200 μg of IgG.

Fragments of the CNS specific MBP, which is very labile during demyelination, have been detected in the cerebrospinal fluid and occasionally in serum of patients with demyelinating disease, and low levels of serum IgG antibodies against MBP have been reported to occur in some MS patients and healthy subjects (Paterson *et al.*, 1981). The presence of IgM anti-MBP antibodies in more than 67% of sera from MS patients with acute or progressive disease may reflect on-going demyelination, while the anti-MBP antibody of IgG isotype found in most neurological diseases may be indicative of the general lability and immunogenicity of MBP (Table 2).

Tubulin occurs in almost all cell types, the highest levels being found in neurons. Microtubules are also present in oligodendrocytes and the cytoplasmic loops of myelin (Peters, Palay & Webster, 1976) and tubulin may be an integral component of rodent brain myelin (Gozes & Richter-Landsberg, 1978; de Néchaud *et al.*, 1983). It is therefore possible that in demyelinating conditions tubulin is released from degenerating oligodendrocytes and myelin or even axons. In contrast to a previous study (Guilbert, Dighiero & Avrameas, 1982), IgG anti-tubulin antibodies were not detected in any normal control sera.

Antibodies to cytoskeletal structures are associated with most organ specific autoimmune diseases. In the present study the major isotype of the anti-tubulin antibody was found to be IgM. The possibility that rheumatoid factor was responsible for this observation was excluded by a comparison of serum samples screened in the presence and absence of β-mercaptoethanol. IgM

autoantibodies against intermediate filaments have been reported in infectious measles, chicken pox and mumps (Toh *et al.*, 1979), in acute viral hepatitis (Pederson *et al.*, 1981), in rheumatoid arthritis (Kurki, Helve & Virtanen, 1983) and in multiple sclerosis (McMillan & Haire, 1979), although the latter finding has not been confirmed (Hyypiä *et al.*, 1982). Increased titres of IgM or IgG antibodies against tubulin have been found in patients with infectious mononucleosis (Mead, Cowin & Whitehouse, 1980) and autoimmune thyroid disorders (Rousset *et al.*, 1983), respectively. In chronic viral and autoimmune diseases antibodies against cytoskeletal elements may occur as a response to pathological tissue damage, and these antibodies may themselves contribute to tissue damage (Grabar, 1975). The occurrence of IgM antibodies against tubulin in about 80% of stable, acute and progressive MS patients suggests either the presence of a constant antigenic stimulus or polyclonal activation.

Immunogenic fragments of CNS particulate fraction proteins may escape into the peripheral circulation via a damaged blood brain barrier, resulting in the development of an extrathecal B-cell response. As fairly high levels of serum immunoglobulins, approximately 1/40 dilution of serum for IgG, were used in conjunction with a sensitive antibody detection technique, these antibodies probably represent only a small fraction of the total serum immunoglobulins. However, they may gain access to the CNS parenchyma through a damaged blood brain barrier where they could contribute to the immunopathology of demyelination.

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