Antibodies to a subpopulation of glial cells and a 66 kDa developmental protein in patients with paraneoplastic neurological syndromes

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Abstract

Background—Paraneoplastic neurological syndromes (PNS) are inflammatory disorders that probably depend on autoimmune processes. Several autoantibodies (anti-Hu, anti-Ri, and anti-Yo) have been characterised in PNS and proved to be helpful in the diagnosis. However, these do not account for all the cases and the possibility that other types of antibodies could be detected was investigated.

Methods and results—Of 45 patients with PNS whose serum was probed on paraformaldehyde fixed rat brain sections, 11 patients were identified whose serum samples recognised a cytoplasmic antigen in a subpopulation of glial cells in the white matter of adult rat brainstem, cerebellum, and spinal cord that were double labelled with a monoclonal antibody specific for oligodendrocytes. All serum samples reacted with a 66 kDa protein of newborn rat brain on western blot analysis. These antibodies were designated as anti-CV2 antibodies. Only one of the 11 patients had one of the well characterised autoantibodies (anti-Hu). Five patients had cerebellar degeneration, three had limbic encephalitis, two had encephalomyelitis, and one had Lambert-Eaton myasthenic syndrome. The tumours were small cell lung cancer or undifferentiated mediastinal cancer in seven patients, uterine sarcoma in two, and malignant thymoma in two. Among 1061 control serum samples, only two patients had anti-CV2 antibodies. One had small cell lung cancer and the other malignant thymoma.

Conclusions—The detection of anti-CV2 antibodies in patients with neurological disorders should be considered as an indication of the presence of an occult cancer.

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Paraneoplastic neurological syndromes (PNS) are rare inflammatory disorders of the central and peripheral nervous system[1] that possibly depend on autoimmune processes directed against antigens shared between the tumour and the nervous system.[2] Several autoantibodies correlated with the neurological disorders and the tumours have been characterised in PNS and proved to be helpful in the diagnosis.[3 4] Thus most patients with the encephalomyelitis-sensory neuropathy complex associated with anti-Hu (or ANNA-1) antibodies have small cell lung cancer.[5 6] Those with paraneoplastic cerebellar degeneration and anti-Yo (or PCA-1) antibodies have gynaecological tumours.[7 8] Most of the patients with anti-Ri (or ANNA-2) antibodies and opsoclonus/ataxia have breast cancers.[9] However, these autoantibodies do not account for all patients with PNS.[10–12] Some of the patients negative for these antibodies could actually harbour autoantibodies that could be identified by other methods, in particular, by using fixing procedures which improve antigen preservation.

Recently, by using paraformaldehyde fixed rat brain sections, we described two patients with PNS and antibodies that reacted with a cytoplasmic antigen in a subpopulation of glial cells in the brainstem and cerebellar white matter.[13 14] To investigate the possibility that these antibodies could be relevent for the diagnosis of cancer in patients with neurological disorders suspected to be paraneoplastic, we screened 45 patients with PNS, 128 patients with tumour, and 933 control patients of various types for the presence of these antibodies.

Materials and methods

PATIENTS

Serum and CSF from 45 patients with PNS were collected and stored at −20°C until use. Control serum samples were obtained from 900 patients with various inflammatory or non-inflammatory neurological diseases without cancer, 128 patients with cancer and no PNS (37 small cell lung carcinoma, 14 other lung cancer, 25 intestinal adenocarcinoma, two lymphoma, 29 gynaecological cancer (breast and ovary), seven various carcinomas, 14 malignant thymoma with myasthenia gravis) and 33 normal control subjects. Control CSF samples were obtained from 30 patients with various neurological syndromes. As positive controls for the identification of anti-Hu, anti-Ri, and anti-Yo antibodies, we used three serum samples obtained from Dr Josep Dalmau (Memorial Sloan Kettering Cancer Center, New York, USA).

TISSUE PREPARATION FOR IMMUNOHISTOCHEMISTRY

Adult rat, newborn rat, and day 22 rat embryonic tissues (E22) were obtained from OFA

rats (IFFA CREDO, France). Human brain samples were obtained from a 71 year old woman without neurological disease and from a 24 week embryo after a spontaneous abortion.

Adult rats were anaesthetised with Nembutal and killed by intracardiac perfusion of 4% paraformaldehyde and 0·2% picric acid diluted in phosphate buffer (0·1 M, pH 7·4). Tissues were postfixed for 24 hours at 4°C in the same solution, rinsed in phosphate buffer for 12 hours, cryoprotected in a solution of 25% sucrose in phosphate buffer, rapidly frozen in isopentane chilled at −60°C in liquid nitrogen, and stored at −80°C. Human brain, rat embryo, and newborn rat brain were fixed at 4°C for four days by immersion in 4% paraformaldehyde and 0·2% picric acid diluted in phosphate buffer (0·1 M, pH 7·4) and then cryoprotected as described above.

IMMUNOCYTOCHEMISTRY
Immunofluorescence
To screen the serum samples we used an indirect immunofluorescence technique. Frozen sections (12 μm) were cut on a cryostat, mounted on gelatin-covered slides, treated for two hours in phosphate buffered saline (PBS) and 1% bovine serum albumin (BSA) with 0·1% Triton X 100, and incubated for 12 hours with the patient's serum in PBS-1% BSA at room temperature (diluted 1/100 on adult rat brain sections and 1/1000 on E22 rat embryo sections). After three washes with PBS-1% BSA-0·1% Triton X 100, the sections were incubated for two hours with 1/100 diluted fluorescein-conjugated rabbit anti-human antiserum (Dakopatts, Denmark) in PBS-1% BSA. After washing in PBS, the slides were mounted in moviol and examined with an Axiophot Zeiss microscope. Control sections were incubated with either fluorescein conjugated antihuman IgG antiserum alone, patient's serum alone, or control serum samples and the fluorescein conjugated antibody at the same dilution. Positive serum samples were then tested to establish end point dilution.

Immunoperoxidase
To confirm the positivity of the serum samples detected by screening with immunofluorescence, we used both indirect and direct immunoperoxidase labelling after biotinylation of the patient's serum IgG.

Indirect immunoperoxidase method—Frozen tissue sections fixed by paraformaldehyde were sequentially incubated with 0·3% hydrogen peroxide (to destroy tissue peroxydase activity) and 10% normal rabbit serum (to prevent non-specific binding of rabbit IgG) or 1% BSA. After incubation for 12 hours with patient's serum diluted 1/1000 and washes, the sections were incubated for two hours with biotinylated rabbit antihuman IgG antiserum (Jackson, Baltimore, USA) diluted 1/1000 in PBS-1% BSA. Bound human IgG was vizualised by incubation with avidin-biotin-peroxidase (Vectastain ABC complex, Vector) and developed with 0·05% diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO).

Control sections were obtained with 15 serum samples from patients without PNS using the same protocol.

Patient's IgG biotinylation—IgG was prepared from the serum samples of four patients (3, 6, 7, and 8) and conjugated with biotin as described by Furneaux *et al.*[15] After treatment with hydrogen peroxide and incubation with 10% normal human serum, the sections were incubated with biotinylated IgG (1 μg/ml) for 12 hours at room temperature. Bound human IgG was vizualised by incubation with avidin-biotin-peroxidase (Vectastain ABC complex, Vector) as described above. Control sections were obtained with biotinylated IgG from a healthy patient.

Double labelling
To determine whether the CV2 reactive cells were oligodendrocytes, we used a double labelling with Rip, a mouse monoclonal IgG1 antibody specific for oligodendroglial cells provided by Dr B Zalc (INSERM U 289, Paris, France).[16]

Double labelling was performed on white matter of adult rat spinal cord. Longitudinal sections of spinal cord were incubated overnight at room temperature, simultaneously with anti-CV2 antiserum (diluted 1/300) and with Rip antibody (diluted 1/20). Then, sections were stained with appropriate fluorescein conjugated antiserum (anti-CV2) and Texas red conjugated antiserum (Rip) by the method previously described.

BIOCHEMICAL AND IMMUNOCHEMICAL METHODS
Subcellular fractionation of brain
Samples of fresh rat or postmortem human brain were homogenised with sodium phosphate buffer (5 mM, pH 7) containing protease inhibitors (1/1000 leupeptine, 1/1000 pepstatine, 2·5/1000 aprotinine (Sigma)), and 0·3 M sucrose, using a hand held teflon glass potter homogeniser (clearance 0·1 mm) and centrifuged for 10 minutes at 1000 g to remove nuclei and large tissue fragments. In some experiments, the supernatant (S1) was then centrifuged for one hour at 6000 g. The pellet (P2) contained a crude membrane fraction. The supernatant (S2) was then centrifuged for 30 minutes at 100 000 g to separate soluble proteins (S3) from a crude microsome fraction (P3). The fractions P2, S3, and P3 were then diluted with buffer containing protease inhibitors to adjust the protein concentration to 1 mg/ml and stored at −20°C.

Immunoblotting
Each fraction was diluted (1:1; v/v) with 250 mM Tris HCl buffer, pH 6·8 containing 2% SDS, 0·01% bromophenol blue, 20 mM dithiothreitol, and 10% glycerol. For each experiment, 18 μg proteins per lane were separated on 12% polyacrylamide gels by electrophoresis and electrically transferred to PVDF membranes (Immobilon membranes, Millipore). Strips of PVDF were first incubated for 30 minutes with 1% BSA and 0·1%

Tween 20 in Tris buffered saline (TBS) to saturate non-specific protein binding sites and then for 12 hours with the patient's serum diluted in the same solution. After three washes with TBS-Tween 20 (0·1% v/v) and 0·5% BSA, bound antibodies were disclosed by incubating the PVDF strips for two hours with biotinylated rabbit antihuman IgG antiserum (Jackson; Baltimore) diluted 1/1000 in TBS-1% BSA, rinsed for 3 × 15 minutes in TBS, and then incubated for one hour with streptavidin-peroxidase (Jackson, Baltimore) 1/2000 in the same buffer. The colour reaction was developed with diaminobenzidine tetrahydrochloride. Apparent molecular weights were estimated from standards (Pharmacia). The presence of anti-Hu, anti-Ri, and anti-Yo antibodies has been confirmed separately by appropriate methods.[3]

Immunoprecipitation

Twenty microlitres of the serum of patients 2 and 5 and of a control serum were incubated for one hour at 4°C with 1 ml S3 fraction containing 1 mg protein. Protein A sepharose (20 mg) was suspended in 200 μl TBS, added to the mixture, and gently agitated at 4°C. After one hour, a cushion of 100 μl 1M sucrose was applied to the bottom of the reaction tubes which were then centrifuged for five minutes at 10 000 g. The upper fraction was discarded along with most of the sucrose and the pellet containing the protein A sepharose was washed three times in 1 ml TBS and centrifuged at 10 000 g for five minutes. As much of the liquid fraction as possible was removed after each wash using a fine pipette. Finally, the protein A sepharose was resuspended in 100 μl SDS-Page sample buffer containing DTT and incubated for 15 minutes at room temperature. Sucrose (100 ml, 1 M was added to the bottom of the reaction tube which was then centrifuged for five minutes at 10 000 g.

Denatured eluted proteins were recovered in the upper phase and sepharose in the lower. Samples of the superior phase were then analysed by western blot as described above.

Results

Among the 45 serum samples of patients with PNS, 17 did not have detectable antinervous system antibodies, 11 had antineuronal nuclei antibodies (eight corresponded to anti-Hu antibodies by western blot and none to anti-Ri antibodies), seven had antibodies against Purkinje cells (five corresponded to anti-Yo antibodies by western blot). Furthermore, 11 patients had antibodies that reacted with a cytoplasmic antigen in a subpopulation of glial cells in the white matter according to a highly specific reproductible pattern characteristic of antibodies that we have designated as anti-CV2. Among them, 10 were negative for anti-Hu, anti-Ri, and anti-Yo antibodies and one had anti-Hu antibodies. The same cytoplasmic glial labelling was also found with the CSF available from seven of these 11 patients. Among the 1061 control serum samples (from normal controls, patients with various neurological diseases, and patients with cancers without PNS) only two serum samples labelled cells in the white matter with a pattern identical to that of the 11 aforementioned patients. One of them had metastatic small cell lung cancer and the other malignant thymoma.

CLINICAL SYNDROMES

Seven of the 11 patients with PNS and anti-CV2 antibodies were examined by either JH or JCA. The clinical records of the others were obtained from their physicians. Patients 1 and 2 have been reported previously.[13 14] The table summarises the data concerning the clinical symptoms, the results of CSF examination,

Clinical data, CSF study and serum and CSF anti-CV2 titres in the 11 patients with paraneoplastic neurological syndromes

Patient no	Age/sex	Delay* (months)	Neurological syndrome	Tumour	WCC (/mm³) (normal < 2)	Protein (mg/100ml) (normal < 45)	IgG (normal < 17)	oligoclonal bands	Antibody titre (immunofluorescence) Serum	CSF
1	66/F	−24	Cerebellar ataxia Sensory motor neuropathy Uveitis and retinopathy	Undifferentiated carcinoma	40	53	15%	yes	1/15 000	1/300
2	58/F	−1	Limbic encephalitis	Malignant lymphoepithelial thymoma	36	30	ND	ND	1/15 000	1/100
3	49/M	−1	Limbic encephalitis	Small cell lung carcinoma	3	62	ND	ND	1/1000	1/100
4	53/M	+7	Myasthenia gravis Encephalopathy	Malignant lymphoepithelial thymoma	7	30	ND	ND	1/5000	ND
5†	69/M	−6	Frontal dementia Cerebellar ataxia Sensory neuropathy	Small cell lung carcinoma	50	150	20%	No	1/100 000	1/2000
6	70/F	+3	Cerebellar ataxia Sensory neuropathy Loss of vision	Uterine sarcoma	8	38	23%	Yes	1/10 000	1/100
7	60/M	−20	Limbic encephalitis Sensory neuropathy	Small cell lung carcinoma	ND	ND	ND	ND	1/10 000	ND
8	69/M	−5	Cerebellar ataxia Sensory neuropathy	Small cell lung carcinoma	200	200	ND	Yes	1/20 000	1/2000
9‡	61/M	−2	Lambert-Eaton myasthenic syndrome	Small cell lung carcinoma	< 2	90	12%	No	1/10 000	1/1000
10	72/F	−4	Cerebellar ataxia	Cervix uterus sarcoma	ND	ND	ND	ND	1/5000	ND
11	65/M	−1	Cerebellar ataxia	Small cell lung carcinoma	50	30	25%	Yes	1/15000	ND

*Negative numbers indicate that the neurological syndrome preceded the diagnosis of tumour.
†Associated with an anti-Hu antibody (1/60 000).
‡Associated with an anti VGCC-antibody.
ND = not done.
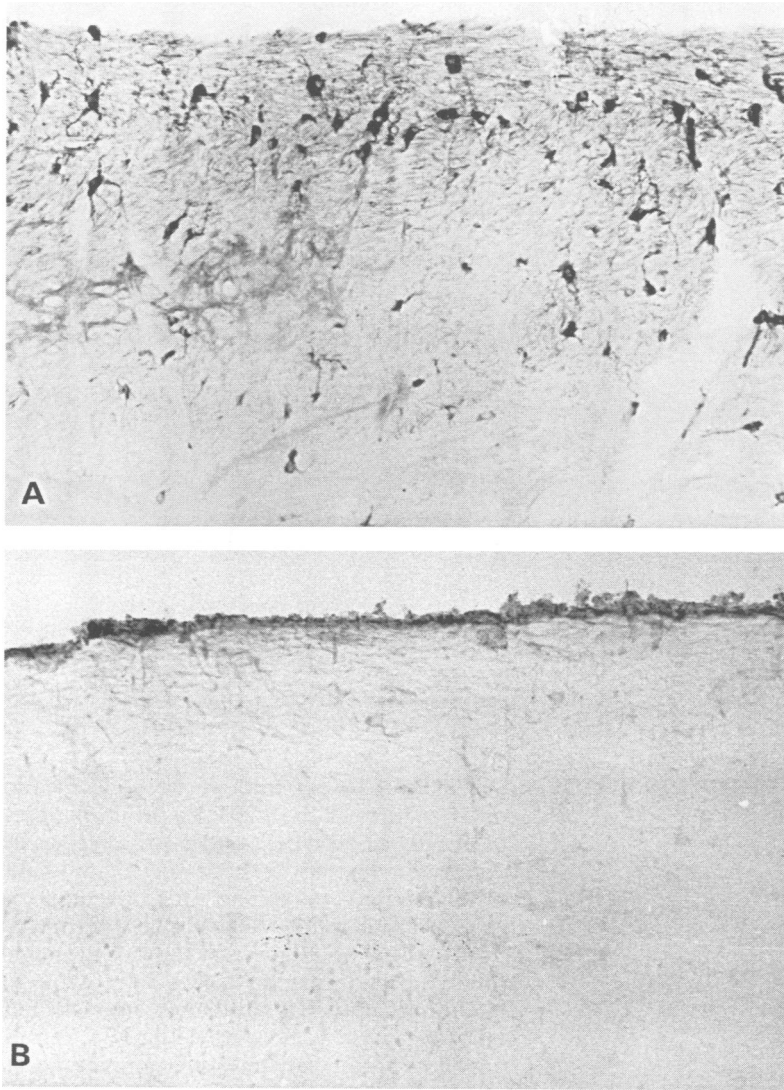
Figure 1   Indirect immunoperoxidase on a six week old rat brainstem (ventral spinocerebellar tract). (A) serum from a patient with anti-CV2 antibodies (patient 1 at 1/1000 dilution) (original magnification × 200). Anti-CV2 antibodies react with the cytoplasm and processes of glial cells in the white matter. (B) serum from a control patient without cancer or neurological disease at 1/100 dilution (original magnification × 200).

Eaton myasthenic syndrome only. In addition to symptoms of CNS dysfunction, sensory or sensory-motor peripheral neuropathy was present in five patients (1, 5, 6, 7, and 8).

Samples were obtained at necropsy for three patients (1, 5, and 8). Two patients (1 and 8) had severe Purkinje cell loss with slight but widespread inflammatory cell reaction and one (patient 5) showed a diffuse inflammatory encephalomyelitis. In these three patients, no primary demyelinating lesions were found in the brain.

IMMUNOHISTOCHEMISTRY ON RAT AND HUMAN BRAIN

On adult rat brain, serum samples and CSF gave identical patterns by indirect immunofluorescence and immunoperoxidase methods. Anti-CV2 antibodies labelled the cytoplasm of cells in the white matter and their processes that sometimes surrounded myelinated fibres homogeneously (figs 1 and 2). Cell nuclei were always negative. The morphology of labelled cells and their clustering like strings of beads along myelinated tracts suggested that they were oligodendrocytes. Double labelling showed that cells positive with anti-CV2 antibodies were also stained with Rip, a mouse monoclonal antibody specific for oligodendrocytes (fig 3),[16] confirming this hypothesis.

Cells stained with anti-CV2 antibodies were numerous in the spinal tract of the trigeminal nerve, the pyramidal tract, and the medial longitudinal fasciculus of the brainstem (fig 4). Immunolabelled cells were also numerous in the cerebellar peduncles and cerebellar white matter, and in all the spinal cord tracts. Labelling obtained with the IgG fraction of four serum samples after biotinylation was identical (fig 2). No labelling of oligodendrocytes occurred in the corpus callosum and cerebral cortex.

The pattern of staining obtained with anti-CV2 antiserum was clearly different from that with anti-Hu antibodies which labelled the nuclei of neurons and at low dilution the nuclei of glial cells (data not shown). By immunofluorescence, the limiting dilution of anti-CV2 serum antibodies from the 11 patients with PNS and the two positive patients with cancer and no PNS ranged from 1/1000 to 1/100 000. The anti-CV2 antibody titre from the seven CSF samples available ranged from 1/100 to 1/2000. In the patient who also had anti-Hu antibodies (patient 5), serum anti-Hu antibody titre was 1/60 000 by our immunohistochemistry technique on rat brain whereas it was 1/100 000 for anti-CV2 antibodies. In addition, four out of the 11 serum samples of patients with anti-CV2 antibodies and PNS labelled cells in the subgranular layer of the dentate gyrus of the hippocampus at 1/1000 to 1/10000 dilution (patient 1, 2, 7, and 11). At low dilution (1/100) some serum samples with anti-CV2 antibodies labelled the cytoplasm of neurons in the thalamus, the cortex, or the hippocampus whereas no labelling was seen on adult rat liver, kidney, testes, ovary, spleen, and adrenal gland.

and the associated tumours. In eight patients (1, 2, 3, 5, 7, 8, 9, and 11), the neurological syndromes preceded the discovery of the tumour by one to 24 months. In the three patients (4, 6, and 10) in whom neurological symptoms occurred after the diagnosis of the tumour, the tumour was not in remission at that time. Seven patients had small cell lung cancer, two had uterine sarcoma, and two had lymphoepithelial malignant thymoma.

In three patients (2, 3, and 7), the main symptoms consisted of epilepsy and recent memory impairment which were highly suggestive of limbic encephalitis. In two of them (patients 2 and 3), brain MRI showed hippocampal high signals on T2 weighted sequences. Five patients (1, 6, 8, 10, 11), had a prominent severe cerebellar ataxia. Two of them also had uveitis or retinopathy (patients 1 and 6). Patient 4 rapidly became bedridden due to a severe encephalopathy with epilepsy, mutism, apragmatism, and stiffness. Patient 5 had dementia, facial dyskinesia, and cerebellar ataxia. The last patient (9) had Lambert-

Figure 2 (A) Immunoperoxidase on a six week old rat cerebellum with biotinylated IgG of patient 7 (1 µg/ml) (original magnification × 400). m = Molecular layer; g = granular layer; wm = white matter. (B): Immunoperoxidase on a six week old rat brainstem (spinal tract of the trigeminal nerve) with biotinylated IgG of patient 7 (1 µg/ml) (× 1000). Some positive processes surround an axon (arrow) suggesting that these cells are oligodendrocytes.
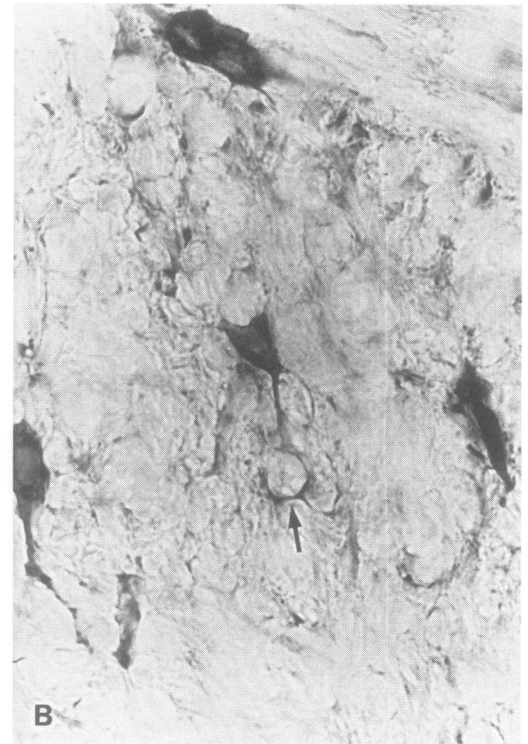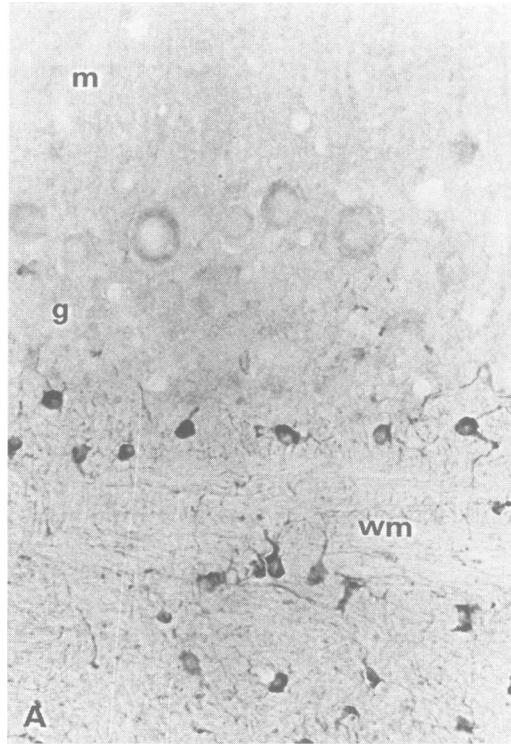
Figure 3   Longitudinal section of white matter of the rat spinal cord (× 1000). (A) Interfascicular cells visualised by indirect immunostaining with Rip monoclonal mouse antibody. (B) The same section double labelled with the serum of a patient containing anti-CV2 antibodies (serum from patient 7, diluted 1/300).

In developing rat embryo, from embryonic day 12 to birth all the anti-CV2 serum samples specifically labelled cells and their processes in postmitotic regions of both the central and peripheral nervous system (figs 5A and 6). Neither proliferating cells in the ventricular zones and the outer granular layer of the cerebellum nor non-neural tissues were stained. By contrast, anti-Hu antibodies only labelled cell bodies in the same regions (fig 5C). Serum from 30 control patients gave no staining at 1/100 dilution (fig 5B). After birth, the staining progressively decreased to a pattern identical to that in adult brain.

We tested seven serum samples of the 11 patients with PNS and anti-CV2 antibodies (patients 1 to 7) by indirect immunoperoxidase on adult human brain. Four serum samples (from patients 1, 2, 5, and 7) which stained rat brain at the highest dilutions, labelled glial cells in the white matter of the adult human brainstem and cerebellum (at a dilution of 1/1000) with a pattern similar to that in the rat brain (fig 7). The other serum samples were negative as were the 30 control samples.

IMMUNOBLOT
Each serum sample was tested on different cellular fractions of adult and newborn rat brain. A principal band of 66 kD apparent molecular weight was recognised at 1/100 dilution by all the 11 serum samples and the seven available CSF samples of patients with PNS and the serum samples of the two positive patients with cancer and no PNS. This protein was particularly enriched in the S3 subcellular fraction of newborn rat brain. Thus this fraction was used to estimate the titre of the serum samples. Among all serum samples only two (patients 6 and 10) were negative at 1/500 dilution (fig 8) and only three serum samples
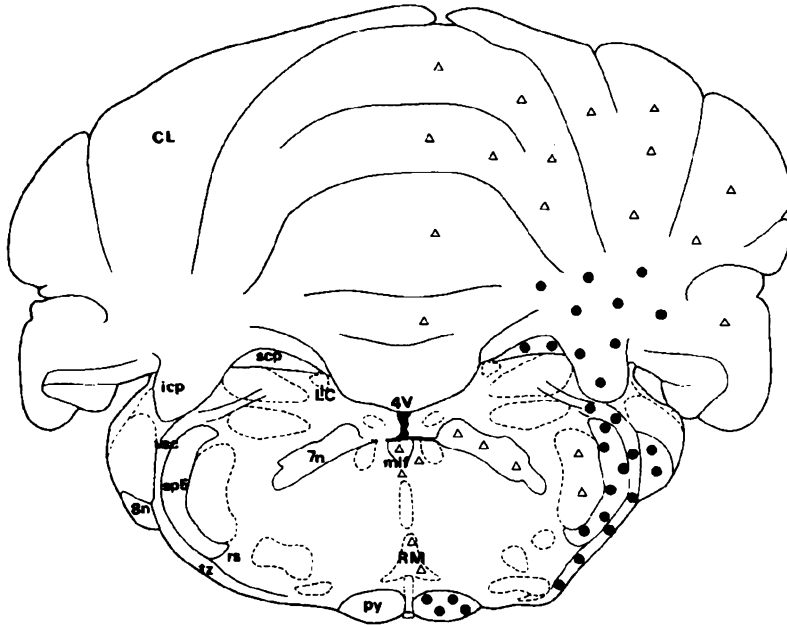
Figure 4 Schematic distribution of CV2 positive cells on a section of rat brainstem.
● *High density of CV2 positive cells and △ low density of CV2 positive cells.*
4V = Fourth ventricle; 7n = facial nerve; 8n = vestibulocochlear nerve; CL = cerebellar lobules; icp = inferior cerebellar peduncle; LC = locus coerulus; mlf = medial longitudinal fasciculus; py = pyramidal tract; RM = raphe magnus nuclei; rs = rubrospinal tract; scp = superior cerebellar peduncle; sp5 = spinal tract trigeminal nerve; tz = trapezoid body; vsc = ventral spinocerebellar tract.

remained positive at 1/1000 dilution (patients 2, 5, and the patient with small cell lung cancer and no PNS). The 66 kDa band was not stained by any of the serum samples in the S3 fraction of rat liver. None of the 150 control serum samples (diluted at 1/100) reacted with a 66 kDa band from the S3 fraction of new born rat brain.

Western blots of the S3 soluble fraction of adult rat brain showed lower levels of expression of the 66 kDa protein than in newborn rat brain and three serum samples gave no staining (patients 6, 8, and 10).

In the S3 fraction of human embryo brain, nine of the 11 serum samples of patients with PNS and the serum samples of the two positive patients with cancer and no PNS labelled a 66 kD band at 1/100 and 1/500 dilutions. Patients 6 and 10 were negative. With the S3 fraction of adult human brain, six serum samples (patients 2, 4, 5, 7, 9, and 11) reacted with a 66 kDa band with variable staining intensities whereas the other serum samples were negative.

IMMUNOPRECIPITATION
To test whether these serum samples recognised the same antigen, we immunoprecipitated the S3 soluble fraction of newborn rat
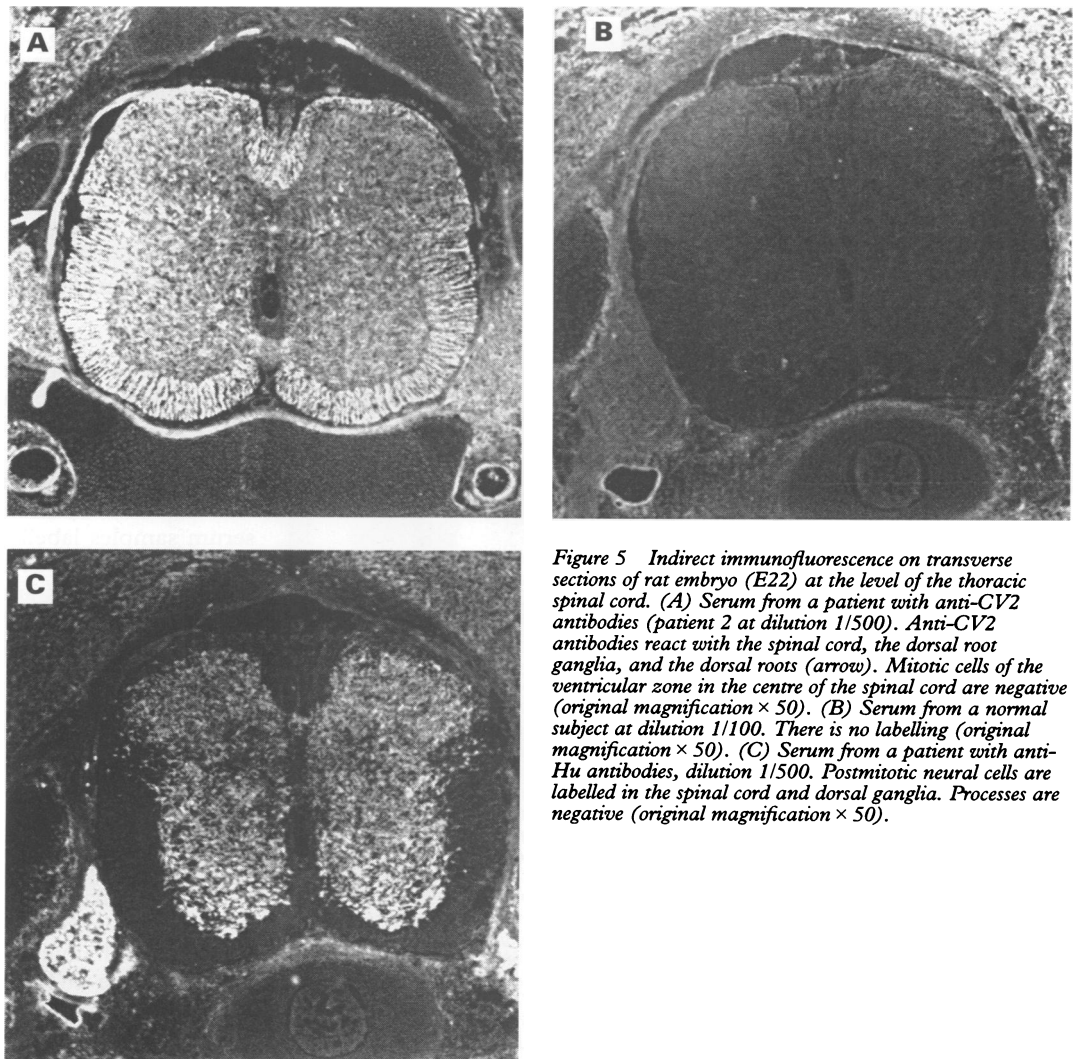
Figure 5 Indirect immunofluorescence on transverse sections of rat embryo (E22) at the level of the thoracic spinal cord. (A) Serum from a patient with anti-CV2 antibodies (patient 2 at dilution 1/500). Anti-CV2 antibodies react with the spinal cord, the dorsal root ganglia, and the dorsal roots (arrow). Mitotic cells of the ventricular zone in the centre of the spinal cord are negative (original magnification × 50). (B) Serum from a normal subject at dilution 1/100. There is no labelling (original magnification × 50). (C) Serum from a patient with anti-Hu antibodies, dilution 1/500. Postmitotic neural cells are labelled in the spinal cord and dorsal ganglia. Processes are negative (original magnification × 50).
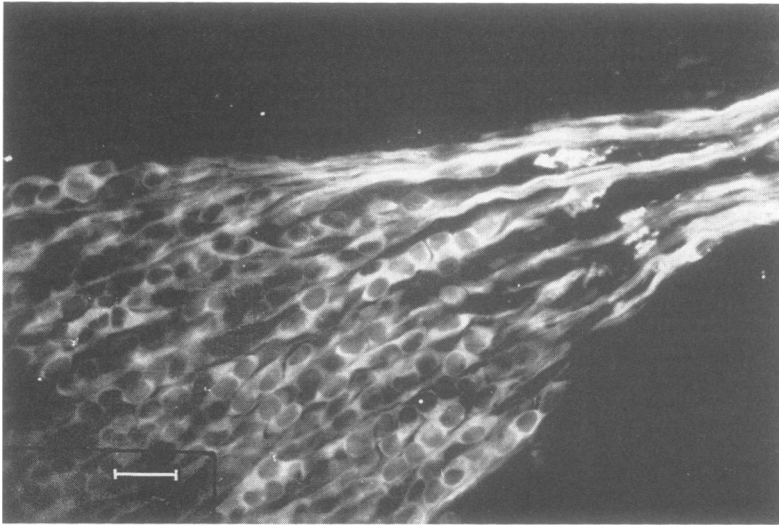
Figure 6   Dorsal root ganglion and peripheral nerve axons in E22 rat embryo stained with serum from a patient with anti-CV2 antibodies (patient 1 diluted 1/500). Cell bodies and processes are labelled (bar = 30 μm).
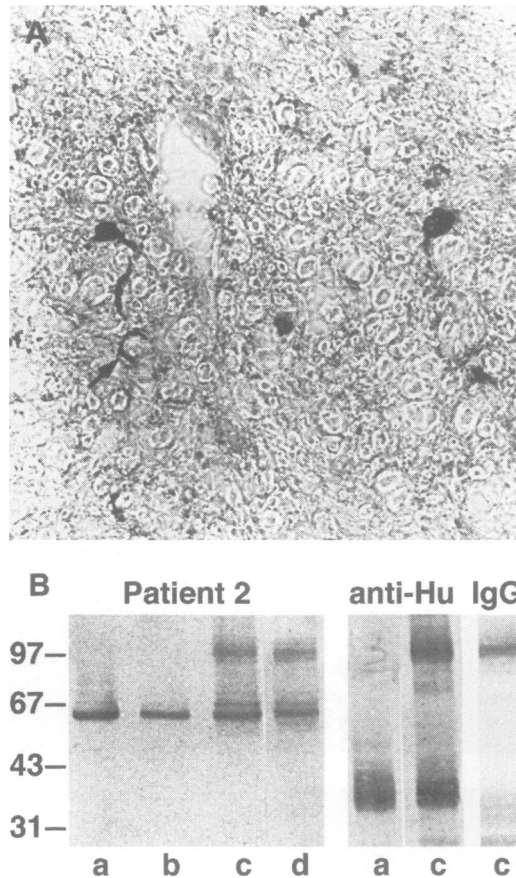

Figure 7   (A) Indirect immunoperoxidase on human brainstem (pyramidal tract) with the serum from a patient with anti-CV2 antibodies (patient 2) at 1/1000 dilution. Glial cells are stained. Some positive processes surround an axon (arrow) suggesting that positive cells are oligodendrocytes (original magnification × 400). (B) Western blots with three different serum samples (6 μg protein per lane): serum from a patient with anti-CV2 antibodies diluted 1/500 (patient 2), serum from a patient with anti-Hu antibodies diluted 1/500 (anti-Hu) and an antihuman IgG rabbit antiserum diluted 1/1000 (IgG). a = S3 fraction of new born rat brain; b = S3 fraction of adult rat brain; c = S3 fraction of human embryonic brain (week 24); d = S3 fraction of adult human brainstem. The serum of patient 2 recognised a 66 kDa band in both human and rat brain extracts. The serum with anti-Hu antibodies and the antihuman IgG rabbit antiserum are negative for the 66 kDa band. Bands of about 97 kDa on human extracts correspond to endogenous IgGs.

brain with the serum samples from patients 2 and 5. On the immunoprecipitated extracts, the seven anti-CV2 antiserum samples tested (from patients 1 to 7) recognised the 66 kDa band (weakly for serum 6; fig 9) and the band was not recognised in either of the two serum samples of normal control patients. No band was recognised by anti-CV2 serum samples in the extracts immunoprecipitated with the control serum.

Discussion

The identification of the well characterised autoantibodies—anti-Hu (ANNA-1), anti-Ri (ANNA-2), and anti-Yo (PCA-1)—associated with PNS relies on immunohistochemical and western blot methods.[3 4] Immunohistochemistry is usually performed on acetone fixed sections from human or rodent brain and only high serum dilutions are considered.[17] For western blot, appropriate antigen enriched preparations of purified neurons or fusion proteins are used and developed at high serum dilutions.[3] However, many patients with PNS have no detectable anti-Hu, anti-Ri, or anti-Yo antibodies.[10-12] Although the presence of other antineuronal cell antibodies has been reported using these procedures, they usually concern individual patients.[18-21] and many patients with PNS have no detectable autoantibodies. Among these antibody negative patients, the PNS could depend on a non-immunological process or could be mediated by cellular immunity. However, it cannot be excluded that antibody negative patients harbour antinervous system autoantibodies not shown by usual procedures—for example, anti-VGCC autoantibodies associated with myasthenic Lambert-Eaton syndromes and small cell lung cancer cannot be shown by standard immunohistochemistry and western blot.[22]

To improve antigen preservation more than with acetone postfixed sections, we tried to detect antibodies by using sections from rat brain fixed by perfusion with paraformaldehyde. With this technique, we identified a subgroup of patients with PNS, negative for anti-Hu, anti-Ri, or anti-Yo antibodies, whose serum samples labelled a particular subpopulation of glial cells in adult rat brain which we have designated as anti-CV2. For several reasons this pattern of staining cannot be confused with non-specific glial cell labelling reported at low dilution with normal control serum samples or serum samples from patients with anti-Hu antibodies.[5] Firstly, anti-CV2 antibodies only stained a subpopulation of glial cells specifically distributed in the white matter of the cerebellum, brainstem, and spinal cord. These were probably oligodendrocytes as shown by double labelling with the Rip monoclonal antibody. Secondly, except in two patients with cancer, we never saw a similar pattern of staining in 1061 control serum samples, even at low serum dilution (1/100). Thirdly, the staining obtained with anti-CV2 antibodies was always found with high serum dilution, ranging from 1/1000 to 1/100 000.
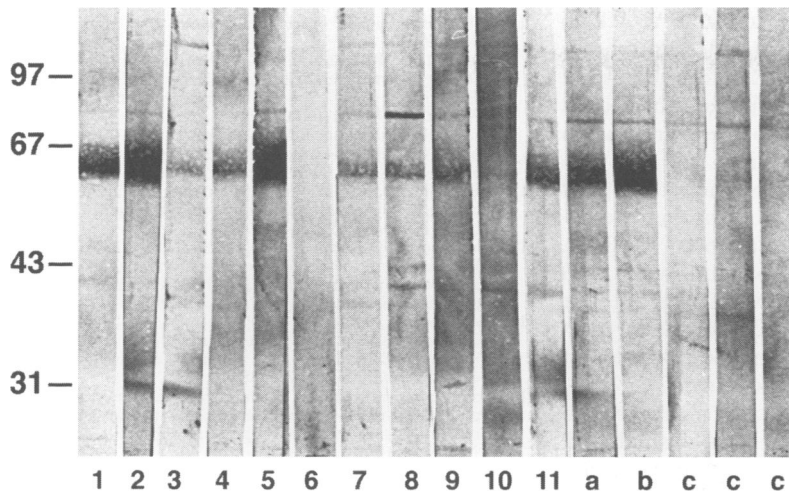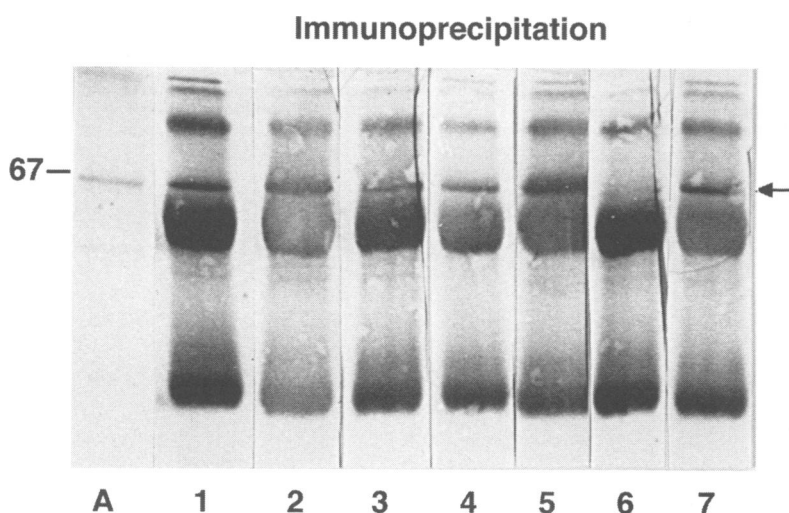
Figure 8   Western blots of 18 µg protein per lane of the S3 soluble fraction of new born rat brain incubated with serum samples (dilution 1/500) and disclosed by immunoperoxidase. 1 to 11 = Serum samples from patients with anti-CV2 antibodies and PNS. a and b = serum samples from the two patients, one with small cell lung carcinoma (a) and the other (b) with malignant thymoma who showed anti-CV2 antibodies by immunohistochemistry. c = Control patients with neurological syndromes and no cancer. All the serum samples with the pattern of anti-CV2 antibodies by immunohistochemistry label a band of apparent molecular weight 66 kDa, except patients 6 and 10 who had uterine sarcoma. These two serum samples were positive at 1/100 serum dilution (data not shown).

Fixation procedures were crucial for the detection of anti-CV2 antibodies, because we found no labelling with acetone fixed sections of rat brain[13] and obtained poor results with less stringent fixation procedures (data not shown). These results could explain the variability of labelling on human brain sections, probably caused by postmortem loss of the antigen or to poor antigen preservation with postfixation. Similar difficulties occur with serum samples from patients with Hodgkin's disease and cerebellar degeneration. In these patients antibodies against Purkinje cells are sometimes more easily detected with mouse brain than with human brain.[23] However, despite variability in staining with the different serum samples, our results show that the CV2 antigen is present in human brain.

Immunoprecipitation

Figure 9   S3 fraction of new born rat brain was immunoprecipitated with the serum of patient 2. The immunoprecipitate was processed for western blot analysis and incubated with the serum samples of seven patients with anti-CV2 antibodies (lane 1 to 7) at 1/100 dilution. All of them recognise the 66 kD band (arrow). Other bands correspond to denaturated immunoglobulins in the precipitate. Lane A is a western blot with the S3 fraction of newborn rat brain incubated with the serum of patient 2.

The progressive restriction of the labelling from a widespread staining of postmitotic neural cells in rat embryo to a subpopulation of glial cells in the adult suggests that CV2 antigen is ontologically down regulated. This regulation could explain the differences of the immunoreactivity by western blot between newborn and adult brain. Indeed, if all anti-CV2 serum samples recognised a 66 kDa band on newborn rat brain extract, the immunoreactivity was different between serum samples with adult brain. Thus we used S3 subcellular fractions of new born rat brain for western blot. However, the high sensitivity obtained by immunohistochemistry contrast with the lower sensitivity on western blot. On silver stained electrophoresis gels with newborn rat brain extract, the 66 kDa protein corresponds to a faint band suggesting that the amount of the antigen in the brain is small (data not shown). This suggests that this protein could be present in a low quantity in the brain which could explain the low sensitivity of western blot. Obviously, further work is needed to purify the 66 kDa protein for routine western blots with higher serum dilution. The availability of a recombinant protein, currently in progress, will probably improve the sensibility of western blots.

Anti-CV2 antibodies are strongly associated with cancer, predominantly in patients with PNS. However, they do not seem to be specific for one type of neurological disorder nor of one type of tumour. Indeed, although anti-CV2 antibodies seem to be mainly associated with small cell lung cancer, they occur in several cases with uterine sarcoma or malignant thymoma and a range of neurological syndromes including Lambert-Eaton myasthenic syndrome, limbic encephalitis, encephalomyelitis, and cerebellar degeneration. However, all of these neurological syndromes are known to be paraneoplastic.[1]

One of the criteria proposed for the characterisation of antibodies associated with PNS is that these antibodies should react with neurons in regions corresponding to the neurological syndrome.[17] This presupposes that the antibodies are the primary mediator of the immune attack. For the moment, this hypothesis needs confirmation.[2] Indeed, although some data suggest that anti-Hu antibodies could provoke lysis and subsequently neuronal death,[24] other studies failed to confirm this hypothesis.[25] Alternatively, the antibodies may be only part of a complex autoimmune reaction involving several tumour and brain antigens and several effectors of humoral and cellular immunity.[26] Thus a polyclonal activation of B cells could lead to the production of antibodies that are not directly involved in the autoimmune process.[4] Antistriated muscle antibodies are a good example of this phenomenon in patients with myasthenia gravis and thymoma.[27] Whether anti-CV2 antibodies result from a polyclonal activation or are directly involved in the PNS remains to be established. Anti-CV2 antibodies react, in adult brain, with a subpopulation of oligodendrocytes, a cell class that is not considered to

be the usual target of the immune attack in PNS. This suggests that anti-CV2 antibodies could result from an incidental immunological reaction. In addition, one patient had both anti-Hu and anti-CV2 antibodies, arguing in favour of a polyclonal activation.

However, whatever the physiopathological relevance of anti-CV2 antibodies, their detection is valuable in the diagnosis of PNS. Indeed, 10 out of the 11 patients with PNS and anti-CV2 antibodies were negative for anti-Hu, anti-Ri, or anti-Yo antibodies. In four cases investigated before the discovery of the tumour, the detection of anti-CV2 antibodies predicted the presence of a cancer with the high probability that it was a small cell lung cancer. This was confirmed in each case by the discovery of mediastinal lymph nodes on chest CT. If a chest CT is negative for thymoma or metastatic lymph nodes, patients with anti-CV2 antibodies should be preferentially investigated for uterine sarcoma.

1 Henson RA, Urich H. Paraneoplastic disorders. In : Henson RA, Urich H, eds. *Cancer and the nervous system.* Oxford: Blackwell Scientific Publications, 1982:311–621.

2 Posner JB, Dalmau J. Clinical enigmas of paraneoplastic neurological disorders. *Clin Neurol Neurosurg* 1995;97: 61–70.

3 Dalmau J, Posner JB. Neurologic paraneoplastic antibodies (anti-Yo; anti-Hu; anti-Ri): the case for a nomenclature based on antibody and antigen specificity. *Neurology* 1994;44:2241–6.

4 Lennon VA. Paraneoplastic autoantibodies: the case for a descriptive generic nomenclature. *Neurology* 1994;44: 2236–40.

5 Graus F, Cordon-Cardo C, Posner JB. Neuronal antinuclear antibody in sensory neuronopathy from lung cancer. *Neurology* 1985;35:538–43.

6 Dalmau J, Graus F, Rosenblum MK, Posner JB. Anti-Hu associated paraneoplastic encephalomyelitis/sensory neuronopathy. A clinical study of 71 patients. *Medecine* 1992;71:59–72.

7 Greenlee JE, Brashear HR. Antibodies to cerebellar Purkinje cells in patients with paraneoplastic cerebellar degeneration and ovarian carcinoma. *Ann Neurol* 1983; 14:609–13.

8 Peterson K, Rosenblum MK, Kotanides H, Posner JB. Paraneoplastic cerebellar degeneration. I. A clinical analysis of 55 anti-Yo antibody-positive patients. *Neurology* 1992;42:1931–7.

9 Luque FA, Furneaux HM, Ferziger R, et al. Anti-Ri : an autoantibody associated with paraneoplastic opsoclonus and breast cancer. *Ann Neurol* 1991;29:241–51.

10 Anderson NE, Rosemblum MK, Posner JB. Paraneoplastic cerebellar degeneration: clinical-immunological correlations. *Neurology* 1988;24:559–67.

11 Hammack JE, Kimmel DW, O'Neill BP, Lennon VA. Paraneoplastic cerebellar degeneration: a clinical comparison of patients with and without Purkinje cell cytoplasmic antibodies. *Mayo Clin Proc* 1990;65:1423–31.

12 Hammack J, Kotanides H, Rosenblum MK, Posner JB. Paraneoplastic cerebellar degeneration. II. Clinical and immunologic findings in 21 patients with Hodgkin's disease. *Neurology* 1992;42:1938–43.

13 Antoine JC, Honnorat J, Koenig F, et al. Posterior uveitis and paraneoplastic encephalomyelitis with auto-antibodies reacting against cytoplasmic proteins of brain and retina. *J Neurol Sci* 1993;117:215–23.

14 Antoine JC, Honnorat J, Thomas-Anterion C, et al. Limbic encephalitis and immunological perturbations in two patients with thymoma. *J Neurol Neurosurg Psychiatry* 1995;58:706–10.

15 Furneaux H, Rosenblum MK, Dalmau J, et al. Selective expression of Purkinje cell antigens in tumor tissue from patients with paraneoplastic cerebellar degeneration. *N Engl J Med* 1990;322:1844–51.

16 Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG. In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, Rip. *Glia* 1989; 2:380–90.

17 Posner JB, Furneaux HM. Paraneoplastic syndromes. In: Waksman BH, ed. *Immunologic mechanisms in neurologic and psychiatric diseases.* New York: Raven Press, 1990: 187–219.

18 Ahern GL, O'Connor M, Dalmau J, et al. Paraneoplastic temporal lobe epilepsy with testicular neoplasm and atypical amnesia. *Neurology* 1994;44:1270–4.

19 Darnell RB, DeAngelis LM. Regression of small-cell lung carcinoma in patients with paraneoplastic neuronal antibodies. *Lancet* 1993;341:21–2.

20 Anderson NE, Budde-Steffen C, Wiley RG, et al. A variant of the anti-Purkinje cell antibody in a patient with paraneoplastic cerebellar degeneration. *Neurology* 1988;35: 1018–26.

21 Darnell RB, Furneaux HM, Posner JB. Antiserum from a patient with cerebellar degeneration identifies a novel protein in Purkinje cells, cortical neurons, and neuroectodermal tumors. *J Neurosci* 1991;11:1224–30.

22 Sher E, Canal L, Piccolo G, et al. Specificity of calcium channel autoantibodies in Lambert-Eaton myasthenic syndrome. *Lancet* 1989;2:640–3.

23 Greenlee JE, Brashear HR, Herndon RM. Immunoperoxidase labeling of rat brain sections with sera from patients with paraneoplastic cerebellar degeneration and systemic neoplasia. *J Neuropathol Exp Neurol* 1988;47: 561–71.

24 Greenlee JE, Parks TN, Jaeckle KA. Type IIa (anti-Hu) antineuronal antibodies produce destruction of rat cerebellar granule neurons in vitro. *Neurology* 1993;43: 2049–54.

25 Hormigo A, Lieberman F. Nuclear localization of anti-Hu antibody is not associated with in vitro cytotoxicity. *J Neuroimmunol* 1994;55:205–12.

26 Jaekle KA. Autoimmune mechanisms in the pathogenesis of paraneoplastic nervous system disease. *Clin Neurol Neurosurg* 1995;97:82–8.

27 Lanska DJ. Diagnosis of thymoma in myasthenics using anti-striated muscle antibodies. *Neurology* 1991;41: 520–4.