Ma1, a novel neuron- and testis-specific protein, is recognized by the serum of patients with paraneoplastic neurological disorders

Josep Dalmau,1,3 S. Humayun Gultekin,1,2 Raymond Voltz,1 Rita Hoard,1 Thomas DesChamps,1 Casilda Balmaceda,4 Tracy Batchelor,5 Elizabeth Gerstner,1 Joseph Eichen,1 Jennifer Frennier,1 Jerome B. Posner1,3 and Myrna R. Rosenfeld1,3

1Department of Neurology and the Cotzias Laboratory of Neuro-Oncology and 2Department of Pathology, Memorial Sloan Kettering Cancer Center, 3Department of Neurology and Neuroscience, Cornell University Medical College, 4Department of Neurology, Neurological Institute, Columbia Presbyterian Medical Center, New York and 5Department of Neurology, Massachusetts General Hospital, Boston, Massachusetts, USA

Summary
The identification of antineuronal antibodies has facilitated the diagnosis of paraneoplastic neurological disorders and the early detection of the associated tumours. It has also led to the cloning of possibly important neuron-specific proteins. In this study we wanted to identify novel antineuronal antibodies in the sera of patients with paraneoplastic neurological disorders and to clone the corresponding antigens. Serological studies of 1705 sera from patients with suspected paraneoplastic neurological disorders resulted in the identification of four patients with antibodies that reacted with 37 and 40 kDa neuronal proteins (anti-Ma antibodies). Three patients had brainstem and cerebellar dysfunction, and one had dysphagia and motor weakness. Autopsy of two patients showed loss of Purkinje cells, Bergmann gliosis and deep cerebellar white matter inflammatory infiltrates. Extensive neuronal degeneration, gliosis and infiltrates mainly composed of CD8+ T cells were also found in the brainstem of one patient. In normal human and rat tissues, the anti-Ma antibodies reacted exclusively with neurons and with testicular germ cells; the reaction was mainly with subnuclear elements (including the nucleoli) and to a lesser degree the cytoplasm. Anti-Ma antibodies also reacted with the cancers (breast, colon and parotid) available from three anti-Ma patients, but not with 66 other tumours of varying histological types. Preincubation of tissues with any of the anti-Ma sera abrogated the reactivity of the other anti-Ma immunoglobulins. Probing of a human complementary DNA library with anti-Ma serum resulted in the cloning of a gene that encodes a novel 37 kDa protein (Ma1). Recombinant Ma1 was specifically recognized by the four anti-Ma sera but not by 337 control sera, including those from 52 normal individuals, 179 cancer patients without paraneoplastic neurological symptoms, 96 patients with paraneoplastic syndromes and 10 patients with non-cancer-related neurological disorders. The expression of Ma1 mRNA is highly restricted to the brain and testis. Subsequent analysis suggested that Ma1 is likely to be a phosphoprotein. Our study demonstrates that some patients with paraneoplastic neurological disorders develop antibodies against Ma1, a new member of an expanding family of ‘brain/testis’ proteins.

Keywords: Ma1; paraneoplastic; brain/testis proteins; antineuronal antibody; cerebellar degeneration

Abbreviations: cDNA = complementary DNA; SDS = sodium dodecyl sulphate

Introduction
Some paraneoplastic syndromes affecting the nervous system are associated with antibodies that react with neuronal proteins and the causal tumour (onconeural antigens) (Greenlee, 1982; Graus et al., 1985; Budde-Steffen et al., 1988; Dalmau and Posner, 1997). Several of these antibodies are markers of specific neurological syndromes associated with distinct types of cancer (Furneaux et al., 1990; Luque et al., 1991; Dalmau et al., 1992). The presence of some
antibodies is so specific that disorders previously identified by brain biopsy or at autopsy can now be diagnosed serologically (Henson et al., 1965; Anderson et al., 1988; Dalmau et al., 1990; Posner, 1995). The expression of neuronal proteins by the tumour is probably a crucial step that breaks the immune tolerance for otherwise normal neuronal proteins (Carpentier et al., 1998). The identity of most onconeural antigens has been established by probing human complementary DNA (cDNA) expression libraries with serum containing antineuronal antibodies (Dropcho et al., 1987; Sakai et al., 1990; Fathallah-Shaykh et al., 1991; Szabo et al., 1991; Buckanovich et al., 1993).

To date, characteristic antineuronal antibodies have been discovered in only a few paraneoplastic disorders. Because these antibodies have important clinical implications and can also be used as probes to isolate neuron-specific proteins, we examined the serum of patients with suspected paraneoplastic syndromes for novel antineuronal antibodies. We report (i) a novel antineuronal antibody (called anti-Ma) identified in the serum of four patients with paraneoplastic neurological syndromes, (ii) the expression of the target antigens in rat and normal human tissues and tumours, and (iii) the cloning of the cDNA for Ma1, encoding a novel neuron- and testis-specific protein recognized by anti-Ma sera.

**Method**

**Patients, sera and tissues**

The sera (or CSF when available) from 1705 patients that were sent to us to be screened for paraneoplastic antineuronal antibodies were used in the study. At the time that these sera were collected, 984 of the patients had a cancer diagnosis. Control sera were from 52 normal individuals, 96 patients with well-characterized paraneoplastic syndromes (44 with anti-Hu-related encephalomyelitis) and 17 with anti-Yo-related cerebellar degeneration, 11 with Lambert–Eaton myasthenic syndrome with P/Q-type voltage-gated calcium channel antibodies, two with anti-Ri-related cerebellar ataxia and oposclonus, six with anti-Tr-related cerebellar dysfunction, five with myasthenia gravis associated with thymoma and 11 with oposclonus associated with neuroblastoma), 179 patients with cancer (44 testicular, 10 colon, 10 ovarian, 40 lung, 22 breast, 20 brain tumours and 33 neuroblastomas) but without paraneoplastic neurological syndromes, six patients with amyotrophic lateral sclerosis without cancer and four with myasthenia gravis without thymoma.

Sera were kept frozen at −70°C. Human nervous system and systemic tissues were obtained from autopsy or biopsy studies of neurologically normal individuals. Fifty-three cancer tissues (15 colon, five breast, five bladder, three parotid, five neuroblastomas, five non-small-cell lung cancer and 15 testicular germ cell tumours) from patients without paraneoplastic symptoms and 13 from patients with antibody-associated paraneoplastic disorders (four of the ovary, four of the lung, two of the uterus, one of the bladder, one of the larynx and one chondrosarcoma) were provided by the Tumor Procurement Service at the Memorial Sloan-Kettering Cancer Center.

Wistar rats were anaesthetized and perfused with saline, and brain and other tissues were removed. Samples of human and rat tissues were kept at −70°C; other samples from the same tissues were embedded in OCT (Optimal Cutting Temperature) medium (Miles, Elkhart, Ind., USA) and frozen in isopentane chilled by liquid nitrogen.

For studies of human tumours and immunohistochemical competition assays, the IgG (immunoglobulin G) from two patients’ sera was isolated using a protein-G Sepharose column (Sigma, St Louis, Mo., USA) followed by labelling with biotin (Furneaux et al., 1990).

For Western blot analysis, human tissues were homogenized in 0.1% Nonidet P-40 and the following protease inhibitors: PMSF (phenylmethyl sulphonyl fluoride) (50 μg/ml), aprotinin (1 μg/ml), pepstatin (1 μg/ml) and leupeptin (1 μg/ml) (all from Sigma).

**Immunohistochemistry**

Frozen sections (7 μm thick) of rat and human brain and cerebellum were fixed in formalin, 100% methanol or cold acetone (4°C) and sequentially incubated with 0.3% hydrogen peroxide in PBS (phosphate-buffered saline) for 10 min, 10% normal goat serum for 20 min, the patient’s serum diluted at 1:500 for 2 h, biotinylated goat anti-human IgG (Vector, Burlingame, Calif., USA) diluted 1:2000, for 30 min, and the avidin–biotin–peroxidase complex (Vector) for 30 min. The reaction was developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma) with 0.01% hydrogen peroxide and 0.5% Triton X-100 in PBS. Patients’ sera and secondary antibody were diluted in 10% normal goat serum in PBS. Between steps, slides were washed with PBS.

To avoid reactivity with endogenous IgG, all immunohistochemical studies on systemic human tissues and tumours used biotinylated IgG isolated from the patients’ sera. All steps were done as above except that preincubation of the sections with 10% normal human serum was used to block non-specific IgG binding, and no secondary antibody was used.

For competition assays, tissue sections were preincubated with the serum of one of the patients (diluted 1:5) for 1 h, followed by incubation with biotinylated IgG isolated from the serum of another patient (diluted 1:25). Tissues preincubated with normal human serum or serum from patients with other antineuronal antibodies (diluted 1:5) served as controls. Sera were considered to compete for the same epitopes when the reactivity of the biotinylated IgG of one patient was abrogated by preincubation of the tissue with serum from another patient.

**Screening of a cerebellar cDNA expression library**

A λZAP human cerebellar library (Stratagene, La Jolla, Calif., USA) was screened at a density of 5 × 10⁶ p.f.u./150 mm plate
(where p.f.u. = plaque-forming unit). After 3 h of incubation at 42°C, plates were overlaid with filters soaked in 10 mM isopropyl β-D-thiogalactopyranoside and incubated for 4 h at 37°C. Plates were then cooled for 20 min at 4°C, and filters were removed, blocked with 1% BSA (bovine serum albumin) for 12 h at 4°C and incubated for 3 h with serum (diluted 1:1000) from a patient with paraneoplastic ophthalmoplegia and cerebellar dysfunction. After washing with Tween-20, filters were incubated with [125I]protein A (0.1 µCi/ml) for 1 h, washed, dried and exposed to XAR5 film for 24 h at –70°C. Clones giving positive results were purified by several rounds of antibody screening until a yield of 100% positive plaques was obtained. Phage clones were subcloned in pBluescript using the in vivo excision phage rescue protocol (Stratagene).

**DNA sequencing**

Sequence analysis was performed with an automated DNA sequencer (ABI 377) using the dye terminator fluorescence method (Lee et al., 1992). DNA sequencing was performed with an automated DNA sequencer (ABI 377) using the dye terminator fluorescence method (Lee et al., 1992). Double-stranded DNA was purified using the Qiagen plasmid midi-prep system (Qiagen, Santa Clarita, Calif., USA) and sequenced on both strands. Internal oligonucleotide primers and SK and KS primers were used (GeneLink, Thornwood, NY, USA).

**Western blot analysis**

Fusion protein and *Escherichia coli* protein extracts were obtained by growing an individual colony to an optical density of 0.6 and inducing with 10 mM IPTG for 3 h at 37°C. Cells were isolated by centrifugation and lysed by resuspension in 0.1% NP-40 and 2% SDS (sodium dodecyl sulphate) in PBS. Lysates of fusion proteins, or proteins extracted from human and rat tissues, were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose (Towbin et al., 1979). After blocking with 5% of the sample was loaded onto a 10% SDS–polyacrylamide gel and transferred to nitrocellulose filters.

**Table 1 Clinical features of four patients with paraneoplastic syndromes associated with anti-Ma antibodies**

<table>
<thead>
<tr>
<th>Patient, sex, age (years)</th>
<th>Time from PNS to tumour diagnosis</th>
<th>First neurological symptom(s)</th>
<th>Paraneoplastic syndrome</th>
<th>Tumour, stage (expression of Ma antigens)</th>
<th>Neurological treatment</th>
<th>Outcome</th>
<th>Autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1 F, 63</td>
<td>6 months</td>
<td>Gait difficulty</td>
<td>Pancerebellar syndrome (unable to walk), dysphagia, oscillopsia, absent reflexes (both knees and right ankle)</td>
<td>Parotid, limited (Ma Ag&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>IVlg, protein A column</td>
<td>Stable, 2 years after first symptom</td>
<td>–</td>
</tr>
<tr>
<td>Patient 2 F, 63</td>
<td>Preceded (? months) recurrence</td>
<td>Ataxia of extremities</td>
<td>Cerebellar dysfunction</td>
<td>Breast, extensive (Ma Ag&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>None</td>
<td>Dead, from systemic complications</td>
<td>Severe loss of Purkinje cells, Bergmann gliosis, Inflammatory infiltrates of T cells in cerebellar white matter</td>
</tr>
<tr>
<td>Patient 3 M, 58</td>
<td>1 year</td>
<td>Dysphagia</td>
<td>Dysphagia, mild proximal weakness, absent ankle reflexes, decreased vibratory and temperature sensation in feet, impotence</td>
<td>Large-cell cancer of the lung, limited. (Ma Ag not studied)</td>
<td>Tumour resection</td>
<td>Lost to follow-up</td>
<td>–</td>
</tr>
<tr>
<td>Patient 4 F, 58</td>
<td>11 months</td>
<td>Diplopia, unsteadiness, pseudobulbar affect</td>
<td>Abolished vertical eye movements; limited adduction of left eye, dysarthria, myokymia and decreased sensation on left side of the face, wide-base gait. Mild cognitive deficit</td>
<td>Colon, limited (Ma Ag&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>IVlg, plasma exchange, prednisone</td>
<td>Dead, from neurological disease</td>
<td>Neuronal loss and gliosis involving brainstem, Purkinje cells, dentate nucleus of cerebellum, T-cell infiltrates and microglial nodules: brainstem (mainly medulla), cerebellar white matter, hypothalamic substantia innominata</td>
</tr>
</tbody>
</table>
Northern blot analysis

Sequence-specific oligonucleotide probes were end-labelled with [γ-32P]ATP using T4 polynucleotide kinase. As the probe for Ma1 we used: 5’-GAAACCCAAGGACACGGG-3’ (cDNA base pairs 647–630), and as the probe for β-actin we used 5’-GCTTGGGATGGCCAGC-3’. Labelled probes were extracted with phenol chloroform and purified over a G-25 Sephadex column. Probes (1 probe was extracted with phenol chloroform and purified for 1 h. Strips were then immersed in an enhanced chemiluminescence solution (Amersham) for 1 min and exposed to Kodak XAR5 film (Sigma). Between steps, strips were washed with 0.05% Tween-20 in PBS. All incubations were done at room temperature.

Results
Clinical and pathological findings

The study of 1705 sera resulted in the identification of four patients who harboured a novel antineuronal antibody (see below), which we call anti-Ma. The clinical information on these patients is summarized in Table 1. Neurological symptoms preceded the diagnosis of the tumour in three patients, and recurrence of a breast cancer diagnosed 6 years earlier in one patient. Three patients had symptoms of cerebellar and/or brainstem involvement; the associated cancers were of the breast, parotid and colon. Another patient with a history of mild dysphagia, proximal weakness and sexual impotence for 1 year underwent mediastinoscopy and biopsy of a large cell carcinoma of the right bronchus; after anaesthesia he developed respiratory muscle weakness. Neurophysiological studies and serological test (P/Q-type voltage-gated calcium channel antibodies) for the Lambert–Eaton myasthenic syndrome were negative. This patient was lost to follow-up and it is not known if he developed other neurological symptoms.

Among the three patients with available clinical information, two received immunomodulatory treatments (intravenous immunoglobulin, protein A column immunoabsorption, plasma exchange), but the neurological deficits were not improved in any of them. One patient is alive and two are dead, one from multiple systemic problems (peritoneal carcinomatosis, sepsis, coagulopathy), the other from progressive brainstem dysfunction.

At autopsy, one patient had extensive systemic metastases of breast cancer, and micronodular cirrhosis. No metastases were identified in the nervous system (the spinal cord was not examined). There was almost complete absence of Purkinje cells in the cerebellum (Fig. 1A), associated with Bergmann gliosis, and mild inflammatory infiltrates in the deep cerebellar white matter. Neuritic plaques were identified in the cortex (mainly in the occipital lobe), but no other abnormalities were found in the cerebral cortex, amygdala and brainstem. This patient had no history suggesting Alzheimer’s disease.

The autopsy of the other patient was restricted to the brain, and the possibility of clinically undetected systemic metastases could not be ruled out. The tectal and tegmental regions of the midbrain, pontine tegmentum and medulla showed extensive perivascular and interstitial inflammatory infiltrates with microglial nodules (Fig. 1B). Severe neuronal loss and gliosis were found in the inferior olivary nucleus and surrounding tissue. There was also focal loss of Purkinje cells and of neurons of the dentate nucleus, with Bergmann gliosis. Inflammatory infiltrates were found in the deep cerebellar white matter. Milder perivascular and interstitial lymphocytic infiltrates were observed in the hypothalamus and substantia innominata.

In both patients, immunohistochemical analysis of the inflammatory infiltrates with markers for B cells (CD20), T cells (CD3) and subtypes of T cells (CD4 and CD8) demonstrated that most (>90%) of the cells were T lymphocytes, mainly CD8+ (>75% of T cells).

Laboratory findings

Anti-Ma antibodies specifically react with normal brain and testis

The sera of the above four patients reacted with all neurons of the central and peripheral nervous system, including sympathetic and dorsal root ganglia and myenteric plexus, in a characteristic pattern (Fig. 2A). Anti-Ma antibodies reacted mainly with the nuclei and nucleoli of neurons, and to a lesser degree with the cytoplasm. Non-neuronal cells did not react. Reactivity was not affected by formalin, methanol or acetone fixation, but it was better preserved in frozen than in paraffin-embedded tissues. In frozen rat tissue, the neuronal nuclei showed a speckled pattern of reactivity, and in many neurons it appeared confined to the nucleoli; in contrast, the cytoplasm reacted in a mild and diffuse, but not granular, pattern. In frozen and paraffin-embedded human tissues, the reactivity appeared more concentrated in the nucleoli of neurons, and there was also mild labelling of the cytoplasm. Human and rat systemic tissues, including lung, liver, kidney, spleen, thyroid gland, pancreas, small intestine, colon, heart, skeletal muscle and ovary, did not react with anti-Ma IgG, but testicular germ
cells, especially spermatocytes and early spermatids, did react (Fig. 2B).

In immunoblots of protein extracts from the same systemic tissues, brain homogenates and purified neurons (cortical neurons and Purkinje cells), the four anti-Ma sera reacted with proteins expressed only in purified neurons and homogenates of brain and testis. In brain, two distinct bands of reactivity were identified at 37 and 40 kDa (Fig. 3A and B). In testis, only the 37 kDa protein was found.

None of the 337 control sera showed the above immunohistochemical and Western blot reactivities.

**Anti-Ma antibodies specifically recognize paraneoplastic tumours**

We were able to obtain paraffin-embedded tumour tissue from three of the four patients with anti-Ma antibodies. After
Fig. 2 Immunohistochemical characterization of anti-Ma antibodies. (A) Anti-Ma serum reacted with rat cerebral cortex. Reactivity is present within subnuclear elements (speckled staining) of neurons, and to a lesser degree with the cytoplasm. The insert shows a human cortical neuron and demonstrates that in human tissue the anti-Ma reactivity is more concentrated in the nucleoli. (B) Anti-Ma serum reacted with rat testis. There is speckled staining selectively involving the germ cells of the seminiferous tubule. No labelling of the Leydig cells in the interstitium is observed. The insert shows human testicular germ cells from the intermediate stages of spermatogenesis (spermatocytes and early spermatids) in the seminiferous tubule; anti-Ma labelling is restricted to a few dots of nuclear reactivity, with milder, diffuse staining of the nucleus and cytoplasm. All sections were lightly counterstained with haematoxylin.
Paraneoplasia and brain/testis proteins

Fig. 3 Immunoblot characterization of anti-Ma antibodies. (A) Immunoblots of human neurons with anti-Ma sera. Lanes A–D correspond to the sera of four patients with anti-Ma antibodies and show reactivity with two bands of approximately 37 and 40 kDa (arrow points to the 37 kDa band). Non-specific bands in lanes B, C and D were also identified in some control sera. Control lanes include serum from a normal individual (N), a patient with anti-Ri antibodies (Ri) and a patient with anti-Hu antibodies (Hu). All sera were diluted 1 : 1000. (B) Immunoblots of normal human tissues with anti-Ma serum. The 37 kDa protein (arrow) recognized by anti-Ma antibodies is specifically expressed in testis and neurons, while the 40 kDa protein is expressed only in neurons. All lanes contain 100 µg protein, except the lane labelled ‘neurons’, which contains 15 µg purified neuronal protein. Serum diluted 1 : 1000.

paraffin removal and antigen retrieval (Cattoretti et al., 1993), tissue from all three tumours (breast, colon and parotid cancer) were found to express antigens identified by anti-Ma antibodies (Fig. 4), but in contrast to the situation in neurons reactivity in the tumours was concentrated in the cytoplasm.

We also examined the expression of Ma antigens in frozen or paraffin-embedded tumours, including 53 tumours from patients without paraneoplastic syndromes and 13 tumours from patients with other antibody-associated paraneoplastic symptoms: none reacted with anti-Ma antibodies.

Initial immunohistochemical findings were reproduced using biotinylated anti-Ma IgG from two patients, and were confirmed by a competition assay in which preincubation of tissues with any of the anti-Ma sera abrogated the reactivity of the biotinylated IgG from another anti-Ma patient (data not shown).

Cloning and characterization of the Ma1 antigen

Screening of a λ ZAP human cerebellar library resulted in the isolation of three recombinant bacteriophage clones. None reacted with normal human serum. The phage clones were subcloned into pBluescript using the phage excision protocol. The resulting bacterial cDNA plasmids contained inserts of 2139 bp, and sequence analysis demonstrated that all clones had identical inserts. Further studies were done using plasmid p8A, which was derived from clone 8–3A1.

The cDNA sequence revealed an open reading frame with two putative initiation AUG codons separated by one codon. The first of these, at nucleotide 272, is likely to be the translation initiator codon as it most closely fits the Kozak consensus rule (Kozak, 1987). The open reading frame extends until the first in-frame stop codon at nucleotide 1258 and encodes a protein of 330 amino acids with a predicted molecular mass of 36.8 kDa. We call this gene product Ma1.

In addition to the open reading frame, the cDNA clone includes 5' non-coding sequence and a 3' polyadenylation signal (GenBank accession number AF037364) (Fig. 5). A search of the EMBL/GenBank databases revealed that Ma1 cDNA nucleotides 272–546 had 97% homology with a human CpG island DNA genomic fragment (GenBank HS19A6R) (Cross et al., 1994), and nucleotides 794–1230 had 98% homology to cDNA clones derived from a human colon carcinoma cell line (GenBank AA314009) (Adams et al., 1995) and infant brain (GenBank HO6341). These clones were derived during screenings for CpG islands and expressed sequence tags; no further characterizations have been published. A search of several databases for protein subsequence motifs revealed that the Ma1 protein contains several potential casein kinase II and protein kinase C
phosphorylation sites but no other readily identifiable domains.

Sera from patients with paraneoplastic symptoms recognize Ma1 fusion protein
Using immunoblots of Ma1 fusion protein, the sera of all four patients with anti-Ma-associated paraneoplastic symptoms reacted with a band of ~37 kDa (Fig. 6A). No reactivity was observed with sham protein (extracts of E. coli containing the parental plasmid without the insert). None of the 337 control sera reacted with Ma1.

To determine whether the antibodies against Ma1 correspond to the same antibodies that react with brain and testis, sections of these tissues and immunoblots of brain were incubated with anti-Ma sera that had been preabsorbed with Ma1 fusion protein or sham protein. Immunoabsorption with Ma1 protein, but not with sham protein, abrogated all the reactivity with testis and 80% of the reactivity with brain (only a few dot-like reactive granules remained positive in the nuclei of neurons). In addition, the serum preabsorbed with Ma1 no longer reacted with the 37 kDa neuronal protein but remained reactive with the 40 kDa band, indicating that the 37 kDa protein corresponds to the cloned Ma1 (Fig. 6B).

Expression of Ma1 mRNA in human tissues
Hybridization of an Ma1-specific oligonucleotide probe to Northern blots of mRNA from multiple human tissues showed that Ma1 mRNA was expressed by brain and testis but not by placenta, lung, liver, spleen, thymus, prostate, ovary, small intestine, colon or peripheral blood leukocytes (Fig. 7). The blots revealed a single band of ~2.6 kb in both brain and testis. The faint signal observed in heart, skeletal muscle, kidney and pancreas could represent either a very low level of Ma1 mRNA expression or a trace of nervous tissue in these organs. In immunohistochemical and immunoblot assays (see above), these tissues did not react with anti-Ma serum, indicating that there is no Ma1 protein expression.

Discussion
We identified a novel antineuronal antibody (anti-Ma) in the serum of four patients with paraneoplastic neurological
Paraneoplasia and brain/testis proteins

Fig. 5 Nucleotide sequence of Ma1 cDNA. The deduced translation product is shown for the longest open reading frame. The putative initiation codon is marked in bold and the termination codon is underlined.

disorders. The target antigens of this immune response are two neuronal proteins of 37 and 40 kDa (Ma antigens). The screening of a human cDNA cerebellar expression library with the serum of one of the patients resulted in the cloning of a gene that encodes a predicted 37 kDa protein (called Ma1) expressed in brain and testis. Immunoblots of Ma1 fusion protein were specifically recognized by anti-Ma sera, but not by sera of normal individuals or cancer patients without paraneoplastic symptoms.

As with the initial reports of other paraneoplastic antineuronal antibodies (anti-Tr, Yo, Hu, Ri) (Trotter et al., 1976; Greenlee and Brashear, 1983; Graus et al., 1985; Budde-Steffen et al., 1988), which described one or two patients, it is premature to define the full spectrum of neurological symptoms or tumours associated with anti-Ma antibodies. Our clinical data, based on the three patients for whom detailed clinical information is available, suggest that the brainstem and cerebellum are the main targets of the disease. This is supported by the autopsy findings of two of these patients, which showed that the neuropathological abnormalities predominated in the brainstem (perivascular and interstitial infiltrates of T cells, gliosis and loss of neurons) and cerebellum (degeneration and loss of Purkinje cells).

Unlike previously characterized antineuronal antibodies, which in general are markers for relatively specific types of tumours (Luque et al., 1991; Dalmau et al., 1992; Peterson et al., 1992; Graus et al., 1997) each of our four patients had a different type of tumour. Three of these tumours (one tumour was not available for study) were found to express Ma antigens, but histologically similar tumours from patients with and without paraneoplastic syndromes did not express these antigens. In contrast to the situation in neurons where the anti-Ma reactivity predominates in the nuclei, the reactivity of the tumour cells is concentrated in the cytoplasm. The significance of this difference in the distribution of immunoreactivity is unknown. One possibility is that it represents the expression by the tumour of an abnormal form of Ma1 protein. Another possibility is that the cytoplasmic sequestering is due to binding of Ma1 to an altered cytoplasmic protein.

A preliminary conclusion of these findings is that the detection of anti-Ma antibodies suggests the presence of an
Fig. 6 Anti-Ma sera specifically recognize Ma1 fusion protein. (A) Lanes A, B, C and D correspond to sera from four patients with anti-Ma-associated paraneoplastic symptoms and lane N to serum from a normal individual. Lanes 1–16 are representative of 337 control sera from patients with cancer but without paraneoplastic neurological symptoms (cancer of the breast, lanes 1–3; colon, lanes 4–6; lung, lanes 7–9; testicular germ cell tumours, lanes 10–12), and patients with paraneoplastic neurological symptoms (anti-Hu-related, lanes 13 and 14; anti-Yo-related, lanes 15 and 16). These 16 sera and the other 321 control sera (not shown) were negative for anti-Ma antibodies. (B) Immunoblots of cortical neurons incubated with anti-Ma serum (dilutions 1:1000 and 1:2000) preabsorbed with 300 µg irrelevant protein (first two lanes) and 300 µg Ma1 (last two lanes). Note that the serum preabsorbed with Ma1 no longer reacts with the 37 kDa neuronal protein.

Fig. 7 Analysis of Ma1 expression in normal human tissues. Northern blot analysis of normal human tissues hybridized with a Ma1-specific oligonucleotide probe demonstrates a 2.6 kb band in brain and testis. The faint signal observed in heart, skeletal muscle, kidney and pancreas could represent a low level of Ma1 mRNA expression, but no protein expression was observed in immunohistochemical and immunoblot analysis of these four tissues (three of them shown in Fig. 3B).
underlying malignancy, but does not direct the search for a tumour to one or a few sites, as is the case with other paraneoplastic antibodies (e.g. anti-Hu and small-cell lung cancer or neuroblastoma). Thus, until the spectrum of associated tumours is more clearly defined, the detection of anti-Ma antibodies should prompt a general search for a systemic cancer and close observation for those patients in whom a neoplasm is not found.

The function of the Ma1 protein is unknown. An extensive search of the available databases did not reveal homology to any known proteins or any particular subsequence motifs. However, preliminary studies indicate that the Ma1 gene is homologous to Ma2, a recently cloned gene. The Ma2 protein corresponds to the 40 kDa antigen identified by anti-Ma antibodies in Western blot of human neuronal proteins (Fig. 3). Preabsorption of anti-Ma sera with either of these proteins did not abrogate the reactivity with the other, indicating that the epitopes recognized by anti-Ma sera in the two proteins are different (J. O. Dalmau, unpublished data). Ma2, but not Ma1, is the target of antineuronal antibodies present in the sera of patients with testicular cancer and paraneoplastic limbic encephalitis or brainstem–cerebellar dysfunction (Voltz et al., 1998).

In normal tissues the expression of Ma1 is restricted to brain and testis, making it a new member of an expanding family of ‘brain/testis’ proteins. Other proteins with expression patterns restricted to the brain and testis have been shown to have a variety of functions, including important roles in neurogenesis and gametogenesis (Noce et al., 1993; Connor et al., 1995; Martin et al., 1996). The predominant nucleolar localization of the Ma1 protein suggests a role in phosphorylation-dependent RNA processing. This is supported by the presence of several putative phosphorylation sites.

The finding that the expression of Ma1 mRNA is highly restricted to brain and testis, as is the Ma1 protein, suggests that the selective tissue expression does not result from post-translational modification, as occurs with the paraneoplastic CDR-62 antigen (Corradi et al., 1997), but is probably transcriptionally controlled.

The brain and testis are immunologically privileged by the existence of a blood–tissue barrier (McLay et al., 1997). In addition to this physical barrier, the Ma1-expressing cells of these organs (neurons and germ cells) lack expression of the major histocompatibility antigens (MHC class I and II) needed for the presentation of surface or intracellular proteins to the immune system (Haas et al., 1988; Neumann et al., 1997). Class I MHC antigens are important for self/non-self recognition. These findings indicate that the expression by a tumour of proteins normally restricted to immunoprivileged tissues (brain or testis) is a crucial step in the development of an immune response that may result in neurological disease. In keeping with this model, a group of ‘cancer/testis’ antigens, such as MAGE-1 and MAGE-3, were originally identified because of their ability to elicit immune responses, usually T-cell-mediated (van der Bruggen et al., 1991; Gaugler et al., 1994; Boel et al., 1995; van den Eynde et al., 1995; Boon and van der Bruggen, 1996). Whether patients with anti-Ma antibodies also develop immunopathological abnormalities in the testis has not been studied, and unfortunately our male patient was lost to follow-up.

In immune-related paraneoplastic neurological disorders, two broad groups of pathogenic mechanisms have been proposed (Verschuuren and Dalmau, 1998). In one group (e.g. lymphoma, Waldenström’s macroglobulinaemia) the neoplastic cells synthesize antibodies that may damage the peripheral nerves (e.g. antibodies against GM1 ganglioside and myelin-associated glycoprotein). In the other group, the expression of neuronal proteins by a tumour contributes to breaking the immune tolerance for these proteins. Depending on the location of the antigen, the latter group comprises two types of disorders. First, those in which the antigens are expressed on the cell surface (e.g. voltage-gated calcium channels in the Lambert–Eaton myasthenic syndrome, voltage-gated potassium channels in neuromyotonia) (Lang et al., 1981; Shiellito et al., 1995). These disorders mainly involve the peripheral nervous system and improve with antibody removal, treatment of the tumour, or both (Lang and Vincent, 1996). In the second type of disorder, usually affecting the central nervous system, the antigens are intracellular, either predominantly cytoplasmic (CDR34, CDR62, amphiphysin, Tr) or nuclear (Hu, Nova proteins); these syndromes rarely improve with treatment (Posner, 1995). The target antigens of anti-Ma antibodies, such as Ma1, should now be included in the latter group. As with the other members of this group, the anti-Ma antibodies, rather than being viewed as pathogenic, should be considered markers of paraneoplasia. Characterization of the anti-Ma immune response expands the number of paraneoplastic disorders with antibodies targeted to neuronal proteins.

**Acknowledgements**

We wish to thank Drs Fred H. Hochberg and Diane Edwards for providing clinical information, Dr Elen Louis for referring one patient to us, Dr Steven Chin for providing pathological material of one of the patients, and Dr Francesc Graus for critical review of the manuscript. Supported by NIH grant NS-26064 (J.B.P., J.D.), ACS Evelyn Frew Clinical Research Professorship (J.B.P.), NIH grant NS01625-04 (M.R.R.) and the Deutsche Forschungsgemeinschaft Habilitationsstipendium Vo 497/2-1 (R.V.).

**References**


Buckanovich RJ, Posner JB, Darnell RB. Nova, the paraneoplastic RI antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. Neuron 1993; 11: 657–72.


Peterson K, Rosenblum MK, Kotanides H, Posner JB. Paraneoplastic


