

A SEROLOGIC MARKER OF PARANEOPLASTIC LIMBIC AND BRAIN-STEM ENCEPHALITIS IN PATIENTS WITH TESTICULAR CANCER

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ABSTRACT

Background In patients with cancer, symptoms of limbic and brain-stem dysfunction may result from a paraneoplastic disorder. Paraneoplastic limbic or brain-stem encephalitis occurs more frequently with testicular cancer than with most other cancers. We sought antineuronal antibodies that might be used in a diagnostic test for this syndrome.

Methods Immunohistochemical and immunoblotting techniques were used to detect serum and cerebrospinal fluid antibodies. Serologic screening of a complementary DNA library and Northern blotting were used to clone the target antigen and determine which tissues expressed it.

Results Of 13 patients with testicular cancer and paraneoplastic limbic or brain-stem encephalitis (or both), 10 had antibodies in serum and cerebrospinal fluid against a 40-kd neuronal protein. These antibodies were used to clone a gene that we call *Ma2*, which codes for a protein (*Ma2*) that was recognized by serum from the 10 patients, but not by serum from 344 control subjects. *Ma2* was selectively expressed by normal brain tissue and by the testicular tumors of the patients. *Ma2* shares homology with *Ma1*, a "brain-testis-cancer" gene related to other paraneoplastic syndromes and tumors.

Conclusions The serum of patients with subacute limbic and brain-stem dysfunction and testicular cancer contains antibodies against a protein found in normal brain and in testicular tumors. Detection of these antibodies supports the paraneoplastic origin of the neurologic disorder and could be of diagnostic importance. (N Engl J Med 1999;340:1788-95.)

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THE identification of antibodies against neuronal proteins in the serum and cerebrospinal fluid of patients with both cancer and a specific neurologic disorder (paraneoplastic syndrome) has uncovered the existence of antigens shared by some tumors and the nervous system (onconeurological antigens).¹ Paraneoplastic syndromes usually precede detection of the tumor, may affect any part of the nervous system, and are often more debilitating than the cancer itself.² The detection of antibodies against onconeurological antigens points to the diagnosis of a paraneoplastic syndrome and focuses the search for an underlying tumor to a few organs.³

Paraneoplastic limbic encephalitis is a syndrome consisting of irritability, depression, seizures, severe memory deficit, and dementia.⁴ These symptoms are

due to dysfunction of the limbic system (hippocampus, amygdala, hypothalamus, and insular and cingulate cortex), which is the area of the nervous system where most of the pathological changes that characterize the syndrome occur. Brain-stem encephalitis and abnormalities in other areas of the nervous system are frequent, but they may be clinically silent.^{5,6}

Paraneoplastic limbic encephalitis is probably underdiagnosed, because of the diversity of its symptoms and the lack of specific diagnostic markers. In patients known to have cancer, symptoms of this paraneoplastic syndrome may be attributed to metastases to the brain, toxic or metabolic encephalopathies, infections, or toxic effects of cancer therapy.² In approximately 60 percent of patients with paraneoplastic limbic or brain-stem encephalitis, the syndrome precedes the detection of the tumor, further complicating the diagnosis of the neurologic disorder.⁶⁻⁸ Abnormalities involving the mesial temporal lobes on magnetic resonance imaging (MRI) studies and the finding of changes due to inflammation in the cerebrospinal fluid (pleocytosis, increased levels of proteins, and oligoclonal bands) may be suggestive of paraneoplastic limbic encephalitis but do not establish the diagnosis.⁹

In 80 percent of patients with paraneoplastic limbic encephalitis, the primary tumor is a small-cell lung cancer, and about half of these patients have antibodies against the Hu family of neuronal RNA-binding proteins (human homologues of the drosophila embryonic lethal abnormal visual, or *elav*, protein) expressed in the brain and the tumor.^{8,10,11} With the exception of these antibodies, there are no other serologic markers of paraneoplastic limbic encephalitis, and the diagnosis relies on brain biopsy or is made at autopsy. In the remaining 20 percent of patients with the syndrome, testicular cancer occurs more frequently than expected. This observation, together with the detection of an antibody against a novel neuronal antigen in a patient with testicular cancer and paraneoplastic limbic encephalitis,¹² led us to investigate other cases of this syndrome in patients with testicular tumors.

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METHODS

Serum and Tissue Samples

We analyzed serum samples (or cerebrospinal fluid samples when available) from 986 men and women with histologically proved cancer that had been sent to us for antineuronal-antibody testing. In addition, we obtained 344 serum samples for use as controls from patients with other paraneoplastic syndromes, patients with a variety of cancers without such syndromes, patients with multiple sclerosis or systemic lupus erythematosus, and normal subjects.

Tumor tissues were provided by referring physicians and the Tumor Procurement Service at Memorial Sloan-Kettering Cancer Center. They included testicular tumors from 4 patients with paraneoplastic limbic encephalitis, brain-stem encephalitis, or both; tumors from 85 patients without paraneoplastic syndromes (65 with testicular germ-cell tumors, 5 with colon cancer, 4 with breast cancer, 3 with lung cancer, 2 with parotid-gland cancer, and 6 with small-cell lung cancer); and tumors from 8 patients with other paraneoplastic syndromes (4 with small-cell lung cancer, 3 with ovarian cancer, and 1 with bladder cancer). Tissue from normal subjects and Wistar rats was processed and stored as reported elsewhere.^{13,14} For Western blot analysis, tissues were homogenized in 0.1 percent Nonidet P-40 and protease inhibitors.¹⁵

Immunohistochemical Analysis

Frozen sections of rat and human tissues that were 7 μ m thick were fixed in 10 percent formalin or a combination of 30 percent methanol and 70 percent acetone at 4°C and incubated with a sample of serum, IgG, or cerebrospinal fluid from a patient with use of an avidin-biotin-peroxidase immunoassay.^{14,15} A monoclonal antibody against human CD8+ T cells (Dako, Carpinteria, Calif.) was used to examine the immunophenotype of the inflammatory infiltrates in brain-biopsy specimens.¹⁰

To avoid interference with endogenous IgG in the immunohistochemical studies with human tissue, we used IgG that had been purified from patients' serum samples and labeled with biotin.¹⁶ The same IgG was used for immune-competition assays: two serum samples were considered to be competing for the same epitopes when preincubation of the tissue with one sample abrogated the reactivity of the IgG isolated from the other sample.

Intrathecal Synthesis of Antibodies against Ma2 Onconeural Antigen

The presence of intrathecal synthesis of antibodies against an onconeural antigen that we have called Ma2 (also called anti-Ta) was assessed according to Schüller's formula.¹⁷ A ratio of Ma2 antibody reactivity in cerebrospinal fluid to Ma2 antibody reactivity in serum of more than 2 indicates that there is intrathecal synthesis of Ma2 antibody.

Cloning, Isolation, and Sequence Analysis of Ma2 Complementary DNA

Serum from a patient with paraneoplastic brain-stem dysfunction was plated at a density of 5×10^4 pfu per 150-mm plate and screened with a λ ZAP human cerebellar phage library (Stratagene, La Jolla, Calif.).¹³ Positive phage colonies were purified by several rounds of antibody screening, followed by subcloning into a pBluescript vector according to the phage-rescue protocol (Stratagene).

Double-stranded Ma2 complementary DNA (cDNA) was purified with the Qiagen plasmid midi-prep system (Qiagen, Santa Clarita, Calif.) and sequenced on an automated DNA sequencer (model ABI377, Applied Biosystems, Foster City, Calif.) with use of the dye-labeled terminator fluorescence method.¹⁸

Western Blot Analysis

Recombinant fusion proteins, *Escherichia coli* proteins, and proteins from human and rat tissues were obtained as described pre-

viously,^{10,14} subjected to 10 percent sodium dodecyl sulfate-polyacrylamide-gel electrophoresis, and transferred to nitrocellulose strips. The nitrocellulose strips were incubated with the patients' serum (dilution, 1:1000) and assessed for reactivity by an enhanced chemiluminescence assay (Amersham, Arlington Heights, Ill.).¹³

Northern Blot Analysis

The following sequence-specific oligonucleotide probes were used: Ma2, 5'GGGAATGGCCGAGACATC3' (cDNA base pairs, 234 to 217); Ma1, 5'GAAACCCAAGGACACGGG3' (cDNA base pairs, 647 to 630); and β -actin, 5'GTCTTTGCGGATGTCCA-CG3'. Probes were end-labeled, purified, and hybridized to human I and II multiple-tissue Northern blots (Clontech, Palo Alto, Calif.) as described previously.¹³

RESULTS

Patients

Among the 986 patients with cancer whose serum samples we examined for antibodies against onconeural antigens, 19 had testicular cancer and a paraneoplastic syndrome. Of these 19 patients, 13 had symptoms of limbic or brain-stem dysfunction (or both), and 10 had antibodies against an onconeural antigen we have named Ma2.

Table 1 shows the clinical features of the 10 patients with antibodies against Ma2. Of these 10 patients, 8 had paraneoplastic limbic encephalitis. Four patients had symptoms of brain-stem encephalitis, two of whom also had limbic encephalitis.

Neurologic symptoms preceded the diagnosis of the tumor in 8 of the 10 patients with anti-Ma2 antibodies (median time from onset of symptoms to diagnosis, 6 months; range, 2 to 36); in the other 2 patients the tumor had been discovered and treated 6 and 12 months before the neurologic disorder became evident. MRI or computed tomographic scans of the head were abnormal in seven patients, all of whom had prominent limbic dysfunction. Four patients underwent brain biopsy; in all cases there were mononuclear inflammatory infiltrates, astrogliosis, and neuronal degeneration. Two patients had relapsing and remitting neurologic symptoms: one has been described previously,¹⁹ and the other had symptoms for 12 months before the detection of serum anti-Ma2 antibodies led to the discovery of the tumor. All 10 patients had testicular tumors (4 seminomas and 6 nonseminomatous or mixed germ-cell tumors). At the time of the diagnosis of the tumor, four patients had systemic metastases.

All 10 patients underwent orchiectomy, 5 received chemotherapy, and 1 received radiation therapy. After treatment of the testicular tumor, four patients had neurologic improvement (two of whom had a clinical remission), the neurologic status remained stable in three and deteriorated in one, and two died (one from complications of chemotherapy and the other as a result of the neurologic disease). In some patients the neurologic symptoms were treated with corticosteroids, plasma exchange, or intravenous immune globulin alone or in combination. In only one of these

TABLE 1. CLINICAL FEATURES OF 10 MEN WITH CANCER, A PARANEOPlastic SYNDROME, AND ANTIBODIES AGAINST MA2 ANTIGEN.

PATIENT NO.	AGE (YR)	INITIAL SYMPTOMS	SYNDROME	DIAGNOSTIC TESTS AND RESULTS*	TIME OF DIAGNOSIS OF TUMOR	TUMOR TYPE	TREATMENT	STATUS
1	28	Resting tremor, slow mentation, anxiety, irritability, depression, dystonia, seizures, memory problems	Paraneoplastic limbic encephalitis, basal-ganglia dysfunction	CT: normal CSF: abnormal	1 yr before the neurologic disease	Nonseminomatous germ-cell tumor	Orchiectomy, plasma exchange, corticosteroids	Neurologic: stable Tumor: NED at 3 yr
2	45	Depression, memory loss, seizures, weight gain (9 kg)	Paraneoplastic limbic encephalitis	MRI: abnormal CSF: normal Brain biopsy: paraneoplastic limbic encephalitis	3 yr after the neurologic disease	Nonseminomatous germ-cell tumor	Orchiectomy, chemotherapy	Neurologic: stable Tumor: NED at 9 yr
3	26	Memory loss, seizures	Paraneoplastic limbic encephalitis	CT: enhancing right-temporal-lobe lesion CSF: abnormal Brain biopsy: paraneoplastic limbic encephalitis	9 mo after the neurologic disease	Nonseminomatous germ-cell tumor	Orchiectomy, chemotherapy	Neurologic: remission Tumor: NED at 3 yr
4	37	Severe memory loss, mild brain-stem signs, hypersomnia	Paraneoplastic limbic encephalitis, paraneoplastic brain-stem encephalitis	MRI: abnormal CSF: abnormal	2 mo after the neurologic disease	Seminoma	Orchiectomy, chemotherapy, corticosteroids	Neurologic: remission Tumor: metastases
5	22	Seizures (left-sided facial twitching, with abnormal taste in mouth)	Paraneoplastic limbic encephalitis	MRI: abnormal CSF: abnormal Brain biopsy: paraneoplastic limbic encephalitis	6 mo after the neurologic disease	Mixed germ-cell tumor	Orchiectomy, chemotherapy, carbamazepine	Died of neurologic deterioration
6	28	Hallucinations, seizures (déjà vu), memory loss, hyperthermia	Paraneoplastic limbic encephalitis, cerebellar syndrome	MRI: abnormal CSF: abnormal	6 mo after the neurologic disease	Mixed germ-cell tumor	Orchiectomy, chemotherapy	Died of complications of chemotherapy
7	45	Ataxia, dysarthria	Paraneoplastic brain-stem encephalitis, cerebellar syndrome	MRI: normal CSF: normal	6 mo after the neurologic disease	Seminoma	Orchiectomy, radiation, carbamazepine	Neurologic: partial improvement Tumor: NED at 3 yr
8	28	Visual and auditory hallucinations, confusion, eye-motility dysfunction, memory loss, hyperthermia	Paraneoplastic limbic encephalitis, paraneoplastic brain-stem encephalitis	MRI: abnormal CSF: abnormal Brain biopsy: paraneoplastic limbic encephalitis	6 mo before the neurologic disease	Nonseminomatous germ-cell tumor	Orchiectomy, corticosteroids, plasma exchange, intravenous immune globulin	Neurologic: deterioration Tumor: NED at 9 mo
9	30	Diplopia, dysarthria, oscillopsia	Paraneoplastic brain-stem encephalitis, cerebellar syndrome	MRI: normal CSF: normal	12 mo after the neurologic disease	Seminoma	Orchiectomy	Neurologic: stable Tumor: NED at 4 mo
10	38	Lethargy, loss of libido, diabetes insipidus, hypothyroidism, urinary incontinence, mutism, hypersomnia, decreased voluntary movements	Paraneoplastic limbic encephalitis, hypothalamic dysfunction	MRI: abnormal CSF: abnormal	5 mo after the neurologic disease	Seminoma	Orchiectomy, dexamphetamine, intravenous immune globulin, corticosteroids	Neurologic: mild improvement Tumor: NED at 3 mo

*CT denotes computed tomography, CSF cerebrospinal fluid, and MRI magnetic resonance imaging. For cerebrospinal fluid examinations an abnormal result indicates the presence of elevated protein levels, pleocytosis, or both. In all patients, the cytologic examination of cerebrospinal fluid was negative for cancer cells. For MRI of the head, abnormalities on T₂-weighted sequences involving one or both temporal lobes were identified in six patients, abnormalities in the suprasellar-diencephalic region in three patients, and abnormal uptake of gadolinium in temporal or diencephalic regions in three patients.

†NED denotes no evidence of disease.

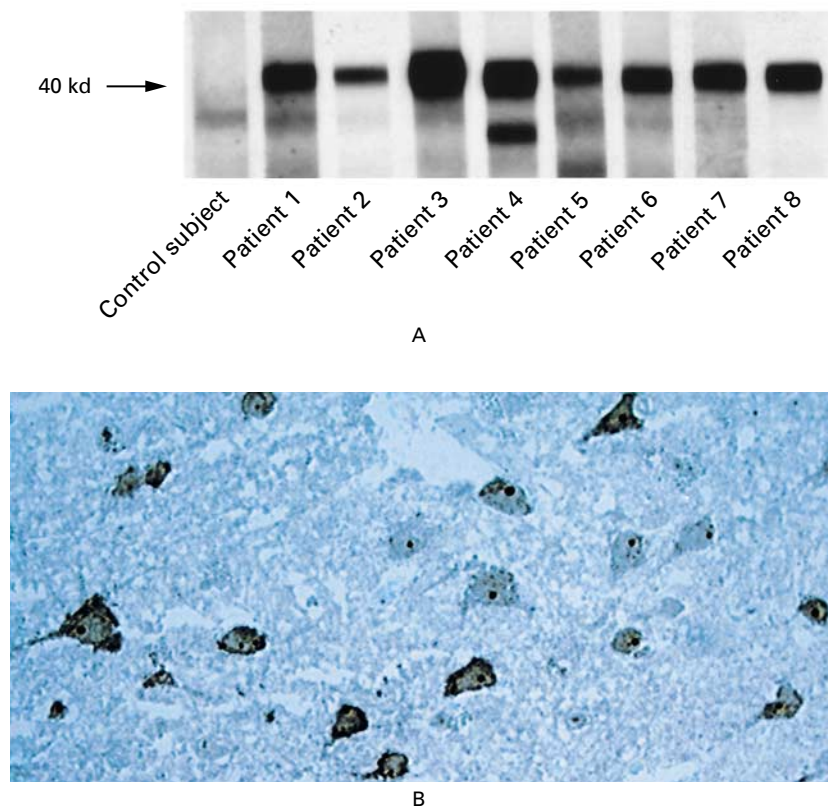


Figure 1. Western Blotting and Immunohistochemical Analysis of Serum Containing Anti-Ma2 Antibodies. Panel A shows the results of immunoblotting of human neuronal proteins incubated with serum from a control subject and eight patients with testicular cancer who also had paraneoplastic limbic or brain-stem encephalitis, or both. Serum from all eight patients reacted with a 40-kd neuronal protein. Panel B shows the pattern of reactivity of the antibody with human brain tissue. The reactivity involves the nuclei and cytoplasm of neurons and is concentrated in the nucleoli and perikaryon (no counterstain, $\times 400$).

patients was treatment (corticosteroids and intravenous immune globulin) followed by improvement.

Three patients who had testicular tumors but no anti-Ma2 antibodies had symptoms suggestive of a paraneoplastic syndrome: two had brain-stem and cerebellar dysfunction, and the other had transient memory loss and confusion. Neurologic symptoms developed in these patients 3 months before and 12 and 24 months after the diagnosis of testicular cancer. In contrast to patients with anti-Ma2 antibodies, in whom the findings on MRI of the brain and studies of cerebrospinal fluid were usually abnormal, these three patients had normal results on MRI of the brain, and one (with brain-stem and cerebellar symptoms) had changes indicative of inflammation in the cerebrospinal fluid.

Characterization of Antibodies against Ma2

Serum samples from the 10 patients with anti-Ma2 antibodies reacted on Western blotting of an extract of purified human neurons with a 40-kd protein

(Fig. 1A). Cerebrospinal fluid samples were available from six of the patients, and all samples also reacted with the protein. No patient had antibodies exclusively in cerebrospinal fluid. The pattern of reactivity of antibodies in the 10 serum samples was examined immunohistochemically with the use of frozen human and rat tissues fixed in methanol–acetone and biotin-labeled purified IgG from each patient's serum. Under these conditions, all neurons of the human and rat brain, spinal cord, dorsal-root ganglia, intestinal autonomic neurons, and adrenal medullary ganglion cells showed discrete subnuclear and cytoplasmic immunoreactive structures (Fig. 1B). Purkinje cells and other neurons of the cerebellar cortex had the weakest reactions. No reactivity was identified in liver, lung, and other non-neuronal tissues.

When formalin-fixed tissue was analyzed, only the areas that had the strongest reactions with methanol–acetone fixation were reactive: hippocampus, amygdala, diencephalic structures (medial thalamic and subthalamic nuclei, and the lateral hypothalamic area),

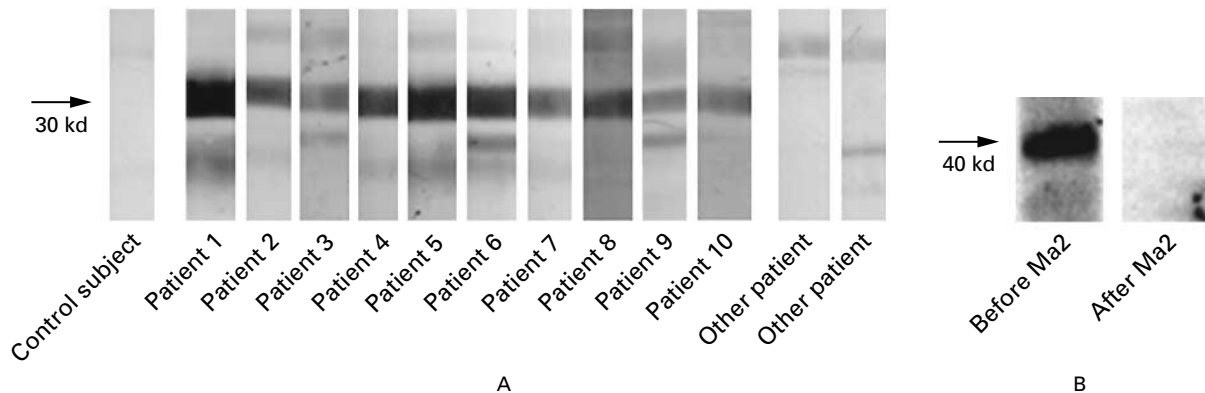


Figure 2. Western Blot Analysis of Recombinant Ma2 Protein.

In Panel A, serum samples from all 10 patients with testicular cancer who also had paraneoplastic limbic or brain-stem encephalitis, or both, reacted with the protein. In contrast, serum samples from two patients with testicular cancer but without paraneoplastic limbic or brain-stem encephalitis and a serum sample from a control subject did not react to the protein. In Panel B, human neuronal proteins were incubated with a serum sample containing anti-Ma2 antibodies before and after exposure to Ma2. The incomplete open reading frame of Ma2 accounts for the protein's smaller size (30 kd) as compared with the neuronal protein (40 kd).

various tegmental nuclei, and the dentate nucleus of the cerebellum. Preincubation of tissues with serum samples from 8 of the 10 patients abolished the reactivity of IgG isolated from serum from the other 2 patients, suggesting that all serum samples had a similar immunohistochemical specificity (data not shown). Antibodies against Ma2 were not identified in any of the serum samples from the 344 control subjects.

Cloning of the Gene Encoding the Ma2 Antigen

Screening of serum containing anti-Ma2 antibodies with a λ ZAP human cerebellar phage library allowed the isolation of a positive clone, which was recovered by subcloning into a pBluescript vector. After purification, a plasmid (p561A) was isolated that contained an insert of 614 bp. The sequence of this insert included an incomplete open reading frame corresponding to 195 amino acids, with a predicted molecular mass of 21.9 kd. We called this gene *Ma2* (GenBank accession number AF037365) because of its partial homology with *Ma1* (GenBank accession number AF037364), a gene that codes for an antigen associated with another paraneoplastic neurologic syndrome.¹³ A search of Genbank data bases revealed a gene that had 60 percent homology with *Ma2* and that had been cloned from adult mouse testis (GenBank accession number AA498105).

Antibodies against Recombinant Ma2 in Serum and Cerebrospinal Fluid

Recombinant Ma2 protein was expressed in *E. coli* from the p561A plasmid.^{10,13} With the use of Western blots containing the recombinant Ma2 fusion protein, all 10 serum samples (Fig. 2A) and all 6 cerebrospinal fluid samples from patients with paraneoplastic

limbic or brain-stem encephalitis (or both) reacted with a band of approximately 30 kd. There was no reactivity with the control protein (an extract of *E. coli* containing pBluescript without an insert). None of 344 control serum samples reacted with recombinant Ma2.

To determine whether recombinant Ma2 corresponds to the 40-kd protein in extracts of purified neurons, we tested Western blots of neuronal proteins with serum samples that had been preincubated with recombinant Ma2. Preincubation with recombinant Ma2, but not with the control protein, abrogated the reactivity of the serum to the 40-kd neuronal protein. These results suggest that the 40-kd neuronal protein is Ma2 or contains Ma2 epitopes (Fig. 2B).

In five patients, the ratio of Ma2 antibody reactivity in cerebrospinal fluid to that in serum was measured and was 0.74, 4.4, 6.2, 16.9, and 23.5, indicating intrathecal synthesis of the antibodies in four patients.¹⁷

Expression of Ma2 by Normal Brain and by Testicular Tumors

Northern blot analysis of messenger RNA (mRNA) from normal human tissues showed that Ma2 mRNA occurs in brain but not in placenta, lung, liver, spleen, thymus, prostate, ovary, testis, small intestine, colon, or peripheral-blood leukocytes (Fig. 3). In brain tissue the mRNA was present as a single transcript of approximately 6.5 kb. Immunohistochemical and Western blot analysis of the same tissues, with biotinylated IgG from patients with Ma2 antibodies used as a probe, showed reactivity (presumably with Ma2) only in brain (data not shown).

Specimens of the tumors from four of the patients with paraneoplastic limbic encephalitis, brain-stem

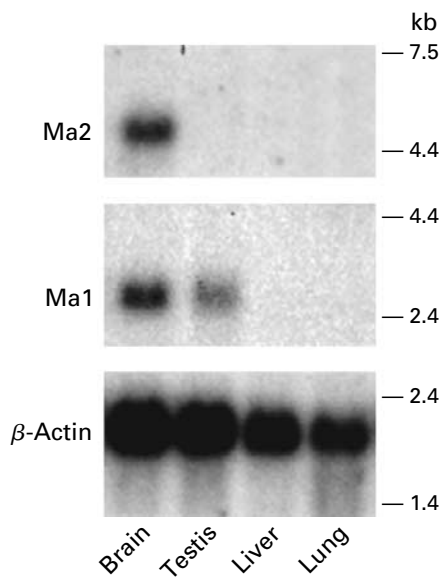


Figure 3. Northern Blot Analysis of Normal Human Brain, Testis, Liver, and Lung for the Expression of Ma2 Messenger RNA.

Poly(A+) RNA (2 μ g each) was obtained from human brain, testis, liver, and lung tissues; electrophoresed; and blotted onto nylon membranes (human I and II multiple-tissue Northern blot, Clontech). Hybridization with γ - 32 P-labeled oligonucleotide probes specific for Ma2, Ma1, or β -actin revealed a single Ma2 transcript of 6.5 kb only in brain tissue, whereas a single Ma1 transcript of 2.6 kb was expressed by both brain and testis tissue.

encephalitis, or both and Ma2 antibodies were available in formalin-fixed, paraffin-embedded blocks. After tissue deparaffination and antigen retrieval by microwave,²⁰ all four tumors reacted with biotinylated IgG containing Ma2 antibodies (Fig. 4). Reactivity was abolished by preincubation of the IgG with recombinant Ma2 protein (Fig. 4). No reactivity against Ma2 was detected in 93 tumor specimens of diverse histologic types (including 65 testicular cancers) from patients without paraneoplastic syndromes or with other paraneoplastic disorders.

Antibodies against Ma1 and Ma2 as Markers for Distinct Paraneoplastic Syndromes

We have previously described four patients with paraneoplastic neurologic syndromes (brain-stem encephalitis and cerebellar degeneration) and serum antibodies (called anti-Ma) against neuronal proteins of 37 and 40 kd.¹³ These anti-Ma antibodies were used to clone *Ma1*, which codes for a 37-kd protein in brain and normal testis. Because of the similarities between the nucleotide sequences of *Ma2* and *Ma1*, we examined whether serum containing anti-Ma or anti-Ma2 antibodies reacted with both of the Ma proteins. Serum containing anti-Ma2 antibodies reacted exclusively with Ma2, but serum containing anti-Ma antibodies reacted with both Ma1 and Ma2 proteins. Pre-

incubation of serum containing anti-Ma antibodies with either of these proteins did not abrogate its reactivity with the other protein, indicating that Ma1 and Ma2 contain distinctive epitopes. In addition, preincubation of rat-brain sections or immunoblots of neuronal or recombinant Ma2 proteins with serum containing anti-Ma antibodies decreased but did not abolish the reactivity of these blots with anti-Ma2 IgG antibodies. These findings suggest that some Ma2 epitopes are recognized by both types of antibodies (data not shown). The clinical and immunologic findings associated with the presence of anti-Ma and anti-Ma2 antibodies are summarized in Figure 5.

DISCUSSION

We found that serum and cerebrospinal fluid from 10 of 13 patients with testicular cancer and paraneoplastic limbic or brain-stem encephalitis (or both) contained antibodies against a 40-kd neuronal protein. A recombinant preparation of this protein (called Ma2, also known as Ta)²¹ was bound by serum samples from all 10 patients, but not by serum samples from patients with testicular cancer who did not have a paraneoplastic syndrome. Moreover, these antibodies reacted not only with the neuronal protein but also with the patients' testicular-tumor tissue.

Antineuronal antibodies in the serum of patients with this paraneoplastic syndrome were used to clone the *Ma2* gene. *Ma2* was found to resemble *Ma1*, a gene that we previously identified using antibodies (called anti-Ma) in serum from patients with paraneoplastic cerebellar or brain-stem dysfunction (or both) associated with lung, breast, parotid-gland, or colon cancer.¹³ Anti-Ma antibodies react with a 37-kd neuronal protein (Ma1)¹³ and a 40-kd protein, which we have identified as Ma2. Unlike the anti-Ma antibodies, which recognize both proteins, anti-Ma2 antibodies react only with Ma2 (Fig. 5).

Ma2 and *Ma1* are most likely members of a novel gene family that includes *KIAA0883*, a gene cloned from the brain of an adult (GenBank accession number AB020690). The *KIAA0883* gene is almost identical to *Ma2*, but the protein it encodes contains additional 3' sequences that have homology with the sequence of the corresponding region of Ma1. Whether *Ma2* represents a truncated or alternatively spliced form of *KIAA0883* is not known.

The function of the Ma1 and Ma2 proteins is unknown, but they are both target antigens in diseases that are probably initiated by an immune response to neuronal proteins expressed by tumors. The intrathecal synthesis of anti-Ma2 antibodies in four of five patients whom we studied indicates that there is an immunologic response against Ma2 within the nervous system of these patients.¹⁷ The absence of intrathecal synthesis of anti-Ma2 antibodies in one patient may have resulted from treatment with corticosteroids and intravenous immune globulin 10

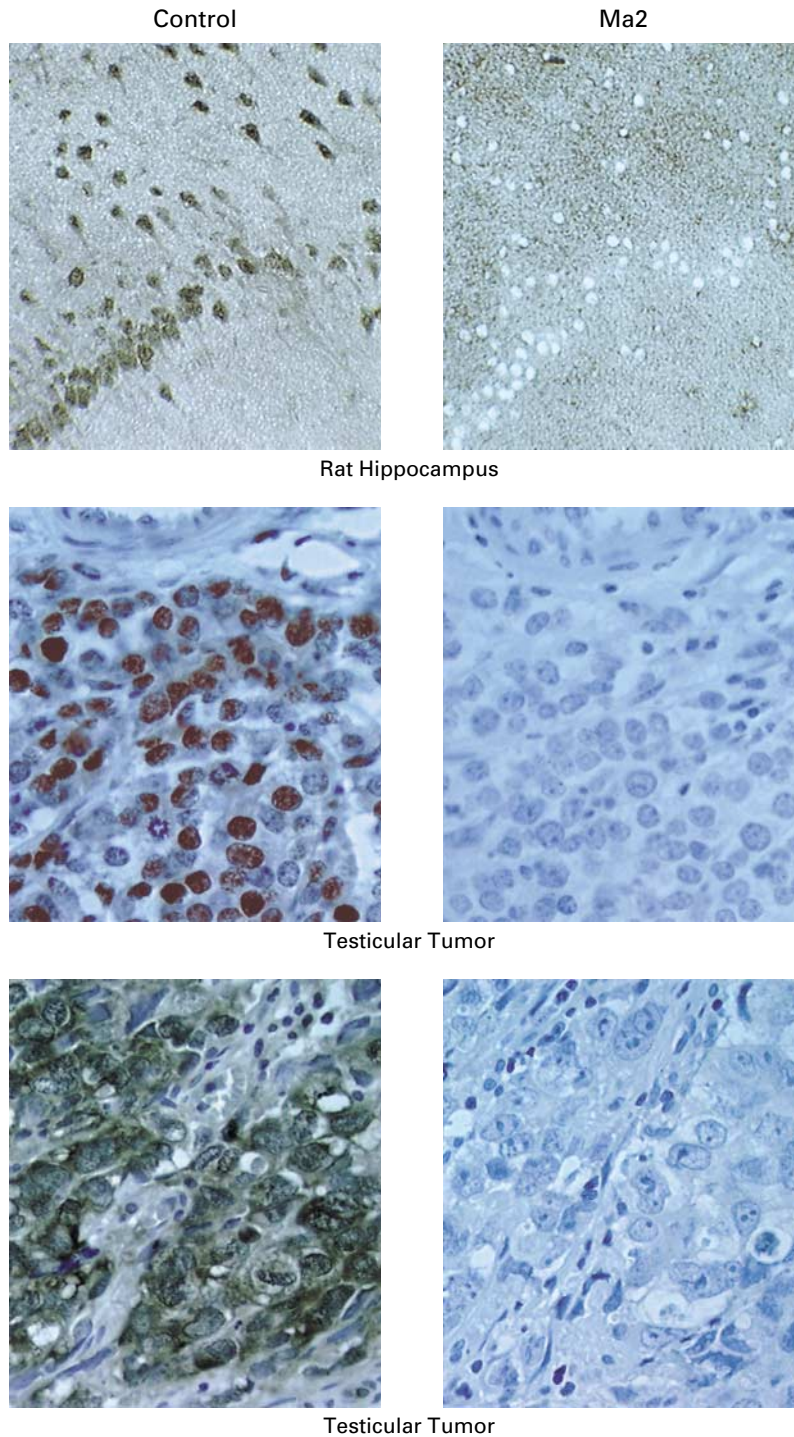


Figure 4. Expression of Ma2 by Rat Hippocampus and by Tumor Tissue from Two Patients with Paraneoplastic Limbic Encephalitis or Brain-Stem Encephalitis and Ma2 Antibodies.

The reactivity of Ma2 antibodies with tissue samples was not affected when Ma2 antibodies were preabsorbed with control protein (left-hand panels). Reactivity was abolished when Ma2 antibodies were preabsorbed with Ma2 protein (right-hand panels). (Top and middle left-hand panels: no counterstain, $\times 200$; other panels: hematoxylin counterstain, $\times 400$.)

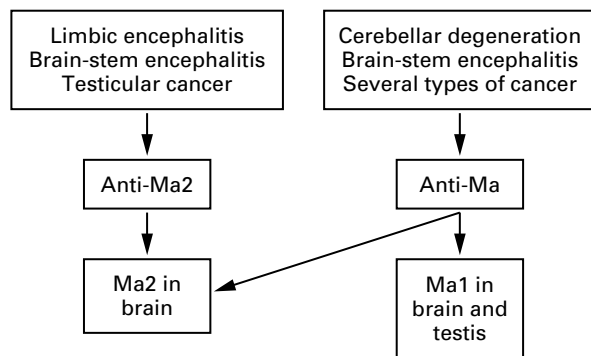


Figure 5. Clinical and Immunologic Findings Associated with the Presence of Anti-Ma and Anti-Ma2 Antibodies in Serum.

Anti-Ma2 antibodies react exclusively with Ma2 and are found in patients with testicular cancer who also have prominent limbic or brain-stem encephalitis (or both). Anti-Ma antibodies react with both Ma1 and Ma2 and are found in patients with diverse kinds of tumors (not testicular) in whom a predominantly paraneoplastic cerebellar degeneration and brain-stem encephalitis occur.¹³

days before testing, which ameliorated the neurologic symptoms and abnormalities on MRI.

We do not know whether anti-Ma2 antibodies, an associated cytotoxic T-cell response, or both cause the neurologic disease. The inflammatory infiltrates of one patient showed neurons closely surrounded by CD8+ T cells, suggesting that cytotoxic T cells may be the effectors of the neuronal damage. Three of the six families of previously identified “cancer-testis” antigens (MAGE, BAGE, and GAGE) were originally identified through the use of cytotoxic T cells to define antigens expressed by the tumor cells of one patient. The other three families of antigens (SSX2, NY-ESO-1, and SCP1) were identified from recombinant cDNA expression libraries with the use of serum samples from patients with cancer.²²

Our findings suggest that patients with symptoms of paraneoplastic limbic or brain-stem encephalitis, particularly if they are young men, should be examined for serum antibodies against Ma2. Detection of these antibodies supports the diagnosis of a paraneoplastic syndrome and guides the search for the tumor to the testis.

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Memorial Sloan-Kettering Cancer Center has an agreement with Athena Diagnostics (Worcester, Mass.) licensing it to use the Ma2 protein for diagnostic testing.

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REFERENCES

1. Darnell RB. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain. *Proc Natl Acad Sci U S A* 1996;93:4529-36.
2. Paraneoplastic syndromes. In: Posner JB. *Neurologic complications of cancer*. Philadelphia: F.A. Davis, 1995:353-85.
3. Dalmau JO, Posner JB. Paraneoplastic syndromes affecting the nervous system. *Semin Oncol* 1997;24:318-28.
4. Corsellis JAN, Goldberg GJ, Norton AR. “Limbic encephalitis” and its association with carcinoma. *Brain* 1968;91:481-96.
5. Bakheit AMO, Kennedy PGE, Behan PO. Paraneoplastic limbic encephalitis: clinico-pathological correlations. *J Neurol Neurosurg Psychiatry* 1990;53:1084-8.
6. Encephalomyelitis. In: Henson RA, Urich H. *Cancer and the nervous system: the neurologic manifestations of systemic malignant disease*. Oxford, England: Blackwell Scientific, 1982:314-45.
7. Dalmau J, Graus F, Rosenblum MK, Posner JB. Anti-Hu-associated paraneoplastic encephalomyelitis/sensory neuropathy: a clinical study of 71 patients. *Medicine (Baltimore)* 1992;71:59-72.
8. Alamowitch S, Graus F, Uchuya M, Reñé R, Bescansa E, Delattre JY. Limbic encephalitis and small cell lung cancer: clinical and immunological features. *Brain* 1997;20:923-8.
9. Dirr LY, Elster AD, Donofrio PD, Smith M. Evolution of brain MRI abnormalities in limbic encephalitis. *Neurology* 1990;40:1304-6.
10. Szabo A, Dalmau J, Manley G, et al. HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and sex-Lethal. *Cell* 1991;67:325-33.
11. Dropcho EJ, King PH. Autoantibodies against the Hel-N1 RNA-binding protein among patients with lung carcinoma: an association with type I anti-neuronal nuclear antibodies. *Ann Neurol* 1994;36:200-5.
12. Ahern GL, O'Connor M, Dalmau J, et al. Paraneoplastic temporal lobe epilepsy with testicular neoplasm and atypical amnesia. *Neurology* 1994;44:1270-4.
13. Dalmau J, Gultekin SH, Voltz R, et al. Ma1, a novel neuron- and testis-specific protein, is recognized by the serum of patients with paraneoplastic neurological disorders. *Brain* 1999;122:27-39.
14. Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB. The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuropathy) antigen in human normal and tumor tissues. *Am J Pathol* 1992;141:881-6.
15. Dalmau J, Furneaux HM, Gralla RJ, Kris MG, Posner JB. Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer — a quantitative Western blot analysis. *Ann Neurol* 1990;27:544-52.
16. Furneaux HM, 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.
17. Schüller E. A new strategy for the study of intrathecal immunity. In: Marrosu MG, Cianchetti C, Tavalato B, eds. *Trends in neuroimmunology*. New York: Plenum Press, 1990:3-12.
18. Lee LG, Connell CR, Woo SL, et al. DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. *Nucleic Acids Res* 1992;20:2471-83.
19. Burton GV, Bullard DE, Walther PJ, Burger PC. Paraneoplastic limbic encephalopathy with testicular carcinoma. *Cancer* 1988;62:2248-51.
20. Cattoretti G, Pileri S, Parravicini C, et al. Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J Pathol* 1993;171:83-98.
21. Bennett JL, Galetta SL, Frohman LP, et al. Neuro-ophthalmologic manifestations of a paraneoplastic syndrome and testicular carcinoma. *Neurology* 1999;52:864-7.
22. Chen Y-T, Gure AO, Tsang S, et al. Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library. *Proc Natl Acad Sci U S A* 1998;95:6919-23.