Major Histocompatibility Proteins, Anti-Hu Antibodies, and Paraneoplastic Encephalomyelitis in Neuroblastoma and Small Cell Lung Cancer

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Background. **Patients with neuroendocrine-related tumors and paraneoplastic encephalomyelitis (PEM) or paraneoplastic sensory neuronopathy (PSN) develop high titers of antibodies, called anti-Hu, against neuronal proteins expressed in their tumors, usually small cell lung cancer (SCLC). These tumors appear to be more indolent than those not associated with anti-Hu antibodies. The aims of this study were to determine 1) if patients with neuroblastoma (NB) also have anti-Hu antibodies, 2) the correlation between antibody titer and survival, and 3) if coexpression of Hu antigens and major histocompatibility proteins (MHC) by the tumor correlates with the development of anti-Hu associated PEM/PSN.**

Methods. **Using immunohistochemistry and Western blot analysis, the sera of 109 patients with NB whose neurologic condition was concealed at the time of the study were examined for the presence of anti-Hu antibodies. The expression of Hu antigens and MHC proteins in 50 nonselected NB and 26 SCLC (16 known to be from seropositive and 10 from seronegative patients) was examined using immunohistochemistry.**

Results. **Four Stage 4 NB patients were seropositive and had longer survival (median 86 months) than 71 seronegative patients in the same age group and with the same tumor stage (median survival, 28.5 months). Seventy-eight percent of NB and all SCLC expressed Hu antigens. Overall, 17 of 20 tumors from seropositive patients expressed both Hu and MHC Class I proteins, but only 4 of 30 tumors from seronegative patients expressed both proteins ($P < 0.0001$).**

Conclusions. **1) Some patients with NB develop anti-Hu antibodies; a search for that type of tumor is indicated in seropositive children, 2) most NBs and SCLCs express Hu antigens but only a few are associated with anti-Hu antibodies, and 3) Class I MHC expressed by some Hu antigen-bearing tumors may play a role in the development of anti-Hu associated PEM/PSN. *Cancer* 1995;75:99–109.**

Key words: anti-Hu antibody, HuD protein, paraneoplastic encephalomyelitis, neuroblastoma, small cell lung cancer, major histocompatibility complex.

Introduction

Patients with anti-Hu associated paraneoplastic encephalomyelitis (PEM) and/or paraneoplastic sensory neuronopathy (PSN) develop an intense immune response against HuD-related proteins (Hu antigens) expressed in their tumors and in neurons of the peripheral and central nervous system.[1–4] This immune response is characterized by high titers of anti-Hu antibodies in the serum and cerebrospinal fluid, deposits of anti-Hu IgG in the nervous system and tumor, neuronal degeneration, and inflammatory infiltrates in the nervous system.[4–7] In most patients (85%), the underlying tumor is small cell lung cancer (SCLC), but other neuroendocrine-related tumors are also involved.[8,9] Approxi-

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mately 15% of patients who have SCLC without neurologic dysfunction have low titers of anti-Hu antibodies in their sera.[4] Most of these patients' tumors are confined to the thorax at diagnosis. In contrast, more than 50% of patients who have SCLC without the anti-Hu antibody have systemic metastases at diagnosis.[4]

Previous studies demonstrated that all SCLC, whether associated with PEM/PSN or not, and four of eight neuroblastomas (NB) examined expressed Hu antigens.[9] The frequency of anti-Hu antibodies in patients with NB is unknown, and it is unclear why only a few patients with tumors expressing Hu antigens develop the anti-Hu immune response. Several studies have demonstrated the importance of major histocompatibility proteins (MHC) in regulating antitumor immune responses.[10–14] In the present study, we undertook to examine 1) the presence of anti-Hu antibodies in the sera of patients with NB and the correlation between antibody titer and survival, 2) the expression of Hu antigens in the tumors of these patients, and 3) the expression of MHC proteins in NB and SCLC either associated or not with PEM/PSN.

Materials and Methods

Sera, Tissues, and Antibodies

One hundred and ninety serum samples were obtained from 109 patients with NB. Tumor tissues from biopsy or autopsy were available from 50 of these patients. Ninety-four of 109 patients with NB were examined and followed clinically by one of the authors (N-K. V.C.). The other authors were masked regarding the neurologic condition of these patients at the time of the serologic and tumor studies. The sera and clinical information of the other 15 patients with NB were provided to us by the patients' physicians.

Sera and tumors were obtained from 26 patients with SCLC whose neurologic conditions and anti-Hu serology were previously known; 15 had PEM/PSN associated with high titers of anti-Hu antibodies in their sera; 10 patients (one of them affected with Lambert–Eaton myasthenic syndrome [LEMS]) had no symptoms of PEM/PSN and no detectable titer of anti-Hu antibody, and 1 patient without neurologic symptoms had a low titer of anti-Hu antibody in his serum.

Serum from seven healthy adults and three healthy children served as controls. Normal tissue samples, including human brain, kidney, and lymph node, were obtained from biopsy or autopsies of neurologically normal adult individuals. Sera were kept frozen at −70°C until use. Tissues were embedded in Optimal Cutting Temperature compound (Miles Inc., Elkhart, IN), snap-frozen in isopentane chilled with liquid nitrogen, and kept at −70°C.

Total immunoglobulin G (IgG) was isolated from sera of patients with anti-Hu associated PEM and sera from normal individuals (blood donors) by adsorption to protein A sepharose gel columns. After IgG isolation and quantitation, both the IgG from anti-Hu patients and from normal individuals were labeled with biotin as previously described.[15] Therefore, the same type and amount of IgG from patients who had the anti-Hu antibody and from patients who did not have this antibody were used.

The expression of MHC proteins by tumor tissue was studied by immunohistochemistry using the mouse monoclonal antibodies W6/32 (Serotec, UK) and HLA-DR (Becton/Dickinson, San Jose, CA).

Informed consent for the respective protocols was obtained from all patients who underwent surgery or received chemotherapy (or their guardians). For serologic studies, serum was obtained as part of the initial and follow-up serum banking, or as part of the diagnostic work-up. There is no formal Institutional Review Board protocol for this retrospective study.

Western Blot

Proteins from a crude neuronal preparation (or HuD recombinant protein) were resolved in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters.[16] After blocking with 5% Carnation evaporated milk (Carnation Company, Glendale, CA) overnight at 4°C, the filters were cut in strips and incubated with patient's serum diluted to 1:500 in 10 mM Tris-HCl (pH 7.4), 1% bovine serum albumin, 0.9% NaCl, 0.5% Triton X-100 buffer for 1 hour at room temperature. Strips were then washed in 10 mM Tris-HCl (pH 7.4), 1% bovine serum albumin, 0.9% NaCl, 0.5% Triton X-100 buffer, incubated with ^{125}[I] protein A (0.1 μCi/ml) for 1 hour at room temperature, washed, dried, apposed to Kodak XAR5 film (Sigma, St. Louis, MO), and exposed for 4 hours at −70°C.

Immunohistochemistry

To study the expression of the Hu antigens by tissues, 7 μm-thick frozen tissue sections were fixed for 10 minutes in cold acetone (4°C), and after washing with phosphate-buffered saline, sequentially incubated with 0.3% hydrogen peroxide (to avoid endogenous peroxidase activity), 10% normal human serum (to prevent nonspecific binding of IgG), and biotinylated anti-Hu IgG (5 μg/ml in 10% normal human serum) for 4 hours at room temperature. After washing, sections were incubated with avidin-biotin peroxidase (Vector Labs, Burlingame, CA) for 30

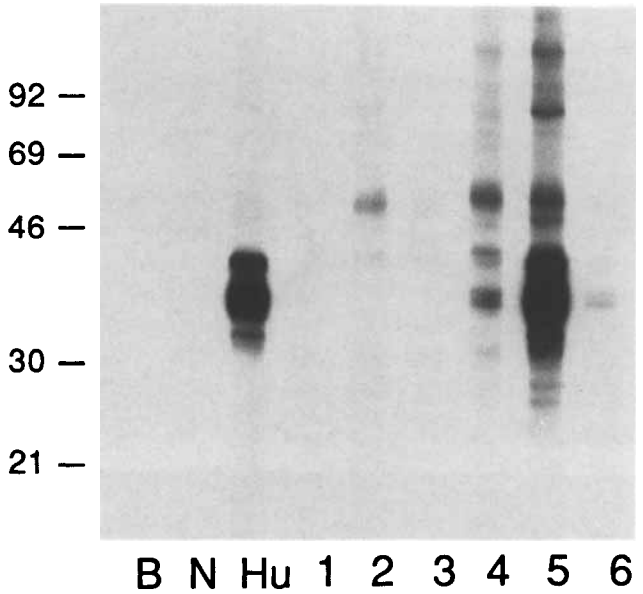

Figure 1. Western blot analysis of the presence of the anti-Hu antibody in the sera of patients with NB. Immunoblots containing 5 μg of crude neuronal preparation were exposed to serum (diluted 1:1000) from six patients with NB (lanes 1–6). The sera corresponding to lanes 1–3 had no detectable anti-Hu antibodies. Lanes 4 and 6 correspond to two patients with low titer of anti-Hu antibodies who did not have paraneoplastic symptoms. The serum shown in lane 5 corresponds to a patient with paraneoplastic brain stem encephalopathy. Negative controls included lane B, which did not contain serum, and lane N, which shows serum from a normal individual. Lane Hu corresponds to serum from a SCLC patient with anti-Hu associated PEM and served as positive control.

minutes at room temperature, and the substrate developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma) with 0.01% hydrogen peroxide. Slides were counterstained with hematoxylin and mounted with Permount (Fisher, Springfield, NJ). Sections incubated with biotinylated normal human IgG served as negative controls.

To study the expression of MHC proteins by tumors, tissue sections were fixed with acetone (4°C) and sequentially incubated with 0.3% hydrogen peroxide, 10% normal horse serum, and the mouse monoclonal antibodies W6/32 (MHC Class I) diluted to 1:60, or HLA-DR (MHC Class II) diluted to 1:60. After washing, sections were incubated with biotinylated horse anti-mouse IgG (diluted 1:2000) and the reaction developed as described above. Sections of normal brain, lymph node, and kidney served as tissue controls.

Medical records of the patients were reviewed after the above studies were completed.

Results

The Presence of Anti-Hu Antibodies in the Sera of Patients with NB

Four of 109 (4%) patients with NB had detectable titers of anti-Hu antibody in their sera (Fig. 1). A 10-fold difference was found between the titer (1270 U anti-Hu/ml) of the anti-Hu serum control (lane Hu) and three of the patients with NB (< 120 U/ml; two shown in lanes 4 and 6). The fourth patient (lane 5) had the highest titer of anti-Hu antibody (3120 U/ml), and was subsequently identified as a patient with brain stem encephalopathy previously reported by us[8] (see below).

Review of the clinical records demonstrated that the patient with the highest titer of antibody had symptoms of brain stem encephalopathy, including head and truncal myoclonus and supranuclear and internuclear gaze paresis. One of the three patients with a low titer of antibodies had paraneoplastic opsoclonus-myoclonus. Bacterial meningitis followed by lethargy of unknown etiology that lasted for 2 months developed in another patient, who recovered spontaneously, and no paraneoplastic symptoms developed in the other patient. In these patients, follow-up anti-Hu serologies remained positive irrespective of whether the sera were obtained at diagnosis or during treatment with intensive chemotherapy and/or radiation therapy (not shown). The 4 seropositive sera were Stage 4 NB. Among the 105 seronegative patients, 8 had Stage 1 NB, 12 had Stage 2, 6 had Stage 3, 71 had Stage 4, 7 had Stage 4s, and for 1 patient the stage was unknown. Paraneoplastic opsoclonus-myoclonus developed in two of the seronegative patients. The four patients with detectable levels of anti-Hu antibodies survived longer (median survival 86 months versus 28.5 months for the seronegative group).

Expression of Hu Antigens and MHC Proteins by NB

Using immunohistochemistry, 39 out of 50 (78%) NBs were found to express the Hu antigens (Fig. 2, top panels). The tumors of all four anti-Hu positive patients expressed the Hu antigen (Fig. 2, Top left). The reactivity of the Hu positive tumors with the anti-Hu IgG was similar in patients with and without the antibody in their sera. There was no correlation between the expression of Hu antigens by tumors and stage of the disease (Table 1). The expression of MHC Class I and II was studied in 24 NB tumors, including the 4 tumors associated with the presence of anti-Hu antibodies and 20 tumors from patients without detectable antibodies (Table 2). Two of the four tumors associated with anti-Hu antibodies expressed MHC Class I proteins (Fig. 2, Bottom left). Among the 20 tumors from patients without detectable anti-Hu antibodies (Fig. 2, Bottom right), only 1 had mild level of coexpression of Hu antigens and MHC Class I proteins (not shown). None of the 24 NB expressed MHC Class II proteins.

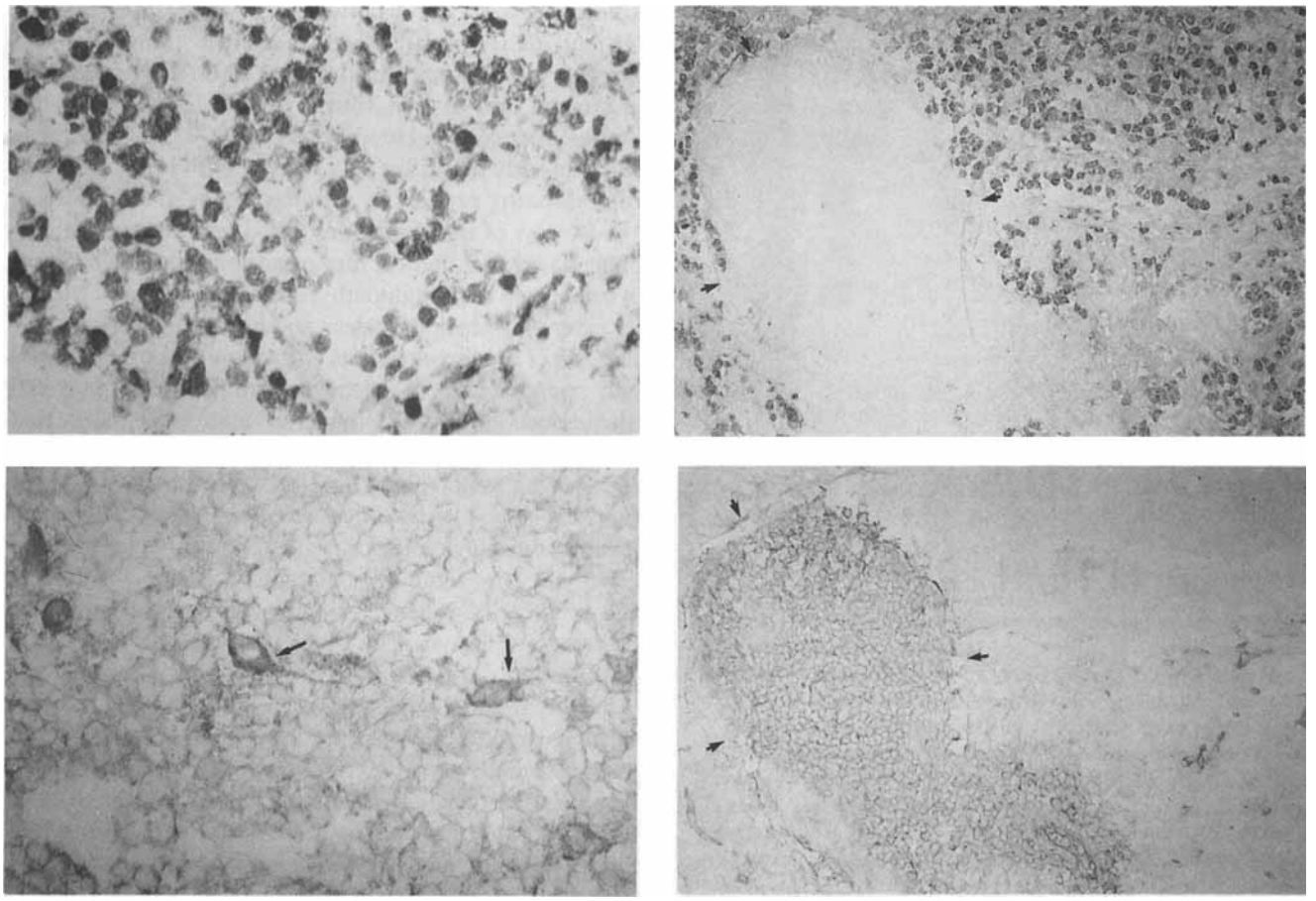

Figure 2. Top left, sections of NB from a patient who had the anti-Hu antibody; the section was immunoreacted with biotinylated anti-Hu IgG to show the distribution of the tumor cells. Bottom left, the section was immunoreacted with the antibody W6/32 and showed immunostaining of the cell surface of the tumor cells indicating expression of MHC Class I proteins. No inflammatory infiltrates and normal tissue were identified in sections A and B (not shown). Note in Bottom left that in addition to cell surface expression of MHC Class I proteins, a few large neuroblastoma cells had expression of these proteins in the cytoplasm (arrows). Top right, consecutive sections of NB from a patient who did not have the anti-Hu antibody demonstrated that although most of the tumor cells contained Hu antigens, they did not express MHC Class I proteins (Bottom right). Arrows in right panels delineate infiltrates of normal cells (lymphocytes) that did not express Hu antigens but had intense expression of MHC Class I proteins. Sections were not counterstained (left panels, original magnification ×200; right panels, ×100).

Expression of Hu Antigens and MHC Proteins by SCLC

All SCLC tumors were found to express Hu antigens (Fig. 3, top panels). In the 15 tumors of patients with PEM/PSN the majority of the cells had intense expression of the Hu antigens, whereas in the 11 non-PEM/PSN tumors (including 1 with LEMS) the expression of the Hu antigens was less intense and more heterogeneous. In these tumors, areas of Hu positive cells were intermixed with areas of morphologically identical cells with less or no Hu reactivity (not shown).

Table 1. Stage of Disease and Expression of Hu Antigens in Neuroblastomas

Stage of disease	Number of tumors	Hu positive	Hu negative
1	5	4	1
2	5	4	1
3	2	2	0
4	34	26*	8
4s	4	3	1

* The four anti-Hu seropositive patients were included in this group.

In 13 out of 15 paraneoplastic SCLC most (90%) neoplastic cells expressed MHC I proteins (Fig. 3, Bottom left); 1 tumor had very weak and heterogeneous expression of these proteins, and 1 tumor did not express them at all (Table 3). By contrast, 7 out of 11 non-PEM/PSN tumors did not express MHC-I molecules (Fig. 3, Bottom right, Table 4), and the other 4 had very weak expression of these proteins in less than 15% of neoplastic cells. One of these four tumors came from a patient without paraneoplastic symptoms who had a

Table 2. Expression of MHC and Hu Antigens in Neuroblastomas of Anti-Hu Seropositive and Seronegative Patients

Total neuroblastoma sera	109
Sera anti-Hu positive	4
Tumor expression of Hu Ag	4/4
Tumor expression of MHC I	2/4
Tumor expression of MHC II	0/4
Sera anti-Hu negative	105
Tumor expression of Hu Ag	39/46
Tumor expression of MHC I	1/20*
Tumor expression of MHC II	0/20*

* These 20 tumors were randomly taken from the 46 NBs not associated with the presence of antibodies.

low titer of anti-Hu antibody in his serum but no detectable antibody in the cerebrospinal fluid; the other three (one of them associated with LEMS) had very weak and heterogeneous expression of MHC Class I proteins and had large areas of anti-Hu reactive cells that did not express MHC-I proteins. Focal expression of MHC Class II proteins was observed in SCLC cells of 6 of 15 PEM/PSN patients, but not in any of the nonparaneoplastic tumors (Tables 3 and 4).

SCLC patients with PEM/PSN and patients without neurologic symptoms were similar in many respects except for the type of treatment administered and the the stage of the tumor at autopsy (Tables 3 and 4). For all the PEM/PSN patients neurologic symptoms developed before the tumor diagnosis (1–40 months, median 6 months). Detection of the anti-Hu antibody directed

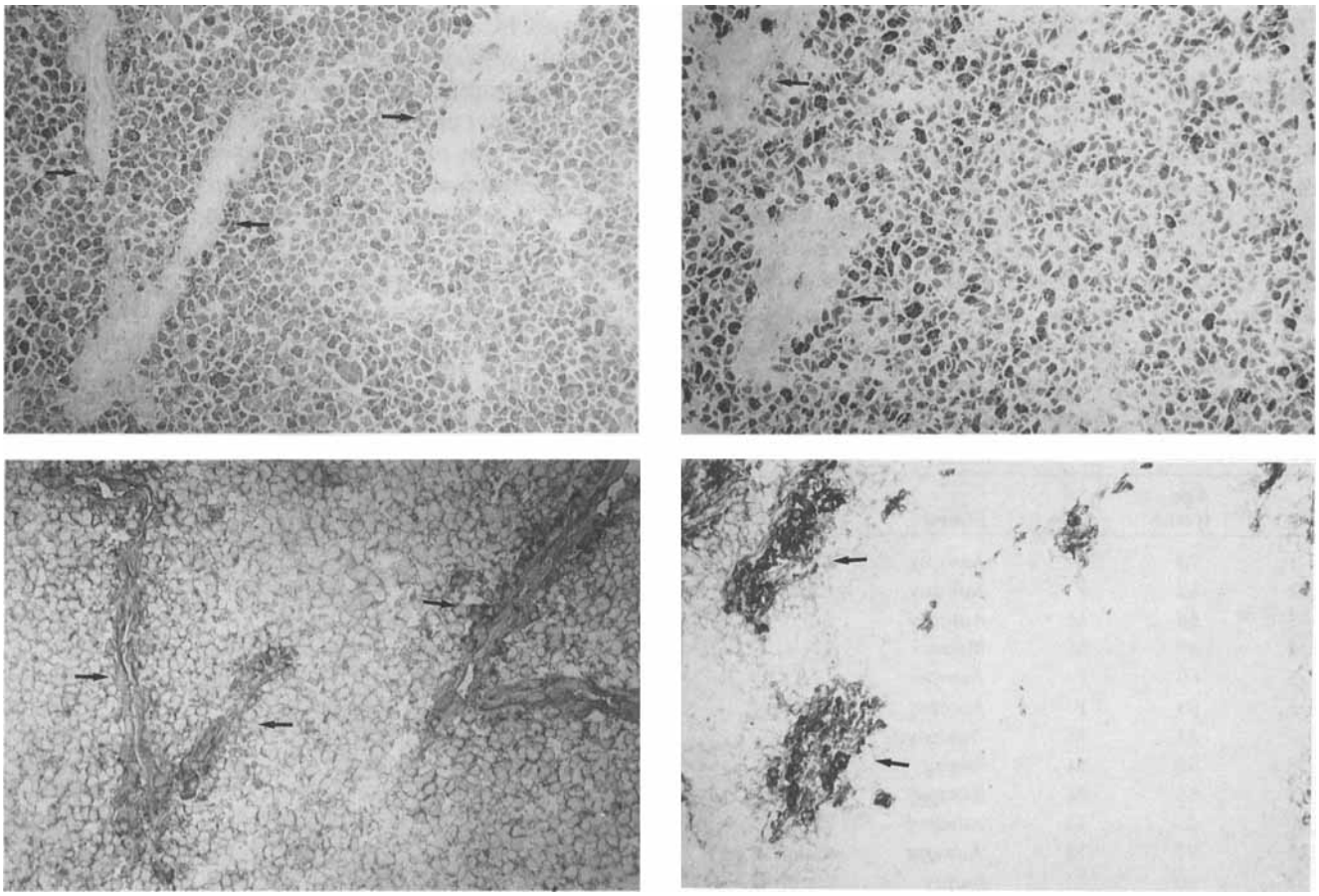

Figure 3. Top left, section of SCLC from a patient with anti-Hu associated PEM; the section was immunoreacted with biotinylated anti-Hu IgG to show the distribution of the tumor cells. Bottom left, the section was immunoreacted with the antibody W6/32 and showed immunostaining of the cell surface of the tumor cells indicating expression of MHC Class I proteins. Top left, normal tissue (blood vessels) indicated by arrows did not react with the anti-Hu antibody but had intense expression of MHC Class I proteins in Bottom left. A similar study using consecutive sections of tumor from a patient who did not have anti-Hu antibodies demonstrated that although most of the tumor cells expressed Hu antigens (Top right), they did not express MHC Class I proteins (Bottom right). Arrows in Top right and Bottom right delineate normal tissue (blood vessels and perivascular lymphocytes) and demonstrate that normal tissue did not express Hu antigens but had intense expression of MHC Class I proteins. Sections were not counterstained (all panels, original magnification ×200).

the search for a SCLC in all patients, but in nine the tumor could not be demonstrated until autopsy. Most (12/15) PEM/PSN patients did not receive chemotherapy because of the severity of their neurologic condition and difficulties in demonstrating the presence of the tumor. However, many of these patients received immunosuppressive treatments, including plasmapheresis, cyclophosphamide, intravenous immunoglobulin, and steroids. Only 3 of the 12 patients with PEM/PSN whose autopsies were available had evidence of disease outside of the thoracic cavity. In the non-PEM/PSN group, the majority of the patients received chemotherapy and had widespread metastasis involving multiple organs (Tables 3 and 4).

Overall, including NB and SCLC, 17 of 20 tumors from seropositive patients were found to express both Hu antigens and MHC Class I proteins, but only 4 of 30 tumors not associated with the anti-Hu immune response expressed both proteins (chi-square, $P < 0.0001$) (Table 5).

Discussion

In 1985, Graus et al.[2] demonstrated that the serum and cerebrospinal fluid of patients with SCLC and PSN contained an antineuronal antibody that was named anti-Hu.[17] The anti-Hu antibody reacts with 35–40 kd proteins expressed in the tumor and neurons of the central and peripheral nervous system.[3] In immunohistochemical analysis of sections of cerebral cortex, the anti-Hu antibody reacts with the nuclei of neurons, sparing the nucleoli, and to a lesser degree with the cytoplasm of neurons.[2,9] Because there are other antibodies with identical immunohistochemical characteristics but no specific tumor association,[18,19] the presence of anti-Hu antibodies in serum and cerebrospinal fluid must be studied by immunohistochemistry and Western blot analysis of cortical neurons, or by Western blot of a cloned recombinant protein, named HuD.[1] Although the exact role of HuD and other related proteins has not been established, their early expression in vertebrate neurogenesis[20–22] and their homology to Drosophila proteins involved in maturation and maintenance of neurons,[1] have suggested that the Hu antigens play a role in neuronal development.

In a study of 71 patients with anti-Hu associated PEM/PSN,[8] neurologic symptoms developed before the diagnosis of the tumor in 83% of the patients. In 60% the detection of the antibody prompted the search for the SCLC, which usually remained localized until death, or was demonstrated only at autopsy. Autonomic and respiratory failure secondary to the paraneoplastic disorder and not the underlying cancer, were the principal causes of death. This and other studies,[4,8,17,23] which included more than 400 controls, demonstrated a specific association between high titers of anti-Hu antibodies and PEM/PSN in SCLC patients.

These findings suggest that the ectopic expression of Hu antigens by the neoplasm triggers an immunologic reaction against the tumor that crossreacts with

Table 3. Expression of MHC Antigens by SCLC of Patients With Anti-Hu Associated Paraneoplastic Encephalomyelitis

No.	Age (yrs)	Sex	Source	Location	MHC I	MHC II	Chemo	Immuno	Stage*
1	70	M	Autopsy	LN	+++	+	No	No	Limited
2	62	F	Autopsy	LN/lung	++	–	No	Yes	Limited
3	58	M	Autopsy	LN	++	–	No	Yes	Limited
4	49	M	Biopsy	LN	+++	+	No	Yes	NED
5	60	F	Autopsy	LN	+++	–	No	No	Limited
6	83	F	Autopsy	LN/lung	+++	+	Yes	Yes	Limited
7	64	M	Autopsy	Lung	++	–	No	No	Extens
8	40	M	Biopsy	Adrenal	–	–	Yes	No	Extens
9	55	M	Autopsy	Lung	+++	+	Yes	Yes	Limited
10	65	M	Autopsy	LN	+++	–	No	Yes	Limited
11	71	M	Autopsy	Lung	+++	–	No	Yes	Extens
12	60	M	Biopsy	Lung	+++	+	No	No	Limited
13	58	M	Autopsy	LN	+++	+	No	No	Limited
14	78	F	Autopsy	LN/lung	+†	–	No	No	Extens
15	61	F	Autopsy	LN/lung	++	–	No	Yes	Limited

LN: lymph node; +++: intense reactivity; ++: moderate activity; + weak reactivity; NED: no evidence of disease; Extens: extensive (disease beyond one hemithorax and the regional lymph nodes); chemo: chemotherapy; Immuno: Immunosuppressive therapies including plasmapheresis, cyclophosphamide, intravenous immunoglobulin, or steroids.

* Tumor staging at the time tissue was obtained for analysis.

† Weak and heterogeneous MHC I reactivity.

Table 4. Expression of MHC Antigens by SCLC of Patients Without Paraneoplastic Encephalomyelitis

No.	Age (yrs)	Sex	Source	Location	MHC I	MHC II	Chemo†	Stage
1	71	M	Autopsy	LN/lung	+*	–	No	Limited
2	65	F	Biopsy	Lung	–	–	Yes	Limited†
3	69	F	Autopsy	LN/lung	–	–	Yes	Extensive
4	68	F	Autopsy	Lung	–	–	Yes	Extensive
5	78	M	Autopsy	LN/lung	+*	–	No	Extensive
6	68	M‡	Biopsy	Lung	+*	–	Yes	Extensive
7	40	F	Biopsy	Lung	–	–	Yes	Extensive
8	62	M§	Autopsy	LN/lung	+*	–	Yes	Extensive
9	59	F	Biopsy	LN	–	–	Yes	Extensive‖
10	56	M	Biopsy	Lung	–	–	No	Limited
11	50	F	Biopsy	Brain met	–	–	Yes	Extensive

Met: metastasis.

* Less than 15% of cells had weak and heterogeneous reactivity.

† Patient in addition to SCLC had a second primary tumor involving the vocal cords.

‡ Patient with low titer of anti-Hu antibody in serum; no anti-Hu in CSF.

§ Patient with LEMS.

‖ This patient in addition to SCLC had two other primary tumors, adenocarcinoma of the lung and squamous cell carcinoma of the base of the mouth.

the nervous system and results in the neurologic disorder. This immunologic reaction is characterized by intrathecal synthesis[24] and deposits of anti-Hu IgG in the nervous system and tumor,[5] and the presence of conspicuous inflammatory infiltrates mainly composed of T-cells in the nervous systems of these patients.[6,7]

However, the exact role of the anti-Hu antibody in the pathogenesis of the disease remains unknown. Passive transfer of anti-Hu IgG to animals does not reproduce the disease. Animals immunized with HuD recombinant protein made in bacteria develop antibodies but do not develop neurologic symptoms.[25]

For the clinicians, however, the anti-Hu antibody is a useful biologic marker. Because anti-Hu associated PEM/PSN usually precedes the diagnosis of the neoplasm, detection of high titers of anti-Hu antibodies indicate the paraneoplastic origin of a neurologic disorder and the presence of an underlying tumor, which in the adult population is almost always SCLC.[8,17,23] If a tumor other than SCLC is detected, but does not express Hu antigens, the presence of a second neoplasm (probably SCLC) must be considered.[8]

Table 5. Correlation Between Tumor Coexpression of MHC Class I and Hu Antigens and Anti-Hu Serology

	Anti-Hu serology	
Coexpression	*Positive**	*Negative*
Positive	17	4
Negative	3	26†

Chi-square: $P < 0.0001$.

* All tumors expressed Hu antigens.

† Thirteen tumors were Hu−/MHC−, 13 were Hu+/MHC−.

Studies including different histologic types of tumors demonstrated that all SCLC, whether they come from patients with paraneoplastic symptoms or not, express the Hu antigens,[9,26] and that 16% of patients with SCLC but without neurologic dysfunction develop low titers of anti-Hu antibodies.[4] There was no overlap between the titers of anti-Hu antibodies in SCLC patients with PEM/PSN (average titer, 4,592 U/ml) and those without neurologic symptoms (average titer, 76 U/ml).[4] All patients with low titer of anti-Hu antibody had the tumor confined to thorax at diagnosis. In contrast, more than 50% of patients with SCLC without the anti-Hu antibody had systemic metastases at diagnosis.[4] These findings and the tendency of SCLC from patients with PEM/PSN to remain localized or undetectable until autopsy[5,9] suggest that the antitumor immune response contributes to the more indolent course of these neoplasms.

The present study demonstrates that although 78% of NBs express the Hu antigens, less than 4% of patients have detectable titers of anti-Hu antibodies in their sera. Paraneoplastic symptoms, when present, are associated with higher titers of anti-Hu antibodies, which also occurs with SCLC patients.[4] The current study, however, does not reflect the incidence of anti-Hu antibody in the sera of patients with SCLC because this group of patients was selected solely for availability of tumor tissue with which to examine the expression of Hu and MHC proteins.

All four anti-Hu seropositive NB patients were age

1 year or older when diagnosed. Patients with Stage 4 NB who were diagnosed when they were older than one year constitute the majority of the patients with this disease.[27] They also constitute 63% of the patients in this study. Because the biology of the tumor in this group is relatively homogeneous, namely resulting in death for most patients (80% of those diagnosed older than 1 year of age, and 90% of those diagnosed older than 2 years of age),[27] the impact of positive titer was analyzed among these subgroups. The median survival of seropositive patients was higher than that of seronegative patients whether one considers Stage 4 diagnosed after one year of age or Stage 4 diagnosed after 2 years of age. However, because the number of seropositive patients is small and the analysis is retrospective, there may be confounding variables that cannot be ruled out. A much larger set of samples needs to be studied before a firm conclusion can be drawn.

Three of 109 patients with neuroblastoma had opsoclonus; one of these patients had low titer of anti-Hu antibodies. Based on studies in adults,[28–31] we believe that the association between opsoclonus and low titer of anti-Hu antibodies is coincidental. Although 30% of adult patients with anti-Hu associated PEM develop brain stem symptoms,[8] there is only one report of anti-Hu associated ataxia, encephalopathy, and opsoclonus-myoclonus.[32] Most adults with paraneoplastic opsoclonus do not have anti-Hu antibodies, even when the underlying tumor is SCLC.[28–30] Another possibility is that opsoclonus and anti-Hu antibodies (related with PEM or not) represent two paraneoplastic disorders with different pathogenic mechanisms. The association of LEMS and anti-Hu associated PEM/PSN[8,33,34] or LEMS and cerebellar degeneration are other examples of this possibility.[35]

In a study of 71 patients with anti-Hu associated PEM/PSN, only one (the same identified in the present study) had NB.[8] Since then, we have examined the sera of three other children with high titers of anti-Hu antibodies associated with paraneoplastic neurologic symptoms. Two of them had neuroblastoma and one had no detectable tumor. One of the two patients with neuroblastoma developed seizures, hearing loss, tonic pupils, areflexia and opsoclonus-myoclonus,[36] and the other had proximal muscle weakness and electrophysiologic signs of denervation (Alan S. Wayne M.D. and Joseph A. Casadonte M.D., Children's Hospital, St. Petersburg, FL, personal communication). Overall, among 130 patients with anti-Hu associated PEM/PSN whose sera we examined, only 3 had NB; SCLC was the tumor involved in 90% (Dalmau et al., unpublished data).[8] Furthermore, the low number (4%) of NB patients with detectable titers of anti-Hu antibodies identified in the present study is substantially smaller than that found in patients with SCLC (16%).[4]

These findings and the present study raise an interesting question. If three fourths of NBs express the Hu antigens, why is the incidence of anti-Hu associated PEM/PSN in these patients much smaller than in patients with SCLC? One theory could be that most NBs express similar, but not identical, Hu antigens to that expressed in SCLC. The Hu antigens identified by the anti-Hu antibody comprise a family of neuronal specific RNA binding proteins, including HuD, HuC, and Hel N-1.[1,37–40] These proteins are the product of three different genes, and they are identical to each other in more than 90% of their amino acid sequence.[1,37–40] Preliminary studies indicate that paraneoplastic and non-paraneoplastic SCLC express HuD, but not HuC and Hel N-1,[39] and that the structure of HuD mRNA expressed in NB cell lines does not differ from HuD mRNA expressed in SCLC[41] (Manley et al., unpublished data).

It is also unclear why most Hu antigen-bearing NBs are not associated with detectable titer of antibodies. One explanation could be that the synthesis of anti-Hu antibodies is transitory and the window of detectability much narrower than in other patients. In this case, a number of patients with NB with transitory titers of anti-Hu would have been missed in our study. However, our experience with patients who have SCLC who remain anti-Hu negative in serial serologic studies (Dalmau and Posner, unpublished observations), and the persistence of detectable titers of anti-Hu antibodies in those who are found positive argue against, but do not completely rule out, this possibility. Another explanation is that factors related to the immunologic system of the patient such as the HLA haplotype may play a role in the development of antibodies in only a small number of patients.

The development of the anti-Hu immune response by some but not all Hu-antigen bearing tumors may depend on the ability of the tumor to present the antigen to the immunologic system. Although only four patients with NB had anti-Hu antibodies, it is notable that the tumors of two of these patients (one of them with high titers of antibody and paraneoplastic brain stem encephalopathy) expressed both MHC-I and Hu antigens, while the tumors of most seronegative patients did not express MHC proteins. The paucity of expression of MHC Class I by most NBs is consistent with previous studies using NB cell lines.[42] To determine if the expression of MHC Class I may play a role in regulating the anti-Hu immune response, we also examined the expression of MHC proteins in SCLC. We selected this type of tumor for two reasons: (1) SCLC is the neoplasm most frequently involved in anti-Hu associated PEM/

PSN,[8] and (2) all SCLC, regardless of their association with the presence of anti-Hu antibodies or not, express the Hu antigens.[9,26] Our findings indicate that there is a correlation between the expression of both MHC Class I and Hu antigens and the development of the anti-Hu immune response. The positive expression of MHC Class I by most anti-Hu paraneoplastic SCLCs contrasts with previous studies of MHC expression by SCLCs. Most of those studies done using SCLC cell lines demonstrated that MHC proteins are never or rarely expressed by SCLCs.[43–45] It is known that tumors with low or no expression of MHC Class I proteins had a growth advantage and increased metastatic potential compared to tumors expressing these proteins.[10–12,46,47] Consistent with these findings, it is notable that SCLC and NB, which usually have low or no expression of MHC Class I also have widely metastatic properties.[42–44,48,49] In contrast to these studies, however, Morris et al.[50] found that nine out of ten SCLC (five of them associated with LEMS) expressed MHC Class I proteins, and that the expression of these proteins was greater in the nonparaneoplastic tumors.

The correlation found in our study between expression of MHC Class I proteins and the anti-Hu immune response in SCLCs and NBs suggest that in addition to a humoral anti-Hu response, there is a potential for a T-cell mediated cytotoxic response. MHC Class I molecules are required for the presentation of viral or tumor neoantigens to cytotoxic T lymphocytes.[51,52] Whether the Hu antigens can function as targets of a cytotoxic T-cell response remains unknown.

There is recent evidence that carcinomas derived from MHC Class II-negative normal tissues can express MHC Class II proteins.[13,53] The abnormal expression of MHC Class II proteins by tissues has been considered secondary to cytokine release from activated infiltrating lymphocytes,[54] or to the endogenous secretion of anti-tumor factors.[14] In models of autoimmune disease, self antigens can be presented by MHC Class II-bearing cells and induce autoimmunity.[55] Furthermore, enhanced expression of MHC Class II proteins after tumor transduction with the interferon-γ gene, promoted antigen presentation to tumor infiltrating CD4+ lymphocytes (helper T-lymphocyte).[56] According to these findings, the expression of MHC Class II proteins by 6 of 15 SCLC associated with anti-Hu PEM may have contributed to the anti-Hu immune response. We did not find, however, that these tumors had larger inflammatory infiltrates than other MHC Class II negative tumors, suggesting that the expression of these proteins was not induced by cytokine release from infiltrating lymphocytes (Dalmau and Graus, unpublished data).

Although anti-Hu related PEM/PSN rarely develops in association with NB, for the clinicians a practical implication of this study is that children in whom neurologic symptoms associated with the anti-Hu antibody develop should be studied for the presence of NB. If the tumor is not detected, periodic follow-up of urine catecholamines and serum tumor markers is recommended.

The treatment of the paraneoplastic disorder with steroids, plasmapheresis, and intravenous immunoglobulin has been unrewarding.[8,57,58] However, the detection of anti-Hu antibodies may lead to the early diagnosis and treatment of the tumor, which may improve the patient's prognosis and, in a few instances, stabilize or improve the neurologic disorder.[59–61]

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