

Identification of β -Tubulin Isoforms as Tumor Antigens in Neuroblastoma

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ABSTRACT

There is currently substantial interest in the identification of human tumor antigens for diagnosis and immunotherapy of cancer. We have implemented a proteomic approach for the identification of tumor proteins that elicit a humoral response in cancer patients, which we have applied to neuroblastoma. Proteins from neuroblastoma tumors and cell lines were separated by two-dimensional PAGE and transferred to poly(vinylidene difluoride) membranes. Sera from 23 newly diagnosed patients with neuroblastoma, from 12 newly diagnosed children with other solid tumors, and from 13 normal individuals were screened for IgG and IgM autoantibodies against neuroblastoma proteins by means of Western blot analysis. Sera from 11 patients with neuroblastoma and from 1 patient with a primitive neuroectodermal tumor, but none of the other controls exhibited IgG-based reactivity against a protein constellation with an estimated M_r 50,000. NH_2 -terminal sequence and mass spectrometric analysis identified the major constituents of this constellation as β -tubulin isoforms I and III. The IgG antibodies were additionally characterized to be of the subclass IgG1. Neuroblastoma patient sera that contained anti- β -tubulin IgG antibodies also contained IgM antibodies specific against the full-length β -tubulin molecule and against COOH-terminal β -tubulin cleavage products. Neuroblastoma patient sera that reacted with β -tubulin I and III isoforms in neuroblastoma tissues did not react with β -tubulin I and III isoforms found in normal brain tissue. Our findings indicate the occurrence of β -tubulin peptides in neuroblastoma, which are immunogenic. The occurrence of immunogenic peptides in neuroblastoma may have utility in diagnosis and in immunotherapy of this aggressive childhood tumor.

INTRODUCTION

There is increasing evidence for a humoral immune response to cancer in humans, as demonstrated by the identification of antibodies against a number of intracellular and surface antigens in patients with various tumors (1–4). For example, somatic alterations in the *p53* gene elicit a humoral response in 30–40% of affected patients (5). The detection of anti-p53 antibodies can predate the diagnosis of cancer (6, 7). The majority of tumor-derived antigens that have been identified and that elicit a humoral response are not the products of mutated genes. They include differentiation antigens and other gene products that are overexpressed in tumors (8). It is not clear why only a subset of patients with a tumor type develop a humoral response to a particular antigen. Factors that influence the immune response may include variability among individuals in major histocompatibility complex molecules. It is also possible that proteins may become immunogenic after undergoing a posttranslational modification, a process that may be variable among tumors of a similar type.

Neuroblastoma is a common childhood tumor of ectodermal origin that exhibits substantial heterogeneity. Some neuroblastomas undergo spontaneous regression and others are highly aggressive. A number of molecular alterations including *N-myc* amplification and chromosomal gains and losses have been identified that may affect tumor behavior. The role of immunological factors in neuroblastoma tumor control remains to be determined. There is evidence that normal human serum contains a natural IgM antibody that is cytotoxic for human neuroblastoma (9). Moreover, serum autoantibodies have been detected in children with opsoclonus-myoclonus syndrome, including some with neuroblastoma (10–13). In a study of children with opsoclonus-myoclonus syndrome, Western blot analysis revealed a distinctive pattern of binding to an antigen with a M_r 210,000, which was identified as the high-molecular-weight subunit of neurofilament (10). These findings raise the possibility that an immune response to neuroblastoma-derived antigens may affect the clinical manifestations of the disease. The identification of autoantibodies against neuroblastoma antigens may have value in neuroblastoma screening or diagnosis or in assessing prognosis. Additionally, identification of neuroblastoma antigens may have utility in antigen-based immunotherapy against the disease.

We have used two-dimensional PAGE to simultaneously separate several thousand individual cellular proteins from tumor tissue or tumor cell lines. With this approach, serum from cancer patients is screened for antibodies that react against separated tumor proteins by Western blotting. Proteins that specifically react with sera from patients with the same tumor type are identified by amino acid sequence and/or mass spectrometric analysis. We have applied this strategy to neuroblastoma. In this study we report the identification of autoantibodies

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to β -tubulin isoforms of both the IgG (specifically IgG1) and IgM classes in serum of patients with neuroblastoma.

MATERIALS AND METHODS

Materials. All of the cell culture reagents, including DMEM (containing L-glutamine, sodium pyruvate, and pyridoxine hydrochloride), Dulbecco's PBS, FCS, and penicillin/streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY). The mouse monoclonal anti- β -tubulin isotype I and II, the anti- β -tubulin isotype III, the antihuman IgG, and the antihuman IgM antibodies were purchased from Sigma Chemical Company (St. Louis, MO). The monoclonal antibodies to human IgG1, IgG2, IgG3, and IgG4 antibodies were obtained from Zymed Laboratories Incorporated (San Francisco, CA). The mouse monoclonal TU-20 (anti- β -tubulin isotype III) antibody was obtained from Accurate Chemical (Westbury, NY). Monoclonal antiacetylated tubulin and monoclonal antiphosphoserine antibodies were from Sigma Chemical Company. The E7 anti- β -tubulin monoclonal antibody developed by Klymkowsky *et al.* was obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Iowa City, Iowa) in the form of hybridoma supernatant. The MABTrap G-II column was obtained from Amersham-Pharmacia, and cyanogen bromide-activated Sepharose 4B beads purchased from Sigma. The horseradish peroxidase-conjugated sheep antihuman IgG and the ECL kits were obtained from Amersham (Arlington Heights, IL). The Immobilon-P PVDF² membranes were purchased from Millipore Corp. (Bedford, MA). The acrylamide used in the first-dimension electrophoresis, urea, ammonium persulfate, and piperazine diacrylamide were purchased from Bio-Rad (Rockville Center, NY). The acrylamide used in the second-dimension electrophoresis was purchased from Serva (Crescent Chemical, Hauppauge, NY), and the carrier ampholytes (pH 4–8) and NP40 were both purchased from Gallard/Schlessinger (Carle Place, NY). The enzymes *O*-glycosidase (BSA-free) and endoglycosidase F (recombinant) were obtained from Roche Diagnostics. All of the other reagents and chemicals were obtained from either Fisher or Sigma and were of the highest purity available.

Cell Culture. SH-SY5Y and KCNR neuroblastoma cell lines were cultured at 37°C in a 6% CO₂-humidified incubator in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 units/ml streptomycin. The cells were passaged weekly after they had reached 70–80% confluence and were used between passages 68 and 82.

Preparation of Extracts from Tumor Tissue and from Cultured Cells. The experimental protocol was approved by the University of Michigan Institutional Review Board for Approved Research Involving Human Subjects. Informed consent was obtained from the patients (or their families) before the study. Both serum and fresh tumor tissue were obtained at the time of diagnosis (*i.e.*, biopsy tissue) from patients with neuro-

blastoma and from other pediatric solid tumors. After excision, the tumor tissue was immediately frozen at –80°C, after which small amounts of tumor tissue were solubilized in solubilization buffer and stored at –80°C until use. Cultured neuroblastoma cells were harvested after reaching confluence and were washed four times in PBS. The cells were lysed by adding 200 μ l of solubilization buffer consisting of 8 M urea, 2% NP40, 2% carrier ampholytes (pH 4–8), 2% β -mercaptoethanol, and 10 mM PMSF and were harvested using a cell scraper. After an additional 100 μ l of solubilization buffer were added, the buffer containing the cell extracts was transferred into microfuge tubes and stored at –80°C until use.

Two-dimensional PAGE and Western Blotting. Proteins derived from the extracts of both cultured cells or solid tumors were separated in two dimensions as previously described (14), with some modifications. Briefly, subsequent to cellular lysis in solubilization buffer, 35- μ l aliquots of solubilized tumor tissue or cultured cells derived from $\sim 2.5 \times 10^6$ cells were applied onto isofocusing gels. Isoelectric focusing was conducted, using pH 4–8 carrier ampholytes at 700 V for 16 h, followed by 1000 V for an additional 2 h. The first-dimension tube gel was loaded onto a cassette containing the second-dimension gel, after equilibration in second-dimension sample buffer [125 mM Tris (pH 6.8), containing 10% glycerol, 2% SDS, 1% DTT, and bromphenol blue]. For the second-dimension separation, an acrylamide gradient of 11 to 14% was used, and the samples were electrophoresed until the dye front reached the opposite end of the gel. The separated proteins were transferred to an Immobilon-P PVDF membrane. Protein patterns in some gels were visualized by silver staining and on some Immobilon-P membranes by Coomassie Blue staining of the membranes. Unstained membranes prepared for hybridization were incubated with blocking buffer [consisting of Tris-buffered saline (TBS) containing 1.8% nonfat dry milk and 0.1% Tween 20] for 2 h, then washed, and incubated either with serum obtained from patients or with normal control serum (300 μ l of serum, at a 1:100 dilution) for 1 h at room temperature. After three washes with blocking buffer, the membranes were incubated with a horseradish peroxidase-conjugated sheep antihuman IgG antibody (at a 1:1000 dilution) for 30 min at room temperature. The membranes were washed five times with TBS containing 0.1% Tween 20 and once in TBS and were briefly incubated in ECL and exposed to XAR-5 X-ray film for 10–30 min. Patterns visualized after hybridization with patient sera were compared directly to the Coomassie Blue-stained blots from the same sample to determine correlation with proteins as well as to patterns obtained from hybridization of blots derived from the same sample with sera from patients with other solid tumors or control sera to determine the specificity of autoantigens. Alternatively, membranes were incubated with a horseradish peroxidase-conjugated sheep antihuman IgM antibody, with horseradish peroxidase-conjugated mouse antihuman IgG antibody to subclasses IgG1, IgG2, IgG3, and IgG4 and processed as for the incubations with antihuman IgG antibody.

Protein Identification. SH-SY5Y cell lysates were subjected to two-dimensional PAGE, after which the separated proteins were transferred to Immobilon-P membranes and subsequently stained with Coomassie Blue. Protein spots of interest were excised from the membrane and subjected to NH₂-terminal

² The abbreviations used are: PVDF, polyvinylidene fluoride; pI, isoelectric point; PNET, peripheral neuroepithelioma.

amino acid sequencing. The resultant sequences were compared with existing databases for protein identification. Additionally, proteins of interest identified on the Coomassie Blue-stained PVDF membranes were subjected to trypsinization, then eluted from the membrane, and resolved by MALDI-TOF on a PerSeptive Biosystems Mass Spectrometer. The peptide data obtained were analyzed with Protein Prospector (<http://prospector.ucsf.edu/>).

Tubulin Detection by Immunoblotting. Three anti- β -tubulin, isotype-specific monoclonal antibodies were used. The mouse monoclonal anti- β -tubulin isotype I and II antibody was raised against a synthetic peptide that corresponds to a protein sequence found in both β -tubulin isotypes I and II but not found in β -tubulin isotype III. Both the mouse monoclonal anti- β -tubulin isotype III and the mouse monoclonal TU-20 antibodies were raised against a synthetic peptide whose protein sequence (CESESQPK) is located at the COOH terminus of β -tubulin isotype III but is not found in β -tubulin isotypes I and II. These primary antibodies were used at a 1:100 dilution.

β -Tubulin Purification Using Affinity Chromatography. The E7 anti- β -tubulin monoclonal antibody (IgG) was purified on a MABTrap G-II column, dialyzed extensively against PBS, and coupled to cyanogen bromide-activated Sepharose 4B beads for 16 h at 4°C. Unconjugated antibody was removed by repeated washing with PBS, and remaining reactive groups on the cyanogen bromide-activated Sepharose 4B beads were blocked by incubation in 0.2 M glycine (pH 8.0) and then washed in PBS.

Whole cell lysates were prepared from SY5Y cells by addition of lysis buffer [50 mM Tris-HCl (pH 7.5) containing 1.5 mM MgCl₂, 1 mM EDTA, 150 mM NaCl], 1% (v/v) Triton X-100, and Complete protease inhibitors (Roche), then scraped from the flask. The lysed cells were briefly sonicated and centrifuged at 15,000 \times g for 5 min at 4°C. The SY5Y whole cell lysates were incubated with the Sepharose 4B beads containing the β -tubulin antibody for 16 h at 4°C. The Sepharose 4B beads were washed with 15 ml of PBS, and β -tubulin was specifically eluted from the columns with 0.2 M glycine (pH 2.3), into an equal volume of 0.2 M Tris-HCl (pH 8.6). The purified tubulin-containing fractions were stored at -80°C until use.

Determination of Posttranslational Modifications.

Three hundred nanograms of dialyzed β -tubulin recovered by affinity chromatography were treated with 1 μ l of *O*-glycosidase enzyme and incubated overnight at 37°C. Similarly 300 ng of purified, dialyzed β -tubulin were treated with 1% SDS and incubated at 100°C for 5 min. One microliter of EndoF-glycosidase enzyme, 0.2 M sodium acetate (pH 4.75), and 2% NP40 were added and incubated overnight at 37°C. The deglycosylated samples were run in separate lanes in one-dimensional gels and compared with deglycosylation of orosomucoid, a known glycoprotein as control, which also was similarly treated with the glycosidases enzymes.

RESULTS

Reactivity of Sera from Neuroblastoma Patients with a Neuroblastoma Protein Detected by Western Blot Analysis.

SH-SY5Y cell proteins were separated by two-dimensional PAGE and transferred onto Immobilon-P PVDF membranes.

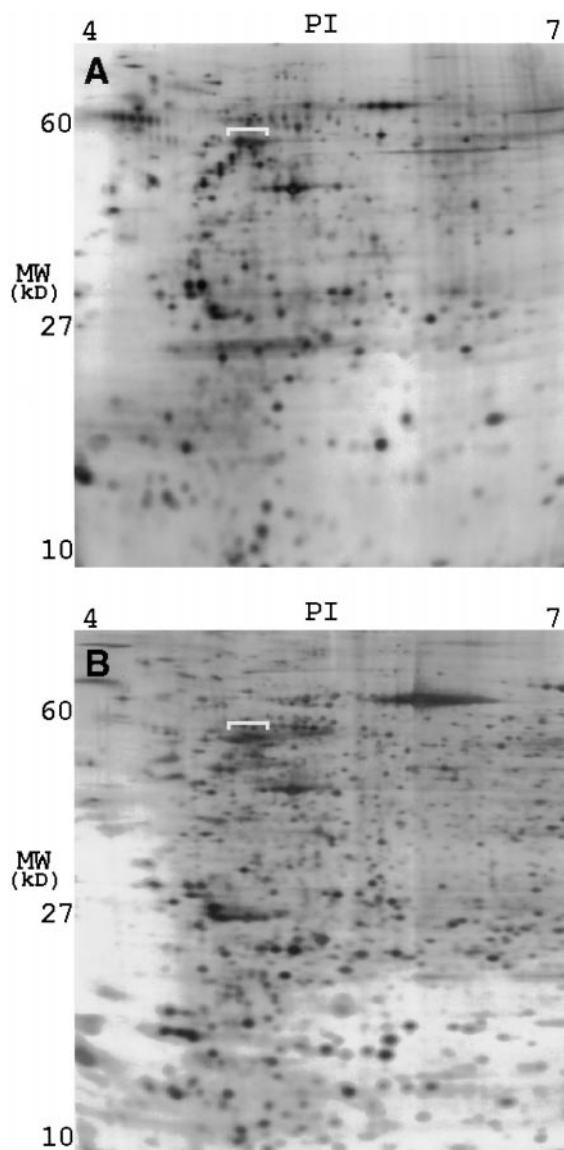


Fig. 1 Silver staining of SH-SY5Y (A) and neuroblastoma tumor (B) proteins separated by two-dimensional PAGE showing protein spot LP1 with a pI of \sim 4.9 and an M_r 50,000. Numbers on left, M_r in thousands.

For Western blot analysis, each membrane was treated with one serum sample. The samples included sera obtained at the time of diagnosis from 23 patients with neuroblastoma (3 with stage I, 3 with stage II, 5 with stage III, 9 with stage IV, and 3 with stage IVS), from 12 patients with pediatric tumors (3 Wilms', 4 PNET, 2 Ewing's sarcomas, 1 rhabdomyosarcoma, 1 undifferentiated sarcoma, and 1 Hodgkin's disease), and from 13 healthy subjects without a prior history of cancer or autoimmune disease. An example of a two-dimensional gel of SH-SY5Y cells stained with silver is shown in Fig. 1A. Hybridization of membranes using patient sera as the primary antibody and sheep antihuman IgG as secondary antibody revealed variable patterns of reactivity among neuroblastoma patient sera. Duplicate hybridizations of serum onto two separate SH-SY5Y blots resulted

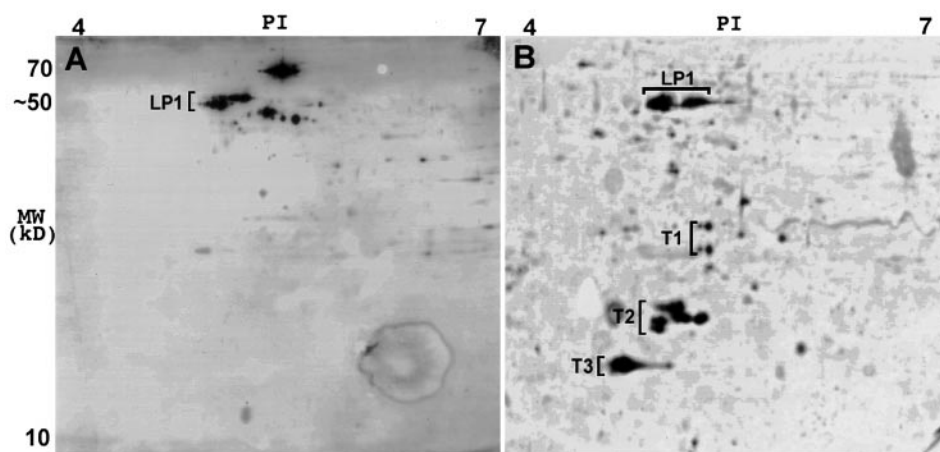


Fig. 2 A, Western blot of a two-dimensional PAGE of neuroblastoma SH-SY5Y cell lysates hybridized with serum of neuroblastoma patient as primary antibody and antihuman IgG as secondary antibody, showing LP1 spot. B, Western blot of a neuroblastoma cell line SH-SY5Y hybridized with serum of neuroblastoma patient as primary antibody and rabbit antihuman IgM as secondary antibody, showing LP1 spot and additional spots corresponding to proteins with a pI 4.4–4.8 and an M_r 15,000–19,000, designated T1, T2, and T3. Numbers on left, M_r in thousands.

Table 1 Clinical staging and patient data of the 11 neuroblastoma patients whose sera were positive for β -tubulin antibodies

Patient	Age	Sex	Site of neuroblastoma	Staging of tumor (INSS)	N-Myc Status ^a
1	16 mo	F	Abdominal	Stage 1	NA
2	6 yr	F	Abdominal	Stage 1	NA
3	5 mo	F	Abdominal	Stage 3	NA
4	3 yr	F	Abdominal	Stage 3	A
5	13 yr	F	Pelvic	Stage 3	NA
6	2 yr	M	Abdominal	Stage 4	A
7	2.5 yr	M	Disseminated	Stage 4	Unknown
8	4 yr	F	Mediastinal	Stage 4	NA
9	9 yr	F	Disseminated	Stage 4	A
10	3 days	F	Mediastinal	Stage 4S	NA
11	6 days	M	Abdominal	Stage 4S	A

^a NA, nonamplified; A, amplified; INSS, International Neuroblastoma Staging System.

in similar patterns. In general, several reactive spots were observed with most sera. Some of the reactive spots were observed with control sera and thus were considered to represent nonspecific reactivity. Others were restricted to neuroblastoma patient sera. Most noticeable among the latter was intense reactivity in a group of contiguous protein spots designated LP1, with a pI of approximately 4.9 and an M_r 50,000 (Fig. 2A), which was observed with sera of 11 of 23 neuroblastoma patients (Table 1) and which was absent in SH-SY5Y membranes hybridized with sera from normal individuals. Of the sera obtained from patients with other tumor types, only one, a patient with PNET showed reactivity against LP1. A protein in this position was readily detectable in silver-stained patterns of SH-SY5Y (Fig. 1A) and in prepared, silver-stained two-dimensional patterns of neuroblastoma tumors (Fig. 1B). Neuroblastoma tumor tissue was available from three patients whose sera were analyzed. The pattern of reactivity of sera with autologous or heterologous tumor tissue was comparable with reactivity with SH-SY5Y for the LP1 spot. Likewise, LP1 reactivity of sera when hybridized to membranes prepared from the neuroblastoma cell line KCNR was similar to reactivity observed with SH-SY5Y membranes (data not shown). In total, 11 of the 23 neuroblastoma sera exhibited reactivity with the LP1 protein(s) in Western blotting of neuroblastoma tumor tissue; SH-SY5Y and KCNR and only

one of the 12 nonneuroblastoma patient sera, from a patient with PNET, showed similar reactivity. Reactive sera were investigated to determine the IgG subtype(s) of anti LP1 antibodies. Of the four IgG subclasses tested (IgG 1–4), only IgG1 reacted with LP1 (data not shown).

IgM Immunoreactivity against Neuroblastoma Proteins. We sought to determine whether sera that exhibited IgG-based immunoreactivity against LP1 protein also exhibited IgM-based immunoreactivity. Sufficient serum from two neuroblastoma patients who exhibited IgG-based immunoreactivity against LP1 was available for this additional analysis. Membranes containing SH-SY5Y lysates as well as membranes containing neuroblastoma tumor lysates were hybridized with patient sera and subsequently incubated with a horseradish peroxidase-conjugated sheep antihuman IgM antibody. The two patients' sera, which exhibited IgG-based reactivity against LP1, were tested and showed, in addition to the LP1 spot, reactivity against a set of protein spots of lower molecular weight than LP1 but with a similar pI, suggesting that the reactive proteins may be cleavage products of LP1 (Fig. 2B).

Specificity of LP1 Reactivity to Neuroblastoma. Neuroblastoma patient sera were hybridized against membranes prepared from either PNET or Wilms' tumor lysates. Sera that were positive for LP1 when hybridized against neuroblastoma

Table 2 LPI spot assignment of MALDI peptide mass map

M/z submitted	MH + matched	Deviation (ppm)	Residues	Peptide sequence
A. Assignment to the human β -tubulin I polypeptide sequence (SWISSPROT accession number P07437)				
1039.6300	1039.5940	34.5	310–318	YLTVAAVFR
1130.6300	1130.5958	30.2	242–251	FPGQLNADLR
1159.6800	1159.6298	43.3	253.262	LAVNMVPPFR
1245.6600	1245.5938	53.1	381–390	ISEQFTAMFR
1258.7500	1258.6908	47.0	242–252	FPGQLNADLRK
1636.9100	1636.8310	48.2	263–276	LHFFMPGFAPLTSR
1959–0600	1958.9823	39.6	104–121	GHYTEGAELVDSVLDVVR
2798.3400	2798.3439	–1.40	78–103	SGPFGQIFRPDNFVFGQSGAGNNWAK
3102.3200	3102.4081	–28.39	20–46	FWEVISDEHGIDPTGTYHGDSLQLDR
B. Assignment to the human β -tubulin III polypeptide sequence (SWISSPROT accession number Q13509)				
1040.6300	1040.5563	70.8	351–359	VAVCDIPPR
1130.6300	1130.5958	30.2	242–251	FPGQLNADLR
1159.6800	1159.6298	43.3	253.262	LAVNMVPPFR
1245.6600	1245.5938	53.1	381–390	ISEQFTAMFR
1258.7500	1258.6908	47.0	242–252	FPGQLNADLRK
1959–0600	1958.9823	39.6	104–121	GHYTEGAELVDSVLDVVR
3102.3200	3102.4746	49.8	283.309	ALTVPELTQQMFDAKNMMAACDPRHGR

lysates did not display reactivity against any protein with a similar molecular weight and pI in membranes prepared from three different Wilms' tumors and two PNET lysates. There was positive but less intense reactivity in the position of LPI only in membranes prepared from a third PNET lysate. A protein in the position of LPI was detectable in silver-stained, two-dimensional patterns of PNET lysates. The LPI protein spot was not present in Wilms' tumor lysates.

Reactivity of Neuroblastoma Sera with Tubulin Isoforms. We sought to determine the identity of the LPI protein(s). To this end, additional membranes were prepared from SH-SY5Y neuroblastoma cell lysates, and the proteins were visualized by Coomassie Blue staining. The LPI spot was excised from the membranes and subjected to direct NH_2 -terminal sequencing. NH_2 -terminal sequence analysis yielded the sequence MREIVHIQAGQCGNQI. A search of this sequence in available databases revealed 100% identity with both human β -tubulin I and III isoforms. The sequence differed from that of β -tubulin II and V isoforms by one amino acid in 7th position, which in β II and β V is Leu instead of Ile. The NH_2 -terminal sequence for β -tubulin IV (MREIVLTQTG) differed from the sequence of the LPI spot at the amino acids in the 6th, 7th, and 9th positions. Mass spectrometric analysis of peptides derived from the LPI spot revealed that β -tubulin I (Table 2A) and III isoforms (Table 2B) were the predominant tubulin isoforms present.

Additional Confirmation of the Tubulin Subtype of LPI and Identification of Tubulin Cleavage Products that Elicited IgM-based Immunoreactivity. To confirm additionally that spot LPI contained a mixture of β -tubulin I and III, membranes prepared from both the neuroblastoma tumors and the SH-SY5Y neuroblastoma cell line were hybridized with either a commercially available mouse monoclonal antibody that reacts with β -tubulin I or the TU-20 mouse monoclonal anti- β -tubulin III antibody. The immunoreactivity patterns obtained were compared with Coomassie Blue-stained blots of the same cell lysate type as well as to the patterns of immunoreactivity observed with sera from patients with neuroblastoma. Both the anti- β -

tubulin I and III antibodies immunoreacted with protein in the LPI spot visualized with neuroblastoma patient sera (Fig. 3, A and B). Strikingly, several additional low-molecular-weight (M_r ~15,000–19,000, with a pI of ~4.4–4.8), immunoreactive spots were observed resulting from the hybridization with both monoclonal antitubulin antibodies used. These spots were identical to the IgM-based immunoreactive spots observed in the hybridizations of either neuroblastoma tumor lysates or SH-SY5Y cell lysates with neuroblastoma patient sera (Fig. 2B). These findings confirm IgM immunoreactivity against β -tubulin I and β -tubulin III cleavage products in patients with neuroblastoma. Immunoblotting of whole cell tumor lysates of other solid pediatric tumors (*i.e.*, two PNET and two Wilms' tumors) with the same tubulin antibodies revealed only slight LPI protein reactivity in one PNET tumor and lack of reactivity in the other three tumors. None of the tumors showed reactivity of tubulin antibodies with the lower-molecular-weight proteins (data not shown). Coomassie Blue-stained membranes of SH-SY5Y cell lysates were used to determine the identity of the low-molecular-weight reactive proteins for the presence of these spots. One of the protein spots (designated T3, Fig. 3) with an M_r ~19,000 and a pI of 4.4 was excised from membranes and subjected to direct NH_2 -terminal sequencing. Analysis of the sequence obtained revealed the NH_2 -terminal sequence to be AVFR-GRMSMKEVDEQMLNVQ. Comparison of this sequence to available databases revealed 100% identity to the human β -tubulin I isoform (starting at residue 315 of the full-length protein) and differed from known sequences of other β -tubulin isoforms by only one amino acid. These data provide additional evidence that T3 is a cleavage product of β -tubulin. Several spots (slightly larger and more basic than T3) were visualized with the mouse monoclonal TU-20 antibody specific for β -tubulin III. These spots therefore contain cleavage products of β -tubulin III.

Western Blot Analysis of Normal Human Brain Tissue. Normal human brain was analyzed for the presence of the β -tubulin I and III isoforms by two-dimensional Western blot analysis using monoclonal antibodies against β -tubulin I and III antibodies. Clearly the LPI spot and the tubulin cleavage prod-

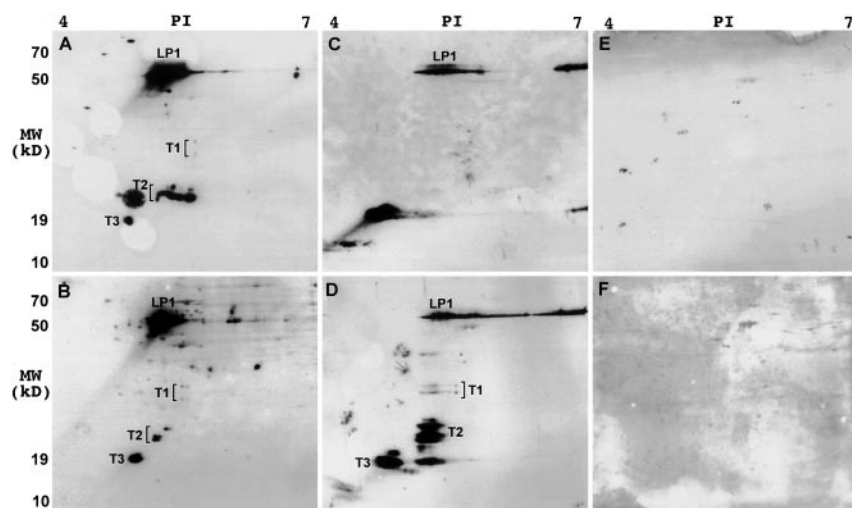


Fig. 3 Western blots of a two-dimensional PAGE of neuroblastoma SH-SY5Y cell lysates treated with the monoclonal antibody to β -tubulin I+II isotypes (A) and the monoclonal antibody to β -tubulin III (B), both showing reactivity with the LP1 and T1, T2, and T3 spots. Western blot of two-dimensional PAGE of normal human brain tissue hybridized with monoclonal antibody to β -tubulin I+II isotype (C) and of normal human brain tissue hybridized with monoclonal antibody to β -tubulin III isotype (D). Western blots of two-dimensional PAGE of normal human brain tissue hybridized with serum of neuroblastoma patient (D and E). E, this serum, which showed LP1, T1–T3 reactivity using antihuman IgG as secondary antibody in Westerns of neuroblastoma proteins, did not show reactivity in Western blots of brain tissue proteins. F, no reactivity was observed against brain tissue proteins using anti-IgM as secondary antibody. Thus, the antineuroblastoma β -tubulin I and III antibodies in the patient serum did not recognize β -tubulin I and III isoforms present in brain tissue. Numbers on left, M_r in thousands.

ucts were demonstrated with both antibodies, indicating the presence of both isoforms of tubulin in normal brain tissue (Fig. 3, C and D).

Given the occurrence of β -tubulin I and III in brain tissue, we examined the reactivity of neuroblastoma sera that contained antitubulin antibodies, with tubulin isoforms in brain tissue. Two neuroblastoma patient sera that contained antitubulin antibodies were used for two-dimensional Western analysis of human brain tissue. The sera did not show any reactivity at the LP1 position or at the positions of T1, T2, or T3 by Western blot analysis using both antihuman IgG and IgM secondary antibodies (Fig. 3, E and F). This result indicates that the antitubulin immunoreactivity of neuroblastoma patient sera was not directed against human brain tubulins and suggests the occurrence of modified β -tubulin isoforms in neuroblastoma.

We sought to determine whether certain posttranslational modifications, namely glycosylation, serine phosphorylation, or acetylation could account for antigenicity of β -tubulin in neuroblastoma. Both SH-SY5Y and human brain tubulin showed a similar pattern of strong reactivity to the acetylated tubulin antibody. Neither SH-SY5Y nor normal human brain tubulin, known to contain phosphoserine, showed any reactivity to the phosphoserine antibody. The antibody used may not recognize phosphorylated serine in certain proteins because of steric hindrance of the recognition site. No cleaved glycosyl residues were detected when we treated β -tubulin purified from SH-SY5Y cell lysates with the enzymes *O*-glycosidase and endoglycosidase F. The control glycosylated protein orosomucoid demonstrated the cleaved glycosyl residue (data not shown). Polyglutamylated as a possible posttranslational modification was considered. However, there was no demonstrable shift in the pI between human brain and neuroblastoma tubulin (Fig. 3).

DISCUSSION

Our findings indicate that patients with neuroblastoma commonly develop a humoral response against tubulin isoforms. Immunoreactivity of neuroblastoma sera in Western blot analysis of SH-SY5Y was localized to a protein spot in two-dimensional gels that contained a mixture of β -tubulin I and III, based on NH_2 -terminal sequence and mass spectrometric analysis of peptides derived from this spot. β -Tubulin, in particular β -tubulin III, is widely used as a neuronal marker. In a study of various cell lines of different origins, a mouse monoclonal antibody prepared against a conserved synthetic peptide from the COOH terminus of β -tubulin III reacted only with a neuroblastoma cell line and with embryonal carcinoma P19 cells stimulated to neuronal differentiation by retinoic acid (15). Interestingly in this same study, immunocytochemical analysis of tumor tissue using this antibody showed reactivity in all of the cases of ganglioneuroblastoma, ganglioneuroma, and medulloblastoma and no reactivity among a large number of nonneuronal tumor types. Given the specificity of certain β -tubulin isoforms to tumors of neuroectodermal origin, the occurrence of autoantibodies to specific tubulin isoforms could be a useful diagnostic marker.

Tubulin is an integral component of microtubules. It occurs mostly as soluble heterodimers consisting primarily of α - and β -tubulin isoforms or as assembled tubulin polymers that form microtubules (16). There is considerable tubulin heterogeneity resulting from a large tubulin gene family encoding numerous isotypic forms and also from numerous posttranslational modifications (17). In vertebrates, expression of the β isotypes is complex. The six β isotypes of tubulin are found in various mammalian tissues. The β isotypes occur at a high level in brain

tissue, and their pattern of expression changes during development of the central and peripheral nervous systems (16, 18, 19). β -Tubulin I is constitutively expressed and constitutes ~3–4% of total tubulin in brain (20). β -Tubulin III is expressed in neurons and Sertoli cells and constitutes ~25% of brain tubulin. Different cells within a given tissue may contain different isoforms of tubulin (21). For example, the epithelial cells of the oviduct express β -tubulin I, and the ciliated cells of the oviduct express β -tubulin IV. This complex distribution of the tubulin isoforms in different tissues and within the same tissue likely reflects a diversity of functions of the various isotypes. β -Tubulin III, which differs from other β -tubulin by containing a phosphorylatable Ser at position 444, may have an important role in neuronal morphogenesis (22–24).

Posttranslational modifications of tubulin include acetylation, phosphorylation, tyrosination, polyglutamylolation, and polyglycylation (25, 26). These posttranslational modifications result from the action of a number of specific modification enzymes. These enzymes can induce rapid changes in tubulin, particularly at the highly variable COOH-terminal region of the molecule (17). Although the significance of tubulin posttranslational modifications is not well understood, it is likely that they have an impact on the role of microtubules in the cell (17). The length of the polyglutamyl side chain appears to regulate the interaction of the α - and β -tubulin with microtubule-associated proteins such as Tau and MAP (27, 28). Posttranslational polyglutamylolation activity has been shown to decrease postnatally. In all, posttranslational modification and differential gene expression contribute to tubulin heterogeneity between tissues (29). This heterogeneity is reflected by the varied migration pattern of tubulin isoforms in two-dimensional gels prepared from different tissues (30).

The specific epitope(s) in β -tubulin isoforms that elicits a humoral response in neuroblastoma remains to be determined. It is interesting in this respect that neuroblastoma patient sera that were reactive against β -tubulin contained both IgG and IgM class anti- β -tubulin antibodies. However, although IgG antibodies reacted with intact β -tubulin, IgM antibodies also reacted with β -tubulin cleavage products. The IgG antibodies were of the IgG1 subclass. No IgG2, IgG3, or IgG4 antitubulin antibodies were detected. A major immunoreactive β -tubulin cleavage product yielded an NH_2 -terminal sequence starting at residue 335, thus encompassing a COOH-terminal portion of intact tubulin. The expected molecular weight of the COOH-terminal portion of tubulin starting at this residue is 15,000. However, this product had an estimated M_r of 19,000, based on its migration in the second dimension of two-dimensional gels. One explanation for the lack of reactivity of IgG antibodies against intact β -tubulin with the COOH-terminal portion of β -tubulin is that the cleavage uncovers an epitope that is distinct from the IgG-related β -tubulin epitope. Another explanation may be that the IgG-reactive β -tubulin and IgM-reactive β -tubulin represent products of separate genes and/or represent β -tubulin isoforms that have undergone distinct posttranslational or structural modifications. The posttranslational modifications we have investigated, namely, acetylation, phosphorylation, and glycosylation, did not appear to be responsible for antigenicity. Differences in glutamylolation of β -tubulin between human brain and neuroblastoma remain a possible explanation for antigenicity (31). How-

ever, such differences, if they occurred, do not appear to alter the pI.

Although, to our knowledge, this is the first report of common occurrence of antitubulin antibodies in a particular cancer type, there have been several prior reports of autoantibodies reactive against tubulin isoforms, notably in demyelinating neuropathies. Selective high-titer anti- β -tubulin antibodies have been associated with Guillain-Barré syndrome and with chronic inflammatory demyelinating polyneuropathy (10, 32, 33). In contrast to our finding of autoantibodies against a COOH-terminal portion of β -tubulin in neuroblastoma, autoantibodies in other acquired disorders have been found to be reactive against epitopes in the central conserved portion of tubulin. Such antibodies have been hypothesized to occur in response to viral antigens that share epitopes with the central portion of tubulin.

Besides β -tubulin isoforms, we have also detected several other proteins against which sera from patients with neuroblastoma exhibited immunoreactivity in Western blots. These proteins have yet to be identified. Other neuroblastoma-associated antigens have been described that are recognized by naturally occurring human antibodies. HNK1, Gal (β 1–3) GalNac carbohydrate epitope, and a neurofilament protein (NFH) with an M_r 200,000 have been found to be targets of naturally occurring human antibodies that are found in low titers in normal individuals (34). These antibodies have been described to occur after viral infections and in patients with demyelinating peripheral neuropathy of motor neuron disease, with degenerative neurological disorders, and with aging (35–37). The occurrence of these antibodies is possibly the result of nonspecific neuronal injury (38). A surface antigen (NB-P260) with an M_r 260,000 has also been described as the target of cytotoxic natural human IgM (39). The expression of this antigen did not show any correlation with retinoic acid differentiation or MYCN amplification.

The demonstration of IgG and IgM antibodies directed against tubulin proteins in serum of patients with neuroblastoma has immunotherapeutic implications. The specific immunogenic tubulin peptide(s), and other antigenic proteins or peptides (when identified) could be pulsed onto antigen-presenting cells (e.g., dendritic cells) to induce a specific and potent immune response. The use of such a combination of tumor-associated antigens for immunotherapy would be particularly effective to overcome problems resulting from variable expression of any particular antigen attributable to tumor heterogeneity. The occurrence of these antibodies and their specificity to neuroblastoma could serve as a diagnostic indicator.

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