Antigen-Specific Recognition of NY-ESO-1 Tumor Antigen

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ABSTRACT

Neuroblastoma cells have been shown to express molecularly defined tumor-associated antigens, which could represent potential targets of T and/or B cell-mediated immunity. However, the existence of a spontaneous immune response to such tumor antigens in neuroblastoma patients has yet to be investigated. In the present work we addressed the issue of whether NY-ESO-1, a germ cell antigen aberrantly expressed in different tumor types, is expressed by neuroblastoma cells and may represent a target for humor and/or cellular immune responses in neuroblastoma patients. We found that a large fraction of neuroblastoma biopsies, independently from the clinical stage and degree of tumor cell differentiation, expressed significant levels of NY-ESO-1 as assessed by reverse transcription-PCR and immunohistochemistry. NY-ESO-1-specific IgG antibodies were detected in the sera of 10% of neuroblastoma patients with stage III or IV disease, but not in patients in clinical remission or with earlier stages. This suggests that antibody production occurred as a late event in the course of disease. NY-ESO-1-specific immune responses were observed for CD4+ and CD8+ T cells from peripheral blood lymphocytes in 4 of 8 neuroblastoma patients, as detected by IFN-γ enzyme-linked immunosorpt assay after in vitro stimulation either with the NY-ESO-1 recombinant protein or with the HLA-A2-restricted peptide NY-ESO-1157–167. NY-ESO-1-specific CD4+ and CD8+ T cells were also able to recognize NY-ESO-1 expressing neuroblastoma cells. The presence of T cells specific for NY-ESO-1 antigen was not associated with the stage of disease, or to the presence or absence of NY-ESO-1-specific antibodies. We conclude that NY-ESO-1 is an immunogenic antigen in neuroblastoma patients and represents a candidate target for immune-based therapy.

INTRODUCTION

Neuroblastoma is one of the most common childhood malignancies. It displays a unique propensity to undergo spontaneous regression in infants and/or differentiation in some older patients. Unfortunately, the majority of patients have metastatic disease at the time of diagnosis, usually followed by rapid tumor progression and fatal outcome. Although advances in supportive care allowing for intensive treatments have increased remission rates, long-term survival for patients with metastatic neuroblastoma has only marginally improved during the last decade (1).

Novel immunotherapeutic strategies are implemented in addition to conventional treatment of advanced-stage neuroblastoma patients (2, 3). In addition to immunological interventions mainly based on the stimulation of nonspecific components of the immune system, such as natural killer or inflammatory cells (4), different immunological strategies aimed at stimulating both the innate and adaptive immunity have been evaluated recently (5–7). Increases in T cell-mediated reactivity against neuroblastoma cells have been detected in a fraction of vaccinated patients, thus implying that neuroblastoma is an immunogenic tumor (5, 8).

Neuroblastoma cells have been shown to express proteins such as MAGE, GAGE, and NY-ESO-1 (9–12) that are able to evoke a T cell- or antibody-mediated response in a variety of malignancies. However, the lack or weak expression of MHC class I and II molecules by neuroblastoma cells, which impairs their recognition by antigen-specific T lymphocytes, has thus far discouraged investigations aimed at assessing the immunogenicity of T cell-defined tumor-associated antigens in neuroblastoma patients.

Nevertheless, T cells can recognize tumor antigens not only directly on tumor cells but also when presented by professional APCs via cross-priming (13). Therefore, the immunogenicity of tumor cells can be examined from a different perspective, as antigens expressed by MHC-negative tumors may evoke a T cell-mediated immune response via cross-priming. Moreover, it has been shown that antigen presentation through cross-priming can trigger antigen-specific CD4+ T cells, which play a central role in maintaining an effective immune response (14). Proinflammatory responses at the tumor site can lead to the in situ production of cytokines that up-regulate HLA expression and would make antigenic determinants expressed by neuroblastoma cells available to recognition by specific T lymphocytes. In fact, in vitro treatment with cytokines such IFN-γ and tumor necrosis factor α has been shown to increase HLA expression on neuroblastoma cells (15–17).

Here we show for the first time that neuroblastoma patients can develop humoral and T cell-mediated immune responses to NY-ESO-1 (18), a tumor antigen already known to be highly immunogenic in melanoma patients (19–24). These data support the use of NY-ESO-1 for specific vaccine therapy in neuroblastoma patients.

MATERIALS AND METHODS

Patients. Neuroblastoma staging was made according to International Neuroblastoma Staging System (1). Parental consent was given for blood sampling for all neuroblastoma patients. LCLs were obtained by B95-8 strain EBV infection of patient peripheral blood B lymphocytes. HLA analysis was performed by sequence-specific primers molecular typing (25).

Cell Lines. All of the cell lines were grown in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% FCS, glutamine, HEPES buffer, penicillin, and streptomycin (BioWhittaker). The HLA-A2+ melanoma cell line Me18732 was generated from a metastasis of a melanoma patient admitted to our Institute. Neuroblastoma cell lines UKF-NB2 and UKF-NB3 were kindly provided by Dr. Jens-Uwe Vogel (J. W. Goethe University, Frankfurt, Germany), GICAN, GIMEN and GILIN by Dr. Valeria Corrias (G. Europe, Verviers, Belgium) supplemented with 10% FCS, glutamine, HEPES buffer, penicillin, and streptomycin (BioWhittaker). The HLA-A2+ melanoma cell line Me18732 was generated from a metastasis of a melanoma patient admitted to our Institute. Neuroblastoma cell lines UKF-NB2 and UKF-NB3 were kindly provided by Dr. Jens-Uwe Vogel (J. W. Goethe University, Frankfurt, Germany), GICAN, GIMEN and GILIN by Dr. Valeria Corrias (G.

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3 Deceased on Sept. 21, 2002.

The abbreviations used are: APC, antigen-presenting cell; LCL, lymphoblastoid cell line; mAb, monoclonal antibody; IL, interleukin; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry; ELISPOT, enzyme-linked immunosorpt, FACS, fluorescence-activated cell sorter.
Gaslini Institute, Genoa, Italy), whereas other lines were purchased from Interlab Cell Line Collection (Genoa, Italy) and from American Type Culture Collection (Rockville, MD): HLA-A2/−ACN and HLA-DR−SK-N-AS cell lines were obtained by retroviral gene transfer of HLA-A*0201 and CTIA genes, and generously provided by Dr. Catia Traversari (Scientific Institute H. S. Raffaele, Milan, Italy) and by Dr. Paul F. Robbins (National Cancer Institute, NIH, Bethesda, MD, respectively). All-trans-retinoic acid (Sigma R2625; Sigma Aldrich, Milan, Italy) was added in culture for 6 days at 10 μM.

**RT-PCR Analysis.** Total RNA was extracted from cells and frozen tumors using RNAzol B (TEL-TEST Inc., Friendswood, TX). RNA (2 μg) was reverse transcribed using Superscript II as detailed by the manufacturer (Invitrogen). Tyrosine hydroxilase and/or dopamine decarboxylase amplifications were carried out as control amplifications as described (26, 27). NY-ESO-1 was RT-PCR amplified as described by Soling et al. (12). The specificity of the NY-ESO-1 PCR product was confirmed by sequencing. PCR products were visualized on 1.8% agarose gels by ethidium bromide staining.

**HIC.** HIC analysis of NY-ESO-1 antigen expression was performed on formalin-fixed, paraffin-embedded tumor samples by using mouse IgG1 mAb ES121 (28). Before overnight antibody incubation at 4°C, tumor sections were heated in autoclave (15 min at 95°C in 5% citrate buffer). Detection was performed by a streptavidin-biotin method (Dakopatts, Glostrup, Denmark), with development in 3-aminooctyl-ethylcarbazole.

**ELISA.** Sera samples were collected between 1995 and 2001 from 55 patients diagnosed at the National Tumor Institute of Milan (Milan, Italy) and 30 patients diagnosed at the G. Gaslini Institute (Genoa, Italy; kindly provided by Dr. Vito Pistoia). The ELISA for the detection of NY-ESO-1-specific IgG antibodies was performed as published (20). Sera were considered positive when the absorbance value exceeded the mean absorbance plus three times SD at 1:4000 serum dilution.

**Lymphocyte Cultures and ELISPOT Assay.** For in vitro sensitization, 3 × 10⁶ PBMCs from patients or normal donors were cultured with NY-ESO-1 recombinant protein or synthetic peptides (5 μg/ml) in 2 ml lymphocyte medium (RPMI 1640 plus 10% human serum) supplemented with 60 IU/ml recombinant IL-2 (Proleukin; Chiron, Amsterdam, the Netherlands). Peptides NY-ESO-1₁₅₇–₁₆₇, ip₁₀₀₂₉–₃₅, and FluNP₅₈–₆₀ were used (Neosystem, Strasbourg, France). NY-ESO-1 recombinant protein was produced in *Escherichia coli* as reported previously (20); recombinant survivin protein, also produced in *E. coli*, was used as a control protein (generously provided by Dr. Dario C. Altierei, Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA). In vitro stimulation was repeated weekly by culturing 5 × 10⁶ lymphocytes with 3 × 10² irradiated (30 Gy) autologous peptide-pulsed PBMCs. Lymphocytes were tested on day 9 or 12 after the last stimulation. To evaluate the blocking effect of anti-HLA-DR-specific mAb L243 and of anti-HLA-class I mAb W6/32, mAbs were added at 1 μl/well to target cells 30 min before culture with lymphocytes. mAbs W6/32 and L243 were obtained from hybridomas purchased from American Type Culture Collection. For IFN-γ ELISPOT assays, MAIP4650 Millipore plates (Bedford, MA) and Mabtech (Nacka, Sweden) mAbs were used as detailed by the manufacturer. As targets, the TAP-deficient HLA-A*0201 lymphoma cell line T2 pulsed with synthetic peptides, or autologous protein-pulsed LCL were used. The number of spots was determined by a computer-assisted ELISPOT reader (Bioline, AID, Torino, Italy). Indicated spot numbers per seeded lymphocyte represent mean values of three or six replicates. Significant increase over baseline was evaluated statistically. Statistical significance of results was evaluated by Student’s t test for unpaired samples. Values of P < 0.05 were considered as significant. ELISA kits from Endogen (Woburn, MA) were used to measure IFN-γ and IL-4.

**Cytotoxic Assay.** Sensitivity of target cells to lysis was evaluated by standard ⁵¹Cr release cytotoxicity assay at different E:T ratio. Cold target inhibition assays were performed by adding to the test cold unlabelled inhibitor target cells at a ratio of 20:1 (cold target:labeled target cells).

**FACS Analysis.** Indirect immune fluorescence was performed using the following mouse mAbs: W6/32, (anti-HLA class-I), BB7.2 (anti-HLA-A2), L243 (anti-HLA-DR), and D8.38 (anti-NY-ESO-1, kindly provided by Dr. Giulio Spagnoli, University Hospital of Basel, Department of Surgery and Research, Basel, Switzerland). As secondary antibody a Fab goat antimouse immunoglobulin FITC was used (Biosource International, Camarillo, CA). The intracellular expression of NY-ESO-1 antigen was evaluated by indirect immunofluorescence performed on cells permeabilized with 5% saponin. Staining was analyzed by flow cytometry (FACScalibur; Becton Dickinson).

**RESULTS**

**NY-ESO-1 Expression in Neuroblastoma Lesions at Different Stages of Differentiation.** We used RT-PCR and IHC to analyze NY-ESO-1 antigen expression in neuroblastoma lesions. By RT-PCR the NY-ESO-1 gene was found to be expressed in 11 of 20 neuroblastoma lesions, including primary tumors surgically resected either before or after chemotherapy, as well as bone marrow samples. By IHC, a higher number of cases (18 of 22) at different disease stage were positive for NY-ESO-1 protein expression (Table 1). Concordance between RT-PCR and IHC results was obtained in 12 of 15 tumors tested by both methods. The three cases resulting negative by RT-PCR showed weak positivity with IHC with few, scanty immunostained cells in a negative ganglioneuromatous context (Fig. 1B). Thus, the lower sensitivity of RT-PCR may be because of a dilution of mRNA encoding for NY-ESO-1 below the threshold of this method.

NY-ESO-1 reactivity detected by IHC was present in the vast majority of the neuroblastic cells in the tumor samples. Staining was shown to mark the neuropilum and the cytoplasm of the neuroblastic component of the tumors, whereas the Schwannian-stroma resulted unreactive (Fig. 1A). The intensity of staining was generally strong, and variations in intensity were independent of the degree of cellular differentiation (Fig. 1, A and B). When present in nodal or bone marrow metastases, only the neoplastic component resulted NY-ESO-1 antigen reactive (Fig. 1, C and D).

In addition, a panel of 16 neuroblastoma cell lines was examined for NY-ESO-1 expression by RT-PCR. As reported in Table 2, 25% of the cell lines resulted NY-ESO-1 positive; treatment with retinoic acid failed to affect NY-ESO-1 expression, although it caused cell growth inhibition and/or cell detachment (data not shown). Cell lines characterized by NMyc amplification and 1p36 deletion were shown to express NY-ESO-1. FACS analysis confirmed the expression of NY-ESO-1 at the protein level in cell lines positive by RT-PCR, as shown in Fig. 6A for ACN cells.

**Neuroblastoma Patients Show Natural Humoral Immunity to NY-ESO-1 Antigen.** To evaluate the presence of antibodies directed against NY-ESO-1, sera collected from 47 patients at diagnosis were tested by ELISA detecting specific IgG antibodies. Three of these subjects with stage III or IV disease were tested positive (Table 3). Of the 17 cases from which autologous neuroblastoma lesions were available, 12 showed expression of NY-ESO-1, as assessed by RT-PCR and/or IHC, including 2 cases seropositive for NY-ESO-1 antibodies. None of the 5 patients with NY-ESO-1 antigen-negative tumor were seropositive. On the contrary, no specific reactivity could be detected in sera from an additional 38 samples collected at different time points after surgery, during treatment, or during remission in the absence of tumor (data not shown).

**Table 1 Expression of NY-ESO-1 antigen in neuroblastomas**

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>NY-ESO-1 positive tumors/total tumors examined*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>3/7</td>
</tr>
<tr>
<td>Stage 2</td>
<td>2/4</td>
</tr>
<tr>
<td>Stage 3</td>
<td>1/2</td>
</tr>
<tr>
<td>Stage 4</td>
<td>5/8</td>
</tr>
<tr>
<td>Total</td>
<td>11/20 (55%)</td>
</tr>
</tbody>
</table>

* RNA was extracted from cryopreserved tumor samples and retrospectively for RT-PCR amplification with NY-ESO-1–specific primers. Tyrosine hydroxilase was used as control amplification for RT-PCR. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumors by using mAb ES121.

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Fig. 1. IHC staining of neuroblastic tumors for NY-ESO-1 antigen. Formalin-fixed, paraffin-embedded tissues were stained with mAb ES121 as detailed in “Materials and Methods.” A, poorly differentiated neuroblastoma: immunoreactivity occurs in cytoplasm of poorly differentiated neuroblasts and in neuroplasm. B, mature ganglion-oneuroma: immunoreactivity occurs in cytoplasm of differentiated, mature ganglion-like cells in an unstained Schwannian stroma. C, lymph node metastasis by poorly differentiated neuroblastoma: mAb ES121 selectively marks the neoplastic component in the lymph node parenchyma. D, bone marrow infiltration by poorly differentiated neuroblastoma: immunoreactivity selectively marks the neoplastic component in the normal bone marrow population.

Table 2 NY-ESO-1 antigen expression in neuroblastoma cell lines as determined by RT-PCR

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>NMYC/1p status</th>
<th>NY-ESO-1 expression after treatment with α-retinoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>NA/wt</td>
<td>+</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>NA/LOH</td>
<td>+</td>
</tr>
<tr>
<td>SK-N-FI</td>
<td>NA/wt</td>
<td>+</td>
</tr>
<tr>
<td>UKF-NB2</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>IMR-5</td>
<td>nd</td>
<td>+</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>nd</td>
<td>–</td>
</tr>
<tr>
<td>LAN-5</td>
<td>A/del</td>
<td>–</td>
</tr>
<tr>
<td>SK-N-BE</td>
<td>A/del</td>
<td>–</td>
</tr>
<tr>
<td>GICAN</td>
<td>NA/wt</td>
<td>–</td>
</tr>
<tr>
<td>LAN-1</td>
<td>A/del</td>
<td>–</td>
</tr>
<tr>
<td>UKF-NB3</td>
<td>nd</td>
<td>–</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>NA/nd</td>
<td>–</td>
</tr>
<tr>
<td>IMR-32</td>
<td>A/del</td>
<td>–</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>NA/wt</td>
<td>–</td>
</tr>
<tr>
<td>GILIN</td>
<td>A/del</td>
<td>–</td>
</tr>
<tr>
<td>GIMEN</td>
<td>NA/wt</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>4/16 (25%)</td>
</tr>
</tbody>
</table>

*NA, nonamplified NMYC gene; A, amplified NMYC gene; LOH, loss of heterozygosity at 1p36.3; del, 1p deletion; wt, wild-type 1p; nd, not determined. This information was obtained from Corrias et al. (29) and Thompson et al. (30).

These data suggest that the antibody response to NY-ESO-1 occurs in a small fraction of neuroblastoma patients. This reactivity appeared to be associated to late stage disease, being detectable only in stage III-IV neuroblastoma, and to the in vivo presence of tumor cells as antigen source, as reported previously in melanoma patients (31). Consistent with this conclusion, the neuroblastoma patient #1 listed in Table 4, while seropositive at diagnosis, was found negative when tested at two later time points during the clinically disease-free period.

Neuroblastoma Patients Show CD4 T Cell-Mediated Response to NY-ESO-1 Antigen. A specific humoral response to NY-ESO-1 has been reported to be associated with T cell-mediated NY-ESO-1 immunity in melanoma patients. Therefore, we checked for an expansion of NY-ESO-1-specific T cells in patient #1, who was seropositive at diagnosis. PBMCs obtained during the disease-free follow-up period were tested for the presence of NY-ESO-1-specific reactivity by IFN-γ ELISPOT assay, after in vitro stimulation with NY-ESO-1 recombinant protein processed and presented by autologous monocytes.

NY-ESO-1-specific, HLA-DR-restricted recognition could be detected in PBMCs of patient #1 as early as 1 week after in vitro sensitization with recombinant protein (Fig. 2). Additional cycles of stimulation increased the frequency of IFN-γ-producing lymphocytes in response to autologous NY-ESO-1-pulsed LCL, with T lymphocytes specific for NY-ESO-1 antigen increasing from 1:100 to 1:50 and 1:40 after the first, second, and third stimulation, respectively. Moreover, at the third week of in vitro culture the NY-ESO-1 reactivity was completely inhibited in the presence of anti-HLA-DR but not anti-class I MHC mAb. To rule out unrelated T cell-mediated reactivity against contaminating bacterial antigens, we additionally tested the immune response to the recombinant protein survivin. The reactivity against this unrelated protein was initially high because of nonspecific immune recognition of autologous LCL, then decreased and disappeared in the second and third cycle of stimulation.

To additionally explore the immunogenicity of NY-ESO-1, 8 additional neuroblastoma patients, at different stages of disease (see Table 4) and having NY-ESO-1-positive tumors, were studied for the frequency of antigen-specific CD4 T cells after in vitro stimulation with the NY-ESO-1 protein. In 4 of 8 patients (# 2, 3, 4, and 5), HLA-DR-restricted NY-ESO-1-specific recognition was induced, albeit less efficiently as compared with the seropositive patient #1, because activation required two to four cycles of in vitro stimulations. In contrast to what we had observed for the antibody response, NY-ESO-1-specific T cell-reactivity occurred independently from the stage of disease, being detectable also in stage I patients (e.g. patient #4; Fig. 3). Nonspecific reactivity against the unrelated protein sur-

Table 3 Antibody response to NY-ESO-1 in neuroblastoma patients

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Antibody positive/sera tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>Stage 2</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>Stage 3</td>
<td>1/9* (10%)</td>
</tr>
<tr>
<td>Stage 4</td>
<td>2/19, 3/28 (15%)</td>
</tr>
<tr>
<td>Total</td>
<td>3/47 (6%)</td>
</tr>
</tbody>
</table>

* Sera collected at diagnosis. Specific ELISA with recombinant NY-ESO-1 protein was used to detect anti-NY-ESO-1 IgG antibodies.

b Tumor biopsy was not available for the assessment of NY-ESO-1 expression.

c Tumor specimens obtained from these 2 patients were positive for NY-ESO-1 expression as evaluated by RT-PCR.
vivin was not detected in the 4 responding subjects. In contrast, in the case of patients #6, 7, 8, and 9, and in the PBMCs of a healthy donor, a nonspecific response was found.

Because sera obtained at diagnosis were available only for 5 patients, the correlation between the antibody and T-cell response against NY-ESO-1 could be only partially assessed. These patients, with the exception of patient #1, were seronegative and did not show HLA-DR-restricted NY-ESO-1-specific T-cell recognition. However, patient #3 displayed a strong NY-ESO-1-specific immunity mediated by CD4⁺/H11001 T cells.

As a positive control we also studied the NY-ESO-1-specific immunity in a melanoma patient who was seropositive at the time of surgery of metastatic lymph nodes (Fig. 3). PBMCs obtained 7 years after surgery, during the disease-free follow up period, showed specific HLA-DR-restricted NY-ESO-1 recognition after three cycles of in vitro stimulation with NY-ESO-1 recombinant protein. This finding supports the conclusion that NY-ESO-1-specific CD4⁺ T-cell precursors are maintained in the peripheral blood of disease-free patients.

Altogether, these data indicate that neuroblastoma patients can develop antigen-specific, CD4⁺ T-cell immunity directed against NY-ESO-1. This T-cell-mediated response could be detected in subjects at early stages of the disease, in absence of tumor burden and of antibody response. Moreover, for some patients the NY-ESO-1-specific CD4⁺ T reactivity was detectable during the disease-free period of follow-up, suggesting the occurrence of a long lasting, antigen-specific immunity.

Table 4 Features of neuroblastoma patients assessed for NY-ESO-1-specific T-cell immunity

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Stage of disease</th>
<th>Antibodya</th>
<th>Clinical status when tested for T-cell reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>+</td>
<td>Disease-free, 4 yrs after treatment</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Not tested</td>
<td>Partial remission during treatment</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>–</td>
<td>Remission, 1 yr after treatment</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>Not tested</td>
<td>Disease-free after surgery</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>Not tested</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>–</td>
<td>Disease-free after surgery</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>Not tested</td>
<td>Disease-free after surgery</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>–</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>–</td>
<td>Disease-free after treatment</td>
</tr>
<tr>
<td>10</td>
<td>2A</td>
<td>–</td>
<td>Disease-free after surgery</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>Not tested</td>
<td>Disease-free after surgery</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>Not tested</td>
<td>Disease-free after surgery</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>Not tested</td>
<td>On treatment</td>
</tr>
<tr>
<td>14</td>
<td>2B</td>
<td>Not tested</td>
<td>Disease-free after treatment</td>
</tr>
</tbody>
</table>

a NY-ESO-1-specific humoral response, assessed at diagnosis.
cells, which prevents them from being directly recognized by specific CD4+ T lymphocytes. Autologous LCL pulsed with lysates obtained from NY-ESO-1-positive neuroblastoma cell lines SK-N-AS and SK-N-FI activated NY-ESO-1-specific CD4+ T cells to release IFN-γ as evaluated by ELISPOT assay (Fig. 4A). On the contrary, no activation occurred when NY-ESO-1-negative SK-N-BE cells were used. Moreover, T-cell triggering required APC-mediated processing and presentation, because no IFN-γ release was obtained by the direct stimulation of T cells with tumor cell-derived protein lysates (Fig. 4A).

To additionally confirm specific NY-ESO-1 recognition, SK-N-AS neuroblastoma cells were induced to express the endogenous HLA-DRB1*0701 alleles by CTIA gene transfer and subsequently evaluated for recognition by the NY-ESO-1-specific T cells obtained from the HLA-DR-matched patient #3. SK-N-AS cells expressing HLA-DR (SKNAS-CTIIA) were recognized in a HLA-DR-restricted fashion by NY-ESO-1-specific T cells, although to a lesser extent than autologous LCL cells pulsed with NY-ESO-1 protein (Fig. 4B). This may be because of the higher level of HLA-DR expression on LCL, as shown by FACS analysis (Fig. 4C).

All together, these data indicate that neuroblastoma cells express NY-ESO-1 protein in a sufficient amount to trigger NY-ESO-1-specific HLA-DR-restricted T cells, providing its processing and presentation by professional APCs and/or the expression of class II HLA on neuroblastoma cells. Moreover, these findings exclude that the reactivity observed against the NY-ESO-1 protein-pulsed LCL could be because of recognition of contaminant bacterial components of the recombinant protein preparation.

**HLA-A2-Restricted NY-ESO-1-Specific CD8+ T-Cell Responses Can Be Generated by in Vitro Peptide Stimulation with NY-ESO-1-Derived Peptides.** In cancer patients, NY-ESO-1 has been shown to induce a CD8+ T-cell response, mainly directed against the HLA-A2 peptides NY-ESO-1-157–167, NY-ESO-1-157–163, and NY-ESO-1-155–163 (19). Thus, to assess the immunogenicity of NY-ESO-1 in the context of class I HLA, we selected patients genetically typed as HLA-A*0201 and having NY-ESO-1-positive tumors, regardless of their serological status. In fact, although HLA-A2-restricted responses have been preferentially detected in subjects showing also NY-ESO-1-specific antibodies (24, 31), HLA-A2-restricted responses have also been found in seronegative patients (22, 32).

PBMCs from 7 HLA-A*0201 neuroblastoma patients were evaluated for the recognition of NY-ESO-1-157–167 peptide after in vitro peptide sensitization. In 3 of 7 cases (patients #4, 10, and 11), T lymphocytes recognized NY-ESO-1-157–167 peptide-pulsed T2 cells in a class I MHC-restricted fashion (Fig. 5). PBMCs from the other 4 patients (#5, 12, 13, and 14) and from a healthy donor, stimulated with NY-ESO-1 peptide under the same conditions, failed to develop a peptide-specific MHC class I-restricted T-cell response. Lymphocytes of these subjects released IFN-γ at significant levels in response to stimulation with T2 cells pulsed with NY-ESO-1-157–167 peptide,
but the addition of the mAb W6/32 failed to inhibit IFN-γ production. Therefore, we concluded that in these cases target cell recognition was not mediated by MHC class I-restricted CD8+ T cells but likely by other lymphocyte subsets present in the in vitro stimulated lymphocyte population, such as CD16+ or CD3-αβTCR+ cells (data not shown).

To determine whether: (a) NY-ESO-1 peptide-specific T cells detected in PBMCs of neuroblastoma patients are able to recognize the naturally processed NY-ESO-1 peptide, and (b) NY-ESO-1-positive neuroblastoma tumor is susceptible to CD8+ T cell-mediated recognition, the NY-ESO-1-positive ACN neuroblastoma cell line (Fig. 6A) was induced to express HLA-A*0201 by gene transfer (Fig. 6B), and subsequently used as a target for NY-ESO-1-specific T cells in IFN-γ ELISPOT and in 51Cr release cytotoxicity assays (Fig. 6C–E). Peptide-specific lymphocytes from patient # 4 were shown to recognize the NY-ESO-1+ ACN-A2+ neuroblastoma cells in a MHC class I-dependent fashion as determined by release of IFN-γ (Fig. 6C). Moreover, these lymphocytes also lysed both T2 cells pulsed with NY-ESO-1157–167 peptide (Fig. 6D) and the NY-ESO-1+ ACN-A2+ neuroblastoma cells (Fig. 6E).

In a cold target inhibition assay, lysis of ACN-A2 cells was strongly inhibited by the addition of cold NY-ESO-1157–167 pulsed T2 cells but remained unaffected in the presence of unlabelled T2 cells pulsed with the irrelevant FluNPs66–66 peptide (Fig. 6E). Moreover, cold ACN-A2+ cells but not NY-ESO-1+ HLA-A2+ melanoma Me18732 cells showed inhibition of the lysis of NY-ESO-1157–167 pulsed T2 cells (Fig. 6F).

Altogether these data indicate that neuroblastoma patients can develop a specific, HLA-A2-restricted T-cell response directed against the NY-ESO-1157–167 peptide and that these effectors are able to recognize the endogenous NY-ESO-1 peptide presented as HLA/peptide complexes on the cell surface of HLA-A*0201-positive neuroblastoma cells.

DISCUSSION

The present study shows that neuroblastoma cells can be naturally immunogenic in vivo and identifies NY-ESO-1 as a neuroblastoma-associated antigen able to elicit both antibody and CD4/CD8 T cell-mediated responses in a subset of patients. NY-ESO-1-specific T-cell immunity appeared to be long lasting, because it was maintained in the peripheral blood of long-surviving patients over the disease-free period. Specific T-cell reactivity was found also in patients with partial remission after treatment or with progressive disease. In contrast, production of antibodies directed against NY-ESO-1 was associated with the presence of the tumor, being detectable only before but not after curative treatment.

Our study was focused on the NY-ESO-1 antigen, identified by Chen et al. (18) by the SEREX method. By IHC analysis we found that the percentage of neuroblastomas expressing NY-ESO-1 antigen was consistently high, reaching 82% of positive cases. Positive IHC staining was restricted to the neuroblastic part of the tumors and was independent of the degree of cellular differentiation, being present in the most undifferentiated neuroblasts, in the neuropilum, in the differentiating neuroblasts up to the differentiated ganglion-like cells. Thus, neuroblastoma can be added to seminomas and synovial sarcomas as exceptions to the heterogeneous pattern of NY-ESO-1 antigen expression observed in the majority of cancer types (28, 33–35). When tested on metastases, NY-ESO-1 immunostaining was a powerful diagnostic tool with selective labeling of the neoplastic cells in the nodal parenchyma or in the bone marrow. Thus, NY-ESO-1 immunostaining as detected by mAb ES121 turned out to be a marker potentially useful also in the routine diagnostics of neuroblastoma tumors and their metastases on formalin-fixed samples.

When assessed in established neuroblastoma cell lines, NY-ESO-1 expression was confirmed, albeit with a frequency lower than that of tumor biopsies. Neuroblastoma cell lines have been shown to consist of NY-ESO-1-157–167 peptide and that these effectors are able to recognize the endogenous NY-ESO-1 peptide presented as HLA/peptide complexes on the cell surface of HLA-A*0201-positive neuroblastoma cells.
of heterogeneous populations of neuroblastic (N-type), Schwannian stromal (S-type), and cells with intermediate (I-type) phenotype that can interconvert or transdifferentiate either spontaneously or after treatment with differentiating agents (36, 37). Retinoids have been shown to exert differentiating and antiproliferative effects in vitro on neuroblastoma cell lines, and clinical trials have shown a significant improvement in high-risk neuroblastoma patients of post-consolidation therapy with 13-cis-retinoic acid (38). Our data indicate that in vitro treatment with retinoic acid did not affect NY-ESO-1 gene expression in neuroblastoma cell lines, thus suggesting that therapy with retinoids would not impair NY-ESO-1 antigen expression in neuroblastoma.

Furthermore, NY-ESO-1 was expressed in cell lines bearing molecular alterations including gene markers of aggressive clinical subtypes, such as NMYC amplifications and 1p32 deletions (1). Thus, the NY-ESO-1 antigen, being widely but selectively expressed by neuroblastic cell subpopulations, can be considered as a potential target for immune-based therapies.

To evaluate the in vivo immunogenicity of NY-ESO-1, sera of many patients were screened for NY-ESO-1 reactivity, including sera obtained at the time of diagnosis and from disease-free subjects at follow-up. Specific IgG antibodies were detected in ~10% of stage III-IV neuroblastoma patients tested at diagnosis. This frequency is in line with the overall frequency of 10% reported in a survey of melanoma, breast, ovary, lung, and bladder carcinoma patients (20). In addition, the association between tumor burden and the presence of anti-NY-ESO-1 serum antibodies reported previously for melanoma and for bladder cancer patients (31, 39) was found also for neuroblastoma patients. NY-ESO-1 appeared more capable of inducing a humoral response than other known tumor-associated antigens, as no antibodies directed against MAGE-1, MAGE-3, MAGE-4, MAGE-10, TP53, CT-7, and CT-10, possibly expressed by the neuroblastoma, could be detected in the patient sera (data not shown).

One of the patients showing antibody response was still in follow-up at our Institute and could be studied for the presence of NY-ESO-1-specific T cell-mediated responses. PBMCs had been obtained 6 years after diagnosis and treatment, when no clinical signs of the disease were detectable. A strong HLA-DR-restricted CD4+ T cell-mediated NY-ESO-1-specific immune response was inducible in this patient by stimulating unseparated PBMCs with recombinant NY-ESO-1 protein. Both IFN-γ and IL-4-producing CD4+ T cells were expanded in this long-lasting systemic response (data not shown). The same assay was performed in 8 additional subjects. In 4 of these patients, including a seronegative one, NY-ESO-1-specific CD4+ T cell-response was detected, although with a lower magnitude as compared with that observed in the seropositive patient. The same long-lasting NY-ESO-1-specific CD4+ T cell-mediated response was also found in PBMCs from a disease-free melanoma patient, who had been seropositive before the surgical resection of tumor draining lymph nodes performed 7 years before. These data indicate that in neuroblastoma patients NY-ESO-1-specific CD4+ T-cell response may develop and be maintained for a long period of time in the absence of the antibody response and tumor burden.

Several peptides have been identified as presented by different MHC class II molecules, and have been shown to be capable of activating both TH1- and TH2-type CD4+ lymphocytes of seropositive cancer patients (40–44); future studies will be aimed at identifying the epitopes recognized by the CD4 T lymphocytes of the studied neuroblastoma patients.

NY-ESO-1-specific CD8+ T-cell responses have been generally observed not only in association with humoral immune response but also in some seronegative individuals (19, 22, 24).

Both CD4+ and CD8+ NY-ESO-1-specific T cells were able to recognize NY-ESO-1 antigen when cross-presented by autologous APC or processed directly by neuroblastoma cells. In fact we showed neuroblastoma cell lines, induced to express HLA-DR or HLA-A*0201 by gene transfer, became susceptible to T cell-mediated recognition, thus implying that NY-ESO-1 protein is endogenously processed.

A major question is how antigen-specific immunity against HLA-negative tumors can contribute in controlling tumor growth in vivo. The mechanism of cross-priming involving the antigen sampling capacity of professional APCs such as dendritic cells should play a role in the induction phase of T and B cell-mediated immunity. Data obtained from murine models clearly show that tumor-specific T cells are directly involved in rejecting MHC-negative tumors and that tumor rejection is associated with the establishment of a systemic immunity (45, 46). One can speculate that a similar immunological network can be activated also in neuroblastoma allowing the induction of NY-ESO-1-specific immunity.

In conclusion, our data indicate that an in vivo NY-ESO-1-specific immune response develops in ~50% of neuroblastoma patients. This immune response involves different components of the immune system, including antibodies, and CD4+ and CD8+ T cells. Responding patients included stage I and stage III seronegative subjects, thus suggesting that T-cell response can be activated before the onset of humoral immunity. Both an antibody response and a strong long-lasting systemic T-cell response were observed in a patient with a favorable clinical evolution.

These findings, together with the widespread but specific expression pattern of NY-ESO-1 in neuroblastoma and the observation that NY-ESO-1-specific T lymphocytes from neuroblastoma patients can recognize neuroblastoma cells, indicate NY-ESO-1 as an interesting candidate antigen for immunological interventions in neuroblastoma patients. New immunogenic NY-ESO-1-derived peptides containing dual class I and class II HLA specificities, as well as peptides promiscuous for HLA binding have been described (43, 44, 47), which should also be considered for dendritic cell-based immunotherapies of neuroblastoma patients.

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