ABSTRACT

The effectiveness of cell-mediated immunotherapy for cancer can be limited by loss-of-antigen mutations that occur during tumor growth. In neuroblastoma, amplification of the MYCN oncogene correlates with rapid tumor progression and a poor prognosis overall. We propose that the MYCN protein, the high-level expression of which is required for maintenance of the malignant phenotype, would be an ideal target for vaccine therapy. The MYCN-derived S9K peptide (amino acids 7–15; STMPGMIKCK), which contains an HLA-A1 binding motif, was used to generate CTLs from the peripheral blood lymphocytes of an HLA-A1+ healthy donor and an HLA-A1+ patient with MYCN-amplified neuroblastoma. These CTL lines specifically lysed HLA-matched, MYCN-amplified neuroblastoma tumor cells. They did not lyse either HLA-mismatched, MYCN-amplified, or matched/nonmismatched, non-MYCN-amplified tumor cells. The CTL activity was inhibited by a monoclonal antibody to a class I HLA monomorphic determinant but not by one specific for HLA class II, consistent with a class I-restricted mechanism of cytotoxicity. Antibodies to CD8, but not those to CD4, also inhibited CTL activity, identifying CD8+ lymphocytes as the effector cell population. These results show that MYCN-derived peptides can serve as tumor-specific antigens and suggest a rational approach to cell-mediated immunotherapy for MYCN-amplified neuroblastoma.

INTRODUCTION

Neuroblastoma is one of the most common extracranial solid tumors in children, accounting for 8–10% of all childhood cancers (1). When diagnosed in infants, the tumor is often localized and in many cases responds well to standard therapy or regresses spontaneously (2). Older children tend to have a more aggressive form of the disease. The MYCN oncogene is amplified in only 10% of patients with low-stage neuroblastoma but in as many as 40% of those with advanced disease (3, 4). High-level expression of MYCN plays an important role in maintaining the malignant phenotype and is the most significant predictor of rapid tumor progression and a poor prognosis overall (5–9).

Several pivotal observations suggest that the immune system plays a clinically significant role in the control and eradication of neuroblastoma. Early work by Hellstrom et al. (10) demonstrated that neuroblastoma cells elicit strong humoral and cellular immune responses in vivo. The clinical relevance of such responses is supported by the finding that patients with a better prognosis are more likely to have lymphocytic infiltrates within their tumors, as well as lymphocytosis and lymphoblastosis in both peripheral blood and bone marrow (11–14). Although CTL responses in neuroblastoma have not been studied in detail, the results of recent clinical trials testing the immunogenicity of IL-2 gene-modified neuroblastoma tumor cells suggest a correlation between antitumor CTL activity and clinical regression after therapy (15, 16). These observations prompted us to attempt to identify tumor-associated antigens capable of inducing tumor-specific cytotoxic immune responses.

In several cancers, the loss of antigens because of fluctuations in gene expression during tumor growth can be a means of evading therapeutic immune responses (17, 18). Hence, oncogene products have been proposed as useful antigenic targets for cancer immunotherapy (19). Because they are needed to establish and maintain the malignant phenotype, such proteins have marked advantages over other tumor-associated antigens, including constitutive high-level expression (20, 21). In recent years, it has become feasible to predict T-cell epitope peptides from a given protein sequence and knowledge of motifs required for binding to class I or class II MHC molecules (22). This peptide-based strategy has been used to elicit antitumor T-cell responses against RAS (23), HER2/NEU (24–26), BCR/ABL (27, 28), and p53 (29, 30).

Because MYCN is expressed at high levels in a substantial portion of high-risk neuroblastomas, we reasoned that specific peptides from the MYCN sequence might recruit CTLs that would effectively kill tumor cells. In this report, we describe a MYCN-derived HLA-A1-binding peptide, selected by a computational approach (22), that elicited peptide- and tumor-specific CTL responses from the PBLs of a normal HLA-A1+ donor as well as from an HLA-A1+ patient with MYCN-amplified neuroblastoma.

PATIENTS AND METHODS

Patients. The neuroblastoma patients participating in this study (Table 1) were recruited from the Texas Children’s Cancer Center at Texas Children’s Hospital in Houston. Informed consent was obtained from the parents or legal guardians of each patient. All of the procedures were approved by an institutional review board. A healthy 26-year-old woman with an HLA type of A1,3; B8(w6),35(w6); Cw4,w7, served as a normal volunteer donor.

Synthesis of Peptides. The MYCN peptides S9K (amino acids 7–15; STMPGMIKCK) and E9K (amino acids 340–348; ESEDAPFPQK) were synthesized by the Merrifield solid phase method (31) using standard T-BOC chemistry. T-BOC blocking groups were removed, and the peptides were hydrolyzed from the resin by hydrofluoric acid treatment at 0°C. The peptides were purified by reverse phase high-performance liquid chromatography.

HLA-A1+ Peripheral Blood Lymphocytes. PBLs were isolated from heparinized venous blood by density gradient centrifugation over Isolymph (Gallard-Schlesinger, Carle Place, NY). They were resuspended in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS and penicillin-streptomycin at 50 μg/ml (Life Technologies), and then cryopreserved in aliquots of 5 × 106 cells. An aliquot of cells from each participant in the study was transformed by infection with EBV according to a standard protocol (32). PHA stimulation was used to generate activated blast cells (PHA blasts) for use in the 3HCr release assays (33). Briefly, PBLs (2 × 105 cells/ml) were treated with PHA (1 μg/ml; Sigma Chemical Co., St. Louis, MO) for 72 h.

Primary Neuroblastoma Cell Cultures. The primary neuroblastoma cell cultures were established from freshly resected surgical specimens. Tumor tissue was digested overnight at 37°C with collagenase (200 units/ml; Sigma) in MEM supplemented with 10% FCS and filtered through a sterile 70-μm cell strainer to obtain a single-cell suspension. Cells were washed twice with MEM and maintained in the same medium supplemented with 40% FCS and penicillin-streptomycin at 50 μg/ml (Life Technologies). MEM medium containing 10% FCS and penicillin-streptomycin was used to maintain established cul-
**Table 1 Patient and neuroblastoma tumor characteristics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>HLA phenotype</th>
<th>INSSa</th>
<th>MYCN</th>
<th>Primary: metastatic site(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>7 years</td>
<td>M</td>
<td>A2, 30; B18(w6), 41(w6)</td>
<td>4</td>
<td>A⁺</td>
<td>Left adrenal: bone, bone marrow, brain</td>
</tr>
<tr>
<td>P9</td>
<td>5 months</td>
<td>M</td>
<td>A2, 3; B7(w6), 51(w4); Cw7</td>
<td>1</td>
<td>NA</td>
<td>Retroperitoneal</td>
</tr>
<tr>
<td>P46</td>
<td>21 months</td>
<td>F</td>
<td>A1, 29; B44(w4), 57(w4)</td>
<td>4</td>
<td>A</td>
<td>Retroperitoneal: bone, bone marrow, lung</td>
</tr>
<tr>
<td>P67</td>
<td>3 months</td>
<td>F</td>
<td>A1, 2; B7(w6), 8(w6); Cw7</td>
<td>4</td>
<td>NA</td>
<td>Right adrenal: contralateral lymph nodes, liver</td>
</tr>
</tbody>
</table>

a INSS, International Neuroblastoma Staging System.

b A, amplified; NA, not amplified.

tures. The cultured tumor cells demonstrated a neuronal phenotype with complex networks of primitive neurites. MYCN amplification was determined using fluorescence in situ hybridization analysis with a MYCN probe. MYCN protein was detected by immunoblotting lysates of neuroblastoma cell cultures with an anti-MYCN mAb (Oncogene, Cambridge, MA). HRP-conjugated goat antimouse Ab (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) was used as the detection Ab (34). ECL reagent (Amersham Pharmacia Biotech) was used for antigen detection in all experiments. SK-N-SH and IMR-32, two neuroblastoma cell lines (American Type Culture Collection, Manassas, VA) served as negative and positive controls, respectively, for MYCN protein expression.

**Generation of CTLs.** Peptide-specific CTLs were generated as follows. Previously frozen PBLs (5 × 10⁵) were thawed, washed, and resuspended in AIM-V medium (Life Technologies) at a density of 1.5 × 10⁶ cells/ml. The lymphocytes were incubated with peptide (10 μg/ml) for 2 h at 37°C, irradiated (30 Gy, 137Cs source), and washed with AIM-V medium. Peptide-treated, irradiated PBLs (2.5 × 10⁵) were mixed with 2.5 × 10⁶ autologous PBLs and plated in a total volume of 200 μl of AIM-V medium in U-bottomed, 96-well microtiter plates. On days 2 and 4, IL-2 (5 units/ml; Boehringer Mannheim, Indianapolis, IN) was added to the cultures. The lymphocytes were restimulated weekly with peptide-pulsed, irradiated autologous PBLs (stimulator: responder ratio, 1:10) for three or four cycles. Every 3 or 4 days, the cultures were fed with fresh medium containing 5 units/ml of recombinant IL-2. When growth-positive wells became apparent, the cells were tested for specific responses to appropriate targets by ⁵¹Cr release assay. The active CTL lines were expanded in vitro with an anti-CD3 antibody (30 μg/ml; Zymed Laboratories, Inc., San Francisco, CA), IL-2 (5 units/ml), and a equal number of irradiated allogenic PBLs and irradiated autologous EBV-LCL in RPMI 1640 (Life Technologies) supplemented with 10% human AB serum (Sigma), according to the protocol of Riddell and Greenberg (35), with modifications.

**Cytotoxicity Assay.** The cytolytic activity of CTL lines was determined by a standard ⁵¹Cr release assay (33). In brief, 1 × 10⁵ target cells were labeled with 150 μCi of ⁵¹Cr (Amersham Pharmacia Biotech, Chicago, IL) for 2 h at 37°C. The labeled target cells were washed three times, and 2500 target cells were incubated with effector cells at 37°C in a total volume of 200 μl of RPMI 1640 supplemented with 10% FCS. In assays with peptide-pulsed cells, the target cells (1 × 10⁶) were treated (after ⁵¹Cr labeling) with 50 μg of peptide for 2 h, and excess unbound peptide was removed by extensive washing prior to the addition of effector cells. After incubation of the target and effector cells for 6 h, supernatants were harvested and counted in a Packard Cobra Quantum gamma counter (Packard Instrument Co., Meriden, CT). The percentage of specific ⁵¹Cr release was calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Maximum and spontaneous release were determined from the supernatants of wells that contained either 5% Triton X-100 or medium alone, respectively. Before their use as targets in the ⁵¹Cr release assay, all neuroblastoma cells were treated with 200 IU/ml of IFN-γ (Genzyme, Cambridge, MA) for 48 h unless otherwise indicated.

**Treatment with mAbs.** To distinguish between the MHC-restricted and nonrestricted cytotoxicity of the CTL lines, we measured the release of ⁵¹Cr in the presence or absence of two mAbs, W6/32 (Sigma) and CR3/43 (Accurate Chemical & Scientific Corporation, Westbury, NY). W6/32 recognizes a monomorphic class I HLA epitope, whereas CR3/43 recognizes a monomorphic class II HLA determinant. The autologous tumor target cells were preincubated with mAb (2 μg of W6/32 or 1 μg of CR3/43 for 1 × 10⁶ cells in 200 μl) for 45 min at 37°C before CTLs were added to the 96-well assay plate. To determine the immunophenotype of the cytotoxic cells, we pretreated the CTL lines with mAb to either CD4 or CD8 (2 μg/300 μl) and washed with CD4 or CD8 (2 μg/300 μl) and washed with PBS, resuspended in PBS containing 1% paraformaldehyde and 0.1% sodium azide. The percentage of fluorescent cells was determined by flow cytometric analysis (FACScan; Becton Dickinson, San Jose, CA).

**RESULTS**

**MYCN Expression in Neuroblastoma Tumor Cells.** The expression of MYCN protein in neuroblastoma primary cultures was investigated by immunoblotting cell lysates with an anti-MYCN mAb (Fig. 1).

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**RESULTS**

**MYCN Expression in Neuroblastoma Tumor Cells.** The expression of MYCN protein in neuroblastoma primary cultures was investigated by immunoblotting cell lysates with an anti-MYCN mAb (Fig. 1).
1; Ref. 34). These results confirm the finding of MYCN amplification in primary tumor cell cultures for patients P4 and P46.

**Peptide-specific Lysis of Target Cells by CTL Generated with the MYCN-derived S9K Peptide.** Using the method of Parker et al. (22), we calculated the half-life for binding of nine amino acid peptides from the MYCN protein sequence with the HLA-A1 molecule. The two peptides with the longest half lives, E9K (amino acids 340–348; ESEDAPPQK) and S9K (amino acids 7–15; STMPG-MICK), were synthesized and used with PBLs from the normal donor to generate CTLs (36). After three rounds of stimulation of HLA-A1+ donor PBLs with autologous peptide-pulsed lymphocytes, we tested the peptide-specific CTL activity at an E:T cell ratio of 50:1, using a 6-h 51Cr release assay. Of the two peptides, only S9K induced a CTL response capable of killing peptide-pulsed autologous PHA blasts. As shown in Fig. 2, CTLs generated with the normal donor’s S9K-stimulated PBLs lysed 43% of the S9K-pulsed autologous PHA blasts compared with only 9% of the controls. The peptide specificity of the S9K-stimulated CTLs was demonstrated by their inability to lyse PHA blasts pulsed with E9K peptides.

Having demonstrated the feasibility of generating MYCN peptide-specific CTLs from normal donor PBLs stimulated with S9K peptides, we tested S9K-specific CTLs produced with PBLs from a HLA-A1+ neuroblastoma patient with MYCN-amplified tumor cells (P46). These cytotoxic lymphocytes lysed 25% of the S9K-pulsed, autologous EBV-LCL but showed no activity against either the medium-treated or E9K peptide-pulsed EBV-LCL (Fig. 2). We used EBV-LCL instead of PBLs in these experiments because of the limited availability of the latter. The results clearly demonstrate that the S9K peptide can be used to generate peptide-specific CTLs from the PBLs of normal donors as well as patients with MYCN-amplified neuroblastoma.

**S9K Peptide-specific CTLs Lyse HLA-matched, MYCN-amplified Neuroblastoma Cells.** S9K peptide-specific CTLs generated after four rounds of stimulation were next tested against HLA-matched, MYCN-amplified tumor cells. At an E:T ratio of 50:1, the CTLs generated with HLA-A1+ PBLs from the volunteer donor lysed 38% of the tumor cells (P46) but were only minimally active (1%) against the natural killer cell targets (K562; Fig. 3). Similarly, S9K-specific CTLs generated with PBLs from the HLA-A1+ patient (P46) lysed 42% of the patient’s MYCN-amplified tumor cells without evidence of natural killer cell activity (Fig. 3). Taken together, the results suggest that the S9K epitope is expressed on MYCN-amplified neuroblastoma cells and therefore provide a lucrative target for cell-mediated immunotherapy.

**Recognition of Tumor Cells by S9K-specific CTLs Depends on MYCN Amplification and HLA Specificity.** To determine whether recognition of neuroblastoma cells by S9K-specific CTLs is dependent on MYCN expression, we performed CTL assays using tumor cells with or without amplified MYCN genes. The targets were tumor cells from patients P46 (HLA-A1+, MYCN-amplified), P4 (HLA-A2+, MYCN-amplified), P67 (HLA-A1+, MYCN-nonamplified), and P9 (HLA-A2+, MYCN-nonamplified). As evident in Fig. 4, the S9K-specific CTLs from the normal donor lysed only HLA-A1+ MYCN-amplified P46 tumor cells, sparing MYCN nonamplified, HLA-A1-matched (P67), or mismatched (P9) tumor cells. The CTLs were also unable to kill MYCN-amplified tumor cells from an HLA-A1-mismatched patient (P4). These results clearly demonstrate the specificity of the CTLs for MYCN-amplified, HLA-A1+ tumor cells.

**Class I MHC Restriction of S9K Peptide-specific CTLs.** The MHC restriction of the CTL lines was confirmed by using target cells preincubated with mAbs to class I or class II HLA molecules. The lysis of P46 tumor cells by S9K peptide-specific CTLs from the normal donor decreased from 45 to 20% when the target cells were preincubated with the anti-class I mAb W6/32, in contrast to a lack of inhibition with use of an isotype-matched mAb (Table 2). Similar results were obtained with S9K-specific CTLs from patient P46. Preincubation of cells with the anti-class II mAb did not reduce killing by either CTL line. Thus, the S9K-specific CTLs kill MYCN-amplified tumor cells by means of a class I MHC-restricted mechanism.

**Peptide-specific Effector CTLs Are CD3⁺ and CD8⁺.** Flow cytometric analysis using mAbs to the CD3, CD16/CD56, CD4, and CD8 lymphocyte markers was performed on S9K-specific CTL lines generated from PBLs of the normal donor or patient P46. A high proportion of cells (>90%) in each of the CTL lines studied consisted of CD3⁺ T cells. The (CD16/CD56)- NK cell component was small (<2%), correlating directly with the inability of the CTLs to lyse standard (K562) NK cell targets. The proportions of CD4⁺ and CD8⁺ cells in the CTL lines generated with normal donor PBLs were 52 and 40%, respectively, compared with 47 and 51% in the lines produced with PBLs from patient P46.

To investigate the immunophenotype of the effector cell population, we incubated cells from each CTL culture with either anti-CD4 or anti-CD8 mAbs before a standard CTL assay. As shown in Fig. 5, ~80% of the activity against HLA-A1+, MYCN-amplified tumor cells was inhibited when the CTLs were preincubated with anti-CD8, in contrast to a lack of inhibition with use of an isotype-matched mAb (Table 2). Similar results were obtained with S9K-specific CTLs from patient P46. Preincubation of cells with the anti-class II mAb did not reduce killing by either CTL line. Thus, the S9K-specific CTLs kill MYCN-amplified tumor cells by means of a class I MHC-restricted mechanism.

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![Image](https://example.com/image1.png)

**Fig. 3.** CTLs stimulated with MYCN peptide S9K kill MYCN-amplified neuroblastoma tumor cells. Target cells were HLA-A1+, MYCN-amplified neuroblastoma cells (P46) or K562 cells used at various E:T ratios in a 6-h 51Cr-release assay. Values shown are means of three separate experiments; bars, SE.

![Image](https://example.com/image2.png)

**Fig. 4.** Recognition of tumor cells by MYCN peptide S9K-stimulated CTLs is MYCN specific and HLA-restricted. The cytotoxicity of CTLs from the HLA-A1+ normal donor was measured with neuroblastoma cells from patients P46 (HLA-A1+, MYCN-amplified), P4 (HLA-A1+, HLA-A2+, MYCN-amplified), P67 (HLA-A1+, MYCN-nonamplified), and P9 (HLA-A1+, HLA-A2+, MYCN-nonamplified) at an E:T ratio of 50:1 in a 6-h 51Cr-release assay. The data represent means from three independent experiments; bars, SE.
contrast to no inhibition with the use of anti-CD4. These observations indicate that the principal cytolytic activity is associated with a CD8+ effector cell population.

Lysis of IFN-γ Untreated Neuroblastoma Cells by 9K Peptide-specific CTLs. The limited expression of class I MHC molecules on neuroblastoma cells in general and MYCN-amplified tumor cells in particular could significantly interfere with MHC-restricted recognition by T cells (37, 38). In agreement with these findings, tumor cells from patient P46 expressed approximately one-tenth the level of HLA-A, -B, and -C observed in autologous EBV-LCL by FACS analysis with mAb W6/32. To address this issue, we first treated HLA-A1+ MYCN-amplified tumor cells with IFN-γ (37, 38), observing a 5-fold increase in the expression of class I MHC antigens (data not shown). We then performed CTL assays with tumor cells that had or had not been exposed to the cytokine. As shown in Fig. 6, the CTLs generated with PBLs from the normal donor and patient P46 killed 21 and 24% of HLA-A1+, MYCN-amplified tumor cells, respectively. Cytokine treatment increased these rates by ~2-fold. Thus, the expression of class I MHC molecules on MYCN-amplified cells appears adequate for CTL recognition and lysis, with or without IFN-γ stimulation.

**DISCUSSION**

Amplification of the MYCN gene is the most significant adverse prognostic factor in children with advanced-stage neuroblastoma (5–9). Because there is no effective therapy for these children, we investigated the feasibility of generating functional MYCN-specific CTLs from the PBLs of patients with neuroblastoma as well as normal donors, using peptides derived from the MYCN protein sequence. The CTLs we produced are peptide specific and kill MYCN-amplified neuroblastoma tumor cells via a classical MHC class I-restricted mechanism.

**Table 2** MHC-restriction analysis of MYCN peptide S9K-specific CTLs generated from PBLs of either the HLA-A1+ normal donor or the HLA-A1+ MYCN-amplified patient (P46).  
P46 tumor target cells were incubated with appropriate mAb for 45 min prior to initiation of the 51Cr release assay. The data represent mean ± SE from three independent experiments.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Normal donor</th>
<th>Patient P46</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>45 ± 2.3</td>
<td>48 ± 1.8</td>
</tr>
<tr>
<td>W6/32</td>
<td>20 ± 1.9</td>
<td>18 ± 2.0</td>
</tr>
<tr>
<td>W6/32 isotype matched</td>
<td>40 ± 1.6</td>
<td>45 ± 1.9</td>
</tr>
<tr>
<td>CR3/343</td>
<td>42 ± 2.4</td>
<td>48 ± 1.7</td>
</tr>
<tr>
<td>CR3/343 isotype matched</td>
<td>42 ± 2.6</td>
<td>47 ± 1.9</td>
</tr>
</tbody>
</table>

a CTL activity (% specific lysis) toward tumor targets from HLA-A1+ MYCN-amplified patient, P46, at E:T ratio of 50:1.

Previous studies (38, 39) have suggested that neuroblastoma cells might be resistant to CTL recognition, possibly because of a low expression of class I MHC antigens. Work from other investigators (40), as well as from our own laboratory,5 suggests that appropriately generated, autologous CTLs can kill these tumor cells in an MHC class I-restricted manner. The primary difference between studies that produced MHC-restricted CTLs and those that generated non-MHC-restricted NK cells may be related to culture conditions (41, 42) that favored NK cell outgrowth (higher concentrations of IL-2) in the latter (39) rather than an overwhelming resistance to MHC-restricted killing in the neuroblastoma cells. Although MHC expression was low on the MYCN-amplified tumor cells, they were nonetheless susceptible to class I MHC-restricted killing by CD8+ CTLs, even without IFN-γ-induced up-regulation of MHC expression. The cytotoxicity of MYCN peptide-specific CTL lines against MYCN-amplified neuroblastoma tumor cells is comparable with the killing observed with melanoma gp100 peptide-specific CTLs against autologous melanoma cells (43) and MHC-restricted virus-specific CTLs with virus-infected cells (44). Taken together, these results imply that MYCN-amplified neuroblastoma cells are sufficiently susceptible to MHC-restricted killing to justify implementing a tumor cell-specific immunotherapy of this disease.

The ability to culture MYCN-specific CTLs from PBLs of a normal individual and a neuroblastoma patient implies that appropriate lymphocyte precursor cells exist, at least in some cases. It should be possible to expand these precursors, either in vivo through a vaccination scheme (15, 16, 45) or in vitro, possibly in conjunction with a bone marrow transplantation protocol (46–49), to produce a CTL population large enough to have a positive therapeutic effect in children with MYCN-amplified neuroblastoma.

Many investigators have demonstrated the close correlation between MYCN expression and the malignant phenotype of neuroblastoma tumor cells (5, 8, 9). Tumor cells with a constitutive high level expression of MYCN have higher levels of the multidrug resistance-associated protein (50). They are less likely to express the favorable nerve growth factor receptor, trkA (51), and are resistant to nerve growth factor-induced neuronal differentiation, even at comparable levels of trkA expression (52). Neuroblastoma cells transfected with the MYCN gene have a higher growth rate (53) secondary to more rapid DNA synthesis and shortening of the G1 phase of the cell cycle (54). These cells are more invasive (increased motility and proteolytic activity) than sibling cell lines with low MYCN levels (55) and lose their ability to stop growing and differentiate in response to retinoic acid (56) or to undergo apoptosis in response to cytotoxic drugs (57).
Thus, if one could reduce the tumor cell population with an immunotherapy directed against an MYCN peptide, the residual malignant cells would likely not express the oncoprotein and might be expected to have a less malignant phenotype overall.

The present study demonstrates that peptide-specific CTLs generated by *in vitro* immunization can kill neuroblastoma cells expressing the MYCN oncoprotein. Vaccination of neuroblastoma patients with class I MHC-binding peptides should engender responses similar to those observed in the present study, although the optimal method of *in vivo* priming remains to be determined. Future studies of MYCN-derived peptides should include *in vitro* studies to define the optimal peptide for each widely held HLA allele, possibly including peptides that have been modified to increase MHC binding, as well as clinical studies to determine the best antigen presentation milieu for generating an effective antitumor response.

ACKNOWLEDGMENTS

We thank Susan Burlingame and Vicky Dulai for technical assistance; John Hicks for histological analysis of tumor specimens; Sue Rowe for analysis of priming remains to be determined. Future studies of MYCN-derived peptides should include *in vitro* studies to define the optimal peptide for each widely held HLA allele, possibly including peptides that have been modified to increase MHC binding, as well as clinical studies to determine the best antigen presentation milieu for generating an effective antitumor response.

REFERENCES


Lysis of MYCN-amplified Neuroblastoma Cells by MYCN Peptide-specific Cytotoxic T Lymphocytes

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