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NY-ESO-1, a germ cell Ag often detected in tumor tissues, frequently elicits Ab and CD8⁺ T cell responses in cancer patients. Overlapping long peptides spanning the NY-ESO-1 sequence have been used to map HLA class I-restricted epitopes recognized by NY-ESO-1-specific CD8⁺ T lymphocytes. To address the antigenicity of long peptides, we analyzed two synthetic 30-mer peptides from NY-ESO-1, polypeptides 80–109 and 145–174, for their capacity to be processed by APCs and to stimulate CD8⁺ T cells. By incubating APCs with polypeptides at different temperatures or in the presence of protease inhibitors, we found that NY-ESO-1 polypeptides were rapidly internalized by B cells, T2 cells, or PBLs and submitted to cellular proteolytic action to yield nonamer epitopes presented by HLA class I. Polypeptides were also immunogenic in vitro and stimulated the expansion of CD8⁺ T cells against naturally processed NY-ESO-1 epitopes in the context of three different HLA class I alleles. Polypeptides can thus serve as exogenous Ags that are cross-presented on HLA class I without requiring the action of professional APCs. These findings support innovative vaccination strategies using NY-ESO-1 polypeptides that would circumvent current limitations of HLA class I peptide vaccination, i.e., HLA eligibility criteria and knowledge of epitope, while allowing for facilitated immunogenicity in the presence of helper epitopes. The Journal of Immunology, 2003, 170: 1191–1196.

A central paradigm of Ag presentation is that proteins added exogenously to cells do not enter class I pathways for epitope loading on MHC class I molecules, except in select professional APCs such as dendritic cells in a process known as cross-presentation (1, 2).

Nevertheless, fragments of proteins are able to overcome this restriction when added exogenously to nonprofessional APCs. Long overlapping peptides, or polypeptides (typically 15–30 aa), have been used in the literature for mapping regions of a protein recognized by CD8⁺ T cells against NY-ESO-1 Ag. NY-ESO-1 is a 180-aa-long protein whose expression in normal tissues is restricted with 18 U.S.C. Section 1734 solely to indicate this fact.

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Exogenous NY-ESO-1 Polypeptides by Nonprofessional APCs

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Nevertheless, fragments of proteins are able to overcome this restriction when added exogenously to nonprofessional APCs. Long overlapping peptides, or polypeptides (typically 15–30 aa), have been used in the literature for mapping regions of a protein recognized by CD8⁺ T cell lines (3, 4). APCs pulsed with these peptides are able to present short (8 –11 aa) epitopes in the context of HLA class I molecules (5, 6). Longer peptides have also been used to immunize mice to obtain MHC class I-restricted cytotoxic T cells (5, 7–9). In vitro, polypeptides have been subjected to purified proteasome preparations to facilitate class I epitope identification (10, 11).

The present study addresses the potential for using polypeptides in vaccination strategies with the NY-ESO-1 Ag. NY-ESO-1 is a 180-aa-long protein whose expression in normal tissues is restricted to germ cells, but it is often aberrantly found in various tumor types (12). NY-ESO-1 can elicit spontaneous humoral (13) and cellular (3, 14) immune responses in a proportion of cancer patients. In clinical trials cancer patients vaccinated with HLA-A2-binding peptides from NY-ESO-1 successfully demonstrated CD8⁺ T cell responses to the immunizing peptides (15). These studies had to be restricted to HLA-A2⁺ patients by design, because of limited information about other HLA-restricted NY-ESO-1 peptides. In the present study we have analyzed the immunogenicity and antigenicity of 30-mer polypeptides derived from NY-ESO-1 and their processing requirements in different cell types, and our findings raise the possibility of a novel general vaccination strategy for NY-ESO-1.

Materials and Methods

Peptides and viral vectors

Synthetic NY-ESO-1 30-mer polypeptides 80–109 and 145–175 (see sequences in Fig. 1) and nonamer peptides 92–100, 157–165, and 159–167 were obtained from Biosynthesis (Lewisville, TX), with a purity of >90% as determined by mass spectrometry. Adenovirus recombinant for full-length NY-ESO-1 (Ad/ESO), wild-type vaccinia virus (v.v. WT), and vaccinia virus recombinant for full-length NY-ESO-1 (v.v. ESO) were previously described (3).

In vitro sensitization with peptides or adenviral constructs

CD8⁺ T lymphocytes were separated from PBLs of cancer patients by Ab-coated magnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway) and seeded into round-bottom, 96-well plates (Corning, Corning, NY) at a concentration of 5 × 10⁵ cells/well in RPMI medium 1640 supplemented with 10% human AB serum (NABI, Boca Raton, FL), l-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 1% nonessential amino acids. As APCs, PBLs depleted of CD8⁺ T cells were either pulsed with 10 μg/ml peptide or infected with Ad/ESO at 1000 infectious units/cell overnight at 37°C in 250 μl of serum-free medium (X-VIVO-15; BioWhittaker, Walkersville, MD). Pulsed or infected APCs were then washed, irradiated, and added to the plates containing CD8⁺ T cells at a concentration of 1 × 10³ APCs/well. After 8 h IL-2 (10 U/ml; Roche Molecular Biochemicals, Indianapolis, IN) and IL-7 (20 ng/ml; R&D Systems, Minneapolis, MN) were added to culture wells, and this step was repeated every 3–4 days until the cells were harvested for testing.

Abbreviations used in this paper: v.v. WT, wild-type vaccinia virus; v.v. ESO, vaccinia virus recombinant for full-length NY-ESO-1.
and at 37°C. At the end of peptide or viral incubation, target cells were washed °20 h) recombinant v.v. (30 PFU/cell) were then added for various times (1

(16), specific for NY-ESO-1 peptide 159–167, restricted by HLA-A*0201. Clone LAU/157 was obtained by limiting dilution from in
centrations of peptides 157–174.

FIGURE 1. NY-ESO-1 polypeptide titration. An ELISPOT assay was performed with clone LAU/157 against T2 cells pulsed with different con-
centrations of peptides 157–165 or 145–174.

Effector cells
The following CD8+ T cell lines specific for HLA class I-restricted epitopes (described in Fig. 1) were obtained by in vitro sensitization (see above): line BE/80, from melanoma patient NW634, specific for NY-
ESO-1 peptide 80–88, restricted by HLA-Cw*0602; line FW/92, from melanoma patient NW29, specific for NY-ESO-1 peptide 92–100, restricted by HLA-Cw*0304; and line PI/159, obtained from vaccinated non-
small cell lung cancer patient PI-E01 (Ludwig protocol LUD00-019) (16), specific for NY-ESO-1 peptide 159–167, restricted by HLA-A*0201.

Target cells
EBV-transformed B lymphocytes expressing HLA-A*0201, HLA-
Cw*0304, or HLA-Cw*0602, and the HLA-A*0201
epitope restriction in superscript, and the name of the CD8+ T cell line
specific for the corresponding epitope in subscript.

Antigenicity of polypeptides
We analyzed two 30-mer polypeptides from NY-ESO-1, 80–109 and
145–174, containing four previously described nonamer
epitopes restricted by three different HLA class I molecules (Fig. 1).
To minimize the possibility of direct hydrolysis or degradation of polypeptides by serum proteases (18), all experiments were con-
ducted in serum-free conditions.

LAU/157, a CD8+ T cell clone specific for HLA-A2-restricted
epitope 157–165 from NY-ESO-1, was tested against HLA-A2
T2 cells pulsed with nonamer 157–165 or polypeptide 145–174 (Fig. 2). Despite the absence of TAP transporters in T2 cells, polypeptide 145–174 was efficiently presented to LAU/157. In ti-
tration assay, LAU/157 could recognize 145–174 at concentrations
down to 1 μM and 175–167 at concentrations down to 10 nM (Fig. 2).
Since HLA-A*0201 was the only allele shared by LAU/157 and the T2 target, recognition of 145–174 probably occurred through presentation of the 157–165 epitope included in its
sequence.

LAU/157 was then tested against HLA-A2+ B-EBV cells pulsed for various times with polypeptide 145–174. Clone LAU/157 could recognize 145–174 when preincubated overnight on tar-
get cells, with similar efficiency to nonamer peptide 157–165 or naturally processed NY-ESO-1 expressed from recombinant vac-
cinia virus (Fig. 3A). Presentation of polypeptide 145–174 was dependent on incubation time, requiring a >3-h pulse on EBV-B
cells.

To determine whether presentation of HLA class I epitopes
included within polypeptides required internalization by APCs, B-
EBV cells were incubated with polypeptide at 4 or 37°C and as-
sayed for recognition by CD8+ T cell lines. Presentation of polypeptide 145–174 to LAU/157 was abrogated at 4°C (Fig. 3A). This was not the case for nonamer peptide 157–165, which was
presented equally well at 4 or 37°C. This suggested that T cell
recognition of polypeptide 145–174 required internalization by tar-
get cells, whereas 157–165 nonamer could bind directly to the
surface of cells.

The second NY-ESO-1 polypeptide, 80–109, was also assessed.
BE/80, a CD8+ T cell line specific for HLA-Cw6-restricted
epitope 80–88 from NY-ESO-1 (3), was tested against HLA-
Cw6+ B-EBV target cells pulsed for various times with polypep-
tide 80–109. In contrast to polypeptide 145–174, polypeptide 80–
109 was presented on target cells after only a 1-h pulse (Fig. 3B).
Recognition of the epitope within 80–109 by line BE/80 was also
dependent on temperature (Fig. 3B), suggesting that polypeptide
80–109 still required internalization despite its rapid presentation
by target cells.
To further understand how APCs take up polypeptides from an exogenous source and load them on HLA class I for presentation to nonamer-specific T cell lines, the processing requirements of polypeptides were analyzed. The action of proteasome or other proteases was investigated by treating target cells with various proteolysis inhibitors.

Processing requirements of polypeptide 145–174 were analyzed using LLnL, a drug that inhibits proteasome and other endoplasmic reticulum peptidases (19). Line LAU/157 was tested on HLA-A2+ T2 targets pulsed with polypeptide 145–174, and recognition of the polypeptide was abrogated by LLnL treatment of target cells (Fig. 4A). The capacity of T2 cells to process epitope 157–165 from polypeptide 145–174 in the absence of TAP transporters was probably explained by the TAP-independent nature of peptide 157–165. Indeed, T2 cells transduced with full-length NY-ESO-1 recombinant v.v. were capable of presenting 157–165 very efficiently despite the absence of TAP transporters (Fig. 4B).

Line FW/92 was obtained from PBLs of patient NW29 specific for peptide 92–100 and restricted by HLA-Cw3 (3). Polypeptide 80–109 was recognized by FW/92 on HLA-Cw3+ B-EBV cells at levels equivalent to nonamer peptide 92–100 (Fig. 4C). Nonhistrocompatible B-EBV targets could not present polypeptide 80–109 to FW/92, ruling out self-presentation by effector T cells (not shown). Presentation of polypeptide 80–109 was rapid and occurred after a 1-h pulse on APCs. Despite this fast process, it appeared that presentation of polypeptide was inhibited by lactacystin treatment of target cells (Fig. 4C). Lactacystin affects Ag processing by inhibiting the action of the proteasome (20). Presentation of synthetic nonamer 92–100 was not affected by lactacystin treatment (Fig. 4C), indicating that its loading on HLA-Cw3 was independent of proteolytic action.

To further confirm the requirements for internalization and cellular processing, recognition of polypeptide 80–109 by FW/92 was tested in the presence of additional inhibitors. Brefeldin A, which blocks the transport of membrane or secreted proteins at the Golgi level, greatly inhibited presentation of polypeptide to effectors (Fig. 5A), implying that polypeptide presentation required newly formed molecules to assemble on the target cell surface. Moreover, LLnL inhibited the presentation of polypeptide 80–109, similarly to its effect on naturally processed NY-ESO-1 from recombinant v.v. (Fig. 5A). On the contrary, chloroquine, a drug that inhibits endosomal-lysosomal enzymes (21), did not appear to affect the presentation of polypeptide or naturally expressed NY-ESO-1 (Fig. 5B). As a positive control, none of the drugs appeared to have an effect on presentation of nonamer 92–100 (Fig. 5A and B).

**Immunogenicity of polypeptides**

Having observed that polypeptides were taken up by APCs and processed into HLA class I epitopes, we assessed the immunogenicity of NY-ESO-1 polypeptides with PBLs from cancer patients with NY-ESO-1 CD8+ T cell reactivity.
CD8⁺ T cells from NW29, a HLA-A2 patient with pre-existing immunity to NY-ESO-1 peptide 157–165, were presensitized using either 157–165 or polypeptide 145–174. After 10 days in culture, CD8⁺ T cells were analyzed with HLA-A2/157–165 tetrameric complexes. We found that polypeptide 145–174 was able to stimulate T cells against the HLA-A2 epitope, as efficiently as the nonamer peptide (Fig. 6A).

Similarly, CD8⁺ T cells from patient NW634 were presensitized with either NY-ESO-1 recombinant adenovirus or polypeptide 80–109. Polypeptide was able to recall NY-ESO-1-specific responses in the context of HLA-Cw6, similar to presensitization with transduced NY-ESO-1 (Fig. 6B).

Finally, we wanted to determine whether the immunogenicity of polypeptides also depended on their processing in a functional assay not dependent on the use of inhibitory drugs. We took advantage of a recently described NY-ESO-1 epitope 159–167, which is immunogenic but not naturally processed by tumor cells (16). In clinical trials, patients develop T cell reactivity to peptide 159–167 as a result of vaccination with NY-ESO-1 peptide 157–167, but fail to recognize B cells transfected with full-length NY-ESO-1 (16).

CD8⁺ T cells from vaccinated patient PI-E01 were presensitized with polypeptide 145–174 or nonamer 159–167. As observed in our previous study, CD8⁺ T cells from PI-E01 responded strongly to 159–167 by ELISPOT assay (Fig. 6C). However, polypeptide 145–174 was not able to stimulate 159–167-specific CD8⁺ T cells in vitro. Furthermore, CD8⁺ T cells specific for 159–167 could not recognize polypeptide 145–174 when pulsed on T2 cells or on EBV-transformed lymphoblastoid cells (Fig. 6C).

Together, these results rule out the possibility of direct presentation of the 159–167 nonamer epitope within the polypeptide or that free contaminating 159–167 nonamer peptide is present within the synthetic polypeptide. Similar to full-length NY-ESO-1, polypeptide 145–174 requires processing, but PBLs, T2, and B cells are not able to process it into nonamer 159–167.

Discussion

Cross-presentation is defined as the processing of exogenous Ags into the MHC class I pathway (2). We show in this study that polypeptides from NY-ESO-1 are cross-presented by B cells, T2 cells, or PBLs without requiring interaction with professional APCs. Melanoma tumor cell lines are also capable of directly presenting exogenous NY-ESO-1 polypeptides in an HLA class I-restricted manner (not shown). Polypeptides appear to be both antigenic, i.e., they are processed by APCs into HLA class-I bound epitopes, and immunogenic, i.e., they induce the proliferation of nonamer-specific CD8⁺ T cells in vitro. Their activity is dependent on internalization by APCs, and their processing by cellular proteases, proteasome, or others appears necessary to yield HLA class I peptides.

Short peptides contaminating the polypeptides or direct binding of polypeptide to HLA class I on the membrane surface are unlikely. Indeed, peptide 159–167, which is immunogenic but not processed from naturally expressed NY-ESO-1 (16), is not presented from polypeptide 145–174 despite high affinity for HLA-A2.
It was shown that intrinsic affinity of nonameric peptides for HLA class I molecules was critical for determining how exogenous peptides are loaded on HLA class I, i.e., via internalization or by direct competition with surface epitopes (22). Polypeptides, in contrast, appear to need some degree of processing by the cell, regardless of the affinity of subepitopes included in their sequences.

The position of class I epitopes within the polypeptide allows for some plasticity. In 18-mer peptides from NY-ESO-1, class I epitopes are recognized from either N- or C-terminal localizations within overlapping polypeptides (3). Also, vaccination strategies with multiple stringed HLA class I epitopes from various Ags show that individual sequences may be swapped within the polypeptide structure without affecting their immunogenicity (23).

The remarkable capacity of polypeptides to be processed and presented on HLA class I as exogenous Ags by nonprofessional APC is still not fully understood. Size appears to be a critical factor, since full-length proteins are not recognized when pulsed on such APCs. Limited size may result in polypeptide configurations that facilitate uptake and processing by APCs, e.g., by hydrophobic transport through the membrane or higher accessibility to cellular proteases. Other possible explanations may include specific help by chaperone molecules receptive to polypeptides (24) or a potential particulate form of Ag (25).

This unique feature makes polypeptides a prime choice for novel vaccination strategies. While short synthetic class I-restricted peptides are very potent at inducing CD8+ T cell responses in vivo, they can be used only in patients with the appropriate haplotype. The use of a full-length protein for vaccination overcomes this restriction, but appears much less efficient for inducing CD8+ T cells (26). Indeed, exogenous proteins require the exclusive action of professional APCs, such as dendritic cells, for the processing of class I epitopes by cross-presentation (1, 27).

The use of polypeptides for vaccination can be compared with a “predigested” protein that would include advantages of peptide vaccination. Polypeptides do not require the action of dendritic cells for presentation of class I determinants included in their sequence. In this respect they resemble short peptides and may have increased efficacy in vaccination. If designed properly, overlapping polypeptides can cover large regions of a protein, including all potential class I epitopes with any allele restriction. Nevertheless, their processing requirements would only allow presentation of naturally processed HLA class I epitopes, even in the presence of serum (data with serum not shown). Furthermore, polypeptides offer the advantage of including class II epitopes, some of which have already been defined for NY-ESO-1 (28–30). Induction of CD4+ T cells by helper peptides was shown to improve the quality and longevity of CD8+ T cell responses in murine tumor models (31). Finally, polypeptides from NY-ESO-1 may even be able to induce an Ab response, which, if nothing else, would prove useful in the monitoring vaccination efficacy.

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References


