RECOGNITION OF OLIGONUCLEOTIDE-ENCODED T CELL EPITOPES INTRODUCED INTO A GENE UNRELATED TO THE ORIGINAL ANTIGEN

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T lymphocytes apparently recognize antigen only in the context of cell surface glycoproteins encoded by class I or class II genes of the MHC (reviewed in references 1 and 2). In many cases these antigens can be mimicked by short synthetic peptides or by protein cleavage products (3–6). Together with the direct demonstration that defined antigenic peptides can associate in vitro with purified class II molecules in an MHC-specific manner (7, 8), these observations support a model whereby ligands for TCRs consist of complexes between MHC molecules and peptides derived from proteolytic cleavage of protein antigens. According to this model, it should be possible to express defined T cell epitopes in the context of genes unrelated to the original antigen. To test this prediction directly, we have inserted synthetic oligonucleotides encoding peptide-defined MHC class I-restricted T cell epitopes into the influenza virus nucleoprotein (NP) gene. We demonstrate here that cells transfected with NP-oligo recombinant genes can be specifically lysed by CTL that recognize synthetic peptides theoretically encoded by the oligomers.

Materials and Methods

Cells. Details for the derivation and culture of P815 cells transfected with HLA class I genes and of K* restricted CTL specific for HLA are presented elsewhere (6, 9–11).

Construction of Recombinant NP Genes. Essential features of the NP wild-type plasmid (pTKNP2) are described elsewhere (12). The Bgl II (B) sites in the NP coding region were used for the construction of hybrid plasmids as depicted in Fig. 1. The larger fragment obtained after cleavage was gel purified and either (a) ligated on itself giving rise to plasmid NP-Bgl II; or (b) ligated to one of two different double-stranded oligomers whose sequences encode amino acid residues 170–182 of either HLA-CW3 or HLA-A24, giving rise to plasmids NP-CW3 and NP-A24, respectively. Complementary oligonucleotides were designed to leave, after annealing, protruding Bgl II termini at both ends. Ligation was performed in the presence of a 100-fold molar excess of unphosphorylated double-stranded oligomer in order to avoid multiple insertions. Screening of transformants was performed by colony hybridization with a 21-mer oligonucleotide straddling the 5'NP-CW3 (or NP-A24) junction. DNA from
positive colonies was further checked by PstI cleavage, and conservation of the correct reading frame was verified by Sanger dideoxy sequencing on double-stranded plasmids.

**Transfection.** Transfection of a high-efficiency transfection variant (13) derived from the P815 cell line was performed as described elsewhere (9, 10). The psvtkneo plasmid (14) carrying a gene for resistance to neomycin (neo) was used for cotransfection and the cells were selected in DME containing 10% FCS and G418 (1.5 mg/ml, Gibco Laboratories, Grand Island, NY). The NP, NP-Bgl II, NP-CW3, and NP-A24 plasmids were linearized with Bgl I before transfection.

**RNA Analysis.** Total RNA extracted by the LiCl method (15) was fractionated on a 1.1% formaldehyde-agarose gel for 4 h at 2.5 V/cm in (3-[N-Morpholino]propanesulfonic acid (MOPS) buffer and then transferred to nitrocellulose filters. The NP probe was a 1,000-bp Hind III fragment corresponding to the 5' region separated on low-gelling temperature agarose after digestion and labeled by the random priming method. Blots were pre-hybridized in 5× SSC, 50% formamide, 5× Denhart, 5 mM EDTA, 0.1% SDS, 50 mM phosphate buffer, pH 7, at 42°C for 5 h. The probe was added to the same mix at 10⁶ cpm/ml and hybridization was carried out overnight at 42°C. The final stringency of washing was 0.2× SSC, 0.1% SDS at 65°C.

**Cytolytic Assay.** P815 transfectant target cells (10⁶) were labeled with 150 μCi sodium [¹⁴C] chromate for 1 h at 37°C and washed three times. Labeled target cells (2 × 10⁵ per well) were incubated with cells from CTL clones in a vol of 200 μl at the CTL to target ratios indicated. After a 4-h incubation at 37°C, the plates were centrifuged and 100 μl supernatant was removed for counting. The percent specific lysis was calculated as (experimental - spontaneous release)/(total - spontaneous release) × 100.

**Results and Discussion**

We have previously shown that Kd-restricted CTL clones raised by immunizing DBA/2 (H-2b) mice with syngeneic P815 cells transfected with cloned HLA-CW3 or HLA-A24 genes can lyse P815 (HLA−) cells in the presence of synthetic peptides containing residues 170-182 of the HLA molecules (6, 11, 16). The two HLA peptides differ only at position 173, which corresponds to lysine for CW3 and glutamic acid for A24. We found that some HLA-specific CTL clones recognized either the CW3 or the A24 peptide, whereas others recognized both, and that the same pattern of specificity was found on P815 cells transfected with the complete HLA genes (16). The oligonucleotides synthesized for insertion into NP were therefore designed to encode amino acids 170-182 of either the HLA-CW3 or the HLA-A24 molecule. After removal of a Bgl II fragment from the coding region of the NP gene, the double-stranded oligomers were inserted to obtain plasmids NP-CW3 and NP-A24 (Fig. 1). As a control, the NP gene with the Bgl II fragment removed was ligated to itself without an insert (plasmid NP-Bgl II). P815 cells were cotransfected with a plasmid carrying a gene for resistance to neomycin (neo) and the HLA-CW3, NP, NP-Bgl II, NP-A24, or NP-CW3 plasmids. Gene expression for individual clones transfected with the NP-derived plasmids was verified by Northern blot analysis with a probe specific for NP (Fig. 2).

P815 cells that expressed the NP-CW3 or NP-A24 recombinant genes were clearly lysed by Kd-restricted anti-HLA CTL clones with a pattern of specificity that corresponded exactly to that obtained on P815 cells transfected with the complete HLA genes (Fig. 3). Controls transfected with the NP or NP-Bgl II plasmids were not lysed by the HLA-specific CTL, but were equally susceptible to lysis by H-2Dk-specific CTL (Fig. 3 and data not shown). We have shown elsewhere (16) that CTL clones CW3/10.1 and A24/10.1 recognize, mutually exclusively, peptides CW3 170-182 or A24 170-182, respectively, whereas CTL clone CW3/1.1 recognizes both. Thus,
FIGURE 1. Schematic representation of recombinant plasmids encoding nucleoprotein (NP) fusion proteins. The critical sequences near the Bgl II (B) sites for each plasmid are shown. The numbers indicate the amino acid residue positions in the original NP plasmid. Amino acids (HLA residues 170-182) are indicated in the one-letter code.

FIGURE 2. Northern blot analysis of RNA isolated from P815 cells transfected with NP recombinant genes. RNA isolated from individual clones of transfected P815 cells was separated on a formaldehyde gel, transferred to nitrocellulose, and probed with the Hind III (H) fragment of NP that is depicted. The lanes correspond to P815 cells transfected with psvtkneo alone (lane 1) or cotransfected with psvtkneo and HLA-CW3 (lane 2), NP (lane 3), NP-A24 (lane 4), NP-CW3 (lane 5), or NP-Bgl II (lane 6).

the specificity of lysis of P815 cells transfected with NP recombinant genes containing HLA oligonucleotide inserts theoretically encoding region 170-182 of the HLA molecules corresponds to the specificity of the inserts.

Our results are in agreement with other studies that suggested that class I-restricted T cells recognize processed or fragmented, rather than native, antigen molecules (5, 6, 17, 18). The present experiments were designed to test directly the prediction that epitopes recognized by class I-restricted T cells could be expressed in the context of unrelated foreign genes, as might be expected if only a peptide fragment
Figure 3. Specific recognition of P815 target cells transfected with NP-CW3 or NP-A24 recombinant genes by HLA-specific (K<sup>b</sup>-restricted) CTL clones. Individual clones from P815 cells transfected with psvtkneo<sup>b</sup> (neo<sup>b</sup>) alone or cotransfected with psvtkneo<sup>b</sup> and NP, NP-Bgl II, NP-CW3, or NP-A24 recombinant genes were labeled with <sup>51</sup>Cr and used as target cells in a 4-h cytolytic assay with CTL clones CW3/10.1, CW3/1.1, and A24/10.1. Control target cells were P815 cells transfected with the thymidine kinase gene and HLA-CW3 (clone 444/C9.3.1) or HLA-A24 (clone 452/D1).

derived from the original antigen were required. The HLA oligonucleotides were inserted into the NP gene that is not expressed at the cell surface. The site for insertion was chosen for its convenience for cloning. It thus seems remarkable that the first NP-HLA oligo recombinants that we constructed could sensitize transfected P815 cells for lysis by HLA-specific CTL. This result implies that the requirements for expression of a given epitope in terms of sites for proteolytic cleavage or biosynthetic pathway of the carrier protein are not very stringent. In this context, it will be interesting to construct other NP-oligo recombinant genes by inserting HLA oligonucleotides into different sites in the NP gene. In view of the apparent differences in the processing and presentation of antigens recognized in the context of class I vs. class II MHC molecules (19), it may be informative to perform a similar analysis for class II-restricted responses. The expression of T cell epitopes encoded by oligonucleotides may be relevant for the design of polyvalent vaccines.

Summary

We have previously demonstrated that H-2<sup>K<sup>b</sup></sup>-restricted CTL specific for HLA-CW3 or HLA-A24 can recognize synthetic peptides corresponding to residues 170–182 of the HLA molecules. Synthetic oligonucleotides encoding region 170–182 of CW3 or A24 were inserted into the influenza nucleoprotein (NP) gene. We demonstrate herein that P815 (H-2<sup>d</sup>) cells transfected with the NP-oligo recombinant genes are
specifically lysed by HLA-specific Kd-restricted CTL clones. Our results imply that there must be a high degree of flexibility for the expression of T cell epitopes in different molecular contexts.

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