Induction of HLA-A2-Restricted CTLs to the Mucin 1 Human Breast Cancer Antigen[1]

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HLA-A*0201/K^b transgenic mice were immunized with oxidized mannan-mucin 1 (MUC1) as a fusion protein (containing five repeats of the 20-amino-acid MUC1 VNTR (variable number of tandem repeats) that generated highly active $CD8^+$ CTLs to MUC1 peptides. In a direct CTL assay, the MUC1 peptides could be presented specifically by both the transgenic murine HLA-A*0201/K^b and human HLA-A*0201 molecules. The 9-mer MUC1 peptide sequences (APDTRPA and STAPPAHGV) were presented by HLA-A*0201, although they did not contain L at P2 and L/V at P9, the preferred motifs; as a consequence, the binding was of relatively low affinity when compared with a high affinity-binding HIV peptide (ILKEPVHGV). In addition, when mice were immunized separately with the HLA-A*0201-binding peptides (STAPPAHGV or APDTRPAP-containing peptides-keyhole limpet hemocyanin-mannan), direct lysis of MCF-7 (HLA-A*0201$^+$, MUC1$^+$) also occurred. The findings are of interest for tumor immunotherapy, particularly as the CTLs generated to low affinity-binding peptides were highly active and could specifically lyse an HLA-A*0201$^+$ human breast cancer cell line without further in vitro stimulation. The findings demonstrate that the range of peptides that can generate CTLs is broader than formerly considered. *The Journal of Immunology,* **1997, 159: 5211–5218.**

There is now considerable emphasis on finding tumor-associated Ags that could serve as targets for immunotherapy; using rDNA techniques, there has been description of many protein sequences that have allowed the preparation of synthetic peptides, recombinant materials, and cytokines to stimulate the immune system. In addition, more efficient Ag presentation is possible using in vitro stimulated macrophages or dendritic cells (1), expressing accessory molecules such as CD28 and B7.1/B7.2 (2), or by using cytokines (3). In this context, we have described recently greatly improved Ag presentation using oxidized mannan-MUC1[3] Ag, which directs the immune response to the T_1 type and induces a high CTL precursor frequency and effective anti-tumor $CD8^+$ CTLs (4–6). Recently, a number of tumor-associated Ags have been described, along with the immunodominant peptides contained therein that are presented by MHC class I molecules, including peptides from melanoma, p53, Her2/*neu*, and the human papilloma virus (7–12). In this context, we recently demonstrated that peptides derived from the breast cancer-associated mucin, MUC1, can be presented by murine class I molecules and detected by CTLs (6). We now show that such peptides can also be presented by HLA class I molecules, particularly HLA-A*0201.

Mucins, which are large heavily *O*-glycosylated transmembrane molecules, are attractive tumor Ags, as they are present in greatly increased amounts (10- to 40-fold) in cancer, and have high cell surface expression, making them useful targets for Abs and non-MHC-restricted T cells, NK cells, and other cells (13). With the increased synthesis of mucins in cancer cells, there should be greater amounts of MUC1 peptides available to be associated with class I molecules. We recently demonstrated that the conjugation of MUC1 VNTR peptides (a fusion protein produced in prokaryocytes) to mannan generated classical $CD8^+$ CTLs, which were MHC class I restricted (5, 6), and could totally protect mice from a subsequent challenge with MUC1$^+$ tumor cells. The findings were that: 1) 15 different mouse strains could be immunized (6); 2) multiple epitopes were found in the 20-amino-acid sequence and were mapped for five different H2 alleles (14); 3) the epitopes did not contain the described anchor motifs for binding to class I molecules, and 4) the peptides bound in the class I groove were detected by anti-MUC1 peptide Abs.[4] On the basis of the immunoreactivity to MUC1 in preclinical studies, clinical trials are now in progress with MUC1 peptides, but it remained to be demonstrated in vivo that MUC1 peptides could associate with HLA molecules. Recently, using stabilization assays, it was shown that 9-mer MUC1 peptides could associate with low affinity to HLA-A*0201 and with higher affinity to HLA-A11, and that in vitro generated CTLs could recognize the MUC1/HLA-A11 complex (15).We now report that HLA-A*0201/K^b mice produce $CD8^+$ HLA-A*0201-restricted CTLs that recognize MUC1 in association with HLA-A*0201, in HLA-A*0201/K^b mice and in human HLA-A*0201-positive cells, and also with the MCF-7 human breast cancer cell line, which can be directly lysed without resorting to extensive in vitro restimulation procedures. A major point of interest is that low affinity-binding peptides can lead to the generation of

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[3] Abbreviations used in this paper: MUC1, mucin 1; FP, human mucin 1 fusion protein with GST attached; KLH, keyhole limpet hemocyanin; M-FP, mannan conjugated to FP in oxidized form; VNTR, variable number of tandem repeats.

[4] V. Apostolopoulos, G. Chelvanayagam, P.-X. Xing, and I. F. C. McKenzie. Anti-MUC1 antibodies react directly with MUC1 peptides bound in the groove of Class I H2 and HLA molecules. *Submitted for publication.*

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highly effective CTLs, studies that form the basis for an immunotherapeutic approach to breast and other MUC1$^+$ cancers in humans.

Materials and Methods

Preparation of MUC1 Ags

Human MUC1 was produced as a fusion protein (FP) (16) for conjugation to mannan (Sigma Chemical Co., St. Louis, MO). Mannan-FP (M-FP) was produced by oxidizing the mannan to a polyaldehyde by treating 14 mg mannan in 1 ml of 0.1 M phosphate buffer, pH 6, with 100 μl 0.1 M sodium periodate in phosphate buffer for 1 h at 4°C; after a further 30-min incubation at 4°C with 10 μl ethanediol, the mixture was passed through a PD-10 column and the mannan fraction was collected; 900 μg of MUC1 FP was added to the oxidized mannan overnight at room temperature (17). Peptides to the MUC1 VNTR, Cp13–32 (C-PAHGVTSAPDTRPAPG STAP) and p1–30 (PDTRPAPGSTAPPAHGVTSAPDTRPAPGST), were synthesized using an Applied Biosystems (Foster City, CA) model 430A synthesizer. The N-terminal cysteine of Cp13–32 allows dimerization, and thus, the Ag consists of two MUC1 VNTR repeats. In addition, 20 overlapping peptides, 9 residues long, spanning the entire tandem repeat of MUC1 protein core and the 7-mer APDTRPA, were synthesized (Chiron Mimotopes, Melbourne, Australia). The 9-mer peptides used for immunizations were coupled to KLH and then to mannan, as above; KLH was used to enable effective conjugation of the 9-mer peptides to mannan. Peptides were dissolved in PBS at a concentration of 1 mg/ml and stored at −20°C until use.

Antibodies

mAbs to murine CD8 (53-6.72) (IgG2a) and HLA-A*0201 (BB7.2) (IgG2b) were used as ascites or tissue culture supernatant. Control isotype Abs were: anti-MUC1 Ab BCP7 (IgG2a) and anti-MUC3 (IgG2b) (18).

Mice and immunizations

Female HLA-A*0201/K^b mice (obtained from The Scripps Clinic and Research Foundation, La Jolla, CA (19)) were between 6 and 8 wk of age. Mice were immunized i.p. with M-FP conjugate (containing 5 μg of FP) weekly for 3 wk. The HLA-A*0201/K^b transgenic mice were crucial for these studies and were constructed to express a chimeric molecule of the α-1 and α-2 domains of the human HLA-A*0201, and the α-3, transmembrane, and cytoplasmic regions are of H2K^b (A2.1/K^b). The α-3 domain of K^b provides suitable interaction sites for murine CD8 molecules, thereby facilitating recognition by murine CD8$^+$ T cells (19).

CTL assays

Spleen cells from mice immunized with M-FP were obtained 7 days after the third immunization assay, as described (4, 5). Target cells used included: EBV-immortalized B cells and PHA lymphoblast target cells derived from HLA-A*0201/K^b mouse spleen cells or from PBMC; PHA blasts function as satisfactory targets in our hands (5, 6), provided the appropriate conditions are used. We optimized the conditions for PHA blasts (PHA-L, leucoagglutinin; Sigma Chemical Co.) and used the culture conditions that generated low spontaneous release of ^{51}Cr and high levels of expression of MHC class I molecules (data not shown). To prepare blast cells, 2×10^6 human PBMC or mouse spleen cells were placed into wells of a 24-well plate with 1 mg/ml of PHA and incubated for 2 days at 37°C in 10% CO_2 to form blast cells. PHA blast cells were then incubated overnight with 20 μM Cp13–32 or p1–30 peptide; 10^6 peptide-pulsed cells were radiolabeled with 100 μCi of $Na_2{}^{51}CrO_4$ (^{51}Cr) (Amersham Corp., Sydney, Australia) for 60 min at 37°C, followed by extensive washing. Human breast cancer cell lines MCF-7 (HLA-A*0201$^+$, MUC1$^+$) and BT-20 (HLA-A*0201$^-$, MUC1$^+$), and melanoma cell line MF272 (HLA-A*0201$^+$, MUC1$^-$) were also used as target cells. Cells were cultured in RPMI 1640 supplemented with 10% FCS (Flow Laboratories, Melbourne, Australia), 2 mM glutamine, 100 U/ml penicillin (Commonwealth Serum Laboratories (CSL), Melbourne, Australia), 100 mg/ml streptomycin (CSL), and 0.05 mM 2-ME (Sigma Chemical Co.).

For inhibition assays, targets were preincubated with various dilutions of mAbs BB7.2 (anti-HLA-A*0201) or M3.2 (anti-MUC3) for ~15 min at room temperature. For blocking experiments with anti-CD4, anti-CD8, or BCP7 Abs, effector cells were preincubated with the Abs (dilution 1/10–1/5000) at room temperature for 15 min, washed, and used in the assay. For inhibition assays involving NK cell-mediated killing, hot ^{51}Cr-labeled MCF-7 cells were incubated with a 20-fold excess of cold K562 cells (human chronic myelogenous leukemia cells, which are sensitive to NK cell lysis).

The assembly assays for peptide binding to HLA-A*0201 were performed as described (20). The peptide transporter-deficient cell line T2 was labeled metabolically with [^{35}S]methionine (100 μCi/10^7 cells, 5×10^6 cell/sample; Amersham Corp.), detergent solubilized in PBS with 5 mM EDTA, 0.5% Nonidet P-40 (Sigma Chemical Co.), 0.5% Mega-9 (Sigma Chemical Co.), 0.02% sodium azide, leupeptin, pepstatin, PMSF, and iodoacetamide in the presence of serial dilutions of peptide, and precleared with Pansorbin (Calbiochem, Sydney, Australia) overnight. The peptide-dependent stabilization of class I MHC heavy chains could then be quantified by immunoprecipitation with the conformation-sensitive Abs BB7.2 (HLA-A*0201), followed by SDS-PAGE and quantitation of the MHC heavy chain bands on a phosphor imager after an overnight exposure of cassettes.

Results

We had shown previously that immunization of mice with M-FP generates MHC class I (H2)-restricted CD8$^+$ CTLs that recognize both MUC1-transfected and MUC1 peptide-pulsed target cells (5, 6, 17). The MUC1 peptides were presented in the context of H2 D, L, or K class I molecules of the b, d, k, s, or z haplotypes, and we were able to identify the MUC1 peptides presented by five different haplotypes (14). We now show that transgenic mice carrying the HLA-A*0201/K^b chimeric human-mouse class I MHC can generate HLA-A*0201/K^b-reactive CTLs.

*Generation of HLA-A*0201-restricted CTLs to MUC1*

Splenocytes from transgenic mice (H2^b; A*0201/K^b) primed with M-FP were tested for their ability to recognize MUC1 Ags presented in vitro by autologous murine spleen PHA blasts, or by human PBMC PHA-induced cells. Both target cells were pulsed with the long MUC1 peptides Cp13–32 or p1–30. Specific lysis of peptide-pulsed autologous murine blast cells occurred (Fig. 1*A*), indicating that the transgenic mice were able to generate an anti-MUC1 CTL response; these findings were in accord with earlier studies with different H2 haplotypes. In addition, HLA-A*0201, 24, PBMC blast cells pulsed with the MUC1 peptides, were lysed by the mouse effector cells, whereas peptide-pulsed targets from HLA-A1, 31; HLA-A11, 11; or HLA-A31, 31 individuals were not lysed (Fig. 1*B*). EBV-transformed B cells were also peptide pulsed and used as targets: there was lysis of EBV-B-HLA-A1, 2 cells, but not of EBV-B-HLA-A1, 29; EBV-B-HLA-A1, 31; or EBV-B-HLA-A11, 32 cells (Fig. 1*C*). In these studies, the presence of the peptide was mandatory; in its absence, no lysis occurred, effectively excluding nonspecific lysis by NK cells (which were also directly examined; see below). These results imply the CTL response was HLA-A*0201 restricted. It should be noted that the CTLs were obtained directly from the spleen and that no in vitro restimulation was required to demonstrate their activity; we ascribe this strong reactivity to the potent induction of cellular responses using oxidized mannan-MUC1.

*Lysis of MCF-7 human HLA-A*0201 breast cancer cell line*

To determinewhether the MUC1 peptides could be endogenously processed and presented by mucin-producing cancer cells, the MCF-7 breast cancer cell line (HLA-A*0201$^+$, MUC1$^+$) was examined together with the HLA-A*0201$^-$/MUC1$^+$ cell line (BT20) or the nonmucin-producing melanoma cell line MF272 (HLA-A*0201$^+$, MUC1$^-$); all three were used as targets for the M-FP in vivo induced CTLs. The HLA-A*0201-expressing tumor (MCF-7) was lysed by the HLA-A*0201-specific CTLs obtained directly from mice (Fig. 2*A*); there was no lysis of BT20 or of MF272 cells (Fig. 2*A*), again demonstrating the HLA-A*0201 restriction and the necessity for the presence of MUC1. The data show that the breast cancer cell line MCF-7 can efficiently present MUC1 peptides, and that MUC1 is not only present in high concentrations on

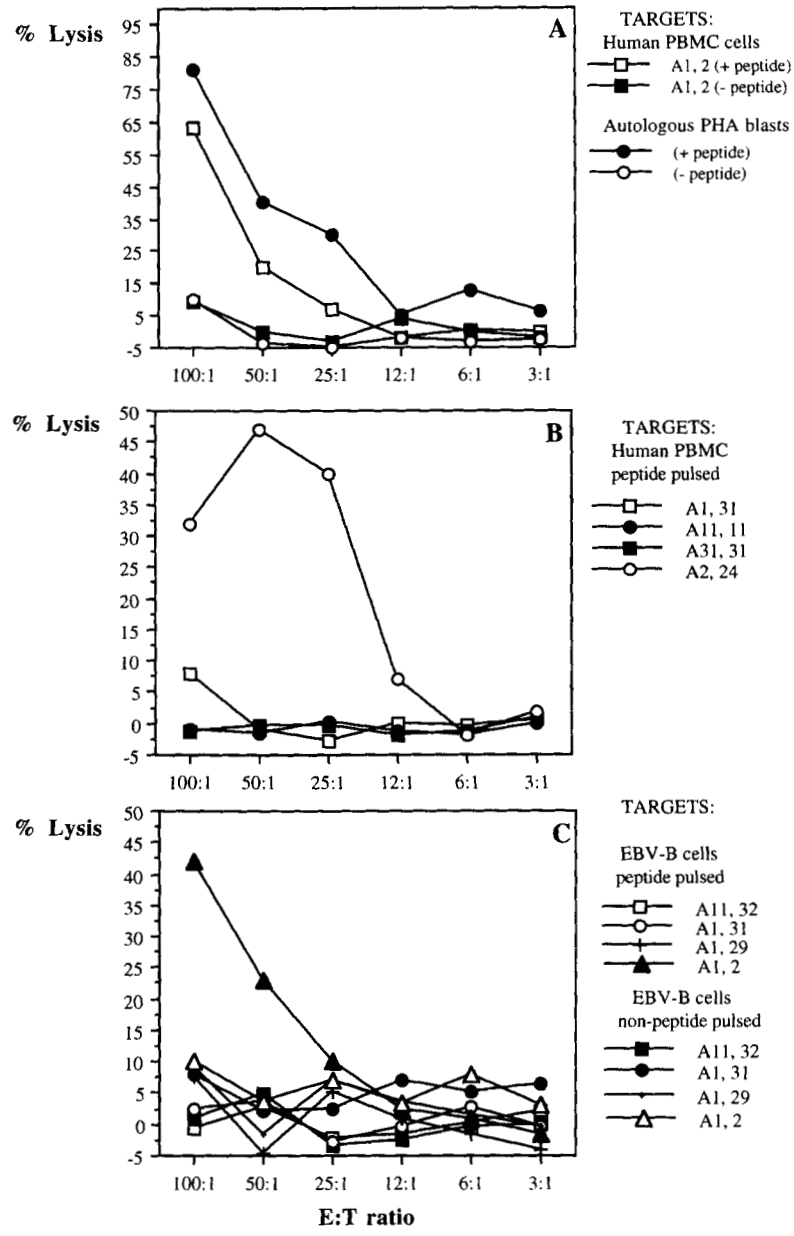

FIGURE 1. CTL assay using CTLs from HLA-A*0201/K^b spleen cells and ^{51}Cr targets, as shown. Ordinate, percentage of lysis; abscissa, E:T ratio.

the cell surface, but is also endogenously processed and presented in association with HLA-A*0201 molecules.

To further examinethe association of MUC1 peptides with class I HLA molecules, blocking experiments were performed with BB7.2, an anti-HLA-A*0201 mAb (Fig. 2*B*). Incubation of target cell (MCF-7) in the presence of increasing concentrations of the BB7.2 Ab (1/10–1/5000) led to the inhibition of the ability of the CTLs to lyse the MCF-7 target cells, whereas anti-MUC3 (M3.2), an isotype Ab control, had no effect. Furthermore, the CTL effector cells were clearly $CD8^+$, as incubating them with increasing concentrations of a known CD8 Ab, before adding to MCF-7 cells, blocked CTL lysis; BCP7, an isotype-matched control, did not (Fig. 2*B*) (the mechanism of the anti-CD8 Ab inhibition is discussed below). The CD4 Ab also did not block (data not shown) (4).

To examine the possibility that the lysis on MCF-7 target cells was NK cell mediated, ^{51}Cr-labeled MCF-7 cells were incubated with a 20-fold excess of cold K562 cells. There was no significant inhibition of killing to MCF-7 cells by the presence of K562 cells (Fig. 2*C*).

We noted a variation of 25 to 60% CTL lysis on MCF-7 target cells from one experiment to the other, most likely due to varying levels of MUC1 and class I expression (FACS analysis, data not shown).

*Mapping the MUC1 peptides binding to HLA-A*0201 molecules*

The results indicated that HLA-A*0201/K^b mice made $CD8^+$ CTLs that recognized MUC1 peptides bound by HLA-A*0201 molecules. It remained to be determined which MUC1 peptides were recognized by CTLs and the relative affinity of the binding of these peptides to HLA-A*0201. Spleen cells from M-FP-immunized HLA-A*0201/K^b transgenic mice were mixed at various E:T ratios with autologous PHA-blast target cells that had been pulsed with 20 overlapping 9-mer peptides from the 20-amino-acid VNTR sequence, so that the response to $H2K^b$, $H2D^b$, and HLA-A*0201 could be examined. In the first study, killing of target cells pulsed with APGSTAPPA, PAPGSTAPP, RPAPGSTAP,

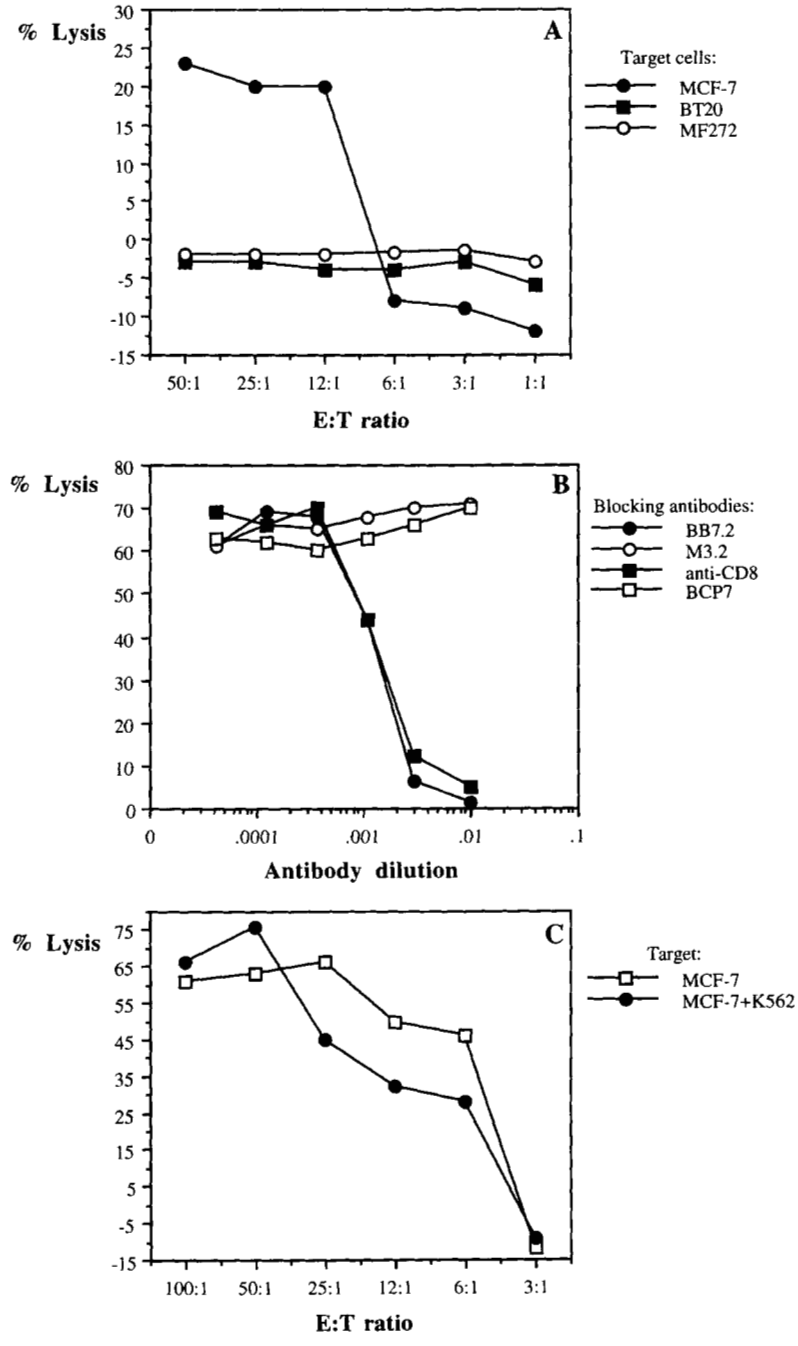

FIGURE 2. *A*, CTL assay using CTLs from HLA-A*0201/K^b mice immunized with M-FP. ^{51}Cr MCF-7 (MUC1$^+$HLA-A*0201$^+$), BT20 (MUC1$^+$HLA-A*02.01$^-$), or MF272 (MUC1$^-$HLA-A*0201$^+$) cells were used as targets. *B*, Inhibition of specific lysis with CD8 Ab. A constant E:T ratio of 50:1 was used, along with dilutions of Ab (1/10–1/5000); BCP7, negative control Ab. ^{51}Cr MCF-7 cells were incubated with anti-HLA-A*0201 (BB7.2) mAb (dilutions 1/10–1/5000); M3.2, negative control Ab. A constant E:T ratio of 50:1 was used. Ordinate, percentage of lysis; abscissa, Ab dilution. *C*, ^{51}Cr MCF-7 cells were incubated with cold K562 cells at a 20:1 hot:cold ratio (K562 is a myelogenous leukemia cell line). Ordinate, percentage of lysis; abscissa, E:T ratio. Effector cells were CTLs from HLA-A*0201/K^b mouse spleens. The above represent experiments performed on different days, and we have reproducibly noted a variation of 25 to 65% CTL lysis on MCF-7 target cells from one experiment to the other.

APDTRPAPG, SAPDTRPAP, TSAPDTRPA, and STAPPAHGV peptides was noted, but not with the other 13 peptides (which also serve as specificitycontrols) (Fig. 3*A*). The lysis of targets pulsed with APGSTAPPA, PAPGSTAPP, and RPAPGSTAP had decreased significantly at an E:T ratio of 25:1, although the other four peptides still had significant target cell lysis (Fig. 3, *A* and *B*). In these studies, the MUC1 epitopes could potentially be presented by K^b and D^b, as well as HLA-A*0201; indeed, in C57BL/6 mice, we have shown previously that the epitopes for K^b are APDTRPAPG, SAPDTRPAP, and TSAPDTRPA, and for D^b are APGSTAPPA, PAPGSTAPP, and RPAPGSTAP.[4] To examine only HLA-A*0201 presentation, EBV-transformed B cells were pulsed with APGSTAPPA, PAPGSTAPP, RPAPGSTAP, APDTRPAPG, SAPDTRPAP, TSAPDTRPA, STAPPAHGV, or APPAHGVTS peptides. Lysis was noted with target cells pulsed with the APDTRPA-containing peptides and with STAPPAHGV (Fig. 3*C*). Although the three APDTRPA-containing peptides are bound by class I molecules, it is likely that the T cell epitope is APDTRPA, as pulsing with the 7-mer APDTRPA gave the same amount of lysis (Fig. 3*C*).

*CTLs generated by immunization with HLA-A*0201-presenting MUC1 9-mer peptides*

As the9-mer MUC1 peptides were the epitopes presented by HLA-A*0201, it was of interest to define whether these could immunize mice to make effective CTLs. Thus, mice were immunized with the HLA-A*0201-binding peptides (STAPPAHGV and the AP DTRPA-containing peptide; peptides were linked to KLH and to mannan), and similar direct lysis of MCF-7 cells occurred (Fig. 4). BT20 and MF272 were negative, showing againthat there was HLA-A*0201 restriction and the necessary presence of MUC1.

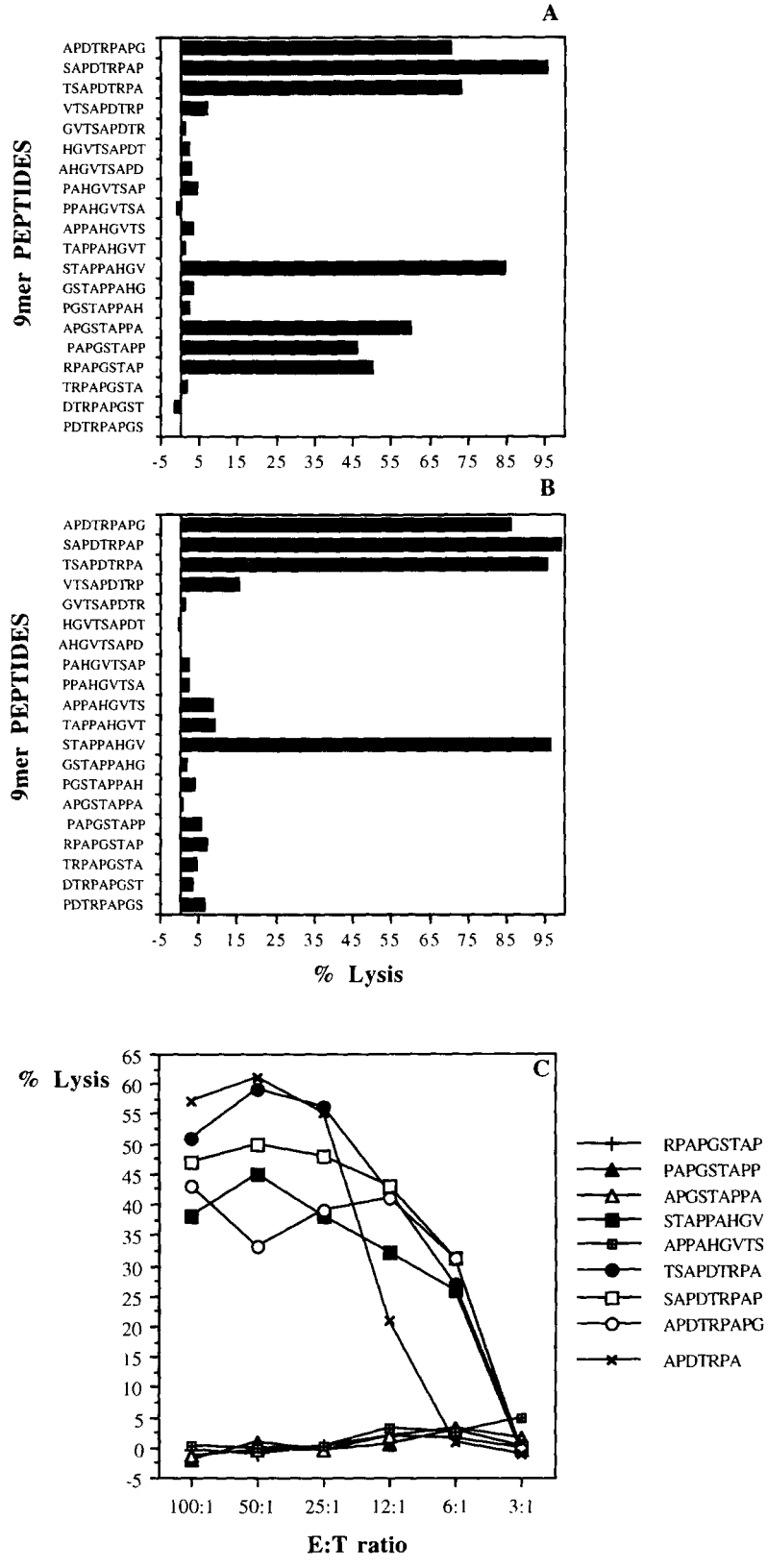

FIGURE 3. CTL assay using CTLs from HLA-A2/K^b spleen cells on autologous PHA blasts pulsed with overlapping 9-mer peptides spanning the MUC1 VNTR. *A*, 100:1, and *B*, 25:1 E:T ratio. *C*, CTL assay using CTLs from HLA-A*0201/K^b spleen cells and ^{51}Cr HLA-A*0201-EBV-B cell peptide-pulsed targets, as shown. Ordinate, percentage of lysis; abscissa, E:T ratio.

*Affinity of binding MUC1 peptides to HLA-A*0201 molecules*

As shown, HLA-A*0201 molecules can present both APDTRPA-containing peptides and the STAPPAHGV peptide (Table I). These peptides are unusual in that HLA-A*0201-binding peptides usually have the hydrophobic amino acid L at P2, and L/V at P9 as anchor residues; the absence of complete anchor motifs (STAPPAHGV has V at P9) could lead to low affinity binding;we therefore tested the binding to HLA-A*0201 of the APDTRPA-containing peptides and the STAPPAHGV peptide. It was apparent that the affinity of binding of the MUC1 peptides to HLA-A*0201 was substantially less than that observed with a known high affinity-binding HIV-binding peptide

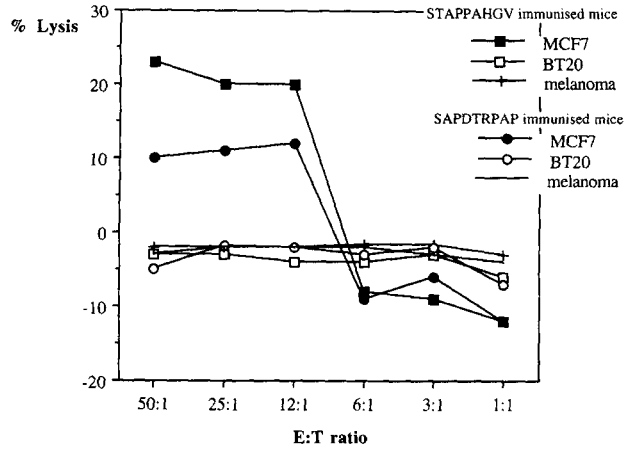

FIGURE 4. CTL assay using CTLs from HLA-A*0201/K^b mice immunized with STAPPAHGV or SAPDTRPAP-KLH-mannan. ^{51}Cr MCF-7 ($MUC1^+$HLA-A*0201^+), BT20 ($MUC1^+$HLA-A*0201^-), or MF272 ($MUC1^-$HLA-A*0201^+) cells were used as targets. Ordinate, percentage of lysis; abscissa, E:T ratio.

(ILKEPVHGV) (Fig. 5). For example, in the binding/stabilization assay, HIV peptide at 1 μM was equivalent in the intensity of the heavy chain band with 40 μM of STAPPAHGV and TSAPDTRPA (Fig. 5). The SAPDTRPAP and APDTRPAPG peptides in the same assay gave substantially less stabilization (Fig. 5). Thus, the MUC1 peptides bind with lower affinity than the known high affinity-binding peptide. In an inhibition system, we also found that the MUC1 peptides bound with a similar affinity to the binding of a low affinity-binding influenza peptide (data not shown). The results are in accord with the requirements for the appropriated binding motifs for HLA-A*0201 (L2, L/V9), both found in HIV (ILKEPVHGV), V9 only found in STAPPAHGV, but neither found in TSAPDTRPA, SAPDTRPAP, and APDTRPAPG peptides. Although the MUC1 peptides bound with low affinity, it was of interest that such CTLs could be generated; presumably the TCR has a compensatory high affinity for the MHC-MUC1 peptide complex.

Discussion

Our studies indicate that mice immunized with MUC1 FP conjugated to mannan (M-FP) produce CTLs that react with human MUC1 VNTR peptides presented by HLA-A*0201 class I molecules. In mice, we had demonstrated previously that the CTL response was class I restricted (6), and that multiple epitopes from the 20-amino-acid VNTR region of MUC1 could be presented (14). In this study, we demonstrate the generation of HLA-A*0201-restricted $CD8^+$ CTLs after immunization of transgenic mice carrying the HLA-A*0201/K^b chimeric human-mouse class I MHC. It was noted that two different MUC1 peptides could associate with HLA-A*0201 with a low affinity binding to the MHC; however, these MUC1 peptides were able to elicit CTLs, in which, presumably, the individual TCRs displayed compensatory high affinity for the MHC-peptide complex. Furthermore, and of importance to immunotherapy, the anti-MUC1 CTLs were able to directly lyse $MUC1^+$/HLA-A*0201^+ breast cancer cells, i.e., the immunization of HLA-A*0201/K^b mice with M-FP can produce CTLs that, without further stimulation, could directly lyse tumor cells.

The HLA-A*0201/K^b transgenic mice were crucial for these studies, and it has been reported that the mice can identify HLA-A*0201-restricted hepatitis C and influenza peptides similar to HLA-A*0201 human CTLs (22, 23). Such studies have demonstrated that substituting the α-3 domain of HLA-A*0201 with its murine counterpart overrides the difficulty in generating HLA-A*0201-restricted CTLs in mice (24, 25). Our findings demonstrate that immunization of HLA-A*0201/K^b transgenic mice with MUC1 fusion protein conjugated to mannan (M-FP) leads to the generation of HLA-A*0201-restricted $CD8^+$ MUC1-specific CTLs. The blocking of $CD8^+$ cells by anti-CD8 Abs suggests the CD8 CTL-class I interaction is of low affinity; however, we note in the description of the HLA-A*0201/K^b mice (26), most T cell clones produced could be blocked with anti-CD8 Ab.

Using long peptides of the MUC1 VNTR (Cp13–32 or p1–30), CTLs from A2.1/K^b-immunized mice were able to lyse autologous PHA-induced splenocytes, HLA-A*0201 PBMC from HLA-A*0201 individuals, and HLA-A*0201 EBV-B peptide-pulsed target cells (Fig. 1). A major point of interest is that the CTLs obtained by M-FP immunization of HLA transgenic mice were able to recognize endogenously processed and presented human MUC1 tumor peptides (Fig. 2) from the MCF-7 breast cancer cell line. Several studies, e.g., for p53 and for human papillomavirus type 16 E6 and E7 peptides, demonstrated that immunizing with peptides generated CTLs that reacted with peptide-loaded target cells (12, 27), but not with the endogenously processed peptides from the respective tumors. Clearly, the M-FP peptide immunization protocol is superior to immunization with peptide alone. How such good lysis occurs is not entirely clear; the MUC1 peptides do appear to bind to class I molecules with low affinity; however, a striking aspect of the study is the generation of large numbers of potent CTLs. Recently, Dahl et al. showed that low affinity-binding peptides can generate high avidity autoreactive CTLs (28), to cyclin D1, mdm2, and p53, the avidity of peptide-induced CTLs differing by >1000-fold. In their study, it was paradoxical that the generation of high avidity CTLs was by low affinity peptides, whereas high affinity peptides generated low avidity CTLs (28). In other studies, the affinity of the peptide-MHC interaction was shown to be an important parameter in the induction of CTLs; low affinity peptide-MHC interactions can lead to sufficient stabilization of MHC class I complexes to induce effective CTLs (29). Thus, the HLA-A*0201-binding peptides may not have the appropriate anchor motifs, but bind with a low affinity and induce high avidity CTLs.

Using overlapping 9-mer peptide-loaded targets (autologous splenic PHA-induced cells), multiple CTL epitopes were demonstrable (Fig. 3) at an E:T ratio of 100:1 (which included peptides that were previously found to associate with $H2D^b$ (14)). When EBV-B-HLA-A*0201 cells were pulsed, it was clear that only the APDTRPA-containing peptides and STAPPAHGV associated with HLA-A*0201 (Fig. 3*C*). This was emphasized further when mice were immunized with 9-mer peptides coupled to KLH and mannan, when specific lysis of peptide-coated cells and of MCF-7 occurred. The STAPPAHGV peptide binding is in accord with recent in vitro studies (15) that demonstrated it to be a low HLA-A*0201-binding peptide; the APDTRPA-binding peptides were not detected in this study. Other studies in our laboratory agree with these findings, as CIR-A2 cells (Hmy2.CIR B cell line, transfected with HLA-A*0201) bind STAPPAHGV, but not AP DTRPA in a stabilization study; but when CIR-A2 cells were used as targets, lysis occurred with both APDTRPA-containing peptides and STAPPAHGV (L. Hwang et al., unpublished data). Furthermore, in a separate study (M. Reddish, Biomira, personal communication) eluted the APDTRPA-containing peptides from HLA-A*0201^+ cells. In his study, $MUC1^+$, HLA-A*0201^+, MCF-7 cells were used, in which the class I molecules were isolated by immunoprecipitation with an anti-HLA-A*0201 Ab, acid treated to release bound peptides, which were then purified and coated on an ELISA plate, in which an anti-MUC1 Ab, BCP8 (which detects the epitope DTR), was used to detect the presence of the peptide

Table I. *Comparison of known HLA-A2 peptides with MUC1 peptides*

HLA-A2-Binding Motifs	Position 1	2	3	4	5	6	7	8	9[b]	Origin of Peptide	Reference[a]
Anchor risdues	L								V		(30)
									L		
Alternative residues	A		P				H				(30)
		P	D								
Examples of binding peptides	S	L	L	P	A	I	V	E	L	protein phosphatase 2A	a
(CTLs not demonstrated)	S	X	P	S	G	G	X	G	V	unknown	a
	S	X	X	V	R	A	X	E	V	unknown	a
	L	L	L	D	V	P	T	A	A	V Q	IP-30 signal sequence	a
		A	L	L	P	P	I	N	I	L	unknown	b
		H	L	I	D	Y	L	V	T	S	carboxypeptidase M91-99	b
		M	L	L	S	V	P	L	L	L G	calruticulum signal sequence	c
T cell epitopes		L	L	F	G	Y	P	V	Y	V	HTLV-1 tax 11-19	d, (22)
(found to be CTL)		I	L	K	E	P	V	H	G	V	HIV-1 RT 476-484	(22), e
		G	L	S	P	T	V	W	L	S	hepatitis B sAg 348-357	f
		A	A	G	I	G	I	L	T	V	Melan A/Mart 1	(9)
		F	I	A	G	N	S	A	Y	E Y	HCMV glycoprotein B	f
		I	A	G	N	S	A	Y	E	Y V	human CMV gB 618-628	f
		D	L	M	G	Y	I	P	L	V	HCV core 132-140	(23)
		I	L	D	G	T	A	T	L	R L	pmel 17/gp 100	(11)
		I	L	G	F	V	F	T	L	T	Influenza MP 59-68	(21)
			A	P	D	T	R	P	A	P G	MUC1 VNTR	
		S	A	P	D	T	R	P	A	P	MUC1 VNTR	
	T	S	A	P	D	T	R	P	A		MUC1 VNTR	
		S	T	A	P	P	A	H	G	V	MUC1 VNTR	

[a] Reviews on known class I molecules: Engelhard et al., 1994; Ref 30; a: Hunt et al., 1992; b: Harris et al., 1993; c: Henderson et al., 1992; d: Utz et al., 1992; Ref. 22; e: Henderson et al., 1993; f: Nayersima et al., 1993.

[b] The underlined amino acids correspond to the same amino acids as those of MUC1 VNTR.

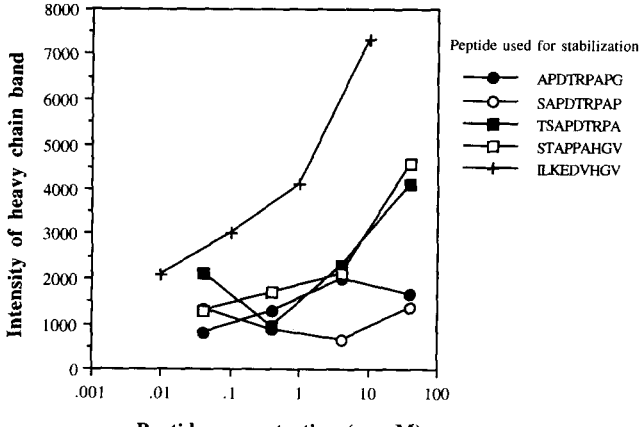

FIGURE 5. Assembly assays for peptide binding to HLA-A*0201. The T2 peptide transporter-deficient cells were labeled with [^{35}S]methionine, detergent solubilized in the presence of serial dilutions of peptide (ILKEPVHGV, 0.01–10 μM) (STAPPAHGV, TSAPDTRPA, SAPDTRPAP, APDTRPAPG, 0.04–40 μM). Class I MHC heavy chains were immunoprecipitated with BB7.2, followed by SDS-PAGE. Ordinate, intensity of heavy chain band; abscissa, peptide concentration (μM).

APDTRPA; STAPPAHGV could not be detected in this system, as it is not bound by the anti-MUC1 Ab.

The MUC1 peptides that bind HLA-A*0201 do not fit with the consensus sequences for the anchor positions, although adjacent amino acids can make contact with the MHC groove. The anchor residues of the HLA-A*0201 are usually large nonpolar amino acids, L2, and L/V9 (although position (P) 9 can accommodate a range of other amino acids) (27). The MUC1 HLA-A*0201-binding sequence SAPDTRPAP has a small nonpolar alanine (A) at P2 (different from the large nonpolar L anchor), but A at P2 is not uncommon, e.g., Melan A/Mart 1 and human CMV gB 619–628 peptides. At P9, however, MUC1 peptides have a small nonpolar P, although V/L is preferred; at the C terminus, the peptide is not anchored,[4] which may serve to explain how MUC1 peptides appear to bind with a relatively low affinity. Although preferred P2-P9 combinations are predictive of strong peptide binding, it is becoming clear that various other combinations give rise to high affinity binding, but may not provide sufficient affinity to enable durable survival at the cell surface. Indeed, the melanoma peptide AAGIGILTV for HLA-A*0201 does not have the standard anchor residues at P2 or P9, but does make the appropriate interactions, and is a high affinity-binding 9-mer peptide (9). Similar findings have been noted with the H2K^b tumor-associated E6 human papillomavirus type 16 protein (31) and the peptide SRDHSRTPM for H2K^b (32), in which the peptides bind with high affinity, but do not provide sufficient stability to the class I complex to be detected by anti-class I Abs.

It was noted that three of the HLA-A*0201-binding peptides were the same related peptides that bound to H2K^b (APDTR PAPG, SAPDTRPAP, TSAPDTRPA),[4] even though the human and mouse class I molecules are different and require different anchoring residuesin the groove. H2K^b differs from the rest of the murine and human class I molecules in that the first anchor residue occurs at P5 in the middle of the peptide sequence rather than at P2, and that H2K^b generally binds 8-mer peptides. Computer modeling of the clefts show that HLA-A*0201 has a flat shallow central cleft region, and therefore accommodates no binding sites for a central anchor residue (33). Furthermore, the crystal structure of the 9-mer SEV peptide bound to H2K^b (34) shows that this peptide is anchored in MHC in the same way as peptides to HLA-A*0201/9-mer complexes (24). Thus, a similar mode of binding to K^b and HLA-A*0201 is possible. Consequently, crystallographic studies of MHC class I peptide complexes have shown that the overall backbone structure of three human (HLA-A*0201, HLA-Aw68,

and HLA-B27) and two murine ($H2K^B$ and $H2D^b$) class I complexes is very similar (33), with an amino acid sequence identity 70% between $H2K^b$ and HLA-A*0201. Whatever the mode of binding of MUC1 peptides, it is apparent that they are of low affinity (Fig. 4), although strong CTLs are induced.

Hopefully, the findings can be applied directly to patients, but obviously mice and humans have different T cell repertoires, and human MUC1 is foreign to mice. However, of relevance are the findings of Finn and colleagues, who could generate anti-MUC1 CTLs in chimpanzees, although whether these were MHC restricted was not determined (34). We have recently immunized cynomologous monkeys with MUC1 and obtained CTLs,[5] and also in patients with adenocarcinoma of breast and other tissues; two patients made anti-MUC1 CTLs, of which both were HLA-A*0201 (35).

The use of HLA transgenic mice in identifying potential antigenic peptides presents a number of advantages, the most obvious being the ability to prime for an immune response in vivo. Although this work restricts its discussion to MUC1, the strategy described herein could be of value to a number of different Ags, and defining the CTL epitopes forms the basis for immunotherapy and vaccine design. The MUC1 peptides were restricted by HLA-A*0201, and clearly the molecule contains many different sequences capable of presentation at least by the murine MHC class I molecules to the TCR. Thus, the MUC1 VNTR peptide has more than one T cell epitope, and the range of epitopes generated is central in the development of a breast cancer (MUC1) vaccine. M-FP is used currently in clinical trials, and from the studies described herein, it is likely that HLA-A*0201 patients have the potential to be targeted by immunotherapy; whether they can be immunized by the self MUC1 peptide will be the aim of the clinical study.

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