Advances in Brief

Naturally Processed Class II Epitope from the Tumor Antigen MUC1 Primes Human CD4+ T Cells[1]

Elizabeth M. Hiltbold, Pawel Ciborowski, and Olivera J. Finn[2]

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Abstract

Epithelial cell mucin MUC1 is expressed on adenocarcinomas in an underglycosylated form that serves as a tumor antigen in breast, pancreatic, ovarian, and other tumors. Two predominant MUC1-specific immune responses are found in patients: CD8+ CTLs, which recognize tandemly repeated epitopes on the MUC1 protein core, and IgM antibodies. There have been no reports to date of MUC1-specific CD4+ T-helper cells in cancer patients. We show here that MUC1-specific CD4+ T cells are neither deleted nor tolerized and that CD4+ T cell responses can be generated when an appropriate soluble form of MUC1 is used. Naive CD4+ T cells from healthy donors were primed *in vitro* to a synthetic MUC1 peptide of 100 amino acids, representing five unglycosylated tandem repeats, presented by dendritic cells. They produced IFN-γ and had moderate cytolytic activity. We identified one core peptide sequence, PGSTAPPAHGVT, that elicits this response when it is presented by HLA-DR3.

Introduction

MUC1 mucin is a highly glycosylated integral membrane glycoprotein that is expressed on the apical surface of ductal epithelia; epithelial adenocarcinomas of the pancreas, breast, ovary, colon, prostate, and lung (1, 2); and multiple myelomas (3). Both humoral and cellular immune responses that are specific for MUC1 have been reported in patients with MUC1+ tumors. The humoral immune responses are primarily of the IgM isotype and specific for a tandemly repeated immunodominant epitope on the MUC1 polypeptide core (4). The predominant CTL responses are MHC unrestricted and directed against the same immunodominant peptide epitope (5–7). MHC-restricted CTL responses have been detected against one epitope derived from the tandem repeat region of the MUC1 polypeptide core, restricted by HLA-A2 (8), and against another epitope from the same region of the molecule, restricted by HLA-A11 and, to a lesser degree by HLA-A3, HLA-A2, and HLA-A1 (9). Neither the antibodies nor the CTL responses reach therapeutic levels. Both responses are T-helper cell independent, and there has been no evidence to date of MUC1-specific T-helper cells in cancer patients.

Tumor cells express high levels of underglycosylated MUC1 molecules on their surface. Some MUC1 molecules are also cleaved from the tumor cell surface and can be found in a soluble form in sera and ascites from patients. These molecules are very large, due to their extensive glycosylation with mostly O-linked sugars terminating in sialic acid. These are the predominant and possibly the only soluble

forms of MUC1 available for processing by APCs.[3] The underglycosylated or completely unglycosylated forms remain tumor associated and unavailable to APCs.

We set out to determine whether a healthy human peripheral T-cell repertoire contained T-helper cells capable of recognizing MUC1 tandem repeat polypeptide core epitopes. The putative epitopes were to be processed by APCs either from a fully glycosylated and sialilated soluble form that is found in cancer patients or from unglycosylated long synthetic peptides. Using *in vitro* expanded DCs as APCs and autochthonous naive CD4+ T cells as responders, we found that we could prime CD4+, MUC1-specific, class II-restricted T cells only in response to the unglycosylated tandem repeat long synthetic peptide. The fully glycosylated MUC1 protein isolated from a patient's ascites did not stimulate specific T-helper cells and resulted only in limited MHC-unrestricted CTL responses, as did the forms of MUC1 found previously in cancer patients (7, 10).

It is tempting to speculate that eliciting efficient T-helper cell responses to this antigen might serve to amplify the tumor-specific CTL responses and to promote isotype switching and increase in titers of tumor-specific antibody, leading ultimately to an efficient antitumor immune response.

Materials and Methods

Peptides. The 100-amino acid peptide of MUC1, (GVTSAPDTRPAPG-STAPPAH)₅, was synthesized in the peptide synthesis facility of the Department of Molecular Genetics and Biochemistry at the University of Pittsburgh School of Medicine (Pittsburgh, PA). The seven peptides designed to bind to HLA-DR3 (peptide 1, APGSTAPPAHGV; peptide 2, SAPDTRPAPGST; peptide 3, PGSTAPPAHGVT; peptide 4, PGSTAPPAHGVTSAPDTRPA; peptide 5, SAPDTRPAPGSTAPPAHGVT; peptide 6, APGSTAPPAHGVTSAPDTR; and peptide 7, TAPPAHGVTSAPDTRPAPGS) were a gift from Corixa Corporation (Seattle, WA).

Antibodies. Mouse antihuman HLA-DR (L243), CD56 (Leu-19), CD8 (Leu-2a), CD3 (Leu-4), CD3-Per cp, CD8-FITC, and mouse IgG isotype control were purchased from Becton Dickinson (San Jose, CA). Mouse antihuman CD45RO (UCLHL) and CD20 were purchased from DAKO A/S (Glostrup, Denmark). Mouse antihuman IFN-gamma-PE, mouse antihuman IL-10-PE, rat antihuman IL-4-PE, mouse IgG-PE, and rat IgG-PE were purchased from PharMingen (San Diego, CA).

Cell Lines. Breast tumor cell line BT-20 (HLA-A24, 31; B15, 7, Bw4) and pancreatic tumor line HPAF (HLA-A1, 2601; B5401/55/56/5901, B8, Bw6) were described previously (5, 7). The SV-40-transformed IB3 epithelial cell line (HLA-A24, 11; B5401/55/56/5901, B51/52/7801, Bw4, Bw22) was a gift from Dr. Jill Siegfried, Department of Pharmacology, University of Pittsburgh (Pittsburgh, PA). The B lymphoma cell line Raji (HLA-A3; B15; C7; DR3; DRw10; DQw1, 2) was purchased from American Type Culture Collection (Manassas, VA). Croft EBV (HLA-A2; B7, 8, Bw6; Cw7; DR1,3, DRw52, 53; DQ1) is an immortalized B-cell line that was established in our laboratory (11). The MUC1-negative epithelial tumor cell line MS (HLA-A3; B7; C7) was derived from breast adenocarcinoma and characterized in our laboratory.

[3] The abbreviations used are: APC, antigen-presenting cell; DC, dendritic cell; PE, phycoerythrin; IL, interleukin.
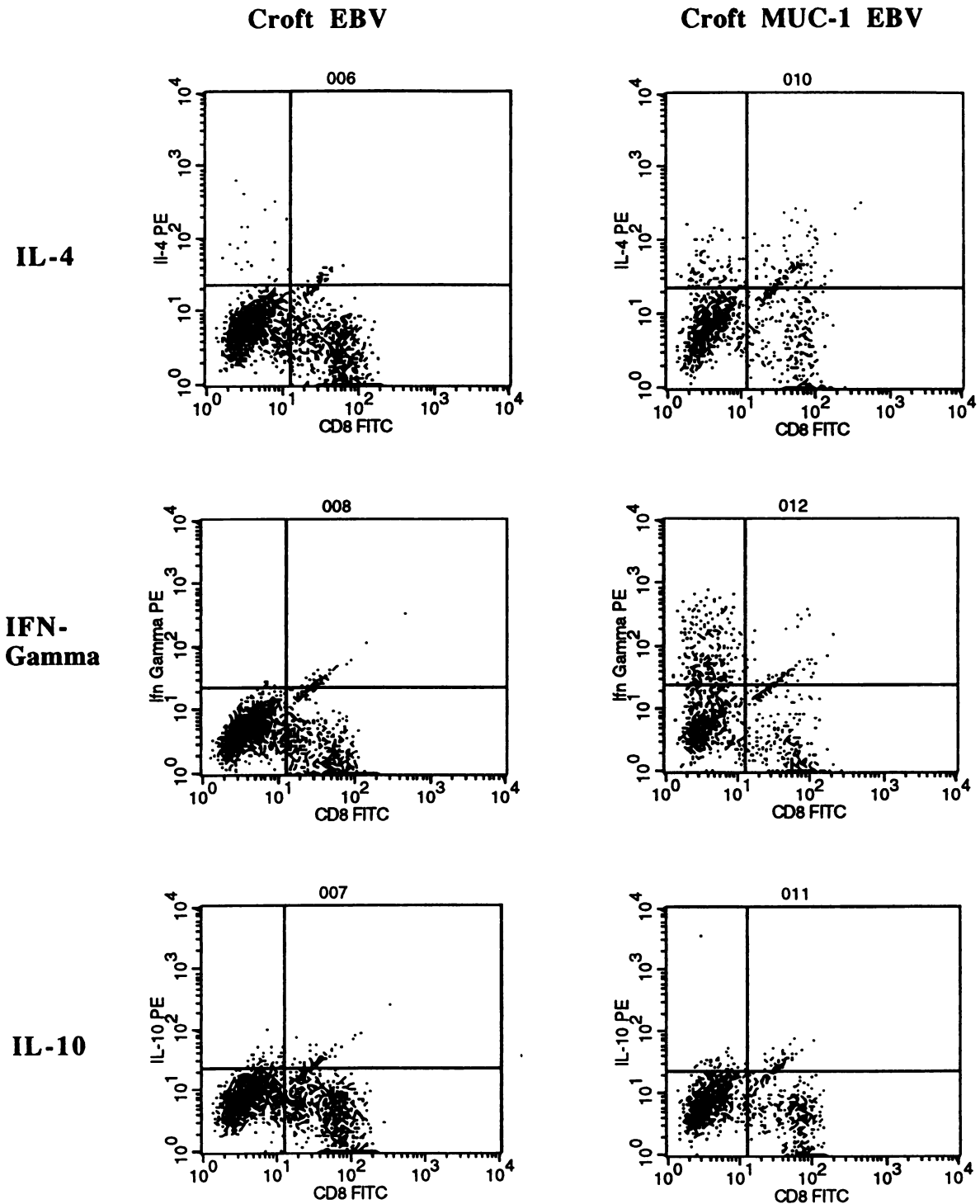
Croft EBV

Croft MUC-1 EBV

Fig. 1. Cytokine production of 100-mer peptide-primed T cells in response to Croft EBV or Croft MUC-1 EBV B cells. T cells were stimulated with either Croft EBV (*left*) or Croft MUC1 EBV (*right*) for 24 h. Production of IL-4, IFN-gamma, and IL-10 was analyzed by intracellular cytokine staining and flow cytometry. Cells were first gated for expression of CD3 and then separated based on the expression of CD8, indicated on the *X axis*. The cells were 75% CD4+/25% CD8+.

In Vitro **Priming and Functional Assays.** T cells, DCs, and macrophages were derived from a leukophoresis product collected by the Central Blood Bank of Pittsburgh (Pittsburgh, PA) from a healthy donor who was HLA-A1, 3; B7, 8, Bw6; DR2, 3; DQ-1, 2.

DCs were cultured and matured *in vitro* for a 7-day culture period according to a previously described method (12). DCs were purified by removing T, B, and natural killer cells using lineage-specific antibodies and magnetic beads coated with goat antimouse IgG (Dynal A/S, Oslo, Norway). The DCs were further matured for a period of 24 h by the addition of tumor necrosis factor-α

(100 units/ml) purchased from Genzyme (Cambridge, MA) in the continuous presence of granulocyte macrophage colony-stimulating factor and IL-4. Antigens were added during this final 24-h culture period to allow for their uptake and processing.

Naive T cells were purified from peripheral blood mononuclear cells by panning to remove cells expressing CD8, CD45RO, HLA-DR, CD56, and B-cell markers CD19 and CD20. The T-cell population obtained was >80% CD4+ and expressed CD45RA. T cells were combined with DCs that had been preincubated with protein or peptide antigen (100 μg/ml) overnight, at a ratio
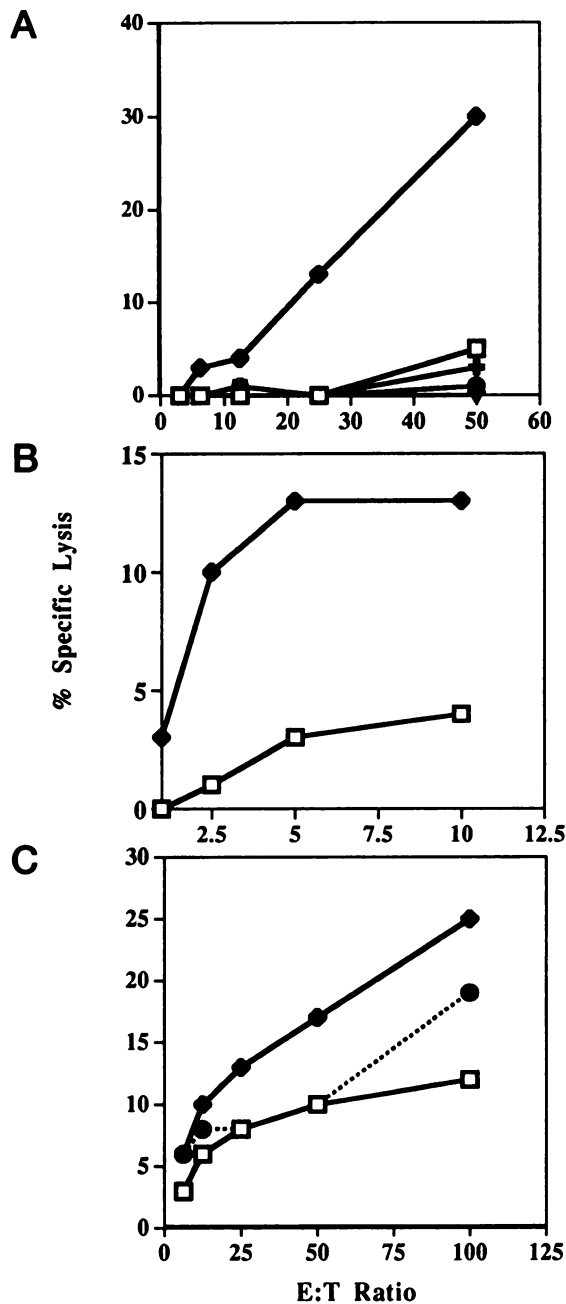
Fig. 2. Cytolytic activity of 100-mer peptide-primed T cells. T cells were stimulated with target cells for 4 h in a standard chromium release assay. *A*, targets matched with T cells at HLA class I loci include BT-20 (▲), HPAF (◆), MS (▽), Croft EBV (□), and Croft MUC1 EBV (◆), and those matched with T cells at class II loci include Croft EBV and Croft MUC1 EBV. IB3 (●), used as a negative control, is a MUC1-expressing target with no HLA match with the T cells. All target cells express MUC1 except MS, which was also used as a negative control. *B*, Raji (HLA-DR3 homozygous target). □, Raji; ◆, Raji MUC1. *C*, Croft EBV was preincubated with the 100-mer peptide for 4 h prior to the cytolysis assay. □, Croft, EBV; ◆, Croft MUC1 EBV; ●, Croft EBV + 100-mer.

of 10:1 and plated in 96-well plates at 10^5 T cells per well in RPMI (Life Technologies, Inc., Gaithersburg, MD) containing 10% human serum AB (Gemini Bioproducts, Calabasas, CA). Cytokines used in the priming culture were 20 units/ml IL-1 (R&D Systems Inc., Minneapolis, MN), 20 units/ml IL-2 (Dupont, Wilmington, DE), and 20 ng/ml IL-4 (Schering Plough, Kenilworth, NJ). T cells were restimulated weekly with antigen-pulsed autochthonous macrophages and maintained in 20 units/ml IL-2. For intracellular cytokine staining, T cells were stimulated, stained for surface markers, permeabilized, and fixed as described previously (13). Cytolysis assays were performed as reported previously (6).

Results and Discussion

Priming of CD4+ MUC1-specific T Cells

Cytokine Production. *In vitro*-generated DCs were pulsed exogenously either with the purified soluble form of MUC1 derived from tumor ascites fluid obtained from a breast cancer patient or the MUC1 100-mer peptide corresponding to five tandem repeats of the molecule. The DCs were given 18 h to process the antigen and present it on their class II molecules, and then they were combined with purified naive, CD4+ (depleted of CD8+ cells) autochthonous T cells in a priming culture. The T cells were restimulated three times at weekly intervals with the same antigen on macrophages before their function and specificity were analyzed. The cultures primed with the fully glycosylated soluble MUC1 protein resulted in vigorous T-cell blastogenesis and proliferation of both CD4+ and CD8+ T cells without generation of any MUC1 specific T-helper responses (data not shown). In the same time period, the cultures primed with the unglycosylated long synthetic peptide showed outgrowth of primarily CD4+ T cells (>75%).

We began to asses the function of the 100-mer peptide-primed T cells by evaluating their cytokine production in response to MUC1 presented by EBV-immortalized B-cell line Croft EBV, matched with the responding T cells at both class I (HLA-B8) and class II (HLA-DR3) and transfected with MUC1. As shown in Fig. 1, stimulation with Croft MUC1 EBV resulted in a specific response in only the CD4+-cell compartment (left quadrants of each plot), evidenced in the production of primarily IFN-γ. There was no specific response by CD8+ T cells. IFN-γ is a hallmark cytokine of a Th1-type CD4+ T-cell population. IL-4, a Th2-type cytokine, was produced at very low levels by both CD4+ and CD8+ T cells, and there was little or no IL-10 detected. There was little or no cytokine response following stimulation with the MUC1-negative parental line Croft EBV. Because different cytokines are optimally expressed at different times after antigen stimulation [IL-10, in particular, is known to be a late responder (14)], we also analyzed the T-cell response at 48, 72, and 96 h poststimulation and found that IFN-γ was still the predominant cytokine produced (data not shown).

Cytolytic Activity. We further analyzed these T cells in CTL assays to determine the specificity, if any, of the minor CD8+ T-cell population that was present in the culture, as well as to gain additional information regarding the specificity of the CD4+ T cells. As targets we used a panel of tumor cell lines and EBV-immortalized B-cell lines selected for their HLA and MUC1 phenotype. Croft EBV and Croft MUC1 EBV matched with the T cells at HLA-B8 and DR3; MUC-1+ breast tumor line BT-20 matched with the T cells at HLA-B7; MUC1+ pancreatic tumor line HPAF matched with the T cells at HLA-A1 and B8; MUC1− control epithelial tumor cell line MS matched with the T cells at HLA-A3 and B7; and another negative control, MUC1+ epithelial cell line IB3, had no HLA matches with the T cells. In several CTL assays carried out with these target cells, we found specific lysis only of the DR3+ Croft MUC1 EBV (Fig. 2*A*). These data suggested that the MUC1-specific cytolytic activity was restricted to HLA-DR3. To confirm this, we added another target, the B-cell line Raji, which is homozygous for HLA-DR3. This target was also specifically recognized by the T cells, but only when transfected with MUC1 cDNA (Fig. 2*B*). Furthermore, we tested the recognition by the T cells of the Croft EBV, Croft MUC1 EBV, and Croft EBV, which had been pulsed with the 100-mer MUC1 peptide overnight (Fig. 2*C*). We found that, as before, the T cells lysed the MUC1+ cells and not the parental cells, and that preincubation of the parental cells with the 100-mer peptide overnight made them susceptible to lysis. Thus, both the MUC1 cDNA-transfected cells as well as cells loaded

Antigen

Antigen

Fig. 3. T-cell responses to tandem repeat peptides. *A*, cytolytic activity of T cells against Raji targets pulsed with individual peptides. Cytolytic activity was assessed in a standard chromium release assay and cytolysis of peptide-pulsed Raji cells was compared to the background lysis of the MUC1-negative target Raji (4%) at an E:T ratio of 1:10. *B*, production of IFN-γ in response to individual peptides. T cells were stimulated with autochthonous macrophages that had been preincubated with one of the seven peptides or no antigen. IFN-γ was detected by intracellular cytokine staining. The fold increase in IFN-γ response was calculated by comparing the percentage of IFN-γ-producing CD4+ T cells resulting from stimulation by macrophages pulsed with individual peptides *versus* the percentage of cytokine-producing cells incubated with macrophages pulsed with no antigen (5%).

A. Fold increase in % specific lysis

B. Fold increase in % of CD4⁺ T cells producing IFN-γ
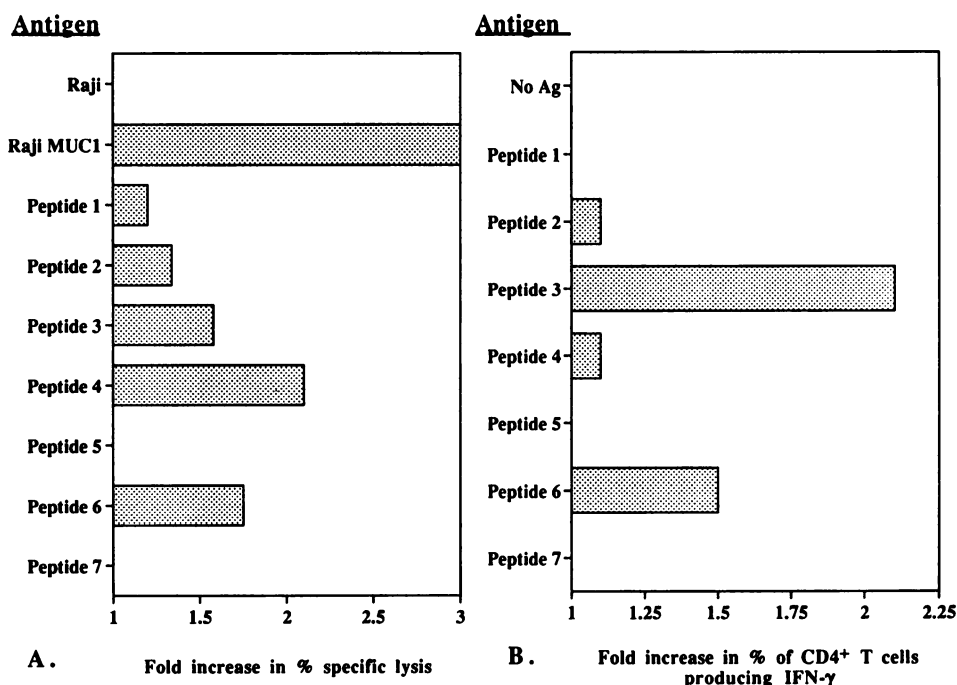
with the large peptide process and present the same class II-restricted epitope.

HLA Class II-restricted Epitope in the MUC1 Tandem Repeat. To better define the DR3-restricted peptide epitope in the MUC1 tandem repeat sequence, we synthesized three 12-mer and four 20-mer peptides that satisfy the approximate peptide length requirement for binding to class II MHC (15–17). These peptides also conform to the previously reported peptide binding motif for DR3 (18–20). This motif includes a large, hydrophobic amino acid at position 1 (n), a negatively charged amide-like amino acid (or threonine) at position n + 3, and a positively charged amino acid at position n + 5 (19). The abilities of these seven peptides to serve as DR3-restricted epitopes for MUC1-specific CD4⁺ T cells were compared using both the cytolysis assay and the cytokine (IFN-γ) production assay. In the cytolysis assays, T cells were exposed to Raji cells (homozygous DR3) that had been preincubated with individual peptides for 2 h prior to the assay. The specific reactivity in response to peptide-loaded Raji cells was compared to the response elicited by positive control Raji MUC1. We found that the peptide that augmented cytolysis of Raji targets the most was peptide 4 (Fig. 3*A*). This peptide is a 20-mer corresponding to the sequence PGSTAPPAHGVTSAPDTRPA. Two other peptides, peptide 6 (APGSTAPPAHGVTSAPDTR) and peptide 3 (PGSTAPPAHGVT), also enhanced the recognition of the target cells. These three peptides share the sequence PGSTAPPAHGVT. In Fig. 3*B*, peptides 3 and 6 specifically elicited cytokine production by the CD4⁺ T cells. For these experiments, T cells were stimulated with autochthonous macrophages pulsed with the indicated peptides.

The combined data from the cytolysis and the cytokine assays that were performed numerous times suggest that MUC1 tandem repeat peptides that contain the sequence PGSTAPPAHGVT elicit HLA-DR3 restricted responses (Table 1). This sequence, represented by the 12-mer peptide 3, can stimulate alone or in the context of longer peptides (peptides 4 and 6). The requirement appears to be that it is located at the NH₂ terminal end of a longer peptide. Peptide 5 is an inverted version of peptide 4, with the PGSTAPPAHGVT sequence on the COOH terminus, and it is not capable of stimulating the DR3-restricted T cells. Similarly, peptide 1 is missing the last threo-

nine of that sequence and has no stimulatory activity. It is of interest to note that this DR3-restricted sequence does not overlap with the immunodominant sequence PDTRP with which the MUC1-specific MHC-unrestricted CTL and MUC1-specific IgM antibodies react. This class II restricted epitope does, however, contain within its sequence the entire Class I binding epitope STAPPAHGV. It is encouraging for future MUC1 vaccine designs that the MUC1 tandem repeat sequence contains multiple CTL and T-helper cell epitopes.

This is the first defined epitope for MUC1-specific, class II-restricted CD4⁺ T-cell responses. These T cells, which could be primed *in vitro* against the naturally processed HLA class II-restricted epitope, exhibited both a strong T-helper 1 activity in the form of IFN-γ production and a cytolytic activity. We have preliminary data to suggest that other class II alleles (*e.g.*, DR4) may also be restricting elements for peptides in the MUC1 tandem repeat region. The ability of the APC to process MUC1, rather than the peripheral T cell repertoire, appears to determine whether MUC1-specific T-helper cells can be generated. In our experiments, this class II-restricted epitope was not efficiently processed by APCs from fully glycosylated MUC1 molecules, and CD4⁺ T cells were not efficiently primed. We propose that a similar situation may exist in cancer patients, in whom most of the underglycosylated tumor MUC1, the

Table 1 *Stimulatory potential of the MUC1 peptides aligned according to their position in the tandem repeat[a]*

Peptide no.		Peptide sequence	
	1	2021	40
Tandem repeats		PDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTSA	
Stimulatory	4	**PGSTAPPAHGVT**SAPDTRPA[b]	
	3	**PGSTAPPAHGVT**	
	6	A**PGSTAPPAHGVT**SAPDTR	
Not stimulatory	2	SAPDTRPAPGST	
	1	APGSTAPPAHGV	
	7	TAPPAHGVTSAPDTRPAPGS	
	5	SAPDTRPAPGSTAPPAHGVT	

[a] Stimulatory potential was defined in cytolysis assays and IFN-γ production assays.
[b] The sequences indicated in boldface type demonstrate the core residues thought to be necessary for stimulation of MUC1-specific T-helper cell responses.

form that is easily processed and presented, remains tumor bound and, thus, unavailable to APC. The circulating form that is available to APC consists of the minority of MUC1 molecules that have been more extensively glycosylated and, therefore, are much less likely to be processed and presented by APCs. Providing a less glycosylated or completely unglycosylated MUC1 in a form of a vaccine should allow generation of T-helper cells.

MUC1+ adenocarcinomas express very low levels of HLA-class II molecules, and normal epithelial ducts are class II negative. Thus, MUC1-specific CD4+ T cells are not expected to have a direct effect on tumor cells, nor are they of concern as potentially autoreactive effector cells. We consider them essential, however, at the site of antigen presentation to the immune system, such as the tumor-draining lymph nodes, where they can help amplify the activated CTL population and allow antibody isotype switching in activated B cells. We have recently demonstrated a similar potential of the MUC1 100-mer peptide to elicit both T-helper cells and CTL in vivo in chimpanzees.[4] This synthetic peptide is currently in clinical trials.

Acknowledgments

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