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Differential expression of MUC1 on transfected cell lines influences its recognition by MUC1 specific T cells

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In adenocarcinomas of the breast and pancreas, underglycosylation of the glycoprotein MUC1, also expressed by normal breast and pancreatic ductal epithelial cells, results in new protein epitopes to which the immune system mounts a cytotoxic T cell response. This cytotoxic immune response is directed primarily against epitopes on the tandem repeat domain of MUC1, and is unconventional in that it is major histocompatibility complex (MHC)-unrestricted. It is therefore necessary to investigate the molecular basis of this immune response in order to enhance and optimize it for immune therapy purposes. In the present study, we characterize new MUC1 transfected human lymphoblastoid cell lines C1R and T2, and a pig kidney epithelial line LLC-PK_1, that express MUC1 with either two repeats (MUC1-2R) or 22 repeats (MUC1-22R), and use them as stimulators and targets for cytotoxic T cells (CTL) *in vitro*. We show that MUC1-2R is processed and glycosylated similarly to MUC1-22R. In contrast to MUC1-22R, MUC1-2R is not recognized by CTL on T2 and C1R cells known for no or low MHC class I expression. It is however recognized when expressed at high density on xenogeneic LLC-PK_1 cells. We propose that in MHC-unrestricted recognition, a large number of MUC1 epitopes is necessary to effectively engage the T cell receptor, and that in the presence of a low number of epitopes, engagement of the CD8 co-receptor by MHC class I molecules may be required for completing the signal through the T cell receptor.

Keywords: MUC1, MHC-unrestricted, tandem repeats, tumour immunotherapy

Introduction

The realization that tumours express antigens that are recognized by cytotoxic T cells (CTL) has opened up a whole new era in tumour immunology. Several tumour antigens have been identified in recent years that can potentially be used for effective immunization against various types of tumours [1]. Many of these antigens are expressed in melanomas and promise to have a potential in immunotherapy. While some of these antigens such as tyrosinase, Melan-A/MART-1, gp100 and gp75 are expressed on both normal melanocytes and melanoma cells, some others such as MAGE-1, MAGE-3 and BAGE are considered to be relatively tumour specific. For the immunotherapy of virus-associated tumours, oncoviral peptides such as those from human papilloma virus (HPV), may prove to be effective. For breast, ovarian and pancreatic tumours, an unusual molecule, MUC1, holds a similar potential for an applicable and efficient immune therapy of these tumours. Our laboratory has been able to isolate CTL from the tumour draining lymph nodes of patients with breast and pancreatic adenocarcinoma that specifically recognize MUC1 as the target antigen on these tumours [2–4].

Of all the tumour antigens that have been described so far, MUC1 is distinguished by two important properties. First, unlike most tumour antigens that are expressed on both tumour and normal cells, the tumour associated MUC1 is truly tumour specific in that it is different than its counterpart on normal cells. In adenocarcinomas, MUC1 is aberrantly glycosylated. The CTL recognize tumour-associated epitopes resulting from the aberrant glycosylation and unmasking of previously hidden peptide sequences on the MUC1 polypeptide core. MUC1 on

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normal cells has many O-linked carbohydrates masking the polypeptide core, thereby blocking recognition by tumour-specific CTL and tumour-reactive monoclonal antibodies (mAb). Second, the epitope recognized by CTL is located on the 5′ end of a 20- amino acid peptide which can be repeated up to 200 times per molecule. Structural analyses of the MUC1 protein core using synthetic peptide analogues have revealed that the CTL epitope assumes a stable ordered structure that protrudes past the extended rod-like structure of the polypeptide backbone [5]. The protruding, evenly spaced T cell epitopes are capable of engaging multiple T cell receptors directly and without processing, thus bypassing MHC restriction.

By defining and understanding the precise requirements for the MHC-unrestricted recognition of MUC1, we can optimize the conditions for activating MUC1 specific CTL, thereby coming a step closer towards effective anti-tumour immunization based on MUC1. There are several aspects of MHC-unrestricted recognition that need to be defined. 1) Is the highly repetitive nature of MUC1 necessary for MHC-unrestricted recognition? If so, is there a threshold for the number of repeats below which no recognition occurs, and does an increased number of tandem repeats lead to better activation of CTL? 2) If the protruding motif of the T cell epitopes allows recognition outside the groove of the MHC, then what role if any do MHC class I molecules play? 3) What role do molecules such as CD8, CD2 and LFA-1 on the CTL play in this type of recognition?

To begin addressing these questions, we previously constructed a MUC1 cDNA expression vector that encodes MUC1 with only two repeats in the tandem repeat domain (MUC1-2R) [6]. We also previously described another MUC1 expression vector that encodes MUC1 with 22 repeats in the tandem repeat domain (MUC1-22R) [7]. We then compared the ability of cells transfected with either construct to serve as targets for MUC1 specific CTL [6]. T cell clones obtained from the peripheral blood of a patient with breast cancer, and stimulated with an allogeneic EBV immortalized cell line transfected with MUC1-22R, killed MUC1-2R expressing allogeneic human B cells even better than MUC1-22R expressing cells. We considered two explanations for this unexpected result. The first was the possibility that MUC1-2R was not glycosylated. This would result in the expression of the naked protein epitope on the cell surface which would readily be recognized by effector CTL. The second was the ability of MUC1-2R transfected cells to form more intimate interactions with the effector CTL. The native MUC1 molecule is a heavily glycosylated molecule that extends far above the cell membrane. It reduces intercellular adhesion such that MUC1 bearing cells have an impaired ability to form conjugates with other cells [8]. Similarly, MUC1 interferes with the close contact between effector cells and target cells [9]. Target recognition by CTL is mediated by a number of accessory molecules such as CD8, CD2 and LFA-1 in addition to the T cell receptor/CD3 complex [10, 11]. Similar to MUC1-22R, the MUC1-2R molecule possesses the proper T cell epitope, but being shorter than MUC1-22R it might allow better interaction of accessory molecules on the effector T cell with their respective ligands on the target cell.

We have performed experiments to address both possibilities. In the present and accompanying study by Poland *et al.* [12], we show by Western blot analysis that MUC1-2R is glycosylated to an extent similar to that of MUC1-22R. To address the role of accessory molecules in the MHC-unrestricted recognition of MUC1 by T cells, we used mutant cell lines T2 and C1R transfected with MUC1-2R or MUC1-22R. These cell lines lack or express low levels of MHC class I molecules, and express low levels of MUC1 after transfection. We also transfected MUC1-2R into a pig kidney epithelial cell line LLC-PK_1, and obtained high levels of MUC1 expression on the cell surface. The xenogeneic nature of these cells allowed us to test whether the mere presence of MUC1 in the absence of human accessory molecules is sufficient for MHC-unrestricted recognition by T cells, or whether accessory molecules need to be present for completing the stimulatory signal through the TCR.

Materials and methods

Transfection of cell lines

T2 [13] and C1R [14] lack or express low levels of MHC class I molecules. These cell lines were obtained from R. Salter and W. Storkus (University of Pittsburgh, School of Medicine, USA) respectively. Expression vectors pDKOF.-muc1 [7] (renamed pDKOF.muc1-22R) and pJBOF.muc1Δ [6] (renamed pJBOF.muc1-2R) were transfected into T2 and C1R cells by electroporation using an electroporator (BioRad) at 250 V using 10 μg DNA. The transfected cells were selected in 800 $\mu g\,ml^{-1}$ G418 (Gibco). LLC-PK_1 cells [15] were obtained from K. Amsler (Robert Wood Johnson Medical School, NJ). Expression vectors pREP4.muc1-22R and pREP4.muc1-2R (see accompanying paper by Poland *et al.* [12]) were transfected into the polarized pig kidney epithelial cell line LLC-PK_1 using 2 μg DNA and 6 μg Lipofectamine (BRL/Gibco) as directed by the manufacturer. Transfected cells were grown in 300 $\mu g\,ml^{-1}$ hygromycin B (Calbiochem).

Inhibition of MUC1 glycosylation in transfected cells

The inhibition of mucin O-glycosylation has been described previously [7] and was performed using phenyl-*N*-acetyl-α-galactosaminide (Sigma) (phenyl-Gal-

NAc), an inhibitor of O-linked glycosylation. Briefly, cells were plated at 10^6 per ml per well in a 24 well plate and incubated for 24–36 h in 5 mM phenyl-GalNAc dissolved in growth medium (no. G418). Inhibition of mucin N-linked glycosylation was performed using 20 μg ml^{-1} Tunicamycin (Sigma) for 17 h.

Establishment of MUC1 specific T cell lines

T cell lines were established as previously described [3] with the following modifications. Tumour draining lymph nodes were obtained from patients with breast and pancreatic cancer. Lymph nodes were teased to release lymphocytes. Lymphocytes were then plated at 10^6 per 2 ml per well in a 24 well plate (Linbro) in AIM V medium (Gibco) supplemented with 10% human serum (Gemini Bioproducts, CA), 5–20 U ml^{-1} of recombinant IL-2 (DuPont, DE), 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin. For MUC1 specific stimulation, we used three different protocols: we either rotated as stimulator cells a number of allogeneic EBV-immortalized B cell lines expressing MUC1-22R, or we used class I negative C1R cells expressing MUC1-22R, or we used xenogeneic PK_1MUC1-2R. The stimulator cells were treated with phenyl-GalNAc except for PK_1MUC1-2R, irradiated at 6000 R, and 10^5 cells added to every 1×10^6 responder T cells. The cultures were not given IL-2 until three days after initiation. They were then started on 5 U ml^{-1} IL-2, and gradually brought up to 20 U ml^{-1} IL-2. T cell cultures were split 1:2 when they proliferated to a density of greater than 2×10^6 cells per well. The cultures were restimulated every 7–10 days using the same protocol.

Antibodies and flow cytometry

The following tumour-reactive, MUC1 polypeptide core specific monoclonal antibodies: SM-3 (IgG1) directed against epitope PDTRP, and HMFG-2 (IgG1) directed against epitope DTR, were a gift from Dr Joyce Taylor-Papadimitriou, London, England; BC-1 (IgG3) and BC-2 (IgG1) and BC-3 (IgM) directed against epitope XPDTR, were gifts from Dr Ian F. C. McKenzie, Melbourne, Australia. The epitopes are present along the 20-amino acid tandemly repeated MUC1 polypeptide core: PDTRPAPGSTAPPAHGVTSA. The anti-class I antibody W6/32 (IgG2a) and the control antibody P3 are affinity purified from tissue culture supernatants of hybridomas obtained from American Type Culture Collection (Rockville, MD). Indirect immunofluorescence was performed as described previously [7]. Cells were analysed by flow cytometry using the Becton-Dickinson FACScan in the Pittsburgh Cancer Institute facility.

Proliferation assays

Two $\times$ 10^5 responder cells were incubated with 10^5 irradiated stimulator cells for 72 h at 37 °C, in complete growth medium supplemented with 10% inactivated human serum. The cells were then pulse-labelled with 1 μCi of ^{3}H-thymidine per well for 18 h at 37 °C at which point they were harvested using a Skatron cell harvester, and counted.

Cytotoxicity assays

Target cells were treated with phenyl-GalNac for 36 h to inhibit MUC1 O-glycosylation. These cells were then labelled for 1 h by incubating 10^6 cells at 37 °C in 100 μCi of sodium ^{51}chromate (Amersham, IL). Cells were then washed extensively to remove free sodium ^{51}chromate. Target cells (2×10^3) were placed in each well in a 96 well plate with varying numbers of effector T cells, and centrifuged at $800 \times \mathbf{g}$ for 5 min to ensure cell-to-cell contact. The plates were then incubated for 4 h in 5% CO_2 at 37 °C. The supernatants were harvested using a Skatron harvesting press and counted in a Micromedic ME plus gamma counter. Maximum release was obtained by adding 100 μl of 1 N HCl to the labelled target cells. Percentage specific killing was calculated using the following equation: % specific killing = 100$\times$ (experimental release–spontaneous release)/(maximum release–spontaneous release).

Western blots

Transfected cells, 2.5×10^5, MUC1, were lysed with octyl-β-glucoside and the lysate proteins electrophoresed on non-reducing 7.5% SDS/Polyacrylamide gels. The proteins were transferred to 0.45 μM pore size nitrocellulose membranes (BioBlot, NC), and non-specific sites on the membrane blocked with 10% non-fat powdered milk (Carnation). MUC1 was detected by immunoblotting using anti-MUC1 antibodies. The membranes were then incubated with goat anti-mouse peroxidase-labelled secondary antibody (Sigma). The immunoblot was developed by the enhanced chemiluminescence (ECL) method (Amersham, IL) as directed by the manufacturer.

Results and discussion

Glycosylation of MUC1-2R is similar to that of MUC1-22R

In order to test our first hypothesis that MUC1-2R may be aberrantly glycosylated thus allowing its recognition by T cells without further treatment with an inhibitor of O-glycosylation, Western blot analyses were performed on MUC1-2R and MUC1-22R transfected cell lysates. Figure 1 shows such an analysis on lysates from the lymphoblastoid cell line T2 that was transfected with either MUC1-22R (Fig. 1A) or MUC1-2R (Fig. 1B). The blots were probed with either anti-MUC1 monoclonal antibody (mAb) BC-3 which recognizes both fully glycosylated as well as underglycosylated MUC1, or they were probed

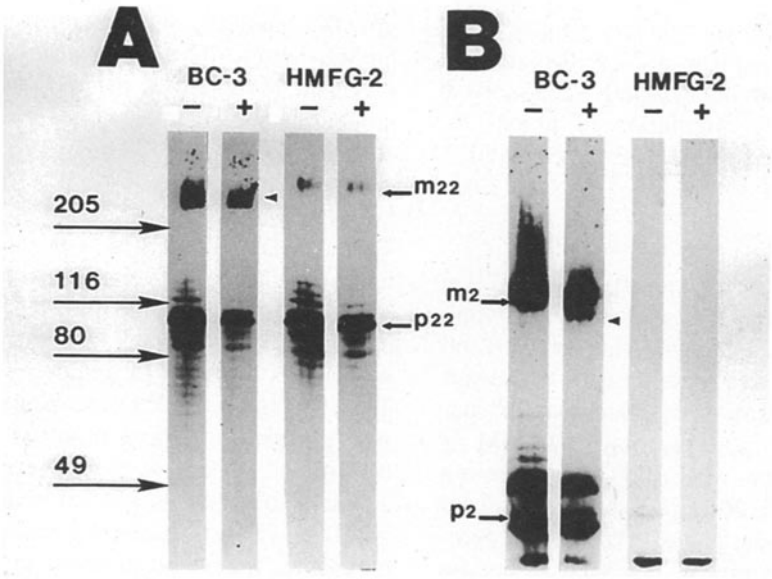

Figure 1. Western blot analysis of MUC1-2R and MUC1-22R transfected T2 cells. T2 cells transfected with the cDNA for MUC1 with either twenty-two (MUC1-22R) (A), or two (MUC1-2R) (B) tandem repeats, and grown for 17 h with (+) or without (–) tunicamycin, were lysed and extracts were subjected to SDS-PAGE. Western blot analysis was performed with either BC-3 or HMFG-2 anti-MUC1 antibodies. Molecular weight markers are shown on the left in kDa. Bands corresponding to the propeptide (p2, p22) or mature (fully glycosylated) (m2, m22) forms of the MUC1-2R or MUC1-22R respectively, are indicated at the sides. New bands resulting from the tunicamycin treatment are indicated by arrow heads.

with anti-MUC1 mAb HMFG-2 which recognizes only underglycosylated or the propeptide MUC1. The immunoreactive forms we detected with BC-3 were consistent with the presence of the 40 kDa propeptide (p2) and the mature (fully glycosylated) forms of MUC1 of 115 kDa (m2) for the MUC1-2R. For the MUC1-22R, we detected with the same mAb BC-3 the 110 kDa propeptide (p22) and 190 kDa mature form (m22) for the MUC1-22R. The pattern of immunoreactivity observed with mAb HMFG-2 which recognizes underglycosylated tandem repeats, also supports this conclusion. A minor reactivity of HMFG-2 with the mature MUC1-22R (m22) reveals the presence of molecules that are only partially glycosylated. This is consistent with microheterogeneity in the O-glycosylation of the MUC1-22R. In the MUC1-2R transfected cells there are ten times fewer glycosylation sites that may allow complete glycosylation of the mature form, and lack of its recognition by HMFG-2. The same results were obtained in several other EBV immortalized B cell lines transfected with MUC1-2R or MUC1-22R (data not shown).

Overnight incubation of the transfected T2 cells with tunicamycin, an inhibitor of N-linked glycosylation, alters the BC-3 immunoreactive pattern of the MUC1-2R similarly to that of MUC1-22R. Both mature forms, m2 and m22 are reduced in size. The difference which is more accurately estimated in m2, is nearly 5–10 kDa (see arrowhead). Several minor bands with M_r slightly greater than the propeptide (p2 or p22) are lost (Fig. 1). These evenly spaced bands are likely to represent variable numbers of N-linked oligosaccharides which are added cotranslationally. Since the level of the major propeptide band is apparently unchanged, this would indicate that most forms of MUC1 are poorly N-glycosylated in these cells, although posttranslational processing of the N-linked oligosaccharides does contribute to the microheterogeneity of the mature forms of MUC1 on SDS-gels. Overall, these data suggest that similar to MUC1-22R, N-linked precursors of MUC1-2R are present, and they disappear after tunicamycin treatment. The persistent higher M_r forms suggest the presence of O-linked sugars as well. Incubation of the transfected T2 and C1R cells with phenyl-GalNAc for 6 h, reveals a decrease in the intensity of the mature forms of MUC1-2R and MUC1-22R (see accompanying article by Poland *et al.* [12]) consistent with effective inhibition of glycosylation in both cases. Therefore, in as much as our data suggest that the processing and glycosylation of MUC1-2R appears to be very similar to that of MUC1-22R, it does not account for their differential recognition by T cells. These experiments, however, do not address whether or not unglycosylated forms, p2 and p22, are present on the cell surface and could account for differences in T cell recognition of the transfected cells. Experiments we have performed to analyse the forms of MUC1 expressed on the cell surface show that both MUC1-2R and MUC1-22R transfected cells express only trace levels of surface p2 and p22 (data not shown). Inasmuch as it is an

unglycosylated epitope and not unglycosylated form that is a target for T cell recognition, there is no requirement for the p2 or p22 forms to be expressed on the cell surface.

Human T cell lines that are enriched for MUC1 specific T cells are cytotoxic to target cells transfected with MUC1-22R, but not with MUC1-2R

T2 [13] and C1R [14] are both mutant cell lines that lack or express very low levels of MHC class I on their cell surface. These cells were transfected with MUC1 to impart on them the added property of MUC1 expression. We used previously established and characterized MUC1 specific T cell lines JR and Sch. Phenotypic analyses of surface markers on these T cells revealed the presence of 36–40% $CD8^+$ T cells which based on previous studies in our laboratory is the MUC1 specific population. The cells were then tested for cytotoxic function by standard chromium release assays. Both T cell lines specifically recognized MUC1-22R expressing transfected cells and not the untransfected control cells C1R and T2 (Fig. 2A and B respectively). However, these MUC1 specific T cells did not recognize MUC1-2R expressed by either C1R or T2 cells. This was surprising because it was in contrast to our previous observations [6] where EBV immortalized B cells transfected with MUC1-2R were recognized efficiently when used as cytotoxicity targets. We considered the potentially important differences between the EBV immortalized B cells transfected with MUC1 and the mutant C1R or T2 cells transfected with MUC1-2R. Both cells express similar levels of MUC1 by FACS analysis (data not shown), and they both express adhesion molecules such as ICAM-1 (CD54) and LFA-1 α and β chains (CD11a and CD18 respectively) (data not shown). The only difference that we were aware of was in the level of MHC class I expression. Using the values of mean fluorescence intensity (MFI) of staining with the anti-class I antibody W6/32, compared to EBV immortalized B cells which have MFI values around 600, C1R mutant cells have values of around 100. It is possible that in the presence of a smaller number of MUC1 tandem repeats (MUC1-2R) and consequently MUC1 epitopes, there is a greater dependence on MHC class I molecules to engage CD8 molecules on the T cells. CD8 molecules mediate adhesion as well as co-receptor function [16], thus enhancing the stimulatory signal via the T cell receptor. We are currently investigating this possibility. We know from our previous work [3] that MUC1 specific killing can be partially inhibited with antibodies against CD8.

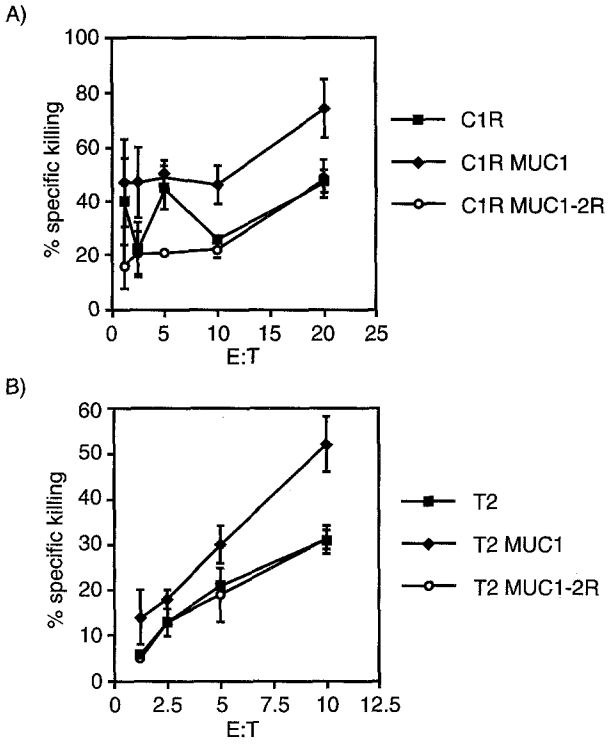

Figure 2. Only MUC1-22R and not MUC1-2R is recognized by T cells on MHC class I negative human transfectants C1R and T2. Two different T cell lines were tested for MUC1 specificity in cytotoxicity assays at several different effector to target (E:T) ratios. All targets were treated with phenyl-GalNAc for 36 h prior to the experiment. T cells were obtained from the tumour draining lymph nodes of patients JR (A) or Sch (B) by stimulation with several alternating human B cell lines transfected with MUC1-22R (Sch) or a single B cell transfectant C1R MUC1-22R (JR). Both types of transfectants used as stimulators were treated with phenyl-GalNAc for 36 h.

Human T cell lines that are enriched for MUC1 specific T cells are capable of recognizing MUC1-2R on transfected PK_1 cells

We established two new T cell lines from the tumour draining lymph nodes of patients JR and SG, by four stimulations with C1R transfected with MUC1-22R, and treated with phenyl-GalNAc. Both cell lines were then tested for cytotoxicity. As shown in Fig. 3, these T cells do recognize and kill xenogeneic PK_1 cells transfected with MUC1-2R. This recognition is MUC1 specific as they do not recognize the untransfected PK_1 controls. Furthermore, two other T cell lines expanded *in vitro* on PK_1 MUC1-2R proliferate specifically to PK_1 MUC1-2R. T cells were obtained from the tumour draining lymph nodes of patients JR and Sch. JR lymph node T cells were stimulated four times with PK_1 MUC1-2R. Sch lymph node T cells were stimulated three times with PK_1 MUC1-2R. Both of these T cell lines were then tested in proliferation assays. Figure 4 shows that both T cell lines proliferated specifically to PK_1 MUC1-2R, but not to untransfected PK_1 controls.

Differential expression of MUC1 in transfected cells

Using several different transfected cell lines and MUC1 of different lengths, we were able to observe the following

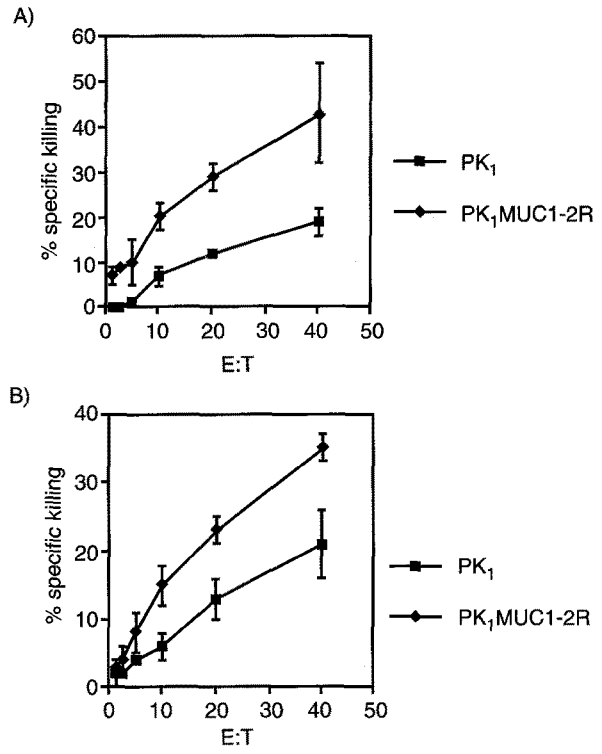

Figure 3. Human T cell lines expanded *in vitro* on C1R MUC1-22R recognize and kill pig kidney epithelial cells transfected with MUC1-2R. Two different T cell lines were tested at several different effector to target (E:T) ratios for cytotoxic activity specific for MUC1-2R transfected pig kidney epithelial cell line PK_1 MUC1-2R using the untransfected parental cell line PK_1 as a control. T cells were obtained from the tumour draining lymph nodes of patients JR (A) and SG (B) by four stimulations with C1R MUC1-22R treated with phenyl-GalNAc for 36 h.

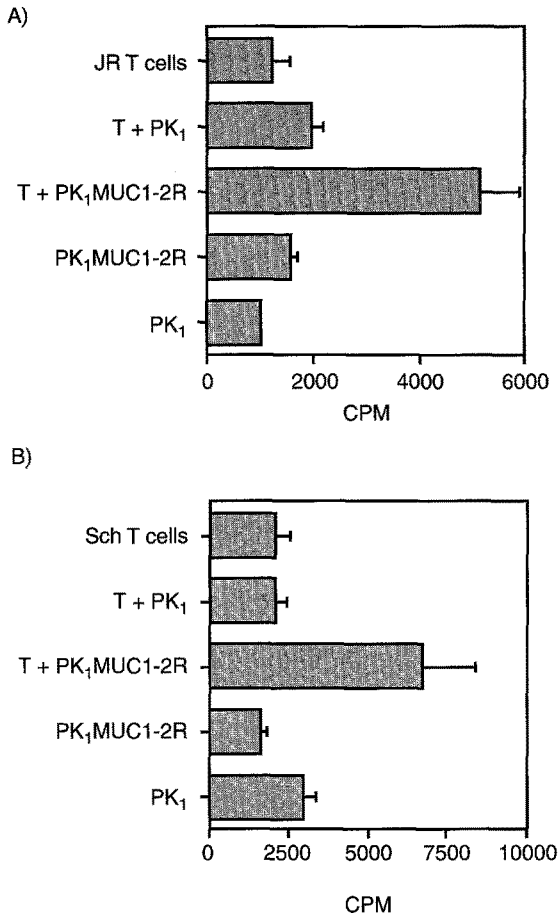

Figure 4. Human T cell lines expanded *in vitro* on PK_1 MUC1-2R proliferate specifically in response to PK_1 MUC1-2R. Two different T cell lines were tested for proliferation specific for the MUC1-2R transfected pig kidney epithelial cell line PK_1 MUC1-2R using the untransfected parental cell line PK_1 as a control. T cells were obtained from the tumour draining lymph nodes of patients JR and Sch. (A) JR lymph node T cells were stimulated four times with PK_1 MUC1-2R. (B) Sch lymph node T cells were stimulated three times with PK_1 MUC1-2R. The responder cells on the Y-axis are as follows: 'JR T cells' or 'Sch T cells' indicates background proliferation of T cells in the absence of any stimulator cells, '$T + PK_1$ MUC1-2R' indicates proliferation of T cells in the presence of PK_1 MUC1-2R, '$T + PK_1$' indicates proliferation of T cells in the presence of PK_1, 'PK_1 MUC1-2R' indicates background proliferation of PK_1MUC1-2R in the absence of T cells, and 'PK_1' indicates background proliferation of PK_1 in the absence of T cells. The level of ^{3}H-thymidine uptake by each responder is represented as counts per minute (CPM) on the X-axis.

phenomenon: both long (22 repeats) and short (two repeats) MUC1 can be used as targets to lyse transfected cells when they have normal expression of MHC class I molecules (EBV/B cells), but only the long MUC1 and not the short MUC1 sensitizes to killing of class I negative B cells (C1R and T2). On the other hand, the short MUC1 works quite well when the target cell is an epithelial cell PK_1 with a foreign (pig) MHC class I. Clearly, the MHC class I – CD8 interactions are important for the recognition of the smaller molecule, as the mutant B cells appear to show, but not absolutely required, as seen by its recognition on the pig epithelial cells. We postulated that the requirement for accessory interactions may vary depending on the density of antigen. Figure 5 shows a comparison of the level of MUC1 expression in the PK_1 MUC1-2R transfected cells stained with BC-3 and HMFG-2, compared to that in T2 MUC1-2R transfected cells stained with BC3 and BC-2 (because of the lack of staining with HMFG-2 as reported in [6], and that in the breast tumour cell line BT-20, stained with BC-3 and HMFG-2. Staining with BC-3 shows that T2 MUC1-2R transfected cells (Fig. 5C), similar to C1R MUC1-2R transfected cells (data not shown), express MUC1 at levels almost 10-fold lower than BT-20 (Fig. 5A). On the other hand, PK_1 MUC1-2R transfected cells (Fig. 5B) express MUC1 at levels comparable to that expressed by BT-20. We initially thought that the reason for the lower expression of MUC1 in T2 and C1R cells was that unlike the PK_1 cells, these cells were transfected with an expression vector that has the human β-actin promoter/

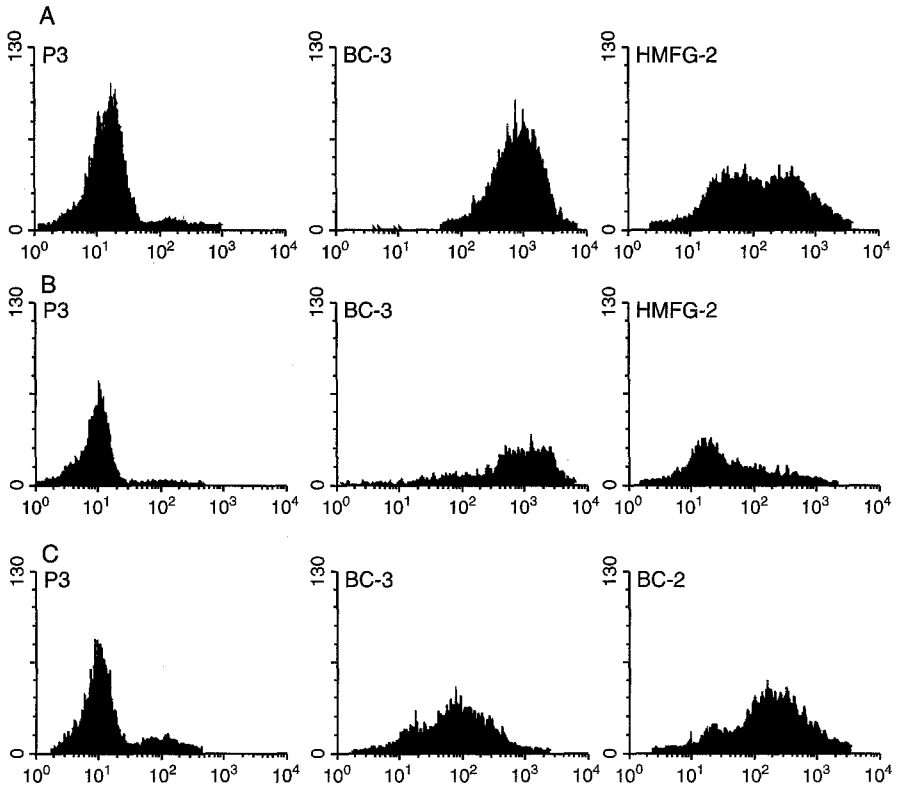

Figure 5. Comparison of MUC1 cell surface expression on PK_1 MUC1-2R cells with that on the breast tumour cell line BT-20 on transfectant T2 MUC1-2R. Breast tumour cell line BT-20 (A), PK_1 MUC1-2R (B) and T2 MUC1-2R (C) were stained with either MUC1 specific monoclonal antibodies BC-3, BC-2 or HMFG-2, or the negative control antibody P3, followed by FITC-conjugated goat anti-(mouse IgG and IgM). A total of 2×10^5 cells were analysed for each sample. The X-axis represents log fluorescence whereas the Y-axis represents cell number.

enhancer upstream of the *muc-1* gene, and that it was probably not as efficient as the RSV promoter in the expression vector that was transfected into PK_1 cells. Our laboratory has since transfected C1R with an expression vector encoding MUC1 downstream from the CMV promoter. The level of MUC1 expression was still 10-fold lower than that in BT-20. Therefore, we attribute the higher level of MUC1 expression in PK_1 cells to the epithelial nature of these cell lines, which better mimics the cell type in which MUC1 is expressed *in vivo*.

Unlike T2 or C1R cells transfected with MUC1 that need treatment with phenyl-GalNAc in order to express HMFG-2 and SM-3 tumour associated epitopes (data not shown), PK_1 transfected cells express HMFG-2 MUC1 epitopes without the need for treatment with phenyl-GalNAc (Fig. 5B). The presence of tumour-associated MUC1 epitopes on these cells is possibly due to the presence of a different set of glycosyl transferases in epithelial cells.

Therefore it is likely that expression of the small MUC1 with fewer tandem repeats but at high density may engage the T cell receptor with an avidity high enough to send a stimulatory signal to the T cell without the need for the CD8 co-receptor function. The other possibility is that porcine MHC class I molecules being similar to human MHC class I molecules, are capable of engaging the human CD8 co-receptor allowing for completion of the activation signal through the T cell receptor. These observations clearly need further study.

In summary, we have explored the possibility of using new cell lines transfected with MUC1 and expressing MUC1 epitopes as *in vitro* stimulators of MUC1 specific T cells from the tumour draining lymph nodes of patients with breast or pancreatic cancer. MUC1 expression in these new cell lines has been fully characterized (see accompanying article by Poland *et al.* [12]). In the present study, we use a strategy that would reduce alloreactivity and increase the probability of enriching for MUC1 specific T cells. Instead of using allogeneic EBV immortalized B cells transfected with MUC1-22R as stimulators of MUC1 specific T cells, we used the MHC class I lacking cell line C1R transfected with MUC1-22R, and a xenogeneic pig kidney epithelial cell line PK_1 transfected with MUC1-2R. We found that these transfected cells do allow the expansion of MUC1 specific T cells. The use of MUC1 transfectants expressing different numbers of tandem repeats as targets of T cell effector function promises to answer several

questions regarding the mechanism of MHC-unrestricted recognition of MUC1 by T cells. We show that MUC1-2R is processed and glycosylated similarly to MUC1-22R, and that any differences in recognition of these MUC1 proteins by T cells is not due to differences in glycosylation, but due to either numbers of presented epitopes, or the presence and extent of participation of accessory molecules in the MHC-unrestricted recognition of MUC1 by the T cell receptor.

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