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Immunogenicity of synthetic peptides related to the core peptide sequence encoded by the human MUC1 mucin gene: effect of immunization on the growth of murine mammary adenocarcinoma cells transfected with the human MUC1 gene

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Abstract. The immune response of CAF1 mice to various synthetic peptides (SP) related to the amino acid sequence (PDTRPAPGSTAPPAHGVTSA) of the tandem repeat of the MUC1 human breast mucin core peptide was evaluated. The most immunogenic preparations of the synthetic peptides were those conjugated to keyhole limpet hemocyanin (KLH) or clustered in a dendritic multiple antigenic peptide (MAP-4) configuration. The mice were immunized subcutaneously with synthetic peptides emulsified in RIBI adjuvant, employing various immunization protocols. Equivalently high IgG responses were induced using SP-KLH conjugates (GVTSAPDTRPAPGSTA-KLH) or an SP – MAP-4 chimeric configuration (SP1-6), which also included a universal malarial CST-3 T-helper epitope (SP1-6 = SAPDTRPAEKKIAKMEKASSVFNVVNS – MAP-4). These IgG antibodies bound both the appropriate MUC1 synthetic peptides and the cell surface expressed MUC1 mucin on murine mammary cells that had been transfected with the human MUC1 gene and a human breast cancer cell line that expresses cell-surface MUC1. A MAP-4 molecule, which included the entire 20-amino-acid sequence of the MUC1 tandem repeat (SP1-5 = PDTRPAPGSTAPPAHGVTSA–MAP-4) induced a poor IgG response. In contrast, all three types of molecule: SP-KLH, SP1-6 and SP1-5, were found to be good immunogens for the induction of specific delayed-type hypersensitivity (DTH) reactions measured using either synthetic peptides or MUC1-transfected cells. In addition, immunization with irradiated MUC1-transfected cells induced strong DTH reactions measured using synthetic peptides that expressed the PDTRP sequence, which has been shown to be, or to overlap, a T cell epitope in humans and a B cell epitope in mice. Finally, it was demonstrated that synthetic MUC1 peptide "vaccines" could be used both prophylactically and therapeutically to inhibit the growth of MUC1-transfected tumor cells and prolong the survival of tumor-bearing mice.

Key words: Cancer peptide immunogens – Active specific immunotherapy

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Introduction

It is becoming increasingly clear that mucins serve more than just a lubrication and protective function for mucosal surfaces. In the case of the mucin encoded by the MUC1 gene, the glycoprotein can be found extracellularly or as an integral cell membrane glycoprotein with an external domain, a transmembrane as well as a cytoplasmic piece [16]. Recent data suggest that cell-membrane-associated mucins or mucin-like molecules may serve important functional roles in cell communication [26, 34] and in maintaining polarity.

The structure of cancer-associated mucins makes them potential target molecules for immunotherapy. Because of their high molecular mass as well as extensive glycosylation, cell membrane mucins exist as flexible rods and protrude relatively great distances from the cell surface forming an important component of the glycocalyx [16] and are probably the first point of contact with antibodies and cells of the immune system. In fact, it has been shown that cell membrane mucins can actually "mask" other cell-surface antigens and protect cancer cells from immune attack [3, 7, 25]. Cancer-associated mucins are also known to be underglycosylated [14] and hence antigenically different from their normal cell counterparts exposing normally cryptic carbohydrate [10, 28, 33], peptide [2] and perhaps even glycopeptide epitopes. Therefore, because cell-surface mucins protrude such relatively great distances into the external environment, they themselves may serve as targets for immune attack [6, 12, 29].

We have used an animal model, the TA3-Ha murine mammary adenocarcinoma, to study how cancer-associated mucins modulate the immune system [5] and have developed novel synthetic "vaccines" for active specific immunotherapy, targeting specific carbohydrate epitopes on cell-surface mucins of TA3-Ha cancer cells [6, 12]. Our results show that (a) epiglycanin, a mucin produced and secreted by TA3-Ha cells, can induce a state of specific suppressor-T-cell-mediated immunosuppression against carbohydrate epitopes expressed on epiglycanin and on the surface of TA3-Ha cells [5], (b) epiglycanin-induced specific immunosuppression can be completely reversed by treatment of the suppressed mice with low-dose cyclophosphamide [5] and (c) cyclophosphamide-treated mice can be successfully immunized with a "vaccine" consisting of a synthetic hapten (mimicking a specific carbohydrate epitope on epiglycanin) conjugated to keyhole limpet hemocyanin (KLH) and emulsified in RIBI adjuvant. Following immunization, the majority of the mice were able to reject an otherwise lethal pre-existing tumor [6].

On the basis of our results with the TA3-Ha animal model, we have embarked on phase I clinical trials [24] evaluating the toxicity and immunogenicity of KLH–carbohydrate-hapten conjugates plus Detox adjuvant in human ovarian and breast cancer patients. Our clinical studies to date have employed two cryptic carbohydrate epitopes, which are well represented on human cancer-associated mucins: the Thomsen-Friedenreich (TF) hapten [4, 28, 31] and sialosyl-Tn [10, 15, 19]. It was found that the formulations were both non-toxic and immunogenic, stimulating the production of high titres of hapten-specific antibodies reactive with and cytotoxic for cancer cells bearing the appropriate TF or sialosyl-Tn epitopes [23, 24].

The human MUC1 gene product has been referred to by various names, including MAM6; milk mucin; human milk fat globule antigen (HMFG); human mammary epithelial antigen, CA 15-3, CA 27.29; episialin and polymorphic epithelial mucin (PEM) (reviewed in [32]). This mucin is strongly expressed on human breast [8], pancreatic [21] and certain ovarian cancer cells [22]. Although the MUC1-encoded mucins expressed on various cancers contain the same tandem repeat core peptide sequence, glycosylation differences do exist [8, 21]. MUC1 is the first mucin gene to be cloned and mapped [9], and has recently been transfected into a murine mammary cell line, 410.4 [20]. MUC1-transfected 410.4 cells express the MUC1 gene product on the cell surface. The pattern of glycosylation is similar to malignant-cell-derived mucins expressing the same cryptic peptide epitopes as expressed by human-cancer-associated MUC1 [32]. Lalani and co-workers [20] have examined the immunogenicity of the 410.4 transfectants in mice. These workers demonstrated that mice that rejected a low dose of transfected 410.4 cells did not develop tumors after a subsequent transplant of a high dose of transfected 410.4 cells, although no effect on tumor development of untransfected wild-type 410.4 cells was seen [20]. In the present study we have evaluated the immunogenicity of synthetic peptides that mimic the core peptide sequence of the tandem-repeat core peptide structure encoded by the human MUC1 gene and have used the 410.4 transfectant model to test whether it is possible to limit the growth of MUC1-transfected 410.4 cells by immunization with various synthetic peptides and glycopeptides related to known MUC1 core peptide sequences of the tandem repeat.

Materials and methods

Synthetic peptides and conjugates

All antigens and conjugates used in this study were obtained from Biomira Inc. (Edmonton, Canada). The various synthetic peptides were designed to contain the well-known B and T cell epitope of the MUC1 core peptide, PDTRP, in various configurations. Various linear sequences were used for serological and delayed-type hypersensitivity (DTH) assays or were conjugated to KLH and used as immunogens. MAP-4 configurations were compared to linear KLH conjugates for immunogenicity. Certain MAP-4 peptides (e. g. SP1-6) were chimeric molecules containing both a MUC1 epitope and a universal T cell helper (T_H) epitope to determine whether the T_H epitope could replace KLH as a carrier for antibody production. One additional configuration, SP1-16, was employed for comparison. SP1-16 is a linear chimeric molecule containing two tandem repeats of a single MUC1 epitope plus a tetanus toxin universal T_H epitope (SP1-16 = SAPDTRPASAPDTRPAY-SYFPSV).

Antigen preparation and immunization

Synthetic peptides (SP). Freeze-dried synthetic peptides were dissolved in phosphate-buffered saline, pH 7.4, and mixed with RIBI Detox adjuvant (RIBI ImmunoChem Research Inc., Hamilton, Montana). The mixture was vortexed vigorously for 10 min. Each immunization consisted of 75 μg peptide plus 4.5 μg adjuvant in a total volume of 200 μl injected subcutaneously into three locations: the base of the tail and both inguinal areas. For induction of DTH responses, the mice were immunized 7 days prior to footpad challenge and for antibody, the test mice were immunized and bled at 2-week intervals.

Cells. MUC1-transfected 410.4 cells, termed E3, and wild-type 410.4 cells were cultured as previously described [20]. E3P3 cells are E3 cells that were rapidly passaged three consecutive times following subcutaneous inoculation. This new cell line was found to grow much more rapidly than the parent E3 cells (our unpublished observations). For immunization, 10^7 irradiated (50 Gy) cells, which were mixed with 4.5 μg RIBI adjuvant, were injected subcutaneously as described above. MCF-7 cells, a human breast cancer cell line, was obtained from the American Type Culture Collection.

DTH assays. DTH tests were conducted as previously described [5, 12]. For the footpad challenge antigen, the doses of synthetic peptides were 50 μg/mouse or 10^3, 10^4 or 10^5 irradiated MUC1-transfected or wild-type cells. Typical DTH time-course kinetics [5, 12] were noted with the MUC1 synthetic peptides and transfected cells.

Tumor challenge experiments

Preimmunization experiments. Various groups of female CAF1 mice, obtained from the University of Alberta Medical Laboratory Animal Services (6–8 weeks of age), were immunized with peptide plus RIBI adjuvant as described above on various days prior to challenge, and challenged on day 0 with E3 or E3P3 cells or wild-type 410.4 cells: 10^6 E3 cells or 5×10^5 E3P3 cells were injected in 100 μl sterile phosphate-buffered saline subcutaneously into the breast pad. Tumor volume was estimated as previously described [20].

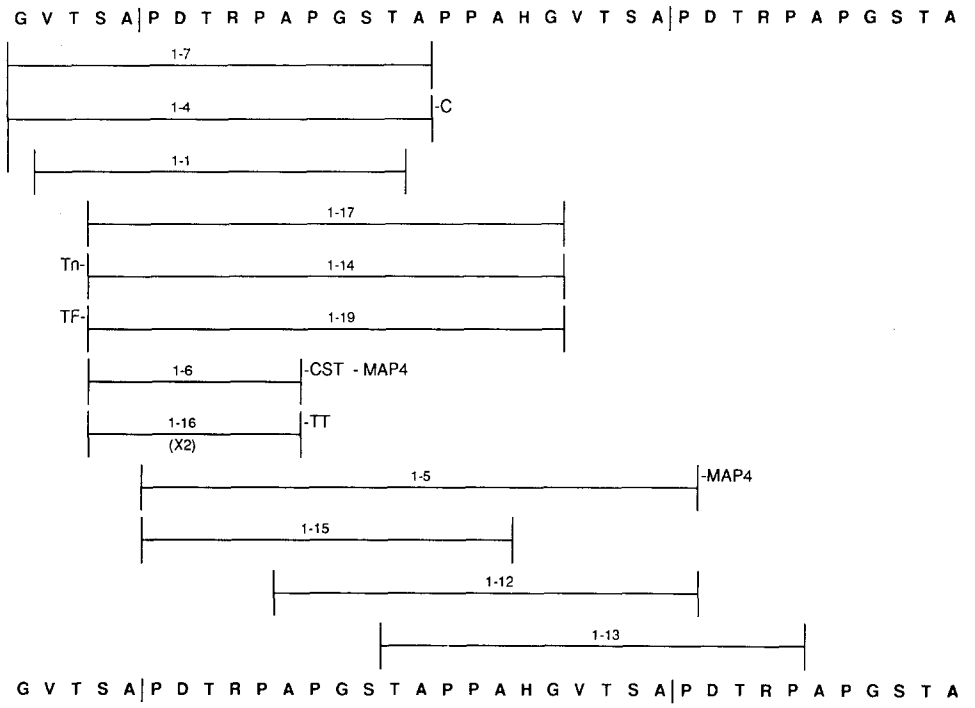

Fig. 1. MUC1 synthetic peptide antigens used in this study. The single-letter codes for amino acids at the *top* and *bottom* of the figure represent the overlapping tandem repeat of the MUC1 core peptide. SP1-14 has a GalNAc residue incorporated as the Tn antigen (GalNAc-*O*-Ser) during the solid-phase peptide synthesis. SP1-19 has a Gal-GalNAc residue incorporated as the Thomsen Friedenreich (TF) antigen. SP1-4 is the same as SP1-7 but with an added cysteine (C) residue

Active specific immunotherapy (ASI) experiments. Various groups of female CAF1 mice were given 10^6 E3 cells into the breast pad as described above. Three days later, mice were injected intravenously with 100 mg/kg cyclophosphamide, a dose that effectively reduces murine T-suppressor cells in another ASI model [6]. Before conducting ASI experiments with the present model, we conducted dose/response experiments with cyclophosphamide in E3-tumor-bearing mice and determined that 100 mg/kg cyclophosphamide did not retard the development of E3 tumors (our unpublished data). On days 5, 14, 30 and 48, mice were immunized subcutaneously with various synthetic peptides in RIBI adjuvant, as described above, and survival was monitored.

Results

IgG response to synthetic MUC1 peptides conjugated to KLH or clustered in a MAP-4 dendritic configuration

Figure 1 illustrates the synthetic peptides, based on the MUC1 tandem repeat sequence, that have been used in this study. Subcutaneous injections of 75 μg of each conjugate plus RIBI adjuvant were administered on days 0 and 14 and the mice were bled on day 28. The SP1-7–KLH conjugate generated specific IgG responses to SP1-7 and SP1-4, which were negative when tested with the unrelated peptide SP1-9 (Table 1).

For comparison, groups of mice were immunized with either of two MAP-4 synthetic peptides, SP1-5 or SP1-6. SP1-5 is a MAP-4 cluster in which each chain is made up of the entire 20-amino-acid sequence of the MUC1 tandem repeat plus an additional two amino acids from the amino-terminal end of the next tandem repeat (see Fig. 1). SP1-6 is a MAP-4 chimeric molecule made up of an 8- amino-acid amino-terminal sequence containing a known MUC1 B-cell epitope (PDTRP) attached to a 19-amino-acid sequence of a universal T-helper epitope from the CST-3 peptide of *Plasmodium falciparum* [30]. The purpose of this experiment was to test whether the CST-3 T_H epitope could provide sufficient help for IgG antibody formation to the well-known PDTRP B cell epitope and to ask whether the tandem repeat itself contained sufficient T_H sequences to stimulate antibody formation against the B cell epitope PDTRP. Table 1 demonstrates that the SP1-6 chimeric molecule generated a vigorous IgG response (log_2 titre >10) when tested with the SP1-7, SP1-4 or SP1-5 synthetic peptides but was negative when tested with the negative

Table 1. IgG antibody response[a] following immunization with various MUC1 synthetic peptides

Immunogen	Mouse no.	Response to test peptide[b]			
		SP1-7	SP1-5	SP1-4	SP1-9
SP1-6	1	6	8	>10	<1
	2	4	7	>10	<1
	3	8	8	>10	<1
	4	4	4	>10	<1
	5	>10	>10	>10	<1
SP1-5	1	<1	<1	<1	<1
	2	<1	<1	<1	<1
	3	<1	<1	<1	<1
	4	<1	2	2	<1
	5	<1	2	2	<1
SP1-7–KLH[c]	1	>10	8	8	<1
	2	>10	>10	6	<1
	3	>10	>10	6	<1
	4	>10	>10	8	<1
	5	NT[d]	NT	8	<1

[a] Serum sample taken 10 days following the second subcutaneous immunization in RIBI adjuvant

[b] Log_2 titre on enzyme-linked immunosorbent assay after background subtraction

[c] KLH, keyhole limpet hemocyanin

[d] Not tested

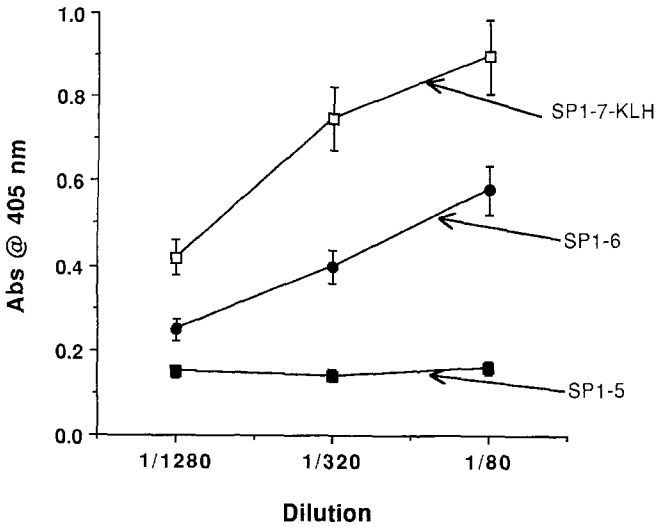

Fig. 2. Reactivity of anti-(synthetic peptide) IgG antibodies with E3 and 410.4 cells. Groups of five mice each were immunized subcutaneously twice with 75 μg SP1-6, SP1-5 or SP1-7–KLH and bled 10 days later; the serum was tested for binding to gluteraldehyde-fixed E3 or 410.4 cells in an enzyme-linked immunosorbent assay format. Preimmune serum gave an absorbance (405 nm) <0.1 on both E3 and 410.4 cells. All immune sera also gave an absorbance <0.1 on 410.4 cells (data not shown). Only sera from SP1-6 and SP1-7–KLH immunized mice demonstrated a significant binding to E3 cells. *KLH*, keyhole limpet hemocyanin

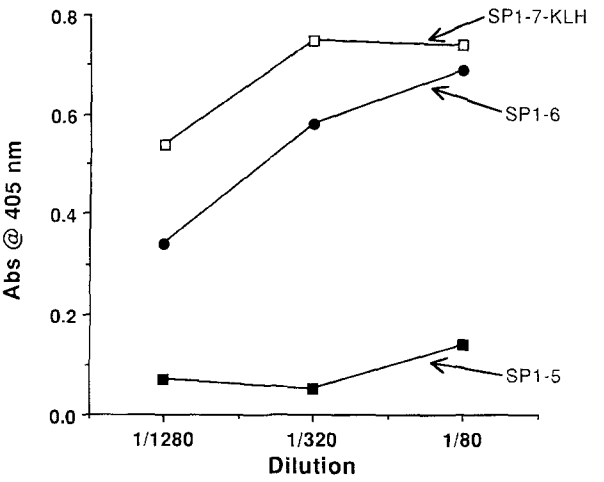

Fig. 3. Reactivity of anti-(synthetic peptide) IgG antibodies with MCF-7 cells

control peptide SP1-9. In comparison, the SP1-5 sequence generated a non-detectable or only a weak IgG response ($\log_2$ titre = 2 or <1). SP1-6 also generated higher IgM antibody responses than SP1-5 (data not shown).

In order to confirm that the IgG antibodies generated against synthetic MUC1 peptide sequences reacted against cell-surface MUC1, they were tested with MUC1-transfected (E3) cells as well as wild-type 410.4 cells. Antibodies generated using SP1-6 and SP1-7–KLH as immunogens were found to react with E3 but not 410.4 cells (Fig. 2). In contrast, sera from SP1-5-immunized mice did not produce any detectable reactivity with either type of cells. Figure 3 demonstrates that the same sera that reacted with E3 cells also reacted similarly with MCF-7 human breast cancer cells.

Generation of DTH reactions to MUC1 synthetic peptides following immunization with MUC1 synthetic peptide conjugates or MAP-4 cluster peptides

Subcutaneous immunization with SP1-7 conjugated to KLH generated specific DTH reactions when mice were challenged in the footpad with single-chain SP1-7. Even stronger reactions were observed when mice immunized with SP1-7–KLH were challenged in the footpad with an SP1-7–HSA (human serum albumin) conjugate (see Table 2). In contrast, the unconjugated single-chain peptide SP1-7 was found to be a poor immunogen for the generation of specific DTH reactions. Similar to SP1-7–KLH, the two MAP-4 clusters, SP1-5 and SP1-6 were able to generate specific DTH reactions when tested with SP1-7 and even stronger DTH reactions following footpad challenge with SP1-7–HSA (Table 2). SP1-5 and SP1-6 share several amino acid sequences in common with SP1-7, which are indicated in Fig. 1 but the only uninterrupted sequence shared in common among the three synthetic peptides is PDTRPA.

Table 2. Delayed-type hypersensitivity (DTH) results with various peptides and carrier peptide constructs

Immunization	DTH challenge	*n*	Increase in footpad thickness at 24 h[a]
SP1-7–KLH–RIBI	SP1-7	6	20±14
	SP1-5	5	15± 9
	SP1-7–HSA[b]	6	38±13
	HSA	6	6± 5
SP1-5–RIBI	SP1-4	5	21± 6
	SP1-7	5	16± 2
	SP1-5	5	20± 5
	SP1-9	5	2± 3
	SP1-7–HSA	5	41±13
	HSA	5	4± 7
SP1-6–RIBI	SP1-4	5	24± 6
	SP1-7	5	13± 4
	SP1-6	5	36± 5
	SP1-9	5	4± 2
	SP1-7–HSA	5	48± 6
	HSA	5	4± 6
SP1-7–RIBI	SP1-4	5	6± 2
	SP1-7	5	6± 4
	SP1-5	5	7± 3
	SP1-9	5	1± 4
	SP1-7–HSA	5	11± 4
	HSA	5	4± 4

[a] Mean ± SD measured as previously described [5, 12]
[b] Human serum albumin

Generation of DTH reactions to MUC1-transfected 410.4 cells (E3 cells) following immunization with various synthetic MUC1 conjugates or MAP-4 peptides

Various groups of mice were immunized subcutaneously with a single injection of 75 μg SP1-5, SP1-6 or SP1-16 in

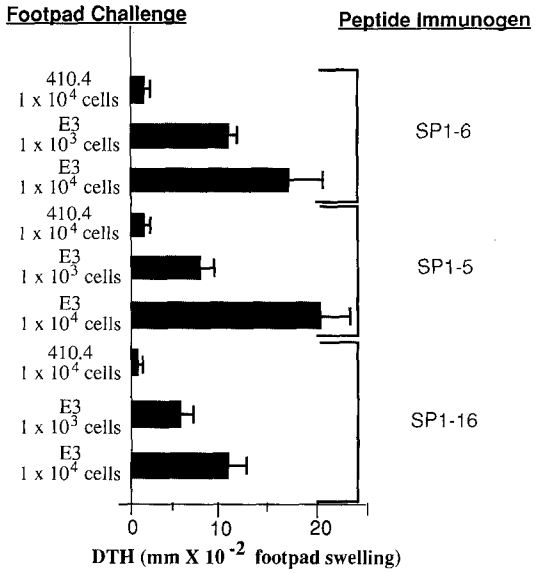

Fig. 4. Effect of peptide immunization on delayed-type hypersensitivity (DTH) reactions elicited by MUC1-transfected cells. Various groups of mice were immunized subcutaneously with various synthetic peptides plus RIBI adjuvant and challenged 7 days later in the footpad with irradiated E3 or 410.4 cells

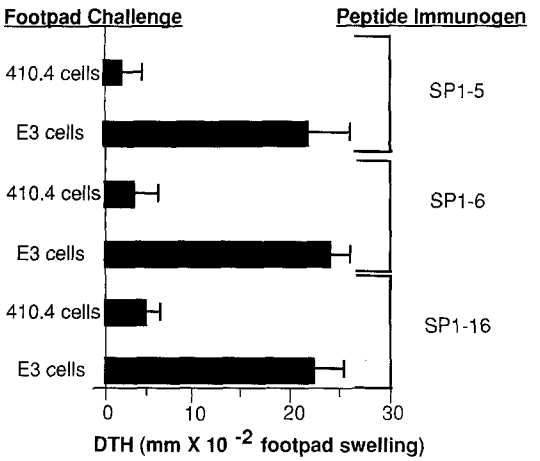

Fig. 5. Effect of peptide immunization on DTH reactions elicited by MUC1-transfected cells. See legend to Fig. 4 for details

RIBI adjuvant and challenged 7 days later in the footpad with either 10^3 or 10^4 irradiated transfected E3 or wild-type 410.4 cells as a negative control. SP1-16 is a linear chimeric molecule made up of two sequential repeats of the MUC1 SAPDTRPA linked to the hydrophobic universal T cell proliferation sequence of peptide 12 [13] of tetanus toxin, YSYFPSV (Fig. 1). Figure 4 demonstrates that no significant DTH was elicited by the negative-control wild-type 410.4 cells. The DTH response was dose-dependent and specific to the MUC1-transfected cells: 10^4 E3 cells elicited stronger DTH reactions than 10^3 E3 cells, and SP1-16 was a less effective immunogen than SP1-5 and SP1-6 at the doses of peptide and challenge cells used in this experiment.

When the number of immunizations was increased from one to two and the challenge dose of cells was increased to

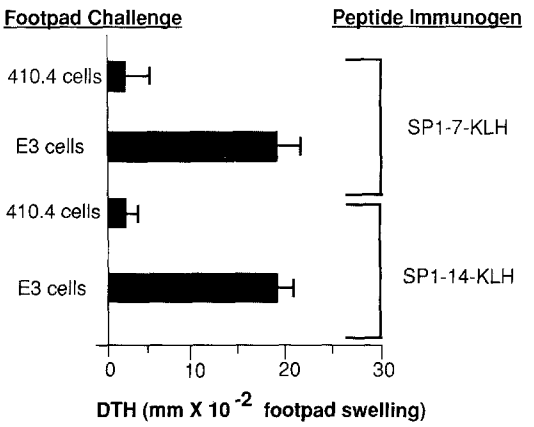

Fig. 6. Effect of peptide immunization on DTH reactions elicited by MUC1-transfected cells. See legend to Fig. 4 for details

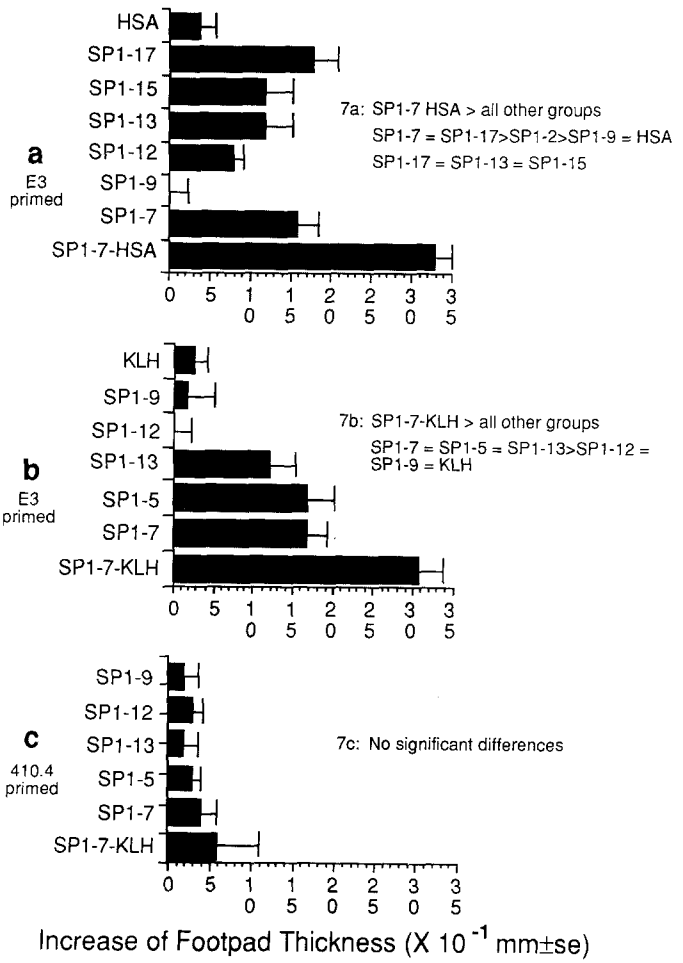

Fig. 7a–c. DTH response elicited by E3 and 410.4 cells in mice immunized with various MUC1 peptides. **a** SP1-7–HSA >all other groups; SP1-7 = SP1-17 >SP1-12 >SP1-9 = HSA; SP1-17 = SP1-13 = SP1-15. **b** SP1-7–KLH >all other groups; SP1-7 = SP1-5 = SP1-13 >SP1-12 = SP1-9 = KLH. **c** No significant differences. HSA, human serum albumin

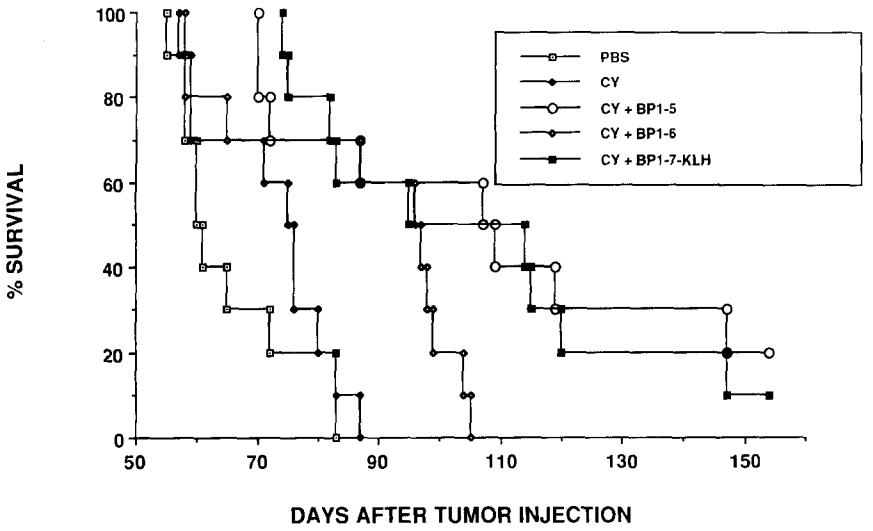

Fig. 8. Active specific immunotherapy of E3 tumors using synthetic MUC1 peptides. All mice were inoculated with E3 cells on day 0. Control mice (group 1) were injected with phosphate-buffered saline plus RIBI adjuvant or cyclophosphamide (*CY*) (group 2). Test mice were treated with cyclophosphamide followed by immunization with SP1-5 (group 3), SP1-6 (group 4) or SP1-7–KLH (group 5). All mice were monitored for survival. The data was statistically analyzed by the generalized Wilcoxon test. Groups 3 and 5 showed the best survival, significantly longer than groups 1 and 2 ($p < 0.02$). Group 4 showed intermediate survival significantly different from control groups ($p < 0.05$)

Table 3. The effect of pre-immunization with various MUC1 synthetic peptides on E3 subcutaneous tumor growth[a]

Immunogen[b]	Tumor size[c] ± SE (mm^3)	P[d]
RIBI adjuvant	1663 ± 331	–
SP1-16	1460 ± 160	>0.40 NS
SP1-6	1087 ± 234	>0.19 NS
SP1-5	793 ± 175	<0.03

[a] 10^6 E3 cells transplanted s.c. on day 0
[b] Peptide immunizations on days –34 and –21, 75 μg in RIBI adjuvant, s.c.
[c] Measured on day 45 following tumor transplantation
[d] Compared to control RIBI group

Table 4. The effect of pre-immunization with various MUC1 synthetic peptides on E3 subcutaneous tumor growth[a]

Immunogen[b]		Dose per immunization (μg)	Tumor size[c] ± SE (mm^3)	P[d]
KLH	(1×)	75	1209 ± 136	–
SP1-7–KLH	(1×)	23	579 ± 94	<0.002
SP1-7–KLH	(2×)	23	650 ± 92	<0.001
SP1-7–KLH	(1×)	75	529 ± 128	<0.002
SP1-7–KLH	(2×)	75	619 ± 153	<0.007
SP1-7	(2×)	75	1254 ± 253	NS
SP1-5	(1×)	75	317 ± 62	<0.0001
SP1-5	(2×)	75	579 ± 101	<0.002

[a] 5×10^5 E3P3 cells transplanted s.c. on day 0
[b] Immunizations on days –17 and –10 in RIBI adjuvant, s.c. for mice immunized twice and on day –10 for mice immunized once
[c] Tumor size measured 2 weeks following tumor transplant
[d] Compared to KLH control

10^5, SP1-5, SP1-6 and SP1-16 induced comparably strong DTH reactions (Fig. 5).

Figure 6 demonstrates that MUC1 peptide (SP1-7) and glycopeptide (SP1-14) conjugates with KLH are also able to induce comparable DTH reactions when tested with 10^4 irradiated E3 cells. Numerous control experiments show

Table 5. Subcutaneous growth of E3[a] tumors following active specific immunotherapy with MAP-4 synthetic peptides

Antigen[b]	Tumor size[c] ± SE (mm^3)	P[d]
CY+SP1-5 (75 μg)	568 ± 76	<0.002
CY+SP1-6 (75 μg)	562 ± 81	<0.002
CY	2090 ± 302	NS
RIBI adjuvant	1603 ± 268	–

[a] 10^6 E3 cells transplanted subcutaneously on day 0
[b] Subcutaneous immunization in RIBI adjuvant on days 5, 15, and 36. SP1-5 and SP1-6 – 75 μg per immunization. CY, cyclophosphamide (100 mg/kg) intravenously on day 3
[c] Measured at day 63 following tumor transplantation
[d] Compared to RIBI adjuvant control group

that immunization with KLH alone plus RIBI does not induce DTH against E3 cells (data not shown). Several other control experiments using unrelated synthetic peptides as negative controls also demonstrate the specificity of the DTH for the synthetic MUC1 peptides used for immunization (data not shown).

DTH responses to synthetic MUC1 peptides following immunization with MUC1-transfected 410.4 (E3) cells

Various groups of mice were immunized three times subcutaneously with 10^7 irradiated E3 cells in RIBI adjuvant. Control groups were immunized three times with 10^7 wild-type 410.4 cells. The mice were challenged in the footpad with 50 μg of various synthetic peptides 7 days following the third immunization and DTH responses were measured. Figure 7 demonstrates that mice immunized with control 410.4 cells did not produce DTH reactions to any of the peptides (Fig. 7c). In contrast, E3-immunized mice produced a moderate to strong DTH to all the peptides that contained an internal or terminal PDTRP MUC1 sequence (SP1-5, SP1-7, SP1-13, SP1-15, SP1-17) but not to SP1-9,

KLH or HSA. SP1-12 induced only a weak (Fig. 7a) or undetectable (Fig. 7b) DTH response.

The effect of pre-immunization with various MUC1 synthetic peptides on E3 subcutaneous tumor growth

Various groups of mice were pre-immunized with either SP1-5, SP1-6, or SP1-16 synthetic peptides (on days –34 and –21) and challenged subcutaneously 3 weeks later with 10^6 E3 cells, and tumor volume was estimated on day 45. Only SP1-5 was found to provide a statistically significant decrease in tumor size (Table 3). The experiment was repeated, including two different doses of SP1-7–KLH and a different immunization protocol. Table 4 demonstrates that one single immunization with SP1-7–KLH at a dose of 23 μg or a single 75-μg dose of SP1-5 induced significant inhibition of growth of E3 tumors.

Active specific immunotherapy of E3 tumors using synthetic MUC1 peptide

The same immunogens employed in the pre-immunization experiment described above were tested in an ASI protocol. Various groups of mice were given 10^6 E3 cells subcutaneously on day 0, followed on day 3 by cyclophosphamide or PBS. On days 5, 15, and 36, the mice were immunized with various MUC1 synthetic peptides, 75 μg in RIBI adjuvant. Tumor sizes were measured on day 63. Table 5 demonstrates that immunizations with SP1-5 or SP1-6 caused a significant decrease in subcutaneous E3 tumor growth. Figure 8 demonstrates an ASI survival experiment whereby mice immunized with SP1-7–KLH and BP1-5 gave the best survival, with BP1-6 conferring intermediate survival compared to controls.

Discussion

The present study represents an extension of our work on the use of synthetic epitopes that mimic important determinants on cancer-associated mucin molecules for active specific immunotherapy of cancer. In our previous studies, we have used synthetic carbohydrate haptens conjugated to KLH as cancer "vaccines" both in animal model studies as well as in phase I clinical trials [23, 24]. In the animal model work [6, 12], we used the TA3-Ha murine mammary adenocarcinoma, which expresses a mucin-like molecule called epiglycanin on the cell surface. TA3-Ha cells also secrete epiglycanin into the circulation. Epiglycanin has repeating TF and Tn carbohydrate epitopes. As both TF and Tn are recognized to be important human-carcinoma-associated epitopes [31] expressed on human carcinoma-associated mucins [4, 28], the TA3/Ha tumor is an excellent syngeneic animal model for ASI using synthetic TF and Tn vaccines [6, 12, 29]. In the animal model, we demonstrated that TF-KLH-containing vaccines can cause rejection of established tumors. Phase I clinical studies employing similar TF-KLH conjugate vaccines demonstrated low toxicity and strong immunogenicity [24]. Other clinical trials using the related sialylated-Tn epitope are currently underway with equally encouraging results [23].

In the present study, we have extended our work on the use of synthetic carbohydrate epitopes to defined synthetic core peptide epitopes. The core peptide expressed by the human MUC1 gene was chosen for study because of its strong association with human breast cancer [8] and the fact that core peptide cryptic epitopes are revealed in cancer cells as a result of underglycosylation of mucins in carcinoma cells [2].

In order to evaluate the capacity of the immune response to defined MUC1 peptide sequences to promote elimination of breast carcinoma cells expressing the MUC1 mucin, we used MUC1 transfectant cells developed by Lalani and co-workers [20]. These workers transfected the human MUC1 gene into the mouse mammary epithelial tumor cell line 410.4 and demonstrated that the MUC1 mucin was expressed both cytoplasmically and in the cell membrane. Interestingly, the glycosylation pattern in the transfected cells appeared to be similar to that seen in human tumor cells. Mice that rejected a low number of 410.4 transfected cells did not develop tumors after a subsequent inoculation of a large number of transfectant cells, although the pre-immunization had no effect on the tumor development of the untransfected 410.4 cells.

In the present study, we have used defined synthetic peptides to demonstrate the immunological specificity of tumor rejection in this model. First, we showed that immunization with defined synthetic MUC1 sequences generated specific DTH reactions against specific MUC1 sequences and transfected 410.4 cells but not against unrelated control peptides or untransfected 410.4 cells. Secondly, we demonstrated that immunization with transfected cells but not untransfected cells generated specific DTH reactions against defined synthetic MUC1 peptides. Finally, it was shown that synthetic MUC1 peptide "vaccines" could be used both prophylactically and therapeutically to inhibit the growth of MUC1-transfected tumor cells and prolong the survival of tumor-bearing mice.

Some interesting differences exist with respect to the types of immune responses that were induced following immunization with the various MUC1 synthetic peptides. For example, SP1-5 was found to be a poor immunogen for specific anti-MUC1 IgG production but a good immunogen for DTH and tumor rejection. In contrast, SP1-6 was found to be an excellent immunogen for IgG production, a good immunogen for DTH and generally less effective than SP1-5 for tumor rejection. This difference is compatible with the hypothesis that cellular immunity is the most effective antitumor rejection mechanism in this model and perhaps that antibody can actually interfere with rejection.

Other workers [1, 17], have cloned cytotoxic T lymphocytes (CTL) from human cancer patients the activity of which is specifically inhibited by the SM3 antibody, reactive with the PDTRP epitope of the MUC1 gene. Moreover the CTL only kill cells expressing mucins carrying this epitope. We have shown a similar preferential reactivity to this sequence in mice immunized with MUC1-transfected (E3) cells. Thus, it appears that the same PDTRP sequence contains (a) immunodominant B-cell epitopes recognized by the murine immune system, (b) immunodominant T cell

epitopes recognized by human T cells (CTL) and (c) immunodominant T cell epitopes recognized by murine (DTH) T cells. This suggests that the immune systems of the strain of mice employed in this study and at least certain humans may "see" similar epitopes on the MUC1 peptide sequence.

In the present studies, most of the mice did not totally reject their tumors but the immunization procedure resulted in delayed tumor development. This is similar to the results of Lalani and coworkers [20]. Tumor cells isolated from 9-week-old tumors of mice immunized with MUC1 synthetic peptide were mostly negative for MUC1 expression (data not shown). Lalani and coworkers [20] suggested that this phenomenon may be due to the immune rejection of MUC1-expressing cells allowing the preferential growth of MUC1-negative "revertants" in the absence of the selectable marker hygromycin. This would give rise to a tumor that is, for the most part, negative for mucin expression. Despite this, we have observed that several mice in our ASI study did apparently reject the entire tumor and have remained tumor-free for over 150 days. This suggests that, despite the tumor heterogeneity created by the loss of MUC1-positive cells, a "bystander" effect was created by the response to the MUC1-positive cells that also killed the MUC1-negative variants.

Thus, we have demonstrated that immunization with MUC1 synthetic peptides can generate specific cell-mediated immune responses against MUC1-encoded mucin expressed at the cell surface. We have also demonstrated that immunization can cause rejection of the transfectants and prolong the survival of mice bearing the transplant. Although, to our knowledge, this is the first demonstration of the use of a synthetic mucin core peptide for effective ASI, others have used murine tumor transfectant models and recombinant vaccinia virus constructs as immunogens to demonstrate effective ASI [11, 18].

Although models that employ rodent tumor cells transfected with human genes [11, 18] provide useful information with regard to immunogenicity of the various peptide "vaccines" and their effects on tumor growth, they all have limitations as models appropriate for human ASI since the "rejection" antigen is human and is substantially different from the homologous murine MUC1 gene [8] expressed in the normal tissues of the mouse. Therefore, stronger immune responses might be expected than if the homologous murine MUC1 sequence had been the target antigen.

We are presently attempting to overcome this objection with the use of mice made transgenic for the human MUC1 gene, which we have shown to be expressed in normal tissues of the mouse. Therefore, we are now evaluating the immunogenicity of MUC1 synthetic peptides in MUC1 transgenic mice. As MUC1 is expressed in the same normal tissues in the transgenic mice as in humans (breast and pancreatic), this should represent an appropriate model to test MUC1 vaccines for ASI and autoimmunity.

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