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Award Number: DAMD17-99-1-9067

TITLE: Vaccine Development Against Novel Breast Cancer Antigens

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REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Annual (2 Aug 99 - 1 Aug 00)

4. TITLE AND SUBTITLE
Vaccine Development Against Novel Breast Cancer Antigens

5. FUNDING NUMBERS
DAMD17-99-1-9067

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The aim of these studies were to produce new antigens overexpressed in breast cancer; to immunise mice with these, particularly using new modes of immunisation including oxidised mannan and to measure the ensuing antibody and cellular responses and ultimately determine the effect of using multiple antigens on tumour growth in mice. In the first year of the project a number of antigens have or are being made including nm23, p53; Her2/neu is in the process of being produced, and an additional antigen, glycosylated whole human MUC1, is in the process of being engineered and produced. Although the original application cited cytotoxicity as the mode of testing, we have been busy establishing an ELISPOT assay and can distinguish between γIFN production by CD4 and CD8 cells. Immunisation methods have been refined, targeting the mannose receptor is being compared with various prime boost strategies and a new mode of immunisation has been designed. Progress is satisfactory and we are poised to push ahead with the studies in years 02 and 03.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Mannose, HLA-A2 mice, CTLs, MHC, Class I molecules	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The therapy of breast cancer is largely static. Thus surgery, radiotherapy, cytotoxic drugs and hormones have almost reached the limit of their use, although Taxol is now making some impression. However, it is appropriate that new immunotherapeutic approaches be examined, and in this light, we have already conducted a number of clinical trials of mannan MUC1 in patients. In these patients we see reasonable immune responses frequently with antibody produced, some cellular responses, but to date no major tumour shrinkage has been seen. Indeed, other than for melanoma, it is a rare event to see solid tumors disappear. We are therefore re-examining all aspects of immunotherapy and in particular to : -

1) Examine the immunotherapeutic role of a number of antigens including nm23, p53, Her2/neu, pim-1 (which is present in breast cancer), Crypto-1, and more recently, fully glycosylated whole MUC1.

2) Modes of immunisation/delivery. We are concentrating on oxidised mannan as a delivery system as it is so potent in mice for T1 type cellular responses, but now are also examining other methods of delivery.

3) Improved assay methods for cellular immunity and this includes the ELISPOT assay to measure the frequency and cell type source of critical cytokines from antigen-specific reactive T cells.

4) These strategies have been assembled together into immunotherapeutic studies, which are in progress.

BODY

Statement of work from application

Vaccine development against novel breast cancer antigens

Task 1 **Produce recombinant proteins Her2/neu, nm23, p53, Bcl2, bax and other antigens for linkage to mannan (months 1-18).**

- Clone cDNAs for Her2/neu, nm23, p53, Bcl2 and bax into the pGEX expression vector (months 1-12).
- Produce small amounts of protein for *in vitro* characterisation of antigens (months 6-14).
- Conjugate the recombinant proteins to mannan for *in vitro* characterisation (months 12-18).
- Examine other antigens: Cathepsin D, Brca1/2, EGFr, Crypto-1, and amphiregulin.

Products/deliverables: Pure recombinant fusion proteins (Her2/neu, nm23, p53, Bcl2, bax and others) and their mannan conjugates.

Task 2 **Study the *in vivo* immune response of mannan-conjugated and non-mannosylated free antigens in mice (months 18-24).**

- Immunise C57BL/6, BALB/c and HLA-A2/K^b transgenic mice with immunogens and study antibody response, CTL and proliferation to the antigens (months 16-24).
- Immunise mice with HLA-A2.1 binding peptides of Her2/neu and p53 conjugated to KLH and mannan and study the immune responses(months 18-24).

Products/deliverables: Her2/neu, p53 peptide-KLH-mannan conjugates.

Task 3 **Study tumor protection in mice immunised with the mannan conjugates (months 24-36) .**

- Immunise C57BL/6 and BALB/c mice with the various conjugates and challenge with murine tumors transfected with the various antigens (months 20-26).
- Immunise HLA-A2/K^b transgenic mice for generating lymphocytes for adoptive transfer into *scid* mice with human $HLA2^+$ breast tumors (months 24-36).
- Study the effects of T1 cytokines(γIFN, TNFα, IL-2 and IL-12) given simultaneously with mannan conjugates on tumor challenge (months 26-32).
- Develop new peptides based on known HLA-A2.1 epitopes of Her2/neu and p53 using molecular modelling and study immune responses in HLA-A2/K^b mice (months 30-36).

Products/deliverables: peptide mimics for known HLA-A2.1 epitopes of Her2/neu and p53 and their KLH-Mannan conjugates.

Task 1: **Produce recombinant proteins Her2/neu, nm23, p53, Bcl2, bax and other antigens for linkage to mannan**

These studies are progressing satisfactorily, in particular nm23 is now in production and small amounts of protein made – the same is true for p53 and Her2/neu. At this time we will go ahead with the production and testing of these prior to starting Bcl2, bax and others. An additional protein has been added – glycosylated whole MUC1. Previous studies from our Institute and elsewhere have used peptides in the MUC1 VNTR. The VNTR is a 20 amino acid repeat, which contains highly immunogenic peptides. However, on analysing the data from the patients who received mannan VNTR it was clear that large amounts of antibody could be made to the peptides, but not to glycosylated MUC1 nor to the patients' own tumour. We thought that tolerance was being broken, however MUC1 peptides do not occur in isolation, they are heavily glycosylated and therefore it is sensible to immunise with glycosylated peptides. To do this we are negotiating with Dr Henrik Clausen in Copenhagen, Denmark to add Gal NAc to synthetic VNTR. While the VNTR does contain epitopes for Class I presentation and recognition by $CD8^+$ cytotoxic lymphocytes there are also epitopes in the other region of MUC1 which we have recently identified (Pietersz et al manuscript) and we therefore have considered it more useful to use the whole extracellular MUC1 to provide more epitopes. In a second approach we are in the midst of cloning whole extracellular MUC1 sequence (see below) into the appropriate vector so it will be expressed in the human embryonic 293 kidney cells – in that way the secreted material will be glycosylated, but not recognised by human natural antibodies (anti-Gal) that react with glycosylated proteins from most animal cells. Several other antigens are on the list; pim-1 and Crypto-1 have been expressed and these will be available for use.

In a recent study using serial analysis of gene expression (SAGE) and DNA arrays, in addition to MUC1, Claudin-7 was shown to be increased >100 fold in primary breast tumours. This is the first time that the association of Claudin-7 with breast cancer was identified and it is another antigen which will be used as a target for developing CTL for breast cancer.

In short, most of the antigens are on track for production, with Bcl-2 and bax delayed pending results from the others, but substituted with whole MUC1 and Claudin-7 and pim-1.

Products/Deliverables:

Recombinant fusion proteins from a number of antigens have already been made (Her2/neu, nm23, p53, pim-1, Crypto-1) – they will soon be tested for their ability to be mannosylated and be appropriate for testing in the next part of the study. Whole human MUC1 should be expressed within the next 3 months and they rapidly determined.

Task 2: **Study the *in vivo* immune response of mannan-conjugated and non-mannosylated free antigens in mice**

Studies have commenced injecting mice with nm23 and whole MUC1 as HMFG and studies will shortly commence with the other antigens. However, several points should be made here.

1) Mannosylation vs other methods of immunisation. While the original program used oxidised mannan, analysis of our clinical results gave a disappointing lack of responsiveness of tumours. Admittedly the patients had advanced disease, but they did make antibody and CTL responses – albeit the latter were fairly weak. We were concerned that the mode of immunisation while superb in mice was not appropriate in humans and we are moving in 2 directions: -

   a) Associate Professor Magda Plebanski in our group has defined a new method of immunisation using antigens conjugated to beads. Using the beads, appropriate immune responses have developed in mice as antibodies, CTLs, but also particularly successful ELISPOT assays have been undertaken. The data for this is shown in Appendix 1.

   b) Prime boost immunisation strategy. The mice are primed by whatever method and route is under study, e.g. mannosylated protein, DNA, peptide and so on. The trick is then to follow the priming with a boost using a live vector such as vaccinia, carrying the antigen of interest. These studies were conducted and the data is shown in Appendix 2. It was of interest that the prime boost strategy for MUC1 was not efficient, but it will be applied to the other antigens

2) Measurement of immune responses. While our initial proposal planned to measure antibodies and cellular responses by way of proliferation and CTLs, we have developed a far more suitable method of measuring cellular responses: ELISPOT assays detecting γIFN production by either CD4 or CD8 cells. These tests can be done immediately, rather than after days of *in vitro* culture, and give an answer on the status of total cellular immunity, as opposed to CTLs which often takes some weeks to obtain an answer, and are limited to one effector mechanism. Typical ELISPOT data are included in Appendices 1 and 2.

Task 3: **Study tumour protection in mice immunised with the mannan conjugates.**

As indicated, these studies have not commenced as yet and will be done in Year 02 and going onto Year 03.

KEY RESEARCH ACCOMPLISHMENTS

The important findings of the study have not yet been published, but will be done so in the next year and will include: -

1) Production of the different antigens, particularly glycosylated whole MUC1, Pim-1 and the other peptides.

2) Defining a new method of immunisation with the antigens conjugated to the beads.

3) Demonstrating that the prime boost immunisation strategy for MUC1-VNTR is suboptimal (this is a negative finding); exploring this finding with glycosylated whole MUC1, and noting that in almost all other systems the prime boost strategy seems to be a preferential mode of immunisation

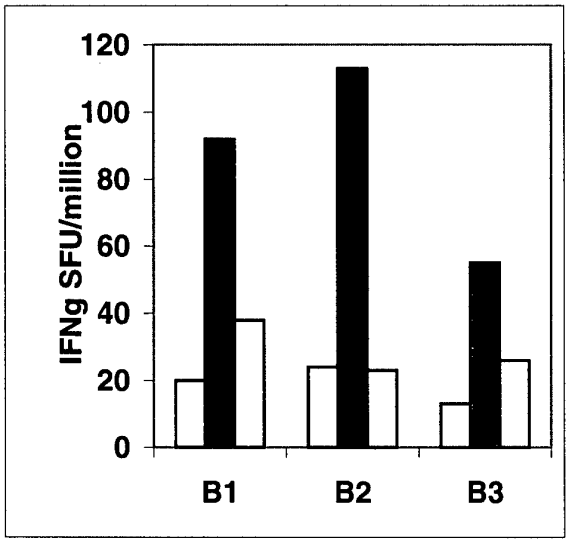
REPORTABLE OUTCOMES

There are no manuscripts submitted as yet, but they will be done so in the next year. It is not unlikely that a patent application will be lodged during the next year.

CONCLUSIONS

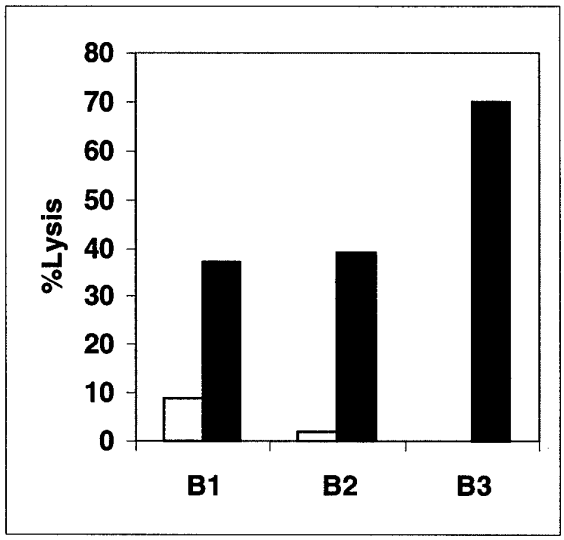
The first point to make is that the study is on track or indeed ahead of milestones. The most important finding is that whole glycosylated MUC1 (HMFG) is immunogenic – these studies had started earlier and have now been taken up in this particular study, making recombinant glycosylated MUC1. The second most important finding was that antigen conjugated to beads give rise to potent immune responses and finally that ELISPOTS, measuring γ-interferon, can satisfactorily measure anti-breast cancer antigen immune responses.

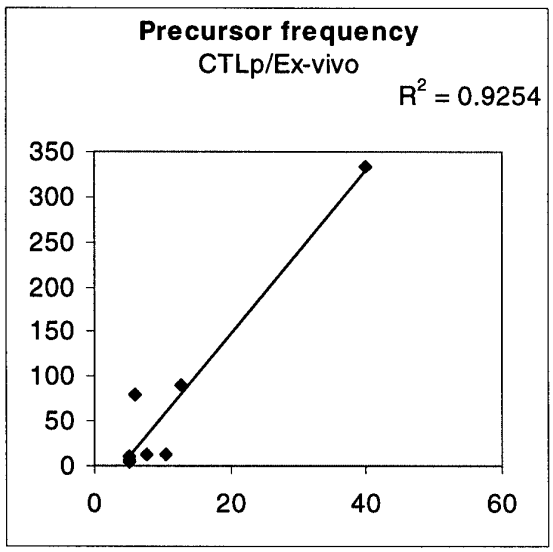
APPENDIX 1



A single intradermal immunization with 100µg of polysterene beads covalently conjugated to ovoalbumin (OVA) is capable of inducing a high precursor frequency of interferon gamma (IFNγ) producing T cells specific for the H-2Kd class I restricted CD8 T cell epitope SIINFEKL (dark bars) but little or no reactivity to soluble OVA (light bars). Spleen cells were taken at day 14 after immunisation for assessment of IFNγ production by the sensitive ELISPOT assays. A representative experiment of 5 is shown. Data is shown as the precursor frequency (spot forming units=SFU) per million spleen cells tested in three mice (B1, B2 and B3). Strong responses were maintained for 6 months after the single dose immunisation (data not shown). SIINFEKL response differences with background are highly significant ($p>0.001$) in all animals.

A single intradermal immunisation with 100µg of polysterene beads covalently conjugated to OVA was also capable of inducing cytotoxic T lymphocyte (CTL) responses, as shown for by % Lysis of SIINFEKL peptide pulsed chromium loaded P815 target cells (dark bars) over cells with no peptide (white bars). In the assay shown the effector:target ratio (E:T ratio) was 20:1. The data shown is representative of separate 3 experiments. Spleens from mice B1, B2 and B3 were tested for cytotoxic activity 14 days after immunization.





The precursor frequency of cytotoxic T cells determined by limiting dilution analysis of SIINFEKL specific T cells after induction with OVA-conjugated beads under optimal and suboptimal induction conditions correlates strongly with the precursor frequency of IFNγ producing SIINFEKL specific T cells detected by ELISPOT. It is therefore possible to use ELISPOT to assess the degree of class I restricted T cell reactivity by immunization in mice.

extracellular region; this is the first description of such CTL.

4. *Intracellular regions.* Three different, non-overlapping intracellular peptides containing amino acids 408–423, 471–493, 507–526, were examined using the approach described above. CTLp frequencies of 1/30,000 (408–423), 1/12,500 (471–493) and 1/22,500 (507–526) were obtained, amino acids 471–493 being the most effective to restimulate cytolytic cells.

To demonstrate that the CTL were specific for MUC1 sequences, and not due to non-specific killing by NK cells or other cells, P815 target cells were used with a non-MUC1 peptide, T4N1, as the pulsed antigen, CTLp either were not detected or the frequencies were ≤1/200,000 and were considered to be negative (not shown). Of the different regions, three were of equivalent immunogenicity (using CTLp frequency as a measure): extracellular (51–70) = VNTR (Cp13–32) = intracellular (471–493), all of which gave a high frequency of ~1/10,000.

In contrast, immunising BALB/c mice with non-conjugated HMFG, and stimulating with the VNTR peptide Cp13–32, the CTLp frequency was 1/80,500. This frequency is similar to the CTLp frequency of 1/95,000 obtained with mannan conjugated to a recombinant bacterial fusion protein containing 5 repeats of the MUC1 VNTR (19) and thus conjugation of HMFG to mannan is necessary for generating a strong CTLp frequency in mice.

3.3. CTL responses to mannan–HMFG in C57BL/6 mice

C57BL/6 were immunised with mannan–HMFG and in vitro stimulated with the same antigens used for the BALB/c mice (Table 2). There was a CTLp frequency of 1/13,500 for whole HMFG and 1/12,500 for the VNTR region peptide p1–30 (Table 2). Of the non-VNTR extracellular peptides, CTL were detected only to one extracellular peptide (344–364) with a frequency of 1/24,500. CTL were not detected to any of the intracellular peptides. Again the specificity of the CTL were confirmed by using a non-MUC1 peptide, T4N1, for stimulation and also using the non-MUC1 transfected parent RMA cell line as the target. Thus, C57BL/6 mice can respond to both VNTR and non-VNTR peptides, but there were no responses to certain peptides to which BALB/c mice responded.

3.4. Cellular immune responses to mannan–HMFG in transgenic HLA-A*0201/K^b mice

Transgenic HLA-A*0201/K^b mice were immunised *once* with mannan–HMFG (not ×3 as used above), stimulated in vitro with either HMFG, the VNTR peptide (p1–30) or one of the extracellular peptides (31-55). The CTLp were measured on human EBV HLA-A*0201$^+$ cells (see below) and frequencies were 1/39,000 (HMFG), and 1/33,000 (VNTR p1–30), which compare favourably with immunisation with mannan–VNTR peptide (1/48,000) (not shown), i.e., whole HMFG is as immunogenic as VNTR (Table 2). Further, when an extracellular peptide (31–55) was used, the CTLp frequency was 1/40,000, i.e., the same as that found for VNTR. Thus, HLA-A*0201 can present extracellular and VNTR peptides. It should be noted that, the target cell being EBV transformed B cells, which expresses HLA-A*0201 but not H-2^b class I molecules (expressed by the immunised mice), the CTLs detected were restricted to HLA-A*0201 presenting MUC1 peptides.

3.5. Cellular immune responses to mannan–HMFG in A2 K^bMUC1 double transgenic mice

To ascertain the ability of MUC1 CTL to lyse MUC1 positive breast cancer cells A2 K^bMUC1 double transgenic mice injected with mannan HMFG 3 times were stimulated in vitro with either HMFG, the VNTR peptide (p1–30), extracellular peptides (31–55, 344–364) or intracellular peptides (408–423, 471–493, 507–526) (Table 2). There was a CTLp frequency of 1/2000 for the whole HMFG and 1/8000 for the VNTR region peptide p1–30. CTL were detected to the extracellular peptides 31–55 and 344–364 with a frequency of 1/2000 and 1/11,000 respectively. Of the intracellular peptides CTL were detected for only peptide 408–423 with a frequency of 1/20,000.

Spleens of the immunised mice were used in a direct CTL assay to ascertain specificity of the anti-MUC1 CTL. As seen in Fig. 2 MUC1 CTL lysed 55% of MUC1$^+$ MCF7 (HLA-A*0201) breast carcinoma cells at an E:T ratio of 12:1 and was reduced to 17% when incubated in the presence of cold K562 targets. The MUC1 CTL were HLA restricted as no lysis was detected when the MUC1$^+$ BT20 (HLA-A1) breast cancer cell line was used. The MUC1 CTL did not lyse the MUC1 −ve melanoma cell line ME272.

Thus, immunisation of A2 K^bMUC1 mice with mannan–HMFG resulted in specific Class I restricted CTL that can lyse tumour cells expressing native MUC1 and moreover anti-MUC1 CTL can be generated in mice in the presence of endogeneously expressed human MUC1.

3.6. T cell epitope prediction and mapping

To precisely map the T cell epitopes involved in CTLp generation, a large number of overlapping 9-mer peptides would have to be synthesised and used in

lutions of anti-MUC1 antibody (BC2 [30]) in bicarbonate buffer overnight and non-specific binding blocked as described above. HMFG or mannan–HMFG was then added to the wells and incubated for 1 h at RT followed by washing extensively with PBS containing 0.05% Tween 20. Fifty μl of radiolabelled concanavalin A, which binds specifically to mannan but not HMFG, was then added and the plate incubated for a further 1 h followed by washing with PBS/Tween 20. Microscint-O (120 μl) was added to the wells, and plates counted in a β-scintillation counter.

3. Results

3.1. Preparation and characterisation of mannan–HMFG

The activity of the HMFG after conjugation to mannan was determined by inhibition ELISA; the 50% inhibitory concentration for HMFG was 22 μg/ml while for the mannan–HMFG was 20 μg/ml [Fig. 1(a)], i.e. HMFG retained full reactivity after conjugation to mannan. The integrity of the mannan–HMFG complex was shown by a sandwich radioimmunoassay using anti-MUC1 antibody bound to the plate and ^{125}I-labelled Con-A for the read out [Fig. 1(b)]. Non-conjugated HMFG did not bind [^{125}I]-Con-A while mannan–HMFG bound demonstrating mannan to be linked to HMFG.

3.2. CTL responses to mannan–HMFG in BALB/c mice

Spleen cells, from BALB/c mice immunised with mannan–HMFG, were stimulated in vitro with different peptides (from both VNTR and non-VNTR regions, Table 1) and CTLp were determined by testing on target cells expressing native MUC1 (Table 2). It was apparent that immunisation with mannan–HMFG leads to CTL reacting with epitopes from the whole of MUC1, i.e., from both the VNTR and non-VNTR region. The responses were:

1. *HMFG.* When whole MUC1 (HMFG) protein was used as the source of stimulating peptides, a CTLp frequency of 1/9700 was obtained. Clearly HMFG is immunogenic for CTL production in BALB/c mice and can be processed to yield peptides presented by Class I molecules.
2. *VNTR.* When VNTR peptides Cp13–32 and p1–30 were used to stimulate, CTLp frequencies of 1/7000(Cp13–32) and 1/13,200 (p1–30) resulted, i.e., by immunising with HMFG, anti-VNTR CTL were produced, results similar to those found previously by immunising with mannan-conjugated VNTR peptides [19]. This is the first discription of such CTL obtained by immunising with native mucin which is glycosylated.
3. *Extracellular regions.* When in vitro stimulation was with peptides containing amino acids 31–55, 51–70, 33–103, 344–364, CTL could be detected with a frequency of 1/19,500 (31–55); 1/10,000 (51–70); 1/20,150 (33–103) and 1/36,800 (344–364). Thus CTL can be produced to non-VNTR regions from the

Table 2
CTLp frequencies in spleens of mice immunised with mannan–HMFG

Restimulating antigen	Peptide details	CTLp frequency			
		Immunized strain			
		C57BL6(K^bD^b)	BALB/c ($K^dD^dL^d$)	HLA*0201/K^b	A2 K^bMUC1
		Target cell			
		MUC1-RMA	Target MUC1-P815	EBV + pep	MCF7
Whole MUC1	HMFG	1/13,500	1/9700	1/39,000	1/2000
Extracellular region	Cp13–32	ND[a]	1/7000	ND	ND
	p1–30	1/12,500	1/13,200	1/33,000	1/8000
	31–55	Not detected	1/19,500	1/40,000	1/2000
	51–70	Not detected	1/10,000	ND	ND
	33–103	ND	1/20,150	ND	ND
	344–364	1/24,500	1/36,800	ND	1/11,000
Intracellular region	408–423	Not detected	1/30,000	ND	1/20,000
	471–493	Not detected	1/12,500	ND	Not detected
	507–526	Not detected	1/22,500	ND	Not detected
Non-MUC1	T4N1	Not detected	Not detected	Not detected	Not detected

[a] ND, not done; no CTLp were detected when RMA, P815 or EBV infected lymphocytes without peptide were used as targets.

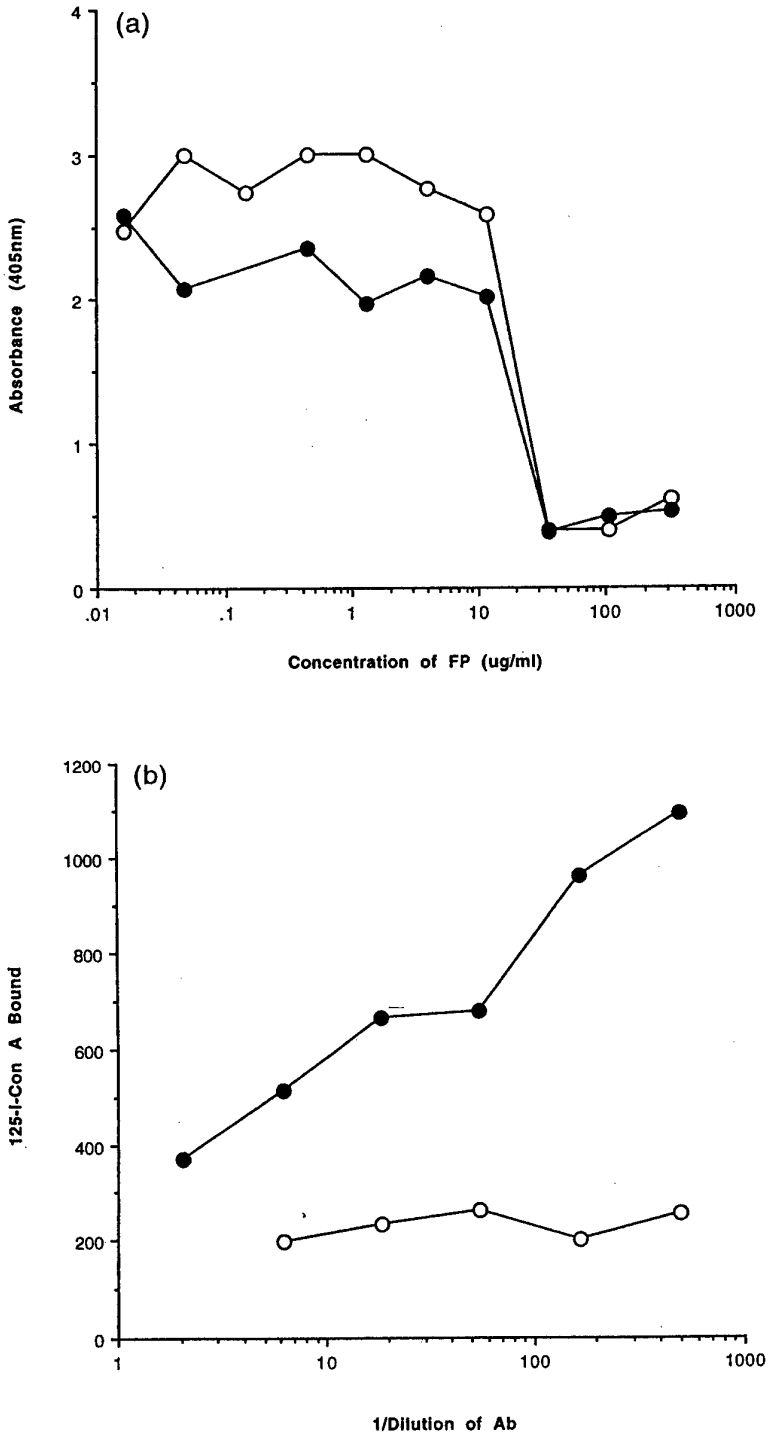


Fig. 1. Assay for HMFG and mannan. (a) Inhibition of binding of anti-MUC1 antibody to HMFG by competitor preparations of HMFG (○) and mannan–HMFG (●). (b) Binding of mannan–HMFG (●) and HMFG (○) to anti-MUC1 antibody and Con A detected by a radio-immunoassay.

2.2. Synthetic peptides

Peptides (Table 1) were synthesised at the ARI; the purity of the peptides (>95%) was determined by mass spectroscopy.

2.3. Conjugation of HMFG to mannan

HMFG was isolated from human milk [19] and coupled to mannan. Mannan (1 ml,14 mg/ml) in phosphate buffer (0.1 M, pH 6.0) was treated with sodium periodate (100 μl, 0.1 M) and incubated at 4°C for 30 min [20]. Ethanediol (10 μl) was added for 30 min at 4° to stop the reaction, and the mixture was passed through a PD10 column (Pharmacia Biotech, Sweden), equilibrated in bicarbonate buffer (0.2 M, pH 9.0) and the oxidised mannan fraction was mixed with 1 mg of HMFG overnight at room temperature to give mannan–HMFG.

2.4. T cell epitope prediction

There are several CTL epitope prediction algorithms available and in this study we used the program developed by Dr Kenneth Parker available on the internet (bimas.dcrt.nih.gov/molbio/hla_bind/) to identify potential T cell epitopes. This program is based on scores given to the amino acids at each of the positions from 1–9 from input sequences by comparison with the reported databases [21,22]. Higher numerical values for the 9-mer predict increased likelihood of being a T cell epitope. For example, the T cell epitope for ovalbumin (K^b, SIINFEKL) and papillomavirus-16 E7 protein (D^b, RAHYNIVTF) gives scores of 17 and 6 respectively.

2.5. Cytotoxic T cell and cytotoxic T cell precursor (CTLp) frequency assays

CTL assays were performed as described [9,11,20]. Briefly, 7–10 days after the final immunisation, splenocytes were harvested, washed and resuspended in growth medium and serially diluted in 96-well microtitre plates. A standard 3 h ^{51}Cr release assay was performed with 1×10^4 peptide pulsed or untreated P815 or RMA cells as targets at various effector:target ratios. Peptide pulsed P815 or RMA target cells were prepared by overnight incubation with 9-mer peptides (25 μg/ml) [9]. For CTL assays with A2 K^bMUC1 double transgenic effectors, MCF7 ($MUC1^+$HLA-A^*0201^+) and BT20 ($MUC1^+$HLA-A^*0201^-) breast cancer cell lines or the ME272 ($MUC1^-$HLA-A^*0201^+) melanoma cell line was used as targets. All of these human tumour cell lines are susceptible to cell mediated lysis [11,23,24]. CTLp frequencies were determined from a minimum of 32 replicates, for at least 6 effector cell numbers (1×10^3–1.28×10^5). Cells were cultured in U-bottomed microtitre trays, with 5×10^5 mitomycin C treated BALB/c (H-2^d), C57BL/6 (H-2^b) or HLA-A^*0201/K^b spleen cells, in DMEM supplemented with 10% foetal calf serum, 5 μM of various MUC1 peptides (Table 1) or HMFG and 10 U/ml rhIL-2. Seven days later, each microculture was assayed for cytotoxicity by replacing 100 μl of culture medium with 100 μl target cell suspension containing 10^4 ^{51}Cr-labelled Tm211 (H-2^d), RMA-MUC1 (H-2^b) Tumour or EBV transformed human B cells (HLA-A^*0201) or MCF7 as targets. As a specificity control non-MUC1 expressing P815(H-2^d) or RMA(H-2^b) cells were used. Cytotoxic activity was considered to be present if in each well ^{51}Cr release was found 3 standard deviations above the mean isotope release from 10^4 effectors cultured with stimulators only or from stimulator cells with peptide only or rIL2 only. A linear relationship ($0.987 \leqslant r^2 \leqslant 1$) existed between the number of responder cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of responder cell dose required to generate 37% negative wells [25–27]. CTLp frequency assays were performed three times and the individual frequencies did not differ by more than 20% from the mean value. It should be noted that the CTLp frequency in immunised mice are directly correlated with tumour protection [28].

2.6. Inhibition ELISA

An antibody inhibition ELISA was performed to compare the activity of HMFG before and after conjugation to mannan. Polyvinyl chloride plates were coated with 70 μl of 10 μg/ml HMFG in bicarbonate buffer (0.2 M, pH 9.0) overnight at 4°C or 1 h at 37°C and non-specific binding was blocked with 2% bovine serum albumin (BSA). Various concentrations of HMFG or mannan–HMFG were incubated with anti-MUC1 antibody (VA2 [29], 1/200 supernatant) for 3 h and 100 μl was added to PVC microtitre well plates coated with HMFG. After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20, 50μl of sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (Amersham, UK) was added and incubated for a further 1 h at RT. After washing with PBS/Tween 20, the plate was developed with the chromogenic substrate 2,2″-azino-di(3-ethylbenzthiazoline) sulphonate (ABTS) (Amersham, UK) and the absorption at 405 nm recorded.

2.7. Radioimmunoassay

A sandwich radioimmunoassay was performed to ascertain that the mannan was covalently linked to HMFG. A microtitre plate was coated with serial di-

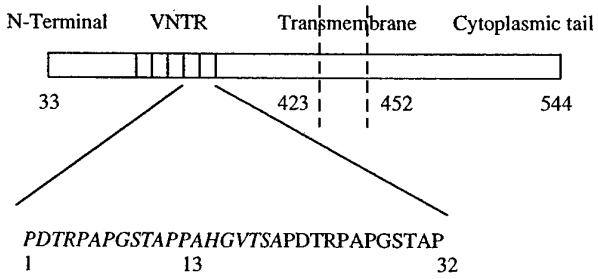
and the finding that non-HLA restricted CTL also are directed to the VNTR, almost all interest in MUC1 for CTL induction has concentrated on VNTR peptides [9,13,14]. However, while this region may be immunogenic for antibody production, there is no reason to conclude that CTL epitopes are restricted to this region. Indeed, an algorithm-based search of the whole MUC1 sequence reveals many potential CTL epitopes outside the VNTR region (see below). With this in mind we now describe the induction of CTL to non-VNTR epitopes, in the extracellular and intracellular parts of MUC1 identified by immunising mice with native mucin (HMFG) obtained from human breast milk.

2. Materials and methods

2.1. Mice and tumour cells

BALB/c (H-2^d), C57BL/6 (H-2^b), human MUC1 transgenic mice (obtained from B. Acres (Transgene, Strasbourg, France)), transgenic HLA-A*0201/K^b mice (H-2^b) (obtained from The Scripps Clinic and Research Foundation, La Jolla, CA) and double transgenic mice (A2 K^bMUC1) were bred at The Austin Research Institute. The human MUC1 in the MUC1 transgenic mice (back crossed to DBA/2) is under the control of the human MUC1 promoter; MUC1 is expressed in the lung bronchioles, β-islets of the pancreas, kidney tubules and stomach [15]. The HLA-A*0201/K^b mice express a transgene composed of the α1 and α2 domains of HLA-A*0201 and the α3 contains the transmembrane and cytoplasmic domains of H-2 K^b [16]. The double transgenic mice were screened for expression of the HLA-A*0201/K^b and human MUC1 transgenes by flow cytometry with antibodies to HLA-A*0201 and MUC1. RMA-MUC1 is a MUC1 transfected (C57BL/6 (H-2^b)) lymphoma cell line [17]. Tm211 is a MUC1 transfected P815 mastocytoma (DBA/2 origin; H-2^d) obtained from B. Acres (Transgene, Strasbourg, France) [18]. All mouse cell lines were maintained in Dulbecco's modified Eagles medium (DMEM) with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum [all from Commonwealth Serum Laboratories (CSL), Melbourne] and human cell lines in RPMI with the same additives in a 7% humidified CO_2 incubator at 37°C. BALB/c, C57BL/6 and double transgenic A2 K^bMUC1 mice were immunised intraperitoneally with 3 injections of 5 μg mannan–HMFG or HMFG on days 0, 10, 17 while HLA-A*0201/K^b mice were injected once.

Table 1
Diagramatic structure of MUC1 and sequences of the synthetic peptides[a]



Extracellular region	
Cp13–32	(C)PAHGVTSAPDTRPAPGSTAP
p1-30	PDTRPAPGSTAPPAHGVTSAPDTRPAPGST
31–55	TGSGHASSTPGGEKETSATQRSSVP
51–70	RSSVPSSTEKNAVSMTSSVL
33–103	Glutathione-S-transferase fusion protein SGHASSTPGGEKETSATQRSSVPSSTEKNAVSMTSSVLS SHSPGSGSSTTQGQDVTLAPATEPASGSAATW
229–237	SAPDNRPAL
344–364	NSSLEDPSTDYYQELQRDISE
Intracellular region	
408–423	TQFNQYKTEAASRVNL
471–493	AVCQCRRKNYGQLDIFPARDTYH
507–526	(C)YVPPSSTDRSPYEKVSAGNG
Mouse CD4	
T4N1	KTLVLGKEQESAELPCECY

[a] In the peptides of the VNTR (sequence in *italics*) p1–30 and p13–32 the numbers refer to amino acid number in the VNTR. Other numbers (31–55, 51–70 etc.) refer to the amino acid position in the published sequence.

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Vaccine 18 (2000) 2059–2071

Vaccine

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Definition of MHC-restricted CTL epitopes from non-variable number of tandem repeat sequence of MUC1

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Received 17 June 1999; received in revised form 24 August 1999; accepted 18 October 1999

Abstract

Mucin1 (MUC1) is expressed ubiquitously on breast cancer cells and is a potential target for the generation of cytotoxic T cells for vaccination against breast cancer. Thus far studies of the immunogenicity of MUC1 have used peptides from the variable number of tandem repeat (VNTR); mice so immunised can generate strong cellular and antibody responses to the VNTR of human MUC1. We now demonstrate that significant CTL and CTLp can be induced to other regions of MUC1. Using the whole native MUC1 molecule, the human milk fat globule membrane antigen (HMFG) linked to mannan, cytotoxic T cell precursors (CTLp) can be generated in BALB/c, C57BL/6, transgenic HLA-A*0201/K^b and double transgenic HLA-A*0201/K^b × human MUC1 (A2 K^bMUC1) mice. By immunising with HMFG and testing selectively on (a) extracellular (non-VNTR); (b) VNTR and (c) intracellular peptides, it was shown that all three regions generated effective CTL. Further, the CTL responses to non-VNTR peptides were as strong as those generated to the VNTR. Epitope prediction algorithms were not particularly helpful to describe CTL epitopes: overlapping peptides had to be synthesised and tested to find the epitopes. Thus, for CTL generation, the whole HMFG molecule is a powerful immunogen when linked to mannan, especially as multiple peptide epitopes for presentation by many Class I molecules are contained within the one molecule. Furthermore, Class I restricted MUC1 CTL were generated in double transgenic A2 K^bMUC1 mice by immunising with mannan-native mucin (HMFG), suggesting that tolerance to MUC1 can be overcome with mannan–HMFG.

Keywords: Mucin 1; Epitopes; Immunotherapy

1. Introduction

Immunotherapeutic approaches for the treatment of breast cancer have included the use of monoclonal antibodies and the generation of cytotoxic T lymphocytes (CTL) [1–6]. The identification of target antigens, the availability of recombinant proteins and cytokines have given impetus to immunotherapy. Thus, there are new means by which to generate an effective cytotoxic T cell response to MUC1-expressing carcinomas of the breast and other tissues [7]. MUC1 is a particularly attractive target for the generation of CTL: it is immunogenic in mice for the production of antibodies and, more recently, we have described $CD8^+$ CTL, and the MHC Class I H-2 and HLA-A*0201 binding peptides have been mapped in the VNTR [8–11]. Furthermore, in cancer cells, there is up to a 100 fold increase in the amount of mucin [12] and there should be a significant amount of MUC1 peptide available to be bound by Class I molecules. The reason for the focus on the VNTR peptides is clear — it is the most immunogenic region in MUC1 when whole tumour cells or mucin extracts (HMFG) are used to immunise mice for the production of antibodies [12]. Because of this focus

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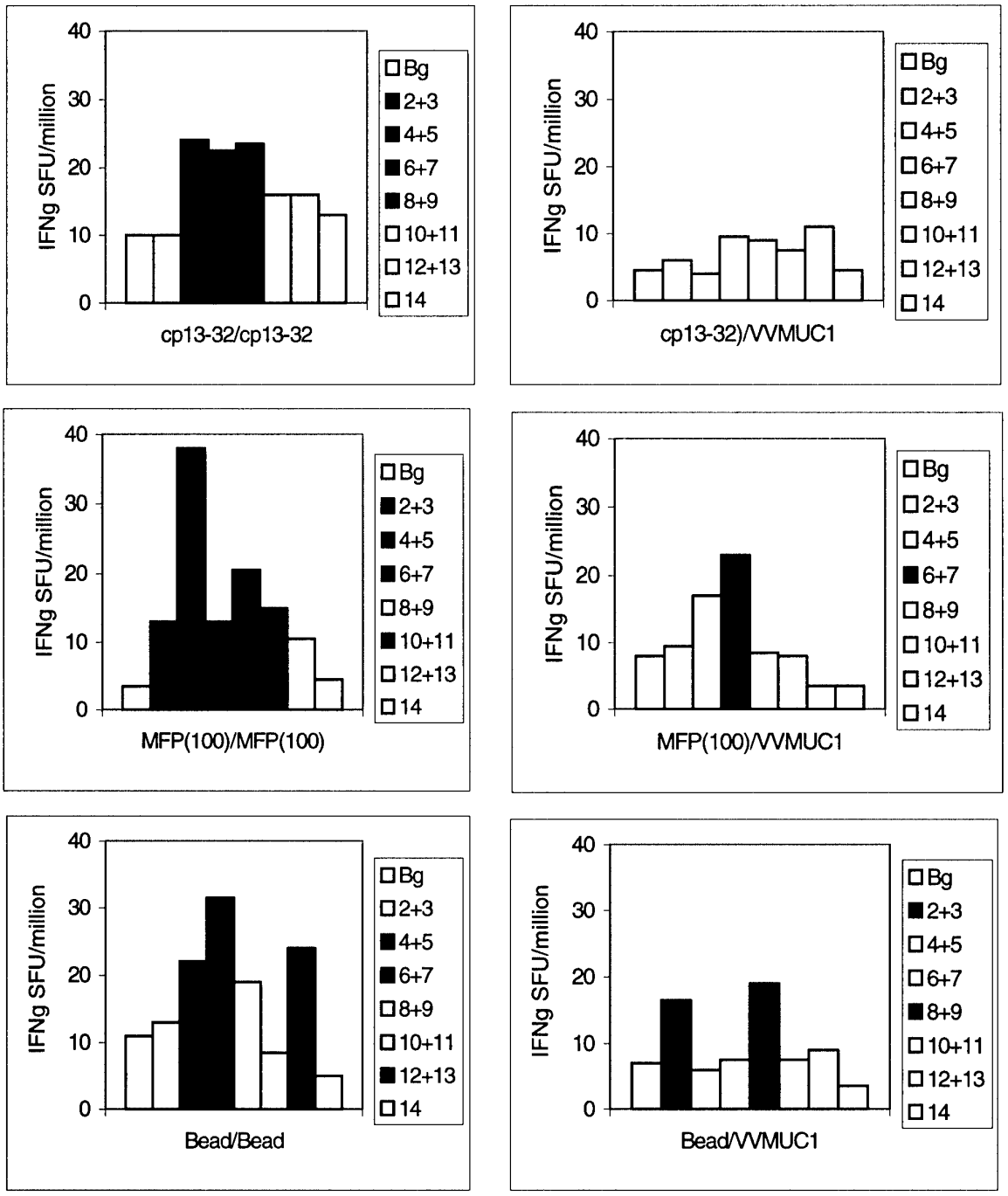
APPENDIX 2



Balb/c mice were primed intradermally with 100 ug of recombinant human mucin-1 VNTR fusion protein conjugated to oxidised mannan (MFP) or to polysterene beads (Bead) or the VNTR region peptide cp13-32 in Freunds complete adjuvant (FCA) and then boosted with a homologous immunisation (in the case of cp13-32 now in incomplete Freunds adjuvant (IFA)) or with a heterologous immunisation with Vaccinia virus expressing human mucin-1 (provided by Prof. Bruce Ackers)(VVMUC1). Spleens cells were tested for reactivity against a panel of overlapping 9-mer peptides representing the VNTR region (1-14). Each peptide overlaps by one aminoacid with the next one. Pools of 2 peptides (each at 25 ug/ml) from peptide 2-13, and peptide 14 on its own were tested in each case. Background (Bg) indicates an absence of added peptide. Shaded bars represent a significant response over background to the specific peptide pool ($p>0.05$). Potential total activity to VNTR may be calculated by adding reactivities to the individual peptides (not shown). In this same experiment, VVMUC1 alone was capable of inducing significant responses to peptides 2+3, 4+5, 10+11 and 12+13 (35,41, 38 and 45 SFU/million respectively). One of three similar experiments is shown. Therefore, although MFP, beads-FP, cp13-36 are all capable of priming MUC-1 VNTR specific responses, vaccinia MUC-1 was incapable of producing a heterologous prime-boost synergistic immune effect, despite being immunogenic by itself.

CTL assays. Instead, a CTL epitope prediction program was used to select putative immunogenic peptides and these were synthesised to test their antigenicity.

3.6.1. Predicted H-2^d-restricted peptides (intracellular region MUC1)

Several peptides [NYGQLDIFP(K^d), YGQLDIFPA(D^d), KNYGQLDIF(L^d)] were contained in 471–493 (CTLp frequency = 1/12,500) and had predicted scores 6, 6 and 10 respectively (Table 3). To ascertain if the predicted 9-mers are presented by the Class I molecules, cytotoxic T cell assays were performed using spleen cells from mannan–HMFG immunised mice as effectors and P815 target cells were pulsed with the synthetic peptides. These were NYGQLDIFP(K^d), YGQLDIFPA(D^d), KNYGQLDIF(L^d). The pulsed cells were not lysed by mannan–HMFG derived CTL from BALB/c mice [Fig. 3(a)], i.e., the CTL epitopes were not predicted accurately by the algorithm. The MUC1 VNTR peptides SAPDTRPAP(D^d) and APDTRPAPG (L^d) identified previously as CTL epitopes in the VNTR region [10], were used as positive controls and 62% and 50% lysis at an E:T ratio of 50:1 was obtained. The listeriolysin K^d peptide (GYKDGNEYI) and HIV Dd peptide RKSIRIQRGPGRAFVTIGKGKGKGY, used as negative controls, did not give rise to lysis [Fig. 3(a)].

3.6.2. Predicted H-2^d-restricted peptides (extracellular region MUC1)

A number of 9 mer peptides in the extracellular region are predicted to be CTL epitopes [(AVSMTSSVL(K^d), TTQGQDVTL(K^d), NAVSMTSSV(K^d), TSATQRSSV(K^d), SSTTQGQDV(K^d), SVPSSTEKN(D^d), EPASGSAAT(L^d), SPGSGSSTT(L^d), VPSSTEKNA(L^d), TPGGEKETS(L^d), TSATQRSSV(L^d), SSTTQGQDV(L^d)] and were contained in peptide 33–103 (CTLp frequency = 1/20,150) with scores of 58, 40, 29, 10, 10, 2.9, 39, 39, 36, 30, 10 and 10) respectively. A subset of these peptides were also contained in the 51–70 peptide(CTLp frequency = 1/10,000) (Table 3). Of these, four were made [AVSMTSSVL(K^d), NAVSMTSSV(K^d), VPSSTEKNA(L^d), SVPSSTEKN (D^d)] and tested. Three of the four peptides were indeed presented and one was not. The synthetic peptides AVSMTSSVL(K^d), NAVSMTSSV(K^d) and VPSSTEKNA(L^d) sensitised P815 target cells with 77%, 80% and 78% lysis at E:T of 50:1 respectively, while SVPSSTEKN (with the lowest predictive value) was inactive [Fig. 3(b)]. Therefore, AVSMTSSVL, VPSSTEKNA and NAVSMTSSV are CTL epitopes in peptides 33-103 and 51-70.

3.6.3. Predicted H-2^b restricted peptides

Even though there were fewer identified peptide epitopes for C57BL/6 mice, there are a large number of potential CTL epitopes present in the peptides, albeit

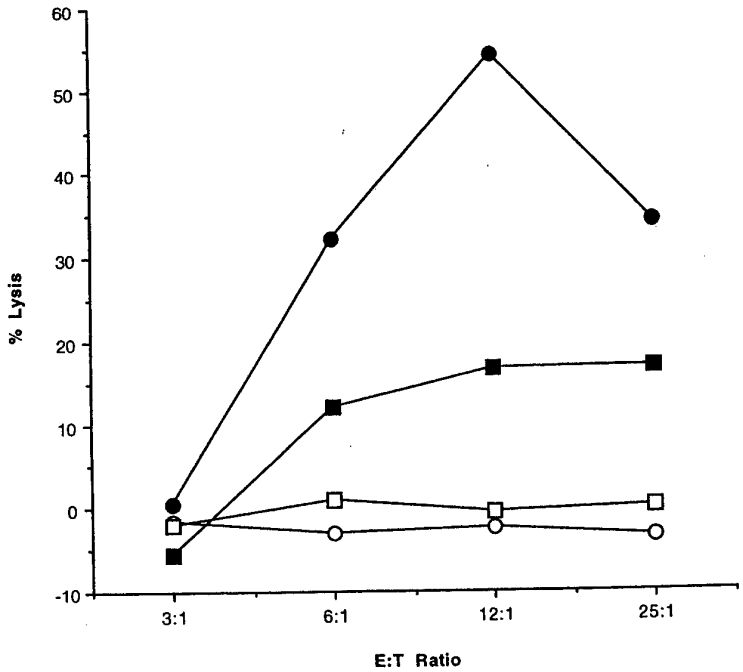


Fig. 2. A2 K^bMUC1 double transgenic mice were immunised with mannan–HMFG and splenocytes were used in CTL assays. Cytotoxic activity of the effector cells were measured on ^{51}Cr-labelled MCF7 with (■) or without cold K562 (●); BT20 (□) or ME272 (○).

Table 3
Mice immunised with mannan–HMFG: CTLp frequencies to various non-VNTR peptides and their predicted CTL epitopes

Peptide used for stimulation	CTLp frequency		Predicted 9-mers and score for the various H2 haplotypes									
	BALB/c	C57Bl/6	K^d	CTL	D^d	CTL	L^d	CTL	D^b	CTL	K^b	CTL
471–493 AVCQCRRKNYGQL DIFPARDTYH	1/12,500	Not detected	NYGQLDIFP 6	0%	YGQLDIFPA 6	0%	KNYGQLDIF 10	0%	CRRKNYGQL 10	20%	CRRKNYGQL 1.4	20%
507–526 CYVPPSSTDRSPYEKVS AGNG	1/22,500	Not detected	CYVPPSSTD 12		VPPSSTDRS 2.4		VPPSSTDRS 45		RSPYEKVSA 0.99		VPPSSTDRS 0.6	
408–423 TGFNQYKTEAASRYNL	1/30,000	Not detected	QFNQYKTEA 29 QYKTEAASR 6.0 TEAASRVNL 5.8		FNQYKTEAA 2.2		TEAASRVNL 6.0		TEAASRVNL 0.18		FNQYKTEAA 0.72	
344–364 NSSLEDPSTDYYQELQR DISE	1/36,800	1/24,500	YYQELQRDI 2880 SLEDPSTDV 14		YYQELQRDI 1.8		NSSLEDPST 5 DPSTDYYQE 3.6 PSTDYYQEL 2.5		SSLEDPSTD 4.4		PSTDYYQEL 2.9 YYQELQRDI 1.1	
33–103 SGHASSTPGGEKETSATQ RSSVPSSTEKNAVSMTSS VLSSHSPGSGSSTTQGQD VTLAPATEPASGSAATW	1/20,150	Not done	AVSMTSSVL 58	77%	SVPSSTEKN 2.9	0%	EPASGSAAT 39		STEKNAVSM 15	19%	AVSMTSSVL 1.2	18%
			TTQGQDVTL 40 NAVSMTSSV 29 TSATQRSSV 10 SSTTQGQDV 10	80%			SPGSGSSTT 39 VPSSTEKNA 36 TPGGEKETS 30 TSATQRSSV 10 SSTTQGQDV 10	78%	AVSMTSSVL 10.1	18%		
51–70 RSSVPSSTEKNAVSMTSSVL	1/10,000	Not detected	AVSMTSSVL 58		SVPSSTEKN 2.9		VPSSTEKNA 36		STEKNAVSM 15		AVSMTSSVL 1.2	
			NAVSMTSSV 29 STEKNVSM 6.0				AVSMTSSVL 5.0 SSTEKNAVS 5.0 NAVSMTSSV 2.0		AVSMTSSVL 10			
31–55 TGSGHASSTPGGEKETSA TQRSSVP	1/19,500	Not detected	TSATQRSSV 10		GGEKETSAT 2.0		TPGGEKETS 30		TSATQRSSV 2.6		KETSATQRS 0.24	
					TGSGHASST 2.0		TSATQRSSV 10					

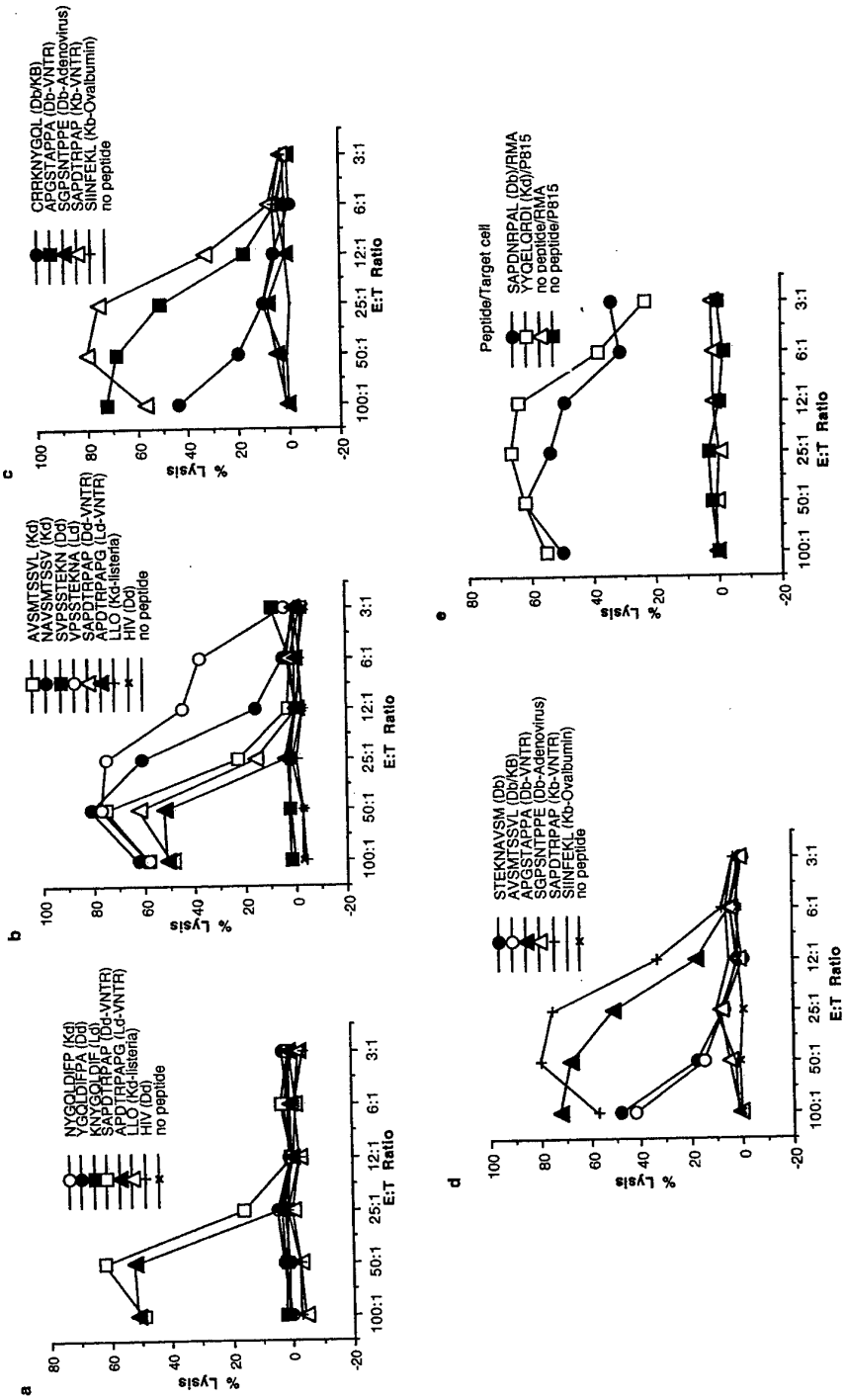


Fig. 3. C57BL/6 and BALB/c mice were immunised with mannan–HMFG and splenocytes were used in CTL assays. Lysis of P815 (a) or RMA (c) cells pulsed with various 9-mer peptides from the intracellular peptide 471–493; Lysis of P815 (b) or RMA (d) cells pulsed with various 9-mer peptides from the extracellular peptides 33–103 and 51–70 and (e) Lysis of P815 cells pulsed with YYQELQRDI and RMA-MUC1 cells pulsed with SAPDNRPAL. As controls for peptide pulsing and antigen-specific cell lysis, known peptide antigens were used and are shown in each panel and described in the text.

with low scores (Table 3). The 9-mer CRRKNYGQL (D^b, K^b) was contained in 471–493 (CTLp not detected) and had scores of 10 and 1.4. It weakly sensitised RMA targets to lysis by mannan–HMFG CTL with 20% lysis at a E:T of 50:1 and 42% lysis at E:T of 100:1 [Fig. 3(c)]. The MUC1 VNTR peptides APGSTAPPA (D^b) and SAPDTRPAP (K^b) were used as positive specificity controls, where lysis of 70% and 80% were obtained while no lysis was detected for the ovalbumin K^b 9-mer SIINFEKL and Adenovirus D^b 9-mer (used as negative specificity controls). The 9-mer peptides STEKNAVSM(D^b), AVSMTSSVL(D^b) and AVSMTSSVL(K^b) were contained in the peptides 33–103 and 51–70 with scores of 15, 10 and 1.2. All of these three peptides weakly sensitised RMA targets to lysis (~20% at 50:1 and ~40% lysis at E:T of 100:1) [Fig. 3(d)]. There were no CTL reactive to peptides 31–55 and 51–70 in C57BL/6 mice.

Two high scoring CTL epitopes predicted from the whole MUC1 molecule from the intracellular region [YYQELQRDI(K^d) score 2880] and extracellular region N-terminal to the VNTR [SAPDNRPAL(D^b) score 4723] with scores of 2880 and 4723 sensitized RMA and P815 target cells to 50% lysis at an E:T of 50:1 [Fig. 3(e)]. Therefore, several T cell epitopes are present in the non VNTR regions of the MUC1 molecule and as 9-mer peptides can be presented by target cells to CTL generated by mannan–HMFG immunisation.

4. Discussion

Our previous immunisation studies used a MUC1 fusion protein containing 5 repeats of the VNTR linked to mannan (MFP) and this generated strong cellular responses to MUC1 characterised by the production of IFN-γ, IL-12, very little IgG_{2a} antibody and protection from tumour growth [8,20]. Immune responses in humans have also shown promise for the therapeutic use of MUC1 antigens as in a Phase I clinical trial using MFP, 4 of 15 patients generated proliferative responses, 13 of 25 showed high levels of MUC1 specific serum antibody and 2 of 10 generated CTL to MUC1 [31]. However, in vitro peptide binding studies and in vivo studies using transgenic HLA-A*0201 mice demonstrated that the VNTR sequences can only be presented by HLA-A*0201 and HLA-A*1101 [11,32], and studies thus far have concentrated on the MUC1 VNTR because of its preferential immunogenicity in mice, at least for antibodies, and because of evidence from humans implicating the VNTR in immune responses. Other protein sequences of MUC1 have not been examined for their cellular immunity. In the past, we have sought monoclonal antibodies to non-VNTR regions in mice immunised with MUC1: none resulted and none were found in an international study. However, there is no a priori reason that VNTR peptides should be better or worse than non-VNTR peptides for the induction of CTL. Indeed, scanning the whole MUC1 sequence for potential T cell epitopes predicted many previously untested peptides. We have therefore immunised mice with mannan conjugated HMFG, to provide all possible MUC1 epitopes but dependent on natural antigen processing for their presentation, and showed that cellular immune responses to the non-VNTR regions of the MUC1 can be generated which are as effective as those generated to the VNTR and further both HLA-A*0201 and A2 K^bMUC1 transgenic mice could be immunised, indicating that humans may also be able to be immunised.

Cellular responses could be detected to the extracellular region of MUC1, the VNTR and also to intracellular peptides in mannan–HMFG immunised BALB/c, C57BL/6, HLA-A*0201/K^b and double transgenic A2 K^bMUC1 mice. Immunised BALB/c mice developed CTL that could respond to more non-VNTR CTL epitopes than C57BL/6 mice, in which only the 344–364 peptide and SAPDNRPAL was recognised by CTL [Table 2, Fig. 3(e)].

Of the various peptides used for restimulation, several possible candidate 9-mer epitopes could be predicted using the peptide motif search program (Table 3). In BALB/c mice, the precursor frequency for the 471–493 peptide was 1/12,500, however the predicted epitope peptides NYGQLDIFP, YGQLDIFPA and KNYGQLDIF were not able to sensitise P815 targets for lysis by mannan–HMFG CTL [Fig. 3(a)]. Therefore, either the stimulating CTL epitope was not correctly identified by the algorithm or these synthetic peptides were not appropriately processed and presented by the target cells. In contrast, several 9-mers present in the 33–103 and 51–70 sequences (AVSMTSSVL, NAVSMTSSV and VPSSTEKNA) were identified as functional CTL epitopes in the lysis assays [Fig. 3(b)].

In C57BL/6 mice, the CRRKNYGQL, STEKNAVSM and AVSMTSSVL peptides from the 51–70 and 471–493 sequences sensitised RMA cells for lysis however no CTLp were identified by restimulation with the larger peptides. This observation could result from the three 9-mers not being processed and presented by the $MUC1^+$ cells.

Further analysis of the entire MUC1 sequence using the T cell epitope algorithm for mouse K^d, D^d, L^d, K^b, D^b, K^k, and human HLA-A1, HLA-A*0201, HLA-A3 and HLA-A24 epitopes show several candidate 9-mers for presentation by mouse or human cells (not shown). Of these 9-mer peptides, SAPDNRPAL (D^b) and YYQELRDI (K^d) were synthesised and both were very efficient in sensitising P815 or RMA cells for lysis

by mannan–HMFG CTL [Fig. 3(e)]. It is apparent in this study and others that the prediction of CTL epitopes is not always accurate. A comparison of the predicted and experimentally determined T cell epitopes for the VNTR region illustrates that the lower scores do not necessarily predict a lack of presentation or antigenicity (Table 4). For example, SAPDTRPAP peptide has been confirmed to be a K^b-restricted epitope by class I stabilisation when incubated with the TAP defective RMA-S cells as well as by lysis of peptide pulsed RMA cells [Fig. 3(c)], however the predicted score is only 0.004 (10). Similarly, the K^k, L^d and D^d was not predicted accurately (10). The HLA-A*0201 T cell epitope, STAPPAHGV identified independently by epitope mapping (11) was predicted albeit with a low score. The prediction algorithms act as a guide, to the probability of antigen presentation, but the in vivo response will be defined by antigen processing, immunodominance, T-cell repertoire, glycosylation and other unknown factors [33,34].

Even though the whole MUC1 protein in purified form has not previously been used to immunise mice to generate cellular immunity, several other immunisation methods have been used. The whole MUC1 protein has been delivered in a vaccinia construct [18,35], as a construct in DNA immunisation [36], in transfected dendritic cells [37] and in transfected EBV-B cells [38]. In none of these studies was the specificity of the CTL ascertained. However, the importance of using glycosylated MUC1 (as HMFG) should be stressed. Other studies, in mice and humans have used non-glycosylated peptides which have led to antibody production in both MUC1 transgenic mice [39] and in humans [31,40,41]; in these studies it was considered that B cell and at times T cell tolerance had been overcome but, with respect to antibodies, the non-glycosylated peptides represent novel antigens and the response is not surprising. However, in the studies described herein, native glycosylated mucin (HMFG) linked to mannan successfully primed CTL in several strains of mice including A2 K^bMUC1 transgenic mice. Mannan–HMFG gave a higher CTLp frequency in A2 K^bMUC1 mice (1/2000) compared to BALB/c or C57Bl/6 mice and could be due to either the different strain of mice or to the presence of a higher affinity HLA-A*0201 CTL epitope. In BALB/c mice HMFG gave a CTLp frequency of 1/80,500. This was comparable to the CTLp frequency in mice immunised with a non-glycosylated form of MUC1 VNTR [19], i.e., both glycosylated and non-glycosylated forms of the VNTR were equally immunogenic provided they are presented with oxidised mannan. Clearly, the carbohydrate coating did not obscure the underlying peptide. Thus, mannan–HMFG is able to break tolerance in A2 K^bMUC1 transgenic mice by producing CTLs to peptides in the VNTR, the extracellular region and the intracellular region in MUC1. These results reinforce the concept that MUC1 should be a useful target in therapy.

Several points are important for the use of mannan–HMFG in humans. Firstly, MUC1 is a present on some normal cells such as pancreas, kidney and it is possible that immune responses may be generated to these tissues and give rise to autoimmunity. Thus far in our clinical trials using MUC1 VNTR conjugated to mannan no autoimmunity was detected, however, careful dose escalation studies and monitoring is necessary [31]. Secondly, the HMFG obtained from donors is unlikely to be approved for use and recombinant material will have to be made. It is not clear how this will be done as the HMFG used herein is highly glycosylated. Presumably, a eukaryotic vector will be necessary. Thirdly, we have recently shown that the VNTR peptides can deviate the immune response towards antibodies, because of a cross reaction with existing, natural human antibodies [42]. Such a deviation may occur when using whole MUC1 and we are currently testing MUC1 lacking VNTRs.

Table 4
Experimentally determined and predicted mouse and human CTL epitopes in the MUC1 VNTR

Haplotype	Experimentally determined T cell epitope	Predicted score for T cell epitope from algorithm	Predicted T cell epitopes	Predicted score from algorithm
K^b	SAPDTRPAP	0.004	APPAHGVTS	0.330
			TAPPAHGVT	0.300
			STAPPAHGV	12.000
K^d	ND			
K^k	PDTRPAPGS	0.200	STAPPAHGV	0.5
L^d	APDTRPAPG	0.900	APPAHGVTS	45.00
			APGSTAPPA	30.00
D^d	SAPDTRPAP	0.086	STAPPAHGV	12.00
HLA-A*0201	STAPPAHGV	0.966	STAPPAHGV	0.966

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