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12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Human mucin glycoprotein MUC1 is a "self" peptide overexpressed on all surfaces of breast cancers and is an attractive candidate immunogen for a breast cancer vaccine. MUC1 is only weakly immunogenic, eliciting a low frequency of cytolytic T lymphocytes (CTL). We proposed to enhance immunity to MUC1 by using peptide mimics of native MUC1 epitopes. Mimic peptides contain amino acid substitutions; some mimics augment the T cell response to the native epitopes. The activity of mimics takes advantage of the degeneracy of T cell recognition, i.e., on the fact that a single T cell receptor (TCR) can recognize many different peptides. To develop MUC1 specific mimic peptides we planned to: 1) establish MUC1 specific CTL lines, 2) use combinatorial peptide libraries in a positional scanning format to determine which amino acid substitutions in the original peptide were recognized best by the CTL, 3) synthesize those mimics; 4) test them on an index cell line and 5) identify those that were stronger immunogens for CTL than the native peptide. First we had to choose the best (most immunogenic) native MUC1 epitope and develop a CTL line from a healthy HLA-A*0201+ individual. Such a line had to be (1) highly specific for chosen peptide, 2) strongly cytotoxic against MUC1 positive adenocarcinoma cells, and (3) contain at least 10 ⁸ T cells. All of these qualities are required for successful analysis of the peptide library. Because MUC1 is a weak antigen, this first step proved to be very difficult, but we ultimately found a leader sequence LLLLTVLTV that consistently led to CTL and developed the CCM4 cell line. With this index cell line, we screened combinatorial nonamer libraries and identified amino acid substitutions that allowed better recognition of the peptides than the native. We then synthesized 96 peptides containing as many as 8 substitutions. Unfortunately the CCM4 line did not survive frozen storage, so a new CTL line must be established. With it and the novel peptides we can find the most immunogenic mimics, to complete the project.			
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Introduction

The human mucin MUC1, a complex glycoprotein, is normally expressed on the apical surface of ductal epithelial cells and is usually cryptic—(hidden from the immune system. However, tumor MUC1, while identical in its protein portion, is over-expressed on all surfaces of a wide variety of ductal adenocarcinomas, including those of breast, pancreas, lung, colon, and prostate ¹. Thus the widespread distribution of MUC1 and its exposure to the immune system make it a potentially attractive immunogen for a cancer vaccine with broad specificity. MUC1 is essentially a “self” antigen, and, as such, is only weakly immunogenic. That is, natural responses against it are characterized by a low frequency of cytolytic T lymphocytes (CTL) and low antibody titers.

Our aim in this program was to enhance the immune response to MUC1 by using mimics of natural MUC1 epitope(s). Mimics were defined as peptides of a different structure from the natural epitope, but which could augment the CTL response to the latter. Responses to a particular mimic might theoretically be an augmentation or a diminution, depending upon the mimic. Immunological stimulation by mimics takes advantage of the “degeneracy” of T cell recognition: that a single T cell receptor (TCR) can recognize many different peptides.

Body of Report

To develop MUC1 specific mimic peptides we planned to 1) establish a MUC1 specific CTL, 2) use combinatorial peptide libraries in a positional scanning format to determine which amino acid substitutions in the original peptide were recognized best by

CTL², 3) synthesize those mimics; 4) test them on the index cell line and 5) identify those that were stronger immunogens for CTL than the native peptide.

First we had to choose the best (most immunogenic) native MUC1 peptide and develop T cell line from a healthy HLA-A*0201+ (HLA-A2) individual. Such a line had to be (1) highly specific for chosen peptide, 2) strongly cytotoxic against MUC1 positive adenocarcinoma cells, and (3) contain at least 10⁸ T cells. All of these qualities are required for successful analysis of the peptide library. The weakness of the immunogenicity of MUC1 made this first step very difficult. After finding it impossible to generate CTL against a number of HLA-A*0201 (HLA-A2)- restricted MUC1 epitopes described in the literature, we were successful in developing a stable CTL line against a peptide in the leader sequence of MUC1 (amino acids 12-20, LLLTVLTV), previously described by Brossart et al.³. This CD8+ CTL line (named CCM4) was characterized by strong specific cytotoxicity against T2 cells labeled with specific peptide and good sensitivity. CCM4 CTL could recognize 10-50 ng/ml of peptide. CCM4 was not only cytotoxic but also secreted a large amount of IFN- γ , which allowed us to use it in both ⁵¹Cr release and IFN- γ secretion assays. In response to specific stimulation almost 100% of CCM4 T cell synthesized IFN- γ , which demonstrated that the line consisted exclusively of MUC1 peptide-specific cells. This feature was important for scanning of the positional libraries, because non-specific cells within the T cell line could produce non-specific "noise" that would make it difficult to interpret the data. CCM4 demonstrated strong, MHC class I- restricted cytotoxicity against the HLA-A2+ breast cancer line MCF7. The same T cells failed to recognize HLA unmatched MSM-M3 melanoma cells; and cytotoxicity against MCF-7 could be blocked by pre-incubation with

anti HLA class 1 W6/32 antibodies. Hence, killing was specific and HLA class I restricted. More importantly, lysis of HLA-Class I matched breast cancer cells showed that the peptide we chose for the *in vitro* immunization and determination of mimics was relevant to anti-cancer immunity.

We generated 2×10^8 CTL and screened two 9-mer-peptide libraries, to identify which amino acids could be substituted in the original native peptide and still be recognized by CCM4. The results of the two scans are shown in Figures 1 and 2. With the CCM4 cell line we were able to screen successfully a combinatorial peptide library. This procedure was repeated 3 times, for some crucial positions 4 times. The index cell line recognized the native amino acids in their native positions, such as position 1-L, position 4-L, position 9-V etc. When substitutions were made randomly, there was still recognition of the resulting peptides, namely: position 1-V, position 2- I or P, position 3- P or N, position 4- F, T or V, position 5-W, position 6- P or F, position 7-S or R, position 8-Y or E, but at position 8 only V. A total of 96 non-native peptides were then synthesized, with as many as 8 substitutions, which are shown in Figure 3. We are now re-establishing our index cell line because none of the aliquots of 5×10^7 cells we froze in anticipation of receiving the libraries from Dr. Wilson in California, could be revived after thawing. Since the first index cell line made against the native leader peptide grew very rapidly, we are confident we will generate another to a sufficiently large number of cells within the next two months. This feature, a large number of cells, is very important, since a low number of T cells in specific lines is one of the major obstacles in the use of positional scanning libraries, the strength of whose signal depends upon the release of ^{51}Cr or IFN- γ .

After we regenerate the index CTL line, we will analyze the potency of those mimics on stimulating the cell line. Then we will use the mimics to immunize naïve HLA-A2+ lymphocytes to determine mimics with stronger agonist activity than the native peptide. It should be noted that some mimics in other systems, such as melanoma and HIV epitopes, have been less potent than the native epitope, but at least 10-20% are more stimulatory. We have in parallel analyzed mimics of the 9-mer tyrosine melanoma peptide YMNGTMSQV, and after similar library analyses, synthesis of candidate mimic peptide and testing on an index CTL line we identified several agonists that were more immunogenic than the original peptide. The strength of immunogenicity was expressed in either of two ways: some mimics stimulated CTL in lower concentration than the native peptide, while others caused much stronger response than the native peptide at similar concentrations. Such mimics are good candidates for clinical immunization against melanoma. By direct extrapolation, similar mimics with potential as immunogens should be identifiable for MUC1 leader peptide.

Key Research Accomplishments

- Development of an index CTL line against MUC1 leader sequence epitope LLLLTVLTV
- Several screens of combinatorial 9-mer peptide libraries with the CTL line
- Identification of amino acid substitutions that still permit recognition of the epitope by CTL, some with an apparently improved degree of sensitivity than the native peptide
- Purposeful synthesis of 97 nonamers with predetermined substitutions of amino acids at various positions, based upon results of screening

Reportable Outcomes

1. Mitchell, M.S., Compagno, D., Glazyrin, A., Kan-Mitchell, J., and Wilson, D. Detection of "superagonist" mimics of breast cancer mucin epitopes. Proc. Dept. of Defense Breast Cancer Res. Program Meeting, Era of Hope, Vol. III, P24-25, 2002.
2. Mitchell, M.S. Immunization against ubiquitous tumor-associated antigens. Proc. 4th Annual Walker's Cay Colloquium on Cancer Vaccines and Immunotherapy, Cancer Immunol. Immunother. 52, Supp., S6, 2003.

Conclusions

It is possible to develop cytotoxic T lymphocytes against MUC1, using a leader epitope from that complex glycoprotein, whereas nonamers from the main portion of the protein were ineffective in consistently generating such a cell line. The CTL index line could be used to screen combinatorial peptide libraries and identify amino acid

substitutions that permitted improved recognition of the substituted epitope compared with the native peptide. Proof of the value rests with 1) recognition of the substituted peptides by the CTL line, 2) subsequent restimulation of the CTL line by the substituted peptides and 3) improved de novo generation of a CTL line recognizing MUC1-bearing breast cancer cell line MCF-7 by immunization with the peptides.

References

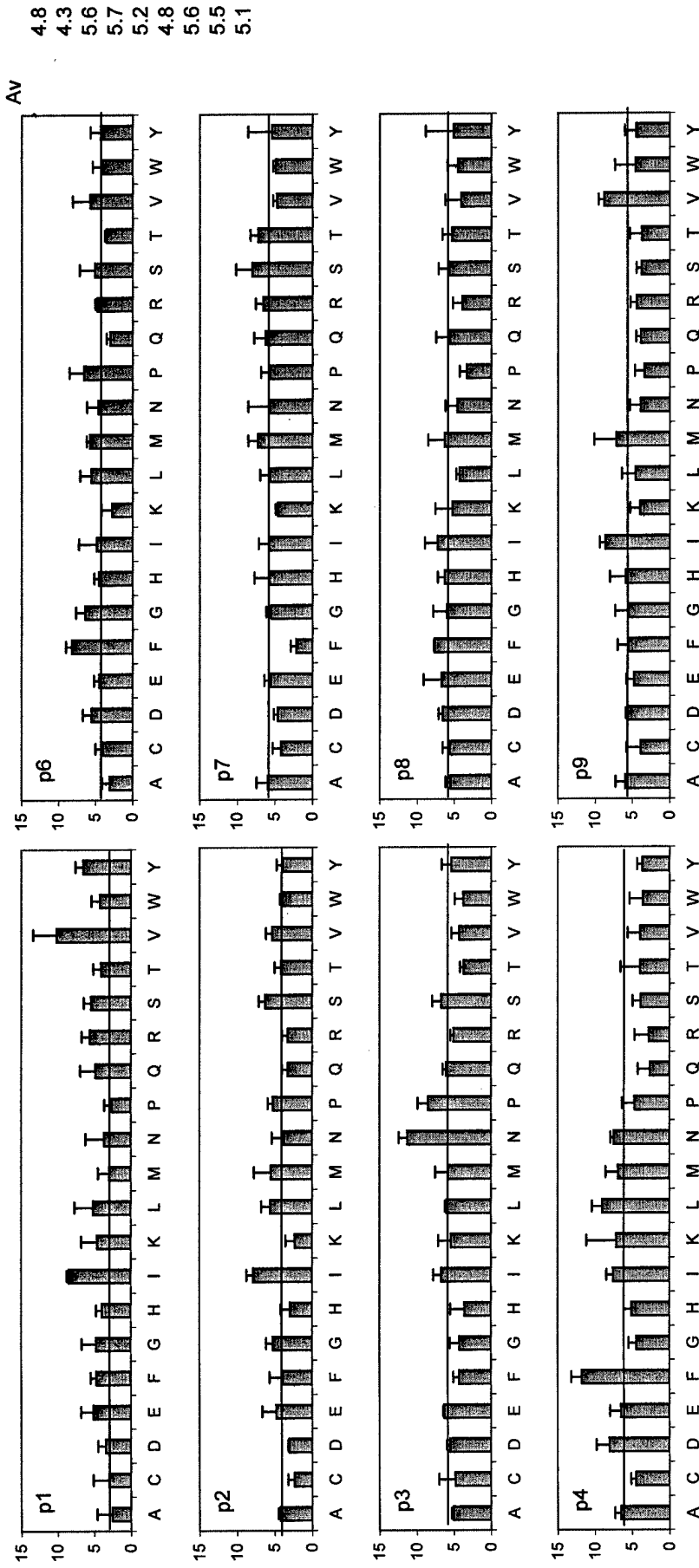
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2. Wilson DB, Pinilla C, Wilson DH, Schroder K, Boggiano C, Judkowski V, Kaye J, Hemmer B, Martin R, Houghten RA: Immunogenicity. I. Use of peptide libraries to identify epitopes that activate clonotypic CD4+ T cells and induce T cell responses to native peptide ligands. *J.Immunol* 163: 6424-6434, 1999
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Appendices

Figure 1. Nonamer library scan #1

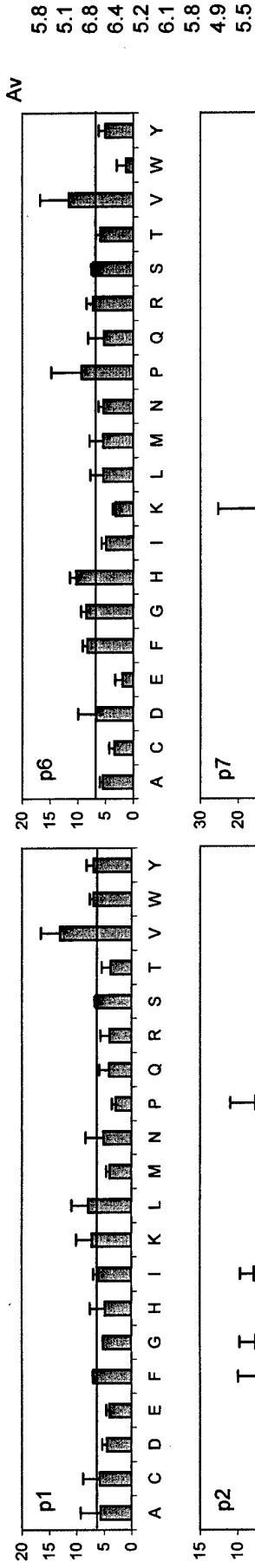
Figure 2. Nonamer library scan #2

Figure 3. Mimic peptides synthesized, with substituted positions indicated at top



4.8
4.3
5.6
5.7
5.2
4.8
5.6
5.5
5.1

020823 MUC 1 921lib(1), Figure 1 Graph



020820 MUC1 921lib(2), Figure 2 Graph

Figure 3

MUC-1 Mimic Peptides

V I P F W P S Y V
P N T F R E
V
1 2 2 3 1 2 2 2 1 96 peptides

	1	2	3	4	5	6	7	8	9		
1	H2V	I	P	F	W	P	S	Y	V	-COOH	
2		V	I	P	F	W	P	S	E	V	
3		V	I	P	F	W	P	R	Y	V	
4		V	I	P	F	W	P	R	E	V	
5		V	I	P	F	W	F	S	Y	V	
6		V	I	P	F	W	F	S	E	V	
7		V	I	P	F	W	F	R	Y	V	
8		V	I	P	F	W	F	R	E	V	
9		V	I	P	T	W	P	S	Y	V	
10		V	I	P	T	W	P	S	E	V	
11		V	I	P	T	W	P	R	Y	V	
12		V	I	P	T	W	P	R	E	V	
13		V	I	P	T	W	F	S	Y	V	
14		V	I	P	T	W	F	S	E	V	
15		V	I	P	T	W	F	R	Y	V	
16		V	I	P	T	W	F	R	E	V	
17		V	I	P	V	W	P	S	Y	V	
18		V	I	P	V	W	P	S	E	V	
19		V	I	P	V	W	P	R	Y	V	
20		V	I	P	V	W	P	R	E	V	
21		V	I	P	V	W	F	S	Y	V	
22		V	I	P	V	W	F	S	E	V	
23		V	I	P	V	W	F	R	Y	V	
24		V	I	P	V	W	F	R	E	V	
25		V	I	N	F	W	P	S	Y	V	
26		V	I	N	F	W	P	S	E	V	
27		V	I	N	F	W	P	R	Y	V	
28		V	I	N	F	W	P	R	E	V	
29		V	I	N	F	W	F	S	Y	V	
30		V	I	N	F	W	F	S	E	V	
31		V	I	N	F	W	F	R	Y	V	
32		V	I	N	F	W	F	R	E	V	
33		V	I	N	T	W	P	S	Y	V	
34		V	I	N	T	W	P	S	E	V	
35		V	I	N	T	W	P	R	Y	V	
36		V	I	N	T	W	P	R	E	V	
37		V	I	N	T	W	F	S	Y	V	
38		V	I	N	T	W	F	S	E	V	
39		V	I	N	T	W	F	R	Y	V	
40		V	I	N	T	W	F	R	E	V	
41		V	I	N	V	W	P	S	Y	V	
42		V	I	N	V	W	P	S	E	V	
43		V	I	N	V	W	P	R	Y	V	
44		V	I	N	V	W	P	R	E	V	
45		V	I	N	V	W	F	S	Y	V	
46		V	I	N	V	W	F	S	E	V	
47		V	I	N	V	W	F	R	Y	V	
48		V	I	N	V	W	F	R	E	V	
49		V	P	P	F	W	P	S	Y	V	
50		V	P	P	F	W	P	S	E	V	
51		V	P	P	F	W	P	R	Y	V	
52		V	P	P	F	W	P	R	E	V	
53		V	P	P	F	W	F	S	Y	V	
54		V	P	P	F	W	F	S	E	V	
55		V	P	P	F	W	F	R	Y	V	
56		V	P	P	F	W	F	R	E	V	
57		V	P	P	T	W	P	S	Y	V	
58		V	P	P	T	W	P	S	E	V	
59		V	P	P	T	W	P	R	Y	V	
60		V	P	P	T	W	P	R	E	V	
61		V	P	P	T	W	F	S	Y	V	
62		V	P	P	T	W	F	S	E	V	
63		V	P	P	T	W	F	R	Y	V	
64		V	P	P	T	W	F	R	E	V	
65		V	P	P	V	W	P	S	Y	V	
66		V	P	P	V	W	P	S	E	V	
67		V	P	P	V	W	P	R	Y	V	
68		V	P	P	V	W	P	R	E	V	
69		V	P	P	V	W	F	S	Y	V	
70		V	P	P	V	W	F	S	E	V	
71		V	P	P	V	W	F	R	Y	V	
72		V	P	P	V	W	F	R	E	V	
73		V	P	N	F	W	P	S	Y	V	
74		V	P	N	F	W	P	S	E	V	
75		V	P	N	F	W	P	R	Y	V	
76		V	P	N	F	W	P	R	E	V	
77		V	P	N	F	W	F	S	Y	V	
78		V	P	N	F	W	F	S	E	V	
79		V	P	N	F	W	F	R	Y	V	
80		V	P	N	F	W	F	R	E	V	
81		V	P	N	T	W	P	S	Y	V	
82		V	P	N	T	W	P	S	E	V	
83		V	P	N	T	W	P	R	Y	V	
84		V	P	N	T	W	P	R	E	V	
85		V	P	N	T	W	F	S	Y	V	
86		V	P	N	T	W	F	S	E	V	
87		V	P	N	T	W	F	R	Y	V	
88		V	P	N	T	W	F	R	E	V	
89		V	P	N	V	W	P	S	Y	V	
90		V	P	N	V	W	P	S	E	V	
91		V	P	N	V	W	P	R	Y	V	
92		V	P	N	V	W	P	R	E	V	
93		V	P	N	V	W	F	S	Y	V	
94		V	P	N	V	W	F	S	E	V	
95		V	P	N	V	W	F	R	Y	V	
96		V	P	N	V	W	F	R	E	V	
97		L	L	L	L	T	V	L	T	V	Native Seq