AD_________________

Award Number: W81XWH-05-1-0012

TITLE: Identification and Characterization of Ovarian Carcinoma Peptide Epitopes Recognized by Cylotoxic T Lymphocytes

PRINCIPAL INVESTIGATOR: Kevin T. Hogan, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904

REPORT DATE: November 2005

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*: 01-11-2005

2. REPORT TYPE: Annual

3. DATES COVERED *(From - To)*: 1 Nov 2004 – 31 Oct 2005

4. TITLE AND SUBTITLE
Identification and Characterization of Ovarian Carcinoma Peptide Epitopes Recognized by Cylotoxic T Lymphocytes

5a. CONTRACT NUMBER

5b. GRANT NUMBER: W81XWH-05-1-0012

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)
Kevin T. Hogan, Ph.D.

E-mail: kh6s@virginia.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Virginia
Charlottesville, VA 22904

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. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The *purpose* of the research is to identify new ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. The *scope* of this work involves (1) identifying the peptide antigens recognized by ovarian reactive cytotoxic T lymphocytes (CTL) by using an antigen-unbiased, mass spectrometric approach to antigen identification; and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian reactive CTL. Two TAG-derived peptides, SLGWLFLLL which is recognized in association with HLA-A2, and LSRLSNRLL which is recognized in association with HLA-B8, have tentatively been identified as novel antigens. These antigens should be useful in the treatment of ovarian cancer patients with TAG^{+} tumor cells. Ten established ovarian cancer cell lines and 29 ovarian cancer patient archival samples have been characterized for expression of class I MHC molecules, Her-2/neu, FBP, and TAG. These characterized materials will be used to generate additional ovarian cancer cell lines and ovarian cancer reactive CTL lines that will be used for the mass spectrometric identification of additional tumor antigens, and they will be used for the specific identification of Her-2/neu, FBP, and TAG derived antigens. The overall significance of this work is that the newly discovered antigens can be incorporated into a therapeutic vaccine for the treatment of ovarian cancer.

15. SUBJECT TERMS
Ovarian carcinoma, immunotherapy, cytotoxic T lymphocytes, epitope, peptide, major histocompatibility complex (MHC)-encoded molecules, class I MHC molecules, HLA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Cover 1

SF 298 2

Table of Contents 3

Introduction 4

Body 4

Key Research Accomplishments 16

Reportable Outcomes 16

Conclusions 16

References 16

Appendices 17

INTRODUCTION

The *subject* of this research is vaccine development for the treatment of ovarian cancer. The *purpose* of the research is to identify new ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. Specifically, we will identify the peptide antigens that associate with class I major histocompatibility (MHC) complex coded molecules, and which are capable of stimulating an ovarian cancer cell reactive cytotoxic T lymphocyte (CTL) response. The *scope* of this work involves (1) identifying the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification; and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian reactive CTL. Although beyond the scope of the present work, it is contemplated that the identified antigens will be tested in future ovarian cancer vaccine trials conducted at the University of Virginia.

BODY

1. Identify the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification (Months 1-36)

1.1. Establish a panel of ovarian cancer cell tumor lines. (Months 1-30)

Nine ovarian cancer cell lines (CAOV-3, CAOV-4, ES-2, OV-90, OVCAR3, SK-OV-3, SW626, TOV-21G, and TOV-112D) were obtained from the ATCC and one line (TTB-6) was obtained from our own repository. Characterization of these cell lines has been ongoing and has included class I MHC molecular typing, class I MHC protein expression analysis by flow cytometry, and PCR analysis of TAG, FBP, and Her-2/neu mRNA expression. Our ability to fully characterize some of these lines has been hampered by the ability to expand them to sufficient numbers *in vitro*.

The class I MHC genotypic and phenotypic analyses of the ovarian cancer cell lines are shown in Table 1. Seven of the lines have been fully characterized for class I MHC expression at both the genomic DNA and protein levels and five of the lines express class I MHC molecules of interest to our studies. Both CAOV-4 and SW626 still require both molecular typing and phenotypic characterization, although SW626 is thought to express HLA-A3 and -B7 (personal communication, Venky Ramakrishna, Argonex, Charlottesville, VA). TTB-6 has the genes for HLA-A2 and B44, but expression of these molecules at the cell surface has not yet been confirmed.

TAG, FBP, and Her-2/neu expression for these same lines is shown in Table 2. TAG mRNA is readily detectable in OVCAR-3 and TTB-6, with lesser amounts detected in two other ovarian cancer cell lines. Seven of the eight ovarian cancer cell lines express high amounts of FBP and Her-2/neu mRNA, and all cell lines express at least low amounts of each of the genes.

Because not every antigen of interest is also concurrently expressed with every class I MHC molecule of interest (for example, no line expresses HLA-A1), we have also obtained cDNA clones for HLA-A1, -A2, -A3, -B7, -B8, and -B44. Each of these genes has now been subcloned into pcDNA3.1 which also contains the neomycin resistance gene, a selectable marker which can be used in conjunction with G418 to select cells that have taken up the plasmid following transfection. The G418 sensitivity of each ovarian cell line (except CAOV-4 and SW626 which are difficult to grow) has also been established. To expand the utility of this panel of cell lines, we are going to begin to individually transfect each of the class I MHC genes into OVCAR3 so that we will have a complete panel of cell lines co-expressing both the relevant class I MHC molecules and the tumor antigens of interest.

In addition to the previously established ovarian cancer cell lines described above, we have also characterized archived ovarian cancer material which can be used for the establishment of

additional cell lines. As shown in Table 3, we have now characterized material from 29 different patients. This characterization included molecular class I MHC typing, and PCR typing for FBP, Her-2/neu, and TAG gene expression. Material from four patients was insufficient for characterization, generally due to poor cell recovery following thawing. Of the remaining 25 samples, two do not express any class I MHC molecule of interest and two were not completely characterized because initial results showed that FBP and/or Her-2/neu was expressed poorly, if at all. To maximize the usefulness of each sample, we will only attempt to generate tumor cell lines from any given sample in conjunction with thawing the sample for CTL generation (1.2 below).

1.2. Establish class I MHC-restricted, ovarian cancer cell reactive CTL lines. (Months 1-30)

As part of the effort to establish ovarian cancer cell reactive CTL lines from patient material we have first elected to characterize our archived patient material for class I MHC and antigen expression as described in 1.1 above. This initial characterization will now allow us to pursue the establishment of CTL in a more expeditious fashion than would otherwise be attainable. Based on the information in Table 3, it is now possible to perform CTL stimulations in cohorts of 4-6 samples each, with each cohort chosen to represent a particular class I MHC molecule (HLA-A1, -A2, etc.). By performing the experiments in this fashion, there is a greatly reduced need for maintaining extensive panels of tumor cells in culture at any one time as a more restricted panel of tumor cells can then be used for both stimulations and as targets in specificity assays. Likewise, it also becomes easier to focus on those CTL that recognize shared antigens rather than unique antigens. Although the stimulations could have been initiated and then cryopreserved until such time that we were ready to analyze them, this would have generated additional work that would have been counterproductive in the longer term. This work will be a major focus of our efforts going forward.

1.3. Identify the peptide antigens recognized by the CTL established in 1.2. Each identification project will last an average of 4-6 months, and a given antigen identification project is expected to yield from one to several new peptide antigens. (Months 6-36)

No work has been initiated under this heading as we have not yet established the CTL in 1.2. It is anticipated that this will be a major effort in the next 24 months.

2.0 Identify peptide antigens within the Her-2/neu, folate binding protein, and TAG proteins that give rise to ovarian reactive CTL (Months 1-36)

2.1. Predict the Her-2/neu, folate binding protein, and TAG-derived peptides that conform to the HLA-A1, -A2, -A3, -B7, and -B8 binding motifs. Have synthesized the HLA-A1 and –A3 peptides that conform to the rules in specific aim #2. The remaining peptides will be synthesized as needed for step 2.4 below. (Months 1-2)

Each of the target proteins was analyzed with the SYFPEITHI and Parker algorithms, and peptides corresponding to the HLA-A1, -A2, -A3, -B7, and -B8 binding motifs were predicted and ranked. This was followed by a visual inspection to remove any predicted peptide that had a poor score, and to remove any that would be difficult candidates to synthesize. The lists were then merged together to give two to six peptides per antigen per class I MHC molecule (Table 4). Each of the listed peptides were then synthesized and are now available for analysis. By having all of the peptides synthesized simultaneously, we were able to take advantage of a multiple-peptide synthesis service that reduced the overall cost of the synthesis.

2.2. PCR isolate the cDNA for Her-2/neu and folate binding proteins, and clone the cDNA for Her-2/neu, folate binding protein, and TAG, into the plasmid pcDNA3.1. Plasmids will be transfected as needed. (Months 1-3)

Based on the fact that many of our established ovarian cancer cell lines already express these genes, we have forgone this objective in favor of obtaining class I MHC genes which can be used to transfect existing cell lines. As noted in objective 1.1 above, we have subcloned the cDNA for HLA-A1, -A2, -A3, -B7, -B8, and -B44 into pcDNA3.1 and prepared plasmid stocks of each construct. These plasmids will be transfected into appropriate antigen expressing cell lines as needed to generate cell lines expressing both the antigen and class I MHC molecule of interest. As indicated in 1.1 above, the G418 sensitivity has been established for most of the ovarian cancer cell lines in our panel, thus allowing these transfections to proceed as required. Based on our characterization of the existing ovarian cancer cell lines under 1.1 above, we believe that this is the most judicious use of resources at this time.

2.3. Stimulate TIL/TAL samples from HLA-A1$^+$ and HLA-A3$^+$ ovarian patients with ovarian peptides predicted to bind to the respective class I MHC molecules. Test the specificity of the ensuing cultures for reactivity with peptide-pulsed target cells and with ovarian cancer cells expressing the appropriate class I MHC molecule and cognate protein. Confirm peptide identity with SRM mass spectrometry. (Months 4-12)

The original plan for this aim was to use ovarian cancer patient-derived lymphocytes as the responder cells and peptide-pulsed B-LCL as the stimulator cells. We also proposed an alternative methodology in which the responding lymphocytes from healthy donors were stimulated with peptide-pulsed autologous dendritic cells (DC). For two reasons, we chose to begin these experiments using the alternative methodology. First, to perform these experiments most expeditiously, it was first necessary to characterize the available patient material for class I MHC, TAG, FBP, and Her-2/neu expression as has now been done under 1.1 above. Second, we had already established methodology in the laboratory to allow for the use of healthy donor lymphocytes for antigen identification. This procedure is based on that of Lu and Celis (1) and is illustrated in Figure 1. Once the lymphocytes have been stimulated four times with peptide, they are assayed for specificity as illustrated in Figure 2.

PBMC from healthy donors were obtained through Virginia Blood Services. Initial processing was done to provide: (1) genomic DNA for class I MHC molecular typing; (2) dendritic cells to which peptide was bound, and were then used as stimulator cells; and (3) lymphocytes which were used as the source of CD8$^+$ responder T cells. The cells were cryopreserved until an average of six donors could be simultaneously tested as described above for their response to a defined peptide/class I MHC molecule combination. Three peptides with an HLA-A1 binding motif (SRDPPASAS, ESERGLPAS, NLEPLVSRD) and one peptide with an HLA-A3 binding motif (LLLLNSTTK) were tested, but none of the four peptides proved to be immunogenic (Table 5). Testing of the HLA-3 binding motif peptide, GLPASTLSR, is ongoing.

Testing of the FBP and Her-2/neu peptides predicted to bind to HLA-A1 and HLA-A3 will begin in the second year and will be accomplished using TIL/TAL from ovarian cancer patients. As this material has now been characterized for both class I MHC and tumor antigen expression we will perform these experiments in groups of responders that have one or the other class I MHC molecule in common which greatly simplifies the restimulation and specificity testing phase of the experimental protocol. Perhaps more importantly, we will be able to selectively use lymphocytes from individuals whose ovarian tumors are known to express the antigen of interest. This is advantageous as these individuals may have already developed an antigen-specific CTL response *in vivo*, which means that we are not trying to elicit a primary response *in vitro*, and thus the likelihood of a successful stimulation is increased.

2.4. Repeat 2.3 for peptides associated with HLA-A2, B7, and B8. The order in which this is done will be dictated by order in which patient material becomes sufficiently available to conduct the experiments. (Months 13-36)

As with 2.3 above, we have initiated this work with the alternative protocol using PBMC obtained from healthy donors. To date, one peptide with an HLA-A2 binding motif (SLGWLFLLL), one peptide with an HLA-B7 binding motif (LPASTLSRL), and three peptides with HLA-B8 binding motifs (LSRLSNRLL, VQRRAEGLL, TVQRRAEGL) have been tested with two peptides (SLGWLFLLL and LSRLSNRLL) demonstrating evidence of immunoreactivity (Table 5). Testing of three HLA-A2 binding peptides (LLLRLECNV, FLLLLNSTT, TLSRLSNRL), and four HLA-B7 binding peptides (LPAQEGAPT, DPPASASLF, LSRLSNRLL, VQRRAEGLL) will be initiated shortly.

As shown in Figure 3, HLA-A2^{+} cell lines (T2 and C1R-A2) pulsed with SLGWLFLLL peptide are recognized by CTL lines generated in response to SLGWLFLLL stimulation. These same cell lines recognize a tumor target expressing both HLA-A2 and TAG, but not tumor targets that are HLA-A2^{+}/TAG^{-} or HLA-A2^{-}/TAG^{+}. Cold target inhibition studies were performed to demonstrate that antigen recognized on the tumor cells was the same as that recognized on the peptide-pulsed cells (Figure 4). When used as a cold target, DM6 blocked recognition of T2 plus the SLGWLFLLL peptide, thus demonstrating that the same CTL were recognizing each target. Unexpectedly, the reciprocal experiment did not result in inhibition and the reason for this discrepancy is under active investigation.

Reactivity was also demonstrated against the LSRLSNRLL peptide in association with HLA-B8 (Figure 5, 6). These same CTL also recognize tumors naturally expressing both HLA-B8 and TAG including DM14 and VMM15. In recent experiments, reactivity to peptide pulsed cells has been lost while reactivity to the tumor targets has been maintained. The specificity of these cells also remains under active investigation.

Testing of the FBP and Her-2/neu peptides predicted to bind to HLA-A2, HLA-B7, and HLA-B8 will begin in the second year and will be accomplished as described in 2.3 above.

2.5 Determine the ability of CTL generated in 1.2 above to recognize target cells transfected or infected with the gene of interest and identify the peptide antigen. Confirm with SRM mass spectrometry. (Months 6-36)

This work will be initiated once we have begun establishing the relevant CTL.

Table 1. Class I MHC Genotypic and Phenotypic Characterization of Ovarian Cancer Cell Lines					
Line	Class I MHC Molecular Typing[a]			Class I MHC Protein Expression[b]	
	A Locus	B Locus	C Locus	W6/32	Specific Class I MHC Antibody
CAOV-3	A*6901	B*4704, 49	CW*07	Medium	A69-Medium
CA0V-4	ND[c]	ND	ND	ND	A2
ES-2	A*03, 68	B*14 (65), 41	CW*07, 08	Medium	A3-medium, A68-low
OV-90	A*02	B*50, 58	CW*06, 07	Low	A2-low
OVCAR3	A*02, 29	B*07, 5805	CW*07	Medium	A2-low, B7-medium
SK-OV-3	A*03, 68	B*18, 35	CW*4, 05	Medium	A3-low, A68-low
SW626[d]	3	7	ND	Medium	ND
TOV-21G	A*11, 26	B*15(62), 40 (61)	CW*02, 04	Medium	No appropriate antibody
TOV-112D	A*03	B*1401 (64)	CW*03 or 07 or 08, 8	Medium	A3-medium
TTB-6	A*02, 68	B*4037, 44	CW*02, 07	ND	ND

[a]Genomic DNA was typed using medium resolution PCR typing kits from One Lambda.
[b]Flow cytometry was used to determine the extent to which the class I MHC molecules are expressed on the surface of the cell lines. mAb W6/32 which binds to all class I MHC molecules was used to assess overall expression levels, while allotype specific antibodies (CR11-351 recognizes HLA-A2, A68, and A69; BB7.2 recognizes HLA-A2 and A69, GAP-A3 recognizes HLA-A3, and ME1-1.2 recognizes HLA-B7) were used to assess the expression levels of selected class I MHC molecules. Expression levels were classified as low, medium or high in comparison to expression of the same molecules on reference B-LCLs.
[c]Not determined.
[d]Typing indicated is a personal communication that has not yet been confirmed.

Table 2. Tumor Antigen Expression of Ovarian Cancer Cell Lines						
Line	Expression by PCR Analysis[a]					
	TAG-1	TAG-2a	TAG-2b	TAG-2c	FBP	Her-2/neu
CAOV-3	*	*	-	-	+	+
CA0V-4	ND	ND	ND	ND	ND	ND
ES-2	-	-	-	-	*	+
OV-90	*	*	-	*	+	+
OVCAR3	+	+	+	+	+	+
SK-OV-3	-	-	-	-	+	+
SW626	ND	ND	ND	ND	ND	ND
TOV-21G	-	-	-	-	+	+
TOV-112D	-	-	-	-	+	+
TTB-6	+	+	+	+	+	*

[a](+)=positive following 30 cycles of PCR; (*)=positive following 40 cycles of PCR; (-)=negative following 40 cycles of PCR. ND=Not determined.

Table 3. Characterization of Archived Ovarian Cancer Patient Ascites Containing Tumor Cells and Lymphocytes[a]

Line	A Locus	B Locus	FBP	Her2/Neu	TAG-1	TAG-2a	TAG-2b	TAG-2c
TPF 5	02, 29	08, 44	-	+/- (w)	*NT*	*NT*	*NT*	*NT*
TPF 6	02, 68	4037, 44	+	+/- (w)	+	+	+/- (w)	+
TPF 29	01	44, 57	+	+	-	-	-	-
TPF 65	03, 23	07, 56 or 82	-	- (30 cycles)	*NT*	*NT*	*NT*	*NT*
TPF 94	01, 11	08, 40	+	+/-	-	-	-	-
TPF 117	02, 68	14, 4901	+	+	+/-	-	-	-
TPF 121	*NT*	*NT*	-	+/-	+/- (w)	-	-	-
TPF 125	02, 24	37, 35 or 7804	+	+	-	-	-	-
TPF 136	03, 29	39, 44	+	++	-	-	-	-
TPF 144	11, 34	44, 51	+	+/-	+/- (w)	+/- (w)	-	-
TPF 185	01, 68	08,44	+	+/-	-	-	-	-
TPF 189	03, 31	37, 44	+	++	+/- (w)	-	-	-
TPF 212	01, 24	15, 40	+	+	-	-	-	-
TPF 227	11, 3406	51, 57	+	++	-	-	-	-
TPF 258	01, 28	18, 41	+	++	-	-	-	-
TPF 501	01	08	+	+/-	IS	IS	IS	IS
TPF 519	01, 02	07, 44	+	++	+	+	+/-	+
TPF 546	03, 29	07, 44	+	++	-	-	-	-
TPF 567	02, 68	44, 47	-	++	-	-	-	-
TPF 568	02, 68	44, 47	-	++	-	-	-	-
TPF 572	02, 24	13, 41	+	-	-	-	-	-
TPF 632	*NT*	*NT*	-	-	+/-	+/- (w)	-	-
TPF 834	32	27, 47	+	-	-	-	-	-
TPF 1006	03, 23	14, 35	+	+/-	+/- (w)	-	-	-
TPF 1130	IS	IS	IS	IS	-	+/- (w)	-	-
TPF 1287	02, 30	13, 27	IS	IS	IS	IS	IS	IS
TPF 1288	03, 68	07, 44	+	+	-	-	-	-
TPF 2097	01, 26	08, 38	IS	IS	IS	IS	IS	IS
TPF 3883	25	15, 18	+	+	-	-	-	-

[a]Class I MHC typing was done using medium resolution PCR typing kits from One Lambda. FBP, Her-2/neu, and TAG gene expression was determined by PCR.
[b]NT = Not Tested.
[c]IS = Insufficient Sample.

Table 4. Peptides Synthesized for Testing[a]						
	HLA-A1	HLA-A2	HLA-A3	HLA-B7	HLA-B8	HLA-B44
Binding Motifs[b]	--E-----Y D	-M------V L L I	-M------Y L K	-PR-----L I	--K-K---- R R	-E------F Y
TAG-1 Peptides	SRDPPASAS ESERGLPAS NLEPLVSRD	LLLRLECNV SLGWLFLLL FLLLLNSTT TLSRLSNRL	LLLLNSTTK GLPASTLSR	LPASTLSRL LPAQEGAPT DPPASASLF LSRLSNRLL VQRRAEGLL	TVQRRAEGL LSRLSNRLL VQRRAEGLL	ASLGWLFLL AESERGLPA SERGLPAST
FBP Peptides	SNEIWTHSY PNEEVARFY	QLLLLLVWV LLLLVWVAV LLLVWVAVV FLLSLALML LLSLALMLL SLALMLLWL	LLNVSMNAK RVLNVPLSK YLYRFNWNH AVVGEAQTR	GPWAAWPFL AQRMTTQLL WPFLLSLAL	IAWARTELL WRKERVLNV APASKRHFI QSWRKERVL	
Her-2/neu Peptides	HLDMLRHLY LLDIDETEY VSEFSRMAR LEEITGYLY		ILWKDIFHK ILIKRRQQK ILKETELRK QLVTQLMPY	AARPAGATL HVRENRGRL DVRLVHRDL LPASPETHL	ESRPRFREL LIKRRQQKI DLLEKGERL SPKANKEIL	

[a]Each protein sequence was analyzed with the SYFPEITHI and Parker algorithms. The highest ranked peptides were then selected and synthesized.
[b]Anchor binding motifs according to the Parker algorithm.

Table 5. TAG-Derived Peptides Tested for CTL Stimulatory Activity Using PBMC and DC from Healthy Individuals

Class I MHC	Peptide	# Pos/#Tested
HLA-A1	SRDPPASAS	0/6[a]
	ESERGLPAS	0/6
	NLEPLVSRD	0/6
HLA-A2	SLGWLFLLL	4/6
	LLLRLECNV	6 PP[b]
	FLLLLNSTT	6 PP
	TLSRLSNRL	6 PP
HLA-A3	LLLLNSTTK	0/6
	GLPASTLSR	6 IP[c]
HLA-B7	LPASTLSRL	0/6
	LPAQEGAPT	3 PP
	DPPASASLF	3 PP
	LSRLSNRLL	3 PP
	VQRRAEGLL	3 PP
HLA-B8	LSRLSNRLL	1/6
	VQRRAEGLL	0/6
	TVQRRAEGL	0/6
HLA-B44	ASLGWLFLL	6 PP

[a]# of cultures from which a positive response was obtained/#of cultures tested.
[b]PP=Peptde-pulsed DC cultures that have been cryopreserved and are awaiting testing.
[c]IP=cultures for which testing is currently In Progress.

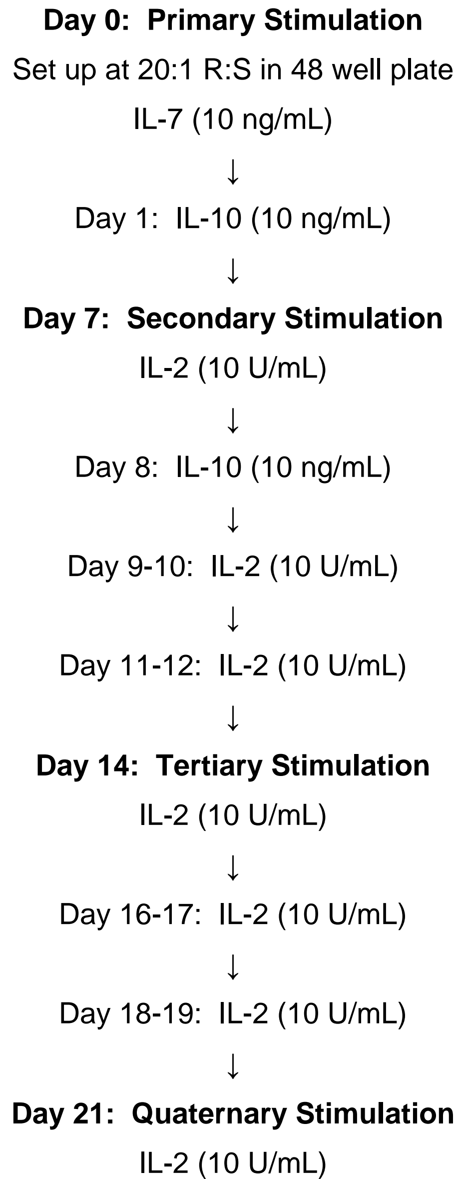

Figure 1. Stimulations were conducted in RPMI 1640 + 10% human serum + 1% penicillin-streptomycin. Subsequent to the primary stimulation, stimulators were used based on availability: mDC (25,000/well), autologous PBMC ($1x10^6$/well), or HLA type relevant B-LCL (250,000-500,000/well).

^{51}Chromium-release assay post 4º stimulation

Each well is tested in duplicate with 2000 target cells per well at 40:1 and 10:1 E:T. Target cells are labeled for 2 hours, washed and peptide pulsed for 1 hour, and co-cultured with effector cells for 4 hours, after which supernatant is harvested. CPM measured via gamma counter.

Positive well selection

A well is generally considered positive when percent specific release is 20% points over background.

5º stimulation

^{51}Chromium-release assay

An assay using a two-fold geometric series dilution in triplicate is conducted as described above.

T cell expansion with anti-CD3 mAb

A T25 flask is seeded with 100,000 cells from positive well + 25 x 10^6 PBMC in RPMI 1640 + 10% FBS + 2 mM L-glutamine + 1% penicillin-streptomycin + 10 ng/mL anti-CD3 mAb + 25 U/mL IL-2.

Further investigation of peptide specificity

Peptide titration, tumor assay, cold target inhibition assay, etc.

Figure 2. Evaluation of peptide specificity procedures.

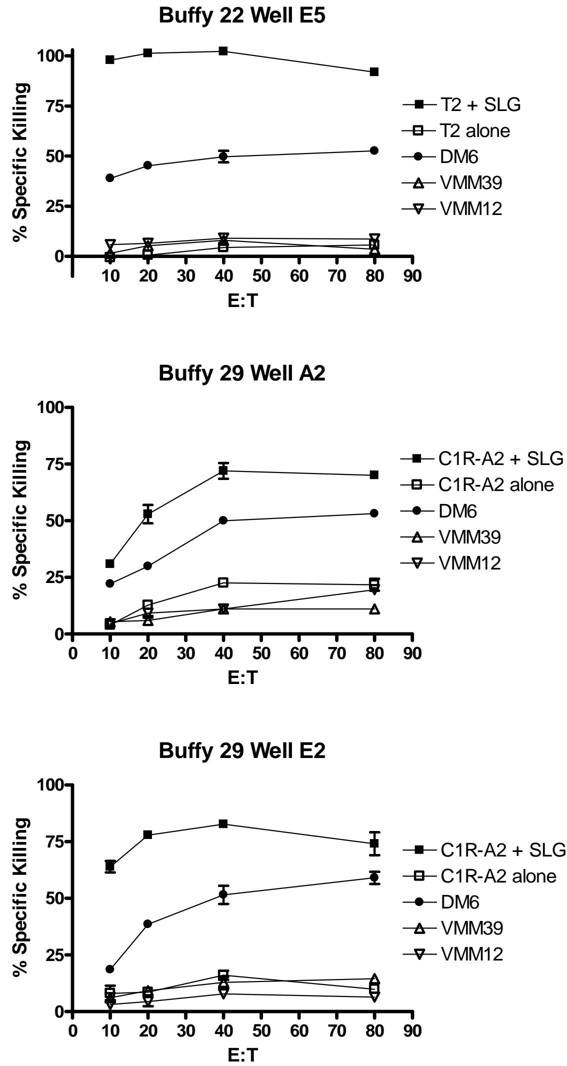

Figure 3. SLGWLFLLL reactive CTL were tested for reactivity against a panel of target cells. As a peptide specificity control either the HLA-A2^{+}/TAG^{-} cell lines T2 or C1R-A2 were pulsed with 10 µg/ml SLGWLFLLL peptide. Melanoma lines DM6 (HLA-A2^{+}/TAG^{+}), VMM39 (A2^{+}/TAG^{-}), and VMM12 (A2^{-}/TAG^{+}) were also tested.

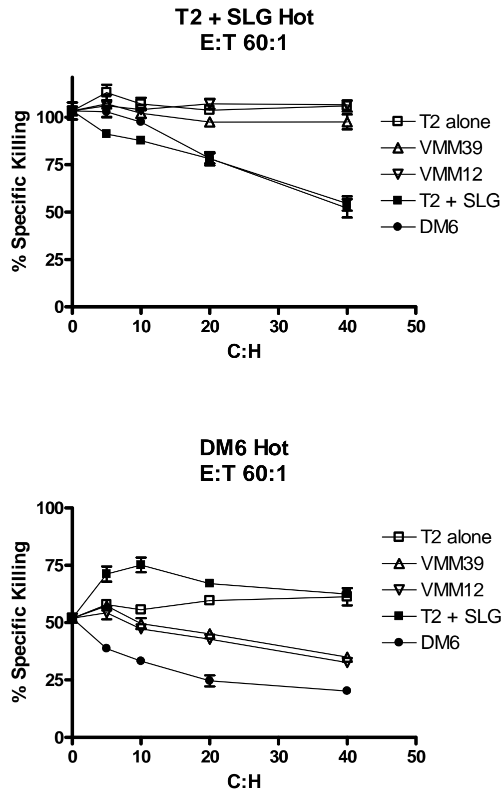

Figure 4. Cold target inhibition experiment. Targets are as described in Figure 3. In the upper panel, T2 was pre-pulsed with SLGWLFLLL peptide, labeled with ^{51}Cr, and used as the "hot" target. In the lower panel, DM6 was used as the "hot" target. Cell lines listed to the right of each panel were unlabeled and used as the cold targets at the indicated cold:hot (C:H) ratios.

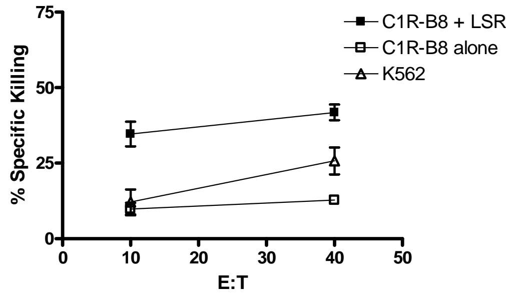

Figure 5. LSRLSNRLL reactive CTL were tested for reactivity against C1R-B8 either pulsed or unpulsed with peptide. K562 was included as a target to measure natural killer cell activity.

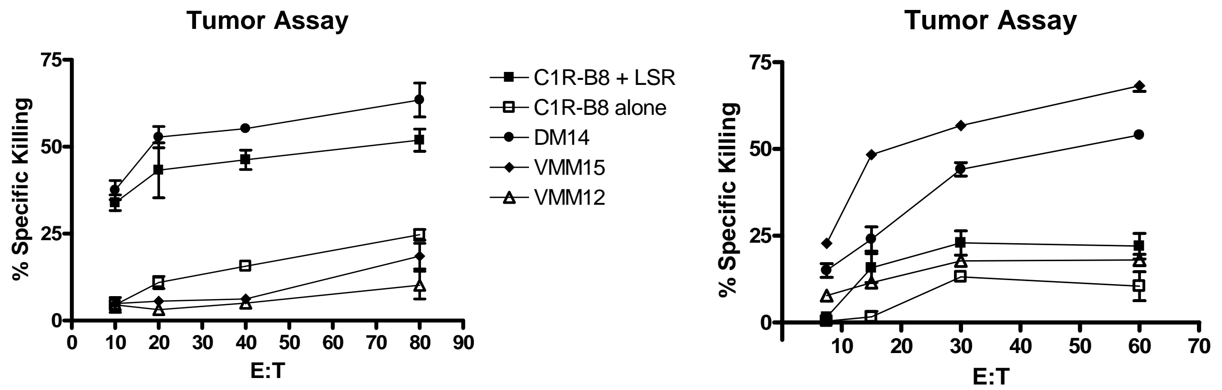

Figure 6. LSRLSNRLL plus HLA-B8 reactive CTL recognition of tumor targets. The CTL were tested against C1R-B8 (HLA-B8^{+}/TAG^{-}) either pulsed or unpulsed with peptide, DM14 (HLA-B8^{+}/TAG^{+}), VMM15 (HLA-B8^{+}/TAG^{+}), and VMM12 (HLAB8^{-}/TAG^{+}).

KEY RESEARCH ACCOMPLISHMENTS

- Two TAG-derived peptides, SLGWLFLLL which is recognized in association with HLA-A2, and LSRLSNRLL which is recognized in association with HLA-B8, have tentatively been identified as novel antigens – these antigens should be useful in the treatment of ovarian cancer patients with TAG^+ tumor cells
- A nearly complete characterization of ten established ovarian cancer cell lines with respect to the expression of the class I MHC molecules and tumor antigens relevant to this study – these cell lines will be critical for use in the stimulation of ovarian cancer cell-specific CTL, the specificity analysis of generated CTL, and the purification and identification of unknown antigens
- Complete characterization of the class I MHC and tumor antigen expression of 29 ovarian cancer patient archival samples – these samples will be used for establishing additional ovarian cancer cell lines and for establishing ovarian cancer cell-specific CTL
- Established a streamlined and efficient methodology for assessing the immunogenicity of peptides predicted to be presented in association with defined class I MHC molecules

REPORTABLE OUTCOMES

Abstracts-

- Fink, M.J., and Hogan, K.T. (2005) TAG, A Cancer/Testis Antigen, Is Widely Expressed in Human Cancers of Diverse Histological Type. *J. Immunother.* 28:639.
- Adair, S.J., Carr, T.M., and Hogan, K.T. (2005) Identification of Cytotoxic T Lymphocyte Epitopes Derived from the Cancer/Testis Antigen, TAG. *J. Immunother.* 28:639.

CONCLUSIONS

The results obtained to date are important because: (i) two novel peptide antigens have been tentatively identified; (ii) existing ovarian cancer cell lines and patient material has been characterized in such a fashion that future experiments can be planned and executed in a streamlined manner; and (iii) they establish the ability of our laboratory to implement the necessary protocols to conduct the planned research.

The identification of additional antigens that can be used in a therapeutic vaccine for the treatment of ovarian cancer is important because there are relatively few antigens that are currently available for such a vaccine. One consequence of this limitation is that if a patient's tumor does not express at least one of the antigens in the vaccine, the vaccine cannot induce a therapeutic effect even if it can stimulate a CTL response. Practically speaking, this means that many patients will be excluded from vaccine trials that measure clinical endpoints as there is no likelihood of a clinical benefit. The two peptide antigens tentatively identified here, and those that will be identified in the future will expand the percentage of women likely to benefit from therapeutic vaccination. Additional peptide antigens in a vaccine also provide two additional theoretical advantages. First, the more antigens towards which the response is directed, the less likely it will be that antigen loss on the tumor will lead to escape from the elicited CTL response. Second, responses against multiple antigens will increase the magnitude of the response and increase the likelihood that the ensuing CTL response will be clinically effective.

REFERENCES

1. Lu J, Celis E. 2002. Recognition of prostate tumor cells by cytotoxic T lymphocytes specific for prostate-specific membrane antigen. *Cancer Research* 62: 5807-12

APPENDICES

TAG, A Cancer/Testis Antigen, Is Widely Expressed in Human Cancers of Diverse Histological Type

Mitsu J. Fink, Kevin T. Hogan. *Department of Surgery, University of Virginia, Charlottesville, VA.*

Although immunization of melanoma patients with tumor antigens is beginning to show promising results, it is likely that truly effective therapy will require vaccination with multiple peptides derived from different proteins and with peptides that target multiple class I MHC molecules. Cancer/testis antigens, which are expressed in a variety of tumors, but not in normal tissue except the testis and placenta, are particularly promising tumor vaccine candidates. Using melanoma-reactive cytotoxic T lymphocytes, we have recently identified a new cancer/testis antigen termed TAG which gives rise to an HLA-A3-restricted epitope (RLSNRLLLR). TAG was cloned and identified using 5′ and 3′ RACE, and was localized to chromosome 5. The gene coding for TAG has multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) and is expressed in 84–98% of the melanoma lines tested. The TAG protein is coded for in an open reading frame that is initiated by one of three nonstandard initiation codons, and the nucleotide sequence coding for the RLSNRLLLR peptide crosses an exon-exon boundary. The TAG gene has homology to two chronic myelogenous leukemia-derived clones and a hepatocellular carcinoma clone in the human expressed sequence tags (EST) database. TAG is not expressed in B- or hybrid T-B-lymphoblastoid cell lines, although all four isoforms are expressed in K562, a myelogenous leukemia cell line. One or more of the TAG genes has also been shown to be expressed by PCR in four of nine (44%) lung cancer cell lines, one of eight (13%) breast cancer cell lines, one of three (33%) ovarian cancer lines, seven of seventeen (41%) ovarian cancer clinical isolates, two of five (40%) colon cancer cell lines, zero of four brain cancer cell lines (0%), and three of nine (33%) gastric cancer cell lines. To characterize TAG expression at the protein level, the TAG-1 and TAG-2a genes have been cloned into the pET100/D vector for expression as His-tagged fusion proteins. Experiments are presently in progress to determine optimal conditions for the expression and purification of the fusion proteins. The purified proteins will then be used for the production of TAG-specific antisera, and the antisera will be used to assess TAG protein expression in tumor lines expressing the TAG genes. The characterization of TAG expression at both the mRNA and protein levels in different tumors will allow us to determine the extent to which TAG will serve as a useful antigenic target. (Supported by NIH/NCI CA90815)

Identification of Cytotoxic T Lymphocyte Epitopes Derived From the Cancer/Testis Antigen, TAG

Sara J. Adair, Tiffany M. Carr, *Kevin T. Hogan. Department of Surgery, University of Virginia, Charlottesville, VA.*

Cancer/testis antigens are excellent candidates for inclusion in cancer vaccines as they are naturally immunogenic, are expressed in a high percentage of tumors of diverse histological origin, and their expression in normal tissue is limited to the testis and placenta. We have recently identified a new cancer/testis antigen termed TAG which is expressed as multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) at the mRNA level. TAG is known to be immunogenic as it gives rise to an HLA-A3-restricted epitope (RLSNRLLLR) that is recognized by cytotoxic T lymphocytes (CTL) that naturally occur in a melanoma patient. To determine if TAG gives rise to additional epitopes recognized by CTL we have used two predictive algorithms (SYFPEITHI [www.syfpeithi.de] and Parker [bimas.cit.nih.gov/molbio/hla_bind] to identify those peptides that have a high predicted binding affinity for HLA-A1, -A2, -A3, -B7, -B8, and -B44. The top three to five predicted binders for each algorithm/HLA combination have been synthesized and are being tested for their ability to elicit tumor reactive CTL from healthy blood donors. Donor lymphocytes are initially stimulated with peptide-pulsed, autologous, mature dendritic cells (mDC), and are then restimulated thereafter on a weekly basis with peptide-pulsed stimulator cells (autologous mDC, autologous peripheral blood mononuclear cells, or class I MHC-matched allogeneic B-LCL). Following a total of four stimulations, the cultures are tested for reactivity against peptide-pulsed, class I MHC-matched target cells. Using this protocol, CTL responses have developed in response to SLGWLFLLL peptide in association with HLA-A2 and LSRLSNRLL peptide in association with HLA-B8, but not to SRDPPASAS peptide in association with HLA-A1, LLLLNSTTK peptide in association with HLA-A3, or LPASTLSRL peptide in association with HLA-B7. SLGWLFLLL reactive CTL also lyse the melanoma line DM6 (HLA-A2$^+$, TAG$^+$) indicating that the SLGWLFLLL peptide is naturally processed. Experiments are in progress to further characterize the CTL response to SLGWLFLLL and LSRLSNRLL, and to determine the immunogenicity of additional TAG-derived peptides. (Supported by DOD W81XWH-05-1-0012 and NIH/NCI CA90815)

© 2005 Lippincott Williams & Wilkins