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14. ABSTRACT

In the proposed studies, novel T cell immunotherapies against breast cancer will be developed based on studies demonstrating a positive correlation between T lymphocytic infiltration of these tumors and favorable clinical outcome. The major goal of the proposed studies is to isolate and characterize cytolytic T lymphocytes (CTL) with *in vivo*-like T cell receptors. The CTL provide the basis for adoptive CTL immunotherapy and active immunotherapy with CTLderived peptides/antigens. During the past 12 months of the study 14 breast carcinoma tissues were cultured in organotypic cultures (reconstructs) and mixed lymphocyte/tumor cultures (MLTC). T cell lines were established from 11 breast cancer specimens. One long-term breast cancer cell line and 7 fibroblast cell lines also were established. Cytolytic activity was demonstrated in 9 of 10 T cell lines tested. Two of 9 tested T cell lines were shown to have helper function.

15. SUBJECT TERMS
Cytolytic T lymphocytes, organotypic culture, immunology, immunotherapy

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Annual Report

INTRODUCTION

The presence of a T cell infiltrate has been associated with a favorable prognosis in patients with breast cancer (1-3). These studies suggest the feasibility of adoptive breast cancer immunotherapy with cytolytic T lymphocytes (CTL) or active immunotherapy with CTL-defined antigens. To develop such therapies, CTL and tumor cells are traditionally isolated and cultured directly on plastic surfaces in a 2-dimensional mixed lymphocyte/tumor cell culture (MLTC), and tumor-specific CTL lines or clones are isolated. However, immunotherapy trials with adoptive CTL transfer and active immunotherapy with CTL peptides in breast cancer patients have not held promise (4-8). The failure of these therapies might be explained by the marked differences between the CTL generated in ML TC *in vitro* compared to the CTL under *in vivo* conditions, as suggested by studies conducted in melanoma patients (9-11). Thus, we are testing the hypothesis that changes in the T cell receptor (TCR) repertoire occur upon culture of breast carcinoma tissues in ML TC *in vitro,* which might explain the absence of clinical responses in adoptive and active immunotherapy of breast cancer patients. In the proposed studies, CTL are generated under tissue-like conditions in an organotypic breast carcinoma culture system (reconstruct), so that the *in vivo* phenotypic and functional characteristics of the cells are preserved as much as possible.

BODY

The specific objectives of this proposal are to:

1. Isolate T cells and tumor cells from fresh, uncultured breast carcinoma tissues, or tissues cultured in the reconstruct or MLTC.

2. Compare phenotype and function of T cells isolated from fresh, uncultured breast cancer tissue, or tissue cultured in reconstruct or MLTC.

3. Demonstrate CTL migration toward tumor cells.

We obtained Human Subject and Human Anatomical Substance Approvals from USAMRAA in July 2004. Thus, the proposed studies were initiated in July 2004 and this report covers the period between September 2004 and September 2005. Progress has been made on Specific Aims 1 and 2.

Establishment of breast carcinoma and T cell lines from fresh tumor tissues

During the past year (September 2004 – September 2005) primary breast carcinoma tissues and heparinized blood were obtained from 15 patients from our collaborator Dr. Eric Miller (Virtua Memorial Hospital, NJ). A summary of patients' characteristics is included in Table 1. This table also includes 4 patients entered into the study between July and September 2004. Patient 7 was diagnosed with a non-malignant lesion after histopathological examination and therefore is not included in the Table. The tissues were minced, and one part was cryopreserved for T cell characterization. The second part served to initiate T cell lines in reconstructs with bovine type I collagen, and in MLTC directly on plastic surfaces. The third part was used to establish long-term

tumor and fibroblast cell lines directly in plastic dishes. Peripheral blood mononuclear cells were separated from blood specimens by Ficoll-Hypaque density centrifugation, and cryopreserved for later use as antigen-presenting cells.

Table 1. Summary of subjects studied[a]

Patient number	Breast cancer tissue obtained	Patient		
		Age	Gender	Ethnic background
BCP-01	Primary lesion	44	female	Caucasian
BCP-02	Primary lesion	54	female	Caucasian
B-3	Primary lesion	63	female	Caucasian
B-4	Primary lesion	57	female	Caucasian
BC-5	Primary lesion	43	female	Caucasian
BC-6	Primary lesion	55	female	Caucasian
BC-8	Primary lesion	57	female	Caucasian
BC-9	Primary lesion	46	female	Caucasian
BC-10	Primary lesion	44	female	Caucasian
BC-11	Primary lesion	44	female	Caucasian
BC-12	Primary lesion	50	female	Caucasian
BC-13	Primary lesion	42	female	Caucasian
BC-14	Primary lesion	48	female	African American
BC-15	Primary lesion	48	female	Caucasian
BC-16	Primary lesion	53	female	Caucasian
BC-17	Primary lesion	40	female	Caucasian
BC-18	Primary lesion	50	female	African American
BC-19	Primary lesion	49	female	Caucasian

[a] The first four specimens were obtained in the previous funding period between July 2004 and September 2004.

Establishment of T cell lines

Since initiation of this study, T cell lines were established from 15 of 18 breast cancer tissues (Table 2). From 12 tissues, T cell lines were established and cryopreserved from both MLTC and reconstruct. From 3 specimens, T cell lines were obtained from reconstruct only. This indicates that utilizing both culture systems increases the success rate of T cell line establishment.

Establishment of tumor cell lines

Tumor cell colonies were growing in all cultures; however, these colonies died within approximately 2-4 months with the exception of one culture (BC13). This cell line has been in culture for six months, and shows morphology characteristic for tumor cells. Tumorigenicity studies in SCID mice will confirm that it is a tumor cell line. It has been extremely difficult to establish breast cancer tumor cell lines. Different investigators report 7 – 17 % success rates. Since autologous tumor cell lines are needed for characterization of T cells we must improve our success rate in establishing breast cancer cell lines. Dr. Elisabeth Repasky (Roswell Park Cancer Institute, Buffalo N.Y.) reported a new technique for establishing breast cancer cell lines with a 25% success rate. She implants the tumor into the gonadal fat pad of female SCID mice and an estrogen pellet is implanted subcutaneously. The site of tumor implantation (female gonadal fat pad) and estrogen supplementation facilitates growth of estrogen-receptor bearing cells. Since a high percentage of breast cancer cells express these receptors (in our patient population 10 of 14 tumors expressed estrogen receptors), we have decided to establish this method in our laboratory. Dr. K. Berencsi, co-investigator on this grant, spent two days in Dr. Repasky's Laboratory in May 2005 to be trained in the new method. The protocol for breast tumor implantation in SCID

mice has been approved by the IACUC of The Wistar Institute. We anticipate that this new procedure will greatly improve our success rate in establishing tumor cell lines from the specimens.

Peripheral blood mononuclear cells (PBMC) were cryopreserved from all patients (Table 2). These cells will be used as antigen-presenting cells.

Fibroblast cell lines have been established from 7 specimens (Table 2).

Functional characteristics of T cell lines derived from fresh tumor tissues

CTL activity of T cells

Ten T cell lines derived from tissues of three patients were tested in ^{51}Cr-release assays for cytolytic function. Since we did not have autologous tumor cell lines we used partially HLA-matched allogeneic breast cancer cell lines as targets. First we selected HLA-A2$^+$ patients and tested their T cell lines for lysis of HLA-A2$^+$ tumor cell lines. HLA-A2$^-$ tumor cell line served as negative control. T cell lines were stimulated with anti-CD3 antibody, and recombinant human IL-2 using allogeneic PBMC feeder cells for 10 days and CTL lysis of ^{51}Cr-labeled allogeneic HLA-A2-matched and mismatched control target cells was determined in 6 h ^{51}Cr-release assay (Table 3; Fig. 1). Lymphocytes from both Rec and MLTC derived from 3 specimens lysed HLA-A2+ allogeneic target cells (Table 3). T cell line BC-17 Rec/1 significantly lysed (>20% specific lysis) 2 of 4 HLA-A2-matched breast cancer cell lines (Fig. 1A), and T cell line BC-11 MLTC/1 lysed only 1 of 4 HLA-A2-matched cell lines (Fig. 1B). Neither T cell line lysed an HLA-A2$^-$ cell line. Absence of lysis of some HLA-A2$^+$ cell lines may be due to absence of the CTL-recognized antigen in these cell lines and/or incomplete matching of HLA-A2 suballeles expressed by T and tumor cell lines. Similar results were obtained with 7 additional CTL lines (Fig. 1C-I).

Helper activity of T cells

Nine T cell lines were evaluated for their helper function, i.e. for their capacity to induce proliferative lymphocyte responses in allogeneic PBMC in transwell plates, which allow the diffusion of factors secreted by Th cells placed into the top chamber through a membrane to the allogeneic responder cells in the bottom chamber, but do not allow contact between the cells in the two chambers. In two T cell lines, B-4 Rec, and BC-14 MLTC, statistically significant Th function was determined. Seven T cell lines showed no helper activity in this assay (Table 4; Fig. 2 A, B).

Phenotypic characteristics of T cells derived from TIL in MLTC or Reconstructs

T cells derived from three specimens were phenotyped with special emphasis on molecules that might be involved in the interactions of these cells with tumor cells and components of the reconstruct (Table 5). Two of three MLTCs are predominantly CD4$^+$, and one is predominantly CD8$^+$. One of three Rec are predominantly CD8, and two express predominantly CD4. All T cells, regardless of their culture condition, express α2 and β1 integrins. LFA-1, CD28 and ICAM-1 are expressed by all T cell lines. The cells do not express FAS ligand (Table 5). CD40L is expressed by 3 of the 6 cell lines. All 6 T cell lines express granzyme, which may be responsible for CTL activity (see Table 3). Thus, similar patterns of surface marker expression are found in T cell lines derived from the two different culture systems.

Table 2. Reconstructs (REC) and MLTC with fresh breast cancer tissues[a]

Patient #/date of specimen[b]	Number of frozen vials							
			Cell lines					
					T cells			
	Tumor tissue	PBMC[c]	Tumor	Fibroblasts[d]	MLTC[e]		REC[e]	
					# of T cell lines	Total # of vials frozen	# of T cell lines	Total # of vials frozen
BCP 001 7/17/04	6	8	Failed	7	2	4	4	7
BCP 002 7/23/04	5	7	Failed	2	0	0	5	10
B-3 8/19/04	7	9	Failed	4	2	8	2	9
B-4 9/23/04	6	10	Failed	0	2	4	4	10
BC-5 11/11/04	6	8	?[f]	5	2	5	4	10
BC-6 11/18/04	3	9	Failed	2	2	3	2	2
BC-8 12/02/04	5	8	Failed	0	0	0	0	0
BC-9 12/08/04	4	7	Failed	2	0	0	2	3
BC-10 12/10/04	4	7	Failed	0	1	1	1	5
BC-11 1/14/05	7	5	Failed	2	2	6	6	10
BC-12 2/04/05	4	8	Failed	2	0	0	1	2
BC-13 3/17/05	3	9	3	2	5	10	1	2
BC-14 4/15/05	3	10	Failed	1	1	2	1	4
BC-15 4/15/05	3	7	?[f]	0	1	3	2	2
BC-16 5/05/05	3	10	Failed	0	1	2	2	2
BC-17 6/30/05	6	4	?[f]	0	5	11	5	12
BC-18 8/12/05	4	10	?[f]	0	?[f]	?[f]	?[f]	?[f]
BC-19 9/08/05	4	3	?[f]	?[f]	?[f]	?[f]	?[f]	?[f]

[a]Reconstructs were initiated by seeding minced tissue into bovine type I collagen in minimal essential medium (MEM) supplemented with 10% human AB serum; 7 days later and then weekly, cultures received RPMI 1640 supplemented with 10% human AB serum and 10% natural IL-2. MLTC were initiated by seeding minced tissues in RPMI 1640 medium supplemented with 10% human AB serum into plastic culture dishes; 7 days later and then weekly, cultures received 10% natural IL-2-containing medium (RPMI 1640 plus 10% human AB serum).

[b] The first 4 specimens were obtained during the previous funding period.

[c] $1x10^7$/vial

[d] $2\text{-}3x10^6$/vial

[e] $2x10^6$/vial

[f]= too early to be determined

Table 3. CTL activity of Rec and MLTC established from fresh breast cancer tissues of HLA-A2$^+$ patients[a]

Specimen #	T cell line	Lysis of HLA-A2-matched breast cancer cell lines
BC-5	Rec	+
	MLTC	+
BC-11	Rec/1	+
	Rec/3	+
	MLTC/1	+
	MLTC/2	-
BC-17	Rec/1	+
	Rec/2	+
	MLTC/1	+
	MLTC/2	+

[a] Rec and MLTC were stimulated for 10 days with anti-CD3 antibody, and IL-2. CTL lysis of ^{51}Cr-labeled allogeneic HLA-A2- matched control target cells was determined in 6 h ^{51}Cr-release assay.
[b] For details see Figure 1.

Table 4. Helper activity of T cell lines established from fresh breast cancer tissues[a]

Cells		Helper activity
Responder	Stimulator	
PBMC	B-4 Rec	+[b]
PBMC	BC-5 MLTC	-
PBMC	BC-9 Rec/1	-
PBMC	BC-11 MLTC	-
PBMC	BC-13 MLTC	-
PBMC	BC-14 MLTC	+[b]
PBMC	BC-14 Rec	-
PBMC	BC-15 MLTC	-
PBMC	BC-15 Rec	-

[a] Helper activity was determined by enhancement of proliferation of allogeneic PBMC. T cells ($1x10^5$) were added to the top chamber of Transwell plates (Costar No.3413; 0.4 μm pore size), and allogeneic PBMC ($2x10^5$) into the bottom chamber. As negative controls medium or EBV-B3453 was added to the top chamber. After 3 days of culture, the top chambers were removed. Proliferative responses of the allogeneic PBMC in the bottom chamber were determined by ^{3}H-thymidine incorporation assay.
[b] For details see Figure 2 A and B.

Table 5. Phenotypic markers of anti-breast cancer T cells derived from tumor-infiltrating lymphocytes (TIL) in reconstructs (Rec) or MLTCs

Markers[a]	% cells positive					
	BCP001		B-3		B-4	
	MLTC	REC	MLTC	REC	MLTC	REC
CD4	96.2	11.6	97.7	56.5	1.2	93.1
CD8	3.9	86.3	37.8	6.1	98.0	28.8
CD40L	30.4	32.0	8.5	4.6	1.4	29.2
CD28	97.0	25.7	29.6	57.2	11.0	88.4
Granzyme	69.0	87.1	69.0	86.9	97.1	90.5
α1 integrin	7.4	58.4	15.9	43.3	95.5	37.0
α2 integrin	97.4	94.2	70.2	87.8	98.0	58.8
β1 integrin	98.9	98.3	98.8	98.8	98.9	97.7
FAS	93.4	83.3	92.3	89.0	98.7	98.3
FASL	0.3	0.6	0.8	1.0	0.46	1.0
ICAM-1	95.7	93.8	98.5	96.5	97.0	95.7
LFA-1	97.8	96.5	98.6	98.0	98.1	96.6
CD44	93.1	79.4	99	98.2	98.7	87.7

[a]Markers were detected by FACS. All values are corrected for irrelevant, isotype-matched control antibody binding. Values >10% are designated positive.

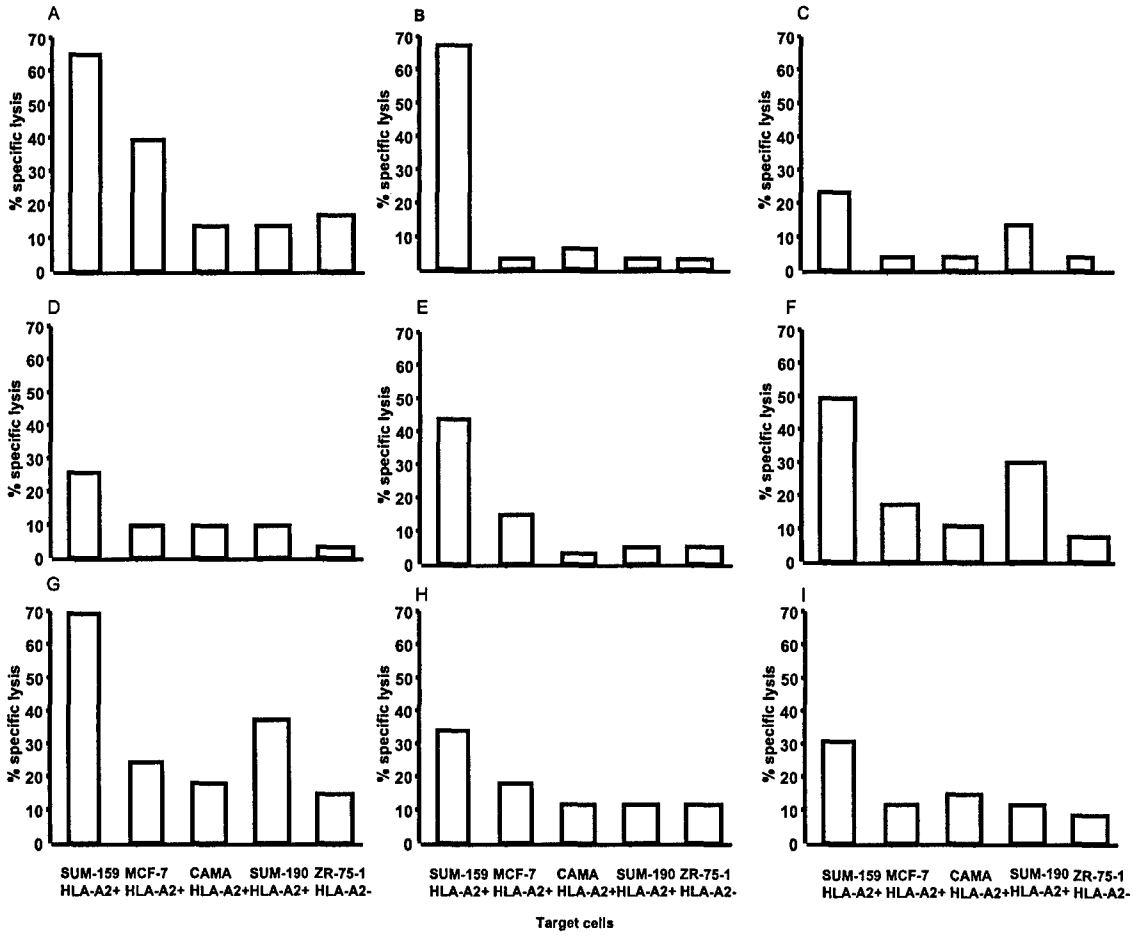

Figure 1. Lysis of HLA-A2-matched breast cancer cell lines by BC-17 Rec/1 (A), BC-11 MLTC/1 (B), BC-11 Rec/1 (C), BC-11 Rec/3 (D), BC-17 Rec/2 (E), BC-17 MLTC/1 (F), BC-17 MLTC/2 (G), BC-5 Rec (H) and BC-5 MLTC (I). Rec and MLTC were established from fresh tumor tissue in reconstructs or MLTC. After four weeks in culture, cells were stimulated with anti-CD3 antibody, and recombinant human IL-2 using allogeneic PBMC feeder cells for 10 days. CTL lysis of ^{51}Cr-labeled allogeneic HLA-A2 matched and mismatched control target cells was determined in 6 h ^{51}Cr-release assay. Effector:Target ratio = 25:1

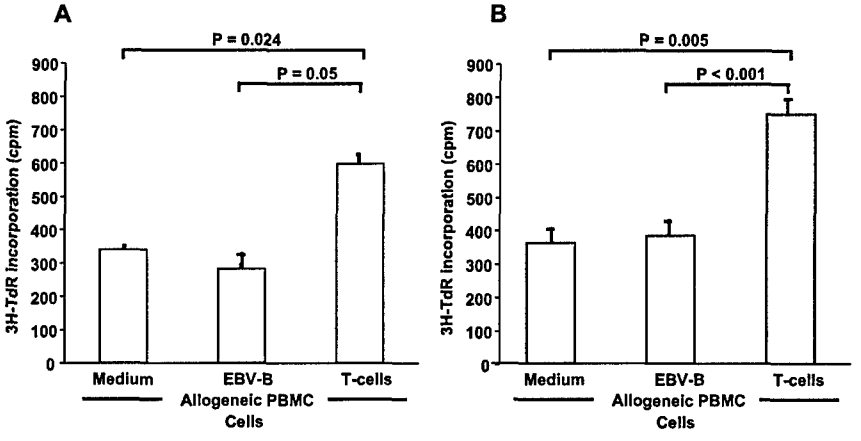

Figure 2. Helper activity of B-4 Rec (A), and BC-14 MLTC (MLTC). Helper activity was determined by enhancement of proliferation of allogeneic PBMC. T cells ($1x10^5$) were added to the top chamber of Transwell plates (Costar No.3413; 0.4 μm pore size), and allogeneic PBMC ($2x10^5$) to the bottom chamber. As negative controls, medium or EBV-B3453 was added to the top chamber. After 3 days of culture, the top chambers were removed. Proliferative responses of the allogeneic PBMC in the bottom chamber were determined by ^{3}H-thymidine incorporation assay. Differences between experimental and control values were determined by Student's 2-sided t-test.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of reconstructs and MLTC from 14 breast cancer tissues.
- Isolation of T cell lines from both MLTC and reconstruct (9 tissues) and reconstruct only (two tissues).
- Establishment of one tumor cell line.
- Establishment of fibroblast cell lines (7 tissues).
- Demonstration of cytolytic activity of 9 of 10 T cell lines tested.
- Demonstration of helper activity of 2 of 9 T cell lines tested.
- Analysis of phenotype of 6 T cell lines.

REPORTABLE OUTCOMES

During this study period, 50 T cell lines from 11 breast cancer tissues, 7 fibroblast cell lines from 7 tissues and one long-term breast cancer cell line were established. CTL activity was demonstrated in 9 of the 10 T cell lines tested. Two of 9 T cell lines tested showed helper activity.

CONCLUSIONS

Nine functional CTL and 2 Th cell lines were established from fresh breast cancer tissues. The T cell lines will be useful for the development of novel immunotherapies against breast cancer based on chemokines and antigen vaccines.

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