

IMMUNOTHERAPY OF CANCER

Generation of CEA Specific CTL Using CEA Peptide Pulsed Dendritic Cells

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1. ABSTRACT

Antigen specific cytotoxic T lymphocytes (CTL) are being studied for their potential immunotherapeutic benefit in the treatment of cancer. Carcinoembryonic antigen (CEA) is an oncofetal protein best known for its overexpression in the majority of colorectal, gastric, pancreatic, non small cell lung, and breast carcinomas. We are using dendritic cells (DC) pulsed with the CEA CTL peptide epitope to generate CEA specific CTL. DC from HLA A2⁺ donors were isolated by culturing plastic adherent PBMC in GMCSF and IL4 for 7 days. As expected these DC expressed the relevant cell surface molecules including HLA DR, CD58, CD80, and CD86. The DC were stripped of their endogenous peptides, pulsed with the A2 restricted CEA peptide, irradiated and used to stimulate autologous CD8⁺ T cells in the presence of IL7. Using this approach we have been able to generate CEA specific CTL from the PBMC of breast and pancreatic carcinoma patients as well as normal donors. These CTL can lyse CEA peptide pulsed T2 targets as well as HLA A2⁺ tumor cells expressing the CEA antigen. This data is being used to support a phase I active immunotherapy clinical protocol using DC pulsed with CEA peptide to treat patients with metastatic malignancies expressing CEA.

2. INTRODUCTION

Immunotherapy protocols for cancer have shown promise in experimental animal models and in the treatment of some human malignancies^{1,2}. While approaches such as vaccination with non specific immunogens or systemic administration of cytokines have been disappointing³, an emerging understanding of antigen specific cellular recognition supports the potential role of specific immunotherapy in human malignancy.

CEA is an oncofetal antigen which is expressed on most adenocarcinomas of the colon, rectum, pancreas, and stomach, as well as on 50% of breast cancers and 70% of non-small cell lung carcinomas⁴. It has also been found on normal adult colonic mucosa. Recently, Schlom and his colleagues have identified peptides corresponding to human MHC class I CTL epitopes within the CEA protein⁵. The immunodominant peptide in this study was a nine amino acid sequence, YLSGANLNL. This peptide was designated CAP-1 and is an HLA-A2 restricted CTL epitope. When CAP-1 was used to stimulate peripheral blood lymphocytes of patients previously immunized with recombinant human CEA vaccinia virus, CEA specific CTL lines could be generated, confirming the immunogenicity of the CEA antigen⁵. However, no CTL could be generated from unimmunized patients.

DC are potent antigen presenting cells (APC) which have been shown to stimulate both a naive and memory T cell response *in vitro*^{6,7}. Recent studies have indicated that DC may be more potent in stimulating an anti-tumor immune response when compared to other approaches, including viral vectors expressing tumor antigen genes, gene modified tumor cells, naked DNA, or peptide emulsified in adjuvant. In this study, we show that DC pulsed with the HLA-A2 restricted CEA peptide can generate a CEA specific CTL response *in vitro* from both unimmunized carcinoma patients and healthy donors.

3. MATERIALS AND METHODS

3.1. Dendritic Cell Isolation

DC were isolated using a modification of Romani *et al.*⁸ Briefly, 1.5×10^8 PBMC from an HLA-A2⁺ donor were allowed to adhere to T150 culture flasks for 2 hours at 37°C. The adherent cells were then cultured in 30 ml of RPMI-10% FCS medium containing 800 units/ml GM-CSF and 500 units/ml IL-4 for 6–7 days.

3.2. Peptide Stripping and Loading

For peptide stripping, DC were washed once in cold 0.9% NaCl with 1% BSA solution, resuspended at 1×10^7 cells/ml in stripping buffer (0.13M L-ascorbic acid, 0.06M sodium phosphate monobasic [pH 3], 1% BSA, 3 mg/ml b2microglobulin, 10mg/ml peptide) and incubated for 2 min on ice. The cells were then neutralized with 5 volumes of cold neutralizing buffer (0.15M sodium phosphate monobasic [pH 7.5], 1% BSA, 3 mg/ml b2M, 10mg/ml peptide) and spun at 1500 rpm for 5 min. Finally, the cells were resuspended in peptide solution (PBS-CMF, 1% BSA, 30 mg/ml DNAase, and 40 mg/ml peptide) and incubated for 4-hr at room temperature. After incubation, the cells were irradiated (3000 Rad) and washed prior to being used for stimulation.

3.3. CTL Generation

CD8⁺ responders for peptide specific CTL were generated by adhering PBMC on AIS (Santa Clara, CA) CD8 T25 MicroCELLector^R flasks. CD8 captured cells were stimulated with irradiated DC loaded with CEA peptides (DC-CEA) at a stimulator to responder (S:R) ratio of 1:3. These cells were cultured in RPMI-10% FCS containing 10ng/ml IL-7. At day 10–12, the lymphocytes were restimulated with DC pulsed with CEA peptide (1:5 S:R ratio). Responders were restimulated weekly to a total of 3–4 restimulations at S:R ratios ranging between 1:5 to 1:15. As controls, CD8 captured cells were stimulated with IL-7 only (10ng/ml) or PHA/IL2 (2ug/ml PHA and 500 U/ml IL-2).

3.4. Cytotoxicity Analysis

HLA-A2 restricted CEA recognition by CTL was assessed by a standard 4-hr ^{51}Cr release cytotoxicity assay. Recognition of CEA peptide by CTL was assessed using T2 cells pre-incubated for 2–4 hr with peptide at 40mg/ml. In addition CEA specific cytotoxicity was evaluated on the CEA expressing cell lines SW403 (HLA-A2⁺) and SW1417 (HLA-A2⁻).

4. RESULTS

4.1. Generation of DC

DC were used as APC to present CEA peptide to CD8⁺ T cells. The DC were differentiated from PBMC adherent progenitors using GMCSF and IL4 as described⁸. The yield of DC (based on FACS analysis) generated in this study was approximately 6% of the starting PBMC population; the purity of these cells was about 60%. DC expressed high levels of HLA DR, the adhesion molecules CD54 (ICAM-1) and CD58 (LFA3), as well as the costimulatory molecules CD80 (B7.1) and CD86 (B7.2). DC did not express CD3, CD14, CD15, CD16, CD19 or CD20 as expected (data not shown).

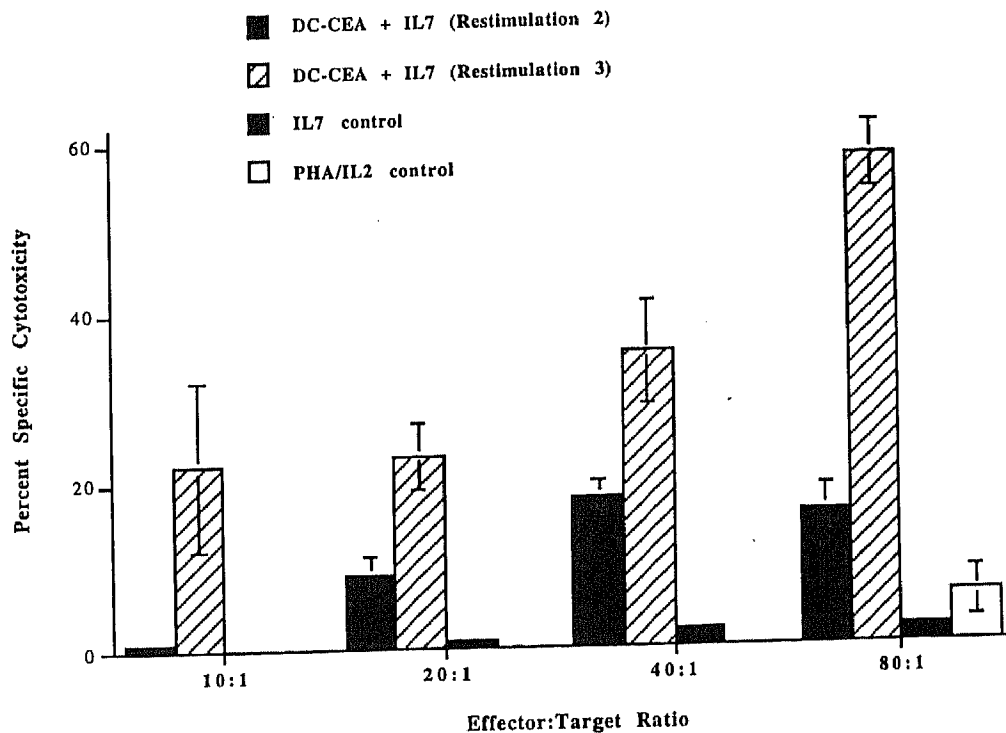


Figure 1. Generation of a CEA peptide response from an HLA-A2⁺ pancreatic carcinoma patient. CD8 captured responders were stimulated with CEA pulsed DC and cultured in 10ng/ml IL-7. Cytotoxicity was assayed after 2 (shaded) or 3 (slash lines) rounds of restimulation with CEA peptide pulsed DC + IL7 at effector:target ratios of 80:1, 40:1, 20:1, 10:1. Control cultures were cultured in 10ng/ml IL7 (■) or PHA/IL2 (□). The background response on empty T2 cells has been subtracted.

4.2. Generation of CEA Specific CTL from HLA-A2⁺ Carcinoma Patients

DC were used to generate CEA peptide specific CTL from an HLA-A2⁺ pancreatic carcinoma patient as described. After two and three rounds of restimulation with DC-CEA + IL7 the CTL effectors were tested for cytotoxicity using T2 cells pulsed with CEA peptide as targets. CEA peptide specific cytotoxicity ranged from 15–20% over background levels after two rounds of restimulation with DC-CEA + IL7 and increased to 60% after one additional round of restimulation (Figure 1). Effector cells that had been stimulated with IL7 only or with PHA/IL2 showed no CEA specific cytotoxicity.

Having shown that CTL generated using DC-CEA + IL7 were CEA peptide specific, we next assessed whether the effector CTL could recognize endogenously processed and presented antigen. DC were isolated as above, pulsed with CEA peptide, and used to stimulate autologous CD8 captured T cells from an HLA-A2⁺ breast carcinoma patient in the presence of IL7. As shown in Figure 2, CEA peptide induced CTL effectors lysed SW403, the CEA⁺ HLA-A2⁺ tumor target, at E:T ratios of 70:1 down to 9:1. Since previous studies have determined that the CEA peptide was HLA-A2 restricted, we assessed the ability of these CTL to kill SW1417, a CEA⁺ HLA-A2⁻ target⁵. Lysis of this tumor target was minimal. Again, effector cells that had been cultured with IL7 only showed no specific cytotoxicity on any target.

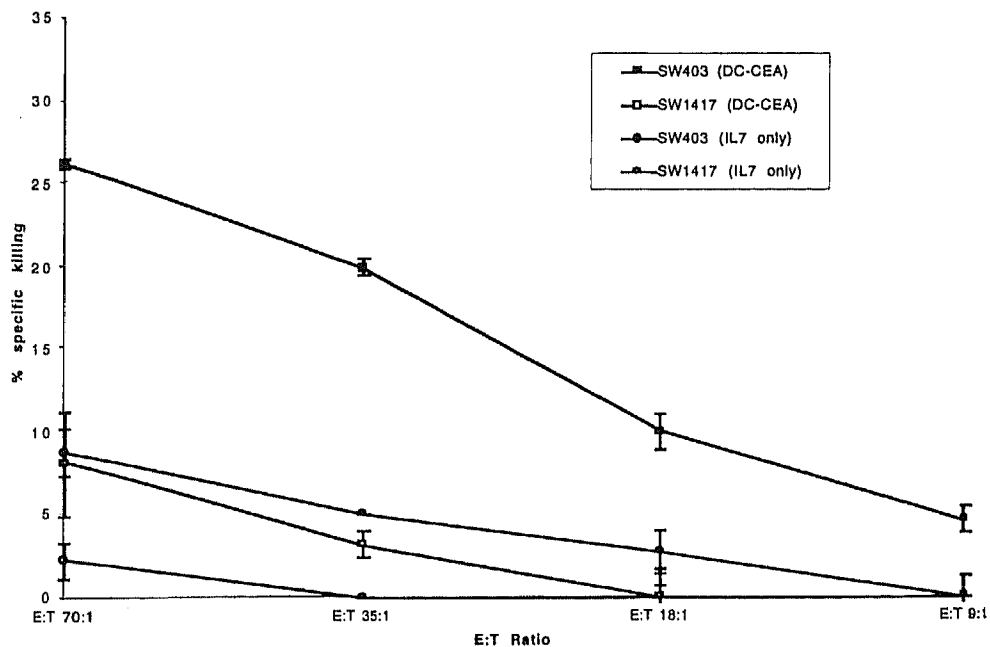


Figure 2. Generation of an HLA-A2 restricted, CEA specific response from an HLA-A2⁺ breast carcinoma patient. The cytolytic activity of CTL generated by stimulation with CEA pulsed DC + IL7 (10ng/ml) was assayed on an A2⁺ CEA⁺ (SW403; ■) and an A2⁻ CEA⁺ tumor cell line (SW1417; □) at effector:target ratios of 70:1, 35:1, 18:1, 9:1. Cytolytic activity of control cells generated with IL7 only was also tested (SW 403; ●) (SW1417; ○). Cytotoxicity was assayed after four rounds of restimulation.

4.3. Generation of CTL from an HLA-A2⁺ Healthy Donor

We next looked at whether CTL could be generated from the blood of healthy HLA-A2⁺ donors. As seen for the carcinoma patients, CEA specific CTL could also be generated from a healthy donor (Figure 3). After 3 rounds of restimulation with DC-CEA in the presence of IL7, 30% killing was detected on T2-CEA as compared to T2 cells pulsed with an irrelevant peptide at E:T of 80:1, and 10% killing was detected at E:T of 40:1. Cells cultured in IL7 only showed no response as expected.

5. DISCUSSION

The CEA antigen which is expressed in many cancer types is a potential target for tumor immunotherapy. However, the generation of an immune response against the CEA antigen has historically been difficult to achieve. Recently, Schlom and colleagues were able to generate CTL lines by stimulating PBL with the CAP-1 peptide and IL2 from patients previously vaccinated with the CEA gene inserted into vaccinia virus⁵. However, no CTL could be generated from unimmunized patients.

In our study, we were successful in generating CEA peptide specific CTL from the blood of both unimmunized patients as well as healthy donors. The use of DC as APC in the CTL protocol may account for the favorable outcome. DC used in our studies express

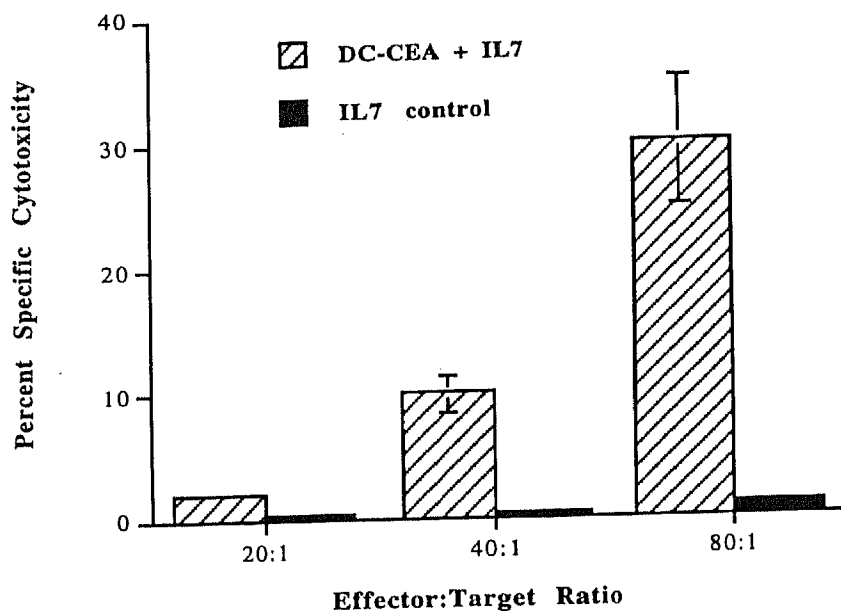


Figure 3. Generation of a CEA peptide response from an HLA-A2⁺ healthy donor. CD8 captured responders were stimulated with CEA pulsed DC and cultured in 10ng/ml IL-7. Cytotoxicity was assayed after 3 rounds of restimulation with CEA peptide pulsed DC + IL7 (slash lines) at effector:target ratios of 80:1, 40:1, 20:1, 10:1. Control cultures were cultured in 10ng/ml IL7 (■). The background response on T2 cells pulsed with an irrelevant peptide has been subtracted.

high levels of HLA-DR, costimulatory molecules such as CD80, CD86 and adhesion molecules such as CD54 and CD58. This may explain the ability of DC to play a critical role in the initiation of the cellular immune response. DC have been shown to stimulate naive T cells to recognize and respond to a variety of antigens *in vitro*^{7,9} and can induce protective or therapeutic immunity when administered as a vaccine to animals *in vivo*¹⁰⁻¹⁴. Recently Bakker *et al.*¹⁵ have demonstrated CTL specific for tyrosinase, gp100, and MART-1 from healthy donors using peptide loaded DC as APC. In our current study we show that CEA specific CTL can be generated from unvaccinated carcinoma patients and healthy donors when the CEA peptide is presented by DC. The CTL generated using peptide-loaded DC were capable of lysing CEA peptide-loaded T2 cells as well as tumor cell lines expressing the CEA antigen. These studies indicate that using a specialized APC, like the DC, may allow for the induction of an effective cellular immune response without the need for prior vaccination. This data is being used to support a phase I active immunotherapy clinical protocol using DC pulsed with CEA peptide to treat patients with metastatic malignancies expressing CEA.

6. REFERENCES

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