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PRINCIPAL INVESTIGATOR: Sylvia M. Kiertscher, Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, California 90095-1405

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The Advantages of Multi-Epitope Tumor Antigens as an Approach to Treating Breast Cancer

Sylvia M. Kiertscher, Ph.D.

University of California
Los Angeles, California  90095-1405

E-Mail: skiertscher@mednet.ucla.edu

Dendritic cells (DC) are an integral part of the immune system's response to cancer. In preparation for treating breast cancer patients with DC, this proposal examines a fundamental issue that needs to be resolved before proceeding with this exciting new therapy. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC is a more efficient and effective way of stimulating T cell responses than current HLA-restricted peptide-based methods. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In the past year, we have continued investigations to compare the various antigen-arming methods. We have determined that T cells stimulated with apoptotic tumor-loaded DC show both tumor-peptide-specific reactivity (as assessed by MHC-HLA-A2-E75 tetramer staining), as well as enhanced responsiveness to Her-2 and other tumor antigens (as assessed by antigen-specific interferon-γ production). In addition, we have established that helper-dependent adenoviral vectors are a viable alternative to E1-deleted adenoviral vectors for the expression of tumor antigens in DC. These research findings support our overall hypothesis, and the work accomplished this year has resulted in two abstracts and two manuscripts.
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INTRODUCTION

Dendritic cells (DC) are an integral part of the immune systems’ response to cancer. When loaded with tumor antigens, DC have great value as immunotherapeutic agents. However, the method for arming the DC with antigens which results in the most effective immune response has yet to be defined. We theorized that the broadest possible mix of tumor antigens might provide the best material for stimulating an effective immune response. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC would be the most efficient and effective way of stimulating T cell responses. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In this study we will compare multiple methods of arming DC with tumor antigens including: 1) purified immunodominant peptides which are specific for a single antigen and a single Class I MHC molecule, 2) transduced cDNA encoding for a single tumor antigen which will allow the recipient DC to intrinsically process and present all possible antigenic peptides (immunodominant and sub-dominant) within the context of all available MHC molecules, and 3) extracts from autologous whole tumor cells which will provide a broad mix of tumor antigens (both defined and undefined antigens) for processing and presentation. The information obtained from this study will further our understanding of the interactions between DC and T cells which lead to the generation of tumor-antigen-specific responses. This understanding will be valuable in the development of immunotherapeutic treatments for breast cancer.
ANNUAL SUMMARY - DAMD17-98-1-8181

RESEARCH ACCOMPLISHMENTS:

Summary of previous accomplishments:
In the previous grant years, we evaluated the phenotype and functional activity of breast cancer patients' DC in preparation for their use in tumor antigen-presentation, developed a rapid method to assess patient's T cell function prior to treatment, and developed methodologies for the cross-presentation of multiple tumor antigens by apoptotic tumor cell-loaded DC. In addition, the optimal culture conditions for stimulating T cells with antigen-loaded DC were determined, and a variety of assays were developed to aid in the assessment of anti-tumor antigen-specific T cell reactivity.

Year 7/01/01 through 6/30/02 accomplishments:
In grant year 7/01/01 through 6/30/02, progress was made on Tasks 1-4 of the statement of work. These experiments focused on the evaluation of the various antigen-arming methods and their ability to promote the development of anti-tumor specific T cell responses (Task 3: To determine the frequency of Her-2-specific T cells generated using the different antigen-arming methods, and Task 4: To characterize the response obtained by stimulation of T cells with DC). Highlights of the data are summarized below, and a manuscript detailing these results is in preparation, with an expected submission for publication date of 8/02. In addition, preliminary work has been completed on the use of adenoviral vectors to express antigens in DC (Task 3), including a comparison of traditional E1-deleted adenovirus and the new helper-dependent adenoviral vector system. Those results are also summarized below, and have been submitted for publication.

T cells from breast cancer patients produce IFN-γ and TNF-α in response to stimulation with E75 peptide-loaded DC
In order to evaluate whether tumor specific responses could be generated from the peripheral blood T cells of breast cancer patients, CD8+ T cells from HLA-A2+ patients were stimulated with DC pulsed with the HLA-A2 E75 Her-2 peptide in a modified limiting dilution assay. T cells in the majority of the wells produced both IFN-γ and TNF-α in response to stimulation with E75-loaded DC. In general, the cells which produced the highest amounts of IFN-γ also produced high amounts of TNF-α. However, the development of specific cytokine production required multiple restimulations in vitro, and the reactivity developed was predominantly peptide-specific rather than tumor-specific. These results are consistent with the work of others in both in vitro and in vivo studies with tumor peptides, and generate concern that peptide-centered treatments may lead to limited responses in vivo, allowing for the selection of tumor variants.

DC take up tumor antigen (p185 neu) expressed by apoptotic tumor cells.
As an alternative to tumor peptide, we hypothesized that the use of whole tumor antigen might be a more effective method to stimulate tumor-antigen specific T cells. In order to assess the feasibility of using apoptotic tumor cells as a source of tumor antigen, we examined the ability of DC to take up apoptotic tumor cells. DC were labeled with the membrane stain PKH67, and then co-cultured with PKH26-labeled SKBr3 cells which had been induced to undergo apoptosis using UVC irradiation. The proportion of DC which had taken up tumor increased over time and was also DC:tumor cell ratio dependent. At lower ratios (0.5:1 and below) there was less uptake, while at higher ratios there were larger numbers of excess tumor bodies which were not taken up by the DC. In addition to
measuring the uptake of cell membrane-labeled cell bodies, we also looked for the presence of tumor antigen protein in the DC. SKBr3 cells, which overexpress p185/neu, were induced to undergo apoptosis using UV irradiation. The apoptotic cell bodies were co-incubated with DC for various lengths of time, followed by fixation, permiabilization and intracellular staining for Her-2. As shown in Figure 1, tumor antigen was present in DC at both time points, but little increase in antigen staining was observed after 22 hours. This may be due to antigen processing by the DC, and the resultant loss of the epitopes to which the anti-Her-2 antibody binds.

Figure 1. DC take up tumor antigen (p185/neu) expressed by apoptotic tumor cells. DC and apoptotic tumor bodies from the p185/neu over-expressing breast cancer cell line SKBr3 were admixed at a 1:1 DC:tumor cell ratio and co-cultured for 2-22hrs. DC were collected, fixed, permiabilized and stained with anti-CD86-PE to identify the DC, and anti-Her-2-FITC to identify the p185/neu protein. Percentages indicate the proportion of DC which were positive for p185/neu. DC stained with mouse IgG1-FITC isotype control in place of anti-Her-2 FITC were < 2% positive. Representative experiment, n=3.

T cells stimulated with apoptotic tumor-loaded DC or E75 peptide-loaded DC show similar MHC-E75-tetramer staining.

In order to compare the T cell response generated by apoptotic tumor vs. tumor peptide, DC were pulsed with either the E75 peptide or apoptotic SKBr3 cells, and then used to stimulate autologous T cells. To evaluate the resulting response, T cells were stained with the HLA-A2 MHC tetramer for the E75 peptide of the p185/neu protein. As expected, a portion of the T cells which had been stimulated with E75 peptide-pulsed DC showed peptide-specific reactivity (Figure 2). Interestingly, T cells which had been stimulated with apoptotic tumor-pulsed DC exhibited a similar number of positive cells, indicating that the p185/neu protein was successfully processed and presented by the DC.

Figure 2. T cells stimulated with apoptotic tumor-loaded DC or E75-peptide-loaded DC show similar MHC-E75-tetramer staining. T cells were stimulated with DC pulsed with apoptotic SKBr3 (Her-2-overexpressing) or DC pulsed with E75 peptide. After 7 days, the T cells were collected and assessed for E75-peptide reactivity by staining with MHC-tetramer and antibodies to CD3 and CD8. Results for the gated CD8+ population are shown. Numbers in parentheses denote the fluorescence intensity of the tetramer-positive cells. Tetramer-binding for non-stimulated T cells was < 1%. Representative experiment, n=4.

T cells stimulated with apoptotic tumor-loaded DC show enhanced responsiveness to Her-2 as assessed by IFN-γ production.

SKBr3 cells express other tumor antigens in addition to the oncoprotein Her-2. When SKBr3 are rendered apoptotic and loaded into DC, all of these antigens are processed and presented to the T cells in the co-cultures. In order to evaluate the proportion of the T cell response which was directed against the p185/neu protein, as opposed to other antigens, we utilized the retrovirally transduced cell lines MCF-7/her-2 and MCF-7/neu. MCF-7/her-2 overexpresses the p185/neu protein at levels comparable to SKBr3. The control cell line MCF-7/neu expresses only minimal amounts of
p185/neu. For these studies, the production of cytokines was measured by intracellular cytokine staining, which allows the cytokine profile of each individual T cell to be evaluated. T cells were first stimulated for 14 days with either E75-pulsed or apoptotic SKBr3-pulsed DC, then challenged in a 5 hour cytokine production assay with DC pulsed with either apoptotic MCF-7/neo or apoptotic MCF-7/her-2. The specific response to Her-2 was assessed by evaluating the difference between the MCF-7/her-2 and MCF-7/neo responses. The T cells which initially had been stimulated with apoptotic SKBr3-pulsed DC showed Her-2 specific IFN-γ production in both CD4 and CD8 T cell populations (Figure 3).

![Figure 3. T cells stimulated with apoptotic tumor-loaded DC show enhanced responsiveness to Her-2 as assessed by IFN-γ production. T cells were restimulated on a weekly basis with DC pulsed with apoptotic SKBr3 (Her-2-overexpressing) cells or DC loaded with the HLA-A2 p185/neu peptide E75. Intracellular IFN-γ production was stimulated by DC pulsed with either apoptotic MCF-7/neo or apoptotic MCF-7/her-2 in a 5 hour assay. The percent positive cells are listed for both CD4 and CD8 T cell populations. Numbers in parentheses denote the fluorescence intensity of the IFN-γ positive cells. The specific response to Her-2 was assessed by evaluating the difference between the MCF-7/neo and MCF-7/her-2 responses. Representative experiment, n=5.]

Some reactivity against MCF-7/neo also was observed, perhaps due to other antigens which are shared by SKBr3 and MCF-7/neo, such as MUC1 and CEA. The generation of reactivity against multiple tumor antigens is the focus of continuing study. In contrast to the results with apoptotic tumor, the T cells which initially had been stimulated with E75 peptide-pulsed DC demonstrated little anti-Her-2 responsiveness as measured by IFN-γ production at this time point. The peptide-stimulated T cells required at least 21-28 days of culture (3-4 stimulations) to produce any measurable IFN-γ. Neither group of T cells produced significant amounts of IL-10, and T cells challenged with non-antigen-loaded DC produced neither IFN-γ nor IL-10 (data not shown). In summary, these results suggest that apoptotic tumor-loaded DC stimulate a larger number of specific T cells at an earlier time point than E75-peptide-loaded DC, and that the multi-epitope approach to antigen-loading of DC is a viable method for enhancing T cell anti-tumor responsiveness.

In addition to the use of tumor peptide and whole tumor cells as a means of loading DC, we are also evaluating the use of adenoviral vectors for the expression of Her-2/p185 protein in DC (Task 3). Adenoviral vectors are an effective means of expressing genes in DC, however pre-existing immunity to adenoviral antigens, and enhancement of this immunity following repeated administration of viral-transduced DC, may limit their clinical efficacy. We hypothesized that helper-dependent adenovirus, in which viral coding regions are replaced by human stuffer DNA, may present an advantageous alternative approach due to their lack of viral genes. In the past year, we have performed preliminary studies to assess the immunogenicity and efficacy of both traditional E1-deleted adenoviral vectors, as well as the helper-dependent vector system. The goal of these studies was to determine how to maximize tumor antigen gene expression, while minimizing potentially deleterious anti-viral reactivity by the immune system.
**Helper-dependent-adenovirus produces higher transgene expression than E1-AdV.**

Human monocyte-derived DC were transduced with equivalent viral particle numbers of either replication-deficient E1-deleted adenovirus or helper-dependent adenovirus. Transduced DC were then compared for their ability to stimulate antiviral T cells, and for their expression of the transgene product. DC transduced with helper-dependent adenovirus expressed higher transgene protein activity under every condition with an average 150-fold increase over E1-deleted adenovirus at the 500 MOI (range 52 to 275-fold increase) (**Figure 4A**). Flow cytometry of FDG-loaded cells confirmed enhanced transgene expression at the single cell level.

**Higher transgene expression by helper-dependent adenovirus occurs as a result of enhanced transcription.**

In order to determine if higher transgene expression was the result of more efficient transduction (vector loading) or a higher level of transcription, genomic DNA and total mRNA were extracted from control and transduced DC and analyzed by semi-quantitative PCR and RT-PCR, respectively. When transduced using the same number of viral particles, identical levels of DNA for the transgene were detected from DC exposed to either vector. In contrast, levels of mRNA encoding for the transgene were always higher in cells exposed to the helper-dependent adenovirus. These results suggest that both vectors exhibit equal transduction efficiencies, but that higher transcriptional activity and/or mRNA stability accounts for the higher protein levels in cells exposed to helper-dependent adenovirus.

**Figure 4.** DC transduced with Hd-AdV express higher levels of transgene but stimulate the proliferation of antiviral T cells to the same level as DC transduced with E1-AdV. Monocyte-derived DC were transduced at a MOI of 500 using either E1-AdV/β-gal or Hd-AdV/β-gal, and 48 hrs later used to stimulate autologous T cells at a DC:T cell ratio of 1:20. Expression of β-gal by transduced DC was determined by luminescence assay (A) and resulting T cell proliferation was determined by [3H]-thymidine uptake after 6 days of co-culture (B). Representative experiment, n=3.

**DC transduced with the same viral particle number of helper-dependent adenovirus and E1-deleted adenovirus activate similar antiviral T cell responses.**

Working under the hypothesis that viral gene products synthesized de novo act as an important source of viral antigens, we expected that E1-deleted adenovirus-transduced DC would stimulate significantly greater anti-viral T cell proliferation than helper-dependent adenovirus-transduced DC. However, both sets of DC stimulated the same level of anti-viral T cell proliferation (**Figure 4B**). The frequency of viral-reactive T cells was determined by intracellular cytokine staining for IFN-γ and TNF-α. T cells were stimulated for 1 week in vitro with either helper-dependent adenovirus-transduced DC or E1-deleted adenovirus-transduced DC, and then challenged with either control DC or DC transduced with an E1-deleted adenoviral vector which did not contain a transgene. This approach allowed us to discriminate viral-specific T cell activation from non-specific activation. As with the proliferation assay, DC transduced with the same amount of either vector stimulated similar frequencies of adenoviral-specific T cells (10.8% of CD4+ cells and 8.2% of CD8+ cells for the E1-deleted; 10.5% of CD4+ cells and 6.9% of CD8+ cells for the helper-dependent).
These results suggest that although helper-dependent adenovirus evokes a vector-related immune response similar to that generated by E1-deleted adenovirus, helper-dependent adenoviral vectors can be used to obtain higher transgene expression in human DC. We conclude that smaller amounts of helper-dependent adenovirus may be required to effectively express antigen in DC, and may therefore limit the degree of potentially deleterious antiviral responsiveness generated. Now that the value of this alternative vector system has been demonstrated, we are preparing Her-2/p185-expressing vectors (Her-2/p185 cDNA generously provided by Dr. Dihua Yu, M.D. Anderson, Houston, TX) to be evaluated for their use in DC-based therapy.

In summary, these research accomplishments support our overall hypothesis, i.e. that the processing and presentation of multiple tumor antigen epitopes by DC may be the most efficient and effective way of stimulating anti-tumor antigen-specific T cell responses. In addition, we have established that helper-dependent adenoviral vectors are a viable alternative to E1-deleted adenoviral vectors for the expression of tumor antigens in DC. The work accomplished this year has resulted in two abstracts (see Appendix) and two manuscripts.

Future plans include further characterization of the anti-tumor T cell responses generated by tumor antigen-loaded DC. Antigen-specific T cells will be isolated based on their tumor-specific production of IFN-γ, and cloned by limiting dilution followed by expansion in vitro. The development of these clones is essential for the complete evaluation of the T cell response, as well as to assess DC antigen-loading in a more quantitative manner. In other studies, we plan on investigating the role that DC may play in the immune response to tumor in patients receiving treatment with chemotherapy and/or Herceptin.

TRAINING ACCOMPLISHMENTS:

Promotion received:
In the past year, I received a promotion from Adj. Assistant Professor Step II to Step III (effective 7/01/02).

Funding applied for:
I will be revising and resubmitting my NIH R01 and American Cancer Society grant applications for the October 2002 deadlines.
KEY RESEARCH ACCOMPLISHMENTS

- Determined that DC take up tumor antigen (p185 neu) expressed by apoptotic tumor cells, using a newly develop procedure to measure intracellular Her-2 antigen.

- Determined that T cells from breast cancer patients produce IFN-γ and TNF-α in response to stimulation with E75 peptide-loaded DC, but that this stimulation does not lead to the development of anti-tumor cytotoxicity.

- Determined that T cells stimulated with apoptotic tumor-loaded DC or E75 peptide-loaded DC show similar MHC-E75-tetramer staining, suggesting that processing of apoptotic tumor by DC leads to the presentation of immunodominant tumor antigen peptides to T cells.

- Determined that T cells stimulated with apoptotic tumor-loaded DC (as compared to tumor peptide-pulsed DC) show enhanced responsiveness to Her-2 and other tumor antigens as assessed by IFN-γ production.

- Determined that the helper-dependent adenoviral vector was 50 to 275-fold more efficient than the E1-deleted adenoviral vector at expressing transgene encoded proteins in human DC.

- Determined that the enhanced expression by helper-dependent adenoviral vectors was due to increased expression of transgene mRNA, suggesting that smaller amounts of helper-dependent may be required to effectively express antigen in DC, and may therefore limit the degree of potentially deleterious antiviral responsiveness generated.

- These research accomplishments support our overall hypothesis, i.e. that the processing and presentation of multiple tumor antigen epitopes by DC may be the most efficient and effective way of stimulating anti-tumor T cell responses.
REPORTABLE OUTCOMES

1. Promotion from Adj. Assistant Professor Step II to Step III (effective 7/01/02).

2. Abstract entitled "A multi-epitope approach to antigen-loading of dendritic cells (DC) enhances T cell immune responses to the Her-2 tumor antigen" was presented at the 93rd Annual Meeting of the American Association for Cancer Research, San Francisco, CA, April 6-10, 2002.

3. Abstract entitled "Helper-dependent adenovirus produce superior transgene expression in human dendritic cells, but still stimulate antiviral immune responses" was submitted for presentation at the 17th Annual Meeting of the Society for Biological Therapy, to be held in San Diego, CA, November 7-10, 2002.

4. Manuscript entitled "Helper-dependent adenoviral vectors efficiently express transgenes in human dendritic cells but still stimulate antiviral immune responses" (MD Roth, Q Cheng, A Harui, SK Basak, K Mitani, TA Low, SM Kiertscher) has been revised and resubmitted for publication.

5. Manuscript entitled "A multi-epitope approach to antigen-loading of dendritic cells (DC) enhances T cell immune responses to the Her-2 tumor antigen" (SM Kiertscher and MD Roth) to be submitted for publication 8/02.
A MULTI-EPITOPE APPROACH TO ANTIGEN-LOADING OF DENDRITIC CELLS (DC) ENHANCES T CELL IMMUNE RESPONSES TO THE HER-2 TUMOR ANTIGEN

Sylvia M. Kiertscher and Michael D. Roth

UCLA School of Medicine
Los Angeles, CA 90095-1690

Advanced stage breast cancer continues as a leading cause of cancer death and innovative approaches to treatment are needed. One obstacle in using DC to treat breast cancer has been the need to identify effective mechanisms for loading them with breast cancer antigens. Prior approaches have focused on pulsing DC with HLA-A2-specific immunodominant peptides derived from the p185/neu protein. While this approach has provided an important proof of principle and advanced our understanding of anti-cancer immunity, clinical responses have been limited. We hypothesize that alternative methods of DC antigen-loading which provide DC access to the entire antigen and allow each person’s DC to process p185/neu antigens regardless of their HLA type, may be a more effective therapeutic choice. One such approach is the use of apoptotic tumor cells as a source of tumor antigen. Purified CD3+ T cells from HLA-A2* donors were stimulated on a weekly basis with monocyte-derived DC pulsed with either apoptotic SKBr3 (Her-2-overexpressing) breast cancer cells or the HLA-A2 Her-2/p185 peptide E75 (p369-377). The T cells were evaluated for IFN-γ and IL-10 production by intracellular cytokine staining following a 5 hour stimulation with unloaded DC, apoptotic MCF-7/neo loaded DC, or apoptotic MCF-7/her-2 loaded DC. The specific response to Her-2 was assessed by evaluating the difference between the MCF-7/neo and MCF-7/Her-2 responses. With apoptotic SKBr3 tumor-loaded DC, the T cells showed measurable IFN-γ production at 10-14 days of culture (2 stimulations), while T cells stimulated with DC pulsed with E75 Her-2 peptide required at least 21-28 days of culture (3-4 stimulations). Neither group demonstrated IL-10 production. The anti-Her-2 reactivity induced after 2 stimulations with apoptotic SKBr3-loaded DC was composed of both CD8 and CD4 responders and was higher than that of the E75-stimulated T cells in terms of both percentage positive (6.2% vs. <0.01% for CD4+, 2.3% vs. < 0.01% for CD8*) and intensity of expression (linear fluorescence intensity of 427 vs. 70 for CD4, 197 vs. 94 for CD8*). These results suggest that apoptotic tumor-loaded DC stimulate a larger number of specific T cells at an earlier time point than E75-peptide-loaded DC, and that the multi-epitope approach to antigen-loading of DC is a viable method for enhancing T cell anti-tumor responsiveness.

HELPER-DEPENDENT ADENOVIRUS PRODUCE SUPERIOR TRANSGENE EXPRESSION IN HUMAN DENDRITIC CELLS, BUT STILL STIMULATE ANTIVIRAL IMMUNE RESPONSES

Roth MD, Cheng Q, Harui A, Basak SK, Mitani K, Low TA, Kiertscher SM

David Geffen School of Medicine at UCLA
Los Angeles, CA, 90095.

Dendritic cells (DC) are antigen-presenting cells that efficiently process antigens and stimulate antigen-specific immunity. Several methods have been used to load DC with target antigens for use in immunotherapy, including transduction with adenoviral (AdV) vectors encoding the protein of interest. However, pre-existing immunity to AdV, and enhancement of this immunity following repeated administration of viral-transduced DC, may limit their clinical efficacy.

We hypothesized that helper-dependent AdV (Hd-AdV), in which viral coding regions are replaced by human stuffer DNA, may present an alternative approach. To evaluate their immunogenicity, human monocyte-derived DC were transduced with equivalent viral particle numbers of either replication-deficient E1-deleted adenovirus (E1-AdV) or Hd-AdV. Transduced DC were then compared for their ability to stimulate antiviral T cells, and for their expression of the transgene product, β-galactosidase.

DC transduced with Hd-AdV stimulated the proliferation of autologous T cells to the same level as those transduced with E1-AdV, with the capacity to stimulate proliferation directly correlating with viral load. Identical frequencies of viral-specific T cell responders were observed by intracellular cytokine analysis. UV-inactivation of the vectors did not prevent T cell activation, suggesting that anti-viral responses were directed primarily against the pre-formed viral capsid proteins and did not require viral gene expression.

Interestingly, Hd-AdV proved to be 50 to 275-fold more efficient than E1-AdV at expressing β-galactosidase protein in human DC. PCR analysis demonstrated similar transduction efficiencies, but RT-PCR revealed much higher expression of β-galactosidase mRNA following transduction with Hd-AdV. These results suggest that smaller amounts of Hd-AdV may be required to effectively express antigen in DC, and may therefore limit the degree of antiviral responsiveness generated. We conclude that although Hd-AdV evokes a vector-related immune response similar to that generated by E1-AdV, Hd-AdV vectors can be used to obtain higher transgene expression in human DC.

Abstract submitted for presentation at the 17th Annual Meeting of the Society for Biological Therapy, to be held in San Diego, CA, November 7-10, 2002.