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Vaccines to Treat Breast Cancer

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Dendritic cells (DC) are highly potent antigen-presenting cells that are currently being tested as adjuvants in cancer vaccine trials. DC have the capacity to take up various types of molecules and can be loaded with tumor-associated antigens (TAAs) in various forms. A novel DC-based approach is vaccination with fused DC-tumor cell hybrids. The fusion hybrid combines the antigen-presenting functions of DC with tumor cell expression of multiple TAAs (both known and unknown), providing for optimal stimulation of a broad repertoire of tumor-specific T lymphocytes. We have applied an optimized procedure that combines the generation of large numbers of clinical-grade DC together with large-scale electrofusion of DC and tumor cells. Because primary breast tumor tissue was not available, we tested electrofusion of DC and tumor cells using the MCF-7 breast cancer cell line. For comparison, a variety of tumor cell lines derived from diverse tumor types were also used. Primary findings are: 1) DC-tumor hybrids can be efficiently and reproducibly generated by our electrofusion method (8-10% hybrid efficiency), and 2) DC-tumor hybrids are functionally active in stimulating antigen-specific CTL responses. These results are promising for the development of DC-tumor hybrids for the treatment of breast cancer, as well as other cancer types.				
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INTRODUCTION

Dendritic cells (DC) are highly potent antigen-presenting cells that are gaining status as a preferred adjuvant for cancer vaccine immunotherapy (1, 2). DC derive their potency from the expression of MHC class I and MHC class II molecules, costimulatory molecules and adhesion molecules that provide secondary signals for the stimulation of naive T cells, CD4+ T-helper cells, CD8+ cytotoxic T lymphocytes (CTLs), NK and NKT cells (1, 2). Because DC have the capacity to take up various types of molecules, the cells can be loaded with tumor-associated antigens (TAAs) in various forms and applied as vaccines (3). A novel DC-based approach is vaccination with DC-tumor cell hybrids generated by fusion of tumor cells with DC to combine sustained tumor antigen expression with the antigen-presenting and immune stimulatory capacities of DC. In animal models, immunization with DC-tumor cell hybrids can effectively provide anti-tumor protection or eradicate established disease (4-7). Hybrids of autologous DC comprised of tumor cell lines or primary human tumor cells (including breast carcinoma cells) have been shown to induce CTL responses against autologous tumor cell types in vitro (8-12). Two recent phase I clinical trials for the treatment of renal cell carcinoma and glioma have demonstrated that vaccination with DC-tumor cell hybrids can safely induce anti-tumor immune responses in patients (13,14). Traditional fusion technology using polyethylene glycol (PEG) is hampered by a lack of reproducibility and difficulties in method standardization. As an alternative, electrofusion has been used for production of DC-tumor cell hybrids (5-7, 13).

Since this proposal was originally submitted, a number of pre-clinical reports have described anti-tumor responses generated by DC-tumor cell fusions. With this consideration we reoriented our project toward clinical trial development. We have applied an optimized procedure that combines the generation of large numbers of clinical-grade DC (15-17) together with large-scale electrofusion of DC and tumor cells. Our inability to obtain primary breast tumor tissue and patient-derived DC resulted in our application of normal donor DC and an allogeneic human breast tumor cell line (MCF-7) for our fusion studies. As a comparison, a variety of tumor cell lines derived from diverse tumor types were also used.

BODY

Production of Clinical-Grade Dendritic Cells.

For these studies, we have incorporated a clinically compatible, GMP process for the differentiation of monocyte-derived DC from leukapheresis product (Immuno-Designed Molecules, Paris, France; 15-17). DC processing was performed in the Immunotherapy and Cell Processing Facility, Arizona Cancer Center (Co-investigator, K. Trevor, Director). A key advantage for therapeutic application is that this culture system is closed-bag, decreasing the likelihood of contamination. The process entails the culture of whole PBMC (5×10^6 cells/ml) derived from leukapheresis product (200 ml) in gas permeable bags in serum-free AIM-V media containing GM-CSF (500 IU/ml) and IL-13 (50 ng/ml). DC are isolated by counter flow elutriation on day 7 with collection occurring when larger DC are detected using a Coulter Z2 Cell Size Analyzer. Up to 10^9 DC has been generated from a single patient leukapheresis product containing 10^{10} PBMC. Quality control and assurance of the DC product includes: 1) yield of DC; 2) DC phenotype as determined by marker antibody immune staining followed by FACS analysis (Arizona Cancer Center Flow Cytometry Facility); and 3) bacterial, fungal and mycoplasma sterility during culture and of the final product.

As determined by FACS forward and side scatter (FSC and SSC, respectively), the initial day 0 leukapheresis product contains mainly smaller cells typical of the high numbers of lymphocytes

present in a leukapheresis sample; the day 7 elutriation product comprises larger cells representative of DC (Fig. 1A).

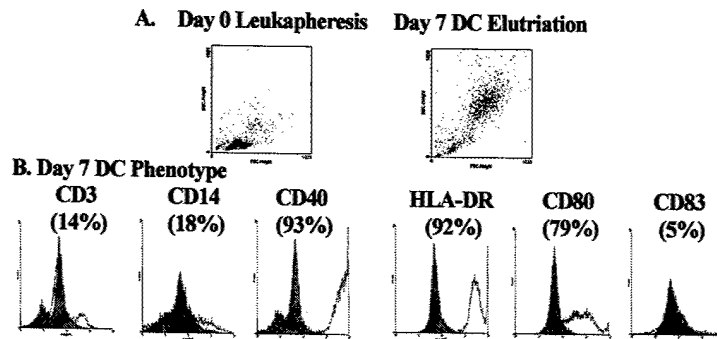


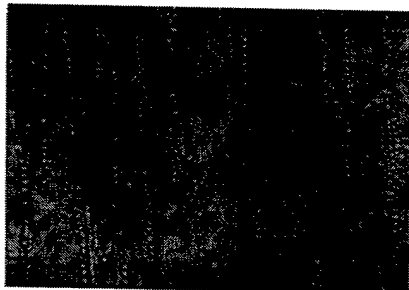
Figure 1. A. FCS and SSC FACS analysis indicating the morphology of the initial day 0 leukapheresis sample and day 7-elutriated DC. B. Marker phenotype analysis of day 7-elutriated DC. Cells were immunostained either with fluorescent-conjugated monoclonal antibodies directed against the indicated markers (open histograms) or fluorescent-conjugated isotype control antibody (gray histograms). The percent positivity for each marker is indicated.

FACS phenotype analysis of day 7-elutriated DC demonstrates that the cells are characteristic of immature DC with low level detection of the monocyte marker CD14, obvious expression of HLA-DR (MHC II) and CD40, low level expression of the co-stimulatory molecule CD80, and no expression of the maturation marker CD83 (Fig.1B). Only low levels of CD3+ cells are present, indicative of the purity of the DC population. This phenotype is representative of 15 independent DC isolations. In addition, the cultures are typically >90% viable. The clinical-grade DC product is to be >80% HLA-DR+, >50% CD80+ and contain >70% larger (DC) cells. Batches of elutriated DC are frozen in liquid nitrogen at 2.5×10^7 cells/ml in AIM V medium containing 10% DMSO, 4% human serum albumin. Cells thawed at 3-4 weeks post-freezing are >90% viable with retention of DC markers.

Electrofusion Methodology.

Electrofusion has several advantages over traditional PEG fusion methods when considering clinical application, including 1) fewer cell manipulations; 2) the ability to standardize the method versus relying on individuals to perform PEG fusion identically each time; and 3) the potential for developing a closed system. Briefly, electrofusion entails a pre-fusion alignment of the cells via application of an alternating current (dielectrophoresis, Fig. 2A) followed by membrane fusion under a direct current pulses. The cells then experience a post-fusion alternating current alignment. For our studies, Cyto Pulse Sciences, Inc. (Columbia, MD) provided the fusion chambers, cytofusion medium, power generator and power supply. We have tested a 6-ml electrofusion chamber, which permits fusion of large quantities of cells ($>2.4 \times 10^7$ cells) at one time. The chamber is a co-axial electrode (4mm gap) that was mathematically modeled for a balance between a highly non-uniform electric field for cell alignment and a uniform electric field desired for cell fusion using high voltage direct current pulses (Fig. 2B).

A.



B.



Figure 2. A. Pre-fusion alignment of cells under alternating current. B. Co-axial fusion chamber.

Characterization of Electrofusion Hybrids.

DC were generated using the VacCell® processing system and frozen in liquid nitrogen until use. Tumor cell lines were cultured in RPMI 1640 with 10% fetal calf serum. The day prior to fusion, cells were plated at ~70% confluency. On the day of fusion, DC were thawed and washed 2X in AIM-V media. Tumor cells were detached using trypsin or 0.02% EDTA solution and subsequently irradiated at 50 Gy. Our previous studies have indicated that there is no difference in the fusion capacity of non-irradiated and irradiated tumor cell populations (data not shown). For purposes of clinical application, irradiated tumor cells would be required.

The DC and tumor cells were mixed at a 1:1 ratio, pelleted and washed 3X in Cytofusion Media™ (isotonic sorbitol solution containing magnesium and calcium). The cells were then resuspended in 3 ml of Cytofusion Media™ (8×10^6 cells/ml, 2.4×10^7 cells total) and placed into the electrofusion chamber. Based on previous studies, the following electrical parameters were applied:

a) pre-fusion alternating current alignment	100 V/cm (30 s), 187.5 V/cm (10 s)
b) direct current fusion pulsing	2000 V/cm (40 us) X 4 pulses, 0.125 s pulse interval
c) post-fusion AC alignment	112.5 V/cm (55 s)
d) frequency	0.8 MHz

Following fusion, the cells were incubated in the chamber for 30 min at room temperature to mature the hybrids. An equal volume of RPMI 1640 medium was then added, and the cells were incubated for a further 15 min. Control cells were treated identically but without experiencing electrofusion. The cells were then either immediately analyzed for hybrid formation or placed in culture for later analysis. Methods of analysis included 1) determination of the percentage of multinucleated cells by cytospin followed by Diff-Quik® dye staining and counting of multinucleated cells and 2) FACS analysis of dual immune-stained cells for DC-tumor hybrids using a PE-conjugated anti-HLA-DR monoclonal antibody (for detection of the DC hybrid parent) and an FITC-conjugated anti-keratin monoclonal antibody (for detection of the carcinoma tumor cell parent). Alternatively, hybrids were detected by FACS analysis after fusion of DC that were pre-stained for 30 min with the "red" CellTracker™ dye CMFTR (2.25 uM, Molecular Probes, Eugene, OR) or tumor cells pre-stained for 30 min with the "green" CMFDA CellTracker™ dye (1.25 uM, Molecular Probes, Eugene, OR).

Figure 3A shows multi-nucleated cells obtained following electrofusion of DC with MCF-7 human breast cancer cells or A549 human lung tumor cells. Large multi-nucleated cells with up to 5 nuclei were observed. Upon visual counting, the percentage of hybrid cells with >1 nucleus ranged between 20-30% of the population. The hybrids would include homologous hybrids (DC-DC cell fusions and tumor cell-tumor cell hybrids) as well as heterologous hybrids comprised of DC-tumor cell hybrids. Dual immunofluorescent antibody staining using anti-HLA-DR antibody and anti-keratin antibody clearly confirmed the presence of heterologous fusion hybrids comprised of both DC and tumor cells (Fig. 3B)

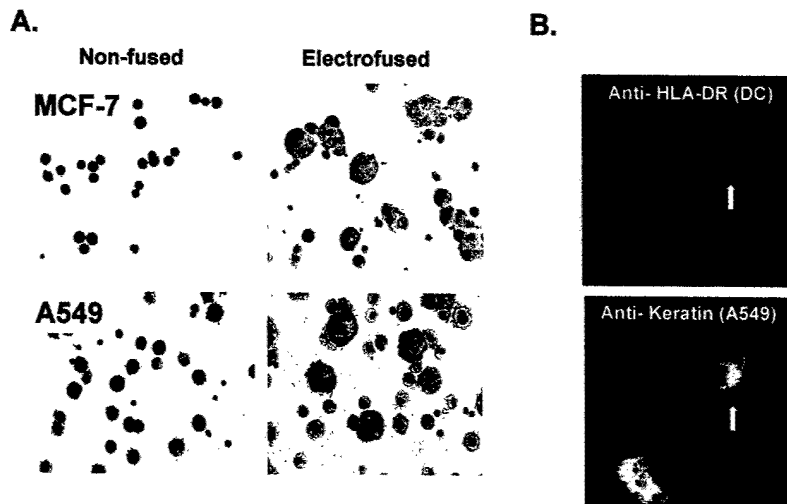
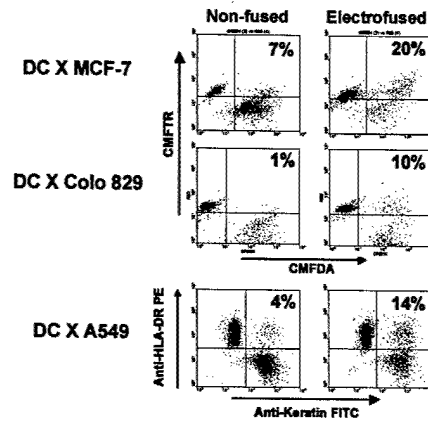


Figure 3. A. Diff-Quik[®] dye staining of non-fused and fused DC-tumor cell populations. Cells were prepared and electrofused as described. B. Dual immunofluorescent staining of DC-A549 hybrid product. Arrow indicates a fused DC-tumor hybrid cell staining for both HLA-DR and epithelial keratin filaments.

FACS analysis was subsequently used to determine total percentage of DC-tumor hybrid formation in the electrofused populations. Dot-plot FACS analyses are shown; the upper right quadrant of the dot plot represents DC-tumor hybrid cells (Fig. 4). Total percent hybrid formation was calculated by subtracting the percentage of background cells present in the upper right quadrant of the non-fused population from that observed in the electrofused population. Electrofused samples comprised of DC and MCF-7 cells contained 13% DC-MCF-7 hybrid cells at 45 min post-fusion while electrofusions of DC and melanoma cells derived from the Colo 829 cell line showed 9% DC-tumor cell hybrids (Fig. 4). For these populations, DC were pre-stained with the CMFTR dye while the tumor cells were pre-stained with the CMFDA dye. For DC-A549 electrofusions, 10% hybrid formation was observed upon dual immunofluorescent staining of populations with anti-HLA-DR antibody and anti-keratin antibody (Fig. 4).

Figure 4. FACS analyses of electrofused DC-tumor cells populations at 45 min post-fusion. For DC-MCF-7 and DC-Colo829 electrofusions, DC were pre-stained with CMFTR dye, and tumor cells were pre-stained with CMFDA dye. DC-A549 populations were immune stained with the indicated antibodies post-fusion.



As summarized in Table I., the DC-tumor hybrid efficiency at 45 min post-fusion averaged between 8-10% for each tumor type tested. There appeared to be no significant difference between the tumor types.

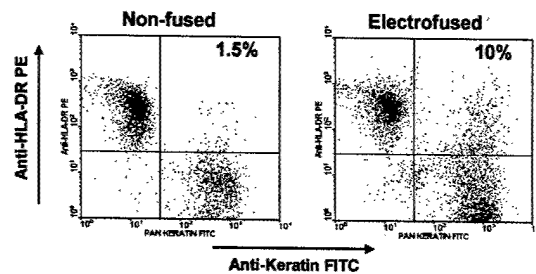
Table I. DC X Tumor Cell Hybrid Efficiencies (45 min post fusion)*.

DC-Tumor Cell Fusion	Average % Hybrid	Range
DC-MCF-7 (n = 3)	10%	9-13%
DC-Colo 829 (n = 10)	8 %	6-15%
DC-A549 (n = 7)	9 %	8-11%

*Percentages determined by FACS analysis.

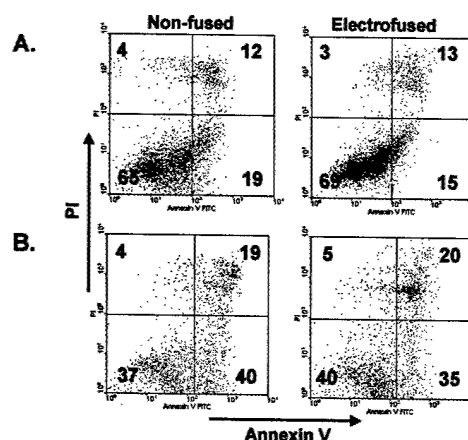
Further analysis of hybrid populations at later times post-fusion revealed the retention of DC-tumor cell hybrids. Figure 5 shows that DC-A549 cell populations retained approximately the same percentage of DC-A549 hybrids (8.5%) as was observed at 45 min post-fusion (Table I.). Multi-nucleated hybrids were also observed by visual inspection at the 24 hr time point (data not shown). Similar results were obtained for DC-MCF-7 and DC-Colo 829 populations (data not shown).

Figure 5. FACS analysis of DC-A549 populations at 24 hr post-fusion. Non-fused and electrofused populations were cultured in RPMI and 10 % FCS directly after electrofusion and subsequently immunostained with the indicated monoclonal antibodies as described.



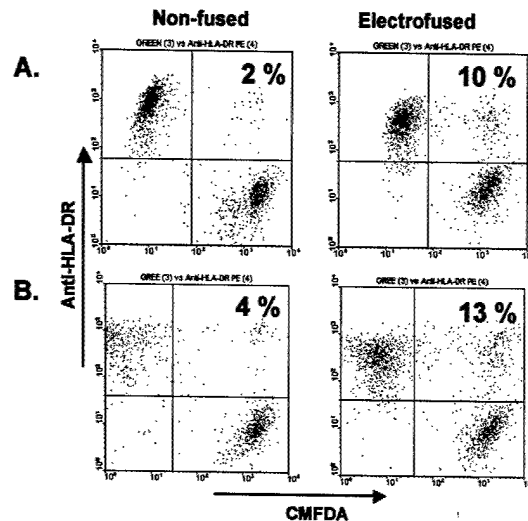
In addition, cell viabilities were examined for non-fused and electrofused populations by determining the numbers of apoptotic and necrotic cells. Propidium iodide (PI) staining was used to detect necrotic cells, and apoptotic cells were detected by staining with FITC-conjugated Annexin V (Apoptosis Detection Kit, Caltag Laboratoeis). For DC-A549 populations, cell viabilities were similar for both the non-fused and electrofused populations at 45 min post-fusion (Fig. 6A, lower left quadrant) and in populations cultured for 24 hr (Fig. 6B, lower left quadrant). However, by 24 hr post-fusion, the percentage of viable cells (lower left quadrant) decreased by ~30% in both the non-fused and fused populations with a concomitant increase in the percentage of early apoptotic cells (Annexin V⁺, lower right quadrant), as well as apoptotic cells that had undergone necrosis (PI⁺ and Annexin V⁺, upper right quadrant). These results indicate that the electrofusion process alone does not alter cell viability. The observed cell death over time is most likely attributed to radiation-induced apoptosis, as the tumor cells undergo 50 Gy irradiation prior to fusion. This particular dose of radiation is relatively low and would be required for the treatment of patients in clinical trials.

Figure 6. Viabilities of non-fused and electrofused DC-A549 populations. A. 45 min post-fusion. B. 24 hr post-fusion. Non-fused and electrofused populations were stained with propidium iodide (PI) and FITC-conjugated Annexin V according to the manufacturer. The percentage of cells positive in each quadrant is indicated: lower left, viable cells; lower right, early apoptotic cells (Annexin V⁺); upper left, necrotic cells (PI⁺); upper right: apoptotic cells that have undergone necrosis (Annexin V⁺, PI⁺).



A further consideration for clinical trial development is whether hybrid populations can be frozen and stored so as to provide multiple, sequential doses of the hybrid cell product. At 45 min post-fusion, both electrofused and non-fused populations of DC-Colo 829 cells were washed in AIM-V medium and frozen in liquid nitrogen at 2.5×10^7 cells/ml in AIM V medium containing 10% DMSO, 4% human serum albumin. One week after freezing, cells were rapidly thawed at 37°C, washed in PBS and analysed. Trypan blue exclusion dye staining indicated that the non-fused and electrofused populations were ~90% viable at 45 min post-fusion and remained at this value after freezing and thawing. As determined by FACS analysis, similar numbers of hybrids (8-9%) were observed in electrofusion products before and after freezing (Fig. 7). Similar results have been obtained for DC-MCF-7 hybrids (data not shown).

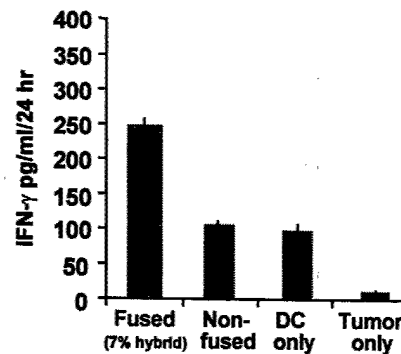
Figure 7. DC-tumor hybrids are sustained after freeze thaw. A. FACS analysis of non-fused and electrofused populations at 45 min post-fusion. B. Analysis of same populations after freezing for one week and thawing. Colo 829 cells were pre-stained with CMFDA prior to fusion. The DC marker, HLA-DR, was detected by immune staining post-fusion. % hybrids are indicated in the upper right quadrant.



T cell immune responses to DC-tumor hybrid populations.

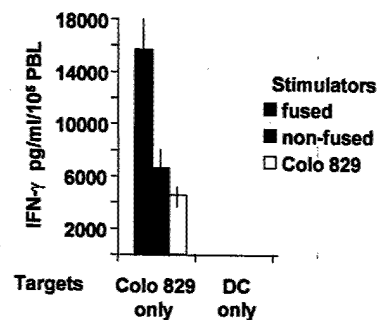
To examine whether DC-tumor hybrids were capable of augmenting specific anti-tumor antigen T cell responses, we obtained a CTL line (H3.1) specific for the melanoma antigen gp100 (kindly provided by F. Marincola, Surgery Branch, National Cancer Institute). The H3.1 CTL line specifically recognizes antigenic gp100 peptide when displayed in the context of the HLA-A2.1 major histocompatibility complex (18). DC were derived from an HLA-A2.1⁺ donor and fused with Colo 829 melanoma cells, which were previously determined to be gp100⁺. In addition, Colo 829 cells are HLA-A2.1⁻ and would not be expected to stimulate the CTL line. Antigen-specific stimulation would only occur via the processing of the gp100 expressed by the DC-Colo 829 hybrid cells and subsequent display of antigenic gp100 peptide in the context of the DC-derived HLA-A2.1⁺ complex. At 45 min post-fusion, the CTL were added to the hybrid population, as well as to non-fused cells, DC alone or Colo 829 tumor cells. After 24 hr, supernatants were removed and tested for IFN- γ expression by ELISA. The highest level of IFN- γ secretion was observed for CTL cultured in the presence of hybrid cells and was ~2.5-fold higher than those levels determined for CTL co-cultured with non-fused cells or DC only (Fig. 8). As expected, only a low amount of IFN- γ was detected when CTL were co-cultured with Colo 829 cells only.

Figure 8. Fusion hybrid induction of an antigen-specific CTL response. H3.1 CTL (5×10^3 cells) were co-cultured with the indicated targets (10^5 total cells present in the electrofused and non-fused DC/Colo829 populations and 5×10^4 DC alone or Colo 829 cells). The electrofused population indicated 7% DC-Colo829 fusion hybrids as determined by FACS analysis. After 24 hr supernatants were removed and tested for IFN- γ production by ELISA. Background IFN- γ production by H3.1 CTL that were cultured separately (5×10^3 cells) was subtracted for each culture (92 pg/ml/24 hr).



To determine whether DC-tumor hybrids are capable of enhancing primary T cell responses, autologous peripheral blood lymphocytes (PBL) were stimulated weekly for 3 weeks with electrofused or non-fused DC-tumor populations, as well as tumor cells only. The stimulated PBL populations were then tested for their ability to respond to tumor cell target as indicated by secretion of IFN- γ . Because primary tumor was not available, the experiment was designed as a mixed lymphocyte reaction (MLR) in that the tumor cell line used for this analysis is allogeneic to the normal donor from whom the DC and PBL were derived. Again, the Colo 829 cell line was used as the tumor fusion partner. When tested against Colo 829 target cells, the greatest amount of IFN- γ secretion was observed for those PBL previously stimulated with the DC-Colo 829 electrofused population. Non-fused mixtures of DC and Colo 829 cells, as well as Colo 829 cells only, were much less capable of stimulating PBL that recognized the Colo 829 target cells, as the detected levels of IFN- γ were 2- to 3-fold lower. When the PBL populations were tested against DC targets, the detected levels of IFN- γ were less than 100 pg/ml, indicating only a limited response to autologous DC, as would be expected. The results indicate that electrofused hybrids are capable of generating an enhanced allogeneic response. We were unable to demonstrate whether this response included the generation of primary TAA-specific T cells (data not shown). Only sparse numbers of antigen-specific T cells would be generated in the primary PBL populations, and the allogeneic response would be expected to dominate.

Figure 9. Autologous PBL (10^5 cells) were stimulated weekly for 3 weeks in the presence of DC-Colo829 electrofused cells, DC-Colo 829 non-fused cells or Colo 829 cells only (10^4 cells). The stimulated PBL cultures were tested against Colo 829 tumor cells or DC cells for activation-induced IFN- γ release (ratio: 10^5 PBL to 10^4 target cells). Supernatants were analyzed by IFN- γ ELISA at 24 hr post-incubation.



KEY RESEARCH ACCOMPLISHMENTS

- We have successfully incorporated two key features important for the clinical development of DC X tumor cell hybrids for vaccine application: 1) a clinically compatible process for the generation of GMP-grade DC and 2) an electrofusion method that is reproducible and provides for the simultaneous fusion of large numbers of DC and tumor cells.
- Applying optimized electrofusion parameters, the average hybrid efficiency for DC-tumor cell electrofusion is routinely 8-10% when using a number of different tumor cell types, including the MCF-7 breast cancer cell line. This results demonstrates that the electrofusion process as delivered by this system is consistent and can be standardized for clinical purposes.
- With an average of 8-10% DC-tumor hybrids in a starting population of 2.4×10^7 cells (8×10^6 cells/ml, 3 ml fusion mix), the therapeutic yield of heterologous hybrids would be $1.9-2.4 \times 10^6$ hybrids.

- By 24 hours post-fusion, DC-tumor cell hybrids are still observed. However, relative to populations early after electrofusion (45 min), there is an increase in the percentage of dead cells (PI⁺, Annex V⁺) and cells showing signs of early apoptosis (PI⁺, Annexin V⁺). This alteration in cell viability is similar in both fused and non-fused populations and may reflect radiation-induced cell death of tumor cells.
- Electrofused populations can be readily frozen for future vaccine application with no loss in viability or decline in the number of DC-tumor hybrids.
- CTL recognizing an HLA-A2-restricted gp-100 peptide are most efficiently stimulated by the DC-Colo 829 melanoma hybrid population. This result indicates that at least one known melanoma tumor-associated antigen is processed and displayed by DC-melanoma hybrid cells in the context of the parental DC HLA molecule.
- As indicated by IFN- γ release, DC-Colo 829 melanoma cell hybrid populations display an increased capacity for stimulating primary T cells that recognize the parental Colo 829 cells. In this setting the observed T cell response is most likely an allogeneic response against the tumor cell line. Even though, the finding demonstrates the functional ability of DC-tumor hybrids to stimulate primary T cell immune responses.

REPORTABLE OUTCOMES

Abstracts

Trevor, K.T., Akporiaye, E.T., Hersh, E.M., Cover, C., Landais, D., Taylor, R. R., King, A.D. and Walters, R.E. Methods for generating dendritic cell-tumor cell hybrids for clinical application. *Proceedings of the American Association for Cancer Research* 43: 3734, 2002.

Trevor, K.T., Akporiaye, E.T., Hersh, E.M., Cover, C., Landais, D., Taylor, R. R., King, A.D. and Walters, R.E. Generation of dendritic cell-tumor cell hybrids for clinical vaccine application. *Society for Biological Therapy. J Immunother* 25: S9, 2002.

Akporiaye ET, Trevor KT, Cover K, Lou S, King A and R. Walters. Generation of dendritic cell-tumor hybrids for clinical application. DOD Breast Cancer Research Program Meeting, Orlando, Florida, September 25-28, 2002.

Trevor KT, Akporiaye ET, Hersh E, Cover C, Lou S and King AD. DC-tumor cell vaccines. Is there a future? 10th SPORE Investigators' Workshop Chantilly Virginia, July 13-16, 2002

CONCLUSIONS

Theoretically, the use of DC-tumor fusion hybrids for cancer vaccine therapy is highly attractive. Unlike other types of DC-based vaccines, the hybrids have the capacity for sustained expression of the entire tumor antigen repertoire within the context of the superior immune stimulatory capacity of the DC. Yet, the application of DC-tumor hybrids has been limited with only two clinical trials reported for the treatment of renal cell carcinoma and melanoma (13,14). Although promising, additional clinical studies are required with the inclusion of other cancer types. With respect to breast cancer, one study has described the successful fusion of DC with tumor cells

derived from human breast cancer cell lines or primary breast carcinoma (8). In contrast to our own report, hybrids were induced using PEG methodology (8).

A major issue for the application of DC-tumor hybrids is the development of a compatible and reproducible procedure that can be readily translated to the clinic. In this study, we have successfully incorporated a GMP process for the generation of large numbers of DC. Furthermore, we demonstrate that 1) large numbers of DC-tumor hybrids can be consistently generated by our electrofusion method for clinical vaccine purposes, and 2) DC-tumor hybrids generated in this system are functional and possess immune stimulatory capacity for generating T cells recognizing target tumor cells.

The percentage of observed heterologous DC-tumor cell hybrids (8-10%) induced by electrofusion appears to be independent of tumor type, which portends well for the translation of this technology to a variety of tumor types, including breast cancer. However, in this study we were unable to attain primary tumor tissue. A general problem for any clinical study requiring primary cancer cells is that the amount, as well as quality, of tumor can rarely be guaranteed. Moreover, the disaggregation and retention of primary tumor cells can be difficult, especially when applying FDA standards. An alternative is to apply allogeneic cell lines, as we did in this study. Because allogeneic cell lines are easily cultured and express TAAs common to primary tumors, allogeneic cell lines are currently being applied in a variety of cancer vaccine trials (19). A disadvantage to their use is the cost associated with certifying the particular cell line(s) for translational purposes. For clinical implementation, we would need to clarify the source of the tumor fusion parent.

Although the DC-Colo829 hybrids can functionally stimulate antigen-specific CTL responses, the DC cells used in our study are classified as immature. More recent studies have demonstrated that immature DC may give rise to immune suppressive effects *in vivo* and that mature DC should be utilized in clinical trials (1,2). Furthermore, mature DC possess an improved functional capacity based on increased expression of MHC complexes, co-stimulatory molecules, and cytokines (Th1-associated IL-12), as well as altered chemokine receptors that facilitate lymph node trafficking (1,2). Further studies are required to determine the maturation ability and functional capacity of DC-tumor hybrids upon treatment with known DC maturation agents, such as TNF- α , poly I:C and cytokine mixtures.

Finally, although DC-tumor cell hybrids are assumed to have an enhanced ability to generate anti-tumor T cell responses, other types of DC-based vaccines have shown efficacy in animal models and humans. These include DC pulsed with whole tumor antigen using necrotic or apoptotic tumor cells, DC pulsed with single TAA or TAA peptides and DC transfected with DNA encoding TAA or transduced with viral vectors encoding TAA (3). One report has indicated that DC X leukemia cell hybrids are more efficient at stimulating *in vitro* CTL activity than are DC pulsed with apoptotic or necrotic leukemia cells (12). Obviously, further comparative studies in this area are required.

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APPENDICES

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