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We postulated that the production of prostanoids, principally prostaglandin E_2 (PGE_2), by human breast cancers contributes to the lack of a significant immune response to breast cancer cells. We have conducted a series of experiments to show that human breast cancer cells commonly secrete soluble agents that directly inhibit T lymphocytes. Following up on our preliminary data, we demonstrated that one of the major inhibitory factors made by breast cancer cells is PGE_2. Specifically, tumor conditioned media containing PGE_2 is able to inhibit T cell proliferation. When PGE_2 is depleted from the media or when PGE_2 synthesis is inhibited, tumor conditioned media no longer inhibits T cell proliferation. We also demonstrate that over expression of cyclooxygenase alone is sufficient to induce PGE_2 synthesis and T cell inhibition. Finally, we have found that membrane proteins in breast cancer cells also contribute to the lack of the immune response to breast tumor derived antigens.

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Human breast cancer derived PGE_2 inhibits B7-1 induced T cell proliferation

Key words: breast cancer, immunotherapy, B7-1, immunosuppressive factors, PGE_2

Introduction

The principal goal of this study is to understand why breast cancer cells are able to evade the host immune system despite the presence of tumor antigens and tumor antigen-specific T lymphocytes. We postulated that the production of prostanoids, principally prostaglandin E_2 (PGE_2), by the tumor directly contributes to the lack of a significant immune response to breast cancer cells. We have conducted a series of experiments to show that human breast cancer cells commonly secrete soluble agents that directly inhibit T lymphocytes. Following up on our preliminary data, we demonstrated that one of the major inhibitory factors made by breast cancer cells is PGE_2. While there have been isolated reports of prostaglandin synthesis by human breast cancers, those studies used only a few cell lines. Therefore, it was important to establish that this is a general phenomenon of human breast cancer cells. Previous investigators have postulated a variety of effects that are mediated by breast cancer produced PGE_2. Most of the prior work has focused on the direct effects of PGE_2 on the growth of human breast cancer. We believe that an equally important function of PGE_2 is to directly alter or suppress the immune response to breast cancer cells. We have shown that the expression of cyclooxygenase (COX) and the resultant production of PGE_2 are sufficient to abrogate the T cell response to tumors cells in a vaccination model. Furthermore, this can be block by inhibiting the COX mediated production of PGE_2..

The initially proposed first year Aims and related Tasks are shown and indicated in the relevant portions of the results sections below.

Aim #1 We will determine the frequency of PGE_2 production in human breast cancers and determine whether inhibition of PGE_2 synthesis eliminates the T cell inhibitory factor in breast cancer media.

Task 1. Analyze tumor conditioned media from human breast and determine the frequency of T cell inhibitory factors. (Months 1-6)

Task 2. Prepare lipid extracts of the tumor condition media and establish that this fraction contains all or part of the inhibitory factors. (Months 1-12)

Task 3. Quantify the PGE_2 levels in the breast cancer conditioned media and correlate their levels with the inhibitory effects produced the conditioned media and authentic PGE_2. (Months 1-12)

Tack 4. Demonstrate that addition of cyclooxygenase inhibitors blocks PGE_2 synthesis with concomitant decline inhibitory activity. (Months 7-12)

Task 5. For those specimens producing PGE_2 and TGFβ we will determine whether blocking both agents has an additive effect. (Months 10-14)

Task 6. We will characterize three murine mammary cancer cell lines (SCK cells, T2994 and MT901 cells) for PGE_2 and TGFβ production and T cell inhibition. (Months 9-12)

Body of the Report

Breast carcinoma cells produce high levels of agents known to alter T cell responses including transforming growth factor β (TGFβ, 1), interleukin 10 (2), and PGE_2 (3). PGE_2 exerts heterogeneous effects on diverse lymphocyte subpopulations. Whereas it increases the expression of IL-12 receptors on lymphocytes (4), promotes immunoglobulin synthesis (5), it also inhibits IL-17 production (6), and reduces cytotoxic activity (7) and lymphocyte proliferation (8-10) In the mouse thymus PGE_2 production plays a critical role in T cell maturation (11,12). Furthermore, PGE_2 has been shown to inhibit differentiation of lymphokine activated killer cells (LAK), suppress of natural killer cell (NK) activity (13-15), and down-regulate humoral immune responses (16). The inhibition of T cell proliferation in lymphocyte cultures by PGE_2 is mediated in part by the down-regulation of MHC class II expression on antigen presenting cells (17-19) and through suppression of cytokine production (20-21). PGE_2 is produced by cyclooxygenase (COX) mediated oxidation of arachidonic acid and has been found in some human breast cancer cell lines (22). There are two isoforms of COX, designated COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, mediates the synthesis of prostaglandins including PGE_2 and is required for constitutive physiological functions, such as maintaining gastrointestinal, kidney and reproductive functions (23). COX-1 was found to be over expressed in 30 of 44 breast tumor tissues compared to normal breast epithelium (24). COX-2 expression can be induced by cytokines, growth factors, oncogenes and tumor promoters (25), is reportedly up regulated in a some metastatic breast cancer cell lines (26) and leads to high levels of PGE_2 production by these cells. Given the development of idiopathic chronic suppurative peritonitis and bowel inflammation in COX-2 knockout mice (27), the involvement of COX-2 in inflammatory and immune responses appears likely but is as yet poorly understood. Similarly, the consequences of COX expression and PGE_2 production for anti-tumor immune responses remain poorly understood.

Breast cancer cells produce soluble factors that suppress lymphocyte proliferation. (Tasks 1, 3 & 6)

We sought to determine if other human breast cancer cell lines produced soluble T cell inhibitory factors. In addition to MCF-7 cells seven of eight breast cancer cell lines tested (BT-20, MCF-10, BT-474, MDA-MB 231, SUM52PE, SUM149PT and SUM190PT) also secreted soluble factors that were capable of inhibiting the proliferation of MN cells (Table 1). By contrast, CM from two cell lines, SUM185 cells and the human breast epithelial HBL-100 cells did not inhibit lymphocyte proliferation (Table 1) under the same experimental conditions. Thus, the production of soluble factors that inhibit lymphocyte proliferation is a common though not universal characteristic of human breast cancer cells.

Human and mouse cell lines	% inhibition of PHA stimulated MN cells	PGE_2 production (pg/ml)
HBL-100* (breast epithelial)	5%	100
BT-20	41%	0
MCF-10	99%	300
MCF-7	97%	40
BT-474	96%	40
MDA-MB 231	90%	260
SUM52PE	66%	30
SUM149PT	56%	>1000
SUM185PE	4%	0
SUM190PT	51%	>1000
SCK (A/J mouse strain)	75%	65
T2994 (BALB/C mouse strain)	15%	40
NT5 (FVB mouse strain)	100%	>1000
MT901 (BALB/C mouse strain	100%	Not determined

Table 1A. PGE_2 levels and inhibition of PHA stimulated MN cell proliferation by CM from a normal human breast epithelial cell line (HBL-100) and human breast carcinoma cell lines (all others).

Human Breast Cancer Cells	PHA	PMA/ionomycin
MCF-7	97%	98%
MCF-10	99%	98%
BT-474	96%	93%
BT-20	41%	0%
MDA-MB-231	90%	0%
T47D	0%	0%
SK-BR-3	0%	0%

Table 1B. The inhibitory effect of conditioned media (CM) from different tumor cell lines on the proliferation of mitogen stimulated mononuclear cells (MN). The percent inhibition was calculated as follows: % inhibition = 100% -(100% x cpm of mitogen stimulated MN cells incubated with sample CM at a dilution of 1:4/cpm of mitogen stimulated MN cells).

Adenoviral transfer of B7-1 to MCF-7 breast cancer cells (Tasks 3 and 4).
We have shown previously that cultured human melanoma cells do not express B7-1 and do not co-stimulate proliferation of T cells in response to PHA (40). However, when transduced with AdhB7-1 these cells acquire the ability to co-stimulate T cells. Similar to other human tumor cells all cultured human breast cancer cells examined in this study do not express endogenous B7-1 at levels detectable by flow cytometric analysis. To test whether adenovirally delivered B7-1 expression affect co-stimulatory activity to these breast cancer cells, we first transduced MCF-7 cells with AdhB7-1. Flow cytometric analysis demonstrated that >90% of MCF-7 cells transduced with AdhB7-1 expressed the B7-1 protein on their cell surface. We observed previously that many human melanoma cells adenovirally transduced to express B7-1 effectively stimulate proliferation of purified human T cells (40). To assess the co-stimulatory activity of B7-1 expressed by MCF-7 cells, we measured the proliferation of allogeneic T cells in co-culture with B7-1 expressing MCF-7 cells in comparison with B7-1 expressing WM9 melanoma cells. Purified human T cells were co-cultured with untransduced, B7-1 transduced, or lacZ transduced tumor cells. B7-1 expressing WM9 cells induced at least a 53-fold increase in T cell proliferation (Table 2) over that achieved by untransduced WM9 cells. This effect was not due to the presence of adenovirus as seen by the low stimulation index (SI) of WM9/lacZ cells (Table 2). In contrast to melanoma cells, MCF-7 cells transduced with AdhB7-1 did not stimulate T cell proliferation. To determine whether the lack of co-stimulation by MCF-7 cells was due to tumor-derived secreted factors, we added medium conditioned by MCF-7 cells (CM) to mononuclear (MN) cultures. MCF-7 CM blocked proliferation of MN preparations stimulated with either PHA (Fig. 1A) or PMA/ionomycin (Fig. 1B) indicating that MCF-7 CM exerts its effect independently of the stimulus used for T cell activation. Inhibition of lymphocyte proliferation was potent and dose-dependent with significant inhibition observed even at 1:4,000 dilution (Fig. 1A, B).

Tumor cells	T cells	cpm	sd	SI
MCF-7	-	39	7	/
	+	55	8	0.3
MCF-7/B7-1	-	48	6	/
	+	551	169	0.9
MCF-7/lacZ	-	37	7	/
	+	43	6	0.1
WM9	-	25	4	/
	+	1287	109	24
WM9/B7-1	-	32	6	/
	+	68374	11736	1340
WM9/lacZ	-	291	55	/
	+	300	236	0.2
	T cells alone	51	6	/

Table 2. Thymidine uptake and relative proliferation of human T cells cultured with B7-1 expressing MCF-7 cells or with B7-1 expressing WM9 cells. Purified T cells were co-cultured with untransduced, AdhB7-1 transduced or AdlacZ transduced tumor cells. Their proliferation is expressed relative that of T cells in the absence of tumor cells as indicated by SI. Thymidine incorporation (mean cpm) and

standard deviation (sd) were obtained from quadruplicates. B7-1 expressing MCF-7 cells failed to stimulate T cell proliferation.

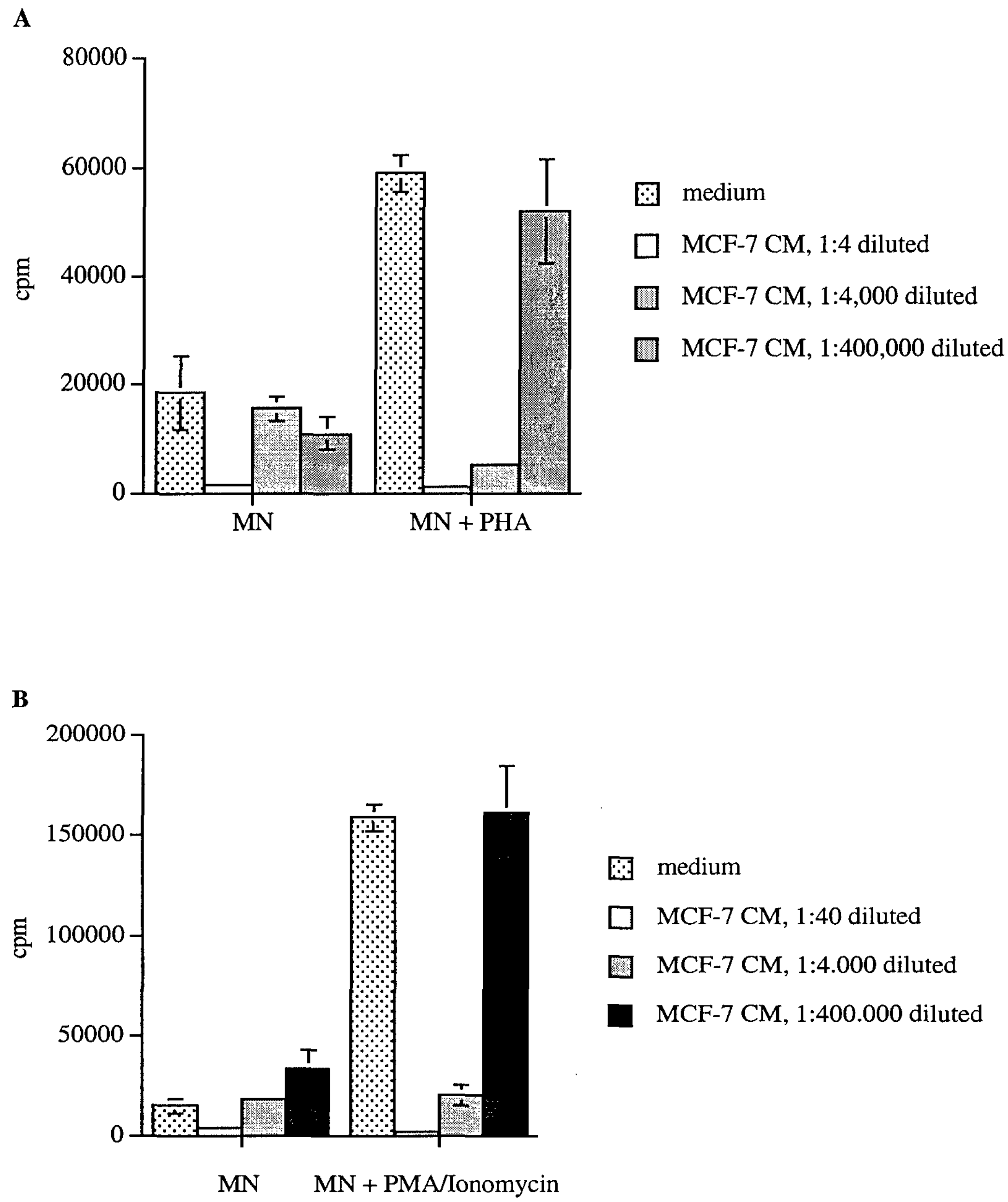


Figure 1. Thymidine uptake (cpm) of MN cells stimulated with (A) PHA or (B) PMA/ionomycin after the addition of MCF-7 CM. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. MCF-7 CM inhibited the proliferation of stimulated MN cells.

Soluble TGFβ alone does not account for the inhibitory effect of MCF-7 CM on the proliferation of MN cells in response to PHA. (Task 5)
Breast carcinoma cells including MCF-7 have been described to express TGFβ which is known to inhibit T cell proliferation (29) Therefore, we sought to determine whether MCF-7-derived TGFβ was responsible for the inhibitory effect of MCF-7 CM on T cell proliferation. We determined that the MCF-7 cells used in the present study also secrete TGFβ at a rate of 1150 pg/1x10^6 cells in a 24 h period. To assess whether TGFβ in MCF-7 CM contributes to the inhibition of lymphocyte proliferation, we evaluated the effect of MCF-7 CM on PHA-dependent MN cell proliferation in the presence of different concentrations of a neutralizing antibody that is reactive with all three known human TGFβ isoforms (Fig. 2). We have described specificity and activity of this antibody earlier (30,31). The TGFβ antibody was used in excess to block the biological effects of TGFβ found in MCF-7 CM. At the highest concentration tested here (100 μg/ml) the TGFβ antibody neutralizes 28 ng/ml of recombinant TGFβ1 as determined by TGFβ bioassay. MCF-7 derived CM contained only 34.5 pg total TGFβ (Fig. 2). At none of the concentrations tested did the neutralizing TGFβantibody affect inhibition of MN proliferation by MCF-7 CM (Fig. 2). These results effectively exclude TGFβas a cause of the inhibitory effect.

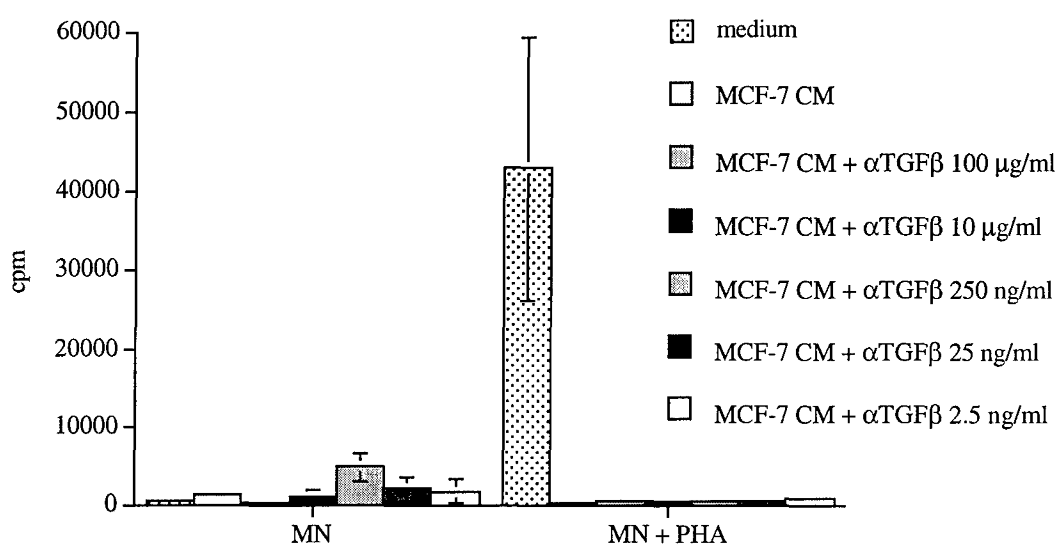


Figure 2. The effect of MCF-7 CM on the proliferation (thymidine uptake in cpm) of PHA stimulated MN cells after incubation with a pan-specific TGFβ neutralizing antibody (100 μg/ml to 2.5 ng/ml). Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. TGFβ did not account for the inhibitory effect of the MCF-7 CM.

PGE_2 production by breast carcinoma cells. (Task 3)
PGE_2, a known modulator of T cell proliferation (17,18) has been shown to be produced by some breast carcinomas (3). We measured PGE_2 production by non-tumorigenic breast epithelial cells and by the breast cancer cell lines used in this study. We found that eight of ten cell lines produced PGE_2 at levels detectable by ELISA assay (Table 1). Interestingly, MCF-7 cells produced relatively low levels of PGE_2 (40 pg/ml) when compared to SUM190PT or SUM149PT cells which produced in excess of 1000 pg/ml PGE_2. With the exception of HBL-100, all of the cell lines that produced PGE_2 also inhibited the proliferation of PHA stimulated MN cells although there was no correlation between the amount of secreted PGE_2 and the inhibitory capacity. In addition, BT-20 CM had a significant inhibitory effect although it did not produce detectable levels of PGE_2. Taken together, these results suggested that PGE_2 may play an important role in the inhibition of a proliferative response of MN cells and lymphocytes. Although commonly expressed by breast cancer cells in culture it may act in concert with other factors other factors to this process.

Indomethacin treatment of breast cancer cells restores lymphocyte proliferation. (Task 4)
We next attempted to inhibit PGE_2 production by breast cancer cells to probe the relative contribution of PGE_2 to inhibition of T cell proliferation by breast carcinoma-derived CM. COX is expressed in some human breast cancers (24, 26) First, we investigated whether the inhibition of COX activity by indomethacin restores lymphocyte proliferation. MCF-7 cells were treated with indomethacin at a concentration of 100 μg/ml for 24 hours. Indomethacin inhibited both the COX-1 and COX-2 enzymes and reduced the PGE_2 content in MCF-7 CM cells to less than 30 pg/ml (the lower limit of detection in this assay). Indomethacin pretreatment abrogated the inhibitory effect of MCF-7 CM on PMA/ionomycin stimulated proliferation of MN cells at a 1:4,000 dilution (Fig. 3). Indomethacin containing medium itself (not exposed to tumor cells) did not influence the proliferation of MN cells in response to PMA/ionomycin. However, the proliferation of MN cells was still inhibited when untreated MCF-7 CM or CM from indomethacin-treated MCF-7 cells that was more concentrated (1:40 dilution) was added to MN cells in the presence of mitogen. This is consistent with our observation that MCF-7 CM from indomethacin-treated cells still contained residual amounts of PGE_2 detectable by liquid chromatograph-mass spectroscopy (LC-MS).

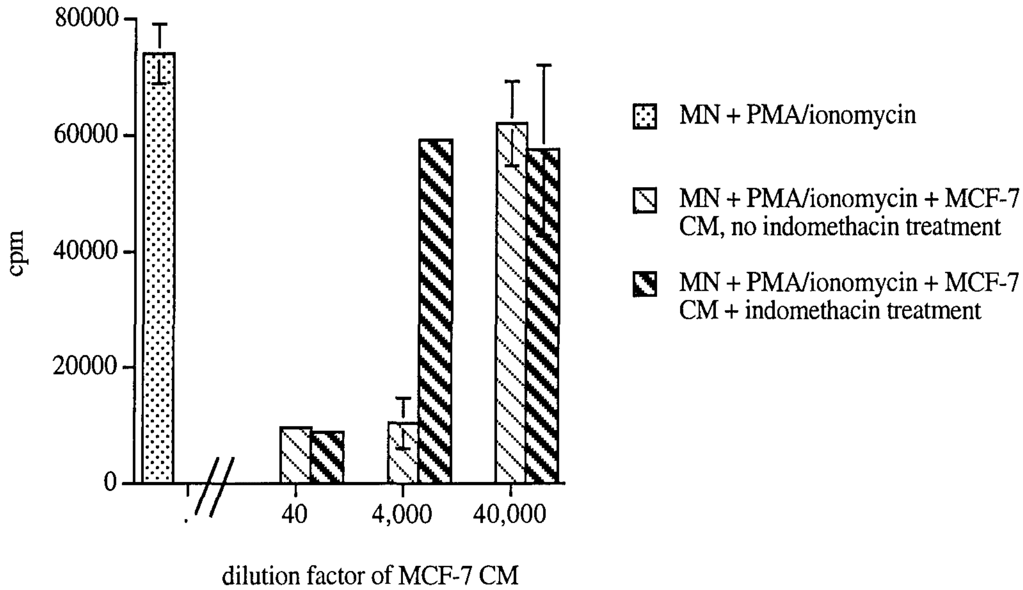


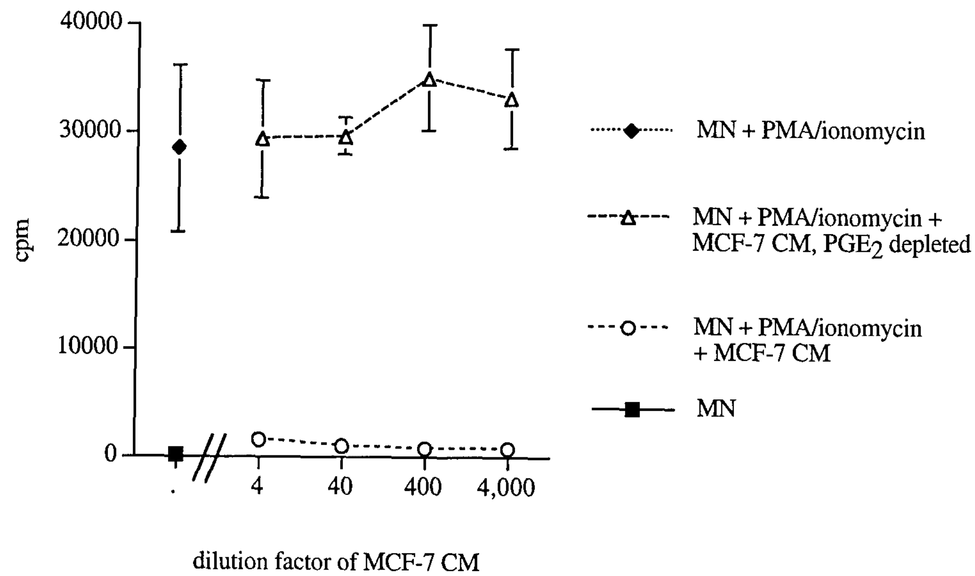
Figure 3. Indomethacin treatment of breast cancer cells restores the proliferation of lymphocytes. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. Thymidine incorporation by MN cells stimulated with PMA/ionomycin in the presence of absence of CM from MCF-7 cells treated with indomethacin. Indomethacin treatment of MCF-7 cells partially alleviated the immunosuppressive effect of MCF-7 CM.

Removal of PGE_2 from breast cancer CM elicits B7-1-dependent stimulation of MN cells and purified T lymphocytes. (Task 4)

Next, we attempted to remove PGE_2 more effectively from MCF-7 CM. We used an immuno-affinity column that binds up to 10 ng PGE_2 (62-fold more than the amount detected in the CM). MCF-7 CM passed through this immuno-affinity column completely lost its inhibitory effect on MN proliferation at all dilutions tested (Fig. 4A). These results indicate that breast cancer derived PGE_2 even at relatively low concentration (40 pg/ml) blocks mitogenic lymphocyte responses.

The effect of PGE_2 on MN responses to mitogens has been reported before (11, 16), whereas the effect of PGE_2 on B7-1 dependent T cell proliferation has not been studied in great detail. To confirm that breast cancer-derived PGE_2 contributes to suppression of the T cell response irrespective of other cell types present in MN cell preparations we performed T cell co-culture assays using MDA-MB 231 breast carcinoma cells. These cells produced markedly higher amounts of PGE_2 than MCF-7 cells (see Table 1). MDA-MB 231 cells were transduced with AdhB7-1 to overexpress B7-1 and treated with either indomethacin or NS398, a COX-2 specific inhibitor. As a control, MDA-MB 231 cells and T cells were co-cultured in ethanol containing medium since ethanol served as a solvent for the COX inhibitors. Untreated B7-1 expressing MDA-MB 231 cells produced 312 pg/ml PGE_2 whereas pretreatment with either indomethacin or NS398 reduced production of PGE_2 to undetectable levels. Untreated or untransduced MDA-MB 231 cells did not stimulate allogeneic T cells in the absence or presence of PHA (Fig. 4B). However, DNA synthesis by T cells was markedly stimulated upon co-culture with B7-1 expressing, indomethacin-pretreated tumor cells. In contrast, there was no thymidine incorporation by T cells co-cultured with ethanol treated or NS398 treated, B7-1 expressing MDA-MB 231 cells or with unmodified MDA-MB 231 cells in PHA containing medium (Fig. 4B). These results indicate that the inhibition of COX enzyme activity by indomethacin restores T cell proliferation in the presence of PHA.

A



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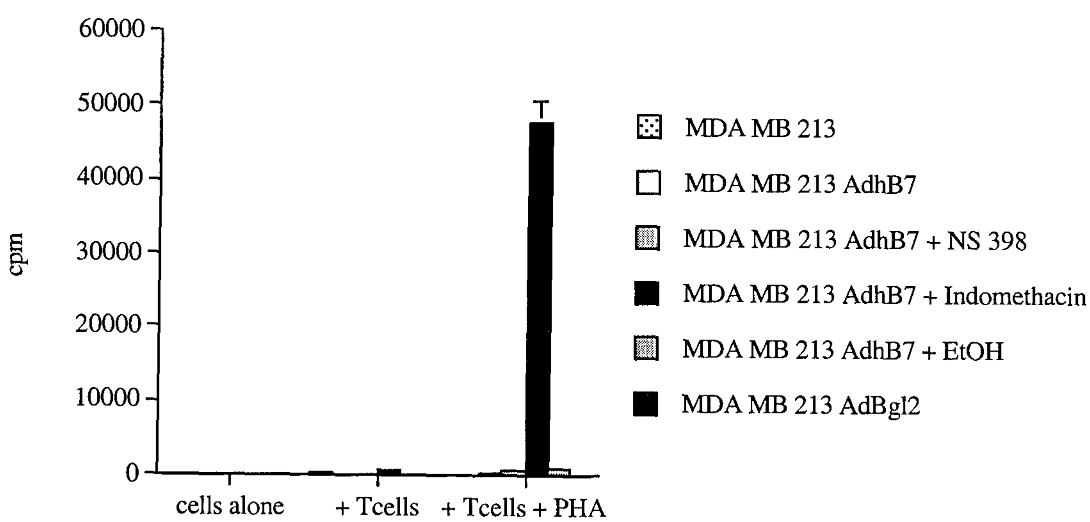

Figure 4. (above) Removal of PGE_2 from breast cancer CM elicits B7-1-dependent stimulation of MN cells and purified T lymphocytes. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. (A) Thymidine incorporation by MN cells (square), by MN cells stimulated with PMA/ionomycin (diamond), by PMA/ionomycin stimulated MN cells incubated with MCF-7 CM (circle) and by PMA/ionomycin stimulated MN cells incubated with PGE_2-depleted MCF-7 CM (triangle). MCF-7 CM inhibited the mitogenic response of MN cells. The inhibitory effect of MCF-7 CM was completely alleviated when PGE_2 was eliminated via PGE_2 affinity column. (B) Thymidine incorporation by T cells co-cultured with B7-1 expressing MDA-MB 231 cells after the treatment with indomethacin, NS 398 or ethanol. In the presence of PHA indomethacin treatment of B7-1 expressing MDA-MB 231 cells abrogated T cell proliferation.

Breast cancer derived PGE_2 inhibits B7-1-dependent T cell proliferation induced by melanoma cells. (Tasks 4 and 5) We sought to confirm the role of PGE_2 on immune responses in a different system in the absence of endogenous PGE_2 and B7-1. To this end, we turned to B7-1 transduced WM9 melanoma cells which produce no PGE_2 and stimulate T cell proliferation. When MCF-7 CM was added to T cells co-cultured with B7-1 expressing melanoma cells the proliferation of T cells was completely inhibited (Fig. 5, diamond). As shown in Fig. 5, the depletion of PGE_2 from MCF-7 CM using the immunoaffinity column restored DNA synthesis of T cells in the presence of PHA (circle), whereas the addition of exogenous PGE_2 to MCF-7 CM blocked thymidine uptake of stimulated T cells (triangle). These data indicate that PGE_2 secreted by tumor cells is essential for the inhibition of B7-1 induced T cell proliferation.

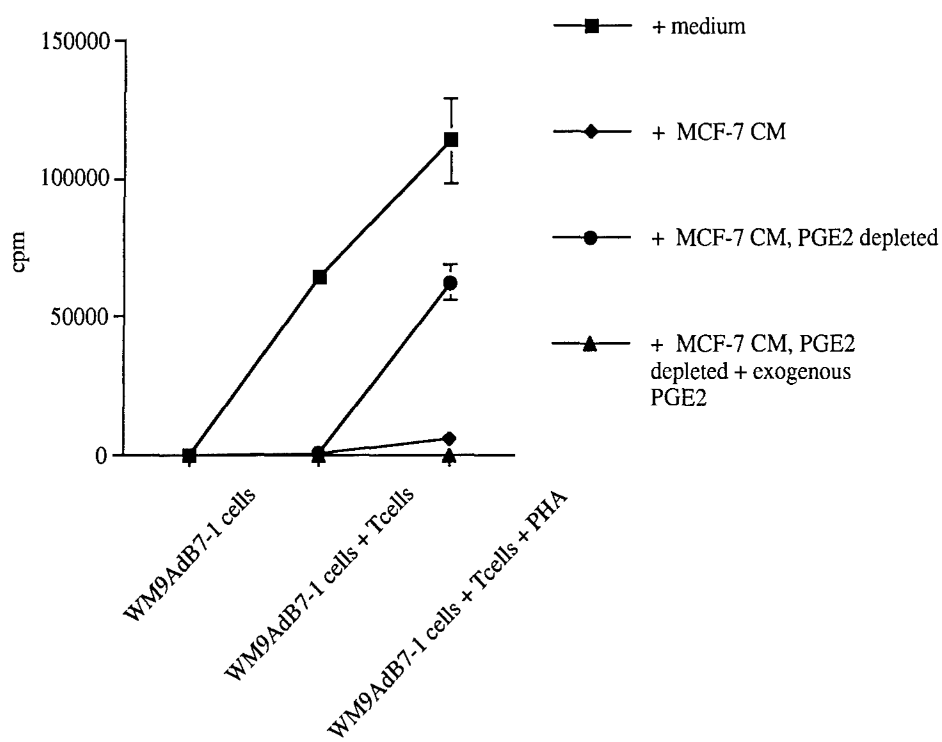

Figure 5. Breast cancer derived PGE_2 inhibits B7-1-dependent T cell proliferation induced by melanoma cells. Thymidine incorporation by T cells co-cultured with B7-1 expressing WM9 cells after the addition of MCF-7 CM (diamond), PGE_2 depleted MCF-7 CM (circle) and MCF-7 CM previously depleted of PGE_2 with exogenous PGE_2 (triangle). In the presence of PHA the proliferation of T cells co-cultured with B7-1 expressing WM9 cells and PHA was inhibited in the presence of MCF-7 CM or in the presence of MCF-7 CM that had been depleted from PGE_2 and substituted with exogenous PGE_2. The proliferation of T cells in response to B7-1 expressing WM9 cells and PHA was restored when PGE_2 depleted MCF-7 CM was added.

Tumor cells transduced to produce PGE_2 block B7-1 dependent T cell proliferation (Task 3).
To further test the idea that tumor cells block B7-1 dependent T cell mediated immune responses primarily through the production of PGE_2, we generated PGE_2 producing and B7-1 positive melanoma WM9 cells by cotransduction with AdCOX-1 and AdhB7-1. Proliferation assays using T cells co-cultured with WM9 cells modified to express B7-1 and to produce PGE_2 were performed. Allogeneic T cells were stimulated to proliferate by co-culture with B7-1 expressing WM9 cells in the absence or presence of PHA (Fig. 6). However, the proliferation of T cells was inhibited when incubated with WM9 cells transduced to express B7-1 and COX-1. There was a four fold increase of PGE_2 in the five days supernatant of T cells co-cultured with B7-1, COX-1 expressing WM9 cells (538 pg/ml) as compared to the supernatant from T cells co-cultured with B7-1 expressing WM9 cells. The allogeneic T cell response of B7-1 expressing WM9 cells was completely restored by the co-culture of T cells with WM9 cells transduced with a mixture of AdhB7-1 and AdBgl2. These results clearly indicate that the

expression of COX-1 and the resulting production of PGE_2 by WM9 cells powerfully inhibits B7-1 induced T cell proliferation.

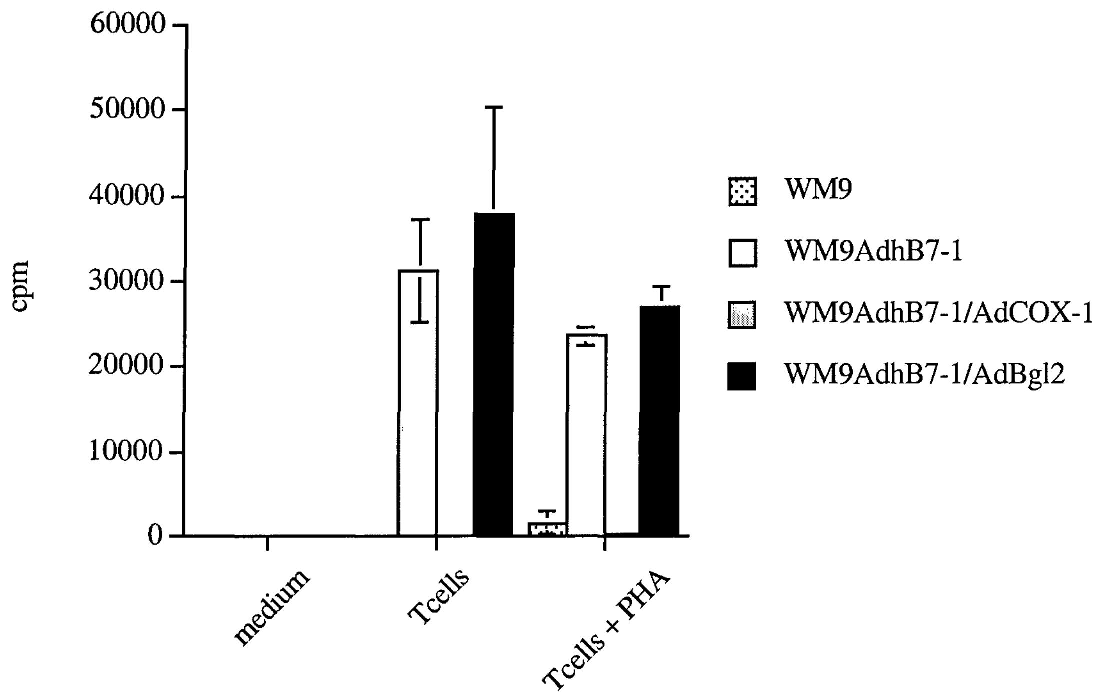

Figure 6. Melanoma cells transduced to produce PGE_2 block B7-1 dependent T cells proliferation. Thymidine incorporation by T cells co-cultured with WM9 cells co-expressing COX-1 and B7-1. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. The proliferation of T cells in response to B7-1 expressing WM9 cells was inhibited by the co-expression of COX-1 on the tumor cells.

Tumor cell membranes inhibits a mixed lymphocyte reaction (Task 4)
We prepared lipid soluble extracts of the tumor condition media but were unable to demonstrate that this fraction contained any inhibitory activity. At this point we believe that this is a technical issue resulting from air oxidation of the lipid fraction during isolation by extraction. However, we have found that membrane containing fractions of tumor cell debris are able to inhibit T cell activation in a mixed lymphocyte reaction. This activity appears to be associated with a protein, not a lipid, constituent of the tumor cell. We plan to pursue this further in our on going studies.

Discussion

The present study demonstrates that expression of the co-stimulatory molecule B7-1 on human MCF-7 breast carcinoma cells fails to elicit proliferative responses of allogeneic T cells. This is in contrast to human melanoma cells which, upon adenoviral transfer of B7-1, efficiently stimulate the proliferation of T cells(28). In addition, murine melanoma cells expressing B7-1 are rejected by syngeneic, immunocompetent mice (28, 32).

One possible cause for the observed failure of B7-1 expressing breast cancer cells to stimulate T cell proliferation is the release of immunosuppressive factors by these tumor cells. In support of this hypothesis we observed that CM obtained from eight out of ten breast cancer cell lines inhibited the proliferation of MN cells in response to PHA. In addition, soluble factors contained in CM derived from these breast cancer cell lines also inhibited the proliferation of MN cells stimulated with PMA/ionomycin. Tumor-derived TGFβ has been implicated in the progression of mammary carcinoma growth and metastasis by immune suppression. Anti-TGFβ antibody treatment inhibited breast cancer tumorigenicity and enhanced the activity of NK cells in an animal model of breast carcinoma (29). Although we found that MCF-7 cells secrete TGFβ, it appears unlikely that TGFβ is the principal source of the inhibition of T cell proliferation. First, titration experiments revealed that the inhibitory factor contained in MCF-7 CM was active at dilutions up to 1:4,000. TGFβ is produced by MCF-7 cells at a rate of approximately 1 ng/$1x10^6$ cells in a 24 h period. A 1:4,000 dilution of the MCF-7 CM would reduce the TGFβ content to concentrations far below the biologically active range. Furthermore, we found that the addition of neutralizing anti-TGFβ antibody to undiluted MCF-7 supernatant did not affect inhibition of T cell proliferation by MCF-7 CM. The concentrations of antibody used were far in excess of those needed to neutralize the effects of TGFβ in a sensitive TGFβ-responsive luciferase reporter assay.

In contrast, PGE_2 was shown to be an important contributor to the T cell inhibitory effect of breast cancer CM. Several lines of evidence support this conclusion. Indomethacin treatment of MCF-7 cells reduced PGE_2 production and partially alleviated inhibition of MN cell proliferation. Indomethacin did not completely remove the inhibitory effect which is consistent with the presence of residual PGE_2 that could be detected by LC-MS. More conclusive evidence for the involvement of PGE_2 in T cell growth inhibition was obtained when we removed PGE_2 from CM using an affinity column that specifically binds PGE_2. The selective elimination of PGE_2 from MCF-7 CM removed its MN cell growth inhibitory activity completely. Thus, inhibition of MN cell proliferation is mediated, at least in large part, by PGE_2 produced by MCF-7 cells. Furthermore, PGE_2 was detected in CM from additional breast cancer cell lines that also inhibited MN cell proliferation. The amount of PGE_2 production did not linearly correlate with inhibition of the proliferation of stimulated MN cells. CM from the immortalized, non-tumorigenic human breast epithelial cell line HBL-100 produced significant levels of PGE_2 (more than MCF-7) but did not suppress MN cell proliferation. Similarly, the CM from two cell lines (SUM149PT and SUM190PT) that produced the most PGE_2 showed only moderate inhibition of MN cell proliferation. Based on these results it appears likely that other factors in the breast cancer CM likely contribute to the immunosuppressive effect. In support of this notion we found that BT-20 cells did not produce significant amounts of PGE_2 but inhibited PHA-dependent proliferation of MN cells by 41%. This suggests that other tumor-derived factors may induce immunosuppression as previously reported (33, 34). Nevertheless, seven of nine breast cancer cell lines produced PGE_2, and all inhibited the proliferative response of MN cells to PHA. Taken together these data suggest that PGE_2 is a necessary but not always sufficient cofactor of tumor-derived immunosuppression, acting in concert with other factors.

The mechanism(s) by which PGE_2 affects MN or T cell proliferation are as yet fully understood. Intracellularly, PGE_2 was found to attenuate p59fyn phosphorylation and its kinase activity, thus suppressing T cell proliferation during burn and sepsis (35). Both suppression of lymphokine production (20,21) and down regulation of MHC class II expression on antigen presenting cells (17,18) by PGE_2 have been demonstrated. PGE_2 inhibits IL-2 production and enhances IL-4 production by T cell, thus shifting a T helper cell 1 response towards a T helper cell 2 response (36). Recently, small cell lung cancer-derived PGE_2 was found to up-regulate IL-10 production by lymphocytes and to down-regulate IL-12 production by macrophages (37) whereas in our study pure T cell cultures were used to investigate the effect of breast cancer derived PGE_2. Furthermore, the expression of CD40L on human memory T cells was blocked by PGE_2 (38). These studies suggest that PGE_2-dependent inhibition of T cell-mediated anti-tumor responses simultaneously affects antigen presentation and shifts the local cytokine milieu to a state unfavorable to an effective immune response (37).

The significance of tumor-derived PGE_2 to inhibition of cells of the innate immune system, e.g., natural killer cells was highlighted by recent reports demonstrating therapeutic efficacy in the prevention and treatment of breast cancer by non-steroidal anti-inflammatory drugs (NSAID) including indomethacin itself (14). Importantly, indomethacin reduces PGE_2 production by inhibiting the enzymatic activity of the COX-1 which is the rate-limiting enzymes in the PGE_2 biosynthetic pathway. Indomethacin has also demonstrated efficacy in the treatment of mouse mammary carcinoma cells. For example, mice injected with mammary adenocarcinoma C3-L5 cells received long-term indomethacin therapy on day 15 followed by two rounds of IL-2 administration for five days. Regression of primary tumors, reduction of lung metastases and prolonged survival were observed in the group receiving the combination therapy as opposed to the group receiving IL-2 treatment alone. Furthermore, the long-term intake of indomethacin in combination with IL-2 was shown to activate tumoricidal lymphocytes in situ (14). Here we show that a combination strategy of B7-1 transfer onto breast cancer cells along with indomethacin treatment activated the proliferation of T cells in vitro.

B7-1 immunotherapy has been reported to be effective in immunogenic tumors (39). Tumor cells are generally termed "immunogenic" when they express antigens that result in the rejection of tumor cells by syngeneic animals previously immunized with the irradiated parental tumor cells (39). Accordingly, non-immunogenic tumors are rejected when similarly tested. However, there is no consensus about the parameters that rendertumor cells more or less immunogenic. Breast cancer cells are known as non-immunogenic tumors and thus escape immune surveillance. Here, B7-1 immunotherapy was not successful in vitro in stimulating T cell proliferation. The majority of non-immunogenic breast cancer cell lines produce PGE_2. We were able to show that the production of PGE_2 specifically inhibited B7-1 induced T cell proliferation. First, B7-1 expressing MDA-MB 231 cells did not secrete PGE_2 at detectable levels after indomethacin treatment and stimulated T cells to proliferate when PHA was present. Second, the elimination of PGE_2 from MCF-7 CM restored the response of T cells to B7-1 expressing melanoma cells only in the presence of PHA indicating that the inhibition by PGE_2 can only be overcome when a strong signal through the T cell receptor was provided. Finally, in the melanoma system the sole modification of co-expression of COX-1 on B7-1 expressing WM9 cells was sufficient to inhibit T cell proliferation. These results underline the potential of a combined modification of breast cancer cells consisting of B7-1 expression and reduction of PGE_2 in order to induce T cell proliferation.

In summary, this study provides evidence that PGE_2 derived from human breast cancer cells can contribute to inhibition of cellular immunity in vitro. Since levels of PGE_2 are elevated in at least some breast cancers (40, 41), the production of PGE_2 may contribute to the impaired anti-tumor immune response. Reversal of tumor-induced immunosuppression offers a potential approach to cancer therapy and may be particularly useful in combination with immunotherapy against breast cancer.

Key Research Accomplishments

- Prostaglandin E2 production is a common but not universal finding in breast cancers
- Prostaglandin E2 production results in inactivation of T cell responses but is likely modified by other factors
- Inhibition of prostaglandin E2 production can restore T cell responsiveness
- Expression of prostaglandin E2 is sufficient to block T cell responses.

Reportable Outcomes

1. A manuscript is being prepared for submission to Cancer Research.
2. Heike Nesbit was awarded a Ph.D based on this worked which was a significant portion of her Ph.D. thesis.
3. Our findings that proteins in the membrane of breast tumor can inhibit an immune response is the basis of an grant application to further pursue this finding. ("GA733-2 Alters MHC Class II Antigen Processing", IDEA Award application to US Army)

Conclusions

We have previously reported that adenoviral transfer of B7-1 to human tumor cells elicits allogeneic T cell responses despite the production of potentially immunosuppressive factors by the tumor cells. In the present study we show that B7-1 expression on human breast cancer MCF-7 cells failed to stimulate effective T cell responses in vitro under comparable experimental conditions. We demonstrate that the failure of B7-1 expressing breast cancer cells to induce T cell proliferation was due in part to soluble immunosuppressive agents produced by the tumor cells. In addition, we provide evidence that tumor-derived PGE_2, but not TGFβ, was essential for curtailing T cell proliferation in this experimental setting. However, in PGE2 negative cells, transfection and overexpression of cyclooxygenase was sufficient to inhibit T cell responses. Furthermore, inhibition of PGE_2 synthesis or removal of PGE_2 restores the T cell response. These data suggest that pharmacologic intervention with COX inhibitors may be a useful adjunctive therapy to breast cancer vaccines. Finally, certain membrane proteins in breast cancer cells Appear to block T cell responses. Further characterization of this effect will be pursued in year 2 of this award.

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Appendix Cirriculum Vitae of Principal Investigator

UNIVERSITY OF PENNSYLVANIA - SCHOOL OF MEDICINE
Curriculum Vitae

June 1999

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Education:

1971-1975	B.A.	Kalamazoo College (Chemistry)
1975-1977	M.S.	Harvard University (Chemistry)
1977-1981	Ph.D.	Harvard University (Chemistry)
1983-1987	M.D.	University of Mississippi School of Medicine

Postgraduate Training and Fellowship Appointments:
1981-1982 Senior Scientist, Monsanto Company, St. Louis, MO.
1982-1987 Res. Associate, Dept. of Biochemistry, Univ. Miss. School of Medicine, Jackson, MS.
1987-1988 Intern in Medicine, University of Michigan Hospitals, Ann Arbor, MI
1988-1989 Residency in Medicine, University of Michigan Hospitals, Ann Arbor, MI
1989-1992 Hematology/Oncology Fellow, Univ. of Michigan Hospitals, Ann Arbor, MI

Military Service: None

Faculty Appointments:
1981-1982 Instructor, St. Louis Community College
1992-1993 Lecturer In Internal Medicine, Division of Hematology/Oncology, University of Michigan.
1993-1994 Instructor, Division of Hematology/Oncology, University of Pennsylvania.
1994-pres. Ann B. Young Assistant Professor of Cancer Research, Division of Hematology/Oncology, Department of Medicine, Univ. of Pennsylvania.

Hospital and Administrative Appointments:
1992 Admissions Committee, The University of Michigan School of Medicine.
1992-1993 Home Infusion Service, Experimental Therapeutics Grant Review Committee. The University of Michigan School of Medicine
1993 Scientific Retreat Committee, The Institute for Human Gene Therapy, The University of Pennsylvania.
1994-1997 Director of Cancer Gene Therapy, The Institute for Human Gene Therapy, The University of Pennsylvania.
1997-pres. Co-Director of Cancer Gene Therapy, The Institute for Human Gene Therapy, The University of Pennsylvania.
1993-present, Director, Gene Therapy Program, The University of Pennsylvania Cancer Center

Specialty Certification:
1990 Board Certified, American Board of Internal Medicine

1996	Board Certified, Hematology
1993	Board Eligible, Medical Oncology

Licensure:

Michigan	(1989-1996)
Pennsylvania	(1993-2000)

Awards, Honors and Membership in Honorary Societies:

1972-1975	Heyl Fellowship In Science, Kalamazoo College
1975	Honors Thesis, Kalamazoo College
1992-1995	Merck-American Fed. for Clinical Research, M.D./Ph.D. Postdoctoral Fellowship
1994-pres.	Anne B. Young Assistant Professor for Cancer Research, July 1, 1994.
1995-1996	Measly Fellowship Award
1998	University of Pennsylvania Nominee for Rita Allen Award.

Memberships in Professional and Scientific Societies:]

Local Societies:

Philadelphia Cancer Research Association
Pennsylvania Chapter of the American Chemical Society

National Societies:

American Association for the Advancement of Science
American Federation of Clinical Research
American Association for Cancer Research
American Chemical Society
The Brain Tumor Society
American Society of Gene Therapy

Peer Review Activities

NIH, NCI PO1 Review, Boston, MA 6/25-27/95
US Army Breast Can. Res. Program, Ad hoc reviewer 11/13-15/95
Breast Cancer Research Program, University of CA 1996-1998
NCI RFA Review Committee, Ad hoc reviewer 6/11-13/96
NIH, Neurosciences 3 Study Section, Ad hoc reviewer 6/26-28/96
NIH, NCI PO1 Review, New York, 7/7-9/96
NIH, NCI, Medicine Branch, Ad hoc reviewer, Wash. D.C. 9/10-12/96
NIMH PO1 Review, Washington, DC. Ad hoc reviewer, 12/96
NIH, NCI Ad hoc Reviewer 6/30/97
State of Massachusetts Breast Cancer Program 10/18-19/97
Chairman, Scientific Review Committee, NCI sponsored North American Brain Tumor Consortium (NABTC) and New Approaches to Brain Tumor Therapy (NABTT) consortium multigroup glioma gene therapy clinical trial. 1997-8
External Reviewer NCI PO1, Massachusetts General Hospital 5/98
NIH, NCI PO1 Review, Los Angeles, 7/27-29/98
State of Massachusetts Breast Cancer Program 10/24-25/98
NCI, Subcommittee D "Clinical Research Studies" 11/30-12/1/98
US Army Ovarian Cancer Study Section 1/20/99-1/22/99
NCI, Subcommittee D "Clinical Research Studies" 4/14-5/99
NCI, RAID Review 3/31/99-4/1/99
NIH, Career Development Award Review 6/21-22/99
NCI, Ovarian Cancer Spore Grants Review 6/27-29/99

Editorial Positions:

Scientific Advisor, Education Committee, Pennsylvania Biotechnology Association, State College, PA 1995
Cancer Gene Therapy, Editorial Board, Simon & Schuster Publisher 1996-.

Gene Therapy, Editorial Board, Stockton Press. 1999-

Ad hoc reviewer for:

Human Gene Therapy, Journal of Immunology, Cancer Research, Journal of Virology, Journal of Gastroenterology, Gene Therapy, Nature Medicine, Annals of Neurology, Cancer Gene Therapy, Proc. Nat'l Acad. Sciences, DNA and Cell Biology, J. Organic Chemistry. J. Nuclear Medicine.

Principal Investigator of Grants:

Ligand Mediated Gene Therapy, American Federation for Clinical Research. 7/92-6/95, $150,000 (3 yrs)

Hepatic Gene Transfer for the Treatment of Metastatic Colon Cancer, Penn Home Infusion Therapy. 7/94-6/95, $23,520 (1 yr)

Stimulation of Breast Cancer Immunity by B7 Expression, 1UO1 CA 65805,
Project 1 NIH. 8/94-12/98, $280,359 (year 3)

Phase 1 Trial of HSVTK Gene Therapy for CNS Tumors, RO1 CA 67799, 8/1/95 -7/31/99, $155,192 (renewal pending review)

Recombinant Adenovirus Vaccine for Colon Cancer 10/1/96-9/30/00, National Cancer Institute, 1RO1CA71915-01 $939,324 (4 yrs)

The CO17-1A/GA733 Antigen in Colorectal Cancer Therapy, 1 PO1 CA 74294-01, Project 2 leader, $218,577 (year 1)

Quantitative Assessment of HSVtk Gene Transfer by PET. Society of Nuclear Medicine ($5,000) 3/1/98 -2/28/99.

The Role of Breast Cancer Derived Prostaglandin E2 in the Elaboration of a Therapeutic Immune Response. US Army Breast Cancer Program, 1997-2000, $75,000/yr

Gene Based Therapies and Imaging of Malignant Gliomas (pending review) NCI $299,973, 3/1/00 –2/28/04

The Mechanism of mEGP: How Tumor Cells Inhibit APCs, RO1 NCI$200,000 2000-2004 (pending review).

GA733 Alters MHC Class II Antigen Processing, US Army Breast Cancer Program, 1999 (pending review)

Academic Committees at the University of Pennsylvania:

Clinical Trials Scientific Review and Monitoring Committee, University of Pennsylvania Cancer Center 1996-present

University of Penn. General Clinical Research Center Internal Review Committee 1996-97

Faculty Grievance Commission 1997-2000

Molecular Life Sciences Advisory Committee 1998-present

Vagelos Scholars Advisory Committee 1998-present

Short Term Experience in Research Advisory Committee 1999

Major Teaching and Clinical Responsibilities at the University of Pennsylvania (last 3 yrs):

1993-1999 Attending Physician, Oncology & Hematology Services, Hospitals of the University of Pennsylvania.

1994-1999 Attending Physician, Oncology & Hematology Services, Philadelphia Veterans Administration. Hospital.

1996, 1998, 1999 Human Biology (Biology 6)

1996 Critical Care Nurse Practitioner Course, "Hematology in the Critical Care Setting"

1995-1999 Selected Topics in Chemistry (Chemistry 700)

1996-1999 The Molecular Basis of Gene Therapy, (CAMB 610)

- Medicine 101C, Differential Diagnosis

1997-1999 Introduction to Gene Therapy (CAMB 610, Fall)

1999-2000 Advanced Seminar in Cancer Gene Therapy (CAMB 633, Spring 1999) Course Director

1997 Wistar Cancer Biology Graduate Student Seminar

1997, 1998 Cancer Biology and Genetics Course (Pathology, Fall)

1998, 1999 Topics in Cancer Pharmacology (PHARM, Fall 1998, 1999)

Lectures by Invitation:

October 19, 1992 "Inhibition of NF-kB by double-stranded oligonucleotides" - I.C.R.F., London, England.

October 25,1993 "Immunotherapy of Breast Cancer by B7 Gene Transfer", Institute for Human Gene Therapy Retreat, Tamiment, PA.

October 30, 1994 "Treatment of Advanced CNS Malignancy with Recombinant Adenovirus HSVtk", Institute for Human Gene Therapy Retreat, Absecon, NJ

January 11, 1995 "Adenovirus Vectors for the Treatment of Brain Tumors", BioEast Conference, Washington DC.

March 10, 1995 "Cancer Gene Therapy", Combined Science Seminar Series, Medical College of Pennsylvania and Hahnemann University, Philadelphia, PA.

April 22, 1995 "Adenoviral Vectors for the Treatment of CNS Tumors", 3rd International Conference on Biologic Therapy of Cancer, Munich, Germany.

April 25, 1995 "Gene Therapy", Medical Grand Rounds, Doylestown Hospital, Doylestown, PA.

May 10, 1995 "Vectors for Cancer Gene Therapy", The Second Symposium of the Philadelphia Cancer Research Association, "Approaches to Active Immunotherapy of Cancer", Thomas Jefferson Univ., Philadel., PA.

May 26, 1995 "Adenovirus-Mediated Cancer Gene Therapy", University of North Carolina, Department of Medicine, D ivision of Hematology and Oncology Research Seminar Series. Chapel Hill, NC.

June 9, 1995 "Adenovirus Mediated Gene Transfer for the Treatment Primary of CNS Malignancy" International Conference on Gene Therapy of CNS Disorders, Philadelphia, PA.

June 14, 1995 "Replication Competent Adenovirus Safety Issues" , Food and Drug Administration, International Conference on Viral Safety and Evaluation of Viral Clearance from Biopharmaceuticals Products. Bethesda, MD.

Sept. 21, 1995 "Clinical Aspects of Cancer Gene Therapy", Pennsylvania Oncologic Society Annual Meeting, Seven Springs, PA.

October 1, 1995 "Treatment of Primary CNS Tumors with Adenovirus Mediated Gene Transfer," The GAAC Meeting on Gene Therapy, Seeon, Germany.

January 18, 1996 "Adenoviral-Mediated Therapy of Brain Tumors", The Preuss Foundation Meeting on Gene Therapy for CNS Malignancies, The Salk Institute, La Jolla, CA.

February 1, 1996 "Cancer Gene Therapy", Cooper Medical Center Medical Grand Rounds, Camden ,NJ.

April 17, 1996 "Gene Therapy", The Estelle Lasko Memorial Lecture, The Twenty-fourth Annual Chester County Cancer Conference, Chester County Hospital, West Chester, PA

April 19, 1996 "Gene Therapy for Inherited and Acquired Diseases", Genetics in the Cause and Treatment of Malignancies Conference, Sacred Heart Hospital, Allentown, PA.

May 13-7, 1996 "Laboratory and Clinical Approaches to Cancer Gene Therapy" 1996 Short Course in Cancer Biology, University of Nebraska Medical Center, Omaha, NE.

June 15, 1996 "Advances in Gene Therapy", The Coalition for Internal Medicine 1996 Scientific Meeting, Hershey, PA.

June 21, 1996 "Gene Therapy: Its Real, It Works and Its Coming to Your Practice," Grand Rounds, North Penn Hospital, Lansdale, PA.

July 11, 1996 "Cancer Gene Therapy" Shering-Plough Corporation, Kenilworth, NJ.

Nov. 14, 1996 "Adenoviral Vectors for Cancer Gene Therapy" Fifth International Symposium on Cancer Gene Therapy, San Diego, CA

January 9, 1997 "Adenoviral Vectors for the Gene Therapy of Cancer", Wayne State University, Center for Molecular Medicine and Genetics, Detroit, MI

March 7, 1997 Gene Therapy for Gliomas and Colon Cancer, University of South Carolina, Department of Microbiology, Charleston, SC

April 25, 1997 "Colon Cancer Vaccines", Megabios Corporation, Burlingame, CA

May 11, 1997 "Methods of Gene Delivery", Plenary Session, American Society of Transplant Physicians, Chicago, IL

Aug. 7, 1997 "Gene Therapy of Malignant Gliomas" Cancer Section, Gorden Conference, Newport, RI.

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Oct. 20, 1997 "Experimental Therapies for Malignant Gliomas", Pathology Grand Rounds, Suburban General Hospital, Norristown, PA

March 13, 1998 "Gene Therapy Strategies for Malignant Glioma Therapy", Contemporary Concepts in Brain Tumor Therapy: From Genes to Patient Care Conference, Conshohocken, PA

March 28, 1998 "Phase I Trial of Gene Therapy for Primary Brain Tumors", Cerebral Vascular Biology 1998 Conference, Portland (Lincoln), OR.

June 29, 1998	"Phase I Trial of Gene Therapy in Primary Brain Tumors", American Society of Gene Therapy, Seattle, WA.
April 28, 1998	New Developments in Cancer Gene Therapy", Hematology/Oncology Research Conference, Children's Hospital of Philadelphia, Philadelphia, PA
August 18, 1998	"New Developments in Cancer Gene Therapy", Grand Rounds, Chestnut Hill Hospital, Philadelphia, PA
May 16, 1999	"Enzyme-Prodrug Gene Therapy for Cancer", American Society of Clinical Oncology Meeting, Atlanta GA.
June 12, 1999	"mEGP Blocks Class II Antigen Presentation" American Society of Gene Therapy, Washington D.C.
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