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EFFECT OF INDOMETHACIN ON TUMORIGENICITY AND IMMUNITY INDUCTION IN A MURINE MODEL OF MAMMARY CARCINOMA

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Indomethacin, an inhibitor of cyclo-oxygenase given orally, reduced the tumorigenicity of cancer cells in a non-immunogenic murine model of mammary adenocarcinoma (4T*1*). In the presence of indomethacin, a dose-dependent immune protection could be induced most effectively by immunizing mice with 1 to 3 doses of irradiated tumor cells inoculated at intervals of 7 days prior to challenge with a tumorigenic cell dose. Three immunizations given without indomethacin resulted in tumor growth in 88% of the recipients, and indomethacin treatment started 28 days prior to the challenge dose and given without immunizations led to tumor onset in 83% of mice. In contrast, tumor was documented only in 12% of mice vaccinated with 3 immunization doses and given concomitantly indomethacin. Moreover, 53% of disease-free survivors resisted a second challenge with a high tumorigenic dose. Induction of an anti-tumor immunity in indomethacin-treated mice was further studied as a therapy for tumor-bearing mice. Complete cure was induced in 50% of mice, and a significant reduction in tumor size as well as prolonged survival time were observed in the remaining animals. Immunostimulation by tumor cell vaccination given in the presence of a tolerable dose of indomethacin, therefore, may be incorporated into immunotherapy protocols to activate an anti-tumor response against residual tumor cells that escaped surgery and/or high-dose chemo/radiotherapy. *Int. J. Cancer* 75:894–899, 1998.
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Indomethacin is a well-known non-steroidal, anti-inflammatory drug, as well as an efficient inhibitor of cyclo-oxygenase, which catalyzes the conversion of arachidonic acid to prostaglandins (PGs), prostacyclin and thromboxanes (Earnest *et al.,* 1992). Increased production of PGE2 in macrophages, fibroblasts and malignant cells was frequently associated with suppressed immune surveillance and depressed cellular immunity (Goodwin and Ceuppens, 1983; Parhar and Lala, 1985; Lala and Parhar, 1988). However, inhibition of cyclo-oxygenase and decrease in PGE2 production by indomethacin often activated cellular immune responses *in vitro* and *in vivo* (Han *et al.,* 1983; Fulton, 1984; Chao *et al.,* 1995; Gogos *et al.,* 1995). Treatment with indomethacin conferred protection against carcinogen-induced cancer in experimental models of colon, urinary bladder and skin (Wild and Degan, 1987; Holmang *et al.,* 1995; Andrews *et al.,* 1991); interfered with human cancer cell growth *in vitro* (Planchon *et al.,* 1995); and reduced effectively tumor load and metastatic diseases in various experimental tumor models of melanoma, mammary adenocarcinoma, lymphoma and colon (Fulton, 1984; Alino *et al.,* 1992; Lala *et al.,* 1986; Liu *et al.,* 1986; Jimbo *et al.,* 1995; Fujiwara *et al.,* 1984). The anti-tumor effect of indomethacin was often connected to its immunostimulating activity and its capability to restore an obstructed immunosurveillance in tumor-bearing hosts (Earnest *et al.,* 1992; Han *et al.,* 1983; Fulton, 1984; Chao *et al.,* 1995; Gogos *et al.,* 1995; Alino *et al.,* 1992; Lala *et al.,* 1986; Liu *et al.,* 1986; Jimbo *et al.,* 1995).

In contrast to other studies that have demonstrated the effect of indomethacin on tumorigenicity, we studied the ability of indomethacin to immuno-activate immunity induction. Over the last decade, induction of anti-tumor immunity was achieved by using chemically, virally or genetically modified tumor cell vaccines. This active, specific immunotherapy could elicit an anti-tumor response and was able to break tolerance against cancer cells bearing self-cell-surface components (Fujiwara *et al.,* 1984; von Hoegen *et al.,* 1989; Colombo and Forni, 1994). We now present our results on increased anti-tumor immunity in mice vaccinated with unmodified syngeneic tumor cells in a model of non-immunogenic mammary adenocarcinoma. These indicate that long-term treatment by indomethacin given together with irradiated tumor cells might be efficient to stimulate an anti-tumor response and to confer long-term protection to a residual tumor load.

MATERIAL AND METHODS

Mice

BALB/c (H-2^d) and F_1 (BALB/c × C57BL/6) (H-$2^{d/b}$) mice aged 10 to 12 weeks were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in our animal house at the Hadassah University Hospital, according to our specific national laws and in full compliance with all regulations for protection of animal rights.

Tumor

4T1 is a cell line established from a cell subpopulation isolated from a single, spontaneously arising mammary tumor of a BALB/cfC3H mouse (Dexter *et al.,* 1978), where f stands for fostering. 4T1 cells were maintained by passage *in vitro* in RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 1% non-essential amino acids. Preparation of cells for injection included harvesting by 0.25% trypsin in 0.05% EDTA, washing with RPMI 1640 and resuspension in Hank's medium for intradermal (i.d.) injection into mice in a 0.1 ml volume. All tissue culture media and reagents were purchased from Biological Industries, (Beit Ha'emek, Israel). Cells were kept at 37°C in a humidified 5% CO_2/air incubator.

Measurement of primary tumor growth in vivo

Tumors were measured once a week in 2 perpendicular dimensions with a Taxol-(r) rimeter caliper. Tumor size (cm) was calculated by the formula $(a \times b^2)/2$, where b is the smaller dimension of the tumor.

Immunization protocol

Cultured 4T1 cells were irradiated (120 Gy) to ensure the absence of proliferating cells from the immunization dose and injected i.d. into naive F_1 or BALB/c mice, 1 to 3 times at intervals of 7 days, as detailed for each experiment. Seven days following the last immunization dose, a challenge of 10^4 to 2×10^4 non-irradiated 4T1 cells was given i.d. A control group of naive, non-immunized mice was inoculated in parallel with 10^4 to 2×10^4 fresh 4T1 cells.

Indomethacin treatment

A stock solution of indomethacin (Sigma, St. Louis, MO) was prepared in absolute ethanol at a concentration of 10 mg/ml and further diluted in sterile tap water added to the drinking bottles to a

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final concentration of 14 or 28 μg/ml as described for each experiment. Drinking bottles were changed twice a week and a fresh stock solution was prepared every week.

Statistical analysis

Statistical significance of tumor size was evaluated by the standard 2-tailed, unpaired Student *t*-test. The Kaplan-Meier method was used to calculate the probability of survival as a function of time following tumor inoculation in indomethacin-treated and untreated mice (Kaplan and Meier, 1958). Statistical significance between pairs of Kaplan-Meier curves was evaluated by the log-rank test (Mantel, 1966).

RESULTS

Effect of indomethacin on tumorigenicity

BALB/c or F_1 (BALB/c × C57BL/6) (F_1) mice inoculated i.d. with 4T1 tumor cells were given indomethacin (14 μg/ml in drinking water) from the day of tumor inoculation until the end of the experiment. Local tumor size in both strains of mice was significantly different ($p < 0.02$) from that observed in control mice not receiving the drug (Fig. 1). Mean ± SE local tumor size of 8 untreated BALB/c or 14 F_1 mice on day 42 following tumor inoculation was 2.09 ± 0.37 and 2.86 ± 0.40 cm^3, respectively, while that of 13 BALB/c or 10 F_1 indomethacin-treated mice was 0.75 ± 0.16 and 0.88 ± 0.26, respectively. F_1 mice given 0.2% ethanol in the drinking water, as a control for the indomethacin solvent, developed large local tumors on day 21 and died within 42 to 55 days (median 48 days), similar to untreated mice (median 54, range 44 to 66 days). Probability of survival of indomethacin-treated mice was significantly higher than that of untreated mice, as shown by Kaplan-Meier curves presented in Figure 2 ($p = 0.033$ and 0.004 in BALB/c and F_1 mice, respectively).

Effect of indomethacin on immunogenicity

F_1 mice were inoculated with 10^7 irradiated 4T1 tumor cells given i.d. once, 2 or 3 times at intervals of 7 days prior to an i.d. challenge with 10^4 non-irradiated 4T1 cells. Indomethacin treatment (14 μg/ml) was started on the day of the first immunization and was continued for >175 days. Over the entire period, mice maintained a healthy profile without any overt clinical changes in weight or fur. Immunization(s) in the presence of indomethacin markedly inhibited tumor growth, as documented by serial weekly measurements of local tumor size in mice vaccinated with 1 (Fig. 3), 2 (Fig. 4) or 3 (Fig. 5) immunizations. Overall, tumor development was totally abrogated in a substantial number of indomethacin-treated mice. Immunity was dose-dependent as 10/16, 14/16 and 15/15 mice survived disease-free following 1, 2 or 3 immunizations in the presence of indomethacin, respectively (Table I). On day 100 following the first challenge, the disease-free survivors of 3 tumor immunizations were inoculated with a second challenge of a highly lethal tumor dose (10^5). Eight of 15 mice remained disease-free as evaluated on day 75 post-second tumor challenge (Table I). A control group of 10 mice inoculated with 10^5 4T1 cells developed large tumors on day 21 and died within 53 days (median). To exclude an effect of indomethacin by long-term treatment prior to challenge, mice were given the drug without concomitant immunizations for 28 days prior to the challenge (Fig. 6). In this group of 36 mice pooled from 3 separate experiments, 30 mice developed tumors. In the control group, where animals were immunized without receiving indomethacin, 36/41 mice developed tumors. In comparison, of 58 F_1 mice (pooled from 4 separate experiments) immunized while being treated with indomethacin, only 7 developed tumors (Fig. 6).

Three immunizations with irradiated 4T1 cells given to a different strain of mice, *i.e.*, BALB/c, also induced immune protection to a challenge of non-irradiated tumor cells, as demonstrated by the mean of tumor size shown in Figure 7. Control BALB/c mice immunized with irradiated 4T1 cells prior to challenge or mice inoculated with a challenge dose only developed

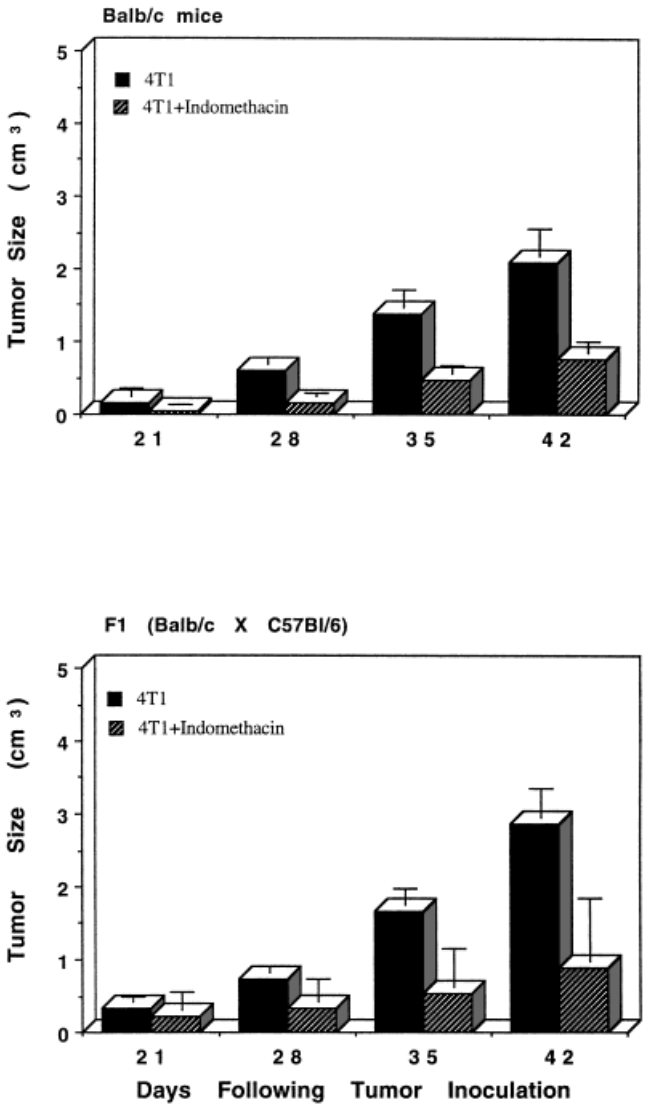
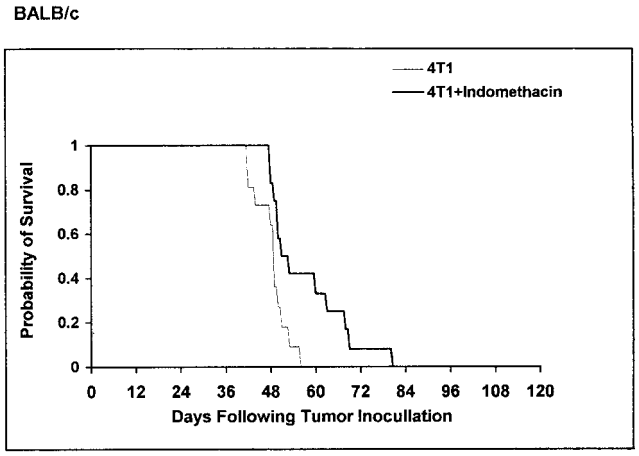


FIGURE 1 – Effect of indomethacin on 4T1 tumor size. BALB/c and F_1 mice were inoculated with 10^4 and 2×10^4 4T1 cells, respectively, and given indomethacin (14 μg/ml in drinking water) from the day of tumor inoculation until the end of the experiment. Results represent the mean ± SE of tumor size of 8 to 13 mice per experimental group.

large local tumors up to 0.76 and 1.99 cm^3, respectively, while the mean local tumor size of indomethacin-treated mice was 0.07 cm^3 on day 50 following challenge. Until day 85, 7/8 indomethacin-treated mice survived disease-free, whereas 10 control mice died within 62 days (median). Mice given 0.2% ethanol in the drinking water, as a control for the indomethacin solvent, developed large local tumors (0.74 cm^3) comparable to the control, untreated, immunized mice (Fig. 7).

Immunization plus indomethacin treatment in a therapeutic model

F_1 mice inoculated with 2×10^4 4T1 cells were vaccinated 3 times at intervals of 7 days with 10^7 irradiated tumor cells starting on day 1 following tumor inoculation. Indomethacin (28 μg/ml) was added to the drinking water on the day of tumor inoculation and for the entire period of the experiment. The results presented in Figure 8 show that tumor development in experimental animals was significantly slower ($p < 0.002$) than that in tumor-bearing mice inoculated with irradiated 4T1 cells only, without drug treatment, or in a control group of mice inoculated with non-



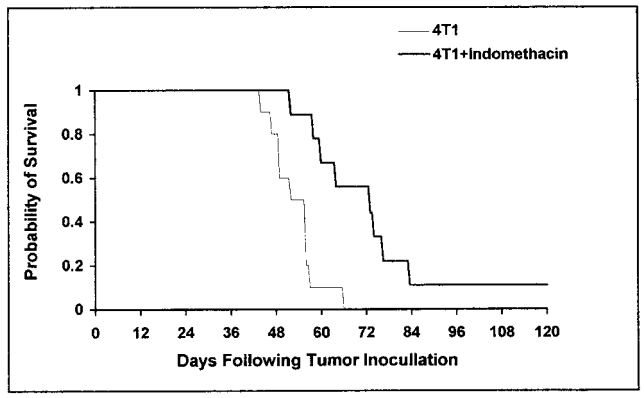


FIGURE 2 – Effect of indomethacin on survival of 4T1-bearing mice. BALB/c and F_1 mice were inoculated with 10^4 and 2×10^4 4T1 cells, respectively, and given indomethacin (14 μg/ml in drinking water) from the day of tumor inoculation until the end of the experiment. Each group consisted of 8 to 13 mice. Probability of survival is presented as Kaplan-Meier curves and statistical significance was calculated by the log-rank test ($p = 0.033$ and 0.004 in BALB/c and F_1 mice, respectively).

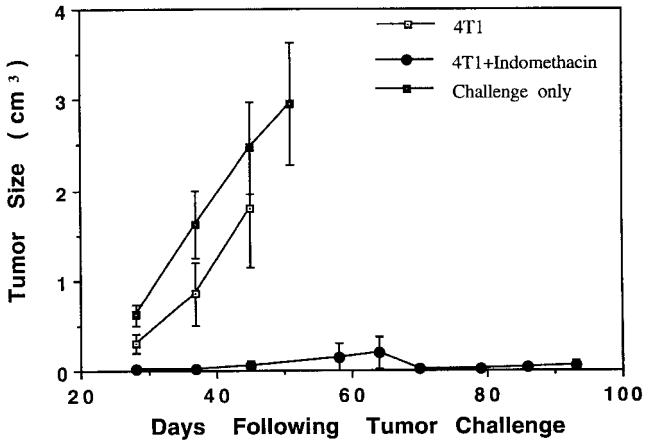


FIGURE 3 – Immunity induction by one immunization. Irradiated 4T1 (10^7) cells were given i.d. (with or without indomethacin) to naive F_1 (BALB/c × C57BL/6) mice prior to an i.d. challenge with 10^4 non-irradiated 4T1 cells. Indomethacin (14 μg/ml in drinking water) was given throughout the experiment. A control group of mice were inoculated with a challenge dose only. Results represent the mean ± SE of 5 to 6 mice per group, from 1 of 2 experiments with a total of 10 to 16 mice per group.

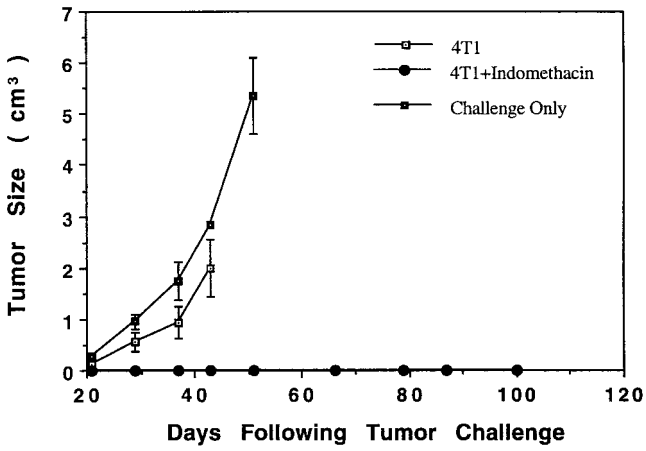


FIGURE 4 – Immunity induction by 2 immunizations. Two doses of 10^7 irradiated 4T1 cells were inoculated i.d. (with or without indomethacin) into naive F_1 (BALB/c × C57BL/6) mice) at intervals of 7 days prior to an i.d. challenge of 10^4 non-irradiated 4T1 cells. Indomethacin (14 μg/ml in drinking water) was given throughout the experiment. A group of control mice was inoculated with a challenge dose only. Results represent the mean ± SE of 5 to 7 mice per group, from 1 of 2 experiments with a total of 15 to 16 mice per group.

irradiated 4T1 cells only. Results pooled from 2 separate experiments showed that all 20 control mice were dead within 42 to 79 days (median 53), whereas 8/13 indomethacin-treated mice were alive by day 90, having only small tumors, and 4/8 mice were tumor-free.

DISCUSSION

The effect of long-term treatment with indomethacin, a non-steroidal, anti-inflammatory drug, on tumor growth and survival time of mice inoculated with a lethal tumorigenic dose of mammary adenocarcinoma cells was tested in 2 strains of mice, one expressing H-2^d and the other H-$2^{d/b}$. Local tumor growth was significantly reduced and a delay in survival time was observed in both strains of mice; however, complete cure was not obtained.

The ability of indomethacin to affect tumor dissemination was reported previously for several experimental models. This anti-tumor effect was related to the well-known effect of the drug to restore obstructed immunosurveillence in tumor-bearing mice (Fulton, 1984; Alino *et al.,* 1992; Lala *et al.,* 1986; Liu *et al.,* 1986; Jimbo *et al.,* 1995). In most of these studies, indomethacin could not totally overcome the metastatic disease and no complete cure was obtained. Therefore, attempts were made to increase its efficiency by adding IL-2 (Lala and Parhar, 1988, 1993; Lala *et al.,* 1990) or by encapsulating the drug in liposomes (Alino *et al.,* 1992). The ability of indomethacin to affect tumor growth in our model prompted us to change the strategy of drug application and to try to improve its anti-tumor effect by induction of an anti-tumor immunity.

Indomethacin, given in the drinking water over the period of 3 immunization(s) with irradiated syngeneic tumor cells, was the most effective way of conferring immune protection to a challenge with a tumorigenic dose. It was crucial to combine vaccination with indomethacin treatment in order to induce anti-tumor immunity in this non-immunogenic tumor model. Neither multiple immunizations given without indomethacin (Morecki *et al.,* 1997; present

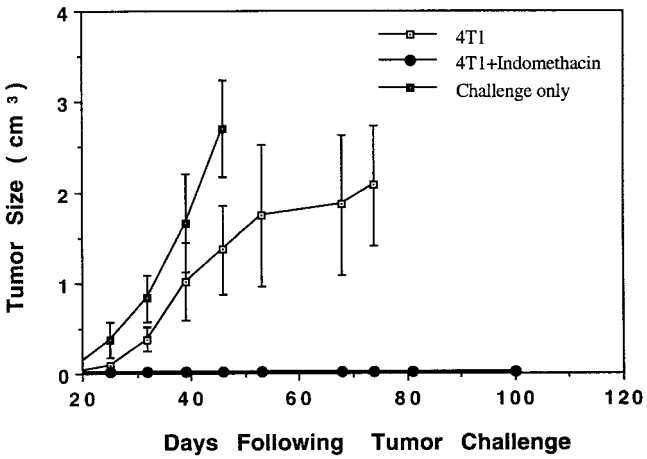


FIGURE 5 – Immunity induction by 3 immunizations. Three doses of 10^7 irradiated 4T1 cells were inoculated i.d. (with or without indomethacin) into naive F_1 (BALB/c × C57BL/6) mice at intervals of 7 days prior to an i.d. challenge with 10^4 non-irradiated 4T1 cells. Indomethacin (14 µg/ml in drinking water) was given throughout the experiment. A group of control mice was inoculated with a challenge dose only. Results represent the mean ± SE of 5 to 7 mice per group, from 1 of 2 experiments with a total of 15 to 19 mice per group.

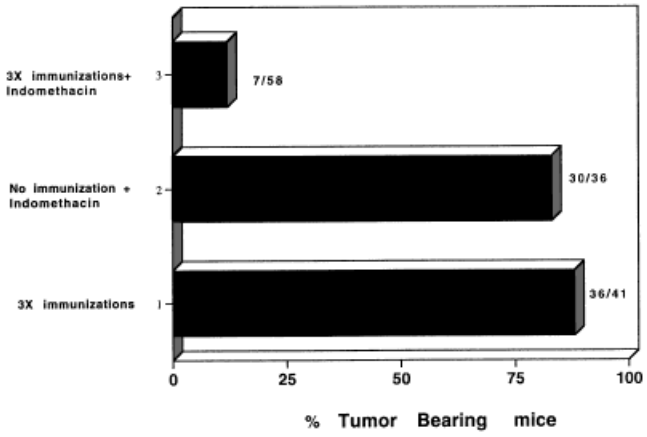


FIGURE 6 – Indomethacin without immunization. Group 1: Forty-one F_1 mice received 3 immunization doses of 10^7 irradiated tumor cells at intervals of 7 days before challenge with 2×10^4 non-irradiated tumor cells. Group 2: Thirty-six mice were treated with indomethacin starting 28 days before inoculation with a challenge dose and continuing throughout the experiment. Group 3: Fifty-eight mice were treated with 3 immunization doses before challenge, indomethacin (14 µg/ml) was administered throughout the experiment.

TABLE I – DOSE-DEPENDENT TUMOR IMMUNITY INDUCED BY IRRADIATED 4T1 CELLS AND INDOMETHACIN

Number of immunizations[1]	Indomethacin treatment	Number of mice with tumors[2]	
		1st challenge	2nd challenge
1	−	8/10	—
1	+	6/16	ND[3]
2	−	11/15	—
2	+	2/16	ND
3	−	15/19	—
3	+	0/15	7/15

[1]Irradiated 4T1 mammary adenocarcinoma cells (10^7) were given at intervals of 7 days prior to a challenge of non-irradiated tumor cells (10^4). The second challenge was given 100 days after the first challenge.–[2]Mice were evaluated on day 75 following the second challenge.–[3]ND, not done.

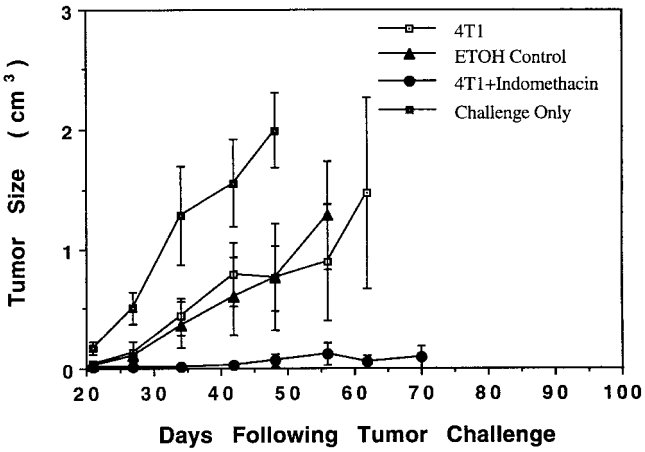


FIGURE 7 – Immunity induction in BALB/c mice. Three doses of 2×10^6 irradiated 4T1 cells were inoculated i.d. (with or without indomethacin) into naive BALB/c mice at intervals of 7 days prior to an i.d. challenge with 2×10^4 non-irradiated 4T1 cells. Indomethacin (14 µg/ml in drinking water) was given throughout the experiment. Results represent the mean ± SE of 10 indomethacin-treated mice and 6 mice in each of the 3 control groups: immunized, immunized + 0.2% ethanol and challenge only.

results) nor indomethacin alone starting 28 days prior to the challenge could induce anti-tumor immunity. This timing of 28 days was chosen to comply with the vaccination schedule (3 doses) in order to test and exclude the effect of indomethacin by long-term administration prior to the challenge. Our results emphasize the necessity for repeated immune stimulation and the importance of continuous administration of indomethacin for immunoactivation.

The ability to induce anti-tumor immunity in a high number of indomethacin-treated, tumor-free mice was further investigated as treatment for tumor-bearing mice. Inoculation with the tumor cell vaccine and concomitant administration of indomethacin in mice that were previously inoculated with a tumorigenic dose led to a complete cure in 50% of the mice and to a marked reduction in tumor size and a prolonged survival time in the remaining mice as compared with untreated animals or mice that were immunized only. These results are encouraging. However, more efforts should be invested in order to improve the cure rate.

Combining our protocol with other immunomodulators or with low-dose, non-toxic lymphokine treatment might improve the cure rate in tumor-bearing mice. Taking into account the large use of indomethacin in the clinic to combat IL-2 side effects and its low extent of toxicity (Rosenberg *et al.,* 1987; West *et al.,* 1987; Peterson *et al.,* 1992), long-term treatment might well be a way to activate an immune anti-tumor response. Several groups have reported a cure rate of 50 to 90% when indomethacin was given together with one or multiple cycles of IL-2 (Lala and Parhar, 1988, 1993; Lala *et al.,* 1990). In these experimental tumor models, indomethacin relieved PGE2-mediated immunosuppression and enabled activation of numerous IL-2-dependent effector lineage cells like natural killer (NK) cells, lymphokine-activated killer (LAK) cells, polyclonal T cells and cytotoxic T cells (CTL) (Lala *et al.,* 1986; Rappaport and Dodge, 1982). Despite the high cure rate, the risk of IL-2 toxicity must be taken into account, particularly when given in multiple cycles. The use of syngeneic unmodified irradiated tumor cells might activate these IL-2-dependent effector mechanisms and, therefore, offer a safe and non-toxic way to achieve activation of an anti-tumor response. Local tumor size of experimental mice inoculated with 4T1 cells and given long-term

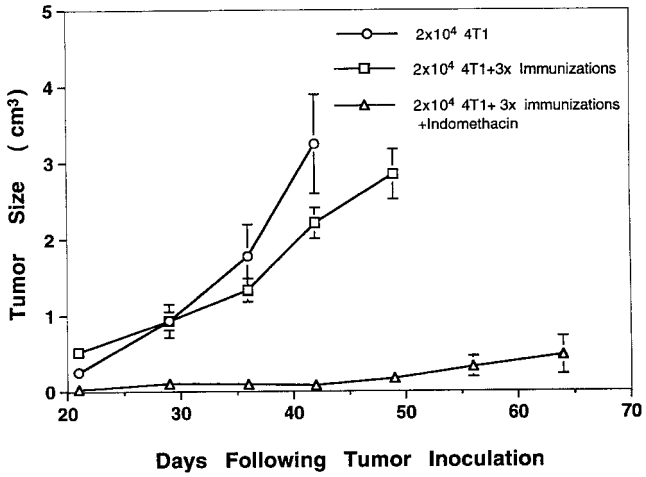


FIGURE 8 – A therapeutic model. F_1 (BALB/c × C57BL/6) mice were inoculated i.d. with 2×10^4 4T1 cells and 24 hr later vaccinated with 3 doses of 10^7 irradiated 4T1 cells given i.d. (with or without indomethacin) at intervals of 7 days. Indomethacin (28 μg/ml in drinking water) was given throughout the experiment for a follow-up period of >250 days. Results represent the mean ± SE of 6 to 8 mice per experimental group, from 1 of 2 experiments.

treatment of indomethacin combined with one cycle of IL-2 was not different from results of treatment with indomethacin alone (data not shown), indicating that diverse tumors may react differently to various treatments. We do not know whether the anti-tumor effect observed in our study is mediated by PGE2 immunosuppression that is being relieved by indomethacin or whether other mechanisms act independently of PGE2 synthesis inhibition (Tilden and Balch, 1982).

Indomethacin can also mediate its anti-tumor effect by acting on suppressors of macrophage or NK activity (Fulton, 1984). In the presence of the immunostimulation provided by the irradiated tumor cells, indomethacin may augment the activation of macrophages and T cells that is required for cell-mediated immune responses. If indomethacin is mediating its anti-tumor activity *via* PGE2 synthesis inhibition, this might lead to an increase in leukotriene levels, which can stimulate histamine release (Uotila, 1993). Histamine is another component known to be a feedback inhibitor of cell-mediated immunity. Both PGE2 and histamine exert an inhibitory effect through their ability to increase cAMP in various immune cell subsets (Earnst *et al.,* 1992; Uotila, 1993). Histamine mediates its effect through histamine-2 receptors and can be abolished by receptor antagonists like cimetidine (Khan *et al.,* 1985). Therefore, activation of an immune anti-tumor response by irradiated tumor cells in the presence of indomethacin should also include cimetidine in order to prevent cAMP elevation.

In summary, immunostimulation by an irradiated syngeneic tumor cell vaccine given concomitantly with long-term indomethacin as an immuno-activator induced an anti-tumor immunity and triggered an efficient anti-tumor effect in tumor-bearing mice. This protocol may be used to prevent and/or treat relapse mediated by residual tumor cells in human patients.

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