

IMMUNE REGULATION OF THE L5178Y MURINE TUMOR-DORMANT STATE

I. In Vivo and In Vitro Effects of Prostaglandin E₂ and Indomethacin on Tumor Cell Growth

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The L5178Y tumor-dormant state is established in DBA/2 mice by subcutaneous immunization and intraperitoneal challenge with a nonmetastatic line of the methylcholanthrene-induced, syngeneic, T cell lymphoma, L5178Y (1, 2). During the tumor-dormant state, clinically normal mice retain small numbers of L5178Y cells in their peritoneal cavity for a prolonged period of time, ranging from 2 to 12 mo. The progressive proliferation of L5178Y cells is restricted by immunologic mechanisms involving cytolytic T lymphocytes and macrophages, acting both individually (3, 4) and in synergy (5). Transfer of peritoneal cells from a tumor-dormant mouse to the peritoneal cavity of a normal mouse results in the rapid formation of an ascitic tumor. L5178Y cells that persist in a tumor-dormant state eventually proliferate to form an ascitic tumor (1). The development of ascitic tumors is preceded by the appearance in the peritoneal cavity of macrophages that have increased immunosuppressive activity (6). These macrophages produce an immunosuppressive factor in vitro, recently identified as PE₂ (our unpublished data), production of which can be inhibited by treatment of macrophage cultures with indomethacin (7). The L5178Y cells that grow out at the end of the tumor-dormant state are less susceptible to immune lysis than the L5178Y cells that are used to initiate the tumor-dormant state (8). This phenotypic shift in the tumor cell population during progression of the tumor-dormant state is caused by a immunoselection process (9, 10).

The production of PGE₂ by macrophages before formation of ascitic tumors suggested that this PGE₂ suppresses the mechanisms by which progressive tumor cell proliferation is restrained during the tumor-dormant state. To test this hypothesis, we performed a series of in vivo and in vitro experiments with PGE₂ and its synthetase inhibitor, indomethacin. This report presents experiments which demonstrate that treatment of tumor-dormant mice with PGE₂ terminates the tumor-dormant state, with formation of ascitic tumors. Also, treatment of cultures of peritoneal cells from tumor-dormant mice with PGE₂ results in

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enhancement of tumor cell growth. Conversely, treatment of tumor-dormant mice with indomethacin results in elimination of all recoverable tumor cells, and treatment of cultures of peritoneal cells from tumor-dormant mice with indomethacin results in inhibition of tumor cell growth.

Materials and Methods

Animals. 8–12-wk-old female DBA/2 mice were obtained from The Jackson Laboratories, Bar Harbor, ME. All mice were fed Purina mouse chow and given acidified tap water ad libitum. They were housed in a temperature-controlled room with a cycle of 12 h of light and 12 h of dark.

Tumor Cells. The L5178Y cell line is a nonmetastatic T cell lymphoma induced in DBA/2 mice with methylcholanthrene (1).

Culture Medium. Eagle's MEM was supplemented with 10% FCS, sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), L-glutamine (2 mM), 15 mM Hepes, sodium bicarbonate (1.125 g/liter) and 50 µg/ml gentamycin sulfate (Schering Corp., Kenilworth, NJ).

Establishment of the L5178Y Tumor-dormant State. This procedure has been described (1). Briefly, 10^5 in vivo-passaged L5178Y cells were implanted s.c. on the mid-ventral surface of DBA/2 mice. 7–10 d later, the resultant 0.5–1.0 cm diam nodule was surgically excised, and 7 d later, mice were challenged i.p. with 5×10^4 L5178Y cells.

Partial Peritoneal Lavage (PPL).¹ Mice received an i.p. injection of 2.5 ml of sterile pyrogen-free PBS, and the abdomen was massaged thoroughly to mix the PBS with the peritoneal contents. The mice were then lightly anesthetized with ether and restrained on a board, ventral side upward. A small area of the ventral surface was shaved, and 0.4–2 ml of the PBS was removed from the peritoneal cavity with a 5 ml syringe fitted with a 25-gauge needle. The volume removed from each sampling was recorded. The recovered peritoneal cells (PC) were pelleted and resuspended in the same volume of MEM.

Serial End-point Dilution Assay (SEPD) for Counting Tumor Cells (9). Single-cell suspensions of peritoneal cell populations containing tumor cells were prepared from tumor-dormant mice. 100 µl of MEM was added to each well of a 96-well flat-bottomed microtiter plate (Costar, Cambridge, MA). 100 µl of each cell suspension was then added to the first well of each row, and a series of twofold dilutions was made through 24 wells. Each PC suspension was titrated in quadruplicate. The plates were incubated at 37°C in a humidified 5% CO₂ atmosphere and examined microscopically after 3 and 14 d incubation for tumor cell growth. Wells containing the highest dilution of each cell suspension that yielded positive tumor cell growth were identified, and the number of tumor cells in each initial cell suspension was calculated.

Classification of Tumor-dormant Mice. Immunized and challenged mice were classified as tumor-dormant if the number of tumor cells in the peritoneal cavity on the 25th d after L5178Y cell challenge days post challenge; as determined by PPL and the SEPD assay, was $1-2 \times 10^5$. Tumor-dormant mice were classified as "tumor-emerging" when the number of tumor cells was $>2 \times 10^5$ and $<10^7$. Tumor-dormant mice were classified as "tumor-emergent" when the number of tumor cells in the peritoneal cavity was $>10^7$. Tumor-dormant mice whose peritoneal cells yielded tumor cell growth when placed in culture at 4×10^5 cells/well in a 96-well microtiter plate are referred to as "in vitro tumor-progressor" tumor-dormant mice. Mice whose peritoneal cells yielded significantly fewer tumor cells after 7 d culture as compared with the inception of culture are referred to as "in vitro tumor-regressor" tumor-dormant mice.

Complete Peritoneal Lavage (CPL). Mice were killed by cervical dislocation, and the PC were removed in two successive 5-ml peritoneal washouts with PBS. The PC were pelleted by centrifugation, resuspended in 4 ml MEM, and counted by hemocytometer. This technique recovered >99% of the PC (9).

¹ Abbreviations used in this paper: CPL, complete peritoneal lavage; PC, peritoneal cells; PPL, partial peritoneal lavage; SEPD, serial end-point dilution assay.

In Vitro Tumor-dormant Peritoneal Cell System. Tumor-dormant mice were killed and CPLs were performed. The recovered PC were brought to a concentration of 4×10^6 cells/ml. A volume of 0.5 ml of the PC suspension from each mouse was used for quantitation of tumor cells using the SEPD assay, and the numbers of tumor cells per 4×10^5 PC was calculated. $\frac{1}{10}$ ml of the remaining PC suspension (containing 4×10^5 PC) from each mouse was added to individual wells of a 96-well microtiter flat-bottomed culture plate (Costar). The wells were then divided into sets as indicated in the text, and the wells of each set received 0.1 ml of MEM, PGE₂, or indomethacin, depending on the experimental design. The plates were incubated for 7 d at 37°C. The cells in the wells were then suspended and the tumor cells were quantitated by the SEPD assay and compared with the number of tumor cells calculated to be in the wells at the start of the cultures.

In Vitro PC Culture Supernatant PGE₂ RIA. Peritoneal cells were removed from individual tumor-dormant mice and the number of tumor cells per 4×10^5 PC for each mouse was determined by SEPD assay. Cultures were then prepared as described in the previous section. After 7 d incubation at 37°C, the cell-free supernatants were removed and tested for PGE₂ by RIA (¹²⁵I-PGE₂ RIA kit purchased from New England Nuclear, Boston, MA). This assay could measure as little as 0.13 pg/ml PGE₂. The cells from the wells supplying the supernatants were then suspended, and the number of tumor cells in each suspension was quantitated by the SEPD assay. The number of tumor cells in each well at the beginning and end of culture was then compared, and each well was classified as yielding tumor cell growth or no growth.

Separation of Nonadherent PC. Nonadherent PC were separated from whole PC by adherence to plastic petri dishes.

PGE₂ or Indomethacin Preparation. PGE₂ (Upjohn Co., Kalamazoo, MI) and indomethacin (Sigma Chemical Co., St. Louis, MO) were dissolved in 100% ethanol in a concentration of 10 mg/ml as stock solution and kept in a -20°C freezer until used. The working solution, 10⁻⁵ M, was prepared by dilution in PBS or MEM.

In Vivo Protocol to Determine Effects of PGE₂ or Indomethacin on Tumor-dormant State. The protocol illustrated in Fig. 1 was used. A PPL was performed on all L5178Y cell-immunized and challenged mice 25 d after L5178Y cell challenge. The number of tumor cells in each lavage was determined by the SEPD assay, and mice were classified as tumor-dormant on the basis of the number of tumor cells in their peritoneal cavity (see section on classification of tumor-dormant mice). The tumor-dormant mice were then divided into groups, with each group having equal numbers of mice with comparable tumor burdens. The groups were then treated with PGE₂ i.p. for 10 d, or with indomethacin as indicated in the text. 4 d after the end of PGE₂ treatment, or as indicated in the indomethacin experiments, the mice were killed and CPLs were performed. 10% of the PC from each mouse was used to quantitate the number of tumor cells in the lavage, and the remaining PC were placed in culture flasks at a low cell/surface area ratio to minimize cell-cell contact and to permit a single tumor cell to grow if present. The tumor cell numbers in each mouse before and after treatment were then compared.

Results

Effect of PGE₂ on L5178Y Tumor-dormant State. To determine whether the PGE₂ produced by peritoneal macrophages in tumor-dormant mice before formation of ascitic tumors is responsible for termination of the tumor-dormant state, we administered 100 μg of PGE₂ i.p. to mice daily for 10 d using the protocol shown in Fig. 1. In this protocol, the number of tumor cells in each mouse was quantitated before treatment by PPL and SEPD assay, and after treatment by CPL and SEPD assay.

In the experiment shown in Table I, 12 tumor-dormant mice received PGE₂ and 12 tumor burden-matched tumor-dormant mice received 5% ethanol as controls. 4 d after the last inoculation of PGE₂, 9 of 12 (75%) mice had tumor

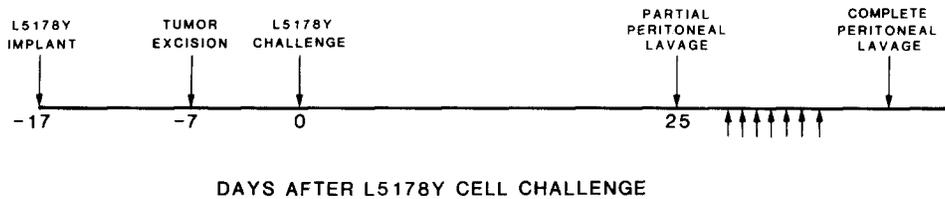


FIGURE 1. Protocol for establishment and treatment of the L5178Y tumor-dormant state. L5178Y cell-immunized and -challenged mice were subjected to a partial peritoneal lavage on the 25th d after challenge. Tumor burdens were quantitated by the SEPD assay, and mice were classified as tumor-dormant if tumor cells were present at $<2 \times 10^5$ cells in the peritoneal cavity. 5 d after partial peritoneal lavage, PGE₂ or indomethacin was administered for the number of days indicated in the text, and CPLs were performed either 4 d after the final day of PGE₂ treatment, or as indicated in the text for the indomethacin experiments. The final tumor burdens in the CPLs were quantitated, and the tumor burdens before and after treatment were compared in individual mice.

TABLE I
Effect of PGE₂ on the L5178Y Tumor-dormant State

Ethanol-treated mice			PGE ₂ -treated mice		
Number of L5178Y cells ($\times 10^3$)		Tumor state*	Number of L5178Y cells ($\times 10^3$)		Tumor state
Before	After		Before	After	
1.6	0	Cure	0.8	>10,000.0	Emergent
2.4	51.2	TD	2.0	409.0	Emerging
3.2	1.2	TD	4.8	102.4	TD
7.2	6.4	TD	4.8	>10,000.0	Emergent
8.0	25.6	TD	6.4	>10,000.0	Emergent
9.6	102.4	TD	7.2	>10,000.0	Emergent
12.8	0.1	TD	8.0	>10,000.0	Emergent
12.8	38.4	TD	9.6	>10,000.0	Emergent
16.0	>10,000.0	Emergent	16.0	6.4	TD
19.2	153.6	TD	28.8	>10,000.0	Emergent
19.2	>10,000.0	Emergent	12.8	>10,000.0	Emergent [‡]
25.6	>10,000.0	Emergent	19.2	>10,000.0	Emergent [‡]
Emergent mice:		3/12 (25%)			9/12 (75%)

PGE₂ was administered i.p. to tumor-dormant mice twice daily for 10 d, 50 μ g per inoculation. Control tumor-dormant mice received inoculations of 0.1 ml of 5% ethanol. Tumor-dormant mice were selected for the experiment if each contained at least 1 and $<200,000$ tumor cells in its peritoneal cavity 25 d after L5178Y cell challenge, as determined by PPLs and SEPD assays. 24 tumor-dormant mice were divided into two groups on the basis of their tumor burdens. Treatment was begun 5 d after PPL. 4 d after the final inoculation of PGE₂, CPLs were performed and the final tumor burdens were determined by SEPD assays.

* Cure: no tumor cells in the peritoneal cavity at the end of the experiment. TD (tumor dormant): at least 1 and $<2 \times 10^5$ tumor cells. Emerging: $>2 \times 10^5$ and $<10^7$ tumor cells. Emergent: $>10^7$ tumor cells. There was a significant difference between the number of emergent mice in the two groups (Mann-Whitney U test, $p < 0.002$).

[‡] These mice died with ascitic tumors before the end of the experimental period.

burdens of $>10^7$ cells, and were classified as tumor-emergent, and one mouse was emerging from the tumor-dormant state. In contrast, only 3 of 12 (25%) of the ethanol-treated mice were tumor-emergent, a level that normally occurs

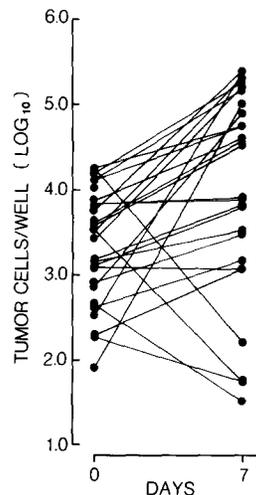


FIGURE 2. Tumor cell growth in PC cultures prepared from different tumor-dormant mice. Peritoneal cells were removed from 26 tumor-dormant mice by CPL. For each mouse, the number of tumor cells per 4×10^5 PC was determined by the SEPD assay, and 4×10^5 PC were planted in each of three wells of a 96-well microtiter plate. After 7 d incubation at 37°C , the number of tumor cells was determined in each of the three wells of each set, and the mean number of tumor cells and SEM per set was calculated. The mean number of tumor cells in cultures prepared from each mouse at the beginning and at the end of incubation is shown.

during such a 3-wk experimental period. In additional experiments, we found that tumor burdens increase in PGE_2 -treated tumor-dormant mice after only 5 d of treatment, as compared with ethanol-treated controls.

Tumor Cell Growth in Cultures of Peritoneal Cells from Tumor-dormant Mice. To study the mechanisms by which PGE_2 produced its tumor cell growth-enhancing effect in tumor-dormant mice, we used an *in vitro* system. In this assay, described previously (1), 4×10^5 PC from a tumor-dormant mouse are placed in individual wells of a 96-well microtiter plate. We reported previously (1) that the small number of tumor cells present in these PC do not grow out during incubation at 37°C .

Fig. 2 shows the growth of tumor cells in PC cultures prepared from a large number of tumor-dormant mice. PC from 26 tumor-dormant mice were harvested, the number of tumor cells per 4×10^5 PC in each mouse was quantitated by the SEPD assay, and the remaining PC were placed in wells of a 96-well microtiter plate, 4×10^5 cells/well, three wells/mouse. The plates were incubated for 7 d at 37°C , and the number of tumor cells in each well was quantitated by the SEPD assay. The mean number of tumor cells in the PC at inception and termination of culture for each mouse are shown in Fig. 2. During the 7-d incubation period, tumor cells proliferated in the PC cultures prepared from some tumor-dormant mice, and the mice providing these PC are referred to as "in vitro tumor-progressor" mice. Tumor cells failed to grow out in the PC cultures prepared from other tumor-dormant mice, and the mice providing these PC are referred to as "in vitro tumor-regressor" mice.

Effect of PGE_2 on Tumor Cell Growth in PC Cultures Prepared from In Vitro Tumor-progressor and In Vitro Tumor-regressor Tumor-dormant Mice. To deter-

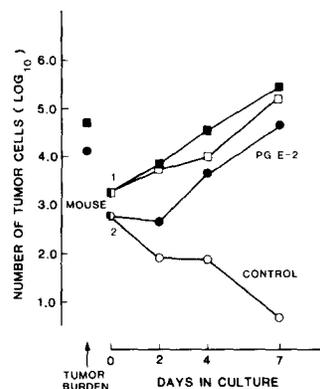


FIGURE 3. The effect of PGE₂ on tumor cell growth in PC cultures prepared from a representative in vitro tumor-progressor tumor-dormant mouse (1, ■) and in vitro tumor-regressor tumor-dormant mouse (2, ●). Peritoneal cells were collected, tumor burdens were determined, and PC cultures were prepared as described in Fig. 2. 12 wells were prepared from each mouse, with 6 of the wells receiving ethanol-containing medium (□, ○) and 6 wells receiving PGE₂ (■, ●) at 10⁻⁶ M. After 2, 4, and 7 d incubation, the tumor cells in two wells of each set were counted by the SEPD assay. The isolated symbols represent the number of tumor cells in the peritoneal cavity of each mouse. Results are mean numbers of tumor cells per set.

mine whether PGE₂ would stimulate the growth of tumor cells in PC populations from in vitro tumor-regressor tumor-dormant mice, we added PGE₂, at 10⁻⁶ M, to 6 of 12 PC wells prepared from a number of tumor-dormant mice; the other 6 wells from each mouse received ethanol-containing MEM. After 2, 4, and 7 d incubation, the cells in two of the PGE₂-treated wells and in two of the untreated wells from each mouse were suspended, and the tumor cells in the suspensions were quantitated. Fig. 3 shows the effects of PGE₂ on tumor cell growth in PC cultures prepared from an in vitro tumor-progressor mouse (mouse 1), and an in vitro tumor-regressor mouse (mouse 2). In mouse 1, tumor cells increased in number in the ethanol-treated control wells, and PGE₂ had no effect on this tumor cell growth. In mouse 2, tumor cells decreased in number during the 7-d incubation period in the control wells, and the addition of PGE₂ resulted in rapid tumor cell growth at a rate equal to the rate of growth of tumor cells in the cultures prepared from mouse 1. Similar results have been found in many matched pairs of in vitro tumor-progressor and in vitro tumor-regressor tumor-dormant mice. In other experiments with pure tumor cell cultures, we found that PGE₂ at 10⁻⁶ M had no direct effect on the rate of tumor cell growth during 72 h incubation at 37°C (data not shown). These data suggest that PGE₂ enhances tumor cell growth in PC cultures from in vitro tumor-regressor tumor-dormant mice by subverting the function of those host cells that normally restrain tumor cell growth.

Production of PGE₂ in PC Cultures from In Vitro Tumor-progressor Tumor-dormant Mice. The parallel growth curves of tumor cells in the PGE₂-treated and untreated wells of PC prepared from mouse 1 in the above experiment suggested that PGE₂ was being produced in the untreated wells. To test this possibility, we prepared PC cultures from 17 tumor-dormant mice, one well/mouse, and after 7 d culture, we collected the cell-free supernatants from each well and measured

TABLE II
Correlation Between Tumor Cell Growth In Vitro and PGE₂ Production in PC Cultures from Tumor-dormant Mice

Total number of wells*	Wells with tumor cell increase [‡]		Wells with tumor cell decrease	
	Number	Wells with PGE ₂ (>3 ng/ml) [§]	Number	Wells with PGE ₂ (>3 ng/ml)
17	11	8 (73%)	6	0 (0%)

* Each well contained 4×10^5 PC from a different tumor-dormant mouse.

[‡] A change in tumor cell numbers equal to or greater than fourfold during the 7-d incubation period.

[§] PGE₂ levels in the culture wells in which tumor cells did not increase were <2.2 ng/ml. 8 of the 11 wells in which tumor cells increased contained >4.3 ng/ml. Statistical analysis by Student's *t* test indicates that the difference in PGE₂ levels between the two groups is significant ($p < 0.001$).

the PGE₂ in the supernatants by RIA. The PC in these wells were then suspended, and the number of tumor cells in each suspension was determined by the SEPD assay. As seen in Table II, there was an excellent correlation between the ability of tumor cells to proliferate and the amount of PGE₂ in each well. However, there was no correlation between the total number of tumor cells in specific wells and the amount of PGE₂ (data not shown).

To identify the cell population that produces PGE₂ in the PC cultures from in vitro tumor-progressor mice, and to exclude the possibility that these cultures contained PGE₂-producing L5178Y cells, we transferred the PC from in vitro tumor-progressor cultures to tissue culture flasks. The L5178Y cells proliferated in the flasks and were passaged until no host cells remained. The supernatants of these tumor cell cultures were then tested for PGE₂ and none was found. We can conclude from this experiment that PGE₂ is produced by host cells rather than by tumor cells.

Effect of Indomethacin on the Tumor-dormant State. The ability of PGE₂ to cause formation of ascitic tumors in tumor-dormant mice and promote tumor cell growth in vitro suggested that administration of an inhibitor of PGE₂ synthetase, indomethacin, might eliminate tumor cells from tumor-dormant mice and inhibit tumor cell growth in vitro. To test this possibility, indomethacin was administered into the peritoneal cavity of tumor-dormant mice from Alzet mini-osmotic pumps. These pumps, implanted subcutaneously on the ventral surface with a size PE 60 catheter leading into the peritoneal cavity, delivered a continuous amount of indomethacin, 50 μ g/d, or ethanol for 14 d; new pumps were not reimplanted in the 21-d-incubation mice. At selected days after implantation of the pumps, mice were killed and all PC were placed in culture, 10% of the PC in a SEPD assay, and the remaining PC in culture flasks at a low cell/culture surface area ratio to minimize cell-cell contact during the incubation period. As seen in Table III, at 7, 14, and 21 d after implantation of the mini-pumps, there were no recoverable tumor cells in the indomethacin-treated mice as compared with the ethanol-treated controls.

Effect of Indomethacin on Tumor Cell Growth in PC Cultures from Tumor-dormant

TABLE III
Effect of Indomethacin on the L5178Y Tumor-dormant State

Treatment	Mice with no recoverable tumor cells vs. total mice* on:		
	Day 7	Day 14	Day 21
Ethanol	0/3 (0%)	0/14 (0%)	2/9 (22%)
Indomethacin	3/3 (100%)	9/14 (64%)	8/11 (73%)

Mice received either 50% ethanol vol/vol or indomethacin (50 μ g/d i.p.) for the designated number of days, delivered at 0.6 μ l/h by catheter from Alzet mini-osmotic pumps, which were implanted subcutaneously on the ventral surface.

* The number of mice that had no recoverable tumor cells on cultivation of all PC at the end of the designated number of days of treatment divided by the total number of mice in each group.

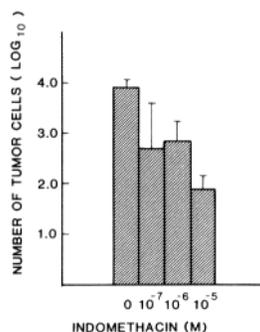


FIGURE 4. The effect of indomethacin on the growth of tumor cells in PC cultures prepared from an in vitro tumor-progressor tumor-dormant mouse. PC were collected and placed in wells of a 96-well microtiter dish, 4×10^5 cells/well. Indomethacin at the indicated molarity was added to wells, three wells per molarity, and the tumor cells were counted after 7 d incubation by the SEPD assay. The results are expressed as the mean numbers of tumor cells (\pm SEM) for each set.

Mice. The production of PGE₂ in cultures of PC from in vitro tumor-progressor tumor-dormant mice suggested that indomethacin added to such cultures at their inception might inhibit tumor cell growth. We therefore added indomethacin at 10⁻⁵, 10⁻⁶, and 10⁻⁷ M to wells of PC from in vitro tumor-progressor tumor-dormant mice, three wells per molarity, and 7 d later, we quantitated the tumor cells in the medium- and indomethacin-treated wells. These concentrations of indomethacin completely inhibit PGE₂ production in PC cultures from in vitro tumor-progressor tumor-dormant mice (data not shown). As seen in Fig. 4, treatment of PC tumor-progressor cultures with indomethacin at 10⁻⁵ and 10⁻⁶ M inhibited tumor cell growth. In experiments with pure tumor cell cultures, we found that indomethacin at 10⁻⁵ M had no direct effect on L5178Y cell growth (data not shown). These data suggest that indomethacin inhibits tumor cell growth by preventing PGE₂-mediated suppression of those host cells that normally restrain tumor cell growth.

Dose Effect of PGE₂ in Presence of Indomethacin on Tumor Cell Growth in PC Cultures Prepared from In Vitro Tumor-regressor Tumor-dormant Mice. We next evaluated the dose effect of PGE₂ on the growth of tumor cells in PC cultures from in vitro tumor-regressor tumor-dormant mice. Indomethacin was added to

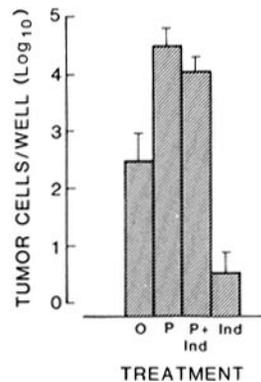


FIGURE 5. The effect of indomethacin, 10^{-5} M, on the tumor cell growth enhancing activity of PGE_2 , 10^{-6} M, in PC cultures prepared from tumor-dormant mice. A pool of PC from two in vitro tumor-regressor tumor-dormant mice was prepared, and indomethacin and PGE_2 were added to wells, three wells per set. Tumor cells were counted after 7 d incubation. The results are expressed as the mean numbers of tumor cells (\pm SEM) for each set.

each well in this experiment in order to inhibit endogenous PGE_2 production. We first determined whether indomethacin would interfere with the tumor cell growth-enhancing effects of PGE_2 . As seen in Fig. 5, PGE_2 enhanced tumor cell growth. Indomethacin at 10^{-6} M inhibited tumor cell growth, but had no inhibitory effect on the tumor cell growth-enhancing effect of 10^{-6} M PGE_2 . Fig. 6 shows the effect of various doses of PGE_2 in the presence of 10^{-6} M indomethacin on tumor cell growth. The addition of 10^{-5} – 10^{-7} M PGE_2 stimulated tumor cell growth, and lower doses of PGE_2 had no effect.

Identification of Host Cells that Restrain Tumor Cell Growth in PC Cultures from In Vitro Tumor-regressor Tumor-dormant Mice. To identify the PC population that was responsible for the restraint on L5178Y cell growth in wells prepared from in vitro tumor-regressor mice, we separated the nonadherent peritoneal cells by a plastic-adherence step, and cultured both the complete and the nonadherent PC at high cell density. Since L5178Y cells are nonadherent, they remained in the nonadherent cell population. PGE_2 was added to one-half of the number of wells of each PC population, and tumor cells were quantitated 7 d later by the SEPD assay.

As seen in Fig. 7, tumor cell growth did not occur in the untreated complete PC cultures, but did occur when PGE_2 was added to the cultures. In the untreated nonadherent PC cultures, tumor cells grew and PGE_2 had no effect on tumor cell growth. These findings indicate that adherent cells are involved in the restraint on tumor cell growth. Yet to be determined is the role of the nonadherent PC in tumor cell growth restraint, and whether the adherent cells lyse or arrest the proliferation of tumor cells, or are required as accessory cells for nonadherent cells to restrain tumor cell proliferation.

Discussion

Prostaglandins of the E series are important regulators of many components of the immune response (11). Among its other effects on the immune system, PGE_2 has been reported to (a) inhibit the production of IL-2 (12, 13), (b) stimulate

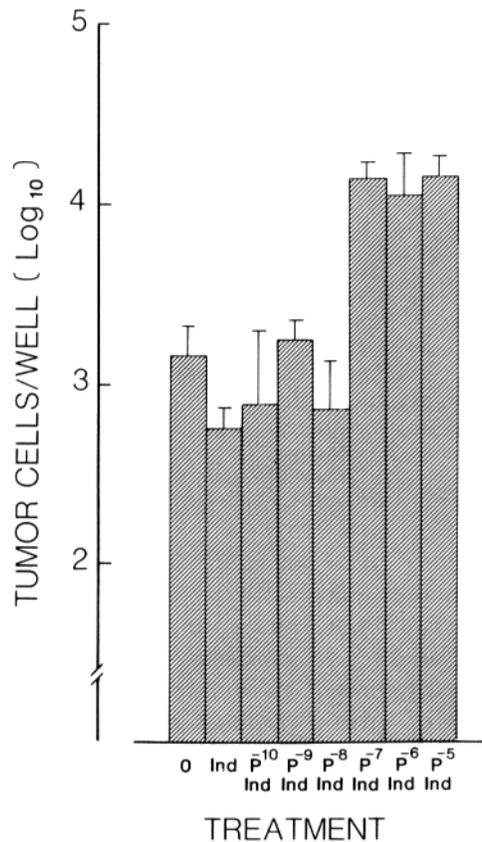


FIGURE 6. The effect of graded doses of PGE₂ in the presence of 10⁻⁵ M indomethacin on the growth of tumor cells in PC cultures prepared from a pool of four in vitro tumor-regressor tumor-dormant mice. The procedure used in Fig. 5 was followed. Results are mean numbers of tumor cells (\pm SEM) per set.

the production of suppressor T lymphocytes (14–16), and (c) inhibit the clonal proliferation of B lymphocytes (17). Macrophages produce large amounts of PGE₂ on appropriate stimulation (18, 19). This PGE₂ can downregulate macrophage tumoricidal activity and may be an important self-regulating feedback mechanism for macrophage activation (20–22). Macrophages from tumor-bearing mice produce increased amounts of PGE₂ (23), and patients with various types of cancer have either increased levels of PGE₂ and PGE₂ metabolites (24–27), or have impaired immune responses that can be stimulated in vitro with indomethacin (28). Administration of inhibitors of PG synthesis in mice has been found to retard or suppress tumor growth (29, 30), and to improve cell-mediated immune functions when added to their spleen cells in vitro (29, 31). However, in some tumors, such as B-16 melanoma, the major cyclooxygenase metabolite is PGD₂ rather than PGE₂, and inhibitors of PG synthesis produce tumor enhancement rather than tumor inhibition (32).

To date, the effects of PGE₂ and indomethacin on tumor growth have been evaluated in animal models in which tumor cells have either been induced or are

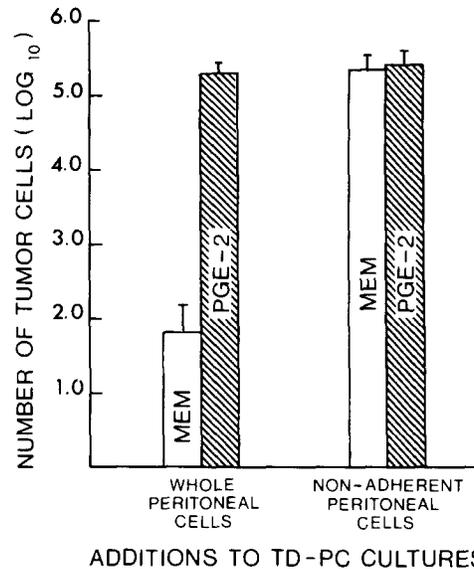


FIGURE 7. Tumor cell growth in cultures of the complete and the nonadherent populations of PC from an in vitro tumor-regressor tumor-dormant mouse. The effect of PGE₂ at 10⁻⁶ M on each set of cultures is shown. The PC were harvested by CPL. One-half of the PC were incubated for 1 h on plastic tissue culture plates and the nonadherent cells (containing the nonadherent tumor cells) removed. Culture wells were prepared with either 4 × 10⁵ PC or the nonadherent equivalent per well, four wells per group. Two wells from each set was then treated with PGE₂, and the cultures were incubated at 37°C for 7 days. The tumor cells in each well were then counted by the SEPD assay. Results are mean numbers of tumor cells (±SEM) per set.

growing after transplantation. Animal models of tumor dormancy are clinically relevant, in that small numbers of lethal tumor cells persist for prolonged periods of time without producing disease (33, 34). Such models may be analogous to those patients who are in clinical remission after treatment of a primary tumor and who will develop a recurrent tumor. Likely examples of tumor-dormant states in human cancer are adenocarcinomas of the breast, which can grow out in the surgical scar tissue of a mastectomy as long as 50 yr after surgical removal of a histologically identical adenocarcinoma (35), and malignant melanomas, which can grow out in the liver many years after removal of a uveal malignant melanoma (36, 37).

In this report we described the effects of PGE₂ on tumor cell growth under conditions in which tumor cells were maintained in a tumor-dormant state. Previous evidence for the regulatory role of PGE₂ in the L5178Y tumor-dormant system consists of our finding (6) that macrophages with increased immunosuppressive activity appear in the peritoneal cavity of tumor-dormant mice just before the appearance of ascitic tumors. These macrophages produce an immunosuppressive factor in vitro, now identified as PGE₂ (our unpublished data), production of which is inhibited by indomethacin (7). Our demonstration that exogenous PGE₂ stimulates formation of ascitic tumors in tumor-dormant mice provides further support for the hypothesis that PGE₂, which is produced by peritoneal macrophages, terminates the tumor-dormant state.

We found that the PC cultures from *in vitro* tumor-progressor tumor-dormant mice (in which tumor cells proliferate progressively) produce more PGE₂ than PC cultures from *in vitro* tumor-regressor tumor-dormant mice (in which tumor cells do not proliferate), and that the addition of PGE₂ to tumor-regressor cultures converts them into tumor-progressor cultures. The variations in the ability of tumor cells to proliferate in PC cultures from tumor-dormant mice may reflect the variations in the duration of the tumor-dormant state among mice. It is possible, however, that the differences between tumor-regressor and tumor-progressor cultures may reflect ongoing cycles within an individual mouse, of macrophage activation and deactivation mediated by IFN- γ released from stimulated T cells, and by PGE₂ released from activated macrophages. If this is so, then an individual mouse may go through cycles of tumor-progression and tumor-regression, the net result of which would be to maintain cytolytic or cytostatic control on tumor cell proliferation without killing all tumor cells. The demonstrations that indomethacin can both eliminate recoverable tumor cells from tumor-dormant mice and inhibit the proliferation of tumor cells in PC cultures from *in vitro* tumor-progressor tumor-dormant mice support the hypothesis that macrophage activation and deactivation is a feature of the tumor-dormant state, and that the inhibition of a suppressor of macrophage activation permits macrophages to kill all tumor cells.

The complete analysis of the L5178Y tumor-dormant model is limited by certain characteristics of the model. Since large numbers of peritoneal cells are required for the preparation of multiple PC cultures, it is necessary to kill the tumor-dormant mouse to obtain these cells. It is therefore not possible, in an individual mouse, to measure the changes in antitumor activity of peritoneal cell populations as that mouse proceeds through the tumor-dormant state towards termination. It is also not possible to compare one tumor-dormant mouse with another because of the large variations in the activity of the immune response and in the duration of the tumor-dormant state among mice that have been immunized and challenged with L5178Y cells at the same time.

The *in vitro* assay described here does provide a system in which the PC population of a single tumor-dormant mouse can be studied under controlled conditions. An important feature of this *in vitro* system is that it is quantitated in the same way as the *in vivo* model, e.g., by tumor cell proliferation. The tumor cell growth-enhancing effects of exogenous PGE₂, when administered both *in vivo* and *in vitro*, and the tumor cell growth-inhibiting effects of indomethacin, when administered both *in vivo* and *in vitro*, support the validity of the *in vitro* system as a correlate of the *in vivo* model. This *in vitro* system, therefore provides an opportunity to evaluate the mechanisms that maintain and terminate the tumor-dormant state *in vivo*. Experiments to identify the mechanisms by which PGE₂ and indomethacin produce their tumor cell growth-enhancing and -inhibiting effects in tumor-dormant mice are in progress, and will constitute a subsequent paper in this series.

Our analysis of the L5178Y tumor-dormant model to date suggests that L5178Y cells are maintained in a tumor-dormant state by peritoneal macrophages and T lymphocytes. These cells are upregulated to a cytotoxic state by lymphokines released from L5178Y-stimulated helper T lymphocytes, and down-

regulated to a noncytotoxic state by PGE₂ released from activated macrophages. The PGE₂ may downregulate macrophage cytolytic activity by acting directly on macrophages and downregulate T lymphocyte cytolytic activity either directly or indirectly by stimulating suppressor T lymphocytes. Both macrophages and cytolytic T lymphocytes may be downregulated before all tumor cells are killed, thereby permitting some tumor cells to escape lysis and persist in a tumor-dormant state. The tumor-dormant state may end when phenotypic variants of the L5178Y cells, which are less susceptible to immune lysis and less immunogenic than the L5178Y population used to initiate the tumor-dormant state, are selected by the immune response and become dominant in the peritoneal cavity of tumor-dormant mice (9, 10). These poorly immunogenic tumor cell variants may not stimulate helper T lymphocytes to produce the regulatory lymphokines that are needed to maintain an effective anti-tumor cell response and maintain the tumor-dormant state. The L5178Y tumor-dormant model can be used to study immunoregulatory circuits that affect tumor growth, and to test therapeutic agents that can be given to patients who are in clinical remission after treatment of a primary tumor and who have a high statistical probability of developing a recurrent tumor.

Summary

Immunization and intraperitoneal challenge of DBA/2 mice with L5178Y lymphoma cells results in the suppression and maintenance of the L5178Y cells in a tumor-dormant state in the peritoneal cavity for many months. Cell-mediated immune responses involving lymphocytes and macrophages are involved in maintenance of the tumor-dormant state. Macrophages that have increased immunosuppressive activity and that produce increased amounts of PGE₂ appear in the peritoneal cavity of tumor-dormant mice before the breakdown of the tumor-dormant state and formation of ascitic tumors.

We report here that the tumor-dormant state can be terminated with formation of ascitic tumors by treatment of tumor-dormant mice with PGE₂. Treatment with indomethacin results in inhibition of tumor cell growth and elimination of all recoverable tumor cells. Cultures of peritoneal cells (PC) from mice harboring L5178Y cells in a tumor-dormant state were used to analyze the PGE₂ and indomethacin effects. Tumor cells did not grow out in the high-cell density PC cultures prepared from many tumor-dormant mice, but addition of PGE₂ to these cultures resulted in tumor cell growth. The tumor cell growth that did occur in the PC cultures from some tumor-dormant mice was associated with PGE₂ production by the associated host cells, and the addition of indomethacin to these cultures inhibited both PGE₂ synthesis and tumor cell growth. Removal of plastic-adherent cells from the PC cultures eliminated the restraint on tumor cell growth. These experiments suggest that L5178Y tumor cells are maintained in a tumor-dormant state by host peritoneal cells, which are under PGE₂ regulation.

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