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# THE ROLE OF TUMOR-DERIVED CYTOKINES ON THE IMMUNE SYSTEM OF MICE BEARING A MAMMARY ADENOCARCINOMA[1]

## I. Induction of Regulatory Macrophages in Normal Mice by the in Vivo Administration of rGM-CSF

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**Using a dimethylbenzanthracene-induced immunogenic nonmetastatic murine mammary adenocarcinoma in BALB/c mice, our previous work has shown that splenocytes from tumor bearers have reduced responses to both mitogens and Ag including tumor-associated Ag. NK and cytotoxic T cell activities are also reduced in splenocytes of tumor bearers. Mac-1$^+$2$^+$ macrophages induced in mammary tumor bearers are capable of down-regulating lymphocyte responses to mitogens and tumor-associated Ag by cell to cell contact interaction and increased $PGE_2$ production. We have found that the tumor constitutively releases a granulocyte-macrophage (GM)-CSF-like factor in vivo and in vitro, which may be responsible for the systemic increase in cells of the macrophage lineage in tumor-bearing mice. A tumor cell line established from the in vivo tumor expresses and releases GM-CSF as shown by Northern and Western blot analyses. Daily i.p. injections for 3 wk of 10,000 U of rGM-CSF into normal mice induces hemopoietic and immunologic alterations similar to those observed in tumor bearers. Mac-1$^+$ and/or Mac-2$^+$ macrophages can also be detected in the spleens and bone marrow of the mice treated with rGM-CSF. Additionally, splenocytes from rGM-CSF-treated mice have reduced responses to mitogens and their peritoneal exudate cells can cause in vitro down-regulation of proliferative responses of lymphocytes from normal mice. The suppression can be partially reversed by the addition of indomethacin to the cultures suggesting that $PGE_2$ may contribute to the effect. rGM-CSF enhances the in vitro release of $PGE_2$ by the spleen, bone marrow, and peritoneal cells of normal mice. These data indicate that the high levels of GM-CSF constitutively produced by the tumor may be responsible for the hemopoietic changes and immunologic alterations observed in tumor-bearing mice.**

GM[3]-CSF is known to stimulate BM precursor cells to proliferate and differentiate into granulocytes and macrophages that participate in a variety of functional activities, including oxidative metabolism, inhibition of migration, antibody-dependent cellular cytotoxicity, increased phagocytosis, and $PGE_2$ production (1–5). Whether this factor can enhance the direct cytotoxic ability of macrophages in vivo and in vitro is still controversial (6–8). Although rGM-CSF treatment in vivo increases the number of granulocytes and macrophages, the role of this cytokine in the in vivo immune response has not been studied in detail. Extensive clinical studies using rGM-CSF have been performed to determine its efficacy in the treatment of cancer or the repopulation of the host's BM after chemotherapy or radiation (8–10). Other clinical studies have shown that many cancer patients have immune alterations, and hematologic abnormalities such as leukocytosis, which appear to be commonly associated with solid tumors in animal models (11–15). A number of solid tumor cell lines have been established that produce CSA in vitro (12–15). Regardless of tumor location or etiology, tumor growth in humans and laboratory animals is frequently accompanied by progressive immunosuppression (16–20), but the mechanisms involved are not clearly understood. Some tumor models suggest that suppressor macrophages may be important (19, 20), and although there are some reports of a relationship between hemopoiesis and suppressor cells (21–24), the consequences to the immune system of hemopoietic alterations have not been elucidated. A lack of in vivo-in vitro combined studies further complicates the picture.

Our previous studies in BALB/c mice, with the use of a dimethylbenzanthracene-induced immunogenic nonmetastatic murine mammary adenocarcinoma, have shown that splenocytes from tumor bearers have reduced immunogenic responses and their NK and cytotoxic T cell activities are also reduced (16, 24–27). The kinetics of the increase in number of a Mac-1$^+$2$^+$ splenic macrophage population tightly parallels the progressive growth of the tumor and the concomitant immunosuppression (24, 27). Furthermore, purified Mac-1$^+$2$^+$ macrophages from tumor-bearing mice suppress the normal T and B cell responses to mitogens and Ag, including tumor-associated Ag. Progenitors of the macrophage lineage are

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[3] Abbreviations used in this paper: GM, granulocyte-macrophage; BM, bone marrow; CSA, colony-stimulating activity; PEC, peritoneal exudate cells.

increased throughout the hemopoietic system in tumor bearers as CSA increases in the serum and tumor cystic fluid. The tumor releases a GM-CSF-like factor that contributes to the alterations in the hemopoietic system and may expand a macrophage population with suppressive function (24, 27). We now present evidence that treatment of normal mice with rGM-CSF induces changes similar to those observed in tumor bearers. Increases of Mac-$1^+$ and/or Mac-$2^+$ macrophages in spleen and BM were also detected in mice treated with rGM-CSF. Splenocytes from rGM-CSF-treated mice, which have reduced responses to mitogens and peritoneal exudates from these mice but not from control mice, suppress the response of splenocytes from normal mice to mitogens. Because the addition of indomethacin can reverse most of this suppression, $PGE_2$ appears to contribute to the down-regulation. In addition, rGM-CSF enhanced the release of $PGE_2$ from spleen, BM, and peritoneal cells of normal mice in vitro. These data support the contention that in tumor bearers the high level of in vivo GM-CSF is responsible, at least in part, for the hemopoietic and immunologic alterations observed.

## MATERIALS AND METHODS

*Mice.* Male and female 10- to 14-week old BALB/c mice, maintained by brother-sister mating in our laboratory, were used in all experiments.

*Tumor.* The mammary adenocarcinoma (D1-DMBA-3) is a transplantable tumor (28) that was derived from a nonviral, noncarcinogen-induced preneoplastic alveolar nodule in a BALB/c mouse after treatment with 7,12-dimethylbenzanthracene. The tumor, which is immunogenic and nonmetastatic, is routinely transplanted in the colony of origin by s.c. injection of $1 \times 10^6$ tumor cells. The tumor appears in 8 days with necrosis around 30 days and animal death after 30 days. The in vitro cell line (DA-3) was derived from the D1-DMBA-3-transplantable tumor, and reinjection of the cell line into BALB/c mice produces a tumor identical to the parent.

*Reagents.* Unless noted otherwise all reagents were obtained from GIBCO Laboratories, Grand Island, NY. The culture medium (RPMI 1640 supplemented) used to grow the cell line and to perform all proliferation assays contains RPMI 1640, 100 U/ml penicillin, 100 μg/ml streptomycin, $5 \times 10^{-5}$ M 2-ME, 2 mM L-glutamine, 1% nonessential amino acids, 1% essential amino acids, 1% sodium pyruvate, and 10% endotoxin-free fetal bovine serum (Hyclone Laboratories, Logan, UT). Where indicated, indomethacin (Sigma, St. Louis, MO) at a concentration of $2 \times 10^{-5}$ M was added to the cultures. Rabbit antisera to GM-CSF and rGM-CSF were a gift from Dr. Diane Y. Mochizuki, Immunex Corp, Seattle, WA. The source of murine rGM-CSF and its antiserum has been described (29). rGM-CSF has a specific activity of $2 \times 10^4$ U/μg GM-CSF with the use of a mouse BM proliferation assay. Rabbits immunized with pure rGM-CSF generated antibodies that were shown to be specific for both rGM-CSF and natural GM-CSF. Other lymphokines, including IL-1, IL-2, IL-3, and human GM-CSF did not react with the antiserum (29). We have previously reported that murine G-CSF and M-CSF did not react with this antiserum (24). The rGM-CSF mixed with 10% normal mouse serum contained less than 0.1 ng/ml of LPS attributable to endotoxin present in normal mouse serum. There was less than 1 pg of endotoxin/injection caused by the rGM-CSF.

*Cell source and preparation.* Splenocytes were separated by passing the spleen though a steel mesh and washing with RPMI 1640. BM cells were collected from the femur by aspiration of RPMI 1640 through a 25-gauge needle. The PEC were collected by washing the peritoneal cavity with 10 ml of cold medium. Orbital plexus blood was collected as a source of white blood cells.

*DA-3 supernatant.* The DA-3 cell line at $5 \times 10^5$/ml was cultured in supplemented serum-free RPMI 1640 (Sigma) for 3 days. The culture supernatant was collected, spun down at $400 \times g$ for 10 min, and passed through a 0.22-μ filter (Millipore Corp., Bedford, MA) to remove the cellular component.

*Northern blot analysis.* The murine GM-CSF probe, generously provided by Dr. N. M. Gough (The Walter and Eliza Institute, Melbourne, Australia) has been described in detail (30). Total cellular RNA was extracted from either $50 \times 10^6$ DA-3 tumor cells or resting splenocytes from normal BALB/c mice by using the guanidinium/cesium chloride method of Chirgwin et al. (31). Total RNA at 10 μg/lane was size fractionated on a formaldehyde-agarose gel and then transferred to a Zeta probe nylon membrane (Bio-Rad Richmond, CA) for 16 h. After transfer, the membranes were hybridized with a $^{32}$P-labeled GM-CSF cDNA probe for 16 h at 42°C in a buffer containing 5× SSPE (1× SSPE is 150 mM NaCl, 10 mM $NaH_2$, $PO_4$, and 1 mM EDTA, pH 7.4), 5× Denhardt's solution, 50% formamide, 100 μg/ml of denatured salmon sperm DNA, and 0.1% of $NaDodSO_4$. The membranes were washed twice in 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS, pH 7.0, at 60°C and twice in 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0, at 60°C. Kodak XAB film with intensifying screens was exposed to the filter for 2 days at −70°C.

*Western blot analysis.* The supernatants of DA-3 tumor cell line or tumor supernatants partially purified by Sephacryl S-300 or rGM-CSF were run on Con A-Sepharose 4B beads (Sigma) to remove nonglycosylated proteins. The purified glycosylated proteins, which were collected from the beads by boiling 10 min, were then added to a 12.5% SDS-PAGE procedure with the modification described by Towbin et al. (32). The protein transfer was performed at room temperature with a trans-blot cell (IDEA Scientific Company, Corvallis, OR). After completion of the electrophoretic protein transfer, nonspecific antibody-binding sites on the nitrocellulose (Schleicher & Schuell, Keene, NH) were blocked by incubation with 5% nonfat milk in PBS (blocking buffer) for 2 h at room temperature with shaking. The strips were incubated with a 1/500 dilution of either normal rabbit serum or rabbit anti-rGM-CSF serum in the blocking buffer for 2 h and were then extensively washed with 0.05% Tween 20 in PBS. $^{125}$I-labeled goat anti-rabbit IgG (ICN, Irvine, CA) in blocking buffer was then added for 2 h after which the filters were again washed. The filters were exposed at −70°C to Kodak XAR film for 2 days.

*CFU bioassay.* A modification of the bioassay in soft agar was used as previously described (24). The murine femur was aseptically removed and aspirated and $1 \times 10^5$ BM and PEC or $5 \times 10^5$ splenocytes were added to 35-mm tissue culture dishes with grids (Miles Scientific, Naperville, IL) that contained a total volume of 1 ml agar (0.3%) with 100 U rGM-CSF, 15% Hyclone FCS, and 10% horse serum (GIBCO). The dishes were left at room temperature for 20 min to allow the agar to gel and then incubated at 37°C in a humidified atmosphere and 5% $CO_2$. Cultures were incubated for 6 or 13 days and colonies (>40 cells) were counted at 20 × magnification.

*Fluorescence staining.* BM cells and splenocytes were stained with rat anti-mouse Mac-1 (M1/70) and anti-Mac-2 (M3/38) from Hybritech, San Diego, CA. Goat anti-rat FITC (Boehringer-Mannheim, Indianapolis, IN) was used as the second step reagent. Monoclonals of irrelevant specificity served as negative controls and all reagents were titrated for the appropriate concentrations. A total of $10^6$ cells was stained and 5,000 to 10,000 cells were analyzed on a FACScan cell analyzer (Becton Dickinson, Mountain View, CA) as previously described (33).

*Lymphocyte transformation assay.* Proliferative assays were performed as previously described (24). Briefly, $2 \times 10^5$ cells were cultured with medium and either 2.5 μg/ml of Con A (Boehringer Mannheim, Indianapolis, IN) or 25 μg/ml of LPS (Difco, Detroit, MI). Cultures were maintained for 48 h, pulsed with 0.5 μCi/well of [$^3$H]TdR (New England Nuclear, Boston, MA) for an additional 18 h and harvested. The [$^3$H]TdR incorporation was measured in a liquid scintillation counter (Beckman Instruments, Irvine, CA). Coculture cells were exposed to 1500 rad before culture as were the normal spleen cells used as controls for crowding.

*RIA for prostaglandin.* The $PGE_2$ levels in serum and cell culture supernatants were assayed by using the protocol described in the $PGE_2$ RIA kit (Seragen, Boston, MA). Sera were extracted with ethylacetate and dimethylformamide and the residue containing the $PGE_2$ was evaporated to dryness under a stream of liquid nitrogen. A total of $5 \times 10^5$ splenocytes or BM cells and $1 \times 10^5$ PEC were cultured for 5 days and the supernatants harvested on day 5. The samples were then subjected to RIA and $PGE_2$ levels were determined by extrapolation from a linear graph of cpm vs a known $PGE_2$ standard.

## RESULTS

Previously we have found that CSA in tumor cell supernatants was totally blocked by anti-rGM-CSF but not anti-rG-CSF or anti-M-CSF, indicating that the antigenicity of the tumor-derived factor is similar to rGM-CSF (24). As shown in Figure 1 (*left*) the tumor cell line was analyzed for GM-CSF mRNA expression by Northern blot analysis. A total RNA extraction of the tumor cell line

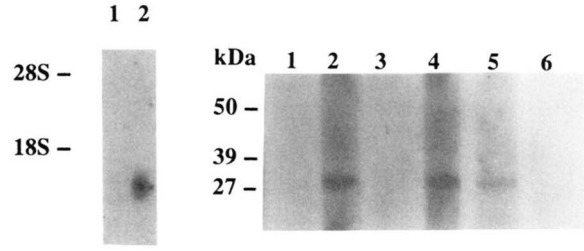


*Figure 1.* Molecular analyses of GM-CSF-related RNA and protein. *Left*, mRNA of GM-CSF detected by Northern blot analyses. Ten micrograms of total RNA from either normal mouse splenocytes (*lane 1*) or DA-3 tumor cell line (*lane 2*) were probed with cDNA from murine GM-CSF. *Right*, Western blot analysis to detect GM-CSF. *Lanes 1* and *2* contain 200 U (10 μg) of rGM-CSF; *lanes 3* and *4* contain unseparated tumor supernatants; *lanes 5* and *6* contain tumor supernatants partially purified by Sephacryl S-300. *Lanes 1, 3,* and *6* were probed with preimmunized rabbit serum; *lanes 2, 4,* and *5* were probed with anti-rGM-CSF rabbit serum.

TABLE I

*Detection of CFU in spleen, BM, and peritoneal cells of normal mice injected with rGM-CSF*[a]

| Cell Source | rGM-CSF[a] | CFU-GM[c] | |
|---|---|---|---|
| | | 6 days | 13 days |
| Spleen | – | 3.3 ± 0.9 | 8.7 ± 3.1 |
| | + | 33.0 ± 2.5[d] | 36.0 ± 2.5[d] |
| BM | – | 64.0 ± 4.1 | 45.0 ± 2.5 |
| | + | 87.0 ± 7.0[e] | 71.0 ± 2.1[d] |
| PEC | – | 0.7 ± 0.5 | 17.0 ± 1.2 |
| | + | 5.3 ± 1.2[e] | 54.0 ± 4.5[d] |

[a] The values indicate the mean number of colonies of triplicate cultures ± SD from individual assayed mice representative of seven separate experiments.

[b] Animals injected with 10% normal mice serum (–) or rGM-CSF plus 10% normal mouse serum (+).

[c] A total of 100 U of rGM-CSF was added per culture. No colonies appeared in the absence of rGM-CSF.

[d] Values are significantly different from control mice, $p < 0.001$.

[e] Values are significantly different from control mice, $p < 0.01$.

contained a RNA fragment approximately 1 kb long, which was able to hybridize to the specific probe for GM-CSF. Furthermore, Western blot analysis in Figure 1 (*right*) shows a characteristic band about 27 to 30 kDa when the anti-rGM-CSF antibody was used to bind the tumor-released CSF as well as the rGM-CSF used as a positive control. These findings confirm our previous conclusions that this tumor cell line is able to constitutively express and release GM-CSF.

To evaluate the possible role of tumor produced GM-CSF on the hemopoietic and immune systems, 10,000 U of rGM-CSF mixed with 10% normal mouse serum were injected into normal mice twice daily for 3 wk. The control group received only 10% normal mouse serum. There is no significant increase of nucleated cells in the spleen, BM, or blood in either group of mice. However, there is a significant increase of nucleated cells in the peritoneal exudates of rGM-CSF treated mice as compared with control animals (2.4 ± 0.7 vs 15.5 ± 2.3 × $10^6$/mouse). Most of the cells within the PEC (77 ± 2% in treated mice vs 51 ± 3% in control mice) appear morphologically to be macrophages as determined by nonspecific esterase staining. To determine whether there is a systemic increase of progenitors of the granulocyte and macrophage lineages, cells from spleen, BM, and peritoneal exudates were cultured in CFU assays. Table I shows that there is a significant increase of CFU generated from all types of cells assayed from rGM-CSF-treated mice, suggesting a systemic increase of cells of the GM lineage after treatment with rGM-CSF by using our protocol. Additional 100 U/well of rGM-CSF in vitro are necessary to drive the progenitor cells to form colonies because medium alone does not generate any colonies in any group. The data also suggest that continued injections of rGM-CSF are required to systemically increase the number of macrophages. The number of colonies in the cultures were counted at 6 and 13 days because both colonies of granulocytes and macrophages are present at 6 days, but the majority of the colonies present at 13 days are caused by cells of the macrophage lineage. The data show that the effects of rGM-CSF on macrophage lineage are detectable in spleen and BM although these two tissues do not exhibit an increase in general cellularity.

Although the total number of splenocytes is not greatly affected, morphologically there is about 3.0-fold increase of splenic cells of the granulocyte-macrophage lineage in rGM-CSF-treated mice as determined by morphology and nonspecific esterase staining. We have previously reported that there is an increase of Mac-$1^+$ and Mac-$2^+$ splenic macrophages in tumor bearers, which may be responsible for immunosuppression (24, 27). The phenotype of 95% of tumor bearers' Mac-$2^+$ spleen cells purified by cell sorting or Percoll gradients is Mac-$1^+$ $Ia^+$ $Thy^-$ $CD4^-CD8^-$ $Ig^-$. These cells are also nonspecific esterase positive and myeloperoxidase negative (27). Thus, we analyzed the splenocytes from rGM-CSF-treated mice by flow cytometric analysis for possible phenotypic changes. Figure 2 shows that there is a pattern of increased Mac-$1^+$ and/or Mac-$2^+$ cells in the splenocytes from rGM-CSF-treated mice (*lower panels*) similar to those of tumor bearers (*upper panels*). We further analyzed the BM cells from tumor bearers and rGM-CSF treated mice. Both have populations of cells expressing increased Mac-$1^+$ and/or Mac-$2^+$ Ag (data not shown). It should be pointed out that the tumor cells themselves do not express either Mac-1 or Mac-2 Ag and do not metastasize into the spleen or the BM. Together, the data suggest that GM-CSF may play an important role on the induction and expansion of populations of Mac-$1^+$ and/or Mac-$2^+$ macrophages.

Because splenocytes from tumor bearers have a reduced capacity to respond to mitogens, the lymphocytes from control and rGM-CSF-treated mice were tested in proliferation assays. The rGM-CSF-treated mice have significantly lower Con A and LPS responses than those of control mice (Fig. 3), as detected by an in vitro incorporation of [$^3$H]TdR assay. Indomethacin, an inhibitor of $PGE_2$ production, could partially reverse the decreased responses. These results are similar to those obtained in studies with tumor bearers. Previously we have reported that macrophages from tumor-bearing mice are capable of down-regulating the lymphocyte responses to mitogens (24, 27). In order to analyze the regulatory functions of peritoneal macrophages from rGM-CSF-treated mice their PEC were added to normal splenocytes in a Con A blastogenic assay. Addition of PEC from rGM-CSF-treated mice significantly inhibited the mitogenic responses in a dose-dependent pattern (Fig. 4). As stated above, PEC from untreated BALB/c mice contained 53% macrophages whereas those from rGM-CSF-treated animals contained 77% macrophages as determined morphologically and by nonspecific esterase activity. To ob-

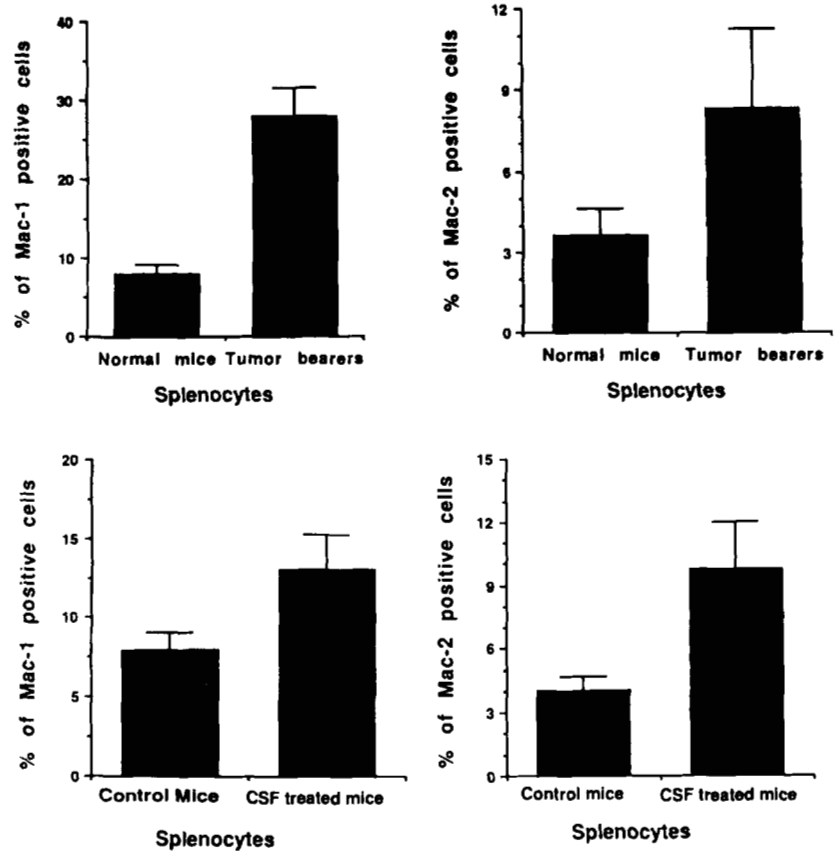


*Figure 2.* The *upper panels* show the levels of Mac-1$^+$ (*left*) and Mac-2$^+$ (*right*) splenocytes as measured by flow cytometry in normal mice vs age-matched mice bearing 4-wk tumors. The *lower panels* show Mac-1 (*left*) and Mac-2 (*right*) expression in splenocytes from either control animals treated with 10% normal mouse serum or animals treated with rGM-CSF. Data are shown as the mean and SD of eight individual mice per group. There is a significant difference from control mice and rGM-CSF-treated mice ($p < 0.01$).

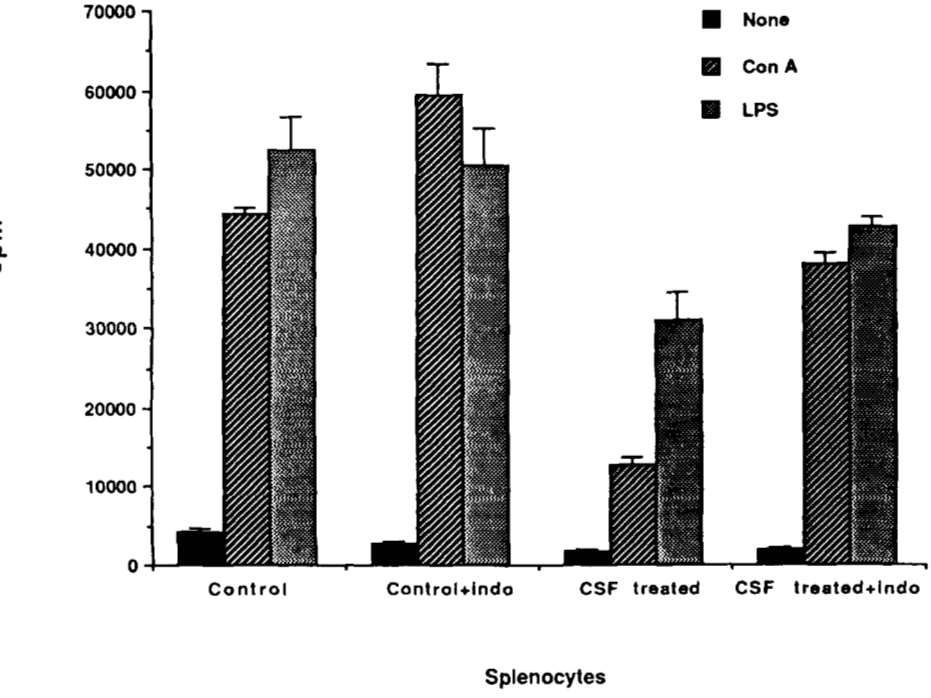


*Figure 3.* Reduced lymphocyte mitogenic responses in mice injected i.p. with 200 ng of rGM-CSF twice daily for 3 wk. Control mice were injected with 200 $\mu$l of 10% mouse serum as a control. The splenocytes were harvested and lymphocyte transformation assays were performed with or without $2 \times 10^{-5}$ M indomethacin as indicated. The results are expressed as mean ± SD of cpm of individual assayed mice representative of four separate experiments. There is a significant difference from control mice and rGM-CSF-treated mice ($p < 0.001$).

tain 50% inhibition of normal splenocyte response to Con A, only 6000 PEC from rGM-CSF-treated mice were needed, whereas 32,000 PEC from untreated mice were necessary to obtain the same levels of suppression. Therefore, these results indicate that the macrophages from the rGM-CSF treated mice are more suppressive than normal PEC, and that the suppression observed is not due merely to the addition of more macrophages per culture.

This inhibitory activity exerted by the PEC from rGM-CSF treated mice is almost completely reversed by the inclusion of indomethacin in the cultures (data not shown). Because it appears that the macrophages from rGM-CSF-treated mice down-regulate lymphocyte proliferation via increased $PGE_2$ production we tested for the level of this molecule in normal and tumor bearing mice

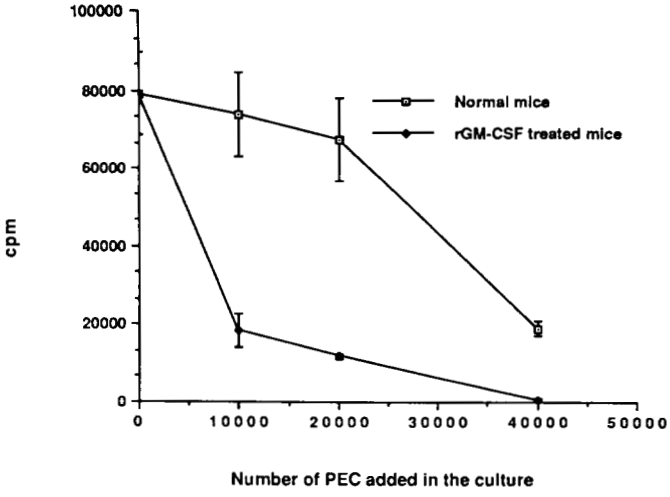


*Figure 4.* The dose-dependent down-regulation of normal splenocyte responses to Con A by the addition of PEC from mice injected i.p. with 200 ng of rGM-CSF twice daily for three wk. Normal mice were injected with 200 $\mu$l of 10% mouse serum as a control. Peritoneal exudates were harvested and placed into wells before the addition of splenocytes to the cultures. The values from individual assayed mice representative of four separate experiments indicate the mean number of cpm of triplicate cultures ± SD.

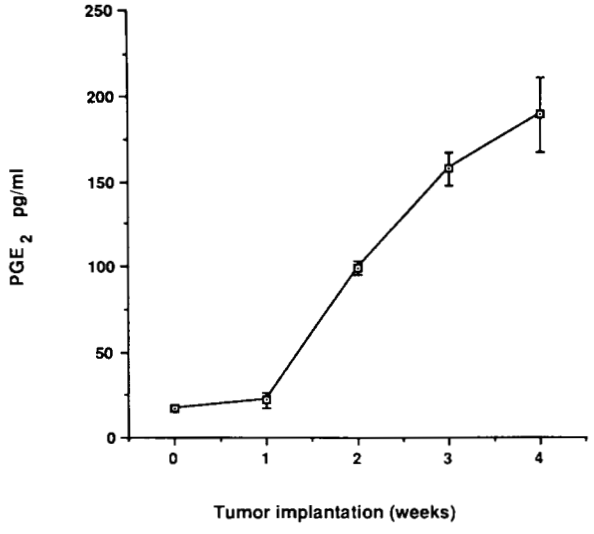


*Figure 5.* Enhanced $PGE_2$ levels in mouse serum during tumor growth. Sera were collected from normal mice or mice with 1-, 2-, 3-, or 4-wk tumors. A Seragen $PGE_2$ [$^{125}$I]-RIA kit was used for all determinations. Extraction efficiency for $PGE_2$ was 82% and all the values were adjusted accordingly. Each point represents the mean ± SD of three replicates from four experiments.

both in vivo and in vitro. $PGE_2$ levels in the serum significantly increase after 1 wk of tumor growth and continue to rise until they attain 10-fold higher levels than those of normal mice (Fig. 5). In further studies, the $PGE_2$ production of macrophages from normal mice and tumor bearers was compared. Cultured macrophages from tumor bearers release much more $PGE_2$ than those from normal mice and this production is reversed by the addition of indomethacin to the culture (Fig. 6). Because we have previously shown that induction of the regulatory macrophages from tumor bearers is caused by tumor-derived GM-CSF, we tested the effect of rGM-CSF on the $PGE_2$ production of various macrophage-containing tissues. A total of 500 U/ml of rGM-CSF was added to cultures of normal spleen cells, BM cells, or peritoneal cells and their supernatants were tested for $PGE_2$ production. In vitro addition of rGM-CSF produced an enhancement of $PGE_2$ levels in spleen cells from 0.8 ng/ml to 3.8 ng/ml; in BM cells from 1.5 ng/ml to 9.8 ng/ml; and in PEC from 0.6 ng/ml to 5.0 ng/ml. The enhanced levels are comparable with those observed in the macrophage cultures from tumor-bearing mice as presented in Figure 6. The data indicate that tumor-derived GM-CSF may contribute to increased $PGE_2$ production from macrophages of tumor bearers and therefore could be a major factor in the down-regulation of immune responses observed in tumor-bearing mice.

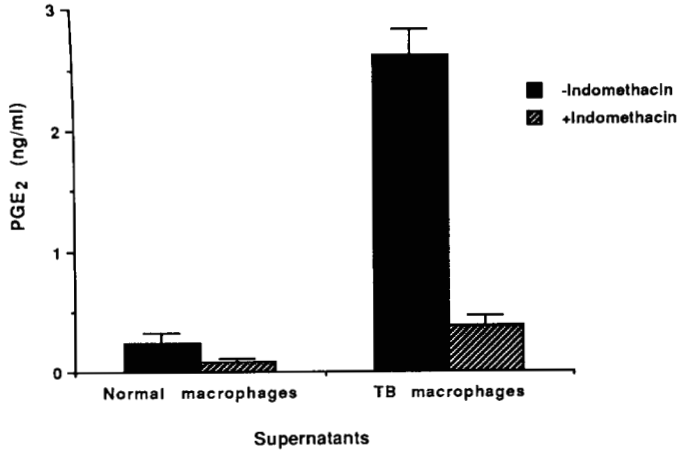


*Figure 6.* $PGE_2$ levels in the supernatants of $1 \times 10^5$ normal and TB splenic macrophages cultured for 24 h as measured by a Seragen $PGE_2$ [$^{125}$I]RIA kit. Extraction efficiency for $PGE_2$ was 82% and all values were adjusted accordingly. Additionally, FCS background $PGE_2$ levels of 17 to 46 pg/ml were subtracted from the macrophage culture determinations. Indomethacin was added at $2 \times 10^{-5}$ M (highest nontoxic concentration giving the greatest $PGE_2$ suppression). Values represent the mean ± SD of three replicates in four experiments. There is significant difference between macrophages from normal mice and mice bearing 4-wk tumors ($p < 0.001$).

## DISCUSSION

Profound hemopoietic changes have previously been shown to be caused by the growth of human and animal tumors, including our murine mammary adenocarcinoma. These changes include increased production of cells of the GM lineage, decreased erythropoiesis, and a decreased primary production of B lymphocytes, NK cells, and thymocytes (11–16). In addition, functional alterations of these cells could be observed. Severe immunosuppression, such as decreased lymphocyte responses to mitogens and Ag, reduced NK and CTL activities, and severe thymic atrophy in tumor-bearing hosts have been reported (16, 24–27, 34–36). Although the roles of tumor-derived cytokines on the interaction between the immune system and the hemopoietic system are not clear, there is some evidence that the enhanced hemopoiesis associated with immunosuppression in tumor bearers is also accompanied by an increase of suppressor macrophages in the BM and/or the spleen (24, 34, 36). We have previously reported that a mammary tumor-derived CSF may be responsible for the expansion of Mac-$1^+2^+$ aberrant macrophages in our system. The CSA from the supernatants of this tumor cultured in vitro can be neutralized by anti-rGM-CSF but not anti-G-CSF or anti-M-CSF (24). There is no IL-1 or IL-3 activity in the tumor cell culture supernatants (data not shown). Partial purification with the use of Sephacryl S-300 chromatography demon-

strated that most of the CSA was present in the 29-33 kDa fractions. The CSA of these fractions in either a soft agar or a liquid proliferation assay could be totally blocked by anti-rGM-CSF, whereas anti-M-CSF or anti-G-CSF had no effect. These data suggest that at least some of the Ag from this growth factor are related to GM-CSF. To further substantiate that the tumor indeed produces GM-CSF, Northern blot analyses were performed by using a specific cDNA probe for GM-CSF. The data presented in this paper show that the tumor transcribes GM-CSF mRNA. Western blot analysis further confirmed that the mammary tumor constitutively releases GM-CSF. Having ascertained the tumor origin of this CSA we sought to determine, in a well defined system, whether the changes observed in the tumor-bearing mice could be caused by GM-CSF. Therefore, we evaluated the effects of long term rGM-CSF injections on hemopoiesis and immune system. Our results suggest that reduction of splenocyte responses to mitogens after treatment of rGM-CSF is associated with increased cells of the macrophage lineage resulting in the subsequent appearance of suppressor macrophages. Furthermore, these macrophages release $PGE_2$ that contributes to the immunosuppression.

Enhanced hemopoiesis in a nontumor model has been linked to the appearance of BM-derived suppressor cells. Levy et al. (21) reported the destruction of murine BM cells treated with $^{89}Sr$-induced suppressor cells. In other studies, macrophage-like suppressor cells associated with enhanced hemopoiesis, were also shown to be present in the BM and spleens of mice given i.p. injections of bacillus Calmette-Guérin (22). BM-derived suppressor cells and enhanced hemopoiesis were also found in mice after total lymphoid irradiation (23). It is likely that the immunosuppression resulting from enhanced hemopoiesis reported in the studies cited above may have been caused by an increase of CSA.

In the present studies we utilized in vivo treatment of normal mice with rGM-CSF before analysis of several immune response parameters. Because in our previous work with tumor bearers the down-regulation of immunity does not occur until 2 wk after tumor implantation, the rGM-CSF inoculations were begun 3 wk before the functional and phenotypic studies. Morrissey et al. (37) have shown that i.p. injections of rGM-CSF 18 h before analysis increased both the number of Mac-$1^+$ macrophages and the density of this Ag on the cell surface. Metcalf et al. (38) employed a 6-day regime of rGM-CSF injections to show an increase in the overall number of cells of the granulocyte-macrophage lineage and a decrease of lymphocytes in the BM. Both of these studies did not analyze the immunologic functions of the resultant cell populations. The data presented in this paper show for the first time an evaluation of several parameters of the murine immune response after long term treatment with rGM-CSF.

The results of the in vivo injections of rGM-CSF resemble many of the immunologic effects observed in animals bearing tumors. There is an increase of immunosuppressive macrophages expressing Mac-1 and/or Mac-2 Ag in rGM-CSF-treated mice similar to that observed in tumor bearers. Sakata et al. (34) recently reported that Mac-2 positive splenic macrophages were responsible for immunosuppression in mice bearing dermal squamous carcinoma. In addition, incubation of splenocytes from normal mice with rGM-CSF or tumor-derived GM-CSF in vitro resulted in the induction of a macrophage lineage expressing Mac-1 and Mac-2 on the cell surface which could suppress T and B cell mitogenic responses of splenocytes (15) (Y-X. Fu and D. M. Lopez, unpublished observations). The spleen is a hemopoietic organ and contains hemopoietic progenitors capable of proliferating or differentiating in response to CSF. Thus, at present, we cannot distinguish whether the hyporesponsiveness of the splenocytes is caused by preexisting suppressive macrophages or by an activation or differentiation of the hemopoietic cells. It is probable that both processes are occurring under our experimental conditions. Because lymph nodes do not support hemopoiesis but may contain a population of suppressor macrophages, planned studies using this source of immunoresponsive cells from r-GM-CSF treated mice will help discriminate between these two possibilities.

Interestingly, Mazzei et al. (39) very recently presented data suggesting that GM-CSF provides a signal to macrophages to become inhibitory macrophages, whereas IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, TNF-$\alpha$, IFN-$\gamma$, G-CSF, and M-CSF do not. Additionally, differentiation of the cells from monoblasts to adherent macrophages is an absolute requirement for induction of suppressive macrophages. It is known that GM-CSF augments the maturation of macrophages in different stages including differentiation of the cells from monoblasts to mature macrophages. There is an increase of monoblasts in tumor bearers and in the rGM-CSF-treated mice in this study. Tumor-derived GM-CSF, therefore, plays at least two roles: first, enhancement of proliferation and differentiation of cells of the macrophage lineage, and second, activation of these macrophages to release regulatory factors. Thus, the study of tumor-derived factors provides new insight into the modulatory effects of neoplasms on the immune system. In cancer therapy, the well known regime of rGM-CSF administration is utilized to restore the depleted BM population of patients after radiation and chemotherapeutic treatments. Our finding that a population of macrophages with down-regulatory properties could arise from long term exposure to this cytokine should be an important consideration in devising future antitumor strategies utilizing rGM-CSF.

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