Regression of an Established Tumor Genetically Modified to Release Granulocyte Colony-stimulating Factor Requires Granulocyte-T Cell Cooperation and T Cell-produced Interferon γ

By Antonella Stoppacciaro,* Cecilia Melani,† Mariella Parenza, contrib Antonio Mastracchio,* Cinzia Bassi,† Carlo Baroni,* Giorgio Parmiani,† and Mario P. Colombo†

From the *Department of Human Biopathology, Immunopathology Section, University of Roma, 00100 Roma; and the †Division of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milano, Italy

Summary

Using the murine colon adenocarcinoma C-26 cell line, engineered to release granulocyte colony-stimulating factor (G-CSF) (C-26/G-CSF), we studied the mechanisms responsible for inhibition of tumor take in syngeneic animals and of regression of an established tumor in sublethally irradiated mice injected with these cells. Immunocytochemistry and in situ hybridization, performed to characterize tumor-infiltrating leukocytes and their cytokine expression, respectively, indicated that polymorphonuclear leukocytes (PMN) were the major cells responsible for inhibition of tumor take and that they expressed mRNA for interleukin 1α (IL-1α), IL-1β, and tumor necrosis factor α (TNF-α). Expression of interferon γ (IFN-γ) and of IL-4 was undetectable, consistent with the absence of T lymphocytes at the site of tumor injection. In mice injected with C-26/G-CSF cells after 600-rad irradiation, the tumors grew to ~1.5 cm in 30 d, regressing completely thereafter in 70–80% of mice. During the growing phase, tumors were infiltrated first by PMN (between days 15 and 20), then by macrophages, and last by T lymphocytes. Both CD4* and CD8* T cells were present but only CD8 depletion significantly abrogated tumor regression. Depletion of PMN by the RB6-8C5 antigranulocytes monoclonal antibody reduced the number of T cells infiltrating the tumor and prevented tumor regression. In situ hybridization performed at the beginning of tumor regression revealed the presence of mRNA for IL-1α, IL-1β, and TNF-α, but also the presence of cells, with lymphoid morphology, expressing IFN-γ. Tumors from mice treated with recombinant IFN-γ (between days 20 and 35) were rejected faster, whereas mice treated with antibodies to IFN-γ (from day 20) died of progressive tumor. Cyclosporin A treatment (started at day 20) also abrogated tumor regression. These results indicate that inhibition of tumor take and regression in this model occurs through different mechanisms that involve PMN and PMN-T cell interactions, respectively, as well as a combination of cytokines that, for tumor regression, require IFN-γ. Thus, gene transfer of a single cytokine gene such as G-CSF into tumor cells appears to be sufficient to trigger the cascade of cell interactions and cytokine production necessary to destroy a cancer nodule.

Studies of peritumoral injection of lymphokine together with cytokine gene transfer into tumor cells show that local availability of cytokines exerts a potent antitumor activity without any apparent local or systemic toxicity (reviewed in references 1 and 2). IL-2, -4, -6, -7, IFN-γ, TNF-α, and G-CSF genes transfected into tumor cells were shown to trigger different immune effector cells that eventually resulted in inhibition of tumor formation (2). However, all these studies were done using tumor cell suspension, and it is known that solid tumor fragments often grow progressively whereas even 10-fold larger numbers of the identical tumor cell type injected as a suspension are rejected (3). Thus the tumor stroma, which consists of vessels, sessile, and migratory cells and extracellular matrix, plays an important role in tumor growth and progression. In addition, tumor cells modified to produce cytokines are likely to have additional regulatory signals that result from cytokine-extracellular matrix crosstalk (4). Thus, the interaction of the tumor with host immune cells
and the features of the effector cells mediating destruction of a tumor injected as a cell suspension might differ from those required to reject an established tumor nodule. Identification and manipulation of the latter may, therefore, provide a more direct approach to destroying solid tumors.

In this report, we describe our analysis of the effector cells and the cytokines they produced during the rejection of a murine adenocarcinoma engineered to express G-CSF (5) and grown subcutaneously in a sublethally irradiated mice for 30 d. The adenocarcinoma C-26/G-CSF does not grow in syngeneic mice when injected as a cell suspension containing >100-fold the number of C-26 cells required for minimal tumorigenic dose because of the PMN recruited and activated in situ by G-CSF released by tumor cells (5). In sublethally irradiated mice, C-26/G-CSF tumors grew progressively until the leukocyte count returned to a normal level and then regressed in most of the mice (5). Here we show that inhibition of tumor take and tumor regression occurs through different mechanisms, the former dominated by PMN, and the latter, by the cooperation of PMN with T cells, mediated by T cell–derived IFN-γ.

Materials and Methods

Tumors and Mice Studies. C-26 is a murine colon adenocarcinoma cell line derived from BALB/c mice treated with N-nitrosodiethyl-N-methylurethane (6). Cells were maintained as tumors in vivo by subcutaneous transplant in syngeneic mice and adapted to culture in DMEM (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco). Construction of the NSV-G-CSF retrovector, production of supernatant containing virus, infection of C-26 cells, and isolation clones producing G-CSF have been described (5). Colony G4, which produces 90 pg/ml G-CSF/10^6 cells per 48 h, was used (5). BALB/c Ch mice were purchased from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. Tumorigenic activity of control and virus-infected C-26 cells was assayed in mice injected subcutaneously in the right flank with cells in 0.2 ml of HBSS. Although a minimal dose of 3 × 10^4 C-26 cells/mouse was required to produce tumors in 100% of injected mice, 10^6 cells were used in the present experiments. In some experiments mice were injected with tumor cells 24 h after sublethal whole body irradiation using a 60Co source (r ~ 4,000 Ci = 148,000 GBq; Theratron 780C; Atomic Energy of Canada Limited, Kanata, Canada) to deliver a dose rate of 28 rad/min for a total dose of 600 rad. On day 20 after irradiation some mice were injected intraperitoneally with 0.2 ml of HBSS containing 100 μg of anti-CD8 (53.6.72 hybridoma, Lyt2), anti-CD4 (GK1.5 hybridoma, L3T4), or anti-Mac-1 (M1/70.15.11.5.HL hybridoma), all obtained from American Type Culture Collection (ATCC; Rockville, MD), antigranulocyte (RB6-8C5 hybridoma; kindly provided by Dr. G. Garotta, Roche, Basel, Switzerland) every 3 d from days 20 to 35 after irradiation. Leukocyte counts and blood composition were automatically determined using an H1 apparatus (Technicon Chemicals Company, Tournay, Belgium).

Morphological Analysis. In nonirradiated mice injected subcutaneously with 10^6 C-26 or C-26/G-CSF cells, at least two mice from each group were killed at 24 h and at 5 d after injection. In irradiated mice injected with 10^6 C-26/G-CSF cells, at least two mice from each group were killed after 15 d and then every 2–3 d, until full rejection was established. The flanks were gently dissected and the肿瘤 mass was recorded. Tumor fragments and draining lymph nodes were embedded in OCT compound (Miles Lab., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C.

Immunocytochemistry. 5-μm cryostat sections were fixed in acetone and immunostained with rat anti–mouse mAbs against CD45 (M1/9.3.4.HL2 hybridoma, T200), CD8 (53.6.72 hybridoma, Lyt2), CD4 (GK1.5 hybridoma, L3T4), Mac-1 (M1/70.15.11.5.HL hybridoma), and Mac-3 (M37/84.6,34 hybridoma) (all from ATCC), CD3 (Boehringer, Mannheim, Germany), and anti–mouse granulocyte antibody (RB6-8C5 hybridoma; Pharmingen, San Diego, CA). Sections were preincubated with rabbit serum and sequentially incubated with optimal dilutions of primary antibodies, rabbit anti–rat IgG (Zymed Labs. Inc., San Francisco, CA) and rat peroxidase–antiperoxidase (PAP; Abbot Laboratories, North Chicago, IL). Each incubation step lasted 30 min and was followed by a 10-min wash in Tris-buffered saline. Sections were then incubated with 0.03% H2O2 and 0.06% 3,3’ diaminobenzidine (BDH Chemicals, Poole, England) for 2–5 min, washed in tap water, and counterstained with hematoxylin. The number of immunostained cells was determined by light microscopy at 400 × in five fields on a 1-mm2 grid and is given as cells/mm2 (mean ± SD).

In Situ Hybridization. The presence of cytokine mRNA was investigated using in situ hybridization with cDNA probes for IL-1α, 1.9-kb BamHI fragment from pIL-1 1301, and IL-1β, 500 bp from pIL-1 130 (7), obtained from Dr. P. Lomedico (Hoffman-La Roche, Nutley, NJ); TNF-α, 860-bp PstI–EcoRI fragment from pUC19 plasmid containing a mouse TNF-α complementary cDNA insert (8), courtesy of Dr. A. Cerami (The Rockefeller University, New York); IFN-γ, 1.3-kb BamHI fragment from pF7/T3-19 (9), obtained from Dr. S. Landolfo (University of Turin, Turin, Italy); and IL-4, 590-bp BamHI–ScaI fragment contained in plasmid p2A-E3 (10) (ATCC 37564). Cryostat sections were harvested on RNA grade slides, air-dried and fixed in 4% buffered paraformaldehyde for 10 min, dehydrated in ethanol, then sequentially rehydrated in PBS, 50 mM MgCl2, washed in 200 mM Tris–HCl–glycine, acetylated in 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.1 M triethanolamine, 0.5% acetic anhydride (pH 8.0), washed in 2× SSC, and dehydrated in ethanol. Slides were then prehybridized for 10 min at 70°C with 2× SSC, 50% formamide, and hybridized overnight at 42°C with 32P-labeled specific cDNA probe, 1.5 × 10^6 cpm/sec, 2× SSC, 500 μg/ml yeast RNA, 5× Denhardt’s solution, 10 mM dithiothreitol (DTT), and 10% dextran sulfate. Unbound and nonspecifically bound probes were removed by sequential washes in 2× SSC, 50% formamide, and 1× SSC, 50% formamide at 45°C, and in 0.1 × SSC at room temperature. Slides were then dehydrated in ethanol, dipped in autoradiographic emulsion NTB-2 (Eastman Kodak Company, Rochester, NY), and exposed for 5 d at 4°C in a light-tight box, developed in D19 (Eastman Kodak Co.), fixed in Rapid Fixer (Eastman Kodak Co.), and counterstained with hematoxylin. RNA-specific binding was controlled by previous digestion with 100 μg/ml ribonuclease A and 10 U/ml ribonuclease T (Sigma, Poole, England). PstI-digested pUC19 plasmid fragments were used as negative controls. Cytosmears of LPS and PHA plus ionomycin–stimulated PBL were inserted as positive controls in each experiment. Cells were considered positive when the number of black grains in the cell was at least five times the mean number of grains present in the negative
The number of positive cells was evaluated at 400× on a 1-mm² grid and reported as cells/mm² (mean ± SD).

Results

Leukocyte Infiltrate Elicited by C-26/G-CSF Cell Suspensions.

Immunocytochemistry analysis and in situ hybridization were performed on cryostat sections of C-26 and C-26/G-CSF tumors, 24 h and 5 d after subcutaneous inoculation, to define the host cells involved in inhibiting the take of C-26/G-CSF tumor cells when injected as a tumor cell suspension. Points were chosen based on our previous observation that C-26-G-CSF cells disappeared from the site of injection 7–10 d after subcutaneous inoculation into syngeneic animals (5). All cases examined at 24 h revealed clustered neoplastic cells surrounded by loose connective tissue with an evident acute neutrophilic inflammatory infiltrate at the inoculation sites (5). After 5 d the host reaction subsided in C-26 tumors, while a large number of CD45+/Mac-1+/RB6-8C5+ cells, both surrounding and infiltrating the neoplastic cells, were present in G-CSF-producing tumors (Table 1). The PMN lineage of the CD45+/Mac-1+/RB6-8C5+ cells was confirmed by morphology, and by hydrogen peroxide–nonresistant endogenous peroxidase analysis. A small percentage of Mac-1+/Mac-3+ macrophages, variable among different mice, was also present but T cells were consistently absent (no staining with anti-CD3 mAb). In situ hybridization performed with IL-1α, IL-1β, TNF-α, INF-γ, and IL-4 cDNA probes 5 d after tumor cell inoculation showed that 10–18% of the reactive cells present in the C-26/G-CSF tumors produced IL-1α, IL-1β, and TNF-α mRNA. (Fig. 1, a–c). The positive cells showed the morphology of PMN and were preferentially distributed at the periphery of the tumor, frequently clustered around blood vessels. No hybridization with IFN-γ and IL-4 probes was detected (Table 1 and Figure 1), confirming the absence of lymphocytes in the cellular tumor infiltrate as demonstrated by immunocytochemistry. Uninfected C-26 cells, which do not produce G-CSF, were virtually without reactive infiltrate at day 5 (Table 1 and reference 5), with rare or undetectable cytokine production (Table 1).

C-26/G-CSF Cellular Tumor Infiltrate in Irradiated Mice.

We have previously shown that C-26/G-CSF tumors grow in sublethally (600 rad) irradiated mice until blood count and composition normalize, resulting in tumor regression (5). Histological and immunocytochemical analyses were performed on tumors during both the growing and the regression phases to determine which cell type infiltrates the tumor first and which cells are present during tumor regression, respectively. Tumor sections obtained during the growth phase (days 7–20) showed a massive solid tumor without reactive infiltrate except for a few large mononuclear Mac-3+ cells that resembled tissue macrophages (Fig. 2 a). Reactive infiltrating cells appeared first at the periphery of the tumor on days 15–20 after inoculation of C-26/G-CSF cells, and

Table 1. Cellular Composition and Cytokine Production of Leukocytes Infiltrating C-26 or C-26/G-CSF Tumors 24 h and 5 d after Tumor Cell Injection

| Cell Type                | C-26  | C-26/G-CSF | C-26  | C-26/G-CSF |
|--------------------------|-------|------------|-------|------------|
| **24 h**                 |       |            |       |            |
| Leukocytes (CD45+)       | 57 ± 15* | 117 ± 45  | 123 ± 64 | 562 ± 87 |
| T lymphocytes (CD3+)     | 0     | 0          | 15 ± 8 | 0          |
| Macrophages and granulocytes (Mac-1+) | 47 ± 5 (82) | 97 ± 14 (83) | 50 ± 13 (41) | 500 ± 50 (89) |
| Macrophages (Mac-3+)     | 9 ± 42 (15) | 10 ± 6 (9) | 48 ± 9 (39) | 62 ± 39 (11) |
| Granulocytes (RB6-8C5+)  | 43 ± 6 (75) | 89 ± 9 (76) | 13 ± 10 (11) | 461 ± 51 (82) |
| Eosinophils              | 2 ± 1 (3) | 11 ± 7 (10) | 8 ± 5 (7) | 45 ± 15 (8) |
| **Cytokine**             |       |            |       |            |
| IL-1α                    | 9 ± 1 (15) | 28 ± 7 (24) | <1    | 67 ± 11 (12) |
| IL-1β                    | 6 ± 3 (10) | 12 ± 3 (10) | 5 ± 4 (4) | 84 ± 11 (15) |
| TNF-α                    | 3 ± 2 (6) | 29 ± 2 (24) | <1    | 67 ± 17 (12) |
| IFN-γ                    | 0     | 0          | 0     | 0          |
| IL-4                     | 0     | 0          | 0     | 0          |

The cell type was determined by immunocytochemistry and the presence of cytokines by in situ hybridization as described in Materials and Methods.

* Mean ± SD of cell number/mm² tissue determined at × 400 in a 1-mm² grid.
1 Positive cells were only occasionally seen on different sections: <1 cell/mm².
5 Numbers in parentheses are ratios of positive cells to total number of CD45-infiltrating leukocytes.
were identified as neutrophils based on their reactivity with Mac-1 and RB6-8C5 mAbs (Fig. 2, b and c), their morphology, and endogenous peroxidase staining. In the next 10 d, the inflammatory infiltrate characteristic of the regression phase appeared. During this period, and after appearance of neutrophils, macrophages became evident, followed by T lymphocytes (Fig. 3). Macrophages and T lymphocytes increased steadily during the time of regression but most of

Figure 1. In situ hybridization of cryostat sections of C-26/G-CSF biopsied 5 d after tumor cell injection. mRNA $^+$ cells were preferentially distributed at the periphery of the tumor where the larger infiltrate was present. The infiltrating cells showed doughnut or segmented nuclei typical of neutrophils. (a) IL-1$\alpha$; (b) IL-1$\beta$; (c) TNF-\alpha; (d) IFN-\gamma. Autoradiograms were counterstained with hematoxylin ($\times$1,000).

Figure 2. Kinetics of tumor-infiltrating leukocytes in mice injected with C-26/G-CSF after 600 rad total body irradiation. Cryostat sections were immunostained using the peroxidase antiperoxidase method. (a) Mac-3 staining; large Mac-3$^+$ cells resembling tissue resident macrophages were present in the tumor as well as the surrounding connective tissues at day 15 after injection and before recovery from radiation-induced immune suppression ($\times$1,000). (b and c) C-26/G-CSF tumors stained with anti-PMN mAb (RB6–8C5) from biopsies performed at days 15 and 20 after injection, respectively, counterstained with hematoxylin ($\times$250).
Figure 3. Immunocytochemical characterization of the reactive infiltrate present in regressing C-26/G-CSF tumors during the regression phase between days 38 and 43 after tumor injection. (a) RB6-8C5; (b) Mac-3; (c) eosinophils stained for endogenous peroxidase; (d) CD3; (e) CD4; (f) CD8; counterstained with hematoxylin (×1,000).

Table 2. Immunocytochemical Characterization of Leukocytes Infiltrating C-26/G-CSF Tumor Growing in Sublethally Irradiated Mice

| Cell type                          | Early phase | Late phase | Rejection |
|------------------------------------|-------------|------------|-----------|
| Leukocytes (CD45+)                 | 375 ± 72†   | 830 ± 46   | 562 ± 67  |
| T lymphocytes (CD3+)               | 0           | 149 ± 7 (18)§ | 243 ± 31 (43) |
| T lymphocytes (CD4+)               | 16 ± 4 (4)  | 178 ± 10 (21) | 126 ± 6 (22) |
| T lymphocytes (CD8+)               | 0           | 85 ± 11 (10) | 143 ± 13 (25) |
| Macrophages and granulocytes (Mac-1+) | 363 ± 42 (96) | 656 ± 35 (79) | 316 ± 18 (56) |
| Macrophages (Mac-3+)               | 13 ± 2 (3)  | 234 ± 21 (28) | 255 ± 19 (45) |
| Granulocytes (RB6-8C5+)            | 350 ± 23 (93) | 411 ± 17 (49) | 88 ± 11 (15) |
| Eosinophils                        | 24 ± 10 (6) | 33 ± 16 (4) | 57 ± 21 (10) |

Kinetics of tumor leukocyte infiltration

- Tumor biopsies, cell immunostaining, and counting were performed as described in Materials and Methods.
- 15–20 d from irradiation and tumor injection.
- 25–27 d from irradiation and tumor injection.
- Virtual disappearance of tumor cells from the site of tumor injection and replacement with granulation tissue.
- Mean number of tumor-infiltrating leukocytes/mm² tissue ± SD.
- Numbers in parentheses are percentages of positive cells among total number of infiltrating leukocytes.
the cells present at the onset of reactive infiltrate formation, and probably favoring the arrival of the late-coming cells, were PMN (Table 2). In fact, PMN depletion by treatment with the antigranulocyte mAb RB6-8C5 (Fig. 4, a and b) reduced the number of both CD4\(^+\) (not shown) and CD8\(^+\) (Fig. 4, c and d) T cells within the tumor (70\% less than in RB6-8C5 untreated mice) and impeded tumor regression (see Exp. 4 in Table 5). In situ hybridization, performed to analyze cytokine gene expression in regressing tumors between days 38 and 43, identified neutrophils and macrophages expressing IL-1\(\alpha\), IL-1\(\beta\), and TNF-\(\alpha\) mRNA (Fig. 5, a–c), and cells with lymphoid morphology expressing mRNA for IFN-\(\gamma\) (Fig. 5 d). IL-4 mRNA was not detected at the tumor site, although IL-4\(^+\) lymphocytes were frequent in the para-cortical area of the draining lymph node (not shown).

Tumors from Irradiated Mice Treated with Anti-CD4, -CD8, and -Mac-1 mAbs or Cyclosporin A or rIFN-\(\gamma\). Because the tumor infiltrate present during regression of C-26/G-CSF tumors in irradiated mice showed a different cellular composition from that in immunocompetent animals after injection of C-26/G-CSF cell suspensions, we analyzed the role of infiltrating cells other than neutrophils in tumor rejection. Irradiated mice bearing C-26/G-CSF tumors were treated with mAbs to CD4, CD8, or Mac-1, and were immunosuppressed with cyclosporin A or treated systemically with rIFN-\(\gamma\). Of the different treatments, those with anti-Mac-1 and cyclosporin A showed the most striking effect on the number of tumor-infiltrating cells, inducing a fourfold decrease (Table 3). However, the two treatments differed in their effects on cellular composition and the role of tumor-infiltrating leukocytes. Irradiated mice treated with anti-Mac-1 mAb showed large areas of tumor necrosis with fibrotic substitution and presence of granulation tissue, and with a reduced number of infiltrating PMN and an increased number of IL-1\(\alpha\) (Fig. 6 a), IL-1\(\beta\), and TNF-\(\alpha\) mRNA–expressing cells (Table 4). Moreover, the number of T lymphocytes infiltrating the tumor remained almost unchanged, while the number of cells expressing IFN-\(\gamma\) mRNA increased (Table 4). In cyclosporin A–treated mice tumor regression was abrogated (Table 5) and the tumors were larger. T lymphocytes were virtually absent and PMN and macrophage numbers were reduced three- to fourfold (Table 3). In situ hybridization of cyclosporin A–treated irradiated mice demonstrated no mRNA expression of the tested cytokines by the residual tumor-infiltrating cells (Table 4). Depletion of CD8\(^+\) cells abrogated tumor regression (Table 5) and in situ hybridization showed the virtual absence of mRNA for IL-1\(\alpha\), IL-1\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\) (Table 4) in these tumors, suggesting that CD8\(^+\) T lymphocytes contribute some crucial function to the milieu of cytokine production by the infiltrating leukocytes. Conversely, depletion of CD4\(^+\) cells did not appear to affect tumor regression (Table 5). Immunocytochemical

Figure 4. Immunocytochemical characterization of the reactive infiltrate present in C-26/G-CSF tumors from mice untreated (a and c), or treated (b and d), with the RB6-8C5 antigranulocytes mAb. (a and b) Stained with RB6-8C5 Abs; (c and d) stained with CD8 mAb. (a, c, and d) \(\times 250\); (b) \(\times 400\) (higher magnification is necessary to show the rare residual PMN; two granulocytes and one eosinophil are visible); without counterstaining.
analysis of the CD4 antibody-treated mice showed an infiltrate at the tumor site similar to that of non-CD4-depleted animals except for the absence of CD4+ cells (Table 3). T cell subset depletion also suggested that CD8+ more than CD4+ T lymphocytes were expressing IFN-γ. Efficient depletion of CD4+ and CD8+ cells at the time of tumor biopsy in mice treated with the appropriate mAb was always confirmed by immunocytochemistry of spleen and lymph node sections.

Based on the observed correlation between the presence of IFN-γ transcript and the effective regression of C-26/G-CSF tumors, we injected rIFN-γ into irradiated mice bearing these tumors; immunohistological analysis performed 15 d after treatment showed large areas of necrosis and a twofold increase in the number of infiltrating cells (Table 3). In situ hybridization revealed an increased number of cells expressing mRNA for IL-1α (Fig. 6 b), IL-1β, and TNF-α (Table 4). At this time point, tumors from mice treated with mAb to Mac-1 or with rIFN-γ were smaller (mean diameters, 8 × 5 mm) than tumors from anti-CD8 or cyclosporin A (mean diameters, 15 × 12 mm). Furthermore, injection of mAb to IFN-γ abrogated tumor regression (Table 5).

Together, the observations that tumor-infiltrating leukocytes of CD8-depleted and of cyclosporin A-treated mice express no detectable cytokine, as well as the improved rejection obtained by rIFN-γ treatment, suggest that G-CSF transduced by tumor cells is not sufficient per se to induce rejection of an established tumor that, indeed, appears to depend on the presence of IFN-γ-producing T cells.

Table 3. Characterization of C-26/G-CSF-infiltrating Leukocytes in Sublethally Irradiated Mice Treated with mAbs to CD4, CD8, and Mac-1 or with rIFN-γ or Cyclosporin A

| Treatment          | None       | CD4         | CD8         | Mac-1       | IFN-γ       | Cy A       |
|--------------------|------------|-------------|-------------|-------------|-------------|------------|
| CD45+              | 1,006 ± 46*| 956 ± 253   | 536 ± 59    | 253 ± 11    | 2,326 ± 149| 239 ± 41   |
| CD3+               | 200 ± 21   | (20)9       | 86 ± 9 (9)  | 112 ± 15 (21)| 154 ± 13 (61)| 581 ± 45 (25)| 2 ± 2 (1) |
| CD4+               | 181 ± 13   | (18)        | 0           | 150 ± 21 (28)| 144 ± 12 (57)| 488 ± 66 (21)| 5 ± 3 (2)  |
| CD8+               | 40 ± 7     | (4)         | 105 ± 17 (11)| 0           | 23 ± 7 (9)  | 209 ± 23 (9) | 2 ± 2 (1)  |
| Mac-1              | 814 ± 97   | (81)        | 871 ± 11 (91)| 402 ± 51 (75)| 83 ± 7 (33) | 1,860 ± 93 (80)| 222 ± 11 (93) |
| Mac-3              | 332 ± 10   | (33)        | 413 ± 19 (43)| 353 ± 13 (66)| 53 ± 5 (21) | 767 ± 14 (33)| 100 ± 9 (42) |
| Rb6-8C5+           | 462 ± 11   | (46)        | 479 ± 15 (50)| 32 ± 6 (6)  | 28 ± 8 (11) | 1,046 ± 35 (45)| 117 ± 9 (49) |
| Eosinophils        | 8 ± 4      | (1)         | 8 ± 2 (1)   | 10 ± 3 (2)  | 0           | 48 ± 9 (2)  | 12 ± 3 (5)  |

Treatments were as described in Materials and Methods; tumor biopsies were obtained between days 38 and 43 after irradiation and tumor injection. Frozen sections were stained with mAb by immunocytochemistry (peroxidase antiperoxidase method).

* Mean number of cells/mm² ± SD positive for immunostaining.

† Numbers in parentheses are percentages of positive cells among total number of infiltrating leukocytes.

157 Stoppacciaro et al.
Figure 6. Effects of anti-Mac-1 mAb (a), rIFN-γ (b), or anti-CD8 mAb (c), on IL-1α mRNA expression. In situ hybridization indicates a significant increase in cells expressing IL-1α mRNA (compare with Fig. 4a for untreated control) in C-26/G-CSF tumors from mice treated with anti-Mac-1 mAb or rIFN-γ.

Discussion

Tumor cells genetically engineered to produce cytokines stimulate host cell immunity in syngeneic mice, eventually resulting in complete tumor growth inhibition. In some studies, the immune cells involved were characterized by immunohistology of tissue at the site of tumor injection (11–16) or by FACS® analysis of isolated infiltrating cells (17) whose functional activity was evaluated by depleting the mice with relevant mAb. The emerging picture is that of a complex interaction among cells involved in nonspecific (granulocytes and macrophages) and specific (T cells) immune responses (reviewed in reference 2). Both cell–cell contacts and flow of cytokines, produced by the infiltrating leukocytes, should influence these interactions. In this paper we provide evidence identifying the cell types that infiltrate the tumor and the cytokine that these cells express in situ. Two experimental models were used. One enabled analysis of the inhibition of tumor take after injection of C-26 cells engineered to produce G-CSF. The second allowed study of the mechanisms of tumor rejection since C-26/G-CSF cells were previously found to form large tumors when injected into sublethally

Table 4. Effects of Anti-CD4, -CD8, and -Mac-1 mAbs or rIFN-γ or Cyclosporin A Treatments on Cytokine mRNA Expression by C-26/G-CSF-infiltrating Leukocytes in Sublethally Irradiated Mice

| Cytokine | None       | CD4        | CD8        | Mac-1     | rIFN-γ    | cy A     |
|----------|------------|------------|------------|-----------|-----------|----------|
| IL-1α    | 5 ± 2      | 2 ± 2      | <1         | 26 ± 9    | 10 ± 2    | <1       |
| IL-1β    | 3 ± 1      | 12 ± 3     | <1         | 21 ± 3    | 36 ± 7    | –*       |
| TNF-α    | 4 ± 3      | 4 ± 4      | <1         | 25 ± 8    | 27 ± 10   | –        |
| IFN-γ    | 9 ± 2      | 1 ± 2      | –          | 13 ± 4    | 2 ± 2     | –        |
| IL-4     | ND         | –          | –          | –         | –         | –        |

mRNA content was detected by in situ hybridization on cryostat sections using 35S-labeled cDNA probes and revealed by autoradiography. Positive cells/mm² were counted at ×400 in a 1-mm² grid.

* Not detectable.
Table 5. Effect of Leukocyte Subpopulation Depletion, Immunosuppression with Cyclosporin A, or Treatment with rIFN-γ

| Treatment                        | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
|----------------------------------|--------|--------|--------|--------|
| None                             | 0/7*   | 4/11   | 1/6    | 1/7    |
| Anti-CD4 plus -CD8                | 5/6    |        |        |        |
| Anti-CD4                         | 2/9    | 0/6    |        |        |
| Anti-CD8                         | 7/9    | 6/7    |        |        |
| Anti-Mac-1                       | 0/2    | 0/1    |        |        |
| Anti-IFN-γ                       |        | 6/6    | 5/5    |        |
| Anti-PMN                         |        |        | 6/6    |        |
| Cyclosporin A                    | 4/4    |        |        |        |
| rIFN-γ                           | 0/3    |        | 0/5    |        |

* Number of mice with tumor, or mice that died because of tumor, after irradiation and C-26/G-CSF cell injection/number of mice observed (randomly chosen from mice treated for immunohistology and in situ hybridization but not killed). In each experiment, mice came from the same pool of irradiated and treated mice.

irradiated mice and then to regress when mouse reconstituted leukocyte function.

Our data confirm that neutrophils represent the majority of the reactive cells involved in C-26/G-CSF tumor inhibition (5). In fact, our previous study demonstrated that when C-26 and C-26/G-CSF cells were mixed together before injection, only C-26/G-CSF cells were eliminated, and PMN were detected at the site of injection until C-26/G-CSF cells were present (18). We now show that the neutrophils present at the site of tumor cell injection express transcripts for IL-1α, IL-1β, and TNF-α, cytokines that might be part of the granulocyte weapon for tumor destruction and not simply reflective of their activated state. Furthermore, PMN that produce cytokines appear to have a pivotal role in the regression of C-26/G-CSF tumors established in sublethally irradiated mice. PMN were clearly the first cells to infiltrate the tumor during leukocyte self-reconstitution. Possibly, the G-CSF produced by large tumors determines the persistence of PMN, and the production of IL-1 and TNF-α by PMN activates vascular endothelial cells, favoring the migration of macrophages and T cells to the tumor site (reviewed in reference 19). However, only a fraction of infiltrating PMN were found to express IL-1 and TNF, consistent with the observation in vitro that PMN express IL-1β mRNA quickly and very briefly when stimulated (20), probably because of their short life span. On the other hand, the preferential distribution of positive cells at the periphery of the tumor and around the blood vessels raises the possibility that cytokine production occurs in newly arrived, young PMN. Note that treatment with antibodies to Mac-1 reduced the number of tumor-infiltrating PMN but increased the number of PMN that expressed IL-1 and TNF-α, suggesting the presence of a Mac-1+ cell population with suppressive effects (21).

Granulocytes, which are usually considered terminally differentiated cells active only during inflammation, have been found to exert antitumoral activity in vivo (5, 22–24). Their ability to synthesize and release cytokines upgrades their role as coregulators of the immune response (25) through antigen presentation ability (26, 27). PMN that express IL-1β, IL-1α (28–30), and TNF-α mRNA (31, 32) also synthesize and secrete the proteins when stimulated (28–31). CSFs, IL-3, IL-8, TGF-α, and TGF-β were found to be expressed by either neutrophilic or eosinophilic PMN (review in reference 25). Similarly, it was IL-6 that is known to stimulate cell proliferation and cytotoxic function in T lymphocytes (33).

Analysis of the antitumor response has provided evidence of PMN-T lymphocyte cooperation. Depletion of PMN by mAb treatment, in rats, resulted in abrogation of specific transplantation resistance to challenge with syngeneic, chemically induced tumors used for immunization (24). Tumors engineered to produce IL-4 or IL-2 are first infiltrated with PMN and subsequently with T lymphocytes (12, 34). This sequential infiltration of different leukocyte types is characteristic of many acute inflammatory processes, suggesting the existence of a general mechanism governing leukocyte traffic that largely involves IL-1α and TNF-α (35). Nevertheless, PMN plays a central role in tumor rejection of both IL-4- (36) and G-CSF-producing tumors. Our data also suggest a reverse direction of PMN-T lymphocyte cooperation, wherein depletion of CD8+ cells reduces the number of infiltrating PMN, thus preventing tumor regression. Expression of IFN-γ mRNA by CD8+ cells infiltrating regressing tumors suggests some role for IFN-γ in sustaining PMN survival and function (37). In fact, we find that antibodies to IFN-γ abrogate tumor regression. T cell expression of IFN-γ mRNA may well be induced unspecifically by PMN- and monocyte-released IL-1 and TNF, as occurs after infection with microbial pathogen (38). Noncytotoxic CD8+ tumor-infiltrating lymphocytes have been reported to eradicate lung tumors in irradiated mice through the release of TNF and
IFN-γ (39). Thus, IFN-γ along with IL-1 and TNF-α appear to provide a favorable setting for tumor destruction, a notion consistent with our observation in tumors of cyclosporin A-treated mice where the remaining leukocytes were negative for cytokine mRNA expression and tumors did not regress. Thus, G-CSF production by neoplastic cells is not sufficient per se to induce regression of an established tumor, whereas it can inhibit tumor take.

Together, our data demonstrated the occurrence of events expected when the appropriate combination of cytokines is released at the tumor site. Such a combination might well be initially triggered by a single cytokine, as in our G-CSF model.

We thank Dr. Gianni Garotta for supplying rIFN-γ and AN18 hybridoma, Dr. Robert Coffman for providing the RB6-8CS hybridoma, and Dr. Luigi Ruco for critically reviewing the manuscript.

This work was supported by grants from C.N.R. Target Projects on Biotechnology and Bioinstrumentation and on Clinical Application of Cancer Research.

Address correspondence to Mario P. Colombo, Experimental Oncology D, Istituto Nazionale Tumori, Via G. Venezian 1, 20133 Milano, Italy.

Received for publication 28 December 1992 and in revised form 19 March 1993.

References

1. Blankenstein, T., D.A. Rowly, and H. Schreiber. 1991. Cytokines and cancer: experimental system. *Curr. Opin. Immunol.* 3:694.

2. Colombo, M.P., A. Modesti, G. Parmiani, and G. Forni. 1992. Local cytokine availability elicits tumor rejection and systemic immunity through granulocyte-T lymphocyte cross-talk. *Cancer Res.* 52:4853.

3. Singh, S., S.R. Ross, M. Acca, D.A. Rowley, and H. Schreiber. 1992. Stroma is critical for preventing or permitting immunological destruction of antigenic cancer cells. *J. Exp. Med.* 175:139.

4. Nathan, C., and M. Sporn. 1991. Cytokines in context. *J. Cell Biol.* 113:981.

5. Colombo, M.P., G. Ferrari, A. Stoppacciaro, M. Parenza, M. Rodolfo, F. Mavillo, and G. Parmiani. 1991. Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma in vivo. *J. Exp. Med.* 173:889.

6. Corbett, T.H., D.P. Griswold, Jr., B.J. Roberts, J.C. Peckham, and F.M. Schabel, Jr. 1975. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assay, with a note on carcinogen structure. *Cancer* 35:2434.

7. Lomedico, P.T., G. Veli, C.P. Hellmann, M. Dukovich, J.G. Giri, Y.C.E. Pan, K. Collier, R. Semionow, A.D. Kuo, and S.B. Mizel. 1984. Cloning and expression of interleukin-1 cDNA in *Escherichia coli*. *Nature (Lond.)* 304:596.

8. Beutler, B., N. Krochein, I.W. Milskark, C. Luedke, and A. Cerami. 1986. Control of cachectin (tumor necrosis factor) synthesis: Mechanisms of endotoxin resistance. *Science (Wash. DC)*. 232:977.

9. Gray, P.W., and D.V. Goeddel. 1983. Cloning and expression of murine immune interferon cDNA. *Proc. Natl. Acad. Sci. USA.* 80:5842.

10. Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann, D. Rennick, N. Roheim, C. Smith, A. Zlotnik, and K. Arai. 1986. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell and mast-cell-stimulating activities. *Proc. Natl. Acad. Sci. USA.* 83:2061.

11. Tepper, R.I., P.K., Pattengale, and P. Leder. 1989. Murine interleukin-4 displays potent anti-tumor activity in vivo. *Cell.* 57:503.

12. Golumbek, P.T., A.J., Lazenby, H.G. Levitsky, L.M. Jaffe, H. Karsuyama, H. Baker, and D.W. Pardoll. 1991. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science (Wash. DC)*. 254:719.

13. Hock, H., M. Dorsch, T. Diamanstein, and T. Blankenstein. 1989. Interleukin 7 induces CD4+ cell-dependent tumor rejection. *J. Exp. Med.* 174:1291.

14. Blankenstein, T., Z. Qin, K. Uberla, H. Rosen, H.D. Volk, and T. Diamanstein. 1991. Tumor suppression after tumor cell-targeted tumor necrosis α gene transfer. *J. Exp. Med.* 173:1047.

15. Russel, S.J., S.A. Eccles, C.L. Fleming, C.A. Johnson, and M.K.L. Collins. 1991. Decreased tumorigenicity of a transplantable rat sarcoma following transfer and expression of an IL-2 cDNA. *Int. J. Cancer.* 47:244.

16. Sun, W.H., R.A. Kreisele, A.W. Phillips, and W.B. Eshleman. 1992. In vitro and in vitro characteristics of interleukin 6-transfected B16 melanoma cells. *Cancer Res.* 52:5412.

17. McBride, W.H., J.D. Thacker, S. Comora, J.S. Economou, D. Kelley, D. Hogge, S.M. Dubinett, and G.J. Dougherty. 1992. Genetic modification of a murine fibrosarcoma to produce interleukin 7 stimulates host cell infiltration and tumor immunity. *Cancer* 52:3931.

18. Colombo, M.P., L. Lombardi, A. Stoppacciaro, C. Melani, M. Parenza, B. Bottazzi, and G. Parmiani. 1991. Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition in vivo. *J. Immunol.* 149:113.

19. Mantovani, A., F. Bussolino, and E. Dejana. 1992. Cytokine regulation of endothelial cell function. *FASEB (Fed. Am. Soc. Exp. Biol.)* J. 6:2591.
20. Ohkawara, S., K. Goto, S. Mori, F. Goto, N. Saia, T. Sagara, and M. Yoshinaga. 1989. Interleukin-1 production by polymorphonuclear leukocytes during the course of acute inflammation in rabbits. *Dermatologica (Basel).* 179:86.
21. Mantovani, A., B. Bottazzi, F. Colotta, S. Sozzani, and L. Ruco. 1992. The origin and function of tumor-associated macrophages. *Immunol. Today.* 13:265.
22. MacPherson, G.G., and R.J. North. 1986. Endotoxin-mediated necrosis and regression of established tumors in the mouse. A correlative study of quantitative changes in blood flow and ultrastructural morphology. *Cancer Immunol. Immunother.* 21:209.
23. Katano, M., and M. Torisu. 1982. Neutrophil-mediated tumor cell destruction in cancer ascites. *Cancer (Phila.)* 50:62.
24. Midorikawa, Y., T. Yamashita, and F. Sendo. 1990. Modulation of the immune response to transplanted tumors in rats by selective depletion of PMN in vivo using a monoclonal antibody: abrogation of specific transplantation resistance to chemical carcinogen-induced syngeneic tumors by selective depletion of PMN in vivo. *Cancer Res.* 50:6243.
25. Lloyd, A.R., and J.J. Oppenheim. 1992. Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunol. Today.* 13:169.
26. Okuda, K., T. Tani, Y. Ishigatsobo, S. Yokota, and C.S. David. 1980. Antigen pulsed neutrophils bearing I antigens can induce T lymphocyte proliferative response to syngeneic or semi-syngeneic antigen primed T lymphocytes. *Transplantation (Baltimore)* 30:368.
27. Del Pozo, V., B. De Andres, E. Martin, B. Cardaba, J.C. Fernandez, S. Gallardo, P. Tramon, F. Leyva-Cobian, P. Palomino, and C. Lahoz. 1992. Eosinophil as antigen-presenting cell: activation of T cell clones and T cell hybridoma by eosinophils after antigen processing. *Eur. J. Immunol.* 22:1919.
28. Tiku, K., M.L. Tiku, and J. Skosey. 1986. Interleukin 1 production by human polymorphonuclear neutrophils. *J. Immunol.* 136:3677.
29. Marucha, P.T., R.A. Zeff, and D.L. Kreutz. 1990. Cytokine regulation of IL-1beta gene expression in the human polymorphonuclear leukocytes. *J. Immunol.* 145:2932.
30. Lord, P.C.W., L.M.G. Wilmoth, S.B. Misel, and C.E. McCall. 1991. Expression of interleukin 1alpha and beta genes by human blood polymorphonuclear leukocytes. *J. Clin. Invest.* 87:1312.
31. Djou, Y.M., D. Serbousek, and D.K. Blanchard. 1990. Release of tumor necrosis factor by human polymorphonuclear leukocytes. *Breed.* 76:1405.
32. Dubravec, D.B., D.R. Spriggs, J.A. Mannick, and M.L. Rodrick. 1990. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor. *Proc. Natl. Acad. Sci. USA.* 87:6758.
33. Kishimoto, T. 1989. The biology of interleukin-6. *Blood.* 74:1.
34. Cavallo, F., M. Giovarelli, A. Gulino, A. Vacca, A. Stoppacciaro, A. Modesti, and G. Forni. 1992. Role of neutrophils and CD4+ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. *J. Immunol.* 149:3627.
35. Binns, R.M., ST. Licence, F.B.P. Wooning, and W.P.H. Duffs. 1992. Active lymphocyte traffic induced in the periphery by cytokines and phytohemagglutinin: three different mechanisms? *Eur. J. Immunol.* 22:2195.
36. Tepper, R.I., R.L. Coffman, and P. Leder. 1992. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science (Wash. DC.)* 257:548.
37. Perussia, B., M. Kobayashi, M.E. Rossi, I. Anegon, and G. Trinchieri. 1987. Immune interferon enhances functional properties of human granulocytes: role of Fc receptors and effect of lymphokinin, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 138:765.
38. Blanchard, K.D., J.Y. Djeu, TW. Klein, H. Friedman, and W.E. Stewart II. 1986. Interferon-γ induction by lipopolysaccharide: dependence on interleukin 2 and macrophages. *J. Immunol.* 136:963.
39. Barth, R.J., J.J. Mulé, P.J. Spiess, and S.A. Rosenberg. 1991. Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8+ tumor-infiltrating lymphocytes. *J. Exp. Med.* 173:647.