Neutrophils, Nitric Oxide Synthase, and Mutations in the Mutatect Murine Tumor Model

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Mutatect MN-11 is a tumor line that can be grown subcutaneously in syngeneic C57BL/6 mice. The frequency of spontaneously arising mutants at the hypoxanthine phosphoribosyltransferase (Hprt) locus was observed to be elevated as a result of *in vivo* growth. The objective of the present study was to identify factors in the tumor microenvironment that might explain this increase in mutant frequency (MF). When tumors were examined histologically, neutrophils were found to be the predominant infiltrating cell type. Quantitative estimates of the number of neutrophils and MF of tumors in different animals revealed a statistically significant correlation ($r = 0.63$, $P < 0.0001$). Immunohistochemical analysis for inducible nitric oxide synthase (iNOS) demonstrated its presence, mainly in neutrophils. Biochemical analysis of tumor homogenates for nitric oxide synthase (NOS) activity indicated a statistically significant correlation with MF ($r = 0.77$, $P < 0.0001$). Nitrotyrosine was detected throughout the tumor immunohistochemically; both cytoplasmic and nuclear staining was seen. To increase the number of infiltrating neutrophils, tumors were injected with chemoattractant interleukin-8 and prostaglandin E2. This produced a statistically significant increase in neutrophil content ($P = 0.005$) and MF ($P = 0.0002$). As in control MN-11 tumors, neutrophil content and MF were strongly correlated ($r = 0.63$, $P = 0.003$). Because neutrophils are a potential source of genotoxic reactive oxygen and/or nitrogen species, our results support the notion that these tumor-infiltrating cells may be mutagenic and contribute to the burden of genetic abnormalities associated with tumor progression. (*Am J Pathol* 2000, 156:509–518)

Chronic or recurrent inflammation is associated with increased cancer risk.^{1,2} In humans, autoimmune diseases such as ulcerative colitis and Crohn's disease are well-

known examples where inflammation is associated with increased cancer risk in the affected organ.³ More recently, an association between chronic inflammation and bladder cancer in schistosomiasis patients and cholangiocarcinoma and liver fluke infection has been suggested.^{4,5} An association has also been found between *Helicobacter pylori*-associated gastritis and increased risk of noncardiac gastric adenocarcinoma and gastric lymphoma.⁶ Rheumatoid arthritis, an autoimmune inflammatory disease, is associated with elevated risk of lymphoma and lung malignancy.⁷ A characteristic of inflamed tissue is infiltration by leukocytes, such as neutrophils, macrophages, and lymphocytes. Many of these same cell types are also found in solid tumors. It has been postulated by many workers that such cells are capable of causing carcinogenic DNA damage.^{1,4,8–10} In animal models, inflammation of the colon resulting from instillation of a chemotactic peptide has been shown to produce infiltration of neutrophils and enhance the development of colon tumors.¹¹ Chronic inflammation of the peritoneal cavity resulting from repeated injection of an inert oil has been shown to induce plasmacytomas.¹² These observations raise the possibility that inflammatory cells may contribute to carcinogenesis through the generation of genotoxic species.

The linkage between inflammation and genotoxicity is strengthened by recent findings that some chronic inflammatory diseases are associated with an increase in mutant frequency (MF) at the hypoxanthine phosphoribosyltransferase (Hprt) locus. This locus is widely used as a marker of genotoxic insult because it is a non-essential gene in which mutational events can be readily scored on the basis of resistance to 6-thioguanine.¹³ T lymphocytes from the peripheral blood of patients with autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, scleroderma and rheumatoid arthritis have an elevated MF.^{14–17} T lymphocytes from the diseased synovium of rheumatoid arthritis patients exhibited an even higher MF.¹⁷ In a lacZ transgenic mouse injected with lymphoma cells to produce inflammation in the spleen and lymphatic tissues, an increase in MF in host cells was seen.¹⁸ Thus, the association between chronic inflamma-

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tion and cancer may be explained by the presence of mutagenic factors in inflammatory milieu.

Genomic instability of cancer cells is well documented, and is a feature of tumor progression. Instability may be manifest as aneuploidy, structural chromosome abnormalities, loss of heterozygosity, homogeneously staining regions (amplification) and accumulation of mutations.¹⁹ The contribution to genetic instability of factors in the tumor microenvironment, such as products of inflammatory cells, has been addressed in only a few experimental studies.²⁰ An increase in mutagenicity in cells grown *in vivo* as subcutaneous tumors compared to the same cells grown in culture has been reported. C3H/10T1/2 cells grown in C3H mice had a very high frequency of mini-satellite sequence instability compared to *in vitro*.²¹ Mutatect cells²² grown in C57BL/6 mice had a fourfold higher MF at the *Hprt* locus.²³ Murine LN12 cells grown in athymic nude mice exhibited a similar increase in MF.²⁴ The nature of the mutagenic species in the tumor microenvironment is unknown, but is suspected of being reactive oxygen species (ROS) or nitrogen species (RNS).^{10,18,23,25–31} Because ROS and RNS are known to be mutagenic, it is possible that they are responsible for the genotoxicity associated with both the inflammatory and the tumor microenvironments.

Solid tumors frequently contain inflammatory cells, whose presence may be explained by chemotactic factors. For example, interleukin-8 (IL-8), a potent chemoattractant for neutrophils of the C-X-C type,³² has been shown in glioblastoma.³³ IL-8 has also been identified in human gastric, colorectal and bronchioloalveolar carcinomas^{34–36} and in B-cell chronic lymphocytic leukemia.³⁷ High expression of several C-C chemokines, in particular, monocyte chemoattractant protein-1, was identified in epithelial ovarian cancers where CD68+ macrophages and CD8+/CD45RO+ T cells were the primary infiltrating cells.³⁸ Recruited neutrophils and hypoxic vascular endothelial cells may be another source of IL-8 and other chemotaxins.^{32,39} Since thrombotic occlusion of blood vessels is common in tumors, clotting-related factors can also contribute to the recruitment of inflammatory cells.³⁸ Activated platelets can secrete chemokines such as monocyte chemoattractant protein-1 and IL-8.^{38,40} Human α -thrombin is a chemoattractant for monocytes.⁴¹ Fibrin-derived polypeptides are chemoattractants for neutrophils.⁴² It would thus appear that many chemotactic factors may be present in tumors; the particular mix of factors present in any individual tumor may dictate the type of leukocytes that infiltrate.

To better understand the contribution of inflammatory cells and their products to genotoxicity in solid tumors, we have used the Mutatect mouse tumor model.²² This is a murine fibrosarcoma that can be propagated as a subcutaneous tumor in syngeneic C57BL/6 mice as well as in culture. This model is unique in that it permits the ready detection of mutations at the *Hprt* locus that may arise *in vivo* or *in vitro*, either spontaneously or induced by agents such as ionizing radiation.^{22,23} In the present study, we show that the number of *Hprt* mutations in individual tumors is a direct function of the

number of infiltrating neutrophils and amount of nitric oxide synthase.

Materials and Methods

Tumor Formation

C57BL/6 female mice, 8 to 10 weeks of age (Charles River Laboratories, St.-Constant, QC, or Taconic, Germantown, NY) were injected subcutaneously (s.c.) in the right flank with 5×10^5 Mutatect MN-11 cells in 0.1 ml PBS (140 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na_2HPO_4 , 1.5 mmol/L KH_2PO_4 , pH 7.4). Tumors were first detectable at day 7, measured about 0.5 cm in diameter at day 11 and were harvested when they were about 1 cm in diameter (day 14–15). Other details and culture conditions are described elsewhere.²³ Experiments were carried out at the Animal Care and Veterinary Service of the University of Ottawa in accordance with guidelines of the Canadian Council on Animal Care.

Detection of S-Phase Cells in Tumors by 5-Bromo-2'-deoxyuridine (BrdU) Labeling

To detect S-phase cell in tumors, BrdU (30 mg/kg) was injected 2 hours before sacrifice. BrdU incorporation into DNA was detected immunohistochemically using a mouse monoclonal anti-BrdU antibody (Sigma Chemical Co., St. Louis, MO), biotinylated horse anti-mouse IgG (Dimension Lab, Inc., Mississauga, ON), avidin-peroxidase (Dimension Lab) and 0.02% diaminobenzidine tetrahydrochloride (DAB).

Determination of *in Vivo* MF

Tumors were allowed to form following s.c. injections as described above. When tumors reached 1 to 1.5 cm in diameter (14–16 days), animals were sacrificed by cervical dislocation. Tumors were removed under aseptic conditions and gently homogenized by passage several times through a syringe. The suspension was allowed to settle for 5 minutes and the supernatant containing predominantly single cells was removed and incubated for 2 to 4 days to establish *ex vivo* cultures. The MF was estimated by culturing 1×10^5 viable cells (determined by trypan blue exclusion) per 10-cm dish in the presence of 50 $\mu\text{mol/L}$ 6-thioguanine. MF is expressed as mutants per 1×10^5 viable G418^R cells (MN-11 cells harbor a *neo* gene that allows them to be readily distinguished from host cells), as described earlier.²³

Histochemical Assays for Detecting Tumor-Infiltrating Host Cells

Tumor tissue was fixed for 24 hours in 10% neutral buffered formalin, embedded in paraffin, sectioned at 3 to 5 μm , and processed for histochemical analysis. Paraffin was removed with xylene and sections were rehydrated with a series of decreasing concentrations of ethanol.

Frozen sections were used where indicated. Fresh tumor tissue was fixed with modified Zamboni's fixative⁴³ for 2 hours. The fixative was removed with ice-cold 100 mmol/L sodium phosphate buffer, pH 7.2 (PB), and the tumor fragment was stored in a 1:1 mixture of 10% sucrose, 3% Triton X-100 in PB for a minimum of 24 hours. Tumor tissue was embedded in Tissue-Tek OCT Compound (VWR-Canlab, Mississauga, ON), frozen immediately in liquid nitrogen, and stored at -80°C until sectioned. Hematoxylin and eosin (H&E) stained sections were examined for infiltrating host cells. Other stains were used as indicated. Neutrophils were identified in frozen sections of mouse tissue by staining for myeloperoxidase activity; this enzyme appears to be inactive in paraffin-embedded sections of mouse and human tissues. Five-micron cryosections were stained using 0.02% DAB, 0.6% H_2O_2 in TBS (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.6) for 10 minutes at room temperature. Sections were washed in running water and counterstained with Mayer's hematoxylin. Brown-stained multinucleated neutrophils were readily identifiable. Macrophages were identified by staining for nonspecific esterase, using frozen sections incubated in the presence of 2% sodium nitrite, 2% pararosanilin (Sigma-Aldrich) for 20 minutes at 37°C .⁴⁴ Mast cells were identified in fixed, paraffin-embedded sections after staining with 1% toluidine blue in 50% isopropanol for 10 minutes at room temperature and destaining in absolute isopropanol for 1 minute.

iNOS Immunohistochemistry

Formalin-fixed tumor tissues were deparaffinized and incubated with TBS containing 1% H_2O_2 and 0.25% Triton-X 100 for 15 minutes at room temperature to destroy endogenous peroxidase activity. Nonspecific immunoglobulins were blocked with 1% normal swine serum at room temperature for 30 minutes. Excess liquid was drained and sections were incubated in a humid chamber with 1:100 dilution (2.5 $\mu\text{g}/\text{ml}$) of rabbit polyclonal antibody to mouse macrophage iNOS (Transduction Laboratories, Lexington, KY). Sections were incubated overnight at 4°C followed by an incubation at room temperature for 30 minutes with 1:200 (7.5 $\mu\text{g}/\text{ml}$) goat anti-rabbit biotinylated IgG (Vector Laboratories). The biotinylated conjugate was detected by incubating sections with avidin-peroxidase 1:1000 dilution (5 $\mu\text{g}/\text{ml}$, Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes. Immunolabeling was detected using DAB as the chromogen, washed, and counterstained with Mayer's hematoxylin.

Nitrotyrosine Immunohistochemistry

Formalin-fixed tumor tissue sections were deparaffinized and heated at $90-100^{\circ}\text{C}$ for 12 minutes in 0.01 mol/L sodium citrate, pH 6.0. Sections were then incubated with TBS containing 3% H_2O_2 for 10 minutes at room temperature. Nonspecific immunoglobulins were blocked with 1% normal swine serum at room temperature for 30 minutes. Excess liquid was drained and sections were incubated in a humid chamber with 2.5 $\mu\text{g}/\text{ml}$ of a rabbit

polyclonal antibody to nitrated KLH (Upstate Biotechnology, Lake Placid, NY). Sections were incubated for 1 hour at room temperature followed by incubation for 30 minutes with DAKO Envision peroxidase conjugated to goat anti-rabbit/anti-mouse Ig antibody (DAKO Corp., Carpinteria, CA). Immunolabeling was detected using DAB as the chromogen, washed and counterstained with Mayer's hematoxylin. Three tests of the specificity of the antibody for nitrotyrosine were carried out. Before incubation with tissue sections, the primary antibody was preincubated for 1 hour at room temperature with either 10 mmol/L nitrotyrosine (Sigma) or 50 $\mu\text{g}/\text{ml}$ nitrated bovine serum albumin (prepared by incubation of albumin (6 mg/ml) for 18 hours at room temperature with 10 mmol/L NaNO_2 , 0.3% H_2O_2 , 9 $\mu\text{mol}/\text{L}$ FeCl_3 and subsequent precipitation with 4 vol ethanol). A third control involved reduction of tissue nitrotyrosine to aminotyrosine with sodium hydro-sulfite.⁴⁵

Nitric Oxide Synthase (NOS) Activity in Tumor Extracts

Fragments of the same tumors collected for MF analysis (*vide supra*) were frozen in liquid nitrogen and stored at -80°C . For assay of NOS activity, a previously described method was followed.⁴⁶ Tumor fragments (100 mg) were thawed and homogenized (Polytron PT-1200, Kinematica, Lucerne, Switzerland) in 250 μl of an ice-cold solution containing 320 mmol/L sucrose, 10 mmol/L HEPES, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 2 $\mu\text{g}/\text{ml}$ aprotinin, pH 7.4. The homogenates were centrifuged at $13,000 \times g$ at 4°C for 30 minutes. The supernatants were removed and assayed for NOS activity by following the conversion of L-[^{14}C]arginine (Amersham, Arlington Heights, IL) to [^{14}C]citrulline.⁴⁶ Forty microliters of supernatant were mixed with 100 μl of assay buffer containing 30 mmol/L KH_2PO_4 , 1 mmol/L MgCl_2 , 0.2 mmol/L CaCl_2 , 1 mmol/L $\beta\text{-NADPH}$, 6 mmol/L L-valine, 18 $\mu\text{mol}/\text{L}$ L-arginine, and 1 $\mu\text{Ci}^{14}\text{C}$ -L-arginine, pH 7.4. Reactions were incubated at 37°C for 20 minutes and terminated by dilution in 1 ml of 1 mmol/L cyclohexanediamine tetraacetate (CDTA, Sigma-Aldrich) and immediately applied to columns containing 0.4 ml Spectra/Gel cation exchanger 50W-X8 to remove [^{14}C]arginine. [^{14}C]Citrulline was recovered by elution with 3 ml of 1 mmol/L CDTA. Fractions were mixed with scintillation fluid (AquaSol-2) and counted in a Packard model 1600 TR liquid scintillation counter. We have not ruled out that arginase may have contributed in part to the observed activity, since inhibition by 1 mmol/L N^G -monomethyl-L-arginine was incomplete (average of 50%). Protein content of tumor homogenates was determined spectrophotometrically with Bradford reagent,⁴⁷ using bovine serum albumin as standard. NOS activity was expressed as pmole of citrulline generated per minute per milligram of protein.

IL-8 Injections

On day 11 after inoculation, tumors were injected directly with IL-8 or *N*-formyl-met-leu-phe (fMLP) in combination with prostaglandin E_2 (PGE_2). Control mice received endotoxin-free sterile saline. Amounts injected were 1×10^{-12} mol IL-8 (Endogen, Woburn, MA), 3×10^{-10} mol PGE_2 (Sigma-Aldrich), and 1×10^{-10} mol fMLP (Pierce, Rockford, IL) in a total volume of 6 μ l using an ethanol-sterilized glass Hamilton syringe and semiautomatic dispenser. On day 15 after tumor inoculation (4 days after IL-8 + PGE_2 injections), animals were sacrificed by cervical dislocation and tumors were excised aseptically. A section of the tumor taken from the middle was washed in PB and fixed in a modified Zamboni's fixative, and myeloperoxidase activity was detected histochemically. The remaining tumor tissue was used for analysis of MF as described.²³

Histological Grading of Neutrophil Infiltration and Necrosis

Neutrophil and necrosis counts were performed by two observers on coded tumor sections. A 2-mm grid was drawn on the coverslip over the tumor section. The four corners of each 2-mm square within the grid were scored for neutrophil and necrosis counts using a 40 $\times$ objective with a 10 $\times$ eyepiece. Between 5 and 14 squares (depending on tumor size) were chosen at random and the same fields were scored for both neutrophil and necrosis counts. The extent of tumor necrosis was determined by the stereological point counting method⁴⁸ and expressed as percentage of tumor necrosis of total area counted. Results were expressed as the mean number of neutrophils and mean percentage necrosis per field. Neutrophils found in blood vessels were not included in the counts.

Statistical Analysis

The MF of individual tumors exhibited considerable variation that appeared to be non-Gaussian. Data were therefore analyzed using nonparametric tests. Comparison between two groups was made using the Mann-Whitney test and correlations using Spearman's rank test. Calculations were done using GraphPad Instat version 3.0. A value of $P \leq 0.05$ was considered to be statistically significant.

Results

Gross and Histological Properties of Mutatect MN-11 Subcutaneous Tumors in C57BL/6 Mice

MN-11 cells readily form subcutaneous tumors in syngeneic C57BL/6 mice. At day 14 after injection of 5×10^4 , 1×10^5 , 2.5×10^5 , or 5×10^5 cells, the percentages of animals (6 per group) bearing a tumor were 17, 67, 67, and 100%, respectively. This percentage did not in-

crease by day 18. The tumor volume was estimated from its length, width, and height according to Isaacs and Coffey.⁴⁹ The tumor volume increased most rapidly between days 10 and 15; tumor volume doubling time following injection of 5×10^5 cells was estimated to be 2.7 days. For example, the average volume was 40 ± 3.5 mm³ ($n = 6$) at day 10 and 256 ± 10.6 mm³ at day 15. The growth fraction in the tumors was estimated using the BrdU labeling technique, in which newly synthesized DNA is detected using an antibody to BrdU. Tumors 15, 19, and 21 days old were examined 2 hours after injection of BrdU. The percentage of nuclei into which BrdU was incorporated was 11.0 ± 1.1 , 8.0 ± 1.2 , and $5.3 \pm 0.3\%$, respectively, at the periphery of the tumors and 3.9 ± 0.8 , 2.8 ± 1.1 , and $1.6 \pm 0.7\%$, respectively, in the core of the tumors. Statistically significant decreases (from day 15) are day 19 and 21 at the periphery and day 21 in the core (analysis of variance, $n = 4$ to 8 tumors in each group, $P < 0.01$). Tumors that were ≥ 2 cm in diameter (usually older than 17 days) tended to have necrotic cores. A low magnification micrograph of a 15-day-old MN-11 tumor is shown in Figure 1A. Tumors were typically surrounded by a capsule and rarely showed evidence of invasion. Histological examination following H&E staining revealed a poorly differentiated fibrosarcoma with considerable variation in cell and nuclear size. Most tumors were very vascular. In some, a heavy neutrophil infiltrate was seen in and around blood vessels (Figure 1B) and in necrotic areas (Figure 1C). Fibrin deposits were also seen in necrotic areas (Figure 1C). Neutrophils were present to a lesser extent in non-necrotic areas and at the periphery of the tumor. Less frequently seen were infiltrating lymphocytes, mainly at the tumor periphery (Figure 1D). Non-specific esterase staining was used to identify macrophages; these were infrequent and found only at the periphery of tumors (Figure 1E). Toluidine blue staining was used to identify mast cells; these were also infrequent and at the periphery of tumors (Figure 1F). On the basis of the examination of 20 Mutatect MN-11 tumors, we concluded that central necrosis was more frequently seen in ≥ 2 cm tumors and that neutrophils were the predominant infiltrating host cell type.

Quantitative Study of Neutrophil Infiltration and MF

To study the relationship between infiltrating neutrophils and MF, a neutrophil count was performed on MN-11 tumor sections, using myeloperoxidase staining to facilitate counting. Thirty-four MN-11 tumors were examined both for MF and for infiltrating neutrophils, as described in Materials and Methods (Figure 2A). There was a statistically significant correlation between the number of infiltrating neutrophils and the MF ($r = 0.63$, $P < 0.0001$, Spearman's rank correlation). The same tumors were examined for necrosis. Again, there was a statistically significant correlation between the number of infiltrating neutrophils and the extent of tumor necrosis ($r = 0.73$, $P < 0.0001$). These findings demonstrate a clear positive

Figure 1. Histochemical analysis of MN-11 tumors. **A:** Low magnification view of a 14-day tumor (1 cm diameter) showing a well defined fibrous capsule ($\times 50$, H&E). **B:** Region of tumor showing blood vessels filled with neutrophils ($\times 200$, H&E). **C:** Necrotic area showing neutrophils and fibrin deposits (lacy, light-staining areas; $\times 200$, H&E). **D:** Tumor periphery showing the presence of lymphocytes with occasional neutrophils ($\times 400$, H&E). **E:** Periphery of tumor showing the presence of macrophages ($\times 400$, nonspecific esterase). **F:** Periphery of tumor showing mast cells (indicated by **arrow**) ($\times 400$, toluidine blue).

correlation between the neutrophils and MF in Mutatect tumors.

Intratumoral Injection of IL-8 + PGE₂ Increases Neutrophil Infiltration and MF

It is possible to increase neutrophils by using chemoattractants such as IL-8 + PGE₂ or fMLP.⁵⁰ The neutrophil content of treated and control 11-day tumors was first examined in a pilot experiment involving 7 tumors per treatment group. Although both fMLP and IL-8 + PGE₂ injected tumors were more heavily infiltrated with neutrophils than control tumors, IL-8 + PGE₂ appeared to be more effective (data not shown). No increase in the number of infiltrating macrophages or lymphocytes was seen.

To confirm and extend these observations, an experiment involving 64 animals was carried out. Thirty-six animals were injected intratumorally with IL-8 + PGE₂ and the remainder with saline on day 11, when tumors were

about 0.5 cm in diameter. On day 15, the tumors were excised and some were examined quantitatively for neutrophil infiltrate (Figure 2B). There was a statistically significant increase in neutrophil content between the IL-8 + PGE₂-injected ($n = 16$) group and the control group ($n = 12$; Mann-Whitney test, $P = 0.0007$). The percentage of tumor necrosis was also significantly increased ($P = 0.01$). Tumors were also analyzed for MF. The IL-8 + PGE₂ injected group ($n = 36$) had a highly statistically significant elevation in MF compared to the control group ($n = 28$) (Mann-Whitney test, $P = 0.0002$; Figure 2C). These data indicate clearly that IL-8 + PGE₂ was effective at recruiting neutrophils to MN-11 tumors and that this was associated with an increase in MF.

For 20 tumors injected with IL-8 + PGE₂, both MF and neutrophil data were available. The relationship between neutrophil content and MF was explored (Figure 2D). As in untreated tumors, there was a strong correlation between the MF and the neutrophil content in this group of

Figure 2. Neutrophil infiltration and mutations in control and IL-8 + PGE₂-treated tumors. **A:** Correlation between neutrophil number and spontaneously arising mutants in Control MN-11 tumors (Spearman's rank test; $r = 0.63$, $P < 0.0001$). **B:** Comparison of neutrophil number in control (median, 0.2) and IL-8 + PGE₂-treated (median, 2.5) tumors (Mann-Whitney U test; $P = 0.005$), where both control and treated animals were drawn from the same lot of mice. **C:** Comparison of the MF in control (median, 11.2) and IL-8 + PGE₂-treated (median, 28.3) tumors (Mann-Whitney U test, $P = 0.0002$), where both control and treated animals were drawn from the same lot of mice. **D:** Correlation between neutrophil number and MF in IL-8 + PGE₂-treated tumors (Spearman's rank test; $r = 0.63$, $P = 0.003$). Mutant frequency is expressed as mutants per 1×10^5 clonable cells; other details are stated in Materials and Methods.

IL-8 + PGE₂-treated tumors (Spearman's rank correlation, $r = 0.63$, $P = 0.003$). Percentage of tumor necrosis and MF were also strongly correlated ($r = 0.70$, $P = 0.0006$).

Because neutrophil count was correlated with percentage of necrosis in the mouse tumors, a similar study of human adenocarcinoma of the lung was carried out. A statistically significant correlation between the number of neutrophils and the extent of necrosis was seen ($n = 30$; $r = 0.70$; $P < 0.0001$). The presence of IL-8 was assessed immunohistochemically in 12 specimens; most tumors showed at least some IL-8-staining areas, localized to the cytoplasm of tumor cells with occasional staining of macrophages and neutrophils (data not shown).

Correlations between NOS Activity and MF in MN-11 Tumors

The Mutatect model was developed to study whether factors such as nitric oxide and/or related species in the tumor microenvironment might be genotoxic and mutagenic. Nitric oxide is produced from arginine by a family

Figure 3. Correlation between NOS activity and mutant frequency in MN-11 tumors. After 14 to 19 days of *in vivo* growth, tumors were excised and mutant frequency and NOS enzymatic activity were determined as described in Materials and Methods. Correlation between NOS activity (pmol/minute/mg protein) and mutant frequency (Spearman's rank test; $r = 0.77$, $P < 0.0001$). Mutant frequency is expressed as mutants per 1×10^5 clonable cells.

of enzymes, NOS. To explore whether NOS activity was present in MN-11 tumors, extracts of 56 individual tumors (15–22 days of *in vivo* growth) were examined. Figure 3 shows that NOS activity varied appreciably among tumors (range, 1.1–33.7 pmol/minute/mg protein). The level of NOS activity was compared to the MF in the corresponding tumor. The data indicate a positive correlation between NOS activity and MF ($r = 0.77$, $P < 0.0001$) in these tumors.

Immunohistochemical Analysis for iNOS

Nitric oxide is a potentially genotoxic reactive nitrogen species formed *in vivo* by nitric oxide synthases. Inducible nitric oxide synthase (iNOS) is an enzyme associated primarily with macrophages. Because MN-11 tumors contain very few macrophages but many neutrophils, it was of interest to determine whether tumor-infiltrating neutrophils expressed iNOS. Tumor sections probed with an antibody to mouse macrophage iNOS demonstrated strong staining of neutrophils. Immunostaining was seen in both non-necrotic (Figure 4A) and necrotic (Figure 4B) areas of the tumor. Macrophages and mast cells at the periphery of the tumor were also stained, but were fewer in number (data not shown). No staining of tumor cells, lymphocytes, or vascular endothelial cells was seen. Specimens of human lung adenocarcinoma were also examined immunohistochemically for the presence of

Figure 4. Immunohistochemical localization of iNOS and nitrotyrosine in MN-11 tumors. **A:** Non-necrotic area showing iNOS (brown stain) in neutrophils (indicated by arrow; $\times 200$, counterstain, hematoxylin). **B:** Necrotic area showing iNOS in neutrophils ($\times 400$). No immunostaining of tumor cells was seen. **C:** Detection of nitrotyrosine in tumor cells; staining of both cytoplasm and nuclei was seen throughout the tumor. **D:** Negative control for C, showing absence of staining when the primary antibody was preincubated with nitrated bovine serum albumin ($\times 600$). Other details are stated in Materials and Methods.

iNOS. In an analysis of 6 cases, iNOS was seen in macrophages and eosinophils but not in neutrophils or tumor cells (data not shown). Thus, it appears that iNOS is present in mouse neutrophils but not in human tumor-infiltrating neutrophils.

Immunohistochemical Analysis for Protein Nitrotyrosine

It is difficult to provide direct evidence for the formation of reactive oxygen or nitrogen species *in vivo* and therefore indirect evidence of oxidative damage to tissues is often used. The availability of antibodies to protein nitrotyrosine has facilitated such analyses.⁵¹ MN-11 tumors probed with an anti-nitrotyrosine antibody revealed diffuse staining throughout much of the tumor (Figure 4C). Both diffuse cytoplasmic staining and nuclear staining was seen. No staining was seen in negative controls in which nitrated serum albumin (Figure 4D), nitrotyrosine amino acid or sodium hydrosulfite was used. These observations supports the notion that peroxynitrite or other reactive nitrogen species capable of nitrating proteins was present in MN-11 tumors.

Comparison of Interanimal and Intra-Animal Tumor MF Variation

A possible source of the interanimal variability in spontaneous tumor MF was investigated in an experiment in which two subcutaneous tumors were grown in each of 14 mice. The MF of tumors grown on the left flank was compared to the MF of tumors grown on the right flank in

this series. There was no significant correlation between the MF of the 2 tumors grown in the same animal (Spearman's rank correlation test, $P = 0.48$) (data not shown). Thus, it does not appear that the variability can be explained by systemic factors and therefore may be due to stochastic events occurring within individual tumors.

Discussion

Mutatect MN-11 tumor cells are derived from a poorly differentiated fibrosarcoma that developed in a C57BL mouse following injection with methylcholanthrene.²² They grow readily in syngeneic C57BL/6 mice after subcutaneous injection of $<5 \times 10^5$ cells to form an encapsulated 1-cm tumor within 2 weeks. If allowed to grow longer, tumors tend to be necrotic and the number of viable cells decreases. BrdU labeling indicated a decrease in S-phase cells after day 15. Mutatect cells are genetically modified to permit sensitive detection of mutations at the Hprt locus.²³ When grown *in vivo*, the MF of cells recovered from tumors is fourfold higher compared to the same cells grown *in vitro*²³ (Sandhu JK, Haqqani AS, and Birnboim HC, submitted for publication). To explain this finding, we directed our attention to factors in the tumor microenvironment. Histochemical examination of tumors revealed the presence of infiltrating host cells, predominately neutrophils, although a low number of macrophages, lymphocytes and mast cells were also seen. When analyzed quantitatively, a strong correlation was observed between the number of infiltrating neutrophils and the MF, suggesting that neutrophil-derived oxidants might be mutagenic in these tumors. Considerable

interexperimental variability was seen in the number of tumor-infiltrating neutrophils and the MF. The explanation for differences observed between different lots of animals is not understood. The variability in MF of two tumors grown in a single animal was not significantly different from the interanimal variability, suggesting that stochastic factors within each tumor, as opposed to systemic factors within each animal, are responsible. To be able to test more directly the role of infiltrating neutrophils in causing genetic damage, historical controls were not used. We were able to demonstrate an effect of IL-8 + PGE₂ by using a single lot of mice. The chemokine produced the expected increase in neutrophil content. Associated with this increase was a corresponding increase in MF. A high neutrophil content was also associated with tumor necrosis. These observations provide the first direct *in vivo* evidence that a high number of infiltrating neutrophils can be associated with a high mutation frequency.

Neutrophils are a potential source of ROS (such as superoxide and hydrogen peroxide) and RNS (such as nitric oxide and peroxynitrite), which are potentially genotoxic.^{10,18,23,25-31} The enzyme responsible for the production of nitric oxide from arginine in tissues is NOS. Immunohistochemical analysis of MN-11 tumors demonstrated iNOS in infiltrating neutrophils and biochemical measurements of tissue extracts showed NOS activity. The presence of iNOS in mouse neutrophils^{52,53} and the presence of high NOS activity in some human cancers has been reported.⁵⁴ Further evidence of the presence of RNS in MN-11 tumors was provided by immunohistochemical analysis for nitrotyrosine; immunoreactivity was observed throughout the tumor, both in the cytoplasm and the nucleus of tumor cells. Nuclear staining has also been observed in human atherosclerotic lesions.⁴⁵ Specificity of staining was proven by blocking with nitrated bovine serum albumin or nitrotyrosine and reduction of nitrotyrosine to aminotyrosine using sodium hydrosulfite. Neutrophils can produce superoxide, nitric oxide and myeloperoxidase, all of which can indirectly nitrate tyrosine.⁵⁵ We have recently shown that a nitric oxide-donating drug, glyceryl trinitrate, can induce mutations in Mutatect tumors and that both spontaneous and drug-induced mutations can be inhibited by dietary vitamin E⁵⁶ (Sandhu JK, Haqqani AS, and Birnboim HC, submitted for publication). Taken together, these data provide strong evidence that RNS/ROS are generated *in situ* in the MN-11 tumor microenvironment, where they are likely to be mutagenic. Because of the complexity of interactions between these reactive species,³¹ the pathways responsible for mutations cannot be identified with certainty.

Neutrophils are commonly observed in human malignancies, but are not thought to be a source of RNS.⁵⁷ In our limited study, we noted a correlation between the number of neutrophils and necrosis in adenocarcinoma of the lung. When these same specimens were stained for IL-8, most showed reactivity in tumor cells but there was no obvious correlation of IL-8 with neutrophil content. In bronchioloalveolar carcinoma,³⁵ IL-8 was found in most specimens but again its presence was not correlated with neutrophil content. In an study of human glioblastoma, Desbaillets et al have observed that IL-8 ex-

Figure 5. Model of the possible relationship between hypoxia, necrosis, and recruitment of neutrophils, which may be responsible for mutations and contribute to tumor progression.

pression was found in hypoxic tumor cells surrounding areas of necrosis.³³ In gastric carcinomas, the presence of IL-8 was correlated with microvessel count.³⁴ A model to depict some of the events that may be occurring in the complex tumor microenvironment is presented in Figure 5. Because tumors typically have very poorly developed blood vessels and have regions of hypoxia,²⁴ small areas of necrosis may arise and produce chemotactic factors for neutrophils, which can in turn generate IL-8.³² Our IL-8 experiments using MN-11 tumors suggest that an influx of neutrophils is associated with necrosis. Thus, both events can occur: necrosis may recruit neutrophils and neutrophils may produce additional chemoattractants. We propose that the poor microvasculature of rapidly growing tumors fosters hypoxia, necrosis, and microthrombi. These can be a source of chemotactic factors, promoting the infiltration of neutrophils and other inflammatory cells. Neutrophil products may in turn cause damage to the tumor microvasculature and promote this cycle of events. Because neutrophils are the only known source of ROS and RNS, they appear to be responsible for the observed high frequency of mutations seen in Mutatect MN-11 tumors.

In summary, our studies have helped to focus on the possible role of tumor-infiltrating neutrophils in tumor progression by contributing to the burden of genetic abnormalities commonly found in tumor cells. The presence of similar cells at sites of chronic inflammation may also help

to explain the association of chronic inflammatory diseases and cancer.

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