Quantitative Immunohistochemical Estimates of $O^6$-alkylguanine-DNA Alkyltransferase Expression in Normal and Malignant Human Colon

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ABSTRACT

A major mechanism of resistance to nitrosoureas is $O^6$-alkylguanine-DNA-alkyltransferase. The alkyltransferase biochemical assay measures mean tissue activity but requires availability of fresh tissue and cannot assess tumor heterogeneity, an important component of tumor resistance to alkylating agents. We assessed the levels of alkyltransferase in human colon carcinoma and normal colon by biochemical assay, Western blot, conventional immunohistochemistry, and quantitative immunohistochemistry (using 5H7 and mT3.1 monoclonal IgGs) to correlate whole tissue levels with cell-specific expression. Alkyltransferase activity was 18.0 ± 4.6 fmol/µg DNA in normal colon and 15.0 ± 6.5 fmol/µg DNA in tumors. By Western blot estimates, alkyltransferase in normal colon was 14.8 ± 4.2 fmol/µg DNA and in tumors was 16.2 ± 7.8 fmol/µg DNA. Alkyltransferase estimates by biochemical and Western blots were correlated strongly ($P < 0.0001$). Conventional immunohistochemistry demonstrated that alkyltransferase was predominantly nuclear and in normal colon was concentrated in glandular epithelial mucosal cells close to the lumen, whereas in tumors, expression was heterogenous but localized to malignant epithelial cells. Two parameters of quantitative immunohistochemistry, integrated gray and mean gray, were correlated strongly with each other ($P = 0.0002$) and with biochemical and Western blot estimates ($P = 0.004–0.04$). Thus, quantitative immunohistochemical estimates of alkyltransferase in fixed tissues are a reasonable alternative to biochemical analysis and have an added advantage of identifying heterogeneity of alkyltransferase expression in tumors.

INTRODUCTION

The DNA repair protein $O^6$-alkylguanine-DNA-alkyltransferase plays an important role in protection against mutagenesis and carcinogenesis by alkylating agents (1–3). This protein is also a major mechanism of drug resistance to commonly used alkylating chemotherapeutic agents, such as BCNU; dacarbazine, triazene, and triazines, which form $O^6$-alkylguanine DNA adducts (4). The alkyltransferase repairs these adducts, but the reaction is stoichiometric, and the protein is not regenerated. Tumors having higher levels of alkyltransferase are thus more resistant to the chemotherapeutic effects of these agents (1, 4).

For the past few years, many laboratories have developed methods to inhibit alkyltransferase activity in human tumors and to increase the therapeutic efficacy of alkylating agents. $O^6$-benzylguanine is one such agent; following depletion of alkyltransferase, the therapeutic effects of alkylating agents such as BCNU are increased (4–6). Phase I clinical trials using $O^6$-benzylguanine to inhibit alkyltransferase and to increase the therapeutic effects of BCNU started at our institution and the University of Chicago Medical Center recently (4).

Clinical estimates of alkyltransferase in tumor samples are often needed without the ability to obtain fresh biopsies. For this reason, we are assessing various methods for quantification of this protein in human tumors. Such information may help assess tumor responses, because it will be important to know which tumors express high levels of alkyltransferase and the degree of heterogeneity that exists. Traditionally, alkyltransferase activity has been measured by a biochemical assay of homogenized tissue. This gives no indication of heterogeneity within tumors at the cellular level and requires fresh or frozen tissue.

We and other groups have shown, by immunohistochemical and in situ hybridization, that the distribution of alkyltransferase is heterogenous (7–10). In the human kidney, alkyltransferase is expressed mainly in the distal tubules and glomerular epithelial cells, whereas the cells of the bowen capsule, collecting ducts, and proximal tubules are deficient (8). Similarly in the human liver, alkyltransferase is located mainly in the hepatocytes and bile duct cells, whereas the portal vein is deficient (9). In the human breast, alkyltransferase is expressed dominantly in the connective tissue and myoepithelial cells, whereas the ductal epithelial cells are relatively deficient (11). There is also heterogenous expression of alkyltransferase among various cells of human tumors such as melanoma, Hodgkin’s disease, and ovarian and colon cancer (7, 9, 12).

Conventional immunohistochemical scoring to quantify an antigen is subjective; the results from different laboratories are difficult to compare; and the data obtained are not quantitative. In many settings, the conventional histochemical assessments are being replaced by computerized quantitative immunohisto-

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3 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MeG; methylguanine; SDPA, SD for percent area; percent area.

4 E. Dolan, personal communication.
chemistry using digital image analyzers. This powerful technique has proved useful in other settings, such as analysis for estrogen and progesterone receptors in human breast and other tumors (13–15). In the present study, we have used similar techniques for alkyltransferase estimation in normal and malignant human colon tissues and show a significant relationship with biochemical and Western methods of estimation.

MATERIALS AND METHODS

Materials. Colon tissue samples were obtained from untreated patients undergoing surgery for colon cancer and were flash frozen in liquid nitrogen. Each sample was collected locally by the Cooperative Human Tissue Network, Western division (Case Western Reserve University). Adjacent pieces of tissue were dissected and used for the analyses outlined below.

Mouse antihuman alkyltransferase monoclonal antibodies 5H7 and mT3.1 have been described earlier (16, 17) and were provided kindly by Dr. B. Li (National University of Singapore, Singapore) and Drs. T. Brent (St. Judes Children Hospital, Memphis, TN) and D. Bigner (Duke University, Durham, NC), respectively. Peroxidase-labeled, goat antimouse IgG (for immunohistochemistry) and normal goat serum were obtained from Dakopatts (Carpinteria, CA). Horseradish peroxidase-labeled, sheep antimouse IgG (for Western analysis) and enhanced chemiluminescence mixture were from Amersham (Arlington, Heights, IL). A polyvinylidene difluoride membrane was obtained from Millipore (Bedford, MA). All other chemicals were used from Sigma Chemical Co. (St. Louis, MO).

Alkyltransferase Assay. Tissue alkyltransferase was measured as described previously (18). Briefly, enzyme activity was measured in tissue extracts by the amount of [3H]O6-MeG present in calf thymus DNA alkylated with [3H]methylnitrosourea (specific activity, 0.039 fmol [3H]O6-MeG/μg DNA). The alkylated [3H]O6-MeG and N7-alkylated with [3H]methylnitrosourea (specific activity, 0.039 fmol [3H]O6-MeG/μg DNA). The alkylated [3H]O6-MeG and N7-MeG bases were separated by HPLC and quantified by liquid scintillation. N7-MeG was used as the internal standard. Alkyltransferase activity was expressed as fmol O6-MeG removed/μg DNA or mg protein. All assays were repeated two to five times/sample.

Western Blot Analysis. Cell extracts were resolved by SDS-PAGE gels (10% polyacrylamide) using a Bio-Rad ( Hercules, CA) minigel apparatus at 130 V for 1 h. Proteins were transferred onto polyvinylidene difluoride membranes, using a Bio-Rad mini-Trans-Blot cell for 1 h at 100 V. The blotted membranes were blocked with 5% dry milk in Tris-buffered saline [10 mm Tris, 150 mm NaCl, and 0.1% Tween-20 (pH 7.5)] and then incubated for 2 h with a mouse antihuman alkyltransferase antibody (mT3.1). After three 5-min washes with Tris-buffered saline plus 0.05% Tween 20, the blots were incubated with a secondary antibody, sheep antimouse horseradish peroxidase IgG for, 1 h. Antibody binding was visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham). Standard curves of samples with known biochemical activity were included in each blot. Fractionated cells extracts were subjected to SDS-PAGE gel electrophoresis and immunoassay as the standard in quantitative Western blotting. Densitometric analysis of film negatives was performed by a SciScan 5000 scanner using BioAnalysis software (US Biologics, Cleveland, OH). Regression analysis indicated that the antibody-antigen curve yielded a linear plot with a correlation coefficient of 0.995, when band intensity was plotted versus fmol protein loaded.

Alkyltransferase Immunohistochemistry. This was done as described previously (19). Briefly, tissues were fixed in Carnoy (60% ethanol, 30% chloroform, and 10% acetone) for 90 min and then transferred to 70% ethanol. Three-μm, paraaffin-embedded sections were brought to water, and endogenous peroxidase activity was blocked by 0.3% H2O2 in methanol. Sections were treated with 0.1% Triton X-100 in PBS for 10 min before overnight incubation with the mouse monoclonal antihuman O6-MeG-DNA-methyltransferase antibody (5H7; 40 μg/ml PBS) at 4°C. Following PBS washes, sections were incubated with the peroxidase-labeled, goat antimouse antibodies for 1 h. The peroxidase reaction was developed with diaminobenzidine for 10 min exactly. Both primary and secondary antibodies were used at the highest possible dilution to give the best estimate of the antigen concentration, as suggested by Rahier et al. (20). These stained slides were used for both conventional and quantitative immunohistochemistry. As a control, isotyping to the primary antibody and omission of the primary antibody were done, and both gave no staining. In addition, with staining of human colon xenografts known to be negative for alkyltransferase activity, there was no signal for either conventional or quantitative immunohistochemistry, whereas xenografts containing various proportions of alkyltransferase positive cells had a linearly intense signal with strong correlations with alkyltransferase activity (r = >0.74; P = < 0.0001). For the conventional immunohistochemistry, a semiquantitative scoring system was used, in which −, +, ++, ++++, and +++++ indicate <5%, 5–30%, 30–50%, 50–80%, and >80% of the cells, respectively, expressing the alkyltransferase.

Quantitative Immunohistochemistry (Image Analysis). Sections were analyzed on a Leica BH-2 microscope linked through a Sony CDD monochrome XC-75 camera to a Leica Quantimax 500MC image analysis program (Leica, Cambridge, England). The video monitor was resolved to 512 x 512 pixels. All measurements were done with a stabilized light source, using a high-resolution, 20X Olympus SPlan-Apo objective, automatic gain control switched off, with the same condenser and threshold settings and by the same operator. For accurate and consistent results before reading each slide, the intensity of light was checked by capturing a blank field, and shade correction was done by computer. This was important, because the intensity of light varied (by 20–40%) with time when the microscope was switched on. The light intensity stabilized in 2 h, and analysis of a same field varied by 10–15% over the next 8 h. Thus, all samples were studied 2 h after switching on the microscope.

Five randomly selected fields (865 μm2 each) were assessed for the density of the immunostain for each sample. The intensity of the labeling was determined by the computer and gave a gray value from 0 (black) to 255 (white). After capturing the image on the computer screen, stained areas were identified

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RESULTS

Alkyltransferase activity was detected in all of the colon samples studied. The mean biochemical alkyltransferase activity in normal colon (n = 9) was 18.0 ± 4.6 (range, 13.2-25.5) fmol/μg DNA. The alkyltransferase activity in colon tumors (n = 18) was 15.0 ± 6.5 (range, 4.1-28.1) fmol/μg DNA (see Table 1).

Western analysis also demonstrated the presence of alkyltransferase in all of the samples studied (see Fig. 1). By this method, alkyltransferase-immunoreactive protein was 14.8 ± 4.2 and 16.2 ± 7.7 fmol/μg DNA in normal and malignant colon, respectively (see Table 1). Alkyltransferase estimation by biochemical assays was correlated highly with the protein determination by Western blot both in the tumors and normal colon tissue (P = 0.0001; see Fig. 2).

Conventional Immunohistochemistry. Alkyltransferase was detected in all of the tissues studied. In both tumor and normal colon, alkyltransferase was detected dominantly in the nuclei; however, in most tissues, cytoplasmic expression was seen as well (see Figs. 3 and 4). In normal colon, alkyltransferase was detected predominantly in the mucosal epithelial cells; there was no detectable alkyltransferase in submucosal tissues except for the muscularis mucosae and submucosal

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**Table 1** Alkyltransferase activity in human colon as determined by biochemical assays, Western analysis, and conventional and quantitative immunohistochemistry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Colonic tissue type</th>
<th>Age/sex</th>
<th>fmol/μg DNA</th>
<th>fmol/mg protein</th>
<th>Conventional immunohistochemistry</th>
<th>Western (fmol/μg DNA)</th>
<th>Integrated gray (×10⁻⁶)</th>
<th>Mean gray (×10⁻⁶)</th>
<th>SDPA ratio</th>
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<td>35.4 ± 0.3</td>
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<td>21.9</td>
<td>979</td>
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<td>24.9</td>
<td>7.3</td>
<td>0.24</td>
<td>29.7 ± 0.6</td>
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<td>Carcinoma</td>
<td>79/F</td>
<td>20.7</td>
<td>956</td>
<td>+++</td>
<td>23.0</td>
<td>10.8</td>
<td>0.4</td>
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<td>744</td>
<td>+++</td>
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<td>5.2</td>
<td>0.10</td>
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<td>805</td>
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<tr>
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<td>16.8</td>
<td>710</td>
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<td>19.5</td>
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<td>14.9</td>
<td>401</td>
<td>+±</td>
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<td>359</td>
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<td>17.7</td>
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<td>524</td>
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<td>5.8</td>
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<td>20.1 ± 7.6</td>
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<td>74/M</td>
<td>10.5</td>
<td>433</td>
<td>+++±</td>
<td>10.7</td>
<td>12.0</td>
<td>0.14</td>
<td>34.0 ± 6.9</td>
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<td>W110</td>
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<td>10.2</td>
<td>501</td>
<td>±</td>
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<td>1.3</td>
<td>0.09</td>
<td>4.0 ± 4.7</td>
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<td>552F</td>
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<td>64/F</td>
<td>9.0</td>
<td>267</td>
<td>+</td>
<td>6.7</td>
<td>7.3</td>
<td>0.12</td>
<td>20.1 ± 2.6</td>
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<tr>
<td>452E</td>
<td>Carcinoma</td>
<td>60/M</td>
<td>7.1</td>
<td>116</td>
<td>+</td>
<td>5.4</td>
<td>1.5</td>
<td>0.07</td>
<td>4.2 ± 6.6</td>
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<td>Carcinoma</td>
<td>68/F</td>
<td>4.3</td>
<td>179</td>
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<td>3.0</td>
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<td>0.25</td>
<td>20.4 ± 1.0</td>
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<td>85</td>
<td>±</td>
<td>6.7</td>
<td>0.2</td>
<td>0.02</td>
<td>0.6 ± 0.3</td>
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Mean ± SD

<table>
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<tr>
<th>Sample</th>
<th>Colonic tissue type</th>
<th>Age/sex</th>
<th>fmol/μg DNA</th>
<th>fmol/mg protein</th>
<th>Conventional immunohistochemistry</th>
<th>Western (fmol/μg DNA)</th>
<th>Integrated gray (×10⁻⁶)</th>
<th>Mean gray (×10⁻⁶)</th>
<th>SDPA ratio</th>
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<tbody>
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<td>1003</td>
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<td>13.2 ± 2.2</td>
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<td>0.10</td>
<td>8.1 ± 4.1</td>
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</table>

Mean ± SD

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α versus β, 0.00001; α versus γ, 0.0001; α versus δ, 0.003; α versus ε, 0.012; δ versus γ, 0.0004; δ versus η, 0.05; δ versus η, 0.0001; δ versus ζ, 0.004; δ versus ζ, 0.006; δ versus η, 0.0001; δ versus ζ, 0.0001; δ versus ζ, 0.002.
**Quantitative Immunohistochemistry for Human Alkyltransferase**

*Fig. 1* Western blot for human alkyltransferase protein in colon adenocarcinoma. A single band of Mr 23,000 protein was detected using mT3.1 monoclonal antibody. The density of the bands was correlated strongly with the amount of alkyltransferase protein. Sample numbers are the same as in Table 1.

*Fig. 2* Correlation between alkyltransferase estimation by biochemical assays and Western blot analysis. A very strong association was found between biochemical alkyltransferase activity and protein determination by Western blots. ■, normal tissue; □, adenocarcinoma.

*Fig. 3* Photomicrographs showing immunohistochemical (peroxidase-dimethylaminoazobenzene) staining for alkyltransferase in normal human colon. *a,* isotype control (× 83). *b,* low-power view showing dominant mucosal staining, with cells at the top of crypts more intensely positive; submucosal tissue is negative except for muscularis mucosae and blood vessels (× 83). *c,* high-power view of *b* showing both nuclear and cytoplasmic staining for cells close to lumen (× 330).

Blood vessels (see *Fig. 3b*). Mucosal epithelial cells toward the base of colonic crypts were relatively deficient in alkyltransferase expression with only nuclear staining. As cells migrate up the crypts, expression of alkyltransferase increased gradually, mostly in the cytoplasm, and cells near the lumen had uniformly strong nuclear and cytoplasmic staining (see *Fig. 3c*).

In colon tumors, alkyltransferase was also detected predominantly in malignant epithelial cells, whereas the stromal cells were relatively deficient (see *Fig. 4*). Alkyltransferase-immunoreactive protein in tumors varied markedly from one patient to another; in some tumors, most of the cells expressed the protein in a relatively homogenous pattern, whereas in others, only a few of the cells were stained either as isolated regions within the tumors or as isolated cells (see *Fig. 4 and*...
Photomicrographs showing immunohistochemical (peroxidase-dimethylaminobenzine) staining for alkyltransferase in human colon adenocarcinoma.

a. nearly all of the malignant epithelial cells are positive for alkyltransferase; the stroma also has moderate staining (X 83).
b. high-power view of the tumor showing the presence of alkyltransferase protein in both the nuclei and cytoplasm (X 330).
c. staining dominantly in parenchyma, with only a few stained stromal cells (X 83).
d. heterogenous expression of alkyltransferase in various regions of the parenchyma; lower right region has no positive staining (X 33).

Table 1. There was also heterogeneity within tumors; some tumors had areas of strong staining adjacent to unstained regions (see Fig. 4d). Conventional immunohistochemistry indicated only a moderate association between biochemical alkyltransferase activity and the immunoreactive protein determination by Western blot (P = 0.1 and 0.09, respectively).

Quantitative Immunohistochemistry. Three different parameters of quantitative immunohistochemistry: (a) integrated gray, (b) mean gray, and (c) percent area, were correlated strongly with each other pairwise (r = 0.57–0.89; P = < 0.002) and can be used, therefore, for quantifying immunostaining (see Table 1 and Fig. 5). Biochemical alkyltransferase activity, measured per µg tissue DNA, was correlated significantly with the mean gray (P = 0.01) and integrated gray (P = 0.03) and to a lesser extent with the percent area (P = 0.09; see Fig. 6). However, alkyltransferase activity as measured per mg protein was not correlated significantly with any of these parameters. Semi-quantitative alkyltransferase estimates by conventional immunohistochemistry (1+ to 4+) was associated significantly with the integrated gray (P = 0.0004), mean gray (P = 0.05), and percentage area (P = 0.0001; see Table 1). The three quantitative immunohistochemistry parameters were also correlated significantly with the amount of alkyltransferase protein per µg tissue DNA determined by Western blot (P = < 0.006; see Fig. 7). These results indicate that the antibody-mediated detection methods are internally consistent.

As indicated previously, alkyltransferase expression in tumors was heterogenous. To quantify this heterogeneity, we used the SDPA ratio, measured in five different fields. In the present study, samples with SDPA ratios of >0.4 were considered very heterogeneous for antigen expression. Using this criteria, the mean ± SD SDPA ratios for tumors was 0.44 ± 0.4 (range, 0.05–1.6). Of these, 7 of 18 tumors samples were very heterogenous, with SDPA ratios >0.4 (tumors 607F, 206A, 179H, W110, 452E, 468E, and W038); this was confirmed by visual inspection as noted above. In contrast, tumors with little heterogeneity (SDPA ratio <0.2; 7 of 18) included tumors 560F, 476N, 552F, 433B, 483H, 457J, and W203. In these samples, the distribution of alkyltransferase-positive cells was quite uniform throughout the tumors. In general, less heterogeneity was observed in normal colon samples (mean ± SD, 0.27 ± 0.1; range, 0.12–0.5), and only 1 of 9 samples had SDPA ratios >0.4. However, the heterogeneity seen in the normal colon was due to the well-ordered pattern of expression, as noted in Fig. 3, compared with the disordered heterogeneity seen in many tumor samples (see Fig. 4).
DISCUSSION

Given the important role of alkyltransferase in human tumor resistance to nitrosoureas and related compounds and the emerging interest in modulating its activity to increase the therapeutic efficacy of alkylating chemotherapeutic agents, it is critical to have better methods to detect heterogenous tissue expression of this protein. Although the biochemical methods of alkyltransferase correlate well with the tumor response to alkylating agents in xenograft models (4, 6), immunohistochemical studies have suggested that there is marked heterogeneity in the level of alkyltransferase among cells within human tumors (7, 9, 10, 12). However, there is no prior data correlating biochemical alkyltransferase activity with histological estimates of its expression in human tumor biopsy samples.

All of the normal and malignant colon samples analyzed had detectable levels of alkyltransferase by biochemical assay and Western analysis, both of which were correlated highly \((r = 0.83; P = 0.0001; \text{see Fig. 2})\). Thus, an immunodetection technique seems to measure mean alkyltransferase activity very accurately. It remains of interest that alkyltransferase is present in all human colon tumors (21) in contrast to other tumors, including melanoma and brain tumors (9, 22), in which a significant proportion of tumors seem to lack alkyltransferase. The mean alkyltransferase activity in normal \emph{versus} malignant tissues was similar, although interindividual activity varied more than 10-fold between samples. Both findings are consistent with earlier studies (1, 21).

We found three parameters of quantitative immunohistochemistry, integrated gray, mean gray, and percentage area, to be correlated strongly with each other \((P < 0.002)\) and with the alkyltransferase estimation by Western blots \((P < 0.004)\) and conventional immunohistochemistry \((P < 0.05)\). Integrated gray and mean gray values were correlated significantly with the biochemical alkyltransferase activity/µg DNA \((P < 0.03)\). We also have used this technique in paraformaldehyde-fixed human tumor xenografts and found a strong correlation between quantitative immunohistochemistry and biochemical alkyltransferase activity \((P < 0.0001)\),\(^5\) suggesting the feasibility of this application to routine pathology slides.

The present study suggests the potential utility of this approach for evaluating clinical samples, particularly when fresh tumor tissue is not available. Furthermore, the histological estimate indicates marked heterogeneity in alkyltransferase expression among different regions of the same tumor. The pres-

mic proteins are measured. Because there is no evidence to
our method correlates well with biochemical alkyltransferase
based only on the nuclear protein. Not surprisingly,
et al. of alkyltransferase. An earlier study by Belanich
analysis (24). This is an important issue in histological estimates
plasm by immunohistochemistry (7, 9, 10) and by biochemical
of presence of alkyltransferase in both the nucleus and cyto-
(see Figs. 3c and 4b). This increase in alkyltransferase seems to be mainly
cytoplasmic. The reason for this variable expression is yet to
determined. It may be related to cellular differentiation of lu-
menal cells, which require higher alkyltransferase expression,
because they are likely to be the first targets for ingested or
endogenous gut lumen-produced nitrosamines (26, 27). High
alkyltransferase may protect the integrity of the mucosa. On the
other hand, the target for gut carcinogenesis seems to be the
proliferating cell located in the lower half of the colonic crypts
(28), as is the case for the glandular stomach (29, 30), and these
express somewhat lower alkyltransferase.

In colonic tumors, alkyltransferase was predominantly in
the malignant epithelial cells, whereas the stroma had lesser
staining (see Fig. 4). The proportion of alkyltransferase-positive
cells and intensity of staining varied strikingly between tumor
samples, as has been observed for melanoma and ovarian tumors
(9, 10, 12). In a number of tumors with lower levels of alkyl-
transferase protein, its expression was quite heterogenous (see
Fig. 4d), with strongly stained cells adjacent to unstained ones.
Because these tumors had low alkyltransferase activity by bio-
chemical assay, the tumor could be classified as sensitive to
nitrosoureas, overlooking small regions of tumor cells expressing
high alkyltransferase that could become the source of resis-
tant tumor cells after nitrosourea exposure (23). Because the
orderly distribution of alkyltransferase seen in normal colon
tissue is lost in tumor samples, it suggests that there is disregula-
tion of gene expression in malignant cells, perhaps due to the
methylation status of the gene (31).

Quantitative, immunohistochemical assessment of alkyl-
transferase in fine-needle aspiration, needle biopsies, and archi-
val histological sections will expand the ability to correlate
tumor response with drug resistance. Its combination with a
biochemical assay and/or Western blot will increase the predic-
tive value of alkyltransferase determination substantially in hu-
man tumors targeted for treatment with alkylating agents.

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Quantitative immunohistochemical estimates of O6-alkylguanine-DNA alkyltransferase expression in normal and malignant human colon.

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