A Comparison of Acridine Orange and Feulgen Cytochemistry of Human Tumor Cell Nuclei

James E. Gill, Leon L. Wheeless, Jr., Carol Hanna-Madden, Richard J. Marisa, and Paul K. Horan

Analytical Cytology Division, Department of Pathology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

ABSTRACT

Specimens of cells derived from tumors of the human female genital tract plus normal cells as standards have been divided into aliquots and stained according to acridine orange or pararosanilin:Feulgen procedures. Acridine orange-stained cells were slit-scanned for 535 nm nuclear fluorescence; Feulgen-stained cells were com-scanned for 580 nm nuclear absorbance. For each specimen examined, the tumor cell:normal cell ratio of mean nuclear fluorescence following acridine orange staining was greater than the tumor cell:normal cell ratio of mean nuclear absorbance following Feulgen staining. The tumor cell:normal cell ratio of mean nuclear fluorescence ranged from 2.3 for a nonkeratinizing squamous cell carcinoma to 3.9 for a keratinizing squamous cell carcinoma. The tumor cell:normal cell ratio of mean nuclear absorbance ranged from 1.4 for a mixed mesodermal sarcoma to 2.3 for a small cell squamous cell carcinoma.

These results indicate that the elevated nuclear fluorescence intensity from acridine orange-stained tumor cells cannot be explained solely on the basis of elevated Feulgen:DNA content. An alternative hypothesis, consistent with these results, is that DNA is the principal binding substrate for intranuclear acridine orange and that the DNA of certain tumor cells is more accessible to acridine orange than is the DNA of normal cells.

INTRODUCTION

Cells derived from many human malignant tissues have altered chromatin content and structure. The cellular Feulgen:DNA content of malignant tissues is generally elevated (1), and the chromatin patterns as seen under the light microscope are different from those associated with normal cells (1), and the chromatin patterns as seen under the light microscope are different from those associated with normal cells (9). Both of these generalizations hold true for cancers of the uterine cervix in particular (13).

Research efforts directed toward cytology automation have attempted to use elevated DNA content (14) and altered chromatin patterns (12) as markers for abnormal cells. Fluorescent dyes believed to bind to DNA within cells have been tested for their ability to discriminate between normal and abnormal cells. In particular, AO3 has been shown to provide effective discrimination between normal and abnormal cells when 535 nm nuclear fluorescence of AO-stained cells has been measured by a slit-scan technique (19).

The purpose of the research was to determine the cytochemical basis for this discrimination. Although it has been reported that intracellular AO:DNA complexes fluoresce green, while AO:RNA complexes fluoresce red, the actual cytochemical behavior of AO is complex (11, 21). The staining protocol used for the experiments reported here was derived (20) from the protocol of Von Bertalanffy (17). It differs significantly from those (15) for which DNA and RNA specificity has been claimed. However, since AO binds to DNA and since the DNA content of abnormal cells is elevated, it appeared plausible that the elevated nuclear fluorescence from AO-stained abnormal cells might reflect elevated DNA content (6). To test this hypothesis, we have examined the AO staining of DNase I- and RNase-treated cells and have compared Feulgen:DNA values and AO nuclear fluorescence values of aliquots of the same clinical specimens. The results of these experiments indicate that DNA is the primary substrate for AO binding within cell nuclei but contradict the hypothesis that the integrated 535 nm (green) fluorescence of AO-stained cells provides a measure of their relative Feulgen:DNA content.

MATERIALS AND METHODS

Collection and Preparation of Specimens. Tumor specimens were collected from surgery, surgical pathology, and autopsy services. Specimens and their sources are summarized in Table 1. Cells derived from solid tumors were rinsed from these tissues into clear Mucosol (Lerner Laboratories, Stamford, Conn.), syringed to break up clumps of cells, and mixed with normal material if none was present in the specimen. Cells taken by cervicovaginal scrape were suspended immediately in clear Mucosol and combined with a suspension of normal cervical material. Normal specimens were collected from an outpatient clinic.

Nuclease Treatment. Cells suspended in Mucosol were centrifuged and resuspended in 20 mM citrate:phosphate, pH 3.0, with 0.1% Triton X-100 for 1 min to facilitate nuclease penetration (5). Cells were then rinsed in pH 7.5 buffer, resuspended in buffer with appropriate salts, and brought to 4 x 10^3 units/ml with DNase I (Sigma Chemical Co., St. Louis, Mo.) or 10^2 units/ml with RNase (Sigma). Cell suspensions were held at 37°C for 30 min. The cells were then centrifuged and stained.

AO Staining. An aliquot of the cell suspension was centrifuged, and the pellet was resuspended in 2.5 x 10^{-5} M AO:0.2 M phosphate buffer, pH 7.0. After 10 min, the cells were centrifuged, resuspended in 3.8% glutaraldehyde:0.14 M phosphate buffer at pH 7, and stored in this suspension until needed (3).

Pararosanilin:Feulgen Staining. A second aliquot of the
Table 1

Description of specimens analyzed

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Tumor material</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed mesodermal sarcoma</td>
<td>Solid</td>
<td>Surgical pathology</td>
</tr>
<tr>
<td>Ovarian Adenocarcinoma</td>
<td>Fluid</td>
<td>Autopsy</td>
</tr>
<tr>
<td>Small cell squamous cell carcinoma</td>
<td>Cervical scrape</td>
<td>Operating room</td>
</tr>
<tr>
<td>Keratinizing squamous cell carcinoma</td>
<td>Solid</td>
<td>Autopsy</td>
</tr>
<tr>
<td>Nonkeratinizing squamous cell carcinoma</td>
<td>Solid</td>
<td>Surgical pathology</td>
</tr>
<tr>
<td>Sarcoma; metastatic from a mixed mesodermal sarcoma</td>
<td>Vaginal scrape</td>
<td>Operating room</td>
</tr>
</tbody>
</table>

a For diagnostic, see Ref. 13.
b No additional normal cells required.

cell suspension was centrifuged, 1 drop of the concentrate was placed on 1 slide, and “pull-apart” smears were generated with a second slide. These slides were immersed in methanol:formalin:glacial acetic acid (85:10:5, v/v) for at least 1 hr and then taken through a Feulgen staining protocol (2).

Schiff’s reagent was prepared according to the method of Graumann (10) with the following modifications. Basic fuchsin (C.I. 42,500), 0.1 g, was dissolved in 15 ml of 1 N HCl and then combined with 85 ml of 2.6 × 10^{-3} M potassium metabisulfite in distilled water and stored overnight in the dark in a capped Teflon bottle. The solution was shaken with activated charcoal (Darco G-60) for 2 min and then filtered through a Millipore prefilter. The resulting staining solution was clear and nonfluorescent.

**Analyses of Stains.** Absorption spectra of stains were obtained with a Beckman Acta V spectrophotometer and compared with published spectra (22) to verify identity and purity. Fluorescence analysis of stains were made with an Amino-Bowman spectrophotofluorometer modified for photon counting (8). Thin-layer chromatography of “purified” AO (Polyscience, Inc., Warrington, Pa.) on silica or on cellulose with a solvent mixture of butanol:ethanol:H_{2}O:NH_{4}OH (32:10:10:0.3, v/v) revealed 1 minor impurity. Other commercial samples of AO had substantial contamination. The “purified” AO was used without further purification.

**Slit-Scan Measurements of Integrated Nuclear Fluorescence.** Slit-scans of individual AO-stained cells were recorded with a Zeiss Axiomat microscope-photometer system, equipped for both epi- and transmitted illumination. For epi-illumination, the light source was an Osram XBO-150 xenon arc lamp. Exciter filters were KP 490 and KP 500 in series; the reflector transition wavelength was 510 nm, and the barrier filters were an LP 528 and a 525 to 566 nm band pass (full width at half-maximum); green fluorescence was selected for measurement. The objective was ×50 with a numerical aperture of 0.95; effective slit width was 5 μm. Epi-illumination intensity was reduced to approximately 5% for location and positioning of cells (to avoid bleaching the specimen) and then increased to the maximum for slit scanning. Scanning of the stage and collection of data were controlled by a PDP 11/40 computer (Digital Equipment Corp., Maynard, Mass.). The fluorescence intensity profile was collected by the computer and analyzed for cell diameter, nucleus diameter, ratio of nucleus diameter to cell diameter, integrated nuclear fluorescence, and integrated cell fluorescence (18).

**Comb-Scan Measurements of Integrated Nuclear Absorbance.** Absorbance measurements of Feulgen-stained nuclei were obtained with the Axiomat system. For transmitted illumination, the light source was a tungsten:halogen lamp operated at 12 V d.c. A linear wedge interference filter was used to select a band of light centered at 580 nm. The field stop diameter was 0.16 mm, giving a 34-μm-diameter circle of illumination in the specimen plane. The objective was ×50 with a numerical aperture of 0.95 to provide an effective photometer spot aperture with a diameter of 0.5 μm in the specimen plane. The image was comb-scanned across this spot aperture by stepping the scanning stage in 0.5-μm increments at approximately 150 Hz under control of the computer. Light passing through the aperture was detected by a photomultiplier tube, and the photomultiplier output was converted to a voltage. This voltage was recorded by the computer after each increment of the stage. The average value from the first line of the comb scan was calculated and considered as background intensity, I_{b}. For each succeeding increment, the computer recorded the measured intensity, I'_{b}, and calculated the specimen absorbance of that position as A' = log (I_{b}/I'_{b}). The total absorbance of the scanned nucleus was calculated as the sum of all A'. Duplicate comb-scan measurements were made of specimen nuclear absorbance, averaged if the values agreed within 20%; otherwise, they were discarded, which was rare. Single slit-scan measurements were made of nuclear fluorescence, since each measurement caused partial fading of fluorescence.

Before specimens were measured for either nuclear absorbance or fluorescence, the absorbance of a marked “standard” Feulgen-stained nucleus was measured repeatedly, and the mean and standard deviation was checked against previous measurements of this nucleus to verify performance of the system. (Standard deviations were typically 5 to 6% of the mean.)

**Statistical Analysis and Protocol.** Analysis of the data was based on the following assumptions: (a) nuclear fluorescence from slit-scan measurements and nuclear absorbance from comb-scan measurements constitute random variables; (b) each of these random variables is associated with a probability density function such that mean and standard deviations exist; (c) if A is the mean of the integrated absorbance of abnormal cell nuclei and ȇ is the mean of the integrated absorbance of normal cell nuclei,
then the ratio of the means, \( \hat{R} = \hat{A}/\hat{B} \), is also a random variable; (d) repeated measurements of \( \hat{R} \) would be normally distributed; (e) if nuclear fluorescence from AO-stained cells represents Feulgen:DNA content, then the ratio of the means of abnormal and normal nuclear fluorescence will be the same, statistically, as the ratio of the means of abnormal and normal nuclear absorbance; (f) the appropriate test for significant difference in the ratios of the means is the t test (23).

The basic protocol for the experiments with human material is given in Chart 1.

**Isolation and Staining of Rat Liver Nuclei.** Isolation followed the procedure of Umana and Dounce (16) with the following modifications. Solutions of 0.44 M sucrose contained 1 mM MgCl₂; the centrifugation through the 2.2 M sucrose was carried out in a Beckman J21C centrifuge with an SW-13 rotor spun at 13,000 rpm for 1 hr. The final nuclear pellets were resuspended in 10 ml of 0.44 M sucrose, and 10 ml of 20% formalin in 0.44 M sucrose were added and mixed by vortexing. The nuclei were fixed in this medium overnight and then stained according to a pararosanilin:Feulgen procedure for cell suspensions (7).

**Flow Cytometry of Rat Liver Nuclei.** Suspensions of Feulgen-stained nuclei in distilled water were diluted with distilled water to a concentration of about 10⁶ nuclei/ml. The analysis and sorting were carried out with a Coulter Model TPS-1 sorter (Coulter Electronics, Hialeah, Fla.).
Histograms of frequency versus integrated fluorescence intensity were obtained (4). The light scatter channel was used to gate out fluorescence signals from small bits of fluorescent debris. Sorted nuclei were examined to verify that peaks in the histogram corresponded to fluorescence from intact single nuclei.

**Quantitative Microscopy of Rat Liver Nuclei.** For measurements of nuclear fluorescence or absorbance with the Axiomat photometer system, 1 drop of the stained rat liver nuclei suspension was smeared on a glass slide and allowed to air dry. The smear was covered with 1 drop of Cargille’s type A immersion oil and coverslipped. Individual nuclei were slit-scanned for integrated fluorescence or comb-scanned for integrated absorbance as described above. Statistical analysis of the ratio of tetraploid to diploid fluorescence or absorbance was as described above.

**RESULTS AND DISCUSSION**

Mean values for normal and tumor cell absorbance or fluorescence and the tumor cell: normal cell ratio of the means have been calculated for each of the 6 specimens tested. Nuclear fluorescence data are summarized in Table 2; nuclear absorbance data are summarized in Table 3. If nuclear fluorescence from AO-stained cells represented nuclear Feulgen:DNA content, then the ratio of the means of nuclear fluorescence would be indistinguishable from the ratio of the means of nuclear absorbance. Statistical analyses of the distributions, summarized in Table 4, indicate that in every case this “null” hypothesis, namely, that the ratios are statistically indistinguishable, can be rejected with a confidence level of at least 95%.

Histograms of data for 2 of the specimens tested are shown in Charts 2 and 3. In each chart A represents the nuclear fluorescence distributions of normal and abnormal cells, and B represents the Feulgen absorbance distributions. Chart 2 represents the mixed mesodermal sarcoma (Tables 1 to 4, Line 1) while Chart 3 represents the small cell squamous cell carcinoma (Tables 1 to 4, Line 3).

Nuclease treatments of normal clinical specimens were performed to test whether AO staining of nuclei was specific for DNA. DNase treatment of cells reduced the nuclear fluorescence below the level of detection by the slit-scan technique. From observation of slit-scan contours, residual nuclear fluorescence of the DNase treatment is estimated to be less than 10% of the value for untreated cells. Fluorescence microscopy of treated cells revealed a lack of nuclear chromatin-like structures. Any remaining nuclear fluorescence was associated with the nuclear membrane. RNase treatment of cells left the nuclear fluorescence statistically indistinguishable from that of untreated cells.

These data indicate that AO staining of normal cell nuclei is 90% or more specific for DNA and does not involve nuclear RNA. However, these data do not indicate whether the amount of nuclear fluorescence from AO-stained cells represents the amount of DNA present. The variability of nuclear fluorescence from normal AO-stained cells (19) indicates that factors other than DNA content must play a role. In short, specificity does not indicate stoichiometry. Experiments involving nuclease treatments of tumor cells are pending.

It was necessary to test the possibility that the Axiomat system was providing fluorescence or absorbance meas-
Confidence levels for rejection of hypothesis that ratios of means are indistinguishable

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Confidence level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed mesodermal sarcoma</td>
<td>6.46 0.9995</td>
</tr>
<tr>
<td>Ovarian adenocarcinoma</td>
<td>4.85 0.9995</td>
</tr>
<tr>
<td>Small cell squamous cell cancer</td>
<td>3.51 0.95</td>
</tr>
<tr>
<td>Keratinizing squamous cell cancer</td>
<td>1.97 0.95</td>
</tr>
<tr>
<td>Nonkeratinizing squamous cell cancer</td>
<td>3.51 0.995</td>
</tr>
<tr>
<td>Sarcoma, metastatic</td>
<td>1.98 0.995</td>
</tr>
</tbody>
</table>

Table 4

Confidence levels for rejection of hypothesis that ratios of means are indistinguishable

<table>
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</tr>
</tbody>
</table>

Table 5

Mean absorbance and fluorescence of Feulgen-stained rat liver nuclei

<table>
<thead>
<tr>
<th>Property measured</th>
<th>Diploid</th>
<th>Tetraploid</th>
<th>Ratio of means</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>100 ± 12.56 (9)</td>
<td>197 ± 19.3 (11)</td>
<td>1.97 ± 0.31</td>
<td>1.28</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>100 ± 18.0 (20)</td>
<td>214 ± 18.6 (26)</td>
<td>2.14 ± 0.43</td>
<td></td>
</tr>
</tbody>
</table>

Measurements that were not proportional to the fluorescence or absorbance of the stained nuclei. For example, the measurements might have indicated that 1 nucleus had 1.5 times the absorbance of a second nucleus when in fact the first nucleus had twice the absorbance of the second. To make such a test, we analyzed Feulgen-stained rat liver nuclei for both fluorescence and absorbance. Additionally, the stained rat liver nuclei were analyzed and sorted on the basis of fluorescence intensity with a flow cytometer.

Pararosanilin:Feulgen staining of nuclei or cells generates both fluorescence and absorbance with the integrated intensities proportional to nuclear DNA content (2, 7). Rat livers contain both diploid and tetraploid cells; hence, fluorescence or absorbance measurements of Feulgen-stained rat liver nuclei can be expected to generate bimodal distributions, with the mean of the high-intensity peak equal to twice the mean of the low-intensity peak. Flow cytometer measurements of fluorescence from the rat liver nuclei generated the histogram shown in Chart 4. The ratio of the means of the 2 peaks, high intensity:low intensity, is 2.06 ± 0.45 (S.D.).

Measurements of absorbance or fluorescence from another aliquot of these rat liver nuclei, made with the Axiomat system, are summarized in Table 5. The ratios of the means of absorbance and fluorescence data are not significantly different from 2.0. This result shows that the Axiomat system does provide measurements that are proportional to the fluorescence or absorbance of stained nuclei over the intensity ranges of interest. The differences between the fluorescence and absorbance ratios of means in Tables 2 and 3 are not due to measurement artifact.

Thus, for each clinical specimen tested, the data indicate that nuclear fluorescence from AO-stained cells is not proportional to Feulgen:DNA content but does provide a measure of some other nuclear property associated with abnormality. If DNA is assumed to be the substrate for AO binding within the tumor as well as normal cell nuclei, it appears that this other property may be increased DNA accessibility within the chromatin of tumor-derived cells.

In work to be published elsewhere, it is shown that propidium iodide as well as AO provides discrimination between normal and tumor cells, based on measurements of nuclear fluorescence. The data indicate that intercalative binding of these fluorochromes occurs in both normal and tumor cells, providing further evidence that DNA is the binding substrate for AO in normal and tumor cells.

An important practical result of these findings, relevant to cytofluorometry, is that integrated nuclear fluorescence at 535 nm from AO-stained tumor cells provides a better indication of abnormality than does Feulgen absorbance (or any other measure of Feulgen:DNA content).

In summary, the increased 535 nm nuclear fluorescence from AO-stained tumor cells is greater than the increase in 580 nm absorbance of Feulgen-stained tumor cells, relative to values for normal cells, with a confidence level of at least 95%. This result can be accounted for on the basis of increased accessibility of DNA to AO within the chromatin of tumor cells.


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REFERENCES

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