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FLOW CYTOMETRIC APPLICATIONS TO TUMOR BIOLOGY: PROSPECTS AND PITFALLS

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Summary.--A brief review of cytometry instrumentation and its potential applications in tumor biology is presented using our recent data. Age-distribution measurements of cells from spontaneous dog tumors and cultured cells after exposure to X-rays, alpha particles, or adriamycin are shown. The data show that DNA fluorescence measurements have application in the study of cell kinetics after either radiation or drug treatment. Extensive and careful experimentation is needed to utilize the sophisticated developments in flow cytometry instrumentation.

Developments in the analysis of cells by flow, commonly termed "flow cytometry" or "flow microfluorometry (FMF)," have provided a variety of instruments having applications in biology and medicine. Some flow-systems developments pertinent to radiobiology and oncology will be reviewed briefly in this paper. The applications and pitfalls are discussed in relation to our recent data.

In certain respects, flow cytometric instrumentation is an extension of the radiation detection and electronic signal processing technology developed to study atomic nuclei (i.e., gamma-ray spectroscopy). Briefly, electronic and optical signals from individual cells are obtained for various cellular parameters such as size, DNA, RNA, protein, or light scatter (Fig. 1). Innovations in cell staining techniques and in optical, electrical, and mechanical developments have provided the present state-of-the-art in cell analysis capabilities. The signals from several parameters of each cell can be processed either individually or in combination using pulse-height analyzers or computers. Those cells of interest can be sorted out for morphological, clonogenic, or other analyses using any one or a combination of these signals. The historical development of flow cytometry instruments has been reviewed by Mullaney et al. (1976), Arndt-Jovin and Jovin (1978), Melamed, Mullaney and Mendelsohn (1979), and Salzman (1979).

The most commonly measured parameter to date by flow cytometric techniques has been DNA content by measuring the fluorescence of cells stained with DNA-specific fluorochromes such as mithramycin (Crissman and Tobey, 1974), propidium iodide (Krishan, 1975), and combined ethidium bromide plus mithramycin (Barlogie et al., 1976). In many cases, this allows quantitation of cells in the G_o/G_1, S, and G_2/M stages of the cell cycle. Since the DNA content of quiescent G_o and cycling G_1 cells and the DNA content of G_2- and

M-phase cells are the same, currently it is difficult to resolve them from one another. However, at least three staining techniques have been developed that promise to improve the G_o/G_1 distinction, including (1) incorporation of bromodeoxyuridine (BUdR) into the cellular DNA of cycling cells to resolve G_o from G_1 cells (Swartzendruber, 1977); (2) improved discrimination between G_o and G_1 cells and G_2 from M cells utilizing acridine orange in which differences in DNA denaturation allow a distinction between them (Darzynkiewicz et al., 1977, 1979); and (3) fluorescent antibody detection of cycling cells that have incorporated BUdR compared to noncycling cells that do not take up this thymidine analog (Gratzner and Ingram, 1979). Although these techniques require some protocol modification for use with different cell systems, however, when properly used, the potential exists for improved discrimination of G_o from G_1 cells and G_2 from M cells.

Most staining techniques presently require fixation of the cells; hence, when using these methods, it is not possible to distinguish clonogenic from nonclonogenic cells. Arndt-Jovin and Jovin (1977) found that the bis-benzimidazole dye Hoechst 33342 could be used to obtain DNA distributions with a minimal decrease in viability or clonogenicity of the stained cells. Twentyman et al. (1979) and Pallavicini et al. (1979) used Hoechst 33342-stained and sorted tumor cells to study the clonogenicity of cells in different stages of the cell cycle. It is important to understand the biochemistry of cell staining, without which the cytometry data could be misleading.

A major limitation in interpreting flow DNA distributions is the heterogeneity of cells within tumors and the overlapping tumor cell and somatic cell DNA contents. Using multiparameter flow analyses with markers such as protein content, Coulter volume, or small-angle light-scatter cell-size measurements,

improved separation of tumor from normal cells can be achieved (Steinkamp, 1977). Tumor cells tend to have a greater protein mass for a given DNA content than normal cells. Therefore, simultaneous measurements of protein and DNA, in certain cases, can distinguish tumor cells from normal cells, even when the DNA contents are the same (Crissman et al., 1978).

RESULTS AND DISCUSSION

We are currently testing for a possible correlation between DNA histograms by flow cytometry of dog tumor biopsies and tumor histopathology in collaboration with the Veterinary School at Colorado State University in Fort Collins, Colorado. Spontaneous dog tumors are being studied at the Los Alamos Scientific Laboratory as models for human tumors. Figure 2 illustrates two examples of DNA distributions obtained from spontaneous dog tumors (i.e., a diploid hemangiosarcoma and a hyperdiploid mammary carcinoma). As of August 1979, we have analyzed 75 dog tumors and numerous normal dog tissues. The DNA index* for these neoplasms has ranged from 1.0 to 3.0, with a mean of 1.4. Those solid tumors with a DNA index $\geq$ 1.4 have had higher fractions of cycling cells ($S + G_2 + M$) and were usually classified histopathologically as more undifferentiated. Interestingly, a previous study by Atkin (1966) indicated that human cervical carcinomas having near-tetraploid DNA contents responded more favorably to radiotherapy than near-diploid tumors while, for breast carcinomas, near-diploid tumors showed better radiation responses than higher DNA content lesions (Atkin, 1966). More definitive and correlative data are needed to establish the relationship of tumor histology, DNA content, and radiosensitivity.

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* DNA index = $\frac{\text{Tumor } G_o/G_1 \text{ Fluorescence Peak Channel}}{\text{Normal Dog Leukocyte } G_o/G_1 \text{ Fluorescence Peak Channel}}$, where the normal leukocyte DNA contents are normalized to 1.0.

DNA distributions are useful for studying cell kinetics and progression effects induced by various therapy modalities. Figure 3A compares the distribution of cells about the life cycle for V79 monolayer cells exposed to X-rays and plutonium alpha particles of matched doses resulting in 10% survival. These results suggest that, after alpha-particle exposure, the retention of cells in S phase is longer in time compared to X-rays. S-phase-specific retention for alpha particles may be coupled with a longer mitotic delay compared to X-rays at a given survival level--consistent with the observations that the RBE for high-LET radiations is higher for the end point of mitotic delay compared to the RBE for cell killing.

Reoxygenation and redistribution of cells treated with fractionated X-rays have been studied using V79-171b Chinese hamster multicellular spheroids. DNA distributions of cells from 10- and 21-day-old control spheroids and irradiated spheroids (333 rad x 1 fraction and 333 x 6 fractions in 2 wk) are shown in Fig. 4, illustrating the increased cycling fraction ($S + G_2 + M$) of cells in the spheroids treated with fractionated radiation due to reoxygenation and entry of arrested hypoxic cells into cycling. This reoxygenation effect was suggested by an increase in the Coulter volume distribution curves and increased cycling fraction of the 21-day-old spheroids treated with fractionated X-rays compared to control spheroids (Figs. 5C and 5D). These results are consistent with the reoxygenation and radio-sensitization of V79-171b spheroid cells observed by Sutherland and Durand (1976) for split-dose irradiation.

It is known that considerable variability exists in tumor response to the drug adriamycin. Figure 6 shows the results of our recent cell progression studies using a regular V79 Chinese hamster cell line and an adriamycin-resistant V79 line developed by Belli et al. (1978). A comparison of the DNA distributions for adriamycin-exposed cells clearly shows that dramatic

cytokinetic perturbations occur in regular V79 cells, compared to practically no change except for some S-phase decrease in adriamycin-resistant V79 cells (Fig. 6). If most of these blocked cells are doomed to die, whi h was found to be the case with these data, then the magnitude and duration of the therapy perturbations might be predictive of the cell-killing response for a given treatment. Since the DNA distributions can be obtained rapidly, such measurements may be helpful in selecting patients who would respond favorably to a given drug, or vice versa. Such measurements on tumor cells from surgical or biopsy specimens assayed in vitro may be useful in predicting tumor response in patients for a given drug. These measurements, however, could be limiting for certain tumors in which a few cells are cycling and accumulate in G_2 perhaps even in culture. Although the role of cell kinetics in tumor biology has been questioned in terms of clinical application, cytometry instrumentation could be a helpful test to resolve this important question.

Light-scatter and low- and high-frequency electrical impedance measurements also have been used to characterize cells. Light-scatter measurements are being used to obtain cell size and cell surface information from unfixed cells, with or without staining. Multiple-angle light-scattering measurements are being used to study the morphology of cells (Salzman et al., 1975). Swartzendruber et al. (1979) showed that, in the mouse teratocarcinoma system, multiple-angle light-scatter measurements were useful for distinguishing among differentiated epithelial cells, differentiated neuronal cells, and their undifferentiated neoplastic precursors. High-frequency rf-impedance flow sensing of cells in the 1- to 10-MHz range has been used to study the resistivities of cells (Hoffman and Britt, 1979). Hoffman and Swartzendruber (1979) demonstrated experimentally that rf-impedance was useful in

distinguishing between differentiated and undifferentiated teratocarcinoma cells of similar size. In addition, preliminary studies in our Laboratory have shown that dual-parameter Coulter dc volume and rf-impedance sensing could detect subtle drug and X-ray-induced damage in V79 monolayer cells that was not evident by cell-size, light-scatter, or DNA fluorescence measurements.

The biological interpretation of flow data obtained by multiparameter studies, as well as light-scatter and/or electrical impedance measurements of cells, is still in its embryonic stage. Only repetitive experiments using simple biological systems in reference to known biological phenomena may lead to an understanding of such data.

CONCLUSIONS

The introduction of flow cytometry instrumentation into biology and medicine has made it possible to examine quantitatively the various cellular and subcellular parameters that, until recently, were difficult or impossible to measure. The initial biomedical research expectations for flow cytometry instrumentation were rather high. Because of the biological complexities, the sophisticated developments of flow instrumentation have far surpassed our ability to solve problems of biological interest. The potential for further improvements in our knowledge in tumor biology is there but requires extensive and careful experimentation to understand the biological meaning of the enormous amount of data collected by these instruments. It is important that we do not fall into the trap that has been so aptly described by the famous Indian poet (Nobel Laureate) Tagore, regarding people with a literary mind: we must not get so busy with our nets that we neglect the fishing.

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FIG. 1. Schematic diagram of a flow cytometry instrument.

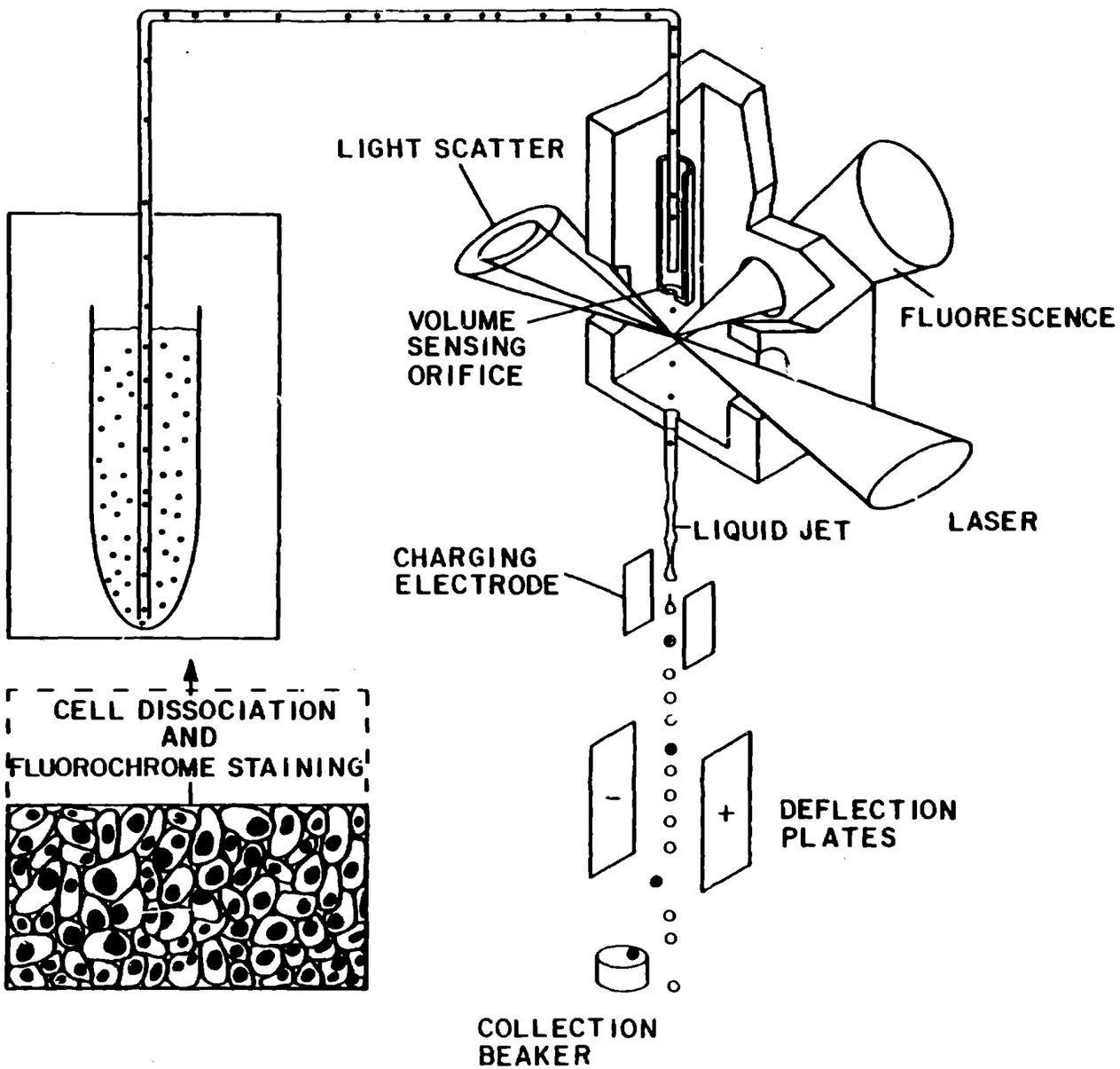

LIGHT SCATTER
VOLUME
SENSING
ORIFICE
FLUORESCENCE
LIQUID JET
LASER
CHARGING
ELECTRODE
CELL DISSOCIATION
AND
FLUOROCHROME STAINING
-
+
DEFLECTION
PLATES
COLLECTION
BEAKER

FIG. 2.--DNA fluorescence distribution data for two spontaneous dog tumors plotted both as DNA distributions and of computer-generated two-parameter contour profiles of DNA fluorescence and Coulter volume (shown in the insert). (Left) Hemangiosarcoma having a low-cycling fraction with DNA index = 1.0. Visual separation between normal and malignant cells was improved using two parameters. (Right) Mammary carcinoma having a high-cycling fraction with a mixed heteroploid DNA index of 1.2 and 1.6.

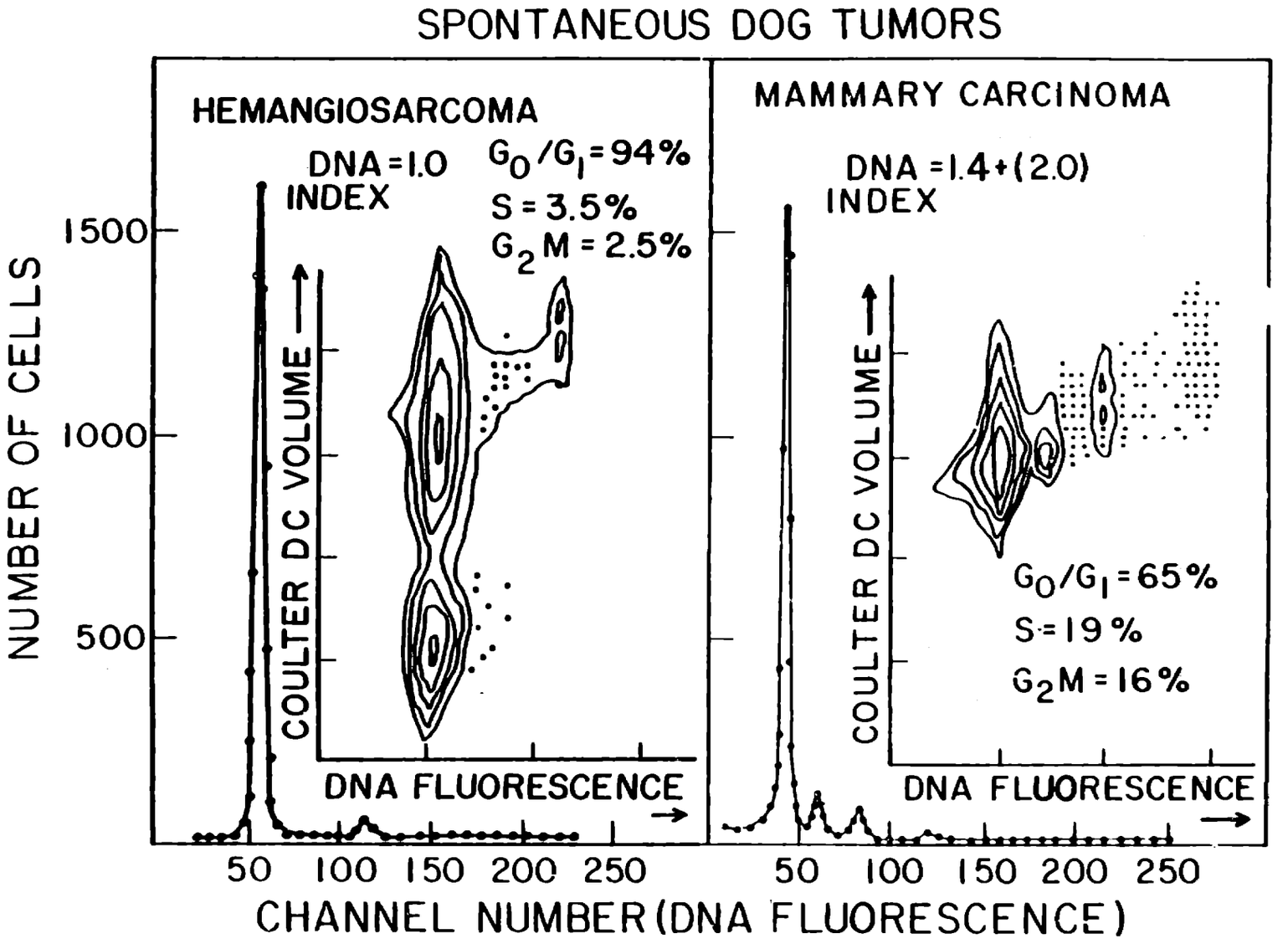

SPONTANEOUS DOG TUMORS
HEMANGIOSARCOMA
DNA = 1.0
INDEX
G_0/G_1 = 94%
S = 3.5%
G_2M = 2.5%
COULTER DC VOLUME
DNA FLUORESCENCE
MAMMARY CARCINOMA
DNA = 1.4+(2.0)
INDEX
G_0/G_1 = 65%
S = 19%
G_2M = 16%
COULTER DC VOLUME
DNA FLUORESCENCE
NUMBER OF CELLS
1500
1000
500
50 100 150 200 250
50 100 150 200 250
CHANNEL NUMBER (DNA FLUORESCENCE)

FIG. 3.--Comparison of the DNA distributions of mithramycin-stained V79 monolayer-grown cells, showing the difference in cell-cycle progression of plutonium alpha particle vs. X-ray-exposed (XRT) cells that received isosurvival (10%) doses. The DNA distributions are shown for control (-O-), XRT-10% (-X-), and α-10% (-◇-) cultures sampled 9 h after treatment. Note the larger fraction of G_o/G_1 cells for XRT-10% compared to α-10% at this time period, representing the shorter mitotic delay for XRT cells.

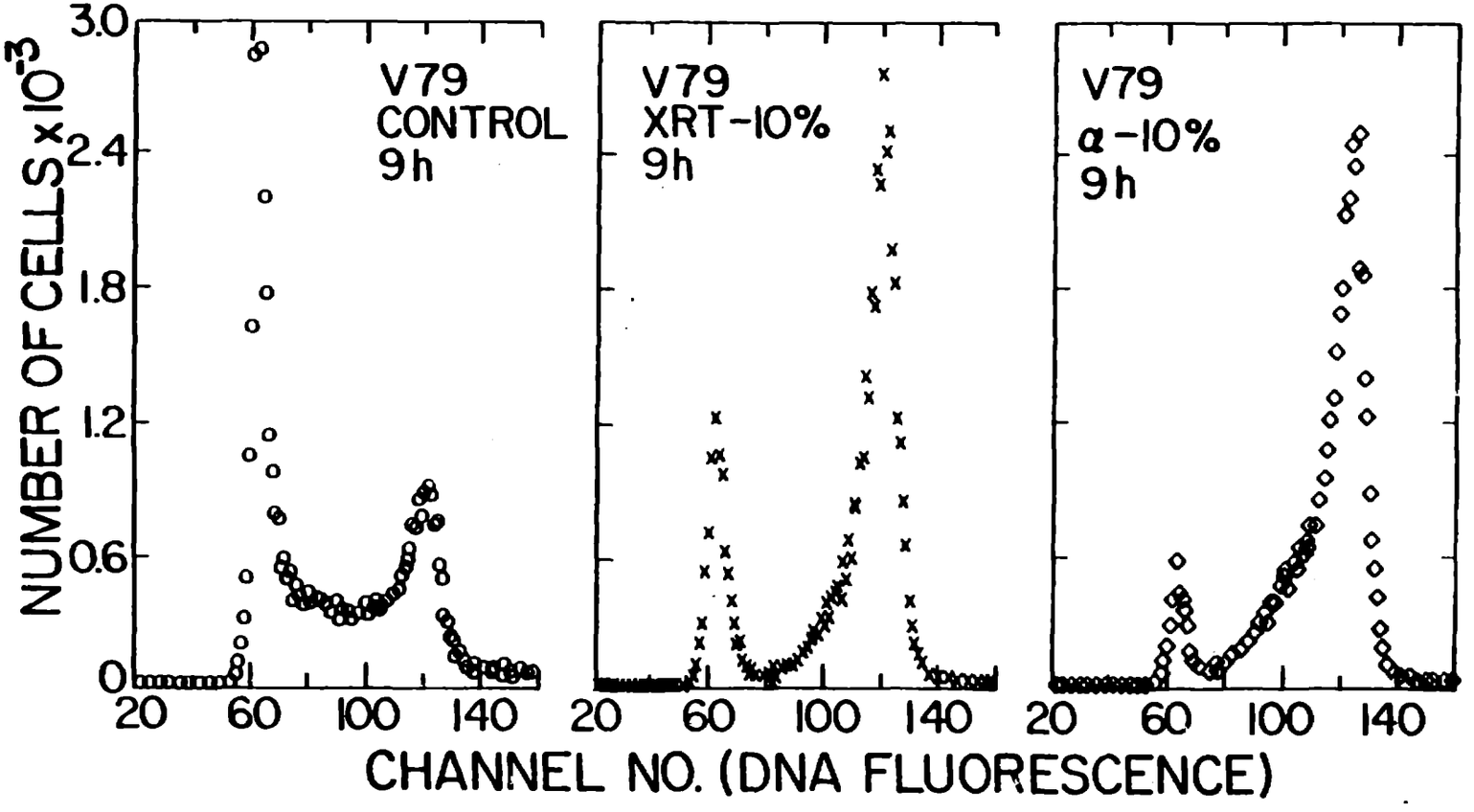

NUMBER OF CELLS x 10^-3
3.0
2.4
1.8
1.2
0.6
0
V79
CONTROL
9h
V79
XRT-10%
9h
V79
α-10%
9h
20
60
100
140
CHANNEL NO. (DNA FLUORESCENCE)

FIG. 4.--The effect of fractionated X-rays (XRT) on V79-171b multicellular spheroid population DNA distributions, showing accelerated growth resulting from XRT-induced reoxygenation and redistribution. (Left) Normalized DNA distributions of 10-day-old unirradiated (-O-) and XRT (-X-), 333 rad x 1 + 3 h, mithramycin-stained cells, showing little change. (Right) Normalized DNA distributions of 21-day-old unirradiated (-O-) and XRT (-X-), 333 rad x 6 in 2 wk, sampled 3 h after the last exposure. Note the increased cycling fraction ($S + G_2 + M$) in the 21-day-old mithramycin-stained spheroid cells given fractionated XRT.

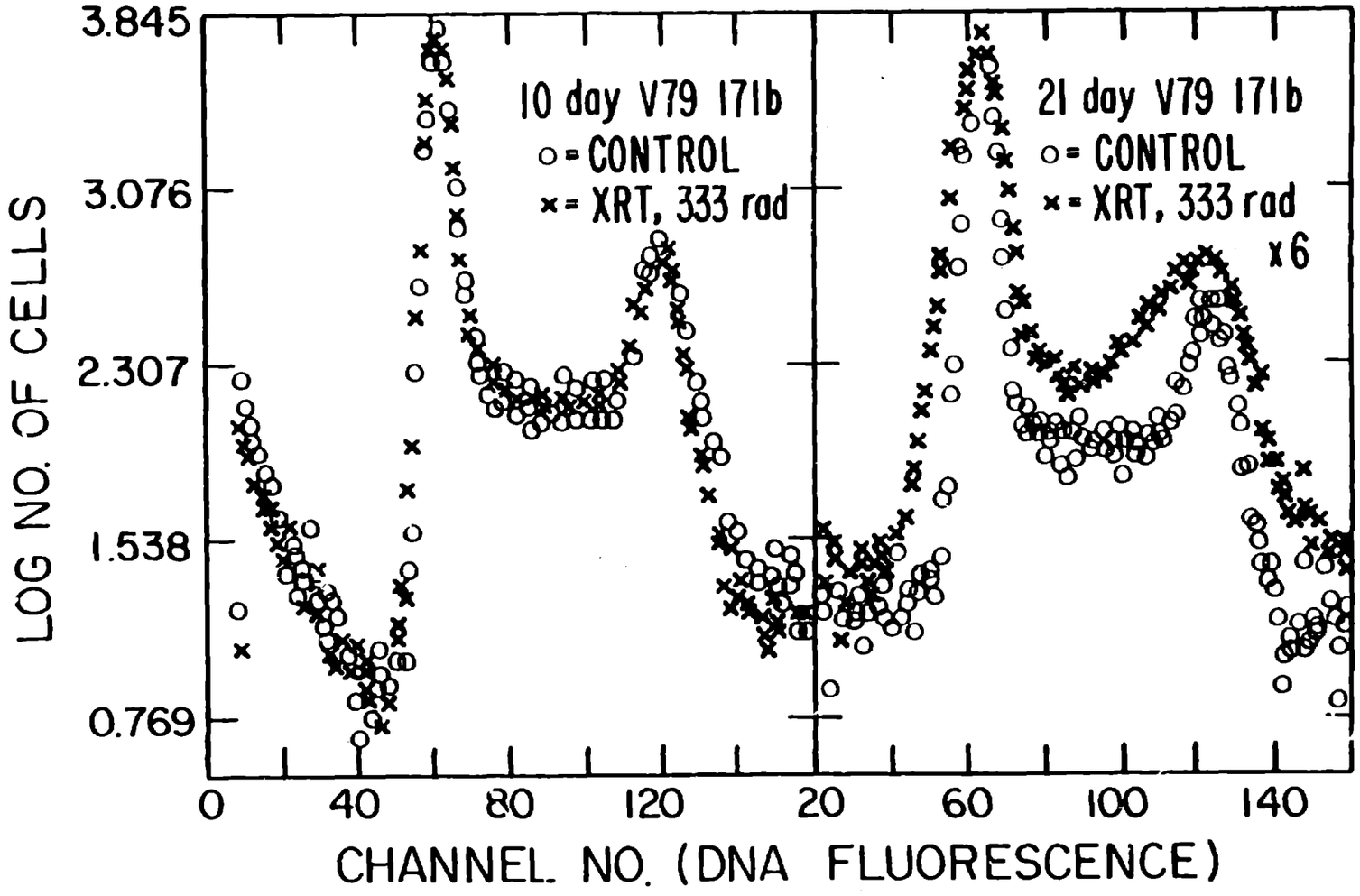

3.845
3.076
2.307
1.538
0.769
LOG NO. OF CELLS
10 day V79 171b
○ = CONTROL
× = XRT, 333 rad
21 day V79 171b
○ = CONTROL
× = XRT, 333 rad
x6
0
40
80
120
20
60
100
140
CHANNEL NO. (DNA FLUORESCENCE)

FIG. 5.--The effect of fractionated X-rays on V79-171b spheroid mithramycin normalized DNA distributions and Coulter volume distributions. Isometric plots of DNA fluorescence (X-axis), Coulter volume (Y-axis), and cell number (Z-axis) are shown for (A) 10-day-old control spheroids; (B) 10-day-old X-rays (333 rad + 3 h) having a small increased cycling fraction; (C) 21-day-old control spheroids; and (D) 21-day-old spheroids sampled 3 h after the last of six fractions (333 rad in 2 wk), showing an increased cycling fraction and a broadened G_o/G_1 Coulter distribution due to transition of small hypoxic cells from G_o/G_1 by reoxygenation and reentry into cycling.

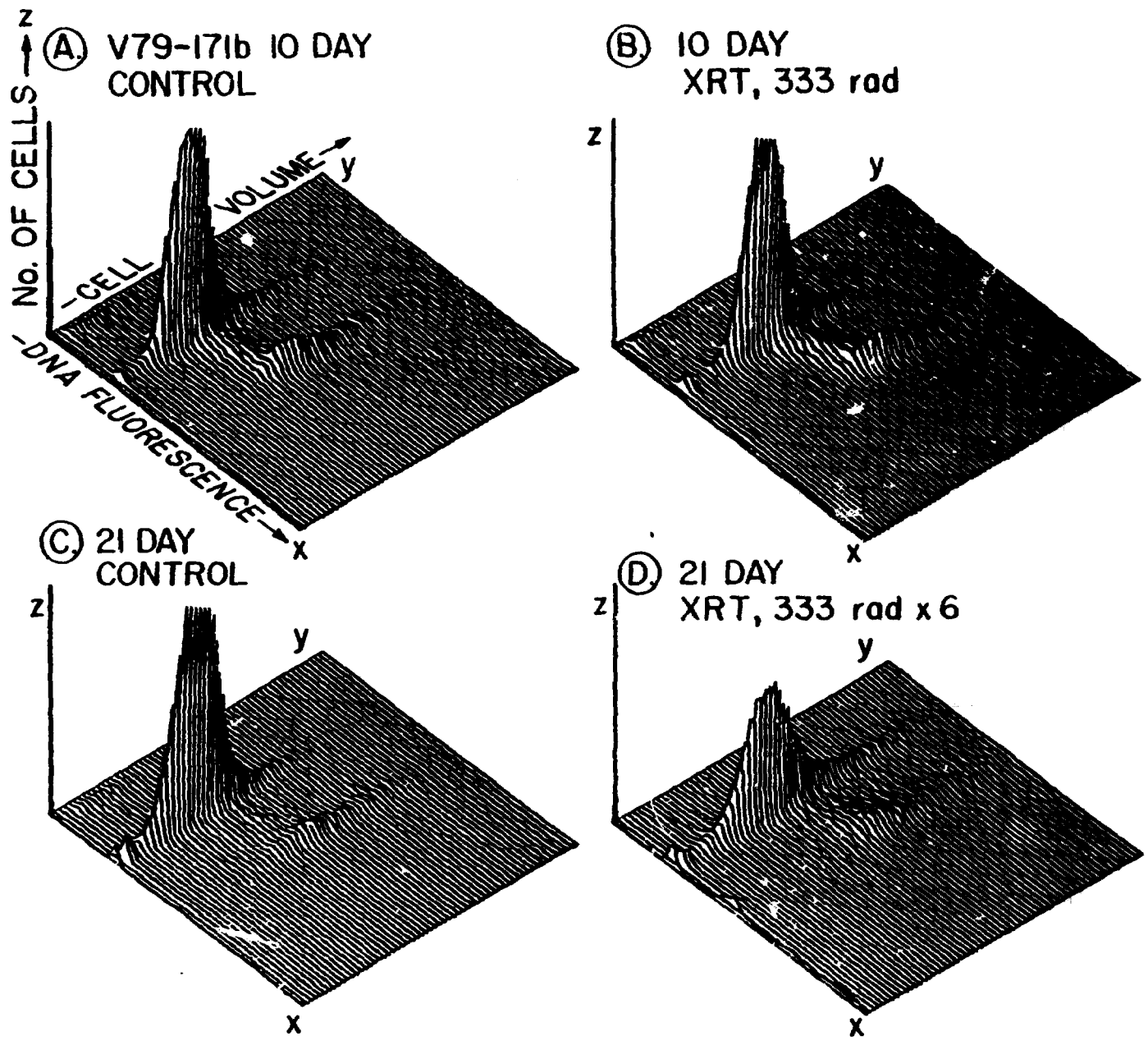
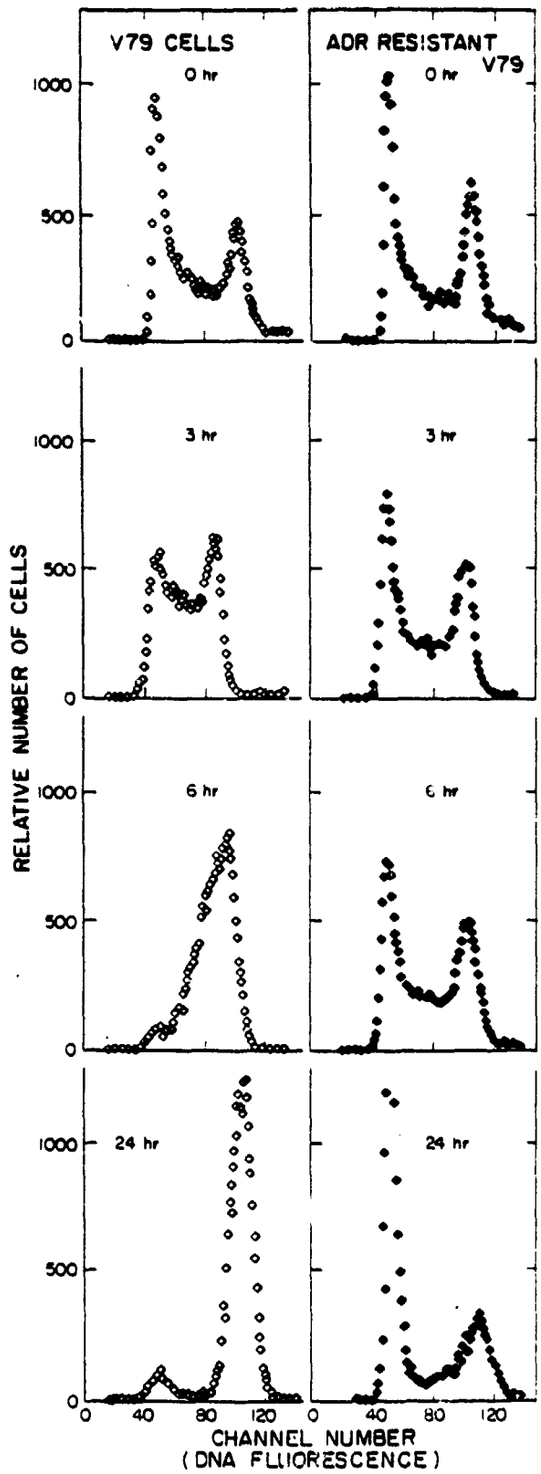

A V79-171b 10 DAY
CONTROL
No. OF CELLS
z
CELL
VOLUME
y
DNA FLUORESCENCE
x
B 10 DAY
XRT, 333 rad
z
y
x
C 21 DAY
CONTROL
z
y
x
D 21 DAY
XRT, 333 rad x 6
z
y
x

FIG. 6.--Comparison of the normalized cell-cycle distribution of regular V79 Chinese hamster cells (-◇-) and adriamycin-resistant V79 cells (-◆-) following exposure to adriamycin (0.5 μg/ml, 1 h) given to exponentially growing monolayer cells. In contrast to the dramatic S and G_2/M blocks that are evident in regular V79 cells (-◇-), only a moderate S-phase decrease was observed by 24 h postexposure in the adriamycin-resistant cells.


V79 CELLS
ADR RESISTANT V79
0 hr
0 hr
3 hr
3 hr
6 hr
6 hr
24 hr
24 hr
1000
500
0
0
40
80
120
RELATIVE NUMBER OF CELLS
CHANNEL NUMBER
(DNA FLUORESCENCE)