Jpn. J. Cancer Res. 83, 477–482, May 1992

Flow Cytometric Bromodeoxyuridine/DNA Analysis of Hyperthermia and/or Adriamycin for Human Pancreatic Adenocarcinoma Cell Line Capan-2

Bunzo Nakata, Yong-Suk Chung,[1] Hideaki Yokomatsu, Tetsuji Sawada, Toshiaki Kubo, Yasuyuki Kondo, Katsusuke Satake and Michio Sowa

First Department of Surgery, Osaka City University Medical School, 1-5-7, Asahimachi, Abeno-ku, Osaka 545

The effects of hyperthermia, adriamycin (ADM), and hyperthermia combined with ADM on pancreatic cancer cells were investigated from the viewpoint of cytokinetics using flow cytometric bromodeoxyuridine (BrdUrd)/DNA analysis. Human pancreatic adenocarcinoma cell line Capan-2 was used. The untreated cells could be clearly divided into G_1, S, G_2M phases on contour plots of BrdUrd/DNA distribution. After heat treatment at 41–43°C, there was an accumulation of cells in the G_2M phase which was correlated with the increase in temperature. After heat treatment at 44 or 45°C, there was marked increase in non BrdUrd-labeled cells in the S phase. ADM caused no change in the percent of non BrdUrd-labeled cells in the S phase, even after treatment with a concentration of 1.0 μg/ml, though that concentration of ADM caused a marked increase in the percent of cells in the G_2M phase. After hyperthermia combined with ADM, the accumulation of the G_2M phase increased remarkably, and was significantly higher than that after each treatment alone ($P<0.005$); however, non BrdUrd-labeled cells in the S phase did not increase. In this study the synergistic effect of hyperthermia combined with ADM in increasing the percent of cells in the G_2M phase could be observed by flow cytometry. The study illustrates the importance of performing *in vitro* flow cytometric BrdUrd/DNA analysis of combined therapy prior to the use of the combined therapy in patients.

Key words: Flow cytometry — Bromodeoxyuridine/DNA analysis— Combined therapy — Adriamycin — Hyperthermia

Pancreatic cancer, lying deep in the abdominal cavity, is not readily treatable with the hyperthermia apparatus currently available. Furthermore, pancreatic cancers are usually hypovascular, so it is difficult to attain a sufficient concentration of chemotherapeutic agents within the cancer. Hyperthermia combined with chemotherapy might be expected to show increased effectiveness through synergy,[1,2] although there are very few reports concerning the use of combined therapy against pancreatic cancer as related to the cell cycle of the cancer. Flow cytometric DNA histogram analysis has sometimes yielded questionable results in cytokinetic studies, due to the occurrence of overlapping of the early S phase with the G_1 phase, and the late S phase with the G_2M phase.[3,4] BrdUrd[2]/DNA analysis was devised in an attempt to avoid this confusion.[5–7]

In the present paper, flow cytometric BrdUrd/DNA analysis was utilized to study the effects of hyperthermia and ADM separately and in combination against pancreatic cancer cells in tissue culture. The potential usefulness of the combined therapy was supported by the results of this study from the viewpoint of cytokinetics.

[1] To whom requests for reprints should be addressed.

[2] Abbreviations: BrdUrd, bromodeoxyuridine; FCM, flow cytometry; ADM, adriamycin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide; CV, coefficient of variation.

MATERIALS AND METHODS

Cell cultures Capan-2 cells derived from a human pancreatic adenocarcinoma[8] were grown as monolayer cultures in 75 cm^2 tissue culture flasks (Falcon 3024: Becton Dickinson Co.) in Dulbecco's modified Eagle's medium (Whittaker Bioproducts Inc.), supplemented with 10% fetal calf serum (Commonwealth Serum Laboratories), 2 m*M* L-glutamine (Flow Laboratories), 2 m*M* sodium pyruvate (Whittaker Bioproducts Inc.), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Flow Laboratories). Cells were plated at a density of 1×10^5 cells/ml and incubated in 5% CO_2 at 37°C with 100% humidity. All experimental treatments were carried out on exponentially growing cell cultures 72 h after seeding. Until the surviving cell number and the cytokinetics were examined 24, 48, and 72 h after the treatments, the Capan-2 cells were cultured subsequently in exchanged fresh medium.

Heat treatment Cell monolayers were exposed for 60 min to elevated temperatures in the range of 41–45°C by placing the culture flask in a waterbath at the appropriately controlled temperature.

ADM treatment Cells were incubated for 60 min in a medium containing 0.1, 0.5 or 1.0 μg/ml concentration of ADM at 37°C.

Heat treatment combined with ADM Cells in a medium containing 0.1 μg/ml of ADM were exposed to heat treatment at 41°C for 60 min.

Staining The modified method of Dolbeare *et al.*[6] was used for these experiments. Capan-2 cells were treated with 5 μg/ml BrdUrd for 30 min. The cells were then harvested, fixed in cold 70% ethanol and stored at 4°C. Ethanol-fixed cells were removed from the ethanol solution, treated with 0.1% ribonuclease (Sigma Chemical Co.) for 15 min at 37°C, resuspended in 2 *N* HCl for 20 min at room temperature, washed twice with sodium tetraborate and then once with PBS, and resuspended for 30 min in a PBS solution containing 0.5% Tween 20 (Sigma Chemical Co.) and 20 μl of FITC-conjugated monoclonal anti-BrdUrd antibody (Becton Dickinson Co.). The cells were then washed twice with PBS and resuspended in 50 μg/ml PI (Sigma Chemical Co.). To eliminate clumping, the stained cells were passed through a 50 μm nylon mesh (NBC Industry).

FCM The BrdUrd/DNA dual-stained cells were analyzed using a FACS-440 (Becton Dickinson Co.). The argon laser was adjusted to emit 700 mW at 488 nm, and a dichroic mirror 560 was used to separate the two fluorescences. Green fluorescence from FITC was measured through a 530 nm band-pass filter and recorded as a measure of the amount of incorporated BrdUrd. Red fluorescence from PI was measured through a 585 nm band-pass filter and recorded as a measure of the total DNA content. Routinely, 10,000 cells were measured for each distribution at a flow rate of less than 500 cells/s. The bivariate BrdUrd/DNA distributions were displayed as contour plots.

Cytokinetic analysis The windows for the G_1 phase, S phase, G_2M phase and non BrdUrd-labeled cells in the S phase were placed on the contour plots for untreated Capan-2 cells. They were adapted for the treated cells, and cytokinetic data were analyzed using the CONSORT 30-Rev. D program (Becton Dickinson Co.) on a Hewlett Packard 9000/300 computer connected to a FACS-440.

Cell count The surviving cell number at the time of flow cytometric analysis was determined using a Coulter counter (Coulter Electronics). The surviving cell number after treatment was expressed as a percentage of the control value, calculated as follows:

$$\frac{\text{No. of cells in treated flask}}{\text{No. of cells in untreated (control) flask}} \times 100\ (\%).$$

The cells used for flow cytometric analysis were almost all alive, as confirmed by dye exclusion using trypan blue.

Statistical analysis Each datum was the mean±SD of the results from five flasks. The statistical significance of the differences between groups was examined by using Student's *t* test.

RESULTS

Cytokinetics of the control cells Untreated Capan-2 cells could be clearly divided into three cohorts of G_1, S, and G_2M phases (Fig. 1), and the proportion of each cell phase could be calculated reproducibly with a small SD value (Table I). The mean ratio of non BrdUrd-labeled cells in the S phase was 2.1%.

Cytokinetics and surviving cell number after heat treatment The cytokinetics of the Capan-2 cells at 48 h after 41°C heat treatment showed an accumulation in the G_2M phase, a result which was significantly different as compared with the control ($P<0.05$) (Table I). The accumulation in the G_2M phase at 48 h increased in accordance with rise in temperature up to 43°C (Fig. 2). The total amount of BrdUrd incorporated into the S phase cells, however, decreased with rise in temperature, and over 44°C, non BrdUrd-labeled cells in the S phase increased and the discrimination of the S phase cells from the G_1 or G_2M phase cells became difficult, even on the contour plots of BrdUrd/DNA distribution (Fig. 3). The values of the ratio of non BrdUrd-labeled cells in the S phase 48 h after heat treatment at 41°C, 42°C, 43°C, 44°C, and 45°C were 2.3±0.4%, 2.6±0.2%, 4.2±0.3%, 8.1±0.6%, and 14.9±2.1%, respectively (mean±SD).

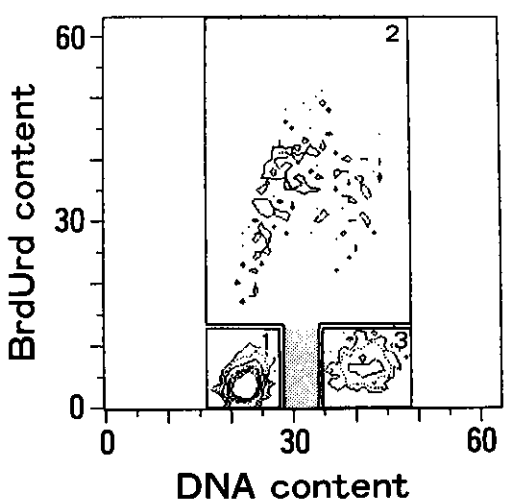

Fig. 1. Contour plots of bivariate BrdUrd/DNA distribution of untreated Capan-2 cells. The abscissa is relative DNA content and the spindle is relative BrdUrd content. 1, G_1 phase; 2, S phase; 3, G_2M phase. Slit area shows non BrdUrd-labeled cells in the S phase.

Table I. Comparison of Cytokinetics and Surviving Cell Number (% of Control) at 48 h after Various Treatments

Treatment	Percent of cells in each cell cycle phase			S[a)]	Surviving cell number (% of control)
	G_1	S	G_2M		
Control (Untreated)	42.0±1.3	41.4±0.8	16.6±1.1	2.1±0.7	100
41°C	37.2±2.1	43.6±2.9	19.2±2.5	2.3±0.4	81.0±4.8
ADM 0.2 μg/ml	35.8±3.2	42.4±1.4	21.8±2.4	2.1±0.1	75.2±2.7
41°C+ADM 0.1 μg/ml	18.3±4.8	22.6±3.9	59.1±8.3	2.4±0.2	40.3±3.6

a) Non BrdUrd-labeled cells in the S phase (% to the total cells). 41°C+ADM 0.1 μg/ml means simultaneous combination of the two treatments. Values represent the mean and SD of five flasks.

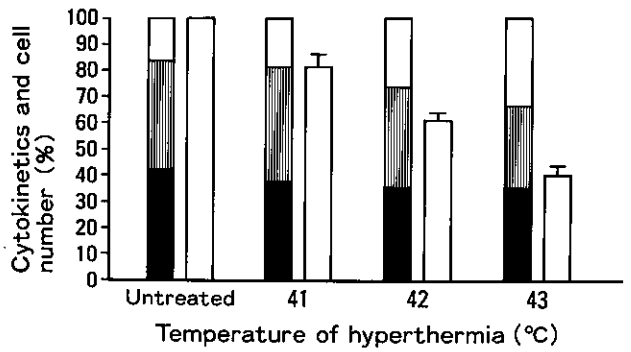

Fig. 2. Cytokinetics and mean surviving cell number (% of control) shown as the open columns with bar, at 48 h after heat treatment at various temperatures for 60 min. ■, G_1 phase; ▥, S phase; □, G_2M phase.

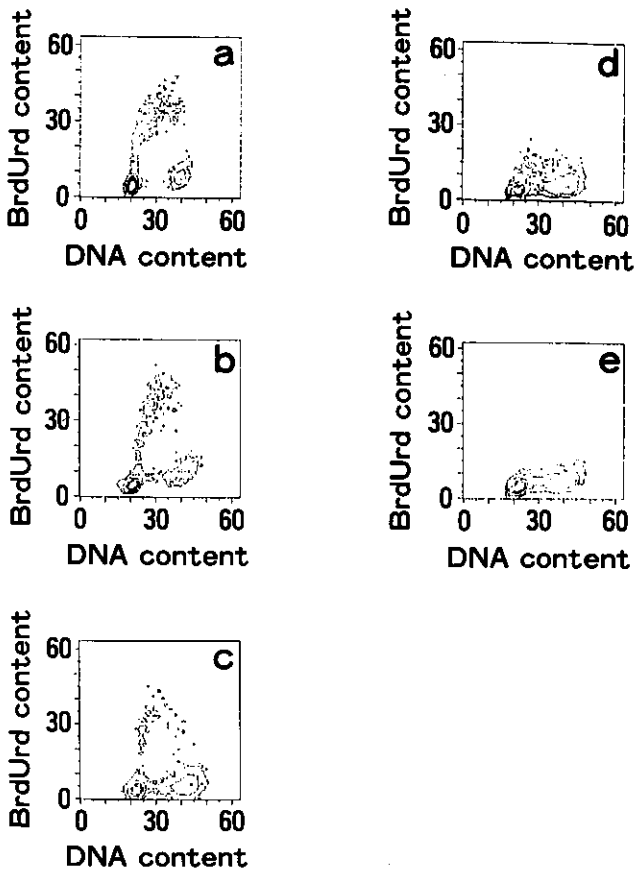

Fig. 3. Contour plots of bivariate BrdUrd/DNA distribution 48 h after heat treatment at various temperatures for 60 min. The abscissa is relative DNA content and the spindle is relative BrdUrd content. a, 41°C; b, 42°C; c, 43°C; d, 44°C; e, 45°C.

The surviving cell number (% of control) decreased in accordance with rise in temperature (Fig. 2).

Cytokinetics and surviving cell number after treatment with ADM The cytokinetics after treatment with 0.1 μg/ml of ADM showed an accumulation in the G_2M phase. The ratio of the G_2M phase at 48 h after treatment was 21.8±2.4%, and was statistically significantly higher than that of the control ($P<0.01$). The accumulation in the G_2M phase increased and the surviving cell number (% of control) decreased as the concentration of ADM rose (Fig. 4). However, non BrdUrd-labeled cells in the S phase did not increase and the values of the ratio at 48 h after treatment with 0.1, 0.5, and 1.0 μg/ml of ADM were 2.1±0.1%, 2.2±0.2%, and 2.2±0.2% respectively (mean±SD). After treatment with 1.0 μg/ml of ADM cells in the late S phase, which were shown to have a continuous relation with the G_2M phase cells on the contour plots of BrdUrd/DNA distribution, became apparent (Fig. 5).

Cytokinetics and surviving cell number after hyperthermia combined with ADM The surviving cell number (% of control) after the combined treatment was severely suppressed and showed the synergistic effect of the combination (Table I).

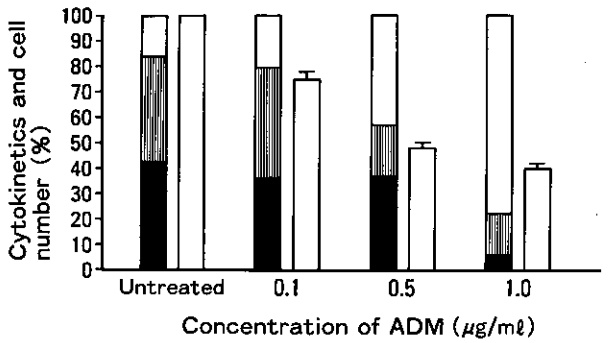

Fig. 4. Cytokinetics and mean surviving cell number (% of control) shown as open columns with bar, at 48 h after treatment with various concentrations of ADM at room temperature for 60 min. ■, G_1 phase; ▥, S phase; □, G_2M phase.

Heat treatment combined with ADM resulted in an increase in the accumulation during the G_2M phase, and was significantly higher than that after each treatment alone ($P<0.005$) (Table I). The mean ratio of non BrdUrd-labeled cells in the S phase at 48 h after treatment was $2.4\pm0.2\%$ in the combination (Table I, Fig. 6).

The cytokinetics after combined treatment indicated an intensive accumulation in the G_2M phase at 24 h after treatment, an accumulation which continued up to 72 h later. In addition, the surviving cell number (% of control) showed long-lasting suppression (Fig. 7).

DISCUSSION

Flow cytometric BrdUrd/DNA analysis was devised by Dolbeare *et al.*[6] The method has been widely used for various investigations, including cytokinetic studies with numerous types of anti-cancer agents. However, there were very few reports on the use of this technique for the study of hyperthermia or combined therapy.

Dolbeare *et al.* stated that no procedure for BrdUrd/DNA analysis exists which is universally optimal for all tissues and experimental endpoints.[7] In the present experiment, we conducted a preliminary evaluation of staining conditions, for example various concentrations and durations of HCl exposure, and under the conditions described above the pancreatic adenocarcinoma cell line Capan-2 could be clearly analyzed by using the BrdUrd/DNA method. Untreated Capan-2 cells were found to have about 2% non BrdUrd-labeled cells in the S phase. However, it was difficult to prove the cells to be non-synthesizing (the S_Q cells of Darzynkiewicz *et al.*[9]), as there was a possibility of changes occurring in the permeability of the cell membrane for BrdUrd after heat treatment. Hence, in the present study, these were simply designated non BrdUrd-labeled cells in the S phase.

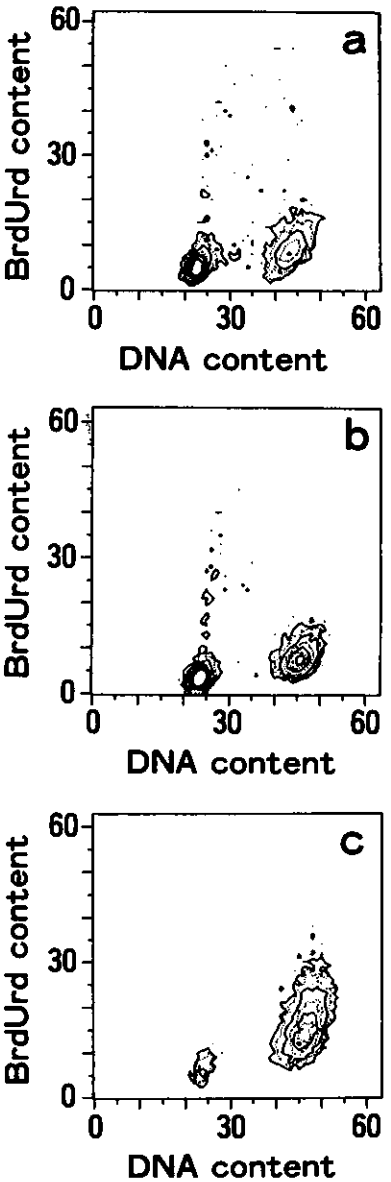

Fig. 5. Contour plots of bivariate BrdUrd/DNA distribution at 48 h after treatment with various concentrations of ADM at room temperature for 60 min. The abscissa is relative DNA content and the spindle is relative BrdUrd content. a, 0.1 μg/ml; b, 0.5 μg/ml; c, 1.0 μg/ml.

In the previous studies on cytokinetics after hyperthermia using FCM, a block in the S phase and in the G_2M phase was revealed by DNA histogram analysis,[10,11] though the ratio of each cell phase was not obtained with sufficient accuracy for statistical evalution. By using

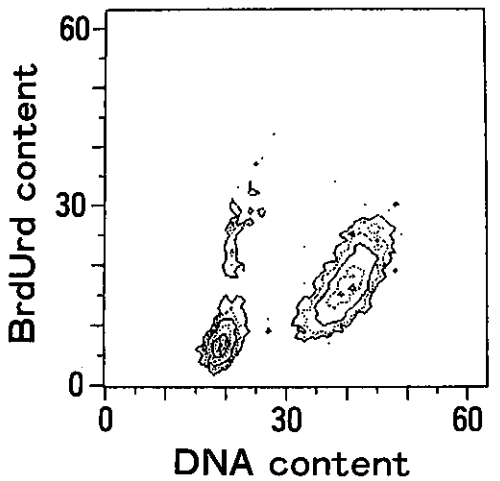

Fig. 6. Contour plots of bivariate BrdUrd/DNA distribution at 48 h after heat treatment at 41°C simultaneously combined with 0.1 μg/ml of ADM for 60 min. The abscissa is relative DNA content and the spindle is relative BrdUrd content.

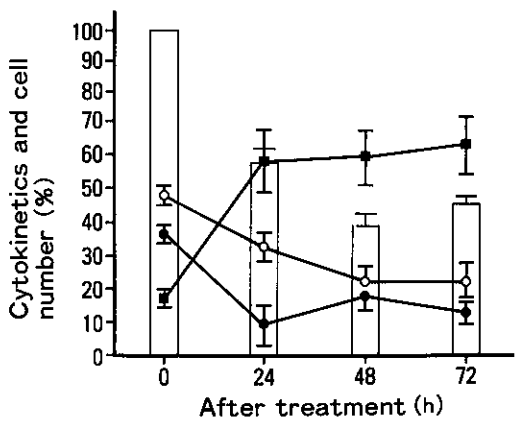

Fig. 7. Cytokinetics and surviving cell number (% of control) at various time lapses after 41°C heat treatment simultaneously combined with 0.1 μg/ml of ADM for 60 min. The open column shows surviving cell number. ●, G_1 phase; ○, S phase; ■, G_2M phase. Values represent the mean and SD of five flasks.

BrdUrd/DNA analysis, a statistically significant difference of accumulation in the G_2M phase after treatment even at lower temperatures, such as at 41°C, was noted as compared with the control. The surviving cell number (% of control) decreased in parallel with the extent of cytokinetic perturbation after hyperthermia. In this study the total amount of incorporated BrdUrd was shown to decrease markedly after treatment at higher temperatures (over 44°C). At 45°C, the incorporation of BrdUrd almost stopped.

ADM is an antitumor antibiotic which causes a block in the G_2M phase in flow cytometric DNA histogram analysis.[12, 13] BrdUrd/DNA analysis quantitatively showed accumulation in the G_2M phase after treatment with low concentrations, such as 0.1 and 0.5 μg/ml of ADM. With higher concentrations, such as 1.0 μg/ml, a greater accumulation in the G_2M phase was noted, as was an increment in the late S phase cells. However, non BrdUrd-labeled cells in the S phase did not increase. The surviving cell number (% of control) also decreased in parallel with the extent of cytokinetic perturbation, especially accumulation in the G_2M phase. These results suggest that the degree of cytokinetic perturbation could indicate the effectiveness of an anti-cancer agent.

In the present study, the effect of hyperthermia combined with ADM was tested for the first time by BrdUrd/DNA analysis, using FCM. The cytokinetics after combined therapy revealed an increase in the accumulation of the G_2M phase which was similar to the cytokinetic changes after exposure to several-fold higher concentrations of ADM alone. The extent of accumulation after combined therapy was significantly higher than that after each respective separate treatment. Non BrdUrd-labeled cells in the S phase, which increased after exposure to higher temperatures, did not increase. This phenomenon appears to support the assumption that the synergism was due to an increment of the effect of ADM by hyperthermia, not of hyperthermia by ADM. *In vitro*, the mechanism of synergism between the effect of hyperthermia and ADM was suggested to be an increased ADM uptake by the cells due to change in the permeability of the cell membrane during heat treatment.[14] The cytokinetics at various time lapses after combined therapy showed a marked effect, with a great accumulation of the G_2M phase up to 72 h after treatment. In addition, from the viewpoint of surviving cell number (% of control), the effect of the combined treatment indicated synergism. Therefore, hyperthermia combined with ADM might prove to be a useful therapy for patients with pancreatic cancer.

To improve the poor prognosis of pancreatic cancer, further basic investigations of combined therapeutic modalities are needed. The experimental model utilized in the present study might be useful in the further study of other variations of exposure, including time intervals and the concentrations of various agents. Flow cytometric BrdUrd/DNA analysis is more expensive and time-consuming than DNA histogram analysis by FCM. However, it is the only method that accurately discriminates S phase cells from other cell phase cells, even in the case of diploid or polypoid cells. It is therefore recommended for utilization in a study of specimens of pancreatic cancer obtained from patients at the time of operation.

ACKNOWLEDGMENTS

The authors wish to thank Professor Emeritus John M. Howard and Associate Professor Appert, Department of Surgery, Medical College of Ohio, for their useful advice. The authors are also grateful to Dr. Takeshi Kono, Department of Dermatology, Osaka City University Medical School, for his helpful discussions.

(Received July 19, 1991/Accepted February 27, 1992)

REFERENCES

1) Overgaard, J. Combined adriamycin and hyperthermia treatment of a murine mammary carcinoma *in vivo*. *Cancer Res.*, **36**, 3077–3081 (1976).
2) Shiu, M. H., Cahan, A., Fogh, J. and Fortner, J. G. Sensitivity of xenografts of human pancreatic adenocarcinoma in nude mice to heat and heat combind with chemotherapy. *Cancer Res.*, **43**, 4014–4018 (1983).
3) Tsurusawa, M., Niwa, M., Katano, N. and Fujimoto, T. Flow cytometric analysis by bromodeoxyuridine/DNA assay of cell cycle perturbation of methotrexate-treated mouse L1210 leukemia cells. *Cancer Res.*, **48**, 4288–4293 (1988).
4) Tsurusawa, M., Niwa, M., Katano, N. and Fujimoto, T. Methotrexate cytotoxicity as related to irreversible S phase arrest in mouse L1210 leukemia cells. *Jpn. J. Cancer Res.*, **81**, 85–90 (1990).
5) Gratzner, H. G. and Leif, R. C. An immunofluorescence method for monitoring DNA synthesis by flow cytometry. *Cytometry*, **1**, 385–389 (1981).
6) Dolbeare, F., Gratzner, H., Pallavicini, M. G. and Gray, J. W. Flow cytometric measurement of total DNA content and incoporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA*, **80**, 5573–5577 (1983).
7) Dolbeare, F., Beisker, W., Pallavicini, M. G., Vanderlaan, M. and Gray, J. W. Cytochemistry for bromodeoxyuridine/DNA analysis: stoichiometry and sensitivity. *Cytometry*, **6**, 521–530 (1985).
8) Fogh, J., Fogh, J. M. and Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Cancer Inst.*, **59**, 221–225 (1977).
9) Darzynkiewicz, Z., Traganos, F. and Melamed, M. R. New cell cycle compartments identified by multiparameter flow cytometry. *Cytometry*, **1**, 98–108 (1980).
10) Kase, K. and Hahn, G. M. Differential heat response of normal and transformed human cells in tissue culture. *Nature*, **255**, 228–230 (1975).
11) Schlag, H. and Lücke-Huhle, C. Cytokinetic studies on the effect of hyperthermia on Chinese hamster lung cells. *Eur. J. Cancer*, **12**, 827–831 (1976).
12) Krishan, A. and Frei, E., III. Effect of adriamycin on the cell cycle traverse and kinetics of cultured human lymphocytes. *Cancer Res.*, **36**, 143–150 (1976).
13) Barlogie, B., Drewinko, B., Johnston, D. A. and Freireich, E. J. The effect of adriamycin on the cell cycle traverse of a human lymphoid cell line. *Cancer Res.*, **36**, 1975–1979 (1976).
14) Hahn, G. M., Braun, J. and Har-Kedar, I. Thermochemotherapy: synergism between hyperthermia (42–43°) and adriamycin (or bleomycin) in mammalian cell inactivation. *Proc. Natl. Acad. Sci. USA*, **72**, 937–940 (1975).