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Flow Cytometric Analysis of DNA Damage and Repair in the Cells Resistant to Alkylating Agents[1]

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ABSTRACT

DNA damage in the cells sensitive and resistant to alkylating agents was determined by flow cytometry analysis of cells stained with anti-DNA monoclonal antibody (MOAB) F7-26. MOAB F7-26 interacted with single-stranded regions in alkylated DNA, and the binding of antibody to the cells increased in proportion to the decrease in cell viability. Development of resistance to L-phenylalanine mustard (L-PAM) in A2780 cells was associated with decreased immunoreactivity of DNA with MOAB F7-26. Fluorescence was significantly lower in resistant cells than in sensitive cells, and the difference in the binding of MOAB between two cell types increased with the dose of L-PAM. The enhancement of L-PAM cytotoxicity to resistant cells by buthionine sulfoximine and hyperthermia was accompanied by a proportional increase of MOAB F7-26 binding to DNA. The same relative potential of sensitization regimens was established by cell survival and MOAB staining. The time course of DNA repair established by decrease of MOAB binding after L-PAM removal was similar in sensitive and resistant cells. Resistance of A2780 cells to L-PAM was associated with low initial level of DNA damage and with decreased cytotoxicity per unit of damage. We conclude that resistant cells could be distinguished from sensitive cells by staining with MOAB F7-26 and that the sensitization of resistant cells could be quantitatively predicted by flow cytometry analysis of MOAB binding.

INTRODUCTION

In previous reports, a method for the measurement of DNA damage in individual cells has been described (1, 2). The method was based on FCM[3] analysis of cells stained with MOAB generated against HN_2-treated DNA. It was shown that the binding of anti-DNA MOAB F7-26 to cells treated with alkylating agents correlated well with cytotoxicity and increased proportionally to the enhancement of cell killing by modulating factors.

The interaction of MOAB F7-26 with alkylated DNA *in situ* was explained by decreased stability of double-helix structure and by the formation of single-stranded regions. The binding specificities of MOAB F7-26 are in agreement with such an explanation. Interaction of MOAB F7-26 with DNA is characterized by an absolute requirement for single-stranded conformation, by the strong preference for deoxycytidine, and by the absence of reactivity with cellular antigens other than DNA.[4] Probably, selective alkylation of guanines by nitrogen mustards and nitrosoureas may be responsible for increased access of MOAB F7-26 to deoxycytidines on the opposite DNA strand.

Increased access of MOAB F7-26 to antigenic determinants expressed on single-stranded regions is in agreement with biochemical and cytochemical studies which demonstrated interaction of alkylated DNA with S_1 nuclease and antinucleoside antibodies (3–9). Although effects of antitumor drugs on DNA conformation were observed by many investigators, biological significance of such changes and their relationship to cytotoxic effects has not been established. Staining of cells with MOAB F7-26 provided a sensitive and reproducible probe necessary to determine the effects of alkylating agents on the structure and stability of DNA *in situ*. In the current work, we have compared the binding of MOAB F7-26 to DNA in sensitive and resistant cells and studied the relationship between sensitization of resistant cells and DNA immunoreactivity. DNA repair in sensitive and resistant cells was evaluated by the changes in antibody binding after drug removal. Quantitative relationship between cytotoxicity and DNA immunoreactivity was analyzed, and the ability of MOAB F7-26 to predict sensitivity to alkylating agents was established.

MATERIALS AND METHODS

Cell Lines. The parent human ovarian carcinoma cell line A2780 ($A2780^S$) and a variant cell line, $A2780^R$, with acquired resistance to L-PAM (10), were grown as monolayers in Opti-MEM medium supplemented with 5% fetal bovine serum and antibiotics (GIBCO, Grand Island, NY). Cultures to be used for experiments were plated into 75-cm^2 culture flasks and treated with L-PAM 2 days later. Monolayers were rinsed with culture medium, removed with 0.1% trypsin-0.2% EDTA, and aliquoted for FCM and survival studies.

Drug Treatments. L-PAM (supplied by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) was dissolved in 1 N HCl, diluted with saline, and stored at −80°C. L-PAM was added to the cultures of sensitive and resistant cells for 1 h. Where indicated, $A2780^R$ cultures were treated with 0.5 mM BSO for 24 h before addition of L-PAM. Hyperthermic conditions during L-PAM treatment were achieved by the immersion of culture flasks into a water bath at 42°C. For repair studies, cell cultures were exposed to L-PAM for 1 h, rinsed with warm culture medium, and incubated in drug-free medium for 3–24 h.

Survival Studies. Drug-induced cytotoxicity was measured by inhibition of colony formation. Control and treated cells were plated into 35-mm Petri dishes (10^2–10^4 cells) or into 75-cm^2 flasks (10^5–10^6 cells) in Opti-MEM medium supplemented with 15% fetal bovine serum. After 7 days ($A2780^R$) or 10 days ($A2780^S$) of incubation, the colonies were visualized by staining with crystal violet and counted. The surviving fraction was calculated as a ratio of plating efficiencies in drug-treated and control cells. The plating efficiency of untreated cells was 40–50% for $A2780^S$ cultures and 80–100% for $A2780^R$ cultures.

Staining with MOAB F7-26. The procedure for the differential staining of control and drug-treated cells with MOAB F7-26 has been described in detail (1, 2). The protocol included three steps: fixation, heating of cell suspension, and indirect immunofluorescence staining with MOAB F7-26.

Cells were rinsed with Ca^{2+}-, Mg^{2+}-free PBS (GIBCO, Cat. No. 310-4190) and fixed by slow addition of 6 ml precooled (−20°C) methanol to 1 ml of cell suspension in PBS. Fixed cells were stored at −20°C for 18–24 h before staining. For heating, 0.5×10^6 cells were suspended in 0.4 ml PBS supplemented with 1.25 mM $MgCl_2$. Tubes were immersed into a water bath at 100°C for 5 min and then transferred into ice-cold water for 10 min. For indirect immunofluorescence staining, cells were exposed for 30 min to MOAB F7-26, rinsed in PBS, and stained for 30 min with fluorescein-labeled goat anti-mouse IgM (Sigma Chemical Co., St. Louis, MO). For double-parameter analysis, cells

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[3] The abbreviations used are: FCM, flow cytometry; MOAB, monoclonal antibody; L-PAM, L-phenylalanine mustard; BSO, buthionine sulfoximine; PBS, phosphate-buffered saline.

[4] O. S. Frankfurt, unpublished results.

were counterstained with DNA fluorochrome propidium iodide.

FCM. Immunofluorescence intensity was measured on a flow cytometer FACScan (Becton Dickinson, Sunnyvale, CA). Green fluorescence of fluorescein-labeled antibody and red fluorescence of propidium iodide were measured for 10,000 cells and recorded in the list mode. Distribution of green fluorescence was displayed on the *x*-axis divided into 10,000 channels using log amplification. Gates were set on forward light scatter to eliminate debris and cell clumps.

Samples were stained in duplicate, and for each cell suspension two log distribution histograms were generated on a flow cytometer. From these histograms, mean fluorescence intensity was determined as a mean channel number using computer Consort 30 interfaced with FACScan. The mean fluorescence expressed as a channel number was used to characterize the binding of MOAB to DNA.

RESULTS

Sensitivity to L-PAM. Cell lines A2780^S and A2780^R were assayed for sensitivity to L-PAM by inhibition of colony formation. Colony survival curves obtained for 1 h exposure to L-PAM are illustrated in Fig. 1. L-PAM produced exponential loss of colony-forming ability with only a small shoulder on the survival curve of resistant cells. The sensitivity of cells to L-PAM was characterized by D_{10}, the dose necessary to decrease surviving fraction by 1 log on the exponential part of the curve. D_{10} for A2780^R cells (4.26 μg/ml) was approximately 7× larger than D_{10} for A2780^S cells (0.64 μg/ml). Thus, the sensitivity difference between parental and resistant lines observed in our experiments is close to the 9× resistance decribed by Hamilton *et al.* (10).

Fluorescence Differences in Sensitive and Resistant Cells. Cultures of sensitive and resistant cells were exposed to L-PAM for 1 h, fixed, and stained with MOAB F7-26. The binding of MOAB was characterized by mean fluorescence measured on a flow cytometer.

Fluorescence as a function of L-PAM concentration is illustrated in Fig. 2. Fluorescence intensity of A2780^R cells was significantly lower than the fluorescence of A2780^S cells at all dose levels of L-PAM. Fluorescence increased by 32 channels for each μg of L-PAM in sensitive cells, but only by 6 channels in the resistant cells.

The effect of resistance on the dose-survival curves and on the dose-fluorescence curves was similar. Steeper slopes for sensitive cells and slower increase of effects with dose for resistant cells were common features for the cytotoxicity and MOAB binding.

The differences in the binding of MOAB F7-26 in sensitive and resistant cells are illustrated by fluorescence histograms and double-parameter contour plots (Figs. 3 and 4). Immunofluorescence of A2780^S cells treated with L-PAM increased significantly, whereas fluorescence of A2780^R cells was only

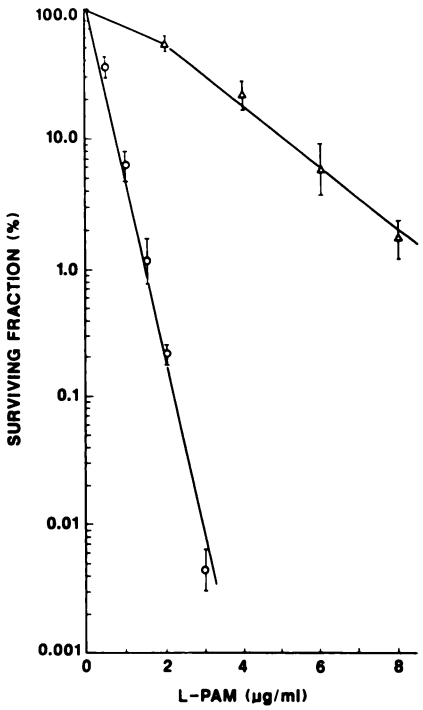

Fig. 1. Survival of A2780^S (○) and A2780^R (△) cells following exposure to L-PAM for 1 h.

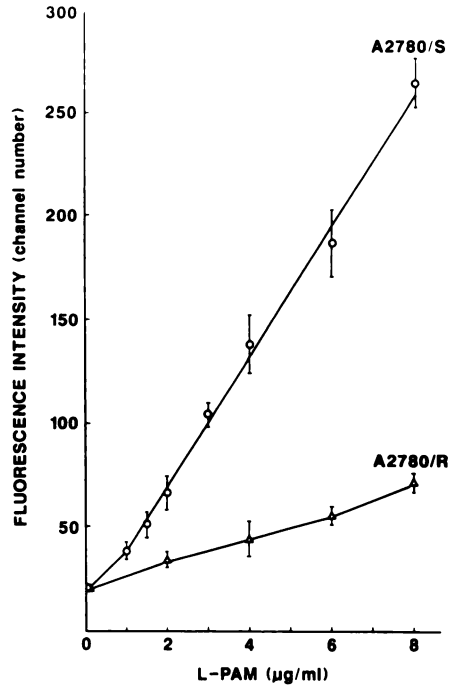

Fig. 2. Effects of L-PAM on the binding of MOAB F7-26 to sensitive and resistant cells. Cultures of A2780^S and A2780^R cells were treated with L-PAM for 1 h, fixed, stained with MOAB F7-26, and measured on a flow cytometer.

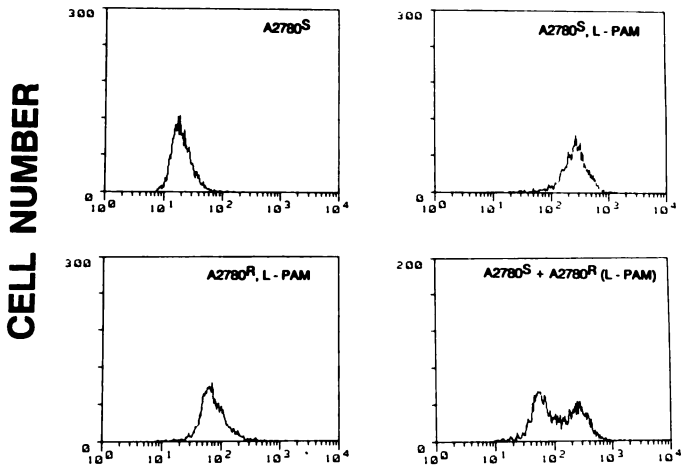

Fig. 3. Fluorescence histograms of untreated A2780^S cells and of A2780^S and A2780^R cells treated with 8 μg/ml L-PAM for 1 h. *Lower right panel,* sensitive and resistant cells were treated with L-PAM and mixed before staining.

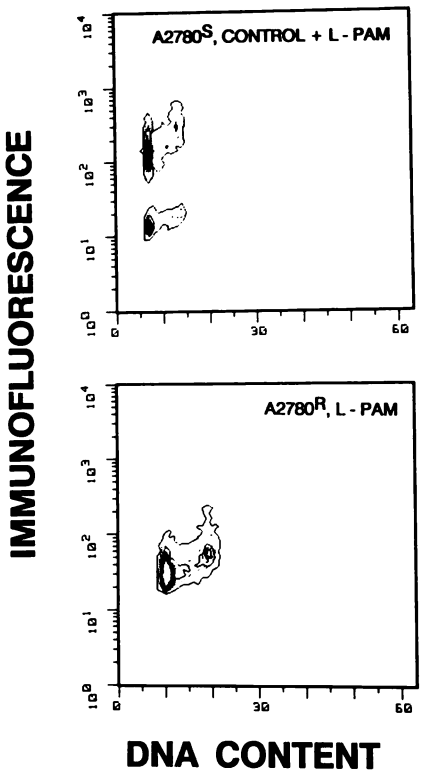

Fig. 4. Two parameter contour plots of A2780^S and A2780^R cells stained with MOAB F7-26 and propidium iodide. Cell cultures were treated with 8 μg/ml L-PAM for 1 h. A2780^S cells were mixed with untreated (control) cells before staining.

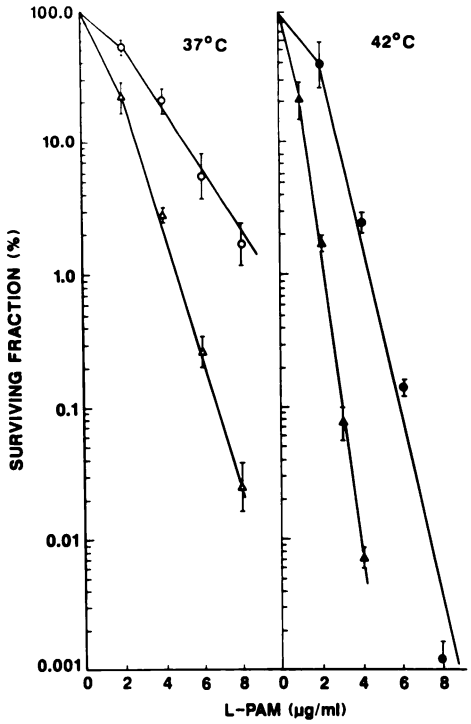

Fig. 5. Survival of A2780^R cells following treatment with L-PAM for 1 h (○); L-PAM for 1 h following pretreatment with 0.5 mM BSO for 24 h (△); L-PAM for 1 h at 42°C (●); and L-PAM for 1 h at 42°C following pretreatment with 0.5 mM BSO for 24 h (▲).

slightly above background levels. Cell populations of sensitive and resistant cells were separated on fluorescence histograms by approximately 1 log difference in fluorescence intensity.

Sensitization of L-PAM-resistant Cells. The effects of BSO and hyperthermia on the survival of A2780^R cells exposed to L-PAM are shown in Fig. 5 and summarized in Table 1. BSO and hyperthermia increased cytotoxicity of L-PAM, in agreement with observations of others (10–14). The maximal decrease of resistance was observed after treatment with L-PAM combined with both BSO and hyperthermia.

The enhancement of L-PAM cytotoxicity was accompanied by a proportional increase of MOAB binding to DNA. Fluorescence was higher in the cells treated with L-PAM combined with BSO or hyperthermia than in the cells treated with L-PAM alone (Figs. 6 and 7). The highest fluorescence and the largest sensitization were observed when BSO pretreatment was combined with L-PAM treatment at hyperthermic conditions.

The enhancement factors calculated from dose-survival curves (Fig. 5) and from dose-fluorescence curves (Fig. 6) were similar (Table 1). These data show that the same relative potential of treatment regimens was established by assessment of cell survival and by FCM analysis of MOAB binding.

DNA Repair. The loss DNA immunoreactivity with MOAB F7-26 was studied as a function of time following drug removal in cultures of sensitive and resistant cells. The doses of L-PAM were selected to achieve approximately similar fluorescence in A2780^S and A2780^R cells after 1 h treatment with L-PAM. The time course of DNA repair, as measured by MOAB F7-26 binding, was similar in A2780^S and A2780^R cells (Fig. 8). Fluorescence had already decreased after 3 h in drug-free medium. Most fluorescence was eliminated during 24 h after drug removal. Thus, the resistance to L-PAM in A2780^R cells was not associated with significant changes in the rate of repair of DNA lesions detected with MOAB F7-26.

Table 1 *Effect of L-PAM on cell survival and MOAB F7-26 binding in A2780^R cells*

Treatment	Cell survival		Fluorescence	
	D_{10} (μg/ml)	Enhancement factor	Channels (per μg/ml)	Enhancement factor
L-PAM	4.26	1.0	6.0	1.0
L-PAM + BSO	2.05	2.1	14.0	2.3
L-PAM 42°C	1.46	2.9	16.0	2.7
L-PAM 42°C + BSO	0.85	5.0	34.0	5.7

Relationship of Fluorescence to Cytotoxicity. To determine the quantitative relationship between the binding of MOAB F7-26 and cytotoxicity of L-PAM, fluorescence was plotted against $\log_{10}$ surviving fraction (Fig. 9). For A2780^S cells, the results of the colony inhibition assay were compared with fluorescence measurements of cells treated with L-PAM (Figs. 1 and 2). To obtain a similar range of cell killing for A2780^R cells, the survival and fluorescence data after treatment with L-PAM alone, and L-PAM with BSO and hyperthermia, were combined (Figs. 5 and 6).

There was a linear relationship between cell killing and fluorescence in A2780^R cells. Thus, the amount of DNA damage per log cell killing remained constant for resistant cells at all levels of sensitization. These data show that the effectiveness of modulating factors can be predicted by measurements of fluorescence.

At a given level of fluorescence intensity, more cell killing was observed in sensitive than in resistant cells (Fig. 9). For example, after L-PAM treatment which induced fluorescence intensity equal to 105 channels, surviving fraction was −4.4 $\log_{10}$ for A2780^S and −2.6 $\log_{10}$ for A2780^R cells. Treatments which decreased cell survival by 4 $\log_{10}$ induced fluorescence equal to 96 channels in sensitive cells and equal to 144 channels in resistant cells. From data shown in Fig. 9, it was calculated that fluorescence increased by 21 channels for each additional

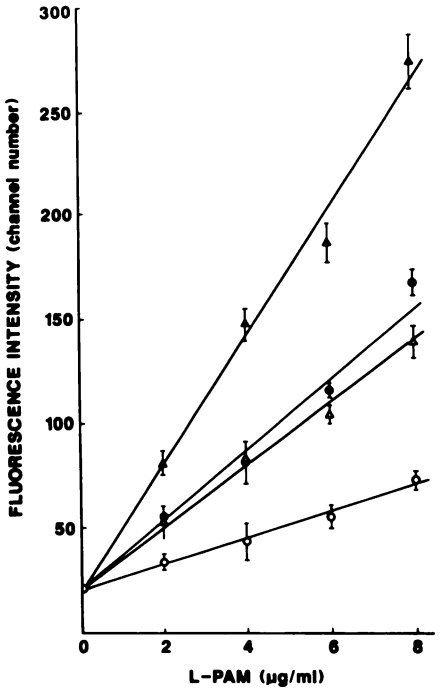

Fig. 6. Fluorescence of A2780^R cells treated with L-PAM for 1 h at 37°C (○); L-PAM for 1 h at 42°C (●); L-PAM for 1 h at 37°C following pretreatment with BSO (△); and L-PAM for 1 h at 42°C following pretreatment with BSO (▲).

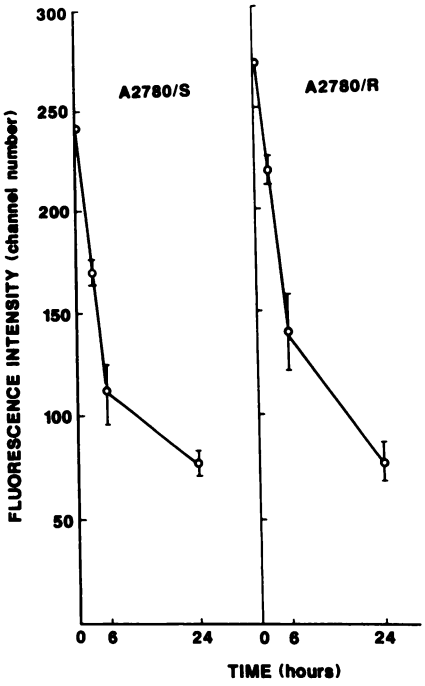

Fig. 8. Changes in DNA immunoreactivity with MOAB F7-26 following removal of L-PAM. A2780^S cells were treated with 8 μg/ml L-PAM for 1 h. A2780^R cells were treated with 12 μg/ml L-PAM for 1 h following pretreatment with 0.5 mM BSO for 24 h.

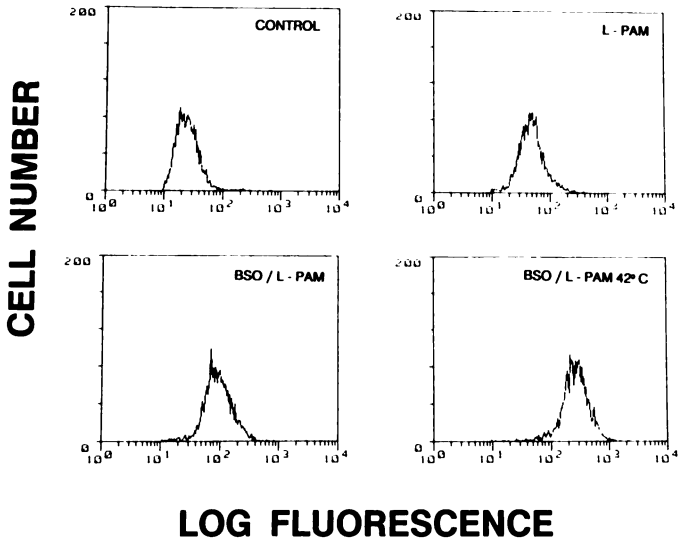

Fig. 7. Fluorescence histograms of A2780^R cells treated with L-PAM combined with BSO and hyperthermia.

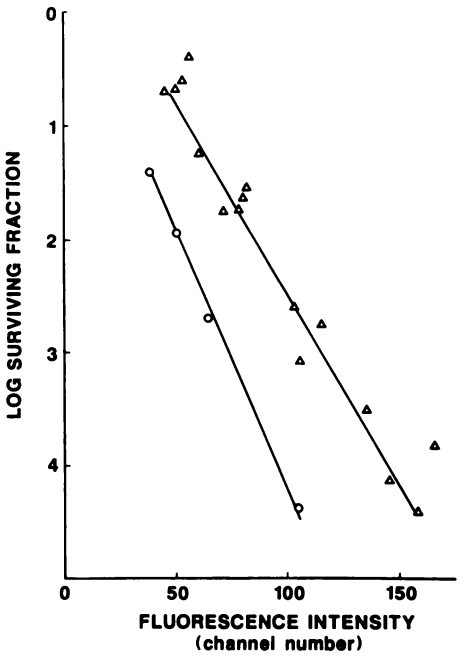

Fig. 9. Relationship between DNA immunoreactivity with MOAB F7-26 and cytotoxic effects of L-PAM in A2780^S (○) and A2780^R (△) cells. The $\log_{10}$ surviving fraction was determined from the data presented in Figs. 1 and 5. Fluorescence data include results presented in Figs. 2 and 6.

$\log_{10}$ cell killing in A2780^S cells and by 30 channels in A2780^R cells. Thus, cytotoxicity per unit of DNA damage, measured by fluorescence of MOAB F7-26, was higher in sensitive than in resistant cells.

DISCUSSION

Treatment of cells with alkylating agents induced binding of MOAB F7-26 to DNA *in situ*. The biological value of this reaction is supported by a strong correlation between the immunoreactivity and cytotoxicity and by the ability of fluorescence measurements to detect resistance and to predict the effectiveness of sensitization in resistant cells.

MOAB F7-26 specifically reacted with deoxycytidines on a single-stranded DNA. It is hypothesized that alkylation of guanines (15, 16) decreased the stability of double helix and increased the access of MOAB to deoxycytidine on the opposite DNA strand. Thus, the intensity of antibody binding probably reflects the amount of monoadducts. This hypothesis is supported by biochemical studies which indicated that the stability of DNA conferred by normal base pairs decreased as the percentage of alkylated bases increased (15).

The binding of MOAB F7-26 to the cells treated with alkylating agent rapidly decreased after L-PAM removal. This time course is different from the kinetics of DNA cross-links, which

are not detected immediately after L-PAM exposure but achieve maximum 6 h later (17, 18). Thus, MOAB F7-26 provides additional approaches for the evaluation of DNA damage: detection of damage directly related to monoadduct formation and evaluation of DNA damage at a single-cell level.

An essential part of MOAB F7-26 methodology was the amplification of single-strandedness in alkylated DNA by heating in the presence of Mg^{2+}. Such a procedure was necessary to increase the sensitivity of MOAB staining for the detection of DNA damage. The ability of Mg^{2+} to stabilize DNA against thermal denaturation was demonstrated by biochemical and cytochemical methods (19–21). In our experiments, presence of Mg^{2+} during heating prevented thermal denaturation in control cells, as defined by the binding of single strand-specific MOAB F7-26, but had only a limited effect on the decreased stability of alkylated DNA. Lower stability of alkylated DNA toward heating was also observed in biochemical studies (22). The amplification of radiation-induced single-strandedness significantly increased the sensitivity of immunochemical measurements of DNA damage (23). Probably, mild denaturation is an essential step in the application of antibodies for effective evaluation of DNA damage.

The rate of DNA repair as characterized by the loss of immunoreactivity was similar in sensitive and resistant cells. The initial level of MOAB F7-26 fluorescence was the best predictor of cytotoxicity and sensitization of resistant cells to L-PAM.

The relation between DNA damage and cytotoxicity was different in sensitive and resistant cells. A similar degree of DNA damage detected by MOAB F7-26 was accompanied by a higher level of cell killing in sensitive than in resistant cells. Thus, the resistance to L-PAM in A2780 cells was associated with two factors: low initial level of DNA damage and decreased cytotoxicity per unit of DNA damage. The study of DNA cross-links also demonstrated that, in some cases, resistance to alkylating agents is accompanied by a higher tolerance to DNA damage and lower cytotoxicity per unit of damage (24–26).

The essential conclusions of this study are that cells resistant to alkylating agents can be distinguished from sensitive cells by staining with MOAB F7-26 and that the sensitization of resistant cells can be quantitatively predicted by FCM analysis of antibody binding.

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