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NOVEL DYNAMIC FLOW CYTOENZYMOLOGICAL DETERMINATION OF INTRACELLULAR ESTERASE INHIBITION BY BCNU AND RELATED ISOCYANATES

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Abstract—We present a novel dynamic flow cytoenzymological demonstration of the potent inhibition by the antitumour chloroethylnitrosourea BCNU of the intracellular hydrolysis of fluorescein diacetate by esterases of viable, intact murine and human tumour cells *in vitro*. The BCNU metabonate chloroethyl isocyanate and the related compound *n*-butyl isocyanate were also potent inhibitors. I_{50} values were in the range 4.2×10^{-5}–2.0×10^{-4} M. Generally similar quantitative results were obtained for intact cells and sonicates by conventional spectrofluorimetry, and inhibition of purified porcine liver carboxyl esterase (EC 3.1.1.1) was demonstrated. Little or no inhibitory activity was seen with the alkylating agents methyl methane-sulphonate, melphalan and nitrogen mustard. The results are consistent with carbamoylation of the esterase molecules by isocyanates, and this may provide a basis for the flow cytometric determination of intracellular carbamoylation in discrete subpopulations of heterogeneous samples.

Chloroethylnitrosoureas (CNUs) are an important class of antitumour agent, exhibiting activity against a variety of human malignancies including lymphomas, brain tumours, melanomas, and lung tumours [1–3]. BCNU (1,3-bis[2-chloroethyl]-1-nitrosourea), CCNU (1-[2-chloroethyl]-3-cyclohexyl-1-nitrosourea, and methylCCNU (1-[2-chloroethyl]-3-[*trans*-4-methylcyclohexyl]-1-nitrosourea), are commercially available and others continue to be developed [4, 5]. A major goal has been to reduce toxicity, particularly myelosuppression, and thereby improve therapeutic ratio.

CNUs rapidly decompose under physiological conditions giving rise to two types of reactive species. Alkylating fragments (chloroethyldiazohydroxides and chloroethylcarbonium ions) are formed, together with organic isocyanates (Fig. 1) [6]. Both are capable of rapid and irreversible binding to cellular macromolecules. Whilst alkylation of DNA is considered the main mechanism of antitumour action, carbamoylation of cellular proteins also has important pharmacological consequences [7, 8]. Carbamoylation has been implicated in inhibition of macromolecular synthesis and RNA processing [8]; interference with DNA repair and enhancement of radiation cytotoxicity [8]; and inactivation of a number of enzymes [9–14].

Carbamoylating activity was analysed originally by measuring the extent of reaction with the amino group of C^{14}lysine after separation by paper chromatography [15], and more recently by reaction with the amino group of 5′-amino-5′-deoxythymidine using HPLC [16]. Inactivation of glutathione reductase has been used to determine the protein carbamoylation potential of CNUs [13]. Other enzymes shown to be inhibited by CNUs or isocyanates include DNA polymerase II [12], alcohol dehydrogenase [11], transglutaminase [10], and the serine proteases trypsin, elastase and chymotrypsin [9, 13]. Results obtained with the lysine assay were not always in agreement with those for glutathione reductase inhibition [14], raising the possibility that carbamoylation of specific targets may predominate over non-specific reactions *in vivo*. Assays based on inhibition of enzymes may therefore have the advantage of greater biological relevance.

We wished to develop an assay for carbamoylation by chloroethylnitrosoureas which would exploit the advantages of reaction with a target nucleophile located not only at a particular amino acid in the active site of the enzyme but also in an intracellular environment, and in addition would utilize the capability of flow cytometry to analyse individual cellular subpopulations in heterogeneous samples. In view of the proven carbamoylation of serine hydrolases

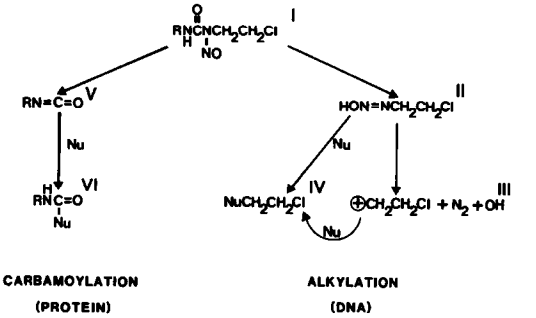

Fig. 1. Decomposition pathways of chloroethylnitrosoureas (I). Alkylating fragments, chloroethyldiazohydroxides (II) and/or chloroethyl carbonium ions (III), react with nucleophiles (Nu), particularly in DNA, to form alkylated products (IV). Organic isocyanates (V) react with Nus, particularly in intracellular proteins, to give carbamoylated products (VI).

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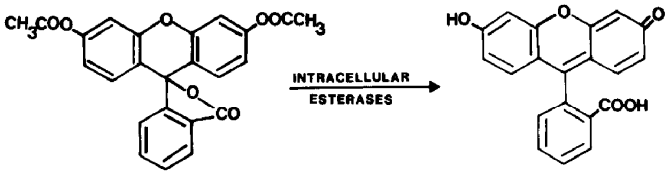

I II

Fig. 2. Esterase enzyme reaction. Intracellular esterases convert the non-fluorescent, lipophilic substrate fluorescein diacetate (I, FDA) to the fluorescent, polar product fluorescein (II) which accumulates in the cell.

by isocyanates [9] and the availability of a flow cytometric assay for cellular esterases in viable, intact cells using the fluorogenic substrate fluorescein diacetate (FDA) (Fig. 2) [18, 19], we selected this reaction as a potential probe for intracellular carbamoylation. Here we describe the use of the flow cytoenzymological technique to demonstrate inhibition of cellular esterases by BCNU, its isocyanate metabonate chloroethyl isocyanate (CEI) [5], and the related compound *n*-butyl isocyanate (n-BI) in cultured EMT6 mouse mammary tumour cells and NCI-H69 human small cell lung cancer cells.

MATERIALS AND METHODS

Cells

EMT6/CaVJAC. This cell line is a tissue-culture adapted derivative of the EMT6 mouse mammary tumour [20]. Monolayers were seeded at 10^5 per 25 cm^2 tissue culture flask (Sterilin), and harvested in log phase by trypsinization 2 days later. Cells were grown in Eagle's Minimal Essential medium with Earle's salts supplemented with glutamine, antibiotics and 20% new born calf serum (all Gibco) and in an atmosphere of 8% CO_2/air. The serum was previously heat-treated (65° for 35 min). For flow cytometry single cell suspensions (10^6 cells per ml) were prepared in medium to neutralize the trypsin. For conventional spectrofluorimetry, medium was then removed by centrifugation and cells washed and suspended in phosphate buffered saline (Dulbecco "A"; PBS, Gibco).

NCI-H69. This cell line was supplied by Dr D. Carney of the US NCI-Navy Medical Oncology Branch. The cells were grown as free-floating aggregates and maintained in RPMI 1640 medium supplemented with 10% foetal calf serum, glutamine and antibiotics (Gibco) and in an atmosphere of 8% CO_2/air. They were seeded in 25 cm^2 tissue culture flasks (Falcon) and harvested in log phase 2 days later. After centrifugation cells were incubated with trypsin/versene (37° for 15 min), following which the trypsin was neutralized by medium. Single cell suspensions were prepared at 10^6 cells per ml, in medium for flow cytometry or PBS for conventional spectrofluorimetry.

For both lines, log phase status was confirmed by flow cytometric analysis using ethidium bromide and Triton-x-100 [21, 22].

Reagents

Fluorescein diacetate (Koch Light) was dissolved at 0.012 M in grade A acetone (Interchem). This was prepared fresh weekly and stored at 4° in the dark. Immediately before addition to cell suspensions, 33.4 μl stock solution was diluted to 100 ml with PBS to give a 2 μM working solution.

BCNU was obtained from the US NCI, and CEI and n-BI from Sigma. Solutions of BCNU and CEI (0.04 M) and n-BI (0.02 M) were made in grade A ethanol (James Burrows). All were stored at −20° and dilutions were made in PBS immediately before use. Melphalan (Chester Beatty Institute), nitrogen mustard (Boots), and methyl methane-sulphonate (MMS, Aldrich Chemical Co.) were prepared immediately before addition to cells; the former was dissolved in 2% HCl (0.1 M) in absolute ethanol and diluted with PBS, while the latter two reagents were dissolved directly in PBS.

Partially purified porcine liver carboxyl esterase (EC 3.1.1.1) was obtained from Sigma as a suspension in 3.2 M $(NH_4)_2SO_4$, pH 8, and containing 10.71 mg protein per ml. It was diluted in PBS immediately before use to 1 μg protein per ml.

Flow cytometry

The dual laser system was designed and built in this laboratory. Full details of the instrumentation, data acquisition and data handling are given elsewhere [19, 23, 24]. Fluorescein produced by the esterase reaction was excited at 488 nm, giving rise to green/yellow fluorescence. The fluorescence emission from each cell was recorded, together with time (from the computer clock) and forward and right angle scatter. Forward scatter provides a measure of size and comparison with right angle scatter gives an indication of cell viability.

In each assay the flow cytometer was instructed to analyse 50,000 cells in a reaction time of 5 min, the flow rate being 166 cells per sec. Aliquots of single cell suspensions (150 μl, 10^6 cells/ml) were placed in Eppendorf tubes (1 ml) and gassed with 5% CO_2/air. Equal volumes of drug solutions were added usually 1 hr prior to analysis and the tubes were regassed and held at room temperature. The reaction was started by addition of 300 μl FDA (2 μM). After rapid mixing the reaction mixture was pumped into the flow cell and sampled continuously at room temperature (20–22°). Data collection was initiated after a preset 20 sec dead time. Untreated control samples were run at regular intervals during the experiment to check stability. Appropriate solvent controls gave identical results to the untreated samples. Data were displayed as 2-D contour maps of fluorescence versus

time and as 3-D plots of fluorescence versus time versus size (e.g. Fig. 3A). Data were analysed as the medians of eight sequential fluorescence versus frequency histograms. These were plotted against time to generate reaction progress curves (e.g. Fig. 3B) from which enzyme reaction velocities were determined by linear regression analysis. Percentage activity remaining was calculated by comparing control and treated samples, and concentrations producing 50% inhibition (I_{50}), with 95% confidence limits, were determined by probit analysis using the GLIM statistical programs of the The Royal Statistical Society of London. In some experiments the period of exposure to BCNU was varied between 0–200 min and in others the serum concentration was varied from 0 to 5%. A more detailed description of the flow cytoenzymological assay is given elsewhere [19].

Conventional spectrofluorimetry

Parallel studies were performed using an MPF-4 spectrofluorimeter (Perkin-Elmer), with monochromators set at 490 nm and 520 nm for excitation and emission respectively, and with a band width of 4 nm, a chart speed of 20 mm/min, a range of 5 mV and a sensitivity of 3–100. The effect of BCNU and CEI on FDA hydrolysis was determined in intact EMT6 cells and their sonicates. Cell sonicates were prepared by pulsing cells on ice for 3 × 10 sec at an amplitude of 12 μm peak to peak. All samples were kept on ice and brought to room temperature (20–22°) 16 min before addition of drug. Aliquots of drug solution (200 μl) were added to an equal volume of cell suspension or sonicate 1 hr prior to analysis. The background fluorescence due to substrate was measured. The reaction was then initiated by adding

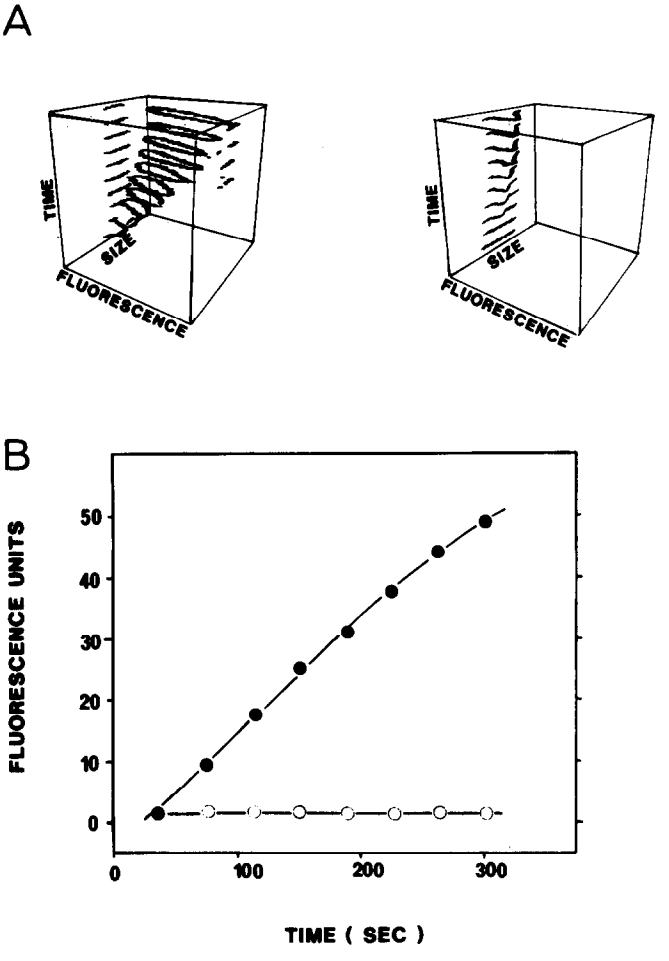

Fig. 3. Flow cytometric monitoring of FDA (1 μm) hydrolysis by intracellular esterases of EMT6 mouse mammary tumour cells, and the effect of BCNU on this reaction. (A) 3-D displays of fluorescence vs time vs cell size. The left-hand panel shows accumulation of fluorescein with time in the absence of inhibitor and the right-hand panel shows the effect of 1 hr pre-exposure to 10^{-3} M BCNU. (B) Progress curves for the above data sets. ●, control; ○, BCNU. Data points are the median values of 6.25×10^3 cells. Results are from a typical experiment.

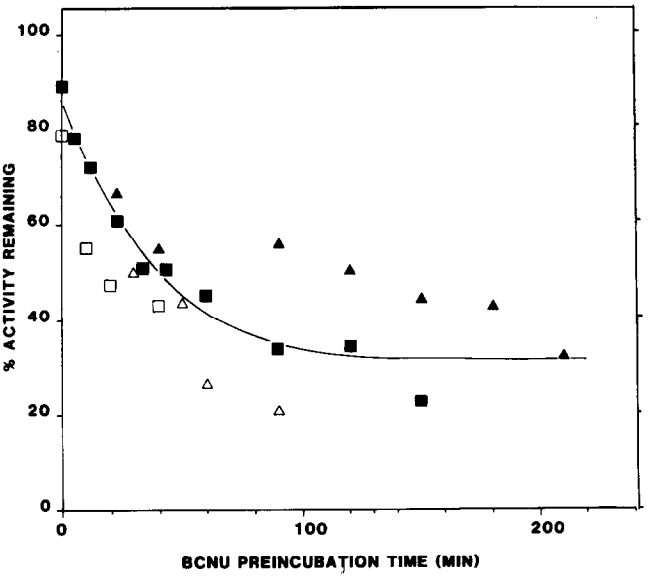

Fig. 4. Effect of pre-exposure time to BCNU (4×10^{-4} M) on the rate of FDA (1 μm) hydrolysis by EMT6 cells as measured by flow cytometry. Different symbols denote four independent experiments.

300 μl preincubation mixture to an equal volume of FDA (2 μM), with rapid mixing in the cuvette of the instrument. The reaction was monitored continuously for 8 min at room temperature. Appropriate solvent controls were included throughout. The initial reaction velocity was determined by the gradient of the linear increase in reaction rate in the first min of the reaction, and % activity remaining and I_{50} were calculated as above. Purified liver carboxyl esterase was assayed identically, using a final concentration of 0.25 μg/ml.

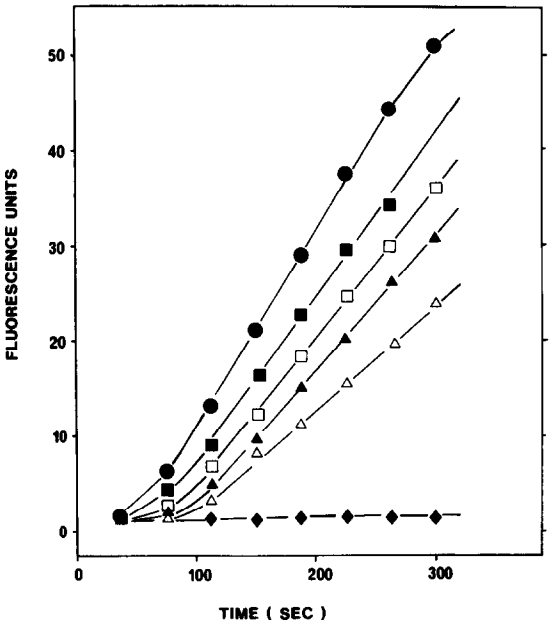

Fig. 5. Effect of a 1 hr exposure to different BCNU concentrations on the hydrolysis of FDA (1 μm) by H69 cells as measured by flow cytometry. Enzyme progress curves are shown for varying inhibitor concentrations. ●, control; ■, 2×10^{-7} M; □, 2×10^{-6} M; ▲, 2×10^{-5} M; △, 2×10^{-4} M; ◆, 2×10^{-3} M. Data points are the median values of 6.25×10^3 cells. Results shown are from one of three similar independent experiments.

RESULTS

Flow cytometry

Figure 3A shows a typical 3-D display of cellular fluorescence versus time versus cell size for the hydrolysis of FDA by EMT6 cells, either with or without pre-exposure to BCNU (1 mM for 1 hr). It can be seen that the frequency contours traverse the 3-D data space as the intracellular reaction product fluorescein accumulates. As predicted from studies on related hydrolytic enzymes (see Introduction), the reaction was markedly inhibited by BCNU. It can also be seen that cell size was unaffected either by the accumulation of fluorescein or by pre-exposure to BCNU. The corresponding reaction progress curves for the same data set are shown in Fig. 3B. As shown previously [19], control progress curves were linear following a short lag phase. Qualitatively similar curves were obtained in all experiments with both EMT6 and H69 cells. Inhibition by BCNU (1 mM for 1 h) was essentially complete.

Figure 4 illustrates the effect of varying the preincubation time on the inhibitory activity of BCNU (4×10^{-4} M). A progressive decrease in esterase activity is seen from 0 to 60 min. Longer preexposure times had proportionally less effect on enzyme activity, and variability was much greater, possibly as a result of reduced cell viability. About 60% inhibition was seen with a 1 hr pre-exposure and this time was used in all subsequent studies.

When serum was omitted from both the preexposure period and the enzyme reaction, the inhibitory activity of BCNU (10^{-4}–2×10^{-4} M) differed by only 3–12% as compared to results obtained with

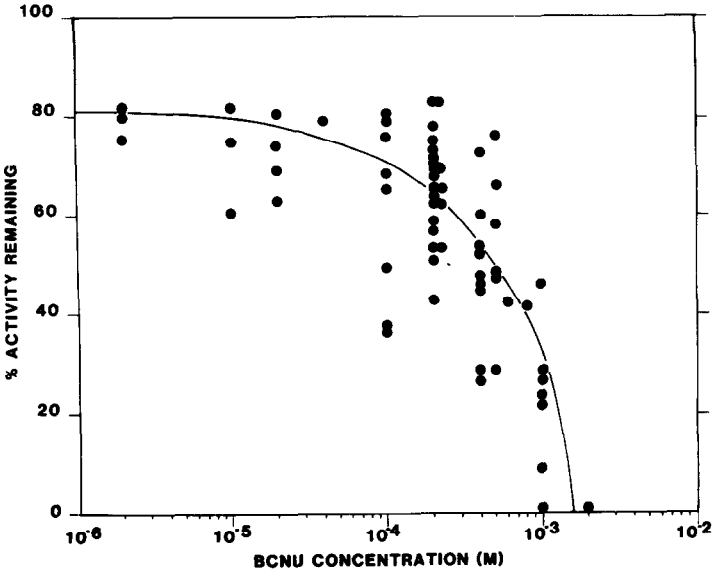

Fig. 6. Dose–response curve for the inhibitory effect of BCNU on the hydrolysis of FDA (1 μm) by EMT6 cells as measured by flow cytometry. Cells were pre-exposed to BCNU for 1 hr. Each point is the mean of duplicate values and results shown are pooled from 11 independent experiments.

serum included throughout (three experiments). Serum was included in all subsequent experiments, as this tended to improve cellular enzyme stability [19].

Figure 5 shows typical progress curves for control and BCNU-treated samples for H69 cells. A concentration-dependent inhibition of FDA hydrolysis was demonstrated in both EMT6 and H69 cell lines (Figs 5 and 6). The dose–response profile for BCNU inhibition of EMT6 cell esterases shows a gradual decrease in enzyme activity from 2×10^{-6} M–10^{-4} M, followed by a sharp decline in activity to complete inhibition at 2×10^{-3} M BCNU (Fig. 6). Similar data (not shown) were obtained for H69 cells. I_{50} values differed by a factor of 5 between EMT6 and H69, more potent inhibition occurring in the human cell line (Table 1).

CEI, the isocyanate decomposition product of BCNU [5], was also shown to cause concentration-dependent inhibition of FDA hydrolysis in EMT6 cells but the dose–response curve exhibited a steeper slope (Fig. 7). The I_{50} value for CEI (Table 1) was, however, closely similar to that of the parent drug.

The simple alkyl isocyanate n-BI also inhibited esterase-catalysed FDA hydrolysis by EMT6 cells in a concentration-dependent fashion. Again, the dose-response profile was of similar shape to those above but with the sharp decline in enzyme activity occurring in the range 8×10^{-6}–2×10^{-4} M. The I_{50} value obtained was fourfold lower than that for BCNU and twofold lower than that for CEI (Table 1).

In several repeat experiments MMS, melphalan and nitrogen mustard each exhibited minimal effect on the reaction following 1 h exposures to concentrations up to 10^{-3} M. For example at the latter concentration the mean % activity remaining (two experiments) was 90% for MMS, 93% for melphalan and 100% for nitrogen mustard.

Table 1. I_{50} values for BCNU, chloroethyl isocyanate (CEI), and *n*-butyl isocyanate (n-BI) inhibition of FDA hydrolysis (1 μm) by EMT6 and H69 cells—comparison of results by flow cytometry and conventional spectrofluorimetry

	I_{50}* (M)
Intact cells by flow cytometry	
BCNU (EMT6)	2.0×10^{-4} (1.5×10^{-4}–2.6×10^{-4})
BCNU (H69)	4.2×10^{-5} (2.4×10^{-5}–7.3×10^{-5})
CEI (EMT6)	1.2×10^{-4} (9.7×10^{-5}–1.5×10^{-4})
n-BI (EMT6)	4.8×10^{-5} (4.2×10^{-5}–5.6×10^{-5})
Intact cells by spectrofluorimetry	
BCNU (EMT6)	7.5×10^{-5} (4.6×10^{-5}–1.2×10^{-4})
CEI (EMT6)	2.4×10^{-5} (1.6×10^{-5}–3.5×10^{-5})
Cell sonicates by spectrofluorimetry	
BCNU (EMT6)	4.1×10^{-5} (2.4×10^{-5}–6.8×10^{-5})
CEI (EMT6)	1.2×10^{-5} (8.7×10^{-6}–1.7×10^{-5})

* 95% confidence limits in parentheses.

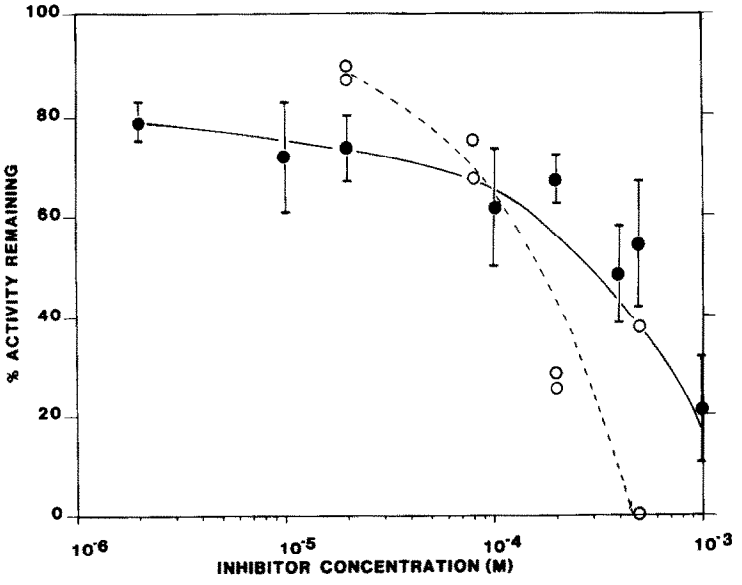

Fig. 7. Dose–response curves for the inhibitory effect of BCNU and CEI on hydrolysis of FDA (1 μm) by EMT6 cells as measured by flow cytometry. Cells were pre-exposed to inhibitor for 1 hr. For BCNU (●), results are the average of 3–14 repeat experiments with bars indicating 2 SE. For CEI (○), results shown are for two independent experiments and each point is the mean of duplicate values.

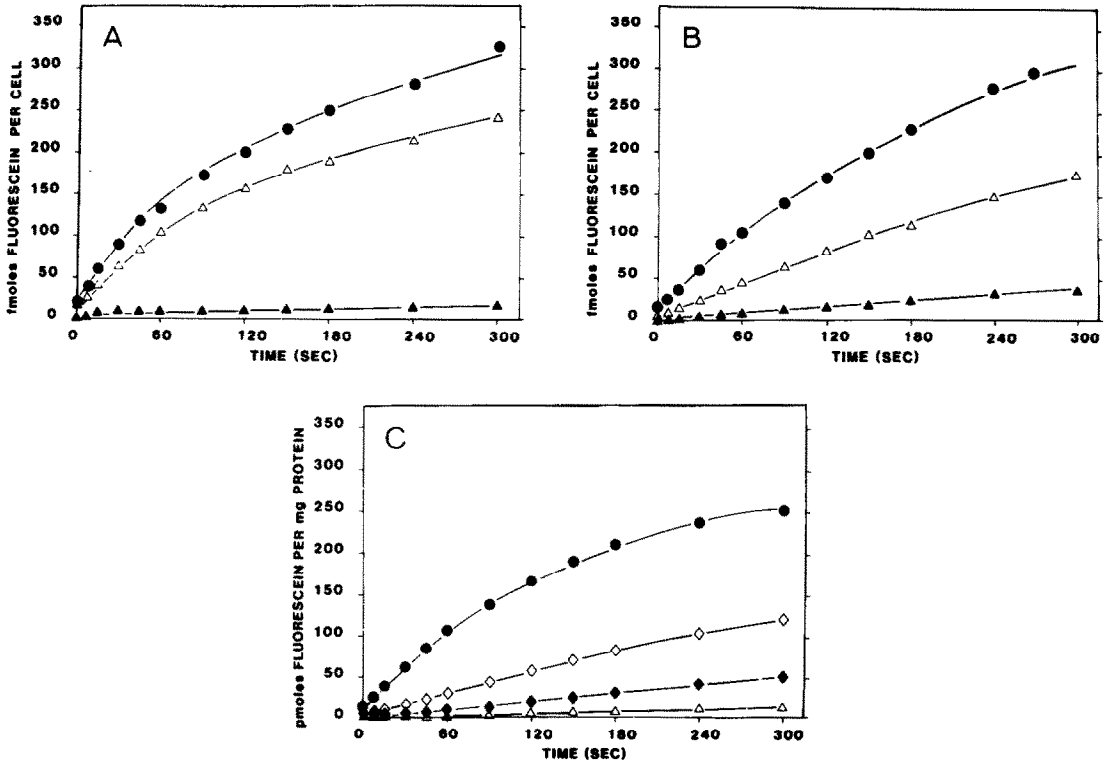

Fig. 8. Enzyme reaction progress curves for the hydrolysis of FDA (1 μm) by (A) EMT6 sonicates, (B) intact EMT6 cells, and (C) purified porcine liver carboxyl esterase, with and without BCNU (1 hr pre-exposure) as measured by conventional spectrofluorimetry. ●, Control; ◇, 2×10^{-6} M; ◆, 2×10^{-5} M; △, 2×10^{-4} M; ▲, 10^{-3} M. Results shown are from a typical experiment and each point is the mean of duplicate values.

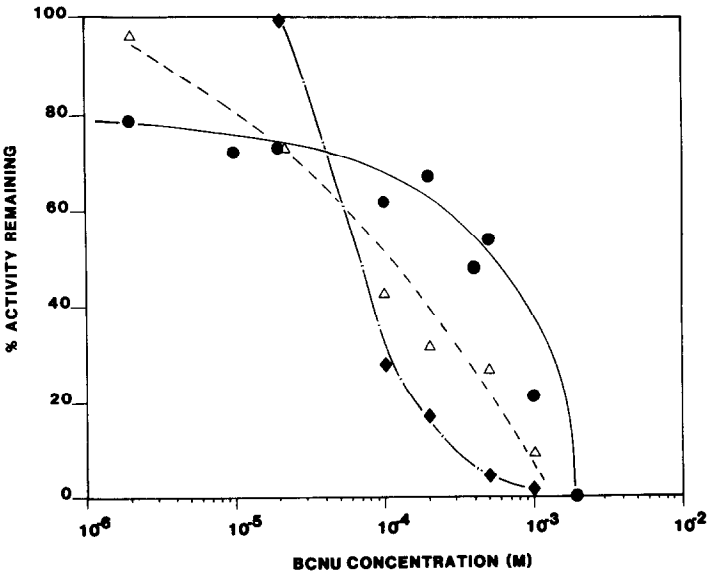

Fig. 9. Dose–response curves for BCNU inhibition of hydrolysis of FDA (1 μm) by intact EMT6 cells and their sonicates: comparison of flow cytometric and spectrofluorimetric assay. Cells were pre-exposed to BCNU for 1 hr. ●, intact cells measured by flow cytometry; △, intact cells measured by spectrofluorimetry; ◆, sonicates measured by spectrofluorimetry. Results shown are mean values from 2–14 independent experiments.

Conventional spectrofluorimetry

Reaction progress curves for FDA hydrolysis by intact EMT6 cells, sonicates, and purified porcine liver carboxyl esterase are shown in Fig. 8. Progress curves for intact cells were linear over the first 2 min followed by a gradual decrease in rate. Cell sonicates exhibited an initial linear rate over the first minute of the reaction, followed by a second slower and more prolonged linear phase. The initial reaction rates in sonicates were always greater than those for corresponding intact cells, and reaction velocities were calculated from these initial rates.

Using 1 hr pre-exposures as above, BCNU showed concentration-dependent inhibition of enzyme activity for both intact EMT6 cells and sonicates (Figs 8 and 9). For intact cells, the dose–response curve was similar in shape to that obtained for the same cells by flow cytometry (Fig. 9), although the I_{50} measured by spectrofluorimetry was 2.7-fold lower (Table 1). The dose–response relationship was rather different for sonicates, with a more dramatic decline in activity between 2×10^{-5} M and 10^{-4} M (Fig. 9). The I_{50} value for sonicates was 1.8- and 4.9-fold lower than that for intact cells measured by conventional spectrofluorimetry and flow cytometry respectively. Interestingly, however, the extent of BCNU inhibition in sonicates was very similar to that in intact cells where both were measured by conventional spectrofluorimetry and where the activity for sonicates was calculated from the second linear phase of the progress curve (data not shown).

Reaction progress curves for FDA hydrolysis by purified esterase were similar in shape to those of EMT6 cells (Fig. 8). BCNU exhibited potent inhibition of this enzyme. The mean % activity remaining was 31% (one experiment) at 2×10^{-6} M, 10% (two experiments) at 2×10^{-5} M, and 2% (two experiments) at 2×10^{-4} M.

DISCUSSION

This paper describes the successful application of a novel dynamic flow cytoenzymological procedure [19], to determine the inhibition of intracellular esterases of viable, intact EMT6 mouse mammary tumour and H69 human small cell lung cancer cells *in vitro* by the chloroethylnitrosourea (CNU) antitumour drug BCNU, its metabonate chloroethylisocyanate (CEI), and the related compound *n*-butyl isocyanate (*n*-BI). Inhibition of various enzymes by nitrosoureas and isocyanates has been described in previous studies where conventional enzyme assays were employed (see Introduction and refs 9–14). Compelling evidence for active site-directed inactivation by nitrosoureas and isocyanates was obtained for chymotrypsin [9, 13], elastase [9], alcohol dehydrogenase [11], transglutaminase [10], and glutathione reductase [14]. Carbamoylation occurred at active site serines for the two serine proteases, while reaction with active site sulphydryl groups occurred with alcohol dehydrogenase and transglutaminase. Where inhibition by CNUs was determined, carbamoylation was attributed to the derived isocyanates.

It was the sensitivity of the above serine proteases to inactivation by carbamoylating agents that led us to propose that the hydrolytic enzymes catalysing FDA hydrolysis would also be susceptible to inhibition by CNUs and isocyanates. In view of the previous results with serine hydrolases, the similarity in the inhibitory potency of BCNU and CEI, the activity *n*-BI and the minimal effect of even quite high doses of alkylating agents MMS, melphalan and nitrogen mustard, it seems likely that the inhibition of FDA hydrolysis follows as a direct result of carbamoylation of the various hydrolases [25] involved in the catalysis of this reaction. In support of this

we have demonstrated BCNU inhibition of FDA hydrolysis by purified porcine liver carboxyl esterase. The flow cytoenzymological technique described here may provide a valuable alternative assay for intracellular protein carbamoylation, and further studies are in progress with a larger series of nitrosoureas and isocyanates to validate this possibility. In experiments carried out to date, we have found that chloroethylnitrosoureas shown to be weakly carbamoylating in other systems (e.g. *cis*-2-hydroxy CCNU, ref. 13) are comparatively ineffective as inhibitors of cellular esterases in our assay.

Compared to the use of cell-free systems, whole cell assays for enzyme inhibition provide the advantage that access to the intracellular enzyme is included as a contributing factor. Two further advantages of flow cytometric assays are that for a given population the median fluorescence can be determined rather than the mean value, which will be weighted in favour of the most active cells, and that multiple heterogeneous populations can be identified. Some differences were noted in the dose–response curves for inhibition of esterase activity using flow cytometric analysis of intact cells and spectrofluorimetric assay of whole cells or sonicates. Nevertheless the results were broadly in good agreement, and the similar behaviour of whole cells and sonicates suggests that intracellular penetration is not a limitation for the agents studied here. This is not, however, the case for more polar carbamoylating agents (unpublished data).

In summary, this novel dynamic flow cytoenzymological technique allows inhibition of intracellular esterases by chloroethylnitrosoureas and isocyanates to be determined in populations of viable, intact cells. Use of this technique may provide valuable information on the cellular pharmacology of such agents. Furthermore, a powerful advantage of flow cytometric analysis for future *in vivo* studies is the unique ability to identify multiple heterogeneous subpopulations differing in these and other biochemical properties.

REFERENCES

1. T. Wasserman, M. Slavik and S. Carter, *Cancer Treat. Rev.* **1**, 131 (1974).
2. R. B. Weiss and B. F. Issell, *Cancer Treat. Rep.* **9**, 313 (1982).
3. A. W. Prestakyo, L. H. Baker, S. T. Crooke, S. K. Carter and P. S. Schein (Eds.), in *Nitrosoureas*, Chap. 14–27, pp. 181–335. Academic Press, New York (1981).
4. A. W. Prestakyo, L. H. Baker, S. T. Crooke, S. K. Carter and P. S. Schein (Eds.), in *Nitrosoureas*, Chap. 32–34, pp. 379–408. Academic Press, New York (1981).
5. T. P. Johnston and J. A. Montgomery, *Cancer Treat. Rep.* **70**, 13 (1986).
6. M. Colvin and R. Brundrett, in *Nitrosoureas* (Eds. A. W. Prestakyo, L. H. Baker, S. T. Crooke, S. K. Carter and P. S. Schein), Chap. 4, pp. 43–48. Academic Press, New York (1981).
7. K. W. Kohn, L. C. Erickson, G. Laurent, J. Ducore, N. Sharkey and R. A. Ewig, in *Nitrosoureas* (Eds. A. W. Prestakyo, L. H. Baker, S. T. Crooke, S. K. Carter and P. S. Schein), Chap. 6, pp. 69–82. Academic Press, New York (1981).
8. H. E. Kann, in *Nitrosoureas* (Eds. A. W. Prestakyo, L. H. Baker, S. T. Crooke, S. K. Carter and P. S. Schein), Chap. 8, pp. 95–104. Academic Press, New York (1981).
9. W. E. Brown and F. Wold, *Biochemistry* **12**, 835 (1973).
10. M. Gross, N. K. Whetzel and J. E. Folk, *J. biol. Chem.* **250**, 7693 (1975).
11. J. Twu and F. Wold, *Biochemistry* **12**, 381 (1973).
12. B. B. Baril, E. F. Baril, J. Laszlo and G. P. Wheeler, *Cancer Res.* **35**, 1 (1975).
13. J. R. Babson, D. J. Reed and M. A. Sinkey, *Biochemistry* **16**, 1584 (1977).
14. J. R. Babson and D. J. Reed, *Biochem. biophys. Res. Commun.* **83**, 754 (1978).
15. G. P. Wheeler, B. J. Bowden, J. A. Grimsley and R. F. Struck, *Cancer Res.* **35**, 2974 (1975).
16. W. F. Brubaker, H. Zhao and W. H. Prusoff, *Biochem. Pharmac.* **35**, 2359 (1986).
17. B. Rotman and B. W. Papermaster, *Proc. natn. Acad. Sci. U.S.A.* **55**, 134 (1966).
18. J. V. Watson, S. H. Chambers and P. Workman, *FEBS Lett.* **81**, 179 (1977).
19. C. Dive, P. Workman and J. V. Watson, *Cytometry*, in press.
20. S. C. Rockwell, R. F. Kallman and L. E. Farjardo, *J. natn. Cancer Inst.* **49**, 735 (1972).
21. I. Taylor, *J. Histochem. Cytochem.* **28**, 1021 (1980).
22. J. V. Watson, S. H. Chambers and P. J. Smith, *Cytometry*, in press.
23. J. V. Watson, *Cytometry* **2**, 14 (1981).
24. J. V. Watson, *Cytometry* **1**, 143 (1980).
25. G. C. Guilbault and D. N. Kramer, *Analyt. Biochem.* **14**, 28 (1966).