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CAN FLOW CYTOENZYMOLOGY BE APPLIED TO MEASURE MEMBRANE-BOUND ENZYME KINETICS?

ASSESSMENT BY ANALYSIS OF γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY

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Abstract—We describe an improved technique which allows the analysis of enzyme reaction kinetics for γ-glutamyl transpeptidase (γ-GT) by flow cytometry. This is technically difficult because of the location of the enzyme on the external surface of the cell membrane leading to the rapid escape of the product. The reaction is determined by monitoring the conversion of γ-glutamyl aminomethylcoumarin to aminomethylcoumarin. Reaction kinetics are described for BL8 hepatocyte and JB1 hepatoma cells lines, together with inhibition kinetics for the active site-directed glutamine analogue L-(α-*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid. We show that it is possible to follow the reaction dynamics in a heterogeneous mixture of BL8 and JB1 cells allowing discrimination of the two cell types based on γ-GT activity. Improvements for further optimizing the assay of this important enzyme are suggested.

The aim of these studies was to develop a flow cytoenzymological assay to measure γ-glutamyl transpeptidase (γ-GT‡) reaction kinetics. This enzyme is of particular interest due to its association with neoplastic transformation [1] and its potential protective role against chemical insult [2]. Elevated levels in tumour subpopulations may provide a possible growth advantage resulting from increased capacity to maintain elevated glutathione levels and more efficient detoxification of electrophilic carcinogens and drugs [3, 4]. The ability to identify within viable heterogeneous cell samples subpopulations which differ in γ-GT activity (rather than simply levels of enzyme) is therefore especially important.

The active site of γ-GT is located on the external surface of the cell membrane [5]. This provides a technological challenge for flow cytometric analysis, difficulties with product diffusion having already been met with dynamic assay of membrane-bound phosphatases [6].

Previous assays of γ-GT include histochemical staining with γ-glutamyl naphthyl analogues [7]. Although the localization of γ-GT activity within tissue preparations can be explored using this type of assay, the potential for quantitation and kinetics is limited. Conventional biochemical assays for γ-GT have been applied which depend on colourimetric determination of γ-glutamyl naphthylamide or nitroanilide metabolism [8, 9]. γ-Glutamyl naphthylamide analogues have also been used for flow cytometric analysis of γ-GT activity, where a coupling reagent was required to trap the reaction product [10]. A much more sensitive spectrofluorimetric assay for γ-GT has been described [11] in which γ-glutamyl aminomethyl coumarin (γ-GAMC) is cleaved by γ-GT in the presence of an acceptor moiety (glycylglycine) to yield a fluorescent product aminomethylcoumarin (AMC) (Fig. 1). The latter assay was subsequently employed to measure cellular γ-GT levels by flow cytometry [12]. However, to our knowledge γ-GT reaction kinetics in individual intact cells have not yet been determined by flow cytometry.

We now describe the successful flow cytometric analysis of the reaction kinetics for the γ-GT reaction in cultured rat hepatoma and hepatocyte cells. Multiparametric resolution of the two populations allowed reaction kinetics to be monitored in both cell types simultaneously. Kinetics of inhibition by L-(α-*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT 125), an active side-directed glutamine analogue, are also described. Complementary studies using conventional spectrofluorimetry for intact cells, sonicates and purified γ-GT are included for comparison.

MATERIALS AND METHODS

Cell lines. BL8 is a rat hepatocyte cell line and JB1 a transformed rat cell line derived from an aflatoxin B1-induced hepatocellular carcinoma. These cells were kindly provided by the MRC Toxicology Unit (Carshalton, U.K.). They were

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‡ Abbreviations: γ-GT, γ-glutamyl transpeptidase; γ-GAMC, γ-glutamyl aminomethylcoumarin AMC, aminomethylcoumarin; AT 125, L-(α-*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; PBS, phosphate-buffered saline.

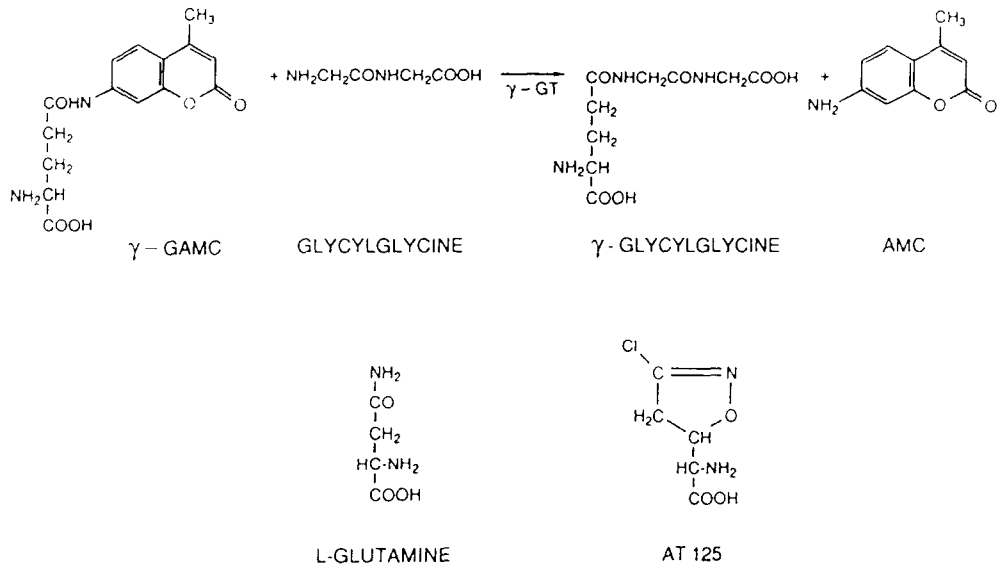

Fig. 1. Reaction scheme for the γ-GT-catalysed transfer of the γ-glutamyl group from the fluorogenic substrate γ-GAMC to the acceptor glycylglycine, releasing the fluorescent product AMC. Also shown are the structures of L-glutamine and its analogue, the active site-directed γ-GT inhibitor AT 125.

grown as monolayers attached to plastic and maintained in Williams' E Medium (Flow Laboratories, Irvine, U.K.), supplemented with glutamine, antibiotics (Gibco, Paisley, U.K.) and 5% foetal calf serum (Sera Laboratories, Crawley, U.K.), at 37° in an atmosphere of 5% CO_2/95% air. Cell monolayers were seeded on day 1 at 10^5/25-cm^2 flask and harvested on day 4 by trypsinization to yield log phase cells. Trypsinization was performed by removing the growth medium and washing monolayers twice with 5 mL trypsin solution (0.1%, Gibco) in phosphate-buffered saline (PBS, Dulbecco "A", pH 7.4, Oxoid, Basingstoke, U.K.). The trypsin was then removed and cells were incubated at 37° for 15 min. Medium was then added to neutralize the trypsin and single cell suspensions were made at 10^6 cells/mL medium for both flow cytometry and conventional spectrofluorimetry experiments.

Reagents. Partially purified γ-GT (EC 2.3.2.2) from bovine kidney (Sigma Chemical Co., Poole, U.K.) was stored at −20° and freshly prepared for each experiment as a solution in medium at 1 mg/mL. This was further diluted to 15 mg/mL in medium immediately prior to use.

γ-GAMC (Cambridge Research Biochemicals, Harston, U.K.) and AMC (Sigma) (see structures in Fig. 1) were prepared as stock solutions in methoxyethanol (Aldrich, Gillingham, U.K.) at 10 mM and stored at −20°. These stock solutions were freshly prepared each week and diluted with PBS immediately before use. The final solvent concentration was 2% (v/v).

AT 125 was kindly provided by the MRC Toxicology Unit. It was further stored at −20°, freshly prepared at 20 mM in PBS for each experiment and further diluted in PBS before addition to cells.

Conventional spectrofluorimetry. Details of instrumentation and general methodology for enzyme reaction and inhibition kinetics have been described in detail elsewhere [13, 14]. Analysis of the emission spectra of γ-GAMC and AMC (both at 100 μM in assay conditions) were performed with excitation wavelengths of 227, 356 and 370 nm (Fig. 2). Wavelengths of 356 and 440 nm were chosen for excitation and emission, respectively. These represent a compromise between the optimal product/substrate fluorescence ratio on one hand and the available flow cytometry wavelengths (see below) on the other. Reaction rates for culture medium alone with γ-GAMC at 100 μM were negligible. Substrate concentrations were varied from 12.5 to 100 μM for reaction with JB1 cells (10^6 cells/mL medium) and corresponding cell sonicates. The inhibitory effects of AT 125 on purified γ-GT (15 μg/mL in medium) were evaluated at inhibitor concentrations of 0.25, 1.0 and 10.0 mM, and preincubation times of 15 and 60 min using a fixed substrate concentration of 100 μM. All experiments were performed at room temperature.

Flow cytoenzymology—instrumentation. The Cambridge MRC flow Cytometer [15, 16] was set to excite with both the 337 and 356 nm UV lines at 100 mW from the krypton laser, and to trigger on orthogonal light scatter. Fluorescence resulting primarily from γ-GAMC was detected between 390 and 420 nm (violet fluorescence). Variable emission wavelengths were used to monitor the reaction with differing substrate to product fluorescence ratios. Either 420–460 nm (indigo fluorescence) or 420–510 nm (indigo through blue fluorescence) were used. In addition, forward and 90° light scatter were recorded for each cell analysed, together with time from the computer clock. Data collection, analysis and display were as described previously [13]. In

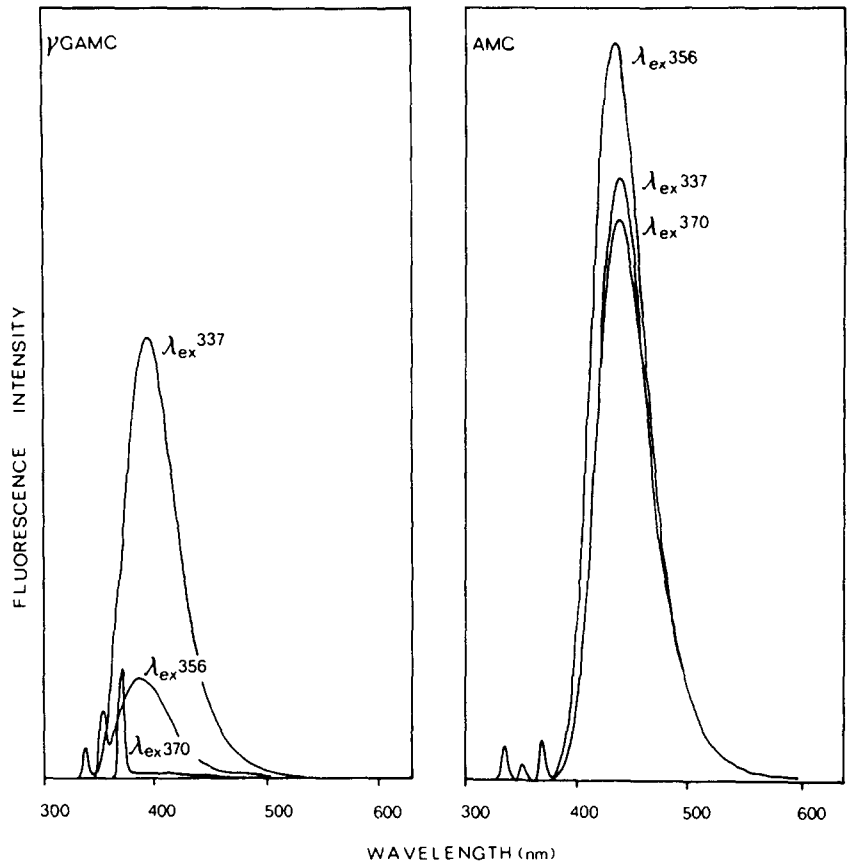

Fig. 2. Typical fluorescence emission spectra for the fluorogenic substrate γ-GAMC and the fluorescent product AMC at 25 μM in PBS, with excitation 337, 356 and 370 nm, as measured by conventional spectrofluorimetry.

brief, 50,000 cells were analysed per sample over a 5-min period with a steady flow rate of 166 cells/sec and a "dead time" of 15 sec. Processed data were analysed as two-parameter cell frequency contour plots of fluorescence and time. The medians of eight sequential fluorescence versus time histograms were plotted against time to generate enzyme reaction progress curves. Initially the analogue to digital data conversion used was 10 bit. During data processing the resolution in the system was reduced to 6 bit due to the wide spread in enzyme activities that was always noted. The fluorescence intensity scales used were therefore 0–63 fluorescence units. However, in data sets where the medians of the fluorescence did not reach full scale, the ordinate scale is marked 0–50.

Flow cytoenzymological assay. Methods for reaction and inhibition kinetics and product efflux kinetics were identical to those described in detail elsewhere for cellular esterases [13, 14]. Cells were initially scraped from the monolayer using a rubber policeman as it was thought this would be less damaging to a membrane enzyme than trypsinization. However, this resulted in clumping of cells and prevented flow cytometric analysis where the production of a single cell suspension is mandatory. Instead, both JB1 and BL8 cultures were subjected to trypsinization as described previously [13]. Fluorescence settings were employed where no autofluorescence was detected. The effect of substrate concentration in the ranges 25–100 μM was determined for JB1 and BL8 cells. Simultaneous analysis of mixed suspensions of JB1 and BL8 cells was carried out at 25 and 50 μM γ-GAMC. The inhibitory effects of AT 125 were investigated at concentrations of 0.25 and 1.00 mM and preincubation times of 15 and 60 min. The rate of loss of cellular fluorescence was measured following a 5-min preincubation of JB1 cells with 100 μM γ-GAMC. All experiments were performed at room temperature.

RESULTS

Conventional spectrofluorimetry

Reaction progress curves for intact JB1 rat hepatoma cells and corresponding sonicates with 12.5–100 μM substrate were linear over the 5-min reaction period. A slightly faster reaction rate was seen for sonicates compared to intact cells (Fig. 3). The plot of reaction velocity (v) versus substrate concentration (s) was curvilinear for both prep-

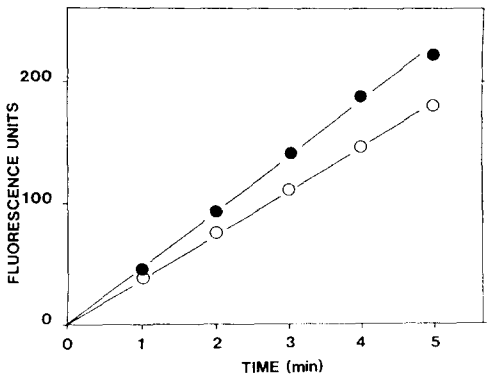

Fig. 3. Enzyme reaction progress curves for the conversion of γ-GAMC (100 μM) to AMC by 1.5×10^5 intact JB1 rat hepatoma cells (○) and corresponding sonicates (●) in medium, as measured by conventional spectrofluorimetry. Results are from one of three independent experiments giving similar results.

arations (Fig. 4A), and the double-reciprocal plots were linear in both cases (Fig. 4B). Apparent K_m values of 286 and 600 μM were obtained from two independent experiments.

Enzyme reaction progress curves for purified γ-GT (15 μg/mL) were linear for 5 min (not shown). AT 125 produced concentration and preincubation time-dependent inhibition. With a 15-min preincubation, 50 and 80% inhibition was seen at 3×10^{-4} M, respectively.

Flow cytometry—reaction kinetics

We had previously analysed JB1 and BL8 cells separately at varying substrate concentrations where JB1 cells demonstrated a 2.5-fold increase in fluorescence at 5 min above BL8 cells (data not shown). Figure 5 illustrates typical data for JB1 and BL8 cells analysed simultaneously in a mixture containing equal proportions of the two cell lines. The fluorescence detection range used was 420–510 nm (which was achieved by removing the 460-nm dichroic mirror to obtain indigo through to blue fluorescence) in order to minimize the effect of substrate fluorescence, as this was thought to be particularly problematic with low activity BL8 cells. There was a clear difference in activity between the non-transformed versus transformed hepatocytes. Only a slight increase in fluorescence with time was observed for BL8 cells at all substrate concentrations. The difference in fluorescence between BL8 and JB1 cells at 5 min was reproducibly 2.5-fold. For JB1 cells enzyme reaction progress curves started from non-zero fluorescence values and were triphasic in the majority of the experiments. A pronounced lag phase or even a decrease in fluorescence was observed over the first 2 min of the reaction. An increase in reaction rate at 2–3 min was then followed by a final decrease in rate up to 5 min. Increasing the substrate concentration resulted in enhanced total fluorescence in all cases, but the progress curves were almost parallel and the rate of reaction was

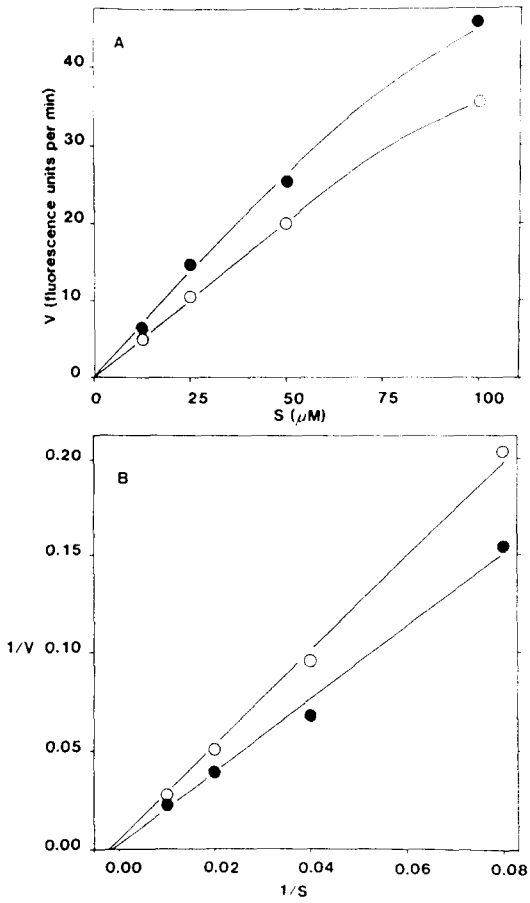

Fig. 4. Reaction kinetics for the conversion of γ-GAMC to AMC by 1.5×10^5 intact rat hepatoma cells (○) and corresponding sonicate (●) in medium as measured by conventional spectrofluorimetry. Panel A: plot of reaction velocity v (fluorescence units per minute) versus substrate concentration s (μM). Panel B: double reciprocal plot of $1/v$ versus $1/s$. Results shown are from one of two independent experiments giving similar results.

not greatly altered. The shapes of the progress curves were not altered in a major way, although the initial lag phase was reduced slightly and there was an indication of faster reaction rates between 3 and 5 min with increasing substrate concentration.

Inhibition kinetics

Figure 6A shows reaction progress curves for control and AT 125-treated JB1 and BL8 cells with 100 μM substrate and detection at 420–510 nm. Results for inhibitor concentrations of 0.25 and 1.00 mM are shown where the preincubation time was 15 min. AT 125 exhibited a concentration-dependent inhibition of the reaction in JB1 cells. Inhibition was also seen for BL8 cells but to a lesser extent. Increasing the preincubation time to 60 min did little to increase the extent of inhibition (not shown). Figure 6B shows the corresponding progress curves where the fluorescence was detected at 390–420 nm, i.e. in the violet region of the spectrum,

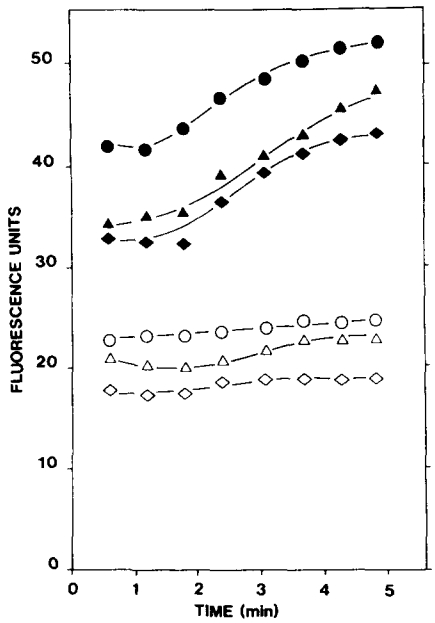

Fig. 5. Flow cytometry determination of γ-GT reaction kinetics for a mixture of equal proportions of JB1 hepatoma cells (closed symbols) and BL8 rat hepatocytes (open symbols) with varying γ-GAMC concentrations. Reaction progress curves are shown for γ-GAMC at 100 μM (circles), 50 μM (triangles) and 25 μM (diamonds). Fluorescence was detected between 420 and 510 nm and was plotted on a linear scale. Each point is the mean value of duplicate samples and a total of 50,000 cells were analysed for each progress curve. Results shown are from one of three independent experiments giving similar results.

where the substrate fluorescence is maximal. Concentration-dependent inhibition of AT 125 was confirmed, but a sharp decrease was seen between 1 and 3 min when the inhibitor concentration was 0.25 mM. In addition, the difference in apparent activity between JB1 and BL8 cells was reduced compared to that at 420–510 nm (Fig. 6A).

Loss of cellular fluorescence

This was measured between 420 and 510 nm for JB1 cells previously reacted with γ-GAMC for 5 min, then washed and resuspended in fresh medium (Fig. 7). Approximately 80% of the fluorescence recorded at 5 min was lost in the 3-min wash period (mean of three similar results). A slight increase in fluorescence was then observed in the subsequent 15 min.

DISCUSSION

The aim of the present studies was to develop methodology to measure enzyme reaction and inhibition kinetics for γ-GT individual viable cells, including those in heterogeneous cell subpopulations. γ-GT is of particular pharmacological interest because of its elevation in transformed cells and its putative role in detoxification. A more general technological objective was to assess the usefulness of flow cytometry for enzymes located at the external surface of the cell membrane, for which γ-GT represents a specific example. By upgrading the method previously described by Manson *et al.* [12] to measure cellular levels of γ-GT we were, nevertheless, successful in determining γ-GT reaction kinetics. However, several factors complicated the analysis.

Linear reaction progress curves were seen using conventional spectrofluorimetry where diffusion of product from cells does not affect measured fluorescence. Non-linear reaction kinetics were observed by flow cytoenzymology and reaction progress curves did not pass through the origin. This is likely to result from significant γ-GAMC substrate fluorescence together with rapid reaction rate before the first seconds when a fluorescence value was obtained. Because of the location of γ-GT the substrate need not penetrate inside the cell and a rapid initial reaction was not unexpected. Using this system it was not possible to determine whether the first 15 sec of the reaction exhibited linear kinetics. The rate of product diffusion from the cell surface was also rapid as seen in Fig. 7. The increase in residual fluorescence recorded for cells previously reacted with substrate, then washed and resuspended in substrate-free medium was probably a result of the conversion of residual unreacted substrate to product. We interpret the plateau in the reaction progress curves seen in Figs 5 and 6 as an indication that equilibrium was attained when the rate of product formation equalled the rate of product diffusion. We did not see increases in fluorescence which paralleled increases in substrate concentration, Fig. 5 shows that increase from 50 to 100 μM GAMC led to a greater than 2-fold elevation in measured cellular fluorescence than that of 25 to 50 μM although substrate fluorescence *per se* was linear with concentration in this range. It is possible that as substrate concentrations approached 100 μM formed product may have entered the cell and hence must be better retained to some degree.

Despite the above disadvantages, assay conditions were optimized for cultured JB1 hepatoma cells and fluorescence detection was manipulated so as to minimize the contribution of substrate fluorescence. The triphasic shape of the resulting progress curves reflected both the production of AMC and the consumption of γ-GAMC by the enzyme. However, evidence to suggest that the observed increases in fluorescence with time were mainly due to γ-GT activity included the observation that activity in JB1 aflatoxin-induced hepatoma cells was far greater than that seen in BL8 untransformed hepatocytes. In addition, potent inhibition of the reaction in JB1 cells was seen with AT 125, an active site-directed analogue of glutamine [4] and a known inhibitor of γ-GT [17–19]. Similar concentrations of AT 125 were shown to inhibit purified γ-GT using conventional spectrofluorimetry.

Interestingly, in JB1 cells when inhibition by AT 125 was measured using fluorescence detection in the region of the spectrum where substrate fluorescence is maximal, a sharp decrease in fluorescence was noted at 2 min (Fig. 6B). This decrease is less apparent in Fig. 6A where the wavelengths better accommodate product rather

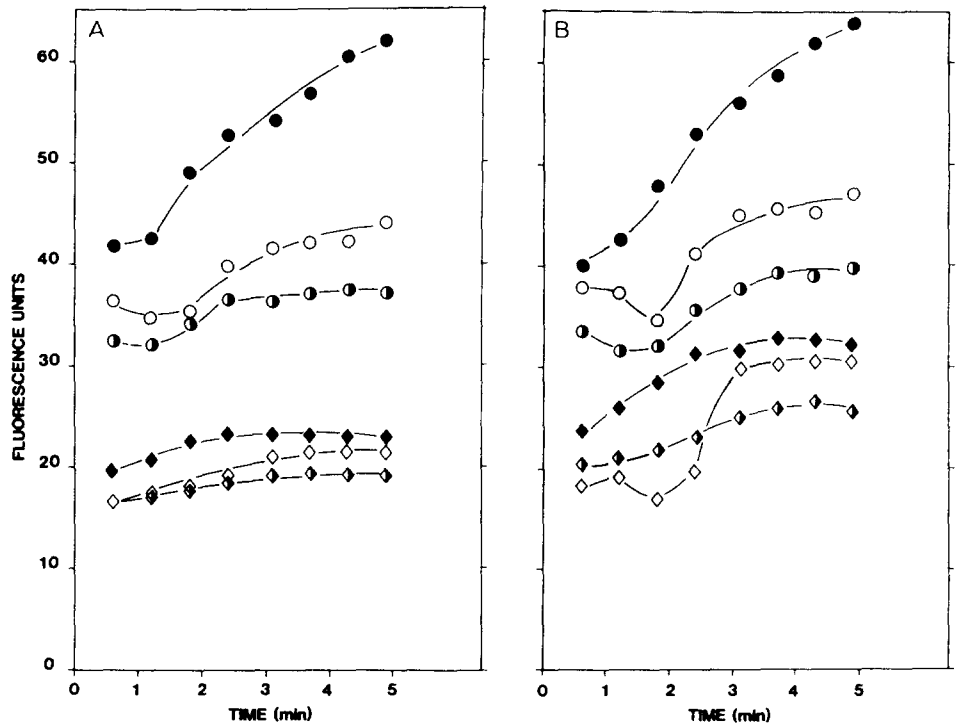

Fig. 6. Flow cytometric determination of the inhibitory effects of AT 125 on the reaction of γ-GAMC (100 μM) with JB1 rat hepatoma and BL8 rat hepatocyte cells. Reaction progress curves are shown for JB1 cells (circles) and BL8 cells (diamonds) in the absence of AT 125 (closed symbols) or after treatment with AT 125 for 15 min at either 0.25 mM (open symbols) or 1 mM (harlequin symbols). Fluorescence was detected from 420 to 510 nm (Panel A) or from 390 to 420 nm (Panel B) and was plotted on a linear scale. Each point analysed is the average of duplicate values and a total of 50,000 cells were analysed per progress curve. Results shown are from one of two independent experiments giving similar results.

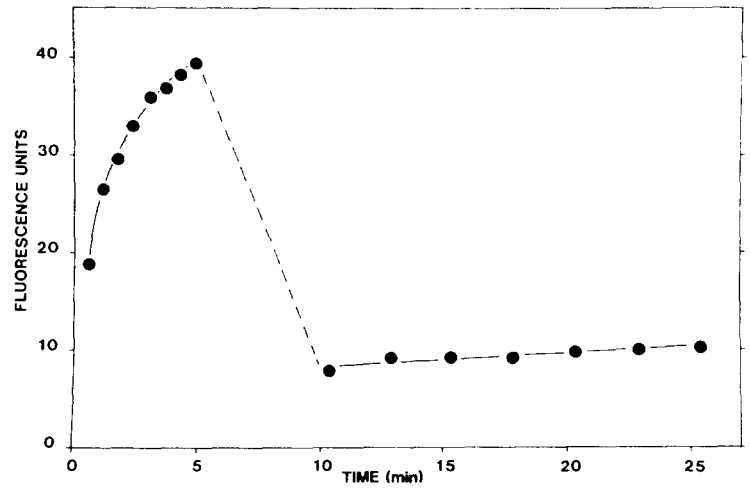

Fig. 7. Flow cytometric determination of the rate of loss of AMC from JB1 rat hepatoma cells. Fluorescence was monitored between 420 and 510 nm during the 5-min loading with γ-GAMC. After this cells were washed with PBS (broken line) and resuspended in substrate-free medium to measure loss. A total of 2.5×10^5 cells were analysed. Fluorescence is plotted on a linear scale and results shown are from one to five independent experiments giving similar results.

than substrate fluorescence. As the reaction proceeds, albeit more slowly in the presence of AT 125, substrate is consumed and substrate fluorescence decreases before the subsequent increase in fluorescence. The latter is largely due to the restricted formation and then diffusion of product (measured with suboptimal filtering). In concurrence with this interpretation of the measured reaction curves is the observation that in low activity BL8 cells treated with 0.25 and 1 mM AT 125 the initial decrease in fluorescence intensity is seen only with the lower inhibitor concentration (compare open diamonds with harlequin symbols in Fig. 6B). This suggests that at 1 mM inhibitor minimal substrate is consumed and minimal product is formed.

A similar difference in enzyme activity was observed between JB1 hepatoma and BL8 hepatocyte cells when the two cell types were examined separately to that observed for two distinct subpopulations clearly distinguished in mixtures containing equal proportions of JB1 and BL8. These flow cytoenzymological experiments confirmed that it was indeed possible to identify each cell type based on γ-GT activity.

Additional modification to the flow cytoenzymological assay for γ-GT activity would improve the potential for accurate kinetics measurements. In particular, the time of the first fluorescence measurement must be significantly reduced. The reaction is rapid and more informative initial reaction velocities are required. Systems currently under development in this laboratory and elsewhere [20, 21] will permit fluorescence analysis 1 sec after initiating the reaction. Structural modification to the available fluorogenic substrates are also envisaged, with the aim of producing a less fluorescent substrate and/or a more fluorescent product, or alternatively one with superior λ_{ex} versus λ_{em} characteristics.

Rapid diffusion of the product from the membrane surface is likely to be a persistent disadvantage for flow cytoenzymological assay of membrane-bound enzymes. Watson *et al.* [6] have described a flow cytometric assay for plasma membrane phosphatase activity and obtained asymptotic enzyme reaction progress curves. The problem of rapid diffusion was partially overcome by use of novel mathematical analysis to calculate kinetic parameters. An approach to eliminate both substrate fluorescence and rapid diffusion would be the synthesis of a non-fluorescence substrate which is cleaved by γ-GT to form a product which binds tightly to the membrane and fluoresces. It should also be borne in mind that flow cytoenzymology does not accommodate investigations into intercellular differences in enzyme activity within the same cell type which might result from microenvironmental conditions within the liver. For a more complete assessment of γ-GT cellular biochemistry, flow cytoenzymology should be complemented by histochemical analysis where tissue architecture is to some extent maintained.

In summary, flow cytoenzymology can be used in to demonstrate reaction kinetics for γ-GT in intact cells. Subpopulations differing in enzyme activity can be characterized simultaneously from heterogeneous samples. Substrate probes with improved fluorescence and disposition characteristics are recommended, together with a decrease in the "dead time" of the assay, in order to improve the quantitation of reaction kinetics for this important enzyme.

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