A Rapid *in Vitro* Assay for Evaluation of Metabolism-Dependent Cytotoxicity of Antiepileptic Drugs on Isolated Human Lymphocytes

Ali R. Tabatabaei,* Robert L. Thies,* Kevin Farrell,† and Frank S. Abbott*

*Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3 and †Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3

Received February 6, 1997; accepted April 9, 1997


*In vitro* assessment of human lymphocyte viability by trypan blue dye exclusion in the presence of an external metabolizing system (microsomes plus NADPH) has been shown to be a useful method in assessing predisposition to idiosyncratic toxicity in response to various anticonvulsant drugs. The trypan blue method, however, is labor intensive, is time consuming, is prone to human error, is not suitable for high-volume toxicity screening, and excludes autolysed cells. The objective of this study was to develop a rapid, high-capacity, objective, and easy *in vitro* cytotoxicity method for the detection of metabolism-dependent cytotoxicity of a test chemical. The *in vitro* system uses an external metabolizing system (rabbit microsomes) in conjunction with isolated human lymphocytes as the target cells. Cellular toxicity was determined by assessing plasma membrane integrity using a membrane-impermeant fluorescent nucleic acid dye (YO-PRO-1) and a multiwell plate scanner for fluorescence. Using this system, cells incubated with either acetaminophen (1500 µg/ml), carbamazepine (62.5 µM), phenytoin (62.5 µM), or phenobarbital (62.5 µM) showed net increases in percentage cell death of 31 ± 5, 11 ± 4, 0 ± 3, and 2 ± 3, respectively. A metabolism-dependent concentration-response was observed for valproic acid-induced cytotoxicity, which approached a plateau at a concentration of 4000 µg/ml with a net percentage cell death of 31 ± 4. This technique resolves various technical difficulties inherent in viability determinations by the trypan blue exclusion method. The YO-PRO-1 method also may be useful in a clinical setting for the assessment of patients with a genetically determined susceptibility to certain drugs and for identifying the responsible drug in patients with idiosyncratic toxicity undergoing multiple-drug therapy.

The role of drug metabolites in idiosyncratic drug reactions has been studied by Spielberg (1980) using an *in vitro* technique in which human lymphocytes were incubated with the test drug in the presence of microsomes prepared from phenobarbital (PB)-treated mice. Human lymphocytes were selected because (1) lymphocytes do not have a significant cytochrome P450 drug-metabolizing capability; (2) lymphocytes contain detoxification enzymes, such as epoxide hydrolase, glutathione peroxidase, and glutathione synthetase, which are involved in the removal of reactive metabolites; (3) genetic variation in these detoxification systems is expressed phenotypically in lymphocytes; and (4) lymphocytes are easily obtained (Spielberg, 1980).

The method described by Spielberg involved the incubation of isolated human lymphocytes with the test drug in the presence of an extracellular microsomal metabolizing system. After 2 hr of exposure, the metabolizing system was removed and the cells were incubated for an additional 16 hr. An aliquot of cell suspension was then removed for assessment of cellular plasma membrane integrity using trypan blue exclusion as an indication of viability.

With this technique, the formation and toxicity of reactive metabolites have been implicated in the pathogenesis of idiosyncratic toxicity exhibited by several antiepileptic drugs. With this *in vitro* system, a higher percentage of cell death has been observed with lymphocytes isolated from individuals with demonstrated idiosyncratic toxicities to PB, phenytoin (DPH), carbamazepine (CBZ), and valproic acid (VPA), compared with lymphocytes from normal donors (Wolkenstein et al., 1995; Friedmann et al., 1994; Gennis et al., 1991; Pirmohamed et al., 1992; Farrell et al., 1988, 1989; Shear and Spielberg, 1988; Gerson et al., 1983; Spielberg et al., 1981). Similarly, a genetic predisposition to idiosyncratic drug toxicity has been demonstrated in patients with DPH toxicity which was reflected in the *in vitro* cytotoxicity assay (Gennis et al., 1991; Strickler et al., 1985).

In previous investigations using human lymphocytes and the trypan blue exclusion method, VPA was shown to produce a concentration-dependent toxicity, which could be inhibited by inactivation of the microsomal metabolizing system (Farrell et al., 1988, 1989). In these
studies, lymphocytes from normal volunteers were less susceptible than lymphocytes from patients having a history of severe VPA hepatotoxicity (Farrell et al., 1988). A further study demonstrated that the lymphocyte toxicity induced by VPA could be inhibited by the addition of glutathione (GSH) or N-acetylcysteine (NAC). This result prompted the use of NAC in the successful treatment of three children who developed severe hepatotoxicity while receiving VPA. The lymphocytes studied from one of these patients showed an unusually high sensitivity to the toxic effects of VPA (Farrell, 1991; Farrell and Abbott, 1991).

The evaluation of lymphocyte viability in the studies described above involved the assessment of trypan blue exclusion, a technique limited by several factors. First, the method identifies only cells that have lost plasma membrane integrity and does not detect cells that may have undergone autolysis (Ankarcrnona et al., 1995). Second, this method is labor intensive and time consuming. Manual counting of the stained and unstained cells is required and, therefore, is highly prone to human error. Third, the trypan blue method allows only for a single sample evaluation at any one time and true side-by-side comparisons of cell toxicity taken from various patients are limited. Finally, the trypan blue exclusion method must be completed within 3–5 min of addition of the dye due to the increasing number of blue-staining cells with time (Jones and Senft, 1985). These problems suggest that the use of the trypan blue exclusion method for assessment of cell viability can lead to a marked inter- and intra-assay variability. Therefore, the comparison of data generated at different times may be unreliable. As a result, the ability to examine the toxic influence of drug metabolites on lymphocytes is severely limited with the use of the trypa blue exclusion method.

The purpose of this study was to develop a rapid, high-capacity, objective, and simplified in vitro cytotoxicity method for the detection of metabolism-dependent cytotoxicity of a drug on isolated human lymphocytes. The method must be easy to perform, require a small sample size, and be reproducible. This method was further optimized for assessment of VPA-induced cytotoxicity in human lymphocytes. The potential use of this assay for in vitro screening of patients sensitive to antiepileptic drug toxicity is discussed.

METHODS

Materials. Hanks’ balanced salt solution (HBSS), Earl’s balanced salt solution (EBSS), RPMI AIM-V, penicillin-streptomycin, and fetal bovine serum were purchased from Gibco BRL (Burlington, Ontario). Ficoll-Paque was the product of Pharmacia Biotech Inc. (Baie d’Urfe, Quebec). Hapes, ribonuclease A, dimethyl sulfoxide, and carbamazepine were obtained from Sigma Chemical Company (St. Louis, MO), valproic acid from Aldrich Chemical Company (Milwaukee, WI), YO-PRO-1 from Molecular Probes Inc. (Eugene, OR), NADPH and fatty acid free bovine serum albumin from Calbiochem (La Jolla, CA), phenobarbital from BDH Inc. (Vancouver, British Columbia), and phenytoin from Novopharm (Scarborough, Ontario).

Animals. Female white New Zealand rabbits (Vancouver, British Columbia) weighing 2.0–2.5 kg were allowed free access to food (Rodent Laboratory Diet, PMI Foods, Inc.) and water. Animals were maintained in polypropylene pens in a room with controlled light (12-hr cycle of light and darkness) and constant temperature (23°C).

Animal treatment and preparation of microsomes. Rabbits were treated intraperitoneally with PB dissolved in normal saline at a dose of 75 mg/kg body wt daily for 5 days to maximally induce hepatic cytochrome P450. Hepatic microsomal fraction was prepared by differential centrifugation as described by Purba et al. (1987) with minor modifications. Briefly, rabbits were killed by an overdose of sodium pentobarbital (65 mg/kg) 24 hr after the last dose of PB. Liver was immediately removed, minced, and homogenized in 4 vol of ice-cold homogenization buffer (20 mM Tris–HCl, 250 mM sucrose, 2 mM EDTA) in a Potter–Elvehjem tissue grinder. Homogenate was centrifuged at 9000g for 20 min at 4°C. The supernatant was centrifuged at 105,000g for 60 min at 4°C. Isolated microsomes were resuspended in wash buffer (2 mM EDTA and 154 mM KCl) and treated with (250 units/ml/g liver) RNase for 15 min at 37°C to remove contaminating RNA. Decontamination of microsomal preparations with enzymatic digestion of RNA was proved necessary in pilot studies due to the cross-reactivity of microsomal RNA with the nucleic acid dye used in the assay. The suspension was diluted with the wash buffer, centrifuged at 105,000g for 60 min at 4°C, and washed once. Microsomes were resuspended in 0.25 M sucrose; aliquots were frozen in liquid nitrogen and stored at −80°C. Cytochrome P450 content was assayed by the method of Omura and Sato (1964). Microsomal protein was determined by the method of Lowry et al. (1951). Aseptic techniques were used throughout the preparation of microsomes.

Lymphocyte preparation. Human lymphocytes were isolated from fresh heparinized blood obtained from normal volunteers. Blood was diluted with an equal volume of HBSS plus 15 mM Hapes. The sample was then layered on a Ficoll-Paque density gradient and centrifuged at 400g for 30 min at 18°C. Lymphocytes were removed from the aqueous–Ficoll interface and washed three times with HBSS. The number and viability of the lymphocytes were determined by exclusion of trypan blue. Viability at this stage was greater than 95%. Aseptic techniques were used throughout the preparation of lymphocytes.

In vitro metabolism-dependent cytotoxicity. The freshly isolated lymphocytes from normal volunteers were used within 2 hr of venipuncture. Lymphocytes were incubated in HBSS containing 15 mM Hapes, PB-induced rabbit microsomes at a concentration of 1 nmol of P450/ml, 1 mM NADPH, and the test drug (i.e., APAP, 1500 μg/ml; VPA, 500–700 μg/ml; CBZ, DPH, and PB, 62.5 μM). Experiments were carried out in 96-well plates (final volume of 300 μl/well) which were maintained under ambient CO2 partial pressure at 37°C. After 2 hr of incubation, plates were centrifuged at 400g for 15 min at 25°C to pellet the cells. The supernatant containing the microsomes was decanted and replaced with the HBSS containing 15 mM Hapes, 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 μM YO-PRO-1 (a cell membrane-impermeant fluorescent nucleic acid stain). The multiwell plates were maintained at 32°C and continuously scanned at 30-min intervals using a Millipore Cytofluor 2350 multiwell fluorescence plate scanner set for 485-nm (22-nm bandpass) excitation and 530-nm (30-nm bandpass) emission. On termination of the experiment, Triton X-100 (final concentration of 0.1%) was added to each well to permeabilize all cells. The percentage of dead cells (as defined by loss of plasma membrane integrity) for each sample well, at every data point, is calculated as % dead = 100(X - A)/(B - A), where A is each well’s initial fluorescence value, B is the fluorescence value for each specific well after addition of Triton X-100, and X is the fluorescence value for each
specific well at any given time. Therefore, each sample well of cells acts as its own control from start to finish, accounting for slight variations in cell number or fluorescence quenching by the various agents (Fig. 1). Unlike trypan blue exclusion and flow cytometry (another method evaluated for possible implementation in this assay), if autolysis occurred, the released nucleic acid would be contained in the well, recognized by the fluorescent nucleic acid stain, and figured into the final calculation of percentage dead cells. Treatment drugs were prepared in HBSS except CBZ and DPH, which were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in HBSS. The final concentration of DMSO (0.17%) had no detectable effects on cell viability. Aseptic techniques were used throughout the assay to avoid potential bacterial nucleic acid contamination.

Data analysis and statistics. To account for individual variations, the net increase in cell death (% cell death in experimental group - % death in control group) was taken as an index. Comparisons between groups were made by repeated-measures analysis of variance. When appropriate, the Bonferroni post hoc test was used. Results are expressed as means ± SE of three to six independent experiments. The numbers of independent experiments and individual donors in each experiment are given in figure legends. Within an experiment, each treatment was performed at least in triplicate.

RESULTS

Maintenance of a Healthy System

A series of experiments were performed to determine conditions and medium necessary to maintain viable human lymphocytes for the duration of the assay (24 hr). Media examined included RPMI, AIM-V (defined, serum free medium), and EBSS containing 15 mM Hepes. Various concentrations of fatty-acid free bovine serum albumin (FFBSA) and FBS were also evaluated. EBSS was replaced with HBSS for experiments conducted at ambient CO₂ partial pressure. Lymphocytes were prepared in HBSS as described under Methods. Freshly prepared lymphocytes were washed once with the medium to be tested. The final incubation contained 200,000 live cells per well in the test media (300-μl final volume per well) and was maintained at 32–37°C. HBSS or EBSS was chosen over RPMI and AIM-V to avoid constituents that may possibly interfere with the assay (e.g., glutathione, cysteine). FBS was required to maintain a reasonable level of cellular viability over the course of the experiment. EBSS with 5% FBS was found to maintain an adequate cell viability (94% over 24 hr) (Fig. 2). All further experiments were performed in HBSS (for ambient CO₂ partial pressure) containing 15 mM Hepes and 5% FBS.

Optimization of Cell Density and YO-PRO-1 and Triton X-100 Concentration

Cell density, as well as dye and detergent concentrations, was varied to optimize conditions for measurement of cell toxicity. An increasing number of lymphocytes were plated and permeabilized by addition of Triton X-100. YO-PRO-1 fluorescence increased linearly with cell
FIG. 2. Evaluation of media for maintenance of lymphocyte viability. Lymphocyte viability was determined 17–24 hr postincubation in various media (for details of the procedures, refer to Methods). (A) Increasing concentrations of FFBSA in EBSS plus 15 mM Hepes. (B) Increasing concentrations of FBS in EBSS plus 15 mM Hepes. (C) Increasing concentrations of FBS in RPMI. (D) The use of defined medium AIM-V. Values are the means ± SE for triplicate determinations in three or four independent experiments.

number up to 1,000,000 cells/well (maximum number tested) (Fig. 3). To reduce the required cell sample size, but provide a sufficient fluorescent signal, 200,000 cells/well was selected for future studies. Fluorescence intensity over baseline increased as a function of YO-PRO-1 concentration up to 8 μM (max concentration tested). A concentration of 4 μM YO-PRO-1 provided a sufficient fluorescent signal when incubated with 200,000 cells and, therefore, was selected for subsequent experiments (Fig. 4). To optimize the concentration of Triton X-100 for permeabilization of lymphocytes, a concentration curve was constructed. The fluorescent signal increased sharply with increasing concentrations of Triton X-100, reaching a maximum at a final concentration of 0.1%. Higher concentrations of Triton X-100 resulted in a decrease in maximum fluorescence (Fig. 5). A final concentration of 0.1% Triton-X was used in all further experiments.

In Vitro Cytotoxicity Evaluation of Antiepileptics and Acetaminophen

The optimized assay parameters were used to examine the cytotoxicity of VPA and reevaluate the cytotoxicity of
Drug Metabolite In Vitro Cytotoxicity

FIG. 3. Relationship between fluorescence of YO-PRO-1 and cell number. Lymphocytes (0–1,000,000 cells/well) were permeabilized by Triton X-100 (0.1%), and the fluorescence of YO-PRO-1 (final concentration of 4 μM) was measured with a fluorescent plate scanner. Each point represents the mean ± SE of triplicate determinations in five independent experiments using lymphocytes from two normal volunteers.

Previously studied antiepileptics. A concentration–response relationship for metabolism-dependent VPA-induced cytotoxicity was assessed over the range 0–7000 μg VPA/ml. A plateau was reached at a concentration of 4000 μg VPA/ml with a net percentage cell death of 31 ± 4 (Fig. 6). Metabolism-dependent cytotoxic effects of APAP (1500 μg/ml), VPA (4000 μg/ml), CBZ (62.5 μM), DPH (62.5 μM), and PB (62.5 μM) were also examined as a function of time using the described method (Fig. 7A) and the final net percentages cell death were determined to be 31 ± 5, 27 ± 4, 11 ± 4, 0 ± 3, and 2 ± 3, respectively (Fig. 7B). Cytotoxicity in the presence of APAP, VPA, and CBZ at the concentrations mentioned above was significantly higher (p < 0.001) than that of the nontreated control cells (treated only with microsomes plus NADPH). In all cases, elimination of NADPH from the mixture resulted in the elimination of toxic effects (data not shown).

**DISCUSSION**

The purpose of this study was to develop a rapid, high-capacity, objective, and simple in vitro cytotoxicity screening method for the evaluation of metabolism-dependent cytotoxicity of drugs on isolated human lymphocytes. A reasonable survival rate of untreated lymphocytes is crucial to in vitro cytotoxicity assays. A minimum of 5% FBS proved necessary to maintain lymphocytes for the duration of the experiment (24 hr). A simple buffered salt solution containing 5% FBS was sufficient to provide 94% viability after 24 hr of incubation. Alternatively, with 0.5% FFBSA in Heps-buffered salt solution, which is commonly used in trypan blue cytotoxicity assays, there was more than 40% cell death of untreated lymphocytes over the duration of the experiment (Fig. 2). This effect may have gone unrecognized in previous techniques due to the exclusion of completely autolysed cells from the viability count by the trypan blue method. Using flow cytometry (a technique that also does not account for autolysed cells), we have previously observed a reduction in the total number of lymphocytes after an overnight incubation in 0.5% FFBSA in Heps-buffered salt solution (unpublished data).

The rare but fatal hepatotoxicity of VPA has been associated with the formation of the unsaturated metabolite 2-n-propyl-4-pentenoic acid (4-ene-VPA) (Baillie, 1988; Cotariu and Zaidman, 1988; Kassahun et al., 1991). The in vitro cytochrome P450-mediated unsaturation of VPA has been reported to be the highest with microsomes prepared from PB-induced rabbits, as compared with preparations from human, mouse, and rat liver in that order (Rettie

FIG. 4. Relationship between fluorescence and increasing concentration of YO-PRO-1. Lymphocytes (200,000 cells/well) were permeabilized by Triton X-100 (0.1%), and the fluorescence of YO-PRO-1 (0–8 μM) was measured with a fluorescent plate scanner. Each point represents the mean ± SE of triplicate determinations in five independent experiments using lymphocytes from two normal volunteers.
FIG. 5. Relationship between fluorescence of YO-PRO-1 and increasing concentration of Triton X-100. Lymphocytes (200,000 cells/well) were permeabilized by increasing concentration of Triton X-100 (0–0.25%), and the fluorescence of YO-PRO-1 (final concentration of 4 μM) was measured with a fluorescent plate scanner. Each point represents the mean ± SE of triplicate determinations in three independent experiments using lymphocytes from two normal volunteers.

et al., 1988). To optimize this assay for the evaluation of metabolism-dependent cytotoxicity of VPA, white New Zealand rabbits were selected as the source of microsomal preparations. Due to the use of a nucleic acid probe in this assay, the enzymatic decontamination of microsomal RNA was found to be crucial to reduce background fluorescence.

Viability determinations using membrane-impermeable nucleic acid probes have been correlated with other well-accepted indicators of viability including (1) the LDH release assay, an indicator of plasma membrane integrity (Nieminen et al., 1992); (2) the thiazolyl blue (MTT) assay, a metabolic indicator of cell death (Becker et al., 1994); and (3) trypan blue exclusion, a common colorimetric method for assessment of plasma membrane integrity as an indication of viability (Moore et al., 1990; Beletsky and Umansky, 1990; Corver et al., 1995). Enzyme release assays are, however, time consuming and the result may vary because of inactivation of the measured enzyme by the xenobiotic or metabolism by products, or possible release of the enzyme from sublethally altered cells, probably due to cytoplasmic blebbing (Allison and Acosta, 1994). The assessment of cellular viability by the trypan blue method is compromised by several factors including the following: (1) the method is laborious and time consuming; (2) autolysed cells are lost from total count; (3) viability assessment is objective and highly prone to human error; (4) the method does not allow one to analyze large numbers of treatment groups simultaneously.

A variety of fluorescent nucleic acid stains are available that are impermeant to the membranes of live cells, but penetrate and increase in fluorescence when they bind to nucleic acids of dead cells with compromised plasma membranes. These dyes include propidium iodide, ethidium homodimer, and a group of new cyanine dyes (TOTO and TO-PRO series of nucleic acid stains). Both propidium iodide and ethidium bromide have been used extensively to detect dead cells (Haugland, 1996; Ankarcrona et al., 1995; Trost and Lemasters, 1994; Vollenweider and Groscurth, 1992; Fady et al., 1995; Corver et al., 1995 Beletsky and Umansky, 1990; Moore et al., 1990). The new cyanine dyes share the following unique properties: (1) high extinction coefficient (10–20 times that of propidium iodide); (2) very low fluorescence intensity except when bound to nucleic acid; (3) very high binding affinity to double-stranded DNA; and (4) ultrasensitivity (>3000-fold increase in fluorescence on binding to nucleic acids) (Haugland, 1996). YO-PRO-1 is a monomeric member of this class of nucleic acid stains that has a narrow emission

FIG. 6. Metabolism-dependent VPA-induced cell toxicity. Cytotoxic effects of increasing concentrations of VPA in the presence of activated rabbit microsomes (1 nmol P450/ml and 1 mM NADPH) (for details of the procedures, refer to Methods). Each point represents the mean ± SE of triplicate determinations in five independent experiments using lymphocytes from one normal volunteer.
With the described method a metabolism- and concentration-dependent cytotoxicity of VPA to lymphocytes was demonstrated (Fig. 6). Significant increases in metabolism-dependent cytotoxicity of APAP, VPA, and CBZ were obtained, while DPH and PB did not cause cytotoxicity in lymphocytes isolated from six normal volunteers (Fig. 7). These results are consistent with the literature whereby APAP and CBZ were reported to induce a concentration-dependent cytotoxicity, whereas DPH and PB were nontoxic to lymphocytes isolated from normal individuals when tested using the trypan blue exclusion method (Gerson et al., 1983; Shear and Spielberg, 1988; Spielberg et al., 1981; Wolkenslein et al., 1995). Our previous attempts to demonstrate a higher level of lymphocyte toxicity in individuals with a history of VPA-induced hepatotoxicity using the trypan blue method were fastidious and labor intensive. Results were highly variable, and side-by-side comparisons of samples were not possible.

In summary, the assay developed is a rapid, high-capacity, objective, and easy in vitro cytotoxicity method for the detection of metabolism-dependent cytotoxicity on isolated human lymphocytes and provides a suitable method to study the mechanisms underlying lymphocyte toxicity exhibited by VPA. Previous investigations, using trypan blue, demonstrated a higher percentage of cell death in lymphocytes isolated from patients having a
The role of glutathione in valproate hepatotoxicity. In *Epilepsia* 29, 700.


Spielberg, S. P., Gordon, G. B., Blake, D. A., Goldstein, D. A., and Herford,
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