Image Cover Sheet

CLASSIFICATION	SYSTEM NUMBER 513827
UNCLASSIFIED	

TITLE

Effect of Intracellular Calcium Modulation on Sulfur Mustard Cytotoxicity in Cultured Human Neonatal Keratinocytes

System Number:

Patron Number:

Requester:

Notes:

DSIS Use only:

Deliver to:

PERGAMON

Toxicology in Vitro 14 (2000) 149–157

Toxicology in Vitro

www elsevier com/locate/toxinvit

Effect of Intracellular Calcium Modulation on Sulfur Mustard Cytotoxicity in Cultured Human Neonatal Keratinocytes

T. W SAWYER * and M G. HAMILTON

Therapy Group, Medical Countermeasures Section, Defence Research Establishment Suffield, Box 4000, Medicine Hat, Alberta, Canada T1A 8K6

(Accepted 26 July 1999)

Abstract—Previous studies in human skin keratinocyte cultures have shown that sulfur mustard (HD) induces an immediate and irreversible increase in internal free calcium levels that was independent of external calcium concentrations. These findings suggested a role for calcium in the aetiology of HD-induced cell death and that modulation of intracellular calcium concentrations may assist in providing protection against this agent. In the current work, actively proliferating and confluent cultures of first passage neonatal human skin keratinocytes were used to assess the effect of altered intra- and extracellular calcium levels on HD toxicity. Treatment of cultures with the endoplasmic reticulum calcium ATPase inhibitor thapsigargin, or the calcium chelator BAPTA-AM, which reduce HD-induced elevation of intracellular free calcium, did not modulate the toxicity of HD. Furthermore, alteration of external calcium concentrations during these same experiments failed to elicit any change in the viability of HD-exposed cells. Treatment of confluent cultures with ionomycin at either low (100 μM) or high (1.2 mM) external calcium concentrations also failed to modulate the toxicity of HD in any way. It appears that in neonatal human skin keratinocytes in culture, HD-induced intracellular calcium perturbation does not play a major role in HD-induced cytotoxicity. ***Crown Copyright***

Keywords: sulfur mustard, bis-2-(chlorethyl) sulfide, HD, intracellular calcium, human skin keratinocytes, protection studies, chemical warfare agents

Abbreviations: BAPTA-AM = 1,2-bis (*O*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, DMSO = dimethyl sulfoxide, HD = bis 2-(chloroethyl) sulfide, sulfur mustard, KSFM = keratinocyte serum free medium

INTRODUCTION

Sulfur mustard [HD; bis 2-(chloroethyl) sulfide] is a strong alkylating agent which, in addition to causing extensive DNA damage, is also capable of exerting deleterious effects in a variety of tissues including skin vesication, eye and respiratory tract injuries and systemic intoxication (Dacre and Goldman, 1996, National Academy Press, 1993; Papirmeister *et al*, 1991, Smith and Dunn, 1991, Warthin and Weller, 1919) Although this compound has attracted a great deal of research attention due to its continued use as a chemical warfare agent, its mechanism of toxic action is as yet unknown A number of hypotheses have been put forward to explain the toxicity of HD, most recently several that propose that HD-induced cell death is either calcium dependent, or is the result of disrupted intracellular calcium homeostasis Although several different laboratories have shown that HD induces a rise in intracellular calcium in cultured cells (Hamilton e*t al*, 1998, Hua *et al*, 1993, Mol, 1994, Mol and Smith, 1996, Ray *et al*, 1994, 1995) or in whole tissue (Lundy *et al*, 1998), the level and the importance of this elevation in calcium has been debated Recent work in our laboratories has shown that secondary cultures of confluent human skin keratinocytes exposed to HD rapidly (≤2 min) exhibited a concentration-dependent, irreversible increase in intracellular calcium that appeared to originate from intracellular stores Removal of the HD from the cultures following a 2-min exposure did not change these findings significantly and the rapidity of the calcium response

*Corresponding author Tel 403-544-4788, fax 403-544-3388, e-mail Thomas Sawyer@dres dnd ca

PII S0887-2333(00)00005-9

paralleled the cytotoxicity of HD, which was expressed maximally at 48 hr after only a few minutes of actual HD exposure (Hamilton *et al*, 1998)

An extensive body of work exists that demonstrates that calcium is widely known to mediate the toxicity of a variety of drugs through activation of lytic enzymes and/or calcium-dependent receptors Furthermore, prevention of induced calcium elevation has been shown in many cases to be protective Previous work showing that HD elevates intracellular calcium suggests that drug regimens aimed at modulating these calcium stores may therefore be of utility in ameliorating HD toxicity

Normal mouse and human skin keratinocyte culture have been shown to be dynamic systems in which the differentiative state of the cells is intimately related to the intracellular calcium environment, which in turn is dependent on both the external calcium concentration in the medium, as well as the confluency of the cultures themselves (Hennings *et al*, 1980, Kruszewski *et al*, 1991, Pillai *et al*, 1990, Sharpe *et al*, 1989, Yuspa *et al*, 1989) We therefore examined the effects of treatments which modulate both external and internal calcium concentrations on HD-induced toxicity in both actively proliferating and confluent cultures of neonatal human skin keratinocytes

MATERIALS AND METHODS

Materials

Trypsin, foetal calf serum and Fungizone were purchased from Flow Laboratories (Mississauga, Ontario, Canada) AlamarBlue™ was acquired from AccuMed International Inc (Westlake, OH, USA) Keratinocyte serum free medium (KSFM) was obtained from Gibco BRL, Grand Island, NY, USA) and dispase from Collaborative Research (Bedford, MA, USA) Pluronic F-127, 1,2-bis (O-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM) and Fura-2 AM were supplied by Molecular Probes Inc (Eugene, OR, USA), while thapsigargin and ionomycin were obtained from Research Biochemicals International (Natick, MA, USA) Sulfur mustard was prepared by the Hazard Avoidance Section, Defence Research Establishment Suffield at greater than 99% purity

Cell culture

Primary cultures of human skin keratinocytes were prepared from neonatal foreskins Tissue was obtained on the day of circumcision and incubated at 4°C for 24 hr in 25 U/ml dispase The epidermis was removed and the cells segregated by a further 5 min incubation in 0 25% trypsin at 37°C The trypsin was deactivated by the addition of 20% serum in medium and the cell suspension was centrifuged The cell pellet was resuspended in KSFM and filtered through 70 μM nylon mesh 75-cm^2 flasks were seeded at 5×10^5 cells/8 ml KSFM supplemented with gentamicin (50 μg/ml) and Fungizone (0 25 μg/ml) and incubated in a 37°C humidified incubator in a 5% CO_2/95% air atmosphere Cultures were re-fed every 2–4 days, as required First passage cultures were seeded from log growth primary cultures at a density of 1000 cells/well in 96-well Costar multiwell plates or onto 13×14 mm glass coverslips (0 15 mm thick, Biophysic Technologies, Inc) in 35-mm culture dishes at 30,000 cells/dish

Chemical treatment and cytotoxicity studies

On the day of chemical treatment the cultures (proliferating 3–4 days *in vitro*, confluent, 7–9 days *in vitro*) were treated with freshly prepared treatment medium so that the desired final HD concentration was reached at 0 25% ethanol (v/v) The viability of HD-exposed cultures was determined at 48 hr In experiments which assessed the effects of BAPTA-AM, ionomycin or thapsigargin on HD toxicity, the compounds were dissolved in ethanol or dimethyl sulfoxide (DMSO, BAPTA-AM) and administered to the cultures 1 hr prior to HD treatment In studies where external calcium was also varied, 1 hr before drug treatment, the cultures were aspirated, rinsed twice with 200 μl phosphate buffered saline and then re-fed with calcium-free medium that had been adjusted to the desired calcium concentration with calcium chloride In these experiments, Fura 2 was not used to quantitate the external calcium and therefore these values represent the *nominal* calcium concentrations In all experiments, test drugs were left in the cultures for the full 48-hr test period The test drug vehicles (ionomycin, 0 046% ethanol, thapsigargin, 0 1% ethanol, BAPTA-AM, 0 15% DMSO) had no effect on the viability of the cultures, even in combination with the ethanol used as the HD vehicle To assess cytotoxicity, alamarBlue™ was added (10%, v/v) and the cultures were allowed to incubate with the dye for the last 2 hr of the treatment time period This assay is based on the reduction of a dye by viable cells to a coloured species which can be measured by absorbance or fluorescence, and has also been found to yield similar results compared to a number of more commonly used dyes and indicators (Fields and Lancaster, 1993) The absorbances (570 nm–600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA, USA) Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing six wells per data point and percent protection was expressed as the LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures ×100 All values represent data obtained from at least three separate experiments

Cytosolic calcium determination

Studies to assess the effect of HD on Fura fluorescence were performed using the cell impermeant

pentapotassium salt of Fura-2 (0.4 μM) in high (1 mM, pH 7 4) and relatively low (225 nM, pH 7 4) Ca^{2+} buffer solutions (Molecular Probes, OR, USA) Fluorescence of Fura-2 was monitored with a Delta-Scan 1 dual excitation/emission fluorometer with front-surface optics sample compartments (model D105, Photon Technology International Inc., Princeton, NJ, USA) The output from the xenon lamp was directed to two excitation monochromators with wavelengths set at 340 and 380 nm (5 nm bandpass), respectively, using a chopper wheel Output from the excitation monochromators was focused on a 1 cm^2 quartz curvette by a fibre-optic cable and the fluorescence collected through an emission monochromator at 510 nm (5 nm bandpass) using a photomultiplier tube

Experiments were performed after loading the cells with the membrane permeant form (Fura-2 AM) of the calcium sensitive dye Fura-2 (Grynkiewicz *et al.*, 1985, McDonough and Button, 1989). The culture medium was removed from cells grown on coverslips

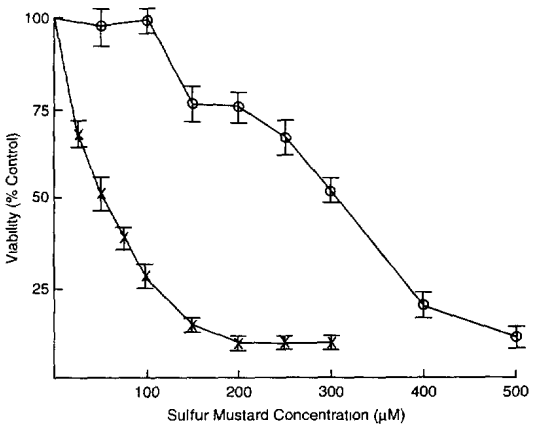

Fig 1 Toxicity of HD in proliferating and confluent cultures of human skin keratinocytes Proliferating (3 days, X) and confluent (7 days, 0) cultures of neonatal human skin keratinocytes were treated with varying HD concentrations and viability was determined 48 hr later using alamarBlue™ Results represent a typical experiment utilizing six wells per data point (mean ± SD)

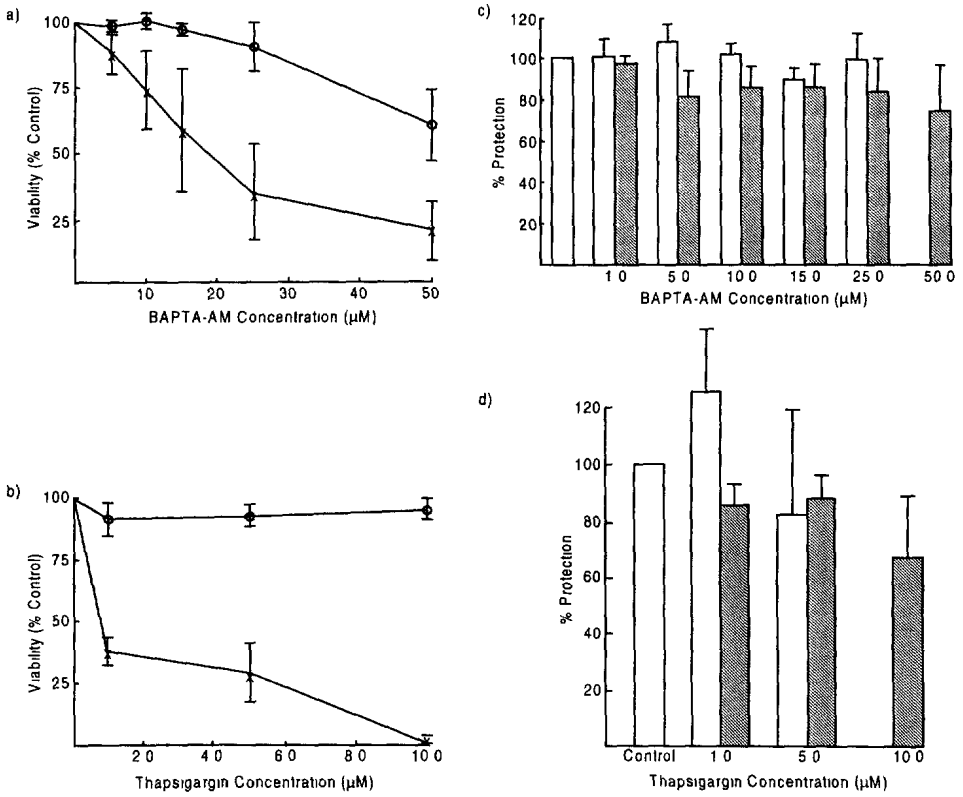

Fig 2 Effects of thapsigargin and BAPTA-AM in human skin keratinocytes The toxicity of BAPTA-AM (a) and thapsigargin (b) was examined in both proliferating (X) or confluent (0) cultures Both compounds were markedly more toxic in proliferating cultures than in confluent cells The modulatory effect of BAPTA-AM (c) and thapsigargin (d) on the toxicity of HD in proliferating (open bars) and confluent (cross-hatched bars) keratinocyte cultures was investigated by incubating test drugs with the cultures for 1 hr prior to HD exposure and assaying for viability 48 hr later Results represent the mean ± SD of three experiments and are normalized against the LC_{50} of vehicle-pretreated, HD-exposed cultures (LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures × 100) Neither treatment had any effect on HD toxicity

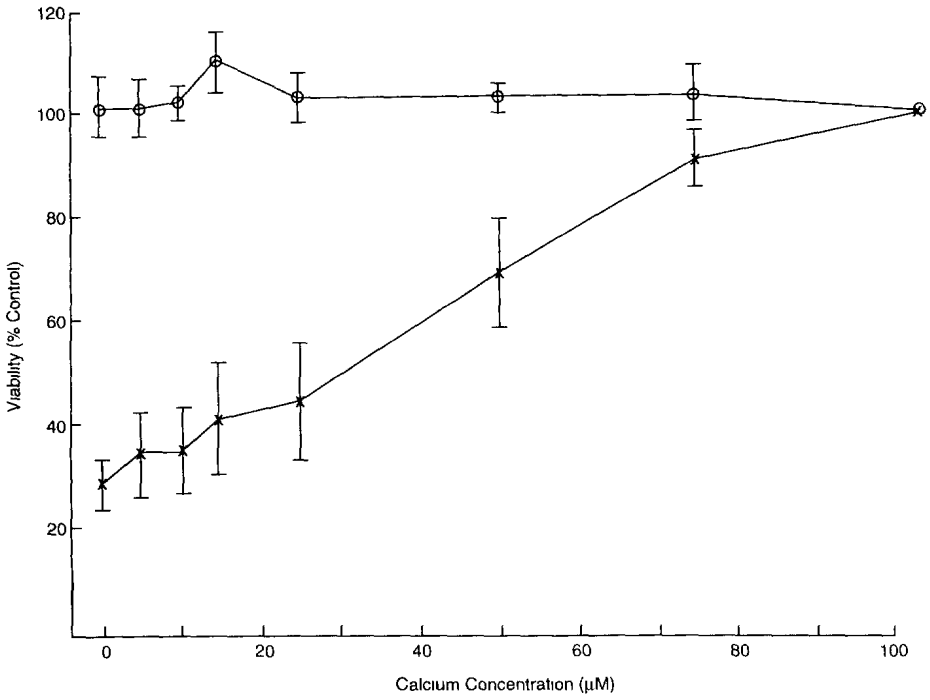

Fig 3 Effect of extracellular calcium concentration on the viability of proliferating and confluent keratinocytes Cultures were grown in KSFM until the day of treatment on day 3 (proliferating X) or day 7 (confluent, 0) of culture At this time the cultures were aspirated, rinsed twice with 200 μl buffer and then re-fed with calcium-free medium that had been adjusted to the desired calcium concentration with calcium chloride The cultures were then assayed for viability 48 hr later Results represent the mean ± SD of three experiments and are normalized against the LC_{50} of cultures incubated at 100 μM calcium Reduced external calcium levels were toxic in proliferating cells but had no effect in confluent cultures

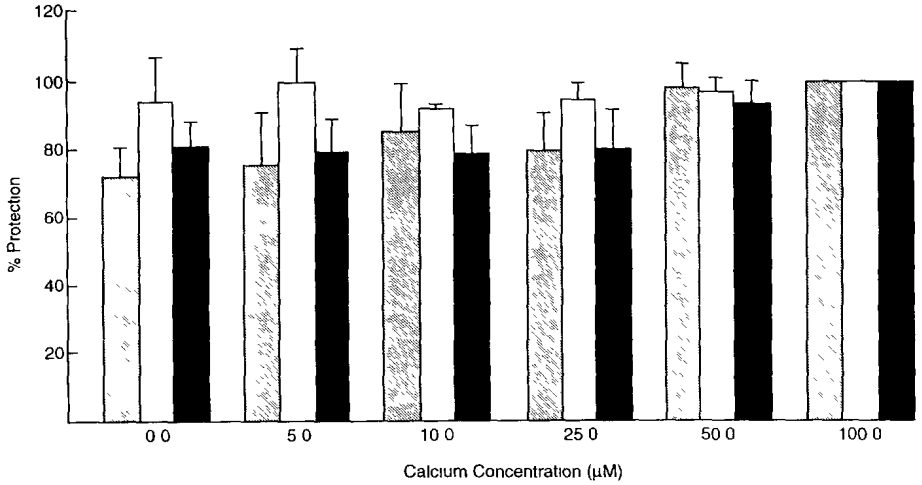

Fig 4 Effect of BAPTA-AM and thapsigargin on HD toxicity in human keratinocyte culture at different extracellular calcium concentrations Confluent keratinocyte cultures grown in normal culture medium were changed to medium adjusted to 0 0 to 100 μM calcium After 1 hr the cultures were incubated with BAPTA-AM (25 μM) or thapsigargin (5 0 μM) for an additional 1 hr prior to HD exposure The figure shows the results of HD treatment only (cross-hatched bars), BAPTA-AM pretreatment (open bars) or thapsigargin pretreatment (closed bars) Results represent the mean ± SD of three experiments and are normalized against the LC_{50} of vehicle-pretreated, HD-exposed cultures incubated in medium containing 100 μM calcium (the LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures × 100) No treatment yielded results that were different than another

and rinsed three times with HEPES buffered Hanks' balanced salt solution (pH 7 4, 1 mM nominal Ca^{2+} concentration) The cells were then incubated in the dark with 3 μM Fura-2 AM in DMSO/pluronic-127 for 45 min at room temperature After incubation, the buffer was changed to buffer containing no Fura-2 AM and allowed to equilibrate for at least 30 min prior to recording fluorescence signals. The coverslip was placed in a quartz cuvette so that the cells faced the excitation light path and the fluorescent emission passed through the coverslip before entering the emission monochromator Measurements were made in 2 4 ml buffer with continuous stirring in a temperature-controlled cuvette holder at 37°C. HD was added to the cuvettes and the signal re-acquired after a delay of 1 min to eliminate an artefact due to the dissolution of the HD in the buffer. Signals were background subtracted using Fura-2 free cells for autofluorescence and, where appropriate, solvent addition.

RESULTS

Figure 1 shows the toxicity of HD in both proliferating and confluent keratinocyte cultures Proliferating cultures were extremely sensitive to HD toxicity with an LC_{50} of 56 ± 23 μM (mean ± SD, n = 3) Sulfur mustard was five to six times less toxic in confluent cultures with an LC_{50} of 300 ± 47 μM (mean ± SD, n = 3). The vehicle (0 25% ethanol) had no discernible effect on the viability of the cultures

BAPTA-AM and thapsigargin were much less toxic in confluent cultures than in proliferating cultures (Fig 2a,b). In confluent cultures no toxicity was apparent with thapsigargin at the concentrations used (1.0–10 0 μM), while BAPTA-AM was non-toxic at concentrations up to 25 μM In contrast, both compounds reduced the viability of proliferating cultures in a concentration-dependent fashion In proliferating and confluent cultures that were treated with these compounds prior to HD exposure, no modulation of toxicity was noted compared to sham-pretreated, HD-exposed cultures (Fig 2c,d)

In studies that examined the effect of extracellular calcium on cell viability, confluent cultures were much less sensitive to changes in external calcium concentration (Fig. 3) Cultures were grown in KSFM (90 μM calcium) and then changed to medium adjusted to various calcium concentrations at or below 100 μM calcium When compared to the cells cultured in maximal calcium concentrations, the viability of confluent cultures did not change after 48 hr, even at a nominal zero calcium concentration In contrast, proliferating cultures were very sensitive to external calcium concentrations in this range and the viability of these cells declined rapidly below 75 μM calcium

Non-toxic concentrations of BAPTA-AM (25 μM) and thapsigargin (10 μM), in combination with varying concentrations of external calcium were examined for their effect on the toxicity of HD in confluent cultures No treatment, or combination of treatments was found to modulate the toxicity of HD (Fig. 4)

After 48 hr of continual exposure, ionomycin was slightly more toxic in cells that were actively proliferating than in confluent cultures (Fig 5a) The effects of ionomycin on the toxicity of HD were examined only in confluent cultures adjusted to either low (100 μM) or high (1 2 mM) calcium (Fig 5b). The cells were grown in normal culture medium and then changed

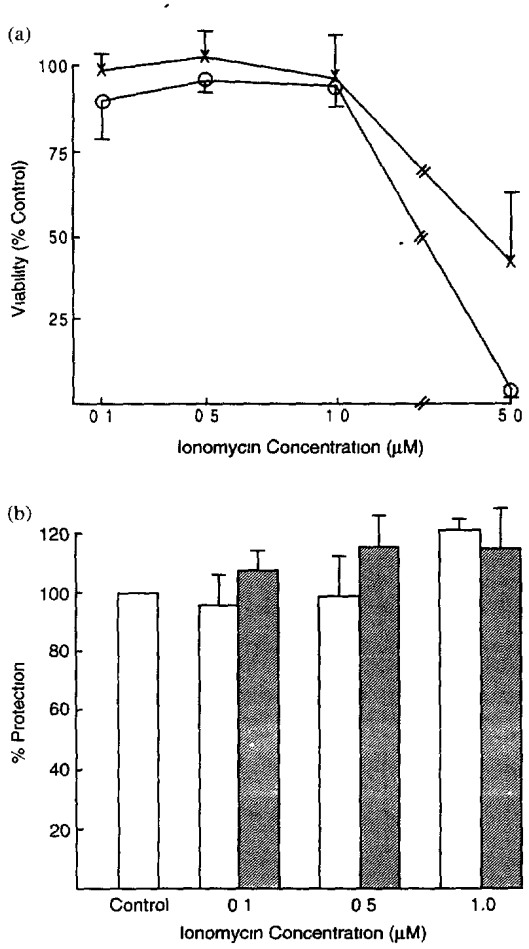

Fig 5 Effect of ionomycin in keratinocyte culture at low or high calcium concentration The toxicity of ionomycin was examined in confluent (X) or proliferating (0) cultures by treating the cells with varying concentrations of test compound and assaying for cell viability 48 hr later (a) The effect of ionomycin on the toxicity of HD was examined only in confluent human keratinocyte cultures The cells were grown in normal culture medium and then changed into low calcium (100 μM, open bars) or high calcium (1 2 mM, cross-hatched bars) medium After 1 hr the cultures were incubated with ionomycin for an additional 1 hr prior to HD treatment (b) Results represent the mean ± SD of three experiments and are normalized against the LC_{50} of vehicle-pretreated, HD-exposed cultures (the LC_{50} of drug-pretreated, HD-treated cultures divided by the LC_{50} of vehicle-pretreated, HD-treated cultures × 100) Ionomycin had no effect on the toxicity of HD

into low calcium or high calcium medium After 1 hr the cultures were incubated with ionomycin for an additional 1 hr prior to HD treatment This compound had no effect on HD toxicity at either calcium concentration in confluent cultures

Figure 6(a,b) shows the effect of various chemical treatments on intracellular calcium levels as measured by Fura-2 HD (1 0 mM) caused a rapid and sustained elevation of intracellular calcium levels (Fig 6a), while thapsigargin (5 0 μM) induced a transient elevation of calcium that eventually returned back to background levels Ionomycin (10 μM) caused an immediate elevation of the calcium signal which was instantly quenched by the addition of the calcium chelator BAPTA (25 mM) Lower concentrations of ionomycin (0 1–1 0 μM) also caused elevations in the calcium signal, albeit at a slower rate and not to the extent that 10 μM ionomycin did (data not shown) The non-ester form of BAPTA-AM was used since it quenches the calcium signal As reported earlier (Hamilton *et al.*, 1998), HD by itself neither affected Fura 2

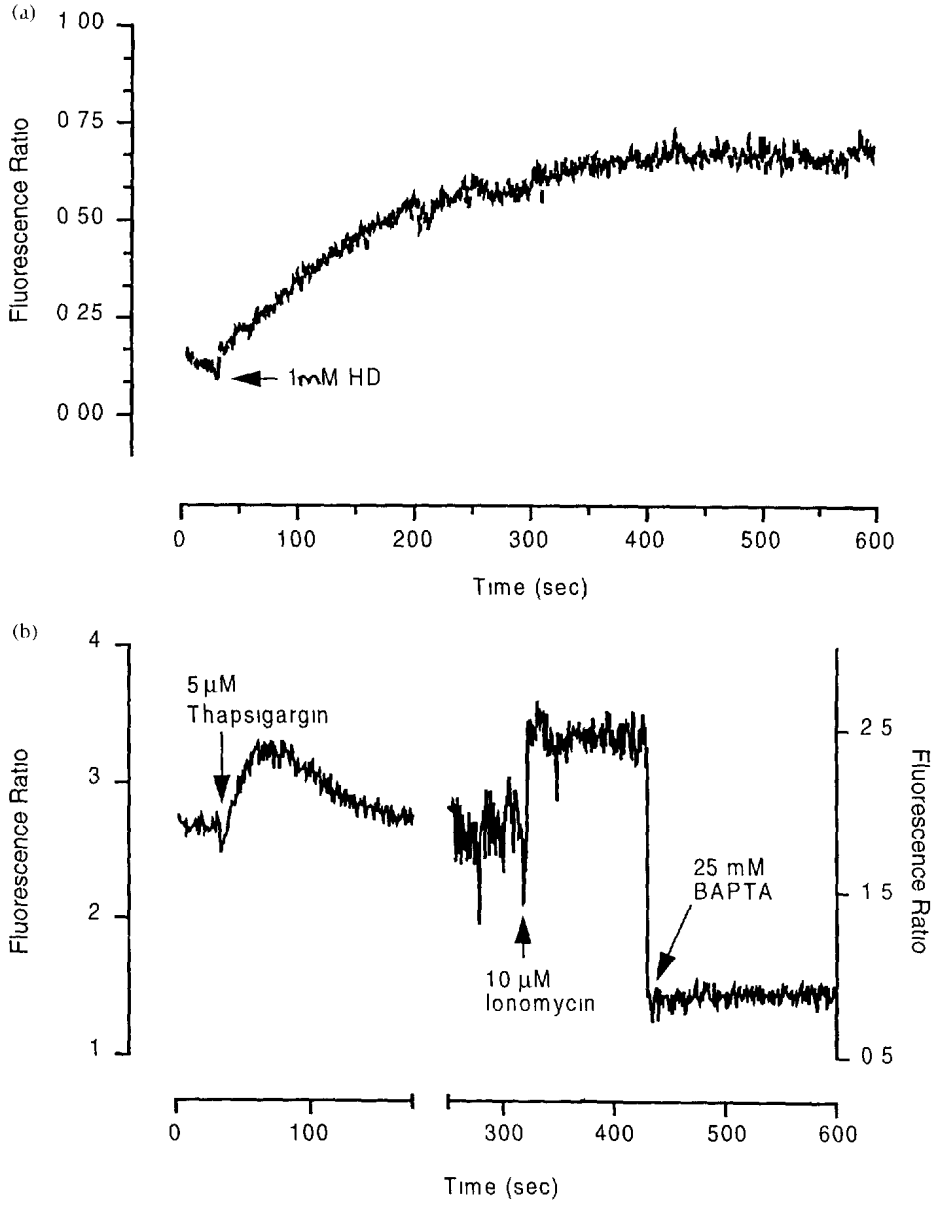

Fig 6 Effect of HD or drug treatment on intracellular free calcium levels in human keratinocytes 1 0 mM HD induced an immediate and sustained rise in intracellular levels as reflected by the rise in fluorescence ratio values (a) The fluroescence ratio value is defined as the signal intensity at 340 nm (maximum for Ca^{2+} associated Fura 2) divided by the signal at 380 nm (minimum for Ca^{2+} associated Fura 2) Thapsigargin caused an immediate rise in intracellular calcium that returned to baseline values In contrast, ionomycin induced a sustained rise in intracellular calcium that was immediately eliminated when the calcium chelator BAPTA was introduced into the cuvette (b)

fluorescence nor exhibited any fluorescence of its own in the excitation/emission ranges used in these experiments

DISCUSSION

A variety of hypotheses have been advanced over the last several decades in efforts to explain the vesicant action of HD (reviewed in Papirmeister *et al.*, 1991) One of the most recent has focused on the role of calcium in chemically induced toxicity and is based on a scheme put forward by Orrenius and co-workers (Orrenius, 1985, Orrenius and Nicotera, 1987, Orrenius *et al*, 1988) They proposed that toxicants that bind thiol compounds not only deplete glutathione, but also inactivate a number of proteins, namely Ca^{2+} ATPases, that are responsible for calcium homeostasis in the cell This combined insult causes a sustained intracellular calcium elevation with resultant activation of endonucleases, phospholipases and proteases that lead to cell death

Several different laboratories utilizing a variety of cell culture systems have examined the effect of HD (approx. 100 μM–1 0 mM) on calcium, and have come to varying conclusions as to the importance of calcium homeostasis in HD-induced cytotoxicity The first laboratory to test the thiol-Ca^{2+} hypothesis found that in mouse fibroblast B77 cells, HD induced a modest, but immediate and sustained rise in intracellular calcium levels that was independent of external calcium concentrations (Hua *et al*, 1993) However, follow-up work by Mol and co-workers (1994) were equivocal In second passage cultures of human skin epidermal keratinocytes grown on 3T3 feeder layers, they found little or no evidence of HD-induced intracellular calcium elevation and dismissed the small increases they detected as reflecting abnormal cellular physiology rather than acute toxicity (Mol, 1994, Mol and Smith, 1996) In contrast, intracellular calcium was consistently found to be elevated 2–6 hr after HD exposure in a mouse neuroblastoma-rat glioma hybid cell line (NG108-15) by Ray and colleagues (Ray *et al*, 1995) who also found a similar elevation in human epidermal keratinocytes (Ray *et al.*, 1994). This same group has gone on to report that the cell-permeant calcium chelator BAPTA-AM not only prevented this intracellular calcium elevation, but was also effective in preventing HD-induced cytotoxocity (Ray *et al.*, 1996, 1997, 1998). Recent studies in our labratory have tended to confirm the initial findings of Hua *et al* (1993) In first passage just-confluent cultures of neonatal human skin keratinocytes, we found that HD does indeed cause an immediate, concentration-dependent and sustained elevation of intracellular calcium that appeared to originate from intracellular stores Furthermore, the sensitivity to HD and the time course of this calcium elevation roughly paralleled the effects of HD on cell viability, although the concentrations of HD that maximally elevated intracellular calcium would be considered supralethal (Hamilton *et al.*, 1998) In order to further assess the link between intracellular calcium and HD-induced cytotoxity we examined the effect of modulating both external and internal calcium levels prior to HD culture exposure, on the resultant toxicity

Human keratinocyte culture is an extremely dynamic system, the sensitivity of which to HD is dependent on a large number of variables, not the least of which is the confluency of the cultures These variables may well be responsible for the different findings concerning the effects of HD on intracellular calcium The differentiative state of both mouse and human keratinocyte culture has been extensively characterized as being dependent on the intracellular calcium environment, which is dependent on a number of factors, including extracellular calcium levels and the confluency of the culture (Hennings *et al*, 1980, Kruszewski *et al*, 1991, Pillai *et al.*, 1990, Sharpe *et al*, 1989, Yuspa *et al*, 1989) For these reasons, we examined the effects of a variety of treatments in both actively proliferating and just-confluent cultures We found that HD was several times more toxic in actively proliferating subconfluent cultures than it was in confluent cultures where a large proportion of the cells would be committed to terminal differentiation We have not been successful using proliferating keratinocytes in our calcium studies because proliferating cells load poorly or not at all with Fura-2. However, the sensitivity of confluent cells to HD toxicity, although reduced, was similar to the concentrations required to elevate intracellular calcium levels (Hamilton *et al*, 1998)

We attempted to replicate the work of Ray and coworkers with BAPTA-AM (Ray *et al*, 1996, 1997, 1998). This cell-membrane permeable calcium chelator should modulate toxicity if intracellular calcium elevation is indeed causal or involved in the cascade of events that lead to HD toxicity. Preliminary studies parallelled this group's work and found that at 25 μM, BAPTA-AM was non-toxic in confluent cultures In contrast, proliferating cells were very sensitive to this compound, with an LC_{50} of 20 7 ± 8.9 μM Unexpectedly, however, pretreatment of either proliferating or confluent cultures with this chelator failed to modulate HD toxicity in any way, even at BAPTA-AM concentrations that were in themselves toxic It is unclear why our results contrast those of Ray *et al* so dramatically We also carried out experiments with thapsigargin, an endoplasmic reticulum Ca^{2+} ATPase inhibitor, that we have previously shown to eliminate the intracellular calcium elevation induced by HD (Hamilton *et al*, 1998) The results were similar to those obtained with BAPTA-AM, proliferating cells were very sensitive to this compound ($LC_{50} = 0\ 8$ μM ± 0.1 μM), while confluent cultures were not affected at the concentrations used Furthermore, thapsigargin pretreatment

failed to modulate HD toxicity in any way at any concentration used It appears that, at least in first passage neonatal human skin keratinocytes, modulation of intracellular calcium is not causal in HD toxicity

We next examined the effects of modulating the calcium concentration of the medium bathing the cultures, since internal calcium levels are known to be sensitive to external calcium Confluent cultures were totally resistant to variations in external calcium, even at nominal zero calcium concentrations However, proliferating cultures were extremely sensitive to changes in external calcium and rapidly lost viability as the calcium concentration decreased below 75 μM Although the concentration-response of HD in confluent cultures did not vary with external calcium concentrations from 0 to 100 μM we examined the effect of varying calcium concentration in conjuction with optimal concentrations of BAPTA-AM (25 μM) and thapsigargin (10 μM) Although unlikely, it was possible that when these compounds exhaust internal stores, external calcium plays a role in HD toxicity at later time points than those measured with Fura-2 This possibility was ruled out when, once again, no treatment or combination of treatments had any effect on HD toxicity

The last series of experiments examined the effects of the calcium ionophore ionomycin Ionomycin concentrations higher than 1 0 μM were toxic in both proliferating and confluent cultures, with the former being the most sensitive Pretreatment of cultures with this compound at low (100 μM) and high (1 2 mM) calcium concentrations were carried out only in confluent cultures The "calcium switch", from low to high calcium, is well known to induce terminal differentiation in proliferating keratinocytes and interpretation of the effects of HD in this situation would be difficult As with every other experimental regimen designed to perturb calcium homeostasis in these studies, ionomycin had no effect on the toxicity of HD, even at 1 2 mM calcium

In summary, earlier work in this laboratory has shown that HD induces a rapid, sustained, concentration-dependent increase in intracellular calcium in neonatal human skin keratinocytes that appears to originate from intracellular stores However, this study shows that perturbation of calcium homeostasis is not causal or directly involved in the development of HD-induced toxicity, at least in this cell culture system

Acknowledgement –This work was supported in part by DAMD17-99-2-9007 from the US Army Medical Research Acquisition Activity

REFERENCES

Dacre J C and Goldman M (1996) Toxicology and pharmacology of the chemical warfare agent sulfur mustard *Pharmacology Reviews* **48,** 289-325

Fields R D and Lancaster M V (1993) Dual attribute continuous monitoring of cell proliferation/cytotoxicity American Biotechnology Laboratory, March

Grynkiewicz G Poenie M and Tsien R Y (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties *Journal of Biological Chemistry* **260,** 3440-3450

Hamilton M G Dorandeu F M, McCaffery M, Lundy P M and Sawyer T W (1998) Modification of cytosolic free calcium concentrations in human keratinocytes after sulfur mustard exposure *Toxicology in Vitro* **12,** 365-372

Hennings H, Michael D, Cheng C Steinert P Holbrook K and Yuspa S H (1980) Calcium regulation of growth and differentiation of mouse epidermal cells in culture *Cell* **19,** 245-254

Hua A, Daniel R, Jasseron M P and Thiriot C (1993) Early cytotoxic effects induced by bis-chloroethyl sulphide (sulphur mustard) $[Ca^{2+}]_i$ rise and time-dependent inhibition of B77 fibroblast serum response *Journal of Applied Toxicology* **13,** 161-168

Kruszewski F H Hennings H, Yuspa S H and Tucker R W (1991) Regulation of intracellular free calcium in normal murine keratinocytes *American Journal of Physiology* **261,** C767-C773

Lundy P M, Sawyer T W and Frew R (1998) Effects of bis 2-chloroethyl sulphide on ATP receptor mediated responses of the rat vas deferens Possible relationship to cytotoxicity *Journal of Pharmacology and Experimental Therapeutics* **285,** 299-306

McDonough P M and Button D C (1989) Measurement of cytoplasmic calcium concentration in cell suspensions correction for extracellular Fura-2 through use of Mn^{2+} and probenecid *Cell Calcium* **10,** 171-180

Mol M A E (1994) Does sulfur mustard cause any changes in the cytoplasmic free calcium levels of human epidermal keratinocytes? In *Proceedings of the NATO RSG-3 Panel VIII Meeting*, pp 25-28 Munich, Germany

Mol M A E and Smith W (1996) Ca^{2+} homeostasis and Ca^{2+} signalling in sulfur mustard exposed normal human epidermal keratinocytes *Chemico Biological Interactions* **100,** 85-93

National Academy Press (1993) *Veterans at Risk The Health Effects of Mustard Gas and Lewisite* ed C M Pechura and D P Rall Washington, DC

Orrenius S (1985) Biochemical mechanisms of cytotoxicity *Trends in Pharmacological Sciences* **15** (Suppl), 18-20

Orrenius S, McConkey D J and Nicotera P (1988) Mechanisms of oxidant-induced cell damage In *Oxy-Radicals in Molecular Biology and Pathology*, ed P A Cerutti, I Fridovichand and J M McCord Alan R Liss, New York

Orrenius S and Nicotera P (1987) Biochemical mechanisms of oxidative liver cell injury *Bulletin of European Physiopathology and Respiration* **23,** 291-295

Papirmeister B, Feister A J, Robinson S I and Ford R D (1991) *Medical Defense Against Mustard Gas Toxic Mechanisms and Pharmacological Implications* CRC Press, Boca Raton, FL

Pillai S, Bikle D D Mancianti M-L, Cline P and Hincenbergs M (1990) Calcium regulation of growth and differentiation of normal human keratinocytes Modulation of differentiation competence by stage of growth and extracellular calcium *Journal of Cellular Physiology* **143,** 249-302

Ray R, Benton B J, Anderson D R Byers S L and Petrali J P (1998) Intervention of sulfur mustard (HD) toxicity by down regulation of cell proliferation and metabolic rates In *Proceedings of the 1998 Medical Defense Bioscience Review* pp 123 US Army Medical Research and Material Command

Ray R Benton B J, Anderson D R, Byers S L, Shih M L and Petrali J P (1996) The intracellular free calcium chelator BAPTA prevents sulfur mustard toxicity in

cultured normal human epidermal keratinocytes. In *Proceedings of the 1996 Medical Defense Bioscience Review* pp 1021–1027 US Army Medical Research and Material Command

Ray R, Benton B J, Broomfield C A, Anderson D R, Byers S L, Petrali J P and Bhat K R (1997) Calcium-dependent mechanisms of sulfur mustard toxicity and its protection In *Proceedings of NATO RSG-3 Panel VIII Meeting* pp 479–486 McLean, VA

Ray R, Legere R H, Majerus B J and Petralli J P (1995) Sulfur mustard-induced increase in intracellular free calcium level and arachidonic acid release from cell membrane *Toxicology and Applied Pharmacology* **131,** 44–52

Ray R, Majerus B J and Broomfield C A (1994) Effect of sulfur mustard on intracellular free calcium level and membrane fluidity in normal human epidermal keratinocytes. *Canadian Journal of Physiology and Pharmacology* **72** (Suppl 1), 3–22

Sharpe G R, Gillespie J I and Greenwell J R (1989) An increase in intracellular free calcium is an early event during differentiation of cultured human keratinocytes *FEBS Letters* **254,** 25–28

Smith W and Dunn M A (1991) Medical defense against blistering chemical warfare agents *Archives of Dermatology* **127,** 1207–1213

Warthin S W and Weller C V (1919) *The Medical Aspects of Mustard Gas Poisoning* Mosby, St Louis, MO, USA

Yuspa S H, Kilkenny A A, Steinert P M and Roop D R (1989) Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro *Journal of Cell Biology* **109,** 1207–1217.