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CD40 ligand, Bcl-2, and Bcl-x_L spare group I Burkitt lymphoma cells from CD77-directed killing via Verotoxin-1 B chain but fail to protect against the holotoxin

J Gordon*[,1] A Challa[1], JM Levens[1,2], CD Gregory[2], JM Williams[3], RJ Armitage[4], JP Cook[5], LM Roberts[5] and JM Lord[5]

[1] MRC Centre for Immune Regulation, The University of Birmingham, Birmingham, UK
[2] School of Biomedical Sciences, The University of Nottingham, Nottingham, UK
[3] Renal Research, Clinical Research Block, Queen Elizabeth Hospital, Birmingham, UK
[4] Immunex Corporation, Seattle, WA, USA
[5] Department of Biological Sciences, University of Warwick, Coventry, UK
* Corresponding author: J Gordon, MRC Centre for Immune Regulation, The University of Birmingham, Vincent Drive, Birmingham, B15 2TT, UK. Tel: 44 121 414 4034; Fax: 44 121 414 3599; E-mail: j.gordon@bham.ac.uk

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Abstract

Owing to its lineage and differentiation stage-restricted expression, CD77 has been mooted as a therapeutic target in Burkitt lymphoma (BL). The recognition that the globotriaosyl moiety of this neutral glycosphingolipid is a receptor for *Escherichia coli*-derived Verotoxin-1 (Shiga-Like Toxin-1) offers a potential delivery system for the attack. Here we show that CD77-expressing Group I BL cells which are normally susceptible to activation-induced death on binding Verotoxin-1 B chain are protected in the presence of CD40 ligand. Ectopic expression of either *bcl*-2 or *bcl*-x_L also afforded resistance to the actions of the B chain. In total contrast, neither of the survival genes nor a CD40 signal – even when acting in concert – protected against killing mediated by the holotoxin. These findings indicate that while therapeutic modalities for CD77-expressing B cell tumors (which include follicular lymphoma) based on the use of Verotoxin-1 B chain might be compromised by the activation of endogenous or exogenous survival pathways, those exploiting the holotoxin should be left unscathed. *Cell Death and Differentiation* (2000) **7,** 785 – 794.

Keywords: lymphoma; Verotoxin; apoptosis; CD77; CD40

Abbreviations: BL, Burkitt lymphoma; CD40L, CD40 ligand; EBV, Epstein-Barr virus; GC, germinal center; TNF, tumor necrosis factor; VT, Verotoxin

Introduction

Verotoxin-1 (VT-1) produced by the enteropathogenic O157 strain of *Escherichia coli* binds selectively to the α-D-gal-(1→4)-β-D-gal-(1→4)-β-D-glucose-(1→) trisaccharide moiety of globotriaosylceramide, also known as CD77.[1–4] Expressed on subsets of endothelial, mesangial, and epithelial cells, the surface representation of CD77 on hematopoietic cells – with the exception of subtypes of erythrocytes and some low level expression on monocytes – is restricted to centroblasts, cells that constitute the proliferating B cell compartment of germinal centers (GC) located within secondary lymphoid organs.[5–11] From its cell line-restricted reactivity and an inability to bind recirculating normal B cells, the prototype CD77 mAb, 38.13, was initially designated as defining a moiety termed 'BLA', or Burkitt Lymphoma Antigen.[12] Based partly on their mutual expression of CD77, BL cells are now deemed to represent phenotypic tumor equivalents of normal GC B cells.[13,14] A large proportion of follicular lymphoma also appears to remain faithful to their normal counterparts by displaying CD77 on the tumor cell surface.[15]

VT-1 is composed of an A subunit non-covalently associated with a pentamer of B subunits. The A subunit itself is further divided into a catalytic A1 region and a B chain-binding A2 region by a furin cleavage site. Cleavage at this site is important for optimal activation of toxin during cellular uptake.[16] CD77 permits the cellular entry of VT-1 through an initial binding of the pentameric B subunits of the holotoxin. Subsequent endocytosis of toxin to the endoplasmic reticulum, and membrane translocation to the cytosol, results in the inactivation of ribosomes by the toxin's catalytic A1 subunit. This inactivation involves a specific depurination of 28S ribosomal RNA which renders the ribosomes unable to function in protein synthesis: an event which ultimately leads to the death of the cell.[16,17] VT-1 has thereby been mooted as a potential delivery system for toxin-based therapy of CD77-expressing lymphoma: indeed, a range of BL cell lines have been shown to die on exposure to the holotoxin in culture.[9,18] In addition to the ribosome-inactivating properties of the A chain, it was found that B chain pentamer – when present at sufficiently high concentration – was, of itself, able to deliver a death signal to BL cells.[18] Subsequent studies using the 38.13 antibody indicated that the cross-linking of CD77 initiated an intracellular signaling cascade (involving a rise in cytosolic Ca^{2+} and the generation of ceramide) which culminated in the induction of apoptosis.[9,19] Thus it appeared that therapeutic attack on CD77-expressing lymphoma independent of VT-1 A chain might now be feasible.

Normal GC B cells display both *in situ* and *in vitro* a high propensity for spontaneous apoptosis, a process reflecting the need to select for high affinity mutations that target the V region genes in proliferating centroblasts.[11] The constitutive B cells of GC fail to express the pro-survival protein Bcl-2, a feature retained by BL cells.[11,20–24] Notably, BL is characterized by a classical 'starry sky'

histology reflecting the presence and activity of tingible body macrophages that normally colonize GC in order to manage the high rate apoptosis occurring at these sites.[11,25–27] Moreover, BL cell lines in early passage that remain faithful to the biopsy phenotype (termed 'group I') are both Bcl-2-negative and can be readily prompted into apoptosis by a variety of signals including those induced by antigen-receptor cross-linking.[23,28–31]

Although BL arising in endemic areas invariably harbor Epstein-Barr virus (EBV), freshly-isolated tumor cells and group I lines derived from them fail to express EBV genes involved in B cell immortalization.[32–34] On long-term culture, however, such lines display 'phenotypic drift' and ultimately give rise to group III lines as a result of full expression of EBV transforming genes.[33,34] This is accompanied by the appearance of Bcl-2 protein and a subsequent resistance to antigen-receptor-dependent apoptosis.[23,29] Introduction of *bcl*-2 directly into group I cells by gene transfection has confirmed the central importance of Bcl-2 to the anti-apoptotic phenotype that emerges.[22,23] Follicular lymphoma is quite different from BL being characterized by intrinsic high survival driven by the influence of deregulated *bcl*-2 that arises from the 14 : 18 chromosomal translocation that defines around 90% of cases.[35,36] This wired-in survival pathway may underlie the observed difficulty in eradicating the tumor population in this disease.[37]

Other survival mechanisms for normal and lymphoma B cells exist. For example, the *bcl*-2 related gene *bcl*-x_L can offer protection against apoptosis in some scenarios.[38–41] Extracellular signals can also engender protection. For both normal GC B cells and BL cell lines, the most potent of these is provided by CD40 ligand (CD40L) activating its receptor, CD40, on the target cell.[20,21,29,31] CD40L – a member of the TNF family – has been characterized as an inducible surface molecule of T-helper cells although a number of studies are now indicating its presence on at least subsets of normal and malignant B cells.[42–45] Thus B lymphoma cells may receive survival prompts both from infiltrating T cells and, potentially, via autocrine/juxtacrine modes.

The design of therapeutic strategies should take account of both the intrinsic and extrinsic means by which a tumor could circumvent the desired result: namely, cell death. Using BL as the model for CD77-expressing lymphoma, we have explored the potential impact of the known survival pathways exploited by B cells on their response to VT-1. The findings demonstrate that these would succeed in protecting lymphoma cells from VT-1 B chain. They would, however, be thwarted when eliciting A chain-dependent killing with the holotoxin.

Results

CD77-dependent sensitivity to cell death is VT-specific

Physiological expression of CD77 on B lymphocytes is restricted to those with an apoptotic phenotype leading to the suggestion that its presence may be an intrinsic component of their capacity for programmed cell death (PCD).[9,46] We assessed here whether the presence of CD77 on BL lines was requisite to their ability to undergo activation-induced PCD while attempting to confirm its necessity for VT-1-mediated killing. This was facilitated by the observation that the L3055 group I BL line maintained in early passage comprised a dual population of $CD77^{pos}$ and $CD77^{neg}$ cells at an approximate 2 : 1 ratio (Figure 1a). Exposure of this line to 100 ng/ml VT-1 for 48 h led to the disappearance of the $CD77^{pos}$ fraction leaving a viable population that was exclusively $CD77^{neg}$ (Figure 1b). It should be noted that with increasing passage, the percentage of $CD77^{pos}$ cells in untreated L3055 cultures fell steadily from around 70% in early passages (<30) to approximately 30% in later ones (>60). The growing proportion of cells that remained viable following exposure to VT-1 closely reflected the increasing number of $CD77^{neg}$ cells present as the cultures progressed (data not detailed). This not only highlights the specificity of VT-1 for CD77-expressing cells but also indicates that the $CD77^{neg}$ population emerging post-VT-1 exposure did not arise simply as a consequence of globotriaosylceramide down-regulation at the cell surface: this conclusion was supported by the finding that VT-1 treatment of a group I line where cells were uniformly $CD77^{pos}$ (Mutu I) failed to yield a viable $CD77^{neg}$ subpopulation (n=4).

When assessed for its ability to respond to VT-1 by the cessation of DNA synthesis (both an indicator of cell killing and a prelude to apoptosis for these cells[24,31]) the wild-type population displayed a dose-dependent inhibition that by 24 h reached a maximum of around 70% with VT-1 concentrations of 10 ng/ml and above (Figure 1c). Extending exposure to 48 h failed to increase significantly the extent of this inhibition. By contrast, the VT-1 selected $CD77^{neg}$ cells were resistant to VT-1-dependent inhibition whether measured at 24 or 48 h (Figure 1d). While the $CD77^{neg}$ cells were similarly refractory to cessation of DNA synthesis promoted by VT-1 B chain, they remained fully sensitive to the inhibitory effects of cross-linking antigen receptor, elicited here by antibody (BU1) to sIgM (data not detailed). The outcome of these treatments as revealed by the inhibition of DNA synthesis was mirrored in a loss of cell viability and/or the appearance of apoptotic nuclei as assessed by visualizing acridine orange-stained cells (Table 1). The latter measurement was significant only for cells exposed to anti-IgM or B chain: the overwhelming majority of cells treated with VT-1 holotoxin had the appearance of lysis or necrosis rather than apoptosis. These data reveal that resistance to killing of group I BL lines resulting from a loss of CD77 is exclusive to VT-mediated pathways.

Expression of *bcl-2* and *bcl*-x_L confer resistance to VT-1 B chain but fail to protect from holotoxin

L3055 cells were forced to express the *bcl*-2 or *bcl*-x_L genes by generating stable transfectants under the influence of the human EF-1α promoter. A clone transfected with empty vector was established as a control. Another group I BL line, Mutu I, was used to establish a similar set of transfectants. Unlike L3055 cells, Mutu is an EBV-harboring BL line that on long

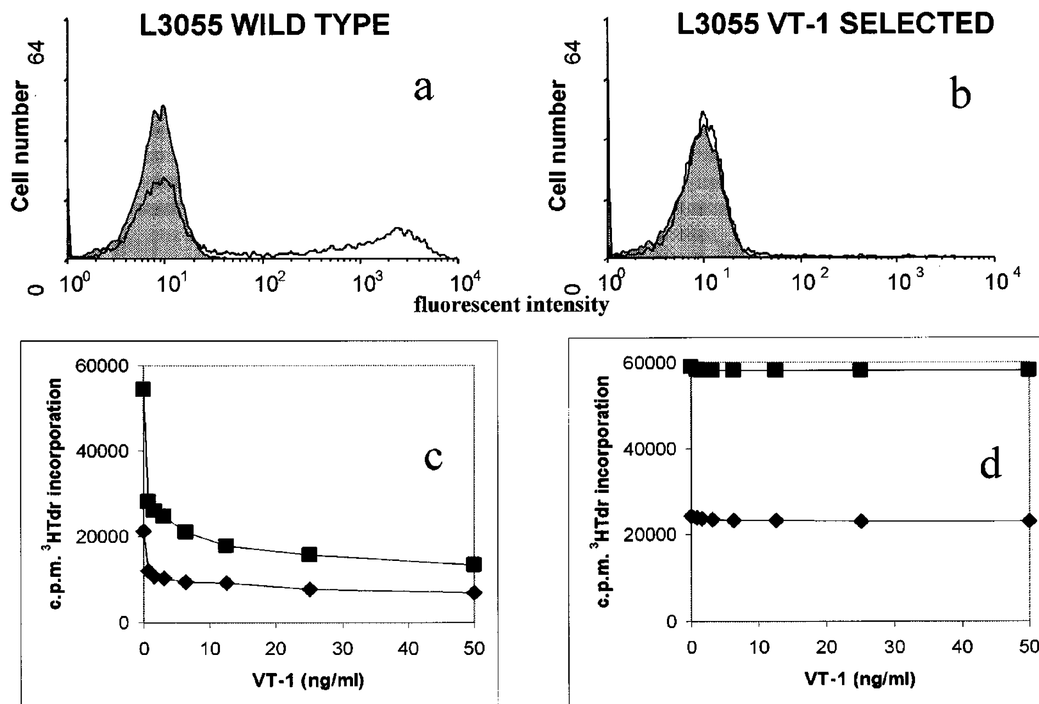

Figure 1 Selection of VT-1-resistant L3055 cells. L3055 wild-type cells (**a,c**) were cultured for 48 h with 100 ng/ml VT-1 after which the viable cells were recovered (**b,d**). Cells were stained for CD77 expression (**a,b**) with directly-conjugated 38.13 mAb (open histograms) or rat IgM control (shaded histograms). The response of the cells to subsequent culture with VT-1 was monitored by DNA synthesis (**c,d**) as assessed by ^{3}HTdr incorporation at 24 (◆) or 48 h (■). Results expressed as means of quadruplicate determinations (S.E.M. always <10% of mean values) are representative of four similar experiments

Table 1 CD77-dependent sensitivity to apoptosis is VT-specific

		% Dead cells				% Apoptotic cells		
Cells	**Time**	**CM**	**BU1**	**B chain**	**VT-1**	**CM**	**BU1**	**B chain**
L3/wild-type	24 h	11 (4)	43 (10)	51 (8)	62 (12)	10 (3)	49 (9)	58 (15)
	48 h	13 (2)	92 (5)	64 (11)	69 (8)	12 (5)	82 (17)	60 (4)
L3/CD77^{neg}	24 h	9 (5)	50 (14)	16 (2)	11 (5)	16 (4)	68 (15)	13 (1)
	48 h	12 (1)	94 (6)	13 (4)	15 (4)	15 (2)	83 (10)	16 (5)

L3 wild-type or CD77^{neg} cells as described in Figure 1 were cultured for times indicated in the presence of: control medium (CM); BU1 (anti-IgM, 500 ng/ml); VT-1 B chain (B chain, 100 μg/ml); or VT-1 holotoxin (VT-1, 30 ng/ml). Results shown are means of triplicate experiments with S.D. indicated in parentheses

term culture progresses to a group III phenotype.[29,34] The Mutu III line was also included for study. The status of Bcl-2 and Bcl-x_L expression among the different lines was confirmed by Western blotting as illustrated in Figure 2. It should be noted that the Mutu III cells contained a significant level of constitutively expressed Bcl-2 protein albeit lower than that found in the *bcl*-2-transfected lines.

Results presented in Figure 3 show that both Bcl-2 and, to a lesser but still significant extent, Bcl-x_L protected L3055 cells from cessation of DNA synthesis induced by antibody to sIgM. They each similarly protected from the inhibitory actions of VT-1 B chain. When assessed for sensitivity to VT-1 holotoxin, neither Bcl-2 nor Bcl-x_L was seen to afford protection to L3055 cells. The clone selected for the control vector showed a somewhat greater susceptibility than that of wild-type L3055 cells to both VT-1 B chain and holotoxin (Figure 3).

While Mutu I cells are relatively resistant to growth inhibition and programmed death mediated via soluble anti-IgM (unpublished observation) they can be encouraged into apoptosis by the calcium ionophore, ionomycin.[29] It can be seen from Figure 4 that Bcl-2 and Bcl-x_L each afforded protection to Mutu I cells from the effects not only of ionomycin but also of VT-1 B chain. Neither Bcl-2 nor Bcl-x_L was able to offer resistance to Mutu I cells from VT-1 holotoxin (Figure 4). Mutu III cells displayed a modest cessation of DNA synthesis in response to holotoxin but were fully resistant to both VT-1 B chain and the calcium ionophore.

Effects monitored by cessation of DNA synthesis were again reflected in the appearance of death and/or apoptotic cells (Figure 5). These results confirmed the protection afforded by Bcl-2 and Bcl-x_L to L3055 cells from apoptotic death induced by cross-linking sIgM and following exposure

to VT-1 B chain. Death still ensued on exposure to VT-1 holotoxin however. Bcl-2 and Bcl-x_L were again seen to protect Mutu I cells from calcium ionophore-induced death and that engendered by VT-1 B chain but failed to protect from killing mediated via holotoxin. Apart from a minor – and variable – population that was susceptible to the holotoxin, Mutu III cells were essentially resistant to all the routes of cell death (Figure 5 and see below). It should be noted that with concentrations of VT-1 holotoxin that were sub-optimal, in that they elicited only partial killing of the populations, the death that ensued – as exemplified here for the L3055 series – remained primarily non-apoptotic irrespective of survival gene status (Table 2).

When assessed for CD77 expression, all lines were clearly positive with the exception of Mutu III cells where a minority of cells stained weakly only: the disappearance of CD77 is a hallmark characteristic of a group I to group III transition among BL lines.[13,34] Interestingly, the bimodal distribution of CD77 expression seen with the L3055 wild-type cells was retained in both the *bcl*-2 and *bcl*-x_L transfectants while the vector controls displayed a uniformly CD77^{high} population (data not detailed). All Mutu I transfectants revealed a unimodal high level staining for CD77 (see, for example, Figure 7).

Thus, among the different lines studied, there was a close correlation between the level of CD77 positivity and susceptibility to VT-1 holotoxin. This association was highlighted by the Mutu III line where the number of dead cells arising in response to VT-1 exposure mirrored closely the proportion of CD77^{pos} cells detected at the time of analysis – thus, over four experiments, the following percentages for 'death' (as measured by trypan blue dye uptake following 24 h treatment with VT-1) *versus* 'CD77-positivity' (determined by FACS analysis at *t*=0) were observed: (i) 13% *vs* 10%; (ii) 26% *vs* 25%; (iii) 27% *vs* 24%; (iv) 43% *vs* 39%. The reason for the variability in CD77 expression seen with Mutu III cells is presently unclear but may relate to the growth phase of the cultures (our own unpublished observations).

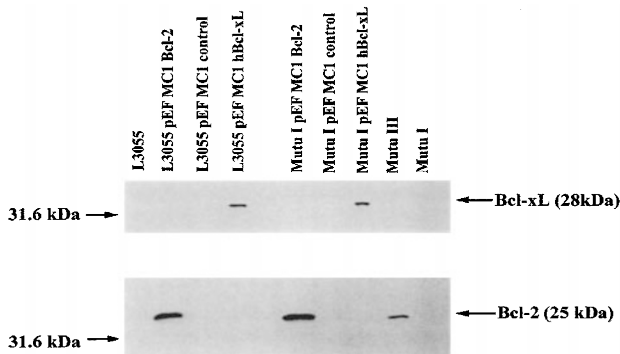

Figure 2 Expression of Bcl-2 and Bcl-x_L in L3055 and Mutu lines. Cell lysates were prepared from pelleted cells and equal amounts of total cellular protein were resolved on 12.5% SDS–PAGE. Western blotting was performed to detect the presence of Bcl-2 (lower panel) and Bcl-x_L (upper panel) with relative molecular weights by reference to standard markers indicated

CD40 signals discriminate against B chain- and holotoxin-mediated death pathways

The presence of soluble CD40L at 50 ng/ml protected both L3055 and Mutu I wild-type cells from cessation of DNA synthesis mediated by soluble anti-IgM and ionomycin respectively, confirming previous observations.[21,24,29] CD40L offered similar protection from VT-1 B-chain but left the actions of VT-1 holotoxin unchecked. CD40 signaling

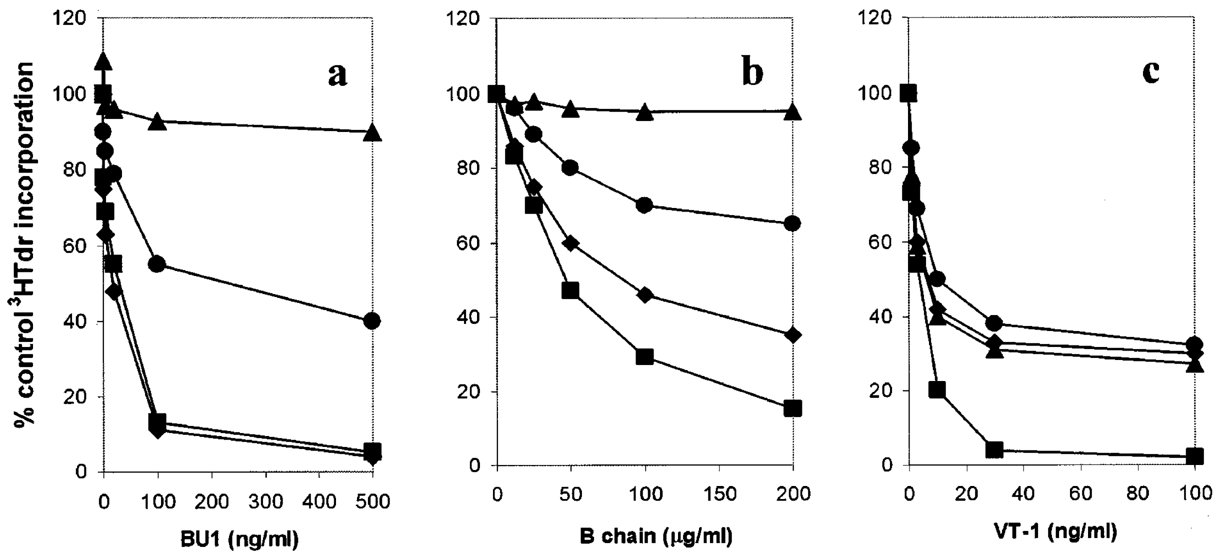

Figure 3 Inhibition of DNA synthesis in L3055 transfectants in response to treatments. Cells were plated with concentrations of: (**a**) BU1 (anti-IgM); (**b**) VT-1 B chain; or (**c**) VT-1 holotoxin as indicated and DNA synthesis assessed at 48 h as in Figure 1. Results are expressed as percentage of control ^{3}HTdr incorporation relative to culture in control medium alone. In this experiment (which is representative of three) the absolute values (as c.p.m. with S.E.M. in parentheses) were: L3 wild-type (◆), 53 257 (3189); L3 vector control (■), 66 209 (2855); L3/Bcl-2 (▲), 86 340 (4002); L3/Bcl-x_L (●) 75 716 (4076). S.E.M. were always <10% of mean values

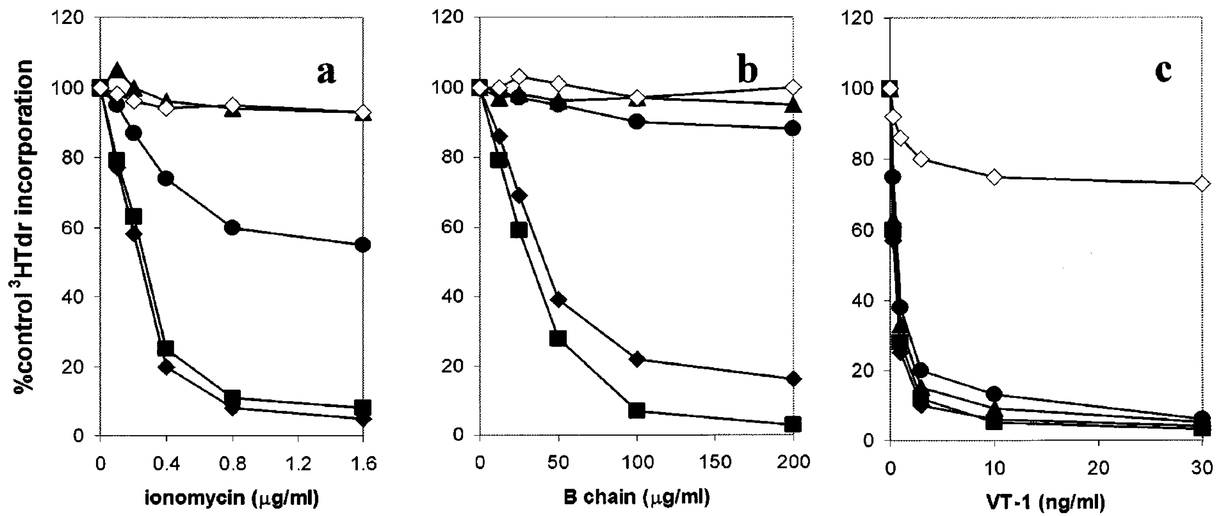

Figure 4 Inhibition of DNA synthesis in Mutu I transfectants and Mutu III cells in response to treatments. As for Figure 3 except cells were plated with concentrations of : (**a**) ionomycin; (**b**) VT-1 B chain; or (**c**) VT-1 holotoxin. In this experiment (representative of three) the absolute values (as c.p.m. with S.E.M. in parentheses) of ^{3}HTdr incorporation in culture medium alone were: Mutu I wild-type (◆), 65 049 (2832); Mutu I vector control (■), 62 477 (3509); Mutu I/Bcl-2 (▲), 99 404 (5878); Mutu I/Bcl-x_L (●), 85 110 (1694); Mutu III (◇), 72 565 (4902). S.E.M. were always <10% of mean values

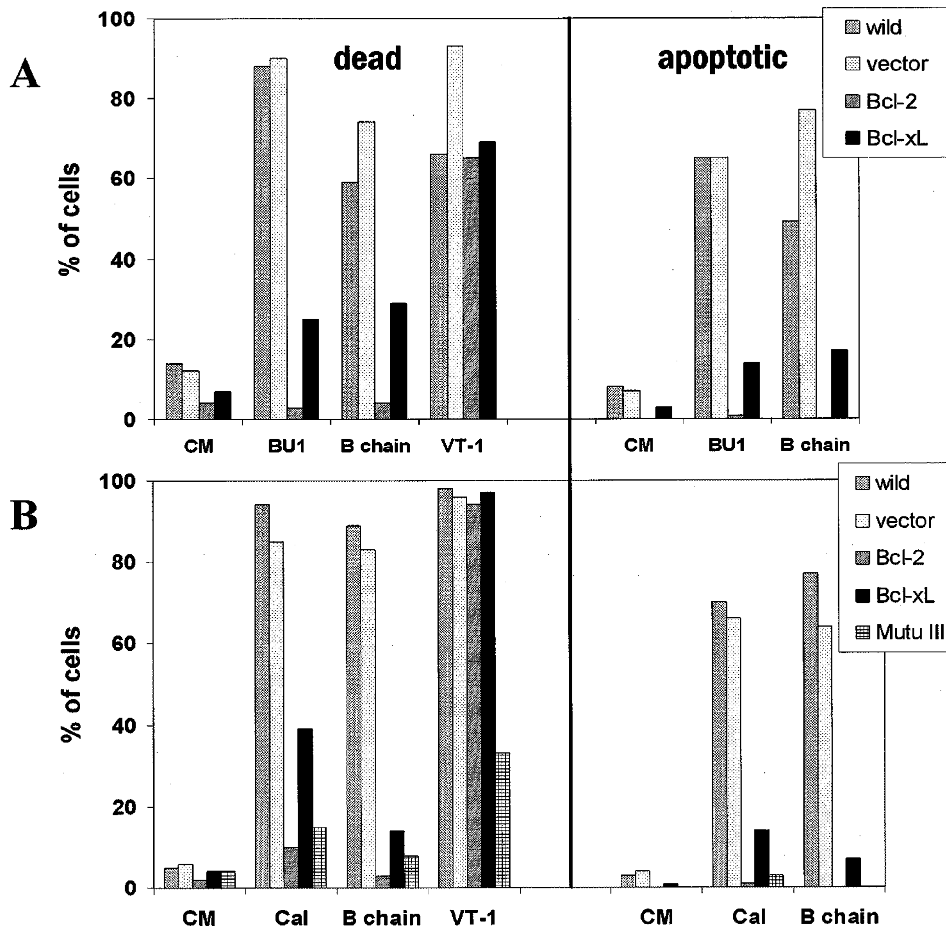

Figure 5 Survival genes protect from B chain- but not VT-1 holotoxin-induced death. Cells were cultured for 48 h in the presence of: control medium (CM); BU1 (anti-IgM, 500 ng/ml); calcium ionophore, ionomycin (Cal, 1 μg/ml); VT-1 B chain (B chain, 100 μg/ml); or VT-1 holotoxin (VT-1, 30 ng/ml) as indicated. (**a**) L3055 series; (**b**) Mutu series. The percentage of dead or apoptotic cells arising following treatments was enumerated as for Table 1. Results given are the means of three separate experiments: S.D. were never >17% and error bars are omitted for clarity

Table 2 VT-1 holotoxin-mediated death is primarily non-apoptotic

	% Dead cells				% Apoptotic cells			
	Following 30 h exposure to VT-1 holotoxin at (ng/ml):							
	0	2.5	5	10	0	2.5	5	10
L3/wild	12 (4)	26 (6)	40 (8)	59 (10)	11 (4)	13 (5)	9 (4)	16 (6)
L3/vector	7 (3)	37 (6)	56 (9)	84 (11)	5 (2)	8 (3)	10 (2)	14 (5)
L3/Bcl-2	1 (1)	22 (5)	35 (7)	54 (7)	0 (0)	0 (0)	1 (1)	1 (1)
L3/Bcl-x_L	5 (2)	25 (4)	38 (9)	58 (12)	2 (1)	1 (1)	4 (2)	7 (4)

As for Table 1 but cells were treated with concentrations of VT-1 holotoxin for 30 h as indicated

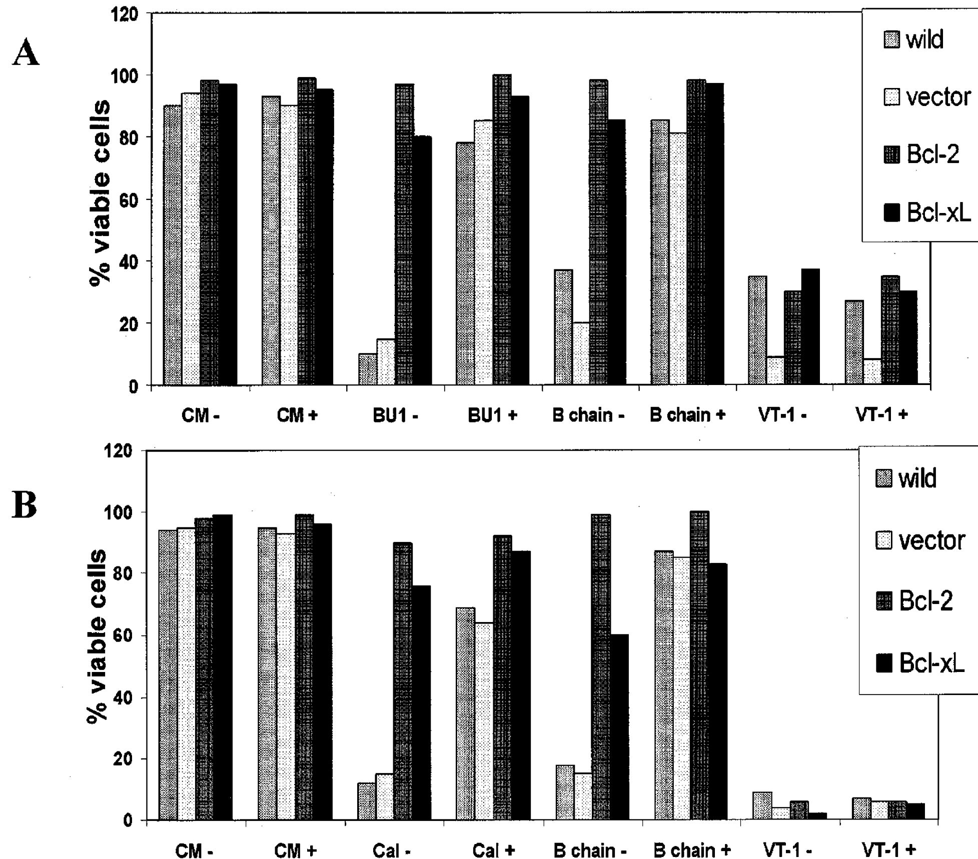

Figure 6 CD40L protects from B chain- but not VT-1-holotoxin-induced death. As for Figure 6 but results presented as percentage viable cells remaining after 48 h. Treatments are indicated without (−) or with (+) sCD40L present at 50 ng/ml. (**a**) L3055 series; (**b**) Mutu series. Results given are the means of three separate experiments: S.D. were never >17% and error bars are omitted for clarity

failed to protect against the holotoxin even for lines expressing *bcl*-2 or *bcl*-x_L (data not detailed). Assessments of cell viability once more confirmed these observations (Figure 6). On studying SAV and BL2 – two other CD77^{pos} group I BL lines sensitive to sIgM-dependent apoptosis – there was an identical outcome in the ability of CD40L to protect from death mediated by soluble anti-IgM or VT-1 B-chain while failing to spare them from killing elicited by the holotoxin (Table 3).

Finally, we asked whether CD40L might be offering protection to the cells from B chain killing by down-regulating CD77 expression, a phenomenon previously

Table 3 CD40L protects from B chain- but not VT-1 holotoxin-induced death

	% Cells remaining viable at 48 h in presence of:			
	CM	sCD40L	CM	sCD40L
Additions	SAV		BL2	
CM	92 (5)	91 (4)	95 (4)	95 (4)
BU1	31 (15)	86 (3)	17 (8)	74 (14)
B chain	20 (12)	80 (10)	15 (7)	84 (3)
VT-1	2 (1)	2 (0)	4 (0)	3 (1)

Cells were treated with additions shown for 24 h exactly as for Table 1. Where indicated, sCD40L (50 ng/ml) was also present. Results shown are means of triplicate experiments with S.D. indicated in parentheses

reported for group I cells exposed to high concentrations (1 μg/ml) of soluble CD40L for 3 days or more.[24] While cells cultured with CD40L remained sensitive to death promoted by holotoxin, the need for extensive cross-linking of CD77 to elicit A chain-independent killing via CD77 might necessitate that high level receptor expression is maintained for B chain-mediated apoptosis to proceed.[9,19] Mutu I cells were used for these studies as they displayed a uniformly high CD77 starting population. It is clear from the results presented in Figure 7 that culture of Mutu I cells (here shown for vector controls) with 50 ng/ml of soluble CD40L for either 24 or 48 h did not lead to a disappearance of CD77. Although CD77 levels did start to fall by day 2, expression remained substantive and was unlikely to be limiting: indeed, even after incubation with soluble CD40L, Mutu I vector controls carried higher levels of CD77 than wild-type cells. A similar outcome was obtained for Mutu I cells carrying *bcl*-2 or *bcl*-x_L (data not detailed).

Discussion

In certain respects, group I BL forced to express *bcl*-2 ectopically models follicular lymphoma. Both B cell tumors display a GC phenotype with the exception of follicular lymphoma being constitutively Bcl-2 positive due to the deregulation of the *bcl*-2 gene arising from the 14 : 18 translocation that characterizes this disease.[14,15,35,36] For any therapeutic modality to succeed against this most common of the lymphoma, it needs to overcome the powerful survival influence afforded to B cells by Bcl-2. Moreover, normally Bcl-2 negative BL cells can be prompted by extracellular signals to express the survival protein.[24] To confound matters further, CD40L can stimulate survival pathways in normal and malignant B cells independently of *bcl*-2.[21] For both human and murine B cells, CD40 engagement can lead to an upregulation of the anti-apoptotic *bcl*-2 family member, *bcl*-x_L[38–41] Though yet to be proven, the potential for CD40 signaling in B cell lymphoma exists by way of infiltrating T cells and via autocrine/juxtracrine CD40L-CD40 interactions.[43–45]

Here we have shown that while activation of either endogenous or exogenous survival pathways spares BL cells from death mediated by the B chain of VT-1, they remain fully susceptible to A chain-dependent killing elicited by VT-1 holotoxin. This extended to cells both ectopically expressing high levels of survival genes and receiving CD40L signals, an important outcome given the finding of Ghia *et al* that CD40-stimulated up-regulation of *bcl*-x_L in follicular lymphoma may contribute to enhanced survival.[47] Moreover, we have recently reported that normal, Bcl-2 positive, non-germinal center B cells induced to express CD77 by high level engagement of CD40L gain full susceptibility to killing by the holotoxin.[48] The complete resistance to B chain killing exhibited by the Mutu III cell line, while remaining fully susceptible to holotoxin, may have been due to more than the relatively low level of Bcl-2 that was found to be expressed: the EBV-encoded Latent Membrane Protein-1 (LMP-1) protein that is turned on as group I cells progress towards a group III phenotype additionally activates the expression of a zinc finger protein (A20) that can provide independent signaling for cell survival:[49,50] moreover, LMP-1 has been shown to behave – in many respects – as a constitutively active CD40 molecule providing further *bcl*-2-independent, anti-apoptotic potential when expressed.[51]

In toto, the above indicate that although therapeutic exploitation of VT-1 B chain for CD77-expressing lymphoma would seemingly have only limited application, that of VT-1 holotoxin appears to hold more universal appeal.

The capacity of the survival pathways to discriminate against B chain- and A chain-dependent killing is likely to reflect the different modes of cell death elicited by the two. High concentrations of pentameric B chain are required to achieve killing of group I BL cells [Mangeney *et al*[18] and data reported herein]. The ensuing cell death has been described previously as arising from an apoptotic pathway.[18] This we confirmed by demonstrating the generation of apoptotic nuclei as revealed by visualization of acridine orange-stained cells. Moreover, the ability to protect against this route of killing by anti-apoptotic signals in itself reinforces the notion that B chain triggers programmed death in these cells. It was recently reported that on cross-linking 38.13 antibody bound to CD77, an intracellular signaling cascade is initiated involving a rise in cytosolic Ca^{2+} and the generation of ceramide.[9,19] Boht have been implicated as components of apoptotic cell death in B lymphoma cells.[28,52]

The A chain of VT-1 promotes cell death by inhibiting protein synthesis as a consequence of modifying ribosomal RNA.[16,17] While still requiring the B chain to gain entry into a target cell, it is questionable whether CD77-dependent pathways additional to those of simple binding and

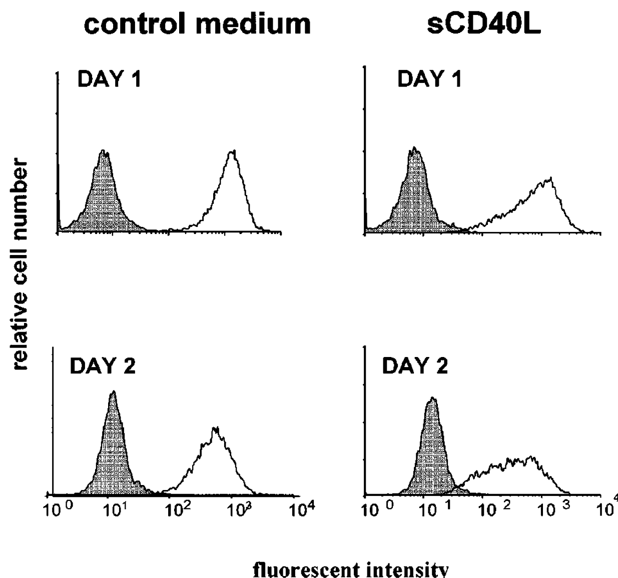

Figure 7 Influence of sCD40L on CD77 expression. Mutu I vector control cells were cultured for 1 or 2 days with sCD40L (50 ng/ml) as indicated. CD77 expression was then assessed by indirect staining with 38.13 mAb (open histograms) or a rat IgM control (shaded histograms). Data are representative of three similar experiments

endocytosis are operative in killing elicited by the holotoxin. This statement is based on the observation that VT-1 holotoxin kills cells at concentrations considerably less (<10 ng/ml) than those required for B chain to be active (>10 μg/ml). The involvement of an apoptotic component to holotoxin-mediated death is not ruled out however. It was recently shown that Pseudomonas endotoxin (PE) – which similar to VT-1 can kill target cells via A chain-dependent inhibition of protein synthesis – also increases caspase activity and induces PARP cleavage in a neoplastic epithelial cell line.[53] Thus, like PE, VT-1 may be exploiting two routes to cell death. Indeed, although for the lines used in the present study we were unable to detect significant induction of fragmented nuclei in response to the holotoxin, apoptotic morphology has been reported for other BL cells exposed to low doses of VT-1.[18] Despite this, as evidenced from the inability of either CD40 signals, Bcl-2, or Bcl-x_L to protect, it seems that even though VT-1 A chain might be capable of stimulating components of apoptosis, this pathway is redundant to the ultimate outcome: i.e., the killing of the cell. A similar conclusion was reached from a study on the VT-1 killing of endothelial cells where caspase inhibitors were able to reverse the measures of apoptosis made while death proceeded as normal.[54]

For group I BL cells, CD77 was found not to be requisite for the expression of an apoptotic phenotype *per se*. This possibility had been raised previously in regard to both BL and their normal GC B cell equivalents:[9,46] indeed, a mechanistic basis for this could be envisaged through a potential pro-apoptotic activity being generated from the intracellular ceramide moiety of CD77. Nevertheless, while the CD77^{neg} fraction of the L3055 line was clearly refractory to killing via both VT-1 B chain and holotoxin they remained fully susceptible to apoptosis induced by cross-linking their sIgM. The retention of an apoptotic capacity by VT-1-selected CD77^{neg} cells is encouraging for the outcome of any VT-based therapy where similar escape variants could potentially arise on treating patients with the toxin *in vivo*.

Having established a preference for exploiting the holotoxin in any CD77-targeted attack on B cell lymphoma, consideration must be given to the danger of damaging other cells, especially CD77^{pos} endothelial cells of the kidney. Verotoxin-producing *Escherichia coli* strains have been strongly implicated in the etiology of hemolytic uremic syndrome, the leading cause of pediatric acute renal failure.[55,56] However, reflecting the age distribution of this problem, some comfort is given by the finding that VT-binding, while it can be observed in the infant glomerulus, is not detectable in the adult where, instead, it is restricted to tubules.[5] We are currently addressing whether even lower concentrations of VT-1 than those used in the present study might act in synergy with other B cell-directed therapeutics and thereby minimize any deleterious effects on non-B cells. CD20 antibodies, already being used with promise in follicular lymphoma, are a consideration here.[57] Finally, we could consider mutating the VT-1 A chain to eliminate the furin-cleavage site which is important for A chain activation during cell cycle entry[16] and to substitute alternative residues that would be susceptible to B cell-specific enzymes. Our finding that VT-1 holotoxin kills lymphoma cells even when confronted with powerful survival factors indicates that these possibilities warrant serious attention.

Materials and Methods

Reagents

The BU1 mouse IgG2a monoclonal antibody to human IgM was produced from the hybridoma in the Department of Immunology, University of Birmingham, and purified by ion-exchange chromatography on DE52 (Whatman Ltd, Maidstone, UK). Asitic fluid containing rat IgM anti-CD77 mAb (38.13) was a kind gift from Dr J Wiels (Institut Gustav-Roussy, Villejuif, France) and control ascitic fluid containing rat IgM anti-DNP was obtained from Serotec (Oxford, UK). 38.13 ascitic fluid and control rat IgM ascitic fluid were conjugated to FITC by standard techniques. In some experiments, unconjugated 38.13 mAb purchased from Serotec was used. Ionomycin was obtained from Calbiochem-Novabiochem (Nottingham, UK). Soluble trimeric CD40L was generated as an isoleucine zipper construct as described by Morris *et al*.[58]

Purification of Verotoxin-1 and VT-1 B chain

Verotoxin-1 was prepared from periplasmic extracts of *E. coli* JM105 transformed with the expression plasmid pSLT (a plasmid encoding the Verotoxin-1 operon under the control of the lac promoter). Cells were grown in Luria broth supplemented with 100 μg/ml ampicillin to an OD_{600} of 0.6 (measured with a Shimadzu UV-160 spectrophotometer). Expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (1 mM final concentration) and allowed to proceed for 3 h. Cells were harvested by centrifugation (10 min at $7500 \times g$), washed in 600 mM sucrose, 300 mM Tris/HCl (pH 8.0), 1 mM EDTA and 0.5 mM $MgCl_2$, and resuspended in 1 mM Tris/HCl, pH 7.5. After incubation on ice for 10 min, cell debris was removed by centrifugation (15 min at $15\,000 \times g$). The supernatant was filter-sterilized by passage through a 0.2 μm filter and applied to a 6 ml globotriose-Sepharose affinity column[59] equilibrated with 0.5 M NaCl in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The column was washed with 40 volumes of 0.5 M NaCl in PBS prior to eluting VT-1 with 6 M guanidine.HCl. Fractions of 1 ml were collected and immediately dialyzed against PBS to remove the denaturant. VT-1 B chain was isolated exactly the same way. The plasmid used here was pSBC32 which contains the coding sequence for the B chain in the high level expression vector pKK233-2 and was a kind gift of Dr SB Calderwood.[60]

Cell lines

Group I BL cell lines L3055 (EBV^{neg}), BL2 (EBV^{neg}), SAV (EBV^{pos}), and Mutu I (EBV^{pos}) were maintained in early passage as previously described.[24,29] Late passage Mutu III cells were also included for study.[29] Cells were maintained in continuous culture with RPMI 1640 supplemented with 10% pre-screened FCS (Bio-Whittacker, Wokingham, UK), 5000 IU/ml penicillin, 5 mg/ml streptomycin (Gibco/BRL, Paisley, UK) and 200 mM glutamine (Gibco/BRL). Stable *bcl*-2 transfectants were obtained as described previously.[22,23] Stable *bcl*-x_L transfectants were derived by electroporation of the plasmid vector pEF-MC1neopA[61] containing the *bcl*-x_L cDNA followed by selection of cells in G418 (Sigma, Poole, UK). Immunoblotting for Bcl-2 and Bcl-x_L was carried out as detailed elsewhere.[23,24] From the lines established, those showing high level expression of the genes transfected were chosen for study.

Selection of CD77^{neg} cells

L3055 cells were cultured at a concentration of 4×10^5/ml for 48 h in the presence of VT-1 at 100 ng/ml. Dead cells were removed by centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Remaining viable cells were collected from the interface, washed three times in culture medium and used as 'CD77^{neg} cells' in the experiments described.

Flow cytometric analysis of CD77 expression

Cells were harvested after culture under the conditions indicated in the text. Cells were washed once in PBS with 0.1% BSA and 0.01% sodium azide (FACS buffer) before direct or indirect immunofluorescence analysis was performed. Briefly, cells were labeled by a 30 min incubation on ice with either directly-conjugated rat 38.13 mAb or unconjugated mAb that was detected by an additional second stage incubation with FITC-conjugated anti-rat IgM Ab (Serotec). In both cases, rat IgM anti-DNP was used as the control. Cells labeled either directly or indirectly were washed once and resuspended in FACS buffer. Flow cytometry data were obtained using an Epics-XL flow cytometer (Beckman Coulter, Miami, FL, USA). Viable cells were gated according to forward (FSC) and side (SSC) light scatter settings. Data were processed and analyzed using WinMDI software (Scripps Research Institute, La Jolla, CA, USA).

Measurement of DNA synthesis

DNA synthesis was determined by [^{3}H]thymidine incorporation.[31] After culture of 5×10^4 cells per 200 μl with additions as specified, wells were pulsed for the final 4 h with [^{3}H]thymidine (Amersham Interanational, Amersham, UK, 10 μCi/ml in culture medium, 50 μl per well) and harvested on a Skatron cell harvester (Helis Bio Ltd, Newmarket, UK). All assays were performed in quadruplicate with replicates usually being within 10% and always within 15% of each other.

Cell viability and apoptosis assays

Cell viability was assessed by a standard trypan blue dye exclusion assay.[31] Apoptosis was determined by staining treated cells with acridine orange and visualizing nuclear morphology exactly as described previously.[22] Viable cells display a homogenous chromatin staining pattern whereas apoptotic cells show characteristically condensed and fragmented chromatin. Each determination was carried out on scoring 100 cells in duplicate.

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