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Differential effects of the antioxidant α-lipoic acid on the proliferation of mitogen-stimulated peripheral blood lymphocytes and leukaemic T cells

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Abstract

The effects of the antioxidant α-lipoic acid (LA) on the proliferation of mitogen-stimulated human peripheral blood lymphocytes (HPBL) were investigated in comparison to its effects on the proliferation of two leukaemic T cell lines, Jurkat and CCRF-CEM. At low mM concentrations, LA inhibited in a dose-dependent manner DNA synthesis of HPBL stimulated with either phorbol myristate acetate (PMA) in combination with ionomycin (IoM), or phytohaemagglutinin (PHA). At similar concentrations, LA inhibited the proliferation of Jurkat and CCRF-CEM cells. However, LA was preferentially cytotoxic to the leukaemic cell lines. The selective toxicity of LA to Jurkat cells was shown by electron microscopy (EM) to be due to the induction of apoptosis. Furthermore, LA had different effects on the secretion of interleukin-2 (IL-2) and steady-state levels of IL-2 mRNA in mitogen-stimulated HPBL depending on the mitogens used. LA dramatically increased the induction of IL-2 mRNA and IL-2 protein secretion in PMA/IoM-stimulated HPBL, whereas it inhibited these in HPBL stimulated with PHA. The differential effects of LA on normal and leukaemic T lymphocytes may indicate a new route towards development of therapeutic agents.

Keywords: Lipoic acid; Leukaemia; Lymphocyte; Interleukin-2; Redox regulation; Apoptosis; Cell proliferation

1. Introduction

The activation and proliferation of T lymphocytes is intimately linked to the intracellular redox state of the cell. A number of studies have shown that various antioxidant compounds, including free radical scavengers, iron chelators, and electron acceptors, can inhibit lymphocyte proliferation (Chaudhri et al., 1986, 1988; Dornand and Gerber, 1989; Hunt et al., 1991; Novogrodsky et al., 1982; Sekkat et al., 1988). These and other observations led to the hypothesis that during T lymphocyte activation, the formation of reactive oxygen species is important in regulating signal transduction by acting as "second messenger" signaling molecules (Chaudhri et al., 1986; Fidelus, 1988; Sekkat et al., 1988). However, T lymphocytes require glutathione (GSH) and other reducing equivalents for optimal immune function and effective lymphocyte proliferation, particularly for DNA synthesis (Iwata et al., 1994; Kavanagh et al., 1990; Staal et al., 1994; Suthanthiran et al., 1990). Thus, while T lymphocyte proliferation requires reducing conditions for optimal growth, a transient pro-oxidant state is thought to be necessary during the early stages of T cell signaling. At high concentrations, however, reactive oxygen species can lead to oxidative stress, overwhelming cellular antioxidant mechanisms and thereby causing cell death (Pahl and Baeuerle, 1994). In addition, lowered intracellular thiol status has been associated with various lymphocyte disorders and pathologies such as AIDS and cancer (Eck et al., 1989; Staal et al., 1992a,b). Thus, antioxidants and compounds that increase cellular thiol levels in lymphocytes have been proposed as therapeutic agents for restoring proper immune function.

One such compound is α-lipoic acid (LA), also known as thioctic acid, which occurs naturally in trace amounts in mammalian cells as lipoamide, an essential cofactor in oxidative metabolism in pyruvate dehydrogenase and α-keto dehydrogenase complexes. The antioxidant properties of both LA and its reduced form, dihydrolipoic acid (DHLA), have been well documented in various in vitro and in vivo systems (Bustamante et al., 1998; Packer et al., 1995, 1997). LA has been shown to be beneficial in various forms of oxidative stress and is of interest as a therapeutic

Abbreviations: DHLA, dihydrolipoic acid; HPBL, human peripheral blood lymphocytes; IL-2, interleukin-2; IoM, ionomycin; LA, α-lipoic acid; PHA, phytohaemagglutinin; PMA, phorbol myristate acetate.

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in ischemia-reperfusion injury, diabetic complications, cataract formation, HIV activation, neurodegenerative disorders and radiation injury (Packer et al., 1995). Added to cell culture medium in vitro, LA readily enters cells and is reduced by mitochondrial and cytosolic enzymes to DHLA, most of which is rapidly effluxed from the cell to the culture medium (Han et al., 1995; Handelman et al., 1994). DHLA can regenerate other antioxidants such as GSH, ascorbate and, indirectly, Vitamin E (Packer et al., 1997). Micromolar concentrations of LA also have been shown to cause an increase in intracellular GSH in Jurkat leukaemic T cells and HPBL in vitro (Han et al., 1995; Handelman et al., 1994; Sen et al., 1997). In addition, concentrations of LA as low as 1 μM have been shown to restore functional defects in HPBL isolated from cancer patients (Mantovani et al., 2000). Thus, LA has been suggested as a particularly beneficial therapeutic agent for various diseases and pathological conditions involving impairment of the immune system due to oxidative stress.

Considering the potential therapeutic benefits of LA, it is important to thoroughly investigate the effects of LA on the proliferative capacity of various lymphocyte populations. The aim of this research was to compare the effects of LA on the proliferation of normal human peripheral blood lymphocytes (HPBL) with its effects on two commonly studied leukaemic T cell lines, Jurkat and CCRF-CEM. Freshly isolated HPBL were used in this study and two commonly used mitogenic stimuli were employed to mimic antigenic activation of the T cells. These were phorbol myristate acetate (PMA) in combination with a calcium ionophore, ionomycin (IoM), and the lectin, phytohaemagglutinin (PHA).

2. Materials and methods

2.1. Isolation of HPBL, cell lines and cell culture

Mononuclear cells were isolated from buffy coat preparations (white blood cell concentrates supplied by the Red Cross Blood Bank, Sydney) by density gradient centrifugation using Ficoll-Paque Plus lymphocyte isolation media (Pharmacia Biotech, Sweden). HPBL were isolated from the mononuclear cells by passaging through a Sephadex G10 (Pharmacia Biotech) column and resuspended in complete RPMI-10 medium. Complete RPMI-10 medium consisted of RPMI-1640 cell culture medium (Gibco BRL, USA) made in bulk from powder with 2 g/l $NaHCO_3$, final pH 7.2, and freshly supplemented prior to use with 10% (v/v) heat-inactivated FCS (CSL, Australia), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10 mM HEPES. HPBL isolated by this procedure have been shown by flow cytometry to be greater than 90% T lymphocytes (Goldstone et al., 1995). Jurkat and CCRF-CEM, two acute T lymphocytic leukaemia (T-ALL) cell lines derived from human peripheral blood, were obtained from the American Type Culture Collection (ATCC, USA). Both cell lines consistently tested negative for mycoplasma contamination. HPBL, Jurkat and CCRF-CEM cells were cultured in complete RPMI-10 medium in a humidified atmosphere of 5% CO_2/95% air at 37 °C. HPBL were cultured for up to 72 h after isolation. Jurkat and CCRF-CEM cells were maintained for up to 3 months at concentrations between 5×10^4 and 1×10^6 cells/ml.

2.2. Cell proliferation studies

HPBL and the leukaemic cells were routinely seeded at 1×10^6 and 1.5×10^5 cells/ml, respectively in complete RPMI-10 medium at 200 μl per well in 96-well, flat bottom microtitre plates. In the proliferation studies, HPBL were stimulated with 10 μg/ml PHA (Sigma, USA), or 20 ng/ml PMA (Sigma), or 20 ng/ml PMA in combination with 100 ng/ml IoM (Calbiochem, USA). The leukaemic cells were used untreated. Stock solutions of PMA and IoM were prepared in absolute ethanol, and PHA was dissolved in PBS. LA (Sigma) was prepared freshly at 10 mM stock solution in complete RPMI-10 media, and added immediately prior to stimulation. The plates were incubated in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Proliferation of HPBL, Jurkat and CCRF-CEM was determined by assaying DNA synthesis, cell density and cell viability.

2.3. DNA synthesis

Tritiated-thymidine incorporation was used to measure DNA synthesis. Four hours prior to harvesting, 0.8 μCi of methyl-[^{3}H]-thymidine (specific activity 20 Ci/mmol; ICN Biomedicals, USA), was added to each well of the test plates. Cells were harvested using a Cambridge PHD Cell Harvester (Cambridge Technologies, USA) onto glass fiber filter discs (ICN). The discs were transferred to plastic scintillation vials (Packard, USA or Beckman, USA) and Emulsifier-Safe scintillant (Packard) was added. The vials were counted on a Beckman LS 6000TA liquid scintillation counter. Maximal readings of 50,000–120,000 counts/min (cpm) were routinely obtained for the test samples. DNA synthesis was expressed as % control. For HPBL, this was calculated as follows: ((cpm of sample − cpm of unstimulatedHPBL)/(cpm of mitogen-stimulated HPBL − cpm of unstimulated HPBL))×100. For Jurkat and CCRF-CEM, this was calculated as follows: (cpm of sample/cpm of) untreated cells × 100.

2.4. Cell viability and cell density

Cell viability was assayed by trypan blue exclusion, which distinguished viable and non-viable cells. Cells were diluted with an equal volume of 0.4% (w/v) trypan blue in PBS and counted with a haemocytometer. To adequately separate clumps of adherent cells, EDTA was added to a final concentration of 0.2 mg/ml, 20 min prior to

counting test samples, without loss of viability. Cell viability was expressed as a percentage calculated as follows: (number of live cells/total number of live and dead cells) × 100. This percentage was then normalized to the % viability of the control cells that were given a value of 100%. Where applicable, the control cells were the HPBL stimulated with mitogen alone, and the untreated or mitogen-treated leukaemic cells. Cell density was calculated as the number of live cells per ml and expressed as % control. For HPBL, this was determined as follows: (live cells in sample/live cells in the control) × 100, where the control cells are the HPBL treated with mitogen alone. For the leukaemic cells, cell density values (% control) subtracting the seeding densities were calculated according to the following formula: ((live cells in sample − seeding cell density)/(live cells in control − seeding cell density)) × 100, where the control cells are the untreated or treated leukaemic cells. Thus, the percent control cell density values for the leukaemic cells more accurately reflect changes in cell density due to proliferation, whereas for HPBL they are a reflection of the changes in cell density overall.

2.5. Light and electron microscopy

Cells were prepared for light microscopy (LM) and electron microscopy (EM) as described previously (Conway et al., 1995). Briefly, pelleted cells were fixed in 2.5% (v/v) glutaraldehyde/22 mM betaine in 0.1 M sodium cacodylate buffer (pH 7.2), rinsed, post-fixed in 2% (v/v) osmium tetroxide followed by dehydration through a series of alcohols and acetone, and embedded in epon-araldite resin cured at 60 °C. Semi-thin sections were stained with toluidine blue and examined with LM. Ultrathin sections were collected on grids, stained with lead citrate and uranyl acetate, and viewed on a Hitachi T2000 transmission EM.

2.6. Differential Jurkat cell counts

Jurkat cells were treated with 4 mM LA for 0, 2, 6, 12, 24 and 48 h. Cell suspensions were diluted in PBS with Dulbecco's B salts (Oxoid, UK) and BSA was added to a final concentration of 50 mg/ml. The cells were then spun onto microscope slides in a cytospin-2 centrifuge (Shandon, USA) for 10 min at 500 rpm, fixed with methanol for 1 min and stained with haematoxylin and eosin (H&E). Differential cells counts were made under LM where Jurkat cells were classified as either viable, apoptotic or necrotic/degenerate based on previously defined morphological criteria (Kerr et al., 1994). Apoptotic cells displayed condensed chromatin or peripheral crescents, and in some cases, condensed cytoplasm. Necrotic/degenerate cells had obvious cytoplasmic swelling and multiple clumps of nuclear chromatin. A total of 300 cells were counted for each cytospin sample. This was determined blind by three independent examiners for three experiments.

2.7. Enzyme-linked immunosorbent assay (ELISA)

A Biotrak human interleukin-2 (IL-2) ELISA system (Amersham, UK) was used according to the manufacturer's instructions to determine IL-2 protein levels in various sample cell preparations.

2.8. Isolation of RNA

Total RNA was extracted from cell suspensions using the acid-guanidium thiocyanate–phenol–chloroform method of Chomczynski and Sacchi (1987). DNA was removed by treating the RNA with RNase-free DNase I for 30 min at 37 °C, followed by phenol–chloroform extraction and ethanol precipitation. The resultant total RNA was resuspended in RNase-free water.

2.9. Reverse-transcriptase (RT)-PCR

RNA was reverse transcribed into complementary DNA (cDNA) using Moloney-Murine leukaemia virus reverse-transcriptase (M-MLV RT; Promega, USA). RNA was heat denatured with an oligo (dT) primer at 70 °C for 5 min, and briefly placed on ice before addition of the remaining reaction components. Typically, 50 μl reactions contained 1 μg RNA, 0.5 μg oligo (dT) primer, 100 μM dNTP, 20–25 units RNasin (ribonuclease inhibitor; Promega), 50 mM Tris–HCl (pH 8.3 at 25 °C), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 200 U M-MLV RT. The samples were incubated at 37 °C for 60 min then boiled for 1 min to stop the reaction. Following reverse transcription, cDNA was amplified using PCR. The PCR primers for IL-2 were GAAGAAGAACTCAAACCTCTGG (F) and CAATAGGTAGCAAACCATACATTC (R), which gave a product of 322 bp. The PCR primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control were CCACCATGGAGAAGGCTGGGGCTC (F) and AGTGATGGCATGGACTGTGGTCAT (R), which gave a PCR product of 241 bp. A typical 20–25 μl reaction consisted of 1 μl of cDNA from the reverse transcription reaction, 50 mM KCL, 10 mM Tris–HCl (pH 9 at 25 °C), 1–1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 1 μM 5′ and 3′ primers, and 1.5 U *Taq* DNA polymerase. Following a 5 min denaturation step at 94 °C, 30 cycles of amplification were performed in a standard reaction. The amplification parameters were: denaturation at 94 °C for 30 s; annealing at 52 and 58 °C, respectively for IL-2 and GAPDH for 30 s; and extension at 72 °C for 1 min; with a final extension of 5 min after the last cycle.

2.10. Northern hybridization

Northern hybridization analysis was performed essentially as outlined in Ausubel et al. (1987). Briefly, total RNA (10–20 μg) was electrophoresed through an agarose/

formaldehyde gel (1–1.3% agarose, 2.2 M formaldeyde in 3-[*N*-morpholino]-2-hydroxypropanesulfonic acid (MOPS) buffer consisting of 40 mM MOPS, ph 7, 10 mM sodium acetate, and 1 mM EDTA, pH 8). Following electrophoresis the RNA was capillary transferred from the gel to a Hybond-N nylon membrane (Amersham) using 20× standard saline citrate (SSC), and fixed to the membrane by UV cross-linking. The membranes were placed in hybridization tubes (Hybaid, UK) and rinsed with 6× SSC. Formamide prehybridization/hybridization (FPH) solution (5× SSC, 5× Denhardt solution, 50% (w/v) formamide, 1% (w/v) sodium dodecyl sulfate (SDS), 100 μg/ml sonicated salmon sperm DNA (freshly denatured prior to use)) was preheated to 42 °C and added to the tubes. The tubes were then placed in a hybridization oven (Hybaid Mini Oven MKII) and prehybridized with rotation for 1–2 h at 42 °C. Hybridization of the radioactively labeled probe was then performed by the addition of freshly denatured probe (10 ng/ml) to the tube and left overnight at 42 °C with rotation. Probes were prepared by incorporating [α-^{32}P]dCTP (NEN, USA) by either random priming using a Random Primer Extension System from NEN Life Sciences Products or by asymmetric PCR. For asymmetric PCR, 50 cycles PCR was performed with a 50 μl PCR reaction essentially as described above, except that 25 ng template DNA was used, 50–100 μCi [α-^{32}P]dCTP was added (no unlabelled dCTP), and only the 3′ (reverse) primer was added (50 μM). Unincorporated [α-^{32}P]dCTP was removed from the probe by spun-column chromatography using Sephadex G50 as outlined in Sambrook et al. (1989). Following hybridization, the membranes were washed in two changes of 2× SSC, 0.1% (w/v) SDS for 20 min at room temperature, two changes of 1× SSC, 0.1% SDS for 20 min at 42 °C, and finally 0.1× SSC, 0.1% SDS for 20 min at 42 °C. After rinsing in 2× SSC, the membranes were sealed in plastic, and the signal was detected by standard autoradiography in a Dupont Cronex cassette exposed to Kodak X-Omat K XK-1 diagnostic film with a Cronex Lightning Plus intensifying screen at −80 °C. Densitometry was performed on the images generated using the GDS 8000 Complete Gel Document & Analysis System from UVP.

3. Results

3.1. LA inhibits HPBL proliferation

LA inhibited in a dose-dependent manner the proliferation of HPBL stimulated with either PMA/IoM (Fig. 1A) or PHA (Fig. 1B) as measured by DNA synthesis at 48 h. The IC_{50} and IC_{90} values, which are the concentrations that inhibit DNA synthesis by 50 and 90%, respectively are summarized in Table 1. The effects of LA on cell viability and cell density of mitogen-stimulated HPBL measured at 72 h are also shown in Fig. 1. The viability of unstimulated and mitogen-stimulated cultures of HPBL over the 72 h time period was between 80 and 85% and there was very little effect of LA on untreated HPBL (data not shown).

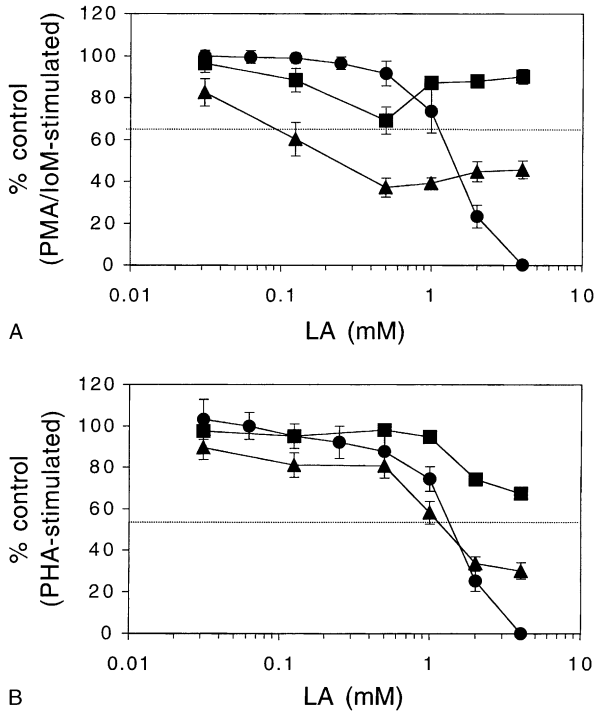

Fig. 1. Effect of LA on the proliferation of (A) PMA/IoM-stimulated, and (B) PHA-stimulated HPBL as determined by DNA synthesis (circles), cell viability (squares) and cell density (triangles). Each value represents the mean ± S.E.M. of *n* experiments. For DNA synthesis, [^{3}H]-thymidine incorporation was measured at 48 h (PMA/IoM-stimulated, $n = 12$; PHA-stimulated, $n = 11$). Cell density and cell viability were measured by trypan blue exclusion at 72 h (PMA/IoM-stimulated, $n = 7$; PHA-stimulated, $n = 5$). The dotted line represents the mean % control cell density of unstimulated HPBL after 72 h (PMA/IoM-stimulated, 64.8 ± 3.6%; PHA-stimulated, 55.4 ± 4.8%). PMA, 20 ng/ml; IoM, 100 ng/ml; PHA, 10 μg/ml.

In PMA/IoM-stimulated HPBL (Fig. 1A), LA had a more potent inhibitory effect on cell density than on DNA synthesis in the concentration range of 0.03–0.5 mM. At these concentrations, while there was only a less than 10% decrease in DNA synthesis, cell density decreased by more than 60%. At the same time cell viability also decreased by 30%. This 30% difference between the effect of LA on cell density and cell viability reflects a 30% loss of cells from the seeding cell density that is approximated by the dotted line on Fig. 1A. This line represents the mean % control cell density of the unstimulated HPBL after 72 h. Above 0.5 mM LA, DNA synthesis was inhibited in a dose-dependent manner with 4 mM LA resulting in complete inhibition. In contrast, however, cell viability increased as the concentration of LA increased from 0.5 mM. At 4 mM LA, HPBL cell viability increased to approximately 90% of the control PMA/IoM-stimulated HPBL. In a similar manner, cell density also increased to approximately 45% of the control as the concentration of LA increased from 0.5 to 4 mM.

Table 1
The concentrations of LA in mM which gave 50% (IC_{50}) and 90% (IC_{90}) inhibition of mitogen-stimulated HPBL, Jurkat and CCRF-CEM cell proliferation[a,b]

	HPBL (PMA/IoM)	HPBL (PHA)	Jurkat	CCRF-CEM
DNA synthesis				
IC_{50} (mM)	1.35 ± 0.17 (12)	1.43 ± 0.09 (11)	1.63 ± 0.09 (9)	2.56 ± 0.07 (6)
IC_{90} (mM)	2.80 ± 0.23 (12)	3.10 ± 0.10 (11)	4.27 ± 0.13 (9)	4.07 ± 0.08 (6)
Cell density				
IC_{50} (mM)	~0.03	~0.50–0.60	0.67 ± 0.05 (5)	0.86 ± 0.09 (3)
IC_{90} (mM)	NA	NA	1.52 ± 0.04 (5)	2.08 ± 0.11(3)
Cell viability				
IC_{50} (mM)	NA[c]	NA[d]	1.69 ± 0.11 (5)	2.65 ± 0.03 (3)
IC_{90} (mM)	NA[c]	NA[d]	4.28 ± 0.12 (5)	4.03 ± 0.09 (3)

[a] The IC_{50} and IC_{90} values were calculated from dose-response curves of individual experiments, summarized in Figs. 1 and 2, except the values for cell density changes in HPBL which were estimated. Each value represents the mean ± S.E.M. of *n* experiments (shown in the parentheses).
[b] NA: not applicable.
[c] 4 mM = 70.5% viable.
[d] 4 mM = 66% viable.

Thus, there was a distinct and reproducible dip in cell viability and, to a lesser extent, cell density at 0.5 mM LA.

There were some striking differences between the effects of LA on PMA/IoM-stimulated and PHA-stimulated HPBL. The dramatic effect of inhibition of PMA/IoM-stimulated HPBL cell density by concentrations of up to 0.5 mM LA was not seen with the PHA-stimulated HPBL (Fig. 1B). Instead, there was a dose-dependent inhibition of cell density and cell viability at 72 h at similar concentrations of LA that inhibited DNA synthesis at 48 h. However, while 4 mM LA completely inhibited DNA synthesis, cell density was inhibited by approximately 60% and cell viability was inhibited by approximately 30%. Note that with 4 mM LA in the PMA-stimulated HPBL cell viability was approximately 90% of the control cells. An estimate of the IC_{50} values for inhibition of proliferative changes in cell density by LA can be made by reading the average concentration that lies halfway between the unstimulated HPBL cell density (the dotted line in Fig. 1A and B) and the mitogen-stimulated HPBL cell density (100% control). Consequently, the estimated IC_{50} values for the effects of LA on proliferative cell density increases are remarkably different depending on the stimulus. From Fig. 1A, the IC_{50} value for PMA/IoM-stimulated HPBL was estimated to be approximately 0.03 mM which is substantially lower than the IC_{50} value for PHA-stimulated HPBL which was estimated to be between 0.50 and 0.60 mM (Fig. 1B). This is a more than 15-fold difference.

3.2. LA is cytotoxic to leukaemic cells

We next determined the effects of LA on the proliferation of two leukaemic T cell lines, Jurkat and CCRF-CEM. From cell density growth curves, the doubling times of the leukaemic cells were estimated to be 18 and 20 h for Jurkat and CCRF-CEM, respectively and the average cell viability of the untreated leukaemic cells was greater than 96% (data not shown). LA inhibited, in a dose-dependent manner, DNA synthesis, changes in cell density due to proliferation (the seeding cell density was subtracted from the calculations, see Section 2), and cell viability in both Jurkat cells (Fig. 2A) and CCRF-CEM cells (Fig. 2B). The IC_{50} and IC_{90} values for LA are summarized in Table 1. The IC_{50}

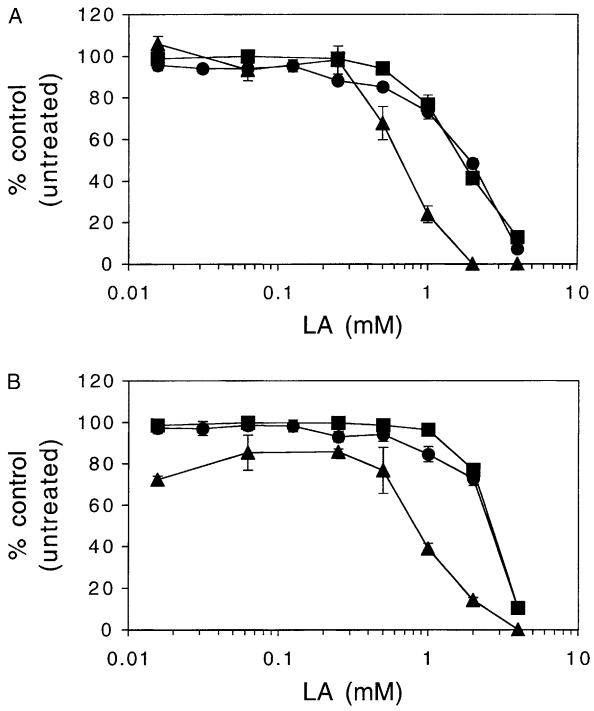

Fig. 2. Effect of LA on the proliferation of (A) Jurkat, and (B) CCRF-CEM cells as determined by DNA synthesis (circles), cell viability (squares), and cell density (triangles). Each value represents the mean ± S.E.M. of *n* experiments. For DNA synthesis, [^{3}H]-thymidine incorporation was measured at 24 h (Jurkat, $n = 9$; PHA-stimulated, $n = 7$). Cell density and cell viability were measured by trypan blue exclusion at 48 h (Jurkat, $n = 5$; CCRF-CEM, $n = 3$). PMA, 20 ng/ml; IoM, 100 ng/ml; PHA, 10 μg/ml.

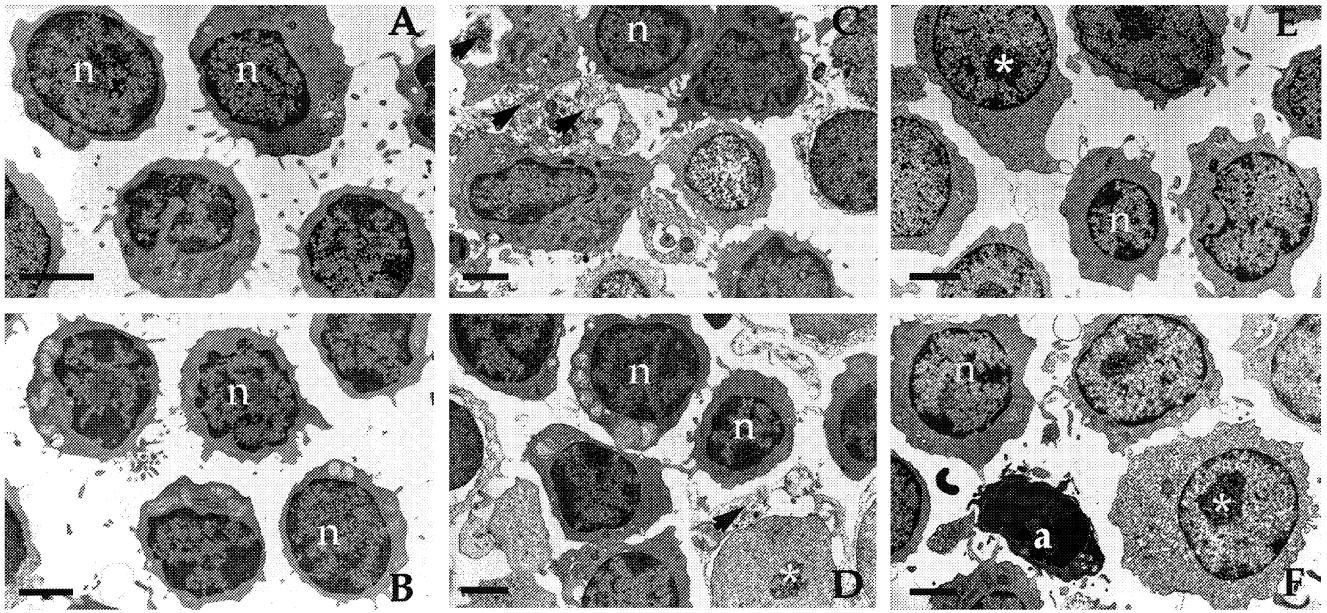

Fig. 3. Electron micrographs of HPBL: (A) control HPBL displayed a characteristic high nuclear to cytoplasm ratio, pale cytoplasm and single-lobed nuclei (*n*); (B) HPBL treated with 4 mM LA for 24 h appeared similar to control cells. Following stimulation with either PMA/IoM (C and D) or PHA (E and F), many cells appeared similar to control HPBL. However, a number of larger cells (open star symbol) with pale cytoplasm and diffuse nuclear chromatin were also seen following PMA/IoM or PHA stimulation (D and F). Some evidence of debris from degenerating cells (arrowheads) was seen in stimulated and LA-treated cell cultures, although very few apoptotic cells (a) were observed. (A) unstimulated control HPBL, 24 h; (B) HPBL + 4 mM LA, 24 h; (C) PMA/IoM-stimulated HPBL, 24 h; (D) PMA/IoM–stimulated HPBL + 4 mM LA, 24 h; (E) PHA-stimulated HPBL, 24 h; and (F) PHA-stimulated HPBL + 4 mM LA, 24 h (bar = 2 μM).

values for inhibition of DNA synthesis were 1.6 ± 0.9 mM in Jurkat cells and 2.6 ± 0.7 mM for CCRF-CEM cells, which are very similar to the IC_{50} values for inhibition of DNA synthesis in mitogen-stimulated HPBL (Table 1). LA also inhibited the increase of Jurkat and CCRF-CEM cell density at similar high μM to low mM concentrations (Fig. 2). However, in stark contrast to the effects of LA on mitogen-stimulated HPBL (Fig. 1), LA was highly toxic to the leukaemic cells. At 4 mM LA there was almost complete loss of cell viability, whereas this concentration inhibited the viability of PMA/IoM-stimulated HPBL by only 10% and PHA-stimulated HPBL by approximately 30%.

3.3. LA induces apoptosis in Jurkat cells

Since mM concentrations of LA were differentially cytotoxic to the leukaemic T cells, we investigated the mode of cell death induced by LA in the Jurkat cells. LM and EM were used to compare the effects of LA on the morphology of Jurkat cells and mitogen-stimulated HPBL. Cell pellets of unstimulated and mitogen-stimulated HPBL as well as Jurkat cells treated with and without 2 and 4 mM LA for various time-points were collected and prepared for LM and EM. Electron micrographs of HPBL are shown in Fig. 3. Unstimulated control HPBL and control HPBL

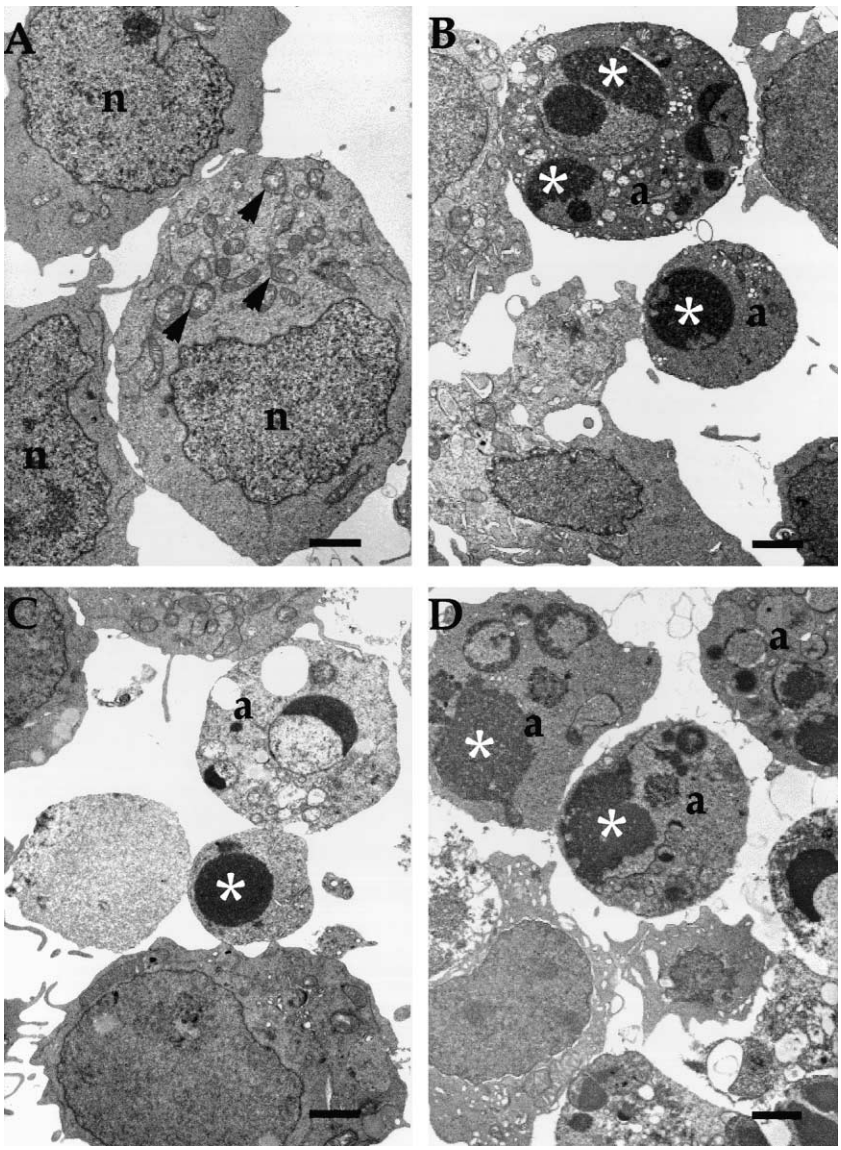

Fig. 4. Electron micrographs of Jurkat cells: (A) control unstimulated Jurkat cells displayed heterochromatic nuclei (*n*) and numerous mitochondria (arrows); (B–D) LA treatment induced dose- and time-dependent apoptotic involution of unstimulated Jurkat cells (see also Fig. 5). Apoptotic cells (a) with characteristic nuclear margination and condensation (open star symbol) were seen interspersed amongst viable Jurkat cells. Several apoptotic bodies at various stages of dissolution were also visible in many cells. (A) untreated control Jurkat cells, 24 h; (B) Jurkat cells + 4 mM LA, 12 h; (C) Jurkat cells + 4 mM LA, 24 h; and (D) Jurkat cells + 2 mM LA, 24 h (bar = 2 μM).

treated with 4 mM LA (Fig. 3A and B) displayed viable cells with characteristic high nuclear to cytoplasmic ratio, pale cytoplasm and single-lobed nuclei. Treatment with 4 mM LA for 24 h (Fig. 3B) had no apparent effect on the morphology of the HPBL, which appeared similar to the control HPBL. The HPBL stimulated with either PMA/IoM (Fig. 3C) or PHA (Fig. 3E) for 24 h mostly appeared similar to the untreated cells, although following mitogen stimulation, a number of larger cells with pale cytoplasm and diffuse nuclear chromatin were visible. These cells were also seen in mitogen-stimulated HPBL treated with 4 mM LA for 24 h (Fig. 3D and F). We also observed evidence of debris from degenerating cells and a few apoptotic cells in these cultures (Fig. 3C, D and F).

Electron micrographs of the Jurkat cells are shown in Fig. 4. Untreated control Jurkat cells were somewhat larger in size than the HPBL and displayed heterochromatic nuclei and numerous mitochondria (Fig. 4A). In contrast to HPBL, addition of 2 or 4 mM LA to the Jurkat cells resulted in the dose- and time-dependent appearance of apoptotic cells with characteristic condensation and margination of nuclear chromatin, and cytoplasmic condensation (Fig. 4B–D). Apoptotic cells and apoptotic bodies were clearly evident interspersed amongst viable Jurkat cells (Fig. 4B–D). By 48 h after LA treatment, there were numerous necrotic/degenerate cells at various stages of degeneration, including dispersal of nuclear chromatin, cytoplasmic swelling and loss of internal cell membranes as well as nuclear membranes.

To further quantify the induction of cell death by LA in Jurkat cells, cytospin preparations of untreated cells and cells treated with 4 mM LA were stained with H&E, and the cells were classified as either viable, apoptotic or necrotic/degenerate by three independent examiners in masked fashion. The percentages of the three cell types were calculated for each time-point and are shown in Fig. 5. A very small proportion of untreated Jurkat cells or cells treated with LA for 2 h displayed features of apoptosis or necrosis/degeneration. After 12 h of LA treatment, the proportion of Jurkat cells classified as apoptotic increased to 18%. This peaked at 36% after 24 h, at which time the proportion of Jurkat cells showing evidence of necrosis/degeneration had also risen to a similar level of 38%. At 48 h, only 10% of the Jurkat cells were viable, supporting viability results shown in Fig. 2A, and the number of apoptotic Jurkat cells decreased to only 8%. At this stage, the proportion of necrotic/degenerate cells had increased dramatically to 80%.

3.4. Differential effects of LA on IL-2 expression in mitogen-stimulated HPBL

The synthesis and secretion of IL-2 is a pivotal event in the activation of T lymphocytes as it is required for initiation of proliferation in these cells (Morgan et al., 1976; Smith, 1992; Taniguchi and Minami, 1993). Therefore, we examined what effect LA had on the production and secretion of IL-2 by mitogen-stimulated HPBL. Supernates were collected at 8 and 24 h from proliferation assays investigating the effects of 4 mM LA on PMA/IoM-stimulated and PHA-stimulated HPBL and the amount of IL-2 secreted was determined using ELISA. Fig. 6A shows that PMA/IoM stimulation of HPBL results in a large increase in secreted IL-2 protein from undetectable levels to almost 3500 pg/ml after 24 h. The addition of 4 mM LA substantially increased the secretion of IL-2 at both 8 and 24 h (Fig. 6A). This is despite the fact that LA inhibited the proliferation of PMA/IoM-stimulated HPBL as measured by DNA synthesis (Fig. 1A). In response to 4 mM LA, the amount of IL-2 secreted was 2.2-fold greater at 8 h and 2-fold greater at 24 h, resulting in approximately 7000 pg/ml IL-2 produced after 24 h. In complete contrast, LA had the opposite effect on the secretion of IL-2 by PHA-stimulated HPBL (Fig. 6B). The amount of IL-2 produced by PHA-stimulated HPBL after 24 h was approximately

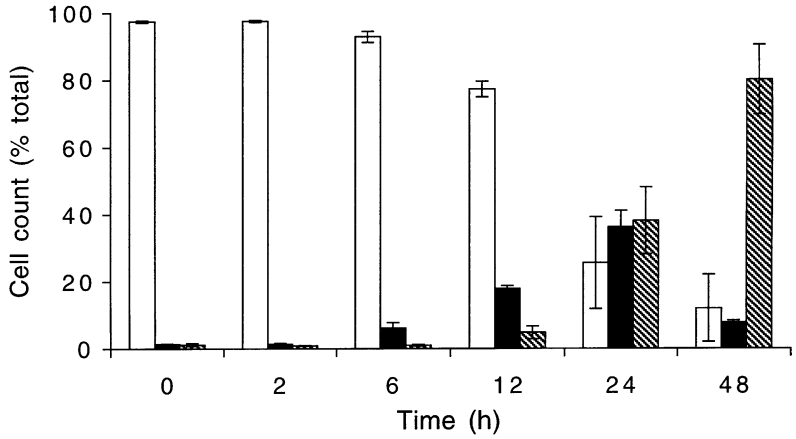

Fig. 5. Effect of LA on the proportion of Jurkat cells that were viable (open bars), apoptotic (solid bars), and necrotic/degenerate (shaded bars) over a 48 h time period. Each bar represents the mean ± S.E.M. of three independent experiments, each counted blind by three individuals. Differential cell counts were determined with LM from H&E stained cytospin slides of Jurkat cells treated with 4 mM LA.

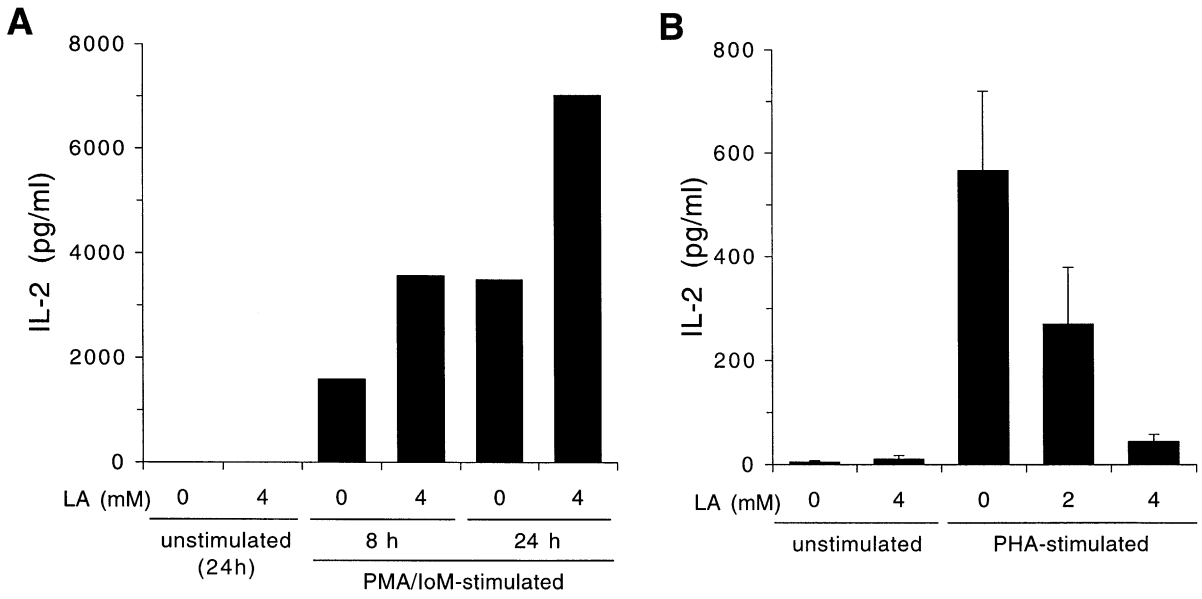

Fig. 6. Effect of LA on the secretion of IL-2 protein by (A) PMA/IoM-stimulated HPBL, and (B) PHA-stimulated HPBL as determined by ELISA. Each bar represents the mean ± S.E.M. of n experiments. The ELISA was performed on supernates collected at either 8 and 24 h ($n = 2$) for PMA/IoM-stimulated HPBL (A) or at 24 h ($n = 5$) for PHA-stimulated HPBL (B) in the presence or absence of LA.

570 pg/ml, which was 6.1-fold less than that produced by PMA/IoM-stimulated HPBL. This was an average value from five replicate experiments, which had quite variable levels of production. Nevertheless, it was clear that addition of 4 mM LA almost completely inhibited the production of IL-2 from these cells, resulting in a 13-fold reduction to less than 44 pg/ml. Lowering the concentration of LA to 2 mM resulted in only a two-fold reduction of IL-2 secretion.

The differential effects of mM concentrations of LA on the production of IL-2 by PMA/IoM-stimulated and PHA-stimulated HPBL were further investigated by analyzing IL-2 gene expression. Total RNA was isolated 2 h following mitogen stimulation of HPBL and the steady-state levels of IL-2 mRNA were analyzed by Northern hybridization and RT-PCR. Fig. 7A shows a representative autoradiograph of total RNA from PMA/IoM-stimulated HPBL run on a denaturing agarose gel, transferred to a nylon membrane and probed with a [^{32}P]-labeled cDNA specific for IL-2 and subsequently a control GAPDH cDNA. The steady-state level of IL-2 mRNA, which was undetectable in unstimulated HPBL, was clearly evident 2 h after PMA/IoM-stimulation. The induction of IL-2 gene expression was increased substantially by the addition of 4 mM LA. Densitometry analysis showed that this increase was 2.4-fold greater than mitogen stimulation alone. Over three independent experiments, an average increase in IL-2 expression of three-fold over PMA/IoM-stimulation alone was observed. This is comparable to the 2.2-fold increase in IL-2 protein secretion at 8 h as determined by ELISA (Fig. 6A). The steady-state level of the control GAPDH mRNA was increased in response to PMA/IoM-stimulation, and this

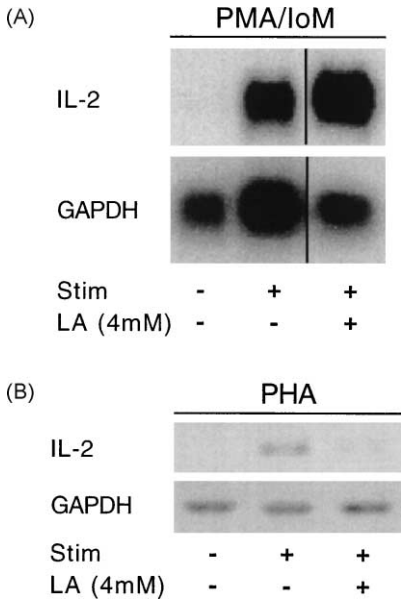

Fig. 7. Effect of LA on the expression of IL-2 mRNA by (A) PMA/IoM-stimulated HPBL as determined by Northern hybridization, and (B) PHA-stimulated HPBL as determined by RT-PCR. Total RNA was isolated 2 h following treatment. (A) Northern hybridization was performed using [^{32}P]-labeled probes specific for IL-2 and a GAPDH control. No IL-2 Northern hybridization signal was obtained for PHA-stimulated HPBL. (B) RT-PCR was performed with primers specific for IL-2 and GAPDH. The results shown are representative of three independent experiments. The vertical bar indicates that some intervening irrelevant lanes were cut out of the same Northern experiment.

increase was inhibited by 4 mM LA. Repeated Northern hybridization analysis failed to detect IL-2 gene expression in PHA-stimulated HPBL. This is reflected in the much lower amounts of IL-2 protein secreted as detected by ELISA after 24 h (Fig. 6B). We, therefore, employed RT-PCR on RNA isolated from PHA-stimulated HPBL using PCR primers specific for IL-2 and the control GAPDH, shown in Fig. 7B. RT-PCR analysis showed that 4 mM LA decreased IL-2 mRNA expression induced by PHA-stimulation of HPBL (Fig. 7B). This is in agreement with the observed effect of 4 mM LA on PHA-stimulated HPBL, which decreased the secretion of IL-2 at 24 h as shown by ELISA (Fig. 6B).

4. Discussion

We have shown that similar concentrations of LA inhibited proliferation of both mitogen-stimulated HPBL and leukaemic cells as measured by DNA synthesis and changes in cell density (Figs. 1 and 2, and Table 1). However, in stark contrast to its cytostatic effects on cell viability of long-term cultures of mitogen-stimulated HPBL (72 h), 4 mM LA was cytotoxic to both Jurkat and CCRF-CEM cells. This toxicity subsequently was shown to be due to the induction of apoptosis in the Jurkat cells, which peaked at 24 h (Figs. 4 and 5). The increased incidence of necrotic/degenerate cells by 24 h, most probably represents secondary degeneration of apoptotic cells. In culture, apoptotic cells predominantly undergo secondary degeneration, rather than uptake by neighboring viable cells as occurs following apoptotic involution in whole tissue (Dive et al., 1992; Kerr et al., 1994).

Consistent with LA being cytostatic to mitogen-stimulated HPBL proliferation, very few apoptotic cells were present in either PMA/IoM-stimulated or PHA-stimulated HPBL treated with 4 mM LA (Fig. 3), although some evidence of cell degeneration was seen. Decreases in Jurkat cell viability in the presence of mM concentrations of LA have been reported, with cell shrinkage, thiol depletion and DNA fragmentation evident (Sen et al., 1997). Sato et al. (1995) have demonstrated that oxidation of cellular thiols can induce apoptosis in T lymphocytes. Differential effects of LA on HPBL and Jurkat cells also have been reported. Prolonged incubation for 72 h of HPBL and Jurkat cells with a concentration of 100 μM LA has been shown to markedly potentiate Fas-mediated apoptosis in the Jurkat cells but not in the HPBL (Sen et al., 1999). While the HPBL were only 35% positive for the Fas receptor, the Jurkat cells were 100% positive and expressed 10-fold greater density of Fas receptors (Sen et al., 1999). In addition, LA treatment did not appear to influence the expression of Fas receptors. While Sen et al. (1999) showed preferential Fas-mediated apoptosis in leukaemic cells using lower μM concentrations of LA, it is unlikely that the observed preferential cytotoxic effects of 4 mM LA alone on Jurkat cells in our studies is mediated through the Fas receptor since no anti-Fas antibody was used in our system. Interestingly, in similar experiments to ours, green tea polyphenols, which exhibit antioxidant activity, recently have been shown to inhibit the proliferation of HPBL while inducing apoptosis in leukaemic cells (Li et al., 2000). However, the mechanism behind the differential effects of the polyphenols on HPBL remains to be elucidated.

A consistent biphasic pattern of inhibition of cell viability, and to a lesser extent cell density, was shown by LA in PMA/IoM-stimulated HPBL, which was greatest at 0.5 mM (Fig. 1A). The reason for this biphasic effect of LA on HPBL cell viability remains to be determined. However, we also have seen biphasic inhibition of proliferation between 0.1 and 2 mM with a number of other thiol compounds, such as *N*-acetylcysteine and cysteamine, on HPBL, Jurkat and CCRF-CEM cells (Pack and Hunt, unpublished observations). Others have shown that significant toxicity of thiol compounds occurs between 0.1 and 2 mM in various cell culture systems (Biaglow et al., 1984; Held and Biaglow, 1993, 1994; Tahsildar et al., 1988; Takagi et al., 1974). In addition, Held et al. (1996) have shown that in some cell lines thiol compounds can cause a biphasic pattern of apoptosis. This phenomenon has been ascribed to the formation of hydrogen peroxide by oxidation of the thiol compound in the cell culture medium at low concentrations, whereas at higher concentrations the thiol compound protects the cell against the hydrogen peroxide formed (Held and Biaglow, 1994). In support of this, we have shown that the aminothiol compound cysteamine inhibits proliferation of CCRF-CEM cells partly due to the production of hydrogen peroxide (Jeitner et al., 1998). Although LA is not a thiol compound itself, it is reduced to the thiol, DHLA, which is rapidly effluxed from the cell to the culture medium (Han et al., 1995; Handelman et al., 1994). It is possible that, in the experimental conditions described for our proliferation assays, LA in the 0.1–2 mM range is causing pro-oxidant inhibition of PMA/IoM-stimulated HPBL. However, this is unlikely since others have shown that 0.1 mM LA causes an increase in intracellular GSH in mitogenically-activated HPBL as well as Jurkat cells (Han et al., 1995, 1997; Sen et al., 1997).

We also have shown a differential effect of LA on the secretion of IL-2 protein and the steady-state level of IL-2 mRNA in mitogen-stimulated HPBL. While LA potentiated by more than two-fold both the induction of IL-2 mRNA at 2 h and the secretion of IL-2 protein at 24 h in HPBL stimulated by PMA/IoM, it significantly inhibited the IL-2 mRNA and protein induced by PHA stimulation. The difference in the site of action of the mitogens most likely explains the differential effect of LA on the induction of IL-2 mRNA expression. Whereas PHA cross-links the T cell receptor to activate T cell proliferation at the cell membrane (Licastro et al., 1993), PMA and IoM are known to bypass the T cell receptor, acting synergistically on downstream signaling molecules (Truneh et al., 1985). The production and secretion of IL-2 protein was inhibited by LA in PHA-stimulated HPBL due to its inhibition of steady-state levels of IL-2 mRNA. Since IL-2 is an autocrine regulator of T cell

proliferation, it is clear that this would inhibit HPBL proliferation. The exact mechanism by which LA inhibits proliferation of PHA-stimulated HPBL is not known, however, it may directly affect T cell signaling associated with the T cell receptor. For example, studies investigating glucose uptake and metabolism have shown that in various cellular systems the stimulation of insulin signaling by LA is dependent on phosphatidylinositol 3-kinase (PI 3-kinase), since the PI 3-kinase inhibitor wortmannin completely abolishes the effects of LA (Estrada et al., 1996; Ramrath et al., 1999; Yaworsky et al., 2000). Moreover, the sub-cellular localization of the membrane anchored linker for activation of T cells (LAT), an adaptor protein important to early signal transduction events from the T cell receptor, is sensitive to changes in intracellular thiol levels (Gringhuis et al., 2000; Pani et al., 2000).

It remains to be determined how LA inhibits the proliferation of HPBL stimulated by PMA/IoM since LA substantially augmented the production of IL-2 from these cells. While we showed that DNA synthesis is inhibited by LA, further analysis of pathways downstream of IL-2 in mitogen-activated HPBL is required. Examples include the effects of LA on the induction of the IL-2 receptor α sub-unit or signaling through the transferrin receptor. Indeed, we cannot rule out the possibility that LA may have a direct effect on DNA synthesis. In more recent experiments, LA has been shown to inhibit DNA synthesis in HPBL even if added 72 h after stimulation with PHA (Hardy and Hunt, unpublished observations). This indicates that the effects of LA in PHA-stimulated HPBL are not specific to the initial signaling cascades and suggests that other steps in the T cell cycle after the early signaling events are sensitive to LA, as indicated by the results in PMA-stimulated HPBL in which proliferation was inhibited despite stimulation of IL-2 protein expression.

The expression of IL-2 mRNA is regulated by the coordinated binding of at least four constitutively expressed and inducible transcription factors (Avots et al., 1995; Hughes and Pober, 1996; Serfling et al., 1995). These include the nuclear factor of activated T cells (NFAT), Rel/nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and Octamer factors 1 and 2 (Oct-1/2). The modulation of AP-1 and NF-κB by redox processes is well known (Sen and Packer, 1996; Sun and Oberley, 1996). In Jurkat cells, LA can block the cytosolic activation of NF-κB by PMA, TNF-α or hydrogen peroxide (Sen and Packer, 1996; Suzuki et al., 1992). LA may act at specific points in the signal transduction pathway leading to NF-κB activation since the ability of LA to inhibit NF-κB activation was not dependent on its ability to increase intracellular GSH levels (Sen and Packer, 1996). DHLA can also block the activation of cytosolic NF-κB. However, in contrast, it acts to enhance the DNA-binding activity of NF-κB, whereas LA is inhibitory (Suzuki et al., 1995). This apparent paradox is explained by the fact that NF-κB has two modes of redox regulation: cytosolic activation by oxidative processes; and DNA-binding by reductive processes. It remains to be determined whether LA has differential effects on the activation and DNA-binding activity of AP-1 or NF-κB, or the expression of c-Fos and c-Jun mRNA and protein in HPBL. Our preliminary results indicate that LA does indeed have differential effects on transcription factor activity (Hardy and Hunt, unpublished observations). The effects of LA on signaling molecules further upstream in the signal cascade leading to IL-2 mRNA expression, such as on the MAP kinases and phosphatases, are also currently being investigated in both mitogen-stimulated HPBL and Jurkat cells.

Modulation of IL-2 is only one aspect of gene regulation by LA. We recently have used mRNA differential display and DNA macroarray technology to explore global effects of LA on gene expression in PMA/IoM-stimulated HPBL (Hardy et al., 2000; Pack et al., unpublished observations). In these studies, LA inhibited the change in expression of a number of genes that were either up- or down-regulated by PMA/IoM. We are currently analyzing the nature and functions of these genes. A comparative analysis of the effects of LA on global gene expression in both PMA/IoM- and PHA-stimulated HPBL, as well as Jurkat cells, may reveal at what point in proliferative signaling cascades, and in cell cycle progression, LA exerts its effects.

In summary, we have clearly demonstrated differential effects of LA on the viability of normal and leukaemic lymphocytes. LA also has been shown to have differential effects on IL-2 expression in mitogen-stimulated HPBL depending on the mitogen used. These in vitro observations suggest a role for LA in the treatment of leukaemia and perhaps other immune system disorders, and suggest that other thiol compounds also may be of benefit.

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