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Original Contribution

Dependence of leukemic cell proliferation and survival on H_2O_2 and L-arginine

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ABSTRACT

The proliferation and/or survival of a variety of cells is dependent on cellular hydrogen peroxide (H_2O_2) production. We tested whether this was true of leukemic cells, using cell lines from leukemic patients (CEM, 697, Mn-60, and Tanoue). We found that addition of catalase inhibited proliferation of all cell lines and induced death in two. However, this turned out to be due to arginase contamination of the catalase. Pure arginase inhibited cell proliferation and survival, which was reversible by adding L-arginine, demonstrating the L-arginine dependency of these cells. The glutathione peroxidase mimetic ebselen killed the cells by a novel, rapid form of death, preceded by cell blebbing and prevented by *N*-acetylcysteine, suggesting toxicity is not due to ebselen's antioxidant activity. Addition of *N*-acetylcysteine to remove endogenous H_2O_2 stimulated survival and proliferation, suggesting that basal levels of H_2O_2 promoted cell death. Consistent with this, leukemic cell death was induced by adding as little as 5 μM H_2O_2. Ascorbic acid, even at 100 μM, induced death through H_2O_2 production. Thus H_2O_2 does not promote proliferation and survival, rather the opposite, and previous literature may have misinterpreted the effects of antioxidants. Arginase, H_2O_2, ascorbic acid, and ebselen might be useful in the treatment of leukemia.

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There is growing evidence that hydrogen peroxide (H_2O_2) can act as a mitogen in some cells, including transformed cells [1,2]. One of the most convincing pieces of evidence for a mitogenic role of endogenously-produced H_2O_2 is that the addition of catalase to cells can eliminate both basal and stimulated proliferation [3–11]. If this were true of cancer cells it would have important implications for treatment.

Although high levels of H_2O_2 are known to induce cell death by apoptosis or necrosis, there is some evidence that lower, potentially physiological levels of H_2O_2 may inhibit cell death by various means [11–15]. Again, if this were true of cancer cells, it would have important treatment implications.

We were interested in whether H_2O_2 could regulate the basal proliferation and survival of leukemic cells. To investigate this we used cell lines that were derived from children with acute lymphoblastic leukemia (ALL). These cells were treated with a number of known antioxidants: *N*-acetylcysteine, glutathione ethyl ester, ascorbic acid, ebselen, and catalase. *N*-acetylcysteine can cross the plasma membrane into the cell cytoplasm, where it is converted into cysteine by acetylase activity [16]. *N*-acetylcysteine acts as an antioxidant both by increasing the intracellular levels of the naturally occurring antioxidant glutathione and through direct scavenging of H_2O_2 [17]. Glutathione ethyl ester is a cell-permeative form of glutathione [18]. Ascorbic acid is an antioxidant that reacts with superoxide, the hydroxyl radical, singlet oxygen, and thiyl radicals, but it can also be a pro-oxidant by producing hydrogen peroxide or the hydroxyl radical in the presence of metal ions [19]. Ebselen and catalase are both catalytic scavengers of H_2O_2. Ebselen is a selenium-containing compound that may work in a manner analogous to glutathione peroxidase: glutathione reduces ebselen to form a selenol, which then reduces H_2O_2 [20]. Catalase is an enzyme that acts directly on H_2O_2, converting two H_2O_2 molecules into two H_2O and one O_2 molecule.

Here we report that most of these antioxidants inhibited the proliferation and/or survival of leukemic cells, consistent with the hypothesis that endogenous oxidant production promotes proliferation and survival of these cells. However, on further analysis we found that these antioxidants were acting on proliferation and survival by means other than their antioxidant activity, in some cases because they were pro-oxidant. Contrary to the above hypothesis, we found that removing H_2O_2 did not inhibit proliferation and survival, and in fact some types of leukemic cells were uniquely sensitive to H_2O_2-induced cell death. Furthermore, we show that the proliferation and

Abbreviations: ALL, acute lymphoblastic leukemia; PI, propidium iodide; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium.

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survival of some leukemic cells seems to be dependent on L-arginine. These results have implications for the treatment of leukemia.

Material and methods

Materials

Cell culture media were from Invitrogen (Paisley, UK). All other chemicals were from Sigma (Poole, UK). The catalases used in this study were from Sigma. The bovine catalase was C1345: powder, 2000–5000 units/mg protein, cell culture tested. The *Micrococcus* catalase was 60634: solution, dark brown, ~170,000 U/ml. The erythrocyte catalase was C3556: ≥90% (SDS–PAGE), buffered aqueous solution, >30,000 units/mg protein.

Cell culture

All cells were bought from the European Cell Culture Collection and were used within 40 passages. Mn-60 (B-ALL (L3)) cells were cultured in F-10 Ham medium and 10% FBS, Tanoue (B-ALL (L3)) cells and 697 (pre-B-ALL) cells were both cultured in RPMI 1640 and 10% FBS, and CEM (T-ALL) cells were cultured in RPMI 1640, 10% FBS, and 1× ITS (contains insulin, transferrin, and sodium selenite) liquid medium supplement (Sigma).

Proliferation assay

Cells were treated and 15,000 cells/well were plated in a 96-well plate and incubated for either 24 or 72 h. After this time the cells were diluted threefold in medium and transferred to a 24-well plate, where they were treated with Hoechst 33342 or propidium iodide (PI). Total cell number and number of cells that had condensed nuclei (apoptotic) or were PI positive (necrotic) were assessed under a fluorescence microscope. For all experiments and treatments, very few condensed nuclei were present, so cell death is presented only as a percentage of PI-positive cells/field.

Each treatment is the combination of at least three independent experiments performed on different days. An independent experiment has each treatment in triplicate, with five fields of view being counted per well. *N* for each treatment is therefore the combination of at least 45 field counts.

Pretreatment of medium with catalase

RPMI was treated with catalase and incubated for either 1 or 72 h and then this medium was spun through a 30-kDa filter to remove the catalase. Tanoue cells were spun, washed in PBS, and treated with 10% FBS and either fresh RPMI or the catalase-pretreated RPMI. Cells were incubated with these media for 72 h and cell number and viability were assayed using Hoechst 33342 and PI as above.

Catalase activity assay

Medium that had either been treated with catalase or been treated with catalase and then spun through the 30-kDa filter was diluted in Krebs–Hepes (118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM Hepes) buffer to the equivalent of 1 U/ml. The initial rate of oxygen produced after addition of 300 μM H_2O_2 was measured using an oxygen electrode.

This experiment was performed once on three independent occasions, and the results were then combined.

Amino acid preparation and analysis

Amino acids were prepared in 1 M hydrochloric acid and then diluted in PBS (final pH 7.4) to 50% of the concentration found in RPMI.

Amino acids in culture medium treated with or without catalase for 72 h were analyzed using the Waters AccQ-Tag system. An aliquot (10 μl) from the medium was treated with α-aminobutyric acid (internal standard: 40 μl, 0.25 mM) and the mixture taken to dryness. The residue was then dissolved in hydrochloric acid (20 μl, 10 mM) and treated with borate buffer (Waters; 30 μl, 0.4 M) and mixed. AccQ. Fluor reagent (Waters; 10 μl) was then added to derivatize the amino acids with a fluorescent moiety; the sample was mixed and left at room temperature for 1 min followed by 10 min at 55°C. The sample was then taken to dryness and the residue was redissolved in an appropriate volume of eluent A (Waters). After centrifugation, an appropriate volume was injected into an amino acid analyzer (Waters AccQ-Tag system) and chromatography was performed on a reverse-phase column (Nova-Pak C18), 4 μm; 3.9×150 mm. Elution was performed with eluent A followed by an increasing percentage of acetonitrile according to the standard program. Peak detection was achieved by measuring fluorescence (λ_{ex} 250 nm, λ_{em} 395 nm). Quantification was performed using Millennium software and calibration curves for each amino acid.

Urea assay

The following assay was adapted from [21]. To 500 μl of catalase-treated RPMI, 500 μl of sulfuric acid/phosphoric acid/water (in the ratio 1/3/7) was added, followed by 40 μl of 9% α-isonitrosopropiophenone. The tubes were heated to 100 °C for 1 h and cooled to room temperature and absorbance at 540 nm was measured. Concentration was determined using a urea standard curve.

The standards and treatments were all performed in duplicate, on three independent occasions, and then combined.

Measurement of arginase activity

RPMI was treated with known activities of pure arginase or with a range of activities of bovine catalase for 2 h. After this time the concentration of urea was measured as above. Urea production was linear with time over the 2-h period. The number of Sigma units of arginase activity in the catalase preparation was 0.21% of the Sigma units of catalase activity.

The standards and treatments were all performed in duplicate, on three independent occasions, and then combined.

Results

Micromolar H_2O_2 inhibits the proliferation and survival of leukemic cells

To test whether leukemic cell proliferation and viability were regulated by H_2O_2 we used four different leukemic cell lines derived from leukemic patients (CEM, 697, Mn-60, and Tanoue). CEM is a T cell leukemia, 697 is a precursor B cell leukemia, and Mn-60 and Tanoue are mature B cell leukemias. For each cell line we tested whether their proliferation and/or survival was altered by adding antioxidants and oxidants in vitro.

To investigate whether H_2O_2 could stimulate the proliferation of leukemic cells, we added a bolus dose of either H_2O_2 or glucose oxidase, which produces H_2O_2 continuously from oxygen and glucose, to the four cell lines. Fig. 1 shows an example of a cell line that was highly sensitive to H_2O_2, 697 (Fig. 1a), and an example of a cell line that was relatively insensitive to H_2O_2, Mn-60 (Fig. 1b). Both the CEM and the Tanoue cells had responses to H_2O_2 and glucose oxidase that were similar to those of the 697 cells; the effects of 10 μM H_2O_2 on proliferation and survival are given in the legend to Fig. 1. Addition of 0.1 or 0.5 μM H_2O_2 had no effect on any of the cell lines (Fig. 1 for 697 and Mn-60, data not shown for CEM and Tanoue). The cells were found to have different sensitivities to 5 and 10 μM H_2O_2, which caused inhibition of proliferation and cell death in all but the Mn-60

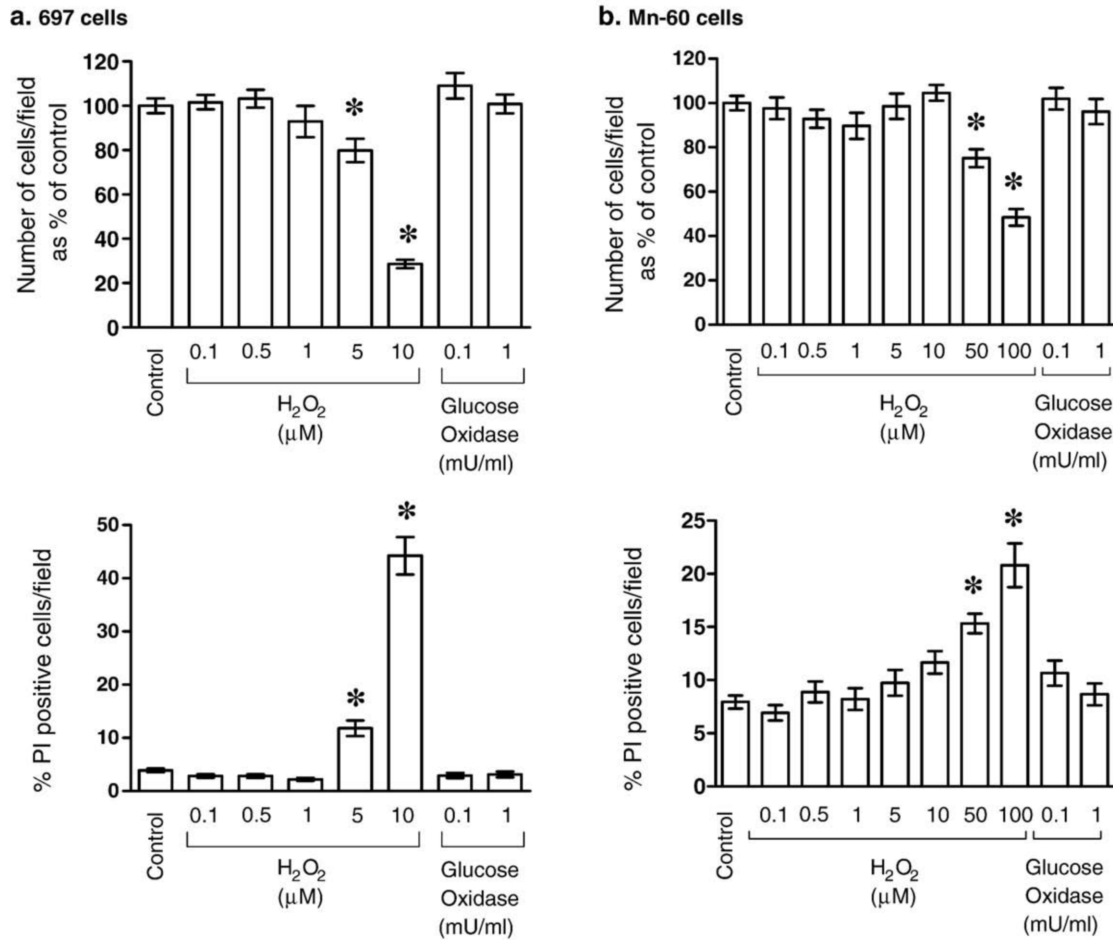

Fig. 1. Effects of H_2O_2 or the H_2O_2-producing enzyme glucose oxidase on the proliferation and survival of leukemic cells. Four leukemic cell lines, (a) 697, (b) Mn-60, and CEM and Tanoue (data for 10 μM below), were treated with increasing concentrations of H_2O_2 or glucose oxidase for 72 h. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. Data for CEM and Tanoue treated with 10 μM H_2O_2—CEM: control, 100.0 ± 3.2 number of cells/field as % of control, 2.0 ± 0.2% PI-positive cells/field; 10 μM H_2O_2, 64.58 ± 3.9* number of cells/field as % of control, 16.53 ± 1.9%* PI-positive cells/field. Tanoue: control, 100.0 ± 3.3 number of cells/field as % of control; 1.5 ± 0.2% PI positive cells/field; 10 μM H_2O_2, 48.67 ± 4.6* number of cells/field as % of control, 27.2 ± 3.3%* PI-positive cells/field. *$p<0.01$, significantly different from control using one-way ANOVA and Dunnett's posttest.

cells (Fig. 1b), with CEM and Tanoue having a response to 10 μM that was similar to that of 697 cells (697, Fig. 1a; CEM and Tanoue, Fig. 1 legend). This suggests that H_2O_2-mediated inhibition of proliferation was a result of increased cell death. Mn-60 cells were more resistant to H_2O_2, with 100 μM inhibiting proliferation and inducing cell death (Fig. 1b). Addition of glucose oxidase did not affect proliferation or survival of the cell lines except in Tanoue, in which 1 mU/ml caused a small but significant increase in cell death and inhibition of proliferation (data not shown). Higher levels of glucose oxidase added to the medium-induced cell death (data not shown). These results indicate that additional H_2O_2 does not stimulate proliferation in these cells, but rather stimulates cell death. However, this does not tell us whether removing endogenous levels of H_2O_2 affects proliferation.

Antioxidants N-acetylcysteine, glutathione ethyl ester, ascorbic acid, and ebselen

We added antioxidants to the leukemic cells to test whether endogenously produced H_2O_2 could affect proliferation and survival. The first antioxidant we used was *N*-acetylcysteine (5 mM), a scavenger of both intracellular and extracellular H_2O_2. *N*-acetylcysteine increased the proliferation of the 697, Mn-60, and Tanoue cells, while having a small but nonsignificant effect on the proliferation of the CEM cells (Table 1). This apparent increase in cell proliferation may simply be due to *N*-acetylcysteine blocking basal cell death: *N*-acetylcysteine significantly reduced the proportion of PI-positive 697 and Mn-60 cells compared to the untreated controls (Table 1).

To test whether the actions of *N*-acetylcysteine were mediated through elevating intracellular glutathione levels, rather than directly scavenging H_2O_2, we supplied the cells with 5 mM glutathione ethyl ester, a cell-permeative form of the antioxidant glutathione. This caused a small decrease in the proliferation of the 697, Mn-60, and

Table 1
Effects of *N*-acetylcysteine and glutathione ethyl ester on leukemic cell proliferation and survival

	5 mM N-acetylcysteine		5 mM glutathione ethyl ester	
	Number of cells/field as % of control	% PI-positive cells/field (control)	Number of cells/field as % of control	% PI-positive cells/field (control)
CEM	115 ± 12	2.6 ± 0.4 (3.0 ± 0.4)	96 ± 10	2.9 ± 0.4 (3.0 ± 0.4)
697	130 ± 9*	3.5 ± 0.6* (10.5 ± 1)	75 ± 5*	8.0 ± 1.0* (4.2 ± 0.5)
Mn-60	150 ± 15*	6.8 ± 0.8* (17.3 ± 1.7)	65.3 ± 4.6*	5.9 ± 0.7* (3.6 ± 0.6)
Tanoue	136 ± 14*	2.0 ± 0.3 (2.5 ± 0.4)	85 ± 4*	1.2 ± 0.2 (1.2 ± 0.3)

CEM, 697, Mn-60, and Tanoue cells were treated with either 5 mM *N*-acetylcysteine or 5 mM glutathione ethyl ester for 72 h. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. For % PI-positive cells/field, numbers shown in parentheses are the % PI-positive cells/field for untreated (control) cells.

* $p<0.05$, significantly different from control using Student's *t* test.

Tanoue cells, but had no effect on the CEM cells (Table 1). In principle this slightly decreased proliferation might be due to mild toxicity as the glutathione ethyl ester was significantly toxic to the 697 and Mn-60 cells (Table 1). The failure of glutathione ethyl ester to stimulate cell proliferation and/or survival, as *N*-acetylcysteine does, suggests that *N*-acetylcysteine may not be acting by supplying glutathione.

Ascorbic acid is a commonly used antioxidant. CEM cells were treated for 24 h with either a high physiological (100 μM [19,22]) or a 10-fold greater (1 mM) concentration of ascorbic acid, to test what effects this antioxidant has on the proliferation and survival of leukemic cells. At 100 μM, ascorbic acid induced around 40% death, whereas at 1 mM ascorbic acid induced nearly 100% death (Fig. 2). Ascorbic acid has been reported to induce death through the production of H_2O_2 [23]. To test whether the toxic effects of ascorbic acid were mediated by H_2O_2, we cotreated the cells with ascorbic acid and purified catalase (derived from human erythrocytes). Addition of catalase completely protected the cells against ascorbic acid-induced death (Fig. 2). This suggests that ascorbic acid is increasing H_2O_2 levels and this is responsible for its toxicity. As noted above, CEM and other leukemic cells are uniquely sensitive to H_2O_2-induced death, which may explain why even physiological concentrations of ascorbic acid were able to induce death.

To test further whether intracellular H_2O_2 affects proliferation and survival of leukemic cells we added ebselen, a catalytic decomposer of intracellular H_2O_2. All four cell lines responded in a similar manner to treatment with ebselen, so only the effects for CEM cells are presented in Fig. 3. Ebselen very slightly inhibited the proliferation of Tanoue cells at 5 μM, but did not significantly inhibit the proliferation of any of the other cell lines. Ebselen had no significant effect on the survival of the cell lines at 5 μM. However, higher concentrations of ebselen (20 μM) caused the death of most cells in all four cell lines. Surprisingly, ebselen started to kill the Tanoue cells within 15 min (untreated control, 2.7 ± 0.7% PI-positive cells/field; 20 μM ebselen, 47.0 ± 3.6% PI-positive cells/field; $p < 0.0001$), and nearly all Tanoue cells were PI positive by 2 h (data not shown).

Large blebs were seen on the cells before the cells became necrotic (PI positive). These blebs were of similar size to the cells, and there were only one or occasionally two per cell (Fig. 4). Some of these blebs appeared to detach from the cells, resulting in a number of membrane ghosts in the culture medium. The rapidity of the death would seem to indicate that ebselen is not acting as a H_2O_2 scavenger. Indeed, the speed and type of death are similar to those of cells treated with high concentrations of H_2O_2 [24]. As ebselen is supplied to the cells in a form that readily oxidizes glutathione, it is possible that it induces death by acting as a pro-oxidant.

The coadministration of *N*-acetylcysteine with ebselen protected the CEM cells from ebselen-induced death after 72 h (20 μM ebselen, 92.7 ± 1.5% PI-positive cells/field; 20 μM ebselen + 5 mM *N*-acetylcysteine, 19.8 ± 1.8% PI-positive cells/field; $p < 0.001$). *N*-acetylcysteine can replace glutathione in reducing ebselen into the H_2O_2-scavenging selenol [25], and thus addition of *N*-acetylcysteine will

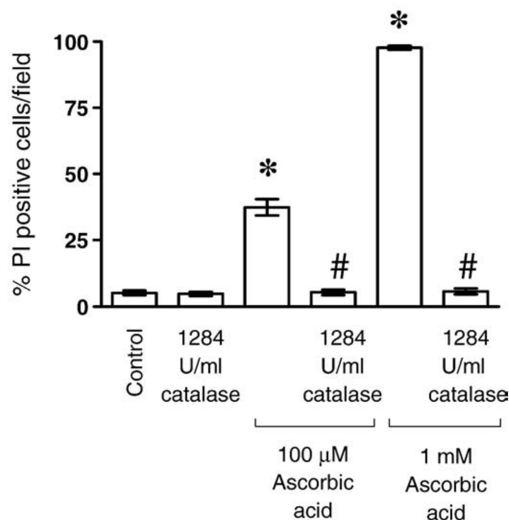

Fig. 2. Ascorbic acid induced death in CEM cells after 24 h through the production of H_2O_2. CEM cells were treated with 100 μM or 1 mM ascorbic acid for 24 h with or without 1284 U/ml erythrocyte catalase. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. *$p < 0.01$, significantly different from control using one-way ANOVA and Dunnett's posttest. #$p < 0.001$, cells treated with either concentration of ascorbic acid and addition of catalase are significantly different from cells treated with the corresponding concentration of ascorbic acid, using one-way ANOVA and Bonferroni's posttest.

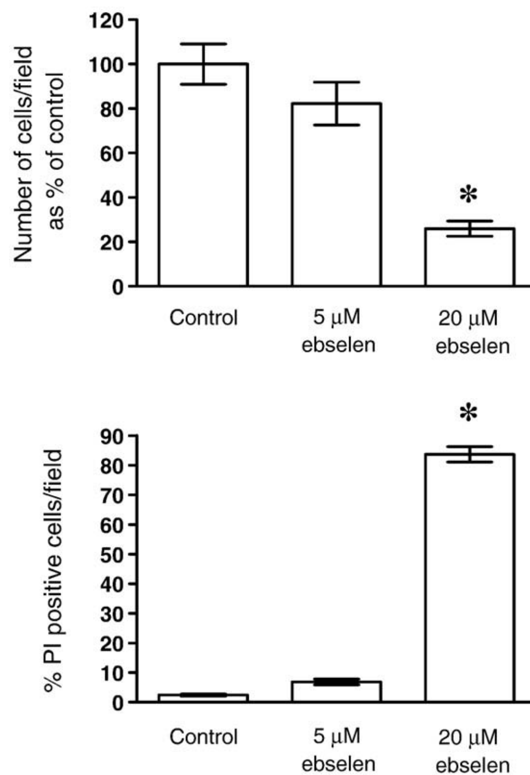

Fig. 3. 20 μM ebselen induced massive cell death, whereas 5 μM ebselen only slightly inhibited cell proliferation and caused no change in cell viability. CEM cells were treated with either 5 or 20 μM ebselen for 72 h. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. *$p < 0.05$, significantly different from control using one-way ANOVA and Dunnett's posttest.

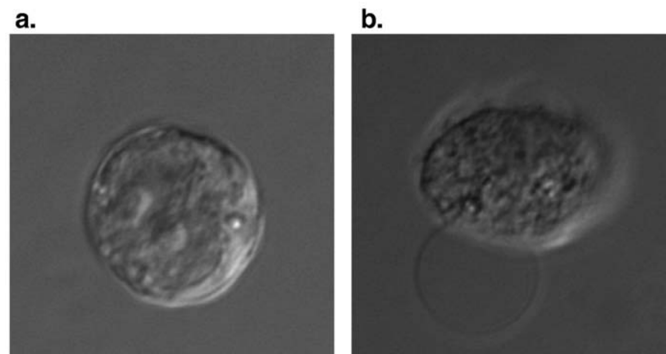

Fig. 4. Blebbing of Tanoue cells treated with ebselen for 15 min. Tanoue cells were treated (a) without or (b) with 20 μM ebselen for 15 min. The cells were then observed under a confocal microscope.

increase the ability of ebselen to scavenge H_2O_2. As we found that N-acetylcysteine prevents the toxic effect of ebselen, this suggests that ebselen toxicity is not mediated by its ability to remove H_2O_2. N-acetylcysteine may be protective by either converting ebselen to the selenol form or increasing the pool of available thiols for ebselen to react with, in both cases potentially protecting the cellular thiols from forming seleno-sulfides with ebselen.

Extracellular catalase

To test whether basal H_2O_2 (produced by cells or medium) affects the proliferation and survival of leukemic cells, we added to the culture medium catalase, an enzyme that decomposes H_2O_2. Semipure catalase preparations were derived either from bovine liver (Sigma C1345) or from *Micrococcus lysodeikticus* (Sigma 60634). Fig. 5 shows examples of a cell line whose proliferation and survival were both sensitive to catalase (697, Fig. 5a) and a cell line whose proliferation but not survival was sensitive to catalase (Tanoue, Fig. 5b). The CEM and Mn-60 cells had similar responses, so only the effects of 1284 U/ml bovine catalase on proliferation and survival are shown in the legend for Fig. 5. High concentrations of extracellular bovine catalase strongly inhibited the proliferation of all four cell lines and induced cell death in the 697 cells and Mn-60 cells only (697 cells in Fig. 5a, Tanoue cells in Fig. 5b, and the effects of 1284 U/ml catalase on CEM and Mn-60 in the legend for Fig. 5). The catalase from *Micrococcus* also inhibited proliferation, but required four times the nominal activity indicated by the supplier to cause the same level of inhibition as the bovine catalase. However, when the activity of the two catalase preparations was measured directly under the same conditions using an oxygen electrode, it was found that the catalase from *Micrococcus* had four times lower activity (bovine catalase, 39.9 ± 4.3, and *Micrococcus* catalase, 10.4 ± 0.8 nmol H_2O_2 consumed per minute by 1 U/ml of stated activity). Thus, these results were consistent with endogenous H_2O_2 being required for the proliferation and/or survival of the leukemic cells.

We tested whether the inhibition of proliferation by bovine catalase was reversible. Tanoue cells were treated with 1284 U/ml bovine catalase for 72 h, after which time the medium was washed off and the cells were recultured to the same density in fresh medium for a further 72 h. Although catalase inhibited the proliferation during the first 72 h (untreated control, $510.2 \pm 57\%$ of initial cell density; catalase treated, $193.6 \pm 18.40\%$ of initial cell density; $p < 0.0001$), the proliferation after 72 h was completely restored after the catalase was removed and fresh medium was added (untreated control, 626.5

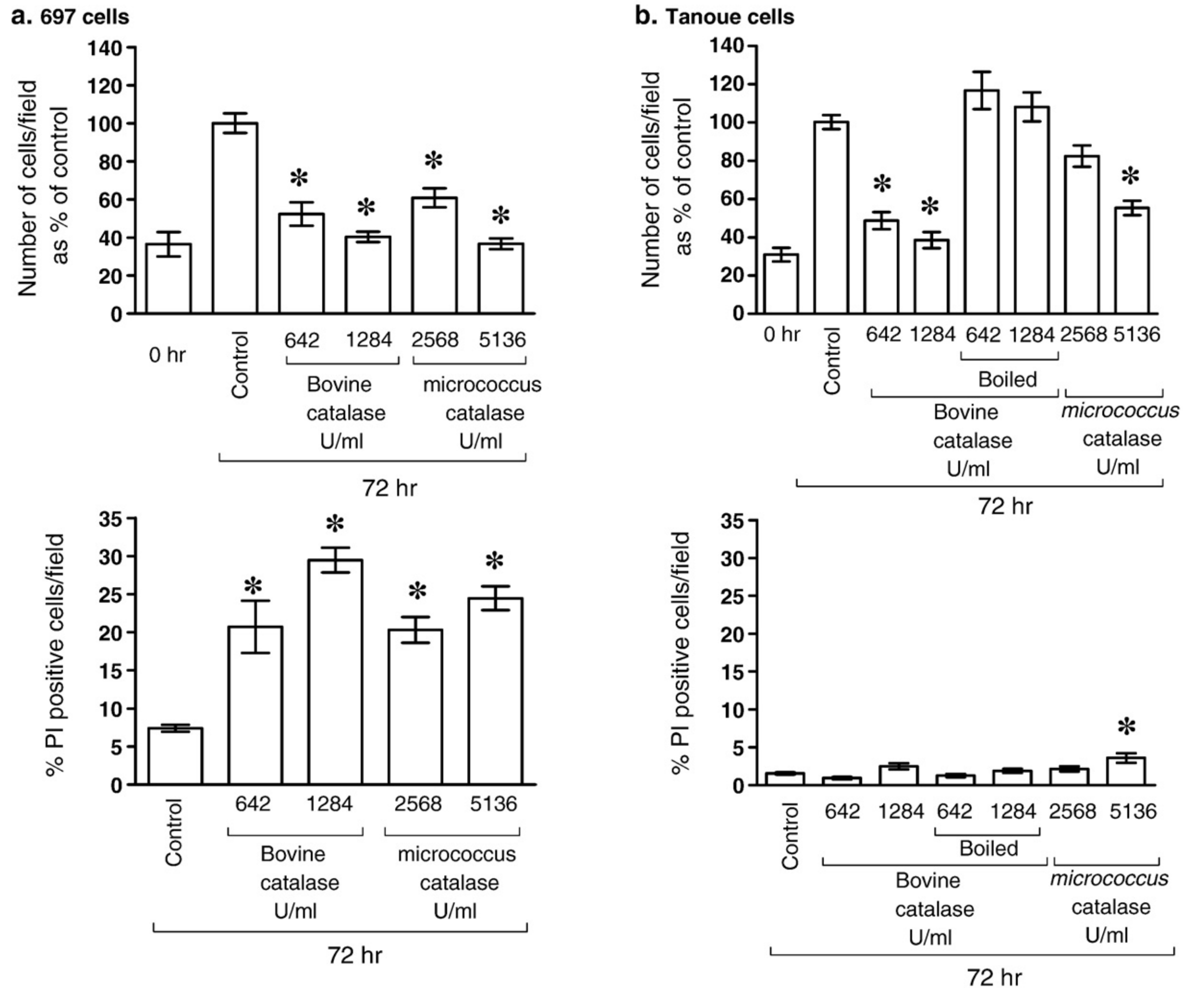

Fig. 5. Leukemic cell proliferation was inhibited, and the viability of 697 and Mn-60 cells was compromised, by the addition of increasing concentrations of either bovine or bacterial catalase. (a) 697, (b) Tanoue, and CEM and Mn-60 (data for 1284 U/ml below) cells were treated with 642 or 1284 U/ml bovine catalase or 2568 or 5136 U/ml *M. lysodeikticus* catalase for 72 h. Tanoue cells were also treated with 642 or 1284 U/ml bovine catalase that had been boiled to inactivate the catalase for 10 min. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. Data for CEM and Mn-60 treated with 1284 U/ml bovine catalase—CEM: control, 100.0 ± 7.6 number of cells/field as % of control, $3.1 \pm 0.3\%$ PI-positive cells/field; 1284 U/ml, 51.9 ± 6.1* number of cells/field as % of control, $2.5 \pm 0.3\%$ PI-positive cells/field. Mn-60: control, 100.0 ± 9.3 number of cells/field as % of control, $4.4 \pm 0.4\%$ PI-positive cells/field; 1284 U/ml, 45.0 ± 5.2* number of cells/field as % of control, $12.5 \pm 1.5\%$* PI-positive cells/field. *$p < 0.05$, significantly different from control using one-way ANOVA and Dunnett's posttest.

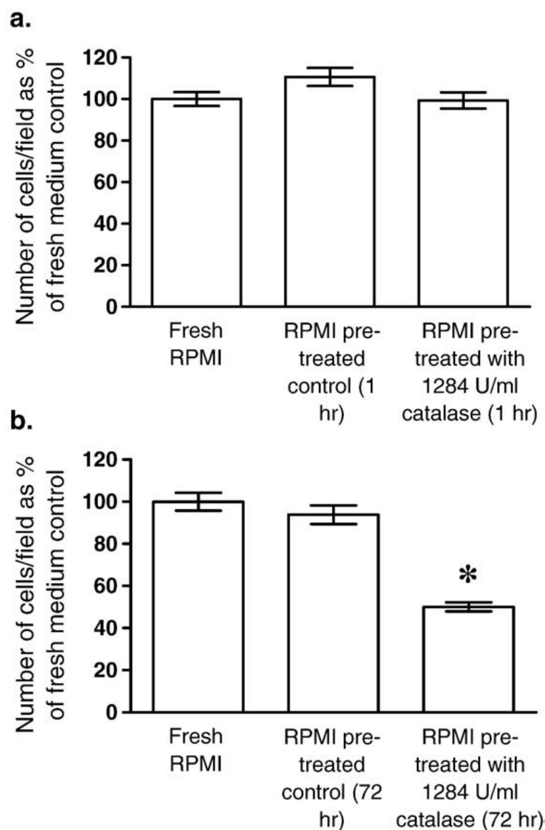

Fig. 6. Effects on proliferation of Tanoue cells by treatment for 72 h with RPMI pretreated for 1 or 72 h with 1284 U/ml bovine catalase. (a) Tanoue cells were treated for 72 h with fresh RPMI, RPMI pretreated for 1 h control, or RPMI pretreated for 1 h with 1284 U/ml catalase. (b) Tanoue cells were treated for 72 h with RPMI, RPMI pretreated for 72 h control, or RPMI pretreated for 72 h with 1284 U/ml catalase. In both experiments, cells were stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. $*p<0.05$, significantly different from control using one-way ANOVA and Dunnett's posttest.

$\pm$45.61% of initial cell density; cells previously treated with catalase but then washed, 635.4$\pm$38.52% of initial density; $p>0.05$). This indicates that "catalase" reversibly arrests the Tanoue cells in their cell cycle, but does not cause them to irreversibly differentiate or senesce.

We investigated this effect of added catalase further. Bovine catalase inhibited the proliferation of Tanoue cells, and this was prevented by heat-inactivating the catalase (Fig. 5b), indicating that inhibition of proliferation was mediated by some activity in the catalase preparation. Catalase has peroxidase activity [26], and this activity could in principle be responsible for the inhibition of proliferation by oxidizing a component of the medium. We wanted to differentiate between catalase inhibition of proliferation mediated either (a) by scavenging H_2O_2 or (b) through peroxidase or some other activity of the catalase responsible for changing components of the medium. The former requires catalase to be present in the medium with the cells, whereas the latter requires catalase to be in the medium only before treatment of the cells. We therefore differentiated between these two mechanisms by pretreating the culture RPMI medium with 1284 U/ml catalase for 1 or 72 h and then removing the 250-kDa catalase protein by filtering through a 30-kDa filter (termed "RPMI pretreated with catalase"). As a control, RPMI that did not have catalase added to it was incubated and filtered in the same way as the RPMI pretreated with catalase (termed "RPMI pretreated control"). Catalase activity before filtering was 17.0$\pm$7.5 nmol O_2/min, and catalase activity after filtering was 0.4$\pm$1.0 nmol O_2/min, indicating that 97.6% of the activity was removed. RPMI pretreated with 1284 U/ml catalase for 1 h did not significantly inhibit proliferation of Tanoue cells that were grown in this medium for 72 h (Fig. 6a), indicating that no low-molecular-weight (<30 kDa) contaminant of the catalase preparation was responsible for the inhibition of proliferation. However, RPMI pretreated with 1284 U/ml catalase for 72 h did inhibit the proliferation of Tanoue cells that were then grown in the medium for 72 h (Fig. 6b). This demonstrated that catalase was inhibiting proliferation not by removing H_2O_2 but rather through the production or removal of something in the medium.

We found that adding fresh RPMI medium to the RPMI pretreated with 1284 U/ml catalase for 72 h could prevent the inhibition of proliferation (cells treated for 72 h with RPMI pretreated for 72 h with 1284 U/ml bovine catalase—50.1$\pm$2.1 cells/field as % of fresh RPMI control, cells treated for 72 h with 50% RPMI pretreated for 72 h with 1284 U/ml and 50% fresh RPMI—99.6$\pm$4.1* cells/field as % of fresh RPMI control, $*p<0.001$), indicating that the catalase treatment was acting by depleting some component of the medium. To identify the component, 12 of the amino acids found in RPMI were added back to catalase-pretreated medium at a concentration of 50% of fresh medium. Table 2 shows the mixes of amino acids used and their concentrations (shown only for the first mix as the amino acids used in the other mixes are at the same concentration). Fig. 7a shows that amino acid mix A could completely reverse the inhibitory effects on proliferation of Tanoue cells treated with the catalase-pretreated medium. Mixes A1 and A2 were used to narrow down which of these 12 amino acids was responsible for the effect (Fig. 7a), with only A1 being able to reverse the inhibition of proliferation. Mixes A1a and A1b were used to further narrow down which of the amino acids in A1 were responsible for reversing the inhibition of proliferation (Fig. 7b). Only A1a could reverse the inhibition, so each of the three amino acids in A1a, L-arginine, L-cysteine, and L-methionine, was added to Tanoue cells treated with RPMI pretreated with 1284 U/ml catalase to determine which of these was responsible. Fig. 7c shows that only L-arginine could reverse the inhibitory effects of the RPMI pretreated with 1284 U/ml catalase. Catalase was therefore inhibiting the proliferation of the Tanoue cells by removing L-arginine. Addition of L-arginine not only prevented the block of proliferation by the RPMI pretreated with 1284 U/ml catalase for 72 h, but also, if added every 24 h, completely prevented the block on proliferation induced by the continuous presence of catalase in the medium with the cells (Fig. 8).

We measured the effect of the catalase treatment on the amino acid content of the RPMI medium. The only amino acid that was depleted was L-arginine, which was undetectable after 72 h treatment with catalase (control RPMI L-arginine concentration 1.118$\pm$0.096 mM; catalase-pretreated RPMI L-arginine concentration 0.005$\pm$0.001 mM). The only amino acid peak that was increased was isoleucine/ornithine (which had indistinguishable peaks on the reverse-phase liquid chromatography column). The increase in the peak was comparable to the decrease in the peak for L-arginine (control RPMI isoleucine/ornithine concentration 0.43$\pm$0.02 mM;

Table 2
Amino acid composition of amino acid mixes used in Fig. 7

Amino acid preparation	Amino acids present (μM)
A	Glycine (66.5), arginine (575), aspartic acid (75), cysteine (41.65), glutamic acid (68), glutamine (1025), isoleucine (191), methionine (50.5), serine (143), threonine (84), tyrosine (90.5), valine (85.5)
A1	Glycine, arginine, aspartic acid, cysteine, glutamic acid, methionine
A2	Glutamine, isoleucine, serine, threonine, tyrosine, valine
A1a	Arginine, cysteine, methionine
A1b	Glycine, aspartic acid, glutamic acid

Amino acids were prepared in 1 M hydrochloric acid and then diluted in PBS (final pH 7.4) to 50% of the concentration found in RPMI. Concentrations are in micromolar in parentheses after each amino acid in preparation A. All other preparations contained the same concentrations of amino acids as given for A.

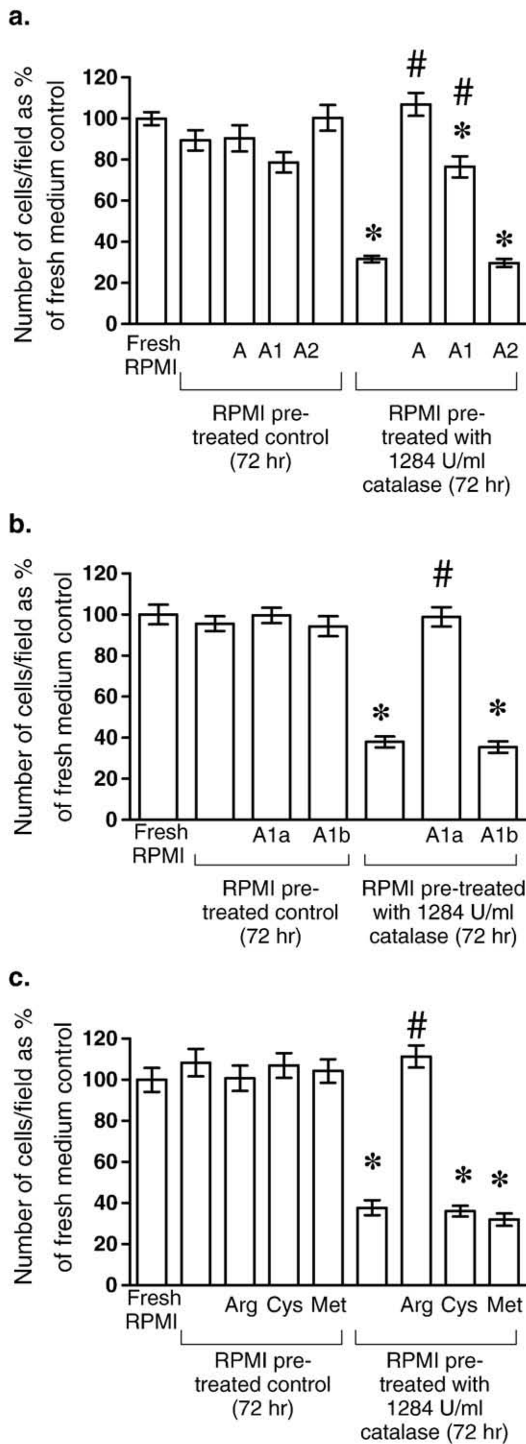

Fig. 7. Effects of amino acid mixes on the proliferation of Tanoue cells treated for 72 h with RPMI pretreated for 1 or 72 h with 1284 U/ml bovine catalase. Tanoue cells were treated for 72 h with fresh RPMI, RPMI pretreated for 72 h control, or RPMI pretreated for 72 h with 1284 U/ml catalase. To the cells treated with the latter two media, an amino acid mix (for composition see Table 2), (a) A, A1, or A2 or (b) A1a or A1b, or (c) L-arginine, L-cysteine, or L-methionine was simultaneously added. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. *$p<0.05$, significantly different from control. #$p<0.05$, amino acid mix-treated cells significantly different from corresponding medium treatment: RPMI pretreated for 72 h control or RPMI pretreated for 72 h with 1284 U/ml catalase. Statistics were performed using one-way ANOVA and Bonferroni's posttest.

catalase-pretreated RPMI isoleucine/ornithine concentration 1.03 ± 0.01 mM). This is consistent with the catalase preparation containing arginase activity, which would breakdown L-arginine to ornithine and urea. We found that medium pretreated for 72 h with catalase had high levels of urea (0.89 ± 0.02 mM) compared to untreated medium (0.02 ± 0.01 mM), indicating that the catalase preparation contained arginase activity, presumably because it contained arginase.

Bovine catalase effect mediated by arginase

We investigated whether the addition of arginase alone could have the same effect as bovine catalase on the proliferation and viability of Tanoue and 697 cells. Purified 1 U/ml arginase inhibited the proliferation of Tanoue cells without altering their survival, but decreased both the viability and the proliferation of 697 cells after treatment for 72 h (Fig. 9). This differential effect of pure arginase replicates that of the crude catalase preparation (Fig. 5). To confirm that the death induced in the 697 cells by arginase was due to the depletion of L-arginine, the cells were incubated with arginase for 24 h together with 10 mM L-arginine. Although 10 mM L-arginine did cause some death in 697 cells, it could reverse the greater death induced by 1 U/ml arginase (10 mM L-arginine, 10.94 ± 1.313%* PI-positive cells/field; 1 U/ml arginase, 16.53 ± 1.520% PI-positive cells/field; 1 U/ml arginase + 10 mM L-arginine, 9.896 ± 1.204%* PI-positive cells/field; *$p<0.01$, significantly different from 1 U/ml arginase-treated cells).

We measured the arginase activity of the catalase preparation by measuring urea production and found that 100 Sigma U/ml of catalase activity contained 0.21 Sigma U/ml of arginase activity. Thus the 642 and 1284 U/ml of bovine catalase that we added to the cells (Fig. 5) contained 1.35 and 2.70 U/ml of arginase activity, respectively. The effects on proliferation and survival of the addition of 1 U/ml purified arginase (Fig. 9) were similar to those of the catalase preparation containing this arginase activity (Fig. 5), consistent with the effects of the catalase preparation being entirely due to its arginase activity.

Micrococcus and erythrocyte catalases

In contrast to the bovine catalase, the catalase preparation from *M. lysodeikticus* seemed to inhibit proliferation by a low-molecular-weight contaminant. Culture medium that was pretreated for 1 h with catalase from *Micrococcus* and then filtered to remove the catalase in

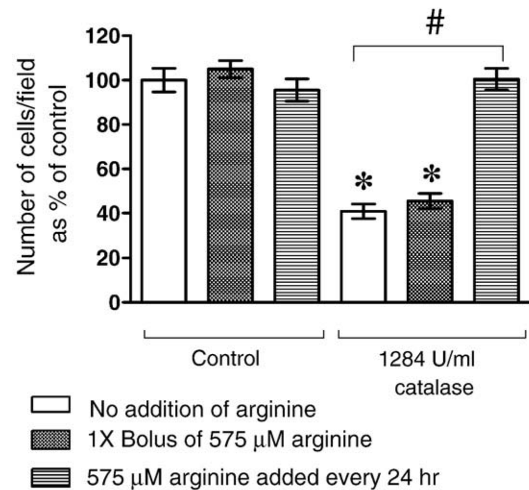

Fig. 8. Inhibition of proliferation by bovine catalase is completely reversed by supplementing the medium every 24 h with L-arginine. Tanoue cells were treated with or without 1284 U/ml catalase for 72 h, and to these cells either no additional L-arginine was added or 575 μM L-arginine was added either once at time 0 or at time 0 and every subsequent 24 h. After the 72-h incubation, cells were stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. *$p<0.01$, significantly different from control using one-way ANOVA and Dunnett's posttest. #$p<0.001$, significantly different from 1284 U/ml catalase-treated Tanoue cells using one-way ANOVA and Bonferroni's posttest.

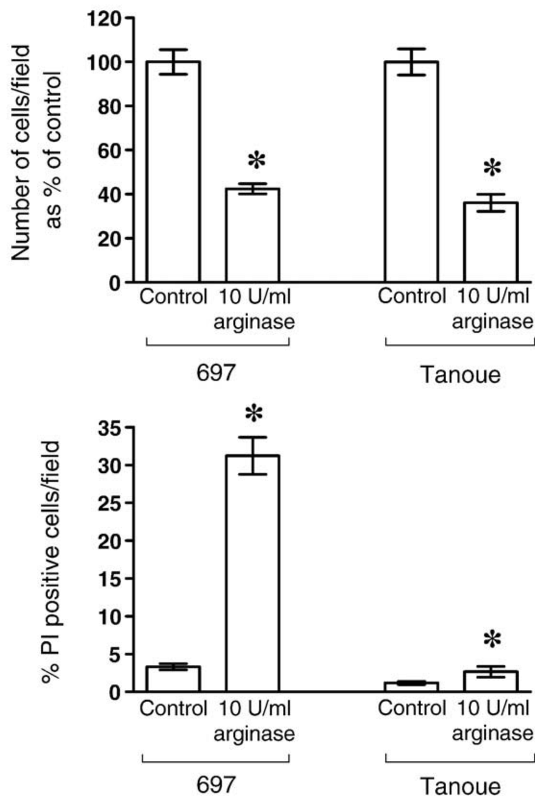

Fig. 9. Addition of arginase inhibited proliferation of both 697 and Tanoue cells, but induced death only in 697 cells. 697 and Tanoue cells were treated for 24 h with 1 U/ml arginase. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. *$p<0.05$, significantly different from control using Student's *t* test.

the same manner as for the bovine catalase still inhibited the proliferation of Tanoue cells incubated with this medium for 72 h (RPMI pretreated control for 1 h, 93.3 ± 4.1 cells/field as % of control; RPMI pretreated with 5136 U/ml *Micrococcus* catalase for 1 h, 49.9 ± 2.5 cells/field as % of control; $p<0.01$). Boiling this catalase preparation did not prevent it from inhibiting proliferation either (data not shown). Thus it is likely that this preparation contains a low-molecular-weight (<30 kDa) contaminant, possibly the ammonium sulfate used in purification, which inhibits proliferation.

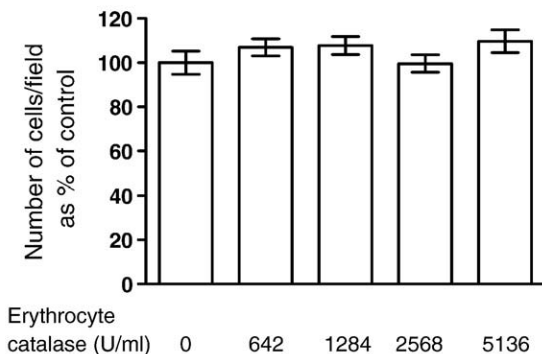

Fig. 10. Tanoue cell proliferation is not inhibited by even very high concentrations of erythrocyte catalase. Tanoue cells were treated for 72 h with 642–5136 U/ml human erythrocyte catalase with ≥90% purity. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope. There was no significant difference ($p>0.05$) for all treatments, using one-way ANOVA and Dunnett's posttest. There was no change in viability of the cells at any concentration tested (data not shown).

Table 3
Effects of human erythrocyte catalase on leukemic cell proliferation

	Control		1284 U/ml erythrocyte catalase	
	Number of cells/field as % of control	% PI-positive cells/field	Number of cells/field as % of control	% PI-positive cells/field
CEM	100.0 ± 4.4	2.4 ± 0.3	109.2 ± 4.6	2.8 ± 0.4
697	100.0 ± 3.4	5.5 ± 0.5	96.73 ± 4.3	4.8 ± 0.5
Mn-60	100.0 ± 3.9	10.7 ± 1.8	117.1 ± 4.6*	12.6 ± 1.2
Tanoue	100.0 ± 5.2	1.4 ± 0.3	107.7 ± 4.1	1.3 ± 0.2

All four leukemic cell lines were treated with 1284 U/ml erythrocyte catalase for 72 h. Cells were then stained using Hoechst 33342 and PI and cell number and viability were assessed using a fluorescence microscope.
* $p<0.05$, significantly different from control using Student's *t* test.

The inhibition of proliferation by bovine catalase seemed to be solely due to the removal of L-arginine. We wanted to confirm that scavenging of H_2O_2 by catalase had no effect on cell proliferation or death. We therefore used another catalase, isolated from human erythrocytes (Sigma C3556), with ≥90% purity as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The erythrocyte catalase had the same measured activity as the bovine catalase: bovine catalase 39.9 ± 4.3 and erythrocyte catalase 37.3 ± 1.6 nmol/min consumption of H_2O_2. Medium treated with erythrocyte catalase had no higher urea levels than control medium after 24 h even at concentrations four times that of bovine catalase (untreated control urea (in mM), 0.096 ± 0.030; 1284 U/ml erythrocyte catalase, 0.16 ± 0.023; 5136 U/ml erythrocyte catalase, 0.12 ± 0.029, and there was no significant difference using a one-way ANOVA and Dunnett's posttest). To put this into context, medium treated with 1284 U/ml bovine catalase for 2 h had already accumulated 0.74 mM urea. At activities four times that of the bovine catalase, there was no inhibition of proliferation in the Tanoue cells by erythrocyte catalase (Fig. 10). Similarly, erythrocyte catalase had no significant effect on the proliferation or survival of any of the other cell lines, except Mn-60, in which 1284 U/ml erythrocyte catalase slightly stimulated proliferation (Table 3).

Discussion

We were interested in whether the proliferation and survival of leukemic cells were dependent on endogenous H_2O_2 acting as a mitogen or survival factor, as has been demonstrated for other types of cells [3–11,14,15,27,28]. However, we found that exogenous H_2O_2 either added as a bolus or continuously supplied by glucose oxidase failed to stimulate proliferation, whereas higher concentrations of H_2O_2 (10 μM) increased cell death. Indeed CEM, Tanoue, and 697 cells were uniquely sensitive to H_2O_2 compared to the extensive literature on H_2O_2-induced cell death in many different cell types: in general the minimum required to induce significant cell death is 100 μM H_2O_2 [1,6], as was found here with the Mn-60 cells.

However, the fact that adding more H_2O_2 does not promote proliferation or survival does not tell us whether removing endogenous levels of H_2O_2 affects proliferation or survival. *N*-acetylcysteine stimulated proliferation and survival, and the increase in proliferation was potentially a result of the increased survival. This, together with the finding that low levels of added H_2O_2 induce cell death, suggests that endogenous H_2O_2 is partially responsible for the background levels of cell death in these cultures, fitting with a model that was suggested to explain *N*-acetylcysteine-induced tumor cell proliferation [28]. Supplementation of glutathione through glutathione ethyl ester either had no effect or was slightly toxic, suggesting that *N*-acetylcysteine effects may not be mediated by glutathione supplementation. In principle, *N*-acetylcysteine might stimulate proliferation by supplying cysteine to the cells, though this seems unlikely as the culture media contain both its biosynthetic precursor methionine and either cysteine itself or its more readily transported oxidized form, cystine.

Ascorbic acid was found to kill CEM cells even at 100 μM, a concentration close to the physiological level in human plasma. However, this death was prevented by the addition of catalase, indicating that the death is mediated by H_2O_2. Ascorbic acid at pharmacological concentrations has previously been found to induce death through the production of H_2O_2 in five human cancers [23], whereas primary cells were found to be insensitive to ascorbic acid. A Burkitt lymphoma (JLP-119) was much more sensitive than the other cancer cells tested. This finding, when taken in conjunction with the data we have reported here, possibly indicates that some lymphocytic cancers are highly susceptible to H_2O_2. Whether ascorbic acid could be used therapeutically to kill leukemic cells would depend on the level of H_2O_2 it could produce in vivo. It has been shown that when ascorbic acid is administered intravenously levels of H_2O_2 in the extracellular fluid can approach 20 μM [29], which would be sufficient to kill three of the leukemic cell lines used here.

An intracellular catalytic scavenger of H_2O_2, ebselen, was toxic to all four leukemic cell lines (80–100% dead at 20 μM). It seems unlikely that this toxicity was due to scavenging of H_2O_2 given the rapidity and type of death and the fact that it was blocked by *N*-acetylcysteine. Ebselen is known to be toxic to a variety of transformed cell lines by mechanisms that are not entirely clear but are thought to involve reaction with protein thiols or glutathione depletion [30–32]. Ebselen contains a selenium atom, which has a high affinity for thiols, such as that of glutathione [19]. It is possible that ebselen could induce death through glutathione depletion and/or oxidation. Ebselen can also exchange onto protein thiols and thereby inhibit a variety of enzymes, such as nitric oxide synthases, cyclooxygenases, lipoxygenases, and the sodium pump [33]. Inhibition of the sodium pump or glutathione depletion might be responsible for the toxicity to leukemic cells, as has been suggested for other transformed cells [30,31]. The type of cell death is interesting in that it is very rapid and proceeded by massive blebbing of the cell, suggesting osmotic swelling or calcium overload of the cell, either of which could be caused by damage to the cell membrane. The mode of death has some parallels with that induced by very high levels of H_2O_2 [24], supporting the idea that ebselen is acting as a pro-oxidant. Given that ebselen is relatively nontoxic to primary cells in vitro and in vivo, and has been used successfully in clinical trials (for stroke) [34,35], its relative toxicity to leukemic cells may suggest its potential for use as a treatment for leukemia.

We show here that a commercial preparation of bovine liver catalase (from Sigma) inhibits the proliferation of Tanoue cells by removing L-arginine from culture medium, and not through the removal of H_2O_2. The bacterial catalase preparation tested was found to inhibit proliferation, probably owing to a low-molecular-weight contaminant. Catalase may deplete intracellular (as well as extracellular) H_2O_2, because first, as H_2O_2 can rapidly cross membranes, scavenging extracellular H_2O_2 should deplete intracellular H_2O_2 and second, catalase can be taken up by some cells [10]. Failure of a high activity of either a purer human erythrocyte preparation of catalase or bovine catalase with L-arginine added every 24 h to inhibit proliferation of Tanoue cells indicates that proliferation of these cells is not dependent on endogenous H_2O_2 but rather on L-arginine availability. The dependence on L-arginine was confirmed using arginase, which was found to cause a response very similar to that to treatment with the bovine catalase.

We also provide evidence that catalase does not cause the cells to irreversibly differentiate or senesce. However, as the effects of catalase on proliferation are due to the removal of L-arginine by an arginase contaminant, it can therefore be inferred that the removal of L-arginine does not induce cells to irreversibly differentiate or senesce, rather it only causes cell cycle arrest (or death in 697 cells).

This is the first time, to our knowledge, that inhibition of cell proliferation caused by exogenous bovine catalase has been shown to be due to an arginase contaminant. Arginase has previously been shown to be present in some catalase preparations from Sigma [36,37], though not specifically the preparation used in this study. Therefore researchers using Sigma bovine liver catalases to examine the effects of H_2O_2 on cell proliferation may instead have been looking at the effects of arginase. As the bovine catalase in this study is sold as "cell culture tested," it is more likely to be used in cell culture studies. The likelihood of observing an arginase instead of a catalase effect in cell culture would be related to the amount of catalase added, time of incubation, and levels of L-arginine in the medium, for example, DMEM has 398 μM, whereas RPMI has 1150 μM.

A number of groups [3–11,27] have found that exogenous catalase inhibits proliferation and/or decreases viability, which has been attributed to H_2O_2 having a regulatory role. These studies had varying amounts of supporting data, such as the use of other antioxidants [4–6,9–11], concomitant addition of H_2O_2 via glucose oxidase [3], or heat-inactivating the catalase [3,7,27]. However, heat inactivation of catalase would also inactivate any enzyme contaminant, and continual production of H_2O_2 could inactivate any contaminating enzyme. Although the use of other antioxidants provides much stronger evidence for a role of H_2O_2 in cell proliferation and/or survival, the data we have presented here show that the observed effects may be independent of their H_2O_2 scavenging activity. Some studies have looked at the effects of modified catalase on tumor growth and dissemination in vivo, e.g., cationized [38,39] or PEGylated catalase [40,41]. However, the modified catalase was one of the Sigma bovine catalases, and as such any arginase contamination would be modified in the same manner. Therefore it is possible that the results of these studies were due to arginase and not catalase activity.

L-Arginine is a semiessential amino acid for the body and an essential amino acid for some cell types, a fact that can be exploited with enzymes that can break down L-arginine to treat some kinds of cancers. Tumors that lack argininosuccinate synthase and argininosuccinate lyase, which convert citrulline into L-arginine, were found to be highly susceptible to L-arginine depletion by a nonmammalian protein originally found in mycoplasm, arginine deiminase [42,43]. Arginine deiminase converts L-arginine into citrulline and ammonia. The problem with using this enzyme therapeutically is that only a subset of cancers seems to lack argininosuccinate synthase and/or lyase, making the rest resistant to treatment [44]. Our finding that some leukemic cells are very sensitive to added arginase suggests this enzyme might be used in treatment, particularly because the precursor B cell leukemic cells (697) were highly susceptible to L-arginine deprivation-induced death. The enzyme asparaginase has been routinely used in remission induction regimens for the treatment of precursor B and T ALL for several decades [45]. The advantage of such therapy over conventional chemotherapy is the relative lack of acute toxicity and the absence of any long-term side effects.

Conclusion

Our data indicate that the basal proliferation of leukemic cells is not regulated by H_2O_2. Rather these cells are unusually sensitive to H_2O_2-induced cell death. Ebselen, ascorbic acid, and arginase may have potential to be developed as anti-leukemic treatments. Our work also indicates the potential problems of using "antioxidants" as investigative tools unless great care is taken to verify that any effects are not mediated by other (non-antioxidant) properties of these molecules.

Acknowledgments

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