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CELL PROLIFERATION AND OXIDATIVE STRESS

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Antioxidants such as mannitol, butylated hydroxytoluene and α-tocopherol enhance the growth of polyoma virus transformed and non-transformed BHK-21 cells. In the case of mannitol this is observed even in the absence of added calf serum. In part these effects may operate to protect cellular growth control mechanisms. On the other hand oxidants such as H_2O_2 and t-butyl hydroperoxide can inhibit growth and overall cellular protein synthesis, through mechanisms that are likely to involve radicals. In the case of H_2O_2 the inhibitory effects can nevertheless be reduced by 'prestressing' the cells with mild heat or with H_2O_2 itself.

Paradoxically very low concentrations (10^{-8} M) of H_2O_2 or t-butyl hydroperoxide can actually stimulate cell growth, even in the absence of serum. These stimulatory effects however do not appear to involve radicals as they are enhanced by inclusion of mannitol or DMSO in the medium.

KEY WORDS: Cell division, oxidative stress, peroxides, antioxidants.

INTRODUCTION

Although the processes that regulate mammalian cell proliferation are already known to be complex, several lines of enquiry suggest that free radical species may have an important modulatory role. For example evidence comes from studies on α-tocopherol levels in regenerating liver in relation to periods of DNA synthesis,[1] levels of cellular lipid peroxidation in relation to cell proliferation,[1-3] the effects of polyunsaturated fatty acids on growth[3] as well as experiments on the effects of lipid peroxide breakdown products on growth and specific intracellular signal transduction mechanisms.[4]

The possible relationship between oxidative stress and cell proliferation has been further explored comparing the growth and cellular protein synthesis of polyoma virus transformed baby hamster fibroblasts (BHK-21/PyY) with corresponding non-transformed cells (BHK-21/C13) following inclusion of either antioxidants or oxidants in the growth medium. Whilst antioxidants generally appeared to enhance proliferation the results with oxidants such as H_2O_2 or t-butyl hydroperoxide (tBuOOH) were unexpected. Both could inhibit growth at high concentrations but in the case of H_2O_2 these effects could be moderated by "prestressing" the cells with mild heat or H_2O_2 itself. On the other hand at very low concentrations (10^{-8}M) both H_2O_2 and tBuOOH were actually stimulatory. Such observations may be of significance in relation to the inflammatory response as well as having biotechnological applications.

EXPERIMENTAL PROCEDURES

Cell Cultures

BHK-21/C13 cells[5] and BHK-21/PyY cells (transformed with polyoma virus[6] were grown as monolayer cultures at 37°C in the Dulbecco (or Glasgow) modification of Eagle's minimal essential medium (DMEM) supplemented with 10% (v/v) calf serum and 10% tryptose phosphate broth (Difco, bacto).[7]

Cell Proliferation

Triplicate monolayer cultures of BHK-21 cells (C13 or PyY) (0.5×10^6 cells per dish) were grown for 24 hr in DMEM supplemented with 10% (v/v) calf serum. Thereafter the medium was removed and the cell monolayers washed three times with serum-free medium and then the media replaced with, or without, serum together with various additions as indicated in the figure legends. After 3 days of growth at 37°C the cells from each dish were removed and the yields of cells assessed using a haemocytometer. Results were expressed as yield of cells per dish $\pm$ s.d.

Incorporation of L-^{35} S-methionine into Protein and the Amino acid Pool

Triplicate monolayer cultures of BHK-21 cells (C13 or PyY) (0.5×10^6 cells per dish) were grown for 24 hr in DMEM supplemented with 10% (v/v) calf serum. Thereafter the medium was removed and the cell monolayers washed with serum-free medium and the medium replaced with, or without, serum together with various additions as indicated in the text for different times. After the experimental period the media and additions were removed, the monolayers washed and the ability of the cells to incorporate ^{35}S-methionine into protein over 30 min at 37°C was assessed as previously described.[8] In certain cases the labelled proteins were also analysed for heat shock proteins by dodecyl sulphate – polyacrylamide gel electrophoresis as previously described.[9] In other cases the incorporation of ^{35}S-methionine into the "amino acid pool" was determined in addition to incorporation into protein. This was achieved after the experimental treatment by first washing the monolayer three times with serum-free medium to eliminate any radioactive medium. Following this, the monolayer was extracted three times, for 10 mins each, with 2 ml portions of ice-cold 10% (w/v) trichloroacetic acid. These extracts were combined, and the content of ^{35}S-radioactivity determined and referred to as trichloroacetic "acid-soluble" radioactivity which represents ^{35}S-methionine in the intracellular "amino acid pool". The remaining monolayers were then dissolved in 880 μl 0.4 M NaOH, neutralised with 20 μl glacial acetic acid and the ^{35}S-radioactivity determined. This is referred to as the trichloroacetic acid 'acid-insoluble' radioactivity and represents ^{35}S-methionine incorporated into protein.

Determination of Catalase Activity

Monolayer cultures, 5×10^6 cells in 75 cm^2 flat bottomed flasks, were grown for 24 hr in DMEM supplemented with 10% (v/v) calf serum. Thereafter the medium was removed and the cell monolayers washed three times with serum-free medium. The cells were then removed and disrupted in 0.5 ml 50 mM potassium phosphate buffer

pH7 using a glass homogeniser. Catalase levels in the cell homogenates were determined spectrophotometrically as described by Claiborne[10] using reaction mixtures containing 2.9 ml 19 mM H_2O_2 in 50 mM potassium phosphate buffer pH7 and 0.1 ml of cell homogenate.

RESULTS

Previous experiments with HeLa cells[2] suggested that oxidative stress might be critical in relation to the growth of cultured mammalian cells. When HeLa cells are deprived of serum their rate of proliferation decreases as does their protein synthetic rate. At the same time there is a significant rise in the level of cellular lipid peroxidation which can nevertheless be abated by the inclusion of mannitol in the serum-free medium.[2]

In light of these observations the effects of including mannitol in the normal growth medium of both BHK-21/C13 and BHK-21PyY cells were explored. Figure 1 shows that the yield of cells after 3 days was enhanced by low levels of mannitol, the stimulatory effect being most noticeable in the "virus transformed" cell line. Vitamin E was less effective (Figure 2). On the other hand butylated hydroxy toluene was particularly stimulatory (Figure 3), again notably in the case of the "transformed" cells. Although these experiments were carried out in normal growth medium contain-

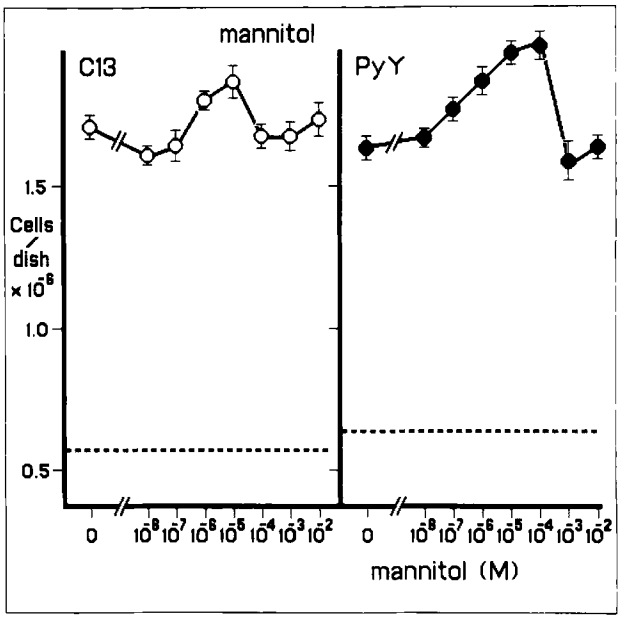

FIGURE 1 The effect of added mannitol on BHK-21 cell yields. Triplicate monolayer cultures (0.5×10^6 cells/dish) were allowed to grow for 24 hr at 37°C in DMEM supplemented with 10% calf serum. The dotted lines indicates the number of cells per dish after that period. The medium in each dish was then removed and the monolayers washed three times with serum-free medium and replaced with DMEM and calf serum along with varying amounts of mannitol. After 3 days at 37°C the cells were removed from each dish and counted as described in EXPERIMENTAL PROCEDURES. Results were expressed as the mean of triplicate determinations ± s.d. (○ BHK-21/C13 cells; ● BHK-21/PyY cells).

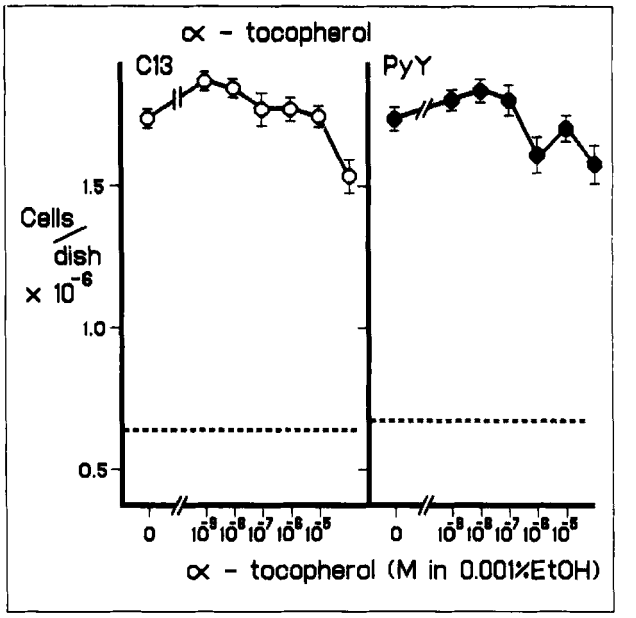

FIGURE 2 The effects of added α-tocopherol on BHK-21 cell yields. Experimental procedures were as described for Figure 1 except that varying amounts of α-tocopherol succinate in 0.001% EtOH were added after the initial 24 hr growth period. (○ BHK-21/C13 cells; ● BHK-21/PyY cells).

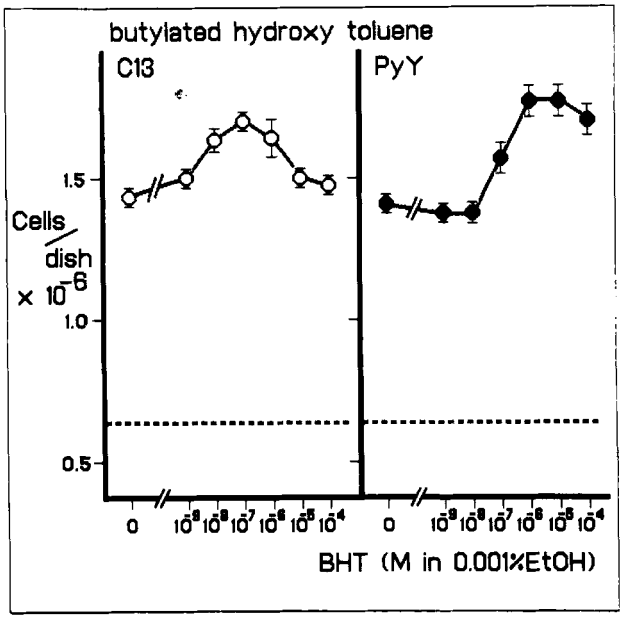

FIGURE 3 The effects of added butylated hydroxytoluene on BHK-21 cell yields. Experimental procedures were as described for Figure 1 except that varying amounts of butylated hydroxy toluene (BHT) in 0.001% EtOH were added after the initial 24 hr growth period. (○ BHK-21/C13 cells; ● BHK-21/PyY cells).

TABLE I
Effects of mannitol on BHK-21 cell growth in serum-free medium

Cell line	Conditions	Cells/dish × 10^{-6} ± s.d.
BHK-21/C13	normal	1.50 ± 0.03
	serum-free	1.28 ± 0.02
	serum-free plus mannitol (10^{-5} M)	1.40 ± 0.02
BHK-21/PyY	normal	1.38 ± 0.02
	serum-free	1.25 ± 0.03
	serum-free plus mannitol (10^{-5} M)	1.32 ± 0.03

Triplicate monolayers of BHK-21 cells (0.5×10^6 cells per dish) were grown for 24 hr in DMEM supplemented with 10% calf serum. As described in EXPERIMENTAL PROCEDURES the medium was removed, the monolayers washed three times with serum-free medium and the medium replaced as indicated above. After 3 days the cells in each dish were removed and counted in a haemocytometer. The results are expressed as the mean of triplicate experiments ± s.d.

ing serum, further investigations indicated that low levels of mannitol would also stimulate cell growth in the absence of serum (Table 1).

Since serum deprivation is also known to reduce protein synthesis, further experiments were carried out to determine whether mannitol addition would moderate this reduction. Table 2 shows that whilst the uptake of ^{35}S-methionine into protein (as represented by the trichloroacetic "acid insoluble" radioactivity) is reduced following serum deprivation, this was minimised in BHK-21/C13 cells if mannitol was added to the serum-free medium. Table 2 however indicates that deprivation of serum also leads to a reduced uptake of amino acid into the amino acid pool (as represented by the cold trichloroacetic "acid-soluble" radioactivity). Again this deleterious effect on amino acid transport into cells is abated by inclusion of mannitol in the serum-free growth medium (Table 2).

In order to explore further the relationship of oxidative stress on cell proliferation the effects of oxidants such as tBuOOH and H_2O_2 were examined. Figures 4 and 5

TABLE 2
Effects of mannitol on L-methionine incorporation into BHK-21/C13 cells

	^{35}S-radioactivity (cpm × 10^{-2})	
Experimental conditions	acid-soluble	acid-insoluble
normal medium (4 hr)	80.60	75.05
plus 10^{-5} M mannitol (4 hr)	83.12	93.62
serum-free (4 hr)	69.93	71.78
serum-free plus 10^{-5} M mannitol (4 hr)	80.03	93.42

Triplicate monolayer cultures of BHK-21/C13 (0.5×10^6 cells per dish) were grown for 24 hr as described in EXPERIMENTAL PROCEDURES. After treatment as above they were assessed for their ability to incorporate ^{35}S-methionine into trichloroacetic acid-soluble material and into trichloroacetic acid-insoluble material as described in EXPERIMENTAL PROCEDURES. The results are expressed as the mean of triplicate experiments.

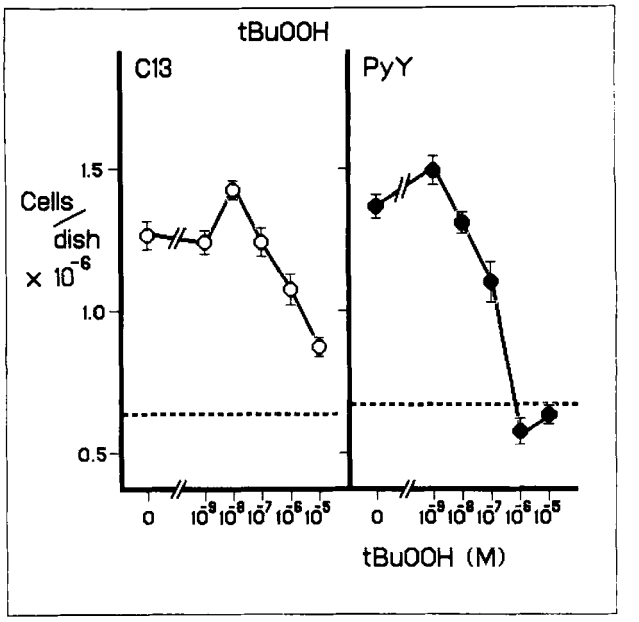

FIGURE 4 The effects of added t-butyl hydroperoxide on BHK-21 cell yields. Experimental procedures were as described for Figure 1 except that varying amounts of t-butyl hydroperoxide (tBuOOH) were added after the initial 24 hr growth period. (○ BHK-21/C13 cells; ● BHK-21/PyY cells).

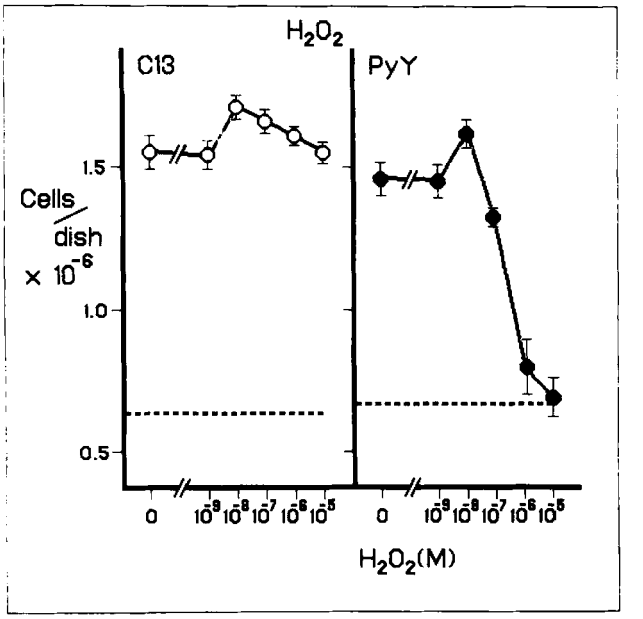

FIGURE 5 The effects of added hydrogen peroxide on BHK-21 cell yields. Experimental procedures were as described for Figure 1 except that varying amounts of hydrogen peroxide (H_2O_2) were added after the initial 24 hr growth period. (○ BHK-21/C13 cells; ● BHK-21/PyY cells).

show the effect of adding these oxidants to the normal growth medium of BHK-21 cells. In the case of tBuOOH, higher concentrations were inhibitory although these effects were greatest in the case of the "transformed" cells. A surprising feature of these experiments was that very low concentrations (10^{-8} M) of oxidant were significantly stimulatory (Figure 4).

In the experiments with H_2O_2 on transformed cells (BHK-21/PyY) high concentrations of H_2O_2 were also inhibitory whilst low concentrations were again notably stimulatory (Figure 5). However in the case of the non-transformed cells (BHK-21/C13) H_2O_2 was stimulatory but not inhibitory even at higher concentrations. When catalase levels in homogenates of the two cell lines were compared there was a small difference (BHK-21/C13 cells 10.4 units mg^{-1}; BHK-21/PyY 17.0 units mg^{-1}). Perhaps more significant however was finding that the enzyme level in BHK-21/C13 cells rises some twelve fold (to 125.8 units mg^{-1}) following prior exposure of the cells to 10^{-5} M H_2O_2 (for 30 min followed by a recovery period of 2 hr). In transformed cells the rise, following similar treatment, is much smaller to 63.7 units mg^{-1}). That H_2O_2 can induce increases in catalase levels may partly explain the apparent resistance of non-transformed cells to high levels of H_2O_2. Another procedure that moderates the inhibitory effects of high levels of H_2O_2 on BHK-21/PyY cell growth is mild heat shock (45°C, 10 min) prior to H_2O_2 exposure (Figure 6).

The differential cellular response to high levels of H_2O_2 is also observed at the level of ^{35}S-methionine incorporation into protein. Figure 7 shows the time course of such

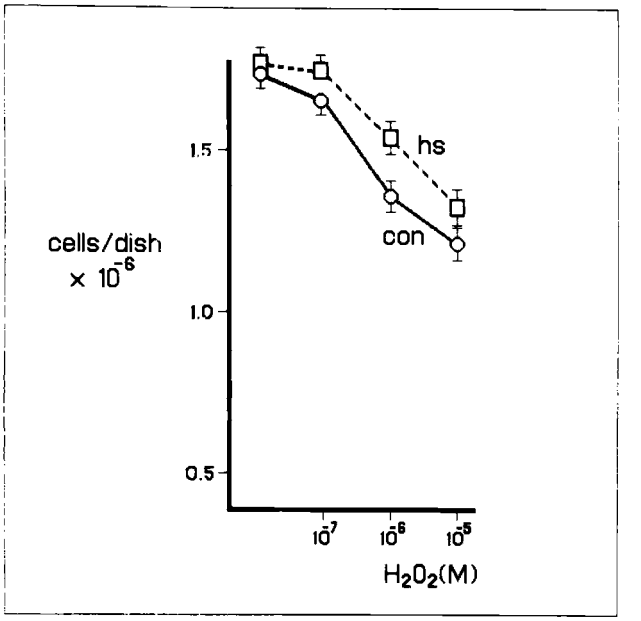

FIGURE 6 Brief heat shock and the subsequent effects of H_2O_2 on BHK-21/PyY cell yields. The procedure is basically that outlined in the legend for Figure 5. However after the initial 24 hr growth period one set of BHK-21/PyY cells was treated at 45°C for 10 min and then allowed to recover at 37°C for 2 hr before addition of varying amounts of H_2O_2. Yield after 3 days (○) of untreated control (con) cells (□) of previously heat shocked (hs) cells.

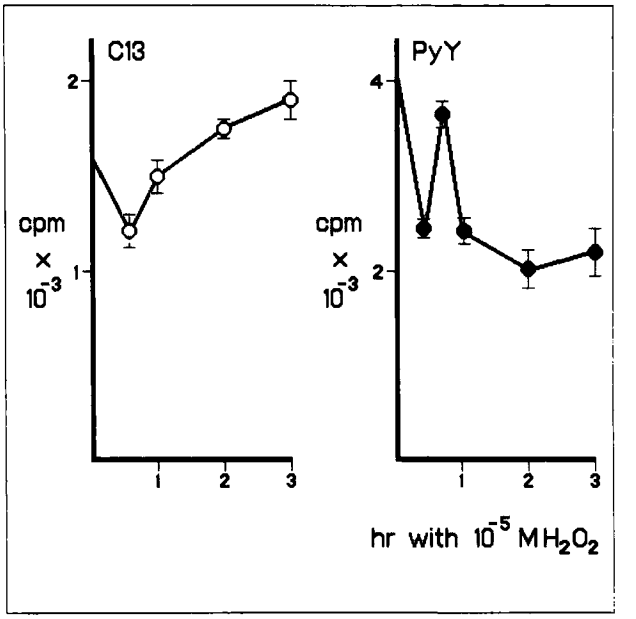

FIGURE 7 The effects of 10^{-5}M H_2O_2 on BHK-cell protein synthesis with time. Triplicate monolayer cultures were treated with 10^{-5}M H_2O_2 for varying periods, and then assessed for their ability to incorporate ^{35}S-methionine into protein (see EXPERIMENTAL PROCEDURES). The results are expressed as the mean of triplicates ± s.d. ○ BHK-21/C13 cells, ● BHK-21/PyY cells.

TABLE III
Effects of low levels of H_2O_2 on the growth of BHK-21 cells in serum-free media

Conditions	Cells/dish after 3 days × 10^{-6} ± s.d. BHK-21/C13 cells	BHK-21/PyY cells
normal medium	1.49 ± 0.03	1.36 ± 0.01
− serum	1.18 ± 0.01	1.13 ± 0.03
− serum + H_2O_2 (10^{-8}M)	1.31 ± 0.03	1.31 ± 0.03
− serum + mannitol (10^{-5}M)	1.41 ± 0.01	1.43 ± 0.03
− serum + mannitol + H_2O_2	1.42 ± 0.01	1.51 ± 0.01
− serum + DMSO (0.001%)	1.25 ± 0.01	1.43 ± 0.02
− serum + DMSO + H_2O_2	1.37 ± 0.02	1.51 ± 0.01

Experimental Procedures as described in Table 1.

incorporation and it is clear that in both C13 and PyY cell lines there is an early decline of methionine incorporation in response to 10^{-5}M H_2O_2. However in both cell lines the decline appears to be arrested with time. Perhaps most significantly in the non-transformed cells there is not only a "recovery" but even a subsequent stimulatory effect. Again these effects may be due to the induction of increased catalase levels, particularly in the PyY cells, following H_2O_2 exposure.

TABLE IV
Effects of low levels of t-butylhydroperoxide on the growth of BHK-21 cells in serum-free media

Conditions	Cells/dish after 3 days × 10^{-6} ± s.d. BHK-21/C13 cells	BHK-21/PyY cells
normal	1.45 ± 0.02	1.49 ± 0.01
− serum	1.18 ± 0.02	1.23 ± 0.03
− serum + tBuOOH (10^{-8} M)	1.21 ± 0.01	1.28 ± 0.01
− serum + mannitol (10^{-5} M)	1.22 ± 0.03	1.28 ± 0.01
− serum + mannitol + tBuOOH	1.29 ± 0.03	1.44 ± 0.01
− serum + DMSO (0.001%)	1.28 ± 0.04	1.31 ± 0.03
− serum + DMSO +tBuOOH	1.40 ± 0.01	1.46 ± 0.01

Experimental conditions as described in Table I.

Despite the differential effects with regard to growth inhibition by higher concentrations of H_2O_2 both cell types were significantly stimulated by low levels of H_2O_2 both in normal and serum-deprived medium (Table 3). Moreover addition of mannitol or DMSO could even enhance the stimulatory effects of low H_2O_2 in certain cases (Table 3). Similar effects were observed with low levels of tBuOOH in the presence and absence of serum (Table 4).

DISCUSSION

In view of the known antioxidants present in serum (e.g. caeruloplasmin, albumin, uric acid, ascorbate[11]) it is perhaps not surprising that mammalian cells cultured aerobically in the absence of serum accumulate increased levels of lipid peroxides.[2] Our observations that mannitol can not only counter this effect[2] but also improve the growth of serum deprived cells is consistent with the notion that oxidative stress, lipid peroxidation and cell proliferation may be interconnected. In situations where the growth media is supplemented with serum it is normally only to the extent of 10% (v/v). Under such conditions it may well be that such cells are still not adequately protected. Indeed we have previously reported low but significant steady-state levels of lipid peroxidation in cultured mammalian cells.[2,12] Thus the stimulatory effects of mannitol or butylated hydroxytoluene may be due to their ability to provide extra protection even in 10% serum supplemented cells.

The above points raise the question of what particular types of growth regulatory mechanisms are impaired in conditions of oxidative stress. A wide variety of cellular signal transduction systems operating at the level of cell plasma membranes, might be primary targets. The data in Table 2 already suggest amino acid transport systems as potentially important. Although there are many other possibilities, it should be stressed that any conditions that can influence overall levels of cellular protein synthesis are ultimately critical for cell division control. Suggestions have been made that mammalian cells require to accumulate a threshold amount of an unstable "trigger" protein to pass a particular restriction point in the cell cycle so as to replicate their DNA and divide.[13-14] Thus conditions that reduce the general rate of cellular protein synthesis would delay the accumulation of threshold levels of this unstable

protein. Other types of experiments point to the importance of proteins produced from specific proto-oncogenes (e.g. c-fos, c-myc, c-ras) in controlling events that enable cells to proliferate.[15,16]

Our observations that oxidants can depress cell growth may simply be an outcome of additional oxidative stress leading to increased damage of cellular components critical to the regulation of cell growth or cellular protein synthesis. Certainly high levels of both H_2O_2 and tBuOOH cause inhibition of cellular protein synthesis (Table 5). Nevertheless in both cases the inhibitory effects of the oxidant in the transformed cells could be abated by the inclusion of mannitol or DMSO in the medium (Table 5) suggesting that radicals could be the actual agents contributing to the inhibition of overall cellular protein synthesis and possibly cell growth. On the other hand the sensitivity of the transformed cells can be moderated by "prestressing", with modest heat. Both mild heat *or* H_2O_2 are known to elevate the synthesis of heat shock proteins,[17–20]. Whether the elevated production of heat shock proteins is directly related to the observed increased tolerance to H_2O_2 is not yet clear. Heat shock proteins are of course known to be related to the acquisition of thermal tolerance.[17–19] Such observations may have significance in relation to the inflammatory response.

Our observations that very low levels of H_2O_2 (and tBuOOH) can actually enhance cell proliferation may also be relevant to the inflammatory response in as much as they may be suggestive of routes to tissue repair or replenishment following tissue damage due to oxidative stress. On the other hand the nature of the mechanisms involved in the stimulation of cell growth which can occur even, in the absence of serum, has yet to be resolved. The experiments with radical scavengers such as mannitol or DMSO Tables 3 and 4) indicate that the mechanisms are unlikely to involve hydroxyl radicals. There is always the possibility that H_2O_2 (or tBuOOH) acts directly to activate components of some cellular signal transduction mechanisms regulating growth. H_2O_2 has previously been reported to have insulinomimetic effects in isolated rat adipocytes.[21] Such effects of H_2O_2 appear to be mediated through the stimulation of insulin receptor phosphorylation although this does not seem to be an effect of H_2O_2 on the insulin receptor itself but requires the participation of non plasma membrane cellular constituents. H_2O_2 has also been shown to stimulate adenyl cyclase activity

TABLE V
H_2O_2, tBuOOH and BHK-21/PyY cell protein synthesis

Pretreatment (2 hr)	^{35}S-methionine incorporation (cpm × 10^{-2})
none	27.6
H_2O_2 (10^{-6} M)	19.9
tBuOOH (150 μM)	20.8
mannitol (10^{-3} M)	24.3
mannitol plus H_2O_2	24.1
mannitol plus tBuOOH	28.8
DMSO (0.001%)	15.7
DMSO + H_2O_2	18.0
DMSO + tBuOOH	16.5

After the pretreatments indicated, the ability of triplicate monolayer culture to incorporate ^{35}S-methionine into protein was assessed over 30 min at 37°C (see EXPERIMENTAL PROCEDURES). The results are expressed as the average of triplicate determinations.

in rat testis Leydig cells (S Sandhu, BA Cooke and C Rice-Evans unpublished observations). Although such observations open up new areas for exploration with regard to mechanisms controlling cell proliferation and their relation to the inflammatory response they also suggest new routes to the formulation of serum-free media, as well as the use of oxidants and antioxidants to enhance cell yield and protein production in biotechnological processes.

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