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OXIDATIVE STRESS AND TUMOUR CELL PROLIFERATION

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The effects of oxidant stress were studied in immortalised hamster (BHK-21) and rat (208F) cell lines before and after transformation to the malignant state with polyoma virus, or activated H-ras, respectively. Whilst intracellular superoxide production was detectable in both transformed and immortalised cells the rate was somewhat higher in the transformed cells which have lower levels of superoxide dismutase. Because growth of transformed cells was particularly depressed in the presence of MTT, a tetrazolium compound reduced by superoxide, the possible role of active oxygen species in the promotion of cell growth was examined. Low levels of hydrogen peroxide were stimulatory towards both immortalised and transformed cells. In the case of H-ras transformed rat cells, paraquat was also stimulatory provided serum was present in the growth medium. In the absence of serum, paraquat was notably inhibitory but inhibition could be alleviated by addition of low concentrations of α-tocopherol (10^{-8}M) to the serum-depleted medium.

Although depletion of serum from the growth medium also leads to lower cell proliferation, subsequent experiments showed that α-tocopherol addition to serum-free medium was sufficient to restimulate growth. In the case of transformed cells, yields of cells were even greater than that encountered in the presence of 10% serum. Thus whilst certain active oxygen species (e.g. hydrogen peroxide) may have a role in promoting the growth of transformed and immortalised cells the necessity for antioxidant protection is important.

KEY WORDS: Cell proliferation, active oxygen species, hydrogen peroxide, paraquat, transformed cells, α-tocopherol.

INTRODUCTION

Although cancers appear to derive from single abnormal cells, considerable information indicates tumorogenesis to be a slow microevolutionary multistep process. Whilst most cancers are probably initiated by a change in the cells' DNA sequence, a single mutation is insufficient to cause cancer. For example it is estimated that between three and seven independent random events, each of low probability are required to transform a normal human cell into a cancer cell. Progression appears to involve successive rounds of mutation and natural selection. In the case of model rodent cell systems, progression towards neoplastic transformation has been envisaged as occurring in two sequential stages brought about by distinct dominant genetic events usually referred to as the 'immortalisation' and 'transformation' phases.[1] Cells in the 'immortalisation' phase have indefinite life span but retain their normal morphology, attachment-dependence and responsiveness to neighbouring cells (such as contact

inhibition at confluency). This is followed by the 'transformation' phase, during which cells exhibit altered morphology and responsiveness to other cells and become capable of forming tumours *in vivo*. Whilst this two-step model maybe an oversimplification there is little doubt that multiple dominant and recessive genetic events are required for full neoplastic transformation.[1]

Some of the genetic elements (oncogenes) responsible for the events leading to full neoplastic transformation have been identified in the genomes of a number of RNA and DNA tumour viruses.[1] In addition normal cells express genes involved in the regulation of cell growth and differentiation that show homology to oncogenes of RNA viruses.[2,3] Indeed such viral oncogenes are believed to be 'captured' versions of these cellular genes that have mutated, resulting in the altered expression or activity of their encoded protein.[1] DNA viral oncogenes such as those from polyoma virus, or SV40, however have no apparent cellular counterparts. They may exert their effects throught the interaction of their encoded oncoproteins with host gene products.[4–6]

RNA and DNA viral oncogenes have been classified into two general groups based on correlations between the cellular location of their product oncoprotein and its resultant cellular effects.[7] The majority of 'nuclear' oncogenes have been implicated in the immortalisation phase[7] (e.g. *myc*, *fos*, E1A adenovirus, polyoma large-T).[8–11] The majority of 'cytoplasmic' oncogenes on the other hand are involved in processes associated with the cellular transformation phase.[7] (e.g. *ras* family, adenovirus E1B and polyoma middle-T).[11–12]

The possible relationship between oxidative stress and cell proliferation has been explored in relation to the above processes of tumorogenesis. The growth of 'immortalised' hamster (BHK-21) and rat (208F) cells under conditions of oxidative stress has been compared before and after 'transformation' with either polyoma virus (middle-T antigen) or activated H-*ras* from human bladder carcinoma. Proliferation of the cells after transformation is particularly enhanced by oxidants, provided serum, or α-tocopherol, is present in the culture medium. It may be that 'transformed' cells are not only the result of an evolutionary process resulting in adaptation to conditions of oxidative stress but also generate active oxygen species some of which may function as important growth promoting agents.

EXPERIMENTAL PROCEDURES

Cell Cultures

Hamster BHK-21/C13 (immortalised) cells[13] and BHK-21/PyY cells (transformed with polyoma virus)[13] 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. Rat 208F (immortalised) cells and RFAGT1 cells (transformed with a plasmid pAGT1 containing T24 activated H-ras from human bladder carcinoma)[14] were similarly grown, as were HeLa cells (derived from a human cervical carcinoma).

MTT-formazan production in cell cultures

Triplicate cell cultures (0.5×10^6 cells per 2.5 cm petri dish) were established as monolayers in 2 ml DMEM supplemented with 10% calf serum by growth at 37°C for 18 hr. The medium was then replaced with (or without serum). 0.25 mg MTT [3—

(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was then added (zero time) and incubation continued at 37°C.[15] At various times thereafter the medium was removed and the remaining monolayers extracted with 2 ml dimethyl sulphoxide (DMSO). The absorbance of DMSO extracts containing the blue formazan that results from the reduction of MTT by superoxide was determined at 570 nm[15] and the results presented as means of the determinations from triplicate cultures ± s.d.

Cell proliferation

This was examined as previously described.[16,17] Triplicate monolayer cultures (0.5×10^6 cells per dish) were given grown for 18 hr in DMEM supplemented with 10% 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 2 days of growth at 37°C, the cells from each dish were removed and the yields of cells assessed using an haemocytometer. Results were expressed as yield of cells per dish ± s.d. ($n = 3$).

RESULTS

In previous studies it was possible to detect a low but steady production of superoxide radicals within cultured human tumour (HeLa) cells.[18] MTT, a tetrazolium based compound (see Experimental procedures) permeates cells and is reduced therein by superoxide radicals to give a blue formazan which is extractable with dimethyl sulphoxide (see ref[16] and Figure 1). Although a possible source of these superoxide

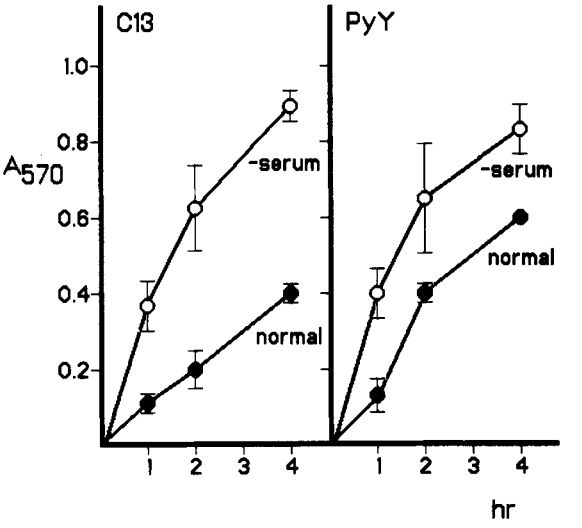

FIGURE 1 MTT-formazan production in cultures of BHK-21/C13 and BHK-21/PyY (polyoma transformed cells. The experimental procedure was as described in EXPERIMENTAL PROCEDURES and the incubation with MTT was carried out in the presence (●) or absence of (○) 10% calf serum. Results presented represent the means of formazan absorbance from triplicate cultures ± s.d.

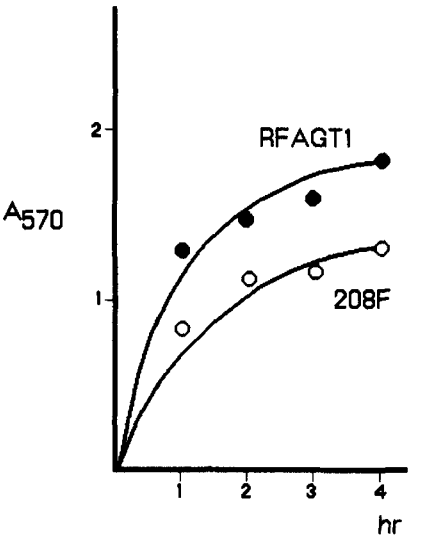

FIGURE 2 MTT-formazan produciton in cultures of rat 208F and rat RFAGT1 (H-ras transformed) cells. The experimental proceedures were as described for Figure 1 although incubation with MTT was only carried out in the presence of 10% calf serum. Results presented represent the means of formazan absorbance from triplicate cultures (0, 208F cells; ●, RFAGT1 cells).

radicals is the enzyme xanthine oxidase[19] there was no change in rate of production if the xanthine oxidase inhibitor allopurinol is included in the culture medium at 50 μM. Due to its poor solubility it may be that allopurinol does not have access to intracellular xanthine oxidase. However another inhibitor oxypurinol is also without effect even at 1 mM. Another likely source of superoxide is mitochondria.[20,21] In normal cells whilst almost all molecular oxygen is fully reduced by cytochrome it is recognised that electrons can 'leak' inappropriately from the respiratory chain with the resultant production of reduced oxygen species such as superoxide rather than water.

From Figure 1 it can be seen that superoxide production is similarly detectable in BHK-21 cells cultured in serum-supplemented medium before and after transformation with polyoma virus. Indeed superoxide production is somewhat greater in the transformed cells. A quite similar situation is observed with superoxide production in rat 208F cells before and after transformation with T24-activated H-ras genes from human bladder carcinoma (see Figure 2). The difference in superoxide production may be a function of the lower levels of superoxide dismutase in transformed cells.[22] For example total superoxide dismutase levels as determined in cell homogenates following the procedures described by Del Maestro and McDonald[23] were as follows: BHK-21/C13, 1507 $\pm$ 131 ng/mg protein; BHK-21/PyY, 1026 $\pm$ 86 ng/mg protein; 208F, 921 $\pm$ 186 ng/mg protein; RFAGT1, 519 $\pm$ 120 ng/mg protein.

In order to explore any possible benefits such superoxide generation might have in relation to cellular physiology, experiments were carried out to assess cell growth in the presence of MTT. From Table I it can be seen that MTT reduces cell growth but particularly growth of transformed cells. MTT also diminishes the growth of HeLa cells (Table I). Such observations suggest that the production of superoxide radicals

TABLE I
Effect of MTT on cell yields

Cell type	Additions	Cells/dish × 10^{-6} ± s.d.
BHK-21/C13 (hamster)	none	1.59 ± 0.01
	MTT	1.44 ± 0.04
BHK-21/PyY (hamster, transformed)	none	1.55 ± 0.02
	MTT	1.06 ± 0.03
208F (rat)	none	1.29 ± 0.04
	MTT	1.17 ± 0.02
RFAGT1 (rat, transformed)	none	1.55 ± 0.03
	MTT	1.16 ± 0.03
HeLa (human cervical carcinoma)	none	1.69 ± 0.04
	MTT	0.94 ± 0.05

Triplicate monolayers of each cell type (0.5×10^6 cells/dish) were grown for 18 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 with fresh serum supplemented medium but also with 250 μg MTT [3-(4, 5-demethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide] as indicated. After 2 days the cells were removed from each dish and counted. The results are expressed as the means of triplicate experiments ± s.d.

may play some important role in the maintenance of cell proliferation, especially in the case of transformed cells.

Whilst previous data of ours,[16,17] showed that the addition of a superoxide radical generating system to the culture medium stimulated growth of transformed BHK-21 cells, it was doubful whether superoxide radicals *per se* were stimulatory. Rather it was likely that H_2O_2 which could be derived from these radicals was more important.[24] Indeed low levels (10^{-8} M) of of exogenous H_2O_2 were stimulatory towards BHK-21 cells.[16,17] With regard to any intracellularly produced superoxide radicals, the action of cellular superoxide dismutases would yield H_2O_2.[24] We have further explored the role of H_2O_2 as a growth promoter with the immortalised rat 208F cells before and after transformation with activated H-ras genes. From Figure 3 it can be seen that H_2O_2 is stimulatory particularly towards the H-ras transformed rat cells (RFAGT1). The data in Figure 4 also shows that low levels of paraquat are also especially stimulatory towards the H-ras transformed rat cells. A major effect of paraquat is reported to be an increased production of intracellular superoxide arising from the reaction of reduced paraquat with molecular oxygen. Paraquat within the cell undergoes repeated cycles of oxidation and reduction thus serving as a continuous intracellular source of additional superoxide radicals.[25-27] Together with data in Table I showing MTT to have a greater inhibitory effects towards the growth of transformed cells, it seems that transformed cells may, by some selective process, have become more adapted to grow under the influence of active oxygen species such as superoxide or H_2O_2. The lower levels of superoxide dismutase in transformed cells may also be a contributory factor in the adaptative processes.

Despite this apparent adaptation, the stimulatory effects of paraquat towards the transformed rat cells (RFAGT1) are not apparent in cells depleted of serum. Rather paraquat is notably inhibitory under these conditions (Figure 5). On the other hand it is possible to protect cells from at least some of these inhibitory effects by the addition of low concentrations of α-tocopherol (10^{-8} M) to the serum-free medium (Figure 6).

The importance of antioxidants in relation to cell growth was suggested from earlier experiments of ours[17] in which the growth of polyoma virus transformed BHK cells

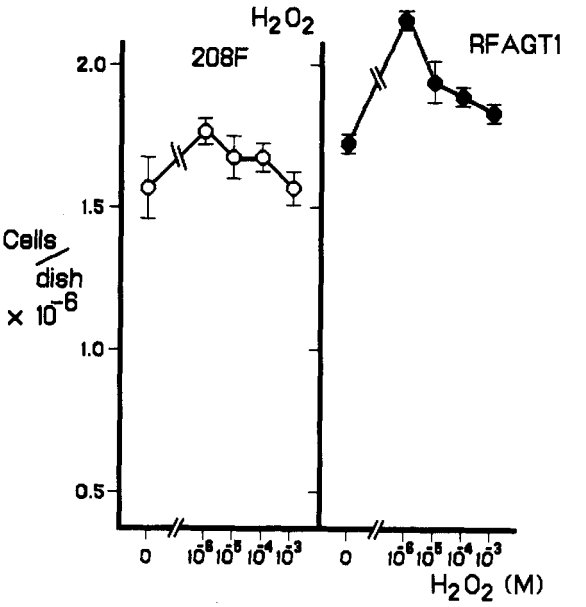

FIGURE 3 The effect of added hydrogen peroxide on rat 208F and RFAGT1 (H-ras transformed) cell yields. Triplicate monolayer cultures (0.5×10^{-6} cells/dish) were allowed to grow for 18 hr at 37°C in DMEM supplemented with 10% calf serum to yield between 0.58×10^6 and 0.60×10^6 cells/dish. The medium was then removed and the monolayers washed three times with serum-free medium and replaced with DMEM and 10% calf serum along with varying amounts of hydrogen peroxide. After 2 days at 37°C the cells were removed from each dish and counted as described in EXPERIMENTAL PROCEDURES. Results are expressed as the means of triplicate determinations ± s.d. (0, 208F cells; ●, RFAGT1 cells).

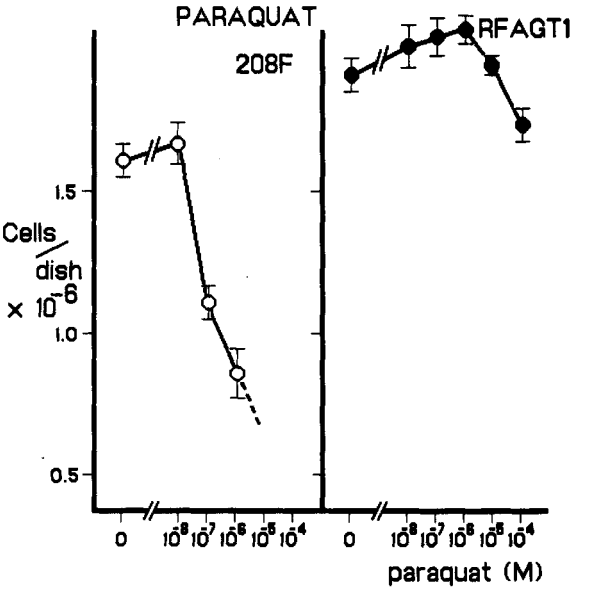

FIGURE 4 The effect of added paraquat on rat 208F and RFAGT1 (H-ras transformed) cell yeilds. Experimental proceedures were as described for Figure 1 except that varying amounts of paraquat were added after the initial 18 hr growth period. Results were expressed as the means of triplicate determinations ± s.d. (○, 208F cells; ●, RFATG1 cells).

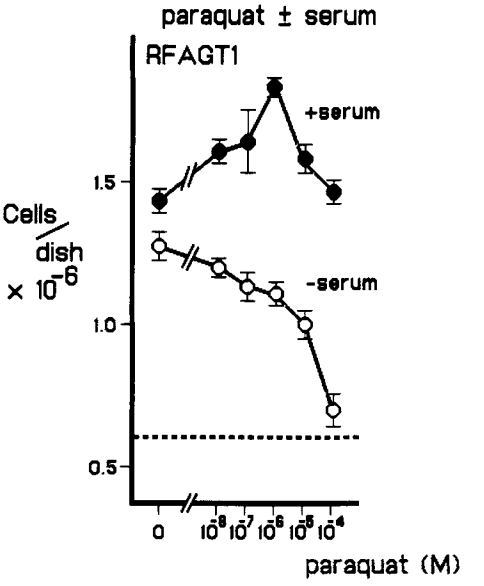

FIGURE 5 The effect of added paraquat on the growth of rat RFAGT1 cells in presence and absence of serum. Experimental proceedures were described for Figure 1, except that varying amounts of paraquat were added, with or without serum after the initial 18 hr growth period in normal medium. The dotted line indicates the number of cells/dish after the initial 18 hr growth period. Results are expressed as the means of triplicate determinations ± s.d. (●, cells with 10% serum, ○, cells depleted of serum).

(BHK-21/PyY) was particularly enhanced by inclusion of antioxidants such as butylated hydroxytoluene inthe normal growth medium. Other early experiments of ours[28] demonstrated that when human tumour cells (HeLa) are deprived of serum, there is a significant increase in intracellular superoxide radical production[18] as well as an increase in cellular lipid peroxidation.[28] Figure 1 shows that deprivation of serum also leads to increased superoxide production in BHK-21 cells. Thus although the level of active oxygen species important for growth promotion might increase in cells deprived of serum, there may also be increased chances of deleterious effects. Because of this, the ability of α-tocopherol to protect immortalised and transformed cells from the effects of serum withdrawal was explored. From Figures 7 and 8 it can be seen that α-tocopherol at low concentrations (10^{-7}–10^{-8} M), is a very reasonable protectant. In the case of the transformed cells (BHK-21/PyY and RFAGT1), it is actually possible to achieve yields greater than in medium supplemented with 10% serum.

DISCUSSION

Whilst MTT, like nitroblue tetrazolium (NBT), has higher specificity for superoxide radicals compared with other reductants, this does not preclude the possibility of interaction with other reductants. Nevertheless it is notable in the case of transformed cells, with lower levels of superoxide dismutase, that the inhibitory effect of MTT on cell growth are more pronounced. Although MTT-formazan may be more toxic to the

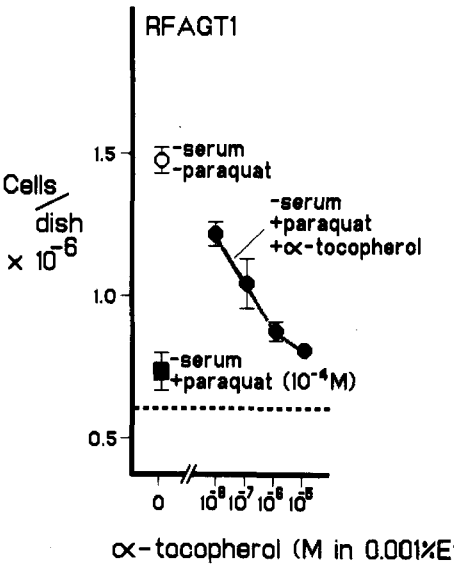

FIGURE 6 The effect of α-tocopherol on the growth of cells in the absence of serum but with added paraquat. Experimental procedures were as described for Figure 1. After the initial growth period of 18 hr in normal serum supplemented medium (the dotted line indicates the number of cells per dish after that period), the medium was replaced with the medium containing no serum and no paraquat but 0.001% ethanol (○),*or* medium containing no serum but 10^{-4}M paraquat and 0.001% ethanol (■) *or* medium containing no serum but 10^{-4} paraquat plus varying amounts of α-tocopherol succinate in 0.001% ethanol (●). After 2 days at 37°C the cells were removed from each dish and counted as in EXPERIMENTAL PROCEEDURES. Results are expressed as the means of triplicate determinations ± s.d.

transformed cells, an alternative explation would be that endogenous production of superoxide is in some way particularly important for the optimal proliferation of 'transformed' cells. This latter possibility raises the question of whether it is superoxide ions, or species derived therefrom, that are important for this physiological aspect of transformed cells. Our observations that exogenously added H_2O_2 is stimulatory are thus of relevance. The additional observations that low levels of paraquat can also be stimulatory to the transformed rat cells (RFAGT1) may also be important, since paraquat will serve to establish an additional source of intracellular superoxide ions[25–27] and thus possibly a further source of H_2O_2.

The fact that H_2O_2 (or superoxide) can have positive effects in relation to cell proliferation raises questions of mechanism. H_2O_2 has been reported to mimic the effects of insulin in rat adipocytes.[29] In rat testis Leydig cells, H_2O_2 serves to stimulate adenyl cyclase (S. Sandhu, B.A. Cooke, & C. Rice-Evans, unpublished observations). In addition previous observations of ours[18] indicated that H_2O_2 at low levels was stimulatory towards protein synthesis of HeLa cells both *in vitro* and *in vivo* as well as serving as inducer of heat shock proteins.[18] Moreover in membrane preparations from HeLa cells it was possible to demonstrate a stimulatory effect of both H_2O_2 and superoxide radicals towards Na/K-ATPase *in vitro*. For instance using the preparative and analytical procedures detailed in references 30 and 31, it was observed that exposure of such preparations *in vitro* to 10^{-4} M H_2O_2 for 2 hr at 37°C resulted in an increase in ouabain-inhibited Na/K-ATPase activity from 0.93 to 6.53 μmol Pi re-

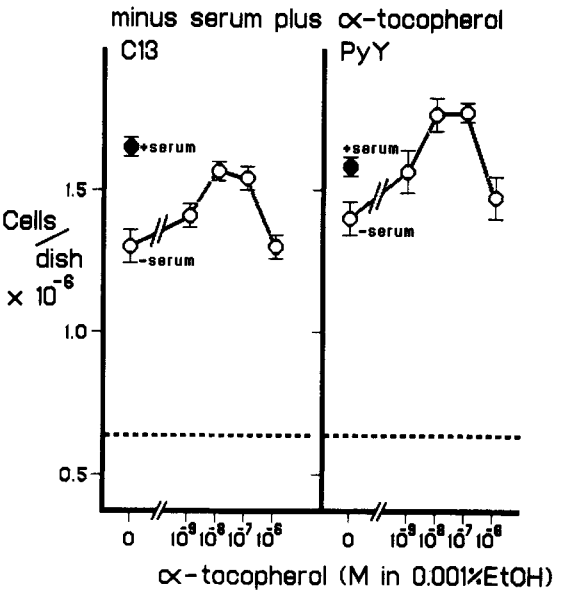

FIGURE 7 The effect of α-tocopherol on yields of hamster BHK-21/C13 and BHK-21/PyY cells deprived of serum. Experimental procedures were as described for Figure 1. After the initial growth period of 18 hr in normal serum supplemented medium (the dotted line indicates the number of cells per dish after that period), the medium was replaced with medium containing serum and 0.001% ethanol (●) or medium lacking serum but containing varying amounts of α-tocopherol succinate in 0.001% ethanol (○). After 2 days at 37°C, the cells as indicated were removed from each dish and counted as in EXPERIMENTAL PROCEEDURES. Results were expressed as the means of triplicate determinations ± s.d.

leased/mg protein/2 hr. If a mixture of xanthine (10^{-4} M) – xanthine oxidase (30 ng/ml) was used in place of H_2O_2, an increased enzyme activity was also detected (to 8.17 μmol Pi released/mg protein/2 hr). The Na/K-ATPase or Na/K-ion pump is known to be important in processes regulating mammalian cell division.[32–35] Whilst the range of potentially relevant stimulatory effects of H_2O_2 on mammalian cellular regulatory systems remains to be fully explored, it is possible to speculate that H_2O_2 (or indeed superoxide) may serve as an important growth regulating agent.

Despite these positive effects of H_2O_2 there is may be a negative side. For example there are data from a number of groups to indicate that H_2O_2 can be mutatagenic and/or toxic towards animal cells at high levels.[36–38] It is suggested by these workers that such deleterious effects may be due to hydroxyl radicals derived from H_2O_2. Certainly an established feature of both transformed and immortalised cells is their genetic instability[39] and mutability.[40–41] This raises the question whether immortalised or transformed cells have any intrinsic protection from possibly deleterious active oxygen species (e.g. hydroxyl radicals). In the case of the transformed rat cells (RFAGT1) whilst low levels of paraquat are stimulatory, this was only in the presence of serum. In the absence of serum (Figure 5) paraquat is distinctly inhibitory at higher concentrations. Such observations suggest that although certain active oxygen species derived from superoxide may serve to promote growth, there may nevertheless be a critical balance these positive effects and possible deleterious effects. This balance can be maintained provided serum is present, although it appears that α-tocopherol can fulfil this role at least partly (Figure 6).

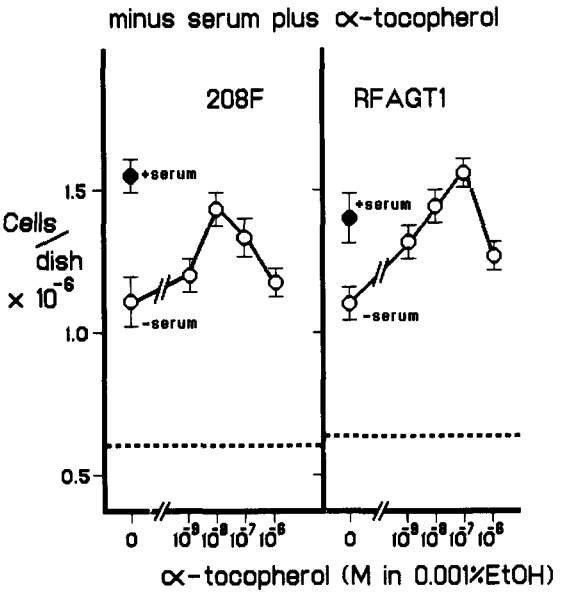

FIGURE 8 The effect of α-tocopherol on yields of rat 208F and RFAGT1 cells deprived of serum. The experimental proceedures followed were the same as those outlined in Figure 7.

The notion that α-tocopherol may contribute towards the maintenance of a critical balance in the transformed and immortalised cells between possible positive and negative effects of active oxygen species, is further supported by the data in Figures 7 and 8. In cells depleted of serum, besides lower growth rates, there is also a rise in intracellular superoxide production (Figure 1) and in the level of cellular lipid peroxidation.[28] However the addition of α-tocopherol, known to inhibit lipid peroxidation,[43] enhances growth of both transformed and immortalised cells in serum-free medium. Such observations raise questions regarding the role of serum. Previous experiments of ours[27] pointed to an antioxidant role in as much as the rise in lipid peroxiation can be reduced by the inclusion of mannitol in the serum depleted medium.[27] Subsequent experiments of ours indicate that α-tocopherol will also serve in this capacity. As yet however we have not sought to identify specifically the actual components of calf serum which provide antioxidant protection in culture. α-tocopherol must nevertheless be a strong candidate. In addition to this proposed antioxidant role serum clearly fulfills other functions. Amongst these is the provision of factors which negatively regulate endogenous superoxide production (e.g. Figure 1). These factors appear to be distinct from antioxidants as neither the addition of mannitol nor α-tocopherol could mimic the effects of whole serum in this context (unpublished observations).

Whilst it can be hypothesised that transformed cells have adapted to a situation where certain active oxygen species such as superoxide and H_2O_2 may be important for their continued proliferation, the cost may well involve some form of 'protection' from the effects of deleterious active oxygen species. This could be provided by α-tocopherol. Other groups[42,43] have shown increased resistance *in vivo* to pro-oxidant stress during hepatocarcinogenesis which may in part be due to α-tocopherol.[43] It is possible that 'transformed' cells are adapted so as to require serum not so much as

a source of polypeptide growth factors, but rather as a protective entity, perhaps by virtue of its content of antioxidants such as α-tocopherol. It should however be pointed out that the degree of antioxidant protection that can be achieved in the presence of serum may vary with the oncogene(s) used for transformation. In the case of activated H-ras transformed rat cells the degree of protection attained is such as to combat even quite high concentrations of H_2O_2 or paraquat (see Figures 3 and 4). On the other hand earlier experiments[16,17] with hamster (BHK-21) cells indicated that they could only withstand low levels of H_2O_2 after transformation with polyoma virus. However because few malignat tumours appear to be associated with papoviruses such as polyoma, the effects of *ras*-type oncogenes may be more important clinically. Besides human bladder carcinoma,[14] *ras* oncogenes are implicated in mammary, skin, lung and colon carcinomas. In any event future studies are required to examine differences between 'immortalised' cells and 'normal' cells in relation to oxidative stress.

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