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REDUCTION OF A TETRAZOLIUM SALT AND SUPEROXIDE GENERATION IN HUMAN TUMOR CELLS (HeLa)

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Experiments have been carried out to explore the use of a tetrazolium salt, MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide in the detection of intracellularly generated superoxide in HeLa cells. From the use of a low molecular weight lipophilic mimic of superoxide dismutase, as well as superoxide dismutase, and inhibitors of superoxide dismutase, it is suggested that at least 20-30% of the intracellular reduction of MTT is due to superoxide. Whilst this may arise from mitochondria another possible intracellular source in HeLa cells may be xanthine oxidase.

The overall rate of intracellular MTT reduction in HeLa cells is inversely dependent on levels of serum in the culture medium. Serum components with a modulatory role in this context are those with anti-oxidant function.

Reduced MTT is also detectable extracellularly in cultures of HeLa cells and at least 80% of this is due to superoxide. Use of inhibitors suggest that whilst a small proportion (30%) may arise through an NADPH-oxidase type enzyme, other sources of extracellular superoxide in HeLa cells remain a possibility.

KEY WORDS: Superoxide, MTT, HeLa cells, superoxide dismutase, CuDIPS, xanthine oxidase

INTRODUCTION

Proliferation of cultured mammalian cells depends amongst other things on oxygen and serum components. Notable amongst the serum components are the polypeptide growth factors which interact with receptors on cell surfaces and thereby elicit transmembrane signalling reactions which regulate growth responses.[1] Serum also contains a number of compounds such as α-tocopherol, ascorbate, albumin, urate and bilirubin with potential antioxidant function.[2] Whilst serum withdrawal has obvious consequences for cellular growth responses, in terms of diminished levels of growth factors, it may also be that the serum antioxidants are important for various aspects of cell homeostasis and/or proliferation. Withdrawal of serum from the growth medium of cultured cells leads not only to diminished growth rates but also to increased levels of cellular lipid peroxidation.[3] Supplementation of the serum deprived medium with low levels of α-tocopherol can nevertheless lead to considerable restoration of growth rates[4] emphasizing the possible importance of antioxidants in the maintenance of cell proliferation.

Recent studies have pointed to the importance of active oxygen species such as superoxide or hydrogen peroxide at low concentrations as promoters of cell

proliferation.[5,6] Besides hamster and mouse fibroblasts these particular active oxygen species can stimulate growth and growth responses in mouse epidermal cells,[7] Balb/3T3 cells[8] and primary human fibroblasts[9]. In the case of Balb/3T3 cells[8] and mouse epidermal cells[7] they stimulate expression of early growth related genes such as the proto-oncogene *c-fos*. These observations have led to suggestions that superoxide and hydrogen peroxide might function as mitogenic stimuli through biochemical processes common to natural growth factors. A considerable variety of non-inflammatory cell types are now known to release superoxide or hydrogen peroxide. These include human primary fibroblasts,[9,10] Balb/3T3/cells,[8] hamster fibroblasts (BHK-21),[11] endothelial cells,[12] smooth muscle cells[13] and pancreatic islet cells.[14] Moreover studies where the addition of exogenous superoxide dismutase or catalase to the culture medium of BHK-21 cells has been growth inhibitory suggest that these active oxygen species are also crucial for the maintenance of cell proliferation.[11,15]

In the various cells where low level release of superoxide or hydrogen peroxide has been detected the source, or sources, have not been clarified. In the case of human primary fibroblasts the possible involvement of a plasma membrane NADPH oxidase is however suggested from inhibitor studies.[16] Such superoxide release has often been detected by following the reduction of nitroblue tetrazolium.[17] On the other hand another tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide (MTT), is able to enter cells and by virtue of intracellular reduction to formazan offers in principle at least a means of also assessing any *intracellular* generation of superoxide.[17]

MTT is known to interact with components of the mitochondrial respiratory chain[18] and its reduction has often been used as a means of assessing cell viability.[19] Evidence is presented to suggest that a significant proportion of its reduction within cultured human tumour cells (HeLa) is due to intracellular superoxide generation possibly involving xanthine oxidase. The overall rate of intracellular MTT reduction to formazan in these cells is nevertheless inversely dependent on the levels of serum in the growth medium. Analysis indicates that serum components with a potentially significant modulatory role in this context are those with antioxidant function rather than any polypeptide growth factors.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa (human carcinoma) cells, were grown as monolayer cultures at 37°C in Eagle's minimal essential medium (MEM) normally supplemented with 10% (v/v) calf serum (Gibco BRL, Paisley). The HeLa cells were a kind gift from Dr M Stewart, Department of Clinical Biochemistry, Glasgow Royal Infirmary.

MTT-formazan production in cell cultures

As previously described[4] triplicate HeLa cell cultures (0.5×10^6 per 3.5 cm petri dish) were normally established as monolayers in 2 ml MEM 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 added and incubation continued at 37°C. 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 and the results presented as means of the determination from triplicate cultures ± s.d.

Inhibitors

Oxypurinol was a gift from Professor D Blake, London Hospital Medical School and diphenylene iodinium was kindly provided by Professor OT Jones, Biochemistry Department, Bristol University. Cu/Zn superoxide dismutase (bovine erythrocytes), MTT, allopurinol and α-tocopherol were from Sigma Laboratories. CuII-(3,5-diisopropyl salicylate)$_2$ was obtained from Aldrich Chemical Company.

RESULTS

MTT-formazan generation in cultured HeLa and the influence of serum

The reduction of nitroblue tetrazolium to blue formazan has often been used as a probe for extracellular superoxide generation and that of other reductants in biological systems.[17] On the other hand another tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide), is able to enter cells[17] and its intracellular reduction to blue formazan has been used as the basis of a rapid cell viability test.[19] Work with rat liver suspensions has indicated points of coupling between MTT and the mitochondrial respiratory chain,[18] but whether any reduction of MTT within cells represents intracellularly generated superoxide requires investigation. Figure 1 shows that when cultures of growing human carcinoma cells (HeLa) are briefly incubated under normal culture conditions with MTT and the level of intracellular blue formazan extractable with DMSO determined, this increases almost linearly with time. Table 1 shows that some blue formazan material is also detectable extracellularly in the growth medium after

TABLE 1
The effects of serum deprivation and exogenous superoxide dismutase on MTT formazan generation in HeLa cells

Expt	Conditions	Blue formazan accumulation (A_{570}) Intracellular	Extracellular
1	MEM + serum	0.292 ± 0.021	0.100 ± 0.013
	MEM − serum	0.497 ± 0.003	0.128 ± 0.009
2	MEM + serum	0.406 ± 0.008	0.071 ± 0.010
	MEM + serum + SOD	0.273 ± 0.021	0.015 ● 0.012

Triplicate HeLa cell cultures (0.5×10^6 cells per 3.5 cm dish) were established as monolayers in 2 ml MEM (minimal essential medium) supplemented with 10% calf serum by growth at 37°C for 18 hr. The medium was then removed and replaced with, or without serum (10%), together with SOD (Cu/Zn-superoxide dismutase) of bovine erythrocytes, Sigma) at 100 μg/ml. After 15 min at 31°C 0.25 mg MTT (Sigma) was added and the incubation continued at 37°C. After 30 min the medium was removed and the remaining monolayers extracted with 2 ml DMSO. The absorbance of DMSO extracts was determined at 570 nm and represents intracellular MTT-formazan generation. The absorbance at 570 nm detected in the medium is referred to as extracellular blue formazan. The results are presented as means of determinations from triplicate cultures ± s.d.

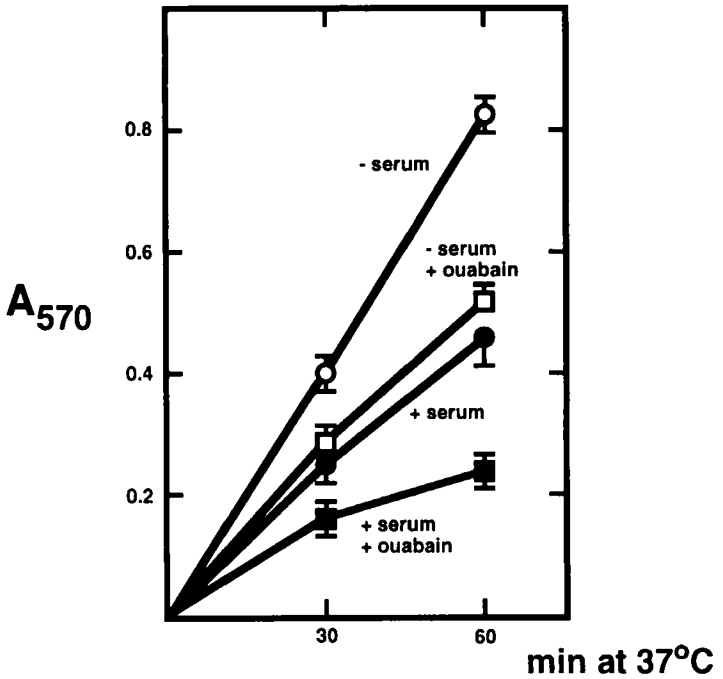

FIGURE 1 The time course of intracellular MTT-formazan generation in cultured HeLa cells and the effects of ouabain.

Triplicate HeLa cell cultures (0.5×10^6 cells per 3.5 cm dish) were established as monolayers in 2 ml MEM supplemented with 10% calf serum by growth at 37°C for 18 hr. The medium was then removed and replaced, with or without serum (10%), together with ouabain (1 mM). After 15 min at 37°C, 0.25 mg MTT was added and the incubation continued at 37°C. After 30 mins and 60 min the medium was removed and the remaining monolayers extracted with 2 ml DMSO. The absorbance of DMSO extracts was determined at 570 nm and represents intracellular MTT-formazan generation. The results are presented as means of determinations from triplicate cultures ± s.d. [●, MEM plus serum; ■, MEM plus serum plus ouabain; ○, MEM − serum; □, MEM minus serum plus ouabain].

incubation of the monolayer cultures with MTT. Moreover formation of this extracellular material is 80% inhibited by superoxide dismutase (Table 1).

Perhaps a surprising initial observation was that removal of serum from the culture medium prior to MTT exposure resulted in a significantly increased rate of intracellular MTT-formazan generation (Figure 1). The increase in the level of extracellular MTT-formazan (Table 1) was small in comparison. Figure 2 shows the effect of varying the level of serum supplementation and demonstrates the suppressive effects of serum to be concentration dependent. Previous experiments of ours have shown that the rate of intracellular reduction of MTT in cultured baby hamster fibroblasts, (BHK-21) is similarly depressed by the inclusion of serum in the growth medium.[4]

The origin of intracellular MTT-formazan

Whilst superoxide will reduce MTT to blue formazan, other intracellular reductants may also contribute.[2] As already mentioned, work with rat liver suspensions has

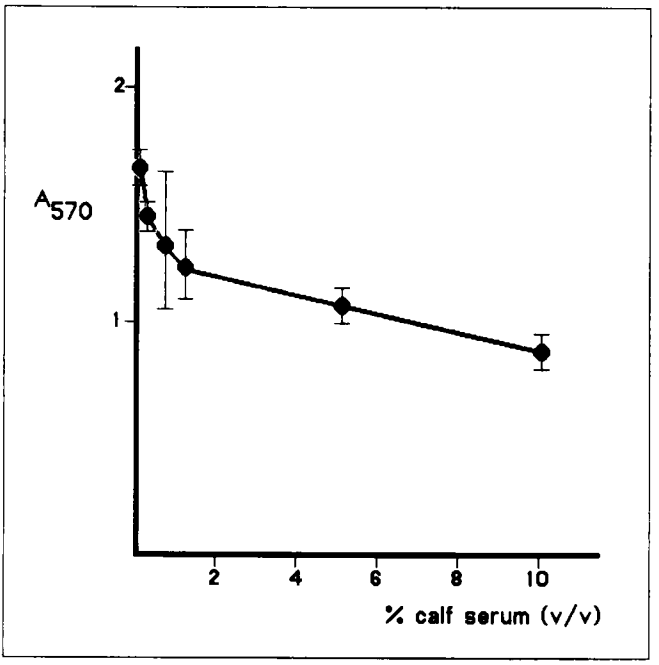

FIGURE 2 The effect of varying serum concentration on MTT-formazan production within cultured HeLa cells.

Triplicate cell cultures were established as described in Figure 1 and the incubation with various levels of calf serum supplementation (v/v) together with MTT (0.25 mg) was at 37°C for 60 min. Results represent the means of blue formazan absorbance from triplicate cultures ± s.d.

demonstrated coupling *in vitro* between MTT possibly at two points in the mitochondrial respiratory chain, one of which is sensitive to antimycin A.[18] Although components of the respiratory chain pass the bulk of electrons to the next component of the chain, some can 'leak' electrons to oxygen.[20-23] Such uni-electron reduction of oxygen will generate superoxide radicals and it could be that mitochondrial oxygen radical generation is of significance even during normal cell respiration.[23]

In their studies *in vitro* with rat liver preparations. Slater *et al*[18] found that antimycin A inhibited succinate-MTT reductase by 40% whereas azide was slightly stimulatory (6%). In our studies with *intact* growing HeLa cells we find that antimycin A modestly depresses intracellular MTT-formazan generation whilst azide, rotenone and the uncoupler FCCP are slightly stimulatory. Surprisingly cyanide treatment however led to quite large increases in intracellular MTT-formazan generation. In view of the fact that cyanide is also an inhibitor of Cu/Zn-superoxide dismutase,[25] the effects of another inhibitor of Cu/Zn-superoxide dismutase were examined. Diethyldithiocarbamate (DDC), a copper chelator, has also been used to inhibit superoxide dismutase *in vivo*.[26] At the concentrations used, it decreases the activity of Cu, Zn-superoxide dismutase *within* HeLa cells by around 75% and its effects on intracellular MTT reduction are also shown in Table 2. Like cyanide, it also stimulated MTT reduction within HeLa cells. Such observations suggest

TABLE 2
The effects of various inhibitors on MTT-formazan generation within HeLa cells

Expt	Additions	Intracellular blue formazan accumulation (A_{570})
1	EtOH (1%)	0.304 ± 0.013
	EtOH (1%) antimycin A (20 μg/ml)	0.255 ± 0.040
	EtOH (1%) rotenone (50 μg/ml)	0.374 ± 0.003
	EtOH (1%) FCCP (10 μM)	0.356 ± 0.039
2	none	0.336 ± 0.042
	Na azide (10 mM)	0.450 ± 0.014
	NaCN (10 mM)	1.280 ± 0.007
	diethyldithiocarbamate (1 mM)	0.496 ± 0.036

Triplicate monolayer cultures of HeLa cells were established as in Table 1. The medium was removed and replaced with normal serum supplemented medium but with the additions indicated. After 15 min at 37 °C, 0.25 mg MTT was added to the medium and after 30 mins at 37°C the level of blue formazan accumulating within cells was assessed as in the EXPERIMENTAL PROCEDURES. The results are presented as means of determinations from triplicate cultures ± s.d.

that a significant proportion of the MTT-formazan generated intracellularly might be due to intracellularly generated superoxide. To approach this question another way, the effects of incubating HeLa cell cultures with a low molecular mimic of superoxide dismutase was investigated. Table 3 shows that CuDIPS, the low molecular weight lipophilic biomimetic superoxide dismutase, copper II-(3,5-diisopropyl salicylate)$_2$,[27] is a significant inhibitor of intracellular MTT-formazan generation in cultured HeLa cells. In the case of serum deprived cells the inhibition observed (33%) was slightly greater than in serum supplemented cells (22%).

Whilst intracellular MTT-formazan generation seems likely, at least in part, to be a measure of intracellular O_2^- generation, there remains the question of likely sources. The possibility that mitochondria are a source of the superoxide contributing to the observed MTT reduction in intact HeLa cells cannot be ruled out, but seems unlikely on the basis of the inhibitor data presented. In *isolated* mitochondria it has been reported that superoxide generation is insensitive to antimycin[24] and can be stimulated by rotenone.[23] Our data however show antimycin to inhibit MTT-reduction and rotenone to be only mildly stimulatory (Table 2). Moreover, whereas hydrogen peroxide production is *isolated* mitochondria can be abolished by uncouplers,[28] FCCP actually modestly stimulates MTT-reduction in intact cells.

An alternative source of intracellular superoxide is nevertheless suggested from experiments in which pretreatment of cells with oxypurinol resulted in greatly decreased intracellular MTT-formazan formation (up to 50%) whether the cells were supplemented with serum or not (Table 4). Oxypurinol is a major metabolite of allopurinol[29] which like allopurinol can act as an inhibitor of xanthine oxidase.[29] Allopurinol also inhibits intracellular MTT-reduction but to a slightly lesser extent (Table 4).

Serum factors regulating the rate of MTT-formazan generation

The observation that the presence of serum can negatively regulate the rate of intracellular MTT-reduction raises the question of the nature of serum components involved. A rapid effect of acute serum withdrawal from cultured human fibroblasts

TABLE 3
The effect of a low molecular weight mimic of superoxide dismutase on MTT-formazan formation in HeLa cells

Conditions	Blue formazan accumulation (A_{570}) Intracellular	Extracellular
MEM + serum	0.201 ± 0.010	0.047 ± 0.005
MEM + serum + CuDIPs	0.157 ± 0.007	0.030 ± 0.008
MEM − serum	0.556 ± 0.029	0.058 ± 0.005
MEM − serum + CuDIPs	0.377 ± 0.037	0.037 ± 0.009

Triplicate monolayer cultures of HeLa cells were established as in Table 1. The medium was removed and replaced with, or without serum (10%), and where indicated 100 μM CuDIPS [copper II-(3,5-diisopropylsalicylate)$_2$] (Aldrich Chemical Co) in 1% EtOH. As a control the cultures not containing CuDIPs were also made 1% with respect to EtOH. After 2 hr at 37°C, 0.25 mg MTT was added to the medium and after 30 min at 37°C the level of blue formazan accumulating was assessed as in EXPERIMENTAL PROCEDURES. The results are presented as means of determinations from triplicate cultures ± s.d.

TABLE 4
Effects of oxypurinol and allopurinol on MTT-formazan formation in HeLa cells

Conditions	Blue formazan accumulation (A_{570}) Intracellular	Extracellular
+ serum	0.407 ± 0.008	0.201 ● 0.015
+ serum + oxypurinol (10 μM)	0.180 ± 0.037	0.158 ± 0.016
+ serum + allopurinol (10 μM)	0.232 ± 0.029	0.188 ± 0.007
− serum	0.959 ± 0.050	0.232 ± 0.005
− serum + oxypurinol (10 μM)	0.671 ± 0.022	0.192 ± 0.009
−serum + allopurinol (10 μM)	0.752 ± 0.050	0.212 ± 0.010

Triplicate monolayer cultures of HeLa cells were established as in Table 1. The medium was removed and replaced with, or without 10% serum, and the oxypurinol or allopurinol (Sigma) as indicated. After 2 hr at 37°C, 0.25 mg MTT was added to the cultures and after 30 min at 37°C the level of blue formazan accumulating was assessed as in EXPERIMENTAL PROCEDURES. The results are expressed as means of determinations from triplicate cultures ± s.d.

is known to be an increased permeability to Na^+ and K^+ ions[30] with a secondary stimulation of ouabain-sensitive Na^+-extrusion mechanisms.[30] Certainly exposure of cultured HeLa cells to the Na^+/K^+-ATPase inhibitor ouabain,[31] was also severely inhibitory towards intracellular MTT-reduction, either in the presence or absence of serum (Figure 1). Another outcome of serum withdrawal from HeLa cells that we have previously observed is an increase in cellular lipid peroxidation.[3] Peroxidation of membrane lipids has structural consequences which affect their fluidity.[32] Another possible consequence is lipid radical modification of membrane ion-pumps such as Na^+/K^+-ATPase.[33] In view of these possible outcomes, a study of the effects of serum components that might limit lipid peroxidation was undertaken. Table 5 shows that the lipid-soluble antioxidant α-tocopherol is effective in minimising the stimulatory effects of serum withdrawal on intracellular MTT-formazan generation. Table 5 shows that other serum antioxidants are also effective, but to varying degrees.

TABLE 5
Effects of exogenous antioxidants on intracellular MTT-formazan formation in HeLa cells deprived of serum

Expt	Conditions	Intracellular blue formazan accumulation (A_{570})
1	+ serum + 0.01% EtOH	0.464 ± 0.015
	+ serum + 0.01% EtOH/10 μM α-tocopherol	0.422 ± 0.020
	− serum + 0.01% EtOH	0.756 ± 0.063
	− serum + 0.01% EtOH/10 μM α-tocopherol	0.506 ● 0.040
2	+ serum	0.288 ± 0.006
	− serum	0.760 ± 0.056
	− serum + ascorbate (10 μM)	0.458 ± 0.012
	− serum + BSA (5 mg/ml)	0.430 ± 0.028
	− serum + urate (20 μM)	0.346 ± 0.024
	− serum + DMSO (0.1%)	0.720 ± 0.106
	− serum + DMSO (0.1%)/bilirubin (10 μg/ml)	0.422 ± 0.032

Triplicate monolayer cultures of HeLa cells were established as in Table 1. The medium was removed and replaced with, or without 10% serum, and with the antioxidants (Sigma) as indicated. After 2 hr at 37°C, 0.25 mg MTT was added to the cultures and after 30 mins at 37°C the level of blue formazan accumulating within cells was assessed as in EXPERIMENTAL PROCEDURES. The results are expressed as means of determinations from triplicate cultures ± s.d. [BSA, bovine serum albumin (Sigma, fatty acid free), DMSO, dimethylsulphoxide].

Extracellular MTT-formazan

As shown in Table 1 an additional outcome of incubating HeLa cells with MTT was the appearance of detectable extracellular MTT-formazan. Although the rate of extracellular MTT-formazan appearance was low compared with that generated intracellularly it was not so dramatically affected by serum (Table 1). Its origin may therefore be different from the MTT-formazan produced within cells. The observation that the level of extracellular MTT-formazan is reduced by 80% in the presence of exogenous superoxide dismutase suggests that the major proportion is due to reduction by superoxide. In order to investigate possible sources of this extracellular superoxide, experiments with various inhibitors were carried out. Inhibitors that did diminish extracellular MTT-formazan, were diphenylene iodonium, an inhibitor of neutrophil NADPH-oxidase[34] (Table 6), and oxypurinol (Table 4).

DISCUSSION

Whilst intracellular MTT-formazan generation has been used as an assay of cell viability,[19] our data shows very clearly that the rate of generation is nevertheless very dependent on the concentration of serum in the growth medium. Surprisingly the antioxidant quality of the serum appears to be of considerable importance in this context. Serum components such as α-tocopherol can decrease the rates of intracellular MTT-formazan generation in serum deprived cells to levels similar to those encountered under conditions of normal serum supplementation. This finding may be of significance when viewed in conjunction with other known effects of acute serum deprivation. For example membrane permeability to ions is increased.[30] Cellular lipid peroxidation is also increased[33] which in turn may lead

TABLE 6
Effect of diphenylene iodonium (DPI) on extracellular MTT-formazan in cultures of HeLa cells

Additions	Blue formazan accumulation (A_{570}) Intracellular	Extracellular
DMSO	0.411 ± 0.015	0.032 ± 0.001
DMSO + DPI	0.478 ± 0.033	0.025 ± 0.002

As in Table 1 triplicate monolayer cultures of HeLa were established. The medium was removed and replaced with serum supplemented medium containing 0.1% DMSO with or without 4 μM diphenylene iodonium (DPI) as indicated. After 2 hr, 0.25 mg MTT was added and the incubation contained for a further 30 min at 37°C. Levels of 'intracellular' and 'extracellular' MTT-formazan were then assessed as described in Table 1. Diphenylene iodonium was a kind gift from Professor O T Jones of Bristol University.

to the generation of radicals that could prejudice the proper function of membrane ion-pumps.[33] Ionic homeostasis certainly appears to be critical in relation to intracellular MTT-formazan generation as judged from the effects of the Na^+, K^+-ATPase inhibitor ouabain. Our observation that α-tocopherol, and other serum antioxidants are effective in neutralising the effects of serum withdrawal suggests that peroxidative membrane damage may be a critical outcome of serum deprivation. Such membrane damage could result in ionic imbalance sufficient to bring about the altered rates of intracellular MTT-formazan generation.

Despite the apparent requirement for serum antioxidants in the modulation of intracellular MTT-formazan production, questions remain regarding the molecular nature of intracellular reductants involved. Whilst reduction does involve respiratory chain components a key question is whether any mitochondrially generated superoxide is involved. A number of *in vitro* studies with various isolated mitochondrial preparations have clearly indicated formation of reactive oxygen species along with mitochondrial respiration.[23] Although components of the mitochondrial respiratory chain pass the bulk of their electrons to the next component of the chain some appear to 'leak' electrons to oxygen,[20-23] uni-electron reduction of oxygen generating superoxide radicals. Our observations that inhibitors of Cu/Zn-superoxide dismutase,[23] greatly enhance intracellular MTT-formazan levels, whereas a lipophilic low molecular weight mimic of superoxide dismutase, CuDIPS[27] is inhibitory suggest that a significant proportion of MTT formazan within cells does result from intracellularly generated superoxide. Moreover data obtained with the superoxide dismutase mimic suggest that this may account for between 22 and 33% of the MTT-formazan generated intracellularly, depending on whether or not the medium is supplemented with serum. The effectiveness of CuDIPS will of course be a function both of the intracellular sites, but a phenotypic property of tumour cells is reduced levels of superoxide dismutase.[4,35] Nevertheless the inhibition in intracellular MTT-reduction observed in presence of CuDIPS (Table 3) may represent a minimal estimate of possible intracellular superoxide generation.

Whilst our inhibitor studies cannot exclude mitochondria as a source of superoxide *in vivo* they do suggest the possible involvement of a xanthine oxidase like system. Such a cytoplasmic source is perhaps more likely in view of the very marked ability of inhibitors of the cytosolic Cu/Zn-superoxide dismutase in increasing levels of intracellular MTT-reduction. An expectation would be that superoxide generated within mitochondria would be directly acted upon *in situ* by the mitochondrial Mn-superoxide dismutase.

Support for the notion that at least 80% of the *extracellular* MTT-formazan is likely to be due to superoxide comes from experiments in which superoxide dismutase added exogenously to the growth medium depressed levels of detectable extracellular MTT-formazan by around that percentage. The possible source of this extracellular superoxide is however not clear. The use of the NADPH-oxidase inhibitor, diphenylene iodonium (DPI) does not resolve the situation (Table 6). Previous experiments of ours using an assay based on the superoxide dismutase inhibitable reduction of cytochrome C has also indicated low levels of superoxide release (3–6 fmol/hr/cell from cultured hamster fibroblasts, BHK-21) but again this was only partly (30%) inhibited by diphenylene iodonium.[11] Although a plasma membrane NADPH-oxidase is attractive as a potential source, other possibilities remain to be properly explored. For example the data in Table 4 using xanthine oxidase inhibitors suggest xanthine oxidase may also be involved in the generation of extracellular superoxide, at least in HeLa cells.

Despite differences in level and lack of serum-responsiveness, a possibility is that a small proportion of 'extracellular' formazan might become associated with cell membrane structures (or even pinocytosed) and thereby be inappropriately included in the 'intracellular' DMSO-extractable fraction. In an early experiment nitroblue tetrazolium (NBT)[17] was used to examine extracellular superoxide from HeLa cells. Whilst this salt does not appear to penetrate cells,[17] the amount of NBT-formazan inappropriately included in the intracellular DMSO fraction was nevertheless less than 10% of that detected in the extracellular fraction.

A surprising feature of experiments involving addition of exogenous superoxide dismutase to the culture medium was not so much the reduction in extracellular MTT-formazan levels, but the notable reduction of the intracellular rate of MTT-formazan generation. This might be because levels of extracellular superoxide have some type of 'feed-back' influence an intracellular MTT-reduction. Alternatively the exogenously added superoxide dismutase may simply be taken up by the cultured HeLa cells even during the relatively brief period (30 min) of exposure. A number of groups have observed the endocytosis of superoxide dismutase[36-39] in various cell types. Results obtained with isolated rat hepatocytes indicate that uptake of the enzyme is required to protect these cells from oxygen radical toxicity.[38] Others have traced the intracellular pathway followed by superoxide dismutase after uptake and found the enzyme reaches lysosomes where it remains for a relatively long time. In vitro superoxide is very resistant to lysosomal hydrolases.[36] *In vitro* therefore it may be the hydrolysis of superoxide dismutase is slow enough to allow significant amounts of this enzyme to remain in the lysosomes a long time after it has been endocytosed.[36] Thus to be scavenged superoxide anions generated within the cell must have access to the intralysosomal milieu. Beckman *et al*[39] have suggested that this may be achieved through anion channels that are present on lysosomal membranes. Despite no specific evidence in our studies for superoxide dismutase endocytosis by HeLa cells, such a phenomenon would nevertheless further support the notion that at least 30% of intracellular MTT reduction may be due to superoxide generated intracellulary and probably from a cytoplasmic source.

In summary the overall rate of MTT-reduction within cultured HeLa cells is very dependant on the extent of media supplementation with serum. The fact that serum antioxidants are important in minimising the rates of intracellular MTT reduction may have implications in relation to plasma antioxidant status in various disease conditions. From experiments with inhibitors and mimics of superoxide dismutase as well as from use of superoxide dismutase itself, it is likely that at least 20–30%

of the intracellular reduction of MTT is due to superoxide. The source of intracellular superoxide is not clear but inhibitor experiments whilst not ruling out a possible role for mitochondria suggest xanthine oxidase as a possible alternative. Outside the cell, reduction of MTT is also observed and experiments with exogenous superoxide dismutase suggest that at least 80% of this reduction is due to superoxide. A possible source of some of this extracellular superoxide may be an NADPH-oxidase type of plasma membrane enzyme, but again other sources such as xanthine oxidase remain a possibility. Whilst xanthine oxidase is detectable in cell-free preparations from HeLa cells, experiments are in progress to establish its distribution between cytosol and membrane fractions.

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