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# THE ROLE OF EXTRACELLULAR MEDIUM COMPONENTS AND SPECIFIC AMINO ACIDS IN THE CYTOTOXIC RESPONSE OF *ESCHERICHIA COLI* AND CHINESE HAMSTER OVARY CELLS TO HYDROGEN PEROXIDE

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A concentration of $H_2O_2$ resulting in mode one killing of *Escherichia coli* is more toxic when exposure to the oxidant is performed in complete medium (K medium), as compared to a saline (M9 salts). Inorganic salts ($MgSO_4$ and $CaCl_2$), thiamine or glucose, when added separately, or combined, to M9 salts had no effect on the cytotoxic response to $H_2O_2$. In contrast, the lethality of the oxidant was highly dependent on the presence of the amino acids in the incubation medium. The addition of glucose further enhanced this response. Among the seventeen amino acids which are present in the complete amino acid mixture, only two, i.e. L-histidine and L-cystine, were found to increase the toxicity of $H_2O_2$. Again, glucose augmented this response.

The effect of these amino acids on the growth inhibitory action of hydrogen peroxide was also tested in Chinese Hamster Ovary cells. It was found that L-histidine was capable of increasing the toxicity of the oxidant whereas all the other amino acids did not affect the toxicity of the oxidant. Glucose only slightly augmented this effect of L-histidine.

DNA single strand breakage produced by $H_2O_2$ was increased by L-histidine and was not significantly modified by the other amino acids. DNA double strand breakage was also shown to occur in cells exposed to $H_2O_2$-L-histidine, and this effect was independent on the presence of glucose.

These results demonstrate that the cytotoxic response of bacterial and mammalian cells to challenge with $H_2O_2$ is highly dependent on the composition of the extracellular milieu. Particularly relevant seems to be the effect of L-histidine, which markedly sensitizes both types of cells to the insult elicited by the oxidant, and that of L-cystine, which increases the sensitivity of *E. coli* cells.

KEY WORDS: hydrogen peroxide, extracellular medium components, amino acids, cytotoxicity, DNA damage.

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## INTRODUCTION

The cytotoxic effect of $H_2O_2$ has been extensively investigated in bacteria (especially in *Escherichia coli*)[1-9] as well as in mammalian cells.[10-16] In bacterial cells, the lethal response elicited by increasing concentrations of the oxidant is characterized by two regions of killing (mode one and mode two killing) which are produced by concentrations of $H_2O_2$ below 5 mM or higher than 12.5 mM, respectively.[2] A region of partial resistance is apparent between these two modes of lethality.[2]

In mammalian cells, the cytocidal response is linear with respect to the concentration of the oxidant utilized. It should be noted, however, that concentration-dependent mechanisms are also likely to be involved in this system. Indeed, although inhibitors of the enzyme poly(ADP-ribose)polymerase markedly reduced the cell killing elicited by millimolar concentrations of the oxidant[16] no effect was exerted by the same inhibitors in the cytotoxic response of mammalian cells treated with micromolar concentrations.[14]

Recently, it has been suggested that extracellular medium components like L-histidine[18-20] or glucose[21] are powerful modifiers of the toxicity of hydrogen peroxide in mammalian cells. To our knowledge, no study has been performed in bacteria.

Understanding the role of the cellular environment in the cytotoxic response of oxidatively injured mammalian and bacterial cells appears critical in that the composition of the biological fluids where target cells are bathed can be markedly affected by various pathological conditions, and this may significantly change the susceptibility of the cells to the oxidative attack.

In this study, we have examined the role of specific extracellular medium components in modulating the oxidative response of *E. coli* and CHO cells.

## MATERIALS AND METHODS

### *Materials*

Radiolabelled compounds were purchased from New England Nuclear Corp., Boston, MA, USA. Free acid EDTA, disodium EDTA, tetrasodium EDTA, sodium dodecyl sulfate, amino acids and other chemicals/reagent-grade biochemicals were from Sigma Chemical Co., St. Louis, MO, USA. $H_2O_2$ was purchased as a 30% solution from J.T. Baker Chemicals B.V. (Deventer, Netherlands); tetraethylammonium hydroxide was from Merck-Schuchardt, Munchen, FRG. Polycarbonate filters were from Nuclepore, Pleasanton, CA, USA. RPMI 1640 medium, foetal bovine serum and trypsin were from Gibco, Grand Island, NY, USA.

### *Bacterial Strain and Growth*

The *E. coli* K 12 wild-type was a gracious gift from Dr R. Tyrrell of the Institut Suisse de Recherches Experimentales sur le Cancer (Epalinges s/Lausanne, Switzerland). Cells were initially grown overnight (16–18 h) at 37°C in K medium (1% glucose, 1% acid hydrolysate of casein, 1 mg/l thiamine hydrochloride, 25 mg/l $MgSO_4 \times 7H_2O$ and 2 mg/l $CaCl_2$ in M9 salts – the composition of M9 salts was 6 g/l $Na_2HPO_4$, 3 g/l $KH_2PO_4$, 0.5 g/l NaCl, 1 g/l $(NaH_4)_2SO_4$). 1 ml of the overnight cell suspension was diluted with 50 ml of fresh K medium and aerobic growth was achieved in an

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Erlenmeyer flask with 200 rpm of shaking. *E. coli* cells grown to an optical density (O.D.) of 0.2 (600 nm), corresponding to about 7–10 × $10^7$ cells/ml, were harvested by centrifugation at room temperature, washed in M9 salts and resuspended at a density of 7–10 × $10^7$ cells/ml in M9 salts.

*Bacterial Cell Survival Experiments*

Treatments with $H_2O_2$ (15 min) were performed in the absence or presence of specific components of the K medium in 3 ml of cell suspension placed in a 20 ml scintillation counting vial with 200 rpm of shaking, at 37°C. At the end of the 15 min treatment, the cell suspension was diluted in M9 salts, cells were plated in quadruplicate in LB agar plates, and incubated for 24 h at 37°C, to allow colony formation.

*Mammalian cell culture and radioactive labelling.* CHO cells were routinely grown in McCoy's 5a medium supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin in an atmosphere of 5% $CO_2$ in air, at 37°C. All experiments were performed with log-phase cells (5 × $10^5$/60 mm dish). Experimental cultures for alkaline elution were plated in 60 mm tissue culture dishes, labelled overnight with methyl-$^{14}$C-thymidine (0.05 μCi/ml) and then chased for 6 h in label-free medium.

*Filter elution assay.* Cells containing $^{14}$C-DNA were exposed for 30 min to increasing concentrations of hydrogen peroxide in Saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose), in the presence or absence of specific compounds, removed from the dishes by trypsinization (1% trypsin for 5 min at ice temperature) and analyzed for DNA damage. The filter elution assay was carried out by a procedure virtually identical to that described by Kohn *et al.*[22] with minor modifications.[13] Briefly, 5 × $10^5$ cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 ml of ice cold Saline A containing 5 mM EDTA (disodium salt). Cells were then lysed with 5 ml of 2% sodium dodecyl sulfate, 0.025 M EDTA (tetrasodium salt), pH 10.1. Lysates were rinsed with 7 ml of 0.02 M EDTA (tetrasodium salt) and the DNA was eluted overnight in the dark with 1.5% tetraethyl ammonium hydroxide/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulfate (pH 12.1), at a flow rate of ca. 30 μl/min. Fractions of approximately 3 ml were collected and counted in 7 ml of Lumagel containing 0.7% glacial acetic acid. DNA remaining on the filters was recovered by heating for 1 h at 60°C in 0.4 ml of 1N HCl followed by the addition of 0.4 N NaOH (2.5 ml) and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 ml of 0.4 N NaOH. This solution was processed for scintillation counting as described above.

Strand Scission Factors (SSF) were calculated from the resulting elution profiles by determining the absolute log of the ratio of the percentage of DNA retained in the filters of the drug treated sample to that retained from the untreated control sample (both after 8 h of elution).

A similar procedure was followed for analyzing DNA double strand breakage except that the eluting buffer had a pH of 9.6 (22). Results from these experiments have been expressed as fraction of DNA retained in the filter after 12 h of elution.

*Mammalian cell growth inhibition studies.* Cells were seeded at a density of about 5 × $10^5$ cells/60 mm dish and, after 6 h, were treated for 30 min with various concentrations of hydrogen peroxide in Saline A, either in the absence or presence of

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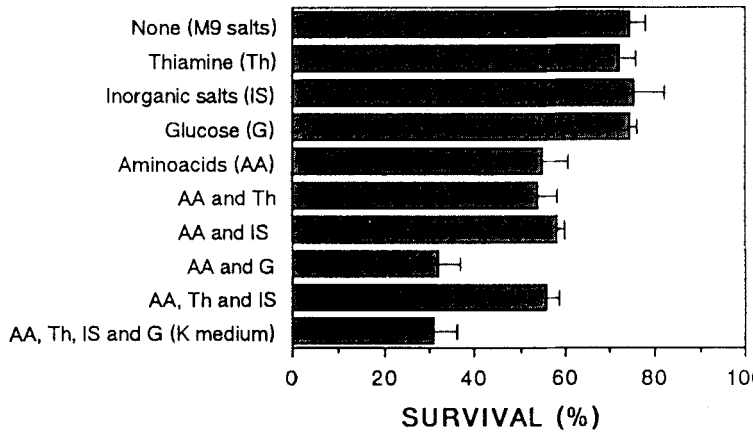


FIGURE 1 Effect of various medium components on the cytotoxic response of *E. coli* to hydrogen peroxide. Cells were exposed for 15 min to 2.5 mM $H_2O_2$ in M9 salts in the absence or presence of various constituents of the K medium (the concentrations of each of the constituents are those of the K medium and are reported in the Methods section). Survival values represent the mean ± S.E.M. calculated from 3–9 separate experiments.

specific compounds. Cell monolayers were then rinsed twice with Saline A and incubated for 48 h in a drug-free medium. Cell number was estimated after trypsinization with a Coulter Counter.

## RESULTS AND DISCUSSION

The effect of various medium components in the cytotoxic response of *E. coli* cells to a concentration of hydrogen peroxide resulting in mode one killing (2.5 mM) has been investigated. Results have demonstrated that, when added separately (Figure 1), or in combination (not shown) to M9 salts, thiamine, inorganic salts ($MgSO_4$ and $CaCl_2$) or glucose, did not affect the cytotoxicity of $H_2O_2$. In contrast, the toxicity of the oxidant was markedly increased by adding the complete amino acid mixture (AA) to the incubation medium. Glucose, unlike thiamine and/or inorganic salts, further enhanced the toxicity exerted by the oxidant in the presence of AA. Note that this response was essentially similar to that obtained in K medium. These results demonstrate that one or more components of the AA enhance/s the killing exerted by $H_2O_2$ and that glucose further increases this effect. We decided therefore to investigate the viability of *E. coli* cells exposed to the oxidant and each of the specific amino acids present in the AA. Since glucose was previously shown to augment the effect of the amino acids on the toxicity elicited by the oxidant, treatments were performed in M9 salts containing 1% glucose. The amino acids were utilized at concentrations as in K medium. Data shown in Figure 2 indicate that the toxicity of $H_2O_2$ in *E. coli* cells was markedly enhanced by L-histidine and L-cystine, whereas all the other amino acids were ineffective. In the presence of L-histidine the toxicity of the oxidant was similar to that obtained in the K medium whereas with L-cystine a stronger response was observed. Thus, the potentiating effects of L-histidine and L-cystine are somehow lessened by one or more components of AA.

These data indicate that the toxicity of $H_2O_2$ is dependent on the presence of

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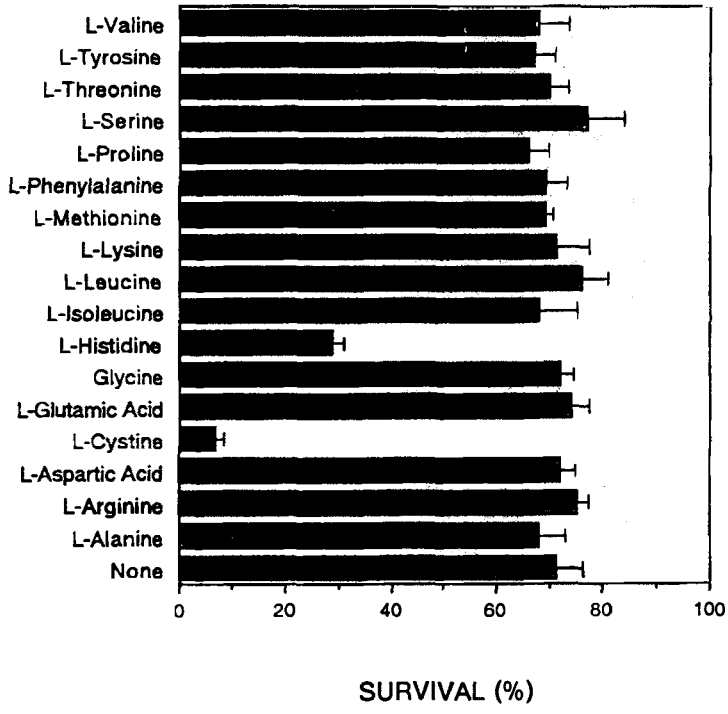


FIGURE 2 Effect of various amino acids on the cytotoxic response of *E. coli* to hydrogen peroxide. Cells were exposed for 15 min to 2.5 mM $H_2O_2$ in M9 salts containing 1% glucose in the absence or presence of various amino acids (the concentrations of each of the amino acids are those of the K medium and are reported in the Methods section). Survival values represent the mean ± S.E.M. calculated from 3–4 separate experiments.

L-histidine and L-cystine in the extracellular milieu, this effect being partially inhibited by other amino acids. The potentiating action of glucose is not surprising since active cellular metabolism is required in order for mode one killing to occur.[2] Glucose, however, was active only when L-histidine, L-cystine (Figure 2) or the AA (Figure 1), were present in the extracellular medium, thus suggesting that the energy-requiring reactions which mediate the cytotoxic action of the oxidant[2] occur only in the presence of specific amino acids.

We have also investigated the effect of some amino acids on the toxicity and DNA single strand breakage produced by hydrogen peroxide in mammalian cells. CHO cells were exposed to 25 $\mu$M $H_2O_2$ in Saline A (30 min) in the absence or presence of a 1 mM concentration of each of the amino acids, and samples were either assayed immediately for DNA damage or, following 48 h of post-challenge growth in a drug-free medium, for cell number. Data shown in Table I indicate that, under the experimental conditions described above, L-histidine, unlike the other amino acids, was capable of increasing the toxicity as well as DNA single strand breakage generated by hydrogen peroxide in cultured CHO cells. In other experiments, we have investigated the effect of glucose, showing that the L-histidine-mediated enhancement of the growth inhibitory effect of hydrogen peroxide was only slightly augmented

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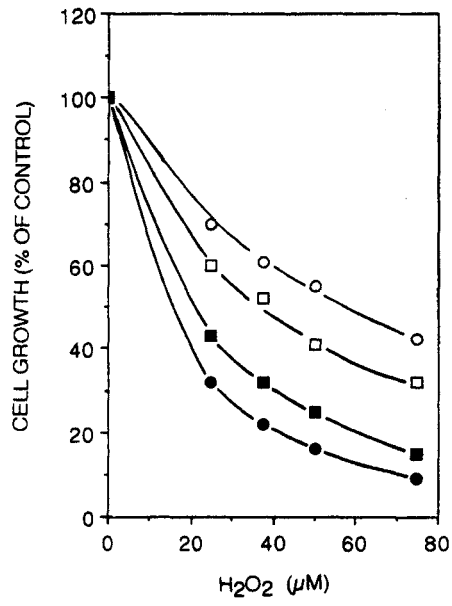


FIGURE 3 The effect of glucose on the L-histidine-mediated enhancement of $H_2O_2$-induced cytotoxicity in CHO cells. CHO cells were exposed to increasing concentrations of $H_2O_2$ (30 min) in the absence (open symbols) or presence (closed symbols) of 1 mM L-histidine. Treatments were performed in Saline A with (○, ●) or without (□, ■) glucose. Cell number was estimated after 48 h of post-challenge growth with a Coulter Counter and results are expressed as percent growth of cells which received $H_2O_2$ with respect to appropriate controls (i.e. cells incubated with or without L-histidine, depending on whether the amino acid was present during incubation of the corresponding oxidant-treated cultures – note that L-histidine, when added alone, did not affect the growth of the cells). Data points are the means of three independent experiments, each performed in duplicate. Standard errors were less than 10%.

(Figure 3). It should be noted, however, that the toxicity elicited by the oxidant alone was slightly lessened by glucose since, as previously suggested, glucose supports the glutathione peroxidase pathway, a first line defence against oxidative stress.[21] Thus, some effect of glucose could be inferred by comparing the growth inhibition curves obtained in the absence or presence of glucose with the appropriate controls. We have previously shown[23] that $H_2O_2$-L-histidine results in the production of DNA double strand breakage, a lesion which cannot be detected even following treatment of the cells with exceedingly high concentrations of the oxidant alone. We now find that glucose does not significantly modify this response (Table II), which may suggest that extracellular glucose is not necessary in order for DNA double strand breakage to occur. It should be kept in mind, however, that small variations in the number of DNA double strand breaks (characterized by barely detectable changes in the elutability of the DNA) may result in profound changes in terms of survival and therefore the experimental data presented in Figure 3 and Table II do not rule out a cause-effect relationship. Indeed, as we have previously suggested,[23] and as indicated by further unpublished results, the L-histidine-mediated enhancement of $H_2O_2$-induced cytotoxicity in mammalian cells is probably related to the appearance of DNA double strand breakage, a lesion often resulting in cell killing.

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TABLE I

The effect of various amino acids on the toxicity and DNA single strand breakage produced by hydrogen peroxide in CHO cells.

| Amino acid | Cell growth (% control)[a] | (SSF)[b] |
|---|---|---|
| – | 65 ± 4.3 | 0.194 |
| L-Valine | 61 ± 3.4 | 0.188 |
| L-Tyrosine | 62 ± 5.6 | 0.194 |
| L-Threonine | 65 ± 4.6 | 0.225 |
| L-Serine | 61 ± 6.2 | 0.187 |
| L-Proline | 59 ± 4.9 | 0.192 |
| L-Phenylalanine | 69 ± 4.4 | 0.270 |
| L-Methionine | 62 ± 4.7 | 0.184 |
| L-Lysine | 65 ± 4.3 | 0.165 |
| L-Leucine | 68 ± 4.0 | 0.163 |
| L-Isoleucine | 58 ± 6.2 | 0.196 |
| L-Histidine | 32 ± 2.9* | 0.373* |
| Glycine | 55 ± 4.4 | 0.203 |
| L-Glutamic acid | 68 ± 4.9 | 0.159 |
| L-Cystine | 60 ± 4.8 | 0.205 |
| L-Aspartic acid | 66 ± 6.1 | 0.158 |
| L-Arginine | 65 ± 4.3 | 0.174 |
| L-Alanine | 62 ± 5.2 | 0.180 |
| L-Glutamine | 62 ± 5.6 | 0.157 |

[a] CHO cells were treated with 25 $\mu$M $H_2O_2$ (30 min) in the absence or presence of 1 mM concentrations of the various amino acids. Cell number was estimated after 48 h of post-challenge growth with a Coulter Counter. Data points are the means ± S.E.M. ($n$ = 4).

[b] CHO cells were treated with 15 $\mu$M $H_2O_2$ (30 min) in the absence or presence of 1 mM concentrations of the specific amino acids. DNA single strand breakage was estimated by the alkaline elution assay and data are expressed as Strand Scission Factor (see Methods). (Standard errors were less than 10%, 3 independent experiments.)

* $p < 0.001$ when compared with corresponding basal value (no amino acid present in the extracellular medium).

TABLE II

The effect of glucose on DNA double strand breakage caused by $H_2O_2$ and L-histidine in cultured mammalian cells*

| | Percent DNA retained | |
|---|---|---|
| L-histidine | + glucose | – glucose |
| – | 96.92 ± 2.1 | 96.79 ± 2.2 |
| + | 79.65 ± 5.1 | 76.32 ± 4.9 |

* CHO cells were exposed for 30 min to 300 $\mu$M $H_2O_2$ with or without 1 mM L-histidine in the absence or presence of 5 mM glucose and then assayed for DNA double strand breakage by the neutral elution technique. Data are expressed as percent DNA retained (see Methods). Means ± S.E.M. of three independent experiments, each performed in duplicate.

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In summary, L-histidine increases the sensitivity to oxidative stress also in mammalian cells (as it did in bacteria), and these results are in keeping with previously published work from various laboratories.[13–18]

Under the experimental conditions utilized in this study, L-cystine did not increase the toxicity of $H_2O_2$ in mammalian cells, as it did in bacteria. Preliminary results (unpublished work) indicate that this amino acid, at concentrations below 1 mM, may actually protect cells against oxidative injury.

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