Study on the Interaction of Ions of Transient Metals with Ascorbic Acid in the Presence of Different Scavengers of Active Oxygen Species in SOS Chromotest

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Abstract: SOS chromotest was employed to study the interaction of ascorbic acid with free ions of transient metals in the presence of added catalase, superoxide dismutase or D-mannitol. Catalase diminished the genotoxic activity of the mixture of ascorbic acid with copper ions in *E. coli* strains PQ37 and PQ 300, but genotoxicity of this mixture was not suppressed by superoxide dismutase and D-mannitol. The results suggest that copper ions diminished the content of peroxide generated by ascorbic acid.

Key words: Vitamin C, transition metal ions, scavengers, SOS chromotest

Introduction

Chemical oxidants and a number of chemicals known to generate reactive oxygen species constitute an important family of genotoxic agents. These genotoxic agents may contribute to aging and cancer. It seems particularly important to understand their mode of action and to identify them among genotoxic agents. They are relatively well detected with the SOS chromotest, a quantitative short-term bacterial assay for the detection of genotoxic activity, using the standard *Escherichia coli* tester strains and derivatives of the SOS chromotest tester strain affected in genes involved in the processing of lesions.

Escherichia coli cells possess two major regulatory mechanisms that protect against oxidative stress. During exponential growth, the expression of several anti-oxidant defense genes is regulated by redox-sensitive OxyR protein, and expression of these defense genes is induced by oxidative agents [1–3].

The SOS chromotest has been described by Quillardet *et al.* [4]. This test is the simple colorimetric assay of the induction of the bacterial gene *sfiA* in *E. coli.* sfiA expression is induced after DNA damage as part of the SOS system. In the test, *sfiA* expression is monitored by assaying β-galactosidase activity in an *sfiA:lacZ* operon fusion tester strain.

Ascorbic acid (vitamin C) can act as either an antioxidant or prooxidant, depending on its concentration and the presence of free transition metal ions (by accelerating the Fenton reaction [5, 6]. The transition metal ions, iron and copper, are the most likely candidates *in vivo* for catalyzing the conversion of $O_2^{\cdot-}$ and H_2O_2 into $^{\cdot}OH$ [7]. Both

Fe(III) and Cu(II) ions are reduced by ascorbic acid via one-electron transfer. The reaction between Cu(II) and ascorbic acid thus leads to the formation of reactive oxygen species, which interfere with many biological systems. The reaction of Cu(II) ions with ascorbic acid to form very reactive radicals has frequently been studied in lipids, DNA, viruses, and cell systems. Fe(III) ions may affect biological systems similarly. An *in vivo* prooxidant role of vitamin C has been suggested, but direct evidence for it is scant.

Our previous study [8, 9] suggested that copper ions intensify SOS response induced in *E. coli* PQ37 and *oxyR* mutants induced by ascorbic acid. The mixture of copper ions and ascorbic acid was more genotoxic and cytotoxic than copper ions alone or ascorbic acid alone. In the present study we tried to evaluate the role of reactive oxygen species in the interaction of free ions Fe(III) and Cu(II) with ascorbic acid by using antioxidant enzymes, catalase and superoxide dismutase, as well as D-mannitol.

Materials and Methods

Tester strains

Escherichia coli PQ37 and PQ300 strains were kindly given by P. Quillardeta and M. Hofnung from Institut Pasteur, Paris. The genetic features of PQ37 have been described by Quillardet *et al.* [4, 10].

The PQ300 has the same gene fusion and the same characteristics as PQ37, but it is an *oxyR* deletion mutant that is more susceptible than PQ37, the standard SOS chromotest strain, to certain types of oxidative genotoxins [11, 12].

Chemicals

Catalase(CAT); superoxide dismutase (SOD)/EC 1.15.1.1; o-nitrophenyl-β-D-galactopyranoside (ONPG), the substrate for galactosidase; p-nitrophenyl phosphate disodium (PNPP), the substrate for alkaline phosphatase; and ascorbic acid (vitamin C) were supplied by Sigma (U.S.A); copper sulfate ($CuSO_4$) was obtained from Riedel De Haën AG Seelze (Germany); ferric sulfate [$Fe_2(SO_4)_3$] was obtained from Fluka Chemie AG Buchs (Switzerland); D-mannitol was obtained from Aldrich.

All substances were dissolved in water and all solutions were prepared immediately before the experiments.

Test procedure

The SOS chromotest was performed as described by Quillardet *et al.* [4]. The test consists of colorimetric assays of enzymatic activities after incubating the tester strain in the presence of various amounts of compound. Briefly, samples (20 µl of the compound to be tested) and an exponentially growing culture of the tester strain (0.6 ml), freshly diluted in medium, were incubated for 2 h at 37°C with shaking. After incubation, β-galactosidase (as an induction assay) and alkaline phosphatase (as a bacterial survival assay) was assayed by kinetic measurement as described by Quillardet *et al.* [10]. The absorbance at 420 nm was read using a spectrophotometer model UV-160A, Shimadzu.

In the test, a compound is classified as genotoxic when the induction factor is larger than 1.5. The induction factor is defined as the ratio of the activity of β-galactosidase to that of alkaline phosphatase at concentration C of the compound divided by its value at concentration 0.

Results and Discussion

The possible contribution of active oxygen species in genotoxicity of ascorbic acid with presence of metal ions Fe(III) or Cu(II) was studied by using different scavengers in the SOS chromotest.

In a series of experiments, we have determined induction factors of ascorbic acid and the mixtures of ascorbic acid with iron ions or copper ions at nontoxic concentrations.

As shown in Figures 1 and 2, the induction factor of ascorbic acid increased with the increasing concentration

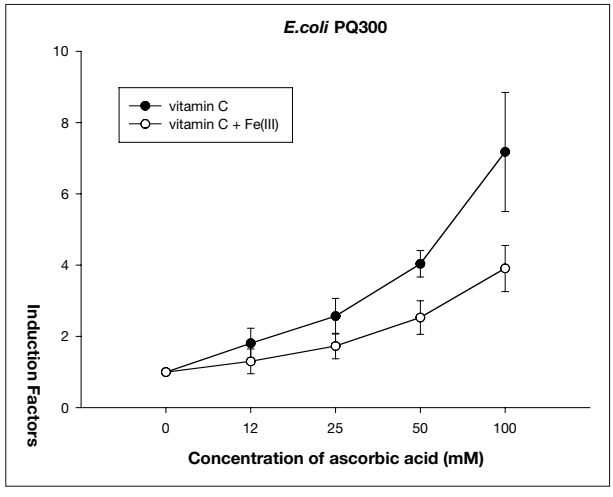

Figure 1: Influence Fe(III) on the induction of SOS response by ascorbic acid in mutant *oxyR E. coli.*
The experiments were performed as described under Materials and Methods. Solutions of ascorbic acid at different concentrations contained 75 nmol/ml of iron ions.
Each value is an average of 6 independent experiments ± SD.

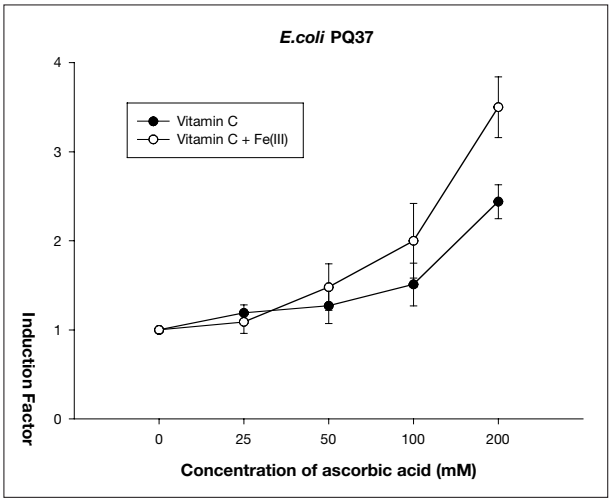

Figure 2: Influence Fe(III) on the induction of SOS response by ascorbic acid in strain *E. coli* PQ37.
The experiments were performed as described under Materials and Methods. Solutions of ascorbic acid at different concentration contained 75 nmol/ml of iron ions.
Each value is an average of 6 independent experiments ± SD.

of the vitamin. In the study using *E. coli* strain PQ37, free iron ions at the concentration of 75 nmol/ml augment the induction factor, in contrast to that which occurs in strain PQ300. In this strain, iron ions (in the same concentration) diminish the induction factor of ascorbic acid in a concentration-dependent manner.

In the next series of experiments for the different mixtures of ascorbic acid with transient metal ions, catalase and superoxide dismutase or D-mannitol were added.

Table I demonstrates the effect of antioxidant enzymes and non- enzymatic scavengers on the genotoxic activity of ascorbic acid at different concentrations, in the presence of iron ions and copper ions at nontoxic concentrations. As can be seen, the presence of iron ion increases the induction factor of ascorbic acid in the PQ37 strain. In addition, copper ions increase induction factors significantly in this strain. The addition of enzymatic scavengers, catalase or superoxide dismutase, led to different effects. In contrast to superoxide dismutase, catalase significantly diminished induction factors of ascorbic acid. D-mannitol was not active.

In the next experiments (Fig. 3 and 4), induction factors of ascorbic acid at the concentration 50 mM in the presence of various nontoxic concentrations of copper ions and in the presence of scavengers were determined.

The genotoxic activity of ascorbic acid plus copper ion in PQ37 and PQ300 was inhibited by addition of catalase, a H_2O_2 scavenger. However, addition of SOD and D-man-

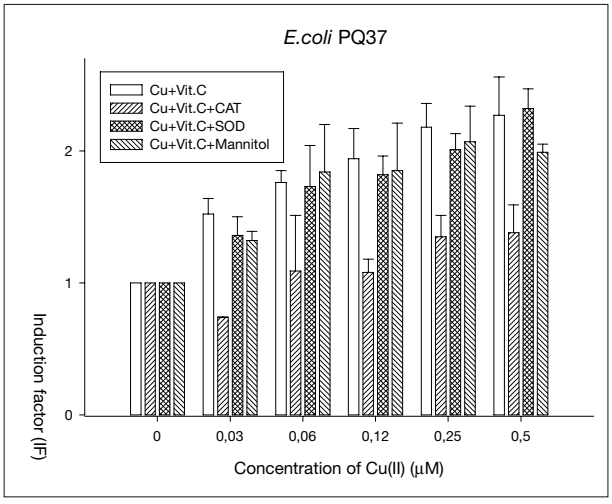

Figure 3: Interaction of ascorbic acid and copper ions at different concentrations in the presence of scavengers.
Solutions of cooper ions at different concentrations contained 50 mM of ascorbic acid. The assay was performed in the presence of scavengers CAT, SOD, and mannitol at the concentrations of 500 U/sample, 1000 U/sample, and 30 mM, respectively.
The value of Induction Factor represents an average of six experiments ± SD.

Table 1: Genotoxicity of ascorbic acid with Fe(III) or Cu(II) in the absence and the presence of scavengers of active oxygen species in E. coli PQ37.

	Induction Factor			
	Ascorbic acid (mM)			
Scavenger	25	50	100	200
Fe(III) 75 nmol/ml	1.29 ± 0.29	1.64 ± 0.33	2.41 ± 0.71	3.92 ± 0.58
D-mannitol 30 mM	1.21 ± 0.29	1.53 ± 0.36	2.14 ± 0.34	3.16 ± 0.32
CAT 500 U/sample	0.459 ± 0.218	0.335 ± 0.057	0.952 ± 0.236	0.537 ± 0.202
SOD 1000 U/sample	1.20 ± 0.18	1.27 ± 0.13	2.18 ± 0.45	3.73 ± 0.72
Cu(II) 0.12 µM	1.63 ± 0.64	2.34 ± 0.65	4.29 ± 0.81	6.41 ± 0.85
D-mannitol 30mM	1.53 ± 0.43	2.19 ± 0.84	3.32 ± 0.43	6.07 ± 1.11
CAT 500 U/sample	0.669 ± 0.201	0.654 ± 0.246	1.53 ± 0.53	2.34 ± 0.48
SOD 1000 U/sample	1.72 ± 0.66	2.83 ± 0.29	3.84 ± 0.18	5.41 ± 0.21

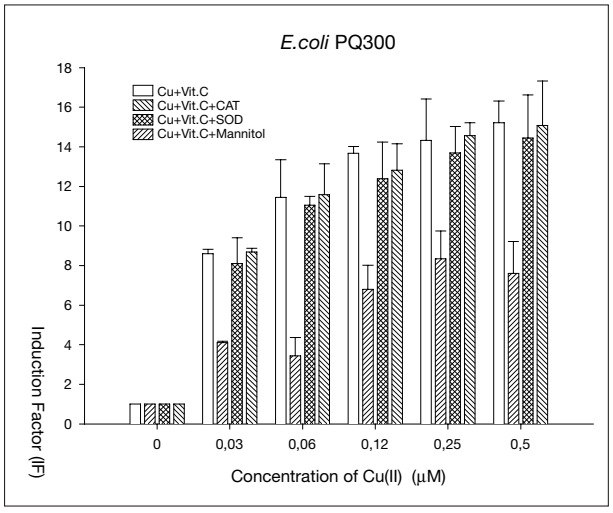

Figure 4: Interaction of ascorbic acid and copper ions at different concentrations in the presence of scavengers.
Solutions of cooper ions at different concentrations contained 50 mM of ascorbic acid. The assay was performed in the presence of scavengers CAT, SOD, and mannitol at the concentrations of 500 U/sample, 1000 U/sample, and 30 mM, respectively.
The value of Induction Factor represents an average of six experiments ± SD.

nitol, scavengers of $O_2^{\cdot -}$ and $^{\cdot}OH$, respectively, did not influence the induction factors of ascorbic acid.

The results demonstrated a drastic, concentration-dependent increase of induction factor of ascorbic acid in the presence of copper ions and a decrease of induction factor by catalase. SOD and D-mannitol remained inactive in this experiment.

This study demonstrated that the interaction of catalase with the mixture of copper ions and ascorbic acid results in the diminishing of the content of H_2O_2, most probably generated by ascorbic acid.

In our study we have shown that in the *E. coli* strains PQ37 and PQ300, the genotoxic activity of ascorbic acid was enhanced by free copper ions and abolished by a H_2O_2 scavenger, catalase. Our data relating to genotoxic activity of the mixture of ascorbic acid with transition metal ions in a bacterial cell system agree with the results obtained by other authors in their *in vitro* studies [13–17].

Various metal ions were investigated for their ability to modify the radical intensity and cytotoxic activity of sodium ascorbate or ascorbic acid [18, 19], using supercoiled and linearized DNA. It has been shown that higher concentrations of ascorbate and Cu(II) ions result in higher frequency of nonspecific DNA cleavage. Genotoxicity of ascorbic acid was increased by Cu(II) ion addition and decreased by catalase [17].

In our experiments, the non-cytotoxic concentration of Cu(II) significantly enhanced the genotoxicity of ascorbate. In this study, the genotoxicity of ascorbic acid plus Cu(II) ions was abolished by catalase. It is of interest that the non-cytotoxic concentration of Cu(II) significantly enhanced the cytotoxicity of ascorbate against the cultured human glioblastoma T98G cell line [18].

In the experiments, when the oxidative effect of ascorbic acid in the presence of transition metals was studied, Cu(II) ions exhibited a higher efficiency compared to Fe (III) ion [20,21]. A possible mechanism of Cu(II) toxicity may be that superoxide, generated by Cu(II), inactivates superoxide-sensitive enzymes such as aconitase, and fumarase, and that the Fe(II) produced by inactivation of these enzymes promotes the Fenton reaction, resulting in the generation of more (OH radicals that attack DNA.

In another set of experiments we examined the dual role of iron ions. The genotoxicity related to the interaction of ascorbic acid with iron ions was enhanced or diminished depending on the strain of bacteria used, and depending on the concentration of the vitamin C. In both cases, genotoxicity was abolished by catalase. The DNA damage is thought to be produced by a Fenton-type mechanism in which transition metals are cycled by first being reduced by superoxide and then oxidized by H_2O_2. The produced $^{\cdot}OH$ will then damage DNA. In the case of added iron ions, catalase diminishes the genotoxic effects of iron ions plus ascorbic acid in the strain PQ37. It may be assumed that iron ions diminish the content of H_2O_2 generated by ascorbic acid. Similar conclusions have been drawn by other authors [17, 22, 23].

In bacterial cell systems, the data led to similar conclusions as those derived from other *in vitro* studies on isolated DNA. Our results and conclusions sustain the opinion [6, 24] that it is necessary to perform experiments on animals and humans to avoid the potential adverse effects danger (phrase may be mis-translated?) of combining ascorbic acid with ions of transition metals.

References

1. Ivanova, A. B., Glinsky. G.V. and Eisenstark, A. (1997) Role of RPOS regulon in resistance to oxidative stress and near-UV radiation in ΔOXYR suppressor mutants of Escherichia coli. Free Radical Biology and Medicine 23, 627–636.
2. Storz, G. and Altuvia, S. (1994) OxyR regulon. Method Enzymol. 234, 217–223.
3. Kullik, I., Toledano, M. B. and Storz, G. (1995) Mutational analysis of the redox-sensitive transcriptional activation. J. Bacteriol. 177,1275–1284.
4. Quillardet, P., Huisman, O., D'Ari, R. and Hofnung, M. (1982) SOS chromotest, a direct assay of induction of an SOS function in Escherichia coli K-12 to measure genotoxicity. Proc. Natl. Acad. Sci. USA 79, 5971–5975.

5. Frei, B., England, L. and Ames, B. N. (1989) Ascorbate is an outstanding antioxidant in human blood plasma. Proc. Natl. Acad. Sci. USA 86, 6377–6381.
6. Kang, S. A. H., Jang, Y. J. and Park, H. (1998) In vivo dual effects of vitamin C on paraquat – induced lung damage: dependence on released metals from the damaged tissue. Free Rad. Res. 28 (1), 93–107.
7. Halliwell, B. and Gutteridge, J. M. C. eds. (1989) Free radicals in biology and medicine, 2nd edition. Oxford: Clarendon.
8. Marczewska, J. and Koziorowska, J. (1998) Badanie dziatania genotoksycznego witaminy C w SOS chromotescie. Biuletyn Instututu Leków 48, 36–42.
9. Marczewska, J., Koziorowska, J. and Anuszewska, E. L. (2000) Influence of ascorbic acid on cytotoxic activity and iron ions in vitro. Acta Poloniae Pharm. 56, 415–417.
10. Quillardet, P. and Hofnung, M. (1985) The SOS chromotest, a colorimetric bacterial assay for genotoxins: procedures. Mutation Res. 147, 65–78.
11. Quillardet, P. and Hofnung, M. (1988) The screening, diagnosis and evaluation of genotoxic agents with batteries of bacterial tests. Mutation Res. 205, 107–118.
12. Müller, J. and Janz, S. (1993) Modulation of the H_2O_2 – induced SOS response in Escherichia coli PQ300 by amino acid, metal chelators, antioxidants, and scavengers of reactive oxygen species. Environmental and Molecular Mutagenesis 22, 157–163.
13. Omura, H., Shinohara, K., Meada, H., Nomaka, M. and Murakami, H. (1978) Mutagenic action of triose reductone and ascorbic acid on Salmonella typhimurium TA100 strain. J. Nutr. Sci. Vitaminol. 24, 185–194.
14. MacRae, W. D. and Stich, H. F. (1979) Induction of sister chromatid exchanges in Chinese hamster cells by the reducing agents bisulfite. Toxicology 13, 167–174.
15. Stich, H. F., Wei, L. and Whiting, R. F. (1979) Enhancement of the chromosome-damaging action of ascorbate by transition metals. Cancer Res. 39, 4145–4151.
16. Rosin, M. P., San, R. H. C. and Stich, H. F. (1980) Mutagenic activity of ascorbate in mammalian cell cultures. Cancer Lett. 299–305.
17. Odin, A. P. (1997) Vitamins as antimutagens: Advantages and some possible mechanisms of antimutagenic action. Mutation Res. 386, 39–67.
18. Satoh, K. and Sakagami, H. (1997) Effect of metal ions on radical intensity and cytotoxic activity of ascorbate. Anticancer Research 17 (2A), 1125–1129.
19. Wang, Y. and Van Ness, B. (1989) Site-specific cleavage of supercoiled DNA by ascorbate/Cu(II). Nucleic Acids Research 17, 6915–6926.
20. Yugay, M. T., Pereira, P. C., Leiria, F. and Mota, M. C. (1996) Oxidative damage to lens membranes induced by metal-catalyzed systems. Ophthalmic Research 28 (suppl 1), 92–96.
21. Kimura, T. and Nishioka, H. (1997) Intracellular generation of superoxide by copper sulphate in Escherichia coli. Mutation Research 389, 237–242.
22. Toyokuni, S. and Sagripanti, J. L. (1992) Iron – mediated DNA damage: sensitive detection of DNA strand breakage catalyzed by iron. Journal of Inorganic Biochemistry 47, 241–248.
23. Vuillaume, M. (1987) Reduced oxygen species, mutation, induction and cancer initiation. Mutation Research 186, 43–72.
24. Halliwell, B. (1996) Vitamin C: antioxidant or prooxidant in vivo? Free Radical Research 25, 439–454.

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