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Cysteine enhances clastogenic activity of dimethylarsinic acid

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The effects of cysteine on dimethylarsinic acid (DMA)-induced cytotoxicity and chromosomal aberration were studied using Chinese hamster V79 cells. The IC 50 of DMA, i.e. the concentration resulting in a 50% decrease in cell population of viable cells, was 130 μg ml^{-1} (0.94 mM), whereas that in the presence of 50 μg ml^{-1} (0.28 mM) cysteine was 20 μg ml^{-1} (0.14 mM). The mitotic index with co-administration of 50 μg ml^{-1} (0.36 mM) DMA and 50 μg ml^{-1} cysteine was 1.4 times that with 50 μg ml^{-1} DMA alone. Whereas 82% of cells divided twice with 25 μg ml^{-1} (0.18 mM) DMA alone, most cells divided only once with co-administration of 25 μg ml^{-1} DMA and 50 μg ml^{-1} cysteine. These results indicated that the increase in mitotic index by cysteine was due to enhancement of mitotic arrest by DMA. With co-administration of 25 μg ml^{-1} DMA and 50 μg ml^{-1} cysteine, tetraploidy was 14.3% higher and fivefold by that with 25 μg ml^{-1} DMA only. Cysteine at 50 μg ml^{-1} enhanced induction of chromosomal aberrant cells by DMA. 100 μg ml^{-1} (0.72 mM) DMA induced 91% chromosomal aberrant cells in the presence of cysteine, and 12% in the absence of cysteine. Chromatid breaks and chromatid gaps were the most frequent types of aberration induced by co-administration of DMA and cysteine or DMA alone. Co-administration of DMA and cysteine produced many attenuated chromosomal figures. The attenuated chromosomal figures always had several chromatid gaps and chromatid breaks. Our findings may provide clues to arsenic carcinogenesis in humans.

KEYWORDS: dimethylarsinic acid; arsenic; cysteine; mitotic arrest; chromosomal aberration; carcinogenicity

INTRODUCTION

Arsenic is ubiquitously distributed in nature. Epidemiological studies have shown that arsenic exposure is correlated with increased incidence of cancers.[1] When inorganic arsenic is introduced into the mammalian body, it is reduced to trivalent arsenic, and is methylated to monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA).[2] In general, the acute toxicity of organoarsenic compounds is much lower than that of inorganic arsenic. Methylation of arsenic has been considered as a mechanism of detoxification (but see Discussion).[3] DMA is a major metabolite of inorganic arsenic in humans.[4]

In vitro studies have indicated that DMA is a mitotic poison, causing mitotic arrest,[5,6] inducing tetraploidy,[5,6] and c-mitosis,[7] and inducing the formation of abnormal spindles in mitotic cultured cells.[8] In an *in vivo* study, DMA significantly induced aneuploidy in mouse bone-marrow cells.[9] It has been reported that DMA acts as a promoter[10,11] or initiator[12] of urinary bladder, kidney, liver and thyroid gland carcinogenesis in rats. Yamanaka *et al.*[13] have shown that DMA is a mutagen in *Escherichia coli* B, and induces DNA damage in mouse and rat lung cells.[14,15] Many studies, however, have indicated that clastogenic activity of DMA is low.[16,17] It has remained unclear whether DMA has any carcinogenic effects in humans.

On methylation of arsenite and MMA, the monothiol cysteine promotes greater activities of methyltransferase obtained from Golden Syrian hamster liver, *in vitro*, than

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either glutathione or dithio-threitol.[18] Cysteine reacts directly with DMA to yield a trivalent organic arsenic, *S*-dimethylarsinocysteine.[19] The thiol glutathione enhanced the induction of the cytotoxic and clastogenic effects of DMA.[20,21] Methylarsonous acid (MMA(III)) and dimethylarsinous acid (DMA(III)) are more toxic than arsenite.[22,23] In the comet assay using human lymphocytes, methylated trivalent arsenicals were very potent.[24] In the present study, we studied the effect of cysteine on the cytotoxicity and chromosome aberration induced by DMA, using Chinese hamster V79 cells.

MATERIALS AND METHODS

Materials

DMA (purity 99.99%) was purchased from Tri-Chemical, Kanagawa, Japan, and dissolved in distilled water. Cysteine hydrochloride monohydrate was purchased from Wako Pure Chemicals, Osaka, Japan. Cysteine solution was freshly prepared. Giemsa's solution was obtained from Merck, Darmstadt, Germany. V79 cells, which originated from Chinese hamster lung, were obtained from the Institute for Fermentation (Osaka, Japan). Leibovitz-15 (L-15) medium was purchased from Sigma–Aldrich, Japan. Fetal bovine serum was obtained from ICN Biochemicals, Costa Mesa, California. 5-Bromodeoxyuridine (BrdU) and Hoechst 33258 were purchased from Wako Pure Chemicals, Osaka, Japan. Trypsin was purchased from Difco, Michigan.

Methods

V79 cells were cultured in L-15 medium supplemented with 7% heat-inactivated fetal bovine serum with kanamycin sulfate (50 μg ml^{-1}) at 37°C in a 5% CO_2 atmosphere. Approximately 4×10^4 cells ml^{-1} were plated in 35 mm diameter Petri dishes with 5 ml L-15 medium. A solution of 10 mg ml^{-1} DMA or cysteine was diluted with distilled water. Then they were mixed equally and the mixture was added into the culture medium. The cells were incubated in 4.5 ml new L-15 medium without serum with 0.5 ml of various concentrations of test compounds (DMA and/or cysteine) and 1 μg ml^{-1} final concentration of BrdU in the dark at 37°C in a 5% CO_2 atmosphere. After 2 h, the medium was replaced by 4.5 ml old medium supplemented with 0.5 ml of various concentrations of test compounds and 1 μg ml^{-1} final concentration of BrdU, and cultured in the dark at 37°C for 26 h in a 5% CO_2 atmosphere. Colcemid was not added in order to avoid its mitotic blocking effect and to determine the net index of DMA–cysteine treatment. The cells were treated with a hypotonic solution of 0.075 M KCl and were fixed with methanol–acetic acids (3:1). The cells were dropped on glass microscope slides. The metaphase figures were stained with 0.1 μg ml^{-1} Hoechst 33258, irradiated with a black lamp (15 W, 2 cm, 20 min) with SSC (0.3 M sodium chloride + 0.03 M citrate) and stained with 2% Giemsa's solution. In twice-divided metaphase (M2), newly synthesized sister chromatids and old chromatids were completely differentially stained, whereas in once-divided metaphases (M1) they were not differentially stained. In threefold-divided (or more) metaphases (M3), they were partially differentially stained. Average generation time (AGT) was computed as 28 h/(1 × M1 proportion + 2 × M2 proportion + 3 × M3 proportion). The mitotic index (percent) was determined as the proportion of metaphase cells in 1000 cells. The number of chromosomes per cells ranged from 20 to 24, and 90% of cells had 22 chromosomes.[25] Hence, metaphase figures with 38 to 50 chromosomes were regarded as tetraploids. Cell proliferation was measured by hemocytometry after the cells were harvested. Cells were examined for vitality by staining with trypan blue. For chromosome aberration experiments, the cells were exposed to DMA–cysteine without BrdU to avoid the toxicity of BrdU. The mitotic figures were stained with Giemsa's solution. Two plates were used for a group in the experiments. The results are shown as the average of two plates. The data of chromosomal aberrations were statistically analyzed by the χ^2 test.

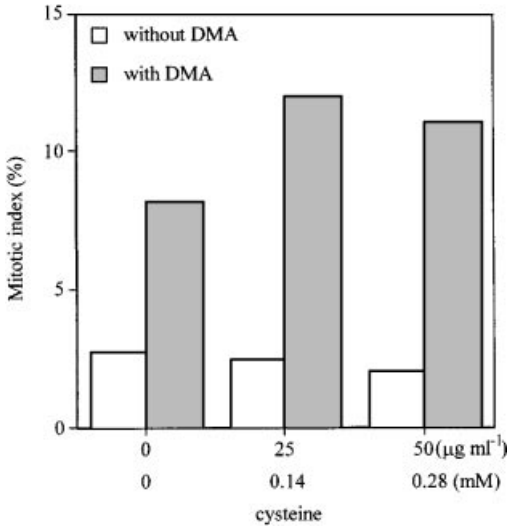

Figure 1. Effects of cysteine on the mitotic index of 50 μg ml^{-1} DMA. The mitotic index (percent) was determined as the proportion of metaphase cells in 1000 cells. V79 cells were exposed to DMA or DMA and cysteine for 28 h (see Materials and Methods).

RESULTS

Effects of cysteine on proliferation, mitosis and tetraploidy of DMA

V79 cells were exposed to 0.5 ml of various concentrations DMA and/or cysteine for 28 h with BrdU. The mitotic indices for V79 cells exposed to 50 μg ml^{-1} (0.36 mM) DMA and 0, 25, 50 μg ml^{-1} (0, 0.14, 0.28 mM) cysteine for 28 h were determined. The mitotic index of the control was 2.8%, and

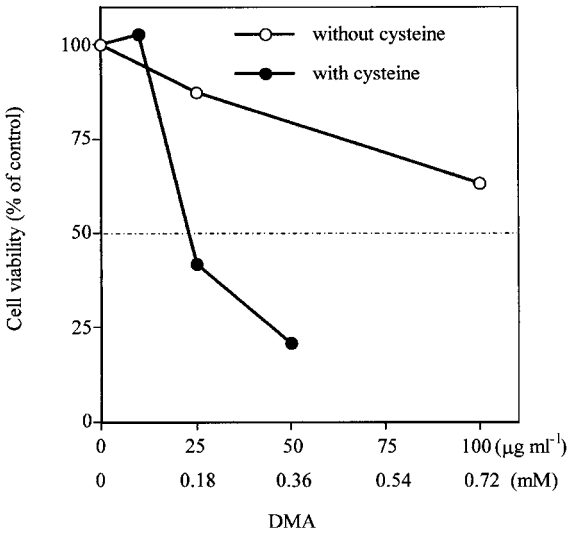

Figure 2. Effects of 50 μg ml^{-1} cysteine on cell viability with DMA. Cells were exposed to DMA alone or DMA and cysteine for 28 h (see Materials and Methods). Cellular proliferation was measured by cytometry.

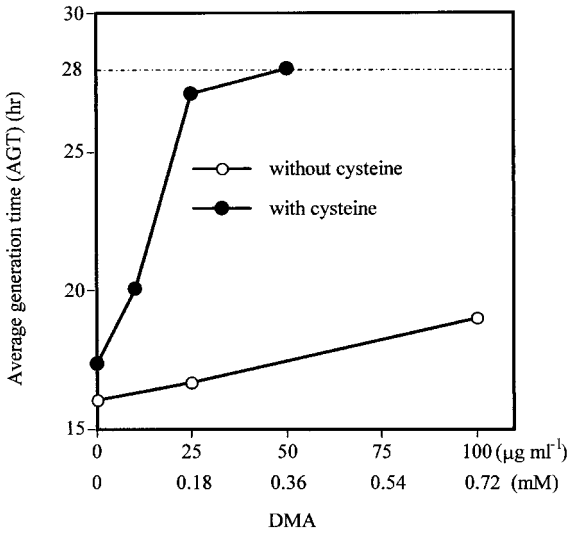

Figure 4. Effects of 50 μg ml^{-1} cysteine on cell-cycle delay in diploid cells with DMA. V79 cells were exposed with DMA or DMA and cysteine for 28 h (see Materials and Methods). The AGT was 28 h/(1 × M1 + 2 × M2 + 3 × 3M).

50 μg ml^{-1} DMA increased the mitotic index to 8.2% (Fig. 1). Cysteine did not affect the mitotic index in concentrations below 50 μg ml^{-1}. Co-administration of 0.5 ml of various concentrations DMA and 25 μg ml^{-1} cysteine increased the mitotic index to 12%. The mitotic index for co-administration of DMA and 50 μg ml^{-1} cysteine was only slightly different from that for co-administration of DMA and 25 μg ml^{-1} cysteine.

DMA inhibited cell proliferation in a dose-dependent manner below 100 μg ml^{-1} (0.72 mM) (Fig. 2). The IC 50 of DMA, i.e. the concentration resulting in a 50% decrease in cell population, was 130 μg ml^{-1} (0.94 mM), whereas that for DMA in the presence of cysteine was 20 μg ml^{-1} (0.14 mM). Inhibition of cell proliferation of DMA with cysteine was 6.5-fold higher than for a single administration of DMA alone.

DMA increased the mitotic index dose-dependently (Fig.

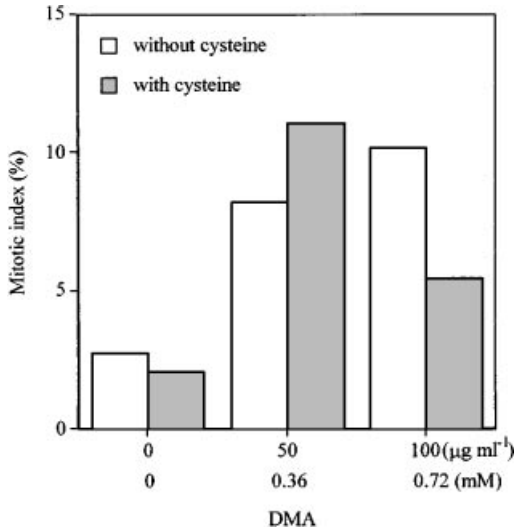

Figure 3. Effects of 50 μg ml^{-1} cysteine on mitotic index with DMA. The mitotic index (percent) was determined as the proportion of metaphase cells in 1000 cells. V79 cells were exposed to DMA alone or DMA and cysteine for 28 h (see Materials and Methods).

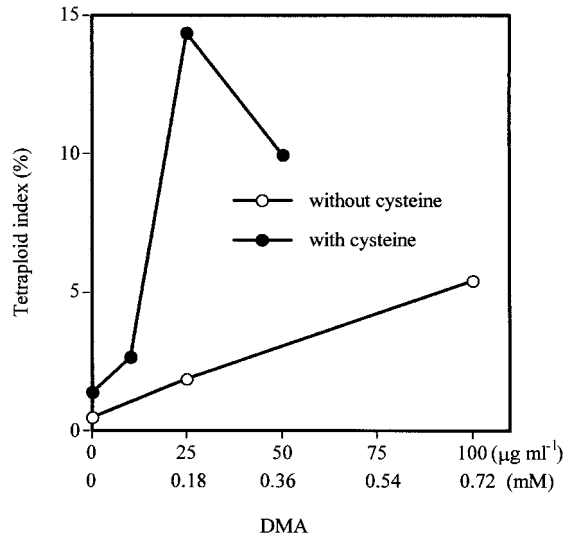

Figure 5. Effects of 50 μg ml^{-1} cysteine on tetraploid index for DMA. V79 cells were exposed to DMA or DMA and cysteine for 28 h (see Materials and Methods). Metaphase figures with 38 to 50 chromosomes were regarded as tetraploids.

Table 1. Effects of cysteine on frequency of aberrant cells induced by DMA[a]

DMA ($\mu g\ ml^{-1}$)	Cysteine ($\mu g\ ml^{-1}$)	Aberrant cells (%)							Total aberrant cells (%)
		ctg	ctb	cte	csg	cse	dic	att	
0	0	2	0	0	1	0	0	0	3
0	50	0	1	0	2	0	0	0	2
50	0	7	5	0	3	0	0	2	8
50	25	33	29	7	1	0	0	0	41*
50	50	36	18	2	0	0	0	0	45*
100	0	8	2	0	1	0	0	3	12*
100	25	44	10	0	4	0	0	12	48*
100	50	88	76	6	2	0	0	60	91*
MMC 0.01		18	7	7	0	0	1	0	28
MMC 0.05		39	25	42	3	5	2	0	60

[a] MMC: mitomycin C (positive control); ctg: chromatid gap; ctb: chromatid break; cte: chromatid exchange; csg: chromosome gap; cse: chromosome exchange; dic: dicentric; att: attenuation. Asterisk indicates.
* $p < 0.01$, compared with control results, using χ^2 test.

3). The mitotic index with co-administration of 50 $\mu g\ ml^{-1}$ DMA and 50 $\mu g\ ml^{-1}$ cysteine was higher than that with 50 $\mu g\ ml^{-1}$ DMA alone. DMA at 100 $\mu g\ ml^{-1}$ in the presence of 50 $\mu g\ ml^{-1}$ cysteine decreased the mitotic index to half of that for 50 $\mu g\ ml^{-1}$ DMA. This decrease in mitotic index might be due to cytotoxic effects of DMA and cysteine.

The AGT of the control was 16 h, whereas that of 100 $\mu g\ ml^{-1}$ DMA was 19 h, indicating that AGT was prolonged 3 h by DMA (Fig. 4). Most cells had divided only once. With co-administration of DMA and 50 $\mu g\ ml^{-1}$ cysteine, the AGT was 27 h. These results indicated that the increase in mitotic index by cysteine was due to enhancement of mitotic arrest of DMA.

With co-administration of 25 $\mu g\ ml^{-1}$ (0.18 mM) DMA and 50 $\mu g\ ml^{-1}$ cysteine, the tetraploid production was 14.3% higher and fivefold that with 25 $\mu g\ ml^{-1}$ DMA alone (Fig. 5). With co-administration of 50 $\mu g\ ml^{-1}$ DMA and 50 $\mu g\ ml^{-1}$ cysteine, the tetraploid index was reduced. This decrease in tetraploid index might also be due to cytotoxic effects of DMA and cysteine.

Effects of cysteine on chromosomal aberrations induced by DMA

Chromosomal aberrations induced in V79 cells treated with DMA and/or cysteine for 28 h without BrdU are summarized in Table 1.

Aberrant cells of control were 3%, and 50 $\mu g\ ml^{-1}$ DMA increased aberrant cells to 8%. This increase was not significant. DMA at 100 $\mu g\ ml^{-1}$ significantly increased aberrant cells to 12%. Chromatid breaks and chromatid gaps were the most frequent aberrations induced by DMA alone. The distribution of frequencies of total aberrant cells with DMA or co-administration of DMA and cysteine treatment

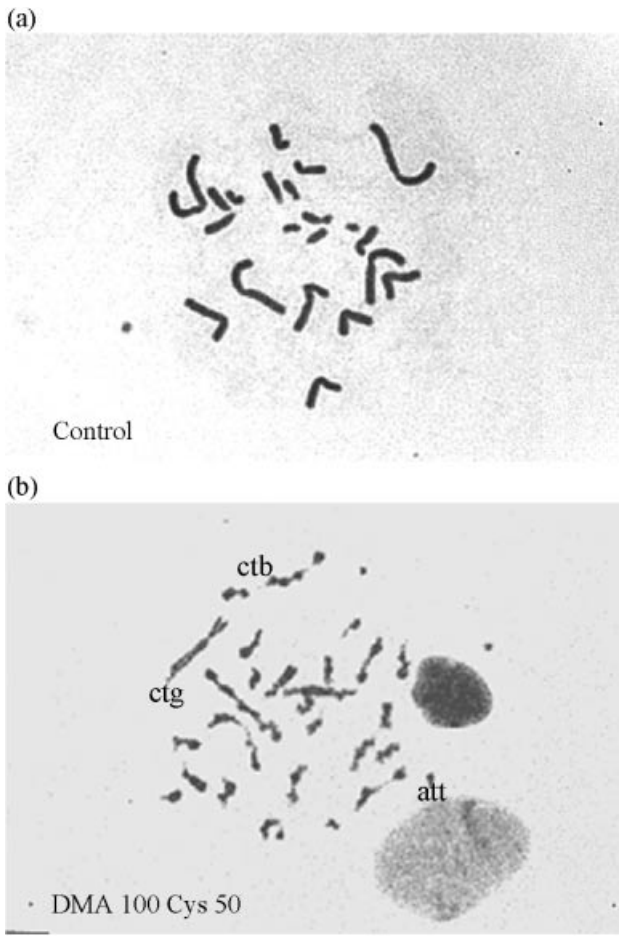

Figure 6. Effects of cysteine on clastogenicity of DMA: (a) control; (b) cells treated with 100 $\mu g\ ml^{-1}$ DMA and 50 $\mu g\ ml^{-1}$ cysteine. The attenuated chromosomal figures always had several chromatid gaps and chromatid breaks.

differed significantly from that control by the χ^2 test ($p < 0.01$).

Cysteine increased the chromosomal aberrations dose-dependently in V79 cells exposed to DMA. Chromosome aberration cells reached 91% at co-administration of 100 μg ml^{-1} DMA and 50 μg ml^{-1} cysteine. The types of chromosomal aberration of DMA with cysteine were the same as with DMA. DMA in the presence of cysteine produced many attenuated chromosomal figures (Fig. 6). Attenuation was observed only in the cells with co-administration of DMA and cysteine. On co-administration of 100 μg ml^{-1} DMA and cysteine, cysteine significantly and dose-dependently increased attenuation. Some 60% of mitotic figures exhibited attenuation in the case of co-administration of 100 μg ml^{-1} DMA and 50 μg ml^{-1} cysteine. All chromosomes with attenuations had chromosomal aberrations, such as gaps and breaks.

DISCUSSION

Co-administration of DMA and cysteine inhibited cell proliferation and increased mitotic index and prolonged AGT (Figs 1–4). These results suggested that cysteine enhanced mitotic arrest of DMA. DMA targets tubulin in mitotic cells to induce abnormal spindles.[8] Cysteine most likely reduces DMA(V) to DMA(III) and enhances those activities of DMA. In the experiment, the cells were exposed to DMA with serum-free medium for 2 h to avoid the compounds binding to serum protein. We compared aberrant cells in serum-free and serum-added medium, and the amounts of aberrant cells were 48% and 39% respectively. It was supposed that serum was not so effective at suppressing factors causing aberrations.

Oya-Ohta *et al.*[21] reported that DMA causes significant chromosomal aberration at a dose higher than 7×10^{-4} M (100 μg ml^{-1}) for 28 h in human umbilical cord fibroblasts. In the study, 12% aberrant cells were observed with 100 μg ml^{-1} DMA. Our results thus supported their findings. Moore *et al.*[17] reported that almost 10 000 μg ml^{-1} DMA was required to induce a genotoxic response in L5178 mouse lymphoma cells, and concluded that DMA was insufficient to be declared clastogenic. However, they exposed cells for only 4 h to DMA.

Attenuation was observed in the case of co-administration of DMA and cysteine (Fig. 6). Since attenuation was supposed to occur in non-staining parts of the chromosome, attenuation in many cases was not classified as a chromosomal aberration. However, all of the chromosomes with attenuation had chromosomal aberrations. It thus appeared that attenuation was due to structural chromosomal aberrations.

Cysteine increased chromosomal aberrations of DMA (Table 1). Glutathione enhanced cytotoxicity[20] and chromosomal aberrations of DMA.[21] Thiol compounds (cysteine, glutathione) that can reduce DMA(V) to DMA(III) are likely donors of electrons for reduction reactions that occur in the course of arsenic metabolism.[26–28] It appeared that cysteine enhanced cytotoxicity and chromosomal aberrations of DMA in the same way that glutathione did.

The concentrations of cysteine and glutathione in L-15 medium are 0.99 mM and 0 mM respectively. We did not know the concentrations of cysteine and glutathione in calf serum. However, it is reported that total cysteine and glutathione in human plasma is 0.12 mM and 0.06 mM respectively. Thus the concentration of cysteine in the medium is assumed to be about 1 mM. In this experiment, test compounds (DMA and/or cysteine) had been mixed before being added to the medium. It is not clear whether cysteine in the medium might enhance the cytotoxicity of DMA.

The main effects of DMA and cysteine on cells seemed to be different between high and low concentrations. Tetraploids appeared most at 25 μg ml^{-1} DMA with cysteine where cell viability was about 50%. At that concentration, toxic effects on cellular metabolism seemed to be weak, because tetraploids had to pass through the DNA synthesis phase twice and the increase of tetraploids is a result of mitotic arrest. Chromosomal aberrations were induced by DMA and/or cysteine when cell viability was reduced below 20%. At the concentrations used, induced chromosomal aberrations led to cell death and reduced cell viability.

DMA(III) has been detected in the urine of some humans exposed to inorganic arsenic in drinking water.[29–31] It was reported that cysteine concentrations in cultured rat astrocytes[32] and in human plasma[33] are 84.7 μg ml^{-1} and 14.4 μg ml^{-1} respectively. The concentration of cysteine used in our experiments is considered to be physiologically relevant. The concentration of cysteine is the highest among thiols in human blood[33] and can be supplied from food. Our results strongly suggest that cysteine reduces DMA(V) in the body to DMA(III). The findings reported here may provide important clues to the nature of human carcinogenesis.

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