EFFECT OF INORGANIC ARSENICS ON CYTOTOXICITY AND MUTAGENICITY OF ULTRAVIOLET LIGHT ON ESCHERICHIA COLI AND THE MECHANISM INVOLVED

SHOJI OKADA, KENZO YAMANAKA, HIROSHI OHBA,* AND YUTAKA KAWAZOE**

Department of Environmental Biochemistry, Shizuoka College of Pharmacy,* 2-2-1 Oshika, Shizuoka, 422, Japan and Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University,** 3-1 Tanabedori, Mizuho-ku, Nagoya, 467, Japan

(Received February 19, 1983)

The genetic effect of arsenite (AsO$_3^-$) and arsenate (HAsO$_4^{2-}$) on the cells of E. coli B (argF$^-$) tester strains was investigated. Both the arsenics, which did not show mutagenic effect on the tester strains, reduced UV-induced mutation frequency of H/r30R (wild-type; Exc$^+$Rec$^+$) cells, while they did not change that of Hs30R (uvrA$^{-}$; Exc$^-$Rec$^+$) cells. To elucidate the mechanism of this antimutagenicity, modifying effect of the arsenics on UV-induced cytoroxicity for Hs30R and NG30 (recA$^{-}$; Exc$^+$Rec$^-$) cells was examined. In a nutrient medium containing the arsenics, the survived cell fraction of NG30 after ultraviolet (UV) irradiation was markedly increased, whereas that of Hs30R was not altered. When the UV-exposed NG30 cells were subjected to recovery incubation in a nutrient medium containing the arsenics, the survived cell fraction was remarkably increased before the cell proliferation in a similar manner as liquid-holding recovery observed in an arsenic-free and non-nutrient medium in which DNA replication was suppressed. The arsenics were found to cause a delay of DNA replication in UV-irradiated NG30 cells. These results indicate that arsenite and arsenate enhance the error-free excision repair of UV-damaged DNA by retarding the DNA replication and thus by prolonging the period for excision repair. This may lead to the reduction in UV-induced mutation.

Keywords—arsenite; arsenate; UV mutagenesis; DNA repair; DNA replication; liquid-holding recovery; E. coli mutant

INTRODUCTION

Since the carcinogenicity of inorganic arsenics on human subjects has been pointed out by epidemiological investigations,$^1,2$ a number of experimental studies have been performed on mutagenicity of the arsenics.$^3$ Most of the studies using microorganisms have provided positive, although not so potent, results for the mutagenicity or co-mutagenicity of arsenics such as arsenite (AsO$_3^-$) and arsenate (HAsO$_4^{2-}$).$^4-5$ On the other hand, antimutagenic effect of arsenite has also been reported.$^6,7$ These findings may suggest that inorganic arsenics have an ability in initiating mutation in itself and, in addition, in modifying mutational processes to result in a decrease or an increase in mutation frequency, as proposed by Léonard and Louwerys,$^8$ and Ross-

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One of the authors (Y. K.)$^9$ recently reported that alkyl isocyanates including isothiocyanates were a sort of modifiers of mutability of microorganisms, i.e., they reduced the mutability of E. coli cells exposed to ultraviolet (UV) light without any relief of the cells from the lethal effect of UV irradiation, and that the mechanism may involve an enhancement of the excision repair of UV-damaged DNA, by prolonging the $G_1$-phase and thus giving a greater chance to carry out the excision repair.

Taking account of such modifying ability of chemicals in mutational and lethal processes, the present study was undertaken to clarify genotoxic effect of inorganic arsenics in connection with their cooperative effect on the mutagenesis and
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the lethality induced by UV irradiation in E. coli
cells.

MATERIALS AND METHODS

Arsenic and Bacterial Tester Strains — The
arsenics used were sodium arsenite (NaAsO₂) and
sodium arsenate (Na₃H₂AsO₄) of reagent grade
produced by E. Merck A. G., Darmstadt, and
Wako Pure Chemicals Co., Tokyo, respectively.
The tester strains of E. coli B (argF⁻) were a gift
from Professor S. Kondo of Osaka University,
School of Medicine; H/r30R [wild-type (Exc⁺Rec⁻)], Hs30R [uvrA⁻ (Exc⁻Rec⁺)] and
NG30 [recA⁻ (Exc⁺Rec⁻)]

UV Irradiation — Bacterial cells layered on an
agar plate or suspended in 1/15 M phosphate
buffer (pH 6.8) were irradiated with a 15 watt
National GL-15 germicidal lamp (Matsushita
Electric Co., Tokyo). The applied energies (J/m²)
were evaluated by reference to a correlation curve
of mutagenicity versus the UV dose applied,
which was calibrated with these tester strains.

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Strains and That under UV Irradiation (Exp. Pro-
cedure I and II) — These cells were grown to the
stationary phase in liquid nutrient broth (NB;
0.8% Difco nutrient broth containing 0.4% NaCl),
then starved in 1/15 M phosphate buffer (pH 6.8),
and an aliquot (0.1 ml) of the diluted
suspension containing approximately 5 × 10⁶ cells
was mixed with 3 ml of 0.8% molten top agar
(Difco agar) and layered on an NB plate containing
various concentrations of the arsenic in an 86
mm Petri dish. The colonies formed after 2 d
incubation at 37°C were counted (Exp. Procedure
I).

When the cooperative effect of the arsenics
with UV irradiation was tested, approximately
5 × 10⁶ cells were layered on an NB plate in the
same manner as described above. Then, the
layered cells were immediately irradiated with
UV light at doses of 7.8 (Hs30R) and 2.6 J/m²
(NG30), which gave 1.7% and 0.01% survivors,
respectively, when the arsenic was absent. The
irradiated cells were incubated at 37°C for 4 d,
and the colonies formed were counted as the
numbers of surviving cells (Exp. Procedure II).

Cooperative Effect of Arsenics on Viability and
Mutability of UV-Exposed H/r30R and Hs30R
Cells (Exp. Procedure III) — An aliquot of the
starved cell suspension was diluted with 1/15 M
phosphate buffer (pH 6.8) to a concentration of
approximately 5 × 10⁸ cells/ml, and 1 ml of this
suspension was mixed with 2 ml of 1.2% molten
top agar and layered on an SEM* plate containing
the arsenic. The layered cells were immediately
irradiated with various doses of UV light, then
incubated for 2 d at 37°C, and the revertant col-
oneys were counted. For the measurement of the
number of surviving cells after UV irradiation, a
diluted cell suspension (ca. 5 × 10⁸ cells/ml) of
1/15 M phosphate buffer, pH 6.8) was added to 3
ml of 0.8% molten top agar, layered on an SEM
plate, and irradiated with various doses of UV
light. After incubation for 1 d at 37°C, the col-
oneys formed were counted. Mutation frequency
was calculated as [M/N – M₀/N₀], where M and
M₀ are the numbers of revertant colonies per ml
on the UV-irradiated and unirradiated plates,
respectively, and N and N₀ are the numbers of the
surviving colonies per ml on the UV-irradiated
and unirradiated plates, respectively.

Recovery Incubation of UV-Exposed Hs30R and
NG30 Cells in Nutrient Medium Containing Arsenic
(Exp. Procedure IV) — A suspension (15 ml) of
the starved cells (ca. 5 × 10⁸ cells/ml of 1/15 M
phosphate buffer, pH 6.8) was irradiated with
UV light with stirring at doses of 5.2 (Hs30R)
and 1.3 J/m² (NG30), respectively, which
resulted in 0.04% survivors of both the strains
of cells. An aliquot (1 ml) of the irradiated cell
suspension was mixed with the NB medium con-
taining the arsenic and incubated at 37°C for 0 to
5.5 h with shaking. Then, the cell suspension was
added to 0.8% molten top agar, layered on an
arsenic-free NB plate, and incubated at 37°C. The

* SEM (semi-enriched medium); a mixture of Difco agar (12 g) and the medium E₁₂ (16 ml) in 750 ml of deionized water
was supplemented with 40% glucose (8 ml) and liquid NB (40 ml).
colonies formed after 1 d were counted.

Recovery Incubation of UV-Exposed NG30 Cells in Non-nutrient Medium Containing Arsenic (Exp. Procedure V) — To 3 ml of the starved NG30 cell suspension was added 12 ml of 1/15 M phosphate buffer (pH 6.8). The mixture containing approximately $1 \times 10^7$ cells/ml was irradiated with UV light at a dose of 1.3 J/m² which gave 0.06% survivors. The irradiated cells were diluted with the phosphate buffer containing the arsenic and incubated at 37°C for 6 h. At appropriate intervals during the incubation, 1 ml of this suspension was added to the molten top agar and layered on an arsenic-free NB plate. The colonies formed after 1 d were counted.

$[^3]$H]Thymidine Incorporation of UV-Irradiated NG30 Cells (Exp. Procedure VI) — The starved NG30 cell suspension (15 ml) was irradiated with UV light at a dose of 1.1 J/m² giving 0.25% survivors. The irradiated cell suspension was diluted with 9 volumes of liquid SEM containing [6-$[^3]$H]thymidine (NEN; 1 μCi/0.01 μmol/ml), 2-deoxyadenosine (P-L Biochemicals; 0.1 mg/ml), and the arsenic. The mixture was incubated at 37°C for 150 min with shaking. During the incubation, aliquots (0.2 ml each) were withdrawn at several times and added to an ice-cold solution (1 ml) of bovine serum albumin (Wako Pure Chemicals; 1 mg/ml), and then precipitated by adding 3 ml of ice-cold 10% trichloroacetic acid. The precipitate, left for 3 h by chilling, was filtered with a Whatman GF/C glass filter, washed with 5% trichloroacetic acid (20 ml) and then with 95% ethanol (2 ml), dried in the air, and counted for the $[^3]$H-radioactivity in the toluene-dioxane-based scintillation fluid with a liquid scintillation counter (Aloka LSC-661).

RESULTS

Lethal Effect of Arsenics on E. coli B Tester Strains (Exp. Procedure I)

When the cells were layered on the NB plate containing arsenite or arsenate at various concentrations, the dose-response curve shown in Fig. 1 was obtained between the survival fractions and the molar concentrations of arsenics. No appreciable decreases in survival fractions were observed for all the strains tested below 5 mM arsenite. As for arsenate, no decreases were found for H/r30R and Hs30R below 10 mM and 30 mM, respectively. For NG30, 10 and 20 mM arsenate caused only a slight decrease in the survival fractions.

FIG. 1. Lethal Effect of Arsenite (●) and Arsenate (■) on E. coli B Tester Strains
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In the experiments for the cooperative effect of arsenics on UV irradiation in the present study, therefore, the concentrations of 2 mM arsenite and 10 mM arsenate were principally used in order to avoid confusions which might come from an appreciable killing effect of arsenics themselves. It is worth noting that these concentrations of arsenics showed no appreciable mutagenicities in any of the tester strains examined.

Effect of Arsenics on Viability and Mutability of UV-Exposed H/r30R and Hs30R Cells (Exp. Procedure III)

Arsenate and arsenate were tested for the cooperative effect on UV mutagenesis of H/r30R

![Graphs showing effect of arsenic on viability and mutability](image)

FIG. 2. Effect of Arsenics on Viability (a and b) and Mutability (c and d) of UV-Exposed H/r30R (a and c) and Hs30R (b and d) Cells

- ○ control, ● 2 mM arsenite, ■ 10 mM arsenate.
(Exc\(^{+}\)Rec\(^{+}\)) and Hs30R (Exc\(^{-}\)Rec\(^{+}\)) tester strains.* UV light was applied to the cells layered on the NB plate containing 2 mM arsenite or 10 mM arsenate. As shown in Fig. 2, a remarkable decrease in mutation frequency of H/r30R cells was observed; 1/10—1/50 with 2 mM arsenite and 1/2—1/5 with 10 mM arsenate compared to control (Fig. 2c). Whereas, the lethality was not appreciably affected by the presence of arsenic (Fig. 2a), indicating that any relief from the lethal effect of UV irradiation was not provided. On the other hand, with regard to Hs30R cells which are deficient of the excision repair system, neither the mutagenic nor lethal effect of UV irradiation was remarkably affected by the presence of arsenic, although the slope of mutation frequency in the presence of arsenate was a little different from that of control (Fig. 2b and 2d). The difference between the effect of arsenics on these two tester strains after UV irradiation may suggest that the arsenics affected the DNA repair processes from UV-induced damage.

**Effect of Arsenics on Viability of Hs30R and NG30 Cells under UV Irradiation (Exp. Procedure II)**

In order to elucidate the mechanism for the arsenic-induced decrease in UV mutagenesis, two types of the repair enzyme-deficient strains, Hs30R (Exc\(^{-}\)Rec\(^{+}\)) and NG30 (Exc\(^{+}\)Rec\(^{-}\)) were used for looking over the repairing events after UV irradiation. The UV irradiation was made at doses of 7.8 J/m\(^2\) for Hs30R and 2.6 J/m\(^2\) for NG30, which gave the survival fractions of 1.7% and 0.01%, respectively. The UV dose which gave such a low survival fraction of NG30 was applied because the arsenics showed more marked effects on this strain at higher doses of UV (data not shown); the cause of this phenomenon is to be elucidated in the further study.

Fig. 3 shows the survival fractions, normalized by those in the absence of arsenic, of both the

FIG. 3. **Effect of Arsenie (●) and Arsenate (■) on Viability of Hs30R (a) and NG30 (b) Cells under UV Irradiation**

UV light was irradiated at doses of 7.8 J/m\(^2\) for Hs30R and 2.6 J/m\(^2\) for NG30 which gave surviving fractions of 1.7% and 0.01%, respectively.

* Mutagenicity on NG30 (Exc\(^{+}\) Rec\(^{-}\)) was not tested because NG30 is known to be unmutable after UV irradiation due to lack of the error-prone post-replication repair system.
strains after the arsenic treatment following UV irradiation. With regard to Hs30R, no appreciable difference was found between the normalized survival fractions of the cells treated with the arsenic after UV irradiation (Fig. 3a) and those just treated with the arsenic without UV irradiation (Fig. 1b). On the other hand, the number of survived cells of NG30 after UV irradiation was markedly increased by the presence of arsenic; either 1 - 10 mM arsenite or 10 - 30 mM arsenate caused 20 - 100 times increase in the number of survived cells (Fig. 3b).

These results suggest that some part of UV-damaged fraction of NG30 cells, which would be destined to death, might have escaped from death. Such the phenomenon might be attributed to an enhancement of the DNA repair capacity of the damaged NG30 cells by the arsenics. Since the excision repair system is proficient in NG30 and deficient in Hs30R, on the latter the arsenic did not affect, it is likely that the arsenics enhanced the capacity in the excision repair of UV-damaged DNA.

**Effect of Recovery Incubation in Nutrient Medium Containing Arsenic on Viability of UV-Exposed Hs30R and NG30 Cells (Exp. Procedure IV)**

To confirm the suggestion that the arsenics enhanced the excision repair of damaged DNA, the time course of recovery of UV-damaged cells was determined by a recovery incubation in the presence of arsenic. The cells of Hs30R and NG30 in phosphate buffer were exposed to UV light, giving 0.04% survivors of both the strains, and were immediately resuspended in the NB medium containing 2 mM arsenite or 10 mM arsenate. The numbers of the survived cells were determined at appropriate intervals by layering the cells on arsenic-free NB plate followed by counting the colonies formed.

As shown in Fig. 4a, UV-exposed Hs30R cells in the arsenic-containing NB medium were delayed by about 2 h to get into the proliferation when compared to the cells in the arsenic-free NB medium. This delay might be attributed to a corresponding delay of DNA replication as described later. On the other hand, the time course changes for NG30 cells were remarkably different from those found for Hs30R cells (Fig. 4b). The number of surviving cells in the arsenic-containing medium increased promptly, whereas those in the arsenic-free medium started their proliferation with a lag time of about 2 h. Since it is unlikely that the cell proliferation would start at such an early stage of the incubation by the presence of arsenic, it seems reasonable to realize that the increase in the number of the cells capable of forming colony comes from the recovery of the cells which would be destined to death without the arsenic.

These results suggest that the arsenic treatment following UV irradiation prevents the cells to get into S-phase for a certain period, resulting possib-
ly in giving the cells a more chance for the excision repair of DNA damage induced by UV irradiation.

Effect of Recovery Incubation in Non-nutrient Medium Containing Arsenic on Viability of UV-Exposed NG30 Cells (Exp. Procedure V)

The Exc⁺Rec⁻ strain (NG30) has been known to have an ability of liquid-holding recovery from UV-induced DNA damage in non-nutrient medium i.e., the excision repair of the damage proceeds during the recovery incubation in non-nutrient phosphate buffer in which DNA replication cannot take place.¹³) This type of recovery incubation might be useful to elucidate the mechanism for the arsenic-induced increase in the excision repair of UV-damaged DNA.

As shown in Fig. 5, regardless of the presence or absence of the arsenics, increasing relieves of the damaged cells from death were observed from the beginning of the incubation. The time courses of the increase in the presence of arsenic were almost identical to that in the absence of arsenic. This finding accounts for the phenomenon observed in the nutrient broth containing the arsenic as follows; the enhancement of excision repair of UV-damaged DNA by the arsenics was caused not by the direct activation of the repair enzyme system but by the suppression of DNA replication so as to give the damaged cells a more chance for the excision repair before DNA replication.

Delay of DNA Replication Synthesis induced by Arsenics (Exp. Procedure VI)

To see the effect of arsenics on the replication synthesis of DNA more directly, the incorporation of [³H]thymidine into DNA of UV-irradiated NG30 cells in SEM containing the arsenic was determined. As indicated in Fig. 6, a remarkable delay of DNA synthesis of the cells treated with the arsenic was observed; more with 2mM arsenite and less with 10 mM arsenate, compared with the control cells. Since [³H]thymidine incorporation mainly reflects DNA replication synthesis in this experimental condition, this result reveals that the arsenics retard the first DNA synthesis after UV irradiation which, in the absence of the arsenic, arose without delay even when a considerable fraction of the cells was

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**FIG. 5. Effect of Recovery Incubation in Non-nutrient Phosphate Buffer Containing Arsenic on Viability of UV-Exposed NG30 Cells**

UV light was irradiated at a dose of 1.3 J/m² giving a surviving fraction of 0.06%. ○ control, ● 1 mM arsenite, ■ 10 mM arsenate.

**FIG. 6. Incorporation of [6-³H]Thymidine into DNA of UV-Irradiated NG30 Cells in SEM Containing Arsenic**

UV light was irradiated at a dose of 1.1 J/m² giving a surviving fraction of 0.25%. ○ control, ● 2 mM arsenite, ■ 10 mM arsenate.
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killed as indicated by the control curve in Fig. 6.

DISCUSSION

The present study demonstrates that the inorganic arsenics, arsenite and arsename, which were not mutagenic as far as the E. coli B tester strains used here are concerned, reduced the UV-induced mutation frequency of the wild-type cells (Hfr30R). The experiments on the modification by the arsenics on the UV-induced cytorotoxicity for NG30 (Exc+Rec−) and Hs30R (Exc−Rec+) strains strongly suggested that this antimutagenicity was derived from the enhancement of the error-free excision repair of UV-damaged DNA of the cells. Furthermore, this enhancement was estimated to be a result from the delay, caused by the arsenics, of DNA replication synthesis after UV irradiation. That is, by retarding the first DNA synthesis for replication, the UV-irradiated cells, which are proficient of the excision repair enzyme system, were given a more chance for the error-free repair of the UV-induced DNA damage, leading to reduction in the mutation frequency and increase in the surviving cell fraction. It is not likely that the arsenics directly stimulated the enzyme system for excision repair, because the experiment on liquid-holding recovery from the UV damage in NG30 cells showed no difference between the presence and absence of arsenic.

These observations on the arsenics are quite similar to those on antimutagenic alkyl isocyanates previously reported. It is, therefore, very probable that a common mechanism is operating for the action of both of the arsenics and the isocyanates, which is of particular interest.

With regard to the arsenics, recent papers by Rossman et al. and Tanee et al. also indicated the antimutagenic effect of arsenite. On the other hand, however, a number of reports have demonstrated that inorganic arsenics are mutagenic or co-mutagenic. A possible speculation, proposed by Rossman, is that arsenite has two actions on bacterial gene, i.e., one is co-mutagenic effect which appears at its low concentrations and the other is antimutagenic effect which appears at its higher concentrations resulting from the inhibition of error-prone SOS repair system. The latter possibility, at least, is not in conflict with our present conclusion, although a more detailed mechanism remains to be solved.

On the other hand, for further considerations on the genetic effect of inorganic arsenics, it may be required to know the actions of arsenic metabolites produced in cells or tissues. Since McBride and Wolfe proposed the metabolic pathway of arsenate, i.e., reduced and methylated to produce dimethyl compounds, a number of investigations have evidenced this type of the pathway in microorganisms and mammals. The effects of these metabolites on mutagenesis are now being investigated in our laboratory and will be reported in the near future.

Acknowledgement We are greatly indebted to Professor S. Kondo of Osaka University, School of Medicine, for the kind gift of E. coli tester strains. We also thank Dr. K. Takahashi of Nagoya City University, Faculty of Pharmaceutical Sciences, and Mr. A. Hirai of Shizuoka College of Pharmacy for their technical contributions.

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