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Adaptive Response of *Vibrio cholerae* and *Escherichia coli* to Nitrofurantoin

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Pretreatment with sublethal doses of nitrofurantoin induced adaptive response in both *Vibrio cholerae* and *Escherichia coli* cells as indicated by their greater resistance to the subsequent challenging doses of the same drug. Adaptive response was maximum corresponding to pretreatment drug concentrations of 0.40 μg/ml and 0.015 μg/ml respectively for *V. cholerae* OGAWA 154 (wild type) and *E. coli* K-12 AB 2463 (*recA*$^-$) cells. Adaptive response was inhibited by chloramphenicol (100 μg/ml) indicating the need of concomitant protein synthesis. Induction of adaptive response in *recA* deficient *E. coli* cells indicated that it was different from the conventional "SOS" response. Melting temperature of DNA of *V. cholerae* cells subjected to adaptive (0.4 μg/ml for 1 hr) and challenging (120 μg/ml for 1 hr) doses of nitrofurantoin (76°C) was closer to that of native DNA (75°C) vis-a-vis DNA isolated from nonadapted and drug treated cells (77.5°C). Also, DNA isolated from *V. cholerae* cells subjected to adaptive and challenging doses of the drug revealed the presence of fewer interstrand cross-links (16% reversible DNA) vis-a-vis DNA from nonadapted but drug treated cells (55% reversible DNA). Photomicrographic studies revealed that *V. cholerae* cells that were nonadapted but drug treated grew into long filamentous forms (4.25 ± 2.97 μm) whereas those subjected to both adaptive and challenge doses of the drug exhibited much less filamentation (2.08 ± 0.84 μm) vis-a-vis native cells (1.42 ± 0.5 μm). Similar results on DNA melting temperature, cross-links in DNA, and filamentation of cells were obtained for *E. coli* AB 2463 (*recA*$^-$) cells subjected to adaptive and challenging treatments with nitrofurantoin. Almost equal degree of resistance against nitrofurantoin could be induced in both *V. cholerae* OGAWA 154 (wild type) and *E. coli* strain PJ3 (AB 1157 *ada*$^-$) when these cells were pretreated with nontoxic doses of hydrogen peroxide or nitrofurantoin. Evidence obtained in this work on the nature of the nitrofurantoin induced adaptive response with particular references to the oxidative and/or alkylating DNA damages were discussed. Nitrofurantoin induced adaptive response appeared similar to that elicited by furazolidone in *V. cholerae* cells and appeared to be directed towards oxidative and not alkylating adaptive repair pathway.

Key words: *adaptive response, Vibrio cholerae, Escherichia coli,* nitrofurantoin

INTRODUCTION

Nitrofurantoin, N-(5-Nitro-2-furfurylidene)-1-aminohydantoin, is one of the members of the group of synthetic nitrofurans and is principally used as an urinary tract disinfectant [Paul and Paul, 1964; Chamberlain, 1976; McCalla, 1983]. Chessin et al. [1978] and Lu and McCalla [1979] reported the relative toxicities of several nitrofurans including nitrofurantoin and showed that the mutagenic activities of these drugs parallelled toxicity in general. Nitrofurantoin was also shown to be *rec*-test positive [Kada et al., 1972; Tazima et al., 1975] in different bacterial systems and was also mutagenic in *E. coli* WP_2 strains [McCalla and Voutsinos, 1974; McCalla, 1983]. Recent studies [Obaseiki-Ebor, 1984; Obaseiki-Ebor and Akerelae, 1986] attempted to explain the basis of mutation caused by nitrofurantoin in both *E. coli* and *S. typhimurium* cells. The drug was shown to inhibit cell division and produced interstrand cross-links in *V. cholerae* and *E. coli* DNA [Mukherjee et al., 1990]. DNA bearing nitrofurantoin induced interstrand cross-links was also shown to have acquired greater thermal stability than untreated or native DNA.

This communication presents results of our investigation which shows that treatment with nontoxic concentrations of nitrofurantoin induced adaptive response in *V. cholerae* OGAWA 154 and *E. coli* strains. The adaptive response was also induced in *E. coli* K-12 strain AB 2463 (*recA*$^-$) and was different from "SOS" response which is dependent on the functional *recA* gene. *E. coli* PJ3 (AB 1157 *ada*$^-$) and *V. cholerae* OGAWA 154 could also be adapted against challenging doses of nitrofurantoin when pretreated with

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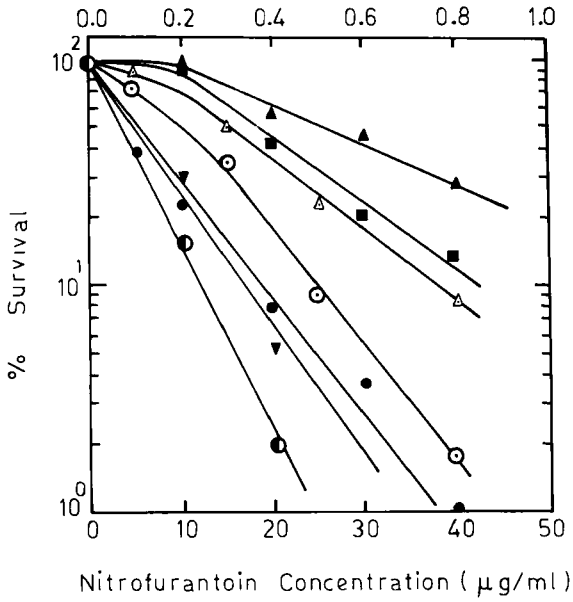

Fig. 1. Survival of adapted or nonadapted wild type cells of *V. cholerae* OGAWA 154 (filled symbols) and repair deficient cells of *E. coli* AB 2463 (*recA*$^-$) (open symbols) upon treatment with challenging doses of nitrofurantoin for 1 hr at 37°C in dark. *V. cholerae* cells were adapted by 1 hr treatment in dark at 37°C with nitrofurantoin at concentrations: 0.0 μg/ml in absence (●—●) or in presence of 100 μg/ml chloramphenicol (▼—▼); 0.2 μg/ml in absence of chloramphenicol (■—■) and 0.4 μg/ml in absence (▲—▲) or in presence of 100 μg/ml chloramphenicol (◐—◐). The adaptive doses of nitrofurantoin to *E. coli* AB 2453 (*recA*$^-$) cells were 0.0 μg/ml (⊙—⊙) and 0.01 μg/ml (△—△) respectively for 1 hr in dark in absence of chloramphenicol. The lower abscissa scale refers to *V. cholerae* and the upper one to *E. coli* cells respectively. Each experimental point in the figure represented the average value of three independent experiments.

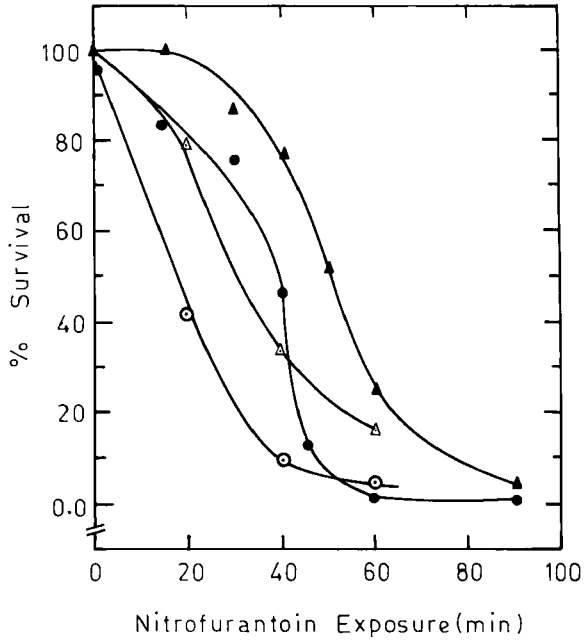

Fig. 2. Loss of viability with time of treatment in dark with challenging doses, 40 μg/ml for *V. cholerae* OGAWA 154 (wild type; filled symbols) and 0.8 μg/ml for *E. coli* AB 2463 (*recA*$^-$) (open symbols) cells, of nitrofurantoin. *V. cholerae* cells were adapted previously to the drug doses of 0.0 μg/ml (●—●) and 0.4 μg/ml (▲—▲) and *E. coli* AB 2463 (*recA*$^-$) cells to the drug doses of 0.0 μg/ml (⊙—⊙) and 0.01 μg/ml (△—△) for 1 hr in the dark.

nontoxic doses of the drug or hydrogen peroxide, which indicated that the adaptive response was not similar to that induced by an alkylating agent; rather nitrofurantoin induced adaptive response appeared similar to that elicited by furazolidone in *V. cholerae* cells and appeared to be directed at oxidative damage. The adapted cells grew more resistant to the subsequent challenging doses of the drug, acquired fewer DNA interstrand cross-links, exhibited much less increase in DNA melting temperature, and underwent significantly less filamentation than the nonadapted cells on being subjected to subsequent challenging doses of the drug.

MATERIALS AND METHODS

Bacterial Strain

The bacterial strains used in this study were 1) *V. cholerae* (classical) strain OGAWA 154, obtained through the kind courtesy of Dr. S.C. Pal, Director, National Institute for Cholera and Enteric Diseases, Calcutta, and 2) *E. coli* K-12 strain AB 2463 (*recA* 13) and *E. coli* PJ3 (AB 1157 *ada*$^-$), obtained through the kind courtesy of Dr. Barbara J. Bachmann, *E. coli* Genetic Stock Center, (New Haven, CT, U.S.A.).

Culture Medium

The culture medium used was nutrient broth (NB) containing 1) bacto-peptone (Difco, Michigan, U.S.A. 10 g); 2) beef extract (Oxoid, Hampshire, England 10 g); 3) NaCl (5 g) in 1 litre distilled water, pH adjusted to 7.0 for *E. coli* strain and 8.0 for *V. cholerae* OGAWA 154 strain. Nutrient agar (NA) plates were prepared by adding 1.3% bacto-agar (Difco) to the NB medium.

Chemicals

Chemically pure nitrofurantoin (NFT) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Chemically pure furazolidone was obtained as a gift sample from Smith, Kline and French (India) Ltd, Bombay. Hydroxyapatite (HAP) crystals (Bio-Gel HTP, DNA grade) was purchased from Bio-Rad Laboratories, U.S.A. Hydrogen peroxide (H_2O_2) and all other chemicals used were of analytical grade.

Cytotoxicity of the Drug

A 1.0 ml aliquot of the log-phase cells was inoculated into 20 ml culture medium containing different amounts of drug. The bacteria in each drug containing medium were incubated at 37°C in the dark for 1 hr and then immediately assayed for viability on drug free nutrient agar plates after

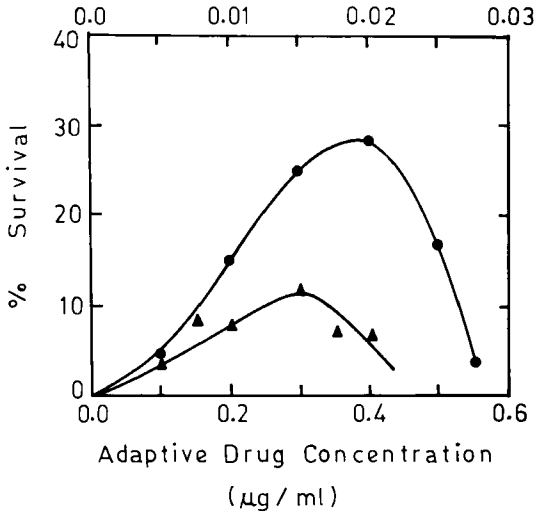

Fig. 3. Changes in adaptive response of wild type *V. cholerae* OGAWA 154 (●—●; lower abscissa scale) and *E. coli* AB 2463 (*recA*$^-$) (▲—▲; upper abscissa scale) cells with change in drug dose used for adaptation. The adapted or nonadapted cells were subjected to a challenging dose of 40 μg/ml *(V. cholerae)* or 0.8 μg/ml *(E. coli)* of nitrofurantoin for 1 hr in dark. For adaptation, treatment with appropriate drug concentration for 1 hr in dark was made. Adaptive response is expressed in terms of percentage increase in survival of the adapted cells over the nonadapted ones.

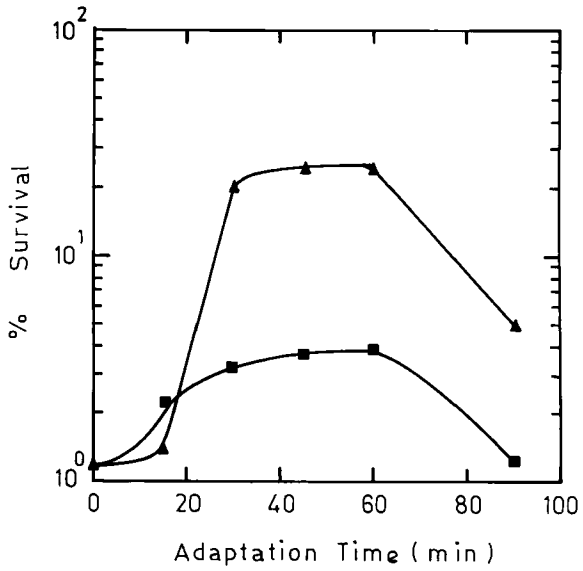

Fig. 4. Dependence of nitrofurantoin induced adaptive response on the adaptation time. *V. cholerae* OGAWA 154 cells were subjected to adaptive drug concentrations of 0.4 μg/ml (▲—▲) or 0.55 μg/ml (■—■) for different periods of time and then to the challenging drug concentration of 40 μg/ml for 1 hr. Adaptive response is expressed in terms of percentage increase in survival of the adapted cells over the nonadapted ones.

appropriate dilution. For determining the kinetics of inactivation, 1.0 ml of log-phase cells in NB medium was inoculated into a fresh 20 ml of the same culture medium containing a fixed amount of the drug and incubated at 37°C in dark. At intervals, aliquots were plated on drug free NA plates and colonies counted after 18–24 hr of incubation at 37°C.

Adaptation treatment of *V. cholerae*, *E. coli* AB 2463 (*recA*$^-$), and *E. coli* PJ3 (AB 1157 *ada*$^-$) cells were performed in dark at 37°C in presence of nontoxic concentration of nitrofurantoin for (1 hr) or hydrogen peroxide (for 30 min) and then challenged with toxic drug concentration for 1 hr. Effect of chloramphenicol during adaptation was also studied in the case of *V. cholerae* cells. In this case after completion of 1 hr adaptation treatment in presence of chloramphenicol, cells were collected by centrifugation, washed in 0.85 M NaCl, resuspended in fresh NB medium, and then challenged with toxic concentration of drug for 1 hr in dark at 37°C.

Light Microscopy

For microscopic observation, cells were harvested by centrifugation (5,000*g*), resuspended, and fixed in phosphate-buffered saline (PBS), pH 7.4 containing paraformaldehyde (4%) for 30 min at 4°C, washed in distilled water, smeared on glass slides, air dried, and stained with methylene blue. The slides were then washed in running water, air dried, and photographed by an optical microscope (Leitz, Wetzlar, Germany). Measurement of cell lengths was done by a micrometer eyepiece (accuracy ± 0.1 mm) on optically enlarged prints.

DNA Isolation and Assay of Reversible DNA

DNA was isolated from bacterial cells (native, nonadapted but drug treated, adapted and drug treated) and the purity was assayed following the methods described earlier [Marmur, 1961; Chatterjee et al., 1983]. The isolated DNA was subjected to thermal denaturation-renaturation treatment (DRT) after brief shearing by ultrasonic radiation [Cole, 1970; Chatterjee et al., 1983]. Cross-linked DNA was estimated in terms of the fraction of the isolated DNA which was rendered reversibly bihelical [Geiduschek, 1961] after DRT, as monitored by thermal chromatography through HAP column [Rutman et al., 1969].

Hydroxyapatite (HAP) Chromatography

Sonically sheared DNA, isolated from untreated or drug treated adapted or nonadapted cells, was subjected to DRT as described above and was then absorbed to HAP column equilibriated to 60°C [Miyazawa and Thomas, 1965]. Fractions (3 ml) were eluted by passing through the column 0.12 M phosphate buffer (pH 6.8) containing 0.1 M saline (for single stranded DNA) [Raptis and Pierre, 1981] or 0.4 M phosphate buffer (pH 6.8) containing 0.1 M saline (for double stranded DNA) and monitored by measurement of absorption at 260 nm. The double or single stranded DNA

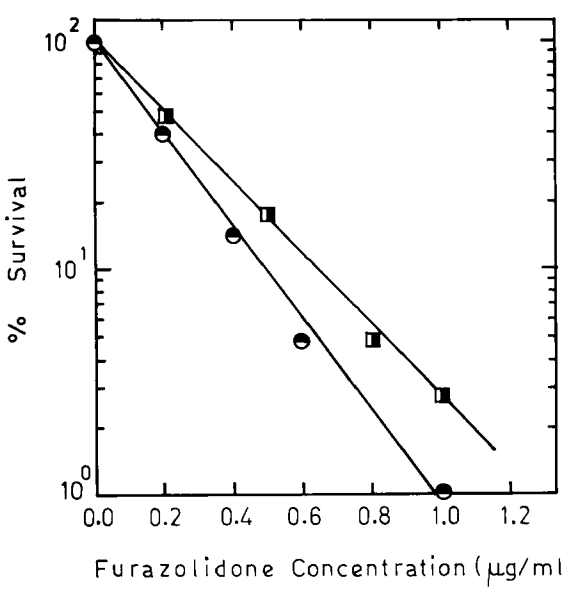

Fig. 5. Survival of adapted (▮—▮) or non adapted (●—●) *V. cholerae* OGAWA 154 cells upon subsequent challenge with different concentrations of furazolidone for 1 hr in dark. Cells were adapted by 1 hr treatment in dark at 37°C with nitrofurantoin of concentration 0.4 μg/ml.

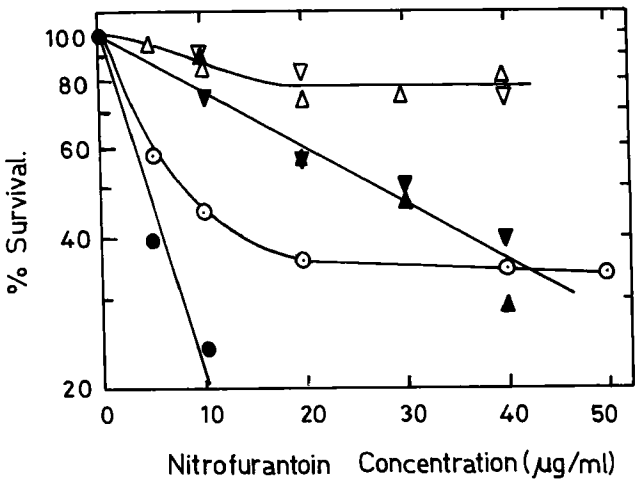

Fig. 6. Survival of adapted (at 37°C in dark) or nonadapted cells of *V. cholerae* OGAWA 154 (wild type) and *E. coli* PJ3 (AB 1157 *ada*$^-$) after treatment (for 1 hr at 37°C, in dark) with different challenging doses of nitrofurantoin. Nonadapted cells of *V. cholerae* OGAWA 154 (●—●); *V. cholerae* OGAWA 154 cells adapted by 30 min treatment with 30.0 μM hydrogen peroxide (▼—▼); and *V. cholerae* OGAWA 154 cells adapted with 0.4 μg/ml nitrofurantoin for 1 hr (▲—▲). Nonadapted cells of *E. coli* PJ3 (AB 1157 *ada*$^-$) (⊙—⊙); *E. coli* PJ3 (AB 1157 *ada*$^-$) adapted with 30 μM hydrogen peroxide for 30 min ((▽—▽) and *E. coli* PJ3 (AB 1157 *ada*$^-$) cells adapted with 1.0 μg/ml nitrofurantoin for 1 hr (△—△).

contents were determined by plani metric measurement of the areas under the respective peaks [Basak and Chatterjee, 1984].

Estimation of Cross-links

Assuming that the cross-links follow a Poisson distribution, approximate estimation of the average number (Z) of cross-links per DNA fragment was made from the relation $e^{-Z} = D/(D + R)$, where D is the percentage denatured or nonreversible (single stranded) and R is the percentage reversible (double stranded) DNA [Cole, 1970; Basak and Chatterjee, 1984]. D and R can be estimated, as described before from HAP chromatographic experiment.

Determination of Melting Temperature

The thermal denaturation and renaturation profile of DNA isolated from untreated and NFT treated adapted and nonadapted *V. cholerae* and *E. coli* AB 2463 (*recA*$^-$) cells were determined by measurement of absorbance at 260 nm by the UV-Vis spectrophotometer, PMQ II (Carl Zeiss, Germany) coupled to a circulating thermostat controlled (±0.01°C) water bath as described previously [Chatterjee and Ghosh, 1979]. The melting temperature (T_m) was defined as the temperature corresponding to which the hyperchromicity, $h(A_T/A_{25})$, is given by $h = 1/2\ (h_{max} + 1)$, where h_{max} is the maximum hyperchromicity.

RESULTS

The nitrofurantoin dose dependent survivals of adapted and nonadapted cells of *V. cholerae* OGAWA 154 and *E. coli* AB 2463 (*recA*$^-$) are presented in Figure 1. For *V. cholerae* cells adapted with 0.4 μg/ml of nitrofurantoin for 1 hr, about a fivefold increase in 37% survival dose (D_{37}) was noted vis-a-vis the nonadapted ones. When the cells were adapted with 0.2 μg/ml of drug for 1 hr, only a threefold increase in D_{37} of the adapted cells was obtained vis-a-vis the nonadapted ones. The D_{37} values of the adapted cells of *E. coli* AB 2463 (*recA*$^-$) increased by about 1.5 times that of the nonadapted ones. For nonadapted *V. cholerae* cells pretreatment or nontreatment with chloramphenicol, 100 μg/ml, did not make much difference in respect of survivals against challenging doses of nitrofurantoin. However, for cells adapted with 0.4 μg/ml of the drug for 1 hr, the D_{37} value of the challenged *V. cholerae* cells adapted in absence of chloramphenicol, 100 μg/ml, was sixfold higher than that of those adapted in presence of chloramphenicol.

The kinetics of loss of viability with time of challenge drug treatment for nonadapted and adapted cells of *V. cholerae* and *E. coli* AB 2463 (*recA*$^-$) are shown in Figure 2. At different times of exposure to a challenging drug dose, the adapted cells exhibited a greater resistance against nitrofurantoin treatment. The adapted (0.4 μg/ml, 1 hr) cells of *V. cholerae* showed practically no loss of viability up to 15 min treatment with the challenging drug concentration of 40 μg/ml.

Figure 3 presents the optimum condition of adaptation for *V. cholerae* OGAWA 154 (wild type) and *E. coli* AB 2463 (*recA*$^-$). For *V. cholerae* cells, maximum adaptive response was obtained corresponding to an adaptive drug concentra-

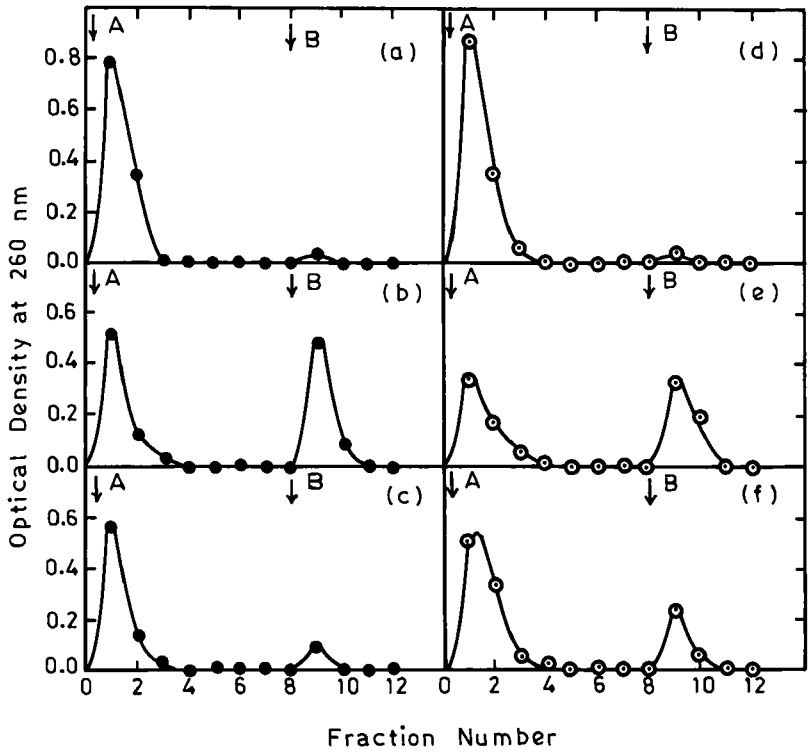

Fig. 7. Thermal chromatography (60°C) through HAP column of DNA isolated from adapted and nonadapted, nitrofurantoin challenged or unchallenged cells of *V. cholerae* OGAWA 154 (●—●) and *E. coli* AB 2463 (*recA*$^-$) (⊙—⊙). Before chromatography, the isolated DNA was sheared by sonication and subjected to thermal denaturation and renaturation treatment (DRT). Single stranded DNA fractions were eluted from HAP column by 0.12 M phosphate buffer containing 0.1 M NaCl and subsequently double stranded DNA fractions were eluted by 0.40 M phosphate buffer containing 0.1 M NaCl. A and B denote the starting points of elution of single and double stranded DNA as obtained after standardization of the column following Bernardi [1969]. The chromatographic patterns are representative of DNA isolated from **a:** *V. cholerae* cells nonadapted and untreated; **b:** *V. cholerae* cells nonadapted but drug treated (120 μg/ml for 1 hr); **c:** *V. cholerae* cells adapted (0.4 μg/ml for 1 hr) and drug treated (120 μg/ml for 1 hr); d: *E. coli* (*recA*$^-$) cells nonadapted and untreated; e: *E. coli* (*recA*$^-$) cells nonadapted and drug treated (80 μg/ml for 1 hr); and f: *E. coli* (*recA*$^-$) cells adapted (0.01 μg/ml for 1 hr) and drug treated (80 μg/ml for 1 hr). Single and double stranded DNA fractions were estimated from the planimetric measurements of areas under the respective peaks.

tion of 0.4 μg/ml for 1 hr treatment and for *E. coli* AB 2463 (*recA*$^-$) cells was 0.015 μg/ml for 1 hr treatment.

For a fixed challenge dose, 40 μg/ml for 1 hr, adaptive response of *V. cholerae* depended on the time of adaptation. For any of the adaptive drug concentrations, 0.4 μg/ml or 0.55 μg/ml, adaptive response of *V. cholerae* was maximum for adaptation times between 30 and 60 min, the response being much higher when the adaptive drug concentration was 0.4 μg/ml than when it was 0.55 μg/ml (Fig. 4). Increased resistance of *V. cholerae* cells to furazolidone treatment resulted when the cells were pretreated with sublethal concentration of nitrofurantoin, 0.4 μg/ml for 1 hr, the respective D_{37} values for nonadapted and adapted cells being 0.215 μg/ml and 0.30 μg/ml, respectively (Fig. 5).

In order to have some idea about the nature of the adaptive response induced by nitrofurantoin treatment, survivals of *E. coli* strain PJ3 (AB 1157 *ada*$^-$) and *V. cholerae* OGAWA 154 (wild type) against different adaptive and challenge doses of nitrofurantoin were investigated (Fig. 6). Unlike the nonadapted cells of *E. coli* AB 2463 (*recA*$^-$) cells (Fig. 1), the nonadapted cells of *E. coli* strain PJ3 (AB 1157 *ada*$^-$) exhibited much greater resistance to nitrofurantoin, the respective D_{37} values being 0.26 μg/ml and 17 μg/ml for 1 hr treatment (Figs. 1 and 6). Both *E. coli* strain PJ3 (*ada*$^-$ mutant) and also the wild type *V. cholerae* OGAWA 154 cells exhibited significant resistance to nitrofurantoin as induced by adaptive doses of H_2O_2. Also it is significant to note that both *E. coli* and *V. cholerae* cells exhibited almost same degree of resistance to nitrofurantoin challenge when pretreated with adaptive doses of H_2O_2 (30 μM, for 30 min) or nitrofurantoin (1 μg/ml for *E. coli* and 0.4 μg/ml for *V. cholerae* for 1 hr).

Photomicrography revealed that the native *V. cholerae* cells measured, on an average, 1.42 ± 0.5 μm (L_1) in length, whereas the nonadapted and treated (50 μg/ml, 1 hr) cells grew longer into filamentous forms and measured 4.25 ± 2.97 μm (L_2) on the average. Some of the cells grew much longer and a significant number grew even to a length greater than 10 μm. But the adapted (0.4 μg/ml, 1 hr) and treated (50 μg/ml, 1 hr) cells retained a length 2.08 ± 0.835 μm (L_3) on the average, which was somewhat greater than control (L_1) but much less than the nonadapted value (L_2).

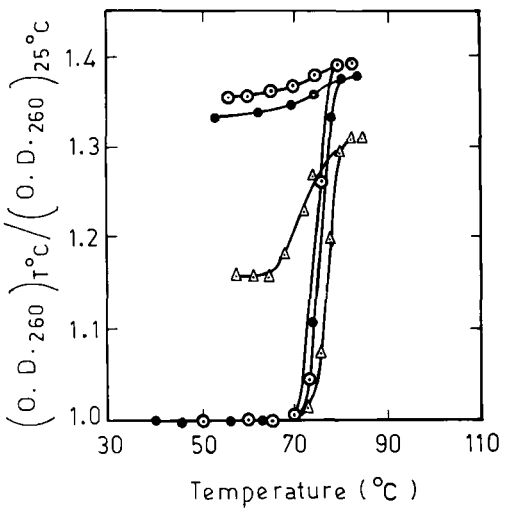

Fig. 8. Thermal transition profiles of DNA in 0.1 × SSC, pH 7.1. DNA was isolated from nonadapted and untreated (⊙—⊙), nonadapted but nitrofurantoin treated (120 μg/ml forl hr) (△—△), and adapted (0.4 μg/ml for 1 hr) and drug treated (120 μg/ml for 1 hr) (●—●) *V. cholerae* cells. Thermal denaturation or melting of DNA was monitored by measurement of the absorption of the DNA solution at 260 nm with progressive increase of temperature, the corresponding absorption at 25°C being used as reference. The renaturation profiles of the corresponding DNA samples on progressive slow cooling after maximum denaturation was attained are also presented here.

Statistical analysis revealed that the differences, $L_1 \sim L_2$, $L_1 \sim L_3$, and $L_2 \sim L_3$ were all significant to a level better than 0.1%. In the case of *E. coli* AB 2463 (*recA*$^-$) cells, treatment of nonadapted cells with nitrofurantoin did not yield any significant filamentation (since the process of filamentation is *recA* dependent), the average lengths of native cells and those nonadapted but drug treated (4.0 μg/ml, 1 hr) being 1.86 ± 0.47 μm and 1.99 ± 0.54 μm, respectively.

Figure 7 shows HAP chromatographic data on *V. cholerae* and *E. coli* (AB 2463) DNA after DRT. DNA from native *V. cholerae* cells was eluted in two fractions, 5% as double stranded and 95% as single stranded (Fig. 7a). DNA from nonadapted and treated (120 μg/ml, 1 hr) *V. cholerae* cells was eluted in almost two equal fractions, 55% as double stranded and 45% as single stranded forms (Fig. 7b). DNA from adapted (0.4 μg/ml, 1 hr) and challenged (120 μg/ml, 1 hr) *V. cholerae* cells was obtained as 16% in double stranded and 84% in single stranded forms (Fig. 7c). Corresponding data for *E. coli* DNA were: 3.2% and 96.8% for native *E. coli* cells, 51.2% and 48.8% for nonadapted but challenged (80 μg/ml, 1 hr) cells, and 29.2% and 70.8% for adapted (0.01 μg/ml, 1 hr) and challenged (80 μg/ml, 1 hr) cells (Fig. 7d,e,f).

Thermal helix-coil transition patterns for *V. cholerae* DNA are presented in Figure 8. The melting temperatures of DNA isolated from native cells, nonadapted but challenged (120 μg/ml, 1 hr) cells, and adapted (0.4 μg/ml, 1 hr) and challenged (120 μg/ml, 1 hr) cells were obtained as 75°C, 77.5°C, and 76°C respectively in 0.1 × SSC (standard saline citrate), pH 7.1. The corresponding values for *E. coli* AB 2463 (*recA*$^-$) were 77.5°C, 84°C, and 81°C, respectively (Table I). While DNA from native *V. cholerae* cells and adapted (0.4 μg/ml, 1 hr) and challenged (120 μg/ml, 1 hr) ones were renatured to a very small extent, that from non-adapted and challenged (120 μg/ml, 1 hr) cells underwent about 48% renaturation.

DISCUSSION

The cytotoxic effects of drug treatment have been monitored in this study by measurements of 1) viability, 2) filamentation, 3) thermal chromatography of DNA isolated from untreated and drug treated cells through HAP column, and 4) melting temperature of DNA isolated from untreated and drug treated cells by UV-absorption spectrophotometry. Earlier studies from this laboratory [Mukherjee et al., 1990] reported that nitrofurantoin produced interstrand cross-links in DNA of treated cells. Geiduschek [1961] first reported that DNA containing interstrand cross-links acquired the property of being reversibly bihelical. Such a DNA becomes bihelical or double stranded after denaturation-quench cooling or renaturation treatment [DRT] when under identical treatment the native DNA (bearing no cross-links) remains single stranded. Fractions of DNA remaining single or double stranded after any treatment can be quantitatively assayed by thermal chromatography through HAP column, which in turn can give an idea of the number of cross-links present in DNA as described earlier (in Materials and Methods). The presence of such cross-links was reported to enhance the melting temperature of DNA [Iyer and Szybalski, 1963; Chatterjee et al., 1983; Mukherjee et al., 1990]. It was generally found that the increase in melting temperature, T_m, is related to the number of such lesions present. It was further reported that nitrofurantoin and also furazolidone induced cross-links in DNA produced filamentation of treated cells [Mukherjee et al., 1990; Chatterjee and Raychowdhury, 1971] subsequent to induction of SOS response [Chatterjee et al., 1983; Sengupta et al., 1990]. These observations thus reflected expression of cytotoxic effects at the cellular and molecular level of cells subjected to drug treatment under different conditions.

This study reported that treatment of both *V. cholerae* and *E. coli* cells with sublethal dose of nitrofurantoin made the cells resistant to subsequent challenge with a high concentration of the drug. The resistance was reflected in the 1) increased viability, 2) significantly less filamentation, 3) comparatively much less formation of double stranded or reversibly bihelical DNA after DRT and hence formation of much lesser number of interstrand cross-links, and 4) comparatively less increase in melting temperature of the DNA isolated from pretreated cells vis-a-vis the nonpretreated ones. Such enhancement of resistance of cells pretreated with a sublethal dose of the drug could be interpreted as being due to the induction of an adaptive response by nitrofurantoin treatment in both *V. cholerae* and *E. coli* cells.

TABLE I. Melting Temperature of and the Number of Drug Induced Cross-Links in the Genome DNA of Adapted and Nonadapted Cells of *V. cholerae* and *E. coli*

Strain	Adaptive dose (μg/ml for 1 hr)	Challenge dose (μg/ml for 1 hr)	Melting temperature[a] (°C)	Percent single stranded DNA	Percent double stranded DNA	Average number of cross-links per genome DNA[b]
V. cholerae	0.0	0.0	75.0	95.0	5.0	5[c]
OGAWA	0.0	120.0	77.5	45.0	55.0	80
154	0.4	120.0	76.0	84.0	16.0	17
E. coli	0.0	0.0	77.5	97.0	3.0	3[c]
AB 2463	0.0	80.0	84.0	49.0	51.0	71
(*recA*$^-$)	0.01	80.0	81.0	71.0	29.0	34

[a]Thermal transition experiments were carried out with DNA in 0.1 × SSC, pH 7.1 buffer.
[b]Number of cross-links were estimated from thermal HAP chromatographic experiments as described in Materials and Methods. The average number of cross-links (Z) per DNA fragment (after sonication) was estimated from the equation given in the Materials and Methods section of the text. Assuming an average molecular weight of DNA fragments as 10^7 and that of the genome DNA as 10^9, the average number of cross-links per genome DNA was estimated as Z × 100. The figures presented in the last column are approximate values since the exact molecular weights of genome DNA and DNA fragments were not used in calculations.
[c]These values correspond to DNA of untreated or native cells and hence represent experimental errors. Untreated cells are not expected to bear any drug induced cross-links in DNA.

It is of interest to note here that a preliminary report of the adaptive response induced in *V. cholerae* by furazolidone, another member of the group of the synthetic nitrofurans, was published earlier [Bhattacharya, 1989]. The inhibition of this response by chloramphenicol, as reported in the present study, indicated its dependence on protein synthesis and its inducible nature.

This study has elucidated several other features of this nitrofurantoin induced adaptive response of a bacterial cell. Since well characterised *recA* mutants of *V. cholerae* cells were not available and since the drug was shown to produce similar type of DNA lesions (interstrand cross-links) in repair deficient *E. coli* AB 2463 (*recA*$^-$) [Sengupta et al., 1990] and *V. cholerae* cells, we used *E. coli* AB 2463 *recA* mutant cells for a parallel study to investigate the dependence, if any, of this adaptive response on the *recA* gene products. The present study showed that adaptive response could be induced in *recA* mutant of *E. coli*. This was indicated in the measured values of all the parameters monitored in this study, viz, 1) D_{37} values, 2) filamentation, 3) formation of reversibly bihelical DNA and interstrand cross-links, and 4) melting temperatures of DNA, of the adapted and nonadapted cells of *E. coli* AB 2463 (*recA*$^-$). It is therefore evident that the nitrofurantoin induced adaptive response is not dependent on the *recA* gene and is different from the inducible "SOS" repair system described by Witkin [1967] and George et al. [1974]. This study further indicated that adaptive responses induced by nitrofurantoin and furazolidone [Bhattacharya, 1989] in *V. cholerae* cells are similar since pretreatment with sublethal dose with nitrofurantoin made the cells more resistance to subsequent challenge by lethal doses of furazolidone.

Recent study led to the discovery of several types of inducible DNA repair systems that are more limited with respect to the types of damaging agents that cause their induction and the types of lesions repaired and are independent of "SOS" response [Volkert, 1988]. These include adaptive response to alkylation damage and the adaptive response to oxidative damage [Samson and Cairns, 1977; Demple and Halbrook, 1983; Volkert, 1988]. The adaptive response to alkylating damage has been fairly well characterised and four genes have been identified as components of this response, *ada*, *alk* A, *alk* B, and *aid* B. In this case, the ada protein functions as a positive regulatory element that controls the expression of the adaptive response to alkylating agents by stimulating expression of the *ada-alk* B operon, and the *alk* A and *aid* B genes. The *ada* gene product, a component of the adaptive response to alkylating damage to DNA, was also reported to prevent interstrand cross-links formation by chloroethyl nitrosourea [Robins et al., 1983]. Some aspects of another kind of adaptive response, elicited by nontoxic levels of hydrogen peroxide, which is distinct from but similar to the adaptive response to alkylating agents and which is directed at oxidative damage, was reported by Demple and Halbrook [1983]. Since the adaptive response investigated presently against nitrofurantoin induced DNA damage could be elicited in *ada*$^-$ mutant of *E. coli* which is incapable of exhibiting adaptive response to alkylating damages [Evensen and Seeberg, 1982], nitrofurantoin induced adaptive response does not appear similar to that induced by alkylating agents. This is supported by additional experiments which showed that pretreatment with nontoxic concentrations of hydrogen peroxide or nitrofurantoin made both the *ada*$^-$ mutant of *E. coli* PJ3 (AB 1157 *ada*$^-$) and the wild type strain of *V. cholerae* OGAWA 154 almost equally resistant to subsequent challenge by nitrofurantoin. Elicitation of the same degree of responses by adaptive treatments with H_2O_2 and nitrofurantoin may lead

to the inference that nitrofurantoin induced adaptive response pathway is similar to that induced by agents causing oxidative DNA damage [Demple and Halbrook, 1983]. However, further experimentation using a mutant unable to induce the peroxide response might help in getting better information as to whether any other adaptive response, besides the one directed against oxidative DNA damage, is induced by nitrofurantoin.

In conclusion, it may be stated that treatment with sublethal dose of nitrofurantoin induced adaptive response in *V. cholerae* and *E. coli* cells. Such a response was independent of the "SOS" repair pathway, appeared similar to the adaptive response elicited by furazolidone in *V. cholerae* cells, and appeared to be directed at oxidative damage and not alkylating damage. Further characterization of the response at the genetic and molecular levels remains to be done. It could be interesting to see whether this adaptive response is involved in 1) the repair or prevention of nitrofurantoin induced cross-links in DNA or 2) detoxifying the drug by inhibiting its activation within the cells or 3) preventing entry of the drug into the cells by activating certain genes or 4) preventing cross-link formation by the binding of certain activated gene products with the activated drug molecules thereby sparing the DNA sites, etc. Any of these processes is likely to reduce the cytotoxicity of the drug, including the reduction of the number of drug induced cross-links in DNA, in the adapted cells.

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