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Selenium Mediated Reduction of the Toxicity Expression of Cigarette Smoke Condensate in *Photobacterium phosphoreum*

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Until about 25 years ago, the primary interest in selenium resulted from its toxic effects in animals after ingesting seleniferous plants (Whanger 1983). Recently, attention has shifted to the potential protective activity of selenium against heavy metal toxicity, cancer and other health disorders (Reddy and Massaro 1983). Currently, cigarette smoke affects the health of more people than any other environmental pollutant (Chortyk and Schlotzhauer 1984). Producing cigarettes fortified with selenium has been proposed as a possible method to develop a safer tobacco product (Chortyk and Schlotzhauer 1984).

Planning product modifications that will reduce the health hazards of cigarette smoking requires bioassays to assess the effects of such modifications. Consequently, it would be informative to determine if the presence of selenium in cigarette smoke leads to increased or decreased toxicity. Luminescent assays have been developed for a wide variety of applications ranging from measuring enzyme activities to monitoring water purity (Shimomura 1983). The purpose of this study was to evaluate the effect of selenium on the toxicity of cigarette smoke condensate using *in vivo* bacterial bioluminescence assays (Yates 1985a).

MATERIALS AND METHODS

Selenium (Se) was tested in the form of sodium selenite (Na_2SeO_3, Aldrich Chemical Company) and concentrations described refer to that of Se and not to Na_2SeO_3. Cigarette smoke condensate (CSC) was prepared under standard Federal Trade Commission smoking machine conditions (Chortyk and Schlotzhauer 1984). Na_2SeO_3 was solubilized in 2% NaCl (diluent, Beckman Instruments, Inc.) and CSC, in dimethyl sulfoxide (Fisher Scientific Company).

Two strains of the bacterium, *Photobacterium phosphoreum*, were used in the experiments. One of the strains was a dark mutant isolated after treatment of wild type *P. phosphoreum* NCMB 844 with N-methyl-N'-nitro-N-nitrosoguanidine and designated K57 (Yates 1985a). Cultures of K57, stored at -80°C, were streaked onto Petri dishes containing solid artificial sea water (ASW) medium. Solid ASW medium was composed of (g/l) NaCl, 30; Na_2HPO_4,

2.5; KH_2PO_4, 2.1; $(NH_4)_2HPO_4$, 0.5; $MgSO_4$, 0.1; agar, 10 and glycerol, 3.0 ml/l. After 48 h, colonies were suspended to a concentration of approximately 2.0 X 10^5 cells/ml of liquid ASW. Liquid medium was prepared the same as solid but without agar. The other strain, P. phosphoreum NRRL B-11177, was bioluminescent and obtained in the freeze-dried condition from Beckman Instruments, Inc. Immediately prior to testing, the bioluminescent strain was reconstituted with ultra-pure water.

The bioassay systems used to study the effects of Se on CSC toxicity were of two types: testing with the dark mutant K57 was designated long-term analysis requiring an assay time of 20 h, while testing with the bioluminescent strain was referred to as short-term analysis requiring an assay time of 20 min.

The procedure for the long-term analysis was similar to that described previously (Yates 1985a). Briefly, CSC in dimethyl sulfoxide was diluted in liquid ASW medium so that when 1.0 ml was added to a 1.0 ml suspension of K57 cells in sterile 7-ml glass scintillation vials, the final concentrations of CSC were 2.03, 4.05, 8.1, 16.2 and 32.4 µg/ml. The reversion rate to an expression of bioluminescence was determined with a Packard Tri-carb 460 CD liquid scintillation system operating in the tritium counting mode. The results are reported as counts per minute (cpm). As described previously, the criteria for determining mutagenicity included an increase in proton emissions in treated samples at least 1 h prior to control samples and this activity must be maintained above control levels for at least 4 h. The effects of CSC and Se alone and in combination were determined. The concentrations of Se added to CSC samples was 0.4 ng/ml and 5 µg/ml. The concentration ratio of 0.4 ng Se to 32.4 µg CSC/ml ASW medium represents the approximate Se:CSC ratio present in CSC from tobacco highly fortified with Se.

In the short-term analysis, light measurements were made with a Microtox Analyzer™ (Beckman Instruments, Inc.) using a 5-step dilution series of CSC in 2% NaCl (diluent, Beckman Instruments, Inc.). The dilutions for CSC were chosen so that the highest concentration resulted in approximately 90% reduction of bioluminescence, while the lowest concentration produced a 10% reduction of bioluminescence. Subsequently, the dilution series for CSC was established in diluent containing Se. The toxicity of CSC (with and without Se) was assayed at 0, 4 and 24 h. The serial CSC dilutions were maintained at room temperature until 15 min prior to assay at which time they were cooled to 15°C and 0.5 ml added to 0.5 ml of freshly reconstituted bacterial suspension. Light measurements were made at 5, 10, 15 and 20 min after addition of CSC test sample to the bacterial suspension. Therefore, two different time factors were investigated in these experiments: 1) the effects of incubating the serial dilutions for 0, 4 and 24 h before addition to bacterial suspension to

determine the kinetics of the interaction of Se and CSC, referred to as chemical incubation, and 2) the effects of test sample after addition to bacterial suspension at 5, 10, 15 and 20 min to determine the kinetics of the bacterial reaction, referred to as response time. In another series of experiments, the bacteria were incubated with Se-containing diluent for 1 h prior to the addition of CSC. This was referred to as bacterial incubation and was used to determine whether prior exposure of bacteria to Se could have a protective action when the cells were treated with CSC.

Data reduction from the short-term analysis was accomplished by calculating gamma (the ratio of light lost to the light remaining, Johnson et al. 1974) by using a correction factor to accomodate the normal change of light by the bacteria without added toxicant. The correction factor was determined by dividing the diluent control blank reading at the time point analyzed (i.e., 5, 10, 15 or 20 min) by the zero time reading. In samples with Se, the diluent control also contained Se at the same concentration as in the samples. The gammas for each sample concentration at a given time point were subjected to power curve analyses with the algorithms developed by Hewlett-Packard for the HP41-C calculator. The power curve was used to derive the concentration of test samples in micrograms per milliter causing 50% light reduction (EC50). The values reported represent the mean of at least three separate experiments. Significant differences between EC50 values for different incubations times for CSC solutions, with and without Se, were determined by analysis of variance followed by Duncan's Multiple Range Test (Klugh 1970).

RESULTS AND DISCUSSION

CSC did not stimulate bioluminescence above control levels during any time period in mutant K57 (Fig. 1a). CSC does not contain mutagenic agents (or at least sufficient quantities of these agents) with a mode of action similar to previously characterized compounds. The P. phosphoreum mutant K57 used in the long-term analysis of CSC and Se has been extensively characterized with regard to the effects of several types of mutagenic agents on the expression of bioluminescence (Yates 1985a, Yates 1985b). Compounds which bind covalently to the guanine sites in DNA, such as mitomycin C and aflatoxin B_1, are able to repair the bioluminescent function so that photon emissions of treated bacteria are 1×10^3 above control levels after a 10 h exposure. In addition ethidium bromide, a DNA intercalating agent, stimulates bioluminescence above control levels after 12 h exposure. Hannan et al. (1980) proposed from work with strains of repair deficient yeast that the effects of tobacco smoke condensate on DNA repair was not specific to any particular pathway, but an effect on all repair processes. Consequently, a mutant, such as K57, with specificity for compounds reacting with guanine sites is evidently too restricted in its response to detect mutagenic constituents with multiple modes of action.

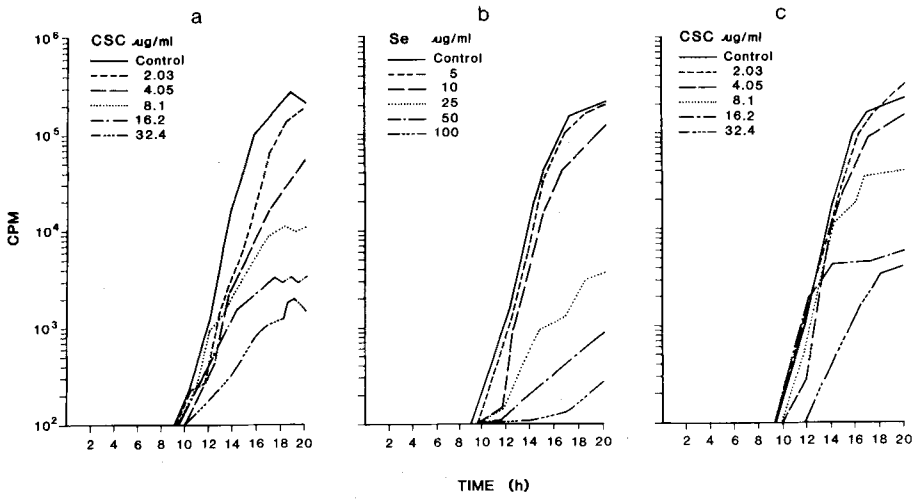

Figure 1. Kinetics of photon emissions from dark mutant K57 cells following treatment with (a) CSC (b) Se (c) CSC containing 0.4 ng Se/ml. The concentrations of CSC and Se are indicated on the figures.

The lack of detectable mutagenic activity of CSC in the current study with Photobacterium phosphoreum could be related to the experimental design in that no exogenous metabolic activation system was used. Various studies have shown CSC to be mutagenic in Salmonella (DeMarini 1981a, Kier et al. 1974, Mizusaki et al. 1977, Sato et al. 1977), Neurospora (DeMarini 1981b), yeast (Hannan et al. 1980) and mammalian cells (Clive et al. 1979) in the presence of a metabolic activating system. However previous studies with P. phosphoreum (Yates 1985a, Yates 1985b) and related species (Ulitzer et al. 1980, Scher and Wecher 1982) indicate that this group of bacteria are capable of demonstrating mutagenic activity of certain agents, such as aflatoxin B_1, without an exogenous metabolic activation system as required with other bioassays. The presence of an endogenous cytochrome P-450 system in Photobacterium (Ismailov et al. 1979) may be responsible for this function.

The effect of CSC on the bioluminescent system of mutant K57 was to inhibit the low level luminescence commonly observed in control samples after 12-14 h (Fig. 1a). With concentrations increasing from 2.03 to 32.4 µg CSC/ml ASW there was a decrease in bioluminescence. This was not a reflection of a decreased cell number as samples treated for 24 and 48 h with up to 32.4 µg CSC/ml ASW had approximately the same number of cells as control samples. The inhibitory effect of CSC on mutant K57 became apparent at 14 h, that is approximately 4 h after photon emissions began to increase in control samples. There was no effect on

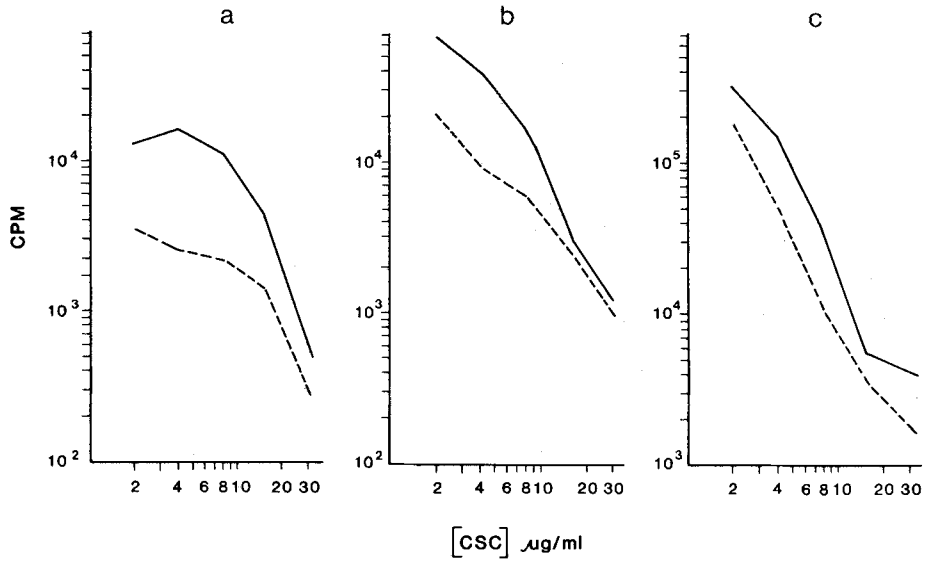

Figure 2. Dose response curves for CSC (---) and CSC containing Se (___) interpolated from kinetic data (Fig. 1) at (a) 14 h (b) 16 H (c) 20 h.

bioluminescence of bacteria treated with less than 2.03 µg CSC/ml ASW. The bacteria did not overcome the CSC induced toxicity for the time period analyzed.

Prior to analyzing the effect of Se or CSC toxicity in mutant K57, Se was tested separately and shown to have little or no effect at concentrations up to 5 µg/ml. However, at 10 µg/ml and above, Se inhibited bioluminescence (Fig. 1b).

Selenium at 0.4 ng/ml of assay solution was effective in reducing the inhibitory effect of CSC (Fig. 1c). The relief in CSC toxicity was most evident at approximately 14 h during the phase of exponential increase in bioluminescence (Fig. 2a) at 2.03, 4.05 and 8.1 µg/ml CSC. At higher concentrations, Se was not as effective in reducing the CSC-generated toxicity. By 20 h, bioluminescence in most samples appeared to have reached a stationary phase of activity and the effect of Se was diminished in comparison to that at 14 and 16 h (Fig. 2). CSC samples were also treated at 5 µg Se/ml ASW medium to determine whether or not higher Se concentrations could further relieve the CSC toxicity (data not shown). However, samples of CSC with the higher level of Se demonstrated profiles similar to those treated at the 0.4 ng Se level.

The EC50 values obtained for CSC and CSC + Se after 0, 4 and 24 h chemical incubation as a function of bacterial response time in the short-term analysis are shown in Table 1. Statistical analyses of the data demonstrated that after 24 h incubation, the EC50 values obtained for CSC + Se were significantly higher than

Table 1. EC50 Values for CSC and CSC + Se

Treatment	Chemical Incubation Time	EC50 (µg/ml) at Bacterial Response Time of: 5 min	10 min	15 min	20 min
CSC	0	2.73[ab]	2.24[a]	2.25[a]	2.27[a]
CSC	4	2.59[ab]	2.40[a]	2.31[a]	2.33[a]
CSC	24	2.88[b]	2.58[a]	2.44[a]	2.46[a]
CSC + Se	0	2.38[a]	2.15[a]	2.12[a]	2.16[a]
CSC + Se	4	2.44[ab]	2.27[a]	2.19[a]	2.15[a]
CSC + Se	24	4.18[c]	3.75[b]	3.57[b]	3.57[b]

[abc]Values in the same column followed by the same superscripts are not significantly different (P>0.01).

CSC alone or CSC + Se at the other chemical incubation times. This increase in the EC50 values reflects a decrease in toxicity and is evident whether the bacterial response was determined at 5, 10, 15 or 20 min. For any given treatment, the toxicity expression appears to be greater at a bacterial response time of 20 min than at 5 min. However, the values obtained as a function of bacterial response time are not statistically different as determined by analysis of variance except in samples treated with CSC incubated for 24 h. The EC50 of 2.88 µg CSC/ml at 5 min is significantly higher than those at 10, 15 or 20 min. Preincubation of the bacteria with Se for 1 h prior to the addition of the CSC did not reduce the toxicity expression of the CSC (data not shown).

The results of the short-term analysis suggest that the mechanism by which Se generates a relief of CSC induced toxicity is to react with the constituents in the CSC itself and not by stimulating a protective mechanism in the cell. In order to demonstrate decreased CSC toxicity by Se, it was necessary to incubate the CSC with Se for 24 h.

Bacterial bioluminescence has been shown to be inhibited by heavy metals (Bulich and Isenberg 1981) and Se has been shown to provide protection to various biological systems against poisoning by heavy metals (Reddy and Massaro 1983). The mechanism of this detoxification process may occur by Se complexing the heavy metals to form insoluble selenides rendering the metals biologically

unavailable (Harr 1978). Chortyk and Schlotzhauer (1984) propose that Se could aid in detoxifying cigarette smoke by such a mechanism; i.e., complexing with toxic trace metals in this environmental contaminant. The data obtained with Photobacterium phosphoreum would indicate that the mechanism of detoxification is a time-dependent process. The lack of activity of low levels of Se as a function of bacterial incubation in the short-term analysis may relate to the uptake or metabolism of Se. Clausen and Tranum (1982) demonstrated that the uptake of selenite by cultivated lymphocytes was a slow process taking more than 3 days to arrive at equilibrium.

The results of the present study utilizing bacterial bioluminescence technology are consistent with the hypothesis that increased levels of Se in fortified tobacco could serve to partially detoxify harmful constituents in CSC. The level of Se in tobacco grown in the United States is about 0.04 ppm and it has been proposed that Se could be increased to 5 ppm without the Se itself becoming a health hazard (Chortyk and Schlotzhauer 1984). The experimental levels of Se described in this study would not elicit even marginal deleterious effects in smokers. However, additional in vitro and in vivo bioassay studies are needed to test the hypothesis that Se does in fact have the potential to reduce the hazards associated with cigarette smoking.

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