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CYTOGENETIC EVIDENCE OF INDUCIBLE PROCESSES LINKED WITH METABOLISM OF A XENOBIOTIC CHEMICAL IN ADULT AND LARVAL *MYTILUS EDULIS**

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ABSTRACT

Sister chromatid exchange (SCE), a sensitive cytogenetic endpoint for measuring the effects of mutagens on chromosomes, was assayed in vivo in adult and larval mussels to determine whether these different life-history stages possess any capability for converting promutagens to mutagenic species. Cyclophosphamide (CPA), a water-soluble promutagen that is dependent for its mutagenic activity in mammals on transformations accomplished via the cytochrome P-450 pathway, was selected for this investigation. CPA was found to cause increased frequencies of SCE in both adult and larval *M. edulis*. In addition, the presence of phenobarbital (PB), an inducer of the microsomal detoxication system in mammals, was found to increase the levels of SCE produced by CPA, indicating that the effect of PB may have been to increase the rate at which CPA was metabolized. Operating as it does at the level of the individual cell nucleus, SCE is thus shown to have potential as an extremely sensitive indicator of stimulation of the microsomal detoxication system in the common mussel. The apparent inducibility of this activity may have important consequences for these organisms when they are exposed to the complex mixtures of xenobiotic chemicals found in the environment.

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INTRODUCTION

Sister chromatid exchange (SCE) is a sensitive cytogenetic endpoint for measuring the effects of mutagens on chromosomes (Latt et al., 1981). SCEs are symmetrical exchanges of apparently homologous chromosomal loci that are detectable microscopically after a differential staining procedure. SCE has recently been studied in several marine species, including fish and invertebrates, with the intention of developing assays for monitoring the marine environment for mutagens (review by Dixon, 1983). The common mussel, *Mytilus edulis*, already an important sentinel organism in environmental toxicology (e.g. Goldberg et al., 1978), has featured prominently in these SCE studies. Cells from adults and larvae have been used to show that *M. edulis* is sensitive to the direct-acting mutagens mitomycin C, methylmethanesulphonate, and 5-bromo-2-deoxyuridine (BrdUrd) at concentrations similar to those reported in the mammalian literature (Dixon and Clarke, 1982; Harrison and Jones, 1982).

As an extension of this work, we have studied SCE in adult and larval *M. edulis* to determine whether they are capable of transforming promutagens to metabolites that are able to induce SCE. Because many of the potentially genotoxic agents entering the natural environment require metabolic activation before becoming toxic (Nagao et al., 1978), it is important from the viewpoint of environmental impact to assess whether the test organism can transform this type of marine pollutant. Furthermore, from the viewpoint of fundamental science, if it could be established that SCE detects the products of metabolic activation, then SCE could serve as a sensitive indicator of monooxygenase activity in adult and larval mussels.

Cyclophosphamide, a water-soluble promutagen dependent for its mutagenic activity in mammals on transformations mediated by the cytochrome P-450 pathway (review by Mohn and Ellenberger, 1976), was selected for this investigation. In some cases, mussels were pretreated with PB, an established inducer of the microsomal detoxication system (Phase I) in mammals. PB has been shown to stimulate the activity of NADPH-neotetrazolium reductase in mussels (Moore, 1979), an enzymic reaction believed to be associated with NADPH-cytochrome P-450 (C) reductase (Masters et al., 1966).

The approach used here can be extended to studies of the metabolism of other xenobiotic chemicals. Such studies could include other classes of xenobiotic chemicals and identification of inducers that are likely to be present in the organisms' natural environment. The results of these studies would provide a basis for assessing the potential for genetic damage to these organisms when they are exposed to promutagens in real environments, and possibly, they would be useful in pointing to metabolic capacities of these organisms that have not been studied previously in detail. They also would extend our understanding of the value that measurement of the frequency of SCE will play in assessing exposure of mussels to mutagens and promutagens in natural environments.

MATERIALS AND METHODS

Mussels were collected from Whitsand Bay, S.E. Cornwall, U.K., and Tomales Bay, N. California, U.S.A., during the period May to September, 1982. Whitsand Bay animals (2—3 cm shell length) were conditioned in the laboratory for several weeks to promote somatic growth (Dixon and Clarke, 1982). Sexually ripe Tomales Bay mussels (5—9 cm shell length) supplied gametes for the larval-dosing experiments and were housed temporarily in a system containing recirculating seawater at 10—12°C.

For the experiments with adult Whitsand Bay animals, the experimental conditions were similar to those described by Dixon and Clarke (1982). Groups of 10 individuals were dosed at each treatment, with the mussels being fed a daily ration of Liquify (Interpret, Dorking) to ensure that cell division continued throughout the experimental period. Where appropriate, PB (sodium salt) was introduced at a daily concentration of 0.3 mg l^{-1}; this dose was based on the average daily intake of this compound used to promote monooxygenase activity in rodents (e.g. Alberts et al., 1978), assuming each Whitsand mussel had a wet weight of 0.5 g. The water containing BrdUrd at a concentration of 5×10^{-5} *M* was changed daily. After a 5-day exposure to BrdUrd (the estimated average time to complete one cell cycle in the gill), with or without PB, each group of mussels was exposed to CPA (10^{-6}—10^{-2} *M*). Exposure to CPA was maintained for 5 days in the continued presence of BrdUrd and, when appropriate, PB. Control groups received either BrdUrd alone or PB plus BrdUrd. Gill tissue was the source of adult cells for SCE analysis.

The experiments with mussel larvae were of shorter duration than those with adults because the average cell-cycle time in larvae is much shorter. The protocol followed was similar to that described by Harrison and Jones (1982) with minor modification. To avoid possible interference with the fertilization process, PB was added 2 h after the sperm were introduced. BrdUrd was introduced to all cultures 12 h after fertilization to give a final concentration of 1×10^{-5} *M*. CPA was also introduced to the larval cultures 12 h after fertilization, together with a second aliquot of PB where appropriate. Mussel larvae were at the late trochophore stage in development when harvested at 24 h. SCE was analyzed in cells of disaggregated larvae.

For the adult studies, SCEs were scored and analyzed following the methods reported in Dixon and Clarke (1982). The method of analysis involved a variance stabilizing transformation of the SCE data, where each individual count was replaced by its square root, followed by one-way ANOVA and 'Tukey' multiple comparisons tests (Scheffe, 1959; Silvey, 1975).

The larval SCE results were analyzed on the basis of the numbers of high frequency cells (HFC) (Carrano and Moore, 1982) as previously reported by Harrison and Jones (1982). This non-parametric measure has been demonstrated to be a sensitive indicator of SCE induction (Harrison and Jones, 1982). Based upon extensive studies of untreated animals, we have defined

an HFC as a cell whose SCE/chromosome ratio exceeds 0.12. Larvae exposed only to BrdUrd are expected (with 95% confidence) to have no more than 5% HFCs as a proportion of their total cell number. We used chi-square analysis to determine whether an observed number of HFC deviated from the number of HFC expected in the controls. On this basis, all sets of control larvae had numbers of HFC comparable to historical controls.

PB was purchased from Ganes Chemicals (New York, NY). All other reagents were products of Sigma Chemical Company (St. Louis, MO).

RESULTS

SCE frequencies of adult mussels exposed to CPA with and without PB are shown as dose—response relationships in Fig. 1. Adult animals exposed in the absence of PB to the lowest CPA concentration (10^{-6} *M*) yielded as a group too few suitable metaphases for analysis. The highest CPA concentration (10^{-2} *M*) was found to be lethal to adult mussels within the first few days of exposure. In the absence of PB treatment, only the highest tolerated CPA concentration (10^{-3} *M*) induced a significantly increased SCE frequency in adult gill tissue (Fig. 1). Differences significant at the 5% level existed in the mean SCE frequency between the group exposed to 10^{-3} *M* CPA and both the seawater control and the group exposed to 10^{-5} *M* CPA. Lower concentrations of CPA (10^{-5} and 10^{-4} *M*) induced elevated SCE frequencies that were not statistically different from controls.

In the PB-pretreated adults there was evidence of a more marked, dose-related effect of CPA on SCE frequency. On the average, PB-pretreated

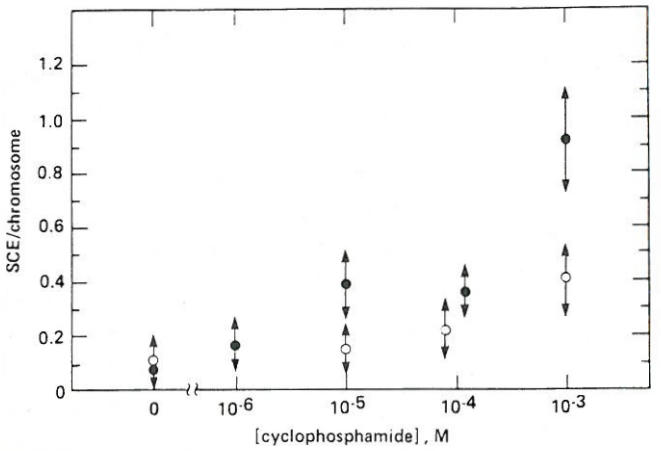

Fig. 1. Induction of sister chromatid exchange (SCE) in *M. edulis* adults as a function of cyclophosphamide (CPA) dose and phenobarbital (PB) treatment. SCE/chromosome was determined for adults exposed for 5 days to CPA, a mutagen requiring activation, either with (filled symbols) or without (open symbols) a 5-day pre-treatment with PB, an inducer of the microsomal detoxication system. Arrows indicate 2 standard errors.

individuals displayed a two-fold greater increase in the level of SCE than the corresponding animals exposed only to CPA (Fig. 1). All pairwise comparisons of SCE frequency of PB-pretreated adults, except comparison between groups treated with 10^{-5} and 10^{-4} *M* CPA, were significantly different at the 5% level.

SCE frequencies of PB-pretreated and untreated adult mussels were significantly different only at 10^{-5} and 10^{-3} *M* CPA, with PB treatment inducing the higher SCE frequency. No comparison was possible for the 10^{-6} *M* treatment. No difference was found between the two control groups, indicating that PB alone did not induce SCE in the adult mussel.

The SCE responses of larval mussels to CPA are presented in Fig. 2 as the response of the proportion of HFCs to CPA dose, with and without PB pretreatment. The results of replicate experiments were pooled except results of the treatment with 10^{-2} *M* CPA-only; it was tested once only. Four sets of larvae were used for the experiments reported, two for those with PB and two for those with no PB pretreatment. CPA was not toxic to the larvae at any concentration tested.

As was true for adult mussels, PB-pretreated larval mussels were more sensitive to SCE induction by CPA than were the untreated larvae. Mussel larvae exposed to CPA alone showed a slight increase in their frequency of HFC. Only the larvae treated with 10^{-3} and 10^{-2} *M* CPA alone had significant increases ($p < 0.014$) in their frequencies of HFCs. In contrast, mussel larvae that were pretreated with PB had significantly elevated ($p < 0.0001$) frequencies of HFCs for all doses of CPA tested, from 10^{-5} to 10^{-2} *M*. The frequency of HFCs for PB-pretreated larvae was in all cases greater than that of larvae exposed only to CPA. As was true of the adult mussels studied, PB alone did not lead to altered SCE frequencies in the larvae.

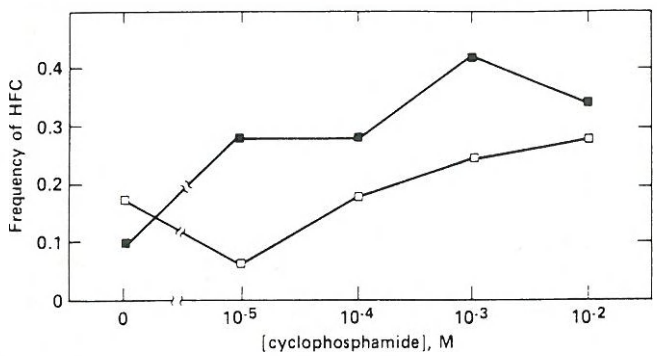

Fig. 2. Induction of high frequency cells (HFC) in *M. edulis* larvae as a function of cyclophosphamide (CPA) dose and phenobarbital (PB) treatment. The frequencies of HFC (cells with SCE/chromosome >0.12) was determined for the larvae exposed to CPA for 12 h with (filled symbols) or without (open symbols) a 10-h pre-treatment with PB.

DISCUSSION

Increases in SCE in the presence of CPA under acute exposure conditions show that both larval and adult mussels have some potential for transforming promutagens into genetically active metabolites. Furthermore, the increases demonstrate the potential usefulness of these test systems for toxicological screening.

In the absence of PB we found significant increases in SCE rate only at the highest doses of CPA. This fact is consistent with results from biochemical studies (Stegeman, 1981; Livingstone and Farrar, 1984) that show only low levels of monooxygenase activity in untreated adult mussels compared with levels typical for fish or mammals. Concentrations of CPA that produced significant increases in SCE in adult mussels also resulted in lower yields of dividing cells, indicating that CPA may have had a directly toxic effect. CPA at a concentration of 10^{-2} *M* was lethal to adult mussels in a few days. Consequently, we cannot dismiss the possibility that increased SCE rates were a reflection of cell injury, possibly caused by the generation of free radicals (Malins et al., 1983), and not directly related to CPA metabolism.

These effects of CPA and PB on SCE in mussel adults and larvae are consistent with results of cytogenetic and biochemical studies of mammalian systems. The requirement for metabolic activation of CPA for SCE induction has been demonstrated in mammalian systems (Perry and Evans, 1975; Allen and Latt, 1976; Au et al., 1980). PB has been demonstrated to be an essentially good inducer of microsomal activation systems for CPA in rat liver (De Raat, 1977). It is not known, however, which metabolites of CPA are responsible for its induction of SCE, nor is it known what metabolic capabilities are involved in the activation of CPA (Au et al., 1980).

The many differences between the assays of the adults and larvae make comparisons of the two life-history stages difficult. However, no evidence existed of toxicity to larvae at any CPA concentration tested, and larvae appeared to have the same metabolic capacities as adults.

With the proviso that no mechanistic link has been established between CPA, PB, and monooxygenases in *M. edulis*, the results presented here indicate that PB-pretreatment increases SCE in mussel adults and larvae at all concentrations of CPA tested. PB appears to be acting in both adults and larvae to increase the rate at which CPA is transformed to metabolites capable of SCE induction. These results are in agreement with those of Moore (1979) who demonstrated increases in the levels of NADPH-neotetrazolium reductase activity in blood cells, intestinal epithelium, and oocytes of adult mussels following exposure to PB. This enzyme has been used as a marker of the microsomal detoxication system in mammals (Lindner and Behyhl, 1978), and the reaction is believed to result from NADPH-cytochrome P-450 reductase (Masters et al., 1966). Because it is not known which metabolites of CPA are SCE inducers, it is not possible to deduce the metabolic targets of PB, or vice versa.

The PB-associated increase of metabolic activation demonstrated here

may represent induction of components of the microsomal detoxication system, and it is a reminder of the complexity of organismal responses to the mixtures of chemicals found in natural environments. Our results indicate that SCE analysis is a valuable tool for investigating the susceptibility of marine organisms to genetic damage by specific or mixed environmental contaminants; as such, it is likely to be useful in a wide variety of natural habitats.

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