Sister Chromatid Exchange Induced by Etheno-ATP Derivatives in Vitro

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ABSTRACT

Genotoxic activities of a series of commercially purchased 1,N'-ethenoadenosine (ε-Ado) and 1,N'-etheno-2-deoxyadenosine (ε-dAdo) derivatives were assessed using the sister chromatid exchange (SCE) assay in murine spleen lymphocytes in vitro. Of the ε-Ado adducts evaluated for SCE induction, ε-ATP and ε-dATP were highly active (5X baseline) SCE inducers over a concentration range of 50-150 μM. Moderate SCE-inducing activities were seen with ε-dAdo, ε-A, and ε-AMP. ε-A was of particular interest in that spleen lymphocytes from a single mouse were highly sensitive to SCE (>50 SCE/cell at 75 μM). ε-Ado was weakly effective and ε-ADP and ε-dAMP did not produce significantly elevated SCEs. Cocanavalin A-stimulated T-lymphocytes and lipopolysaccharide-stimulated B-lymphocytes exhibited comparable SCE responses to ε-A, ε-AMP, and ε-dATP. However, B-lymphocytes were considerably less sensitive than T-lymphocytes to ε-dAdo and ε-ATP.

Evaluation of the purities of specific ε-Ado derivatives, as performed by high-performance liquid chromatography and thin layer chromatography, failed to detect potential contaminants as cytogenetically active agents. However, a difference (about threefold) in cytogenetic activities of two lot numbers of ε-ATP paralleled the difference in UV absorbance of equivalent concentrations (mg/ml), prepared according to the manufacturers stated purity. Any impurities likely to be present were consistent with inactive nonchromophoric compounds such as buffer salts.

Because of the direct genotoxic activity of ε-A in intact mammalian cells, we suggest that intracellular adenylate pools, including the prominent ε-purine adducts ε-ATP, are non-DNA targets for ε-modification by active metabolites and the resulting ε-adducts are likely to be active moieties in SCE induction and in neoplastic transformation produced by ethyl carbamate.

INTRODUCTION

The carcinogenic activity of ethyl carbamate (urethane) is well documented (1). Ethyl carbamate produces lung tumors, lymphomas, hepatomas, melanomas, and vascular tumors in mice, rats, and hamsters following administration by oral, inhalation, s.c., or i.p. routes. It is an initiator in skin carcinogenesis and is carcinogenic in single dose experiments. However, short term in vitro assays have, with few exceptions (e.g., micronucleus and cell transformation assays), failed to detect genotoxic activity of ethyl carbamate (2).

In contrast to other short term assays, in vivo SCE is unique in providing definitive evidence of the genotoxic potential of ethyl carbamate (3). At dose levels previously found to produce pulmonary tumors in strain A mice (4), ethyl carbamate produced highly elevated SCE levels in regenerating liver (5, 6), alveolar macrophage cells (6), and bone marrow of various murine strains (7, 8), including strain A mice (9). The primary hepatic DNA adduct of ethyl carbamate exposure in vivo is 7-(2-oxoethyxyguanosine (13, 14). Because the latter DNA adduct leads to depurination, it is not likely the source of the highly persistent SCEs observed in our studies, as purinic sites do not appear to be associated with SCE induction (15). In contrast to DNA adducts, exocyclic RNA adducts are produced in vivo by metabolites of ethyl carbamate (16). Similar exocyclic adducts of adenine (ε-A), cytidine (ε-Cyt), and guanine (ε-Gua) are produced in vitro by reaction of the respective base with the vinyl chloride metabolite, chloroacetaldehyde (17, 18). Of the ε- and non-ε nucleic acid adducts, only ε-Gua has been found to be a highly efficient mutagenic lesion that could function either as Gua or Ado in transcription in vitro and, consequently, may be involved in initiation of carcinogenesis (19). However, because steric effects inherent in double-stranded DNA limit the formation of ε-adducts, such DNA adducts have not been detected in tissues of animals treated with ethyl carbamate.

In contrast to conventional approaches that focus on DNA as the primary target for initiation of neoplastic transformation, we are investigating the possibility that, because of their high intracellular concentration in a relatively unhindered form, cellular purine pools, in general, are a primary target of the electrophilic metabolite of ethyl carbamate. Moreover, ε-adducts in nucleotide pools may be involved at various stages in neoplastic transformation, including production of alterations in DNA. This hypothesis would be strengthened by demonstrating that ε-purine adducts per se are genotoxic.

In the present study, genotoxic activities of a series of commercially available ε-Ado and ε-dAdo derivatives were assessed using the SCE assay in murine spleen lymphocytes in vitro. Evaluation of the purities of specific ε-Ado derivatives was performed by HPLC and thin layer chromatography.

MATERIALS AND METHODS

Male BDF1, mice bred from parental strains, C57BI/6J and DBA/2J, (purchased from The Jackson Laboratory, Bar Harbor, ME) and used at age 3–3.5 months. The following ε-adducts were purchased from Sigma Chemical Co.: ε-ATP, ε-ADP, ε-AMP, ε-A, ε-Ado, ε-dATP, ε-dAMP, and ε-dAdo. Solutions (25–250 μM) were prepared in RPMI 1640 culture media (GIBCO), without subsequent adjustment of pH, or in 50 mM ammonium phosphate buffer (pH 6.0) just prior to use in SCE or HPLC studies, respectively.

Lymphocyte Isolation and Culture. Isolation and culture of spleen lymphocytes was as previously described (10, 12). Briefly, spleens, isolated aseptically from replicate mice, were gently teased to release lymphocytes into sterile wash solution (phosphate buffered saline con...
taining 2% heat inactivated FBS). Following an additional wash, lymphocytes were suspended in RPMI, stained with Turk’s stain, and counted with a hemocytometer. 10⁶ lymphocytes/well (Linbro, 24-well microculture plates) were seeded into 1 ml of complete media containing 20% FBS (heat inactivated for 30 min at 56°C) in RPMI 1640 (pH 7.3) and, 2 mM l-glutamine, penicillin/streptomycin, 2.5 x 10⁻⁵ M 2-mercaptoethanol, and 5-bromo-2′-deoxyuridine (3.5 μM). Either 60 μM/μl of lipopolysaccharide (Sigma Chemical Co.) or 6 μg/ml of Con A (Sigma, type IV) was used as a mitogen. Immediately prior to initiation of culture specific ε-adducts were added in varying amounts to lymphocytes of replicate mice. No adjustments were made in final media pH prior to initiation of culture. Cells were cultured in complete darkness and 4–5% CO₂. After 21–24 h an additional 1 ml of complete media containing the appropriate mitogen, but without ε-adduct, was added and the cultures were continued for a total of 48 h. Three h prior to harvest, 0.5 ml colcemid (GIBCO, 10 μg/ml) was added to each well. Cell harvest and differential staining were by routine methods. 20 cells/culture were evaluated for SCE and 100 consecutive metaphases were scored for cell cycle kinetic analysis (i.e., relative frequencies of first, second, and third division cells).

Analytical Methods. A Hewlett-Packard HP 1090 HPLC was used to monitor the purity of commercial ε-adducts. This instrument is equipped with a diode array UV detector system and supports programming for simultaneous monitoring at eight wavelengths, thus optimizing UV detection of individual peaks. The wavelengths monitored in this study ranged from 210 to 270 nm in 10-nm increments. Automated scanning of UV spectra enhanced identification and purity assessment of individual peaks. Because of their highly fluorescent nature, ε-Ado derivatives are readily detected by fluorescence analysis. A parallel HP 1046 programmable fluorometric detector was used for fluorescence monitoring (262-nm excitation; 410-nm emission).

A programmed elution gradient from 100%, 50 mM ammonium phosphate buffer (pH 6.0) to 70% buffer/30% methanol over 30 min and a HP reversed-phase C₁₈ column (Hypersil ODS, 5 μM, 200 mm) produced excellent separation of a standard mixture of ε-Ado ribonucleotide and deoxyribonucleotide mono-, di-, and triphosphates; ribonucleosides, deoxyribonucleosides; and free nucleobases. With the methodology used in the present study, standard elution times were: ε-ATP (7.0 min), ε-ADP (7.7 min), ε-dATP (10.2 min), ε-AMP (12.2 min), ε-dAMP (17.1 min), ε-Ado (18.9 min), ε-AMP (22.7 min), and ε-dAdo (24 min).

As a further check of purity, samples of nucleosides and nucleotides, and base standards were chromatographed by two methods of thin-layer chromatography. Highly concentrated standards in water were applied to Brinkman CEL 300-50 thin-layer plates (with and without fluorescent indicator) and plates were developed with n-butylric acid/water/ammonium hydroxide (66%/33%/1%) (20) or to Whatman KC 18F plates and eluted with 80% ethanol/20% H₂O. Spots were visualized by fluorescence using an ultraviolet blacklamp and were then stained by nonspecific iodine staining.

**RESULTS**

**In Vitro Cytogenetic Studies.** Fig. 1 illustrates the SCE dose-response curves produced in spleen lymphocytes by active ε-adducts. Of the ε-Ado adducts evaluated for SCE induction in cultured spleen lymphocytes in vitro, ε-ATP and ε-dATP were highly active SCE inducers over a concentration range of 50–150 μM. Chromosome aberrations were not specifically scored in this study but are the subject of another study. Nevertheless, many normally rare aberrations were observed in the course of scoring SCE and cell cycle kinetics. Very complex chromosomal aberrations including triradials, quadradrials, endoreduPLICATIONS, as well as multiple complex chromosomal breakage and rearrangements within a single cell, were commonly noted on slides corresponding to ε-ATP and ε-dATP at doses of ≥75 μM. Moderate SCE-inducing activities were seen with ε-dAdo, ε-A, and ε-AMP. ε-A was of particular interest in that spleen lymphocytes from a single mouse were highly sensitive to SCE (>50 SCE/cell at 75 μM) induction. Because of the high responses from the single mouse over the entire concentration range, it was not included in the ε-A dose-response curve illustrated in Fig. 1. ε-Ado was only weakly effective and ε-ADP and ε-dAMP did not produce significantly elevated SCEs or chromosomal aberrations.

Con A-stimulated T-lymphocytes and LPS-stimulated B-lymphocytes exhibited comparable SCE responses to ε-A, ε-AMP, and ε-dATP. However, B-lymphocytes were considerably less sensitive than T-lymphocytes to ε-A and ε-ATP. In general, no significant deviations in cell cycle kinetics (as compared with respective control cell cycle kinetics) were observed over the dose ranges of ε-adducts studied.

**HPLC Analysis of ε-Ado Samples.** The widely held assumption that cell membranes are impermeable to nucleotides, and the fact that cytogenetic effects of the magnitude observed in our studies are generally associated with direct-acting alkylating agents, raised concerns that impurities in the commercially purchased samples may be responsible for the observed cytogenetic effects. Commercial ε-Ado samples were analyzed by HPLC analysis. ε-Ado derivatives were readily identified by their characteristic fluorescence and UV-absorbance spectra. UV spectral recordings were indicative of a single component being present in all major peaks.

Single peaks were observed in the UV and fluorescence chromatograms of ε-dAdo, ε-Ado, ε-dAMP, and ε-AMP samples. ε-A was present as a single minor impurity in both the fluorescence and UV chromatograms of ε-A. ε-AMP was a minor component in samples of ε-ADP. The nucleotide and deoxyribonucleotide triphosphates, ε-ATP and ε-dATP contained minor components that eluted at times corresponding to their respective di- and monophosphates as well as a very early (2.4 min) eluting UV absorbing (0.83% and 2.3%) peak in the chromatograms of ε-dATP and ε-ATP, respectively. The latter minor peak coincides in elution time with components commonly found in non-HPLC grade buffer salts (21). For example, a major peak at 2.4 min is consistently present in reagent grade...
sodium acetate, a buffer widely used in the synthesis of ε-adducts. Our assignment of minor ε-components as impurities in specific ε-samples is confirmed by HPLC chromatograms of composite mixtures of ε-A, ε-Ado, and ε-Ado nucleotides or ε-dAdo nucleosides and nucleotides (Fig. 2), in which excellent resolution of individual samples was achieved within 30 min with no evidence of additional extraneous peaks.

The purities of standard ε-Ado samples were also confirmed by thin-layer chromatography. Single fluorescent spots were produced by ε-Ado (R$_s$ 0.90) and ε-dAdo (R$_s$ 0.90) on Whatman KC 18F plates using ethanol/water (80/20), whereas ε-A samples exhibited a major spot (R$_s$ 0.69) and a minor spot (R$_s$ 0.90) that corresponded to ε-Ado. Chromatography of nucleosides and nucleotides was achieved with Brinkman CEL 300-50 plates and isobutyric acid/water/ammonium hydroxide (66%/33%/1%). Single fluorescent spots were observed for ε-Ado (R$_s$ 0.75), ε-AMP (R$_s$ 0.61), ε-Ado (R$_s$ 0.68), and ε-dAMP (R$_s$ 0.66). ε-dATP exhibited a single layer poorly resolved spot (R$_s$ 0.41) and ε-ATP produced a major spot (R$_s$ 0.40) and two minor spots (R$_s$ 0.49 and 0.59). In both thin-layer systems nonspecific iodine visualization failed to detect additional contaminating components.

Comparison of Two Lot Numbers of ε-ATP. Cytogenetic effects of ε-ATP were observed at concentrations of 50–150 μM. Recent shipments of ε-ATP were found to be of a different lot number and a concentration of 250 μM (3.3-fold higher) was required to produce effects comparable to 75 μM of the old lot number. HPLC chromatograms of the two samples were qualitatively similar and differed only slightly in the relative areas of integrated peaks. Stock solutions of the old and new lot numbers of ε-ATP were prepared in water, the pH of the solutions were determined, and UV absorbance spectra (Varian SuperScan 3 recording spectrophotometer) were recorded. Using the extinction coefficients of ε-ATP adjusted for pH (22), it was calculated that the actual stock solutions of the new and old lot numbers were 0.35 (35% pure) and 0.94 mg/ml (94% pure), respectively. The impurities were consistent with inactive nonchromophoric compounds, most likely buffer salts and the relative nucleotide content was in agreement with the differences in cytogenetic activities.

DISCUSSION

The present study has identified commercially purchased ε-adducts of ATP, dATP, AMP, Ado, and A as cytogenetically active agents when added to murine spleen cells cultured in vitro. Our finding of the highest genotoxic activities associated with the nucleotide triphosphates, ε-dATP and ε-ATP was surprising in view of the widely held assumption that cell membranes are impermeable to such highly polar low molecular weight compounds. An obvious explanation for the unexpected genotoxic activities of the ε-nucleotide adducts is the possibility that an impurity is present in the samples and is responsible for the observed genotoxic activities. Previously reported studies concerning assessment of the mutagenic activities of ε-adducts in Salmonella cautioned against the use of commercially purchased agents that may contain impurities as the actual mutagenic components (23). Thus HPLC and thin-layer chromatographic methods were used to evaluate the purities of commercially purchased samples used in our studies.

The commercially purchased samples were generally of high purity with extraneous minor peaks being attributed to other ε-adenine containing components. The only exceptions were ε-dATP and ε-ATP samples that contained minor early eluting peaks (highly polar components) consistent with contaminants widely found in non-HPLC grade buffer salts (21). Two lot numbers of ε-ATP used in the present study, differed by three-fold in their SCE-inducing activities and appeared to be of comparable purity as assessed by HPLC analysis. However, equivalent mg/ml solutions were found to differ by a factor of 2.7-fold in actual nucleotide concentration as determined by UV extinction coefficients.

Thin-layer chromatographic methods also failed to detect non-ε-Ado contaminants. Together, the HPLC and thin-layer chromatographic methods used in the present study will detect most organic agents with the possible exception of saturated aliphatic halides and alcohols.

SCE data in the present do not support the conclusion that impurities are responsible for the cytogenetic effects produced by ε-Ado derivatives. SCE elevations of up to five times baseline levels were observed, in the absence of notable cytotoxicity, at concentrations of ε-Ado nucleotides as low as 10$^{-4}$ M. Such SCE increases are generally associated with potent direct-acting alkylating agents (24). In contrast to alkylating agents, exogenously supplied ε-Ado derivatives (as analogues of normal cellular constituents) might be expected to exhibit diversity in their intracellular genotoxic activities. SCE induction by ε-adducts will depend not only on entry of the specific adduct into the cell but also cellular utilization of the substrate. Assuming uptake of specific ε-Ado derivatives in biologically significant amounts, specific cellular utilization will be a function of the activities of various adenose cycle (metabolic/catabolic) enzymes. ε-Ado derivatives have been demonstrated to substitute for the normal substrate in many enzymatic reactions that utilize substrates containing an adenine moiety (25). That ε-adducts may in general serve as substrates for adenosine cycle enzymes must be considered. For example, ε-A as a potential substrate for APRT and/or HGPRT may explain the hypersensitivity of the single mouse in our study to SCE induction by ε-A.

The observed differential SCE responses of B- and T-cells reflect previously reported differences in activities of specific
cellular enzymes that normally act on adenine containing substrates. Most notable is our finding of greater sensitivity of T-cells to SCE production by e-ATP. The relatively lower responses of B-cells to e-ATP may be due to differential membrane permeability and/or greater activity of the hydrolytic enzyme, ATPase. Peripheral blood B-cells exhibit greater ATPase activity (membrane and mitochondrial associated) than do T-cells (26) and splenic B-cell regions are associated with greater ATPase activity than are T-cell regions (27). e-ATP is an effective substrate for ATPase (20). Less extensive hydrolysis and consequently higher intracellular levels of e-ATP in T-cells may interfere with numerous critical ATP-dependent cellular processes.

Differential membrane permeability is not likely a factor in the differential sensitivity of B- and T-cells to nucleotides, as comparable responses were observed to the nucleotides, e-dATP and e-ATP. e-dATP was particularly active in both B- and T-cells. Perhaps only minor degradation of e-ATP occurs intracellularly, as is the case for de novo synthesized deoxy nucleotides (28). As an analogue of dATP, e-dATP may be incorporated into DNA (29) and/or function as a general inhibitor of ribonucleotide reductase, an enzyme whose activity is similar to B- and T-lymphoblasts (28). The latter mechanism would lead to a general deficiency in deoxyribonucleotide precursors of DNA and disrupt normal DNA replication. The somewhat lower activity of e-AMP may be derived following its conversion to e-dATP and/or e-ATP. The contrasting lack of activity of e-dAMP, however, is consistent with enhanced substrate specificity of cytosolic 5'-nucleotidase for hydrolysis of deoxyribonucleotide monophosphates (28) deoxyribonucleosides as compared with the preference of ecto-5'-nucleotidase for ribonucleotide monophosphates.

In turn, the greater susceptibility of T-lymphocytes relative to B-lymphocytes to SCEs induced by e-Ado may be attributed to differences in the relative cellular activities of phosphorylation/dephosphorylation enzymes. Kinases that are efficient in phosphorylating dAdo are predominant in lymphoid tissues (30). However, differences exist among subpopulations of lymphocytes. As compared to dividing B-lymphoblasts and nonlymphoid tissue, immature T-cells contain higher activities of dAdo phosphorylating enzymes (particularly dCyt kinase and to a lesser extent adenosine kinase) than dAdo dephosphorylating enzymes (mainly, 5'-nucleotidase) (28). Enhanced T-cell responses to e-dAdo observed in the present study mimics greater sensitivity of T-cells, relative to B-cells, to eAdo cytotoxicity in the ADA deficiency disease state (28, 30).

ADA is a purine catalytic enzyme involved in differentiation of B- and T-cells (31). The highest levels are found in thymus and spleen, organs of importance to the immune system (32). e-Ado derivatives are chemical analogues of ADA deficiency. By virtue of their anellated modification at the N7-position of adenine, e-Ado and e-AMP escape the primary route of purine degradation, i.e., deamination via ADA (33) or adenylate deaminase enzyme, respectively. The latter mechanism may assure persistence of e-modified purines at the expense of normal purine pools. High ADA activities may impart unusual susceptibility to lymphoid tissues via production of a high ratio of e-Ado to unmodified adenine-containing substrates following exposure to e-Ado derivatives. Such a mechanism is certainly of relevance to long-term consequences of exposure to agents that produce such adducts. In turn, the highly persistent nature of the SCE-inducing damage produced by ethyl carbamate is consistent with formation intracellularly of e-Ado adducts that persist for prolonged periods of time and actively induce SCE (11, 12) and chromosome aberrations.

Of the e-adducts evaluated in the present study, the nucleotides, e-dATP and e-ATP, were most active in spite of expected lower cellular uptake. Moreover, concentrations of the parent nucleotides in the culture media varied over time due to sequential enzytogenetic hydrolysis of tri-, di-, to monophosphates and the relatively slower hydrolysis of e-AMP but not of e-dAMP to their respective nucleosides via ecto-5'-nucleotidase. Because e-ATP and e-dATP exhibited greater cytogenetic activities than did any of their respective hydrolysis products, we must conclude that the observed cytogenetic activities are due to an external membrane effect and/or entry into the cell of the intact nucleotide.

It is of interest to note that concentrations of unmodified ATP similar to those used in the present study, i.e. ≤150 μM, have been reported to have a permeabilizing effect on the membranes of several cell types, including lymphocytes (34–37). Indeed, considerable evidence is accumulating concerning inherent membrane permeability of specific cell types to substances such as ATP that are normally presumed to be impermeant (38, 39).

The present and accompanying studies are the first to demonstrate direct genotoxicity of e-adducts in intact mammalian cells. Moreover, e-A adducts are readily detected in acid extractable nucleotide pools from spleen lymphocytes of mice treated in vivo with ethyl carbamate, in contrast to previous reports concerning the lack of similar adduct production in hepatic DNA (40). We suggest that intracellular adenylate pools, including the prominent ubiquitous nucleotide, ATP, are non-DNA targets for e-modification by active metabolites, and the resulting e-adducts are likely to be active moieties in SCE induction, and in neoplastic transformation produced by ethyl carbamate and other carcinogens that produce similar adducts.

REFERENCES


* Unpublished data from our laboratory.

† Unpublished observations.

‡ Manuscript in preparation.


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