Suppressive effects of methyl methacrylate on the mutagenicity and DNA adduct formation induced by 1-nitropyrene and benzo[a]pyrene

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Methyl methacrylate (MMA) is widely used as a cement in dentistry, orthopaedic surgery and ophthalmology. Studies based on short-term genotoxicity tests have produced conflicting results in the last two decades. In the present study, the effects of MMA on the mutagenicity of 1-nitropyrene (1-NP) and benzo[a]pyrene (B[a]P) were evaluated with the Salmonella typhimurium TA98 strain in the absence and presence of S9 mix. The direct-acting mutagenicity of 1-NP was markedly decreased by MMA in a dose-dependent manner. However, a low inhibitory effect of MMA on the metabolic-acting mutagenicity of B[a]P was observed. MMA did not show mutagenicity within the concentrations of 4.7-37.6 µM either with or without S9 mix. The inhibitory effect of MMA was not due to its cytotoxicity because very low and/or no cytotoxicity of MMA to S. typhimurium TA98 was observed. To confirm the antimutagenicity of MMA against 1-NP and B[a]P, a 32P-postlabeling method was used to determine whether MMA modified DNA adduct formation produced by both compounds in calf thymus DNA. MMA inhibits the formation of 1-NP- and B[a]P-DNA adducts in a dose-dependent manner. The DNA adduct of 1-NP reduced by MMA was greater than that of B[a]P. Thus, we suggested that MMA was possibly acting as an inhibitor of chemical carcinogenesis.

Introduction

Methyl methacrylate (MMA) is widely used in orthopaedic surgery as a cement for arthroprostheses due to its excellent biocompatibility and haemocompatibility. It is also often used as a cement in dentistry and ophthalmology. Human exposure to MMA is quite frequent for both professional and medical reasons. In the last two decades, the genotoxicity of MMA has been investigated using short-term tests including the Salmonella/microsomal test (Poss et al., 1979; Waegenmaekers and Bensink, 1984), genotoxicity testing in L5178Y mouse lymphoma cells (Moore et al., 1988), chromosomal aberration, the sister chromatid exchange (SCE) assays (Bigatti et al., 1989; Marez et al., 1991) and the micronucleus test (Bigatti et al., 1994). However, conflicting results regarding its genotoxicity have been reported. Some previous reports showed that MMA was mutagenic in the Salmonella/microsomal test using a preincubation procedure (Poss et al., 1979; Lijinsky and Andrews, 1980; Waegenmaekers and Bensink, 1984), while other published data indicated that MMA was not mutagenic in Salmonella typhimurium using the plate incorporation assay (Lijinsky and Andrews, 1980; Waegenmaekers and Bensink, 1984; Zeiger et al., 1987). In carcinogenicity testing, inhalation of MMA for 102 weeks did not induce any increased incidence of neoplasms in male and female rats or mice (Chan et al., 1988). Exposure to MMA up to 2028 p.p.m. resulted in no embryo or fetal toxicity or malformations even at exposure levels that resulted in maternal toxicity (Solomon et al., 1993). In the present study, the antimutagenicity of MMA against benzo[a]pyrene (B[a]P) and 1-nitropyrene (1-NP) was first investigated with the Salmonella/microsomal test using the S. typhimurium TA98 strain in the presence and absence of S9 mix. The 32P-postlabeling method was used to confirm the influence of MMA on the formation of B[a]P- and 1-NP-DNA adduct in calf thymus DNA.

Materials and methods

Chemicals

MMA was obtained from Fluka Chemical Co. (Buchs, Switzerland). B[a]P, glucose-6-phosphate, lysozyme, micrococcal endonuclease and NADP+ were purchased from Sigma Chemical Co. (St. Louis, MO). 1-NP was obtained from Aldrich Chemical Co. (Milwaukee, WI). [γ-32P]ATP was purchased from Amersham International Plc (Amersham, UK). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). Proteins A, RNase A, RNase T1, calf thymus DNA and spleen phosphodiesterase were purchased from Boehringer-Mannheim GmbH (Mannheim, Germany).

Mutagenicity assay

The procedures used were basically the same as those of the plate-incorporation assay described by Maron and Ames (1983). To 2 ml of molten top agar was added 8.1 nmol of 1-NP or 39.6 nmol of B[a]P in 50 µl of dimethylsulphoxide (DMSO) without or with S9 mix, respectively. Four doses of MMA in 50 µl of DMSO and 100 µl of each overnight culture of TA98 were gently mixed with 2 ml of top agar and poured onto minimal glucose agar plates. The plates were then incubated at 37°C for 48 h and the revertant colonies counted. All experiments were performed at least twice with triplet plates for each dose. The results are expressed as means with standard deviation. The percent inhibition (PI) was calculated as described previously (Lee et al., 1994a). S9 mix from liver of Aroclor 1254-treated Sprague-Dawley rat was prepared as described by Maron and Ames (1983).

The toxicity assay

The toxicity of MMA was tested by plating 50 µl of an overnight culture geometrically diluted by a factor of 106 with sterile phosphate-buffered saline (PBS) on nutrient agar plates. containing various doses of MMA and/or S9 mix (Maron and Ames, 1983). As in the mutagenicity assay, all experiments were performed at least twice with triplet plates for each dose of MMA. The mean number of colonies from these plates were used to determine the percentage survival.

32P-postlabeling method

To determine the effect of MMA on the 1-NP-DNA adduct formation, 0.5 ml of a reaction mixture was used which contained 0.5 mg of calf thymus DNA, 125 µg of hypoxanthine, 2.5 µg of catalase, 0.1 U of xanthine oxidase, 100 µM 1-NP, 50 mM KH2PO4 and various doses of MMA. The mixture was incubated with shaking for 3 h at 37°C. Hypoxanthine (125 µg) and xanthine oxidase (0.1 U) were then added to the reaction mixture and incubated at 37°C for 18 h. The DNA was isolated using the phenol method as described previously (Lee et al., 1994b). A 10 µg quantity of DNA was hydrolysed to deoxyribonucleotide 3'-monophosphates by 1 U of micrococcal endonuclease and 10 µg of spleen phosphodiesterase in 50 µl of sodium succinate buffer (10 mM, pH 6.0) containing 5 mM CaCl2 at 37°C for 4 h. The hydrolysates were dissolved in 100 µl of water containing 10 mM ammonium formate (pH 3.5) and 1 mM tetrabutylammonium chloride. To concentrate the adducts, the mixture was extracted twice with 1 vol of 1-butanol. The butanol extract...
was evaporated using a speed Vac concentrator (Savant Instrument, Inc., Hicksville, NY) and dissolved in 18 μl of water and 6 μl of kinase mixtures (1.2 μl of 1 M Tris buffer, 2.4 μl of 10X labelling buffer, 0.5 μl of S.T4 polynucleotide kinase and 1.0 μl of 10 μCi r-32P[ATP]) followed by incubation at 38°C for 1 h. DNA adducts were analysed by four-directional PEI-cellulose TLC (Polygram CEL 300 PEI, Macher-Nalgel Co., Duren, Germany), as described previously (Lee et al., 1994b). The adducts were located by screen-enhanced autoradiography at ~80°C, and the levels were calculated using the formula as described by Gupta (1985).

The protocol used to investigate the effect of MMA on B[a]P-induced DNA adduct formation in calf thymus DNA was similar to that used with 1-NP-induced adducts with the exception of the metabolic activation system. Mixtures of 6% Aroclor 1254-induced rat liver S9, 50 μM B[a]P, 0.5 mg calf thymus DNA and various doses of MMA were prepared and incubated at 37°C for 3 h before extraction of DNA by the phenol method. The DNA was then hydrolysed by micrococcal endonuclease and spleen phosphodiesterase. To enrich the DNA adduct levels, normal nucleotides were removed by incubation with 6 μg of nuclease P1 at 37°C for 1 h. The 32P-labelling procedure was the same as that described above. TLC analyses were performed on the 32P-labelled DNA adducts according to the method described by Moller et al. (1993).

Results

Dose-dependent inhibition of MMA (4.7–37.6 μmol/plate) on the direct-acting mutagenicity induced by 1-NP was shown in S.typhimurium TA98 without S9 mix. However, a low inhibitory effect of MMA on the metabolic-acting mutagenicity of B[a]P was observed at the same concentrations (Table I). No mutagenic activity of MMA at the concentrations tested was detected in the presence or absence of hepatic microsomal activation (Table I).

The cytotoxicity of MMA to S.typhimurium TA98 in the same dose range was evaluated (Table II). Very low or no cytotoxicity was observed with and without S9 mix. Therefore, the inhibitory effect of MMA on the mutagenicity of 1-NP and B[a]P was not mediated by the cytotoxicity.

In order to confirm the antimutagenicity of MMA against 1-NP and B[a]P, the 32P-postlabelling method was used to determine whether MMA modified the DNA adduct formation in calf thymus DNA induced by 1-NP and B[a]P. MMA inhibited the formation of 1-NP- and B[a]P-DNA adducts in a dose-dependent manner (Table III). The inhibitory effects of MMA on the DNA adduct levels of 1-NP were greater than those of B[a]P. The autoradiograms of 1-NP and B[a]P in calf thymus DNA with or without MMA are shown in Figures 1 and 2. No DNA adducts were detected in calf thymus DNA induced by MMA alone.

Discussion

The results presented here are consistent with previous reports (Waegemaekers and Bensink, 1984; Zeiger et al., 1987; Li et al., 1989). However, MMA shows a positive response in the Ames test using a preincubation procedure (Poss et al., 1979; Lijinsky and Andrews, 1980; Waegemaekers and Bensink, 1984). Some lines of evidence support the statement that MMA is probably clastogenic and genotoxic in animal and human cells (Moore et al., 1988; Marez et al., 1991; Bigatti et al., 1994), although those properties are apparent only at concentrations >2000 μg/ml for L5178Y/TK+/- mouse lymphoma cells and 32 p.p.m. for human lymphocytes, respectively. At high atmospheric concentrations (1000 p.p.m.), MMA induced chromosome damage in mice after a single, but not multiple, exposure (Chan et al., 1994). Following inhalation exposure of MMA (0, 500 or 1000 p.p.m.) for 102 weeks, there were no dose-related increases in tumour incidence in exposed rats and mice (Chan et al., 1988). Moreover, a lower incidence of several tumour types in the MMA-exposed animals was observed in comparison with the control group. For example, a significant dose-related decrease was observed in the incidence of alveolar/bronchiolar tumours (11/50, 1/50 and 4/50) in MMA-exposed male mice and pituitary gland neoplasms (12/49, 3/44 and 2/39) in female mice. Our results show that MMA was probably functioning as an inhibitor of mutagenicity and DNA adducts induced by chemical carcinogens. These may partly be responsible for the reduction in the tumour incidence in MMA-exposed animals.

Both 1-NP and B[a]P are generally considered to be the most important mutagenic and carcinogenic compounds in airborne particulate matter and cooked foods (Lijinsky and Shubik, 1964; Kinouchi et al., 1986; Lee et al., 1994b). MMA is frequently found in dental clinics and surgical rooms because of its volatility at room temperature. In addition, MMA has...
Methyl methacrylate inhibits 1-NP and B[a]P mutagenicity

Table III. The inhibitory effect of MMA on the formation of 1-NP- and B[a]P-DNA adducts in calf thymus DNA

<table>
<thead>
<tr>
<th>MMA (µmol)</th>
<th>DNA adducts/10⁸ nucleotide</th>
<th>PI (%)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1-NP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15.1</td>
<td>16.0</td>
</tr>
<tr>
<td>9.4</td>
<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>18.8</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>37.4</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>B[a]P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>446.3</td>
<td>807.5</td>
</tr>
<tr>
<td>9.4</td>
<td>251.2</td>
<td>620.5</td>
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<tr>
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</tr>
<tr>
<td>37.6</td>
<td>20.7</td>
<td>47.4</td>
</tr>
</tbody>
</table>

¹PI (%) = 100 - (total DNA adduct levels in the presence of MMA)/(total DNA adduct levels in the absence of MMA) × 100.

Fig. 1. ³²P-postlabelling assay autoradiograms of 1-NP in calf thymus DNA in the absence and presence of various concentrations of MMA. (A) 1-NP alone, (B) MMA alone, (C) 0.5 µl MMA and 1-NP, (D) 1.0 µl MMA and 1-NP, (E) 2.0 µl MMA and 1-NP and (F) 4.0 µl MMA and 1-NP.

been found in ambient air, commercial polystyrene plastics, industrial sewers, waste water, river water and drinking water (Chan et al., 1988). The highest concentrations of MMA (50-100 p.p.m.) have been measured in the operating room during joint replacement surgery (Darre et al., 1992). Although the lowest concentration of MMA used in this study was 2.5- to 5.0-
fold higher than that in the operating room, in our preliminary experiment, a similarly low concentration of MMA (50 p.p.m.) was used to test antimutagenicity. Inhibition levels of the mutagenicity of 1-NP and B[a]P of 10.8 and 9.4% were observed respectively (data not shown). Thus, our data suggest that the release of MMA from bone cement or inhalation may not be harmful to human health. On the contrary, MMA may be helpful in the prevention of mutagenicity induced by environmental carcinogens.

Covalent binding of carcinogens to target cell DNA is thought to be the first event in the tumour-initiating process. 1-NP and B[a]P require the involvement of nitroreductase in *S. typhimurium* and aryl hydrocarbon hydroxylase in cytochrome P-450 IA1 of rat liver microsomes (S9) to express their mutagenicity (Fu, 1990; Conney, 1982). The specific DNA adduct spot 2 of 1-NP and spot 1 of B[a]P formed in calf thymus DNA in the presence of xanthine oxidase and Aroclor 1254-induced rat liver S9 mix, respectively, appeared to be the major DNA adduct C8-deoxyguanosine-aminopyrene and N2-deoxyguanosine-B[a]P found in the Salmonella/microsomal test system. This is because a TLC chromatographic profile was observed similar to those in previous reports (Roy *et al.*, 1989; Devanesan *et al.*, 1992). The specific DNA adduct spot of both mutagens almost disappeared with the addition of higher doses of MMA (Figures 1 and 2). These results suggest that the inhibitory effects of MMA on the mutagenicity of 1-NP and B[a]P might be mediated through altering their metabolic activation. However, the mechanisms of action for the inhibition of MMA on the mutagenicity and formation of DNA adduct induced by 1-NP and B[a]P need further investigation.

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