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Evaluation of Low-Dosage Environmental Mutagens with a Long-Term, Cultured Epithelial Cell Line

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Polycyclic or polynuclear aromatic hydrocarbons (PAHs) are a group of compounds consisting of at least two fused aromatic rings and are common environmental contaminants in soil, water, and air (Menzie et al., 1992). Additionally, some PAHs are considered to be mutagenic, carcinogenic, and cytotoxic compounds (Klaassen et al., 1986). BaA (Benz(a)anthracene) and BaP (Benzo(a)pyrene) are the two major unsubstituted PAHs identified by the USEPA as priority pollutants (USEPA, 1985). BaA is suspected to be a human carcinogen by the US Department of Health and Human Services (1983), whereas BaP is regarded as an animal carcinogen (Vainio et al., 1985). It is estimated that the emission concentrations of BaA and BaP from mainstream cigarette smoke are 20-70 ng per g cigarette and 20-40 ng per g cigarette, respectively, with an average sidestream to mainstream weight ratio of 3 (NRC, 1986). In addition, BaA and BaP are also the main emissions from diesel particulate extracts with mean concentrations of 500 ppm and 40 ppm, respectively (NRC, 1983). From a recent report (Menichine, 1992) concerning PAHs in urban areas all over the world, the airborne concentration of BaP was typically found to be in the range of 1-20 ng/m^3 in Europe and 1 ng/m^3 in the U.S.A. For BaA, the concentrations commonly ranged from 1 to 50 ng/m^3 in Europe and from 0.1 to 1 ng/m^3 in the U.S.A. Therefore, the long term exposure to these two compounds may cause health effects.

To evaluate the potential carcinogenic, mutagenic, and cytotoxic effects of PAHs, Ames tests and animal tissue cell assay are the most commonly used methods (Hass et al., 1986). In regard to the *Salmonella typhimurium* assay, the dosage range of BaP and BaA was found to be generally between 1 µg/plate and 100 µg/plate (Fu et al., 1990). The range of the revertants /plate was observed to be in the range of 300-1000 and was highly dependent on the type of added enzyme (S9). It is very difficult to use the results of the Ames tests to estimate the realistic mutagenicities of test agents on human cells using such high dosages on bacterial cells. It was also found that a nontransformed pure rat tracheal epithelial (RTE) cell line was transformed *in vitro* by BaP (Steele et al., 1980). In addition, Steele and coworkers (1989) evaluated the respiratory carcinogens of a series of 17 chemicals using the RTE cells. The results indicated that the transformation frequency of BaP was 0.14 at a dosage of 15.5 µg/ml which was six times higher than the control frequency (Nesnow et al., 1986). Danheiser and his coworkers (1989) also evaluated low-dose BaP-induced HGPRT gene mutations with human

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lymphoblasts. However, these evaluation assays mentioned used the animal tracheal epithelial cells or lymphoid cells, not human epithelial cells. These previous studies might not be able to assess the direct effects of PAHs on human health due to the much lower ambient concentrations to which the general population is exposed and to the uncertain toxicological effects on human cells. Furthermore, the respiratory epithelium is a known major target site for many kinds of inhaled PAHs. Human epithelial cells might not have the same mutagenic responses as animal or bacteria cells (Van Dam et al., 1992).

In this investigation, the long-term cultured epithelial cell line were exposed to BaP or BaA, *in vitro*, and the frequency of HGPRT- mutants (Albertini et al., 1982) was evaluated by using limiting dilution analysis (Lefkovits and Waldmann, 1984) in 6-TG (6-thioguanine, 2-amino-6-mercapto-purine) containing medium.

MATERIALS AND METHODS

BaA and BaP were dissolved in cyclohexane (solvent) at a concentration of 10 µg/ml and were purchased from Laboratory Dr. Ehrenstorfer (Augsburg, Germany). Cyclohexane was purchased from Merck (Darmstadt, Germany). Hela cells, which are an epithelial cell line derived from human tissue, were obtained from ATCC. The cells were further subcloned in our laboratory. One of the subcloned cell lines, HS1, was used in this study. Under microscopy analysis, we did not observe the shape difference between HS1 an the original Hela cell line. Without adding mutagen, the spontaneous HGPRT mutation rate of HS1 is lower than those of the original Hela cell line. The cells were cultured in 75 ml plastic tissue culture flasks (Costar, Cambridge, MA) in 20 ml RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 95% humidified, 5% CO_2 incubator at 37°C. The cells were seeded at $5x10^5$ per flask and subcultured when they reached 80% confluence.

In order to choose the test concentrations (non-toxic) of BaP and BaA for the mutagenicity experiments, preliminary cytotoxicity assays were performed. 96-well microtiter tissue culture plates (Costar, Cambridge, MA) were inoculated with 50 µl of RPMI supplemented culture medium with various dosages of the tested compounds in triplicate. HS1 cells (10^4 cells per well) were then added in 50 µl volumes of culture medium. Three days later, the wells were pulsed with 0.5 µCi H^3-Thymidine (Du Pont, Boston, MA) 6 hours prior to harvesting with a semiautomatic harvester (Cambridge Technology Inc., Watertown, MA). The H^3-TdR incorporation was measured in a scintillation counter.

The 10^6 fresh HS1 cells were cultured in the presence of 1 or 10 ng/ml of fresh prepared test compounds (BaP and BaA) and an equivalent dosage of cyclohexane (1 mg/ml or 10 mg/ml) for three days in 50 ml culture flasks with a total volume of 6 ml. After being washed with PBS three times, the cells ($5x10^3$, 10^4, and $2x10^4$ per well) were then incubated in 100 µl of culture medium with the optimal selective dose, 5 µg/ml 6-TG (Sigma, St. Louis, MO), in 96-well plates for seven days. The assay method mentioned above was based on our primary experiments, which indicated that 5 µg/ml 6-TG is the optimal concentration to select HGPRT-negative mutant of HS1. Below the 5 µg/ml concentration, some HAT (hypoxanthine, aminopoerin, thymidine) medium-resistant clones were grown up. Moreover, with seven-day expression time, the 6-TG resistant cells will have grown to become visible colonies (generally 20 to 30 cells per colony). The rest

of the cells will have died out and changed their form into a small round shape. Their shape could be identified easily by an inverted microscope. Ten days after incubation, the number of colonies was found to be identical to that observed at seventh day. Each point as shown in the figures is the average value of triplicate observations.

Based on the Poisson probability distribution, frequency estimates were obtained from the slope of the best fit line determined by the maximum likelihood method for the relationship between the number of cells per sample and the logarithm of the percentage of negative samples (Lefkovits and Waldmann, 1984). Wells containing any growing colonies were counted as positive samples. The total number of cultures (wells) for each experimental point was 60 in this investigation. A semilog plot was used to estimate the frequency of mutant cells. By interpolating the mutation level at 37% negative samples, the size of the sample containing an average of one mutant cell can be estimated.

RESULTS AND DISCUSSION

To prevent the cytotoxic effects of the tested compounds, preliminary range-finding cytotoxicity assays were conducted. The dosages for toxicity tests were chosen to 5 log doses of the test agents (0, 0.1, 1, 10, and 100 ng/ml). Significant cytopathic effects were observed when the concentrations of BaP and BaA were higher than 10 µg/ml. Figure 1 shows the representative results of the proliferation of BaP, BaA, and cyclohexane treated cells. The background (without tested compounds) response was found to be approximately 83700 counts per minute (cpm). The number of counts at the four different doses were observed to range from 70000 cpm to 98000 cpm. It was suggested that there were no cytotoxic effects of BaP, BaA, and solvent found at these four different dosages. From the results of these tests, the concentrations of BaP and BaA for mutagenicity tests were selected to be 1 ng/ml and 10 ng/ml.

The HS1 cells pretreated with BaP, BaA, cyclohexane or nothing were cultured at limiting dilutions with 5 µg/ml of 6-TG. It was observed that the mutagenic response (the number of positive wells) is well linear with the cell numbers per well (data not shown). Moreover, it was found that there was a log-linear relationship between cell number concentrations ($5x10^3$, 10^4, and $2x10^4$/well) and the percentage of negative wells. The mutant frequencies of cells in the background and solvent (1/1000, equivalent to 10 ng/ml test agent containing cyclohexane) conditions were found to be similar, approximately 2x10-5. At doses of 1 ng/ml and 10 ng/ml of BaP (as shown in Figure 2), the mutant frequencies were observed to be very close, $5x10^{-5}$, which is more than two times as that found in the background and cyclohexane conditions. In regard to the mutagenesis of BaA, Figure 3 indicates that the mutation frequencies were found to be $3.7x10^{-5}$ at 1 ng/ml and $5.6x10^{-5}$ at 10 ng/ml. The mutant potency of BaA was found to be very close to that of BaP. A 100% increase in the mutant frequencies was observed at 1 ng/ml and 10 ng/ml of both BaP and BaA.

From our current investigation, it was demonstrated that BaP and BaA, even at quite low concentrations, had mutagenic effects in long-term cultured epithelial cell line. The mutant frequencies of cells incubated with BaP and BaA were found to increase significantly compared with those in the background conditions. Moreover, the dosages of BaP and BaA performed in our study were much lower than those conducted in the mutagenicity investigations described previously. BaP and BaA are the predominant emitted PAHs from cigarette smoke and diesel

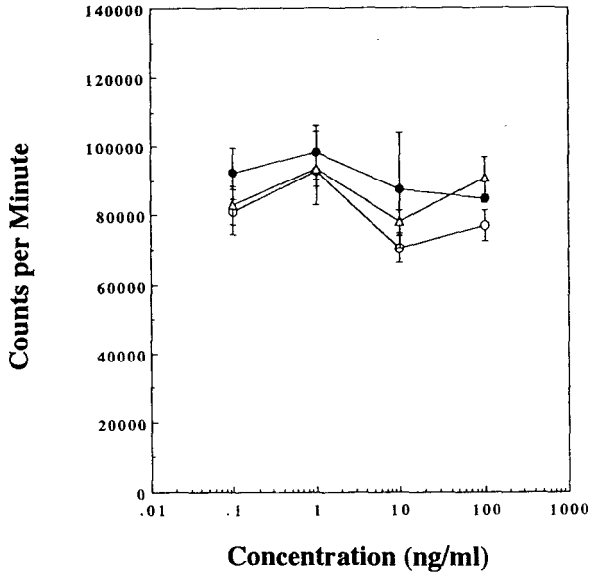

Figure 1. Cytotoxicity test of BaP and BaA. HS1 cells (10^4 cells per well) were incubated with various concentrations (0.1-100 ng/ml) of BaA(-O-), BaP(-●-), and equivalent doses of cyclohexane(-△-). The average background count in untreated cells was found to be 83700 cpm in triplicate cultures.

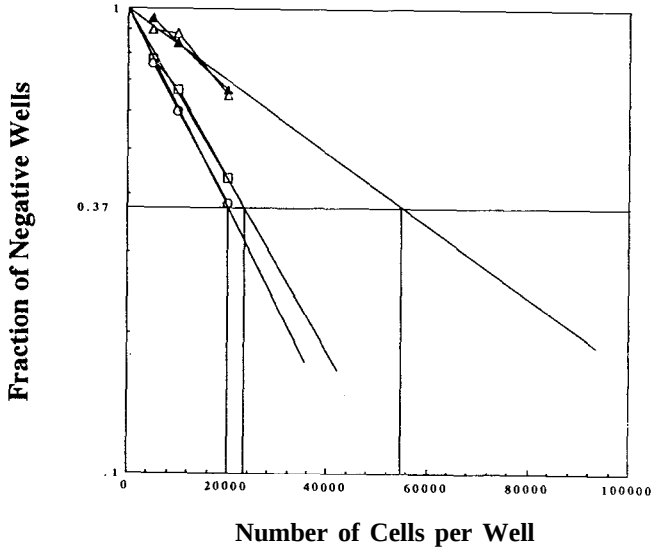

Figure 2. Growth of BaP treated cells in the presence of 5 µg/ml 6-TG. HS1 cells treated with 10 ng/m BaP (-O-) 1 ng/ml BaP (-□-)„ 1/1000 of cyclohexane (-△- as well as nontreated cells (-▲-). The calculation of fraction negative wells is described in the text.

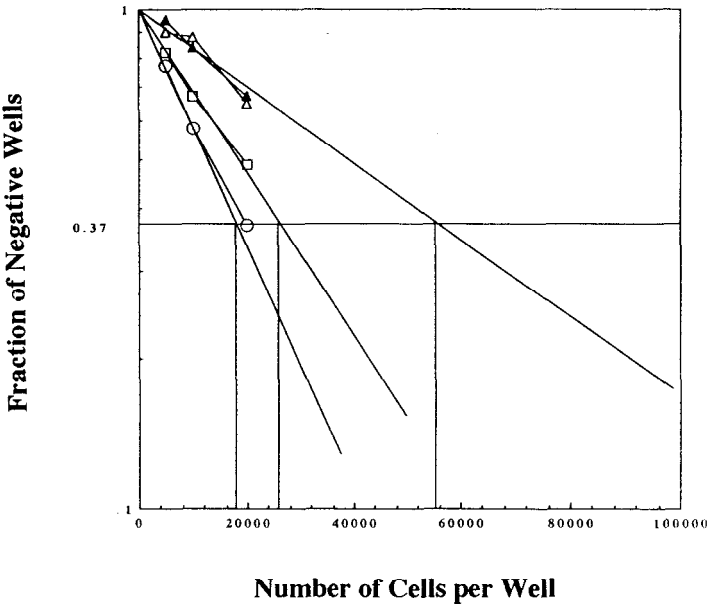

Figure 3. Growth of BaA treated cells in the presence of 5 μg/ml 6-TG. HS1 cells treated with 10 ng/ml BaA(-O-), 1 ng/ml BaA 1/1000 of cyclohexane(-Δ-) as well as nontreated cells (-▲-).The calculation of fraction negative wells is described in the text.

exhaust. Our results indicated that it is necessary to pay more attention to BaP and BaA, even though their emission quantities are very low.

Being the target cells for respiratory mutagens, long-term cultured epithelial cells (HS1 cells) were used in our investigation for the mutagenic assay. It is very important to evaluate the health effects of inhaled environmental chemicals in the respiratory tract, especially because the mutagenic effects of the inhaled chemicals on the epithelial cells are regarded to be related to the occurrence of respiratory cancer. It is very promising that a very sensitive epithelial cell culture assay system is now developed and can be used to evaluate and identify potential carcinogens at more realistic concentrations to which the general population is exposed.

The Ames test is the most commonly used method for detecting chemical carcinogens (Zeiger et al., 1992). However, it was suggested that at least fourteen factors are capable of influencing the results of the mutagenic potency of chemicals in *Salmonella typhimurium* (Ashby and Styles, 1978) in addition to the fact that the responses from the bacteria might not be the same as those from human cells. Furthermore, the addition of extra enzymes, such as S9 mix, is necessary for the metabolic conversion of carcinogens to their active mutagenic forms.

A long-term cultured epithelial cell assay system developed from this investigation is very sensitive to very low concentrations of BaP and BaA. Although, the biological functions of Hela cells might not be the same as those of normal cells, Hela cells are still a promising cell line for evaluation. In addition, Hela cells are a cloned cell line and can easily grow in large quantities. This epithelial cell assay system may be a very useful tool for evaluating more environmental chemicals for their mutagenic effects on the respiratory tract. Furthermore, there is a need for further studies of this technique to examine the questions of how good a predictor of carcinogenicity it is.

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