Multi-endpoint genotoxic assay using L5178Y (Tk^{+/-} – 3.7.2c) cells

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ABSTRACT — When the mouse lymphoma Tk assay (MLA) provides a positive result, its cause can be roughly estimated by examining colony sizes. An increase in the number of large colonies means that the compound tested has point mutational potential, while an increase in small colonies indicates the potential for chromosome aberration. However, it was found to be difficult to clearly judge this in the case of caffeine known as a clastogen lacking the potential of point mutation. In our study, caffeine significantly increased the thymidine kinase (Tk) mutation frequencies derived from large colonies as well as those from small colonies in the standard protocol, although the frequencies derived from a small colony were higher than those from large colonies at higher doses. Therefore, we prolonged the expression period from 2 days, a standard period, to 6 days after treatment and then examined the Tk and Hprt mutations simultaneously. The result showed that caffeine gave a completely negative result on a mutation test for both Tk and Hprt. On the other hand, ethyl methanesulfonate (EMS), a genotoxic carcinogen, showed a positive result for both. Moreover, caffeine and EMS significantly increased the frequencies of micronucleated cells. In conclusion, when MLA gives a positive result and the cause is ambiguous, in order to identify the exact cause of the positive response, it is helpful to perform a confirmatory test investigating the potential of Tk and Hprt gene mutation simultaneously after 6-day expression and to perform an in vitro micronucleus assay during the expression period.

Key words: MLA, L5178Y, Caffeine

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standard period, to 6 days after treatment, and then prepared micro-titer plates for detecting Tk and Hprt mutations simultaneously. Further, we prepared slides for the in vitro micronucleus (MN) assay to confirm the clastogenic potential.

**MATERIALS AND METHODS**

Chemicals: caffeine (Cas No.: 58-08-2) and 6-thioguanine (6-TG) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethyl methanesulfonate (EMS) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Trifluorothymidine (TFT) was purchased from Sigma-Aldrich Chemicals Inc. (St. Louis, MO, USA). RPMI1640 medium and surface active agent (Pluronic) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Horse serum was purchased from HyClone (Logan, UT, USA). Caffeine was dissolved in RPMI0 to prepare the final concentrations of 325, 625, 1250, 2,500 and 5,000 µg/ml. EMS was dissolved in physiological saline and aliquots of solution were added to the medium so as to be the final concentrations of 32.5, 62.5, 125, 250 and 500 µg/ml.

Cell lines: L5178Y (Tk+/− 3.7.2c) cells were obtained from Hadano Research Institute Food and Drug Safety Center. Cells were cleansed using HAT medium (including hypoxanthine, aminopterin and thymidine) and THMG medium (including thymidine, hypoxanthine, methotrexate and glycine) to remove the spontaneous mutants Hprt or Tk, respectively. They were maintained in liquid nitrogen at a density of 1 x 10^6 cells/ml in RPMI1640 medium containing 5% DMSO. Before the experiment, the stocked cells were thawed and cultured for 3 or 4 days, followed by confirming the doubling time was about 9 hr.

Mediums: Basic medium (RPMI0), RPMI1640 medium containing 200 mg/ml sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.5 mg/ml Pluronic, was used to wash cells after treatment. Growth medium (RPMI1640) consisted of RPMI, with 10% heat-inactivated horse serum. Cloning medium (RPMI1640) for colony formation in micro-titer plates (96-well) consisted of basic medium with 20% serum without Pluronic.

**Mouse lymphoma assay**

We followed the micro-titer method described in the reports by Honma et al. (1999). On treatment, the cells were suspended at a density of 2 x 10^5 cells/ml in 50 ml of growth medium in a 250 ml polystyrene flask, and then treated with compounds for 24 hr without S9 mix. A single flask was used for each concentration of compounds and duplicate flasks were used for solvent control. After plates were prepared to select Tk mutants in the standard manner after 2-day expression, the cell culture was continued for another 4 days, maintaining the cell density at less than 1.2 x 10^6 cells/ml. After the full 6-day expression, the plates for selecting Hprt as well as Tk mutants were simultaneously prepared. Duplicate plates were prepared for each concentration to select mutants and to measure plating efficiency respectively, and then were incubated for 12 days. About 6-day expression is routinely employed for Hprt mutation assay (Jenssen, 1984). The method employed for preparing the plates for Hprt mutant selection was the same as that for Tk mutants, except adding 16.7 µg/ml of 6TG to the medium instead of TFT as a selective agent (Chen et al., 2002). When observing the plates by a backlight colony counter with the aid of a stereomicroscope, mutant colonies were classified by size for Tk but not for Hprt mutants. When the diameter of a colony was 1/4 the diameter of the well or larger, and the edge of the colony was diffuse, it was classified as a large colony. When the diameter of the colony was less than 1/4 the diameter of the well, and the edge was well defined, it was classified as a small colony.

The relative total growth (RTG) was calculated to estimate the cytotoxicity of test compounds. Mutation frequencies were evaluated by statistically using multiple comparisons following Dunnett’s procedure.

**Micronucleus assay**

Twenty-four and forty-eight hr after treatment in MLA, a small part of the cell suspension during the expression period was sampled to prepare the micronucleus slides. Centrifuged cells were re-suspended in hypotonic solution (0.075 M KCl) for 15 min, and then fixed in ice-cold methanol/glacial acetic acid (3:1, v/v). Before dropping the cell suspension onto the slides, the fixative was substituted for methanol including 1% acetic acid. After staining by acridine orange solution, 2,000 cells per concentration were observed using a fluorescent microscope (x 600) to calculate micronucleated cell frequencies (Matsuoka et al., 1992). The frequencies were evaluated by statistically using Kastenbaum & Bowman’s test. The relative cell growth in cell suspension at each sampling time was used to evaluate the cytotoxicity.

**RESULTS**

**Mouse lymphoma assay**

Caffeine increased Tk mutation frequencies significantly after 2-day expression. The frequencies calculated only from the number of wells with large colonies
also increased significantly, as well as those only from small colonies. The frequencies derived from small colonies were higher than those from large colonies at higher concentrations. The higher two concentrations, 1,250 and 2,500 µg/ml, were very toxic because their RTG were less than 10% (7.5% and 5.8%, respectively). At the 5,000 µg/ml, the cytotoxicity was so severe that the number of cells were not enough to prepare the plate. After 6-day expression, there was no increase in the Tk mutation frequencies derived from either the large or small colonies, as well as those from the total number of colonies at all the concentrations even including toxic ones (Fig. 1A). Moreover,

Fig. 1. Tk mutation frequencies induced by caffeine (A) and EMS (B) after 2-day (left) and 6-day (right) expression. The cells were treated for 24 hr without S9 mix. After plates were prepared to select Tk mutants at 2-day expression in the standard manner, the cell cultures were continued for another 4 days. After the full 6-day expression, the plates for selecting Tk mutants were prepared in the same way. No cells treated with 5,000 µg/ml of caffeine was survived enough to prepare the plates after 2-day expression.

Tk mutation frequencies calculated from the total number of wells with colonies.
Tk mutation frequencies calculated from the number of wells with large colonies.
Tk mutation frequencies calculated from the number of wells with small colonies.

▲: Relative total growth.
*, **: Significantly different from control, p < 0.05, 0.01, respectively (Dunnett).
Hprt mutation frequencies showed no increase compared to the solvent control level (Fig. 2A).

EMS significantly increased the Tk mutation frequencies derived from either the large or small colonies as well as those from total colonies after both 2- and 6-day expression, although 500 µg/ml was a very toxic concentration (RTG was 1.7%). Frequencies derived from the large colonies were higher than those from the small colonies at all concentrations (Fig. 1B). The Hprt mutation frequencies showed a significant increase (Fig. 2B).

Micronucleus assay

Caffeine and EMS increased the frequencies of micronucleated cells both at 24 and 48 hr after treatment (Fig. 3). The frequencies at 24-hr sampling were higher than 48-hr sampling except at the highest concentration of caffeine.

The relative cell growth in cell suspension was less than 40% at 625 µg/ml and more of caffeine for both sampling times and at 500 µg/ml of EMS for 48-hr sampling time. Caffeine showed relatively higher cytotoxicity in MN assay than that in MLA. On the other hand, the cytotoxicity even at the highest concentration of EMS for 24-hr sampling time (relative cell growth: 42.3%) was not so severe compared to the RTG which indicated to be very toxic in MLA.

DISCUSSION

Caffeine is reported to exhibit a clastogenic potential in almost all of the mammalian cell lines, although it is non-carcinogen (Ishidate et al., 1988; Mohr et al., 1984; Takayama and Kuwabara, 1982). The result of MLA using the agar plate method is also positive (Clive et al., 1990). The mechanism has been recognized that the caffeine hinders post-replication repair in G2 by inducing premature mitosis in G2-blocked cells carrying previously formed aberrations as well as lesions (Ceccherini et al., 1988). A recent report stated that caffeine inhibits the rescue of stalled replication forks by translesion DNA synthesis, thereby causing a switch to bypass via homologous recombination, which results in chromosomal aberrations (Johansson et al., 2006). Therefore, considering that caffeine has no point mutational potential either in the bacteria or mammalian cells, its clastogenic potential can be thought to originate from the indirect DNA damage (Mortelmans et al., 1986; Amacher and Zelljadt, 1984). However, the results in MLA employing a stand-
ard expression time, 2 days, showed that caffeine has a point mutational potential in mammalian cells, because Tk mutation frequencies derived from large colonies, which are considered to be an indication of point mutation, increased significantly, as well as those from small colonies. So, it is difficult to clearly judge the cause of the positive response in MLA and to reject the possibility of a point mutational potential only from this result, although it can be roughly estimated that clastogenic potential affected this positive result by noticing the higher mutation frequencies derived from small colonies. On the other hand, neither Tk nor Hprt mutation was found at

Fig. 3. *In vitro* MN frequencies induced by treatment with caffeine (A) and EMS (B) for 24 hr without S9 mix.

- MN frequencies 24 hr after treatment.
- MN frequencies 48 hr after treatment.
- ▲: Relative cell growth 24 hr after treatment.
- △: Relative cell growth 48 hr after treatment.
- **: Significantly different from control, 0.01 (Kastenbaum & Bowman).
up to toxic concentrations after 6-day expression, which coincided with the fact that caffeine does not have a point mutational potential; in other words, no potential of direct DNA damage. The $TK^{-}$ mutants induced by point mutation can grow as fast as intact cells, so those cells form colonies in the medium including a selective agent after 6-day expression. However, the growth of cells with large deletion is so slow that the fraction of these cells would be diluted enough to be almost disappeared after 6-day expression. Therefore, it is speculated that the seeming increase in large colonies found after 2-day expression might be ascribable to the clastogenic potential. The difficulty of measuring the appearance or size of a colony formed at the edge of the well in the micro-titer plates may affect the colony observation, unlike the agar method.

EMS, a genotoxic carcinogen, is reported to show positive responses in almost all of genotoxic assays, such as the Ames test, gene mutation test using mammalian cells, and chromosome aberration test (Clive et al., 1979; Kier et al., 1986; Li et al., 1988; O’Donovan, 1990; Nesnow et al., 1987). In the positive result for $TK$ mutation, the dominant ratio of frequencies derived from large colonies at 2-day expression suggested that EMS has a point mutational potential. After 6-day expression, an increase in $Hprt$ mutation was clearly noted, as well as for $TK$, which confirmed a point mutational potential of EMS.

From the result of in vitro MN assay, the compound with potential of point mutation, like EMS, may be thought to start inducing chromosomal aberration from the non-toxic concentrations which might eventually be toxic in MLA, while the clastogen lack of direct DNA damaging potential, like caffeine, may induce chromosomal aberration at relatively toxic concentrations. MN induction was more sensitive at 24 hr than 48 hr after treatment both for caffeine and EMS. This result is reasonable given that 24 hr corresponds approximately to two or three times the doubling time of this cell line, which is suitable for the recovery time of micronuclei generation.

In conclusion, when MLA gives a positive result and the cause is ambiguous, in order to identify the exact cause of the positive response, it is helpful to perform a confirmatory test investigating the potential of $TK$ and $Hprt$ gene mutation simultaneously after 6-day expression and to perform an in vitro MN assay during expression period.

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