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Short communication

A method to distinguish between the de novo induction of thymidine kinase mutants and the selection of pre-existing thymidine kinase mutants in the mouse lymphoma assay

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Abstract

The mouse lymphoma assay (MLA) is the most widely used in vitro mammalian gene mutation assay. It detects various mutation events involving the thymidine kinase (*Tk*) gene in L5178Y/$Tk^{+/-}$ −3.7.2C mouse lymphoma cells. Mutants are detected using a thymidine analogue that arrests the growth of cells containing a functional *Tk* gene. However, there are a number of potential test chemicals that are thymidine analogues, and there is a problem when using the MLA to evaluate the mutagenicity of these chemicals. Thymidine analogues are activated by Tk before eliciting their toxicity. Therefore, any pre-existing $Tk^{-/-}$ mutants may avoid the toxicity of the test chemical and obtain a growth advantage over the $Tk^{+/-}$ cells, increasing the *Tk* mutant frequency (MF) in the culture via a selection mechanism. This potential mutant selection effect needs to be distinguished from de novo mutant induction in order to properly evaluate the mutagenicity of these chemicals. Here we describe a simple MLA study design that can differentiate between the selection of pre-existing mutants and de novo mutant induction. Trifluorothymidine (TFT), a thymidine analogue and the selection agent normally used in the MLA, and 4-nitroquinoline-1-oxide (4-NQO), a potent mutagen, were used to treat cells from two different $Tk^{+/-}$ mouse lymphoma cell cultures with different background MFs (approximately 112 and 305×10^{-6}). Both agents significantly increased the *Tk* MFs in both the normal and high background cultures ($p < 0.01$). In 4-NQO-treated cultures, the induced MFs (MF of treated culture − MF of control) for the cultures with different background MFs were about the same ($p > 0.1$), while in TFT-treated cultures, they were significantly different ($p < 0.01$). In TFT-treated cultures, the fold-increases of MF (MF of treated culture/MF of control) for the cultures with different background MFs were about the same ($p > 0.1$), while in 4-NQO-treated cultures, they were significantly different ($p < 0.01$). This study confirms that, when de novo mutations are induced, the induced MF is the same for cultures with normal and artificially high background MFs. In situations where the increase in MF is due solely to selection of pre-existing mutants, the "induced" MF will be a multiple of the background MF and the magnitude of the increase of the induced MF will depend upon the magnitude of the background MF. Our results demonstrate that it is possible, using this experimental design, to distinguish between chemicals acting primarily via the selection of pre-existing *Tk* mutants and those inducing de novo mutants in the MLA.
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Keywords: Mouse lymphoma assay; Thymidine analogue; Mutant selection; Mutant frequency

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1. Introduction

The mouse lymphoma assay (MLA) is widely used as a part of the core battery of genotoxicity tests [1]. It can detect mutations that affect the expression of the thymidine kinase (*Tk*) gene in L5178Y/$Tk^{+/-}$ −3.7.2C mouse lymphoma cells. It is the recommended in vitro mammalian cell mutation assay because it detects a wide range of genetic alterations, including both intragenic mutations and chromosomal alterations, which are involved in the etiology of cancer and other human diseases [2–5].

Mutant selection in the MLA is accomplished by plating cells into medium containing trifluorothymidine (TFT). $Tk^{+/-}$ cells have an active thymidine salvage pathway and are growth arrested by a toxic metabolite of TFT, while $Tk^{-/-}$ mutants survive and form clones that can be observed and counted [3,6]. Although this is a very efficient mechanism to detect mutation, it results in a problem when the MLA is used to evaluate the mutagenicity of chemicals, such as thymidine analogues, which are differentially toxic to Tk-competent and Tk-deficient cells.

Thymidine analogues are widely used in the treatment of viral infection and cancer due to their ability to inhibit RNA reverse transcriptase, or incorporate into the DNA chain to inhibit DNA synthesis. For example, zidovudine (AZT) and stavudine (d4T) are thymidine analogues that are major components of the current therapeutic regimens for AIDS [7–9].

Before eliciting their therapeutic effects as well as their toxicity, thymidine analogues are activated by a series of phosphorylation reactions. The first step is phosphorylation by Tk [10–12]. When using the MLA to evaluate their mutagenicity, thymidine analogues presumably cannot be activated in the pre-existing $Tk^{-/-}$ mutants due to the lack of a functional *Tk* gene. Therefore, pre-existing mutants may either completely or partially avoid the toxicity caused by the thymidine analogues in Tk-competent cells and obtain a growth advantage over $Tk^{+/-}$ cells. As a result of this differential growth, the mutant frequency (MF) will increase solely due to differential cytotoxicity. If this type of selection occurs, the increase in MF will be misleading; that is, the test chemical will appear to be a mutagen when, in fact, it is not. Therefore, it has been recommended that the standard MLA should not be used to evaluate the mutagenicity of thymidine analogues [13]. However, under some circumstances it is unclear whether a compound with structural similarities to thymidine may, in fact, fall into this category. It would be useful to have a method that could distinguish between de novo mutant induction and mutant selection in the MLA.

One strategy is to apply molecular genetic methods to analyze and compare the mutation spectra of spontaneous and "induced" *Tk* mutants and differentiate the possible mutant-selection effect from mutant-induction [14–16]. If the MF elevation is primarily due to mutant selection, then the mutation spectrum of *Tk* mutants from the chemical-treated culture would be similar to the spectrum of *Tk* mutants from the untreated culture and would demonstrate a high degree of clonality. If the mutation spectrum of mutants from the treated culture is significantly different from that of the untreated culture and the mutants are largely independent of one another, it indicates that de novo mutant induction plays a major role in the elevation of the MF. However, in order to employ this approach, a significant number of mutants must be analyzed, which is a very resource-intensive process. We therefore developed a simple method to distinguish between the selection of pre-existing mutants and the induction of de novo mutants. We used the well-known *Tk* mutant-selecting agent TFT and the potent mutagen 4-nitroquinoline-1-oxide (4-NQO) to evaluate our method.

2. Materials and methods

2.1. Cell culture

Using published methods [17], L5178Y/$Tk^{+/-}$ mouse lymphoma cells were cultured in Fischer's medium for leukemic cells of mice (Quality Biologicals, Gaithersburg, MD) supplemented with 10% heat-inactivated horse serum, 200 μg/ml sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.05% (v/v) pluronic F68 (all from Invitrogen, Carlsbad, CA). The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 and saturated humidity and maintained in logarithmic growth.

2.2. Tk mutation assay

The protocol for the microwell version of the *Tk* mutation assay described by Chen and Moore [17] was followed. 4-NQO and TFT were obtained from Sigma (St. Louis, MO). A high background MF culture was generated by adding $Tk^{-/-}$ mutants into a culture with a "normal" background MF. Large colony $Tk^{-/-}$ mutants from previous experiments were used to generate the high background culture; the mutants were selected because they have the same growth rate as the $Tk^{+/-}$ cells (doubling time of approximately 9 h), thus keeping the high background MF stable. Briefly, a culture with a normal background MF and a culture with a high background MF were centrifuged and the cells were resuspended at a concentration of 5×10^6 cells/ml in 20 ml of medium in 50 ml polystyrene

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