Collaborative Study of Thresholds for Mutagens: Hormetic Responses in Cell Proliferation Tests Using Human and Murine Lymphoid Cells

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Abstract

Background: We previously showed that hormetic responses can be established in cell activity tests using human and murine adherent cells. This time, we examined whether hormetic responses can be established in cell proliferation tests using suspended human and murine lymphoid cells.

Methods: Human lymphoblastoid cells (TK6) and mouse lymphoma cells (L5178Y) were cultured in multi-well culture plates and treated with mitomycin C, ethyl methanesulfonate, hygromycin B, aclarubicin or colchicine at various dose levels and the number of cells was measured at varied times using a flow cytometer.

Results: When the ratio of the number of cells treated with a test chemical to those in the negative control was plotted, the dose-response relationship typically showed a reverse U-shaped curve, indicating the occurrence of hormesis and existence of thresholds in cell toxicity. The hormetic responses depended largely on the test chemical, dose level and exposure time. When examining responses over the course of time, a J-shaped or fallen S-shaped curve was also observed.

Conclusions: The dose-response relationship showed a reverse U-shaped curve, a hallmark of hormesis, at least some time points for all chemicals tested here, indicating that chemical hormesis can be established in in vitro cell proliferation tests.

Keywords

aclarubicin, adaptive response, ethyl methanesulfonate, hormesis, hygromycin, mitomycin C

Introduction

It is of interest to learn that a single chemical frequently induces opposite biological responses depending on its dose level—stimulation or benefit at lower dose levels and inhibition or harm at higher dose levels. This phenomenon is called an hormetic or adaptive response. The dose-response relationship in hormetic responses typically shows biphasic dose responses of J- or reverse U-shaped curves.

There are so many kinds of chemicals and their physical and chemical properties vary greatly. Experiments using microorganisms, insects, and plants were carried out and biphasic dose responses were obtained, but specific, dissimilar explanations were proposed. Therefore, it seemed difficult to organize these findings under a standardized concept like chemical hormesis. Townsend and Luckey collected more than 100 biphasic dose-
responses, published in pharmacological literature.  

Later, Calabrese and Blain analyzed 5,632 dose-responses from approximately 900 compounds in more than 300 journals, including the analysis of 1,654 radiation/radionuclides (29%), 1,308 inorganics (23%), and 2,670 organics (47%). Test systems consisted of plants, viruses, bacteria, fungi, insects, fish, birds, rodents, and primates and endpoints were broad, including growth, longevity, numerous metabolic parameters, disease incidences (including cancer), and various behaviors such as cognitive functions and immune responses. Although the intensity of the hormetic responses varied among the test systems, radiation and chemical hormesis seems to be universal.

As members of the Japanese Environmental Mutagen and Genome Society (JEMS), the Collaborative Study Group of Thresholds for Mutagens was organized to confirm hormetic responses using cell activity tests. In this study, we used several mutagens and chemicals to determine whether or not they show hormesis in in vitro cell proliferation tests. For this, we used human and murine cultured lymphoid cells, because suspended cells were convenient for cell counting.

Materials and Methods

Cells

Human lymphoblastoid cells (TK6) and mouse lymphoma cells (L5178Y) were obtained from the JCRB Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Japan. These cells are cited in OECD guidelines for mutagenicity testing. Cells were cultured with RPMI 1640 medium containing 10% fetal bovine serum spiked with penicillin and streptomycin (25 µg/mL each) in a CO2 (5%) incubator at 37 °C. Cells were cultured in dishes 60 mm in diameter and inoculated in multi-well plates with 96, 48, or 24 wells (culture volumes of 100, 250, or 500 µL per well). At inoculation, cells were counted with a hemocytometer.

Flow Cytometer

A Novo Cyte™ flow cytometer (ACEA Biosciences, Inc., San Diego, CA) was used to determine cell numbers. An aliquot (50 or 100 µL) of each culture was taken and mixed with sheath fluid (950 or 900 µL) containing 0.5% paraformaldehyde, a fixative.

Chemicals

Mitomycin C (MMC) (CAS: 1950-07-7), acrariubcin HCl (CAS: 75443-99-1), and hygromycin B (CAS: 31282-04-9) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ethyl methanesulfonate (CAS: 62-50-0) was from NAKALAI TESQUE, INC. (Kyoto, Japan) and colchicine (CAS: 64-86-8) was from Sigma-Aldrich Co. LLC (St. Louis, MO). For treatment of the cells, a 10-fold concentrated chemical solution was prepared, from which a series of diluted solutions were prepared using a dilution coefficient of 2. The prepared solutions were then added to the cell cultures at a ratio of 1:10, meaning that the actual chemical concentrations were 90.9% of values indicated in the figures.

Rationale and Statistics

In the present study, opposing effects of the same chemical—enhancing effects at lower dose levels and suppressing effects at higher ones—were examined. If dose levels of a narrow hormetic range are selected, all responses would be enhancing. If toxic dose levels are chosen, all would show suppressing effects. Thus, enhancing or suppressing responses could be selected arbitrarily by choosing particular dose levels. Therefore, dose-range-finding tests were conducted to select dose ranges that included both enhancing and suppressing concentrations. In addition, hormetic responses are transitional depending on dose level, exposure time, and type of hormesis. Calabrese and Baldwin defined 2 types of hormesis, direct stimulation hormesis (DSH) and overcompensation stimulation hormesis (OCSH). DSH represents a type of steady-state adaptive response that reflects normal physiological dynamics. OCSH is initiated with the disruption of homeostasis, modest overcompensation, reestablishment of homeostasis and the adaptive nature of the process. A typical dose-response in DSH induces a reverse U-shaped curve. When OCSH occurs, a fallen S-shaped curve consisting of concave and convex curves, a J-shaped curve, or a typical reverse U-shaped curve is observed. Since numerous mechanisms are involved in the induction of hormesis, expression patterns are also complicated and responses are time-dependent. In the present in vitro cell proliferation tests, a DSH-type response is primarily assumed and a reverse U-shaped curve with a gentle slope which crosses the x-axis is defined as an indicator of hormesis. Detailed rationale is discussed in our previous paper (see Figure 1A). Although precise and sophisticated analyses of hormetic responses have been published to date, they are not suitable for application to the present tests, in which dose-response patterns fluctuate largely and change transiently as shown by fallen S-shaped and J-shaped curves in addition to typical reverse U-shaped curves. Here, paying attention only to reverse U-shaped curves, we propose tentative, simplified method focusing on the maximum hormetic response. If a reverse U-shaped curve is obtained, trace the straight line, which can be made by connecting the point from 0 (dose level) to that of the most toxic dose level. An expected suppression % at the maximal hormetic dose level on the reverse U-shaped curve is then estimated from this straight line. This % is multiplied by the number of cells at 0 (dose level) to get the expected cell numbers, which are compared to those of the dose level with the maximal hormetic response by the Student’s t-test. When P values are less than 0.05 or 0.01, they are statistically significant at 5% or 1%, respectively.

Results

Preliminary Examination of Culture Conditions

Edge effects. In the previous report, adherent cells were cultured in a 96-well plate and the edge effect—different culture conditions...
between outer and inner wells because water evaporates more rapidly from outer wells than inner ones—had to be taken into consideration.3 Although the effect would only have been minimal, the outer 24 wells were not used. In the present study, 48- or 24-well plates were used, since 2 to 4 or more cell samples can be taken consecutively from a single well. When L5178Y cells (100,000 cells/0.25 mL/well) were inoculated in a 48-well plate and the average number of cells in the outer 24, mid 16, and inner 8 wells was compared 12, 15, and 30 h after inoculation, no significant differences were observed (Table 1).

**Table 1. Edge Effect in a 48-Well Plate.**

| Detection time (h) | Wells (number) | Mean** | SD | P value*** |
|-------------------|----------------|--------|----|------------|
| 12 h              | Outer (24)     | 4223   | 264| 0.217      |
|                   | Mid (16)       | 4082   | 141| 0.123      |
|                   | Inner (8)      | 4083   | 138| 0.017      |
| 15 h              | Outer (24)     | 4216   | 505| 0.015      |
|                   | Mid (16)       | 4137   | 538| 0.641      |
|                   | Inner (8)      | 3635   | 213| 0.776      |
| 30 h              | Outer (24)     | 4453   | 369| 0.076      |
|                   | Mid (16)       | 4407   | 383| 0.664      |
|                   | Inner (8)      | 4393   | 187| 0.664      |

*L5178Y cells were used. Cell numbers in mid and inner wells were compared with those in outer wells.**Cell numbers in 50 μL of cell suspensions that were prepare by adding 50 μL of a cell culture into 950 μL of fixative.***Student’s t test.

Figure 1. Hormetic responses observed in TK6 cells treated with MMC. Cells (50,000/well/500 μL) were inoculated in a 24-well plate, treated with MMC (highest dose level of 20 ng/mL) 12 h after inoculation, and 50 μL were sampled from the same well at 12, 18, and 24 h after treatment. Three wells were analyzed at each time point. Vertical bars denote standard deviations from the mean.

Coagulation of cells. Even when lymphoid cells are suspended, as the culture process proceeds, cells form clusters similar to bunches of grapes. To disperse these cells, pipetting is often effective. L5178Y cells (200,000 cells/0.5 mL/well) were inoculated in a 24-well plate and an aliquot (50 μL) was taken after pipetting 0, 2, 5, and 10 times from 6 wells each 5 and 7 h after inoculation. Since cell numbers without pipetting were higher than those with pipetting, pipetting was determined not to be necessary (Table 2). On the contrary, vigorous pipetting was responsible for an increase in damaged cells.

**Table 2. Effect of Pipetting on Cell Counting.**

| Pipetting | 5 h Mean** | SD | P value*** | 7 h Mean** | SD | P value*** |
|-----------|------------|----|------------|------------|----|------------|
| 0         | 2784       | 159| 0.015      | 3372       | 331| 0.003      |
| 2         | 2551       | 161| 0.007      | 2695       | 228| 0.158      |
| 5         | 2358       | 156| 0.036      | 3099       | 231| 0.204      |
| 10        | 2491       | 234| 0.305      | 3055       | 419| 0.204      |

*Cell numbers after pipetting 2, 5, or 10 times were compared with those of no pipetting 5 and 7 h after inoculation.**Cell numbers in 50 μL of cell suspensions that were prepare by adding 50 μL of a cell culture into 950 μL of fixative.***Student’s t test.

Dose-Response of TK6 Cells Treated With MMC

When TK6 cells were inoculated in a 24-well plate (100,000 cells/0.5 mL/well), treated with 7 dose levels of MMC (from 15.6 to 1,000 ng/mL, dilution coefficient of 2) 24 h after inoculation and cell numbers were counted 24, 36, and 48 h after treatment, no hormetic responses were observed. When a similar experiment was carried out with a different inoculum size (50,000 cells/0.5 mL/well) and different dose levels (from 1.56 to 100 ng/mL), no clear responses were detected 15, 20, 25, or 39 h after treatment. Therefore, another experiment with a larger inoculum size (200,000 cells/0.5 mL/well) was conducted and cell numbers were counted 12, 18, and 24 h after treatment with different dose levels (from 0.625 to 20 ng/mL). The reverse U-shaped curve with a smooth slope indicates a hormetic response 12 h after treatment (Figure 1). However, when the simplified analysis described in the above “Rationale and statistics” section was applied, no statistical significance was noted. The window of hormetic responses appeared to be narrow and the exposure time of 12 h was likely to have been too long for optimal hormesis.

Since human cells seem to be more resistant to toxicants than rodent cells, murine cells were examined. Higher sensitivity of murine cells was hinted in the previous study in which murine CHL cells started to show suppression of cell activity at around 2 ng/mL (see Figure 2A and C), while human TK6 cells started at around 20 ng/mL (see Figure 2D and E).
Doubling Time of TK6 and L5178Y and Dose-Response of L5178Y Cells Treated With MMC

Even though hormetic responses depicted in Figure 1 are very mild, important indications are that 1) some lower doses induced higher cell numbers than the negative control and 2) a reverse U-shaped curve of a gentle continuous convex. An unexpected finding in Figure 1 was that hormetic responses occurred 12 h after treatment, i.e. less than the possible generation time. So, the generation time of TK6 and L5178Y cells was determined by cell counting. L5178Y showed more rapid growth than TK6, with a generation time of approximately 20 h for TK6 and 12 h for L5178Y (Figure 2A). Figure 2A indicates that both TK6 and L5178Y cells start logarithmetic growth 6 h after inoculation. Since higher responses can be expected, L5178Y cells were used instead of TK-6 cells hereinafter.

When L5178Y cells (100,000 cells/0.5 mL/well) were treated with MMC 12 h after inoculation and cell numbers were counted 6 and 9 h after treatment, hormetic responses were clearer at 6 h than 9 h (Figure 2B). For reference, when cell numbers in the 0 and 1.25 mg/mL groups were statistically analyzed, the P value was 0.0012. Hormetic tendency was also seen in increased inoculum sizes of 200,000 cells/0.5 mL/well (Figure 2C) and 400,000 cells/0.5 mL/well (not shown). Reverse U-shaped curve hormetic responses were seen at 6 and 9 h after treatment, but no statistically significant differences were noted. Although the increased cell number observed at 2.5 mg/mL in Figure 2C is statistically significant, the response at 12 h did not show a U-shaped curve, therefore the difference was judged to be incidental.

Higher responses at 6 h were confirmed by cell counting at 6, 8, and 10 h (Figure 3A). Figure 3A showed a reverse U-shaped curve with a gentle slope at 6 h after treatment and the response at 1.25 mg/mL was statistically significant, indicating a hormetic response. No higher responses were observed at 3 h in Figure 3B, but reverse U-shaped curves with a gentle slope were seen at 4, 5, and 6 h after treatment. These are hormetic responses according to our definition, but no statistically significant differences were detected. When cell activity was measured using a coloration reagent, CCK-8, stimulation of cells began 3 h after treatment (Figure 3C). Cell activity was suppressed at higher dose levels (200 and 400 ng/mL) at 3-4 h, but stimulated at 5-6 h and 7-8 h. Generally, hormetic responses appear earlier at lower dose levels and later at higher ones.3 Substantially suppressed cells that were not killed and still possessed the energy to recover were noted to recover and actually show high activity thereafter.

Dose-Response of L5178Y Cells Treated With EMS

MMC showed peak responses around 6 h after treatment (Figure 2B and C, Figure 3A and B); however, EMS showed fluctuations depending on the experimental conditions. An experiment with a small number of cells (100,000) treated with lower dose levels (from 3.12 to 200 mg/mL) showed a slightly higher hormetic response at 6 h and responses gradually decreased at 8 and 10 h (Figure 4A). Another experiment with a larger number of cells (200,000) treated with higher dose levels (from 12.5 to 800 mg/mL) showed a higher response at 9 h than 5 and 7 h (Figure 4B). At 9 h, a reverse U-shaped curve with a gentle slope was observed and the response at 50 μg/mL
was statistically significant. When dose levels and exposure time were further increased (from 62.5 to 4,000 μg/mL for up to 24 h), triple hormetic responses were seen at 5, 12, and 24 h (Figure 4C). Generally, hormetic responses tended to appear earlier when treated at lower dose levels and later at higher dose levels. Furthermore, EMS seemed to be more stable than MMC under the present experimental conditions and cellular responses to EMS might vary dynamically depending on cell numbers, dose levels, and exposure time. When the time course of dose-responses at 5, 8, 12, and 24 h is plotted, a J-shaped curve can be observed (Figure 4C), hinting the occurrence of an OCSH-type response. Although cellular responses to EMS fluctuated to some extent, hormetic responses were noted.

Dose-Response of L5178Y Cells Treated With Hygromycin B, Aclarubicin, and Colchicine

Hygromycin B is an aminoglycoside antibiotic produced by Streptomyces hygroscopicus. It kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. The results of an experiment with 200,000 cells treated with hygromycin B...
A clear hormetic response was seen 8 h after treatment. Higher dose levels seemed to induce hormesis 24 h after treatment (Figure 5A). The delayed hormetic response with statistical significance shows not a typical reverse U-shaped but a J-shaped curve (Figure 5A, 24 h). Here again, the occurrence of an OCSH-type response is suggested.

Aclarubicin is an anthracycline produced by *Streptomyces galilaeus* and used for the treatment of cancer. Hormetic responses are mild, but convex-like curves can be seen at 5, 7, and 9 h. A small convex curve can be seen at higher dose levels 24 h after treatment (Figure 5B). This is a kind of J-shaped curve and hints the occurrence of an OCSH-type response as seen in Figure 5A, 12 h. These responses were not always statistically significant, but hormesis is considered to have been demonstrated as a whole.

Unexpectedly, however, a kind of J-shaped curve was obtained after longer exposure times for hygromycin B (Figure 5A, 24 h), and aclarubicin (Figure 5B, 24 h), and also in the cell activity test (Figure 3C, 7-8 h). When the time course of dose-responses at 5, 8, 12, and 24 h is plotted, a J-shaped curve can be observed (Figure 4C). The cell activity test also shows this pattern (Figure 3C). Similarly, dose-responses of hygromycin B at 5, 8, 12, and 24 h seem to follow an up-down-up response or a fallen S-shaped curve (Figure 5A). These complicated response patterns cannot be explained by a simple concept that stimulation occurs at lower dose levels and inhibition at higher dose levels, which is responsible for a reverse U-shaped curve. To solve this problem, the introduction of 2 types of hormesis, i.e. DSH (direct stimulation hormesis) and OCSH (overcompensation stimulation hormesis), is helpful. A typical reverse U-shaped curve was found for mitomycin C (Figure 1, 12 h; Figure 2B, 6 h; Figure 2C, 6, 9 h; Figure 3A, 6 h; Figure 3B, 4, 5, 6 h), ethyl methansulfonate (Figure 4A, 6 h; Figure 4B, 9 h; Figure 4C, 5, 12, 24 h), hygromycin B (Figure 5A, 8 h), aclarubicin (Figure 5B, 5, 7 h), and colchicine (Figure 5C, 12 h). These responses were not always statistically significant, but hormesis is considered to have been demonstrated as a whole.

A cell is a microcosm. Cell division is achieved by a refined mechanism under genetic control. Following the addition of test chemicals, stimulation of cell activity started at least 3-4 h after treatment at lower concentrations and 5-6 h or 7-8 h after treatment at higher concentrations (Figure 3C). It is
obvious that metabolic and/or genetic adaptive responses occur, but what genes are involved? The Keap1-Nrf2 pathway is a cellular defense system against oxidative and xenobiotic stresses derived from reactive oxygen species (ROS) and electrophiles, respectively.\(^8\) Calabrese and Kozumbo recently reviewed the importance of Nrf2 activation in the hormetic dose-response mechanism.\(^9\) For example, when PC12 cells were treated with protodioscin, an Nrf2-activating agent, this induced a hormetic dose-response against oxygen and glucose deprivation-induced stress, an in vitro model of hypoxia, through the miR/AKT/Nfr2 pathway.\(^10\) Nrf2-knockout transgenic mice and rats provide deep insight into the role of Nrf2 in chemical toxicity in vivo.\(^11,12\)

NF-κB proteins are a family of transcription factors which regulate the expression of hundreds of genes that play important roles not only in ROS treatment but also in inflammation, immunity, cell growth, differentiation, development, and apoptosis.\(^13\) Biological defense systems are not confined to the Keap1-Nrf2 and NF-κB/IκB systems. For example, stresses from UV, heat, or hypoxia are sensed by the respective sensors and we adapt to them. The present study is preliminary research and tests only the fluctuations in cell numbers after chemical treatment. To understand the mechanisms of hormetic responses further, we need to investigate the expression and suppression of the relevant genes, such as the abovementioned Nrf2, Keap1, NF-κB, and IκB and p53, which are associated with the cell cycle, apoptosis, and DNA repair. Therefore, we started to examine changes in the expression of several enzymes using RT-PCR as part of collaborative studies.

**Conclusion**

In hormesis or adaptive responses, where the dose-response relationship is stimulatory at lower dose levels and inhibitory at higher ones, a reverse U-shaped curve occurs and a threshold can be established at the intersection of the curve and the x-axis. When human lymphoblastoid cells (TK6) or mouse lymphoma cells (L5178Y) were cultured in multi-well plates and treated with mitomycin C, ethyl methansulfonate, hydroxyquinol B, aclacinomycin, or colchicine, dose-response curves generally showed reverse U-shaped curves, indicating that hormetic responses occurred and that there are thresholds in cell toxicity. When the time course of responses is plotted, a J-shaped or fallen S-shaped curve was also observed, hinting the occurrence of OCRS (overcompensation stimulation hormesis) in addition to DSH (direct stimulation hormesis).

**Acknowledgments**

All authors belong to the Collaborative Study Group of Thresholds for Mutagens, Mammalian Mutagenicity Study Group (MMS), Japanese Environmental Mutagen and Genome Society (JEMS). This group published an associated paper (Sutou et al Genes and Environment (2018) 40:20 https://doi.org/10.1186/s41021-018-0108-1) in Genes and Environment. We would like of this group to be searchable through PubMed records; the group name is added to the authors. We are grateful to MMS, which has supported publishing costs. Our cordial thanks are due to Ms. Penelope Naruta of Ina Research Inc. for her editing this manuscript.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Each organization to which participants belong supported research fund individually.

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