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Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs

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ABSTRACT

We established a luciferase reporter assay system, the Multi-ImmunoTox Assay (MITA), to evaluate the effects on key predictive *in vitro* components of the human immune system. The system is composed of 3 stable reporter cell lines transfected with 3 luciferase genes, SLG, SLO, and SLR, under the control of 4 cytokine promoters, IL-2, IFN-γ, IL-1β, and IL-8, and the G3PDH promoter. We first compared the effects of dexamethasone, cyclosporine, and tacrolimus on these cell lines stimulated with phorbol 12-myristate 13-acetate and ionomycin, or lipopolysaccharides, with those on mRNA expression by the mother cell lines and human whole blood cells after stimulation. The results demonstrated that MITA correctly reflected the change of mRNA of the mother cell lines and whole blood cells. Next, we evaluated other immunosuppressive drugs, off-label immunosuppressive drugs, and non-immunomodulatory drugs. Although MITA did not detect immunosuppressive effects of either alkylating agents or antimetabolites, it could demonstrate those of the off-label immunosuppressive drugs, sulfasalazine, chloroquine, minocycline, and nicotinamide. Compared with the published immunological effects of the drugs, these data suggest that MITA can present a novel high-throughput approach to detect immunological effects of chemicals other than those that induce immunosuppressive effects through their inhibitory action on cell division.

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1. Introduction

Environmental contaminants, food additives, and drugs can target the immune system, resulting in adverse health effects, such as the development of allergies, autoimmune disorders, cancers, and other diseases. Accordingly, immunotoxicity, which is defined as the toxicological effects of xenobiotics on the functioning of the immune system, has raised serious concerns from the public as well as regulatory agencies. Currently, the assessment of chemical immunotoxicity relies on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as expense, ethical concerns, and eventual relevance to risk assessment for humans. Therefore, European policy is promoting alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals employed for scientific studies (Balls et al., 1995).

A workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (Galbiati et al., 2010; Gennari et al., 2005; Lankveld et al., 2010). In that workshop, a tiered approach was proposed, since useful information can be obtained from regular 28-day general toxicity tests. Namely, prescreening for direct immunotoxicity starts with the evaluation of myelotoxicity. Compounds that are capable of damaging or destroying the bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they are tested for lymphotoxicity. Then, they are tested for immunotoxicity by approaches such as human whole-blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction, natural killer cell assay, T-cell-dependent antibody response, dendritic cell maturation, and fluorescent cell chip. Among these assays, HWBCRA has undergone formal prevalidation, although other techniques are being examined or have been previously examined in a rigorous prevalidation effort by ECVAM and other groups.

The principle of HWBCRA, described by Langezaal et al. (2002), is based on the well-known human whole-blood method for pyrogen testing (Hartung, 2002). In brief, human blood is treated with lipopolysaccharide (LPS) or staphylococcal enterotoxin B (SEB),

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which causes monocytes and Th2 lymphocytes to produce IL-1β and IL-4, respectively. After incubation for 40 h in the presence or absence of immunotoxic and non-immunotoxic test compounds, the levels of IL-1β and IL-4 in the supernatant are quantified, and the 50% inhibitory concentration (IC50) and the fourfold stimulating concentration (SC4) are calculated to establish the immunotoxic potency (Langezaal et al., 2002). According to the ECVAM workshop, this method has several advantages, such as the avoidance of species differences between humans and animals, employment of human primary cells, simple culture techniques, and reduced expense and time requirements as compared to animal experiments. The interindividual variation in leukocyte numbers and their response to stimuli is a major concern when using HWBCRA. Although cryopreservation techniques for human whole blood can overcome these problems (Schindler and Hartung, 2002), this method is not suitable as a high-throughput assay to evaluate vast numbers of chemicals.

In the present study, to develop a high-throughput screening system to evaluate chemical immunotoxicity, we first established 3 stable reporter cell lines transfected with luciferase genes under the control of IL-2, IFN-γ, IL-8, and IL-1β promoters. We selected these 4 cytokines because IL-2 and IFN-γ are mainly produced by T cells and reflect T-cell function, while IL-8 and IL-1β are mostly produced by monocytes or dendritic cells and correspond with their activity. Next, we examined the effects of 3 well-characterized immunosuppressive drugs, dexamethasone (Dex), cyclosporine A (CyA), and tacrolimus (Tac), on luciferase activities of these three cell lines stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) or lipopolysaccharide (LPS). Then, we compared the results with their effects on mRNA expression by the mother cell lines, Jurkat cells or THP-1 cells, under the relevant stimulation. Furthermore, we also compared their effects on luciferase activities with mRNA expression by human whole blood cells stimulated with PMA/Io or LPS in the presence of these immunosuppressive drugs. Finally, we treated these cell lines with immunosuppressive drugs, immunomodulatory drugs, or drugs without known immunomodulatory effects and estimated the performance of our screening system for immunotoxicity.

2. Materials and methods

2.1. Reagents

Water-soluble dexamethasone (Dex), cyclosporin A (CyA), tacrolimus (FK-506), rapamycin, cyclophosphamide (CP), azathioprine (AZ), mycophenolic acid (MPA), mizoribine (MZR), methotrexate (MTX), sulfasalazine (SASP), colchicine, chloroquine (CQ), minocycline (MC), nicotinamide (NA), acetaminophen (AA), digoxin, warfarin, phorbol 12-myristate 13-acetate (PMA), ionomycin (Io), and lipopolysaccharides from E. coli 026:B6 (LPS) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell lines and reporter cell lines

The human acute T lymphoblastic leukemia cell line Jurkat and the human acute monocytic leukemia cell line THP-1 (ATCC, Manassas, VA) were cultured in RPMI-1640 (Gibco, Carlsbad, CA) with antibiotic–antimycotic (Invitrogen, Carlsbad, CA) and 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) (Growth medium) at 37 °C with 5% CO_2. We previously established 2 reporter cell lines, #2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by IL-2 promoter, stable luciferase orange (SLO) regulated by IFN-γ promoter, and stable luciferase red (SLR) regulated by G3PDH promoter (Saito et al., 2011) and THP-G8 cells derived from THP-1 cells containing SLO regulated by IL-8 promoter and SLR regulated by G3PDH promoter (Takahashi et al., 2011).

In the present study, we further established THP-G1b cells derived from THP-1 cells containing SLG regulated by IL-1β promoter and SLR by G3PDH promoter. Full details are available in Supplementary Methods.

2.3. Chemical treatment

Based on the previous reports (Saito et al., 2011; Takahashi et al., 2011), #2H4 cells (2×10^5 cells/50 μl/well), THP-G1b cells, or THP-G8 cells (5×10^4 cells/50 μl/well) in 96-well black plates (Greiner bio-one GmbH, Frickenhausen, Germany) were pretreated with different concentrations of chemicals for 1 h. The optimum cell numbers at seeding were based on the previous reports. Afterwards, #2H4 cells were stimulated with 25 nM of PMA and 1 μM of ionomycin (PMA/Io) for 6 h, while THP-G1b cells or THP-G8 cells were stimulated with 100 ng/ml of LPS for 6 h. In some experiments, we changed the stimulation time to determine the optimum incubation period for the luciferase assay. Three luciferase activities, SLG luciferase activity (SLG-LA), SLO luciferase activity (SLO-LA), and SLR luciferase activity (SLR-LA), were simultaneously determined by using a microplate-type luminometer with a multicolor detection system, Phelios (Atto Co., Tokyo, Japan), and the Tripluc luciferase assay reagent (TOYOBO) according to the manufacturer's instructions. To rule out the variation of cell number or cell viability after chemical treatment, we obtained normalized luciferase activity as follows:

Normalized SLG-LA (nSLG-LA) or normalized SLO-LA (nSLO-LA) = SLG-LA or SLO-LA/SLR-LA.

We also calculated percent suppression as follows:

% suppression = (1 − nSLG-LA or nSLO-LA of the reporter cells treated with drugs/nSLG-LA or nSLO-LA of non-treated reporter cells) × 100.

To eliminate the data affected by cytotoxic effects of drugs or cell death, we also defined the inhibition index of SLR-LA (II-SLR-LA) as follows:

II-SLR-LA = SLR-LA of reporter cells that were treated with chemicals/SLR-LA of untreated reporter cells.

Since our previous study has reported that, in the treatment showing more than 5% in II-SLR-LA, more than 75% of cells are PI-excluding living cells (Takahashi et al., 2011), we presented only the data that demonstrated more than 5% in II-SLR-LA in this study.

2.4. Human whole-blood cytokine mRNA expression test (HWBCMET)

The human whole-blood cytokine mRNA expression test (HWBCMET) was performed by modifying the HWBCRA protocol by Langezaal et al. (2002)) and Thurm and Halsey (2005). The following studies were approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan, and conducted according to the Declaration of Helsinki principles. Full details are available in Supplementary Methods.

2.5. mRNA expression by Jurkat and THP-1 cells

Jurkat or THP-1 cells (3×10^6 cells) in 6-well plates were pretreated with different concentrations of drugs for 1 h and then stimulated with PMA/Io or LPS for 6 h, respectively. Total RNA was extracted by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The total RNA concentration was measured by using a NanoDrop spectrophotometer.

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