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A Three-step Method of Immunotoxicity Assessment

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ABSTRACT *: The immunosuppressive effects of thirty nine chemicals chosen by their potential toxicity were evaluated using a three-step testing method. The immunotoxicity test method developed in this study consisted of three simple assays of lymphoproliferation, mixed leukocyte response, and interleukin (IL)-2 production. The first step was mitogen-induced proliferation assay. Ten chemicals showed the inhibitory effects on the mitogen (lipopolysaccharide or concanavalin A)-induced proliferation in dose-dependent manners. The second step was mixed lymphocyte response. This step crosschecked the growth-suppressive effects detected at the first step. All of 10 chemicals, which showed suppression of lymphoproliferation, also exhibited the suppressive effects on the mixed lymphocyte response in the similar range of chemical concentration. The third step was planned to determine whether or not this growth suppression was mediated through an early activation of T-cell, which could be represented with IL-2 production. Six out of 10 chemicals decreased the interleukin-2 production in the similar concentration range used in the step 1 and 2. These results suggest that those 6 chemicals might have their targets on the signal transduction pathway toward the IL-2 production. In the meantime the other 4 chemicals might have their targets after the IL-2 production signal. Taken all together, the three-step test would be simple, fast, and efficient to determine whether or not the chemical has immunosuppressive effects.*

Key Words *: Immunotoxicity, Three-step test, Lymphoproliferation, Mixed lymphocyte response, Interleukin-2*

I. INTRODUCTION

A full battery of immunotoxicity tests recommended by the National Toxicology Program (Luster *et al.*, 1988) requires a long period of time, lots of animals, and a well-equipped laboratory. These tests consist of Tier I and II, including a variety of procedures for screen and comprehensive tests, respectively. Although the parameters in Tier I are for screening, they are composed of many items; immunopathology (i.e., hematology, body and organ weights, cellularity, and histology), humoral-mediated immunity (i.e., IgM plaque-forming cell assay and lipopolysaccharide mitogen response), cell-mediated immunity (i.e., concanavalin A-induced proliferation and mixed lymphocyte response), and nonspecific immunity (i.e., natural killer cell activity). Tier II testing, which represents an in-depth evaluation, is normally included only if functional changes are seen in Tier I with additional assays for immunopathology (i.e., splenic B and T cell quantitation), cell-mediated immunity (i.e., IgG antibody response to sheep RBC), humoral-mediated immunity (CTL cytolysis and delayed type hypersensitivity response), and nonspecific immunity (macrophage function), as well as an examination of host resistance (i.e., resistance to tumor cells, bacteria, virus, or parasites). In addition, all those parameters require the application of toxicants to animals. The immunotoxicity of drugs and chemicals is a very important parameter to be considered while they were in the developing stage. However, the hardship described above has been a hurdle for immunotoxicity test to be popular. There has been an increasing need for the simple and easy assessment method that most researchers can follow without special laboratory equipments. In this study, we are suggesting a

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simple three-step method of immunotoxicity assessment that uses splenocyte cultures with no isotopes, and that can be easily applied to the drug-screening step. It will help to narrow down possible drug candidates to a manageable number.

II. MATERIALS AND METHODS

1. Animals

Specific pathogen-free male BALB/c mice (4-5 weeks old) were purchased from the Experimental Animal Breeding Facility at Korea Research Institute of Chemical Technology (Taejon, Korea). On arrival, the mice were transferred to plastic cages containing sawdust bedding and quarantined for at least 1 week. Mice were given pelleted food (Purina Chow) and water *ad libitum*. The animal room was kept at 23± 3°C and 55±15% relative humidity with a 12 h light/dark cycle.

2. Test materials

Thirty-nine compounds including well-known environmental toxicants, pesticides, and immunosuppressants were tested through the first step screening, lymphoproliferation. Ten chemicals showing the suppression of lymphoproliferation were selected as follows; 2-acetylaminofluorene, thioacetamide, cobalt chloride, cyclophosphamide, ethionine, mancozeb, gallic acid, 9-aminoacridine, p-methoxyphenol, and paraquat dichloride from Sigma Chemical Co. (St. Louis, MO, USA) and mancozeb from Fluka Chemie AG (Buchs, Switzerland).

3. Lymphoproliferation

Splenocytes were isolated from untreated mice and the cell numbers were adjusted to 1×10^6 cells/m*l* in RPMI medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma), antibiotic (100 units penicillin, 100 μg streptomycin, and 0.25 μg fungizone/m*l*, Gibco), and 50 μM 2-mercaptoethanol (Sigma). The test chemicals were added directly to cells, which were cultured (2×10^5 cells/well) in the presence of either lipopolysaccharide (LPS, 100 μg/m*l*, Sigma) or concanavalin A (Con A, 1 μg/m*l*, Sigma) in 96-well microtiter plates (Costar, Cambridge, MA, USA) at 37°C and 5% CO_2 for 72 h. The cultures were treated with 20 μ*l* of MTS solution in Cell Titer 96® AQueous Nonradioactive Cell Proliferation Assay Kit (MTS solution, Promega, Madison, WI) for the last 4 h of culture period. The optical density was measured at 490 nm by an ELISA reader.

4. Mixed leukocyte response (MLR)

The responder spleen cells were isolated from male BALB/c mice and the cell numbers were adjusted to $1x10^6$ cells/ml in RPMI medium. DBA/2-derived splenocytes served as the stimulator cells. Prior to use, stimulator cells (5×10^6 cells/m*l*) were treated with 40 mg/ml mitomycin C at 37°C for 45 min, washed four times with RPMI 1640, and resuspended to 4×10^6 cells/m*l* in medium. Responder cells (1×10^5) and stimulator cells (4×10^5) were cultured in 200 μ*l*/well at 37°C and 5% CO_2 for 5 days in the presence of test materials. The proliferation levels were determined as described in the lymphoproliferation section.

5. Measurement of interleukin-2 (IL-2)

The culture supernatant of chemical-treated splenocytes at 24 h were collected and assayed in triplicate using the mouse lymphokine ELISA assay kits (Genzyme Corp., Cambridge, MA, USA). Briefly, samples were added to antibody pre-coated microtiter wells and incubated for 1 h at 37°C. The wells were washed and incubated with biotinylated anti-IL-2 antibody for 1 h, then with horse radish peroxidase-conjugated streptavidin for 40 min at 37°C. After a 10 min-incubation with the substrate solution provided by manufacturer, the optical densities were measured at 450 nm. The amounts of IL-2 were determined by comparison to the standard curves of recombinant mouse IL-2.

6. Statistical analysis

The mean±SD was determined for each treatment group of a given experiment. Each experiment was repeated at least twice and confirmed for its reproducibility. When significant differences occurred, treatment groups were compared to the controls using

Dunnetts two-tailed t-test (Dunnett, 1955).

III. RESULTS AND DISCUSSION

In order to evaluate the feasibility of our three-step assessment of immunotoxicity, 39 chemical toxicants (names not listed) were tested via the first step, the lymphoproliferation induced by either Con A or LPS. Although the high concentrations (up to 10 mM) were applied, only 10 chemicals showed suppressed prolif-

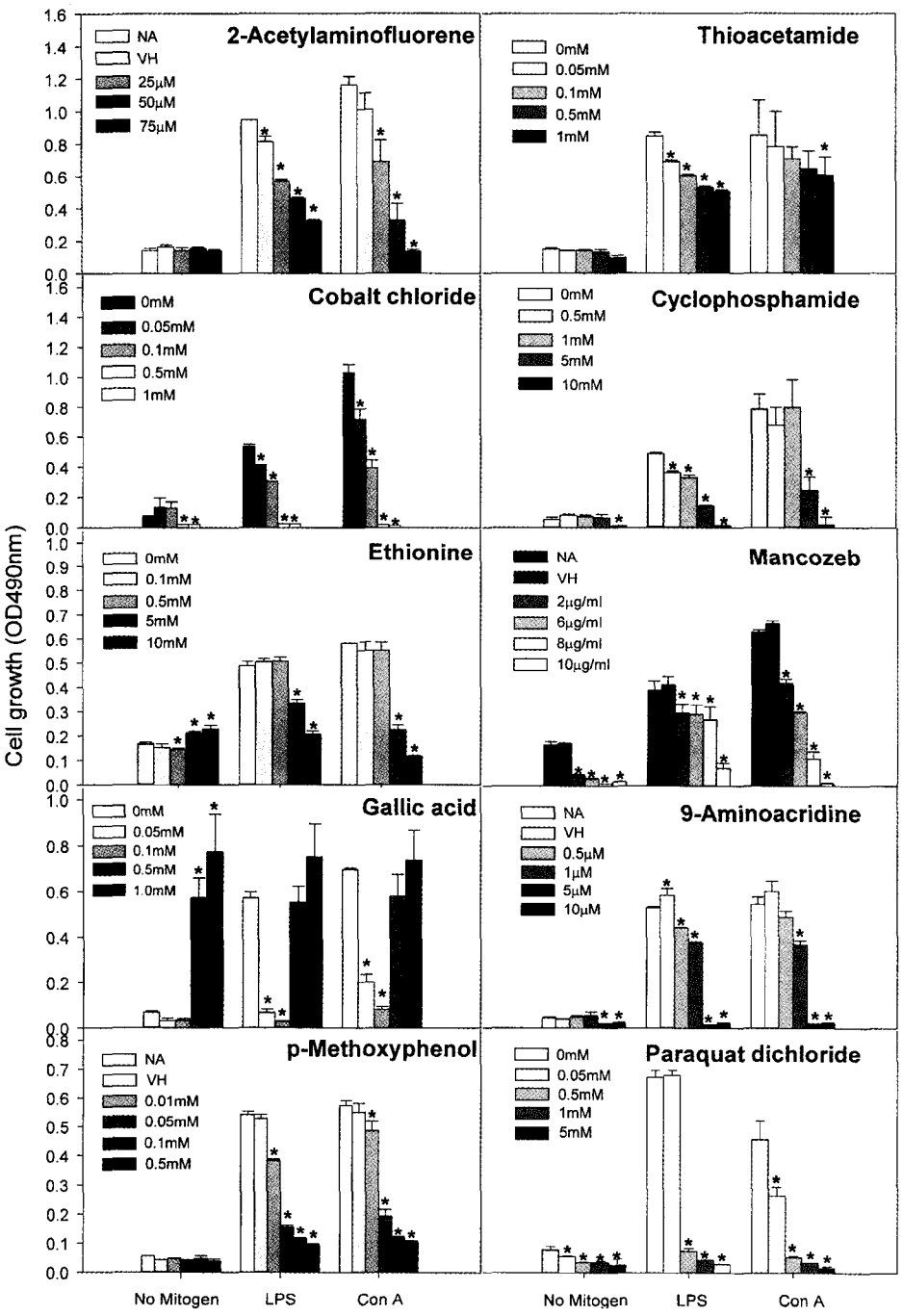

Fig. 1. Lymphoproliferation: Splenocytes (1×10^6 cells/ml) were treated with the indicated concentrations of test chemicals and then stimulated with Con A (2 μg/ml) or LPS (100 μg/ml) for 72 h. The cultures were treated with 20 μl of MTS solution for the last 4 h of culture period. The optical density was measured at 490 nm by an ELISA reader. *$P<0.05$ as determined by Dunnetts t-test as compared to the control group. NA; naive, VH; vehicle.

erations (Fig. 1). Cyclophosphamide, a positive control in this study, is an immunosuppressant that has been using in clinical treatments (Allison, 2000). 2-acetylaminofluorene (Kim *et al.*, 1989; Koh *et al.*, 1995; Lee *et al.*, 1996; Jeon *et al.*, 1999) and thioacetamide (Jeong *et al.*, 1999; Kim *et al.*, 2000) were previously reported to be immunosuppressive. As anticipated, all three chemicals showed dose-dependent suppression of cell proliferation. We used the MTS solution instead of [^{3}H]-thymidine to measure

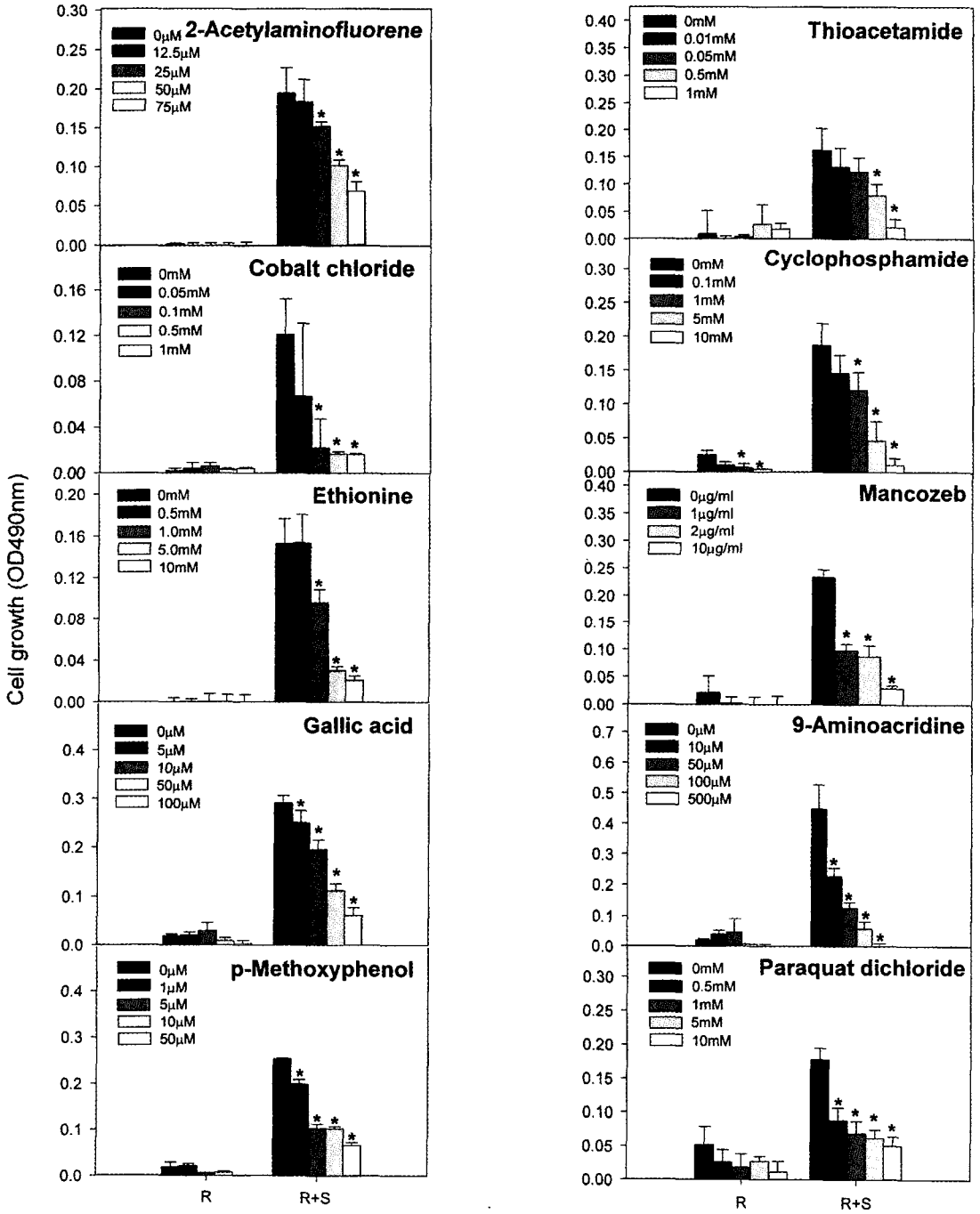

Fig. 2. Mixed leukocyte response: Responder cells (BALB/c splenocytes, 1×10^5) alone or with stimulator cells (mitomycin C-treated DBA/2 splenocytes, 4×10^5) were cultured in 200 μl/well at 37°C and 5% CO_2 for 5 days. The proliferation levels were determined as described in the lymphoproliferation section of the Materials and Methods. *P<0.05 as de- termined by Dunnetts t-test as compared to the control group.

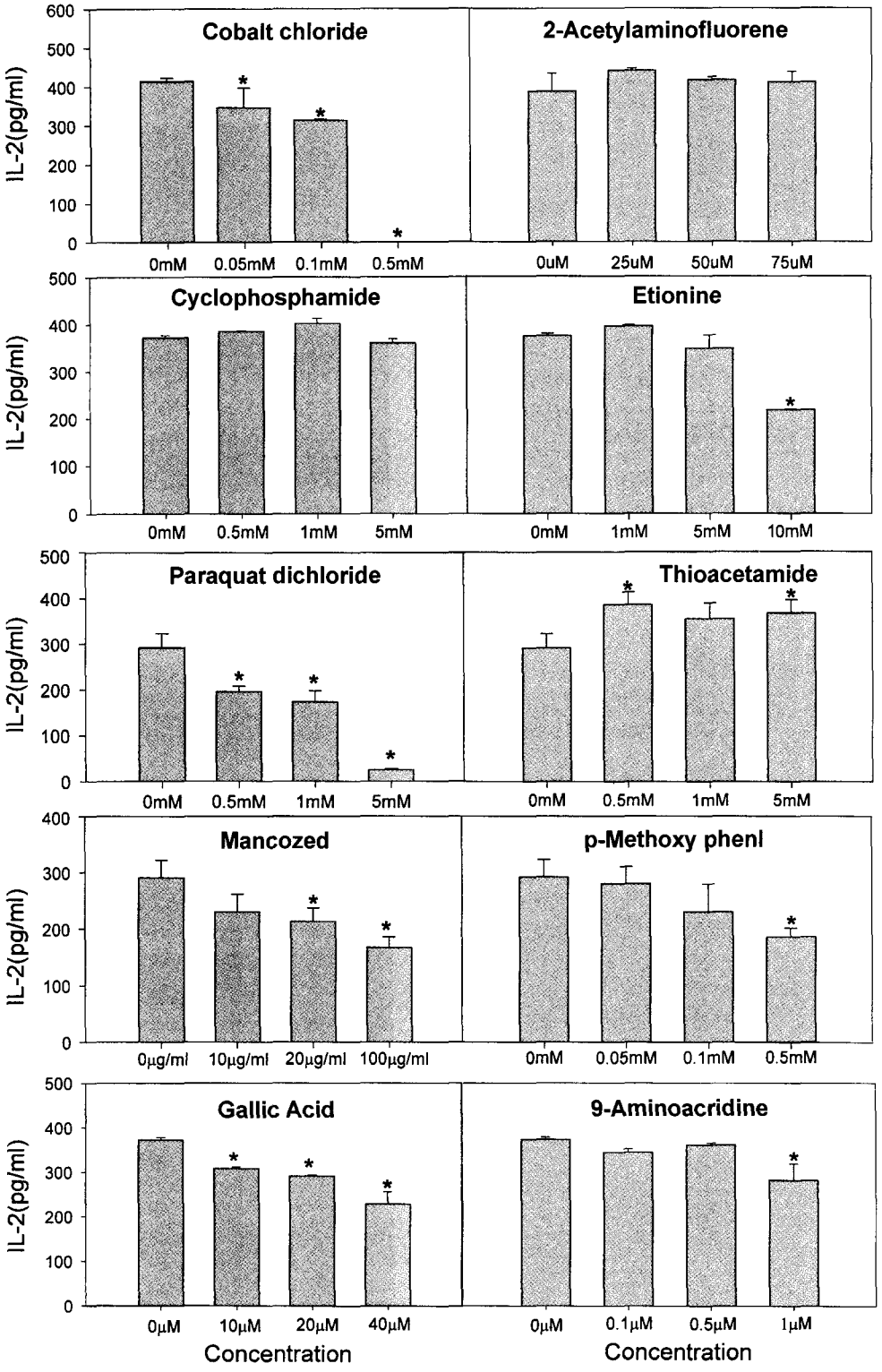

Fig. 3. IL-2 measurement: The amounts of IL-2 in the culture supernatants at 24 h were determined in triplicate us- ing the mouse IL-2 ELISA kit. Samples were added to antibody-precoated microtiter wells and incubated for 1 h at 37°C. The wells were washed and incubated with biotinylated anti-IL-2 antibody for 1 h, then with horse radish peroxidase-conjugated streptavidin for 40 min at 37°C. After a 10 min incubation with substrate solution provided by the manufacturer, the optical densities were measured at 450 nm. *$P<0.05$ as determined by Dunnett's t-test as compared to the control group.

the proliferation levels. The MTS is bioreduced by cellular dehydrogenase and produces a MTS formazan that is soluble in culture medium with an orange-red color. The absorbance at Y axis in Fig. 1 represents the activity of cellular dehydrogenase and is proportional to rather viable cell number than the proliferation level. So it was possible to measure the cell viability together. The proliferation without a mitogen in Fig. 1 indicates the cell viability change induced by chemicals. Treatment with cobalt chloride, mancozeb, 9-aminoacridine, p-methoxyphenol, or paraquat dichloride decreased in cell viability, indicating their nonspecific cytotoxicity. In other words, the suppression of cell proliferation by these chemicals appears due to their cytotoxicity not due to immunotoxicity. Although using the MTS solution has many advantages, such as no needs of radioactive materials, fastness, convenience, and additional cell viability measurement, it also has a disadvantage that MTS can be chemically reduced by reducing agents. Gallic acid is known as an antioxidant and produces reactive oxygen species in water-based solution like another antioxidant ascorbic acid. Thus the antioxidant itself reduced MTS to MTS formazan and produce orange-red color, which explains the reason the high concentrations (0.5 mM and 1 mM) of gallic acid increased the observance at 490 nm (Fig. 1). Although ethionine decreased proliferation, it seems to be a very weak immunosuppressant since the suppressive concentrations were very high (5 mM and 10 mM).

MLR is one of the cellular immunities and is a proliferation induced by the alloantigen stimulation. This proliferation is mainly induced by dendritic cells and macrophages not like Con A or LPS that directly stimulates T- or B-cells, respectively (Sunshine *et al.*, 1982). If a chemical inhibits MLR but not Con A-induced T-cell proliferation, it is reasonable to guess that the chemical inhibits dendritic cell or macrophage functions. All 10 chemicals that had shown the suppression of lymphoproliferation also suppressed the MLR in a dose-dependent manner (Fig. 2). Since, like in the lymphoproliferation, the MTS was used instead of [^{3}H]-thymidine to measure proliferation levels, it does not require radioisotope and special equipment such as the cell harvester.

T-cells stimulated with a mitogen produce and secrete IL-2. Measurement of IL-2 in culture supernatant gives information about where the target of chemical locates. If the target is in the signal transduction pathway toward IL-2 production, the production of the IL-2 is inhibited. On the contrary, if the chemical blocks the pathway after IL-2 production and toward cell growth, it does not change the IL-2 production. Figure 3 showed the effect of chemical exposure to the spleen cell culture on the IL-2 production. Decrease of IL-2 was observed in the culture supernatant treated with cobalt chloride, ethionine, mancozeb, gallic acid, paraquat dichloride, p-methoxyphenol, or 9-aminoacridine. Among these chemicals cobalt chloride, mancozeb, paraquat dichloride, p-methoxyphenol, and 9-aminoacridine were cytotoxic in the same concentration range. Thus this decrease of IL-2 production is considered to be due to the cytotoxic not immunotoxic effect of chemicals. Cyclophophamide, 2-acetylaminofluorene, or thioacetamide treatment did not produce the change of IL-2 accumulated in the culture supernatant, implying these compounds do not modulate the signal transduction pathway toward the IL-2 production.

In this report, we are proposing a simple immunotoxicity assessment composed with three assays; lymphoproliferation, MLR, IL-2 measurement. All three assays use no radioisotope but color development and measurement with an ELISA reader. However, it also has disadvantages in the analysis of chemicals with antioxidant activity. It is basically neither as sensitive as antibody forming cell response in the existing methods nor can produce the information regarding immunopathology or nonspecific immunity. Disregarding these disadvantages, it is very fast and easy, and assesses the effect of chemical on the cellular and humoral immunity, distinguishing between the immunotoxicity and cytotoxicity. Thus, it can be easily employed as a simple immunotoxicity test, such as a screening step during drug development that does not require a highly sensitive and full-scale assessment.

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