A Simple Bioassay for Evaluating Immunotoxic Properties of Chemicals by Use of *in Vitro* Antibody Production System

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Antibody production is one of biomarkers sensitive to chemical toxicity. A simple assay system for evaluating toxic effect of chemicals on antibody production was developed using pokeweed mitogen (PWM)-induced IgM production by spleen cell cultures obtained from BALB/c mice. It was evaluated with reference immunomodulating chemicals. Cultured spleen cells significantly and day-dependently produced IgM after PWM stimulation. Test chemicals were added to the cultures simultaneously with PWM. At 4 day-culture, medium IgM level and cell proliferation were determined by ELISA method and WST-8 method for mitochondrial dehydrogenase activity, respectively. Results showed that PWM-induced IgM production was dose–dependently suppressed by cyclosporin A, a typical immunosuppressor, with more susceptible inhibition of cell proliferation. On the contrary, it was increased by lead nitrate, an immunoenhancer, around 100 μM with little change in the cell proliferation. Moreover some estrogen-related compounds such as estradiol, ethynylestradiol, diethylstilbestrol and bisphenol A dissolved in dimethyl sulfoxide were applied to the assay system. Those below 1 μM affect neither the IgM production nor the cell proliferation in spleen cell cultures irrespective of mouse sex. Further for detecting active metabolites, the preincubation of a chemical with S9 mix was coupled with the assay system. The availability of the procedure was confirmed by inhibitory effect of cyclophosphamide after preincubation with S9 mix, which had no effect without S9 mix. Thus the newly developed *in vitro* IgM production system by mouse spleen cells is effective for evaluating toxic or modulating effects of chemicals on antibody production.

Key words —— immunotoxicity, *in vitro* IgM production, mouse spleen cell, pokeweed mitogen

INTRODUCTION

Immune system is composed of a variety of cellular components such as macrophages/monocytes, T and B lymphocytes, natural killer cells. Immune function, antibody production in particular, is dependent upon a highly regulated network of many types of immune cells and soluble factors such as cytokines and antibodies in lymphoid organs and peripheral circulation. This multi-step process in the immune function is considered to be greatly susceptible to toxic insult and has been used to evaluate risk of chemical exposure.[1,2]

In vitro culture systems of immune cells have been used extensively to study the mechanisms by which agents cause immune dysfunction. has been also tried to develop *in vitro* test systems using immune cells to efficiently predict and evaluate the effects of xenobiotics on immune responsiveness.[3] The *ex vivo* plaque-forming cell (PFC) assay after *in vivo* sensitization with an antigen and exposure to chemicals is widely used as a very sensitive functional test for detecting toxicants to antibody production. The *in vitro* PFC assay including both of antigen sensitization and chemical exposure *in vitro*, however, is unfavorable by less population of sensitized cells and laborious procedures for efficient evaluation of many chemicals.

Proliferation and differentiation of lymphocytes have been reproduced *in vitro* by use of mitogens that induce polyclonal activation of B and/or T lymphocytes. Pokeweed mitogen (PWM) is a plant-lectin obtained from *Phytolacca americana*, and can induces polyclonal B lymphocyte activation, conse-

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quently to induce immunoglobulin (Ig) production.[4] The cellular interactions for Ig production induced by PWM resemble those in antigen-induced process for the generation of a specific antibody. Both processes require the presence of macrophages/monocytes and T lymphocytes.[4] It means that the PWM-induced antibody production can be a convenient *in vitro* model for identifying toxicants targeting immune cells such as B and T lymphocytes and macrophages. In fact an assay for the PWM-induced IgG production in cultures of human peripheral blood mononuclear cells (PBMC) has been developed as an *in vitro* model for assessing drug-induced disruption of antibody production process.[5,6] This model is valuable, but it is difficult to routinely use human PBMC for testing many chemicals. Moreover it takes too much time because IgG production is determined after 7 or 8 day-culture including replacement with fresh medium. IgM is the antibody produced primarily by activated B lymphocytes and can be detected in cultures before IgG.

Then it is investigated here to apply the *in vitro* model of the PWM-induced Ig production to mouse spleen cell cultures and measure the production of IgM instead of IgG. Thus a new *in vitro* model of mouse spleen cell cultures was developed for PWM-induced IgM production and cell proliferation, and it was validated with reference immunomodulating chemicals.

MATERIALS AND METHODS

Materials —— Male BALB/cAnN mice (7–8 weeks old) were obtained from Charles Liver Japan Inc. (Atsugi, Japan). Animals were used at 8–9 weeks of age after being rested for at least one week under specific pathogen-free conditions. Pokeweed (*Phytolacca americana*) mitogen (PWM) and reference immunomodulating chemicals such as cyclosporin A (immunosuppressor), chlorpromazine (immunoenhancer) and cyclophosphamide (immunosuppressor after metabolic activation) were purchased from Sigma Co. (St. Louise, U.S.A.). Lead nitrate (immunoenhancer) was obtained from Wako Pure Chem. Ind., Osaka, Japan). Other chemicals were estrogens (17β-estradiol, 17β-estradiol acetate and ethynylestradiol) purchased from Sigma Co. (St. Louise, U.S.A.), xenoestrogens (diethylstilbestrol and bisphenol A) obtained from Wako Pure Chem. Ind. (Osaka, Japan). Dimethyl sulfoxide (DMSO, spectrum analysis grade) purchased from Dojindo Labs. (Kumamoto, Japan). Rat S-9 mix (S-9 cofactor C set) for metabolic activation of chemicals was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Cell Culture of Spleen Cells —— Spleens were aseptically removed from BALB/c mice and spleen cell suspensions were prepared as described previously.[7] After washed in Hanks' balanced salt solution supplemented with 10 mM HEPES buffer (pH 7.4), spleen cell suspensions were adjusted to give a cell number of 10^6 cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.4 M sodium pyruvate, 20 mM mercaptoethanol, non-essential amino acids and antibiotics. One ml aliquots of cell suspension were cultivated (5% CO_2-air, 37°C) in each well of 24-well culture plate (Costar #3512, U.S.A.) under 5% CO_2-air at 37°C. Under the presence of PWM spleen cells were cultured for 2 to 6 days. A test chemical dissolved in Ca, Mg-free phosphate buffered saline (PBS) or DMSO, or the vehicle solvent (10 μl each) was added to the cultures at the beginning of culture. Each plate usually includes quadruplicate control and test chemical-added cultures at 5 different concentrations. On different culture days, 700 μl of culture media were collected for total IgM assay and the residual cells in 300 μl medium were used for cell proliferation assay.

Cell Proliferation Assay —— The cell number in cultures was measured as optical density at 450 nm of the medium by the modification (WST-8 method) of MTT assay for mitochondrial dehydrogenase activity in viable cells.[8] In brief, the cultured cells in 300 μl residual medium for cell proliferation assay were further incubated for 2 hrs under 5% CO_2-air at 37°C after addition of 30 μl of TetraColar One reagent (Seikagaku Corp., Tokyo, Japan). Mitochondrial dehydrogenase activities in viable cells were measured as the optical density at 450 nm of the medium containing the water-soluble tetrazolium salt produced by the enzyme reaction.

Measurement of Total IgM —— Total IgM levels in the media were determined by indirect ELISA method using peroxidase-conjugated anti-IgM antibody. Briefly, 96-well microtiter immunoplates (Nunc, U.S.A.) were precoated overnight at 4°C with 100 μl of rabbit anti-mouse IgM antibody (IgG) (Caltag Labs., U.S.A.) dissolved in PBS, pH 7.4. After washing with PBS containing 0.05% Tween20, plates were blocked overnight at 4°C with 150 μl of PBS containing 1% bovine serum albumin (BSA). After washing with PBS, 100 μl aliquots of culture

media (usually 11-fold diluted media) or reference mouse serum or standard mouse IgM (Seikagaku Corp., Tokyo, Japan) diluted with 1% BSA-PBS were added into each well and was left for 60 min at room temperature. After washing, 100 μl aliquots of horseraddish peroxidase-conjugated goat anti-mouse IgM antibody (IgG) (Caltag Labs., U.S.A.) in PBS were added and incubated for 60 min at room temperature. Finally after washing again, 150 μl aliquots of substrate solutions containing 0.4% *o*-phenylenediamine and 0.01% hydrogen peroxide in 0.1 M citrate-0.2 M phosphate buffer were added and incubated 15 min at room temperature. The enzyme reaction was terminated by addition of 50 μl 0.5 M H_2SO_4. The optical density at 490 nm of each well was read using a microplate reader (NJ-2000, Intermed Co., Tokyo, Japan). Antibody concent-rations in culture media were calculated by the standard curve for IgM.

Statistical Analysis —— Results are analyzed by Dunnett's *t*-test.

RESULTS

In Vitro IgM Production by Spleen Cells Stimulated with PWM

Unstimulated spleen cell cultures without PWM neither induced cell proliferation nor accumulated IgM in the medium. When spleen cells at different cell concentrations were cultured with 5 μM PWM, however, cell proliferation and IgM production were remarkably enhanced by PWM after Day 2 of culture (Fig. 1). In cultured cells inoculated at 10^6 cells/ml, IgM in the medium accumulated proportionally with increased culture days during 2 to 6 days. Viable cells also were most at 4 day-culture, and later became less. The inoculation of cells at higher cell concentration resulted in earlier saturation of both cell proliferation and medium IgM levels. It means that IgM levels in the medium were optimal at 4 day-culture of spleen cells at 10^6 cells/ml for detecting qualitative and quantitative alterations of IgM production including its enhancement or suppression. Moreover, concentration-effect relationship showed that PWM was most effective at 0.5 μM for both cell proliferation and IgM production (Fig. 2). Then in order to detect immunotoxic effects *in vitro* IgM production was mostly assayed at 4 day-culture in the presence of 0.5 μM PWM, although 3 to 5 day-culture protocol was required for analyzing the mechanism of altered IgM production.

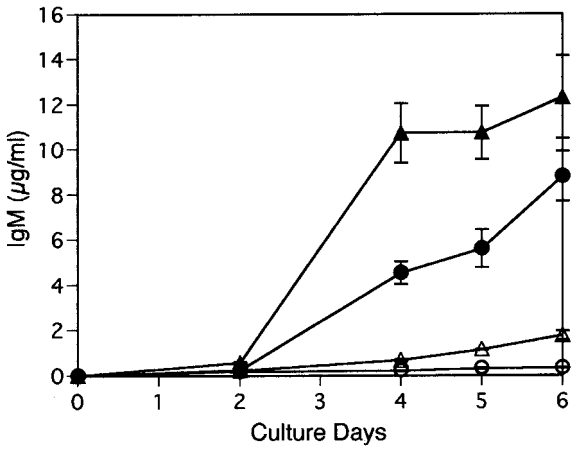

Fig. 1. IgM Levels in the Medium of Mouse Spleen Cell Cultures Treated with PWM

Each symbol and vertical line represent mean and standard deviation (SD) of quadruplicate cultures. Open symbols: control cultures without PWM and closed symbols: cultures treated with 5 μM PWM. Inoculated cell densities were 1×10^6 cells/ml for circles and 2×10^6 cells/ml for triangles. IgM levels in culture media were measured by ELISA as described in Materials and Methods.

Effect of DMSO on *in Vitro* IgM Production System

Water-soluble chemicals were diluted with PBS and were added to spleen cell cultures. Many organic chemicals however are less water-soluble. Such chemicals were dissolved in DMSO and added to the cultures for the test. Then effect of DMSO as solvent control on spleen cell cultures was first examined. DMSO is usually added to cultured cell lines at final concentration of 0.1 to 0.5%, but at such concentrations it promoted PWM-induced cell proliferation in primary cultured spleen cells. On the contrary it suppressed IgM production. Ethanol also gave similar results (data not shown).

Experiments on concentration-effect relation of DMSO showed that 0.1% to 0.02% DMSO still slightly inhibited PWM-induced IgM production in spleen cell cultures (Fig. 3).

Effect of Chemicals on IgM Production *in Vitro*

Various chemicals including some known immunotoxicants and estrogen-related compounds were examined with the *in vitro* IgM production system. Chemicals selected as immunotoxicants were cyclosporin A[6] as an immunosuppressor, chlorpromazine[6] and lead nitrate[8] as an immunostimulator.

As shown Fig. 4A, cyclosporin A evidently suppressed both IgM production and cell proliferation above 1 μM. Cell proliferation was more susceptible to inhibitory effect of cyclosporin A as compared to the antibody production cell, because the drug at

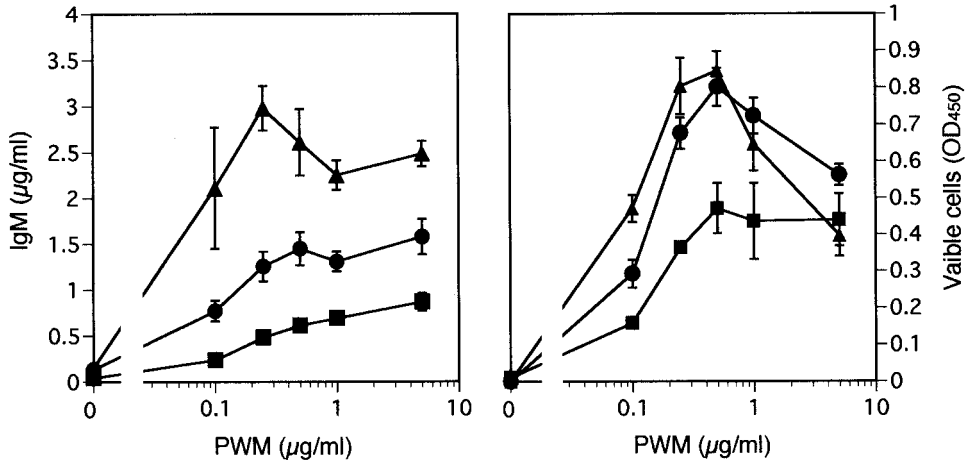

Fig. 2. Dose–Effect Relation for PWM-Induction of IgM Production and Cell Proliferation in Spleen Cell Cultures

IgM levels (left) and viable cells (right) were measured by ELISA and WST-8 methods, respectively, as described in text. BALB/c mouse spleen cells inoculated at 1×10^6 cells/ml were cultured with or without PWM for 3 days (squares), 4 days (circles) and 5 days (triangles). Each symbol and vertical line represent the mean and S.D. (n=4).

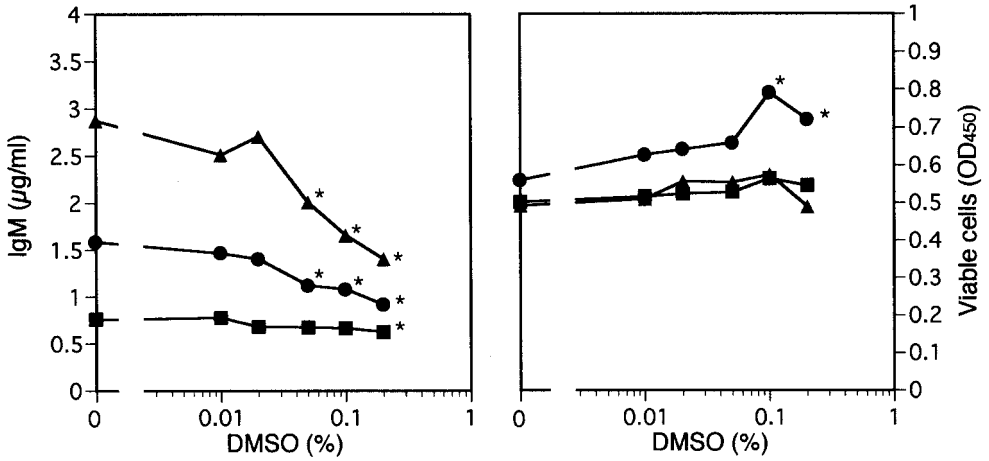

Fig. 3. Effect of DMSO on IgM Production (Left) and Cell Proliferation (Right) in Spleen Cell Cultures Treated with PWM

Spleen cells were cultured for 3 days (squares), 4 days (circles) and 5 days (triangles). Asterisks indicate statistically significant differences ($p < 0.05$) from DMSO-free controls.

0.5 μM inhibited cell proliferation but not IgM production. Chlorpromazine (Fig. 4B) also suppressed both IgM production and cell proliferation above 1 μM, although a slight increase was observed around 0.5 μM. In contrast, lead nitrate around 100 μM enhanced IgM production without a significant effect on cell proliferation. Lead nitrate at more than 500 μM turned to downregulation of the both indicators. The result indicate that enhancement of antibody production induced by lead nitrate can be due to a specific promotion of antibody formation process.

Estrogens such as 17β-estradiol, 17β-estradiol acetate and ethynylestradiol, and xenoestrogens such as diethylstilbestrol, bisphenol A in DMSO solution were also tested for IgM production. These estrogenic compounds were treated at concentrations up to 1 or 10 μM, as they induced proliferation of estrogen-dependent MCF-7 cells.[9,10] None of estrogens and xenoestrogenic compounds tested however affected IgM production and cell proliferation induced by PWM (data not shown). Further spleen cells prepared from female BALB/c mice (8 week-old) were cultured and treated with 17β-estradiol and diethylstilbestrol at the same conditions. Those estrogens produced no effects on the cultures (data not shown).

Coupling with the Metabolic Activation System Using S9 Mix

As the activity of metabolic activation is low in spleen cells, *in vitro* IgM test system by itself cannot detect chemicals that affect immune cells in form

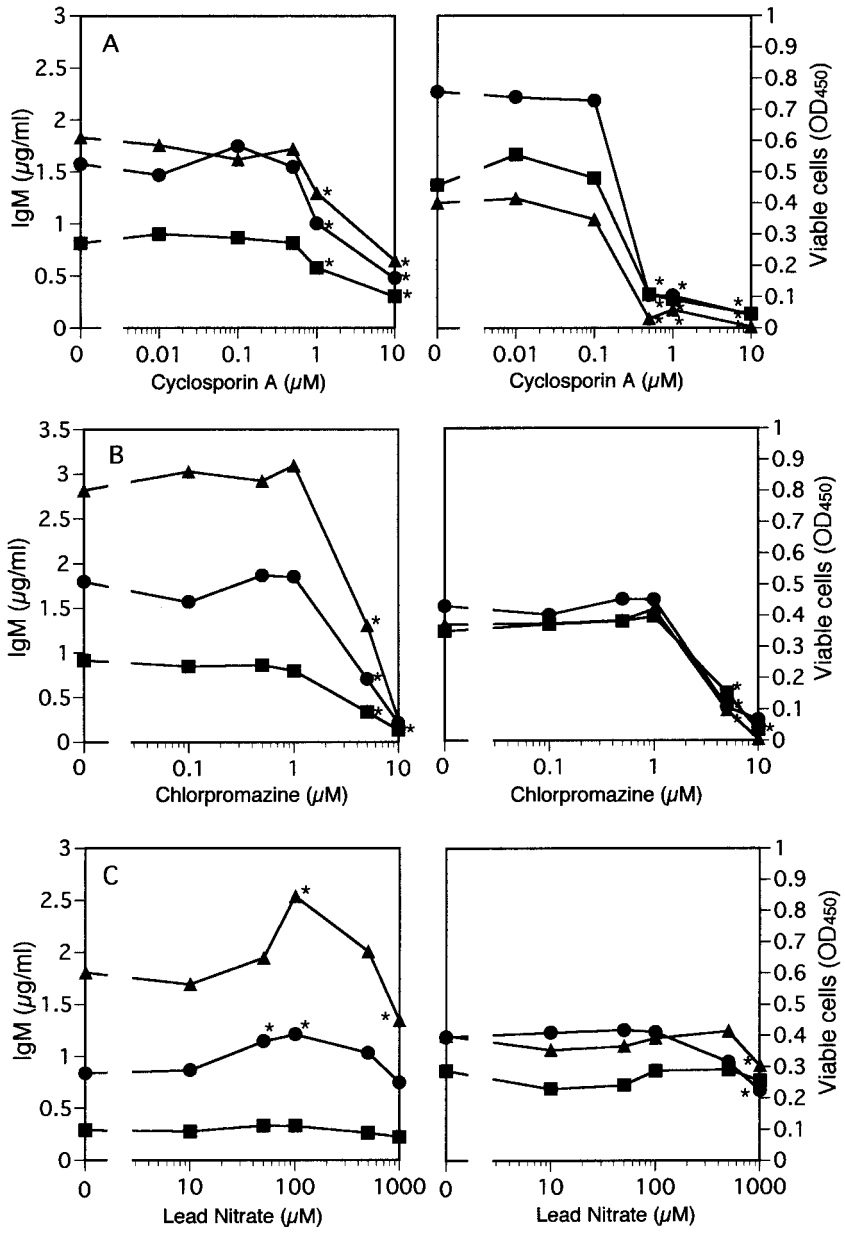

Fig. 4. Effects of Chemicals on IgM Production (Left) and Cell Proliferation (Right) in Spleen Cell Cultures Treated with PWM

A(top): cyclosporin A, B(middle): chlorpromazine, C(bottom): lead nitrate. Details of experimental procedures are the same as in Fig. 2. Spleen cells were cultured for 3 days (squares), 4 days (circles) and 5 days (triangles). Asterisks indicate statistically significant differences ($p < 0.05$) from vehicle controls.

of metabolites. In fact cyclophosphamide (CP), a typical immunosuppressant *in vivo* could neither suppress IgM production nor cell proliferation induced by PWM in the *in vitro* system. Then the IgM production system was investigated to couple with metabolic activation process: CP dissolved in 1 ml of rat liver S9 mix (mixture of 1 ml S9 fraction and 2.35 ml cofactor C) was preincubated for 30 min at 37°C. The reaction mixture was filtrated with the Millipore filter (pore size 0.2 μm). The filtrate was added to the spleen cell cultures. As cofactors in S9 mix were much cytotoxic, the S9 mix after preincubation was 10 to 50-fold diluted with PBS (final concentration of S9 mix in the medium was 0.1 to 0.05%). Such diluted S9 mix had no significant effect on cell proliferation and IgM production (Fig. 5).

The reaction mixture of CP was diluted 10 times with PBS and added to the IgM production system. Figure 6 shows that in contrast to the culture with no metabolic activation, CP above 100 μM after preincubation with S9 mix significantly suppressed both IgM production and cell proliferation. It means that

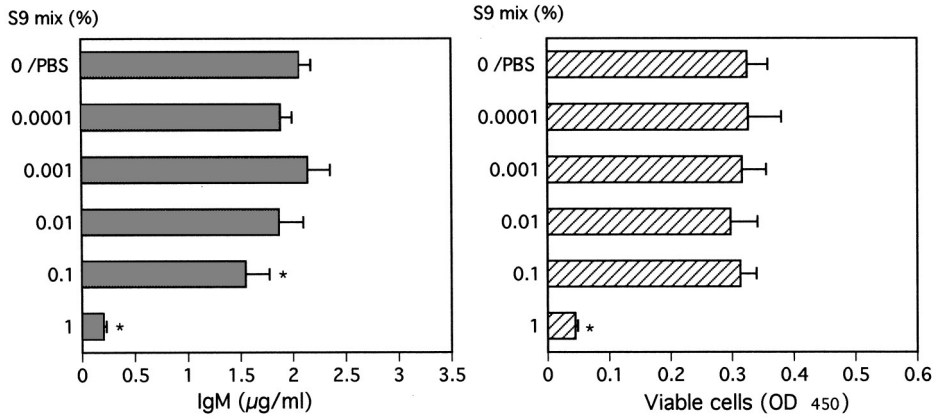

Fig. 5. Effect of the S9 Mixture on PWM-Induced IgM Production (Left) and Cell Proliferation (Right) in Spleen Cell Cultures at 4-Day Culture

S9 mixture at different dose was added to cultures at initiation of cultivation. Horizontal lines represent S.D. (n=4). Asterisks indicate statistically significant differences ($p < 0.05$) from vehicle controls (0/PBS).

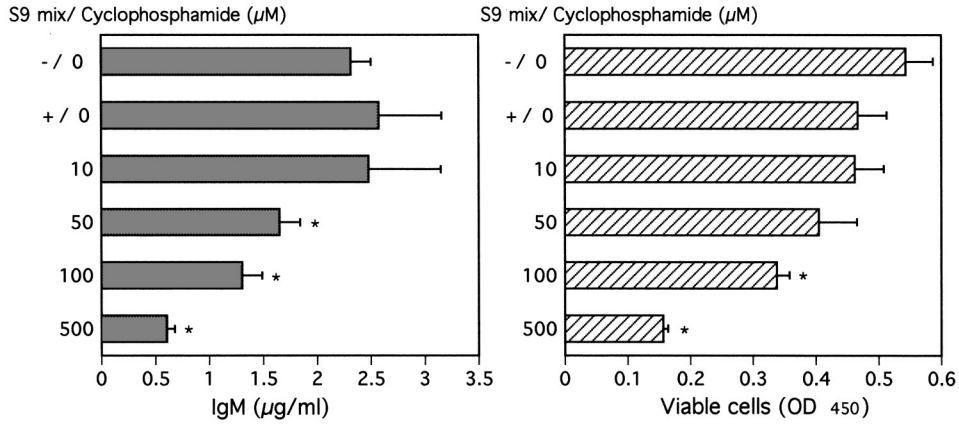

Fig. 6. Effect of Cyclophosphamide on PWM-Induced IgM Production (Left) and Cell Proliferation (Right) in Spleen Cell Cultures at 4-Day Culture

S9 mixture at final concentration of 0.01% and cyclophosphamide were added at the initiation of cultivation. Horizontal lines represent S.D. (n=4). Asterisks indicate statistically significant differences ($p < 0.05$) from vehicle controls with S9 mixture (+/0).

in vitro IgM production system coupled with metabolic activation process is applicable to detect toxic metabolites.

DISCUSSION

In this study an *in vitro* simple test system using PWM-induced IgM production instead of the PFC response was developed as an *in vitro* model of primary antibody production response, in order to detect and to characterize the chemical-induced disruption of antibody production. IgM production was optimized for PWM concentration and culture duration (Figs. 1 and 2). From these results, the standard conditions for active cell proliferation and IgM production at 4 day-culture are confirmed as that 1) spleen cells are inoculated at 10^6 cells/ml medium, and 2) PWM are treated at 0.5 μM. In fact, under these culture conditions reference compounds like cyclosporin A (Fig. 4A) and lead nitrate (Fig. 4C) showed suppressive and stimulative effects on IgM production, respectively, well correspondent with reported findings.[5,11] Different from the stimulatory effect reported else,[6] chlorpromazine produced enhancement of neither cell proliferation nor IgM production induced by PWM. When compared with human PBMC, mouse spleen cells may be less sensitive to the stimulatory effect of this compound, because suppressive effect of the compound at higher concentrations was observed in mouse spleen cell cultures as well as human PBMC cultures.

It is also recommended that DMSO should be used at final concentration of 0.01% (at most 0.1%) for spleen cell cultures to minimize effect of vehicle alone, although it lowers the upper limit of test concentration of chemicals dissolved in DMSO. The present study showed that none of tested estrogens and xenoestrogenic compounds dissolved in DMSO affected PWM-induced IgM production and cell proliferation of spleen cells obtained from both sex mice. It is reported that a major metabolite of 17β-estradiol, 2-OH estrone, was more potent than the parent compound at suppressing lymphocyte proliferation *in vitro* and *in vivo*.[12] Then estrogens such as 17β-estradiol, 17β-estradiol acetate and diethylstilbestrol were reexamined after preincubation by the *in vitro* IgM production test. All reactants of parent estrogens tested at 1 μM did not cause any significant change in PWM-induced IgM production by spleen cell cultures. Thus it is not likely that tested estrogens suppress antibody production in form of their metabolites. Estrogens are thought to suppress immune functions mainly *via* clonal depletion of T cells derived of thymus involution.[13] The results seem to indicate that estrogen and estrogenic compounds below 1 μM do not act on lymphocytes directly. They may affect splenic lymphocytes indirectly *via* effect on thymocytes.

In conclusion, *in vitro* immunotoxicity test model on antibody production was developed as *in vitro* IgM production test. PWM-induction of IgM was a good indicator of the series of immune events that culminate *in vivo* in antibody production, and it can be useful for efficient detection of immunotoxic chemicals and assessing of their concentration-effect relation. When coupled with metabolic avctivation system, *in vitro* IgM production test is available also for chemicals producing toxic metabolites.

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