

Studies on primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin (KLH), in immunotoxicology evaluation

Ryota Kawai

2014

Contents

General introduction	1
 Chapter 1	 4
Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats	
 Chapter 2	 27
Evaluation of canine T-cell dependent antibody response to the primary and secondary immunization with keyhole limpet hemocyanin	
 Chapter 3	 52
Mechanism of immunosuppressive effects of methotrexate on primary and secondary T-cell dependent antigen response to keyhole limpet hemocyanin in rats	
 Summary	 78
 Acknowledgements	 82
 List of publications	 83

General introduction

Many types of food chemicals, environmental chemicals and drugs cause diverse immune responses and increase the risk of serious immune disorders including infections with reduced host resistance to infectious agents or tumor cells in humans [1-3]. In addition, the enhancements to immune responses brought about especially by drugs produce severe hypersensitivity and autoimmune diseases as antigens or modulators [4-6], resulting in the withdrawals of the drugs from clinical or the market [7]. Thus, it is critical that the investigators consider appropriate approaches for evaluating the immunotoxic potential of new chemicals adequately from the nonclinical perspective. As nonclinical immunotoxicity testing, traditional standard toxicity studies using rodents and non-rodents such as antigenicity studies, and sensitization studies, have been routinely used to date [8, 9]. However, these assessments cannot identify the potential immunotoxicity of new chemicals for certain because they interact extensively with the immune system, which is composed of several types of cells and organs, through complicated cell-cell communication. Therefore, comprehensive assessments for evaluating the potential immunotoxicity of new chemicals have been sought to clarify the occurrence of potential adverse effects and discuss the countermeasures against these effects.

The T-cell-dependent antibody response (TDAR) assay has recently been recommended as a first choice for an immune function test to evaluate the potential immunotoxicity of new chemicals [10-12]. However, no

standardized protocol for TDAR assays has been established so far since this assay system has many drawbacks. These include species and strain diversities and variations of practical procedures such as antigens used, experimental protocols, and assay platforms.

In the present study, we evaluated both serum keyhole limpet hemocyanin (KLH)-specific IgM and IgG levels by an enzymed-linked immuno sorbent assay (ELISA) following primary and secondary immunizations with KLH in rats and dogs to develop a practical study design incorporating both the primary and secondary responses in TDAR assays. Next, the effects of the reference immunosuppressive drugs on TDAR were evaluated to confirm the utility of the primary and secondary TDAR models. Furthermore, follow-up testing, including immunophenotyping of lymphocytes and functional testing of splenocytes and macrophages in addition to TDAR, was conducted to propose comprehensive approaches for evaluating the 'true' immunotoxicity potential of new chemicals from a nonclinical perspective.

References

- [1] Hinton, D.M., US FDA "Redbook II" immunotoxicity testing guidelines and research in immunotoxicity evaluations of food chemicals and new food proteins. *Toxicol Pathol*, 2000. 28(3): p. 467-78.
- [2] Vos, J.G., Immune suppression as related to toxicology. *CRC Crit Rev Toxicol*, 1977. 5(1): p. 67-101.
- [3] Dean, J.H., J.R. Hincks, and B. Remandet, Immunotoxicology

- assessment in the pharmaceutical industry. *Toxicol Lett*, 1998. 102-103: p. 247-55.
- [4] Bacon, K.B. and T.J. Schall, Chemokines as mediators of allergic inflammation. *Int Arch Allergy Immunol*, 1996. 109(2): p. 97-109.
 - [5] Mantovani, A., et al., Chemokines and chemokine receptors during activation and deactivation of monocytes and dendritic cells and in amplification of Th1 versus Th2 responses. *Int J Clin Lab Res*, 1998. 28(2): p. 77-82.
 - [6] Riedl, M.A. and A.M. Casillas, Adverse drug reactions: types and treatment options. *Am Fam Physician*, 2003. 68(9): p. 1781-90.
 - [7] Moore, T.J., M.R. Cohen, and C.D. Furberg, Serious adverse drug events reported to the Food and Drug Administration, 1998-2005. *Arch Intern Med*, 2007. 167(16): p. 1752-9.
 - [8] Mota, I. and D. Wong, Homologous and heterologous passive cutaneous anaphylactic activity of mouse antisera during the course of immunization. *Life Sci*, 1969. 8(16): p. 813-20.
 - [9] Magnusson, B. and A.M. Kligman, The identification of contact allergens by animal assay. The guinea pig maximization test. *J Invest Dermatol*, 1969. 52(3): p. 268-76.
 - [10] ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use), Immunotoxicity Studies for Human Pharmaceuticals S8. 2006.
 - [11] Bugelski, P.J. and C. Kim, T-Dependent Antigen Response (TDAR) Tests: Meta-Analysis of Results Generated Across Multiple Laboratories. *J Immunotoxicol*, 2007. 4(2): p. 159-64.
 - [12] Gore, E.R.-P., Immune function tests for hazard identification: a paradigm shift in drug development. *Basic Clin Pharmacol Toxicol*, 2006. 98(4): p. 331-5.

Chapter 1

Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats

Introduction

A T-cell-dependent antibody response (TDAR) assay is recommended as a first choice for an immune function test to evaluate the potential immunotoxicity of new chemicals [1]. As the traditional models of TDAR assays were developed in the last century, the primary immune response to sheep red blood cells (SRBC) measured by a splenic plaque-forming cell (PFC) or an enzyme-linked immunosorbent assay (ELISA) had been widely used [2, 3]. However, these assay systems with SRBC have inherent drawbacks, including complicated procedures of PFC assay and several variations come primarily from the lots of SRBC employed and the different procedures of SRBC preparation for plate coating among laboratories.

Recently, TDAR assay using a soluble immunogenic protein, keyhole limpet hemocyanin (KLH) instead of SRBC, has become an experimental trend [4-8], since KLH is easily applied to ELISA and a lyophilized KLH which can be preserved to remain stable for the long-term is commercially available [9, 10]. The primary antibody response to KLH in rats measured by an ELISA has been reported to be a reliable and readily standardized alternative method to the conventional SRBC PFC assay [5]. Gore et al. have

recommended that evaluation of the IgG response, in addition to the IgM response, provides a more comprehensive assessment of the primary response and increases the likelihood of identifying ‘true’ potential [5]. They have also suggested that evaluation of the secondary antibody response could be included in the TDAR assay to identify the potential immunotoxicity of new medicinal candidates, although the secondary antibody response, consisting predominantly of IgG, would require additional immunizations with the antigen or a longer dosing period [5]. Despite the important nature of the secondary response, no standardized protocol for TDAR assay incorporating both primary and secondary responses has been established to date.

To develop a practical study design incorporating both the primary and secondary responses in TDAR assays in rats, we evaluated both serum KLH-specific IgM and IgG levels by ELISA following primary and secondary immunizations with KLH (300 µg/rat, IV) during a 14-day study period. Although the ICH S8 Guideline stated that a 28-day study period was generally accepted to assess drug-induced immunotoxicity in rodents [1], the study period was selected to be closely-linked to the period routinely used in short-term 14-day general toxicity studies used to support short-term clinical trials for human pharmaceutical candidate drugs (which also last up to 14-days, as per the ICH M3(R2) Guideline [11]). In the present study, four representative strains of rats (Sprague-Dawley (SD), Wistar, Fischer, and Lewis rats) were employed since the differences in strains and inter-individuals have been pointed out as practical issues in TDAR assays

[12]. Additionally, the utility of this model to detect immunosuppressive effects was confirmed in the above four strains with the reference immunosuppressive drug, cyclophosphamide (CPA) by a 14-day exposure. Furthermore, the results obtained from the experiment with CPA were compared to those of the primary response obtained by PFC assay or SRBC-ELISA in order to validate the sensitivity of this TDAR assay.

Materials and methods

Animals

Female SD, Wistar, Fischer, and Lewis rats (6-weeks-old) were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Rats were housed individually in stainless steel wire mesh bracket cages for at least 5 days for acclimation to the laboratory environment (temperature at 23°C; relative humidity of 55%; lighting cycle of 12 h/day). Commercial rodent diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were available *ad libitum*. All animal procedures were performed in accordance with our institutional guide for the care and use of laboratory animals.

Chemicals and antibodies

KLH, bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CPA and Sigma Fast™ o-phenylenediamine (OPD) dihydrochloride tablet sets were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated anti-rat IgM and anti-rat IgG, as well as guinea pig

complement were supplied as lyophilized products by Rockland Immunochemicals, Inc. (Gilbertsville, PA). Anti-KLH rat IgM (purified rat IgM [κ isotype] control) and anti-KLH rat IgG (purified rat IgG1 [κ isotype] control) antibodies were purchased from BioLegend (San Diego, CA). Sheep whole blood (in sterile Alsevers solution) was obtained from Nippon Bio-Supp. Center (Tokyo, Japan). RPMI medium was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). The BCA protein assay reagent kit was purchased from Thermo Scientific Pierce (Rockford, IL).

Preparation of CPA and KLH solution

CPA was dissolved in distilled water at concentrations of 0.2, 0.6 and 1.2 mg/ml. KLH was dissolved in physiological saline at a concentration of 1.5 mg/ml. The solution was filtered using a membrane filter (0.45 μm, Sartorius Stedim Biotech GmbH, Gottingen, Germany) prior to injection.

Treatment protocol

CPA was orally administered to rats for 14 consecutive days at 5 ml/kg body weight (Figures 1a and b). The dose levels were set at 0 (vehicle control), 1, 3, or 6 mg/kg, based on a preliminary study (data not shown). An intravenous dose of KLH-saline solution delivering 300 μg/rat was selected based on the earlier studies of Gore et al. [5] and White et al. [13]. The results of the preliminary study which included a time-course response revealed that intravenous dosing of KLH at 300 μg/rat produced sufficient

levels of anti-KLH specific antibody that were, in turn, considered to be utilizable for detecting the immunosuppressive potential of the test articles, even in the absence of any other overt toxicity findings. In the study here, the rats were immunized with intravenous injections of KLH (300 µg/rat) twice, i.e., on Days 5 and 9 of the CPA dosing regimen. Blood was collected from the jugular veins of individual rats on Days 1, 9, and 15 in order to perform a TDAR assay (Figure 1a). On Day 9, the blood was collected prior to the secondary KLH immunization to avoid affecting the primary TDAR outcome.

For the SRBC-PFC assay or SRBC-TDAR assay, rats were sensitized once with an intravenous SRBC injection (2×10^8 cells/animal) just after the dosing of CPA on Day 11. On Day 15, the rats were sacrificed humanely by exsanguination after blood collection from the abdominal aorta, under anesthesia. The spleen was then removed for use in the PFC assay (Figure 1b). Serum samples were prepared by centrifugation at 3000 rpm and 4°C for 10 min and then stored at -80°C until analysis.

Preparation of SRBC

Sheep whole blood in Alsever's sterile solution was washed three times with saline and the SRBC were then re-suspended in saline at 1×10^6 cells/µl.

ELISAs with anti-KLH antibodies

KLH (10 µg/ml) dissolved in coating (50 mM NaHCO₃) buffer was applied

to a 96-well microplate (Nalge Nunc Intl., Roskilde, Denmark) and then incubated overnight at 4°C. After blocking with 5% BSA in 0.05% Tween-20 (v/v) in PBS (TPBS) at 37°C for 2 h, anti-KLH rat IgM or anti-KLH rat IgG in duplicate was added to the plate for standard curves (4-parameter logistic model). The assay range for the IgM- and IgG-specific ELISAs was 1.56–100.00 ng/ml and 7.80–500.00 ng/ml, respectively. To evaluate each serum sample within the assay range, each serum sample was diluted ($\geq 1:1000$) with the diluent (1% BSA in TPBS). Each diluted serum sample in duplicate was added to the plate and was equilibrated at 37°C for 2 h. After gentle washing to remove non-adherent antibody and other serum factors, the plates were then treated with anti-rat IgM or IgG detection antibody conjugated with HRP at pre-determined optimal dilutions in the diluent and incubated at 37°C for 2 h. The optical density (OD) after stopping the OPD color reaction with 50 μ l 1M sulfuric acid was measured at 492 nm in a SpectraMax Pro micro-plate reader (Molecular Devices LLC, Sunnyvale, CA). Standard curves and anti-KLH Ig levels for each serum sample were calculated using SoftMax Pro software (Molecular Devices LLC).

PFC assay

A PFC assay was conducted according to the methods in Cunningham (1965) and Cunningham and Szenberg (1968). Briefly, the excised spleen was put in a dish with 10% FBS-RPMI medium, and a portion of the spleen was gently minced with tweezers. A single-cell suspension was obtained by passage through a cell strainer (70 μ m, BD Falcon™, Becton Dickinson and

Company, Ltd.). After two washes, the cells were re-suspended in 10% FBS-RPMI medium (cell viability was > 90% in trypan blue dye exclusion test). Subsequently, 400 μ l of the suspension, 50 μ l of 40% (v/v) SRBC, and 50 μ l of guinea pig complement were mixed in a test tube, and the mixture was put in a Cunningham chamber. The sealed chamber was incubated at 37°C for 1.5 h before the number of PFC was counted microscopically; results were then calculated and expressed in terms of specific activity (PFC/ 10^6 splenocytes). In addition, total spleen activity (PFC/whole spleen) was calculated based upon the whole/partial spleen weight ratio. In this paper, only specific activity (PFC/ 10^6 splenocytes) is reported because the outcomes were comparable to those for total spleen activity.

Extraction of SRBC membrane proteins

Erythrocyte ghosts were prepared according to the reported method [14, 15], with some modification. After centrifugation of sheep whole blood in Alsever's sterile solution, the SRBC pellets were washed with saline and then re-suspended in 1 mM $\text{Na}_2\text{-EDTA}$ /5 mM Tris-HCl (pH 7.0). After washing, the pellet was re-suspended with 3M KCl, the solution sonicated for 1 h at 4°C, and then stored for 2 days at 4°C. After centrifugation, the pellet was re-suspended with 0.1% SDS (w/v) and then stored at 4°C for 2 days. The solution was centrifuged and the supernatant dialyzed against PBS at 4°C overnight. The collected SRBC membrane extract solution was diluted with PBS and protein concentration measured with the BCA Protein Assay Reagent Kit. The protein concentration of the solution was adjusted to

1 mg/ml with PBS and the diluted protein samples stored at -20°C until use.

Anti-SRBC antibody ELISA

Serum anti-SRBC IgM was measured by an ELISA using the method of Temple et al. [16], with some modification. Extracted SRBC membranes (20 $\mu\text{g}/\text{ml}$) suspended in coating buffer (0.1 M NaHCO_3) were applied to a 96-well microplate and incubated at 4°C overnight. After blocking with 1% skimmed milk (in water) at room temperature for 2 h, plates were incubated at room temperature for 2 h with serial dilutions (1:4 to 1:65,536) of the serum samples. The plates were then incubated with an anti-rat IgM detection antibody conjugated with HRP at room temperature for 2 h. After gentle washing with buffer, each well received 100 μl OPD substrate and the plate was incubated for 8 min at room temperature. The color reaction was then stopped by addition of 50 μl of 1M sulfuric acid to each well and the absorbance in each was measured at 492 nm with the micro-plate reader. The 4-fold dilution of serum vs. the absorbance was used to fit a 4-parameter curve. A dilution factor (which indicates dilution of serum needed to produce an $\text{OD} = 1.0$) was extrapolated from the equation resulting from the 4-parameter curve using SOFTmaxPRO software (Molecular Devices LLC).

Statistical analysis

The mean and standard deviations (SD) for each parameter were calculated at each timepoint. They were statistically compared between vehicle control and treated groups. First, the parameter was analyzed by a

Bartlett's test (significance level = 1%) to evaluate homogeneity of variance. The parameter was further analyzed by a Dunnett's test when the variance was homogenous and by a Dunnett's rank test when not. The analyses were each performed using a 5% significance level.

Results

Primary and secondary TDAR to KLH in four rat strains

During the course of the study, the primary injection of KLH (300 µg/rat) on Day 5 produced detectable serum amounts of anti-KLH IgM antibodies on Day 9 in the control animals of all strains (Figure 2). However, serum amounts of anti-KLH IgG antibodies were less than those of the IgM type. The second injection of KLH at the same dose on Day 9 led to further increases in anti-KLH IgM antibody production on Day 15 in SD and Wistar rats (1.8- and 2.2-times, respectively, compared to Day 9 levels), but slight decreases in the Fischer and Lewis hosts (0.6-times in both strains). In contrast, anti-KLH IgG productions markedly increased in all strains (ranging from 17–53-times). A large inter-individual variability in anti-KLH antibody responses was observed in the control animals of all strains (Figure 3). The coefficients of variance (CV) in serum anti-KLH IgM levels on Day 9 were 52.6, 56.7, 21.9, and 16.6% in SD, Wistar, Fischer, and Lewis rats, respectively. The CV in serum anti-KLH IgG levels on Day 9 were 54.3, 51.4, 17.7, and 22.5%, respectively. The CV in serum anti-KLH IgM levels on Day 15 were 37.2, 62.0, 29.4, and 17.9%, respectively. Lastly, the CVs in serum anti-KLH IgG levels on Day 15 were 56.5, 49.5, 36.1, and 48.6%, respectively.

A tendency for a wide range of CVs in the primary responses was noted in SD and Wistar rats compared to Fischer and Lewis rats with respect to both anti-KLH IgM and IgG productions on Day 9.

Immunosuppressive effects on primary and secondary TDAR to KLH in four rat strains during 14-day CPA exposure

On Day 9 in all strains, suppressed serum anti-KLH IgM antibody production was noted at 6 mg/kg of CPA; serum anti-KLH IgG antibodies were reduced at 3 and 6 mg/kg (Figure 4). On Day 15 in all strains, CPA at 3 and 6 mg/kg suppressed the anti-KLH IgM antibody production; serum anti-KLH IgG antibodies formation was maximally suppressed at 6 mg/kg of CPA. Although the data shown in Figure 4 seem to suggest to us that the suppressive effects on CPA occur in a dose-related manner, this outcome was not apparent across the strains (no consistent dose-trend relationship in Wistar rats) or as a function of time (see loss of apparent dose-relatedness for IgG from Day 9 to Day 15).

Immunosuppressive effects on primary antibody response to SRBC in four rat strains during 14-day CPA exposure

A single intravenous injection of SRBC (2×10^8 cells/body) on Day 11 resulted in the presence of anti-SRBC IgM-producing cells in the spleen and serum anti-SRBC IgM antibody on Day 15 in the control animals of all strains (Figure 5). CPA at 3 and 6 mg/kg significantly suppressed PFC production and serum anti-SRBC IgM antibody levels at the same time. In

this case, the data more clearly suggested there was a dose-related effect for the CPA on these parameters.

Discussion

In recent years, the utility of the primary response to KLH (i.e., formation of anti-KLH IgM antibody) has been demonstrated to serve as a toxicological end-point to detect any immunosuppressive potential of new drug entities. Additional end-points such as the secondary response (consisting predominantly of IgG) still remain to be addressed. To develop a practical study design incorporating both the primary and secondary responses in a TDAR assay, we characterized anti-KLH IgM and IgG antibody responses following primary and secondary immunizations with KLH (300 µg/rat) during a 14-day course of study in four rat strains. Under these conditions, the primary injection of KLH markedly elevated serum anti-KLH IgM levels 4 days after the injection (in a range from 137–327 µg/ml in all strains); however, the secondary injection caused only slight changes in the anti-KLH IgM responses ($\approx$ 2-times in SD and Wistar rats or $\approx$ 0.6-times in Fischer and Lewis rats, compared with counterpart primary responses). In contrast, remarkable increases in anti-KLH IgG levels (17- to 53-times compared with primary response) were observed 6 days after the secondary KLH injection in all rat strains. In addition, all strains yielded serum levels of $\approx$ 1000 µg/ml of anti-KLH IgG antibodies; these levels were $>$ 4-times those of the anti-KLH IgM antibodies, clearly indicating that IgG predominant reactions were induced by the secondary immunization.

Gore et al. [5] demonstrated that the production of anti-KLH IgM antibody in SD rats reached the maximum level (i.e., 388 $\mu\text{g/ml}$) 5 days after a single intravenous immunization of KLH (300 $\mu\text{g/kg}$), whereas the production of anti-KLH IgG production was slight ($\sim 10 \mu\text{g/ml}$). In that study, anti-KLH IgG antibody production reached the maximum level (230 $\mu\text{g/ml}$) 14 days after the immunization, despite the level still being lower than that for anti-KLH IgM. Consistent with that report, the present study indicated that a single immunization with KLH induced an IgM-predominant response, with the IgG response being induced to a lesser extent. From an immunology point of view, the main antibody involved in a primary response is IgM, which eliminates pathogens in early stages of humoral immunity without sufficient IgG. Generally, IgG antibodies, which are involved predominantly in the secondary immune response, are known to play important roles in complement-mediated activation, opsonization of antigens for phagocytosis, and antibody-dependent cell-mediated cytotoxicity. A presence of specific IgG corresponds to maturation of the antibody responses that are regulated by multiple mechanisms such as Ig class switch and effectors of memory cells. Therefore, incorporating both the primary and secondary responses in a TDAR assay would be crucial to comprehensively evaluate the immunotoxic effect of a new drug.

IgG analysis was demonstrated to show greater sensitivity for the detection of immuno-suppression than IgM analysis in several TDAR studies. An immunosuppressant, FK506, inhibited the production of anti-KLH IgG in KLH-immunized rats with no significant change in the IgM response [6]. The

immunosuppressant cyclosporine also reduced antigen-specific IgG responses more than IgM responses in rats after a single immunization with KLH [5] or dual immunizations with nitrophenyl-chicken γ -globulin [4]. To address this point, we examined the immunosuppressive effects of CPA in the present study. In our study, CPA at ≥ 3 mg/kg suppressed anti-KLH IgM and IgG production in all four rat strains. In three of the test strains, these outcomes appeared to occur in a dose-related manner. Although the mechanisms causing these differences between IgM and IgG responses in the immunosuppressive effects remain unclear, the sensitivity in the IgG suppression of the drug may be associated with its pharmacological properties and dosing duration. FK506 and cyclosporine predominantly suppress T-cell activity, resulting in decreasing cytokine production related to B-cell differentiation and IgG class switch [17]. This specificity of these drugs may provide profiles different from CPA, which simply affects all immune cells as a cytotoxin.

The differences among rat strains have been claimed to be one of the confounders in TDAR assays. In fact, two immunosuppressive compounds, e.g., 2-methoxyethanol and 2-methoxy-acetic acid, have shown different susceptibility in the PFC assay among rat strains [18]. Similar strain differences in anti-KLH IgM responses have been reported to exist in a KLH-ELISA-based TDAR assay using mice and rats [13]. In addition, the inherent inter-individual variability of the antibody responses especially in the outbred strains has been assumed to be affected by variations in analytical methods, i.e., ELISA [12]. Under our sensitizing conditions of KLH

to rats, steady responses of IgM and IgG to KLH were observed in all strains without non-responders, although the primary responses of IgM and IgG in the outbred strains, SD rats and Wister rats, exhibited relatively larger inter-individual variability than those in the inbred strains, Fischer and Lewis rats. Our results indicated that these sensitizing conditions with KLH could practically apply these typical rat strains for induction of the primary and secondary responses enough to detect the immunosuppression by CPA.

The differences in the sensitivity of TDAR methodologies have also long been a topic for discussion [13, 19]. Traditional primary SRBC plaque responses have been reported to be more sensitive to detect the immunosuppressive effects of CPA and cyclosporine A than the primary KLH-ELISA responses in rodents [13]. In the present study, CPA inhibited both KLH and SRBC-induced primary antibody responses at the same dose in either assay system. The data demonstrated that the KLH-ELISA under our experimental conditions has similar capability to detect CPA-induced immunosuppression with regards to the primary IgM response as compared to the primary response in the PFC assay or the SRBC-ELISA. According to a meta-analysis to compare data from several laboratories using SRBC and KLH with different assay formats, both antigens and assay formats showed the same pattern of responses to strong immunotoxicants, indicating that standardizing the choice of an antigen or assay format may not be critical in evaluation of immunotoxicity potential [20].

The duration of a study is another important point for the evaluation of pharmaceutical immunotoxicity. The non-clinical immunotoxicity guideline

for human pharmaceuticals [1] is based on a cause of concern approach using a weight-of-evidence review of various factors, which includes findings in standard toxicity studies. Consequently, in many cases, a 28-day study period could be employed for the TDAR assay, consistent with the conventional general toxicity studies. In the present work, we selected the 14-day study period in order to be able to eventually link outcomes here to those in short-term 14-day general toxicity studies used to support short-term clinical trials for human pharmaceutical candidate drugs (which also last up to 14-days, as per the ICH M3(R2) Guideline[11]). We also believe that our study design with the KLH immunization is applicable to the 28-day repeated dose study design recommended by the ICH S8 Guideline. We feel this is the case because our recent work had also shown that the primary and secondary responses to KLH immunizations on Days 14 and 23 were sufficient to detect immunosuppressive effects of CPA on Days 20 and 29 [21].

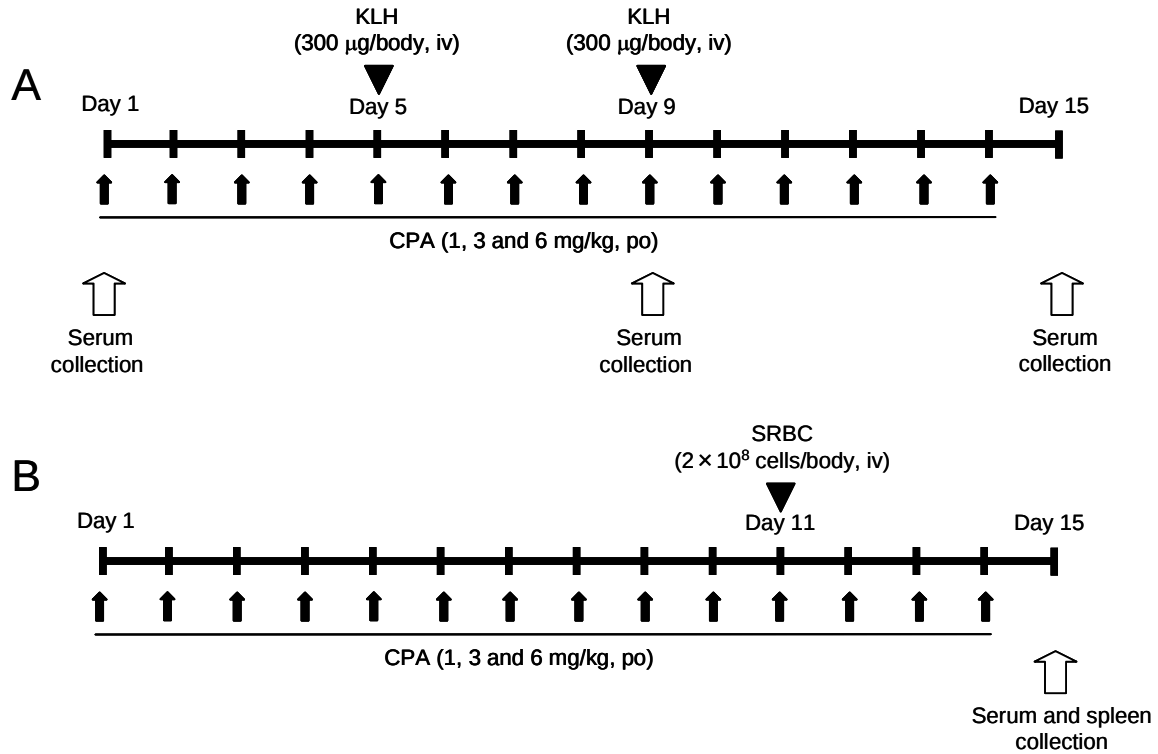


Figure 1. Study designs of TDAR assays.

Cyclophosphamide (CPA) was dosed orally to female rats for 14 consecutive days (0, 1, 3, and 6 mg/kg). (A) Primary and secondary KLH-TDAR assay: Animals were immunized twice by intravenous injection of keyhole limpet hemocyanin (KLH, 300 μ g/rat) on Days 5 and 9 during the 14-day CPA treatment. ELISA was used to determine serum anti-KLH IgM and anti-KLH IgG levels on Days 1, 9, and 15. (B) Traditional SRBC-PFC or SRBC-TDAR assay: Animals were immunized with intravenous injection of SRBC (2×10^8 cells/body) on Day 11 during 14-day CPA treatment. Serum anti-SRBC IgM levels or anti-SRBC IgM producing cell counts were determined on Day 15 by ELISA or PFC assay, respectively.

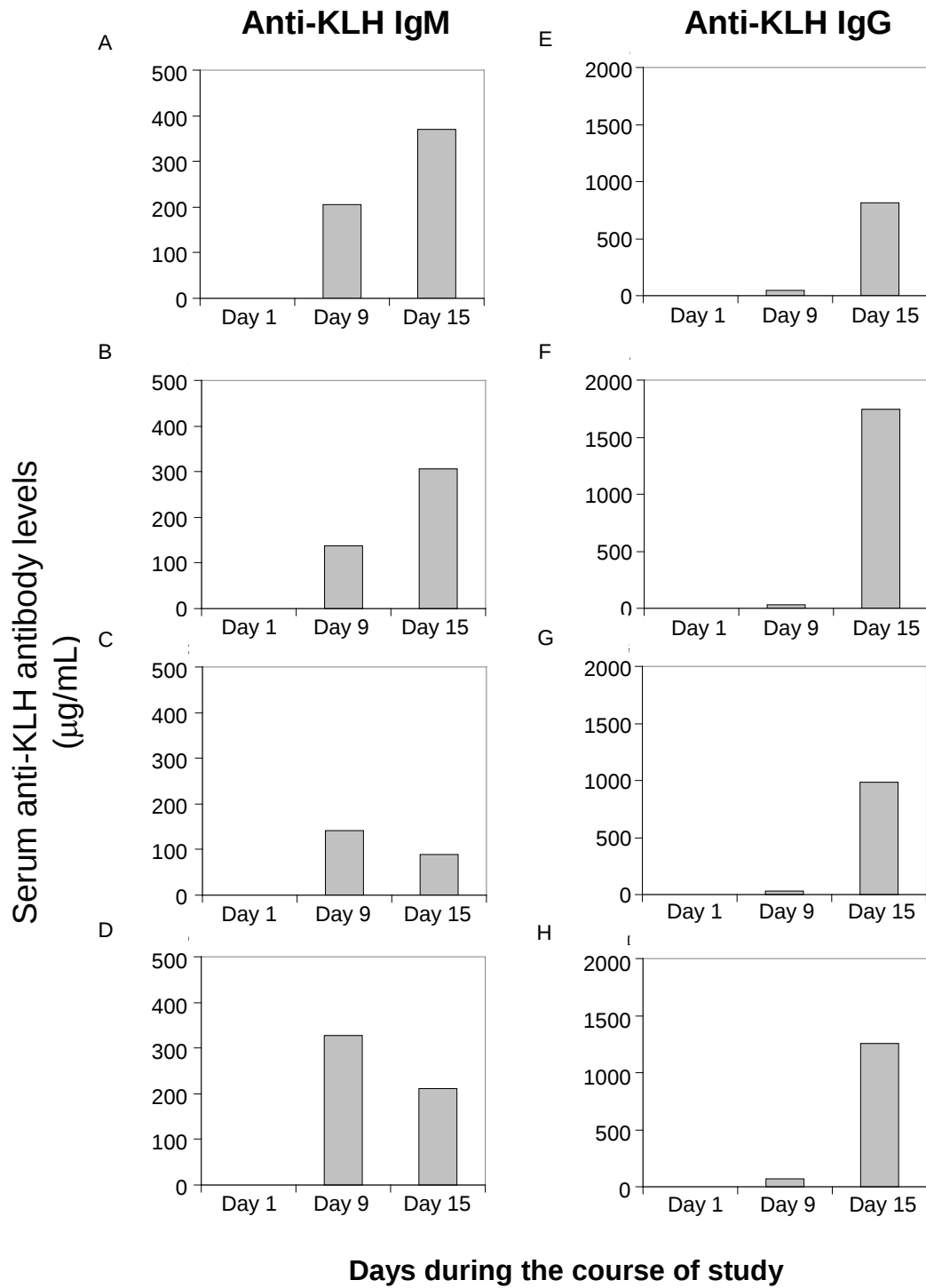


Figure 2. Primary and secondary antibody response to KLH in rats.

Female rats were immunized twice with intravenous injection of KLH (300 µg/rat) on Days 5 and 9 during the course of the study. Left panel: Serum anti-KLH IgM levels, Right panel: Serum anti-KLH IgG levels, (A) and (E): SD rats; (B) and (F): Wistar rats; (C) and (G): Fischer rats; (D) and (H): Lewis rats. Values shown represent the mean of 8 control animals in each strain.

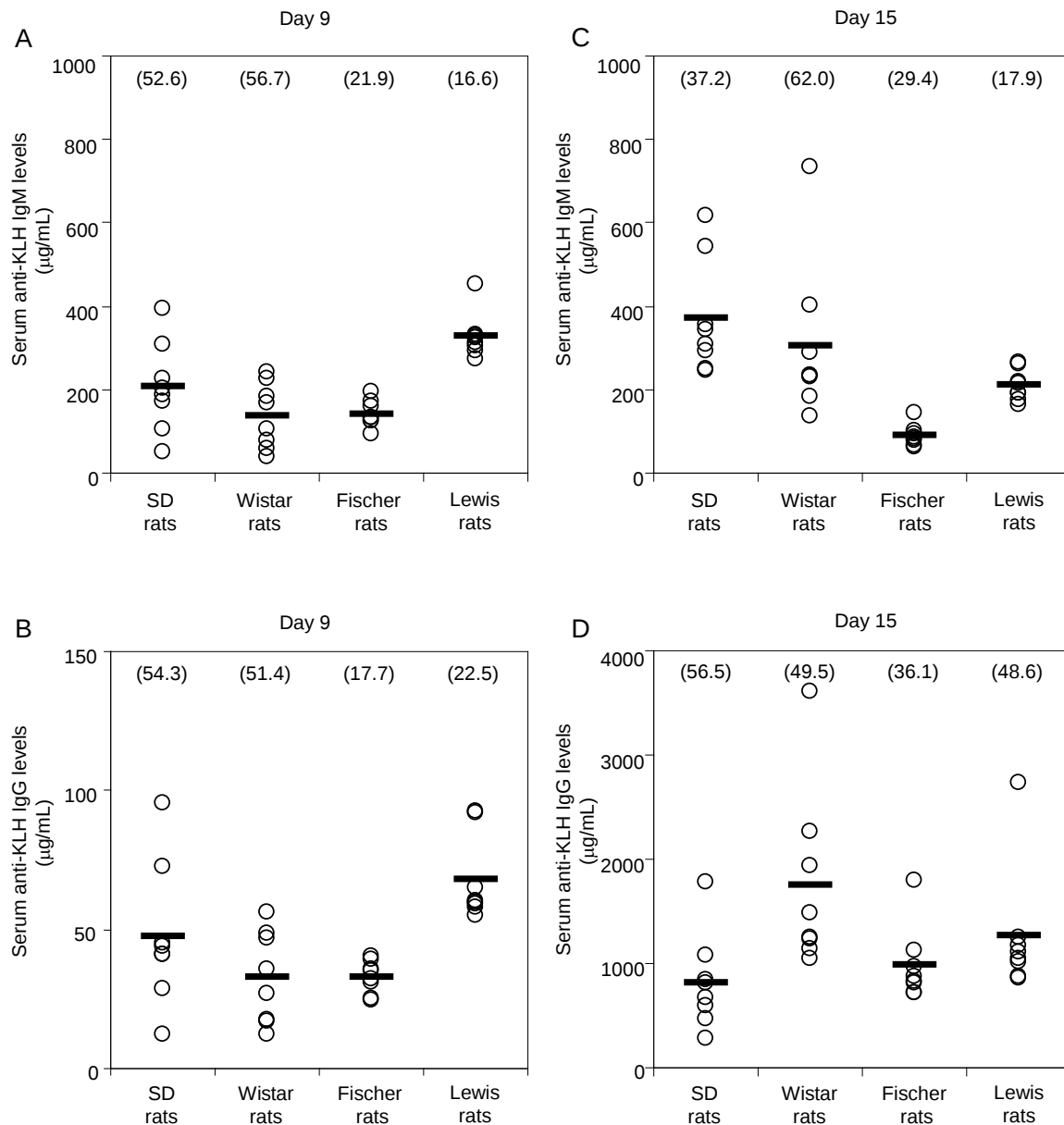


Figure 3. Inter-individual variation of anti-KLH antibody responses in rats.

Female rats were immunized twice with intravenous injection of KLH (300 µg/rat) on Days 5 and 9 during the course of the study. (A) Serum anti-KLH IgM levels on Day 9. (B) Serum anti-KLH IgG levels on Day 9. (C) Serum anti-KLH IgM levels on Day 15. (D) Serum anti-KLH IgG levels on Day 15. Each circle and bar represent the individual value and mean of 8 control animals in each strain. Each value in parentheses means the coefficient of variance.

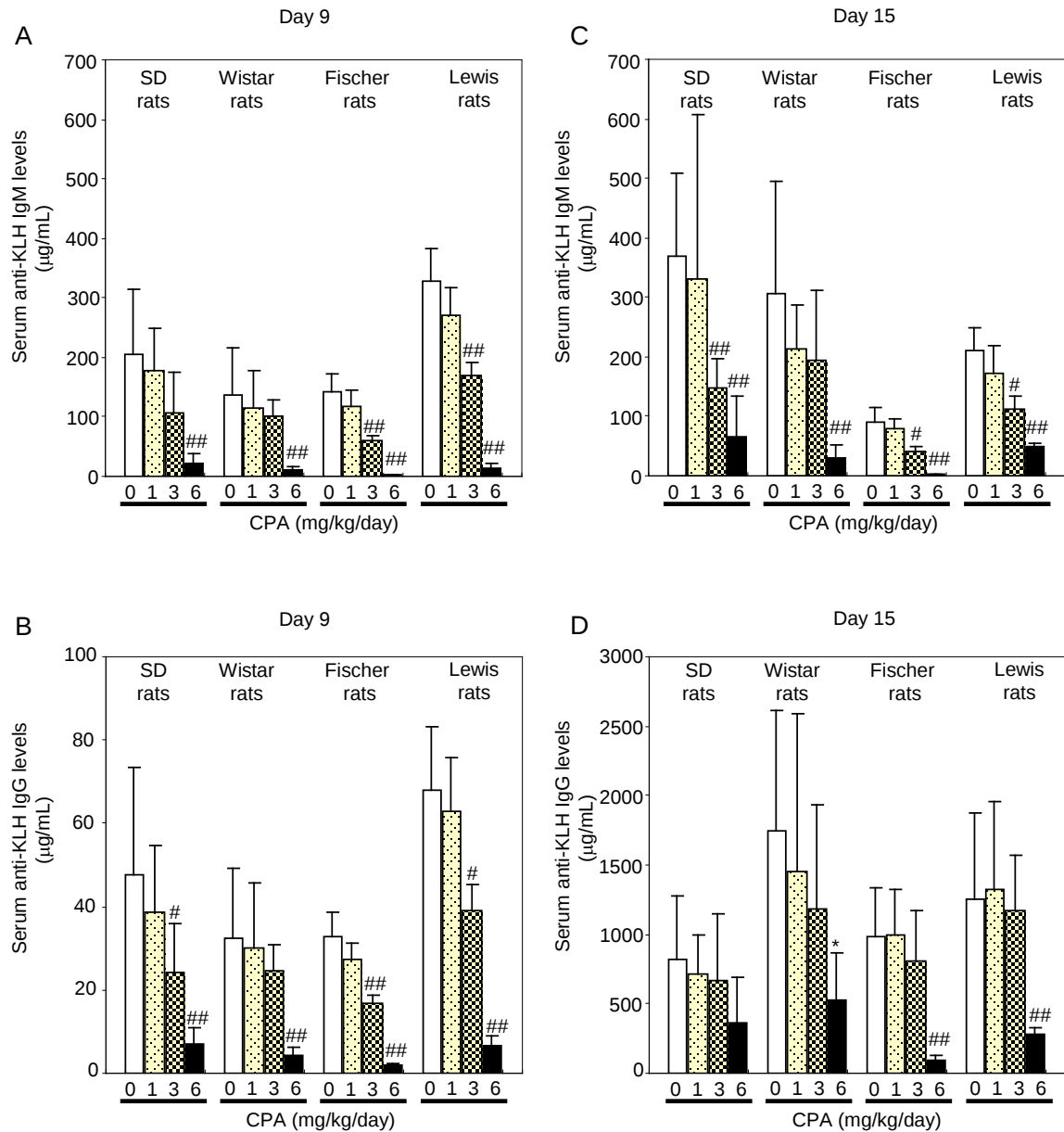


Figure 4. Effect of CPA treatment on anti-KLH antibody responses in rats.

CPA was dosed orally to female rats for 14 consecutive days (0, 1, 3, and 6 mg/kg). Animals were immunized twice with intravenous injection of KLH (300 $\mu\text{g}/\text{rat}$) on Days 5 and 9 during the CPA treatment. (A) Serum anti-KLH IgM levels on Day 9. (B) Serum anti-KLH IgG levels on Day 9. (C) Serum anti-KLH IgM levels on Day 15. (D) Serum anti-KLH IgG levels on Day 15. Each column and bar represents the mean $\pm$ SD of 8 animals. # $p < 0.05$, ## $p < 0.01$: Significantly different from the respective control group in each strain (Dunnett test), * $p < 0.05$: Significantly different from respective control group in each strain (Dunnett rank test).

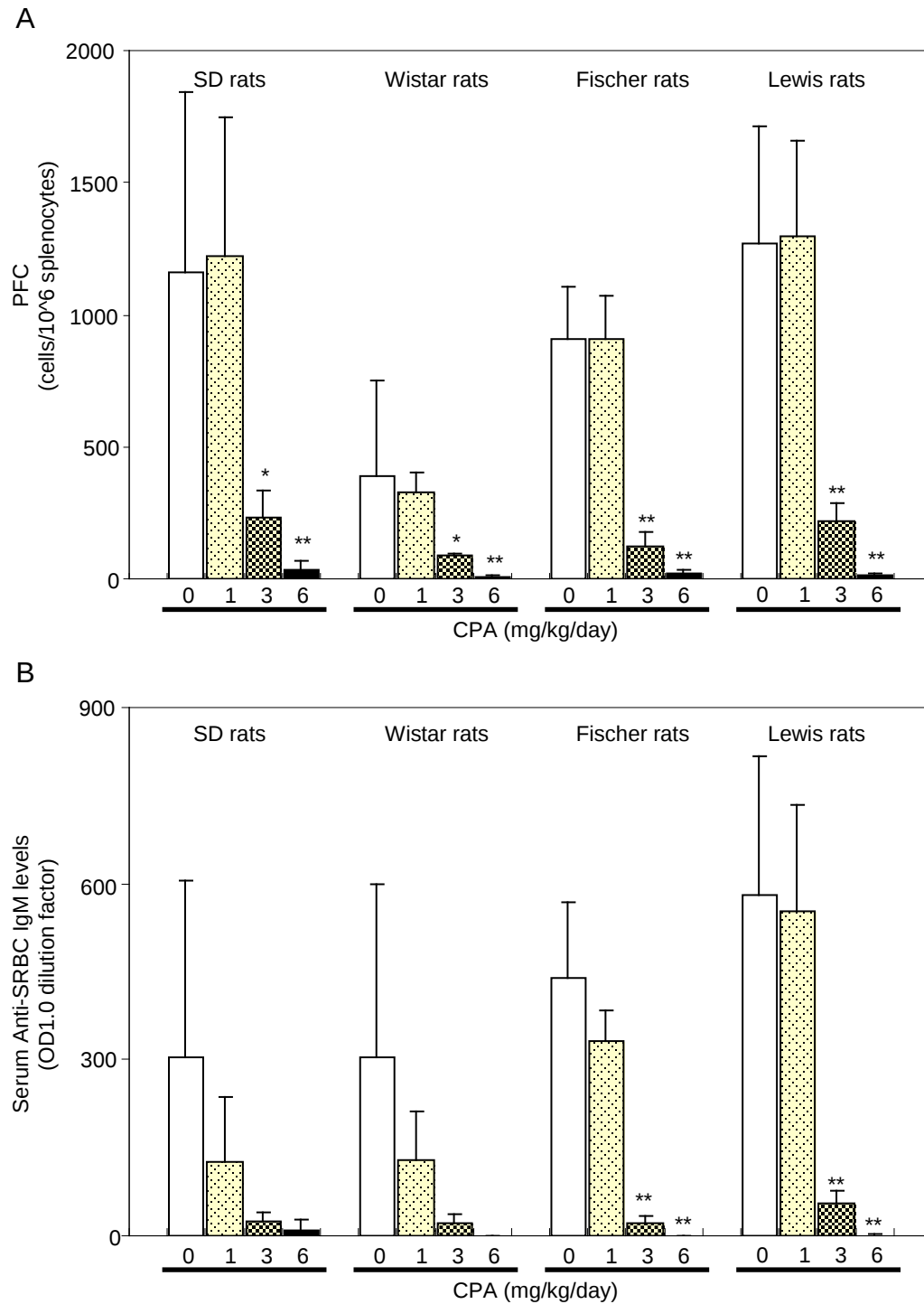


Figure 5. Effect of CPA treatment on plaque-forming cells (PFC) assay and anti-SRBC IgM response by SRBC immunization in rats.

CPA was dosed orally to female rats for 14 consecutive days (0, 1, 3, and 6 mg/kg). Animals were immunized with single intravenous injection of SRBC (2×10^8 cells/rat) on Day 11 during the CPA treatment. (A) PFC on Day 15. (B) Serum anti-SRBC IgM levels on Day 15. Each column and bar represents the mean $\pm$ SD of 4-to-8 animals. ** $p < 0.01$, * $p < 0.05$: Significantly different from respective control group in each strain (Dunnett rank test).

References

- [1] ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use), Immunotoxicity Studies for Human Pharmaceuticals S8. 2006.
- [2] Luster, M.I., et al., Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam Appl Toxicol*, 1992. 18(2): p. 200-10.
- [3] Institoris, L., et al., Immunotoxicological investigation in rats dosed repeatedly with combinations of cypermethrin, As(III), and Hg(II). *Toxicology*, 2002. 172(1): p. 59-67.
- [4] Smith, H.W., et al., A predictive F344 rat immunotoxicology model: cellular parameters combined with humoral response to NP-CgammaG and KLH. *Toxicology*, 2003. 194(1-2): p. 129-45.
- [5] Gore, E.R., et al., Primary antibody response to keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. *Toxicology*, 2004. 197(1): p. 23-35.
- [6] Ulrich, P., et al., Validation of immune function testing during a 4-week oral toxicity study with FK506. *Toxicol Lett*, 2004. 149(1-3): p. 123-31.
- [7] Finco-Kent, D. and T.T. Kawabata, Development and validation of a canine T-cell-dependent antibody response model for immunotoxicity evaluation. *J Immunotoxicol*, 2005. 2(4): p. 197-201.
- [8] Piccotti, J.R., et al., T-cell-dependent antibody response: assay development in cynomolgus monkeys. *J Immunotoxicol*, 2005. 2(4): p. 191-6.
- [9] Stenzel-Poore, M.P. and M.B. Rittenberg, Clonal diversity, somatic mutation, and immune memory to phosphocholine-keyhole limpet hemocyanin. *J Immunol*, 1989. 143(12): p. 4123-33.
- [10] Friedman, E.M., et al., Reduced primary antibody responses in a

- genetic animal model of depression. *Psychosom Med*, 2002. 64(2): p. 267-73.
- [11] ICH, Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3(R2). 2009.
 - [12] Gore, E.R.-P., Immune function tests for hazard identification: a paradigm shift in drug development. *Basic Clin Pharmacol Toxicol*, 2006. 98(4): p. 331-5.
 - [13] White, K.L., Jr., C.M. Sheth, and V.L. Peachee, Comparison of Primary Immune Responses to SRBC and KLH in Rodents. *J Immunotoxicol*, 2007. 4(2): p. 153-8.
 - [14] Dodge, J.T., C. Mitchell, and D.J. Hanahan, The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys*, 1963. 100: p. 119-30.
 - [15] Hanahan, D.J. and J.E. Ekholm, The preparation of red cell ghosts (membranes). *Methods Enzymol*, 1974. 31(Pt A): p. 168-72.
 - [16] Temple, L., et al., Comparison of ELISA and plaque-forming cell assays for measuring the humoral immune response to SRBC in rats and mice treated with benzo[a]pyrene or cyclophosphamide. *Fundam Appl Toxicol*, 1993. 21(4): p. 412-9.
 - [17] Henry, M.L., Cyclosporine and tacrolimus (FK506): a comparison of efficacy and safety profiles. *Clin Transplant*, 1999. 13(3): p. 209-20.
 - [18] Smialowicz, R.J., M.M. Riddle, and W.C.-P. Williams, Species and strain comparisons of immunosuppression by 2-methoxyethanol and 2-methoxyacetic acid. *Int J Immunopharmacol*, 1994. 16(8): p. 695-702.
 - [19] Herzyk, D.J. and E.R.-P. Gore, Adequate immunotoxicity testing in drug development. *Toxicol Lett*, 2004. 149(1-3): p. 115-22.
 - [20] Bugelski, P.J. and C. Kim, T-Dependent Antigen Response (TDAR) Tests: Meta-Analysis of Results Generated Across Multiple Laboratories. *J Immunotoxicol*, 2007. 4(2): p. 159-64.

- [21] Kawai, R., et al., The inter-laboratory KLH-TDAR assay validation study II– investigation using an immunosuppressive agent.
Proceedings of the 17th Annual Meeting of The Japanese Society of Immunotoxicology, 2010: p. 71-6.

Chapter 2

Evaluation of canine T-cell dependent antibody response to the primary and secondary immunization with keyhole limpet hemocyanin

Introduction

Dogs are widely used as a non-rodent species in nonclinical toxicity testing in drug development. If standard toxicity studies indicate immunotoxic potential of a drug, an additional immunotoxicity testing using a relevant species may be required. Although rodent models are generally acceptable for immunotoxicity assessment, canine models can be employed considering consistency with the standard toxicity study in which an adverse immune effect is observed. Because of its size, ease in sampling, and a well-understood physiology with similarities and differences to humans, the dog has become and will continue to be a valuable species in the characterization and prediction of toxicity [1]. In some drug candidates, the utility of canine models for immunotoxicological investigations may be warranted due to pharmacokinetic or drug metabolism similarities with humans [2]. Moreover, the utility of the dog cannot be ruled out due to the increased emergence of new chemical entities targeting signal transduction pathways that impact immune functions [2].

To address drug-related immunotoxicity, immune function testing is more relevant for in-depth assessment [3]. In particular, a T-cell dependent

antibody response (TDAR) assay is recommended as a first choice for an immune function test to evaluate the potential immunotoxicity on new chemicals. The evaluation of the secondary response consisting predominantly of IgG in addition to the primary response is crucial to identify the comprehensive immunotoxic effect of the drug [4]. We have previously developed a rat TDAR model incorporating both the primary and secondary responses to keyhole limpet hemocyanin (KLH) [5]. In addition, the rat study design with the KLH immunization is confirmed to be applicable to the short term (14-day or 28-day, as per the ICH M3(R2) Guideline [6]) repeated dose study design [5, 7]. In dogs, primary TDAR responses (IgM and IgG) to KLH for the immunotoxicity evaluation have been reported [8-11], however, both primary and secondary responses to KLH in canine models for evaluation of drugs have not been fully characterized.

The route of immunization of KLH and kinetics of KLH-specific IgM and IgG are one of the key factors to determine the study design. Several authors have addressed the issue to determine the optimal dose, route of immunization and timing of blood sampling point via the pulmonary route for KLH immunization [12, 13], and intravenous route [14] in the canine models. In addition, Finco-Kent and Kawabata [9] indicated that the antibody response with the intramuscular route was slightly higher than that obtained with the subcutaneous route when exposed to KLH immunization via the intramuscular and subcutaneous routes in dogs. Thus, standardized specifications and protocols are described either poorly or not at all, and the investigators should consider the route of the antigen

depending on its mode of action and the unexpected adverse effects. There is also a need to increase the knowledge and awareness of available and reliable canine assay models identifying immunotoxic potential of the drug as well as a practical study design consistent with the standard toxicity study.

In the present study, we evaluated the kinetics of IgM and IgG responses to the primary and secondary immunization with KLH (10 mg/body) by intravenous or intramuscular route in dogs to determine the timing of blood sampling and TDAR assessment. Since the anti-KLH IgM response with intravenous route was higher than those with intramuscular where no anaphylactic-like response was observed, subsequent experiments were conducted by use of the intravenous route. To confirm the utility of this canine primary and secondary TDAR model, we investigated the effect of cyclophosphamide (CPA) on TDAR to intravenous immunization with KLH during the 28-day treatment, because CPA was shown to exert immunosuppressive effects in dogs [15, 16]. Furthermore, analysis of peripheral lymphocyte subsets and immunoglobulin levels were carried out to confirm the sensitivity of this TDAR model.

Materials and methods

Animals

Male and female beagle dogs (11-months-old) were obtained from Nihon Nosan Kogyo K.K. (Kanagawa, Japan). Dogs were housed individually in stainless steel cages and acclimated to the laboratory environment

(temperature at 22°C; relative humidity of 55%; lighting cycle of 12 h/day) for 1 week before the initiation of the treatment. Commercial canine diet (Certified Canine Diet 5007, PMI Nutrition International, Inc., Saint Paul, MN, USA) and tap water were available *ad libitum*. All animal procedures were performed in accordance with our institutional guide for the care and use of laboratory animals.

Chemicals and antibodies

KLH, bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CPA and Sigma Fast™ *o*-phenylenediamine (OPD) dihydrochloride tablet sets were purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-dog IgM and antidog IgG were supplied as lyophilized products by Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). RPMI medium was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA, USA). OptiLyse C was purchased from Beckman Coulter, Inc. (Brea, CA, USA) FITC-anti-CD3 (Clone: CA17.2A12), PE-anti-CD4 (Clone: YKIX302.9), Alexa Fluor 647-anti-CD8a (Clone: YCATE55.9) and PE-anti-dog B cell (Clone: CA2.1D6) were purchased from AbD Serotec (Oxford, UK). PharMLyse was purchased from BD PharMingen.

Preparation of CPA and KLH solution

CPA was dissolved in distilled water at a concentration of 0.2 mg/ml.

KLH was dissolved in physiological saline at a concentration of 2.0 mg/ml. The solution was filtered using a membrane filter (0.45 μ m, Sartorius Stedim Biotech GmbH, Gottingen, Germany) prior to injection.

Kinetics of anti-KLH IgM and anti-KLH IgG response

To determine the anti-KLH IgM and IgG responses, dogs were immunized following primary and secondary injections of KLH via intravenous or intramuscular route. Four dogs per group (3 males and 1 female) were immunized with 10 mg/body of KLH through either the intravenous or intramuscular route. The dose of KLH was set at 10 mg/body of KLH for both routes based on the report by Haggerty [10]. For the intravenous route, a solution of KLH (5 ml/body) was injected intravenously into the cephalic vein at an injection rate of 5 ml/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA). For the intramuscular route, a solution of KLH (5 ml/body) was injected intramuscularly into the gluteal region. The day of primary injection was designated as Day 1. The preliminary experiment demonstrated that anti-KLH IgM production reached the plateau above higher levels, and anti-KLH IgG started to increase 6 to 8 days after the immunization (Day 7 to 9) following a single intravenous or intramuscular KLH immunization. Therefore, secondary immunization was set at intervals of 8 days after the primary immunization. Blood was collected from the jugular veins on Days 1, 5, 7, 9, 12, 14, 16, 22 and 28 for the analysis of anti-KLH IgM and anti-KLH IgG production. On Days 1 and 9, the blood was collected prior to the KLH immunization to

avoid affecting the outcome in order to perform a TDAR assay.

Treatment protocol

CPA was orally administered (10 ml/kg body weight) to 6 dogs (3 males and 3 females) for 28 consecutive days. The dose level of CPA was set at 2 mg/kg which has previously been shown to induce a significant decrease in humoral immune response to sheep red blood cells [15]. The control group of 6 dogs (3 males and 3 females) was orally administered with the vehicle (distilled water) under the same condition as the CPA-treated group. Based on the kinetics of anti-KLH IgM and anti-KLH IgG responses following an intravenous or intramuscular immunization with KLH, the intravenous route was selected for immunization with KLH because of a higher anti-KLH IgM response. The animals were immunized twice by intravenous injections of KLH (10 mg/body) at an interval of 8 days (on Days 15 and 23) during the CPA treatment. During the course of the study, observations of survival and clinical signs, and measurement of body weight and food consumption were conducted. For the TDAR evaluation, blood was collected from the jugular veins on Days 15, 21, and 28. Serum samples were prepared by centrifugation and then stored at -80°C until analysis. For the evaluation of serum immunoglobulin levels, hematology and immunophenotyping of peripheral lymphocytes, blood samples were also collected on Days -6, 1, 8, 15, 21 and 28. At the termination of the treatment, all animals were euthanized on Day 29 for pathological examination. The study design of TDAR assay is shown in Figure 1.

ELISAs with anti-KLH antibodies

KLH (10 µg/ml) dissolved in coating (50 mM NaHCO₃) buffer was applied to a 96-well microplate (Nalge Nunc Intl., Roskilde, Denmark) and then incubated overnight at 4°C. After blocking with 5% BSA in 0.05% Tween-20 (v/v) in PBS (TPBS) at 37°C for 2 h, plates were incubated at RT for 2 h with serial dilutions (1:5² to 1:5¹³; 12 serial, 5-fold dilutions) of the serum samples in duplicate. After gentle washing to remove non-adherent antibody/other serum factors, the plates were then treated with anti-dog IgM or IgG detection antibody conjugated with HRP at pre-determined optimal dilutions in the diluent and incubated at 37°C for 2 h. After gentle washing with buffer, each well received 100 µl OPD substrate and the plate was incubated for 8 min at RT. The color reaction was then stopped by addition of 50 µl of 1M sulfuric acid to each well and the absorbance in each was measured at 492 nm with a SpectraMax Pro micro-plate reader (Molecular Devices LLC, Sunnyvale, CA, USA). The levels of KLH specific IgM and IgG were expressed as serum dilution factor according to the method by Temple et al. [17]. The five-fold serial dilutions of each serum sample vs. the absorbance was used to fit a 4-parameter curve. A dilution factor (which indicates dilution of serum needed to produce an OD = 1.0 for Anti-KLH IgM; OD = 0.5 for Anti-KLH IgG) was extrapolated from the equation resulting from the 4-parameter curve using SOFTmaxPRO software (Molecular Devices LLC).

Hematology

White blood cells, neutrophil, and lymphocyte counts using EDTA-2K treated blood samples were performed using an ADVIA 120 (Bayer Diagnostics, Switzerland).

Serum immunoglobulin levels

Each class of immunoglobulin (IgA, IgE, IgG and IgM) levels was quantified with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Inc., Montgomery, TX) using a SpectraMax Pro microplate reader (Molecular Devices LLC). Each serum immunoglobulin level was calculated using SOFTmaxPRO software (Molecular Devices LLC).

Immunophenotyping of peripheral and thymic lymphocytes

After lysis of peripheral blood erythrocytes by PharMLyse, the remaining white blood cells were centrifuged at 8000 rpm for 1 min. After washing with wash buffer (3 vol% FBS in PBS), cells were stained with either 3 colors (FITC-anti-CD3, PE-anti-CD4 and Alexa Fluor 647-anti-CD8a), or 2 colors (PE-anti-dog B cell and PECy7-anti-CD16) antibodies. Analyses were performed by a flow cytometer (BD FACSCanto: Becton Dickinson and Company, Ltd.). The ratio of the corresponding positive cells among the total number of lymphocytes were analyzed by FACSDiva software (Becton Dickinson and Company, Ltd.). The ratio of each T cell subset (CD3⁺CD4⁺CD8⁻, CD3⁺CD4⁺CD8⁺, CD3⁺CD4⁻CD8⁻ or CD3⁺CD4⁻CD8⁺) was

calculated by multiplying the ratio of CD3⁺ cells by each ratio of T cells gated by the CD4/CD8 profile. The absolute numbers of each lymphocyte population were calculated by multiplying the relative percentages by the lymphocyte counts obtained in hematology.

The thymus samples were placed in 10 vol% FBSRPMI Medium. A single-cell suspension was prepared by mechanical dissociation. For each sample, 1×10^6 cells were stained with 2 colors (PE-anti-CD4 and Alexa Fluor 647-anti-CD8a) antibodies. The ratio of each T cell subset (CD4⁺CD8⁻, CD4⁺CD8⁺, CD4⁻CD8⁻ or CD4⁻CD8⁺) was analyzed by the CD4/CD8 profile.

Pathological examination

All the animals were systemically anesthetized intravenously into the cephalic vein with 35 mg/kg of pentobarbital sodium (Somnopentyl Injection: Kyoritsu Seiyaku Corporation, Tokyo, Japan), and euthanized by exsanguination from the carotid arteries. After measurement of the organ weight, histopathological specimens for immune organs including spleen, submandibular lymph node, mesenteric lymph node, thymus and bone marrow were microscopically examined.

Statistical analysis

The mean and standard deviations (SD) for each parameter were calculated at each time point. Since sex difference was not observed for the CPA treatment study, the values obtained from both 3 males and 3 females were summed and then statistically compared between the vehicle control

and treated groups. First, the parameter was analyzed by an F test to evaluate the homogeneity of variance. The parameter was further analyzed by a Student's t-test when the variance was homogenous and by an Aspin Welch's t-test when not. The analyses were each performed using a 5% significance level.

Results

Kinetics of primary and secondary anti-KLH IgG and IgM responses by different routes of immunization with KLH

No abnormal clinical signs such as abnormal respiration, excitation, or cyanosis were observed in any dog following intravenous or intramuscular KLH injection. A single intravenous injection of KLH produced a prominent increase in anti-KLH IgM antibody levels in dogs (Figure 2A). Anti-KLH IgM antibody levels peaked on Day 7 (mean $\pm$ SD: 3830 ± 1327) and then slightly decreased on Day 9 (mean $\pm$ SD: 3200 ± 1075). Anti-KLH IgG levels started to increase on Day 7 and apparently increased on Day 9 (Fig. 2B, mean $\pm$ SD: 10764 ± 7927). Secondary intravenous injection of KLH produced a slight increase in anti-KLH IgM antibody levels that peaked on Days 14 to 16 (5 to 7 days after the secondary intravenous immunization). Then, the anti-KLH IgM antibody slightly decreased, however, it remained at steady levels on Day 28 (mean $\pm$ SD: 2994 ± 1030). Anti-KLH IgG antibody drastically elevated 5 to 7 days after the secondary intravenous immunization (Day 14, mean $\pm$ SD: 96771 ± 32037) and then slightly decreased. The KLH IgG antibody levels remained steady on Day 28 (mean $\pm$ SD: 52570 ± 17709).

Intramuscular injection of KLH to dogs also showed similar anti-KLH IgM and IgG kinetics patterns (Figure 2). Namely, anti-KLH IgM antibody levels elevated 6 to 8 days after the primary intramuscular immunization (Day 9, mean $\pm$ SD: 2217 ± 699). Anti-KLH IgG levels also started to increase on Day 7 and apparently increased on Day 9 (Figure 2B, mean $\pm$ SD: 6247 ± 1333). Secondary intramuscular injection of KLH did not affect the anti-KLH IgM antibody levels and remained steady until Day 28 (mean $\pm$ SD: 2223 ± 492). Anti-KLH IgG antibody drastically elevated 5 to 7 days after the secondary intramuscular immunization (Day 14, mean $\pm$ SD: 73142 ± 19942) and then slightly decreased. The KLH IgM antibody levels remained steady on Day 28 (mean $\pm$ SD: 66321 ± 20250). It is noted that there were no large inter-individual differences in anti-KLH IgM and IgG responses for these experiments.

Based on these kinetics profiles, the intravenous route was considered to be more reasonable for the immunization method because of distinct primary and secondary responses and high levels of anti-KLH IgM antibody response.

Immunosuppressive effects of CPA

No toxic changes in clinical signs, body weight or food consumption were noted in any dog given CPA during the course of the study. In hematology, CPA-treatment induced decreased tendency in white blood cells (around -30%, compared with control group) mainly originated from decreases in lymphocytes and neutrophils from Day 8 through Day 28 (Table 1). In addition, CPA diminished both T cells and B cells (at the maximum around

-25%, compared with control group [on Day 21]) from Day 8 to Day 28. On the other hand, CPA did not have a remarkable influence on the ratio of lymphocyte subset ($CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ T lymphocytes or B lymphocytes).

No abnormal fluctuation was noted in serum IgG and IgM levels following CPA treatment or KLH immunization (Table 1), although statistically significant changes were noted from the pre-treatment values in IgM level. Also, no change was noted in serum IgA and IgE levels during the course of the study (data not shown).

In TDAR analysis shown in Figure 3, primary immunization with KLH to dogs in the control group drastically increased anti-KLH IgM and slightly increased anti-KLH IgG levels on Day 21. Secondary immunization with intravenous injection of KLH also increased both anti-KLH IgM and IgG on Day 28. CPA suppressed both anti-KLH IgM and anti-KLH IgG antibody production on Days 21 and 28, and both antibody levels by half of the control group on Day 28. In pathological examination, KLH immunization induced germinal center development in the spleen in all dogs, and CPA treatment induced a decreased size of marginal zone in the spleen with decreased organ weights. In the thymus, the percentage of each subset did not change with the CPA treatment (Table 2).

Discussion

Despite the importance of utilization of dogs as a non-rodent species in the immunotoxicity assessment of drug candidates, only primary response

data have been available on techniques and methods applicable to the canine models so far [2, 9, 11, 18, 19]. To develop a practical study design incorporating both the primary and secondary responses in a TDAR assay in dogs, we evaluated kinetics of anti-KLH IgM and IgG antibody responses following primary and secondary immunizations with KLH (10 mg/dog) by intravenous or intramuscular route.

In the present study, a primary immunization with KLH by both the intravenous and intramuscular routes induced a maximum IgM response on Days 7 to 9 (6 to 8 days after the primary KLH immunization), whereas the IgG response started on Days 7 to 9. Bigwarfe et al. [8] demonstrated that the production of anti-KLH IgM antibody reached the maximum level from 7 to 9 days and kept high levels until 21 days, whereas the anti-KLH IgG levels started to increase from 7 to 9 days, and peaked 21 days, following a single intramuscular immunization of KLH (5 and 10 mg/dog) to beagle dogs. Taking into consideration our results and the report by Bigwarfe et al. [8], a single immunization with KLH induced an IgM-predominant response on Day 9 (8 days after the primary KLH immunization). It also noted that the anti-KLH-IgG antibody value level on Day 9 was assumed to increase enough to evaluate a primary response with the IgG response being induced to a lesser extent. The timing of secondary immunization is an important issue because the secondary immunization was expected to affect the IgM and IgG response induced by the primary immunization. Neither intravenous nor intramuscular immunization affected anti-KLH IgM antibody levels induced by the primary immunization and the IgM levels

remained steady even after the secondary immunization. In contrast, remarkable increases in anti-KLH IgG levels (about 10-times compared with the primary response) were observed 5 to 7 days after the secondary KLH immunization (Days 14 to 16) by both the intramuscular and intravenous routes. Furthermore, the anti-KLH IgG levels peaked earlier than those of single immunization [8], indicating that IgG predominant reactions were induced by the secondary immunization in the present study. When the kinetics of the primary and secondary of anti-KLH IgM and IgG responses by the intravenous or intramuscular route were compared, the intravenous route showed higher baseline titers of primary and secondary anti-KLH IgM responses. Two subunits of KLH1 and KLH2 constructs complex mixture of multi-decamer [20]. After an intravenous immunization, large amounts of the huge complex mixture of KLH systemically circulates and are likely to elicit the reactions of the spleen which is the major site of antibody production. On the other hand, the KLH injected intramuscularly may remain at the injection site and gradually enter the systemic circulation. Generally, the antigen retained in the muscle causes reactions in regional lymph nodes. Kinetics of circulating antigens and lymphoid tissues associated with the immunization route could influence antibody production. Higher primary IgM production induced by intravenous immunization may be related to higher systemic exposure to the KLH resulting in the splenocyte reactions. Similarly, KLH immunization by the intravenous route in rats resulted in a greater anti-KLH IgM production than the subcutaneous or footpad routes [4]. In addition, no immune-mediated

adverse effects were observed in any dog following intravenous injection with KLH unlike sheep erythrocytes which induced an anaphylactic-like response in dogs following intravenous dosing [10]. These results suggest that intravenous immunization of KLH might be more appropriate to induce robust responses. It does not, however, deny that the intramuscular route for immunization could be selected considering the study designs including the dosing route of the test article.

The inherent inter-individual variability of the antibody responses has been pointed out in rodents, especially in outbred rat strains, as practical issues in TDAR assays [21]. However, there was no remarkable inter-individual variability in our canine models. In addition, basal levels of anti-IgM and anti-IgG response following the primary and secondary KLH immunizations were steady without non-responder and were sufficient to detect the immunosuppressive effect of the drug demonstrated by the CPA experiment described below. Therefore, the continuous accumulation of TDAR results in dogs should be necessary to clarify the variability in the canine models.

CPA, alkylating agent which inhibit synthesis of DNA, induces pancytopenia and bone-marrow suppression by cytostatic activity [22]. In the present study, oral administration of CPA at 2 mg/kg for a consecutive 28 days significantly decreased primary and secondary KLH responses. The CPA-treated animals showed a slight decrease in lymphocytes. In addition, terminal pathological examination in dogs given CPA revealed an atrophic change of the spleen in some animals. These findings may be explained by

the cytotoxic action of CPA on immune cells is consistent with other reports [15, 16]. In contrast, CPA at 2 mg/kg did not have an influence on the ratio of blood T and B cells, the ratio of thymic mature and immature T cells, immunoglobulin levels, or morphological changes in the mesenteric lymph nodes or bone marrow compared to the control group. These results indicated that these above examinations could not identify the potential toxicity of CPA on the immune function under the present experimental condition. In some cases, drug-induced alterations in TDAR have been detected in the absence of adverse hematologic and/or histopathological findings indicative of different sensitivity [23, 24]. Furthermore, including IgG analysis for the secondary response beyond the primary response may improve the sensitivity of the TDAR. It is important to note that IgG analysis demonstrated greater sensitivity for the detection of immunosuppressive effects by FK506 and cyclosporine than IgM analysis in several rat TDAR studies [4, 25, 26]. Indeed, we have demonstrated that the secondary IgG response to KLH was the most sensitive indicator to detect cyclosporine induced immunosuppression in our rat TDAR model with two immunizations [7].

The dosing regimen and duration are also important points for the detection of pharmaceutical immunotoxicity. Two previous TDAR studies in dogs have shown different sensitivity for the immunosuppression of CPA at the same dose level. In a study by Putnam et al. [15], repeated oral administration of CPA at 2 mg/kg induced a significant decrease in humoral immune response to sheep red blood cells (SRBC). In contrast, Legrand et al.

[11] has reported that CPA at 2 mg/kg on 4 consecutive days each week for 4 weeks did not affect the primary anti-KLH IgM and IgG responses in dogs. These inconsistent results might be explained by the different study design, in terms of antigens; SRBC as particulate antigens vs. KLH as a soluble antigen, and dosing regimen for CPA as the author pointed out [11]. In contrast, our results would lead to the suggestion that the outcomes of the TDAR studies were influenced by the dosing regimen, in this case, rather than the antigen. Our study design includes 14-day consecutive dosing prior to KLH immunization when conducting a 28-day repeated dose study recommended by the ICH S8 Guideline [3]. Thus, KLH immunization in the latter half of the drug-treatment (e.g. Days 15 and 23) was suggested to be adequate timing for evaluating potential immunotoxicity of drug candidates.

In conclusion, our results and other reports support the view that the dog TDAR model is useful for identifying drugs with a potential for immunotoxicity. Although further examination would be needed to discuss its value for the evaluation of various immunotoxic drugs, the experimental designs stated in this paper could provide valuable information about the influence on both the primary and secondary humoral immune responses in dogs when exposed to potential immunomodulatory drugs.

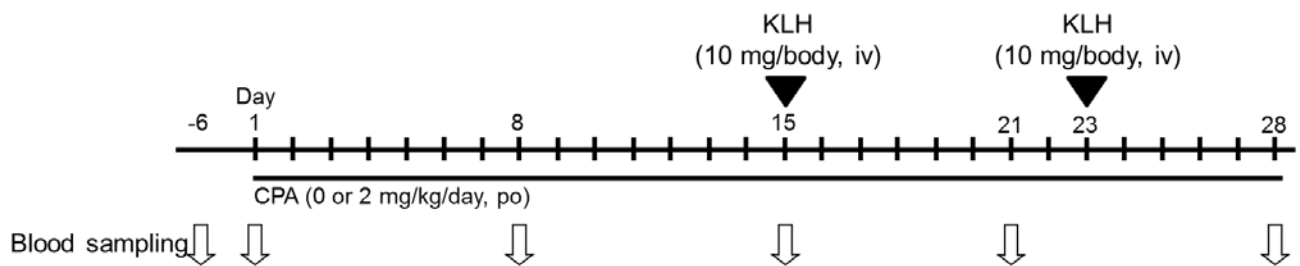


Figure 1. Study design of TDAR assay.

Cyclophosphamide (CPA) was dosed orally to 3 male and 3 female dogs for 28 consecutive days (0 and 2 mg/kg). Animals were immunized twice by intravenous injection of keyhole limpet hemocyanin (KLH, 10 mg/body) on Days 15 and 23 during the 28-day CPA treatment. Blood samples were taken on Days 15, 21, and 28 for analysis of anti-KLH IgM and IgG levels. Blood samples were also collected on Days -6, 1, 8, 15, 21 and 28 for analysis of serum immunoglobulin levels, hematology or immunophenotyping of peripheral lymphocytes. On Day 29, animals were euthanized for pathological examination.

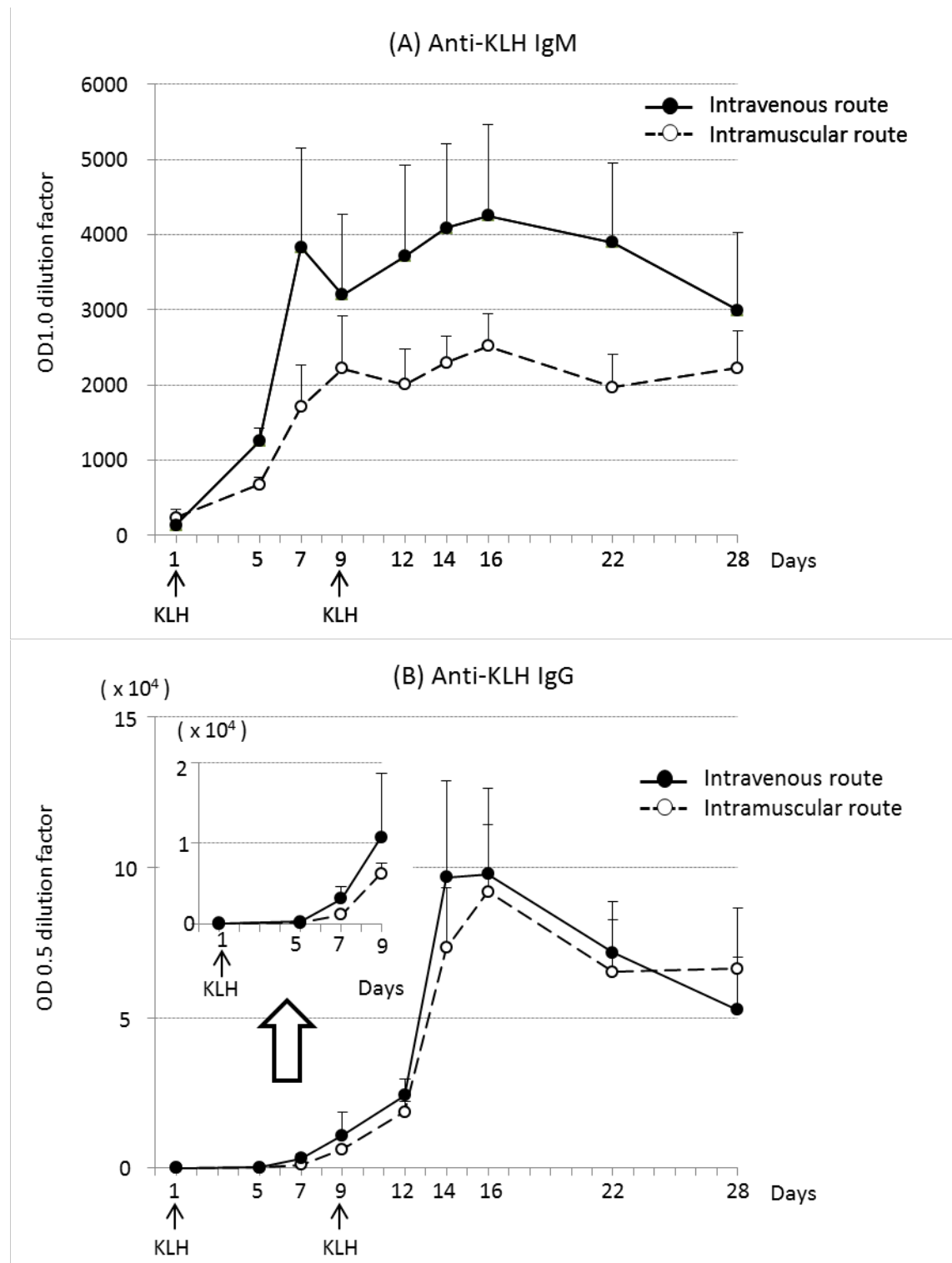


Figure 2. Kinetics of primary and secondary anti-KLH IgG and IgM responses by different routes of immunization with KLH.

Dogs were immunized twice with KLH (10 mg/body) by the intravenous or intramuscular route on Days 1 and 9. The anti-KLH IgM (A) and IgG (B) antibody levels were determined on Days 1, 5, 7, 9, 12, 14, 16, 22 and 28. Each circle and bar represents the mean $\pm$ SD of 4 animals (3 males and 1 female) in each group.

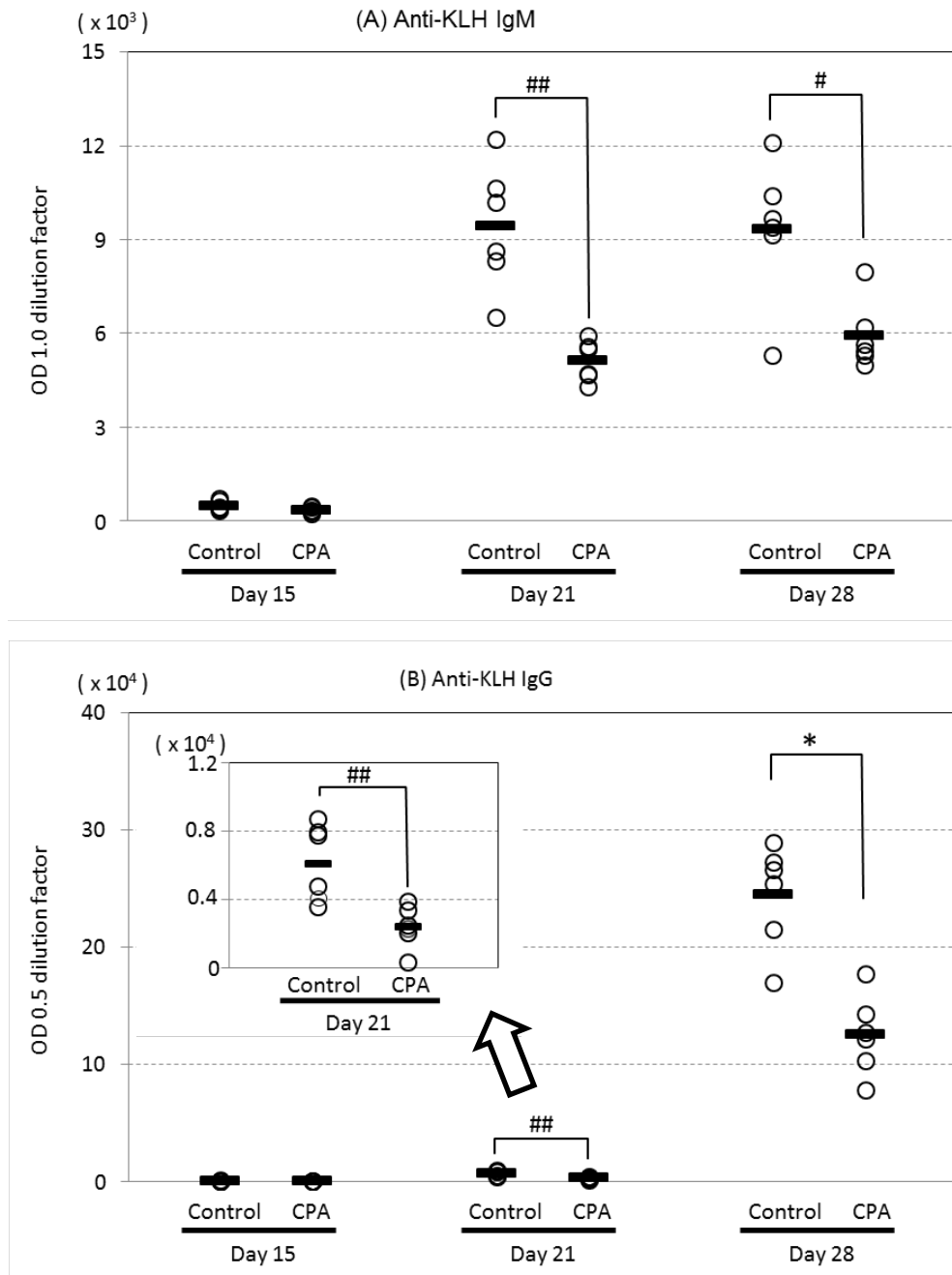


Figure 3. Effect of CPA on anti-KLH antibody responses in dogs.

CPA was dosed orally to dogs for 28 consecutive days (0 and 2 mg/kg). Animals were immunized twice with intravenous injection of KLH (10 mg/body) on Days 15 and 23 during the CPA treatment. (A) Serum anti-KLH IgM levels (B) Serum anti-KLH IgG levels. Each circle and bar represent the individual value and mean of 6 animals (3 males and 3 females). #p < 0.05, ##p < 0.01: Significantly different from the control group (Aspin Welch's t-test). *p < 0.05: Significantly different from the control group (Student's t-test).

Table 1. Effect of CPA on hematology, lymphocyte subset in blood and serum immunoglobulin levels in dogs.

Examination Items	Group	Days after the treatment					
		-6	1	8	15	21	28
Hematology							
White blood cells (x10 ³ /μL)	Control	8.0 ± 1.18	8.6 ± 1.48	8.7 ± 2.47	8.4 ± 2.45	8.9 ± 3.04	9.4 ± 2.61
	CPA	8.5 ± 1.87	8.7 ± 1.31	6.6 ± 1.28	6.0 ± 1.07	5.8 ± 1.58	6.1 ± 1.63 *
Lymphocytes (x10 ³ /μL)	Control	2.7 ± 0.76	3.0 ± 0.98	2.9 ± 1.04	2.7 ± 1.03	2.7 ± 0.75	2.6 ± 0.87
	CPA	3.0 ± 0.59	3.0 ± 0.61	2.3 ± 0.43	2.2 ± 0.41	2.1 ± 0.31	2.3 ± 0.56
Neutrophils (x10 ³ /μL)	Control	4.5 ± 0.45	4.9 ± 0.58	5.0 ± 1.47	5.0 ± 1.62	5.4 ± 2.35	6.0 ± 1.77
	CPA	4.8 ± 1.22	4.8 ± 0.66	3.7 ± 0.72	3.2 ± 0.55 #	3.1 ± 1.18	3.3 ± 0.93 #
Blood Lymphocyte Subset							
Total T cells (x10 ³ /μL)	Control	2.15 ± 0.723	2.58 ± 0.893	2.43 ± 0.796	2.17 ± 0.811	2.24 ± 0.577	2.07 ± 0.724
	CPA	2.50 ± 0.502	2.58 ± 0.519	1.78 ± 0.327	1.62 ± 0.334	1.70 ± 0.266	1.85 ± 0.455
CD3+ (%)	Control	78.2 ± 6.70	84.8 ± 2.85	83.1 ± 2.91	80.5 ± 2.17	82.2 ± 3.33	79.2 ± 2.23
	CPA	83.6 ± 4.90	85.1 ± 1.98	78.4 ± 4.50	73.5 ± 2.93**	80.8 ± 2.20	81.1 ± 1.21
CD3+CD4+CD8- (%)	Control	51.9 ± 7.44	59.2 ± 5.37	58.8 ± 5.73	57.3 ± 4.86	56.8 ± 5.15	54.3 ± 3.20
	CPA	54.8 ± 7.99	53.1 ± 6.81	51.8 ± 7.63	48.3 ± 5.50*	54.6 ± 5.46	54.2 ± 4.76
CD3+CD4-CD8+ (%)	Control	16.4 ± 2.88	18.5 ± 2.17	16.2 ± 1.72	16.1 ± 2.01	16.7 ± 1.46	16.0 ± 1.19
	CPA	20.8 ± 4.41	19.9 ± 4.44	18.9 ± 3.66	18.2 ± 3.57	18.9 ± 3.26	19.3 ± 2.98#
Total B cells (x10 ³ /μL)	Control	0.16 ± 0.070	0.12 ± 0.053	0.29 ± 0.195	0.26 ± 0.134	0.27 ± 0.126	0.28 ± 0.152
	CPA	0.17 ± 0.052	0.19 ± 0.051 **	0.27 ± 0.097	0.25 ± 0.076	0.21 ± 0.029	0.24 ± 0.069
B cell (%)	Control	5.9 ± 1.44	3.8 ± 1.02	8.9 ± 2.85	9.1 ± 1.83	9.4 ± 2.57	10.1 ± 2.27
	CPA	5.6 ± 1.32	6.2 ± 1.06	11.7 ± 2.82	11.1 ± 3.20	9.9 ± 1.46	10.7 ± 2.77
Serum immunoglobulins							
IgM (μg/mL)	Control	2891.6 ± 241.38	2390.4 ± 385.42	2936.3 ± 534.48	2744.3 ± 320.28	2726.8 ± 307.96	2376.1 ± 148.31
	CPA	2100.4 ± 619.68*	1731.4 ± 434.56*	1569.1 ± 225.70**	1980.0 ± 798.56	1968.0 ± 795.45	1939.8 ± 257.69**
IgG (μg/mL)	Control	16905.9 ± 3225.91	13171.4 ± 5168.24	13062.0 ± 5209.94	18233.8 ± 7335.65	9772.3 ± 2766.55	13267.3 ± 3373.45
	CPA	15897.2 ± 4245.17	10752.1 ± 2548.80	10683.5 ± 2607.79	12044.9 ± 3733.83	8922.0 ± 5300.53	13450.4 ± 2867.35
IgA (μg/mL)	Control	2476.6 ± 1423.02	1845.3 ± 994.00	1799.2 ± 696.78	2442.0 ± 1030.38	1775.6 ± 783.55	1423.0 ± 627.10
	CPA	1587.7 ± 1088.60	1275.9 ± 531.37	831.8 ± 322.84*	918.5 ± 395.85**	992.8 ± 417.51	948.7 ± 404.68
IgE (μg/mL)	Control	7.8 ± 5.46	7.6 ± 6.47	7.1 ± 6.33	7.3 ± 6.62	5.9 ± 5.58	5.8 ± 3.97
	CPA	8.6 ± 6.89	7.5 ± 6.71	6.2 ± 5.43	6.2 ± 5.63	5.8 ± 5.40	6.2 ± 5.98

#p < 0.05, ##p < 0.01: Significantly different from the control group (Aspin Welch's t-test).

*p < 0.05, **p < 0.01: Significantly different from the control group (Student's t-test).

Table 2. Effect of CPA on histopathology, organ weight and thymus lymphocyte subset in dogs.

Items	Control	CPA
Histopathology		
Spleen		
Germinal center development	6	6
Decreased size of marginal zone	0	2
Organ weight		
Spleen	31.3 ± 4.08	25.2 ± 2.79#
Thymus Lymphocyte Subset		
CD4+CD8- (%)	8.8 ± 2.12	9.0 ± 1.48
CD4+CD8+ (%)	61.5 ± 14.40	61.3 ± 9.31
CD4-CD8- (%)	20.9 ± 9.95	19.5 ± 4.33
CD4-CD8+ (%)	8.8 ± 3.39	10.2 ± 4.13

#p < 0.05: Significantly different from the control group (Aspin Welch's t-test).

References

- [1] Tibbitts, J., Issues related to the use of canines in toxicologic pathology--issues with pharmacokinetics and metabolism. *Toxicol Pathol*, 2003. 31 Suppl: p. 17-24.
- [2] Lebrech, H., et al., Survey: immune function and immunotoxicity assessment in dogs. *J Immunotoxicol*, 2012. 9(1): p. 1-14.
- [3] ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use), Immunotoxicity Studies for Human Pharmaceuticals S8. 2006.
- [4] Gore, E.R., et al., Primary antibody response to keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. *Toxicology*, 2004. 197(1): p. 23-35.
- [5] Kawai, R., et al., Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats. *J Immunotoxicol*, 2013. 10(1): p. 40-8.
- [6] ICH, Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals M3(R2). 2009.
- [7] Kawai, R., et al., The inter-laboratory KLH-TDAR assay validation study II– investigation using an immunosuppressive agent. *Proceedings of the 17th Annual Meeting of The Japanese Society of Immunotoxicology*, 2010: p. 71-6.
- [8] Bigwarfe, T., et al., T-cell-dependent antibody responses to keyhole limpet hemocyanin (KLH) in pre-clinical immunotoxicity testing. *The Toxicologist/Toxicol. Sci.*, 2004. 78(S1): p. 429.
- [9] Finco-Kent, D. and T.T. Kawabata, Development and validation of a canine T-cell-dependent antibody response model for immunotoxicity evaluation. *J Immunotoxicol*, 2005. 2(4): p. 197-201.
- [10] Haggerty, H.G., Immunotoxicity testing in non-rodent species. *J Immunotoxicol*, 2007. 4(2): p. 165-9.

- [11] Legrand, J.J., et al., Immunotoxic effects of cyclophosphamide and cyclosporine in the dog. *J Immunotoxicol*, 2013. 10(1): p. 90-5.
- [12] Weissman, D.N., et al., Primary immunization in the canine lung. Soluble antigen induces a localized response. *Am Rev Respir Dis*, 1992. 145(1): p. 6-12.
- [13] Jones, S.E., et al., The effects of age on immune responses in the antigen-instilled dog lung. Antibody responses in the lung and lymphoid tissues following primary and secondary antigen instillation. *Mech Ageing Dev*, 1993. 68(1-3): p. 191-207.
- [14] Mates, A. and N.A. Hinton, Immune response in dog. 3. Antibody formation in dogs and rabbits to human serum proteins and keyhole limpet haemocyanin. *Microbios*, 1976. 17(70): p. 175-87.
- [15] Putnam, C.W., et al., Immunosuppression with cyclophosphamide in the dog. *Clin Exp Immunol*, 1975. 22(2): p. 323-9.
- [16] Medleau, L., D.L. Dawe, and C.A. Calvert, Immunosuppressive effects of cyclophosphamide, vincristine, and L-asparaginase in dogs. *Am J Vet Res*, 1983. 44(2): p. 176-80.
- [17] Temple, L., et al., Comparison of ELISA and plaque-forming cell assays for measuring the humoral immune response to SRBC in rats and mice treated with benzo[a]pyrene or cyclophosphamide. *Fundam Appl Toxicol*, 1993. 21(4): p. 412-9.
- [18] Jones, R.D., D.M. Offutt, and Longmoor, Capture ELISA and flow cytometry methods for toxicologic assessment following immunization and cyclophosphamide challenges in beagles. *Toxicol Lett*, 2000. 115(1): p. 33-44.
- [19] Thiem, P.A., L.K. Halper, and J.C. Bloom, Techniques for assessing canine mononuclear phagocyte function as part of an immunotoxicologic evaluation. *Int J Immunopharmacol*, 1988. 10(6): p. 765-71.
- [20] Harris, J.R. and J. Markl, Keyhole limpet hemocyanin (KLH): a biomedical review. *Micron*, 1999. 30(6): p. 597-623.

- [21] Gore, E.R.-P., Immune function tests for hazard identification: a paradigm shift in drug development. *Basic Clin Pharmacol Toxicol*, 2006. 98(4): p. 331-5.
- [22] Kovarsky, J., Clinical pharmacology and toxicology of cyclophosphamide: emphasis on use in rheumatic diseases. *Semin Arthritis Rheum*, 1983. 12(4): p. 359-72.
- [23] Ladics, G.S., et al., Possible incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats on standard toxicology study. *Toxicology*, 1995. 96(3): p. 225-38.
- [24] Ladics, G.S., et al., Further evaluation of the incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats in a standard toxicology study. *Toxicology*, 1998. 126(2): p. 137-52.
- [25] Ulrich, P., et al., Validation of immune function testing during a 4-week oral toxicity study with FK506. *Toxicol Lett*, 2004. 149(1-3): p. 123-31.
- [26] Smith, H.W., et al., A predictive F344 rat immunotoxicology model: cellular parameters combined with humoral response to NP-CgammaG and KLH. *Toxicology*, 2003. 194(1-2): p. 129-45.

Chapter 3

Mechanism of immunosuppressive effects of methotrexate on primary and secondary T-cell dependent antigen response to keyhole limpet hemocyanin in rats

Introduction

A T-cell dependent antigen response (TDAR) assay is recognized as a sensitive indicator of immunotoxicity to comprehensively evaluate the immune function associated with several types of cells including T-cells, B-cells and antigen-presenting cells [1, 2]. However, this assay platform is limited when detecting target cells of the test compound throughout the complicated immune process related to TDAR. Therefore, further investigations for identifying the target cells of the drugs and the underlying mechanism where the drug causes the immune response should clarify the occurrence of potential adverse effects and discuss the countermeasures against these events, if they impact on TDAR.

Methotrexate (MTX) is used worldwide as a first-line drug for the treatment of cancer and rheumatoid arthritis. In contrast, this drug has potential to inhibit cell proliferation and immune function, resulting in the occurrence of several types of adverse effects such as gastrointestinal disorders (i.e. oral ulcer and dyspepsia), hematopoietic disorders (i.e. megaloblastic anemia and cytopenia), and pneumonitis [3]. Despite the

well-known immunosuppressive potential of MTX, the effects of MTX on TDAR and immune function have not been fully evaluated to date.

In the present study, we evaluated the effect of MTX on TDAR to intravenous immunization with keyhole limpet hemocyanin (KLH) in rats during a 14-day intermittent treatment with MTX. Sprague-Dawley (SD) rats were utilized here because the susceptibility of this strain to KLH-TDAR were comparable to those of other strains [4]. Next, the effects of MTX on hematology, immunophenotyping of lymphocytes, and mitogenic response of splenocytes were evaluated in rats. Furthermore, the effects of MTX on macrophage function were evaluated in vitro using peritoneal macrophage to clarify the mechanism of MTX-induced suppressive effect of KLH-TDAR

Materials and methods

Animals

Male SD rats (5-weeks-old) were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Rats were housed individually in stainless steel wire mesh bracket cages for at least 5 days for acclimation to the laboratory environment (temperature at 23°C; relative humidity of 55%; lighting cycle of 12 h/day). Commercial rodent diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were available *ad libitum*. All animal procedures were performed in accordance with our institutional guide for the care and use of laboratory animals.

Chemicals and antibodies

Methotrexate hydrate (MTX), concanavalin A (ConA), phytohaemagglutinin (PHA-L), lipopolysaccharides (LPS) and Sigma Fast™ *o*-phenylenediamine (OPD) dihydrochloride tablet sets were obtained from Sigma (St. Louis, MO). KLH, bovine serum albumin (BSA) and sodium dodecyl sulfate (SDS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Horseradish peroxidase (HRP) conjugated anti-rat IgM and anti-rat IgG were supplied as lyophilized products by Rockland Immunochemicals, Inc., (Gilbertsville, PA), and anti-KLH rat IgM and anti-KLH rat IgG were from BioLegend (San Diego, CA). FITC-anti-CD45 (Clone: OX-1), APC-anti-CD3 (Clone: 1F4), PE-anti-CD4 (Clone: OX-35), PerCP-anti-CD8a (Clone: OX-8), PE-Cy5-anti-CD45RA (Clone: OX-33), PE-anti-NKR-P1A (Clone: 10/78) were obtained from BD Biosciences (San Jose, CA). RPMI medium was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS), and 2-mercaptoethanol, penicillin G sodium, and streptomycin sulfate were obtained from Invitrogen (Carlsbad, CA). OptiLyse C was from Beckman Coulter, Inc. (Brea, CA). PharMLyse was from BD Biosciences (San Jose, CA).

Preparation of MTX suspension and KLH solution

MTX was suspended in 0.5% methylcellulose solution at concentrations of 0.2, 0.4 and 1.0 mg/ml. KLH was dissolved in physiological saline at a concentration of 1.5 mg/ml. The solution was filtered using a membrane filter (0.45 µm, Sartorius Stedim Biotech GmbH, Gottingen, Germany) prior to

injection.

Treatment protocol

MTX was orally administered to rats for a 14-day intermittent repeated dosing (3 times a week, total 6 times) at 5 ml/kg body weight (Figure 1). The dose levels were set at 0 (vehicle control), 1, 2, or 5 mg/kg, based on a preliminary study in which the drug induced immunosuppressive response without any other toxicity findings (data not shown). An intermittent dosing regimen was utilized according to the clinical dose regimen for the treatment of rheumatoid arthritis (0.1 to 0.5 mg/kg/week, Cegiela et al. 2005).

1. Primary and secondary KLH-TDAR assay

The rats were immunized with intravenous injections of KLH (300 µg/rat) twice, i.e., on Days 4 and 8 of the MTX treatment. Blood was collected from the jugular vein under unanesthetized condition on Day 8 and from the abdominal aorta under anesthetized condition on Day 14 in order to perform a TDAR assay (Figure 1). On Day 8, the blood was collected prior to the secondary KLH immunization to avoid affecting the primary TDAR outcome. The animals were euthanized humanely by exsanguination after blood collection under anesthesia. The spleen was removed for use in cell proliferation assay. Serum samples were prepared by centrifugation at 3000 rpm and 4°C for 10 min and then stored at -80°C until analysis.

1.1. ELISAs with anti-KLH antibodies

KLH (10 µg/ml) dissolved in coating (50 mM NaHCO₃) buffer was applied to a 96-well microplate (Nalge Nunc Intl., Roskilde, Denmark) and then incubated at 4°C overnight. After blocking with 5% BSA in 0.05% Tween-20 (v/v) in PBS (TPBS) at 37°C for 2 h, anti-KLH rat IgM or anti-KLH rat IgG in duplicate was added to the plate for standard curves (4-parameter logistic model). The assay range for the IgM and IgG-specific ELISAs was 1.56–100.00 ng/ml and 7.80–500.00 ng/ml, respectively. To evaluate each serum sample within the assay range, each serum sample was diluted ($\geq 1:1000$) with the diluent (1% BSA in TPBS). Each diluted serum sample in duplicate was added to the plate and was equilibrated at 37°C for 2 h. After gentle washing to remove non-adherent antibody and other serum factors, the plates were then treated with anti-rat IgM or IgG detection antibody conjugated with HRP at pre-determined optimal dilutions in the diluent and incubated at 37°C for 2 h. The optical density (OD) after stopping the OPD color reaction with 50 µL 1M sulfuric acid was measured at 492 nm in a SpectraMax Pro micro-plate reader (Molecular Devices LLC, Sunnyvale, CA). Standard curves and anti-KLH Ig levels for each serum sample were calculated using SoftMax Pro software (Molecular Devices LLC).

1.2. Functional analysis of splenocytes

The spleen was gently minced with a glass stick and single-cell suspension of splenocytes was obtained by passage through a cell strainer (70 µm, BD Falcon™, Becton Dickinson and Company, Ltd.). The

suspensions were mixed with 5 mL of 0.2% NaCl and then with 5 mL of 1.6% NaCl to sediment red blood cells. After washing with RPMI1640, the cells were re-suspended in culture medium (RPMI medium containing 10 vol% FBS, 55 μ M 2-mercaptoethanol, 100 units/mL penicillin G sodium and 0.01% streptomycin sulfate). The number of splenocytes in the suspensions was measured with a cell viability analyzer, Vi-CELL XR (Beckman-Coulter Co., Ltd., Fullerton, CA). Aliquots of 100 μ L of cell suspensions (2×10^5 cells) were applied to a 96-well plate and incubated with KLH at concentrations of 0, 50, 100, 200 or 400 μ g/ml for 96 h at 37°C with 5% CO₂.

1.2.1. Cell proliferation assay

After the incubation of the cell suspension of splenocytes (2×10^5 cells) with KLH for 94 h at 37°C with 5% CO₂, 100 M of BrdU at a final concentration of 10 μ M was added to the plate and incubated for a further 2 h. Then, the plate was centrifuged and the supernatants were removed. The plates were then dried and stored in a refrigerator until analysis. Cell proliferation activity was measured as the optical density (OD) at 450 nm using Cell proliferation ELISA, BrdU (colorimetric, Roche Diagnostics GmbH, Basel) and a microplate reader (SpectraMax PLUS: Molecular Devices LLC, Sunnyvale, CA).

1.2.2. Cytokine production

Cytokine levels (IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α) in the supernatants obtained by the above investigations were

measured by a commercially available assay kit Bio-Plex Rat Cytokine 9-Plex Panel (Bio-Rad Laboratories, Inc., Hercules, CA). Fluorescence intensity of the beads was measured using a beads-array reader (Bio-Plex Suspension Array System, Bio-Rad Laboratories, Inc.). Each cytokine level was calculated using Bio-Plex Manager 4.0 software (Bio-Rad Laboratories, Inc.).

2. Mechanism of immunosuppressive effects of MTX

To clarify the mechanism of the MTX-induced suppressive effect on KLH-TDAR, the effects of MTX on hematology, immunophenotyping of lymphocytes and functional analysis of splenocytes were evaluated in rats treated orally with MTX for a 14-day intermittent dosing. Namely, the animals were euthanized humanely by exsanguination the day after 14-day dosing, blood samples and spleen, thymus, mesenteric lymph node and bone marrow were obtained for the following investigations

2.1. Hematology

White blood cells, neutrophil, and lymphocyte counts using EDTA-2K treated blood samples were performed using ADVIA 120 (Bayer Diagnostics, Switzerland) and blood cell autoanalyzer (MICROX HEG-120NA, Omron Corporation, Kyoto).

2.2. Immunophenotyping of peripheral, thymic, splenic and mesenteric lymphocytes

After lysis of peripheral blood erythrocytes by PharMLyse, the remaining white blood cells were obtained by centrifugation at 8000 rpm and room temperature for 1 min. After washing with wash buffer (3% FBS in PBS), the cells were stained with 4 colors (FITC anti-CD45, APC-anti-CD3, PE-anti-CD4 and PerCP-anti-CD8a) or 3 colors (FITC-anti-CD45, PE-Cy5-anti-CD45RA and PE-anti-NKR-P1A) antibodies, and were measured by a flow cytometer (FACSCanto, Becton Dickinson and Company, Ltd.). The ratio of the corresponding positive cells among the total number of lymphocytes was analyzed using FACSDiva v4.0. software (Becton Dickinson and Company, Ltd.). The ratio of each T-cell subset ($CD4^+CD8^-$, $CD4^+CD8^+$, $CD4^-CD8^-$, or $CD4^-CD8^+$) was calculated by multiplying the ratio of $CD3^+$ cells by each percentage of T-cells gated by the CD4/CD8 profile. The absolute number of each cell type was calculated by multiplying the relative cell count by the lymphocyte count.

The thymus, spleen, and mesenteric lymph node samples were placed in 10% FBS-RPMI Medium. Single-cell suspensions were prepared by mechanical dissociation with a glass stick aforementioned. The number of cells of each suspension was counted with an automated blood cell counter (Celltac MEK-5153: Nihon Kohden Corporation, Tokyo, Japan). Cell suspensions of each sample were stained with 4 colors (FITC anti-CD45, APC-anti-CD3, PE-anti-CD4 and PerCP-anti-CD8a) or 3 colors (FITC-anti-CD45, PE-Cy5-anti-CD45RA and PE-anti-NKR-P1A) antibodies

in the same manner as described for peripheral blood. The ratio of each T-cell subset (CD4⁺CD8⁻, CD4⁺CD8⁺, CD4⁻CD8⁻, and CD4⁻CD8⁺) was calculated by multiplying the percentage of CD3⁺ cells by each percentage of T-cells gated by the CD4/CD8 profile. The absolute number of each cell type was calculated by multiplying the relative cell count by each cell count. Analysis of B and NK cells were not conducted for thymocytes.

2.3. Functional analysis of splenocytes

Single-cell suspension of splenocytes was obtained in the same manner as described in Section 1.2. The number of splenocytes in the suspensions was measured with a cell viability analyzer, Vi-CELL XR (Beckman-Coulter Co., Ltd.). Aliquots of 100 µl of the cell suspensions (2×10⁵ cells) were applied to a 96-well plate incubated with ConA (10 µg/ml) for 72 h, PHA-L (25 µg/ml) for 72 h or LPS (10 µg/ml) for 96 h at 37°C with 5% CO₂.

2.3.1. Cell proliferation assay

Cell proliferation assay of splenocytes was conducted in the same manner as described in Section 1.2.1.

2.3.2. Cytokine production

Cytokine levels in the supernatants obtained by the above investigations were measured in the same manner as described in Section 1.2.2.

2.4. Serum immunoglobulin levels

Each class of immunoglobulin (IgA, IgE, IgG and IgM) levels was quantified with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Inc., Montgomery, TX) using a SpectraMax Pro microplate reader (Molecular Devices LLC, Sunnyvale, CA). Each serum immunoglobulin level was calculated using SOFTmaxPRO software (Molecular Devices LLC).

2.5. Pathological examination

After measurement of the organ weight, histopathological specimens for immune organs including the spleen, submandibular lymph node, mesenteric lymph node, thymus and bone marrow were microscopically examined.

3. Effect of MTX on peritoneal macrophage function

3.1. Preparation of peritoneal macrophages and treatment

Peritoneal macrophages were obtained by direct lavage with thioglycolate as previously reported [5, 6] with some modifications. In brief, the suspensions were mixed with 5 mL of 0.2% NaCl and then with 5 mL of 1.6% NaCl to sediment red blood cells. After washing with RPMI1640, the cells were re-suspended in culture medium (RPMI medium containing 10 vol% FBS). Aliquots of 100 μ L of cell suspensions (2×10^5 cells) were applied to a 96-well plate and incubated with MTX at concentrations of 0, 0.1, 1, 10 or 100 μ M for 24 h at 37°C with 5% CO₂.

3.2. Phagocytosis

Peritoneal macrophages (2×10^5 cells) were incubated with MTX (0, 0.1, 1, 10 or 100 μ M) for 24 h and were further incubated with Fluoresbrite plain YG 0.2 micron microspheres (Polyscience, Inc., Warrington, PA). Phagocytic activity of macrophages was evaluated using a flow cytometer (FACSCanto, Becton Dickinson and Company, Ltd.). The ratio of the beads containing macrophages was analyzed using FACSDiva v4.0. software (Becton Dickinson and Company, Ltd.).

3.3. Cytokine production

Peritoneal macrophage (2×10^5 cells) were incubated with MTX at 0, 0.1, 1, 10 or 100 μ M for 24 h, and then further incubated with LPS at 2 μ g/ml for 48 h. Cytokine levels in the supernatants were measured in the same manner as described in Section 1.2.2.

3.4. NO production

NO-producing ability in the supernatants obtained in Section 3.3 were evaluated using a NO₂/NO₃ Assay Kit-C II (colorimetric)-Griess Reagent Kit (Dojindo, Kumamoto, Japan).

Statistical analysis

The mean and standard deviations (SD) for each parameter were calculated at each time point. They were statistically compared between the corresponding control and treated groups. First, the parameter was analyzed

by a Bartlett's test (significance level = 1%) to evaluate homogeneity of variance. The parameter was further analyzed by a Dunnett's test when the variance was homogenous and by a Dunnett's rank test when not. The analyses were each performed using a 5% significance level.

Results

1. Immunosuppressive effects of MTX on primary and secondary TDAR to KLH

MTX at ≥ 1 mg/kg prominently suppressed serum anti-KLH IgM (about 70% decrease) and serum anti-KLH IgG antibodies (about 50% decrease) both on Days 8 and 14 (Figure 2).

1.2. Functional analysis of splenocytes

MTX at ≥ 1 mg/kg dose-dependently decreased cell proliferation of splenocytes stimulated by KLH. Statistically significant reduction of cell proliferation to KLH (400 μ g/mL) was observed at ≥ 2 mg/kg of MTX (Figure 3). MTX did not influence cytokine production from splenocytes stimulated with KLH (data not shown).

2. Mechanism of immunosuppressive effect of MTX

2.1. Hematology

MTX at 5 mg/kg decreased white blood cells and lymphocytes (Table 1).

2.2. Immunophenotyping of peripheral, thymic, splenic and mesenteric

lymphocytes

MTX at ≥ 1 mg/kg showed decreases in peripheral T cells. The decrease at 5 mg/kg was statistically significant. Similar findings were noted in thymic and splenic lymphocytes although MTX tended to preferentially decrease thymic T cells (approximately 80% decrease) compared to peripheral and splenic T cells. No changes were observed in mesenteric lymphocytes (Table 2).

2.3. Functional analysis of splenocytes

MTX did not influence cell proliferation of splenocytes stimulated by any of mitogens (data not shown). On the other hand, MTX dose-dependently reduced IL-1 α , IL-1 β , IL-6, IFN- γ , and TNF- α production from splenocytes stimulated by LPS but not ConA or PHA-L. Additionally, MTX at 5 mg/kg significantly increased IL-10 production from splenocytes stimulated by LPS (Figure 4).

2.4. Serum immunoglobulin levels

MTX at ≥ 1 mg/kg significant decreased serum IgM levels. In addition, MTX at 5 mg/kg significantly decreased IgA levels (Table 2).

2.5. Pathological examination

MTX at ≥ 2 mg/kg produced decreased cellularity and increased megakaryocytes in the bone marrow. In addition, MTX at 5 mg/kg reduced lymphoid in the cortex of the thymus with decreased organ weight (about

50% decrease). No treatment-related findings were noted in other organs (Table 3).

3. Effect of MTX on peritoneal macrophage function

MTX did not impact on phagocytic ability of peritoneal macrophages. In contrast, MTX at 100 μ M significantly decreased IL-1 α , IL-1 β , IL-6, IFN- γ and TNF- α production from peritoneal macrophages stimulated by LPS. Furthermore, MTX at 100 μ M increased IL-10 production from peritoneal macrophages (Figure 5). MTX did not influence NO production from peritoneal macrophages (data not shown).

Discussion

A TDAR assay was suggested to evaluate both IgM-predominant primary and IgG-predominant secondary responses, and the influence on antigen-specific IgM and IgG antibody should be different related to the pharmacological properties of test compounds [4]. An immunosuppressant, FK506, inhibited the production of anti-KLH IgG in KLH-immunized rats with no significant change in the IgM response [7]. The immunosuppressant cyclosporine also reduced antigen-specific IgG responses more than IgM responses in rats after a single immunization with KLH [8] or dual immunizations with nitrophenyl-chicken γ -globulin [9]. These FK506 and cyclosporine predominantly suppress T-cell activity, resulting in decreasing cytokine production related to B-cell differentiation and IgG class switch [10]. Generally, IgG antibodies, which are involved predominantly in the

secondary immune response, are known to play important roles in complement-mediated activation, opsonization of antigens for phagocytosis, and antibody-dependent cell-mediated cytotoxicity. On the other hand, cyclophosphamide, which simply affects all immune cells as a cytotoxin, suppressed the anti-KLH IgM and IgG production was at a similar level, although the antibody specificity was not observed [4]. In the present study, MTX dose-dependently suppressed both anti-KLH IgM and IgG production on Days 8 and 14 to a similar extent. It has been reported that MTX decreased T-lymphocytes in rats [11]. In line with this report, MTX decreased peripheral, thymic and splenic T-cells at the same dose which the drug suppressed KLH-TDAR. In addition, MTX suppressed splenocyte proliferation to KLH and reduced inflammatory cytokine production including IL-1 α , IL-1 β , IL-6, IFN- γ , and TNF- α and increased anti-inflammatory cytokine IL-10 production from splenocytes stimulated by LPS. In contrast, MTX did not influence the number of B cells or NK cells even at the highest dose. Taken together, MTX inhibited KLH-TDAR through decreased T-cells and suppression of T-cell functions like proliferation activity and cytokine production.

It is well known that several types of immune cells like macrophages along with T cells and B cells are involved in TDAR [12, 13]. In fact, the outcome of TDAR assay is suggested to correlate with that of the host resistance model to infection [12, 13]. However, it is difficult to identify target cells of the drug in TDAR composed of a complicated immune process from recognition of the antigen to specific antibody production. In contrast, MTX

was reported to inhibit IL-1 or PGE₂ production from macrophages [14, 15]. Additionally, MTX inhibited angiogenesis [16] or peripheral mononuclear cell proliferation [17], and anti-inflammatory effects associated with macrophages [18]. Therefore, the effects of MTX on macrophage functions were also evaluated in the present study. As a result, MTX at 100 μ M declined inflammatory cytokine production including IL-1 α , IL-1 β , IL-6, and IFN- γ , and promoted anti-inflammatory cytokine IL-10 production from peritoneal macrophages stimulated by LPS. However, MTX did not impact on phagocytic ability of peritoneal macrophages or NO-production from peritoneal macrophages stimulated by LPS. These results suggested that MTX did not influence antigen-presenting capacity of macrophages, but the drug decreased cytokine production from them. It has been reported that MTX inhibited the development of adjuvant-induced arthritis and LPS-induced NO production from peritoneal macrophages [19]. In contrast, consistent with our results, MTX did not affect LPS-induced NO production from peritoneal macrophages of naive or adjuvant-induced arthritis rats [19].

The present results suggested that MTX inhibited KLH-TDAR through the decreased T-cells and suppression of T-cell functions including cell proliferation and cytokine production. Furthermore, decreased macrophage function due to MTX could be at least in part involved in MTX-induced KLH-TDAR suppression. We believe the present work incorporating TDAR assay and follow-up testings could provide valuable information for practical and appropriate approaches for evaluating of 'true' immunotoxicity potentials of new chemicals from nonclinical perspective.

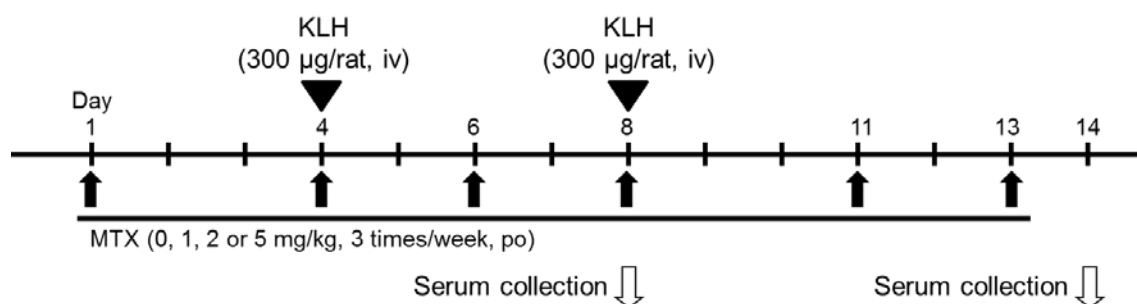


Figure 1. Study design of TDAR assay.

Methotrexate (MTX) was dosed orally to male rats by a 14-day intermittent repeated dosing (0, 1, 2, or 5 mg/kg; 3 times a week, total 6 times: Days 1, 4, 6, 8, 11 and 13). Primary and secondary KLH-TDAR assays: Animals were immunized twice by intravenous injection of keyhole limpet hemocyanin (KLH, 300 µg/rat) on Days 4 and 8 during the MTX treatment. ELISA was used to determine serum anti-KLH IgM and anti-KLH IgG levels on Days 8 and 14.

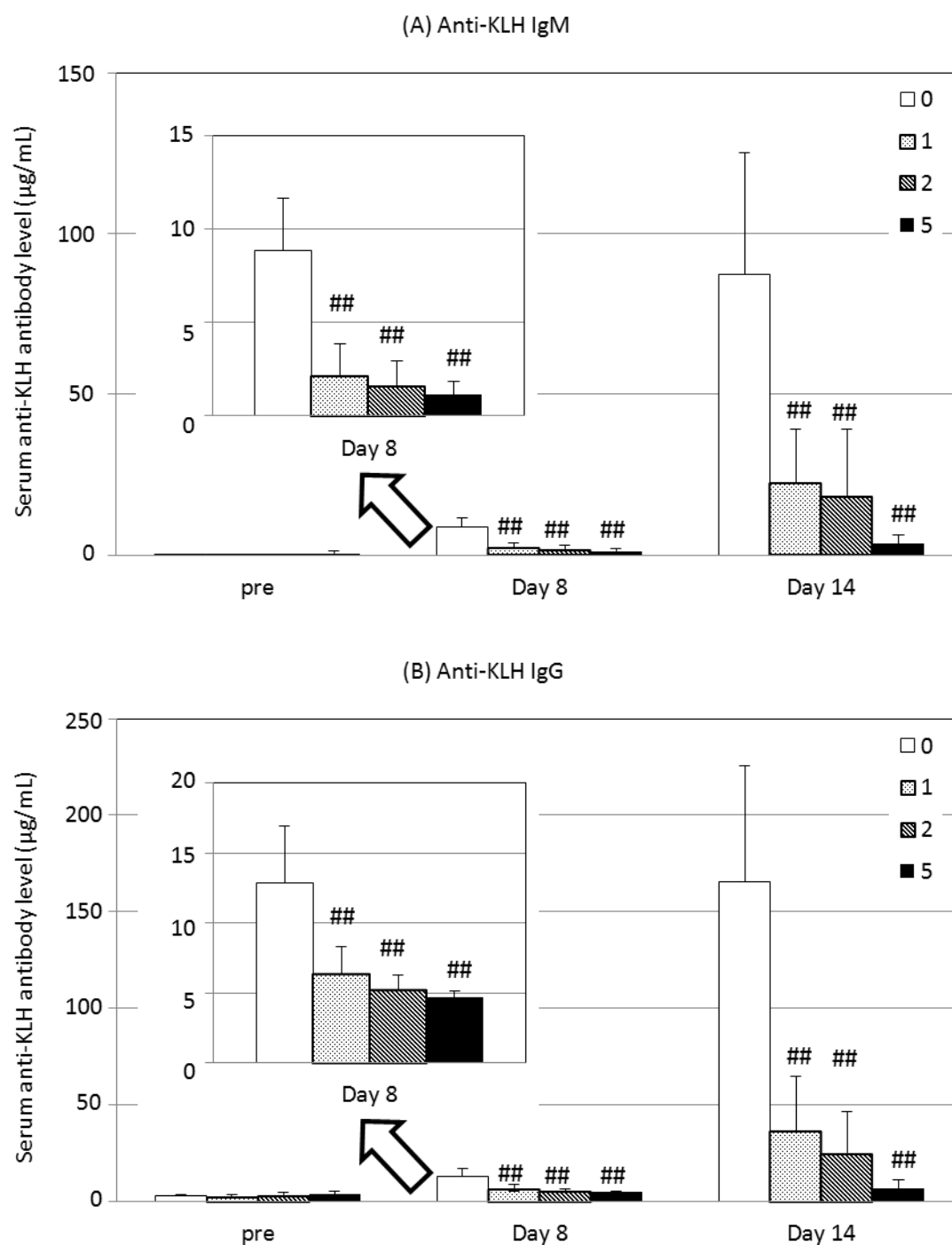


Figure 2. Effect of MTX on anti-KLH antibody responses in rats.

MTX was dosed orally to male rats by a 14-day intermittent repeated dosing (0, 1, 2, and 5 mg/kg, 3 times a week, total 6 times). Animals were immunized twice with intravenous injection of KLH (300 µg/rat) on Days 4 and 8 during the MTX treatment. (A) Serum anti-KLH IgM levels. (B) Serum anti-KLH IgG levels. Each column and bar represents the mean $\pm$ SD of 5 animals. ## $p < 0.01$: Significantly different from the control group (Dunnett test).

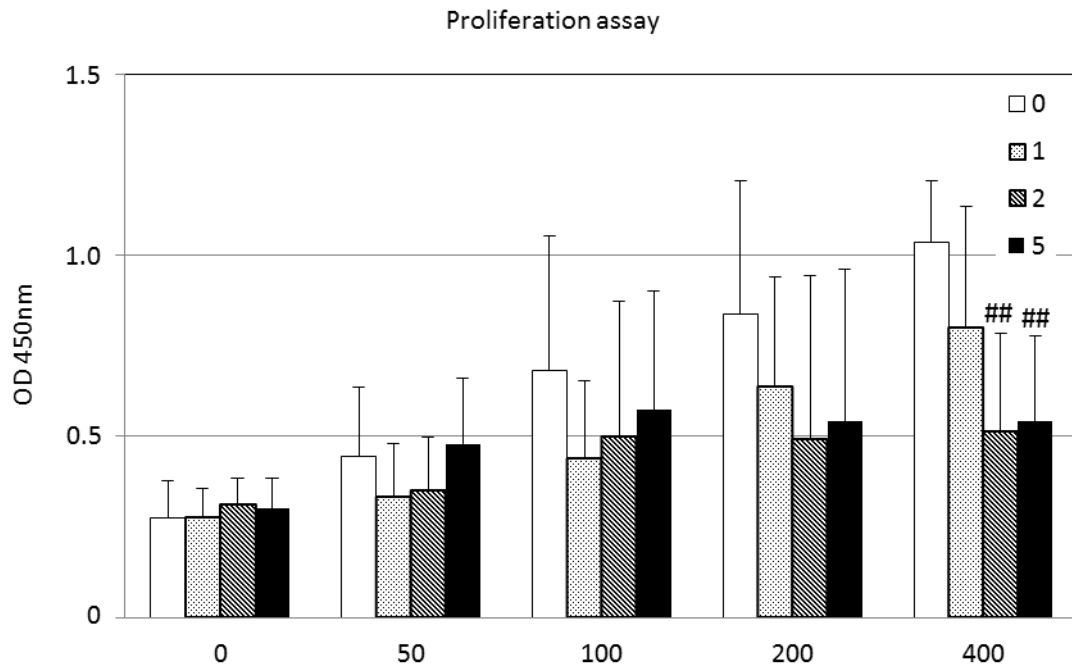


Figure 3. Effect of MTX on cell proliferation of splenocytes stimulated by KLH. Splenocytes (2×10^5 cells) obtained from KLH-immunized rats treated with MTX were incubated with KLH (0, 50, 100, 200 or 400 $\mu\text{g/mL}$) for 96 h. Cell proliferation activity was measured with BrdU incorporation assay. Each column and bar represents the mean $\pm$ SD of 5 animals. ## $p < 0.01$: Significantly different from control group (Dunnett test).

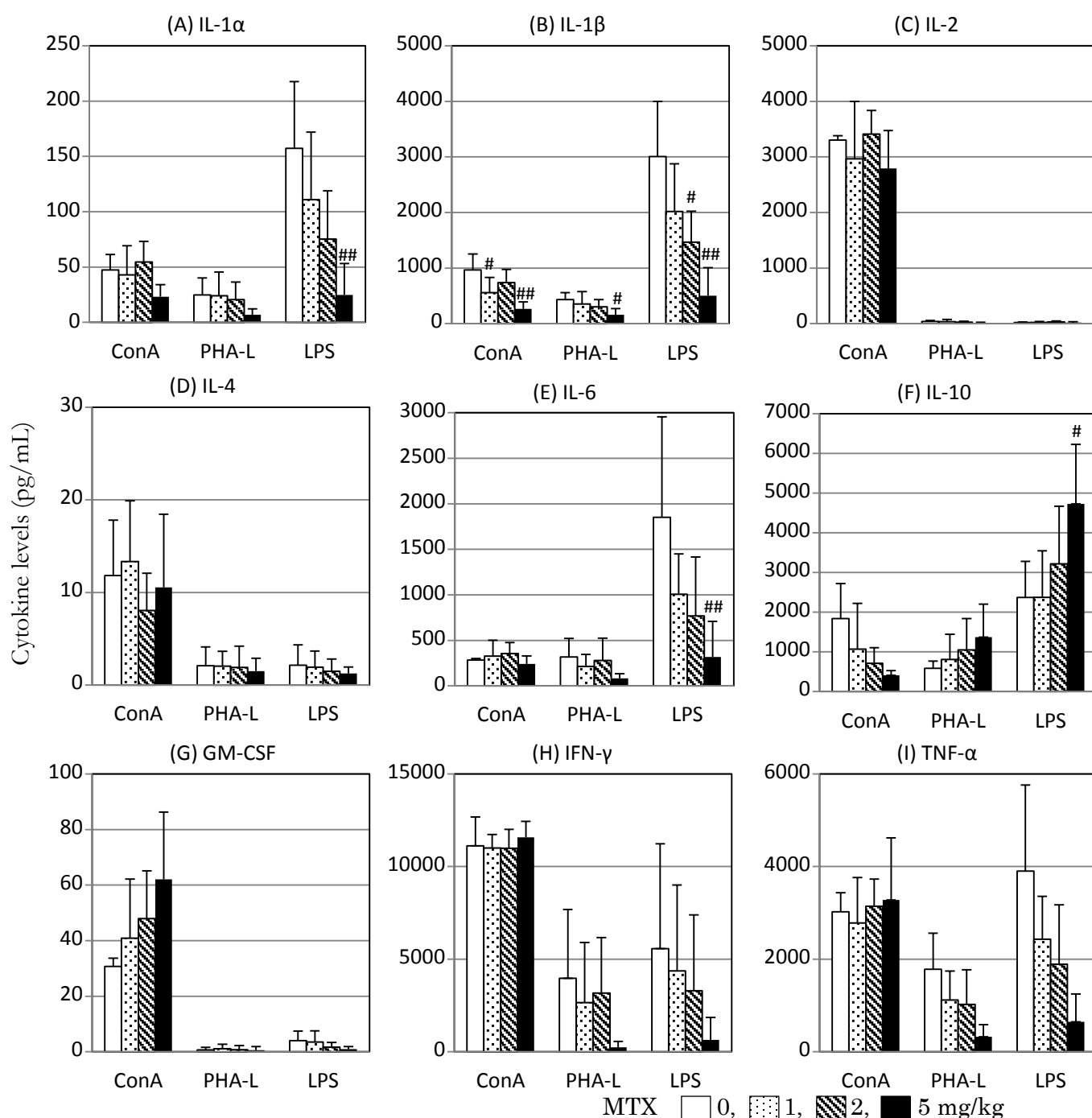


Figure 4. Effect of MTX on cytokine production from splenocytes stimulated by various mitogens.

Splenocytes (2×10^5 cells) obtained from rats treated with a 14-day intermittent repeated dose of MTX (0, 1, 2, or 5 mg/kg) were incubated with concanavalin A (ConA, 10 μ g/mL) for 72 h, phytohaemagglutinin (PHA-L, 25 μ g/mL) for 72 h, or lipopolysaccharide (LPS, 10 μ g/mL) for 96 h. (A)IL-1 α , (B)IL-1 β , (C)IL-2, (D)IL-4, (E)IL-6, (F)IL-10, (G)GM-CSF, (H)IFN- γ and (I)TNF- α . Each column and bar represents the mean $\pm$ SD of 5 animals. # $p < 0.05$, ## $p < 0.01$: Significantly different from control group (Dunnett test).

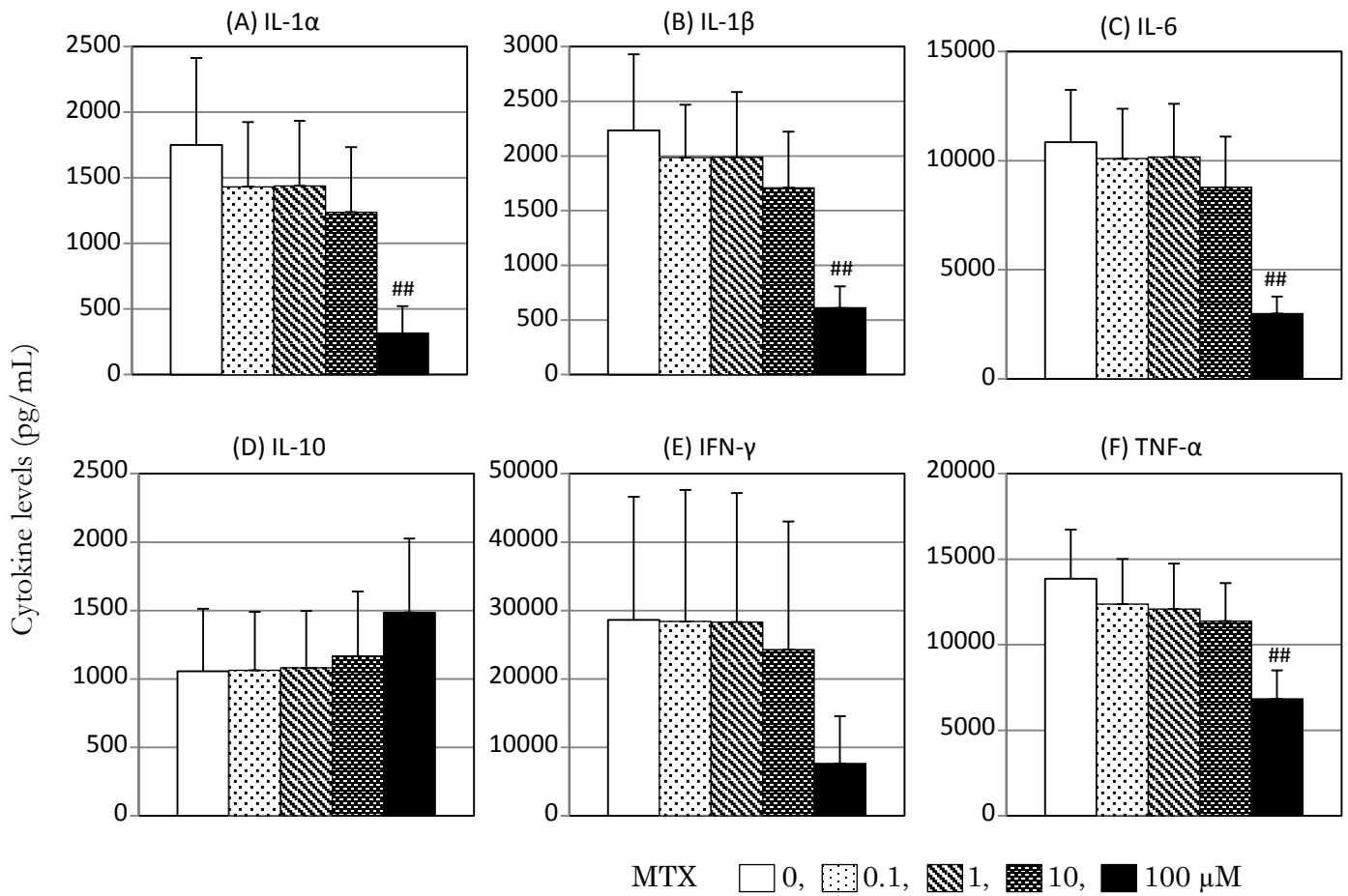


Figure 5. Effect of MTX on cytokine production from peritoneal macrophage stimulated by LPS.

Peritoneal macrophage (2×10^5 cells) were incubated with MTX (0, 0.1, 1, 10 or 100 μ M) for 24 h and additionally incubated with lipopolysaccharide (LPS, 2 μ g/mL) for 48 h. (A)IL-1 α , (B)IL-1 β , (C)IL-6, (D)IL-10, (E)IFN- γ and (F)TNF- α . Each column and bar represents the mean $\pm$ SD of 5 animals. ## p < 0.01: Significantly different from control group (Dunnett test).

Table 1. Effect of MTX on hematological parameters.

Items	Dose (mg/kg)			
	0	1	2	5
WBC parameters				
White blood cell count ($\times 10^3/\mu\text{L}$)	16.406 ± 3.906	11.258 ± 5.184	12.054 ± 1.941	$7.592 \pm 2.238^*$
Lymphocyte count ($\times 10^3/\mu\text{L}$)	14.44 ± 4.09	10.50 ± 4.66	11.52 ± 1.98	$7.20 \pm 2.07^*$

** $p < 0.01$, * $p < 0.05$: Significantly different from control group (Dunnett test)

Table 2. Effect of MTX on peripheral, thymic, splenic and mesenteric lymphocytes, and serum immunoglobulin levels.

Lymphocyte subsets	Dose (mg/kg)			
	0	1	2	5
Peripheral blood				
Total T cells (x10 ³ /μL)	6.988 ± 2.170	4.730 ± 1.646	5.318 ± 0.558	3.750 ± 1.279*
CD4+CD8 ⁻ T cells (x10 ³ /μL)	4.368 ± 1.515	2.932 ± 0.979	3.334 ± 0.412	2.352 ± 0.892*
CD4+CD8 ⁺ T cells (x10 ³ /μL)	0.110 ± 0.058	0.082 ± 0.032	0.074 ± 0.018	0.058 ± 0.013
CD4 ⁻ CD8 ⁻ T cells (x10 ³ /μL)	0.046 ± 0.024	0.042 ± 0.030	0.036 ± 0.015	0.022 ± 0.008
CD4 ⁻ CD8 ⁺ T cells (x10 ³ /μL)	2.462 ± 0.614	1.668 ± 0.690	1.868 ± 0.221	1.314 ± 0.484*
NK cells (x10 ³ /μL)	1.054 ± 0.279	0.824 ± 0.384	0.974 ± 0.164	0.650 ± 0.252
B cells (x10 ³ /μL)	4.506 ± 1.301	4.024 ± 2.684	3.724 ± 1.170	1.988 ± 0.760
Thymus				
Total thymic cells (x10 ⁸ /organ)	15.76 ± 1.88	10.74 ± 5.63	10.72 ± 2.61	3.56 ± 2.59**
CD4+CD8 ⁻ (x10 ⁷ /organ)	9.758 ± 2.201	8.434 ± 3.282	9.372 ± 3.270	4.184 ± 3.632*
CD4+CD8 ⁺ (x10 ⁷ /organ)	140.060 ± 16.231	92.196 ± 50.494	90.614 ± 20.201	28.166 ± 20.628**
CD4 ⁻ CD8 ⁻ (x10 ⁷ /organ)	2.110 ± 0.446	1.660 ± 0.794	1.848 ± 0.769	1.020 ± 0.482*
CD4 ⁻ CD8 ⁺ (x10 ⁷ /organ)	5.644 ± 0.890	5.106 ± 2.244	5.390 ± 2.303	2.242 ± 1.622*
Spleen				
Total spleen cells (x10 ⁸ /organ)	5.54 ± 0.94	5.52 ± 1.00	4.74 ± 1.02	3.72 ± 0.99*
Total T cells (x10 ⁷ /organ)	22.112 ± 4.288	21.512 ± 4.154	19.312 ± 4.903	14.706 ± 3.481*
CD4+CD8 ⁻ T cells (x10 ⁷ /organ)	13.030 ± 2.684	12.756 ± 2.471	11.322 ± 2.766	8.152 ± 1.646*
CD4+CD8 ⁺ T cells (x10 ⁷ /organ)	0.394 ± 0.102	0.680 ± 0.552	0.410 ± 0.140	0.316 ± 0.116
CD4 ⁻ CD8 ⁻ T cells (x10 ⁷ /organ)	0.534 ± 0.151	0.482 ± 0.204	0.376 ± 0.084	0.350 ± 0.167
CD4 ⁻ CD8 ⁺ T cells (x10 ⁷ /organ)	8.150 ± 1.561	7.600 ± 1.410	7.216 ± 2.028	5.896 ± 2.206
NK cells (x10 ⁷ /organ)	2.068 ± 0.537	1.882 ± 0.413	1.716 ± 0.530	1.556 ± 0.162
B cells (x10 ⁷ /organ)	28.204 ± 5.270	29.362 ± 9.978	23.404 ± 5.264	17.940 ± 6.722
Mesenteric lymph node				
Total T cells (%)	64.54 ± 5.33	60.12 ± 9.89	61.88 ± 2.36	64.38 ± 11.93
CD4+CD8 ⁻ T cells (%)	43.24 ± 4.39	41.58 ± 6.48	43.32 ± 1.25	41.90 ± 8.34
CD4+CD8 ⁺ T cells (%)	1.44 ± 0.82	1.08 ± 1.14	0.72 ± 0.13	0.96 ± 0.76
CD4 ⁻ CD8 ⁻ T cells (%)	3.22 ± 1.81	2.06 ± 1.48	1.54 ± 0.13	1.96 ± 0.76
CD4 ⁻ CD8 ⁺ T cells (%)	16.62 ± 1.73	15.42 ± 2.09	16.30 ± 2.44	19.54 ± 7.51
NK cells (%)	0.54 ± 0.05	0.66 ± 0.11	0.56 ± 0.15	0.66 ± 0.26
B cells (%)	32.04 ± 3.68	35.98 ± 9.21	34.86 ± 5.55	32.78 ± 10.68
Serum immunoglobulins				
IgM (μg/mL)	230.16 ± 25.07	152.20 ± 37.14**	139.44 ± 29.70**	85.82 ± 23.86**
IgG (μg/mL)	2371.96 ± 1356.38	2432.42 ± 963.20	1353.46 ± 579.10	1150.10 ± 673.40
IgA (μg/mL)	19.38 ± 4.68	18.44 ± 4.06	14.04 ± 2.08	11.38 ± 3.84*
IgE (μg/mL)	0.10 ± 0.14	0.00 ± 0.00	0.08 ± 0.08	0.14 ± 0.22

** p < 0.01, * p < 0.05: Significantly different from respective control group (Dunnett test)

Table 3. Effect of MTX on pathological examinations.

Organs	Dose (mg/kg)			
	0	1	2	5
Bone marrow				
Decreased cellularity	-	-	4/5	5/5
Increase in megakaryocyte	-	-	-	3/5
Thymus				
Lymphoid depletion in cortex	-	-	-	3/5
Organ weight				
Thymus (mg)	691.18 ± 80.70	547.78 ± 164.50	549.04 ± 63.70	318.06 ± 91.92**

** p < 0.01: Significantly different from control group (Dunnett test)

References

- [1] Dean, J.H., et al., Selective immunosuppression resulting from exposure to the carcinogenic congener of benzopyrene in B6C3F1 mice. *Clin Exp Immunol*, 1983. 52(1): p. 199-206.
- [2] Luster, M.I., et al., Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam Appl Toxicol*, 1992. 18(2): p. 200-10.
- [3] Alarcon, G.S., et al., Risk factors for methotrexate-induced lung injury in patients with rheumatoid arthritis. A multicenter, case-control study. Methotrexate-Lung Study Group. *Ann Intern Med*, 1997. 127(5): p. 356-64.
- [4] Kawai, R., et al., Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats. *J Immunotoxicol*, 2013. 10(1): p. 40-8.
- [5] Uff, C.R., A.G. Pockley, and R.K. Phillips, A rapid microplate-based fluorometric assay for phagocytosis. *Immunol Invest*, 1993. 22(6-7): p. 407-13.
- [6] Turyna, B., M. Bos, and J. Jurek, The influence of in vivo stimulation on functional activity of rat macrophages. *Folia Histochem Cytobiol*, 1994. 32(2): p. 101-5.
- [7] Ulrich, P., et al., Validation of immune function testing during a 4-week oral toxicity study with FK506. *Toxicol Lett*, 2004. 149(1-3): p. 123-31.
- [8] Gore, E.R., et al., Primary antibody response to keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. *Toxicology*, 2004. 197(1): p. 23-35.
- [9] Smith, H.W., et al., A predictive F344 rat immunotoxicology model: cellular parameters combined with humoral response to NP-CgammaG and KLH. *Toxicology*, 2003. 194(1-2): p. 129-45.

- [10] Henry, M.L., Cyclosporine and tacrolimus (FK506): a comparison of efficacy and safety profiles. *Clin Transplant*, 1999. 13(3): p. 209-20.
- [11] Murakami, Y., et al., A one-month repeated oral dose toxicity study of methotrexate in unilaterally nephrectomized rats. *J Toxicol Sci*, 1998. 23 Suppl 5: p. 681-99.
- [12] Ladics, G.S., et al., Possible incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats on standard toxicology study. *Toxicology*, 1995. 96(3): p. 225-38.
- [13] Ladics, G.S., et al., Further evaluation of the incorporation of an immunotoxicological functional assay for assessing humoral immunity for hazard identification purposes in rats in a standard toxicology study. *Toxicology*, 1998. 126(2): p. 137-52.
- [14] Segal, R., et al., The effects of methotrexate on the production and activity of interleukin-1. *Arthritis Rheum*, 1989. 32(4): p. 370-7.
- [15] Johnston, C.A., et al., Measures of immunologic and inflammatory responses in vitro in rheumatoid patients treated with methotrexate. *J Rheumatol*, 1986. 13(2): p. 294-6.
- [16] Hirata, S., et al., Inhibition of in vitro vascular endothelial cell proliferation and in vivo neovascularization by low-dose methotrexate. *Arthritis Rheum*, 1989. 32(9): p. 1065-73.
- [17] Olsen, N.J. and L.M. Murray, Antiproliferative effects of methotrexate on peripheral blood mononuclear cells. *Arthritis Rheum*, 1989. 32(4): p. 378-85.
- [18] Cronstein, B.N., D. Naime, and E. Ostad, The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. *J Clin Invest*, 1993. 92(6): p. 2675-82.
- [19] Omata, T., et al., Methotrexate suppresses nitric oxide production ex vivo in macrophages from rats with adjuvant-induced arthritis. *Res Exp Med (Berl)*, 1997. 197(2): p. 81-90.

Summary

Chapter 1

To develop a rat T-cell-dependent antibody response (TDAR) model evaluating both primary and secondary antibody responses, keyhole limpet hemocyanin (KLH) was used to immunize rats twice during a 14-day course of study, a pattern closely linked to that of a short-term general toxicity study. Female rats of four representative strains (Sprague-Dawley, Wistar, Fischer, and Lewis) were immunized twice with intravenous administrations of KLH (300 µg/rat) on Days 5 and 9 during a 14-day treatment regimen with cyclophosphamide (CPA) at 1, 3, or 6 mg/kg/day. The primary and secondary immunizations of KLH markedly elevated serum anti-KLH IgM and IgG levels in all strains on Days 9 and 15. Remarkable higher levels of anti-KLH IgG (≈ 1000 µg/ml) were noted in all strains, which were more than 4-times compared with those of anti-KLH IgM levels at Day 9, indicating that predominant IgG reactions were induced by the dual immunizations. A large inter-individual variability in KLH-specific IgM and IgG production was observed in all strains. However, levels of the KLH-specific antibodies were considered sufficient for the evaluation, even in Sprague-Dawley and Wistar rats reported as strains with a wide range of variability since immunosuppression of CPA on responses in both anti-KLH IgM and IgG were observed in all strains to the same extent. In addition, the sensitivity of

the KLH-ELISA assay system detecting the immunosuppressive effects of CPA was comparable to other assay systems with PFC assay or ELISA using SRBC. The results here demonstrated that these experimental designs could provide valuable information about the influence on both the primary and secondary humoral immune responses in rats when exposed to potential immunomodulatory drugs. Furthermore, the design of the presented TDAR study would support comprehensive evaluation together with the outcome of the conventional general toxicity study.

Chapter 2

T-cell dependent antibody response (TDAR) incorporating both primary and secondary responses to keyhole limpet hemocyanin (KLH) in canine models have not yet been fully understood. To develop a practical dog TDAR model, we characterized primary and secondary antibody responses by intravenous or intramuscular immunization of KLH twice at intervals of 8 days during a 28-day course of study. Primary immunization with KLH by both routes induced a maximum IgM response on 6 to 8 days after the treatment, whereas the IgG response started 6 to 8 days after the treatment with relatively low levels. Remarkable increases in anti-KLH IgG levels (about 10-times compared with the primary response) were produced 5 to 7 days after the secondary KLH immunization by both routes. These results indicate that IgM-predominant and IgG-predominant responses were respectively induced by the primary and secondary immunization.

Furthermore, the intravenous route showed higher baseline titers of primary and secondary anti-KLH IgM responses, suggesting that intravenous immunization of KLH might be a more suitable method for immunotoxicity evaluation. No remarkable inter-individual variability was noted in our canine models. Treatment with cyclophosphamide (CPA) at 2 mg/kg/day for a consecutive 28 days significantly suppressed primary and secondary anti-KLH IgM and IgG responses induced by KLH injection on Days 15 and 23 of CPA treatment. These results demonstrate that these experimental designs could provide valuable information about the influence on both the primary and secondary humoral immune responses in dogs when exposed to potential immunomodulatory drugs.

Chapter 3

To evaluate the potential immunotoxicity of methotrexate (MTX), we evaluated the effect of MTX on a T-cell dependent antigen response (TDAR) to intravenous immunization with keyhole limpet hemocyanin (KLH) in Sprague-Dawley rats during a 14-day intermittent treatment with MTX (1, 2 and 5 mg/kg; 3 times a week, total 6 times). MTX dose-dependently reduced both serum anti-KLH IgM anti-KLH IgG antibodies on Days 8 and 14. In addition, MTX decreased peripheral, thymic and splenic T-cells at the same dose which the drug suppressed TDAR. In contrast, MTX did not influence on B-cells even at the highest dose. Furthermore, MTX suppressed proliferation response of splenocytes to KLH and inflammatory cytokine

production including IL-1 α , IL-1 β , IL-6, IFN- γ , and TNF- α from splenocytes stimulated by lipopolysaccharides (LPS). In an in vitro assay, MTX at 100 μ M declined inflammatory cytokine production including IL-1 α , IL-1 β , IL-6, and IFN- γ , and promoted anti-inflammatory cytokine IL-10 production from peritoneal macrophages stimulated by LPS. In contrast, MTX did not impact on phagocytic ability of peritoneal macrophages or NO-production from peritoneal macrophages. The present results suggested that MTX inhibited KLH-TDAR come mainly from decreased T-cells and suppression of T-cell functions including cell proliferation and cytokine production. Furthermore, decreased macrophage function due to MTX could be at least in part involved in MTX-induced KLH-TDAR suppression.

Acknowledgements

I would like to express my sincere gratitude to Professor Dr. Teruo Kawada, Assistant professor Dr. Nobuyuki Takahashi, and all members of Graduate School of Agriculture, Kyoto University, for finishing this manuscript.

I especially would like to express my deepest appreciation to my supervisors, Drs. Kazuhiko Mori and Tetsuo Aida for their elaborated guidance, considerable encouragement and invaluable discussion that make my research of great achievement and my study life unforgettable.

I would like to give my thanks to Drs. Sunao Manabe, Atsushi Sanbuissho, Wataru Takasaki, and Tadashi Furukawa, Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., for giving me the chance to do these researches and many supports.

I would also like to give my thanks to Ms. Satomi Komatsu, Shiho Ito, Motoko Ajioka, Masae Yagi, and Dr. Hiroyuki Hattori for their technical assistance, helpful advices, and hearty encouragement.

Finally I would like to extend my indebtedness to my wife, Yuki, and my daughter, Sara, for their endless love, understanding, support, encouragement and sacrifice throughout my study.

Ryota Kawai

List of publications

1. Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats.
Kawai R, Ito S, Aida T, Hattori H, Kimura T, Furukawa T, Mori K, Sanbuissho A, Kawada T.
J Immunotoxicol. 2013 Jan-Mar;10(1):40-8.
2. Evaluation of canine T-cell dependent antibody response to the primary and secondary immunization with keyhole limpet hemocyanin.
Kawai R, Aida T, Hattori H, Furukawa T, Mori K, Takasaki W, Takahashi N, Kawada T.
J Toxicol Sci. 2013;38(4):571-9.
3. Mechanism of immunosuppressive effects of methotrexate on primary and secondary T-cell dependent antigen response to keyhole limpet hemocyanin in rats
Kawai R, Ito S, Aida T, Hattori H, Mori K, Takasaki W, Takahashi N, Kawada T.
J Toxicol Sci. Under preparation

Related papers

1. Methemoglobinemia induced by 1,2-dichloro-4-nitrobenzene in mice with a disrupted glutathione S-transferase Mu 1 gene.
Arakawa S, Maejima T, Kiyosawa N, Yamaguchi T, Shibaya Y, Aida Y, **Kawai R**, Fujimoto K, Manabe S, Takasaki W.
Drug Metab Dispos. 2010 Sep;38(9):1545-52.
2. Toxicoproteomic investigation of the molecular mechanisms of cycloheximide-induced hepatocellular apoptosis in rat liver.
Kumagai K, Ando Y, Kiyosawa N, Ito K, **Kawai R**, Yamoto T, Manabe S, Teranishi M.
Toxicology. 2006 Dec 7;228(2-3):299-309.