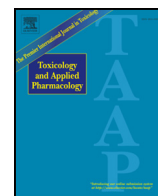




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Oral administration of drugs with hypersensitivity potential induces germinal center hyperplasia in secondary lymphoid organ/tissue in Brown Norway rats, and this histological lesion is a promising candidate as a predictive biomarker for drug hypersensitivity occurrence in humans

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ABSTRACT

It is important to evaluate the potential of drug hypersensitivity as well as other adverse effects during the pre-clinical stage of the drug development process, but validated methods are not available yet. In the present study we examined whether it would be possible to develop a new predictive model of drug hypersensitivity using Brown Norway (BN) rats. As representative drugs with hypersensitivity potential in humans, phenytoin (PHT), carbamazepine (CBZ), amoxicillin (AMX), and sulfamethoxazole (SMX) were orally administered to BN rats for 28 days to investigate their effects on these animals by examinations including observation of clinical signs, hematology, determination of serum IgE levels, histology, and flow cytometric analysis. Skin rashes were not observed in any animals treated with these drugs. Increases in the number of circulating inflammatory cells and serum IgE level did not necessarily occur in the animals treated with these drugs. However, histological examination revealed that germinal center hyperplasia was commonly induced in secondary lymphoid organs/tissues in the animals treated with these drugs. In cytometric analysis, changes in proportions of lymphocyte subsets were noted in the spleen of the animals treated with PHT or CBZ during the early period of administration. The results indicated that the potential of drug hypersensitivity was identified in BN rat by performing histological examination of secondary lymphoid organs/tissues. Data obtained herein suggested that drugs with hypersensitivity potential in humans gained immune reactivity in BN rat, and the germinal center hyperplasia induced by administration of these drugs may serve as a predictive biomarker for drug hypersensitivity occurrence.

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Introduction

Although drug hypersensitivity is uncommon, such reaction is a serious adverse event that sometimes results in death (Adkinson et al., 2002). It includes various cutaneous symptoms of different severity, ranging from simple, uncomplicated skin rash to potentially fatal, severe eruption, such as Stevens–Johnson syndrome and toxic epidermal necrolysis (TEN) (McKenna and Leiferman, 2004; Roujeau, 2005). Drug hypersensitivity generally occurs during the first exposure to the drug, with initial symptoms starting 2–6 weeks later. In patients with a history of drug hypersensitivity reaction, re-exposure after a previous reaction may cause symptoms within 1 day (Pirmohamed et al., 2002).

Drug hypersensitivity is considered to be an immune-mediated reaction (Hausmann et al., 2010; Khan and Solensky, 2010; Rieder, 2009). According to classical immunology, small molecules are not complete antigens and are incapable of directly inducing immune response by priming of Naïve T cells in their native state (Landsteiner and

Jacobs, 1935). Some drugs, such as penicillin, are known to be directly chemically reactive to bind spontaneously in a covalent way to other molecules, mostly proteins, and become immunogenic (Uetrecht, 2006). Other drugs, however, must be metabolized or bioactivated to a chemically reactive form before they bind to such molecules (Griem et al., 1998; Naisbitt et al., 2000).

Unfortunately, drug hypersensitivity reactions are often unnoticed until a drug has been marketed and used in the general population for some time (Olson et al., 2000). The occurrence of such drugs not only has an adverse impact on public health, but also causes financial damage to the pharmaceutical company involved (Ratajczak, 2004). Traditional toxicological studies, however, fail to predict the potential of drug hypersensitivity (Bala et al., 2005; Descotes, 2006), and therefore there is a need to develop predictive models that prevent the introduction of drugs with hypersensitivity potential to the market.

Some models have been attempted, but these have been poor predictors or were not based on mechanisms relevant to drug hypersensitivity reactions mediated by systemically administered drugs. Antigenicity assay, which was developed using guinea pigs and mice, can be used to determine the ability of drugs to induce specific antibody response in vivo. However, it has been demonstrated that most drugs administered

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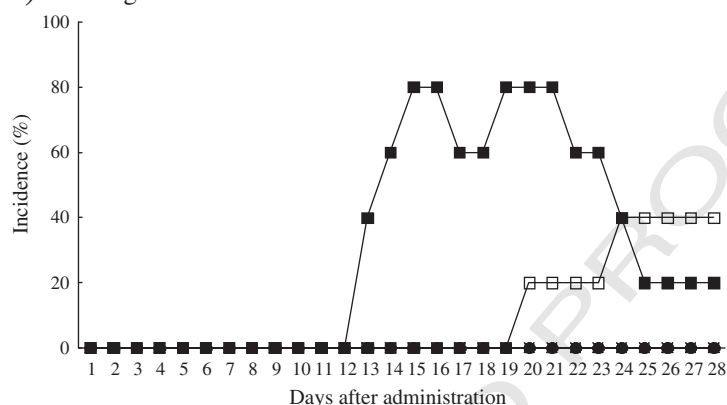
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orally are not immunogenic, even those that are known to induce drug hypersensitivity in humans (Weaver et al., 2003). The popliteal lymph node assay (PLNA) arose from the studies that found that subcutaneous administration of phenytoin and other compounds known to produce autoimmune-like diseases in humans resulted in an enlargement of the popliteal lymph node (Gleichmann, 1981; Pieters and Albers, 1999). For orally administered drugs, however, subcutaneous injection in the

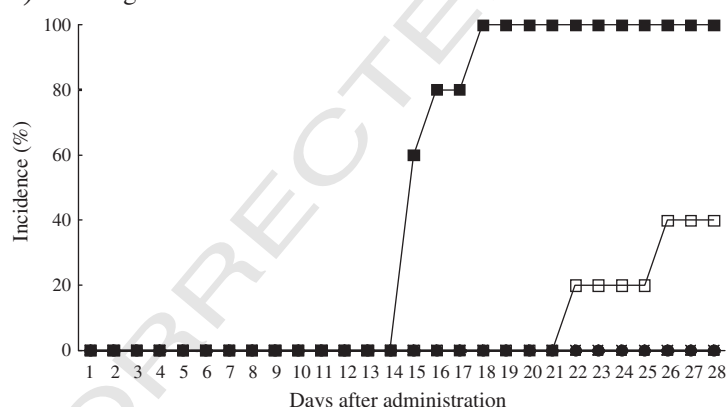
footpad may not be relevant, because many drugs require metabolic activation to induce drug hypersensitivity reaction.

Brown Norway (BN) rat is a strain that seems susceptible to substances that induce immune-mediated adverse reaction, and thus this particular strain has been used in studies on chemical-induced autoimmunity and food allergy (Knippels and Penninks, 2005; Pieters et al., 2002). The offending substances have been shown to induce various

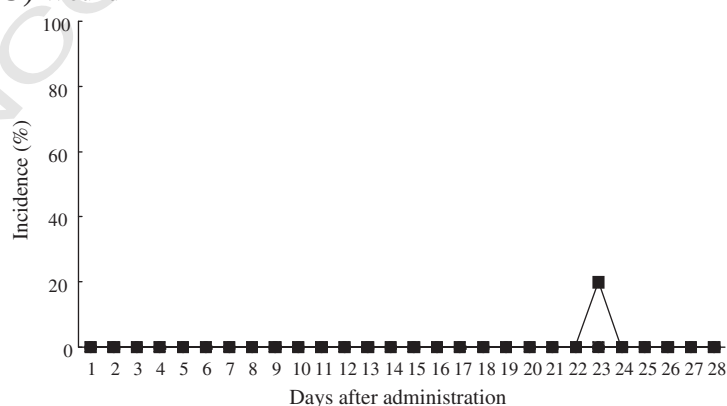
A) Flushing



B) Swelling



C) Wound



—x— Vehicle 0 —□— D-Pen 100 —■— D-Pen 500 —◇— PHT 300 —◆— PHT 450 —△— CBZ 300 —▲— CBZ 500
 —○— AMX 500 —●— AMX 1000 —*— SMX 250 —+— SMX 500 —— LDC 100 —+— LDC 200

Fig. 1. Incidence of cutaneous symptoms in BN rats treated with drugs for 28 days. Incidence of animals in each group that developed (A) flushing, (B) swelling, and (C) wound was recorded. Groups of animals ($n = 5/\text{group}$) were treated either with vehicle, d-Pen at 100 or 500 mg/kg/day, PHT at 300 or 450 mg/kg/day, CBZ at 300 or 500 mg/kg/day, AMX at 500 mg/kg/day or 1000 mg/kg/day, SMX at 250 mg/kg/day, or LDC at 100 or 200 mg/kg/day by gavage for 28 days, and were observed daily for clinical signs.

disease phenotypes, including glomerulonephritis, to skin- and lung pathology in BN rat, D-Penicillamine (D-Pen) and nevirapine, which are known to induce drug hypersensitivity reaction in humans, cause skin rash in BN rats (Uetrecht, 2005). These rashes are characterized by skin infiltration of inflammatory cells, accompanied by an increased lymphoproliferation and hyperimmunoglobulinemia. Except for these two, however, there have been no reports on other drugs with hypersensitivity potential so far.

In the current study, we investigated the effects of phenytoin (PHT; 5,5-diphenylhydantoin sodium salt), carbamazepine (CBZ), amoxicillin (AMX), and sulfamethoxazole (SMX), which have the potential of drug hypersensitivity in humans, and lidocaine (LDC), which only rarely induces allergic reactions in humans (Jackson et al., 1994), as well as D-Pen as a positive control of drug-induced skin rash (Uetrecht, 2005), on BN rats by oral administration of these drugs for 28 days. The results indicated that the test drugs with the potential of drug hypersensitivity commonly induced germinal center hyperplasia in secondary lymphoid organs/tissues in these animals, whereas LDC did not. Data obtained herein suggests drugs with potential hypersensitivity gained immune reactivity in BN rat, and the germinal center hyperplasia induced by administration of these drugs may serve as a predictive biomarker for drug hypersensitivity.

Materials and methods

Test drugs. D-Pen, PHT, CBZ, AMX, SMX, and LDC were purchased from Sigma-Aldrich Co.

Animals and housing. The experiments were approved by the animal ethics board of Daiinippon Sumitomo Pharma Co., Ltd. Brown Norway rats were purchased from Charles River Japan, Inc. (Shiga, Japan). Rats aged 5 weeks were received and acclimated to the laboratory environment for 7 days before use. During the experiment, the rats were housed one or two per cage in suspended aluminum cages with stainless-steel wire-mesh front and floor under controlled environmental conditions. Drinking water and pellet rodent diet were available ad libitum. After a quarantine period, animals in good health (based on clinical signs and

body weights) were selected for the experiments. Prior to administration of test drugs, rats were assigned to groups by a stratified randomization method using a computer program, based on body weight and body weight gain during the quarantine period, so that no significant differences in mean body weight would be present among the groups. At the commencement of treatment, it was confirmed that no significant difference existed in mean body weight among the groups.

Study design. In evaluation of the effects of D-Pen, PHT, CBZ, AMX, SMX, and LDC on clinical signs, hematology, serum IgE concentrations, and histology of second lymphoid organs/tissues in BN rats, each group consisted of five animals for each experiment. Before starting the present study, dose range finding studies were performed (data not shown). Based on these studies, the high doses were selected with expectation to give a positive response but not to exceed maximum tolerated dose, and the low doses of AMX and SMX were selected to be approximately a half of the high doses in the present study. For D-Pen, PHT and CBZ, the low doses were selected with expectation to give a weak response. The dose levels of the test drugs were as follows: D-Pen: 100 or 500 mg/kg, PHT: 300 or 450 mg/kg, CBZ: 300 or 500 mg/kg, AMX: 500 or 1000 mg/kg, SMX: 250 or 500 mg/kg, LDC: 100 or 200 mg/kg. Test drugs were suspended or dissolved in vehicle (0.5% methylcellulose solution) and orally administered for 28 days. The daily amounts administered were 5 ml/kg body weight for AMX and SMX treatments or 10 ml/kg body weight for D-Pen, PHT and SMX treatments, calculated based on body weights of each animal measured once a week. Clinical signs, including any abnormal appearance or behavior, were recorded twice a day in each animal. For hematology, rats were fasted from around 16:00 of the day before they were euthanized. Blood was withdrawn from the abdominal aorta using a syringe fitted with a needle under isoflurane inhalation anesthesia. Serum was prepared from the collected blood and stored in a deep freezer until measurement of serum IgE concentrations. After sampling of blood, spleen, mesenteric lymph nodes, and small intestine (Payer's patch) were collected from all animals and fixed in 10% neutral-buffered formalin. These were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination. Hematology was performed

Table 1

Effects on hematological parameters and serum IgE concentrations in BN rats treated with drugs for 28 days.

	Dose (mg/kg/day)	WBC ($\times 10^3/\mu\text{L}$)	Neut ($\times 10^3/\mu\text{L}$)	Mono ($\times 10^3/\mu\text{L}$)	Eos ($\times 10^3/\mu\text{L}$)	Baso ($\times 10^3/\mu\text{L}$)	Lymph ($\times 10^3/\mu\text{L}$)	LUC ($\times 10^3/\mu\text{L}$)	IgE (ng/mL)
Experiment 1									
Vehicle	0	4.74 $\pm$ 0.446	0.36 $\pm$ 0.007	0.11 $\pm$ 0.036	0.10 $\pm$ 0.021	0.00 $\pm$ 0.005	4.13 $\pm$ 0.419	0.03 $\pm$ 0.011	402.3 $\pm$ 218.1
D-Penicillamine	100	14.59 $\pm$ 21.82	4.60 $\pm$ 9.390	0.40 $\pm$ 0.655	1.35 $\pm$ 2.674	0.06 $\pm$ 0.114	8.00 $\pm$ 8.678	0.19 $\pm$ 0.347	6003.6** $\pm$ 1956.5
D-Penicillamine	500	6.82* $\pm$ 1.943	1.99* $\pm$ 0.645	0.24 $\pm$ 0.162	0.45* $\pm$ 0.257	0.01 $\pm$ 0.008	4.07 $\pm$ 1.509	0.06 $\pm$ 0.036	34552.7** $\pm$ 12123.2
Experiment 2									
Vehicle	0	3.75 $\pm$ 0.629	0.40 $\pm$ 0.223	0.07 $\pm$ 0.012	0.09 $\pm$ 0.054	0.01 $\pm$ 0.005	3.17 $\pm$ 0.505	0.02 $\pm$ 0.009	576.5 $\pm$ 125.1
Phenytoin	300	3.49 $\pm$ 0.275	0.33 $\pm$ 0.077	0.08 $\pm$ 0.032	0.11 $\pm$ 0.063	0.01 $\pm$ 0.004	2.94 $\pm$ 0.238	0.03 $\pm$ 0.019	425.9 $\pm$ 272.3
Phenytoin	450	3.08 $\pm$ 1.029	0.19 $\pm$ 0.057	0.06 $\pm$ 0.024	0.10 $\pm$ 0.091	0.00 $\pm$ 0.005	2.70 $\pm$ 0.903	0.02 $\pm$ 0.010	797.3 $\pm$ 459.1
Carbamazepine	300	3.75 $\pm$ 0.695	0.35 $\pm$ 0.182	0.07 $\pm$ 0.028	0.04 $\pm$ 0.033	0.00 $\pm$ 0.004	3.27 $\pm$ 0.510	0.02 $\pm$ 0.005	2562.8* $\pm$ 845.1
Carbamazepine	500	5.02 $\pm$ 1.522	0.40 $\pm$ 0.201	0.10* $\pm$ 0.007	0.04 $\pm$ 0.014	0.01 $\pm$ 0.005	4.44 $\pm$ 1.310	0.03 $\pm$ 0.023	4310.0* $\pm$ 981.5
Experiment 3									
Vehicle	0	7.55 $\pm$ 2.118	0.48 $\pm$ 0.179	0.12 $\pm$ 0.074	0.12 $\pm$ 0.054	0.03 $\pm$ 0.021	6.74 $\pm$ 1.833	0.06 $\pm$ 0.042	889.9 $\pm$ 956.0
Amoxicillin	500	6.65 $\pm$ 1.029	0.47 $\pm$ 0.172	0.09 $\pm$ 0.043	0.08 $\pm$ 0.080	0.03 $\pm$ 0.005	5.93 $\pm$ 1.054	0.05 $\pm$ 0.021	242.8 $\pm$ 60.9
Amoxicillin	1000	6.95 $\pm$ 0.729	1.21 $\pm$ 0.792	0.11 $\pm$ 0.041	0.04 $\pm$ 0.013	0.02 $\pm$ 0.005	5.52 $\pm$ 0.512	0.05 $\pm$ 0.013	400.5 $\pm$ 261.5
Sulfamethoxazole	250	6.35 $\pm$ 2.128	1.29 $\pm$ 0.588	0.15 $\pm$ 0.032	0.17 $\pm$ 0.039	0.01 $\pm$ 0.008	4.71 $\pm$ 1.622	0.03 $\pm$ 0.007	796.5 $\pm$ 606.9
Sulfamethoxazole	500	7.21 $\pm$ 0.729	2.75** $\pm$ 0.792	0.12 $\pm$ 0.041	0.10 $\pm$ 0.013	0.02 $\pm$ 0.005	4.18* $\pm$ 0.512	0.04 $\pm$ 0.013	3515.0* $\pm$ 1881.0
Experiment 4									
Vehicle	0	4.48 $\pm$ 1.277	0.42 $\pm$ 0.153	0.10 $\pm$ 0.031	0.07 $\pm$ 0.031	0.00 $\pm$ 0.004	3.86 $\pm$ 1.080	0.03 $\pm$ 0.018	384.2 $\pm$ 384.74
Lidocaine	100	5.43 $\pm$ 1.988	0.38 $\pm$ 0.067	0.09 $\pm$ 0.038	0.13 $\pm$ 0.140	0.01 $\pm$ 0.009	4.80 $\pm$ 1.758	0.04 $\pm$ 0.019	198.1 $\pm$ 90.96
Lidocaine	200	6.38 $\pm$ 1.328	0.48 $\pm$ 0.126	0.11 $\pm$ 0.015	0.13 $\pm$ 0.067	0.01* $\pm$ 0.000	5.62 $\pm$ 1.170	0.04 $\pm$ 0.016	226.3 $\pm$ 74.11

Abbreviations: WBC, white blood cells; Neut, neutrophils; Mono, monocytes; Eos, eosinophils; Baso, basophils; Lymph, lymphocytes; LUC, large unstained.

Note. Mean $\pm$ SD, n = 5.

BN rats were either treated with vehicle, D-penicillamine, phenytoin, carbamazepine, amoxicillin, sulfamethoxazole, or lidocaine by gavage for 28 days, and were evaluated for hematology and serum IgE levels on the day following the last day of administration.

* Significantly different from the control group (p < 0.05).

** Significantly different from the control group (p < 0.01).

Table 2
Histological findings in BN rats treated with drugs for 28 days.

Organ, tissue findings	Test article	Vehicle	D-Penicillamine		Phenytoin		Carbamazepine		Amoxicillin		Sulfamethoxazole		Lidocaine	
	Dose (mg/kg/day)		100	500	300	450	300	500	500	1000	250	500	100	200
	Score	(5)	(2)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
Spleen														
Extramedullary hematopoiesis	0	5	0	2	5	5	5	5	5	5	5	5	5	5
	1	0	1	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	2	0	0	0	0	0	0	0	0	0	0
	3	0	1	0	0	0	0	0	0	0	0	0	0	0
Germinal center hyperplasia	0	5	1	0	4	3	2	0	5	5	3	0	5	5
	1	0	1	2	1	2	3	1	0	0	2	1	0	0
	2	0	0	3	0	0	0	4	0	0	0	3	0	0
	3	0	0	0	0	0		0	0	0	0	1	0	0
Mesenteric lymph node	Score	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
Cellular infiltration of eosinophils	0	5	5	5	5	5	5	5	5	5	4	4	5	5
	1	0	0	0	0	0	0	0	0	0	1	0	5	5
	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Granulomatous inflammation	0	5	5	5	5	5	5	5	5	5	5	4	5	5
	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0	0	1	0	0
Germinal center hyperplasia	0	5	0	0	5	5	5	5	5	3	2	2	5	5
	1	0	1	1	0	0	0	0	0	1	2	1	0	0
	2	0	4	4	0	0	0	0	0	1	1	2	0	0
Payer's patch (small intestine)		(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)
Within normal limits		5	5	5	5	5	5	5	5	5	5	5	5	5

Note. (N): number of animals examined, N: number of incidence.

(Score) 0: within normal limits, 1: slight, 2: mild, 3: moderate.

BN rats were either treated with vehicle, D-penicillamine, phenytoin, carbamazepine, amoxicillin, sulfamethoxazole, or lidocaine by gavage for 28 days, and were evaluated for histology.

to determine numbers of white blood cells, neutrophils, monocytes, eosinophils, basophils, lymphocytes and large unstained cells with an ADVIA 120 Hematology System (Siemens Healthcare Diagnostics Manufacturing Limited). Concentrations of serum IgE were measured using commercially available ELISA kits (Shibayagi Co., Ltd.).

In evaluation of the effects of D-Pen, PHT and CBZ on weights and lymphocyte subsets of spleen in BN rats, each group consisted of three animals for the experiment. In this experiment, the dose levels set were the same as the high doses selected in the above experiments. Test drugs were orally administered for 7 or 28 days in the same fashion as above. After the administration period, animals were euthanized and the spleen was removed. Histology of spleen was performed to confirm the presence or absence of germinal center hyperplasia in this experiment.

Histological evaluation. The severity scores of histological lesions were graded according to the number, size, and distribution, with a range of 0 to 3. In particular, germinal center hyperplasia was graded as follows: The primary grading criterion was the number of germinal centers in the section. The size of germinal center was the secondary criterion. The following are the definitions of the germinal center hyperplasia severity scores: 0 = 2–3 germinal centers, all normal in size (as noted in control animals); 1 = 4–10 germinal centers, several of which are slightly enlarged; 2 = 11–20 germinal centers, many of which are obviously enlarged; and 3 = 20–30 germinal centers, most of which are obviously enlarged.

Flow cytometric analysis. For flow cytometric analysis, 1×10^6 splenocytes were incubated with either FITC-, PE, PE-Cy5.5, APC-conjugated anti-CD3, -CD4, -CD8, -CD25, and -CD45RA mAbs (BD Biosciences) in 96 well plates in darkness for 30 min at 4 °C. To evaluate

intracellular Foxp3 expression, lymphocytes were stained with anti-CD4 and -CD25 mAb, fixed and permeabilized with the buffers included in a Foxp3 detection kit (BD Biosciences). After staining with anti-Foxp3 mAb, the lymphocytes were analyzed with a flow cytometer.

Statistics. The data for hematology and serum IgE concentrations were subjected to multi-comparison procedures. Mean and standard deviations for each group were calculated and the homogeneity of variance was analyzed by Bartlett's test. When the variance was homogeneous, values for the treated and control groups were analyzed by Dunnett's multiple comparison test. When not homogeneous, Steel's multiple comparison test was used. The data for weights and lymphocyte subsets of spleen and flow cytometric analysis were analyzed by Student's *t*-test.

Results

Skin rash was only noted in BN rats treated with D-Pen, and not with PHT, CBZ, AMX, SMX, or LDC.

At the initiation of study, we reproduced the D-Pen-induced drug hypersensitivity model. As previously reported (Uetrecht, 2005), hypersensitivity-like cutaneous symptoms were noted in BN rats treated with D-Pen at 100 and 500 mg/kg/day (Fig. 1). Symptoms such as flushing and swelling occurred in these animals about after two weeks of administration and lasted until the end of the dosing period with varying frequency. These symptoms preceded and occurred more frequently in the animals at 500 mg/kg/day than those at 100 mg/kg/day. Although the relevance to drug hypersensitivity was unclear, wound was noted in one animal treated at 500 mg/kg/day (Fig. 1). Besides these symptoms, ataxic gait was noted in some animals at 500 mg/kg/day (data not shown). We then examined whether such cutaneous symptoms occurred in BN rats by administration of the

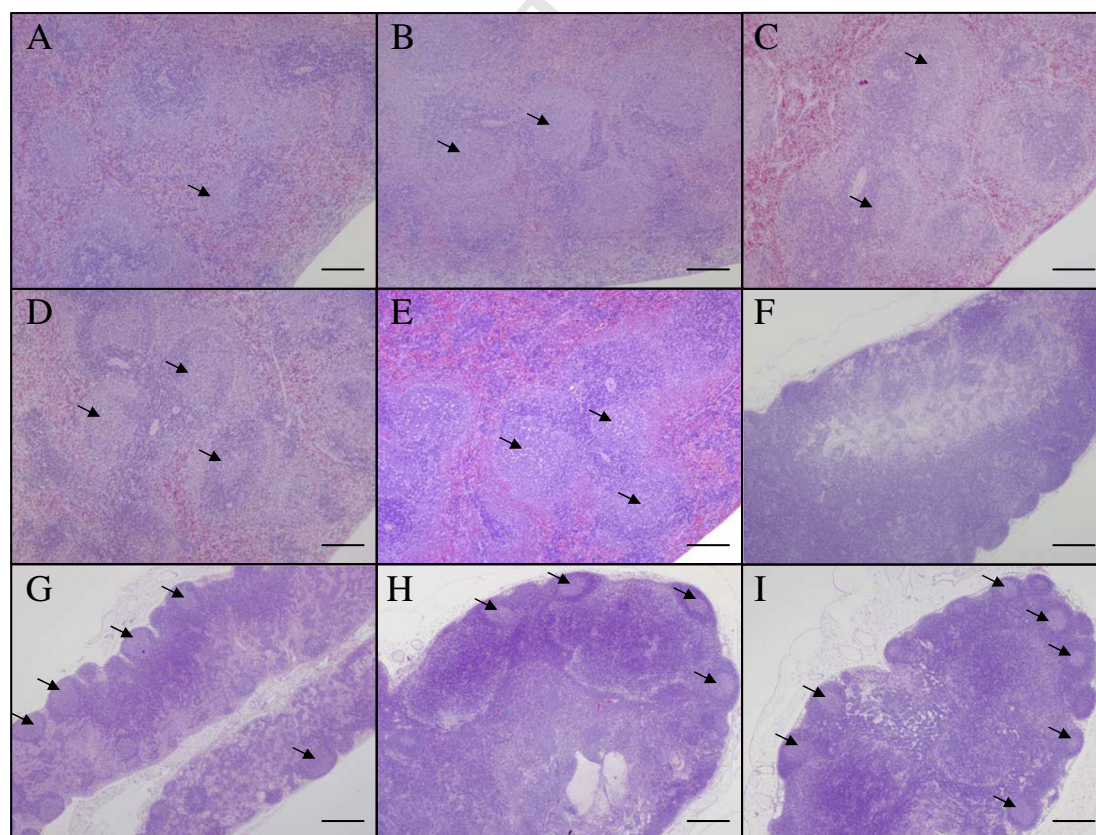


Fig. 2. Germinal center hyperplasia in the spleen and mesenteric lymph node from BN rats treated with drugs for 28 days. Photographs of spleen from BN rat treated with vehicle (A), D-Pen at 500 mg/kg/day (B), PHT at 450 mg/kg/day (C), CBZ at 500 mg/kg/day (D), or SMX at 500 mg/kg/day (E) by gavage for 28 days. Photographs of mesenteric lymph node from BN rat treated with vehicle (F), D-Pen at 500 mg/kg/day (G), AMX at 1000 mg/kg/day (H), or SMX at 500 mg/kg/day (I) by gavage for 28 days. Sections were stained with H&E. Arrows indicate area of germinal center. Bar represents 0.5 (A–E) or 1 (F–I) mm. Photographs are representative of five samples.

other test drugs under the same conditions other than dose levels. These symptoms, however, were not observed in BN rats treated with PHT, CBZ, AMX, SMX or LDC at any dose level, throughout the dosing period in the present study (Fig. 1). These results indicated that clinical observation could not detect the potential of drug hypersensitivity of test drugs in BN rats.

Increases in cell numbers of leucocytes were noted after administration of test drugs other than PHT, and increases in serum IgE concentrations were noted after administration of those other than AMX or LDC.

It has been reported that the number of circulating leukocytes including inflammatory cells increased in patients with drug hypersensitivity reactions, and that serum IgE levels increased in BN rat treated with D-Pen or nevirapine (deShazo and Kemp, 1997; McKenna and Leiferman, 2004; Uetrecht, 2005). Thus, we performed hematological examination designed to examine effects on peripheral blood leukocytes and measurement of serum IgE concentrations in BN rats treated with test drugs for 28 days. In hematological examination, increased numbers of white blood cells, neutrophils, and eosinophils were noted in D-Pen-treated animals (Table 1). These changes showed statistical significance at 500 mg/kg/day, but did not show statistical significance at 100 mg/kg/day. Besides these, increased numbers of monocytes, basophils, lymphocytes, and large unstained cells were also noted at 100 mg/kg/day without statistical significance. Meanwhile, hematological changes were also noted in BN rats treated with several other drugs (Table 1). The number of monocytes, neutrophils, and basophils increased with statistical significance in BN rats treated with CBZ at 500 mg/kg/day, SMZ at 500 mg/kg/day, and LDC at 200 mg/kg/day, respectively. Although there were no statistically significant differences, increased numbers of white blood cells and lymphocytes in BN rats treated

with CBZ at 500 mg/kg/day, and neutrophils in those treated with AMX at 1000 mg/kg/day and SMX at 250 mg/kg/day were noted. In the animals treated with PHT, however, no changes were noted in any leukocyte population. In measurement of serum IgE concentrations, increases were noted in BN rats treated with D-Pen at 100 and 500 mg/kg/day, CBZ at 300 and 500 mg/kg/day, and SMX at 500 mg/kg/day with statistical significance, and in those treated with PHT at 450 mg/kg/day without statistical significance (Table 1). However, these changes were not noted in BN rats treated with AMX or LDC at any dose level (Table 1). These results indicated that hematological parameters could be affected by administration of drugs with less potential of drug hypersensitivity, and that serum IgE concentration was not necessarily affected by the administration of drugs with the potential of drug hypersensitivity in BN rats.

Germinal center hyperplasia in the spleen and/or mesenteric lymph nodes was commonly noted in BN rats treated with D-Pen, PHT, CBZ, AMX, and SMX, but not with LDC.

Next, we performed histological examination of secondary lymphoid organs and tissues. As the object of this examination, spleen, mesenteric lymph nodes, and Peyer's patches were selected. In macroscopic examination, enlarged spleen in BN rats treated with D-Pen, enlarged Peyer's patches in those treated with PHT and CBZ, and enlarged mesenteric lymph nodes in those treated with SMX were noted (data not shown). In microscopic examination, extramedullary hematopoiesis in the spleen as well as germinal center hyperplasia in the spleen and mesenteric lymph nodes was noted in BN rats treated with D-Pen at 100 and 500 mg/kg/day (Table 2). Histological change such as follicular hyperplasia was not observed in the spleen or mesenteric lymph nodes in these animals (Fig. 2). Meanwhile, some findings were noted in animals

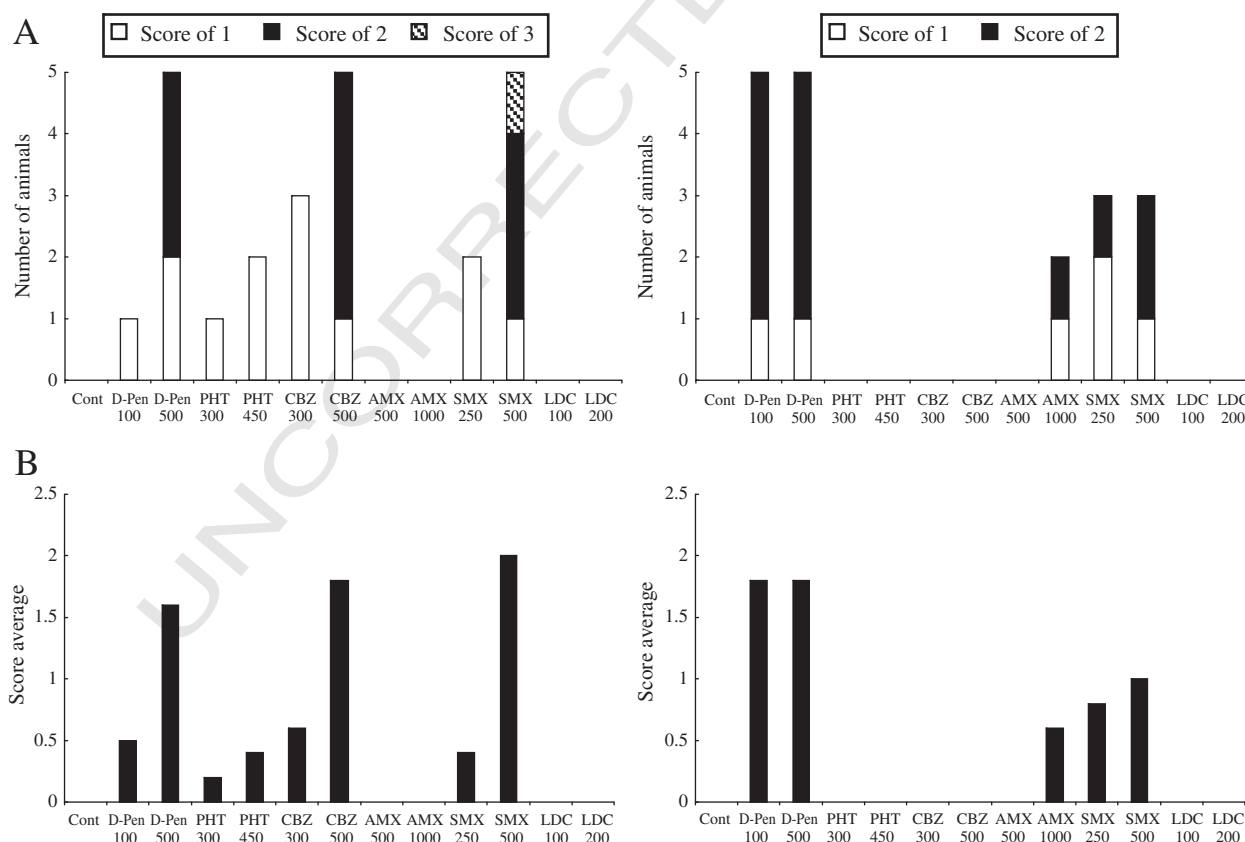


Fig. 3. Effects on severity of germinal center hyperplasia in BN rats treated with drugs for 28 days. (A) Numbers of animals with each germinal center hyperplasia severity score, and (B) score average of germinal center hyperplasia severity in the spleen (left) or mesenteric lymph node (right) of each group. Groups of animals ($n = 5$ /group) were either treated with vehicle, D-Pen at 100 or 500 mg/kg/day, PHT at 300 or 450 mg/kg/day, CBZ at 300 or 500 mg/kg/day, AMX at 500 or 1000 mg/kg/day, SMX at 250 or 500 mg/kg/day or LDC at 100 or 200 mg/kg/day for 28 days. Data were obtained from five animals from each group, except for spleen from a group of D-Pen at 100 mg/kg/day, for which data were obtained from two animals. The severity scores of germinal center hyperplasia were assigned using the scale as presented in Materials and methods.

279 treated with the other test drugs. Cellular infiltration of eosinophil and
 280 granulomatous inflammation were noted in those treated with SMX at
 281 250 and/or 500 mg/kg/day (Table 2). Besides these findings, germinal

center hyperplasia was commonly noted in BN rats treated with PHT, 282
 CBZ, AMX, and SMX (Table 2). This histological change was noted in 283
 different organs/tissues in these animals: in the spleen in the animals 284

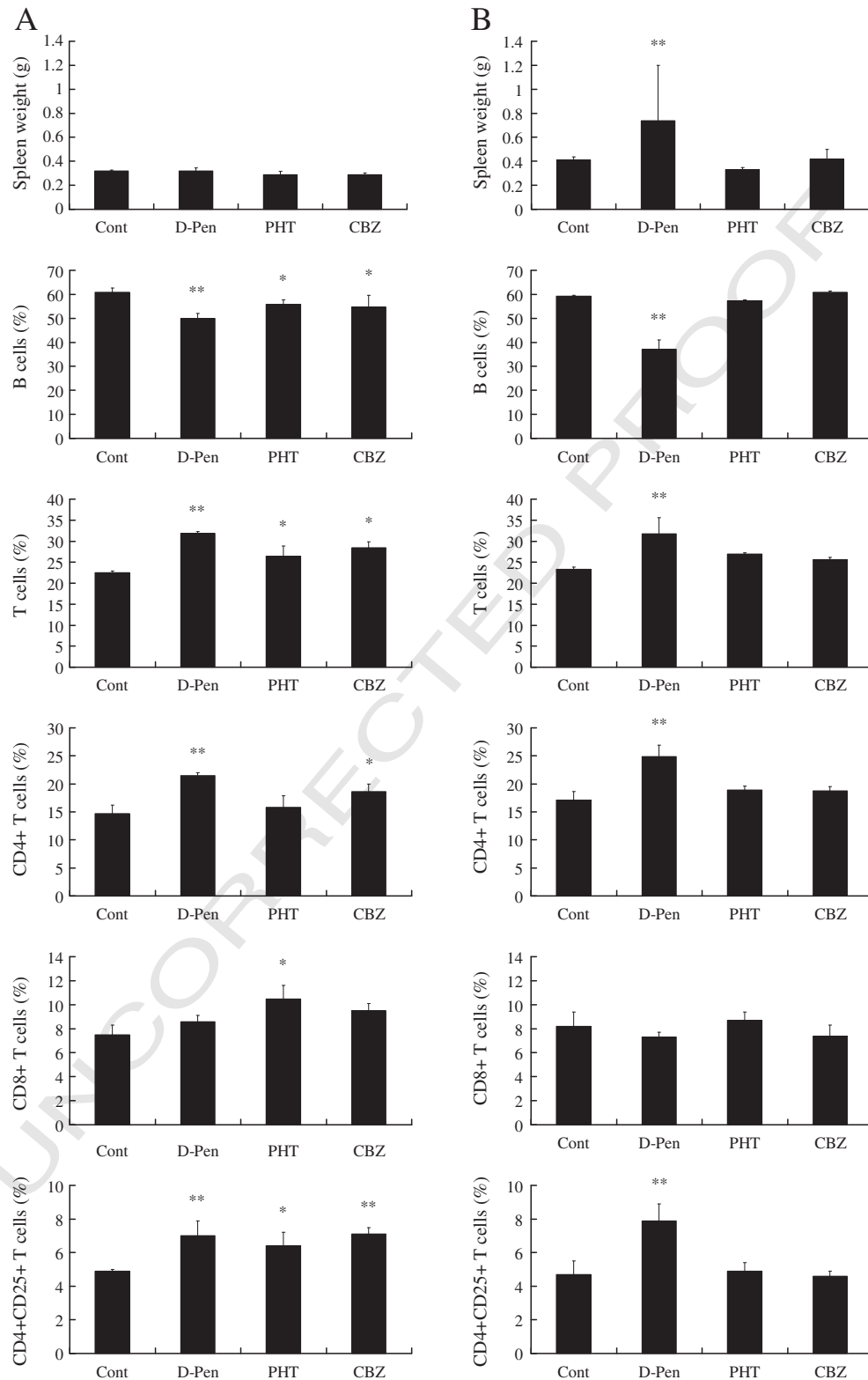
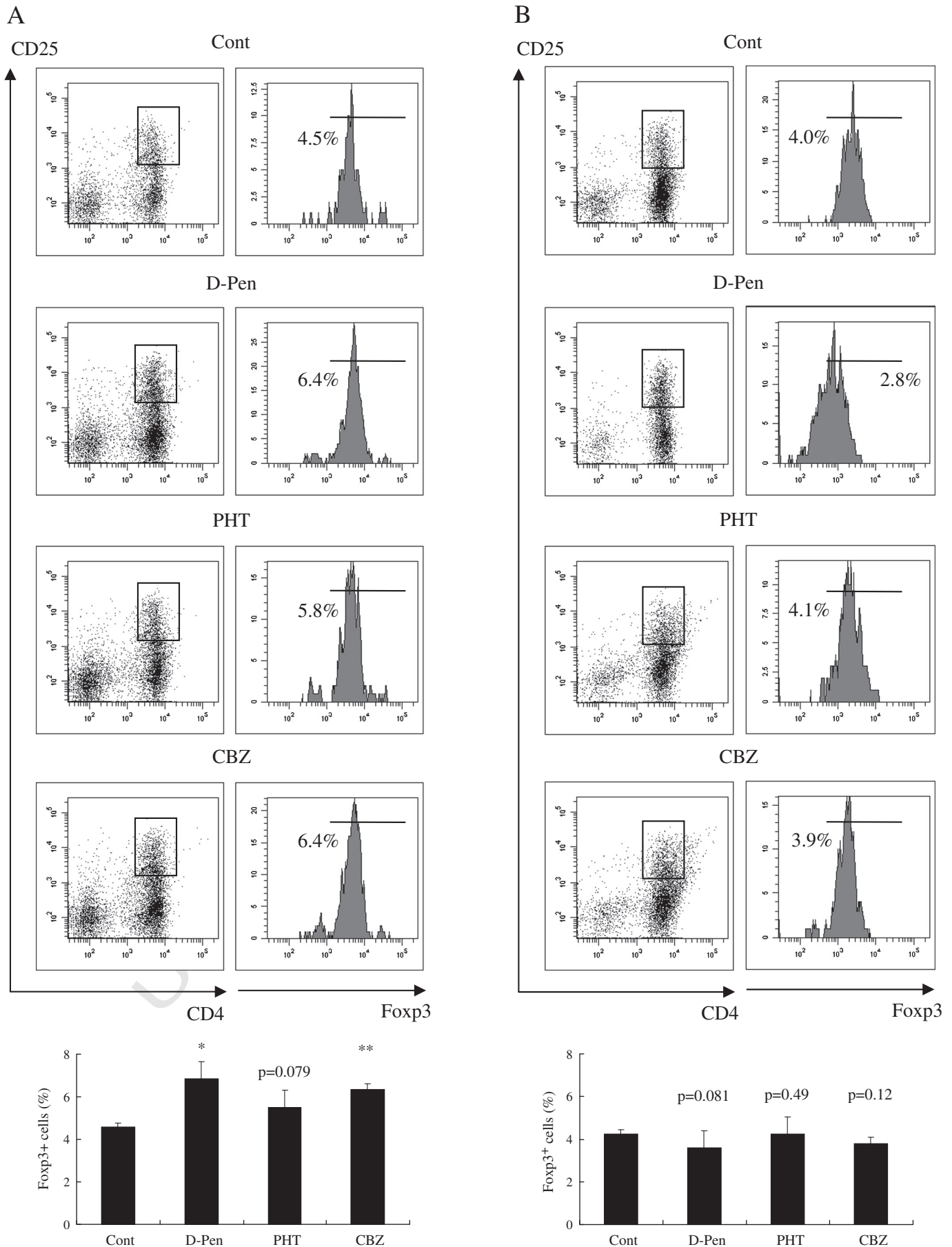


Fig. 4. Effects on spleen weights and proportions of lymphocyte subset in the spleen from BN rats treated with D-Pen, PHT, or CBZ for 7 or 28 days. Spleen weights and lymphocyte subsets of the spleen in BN rats treated with vehicle (Cont), D-Pen at 500 mg/kg, PHT at 450 mg/kg, or CBZ at 500 mg/kg for 7 (A) or 28 (B) days. Spleen weights were measured and proportions of lymphocyte subsets in the spleen were determined by flow cytometric analysis. Spleen weights and proportions of B (CD45RA⁺) cells, T (CD3⁺) cells, CD4⁺, CD8⁺, and CD4⁺CD25⁺ T cells are shown as mean $\pm$ SD of percentage of each lymphocyte subset among total lymphocytes from three rats in each group. * $p < 0.05$; ** $p < 0.001$ administered vs control BN rats.



treated with PHT at 300 and 450 mg/kg/day, CBZ at 300 and 500 mg/kg/day, and SMX at 250 and 500 mg/kg/day; and in mesenteric lymph nodes in those treated with AMX at 1000 mg/kg/day and SMX at 250 and 500 mg/kg/day. Follicular hyperplasia was not noted in the spleen or mesenteric lymph nodes in any of these animals, similarly with the d-pen-treated animals (Fig. 2). The incidence and score average of germinal center hyperplasia of spleen increased in a dose-dependent manner, but not those of mesenteric lymph nodes (Fig. 3). In contrast to these drugs, LDC administration did not induce germinal center hyperplasia in the spleen or mesenteric lymph nodes in BN rats (Table 2). In Peyer's patches, there were no microscopic findings in BN rats treated with any drug (Table 2). These results indicated that histological examination could detect the potential of drug hypersensitivity, and germinal center hyperplasia was only induced by the administration of test drugs with high potential of drug hypersensitivity in BN rats.

Changes in proportions of B cells and T cells were noted in the spleen of BN rats treated with PHT, CBZ, and d-Pen during an early period of administration, but these changes were only sustained in that treated with d-Pen.

To examine immunological changes related to germinal center hyperplasia, we performed flow cytometric analysis of lymphocyte subsets after administration of test drugs. For this purpose, we tested splenocytes from BN rats treated with d-Pen, PHT, or CBZ, prepared 7 and 28 days of administration. Concurrently, we performed histological examination and confirmed that germinal hyperplasia was induced in BN rats treated for 28 days, whereas not in those treated for 7 days (Supplementary Table 1). After 7 days of administration, the effect on lymphocyte subsets was noted in BN rats treated with d-Pen, PHT, and CBZ (Fig. 4). Decreases in proportion of B cells and increases in proportion of T cells were noted by the administration of these drugs. The proportions of CD4⁺ T cells increased by the administration of d-Pen and CBZ, and the proportion of CD8⁺ T cells increased by the administration of PHT. Also, the proportions of CD4⁺CD25⁺ T cells increased by the administration of all these drugs. Meanwhile, after 28 days of administration, the effect was limited to BN rats treated with d-Pen (Fig. 4). The proportion of B cells decreased, and the proportions of T cells, CD4⁺ T cells, and CD4⁺CD25⁺ T cells increased in BN rats treated with d-Pen. However, these changes were not observed in BN rats treated with PHT or CBZ. These results indicated that certain lymphocyte subsets were commonly affected by the administration of d-Pen, PHT or CBZ in BN rats, but the changes of those observed in PHT or CBZ-treated animals were transient, and thus they were not considered related to germinal center hyperplasia.

CD4⁺CD25⁺ T cells in the spleen of BN rats after 7 days of administration of d-Pen, PHT, and CBZ commonly expressed Foxp3, but not after 28 days of administration of d-Pen.

The proportions of CD4⁺CD25⁺ T cells increased in the spleen of BN rats treated with PHT or CBZ for 7 days, but the proportions of these cells were comparable with those in control animals after 28 days of administration. On the other hand, the proportions of CD4⁺CD25⁺ T cells in BN rats treated with d-Pen increased after both 7 and 28 days of administration. Therefore, in an attempt to investigate the possibility that CD4⁺CD25⁺ T cells in BN rats treated with PHT or CBZ is functionally different from those treated with d-Pen, we analyzed their expression of Foxp3, which is known to be a maker of regulatory T cells (Sakaguchi, 2005). CD4⁺CD25⁺ T cells in the spleen of BN rats treated with d-Pen, PHT or CBZ for 7 days commonly expressed Foxp3, and the proportion of Foxp3⁺CD4⁺CD25⁺ T cells increased compared with those in control animals in parallel with the proportions of CD4⁺CD25⁺ T cells (Fig. 5). On the other hand, expression levels of Foxp3 in

CD4⁺CD25⁺ T cells were impaired in BN rats treated with d-Pen for 28 days, and the proportion of Foxp3⁺CD4⁺CD25⁺ T cells in these animals slightly decreased compared with those in control animals (Fig. 5). Meanwhile, expression levels of Foxp3 in CD4⁺CD25⁺ T cells in BN rats treated with PHT or CBZ for 28 days were almost the same as those in control animals, and the proportions of Foxp3⁺CD4⁺CD25⁺ T cells in BN rats treated with these drugs were comparable with those in control animals (Fig. 5). These results indicated that CD4⁺CD25⁺ T cells in BN rats treated with PHT or CBZ for 7 days had the same function as those treated with d-Pen for 7 days, but CD4⁺CD25⁺ T cells observed in the animals treated with d-Pen for 28 days were at least partly different in function from those treated with these drugs for 7 days.

Discussion

BN rat has been used in studies of chemical-induced autoimmunity as well as food allergy (Knippels and Penninks, 2005; Pieters et al., 2002). However, most of these studies focused on cutaneous symptoms and production of allergen-specific IgE in assessment of their potential occurrence. In this study, we have included histological examination of second lymphoid organs/tissues as a new endpoint, and for the first time demonstrated that germinal center hyperplasia is commonly induced by administration of drugs with hypersensitivity potential. Furthermore, while it has been reported that BN rat displayed skin rash and hyperimmunoglobulinemia (mainly IgE) following exposure to d-Pen and Nevirapine (Uetrecht, 2005), we showed here that drugs with potential hypersensitivity other than these drugs also stimulated immune system in BN rat, and the results obtained suggest that germinal center hyperplasia can be used for a predictive biomarker for drug hypersensitivity.

The histological feature of secondary lymphoid organ/tissue observed by the administration of test drugs with hypersensitivity potential in this study was characterized by germinal center hyperplasia. This is different from follicular hyperplasia, which is frequently observed by exposure to immune-stimulating chemicals. The germinal center hyperplasia was noted in the spleens of BN rats treated with mercuric chloride (HgCl₂), and it was also shown that treatment with anti-CD80 and anti-CD86 antibodies prevented germinal center hyperplasia in these rats (MacPhee et al., 2001). These results suggested that germinal center formation induced by exposure to foreign substances requires co-stimulatory signals via CD80 and CD86. It is proposed that distinct co-stimulatory signals, in addition to the antigen, are required for T cell sensitization (Matzinger, 1994), and it is reported that antigen-presenting cells (APCs) respond to haptens, not to chemical irritants, by increasing the surface expression of CD80 and CD86 (Aiba et al., 1997; Azam et al., 2006; Hulet et al., 2005). Indeed, d-Pen treatment was shown to increase the proportion of both CD80 and CD86-expressing macrophages in the spleen in mice (Masson et al., 2004). Taken together, these data suggested that germinal center hyperplasia is a unique immune reaction induced by drugs with a potential to provoke hypersensitive reactions, and it may serve as a useful biomarker for discriminating between sensitizing and nonsensitizing drugs in BN rats.

The germinal center is an area in which B cells proliferate in preparation for antibody production by interacting with T cells that recognize an identical antigen. In the present study, there was a correlation between the incidence of germinal center hyperplasia noted at least in the spleen and dose levels of drugs. Additionally, germinal center hyperplasia was not noted in BN rats after treatment with PHT or CBZ for only 7 days. These results indicated that germinal center hyperplasia depended on the exposure level to drug as well as duration of drug administration, as

Fig. 5. Foxp3⁺CD4⁺CD25⁺ T cells in the spleen of BN rats treated with d-Pen, PHT or CBZ for 7 or 28 days. Proportions of CD25⁺Foxp3⁺CD4⁺ T cells in the spleen from BN rats treated with vehicle (Cont), d-Pen at 500 mg/kg, PHT at 450 mg/kg, or CBZ at 500 mg/kg for 7 (A) or 28 (B) days were determined by flow cytometric analysis. Gating profiles used for identification of CD4⁺CD25⁺ T cells and Foxp3⁺CD4⁺CD25⁺ T cells (percentages) are shown in the panels. Panels are representative of three samples. Proportions are shown as mean ± SD of percentage of Foxp3⁺CD4⁺CD25⁺ T cells among total lymphocytes from three rats in each group (bottom). *p < 0.05; **p < 0.001 administered vs control BN rats.

allergic drugs require those to provoke drug hypersensitivity reactions (Gilliland, 1991; Gomes and Demoly, 2005), but it remained unclear whether the drug/metabolite–protein conjugates were targeted antigens. It is reported that exposure to 3-carbamoyl-2-phenylpropionaldehyde, an intermediate of reactive metabolite of Felbamate, which is supposedly causative in induction of immune-mediated toxicities such as aplastic anemia and hepatotoxicity, to mice induces germinal center formation in lymph nodes (Popovic et al., 2004). This observation implies that the germinal center hyperplasia induced in this study was also targeted by drugs with hypersensitivity potential and/or their metabolites as haptens, but further studies are needed to clarify this.

The location of the germinal center hyperplasia noted in this study differed among drugs. The reason remains unknown, but it is supposed that it associates with the organ and/or tissue in which sufficient amount of immunogenic products and/or co-stimulatory signals to induce immune response was present. For orally administered drugs, the mesenteric lymph nodes and spleen are considered the draining lymphoid organs/tissues exposed to higher concentration of drugs and metabolites, respectively. Most drugs may gain immune reactivity through the hepatic metabolism process, but it was shown that such a process was not indispensable for T cell sensitization induced by AMX and SMX in animal models (Elsheikh et al., 2010; Rozieres et al., 2010). Thus, it is considered that these data explain why these drugs could induce germinal center hyperplasia in mesenteric lymph nodes in this study, but there is still no clear explanation why it was not observed in the spleen of BN rats treated with AMX.

Drug hypersensitivity reactions induced in humans by administration of drugs including those tested in the present study most commonly affect the skin, as reported drug rashes or drug eruptions (McKenna and Leiferman, 2004; Roujeau, 2005). The eruption consists of erythematous macules or papules, and the clinical pattern of the eruption often changes with body site and duration. Urticaria is an eruption of edematous papules and plaques. When dermal and subcutaneous tissues are involved, this reaction is known as angioedema, consisting of pale or pink swelling of skin. Biological features of these reactions vary from one case to another (McKenna and Leiferman, 2004; Roujeau, 2005). Hyperleukocytosis with elevated neutrophil count and eosinophilia is reported in some cases. In the present study, D-pen induced flushing and swelling at various sites on the body, which resembled clinical symptoms in humans, whereas the other drugs did not. Hematological changes, such as increased number of inflammatory cells were observed in BN rats treated with D-Pen, CBZ, AMX, and SMX, which reflected clinical manifestations of drug hypersensitivity, but not with PHT. These data suggested that observation of cutaneous symptoms and hematological examinations were not adequate to discriminate between the drugs with higher and lower potentials of drug hypersensitivity in this model. Meanwhile, there has been no report of germinal center hyperplasia associated with drug hypersensitivity in humans so far. However, it was shown that high exposure to cat or bee venom allergens induced germinal center formation, and that its formation was not necessarily accompanied with IgE production in this situation (Aalberse and Platts-Mills, 2004). These data are consistent with the results obtained in this study, and support our proposal of germinal center hyperplasia as a relevant biomarker for assessing human risk of drug hypersensitivity. However, it remains to be established whether this histological lesion can be predictive of clinical responses that are elicited by allergic drugs.

In flow cytometric analysis, there were some common changes in BN rats treated with D-Pen, PHT, and CBZ during an early period of administration, but not after 28 days of administration. These changes were not considered to be related to germinal center hyperplasia, but it should be noted that CD4⁺CD25⁺ T cell population increased in a transient manner. Further analysis revealed that this population expressed Foxp3, and the result suggested that this type of cells was possibly regulatory T cells. The significance of CD4⁺CD25⁺ T cells in drug-induced autoimmunity has been studied, and the data provide evidence that they were primed by drugs and capable of preventing autoantibody formation,

which is associated with T cell sensitization (Layland et al., 2004). In the present study, a decreased proportion of Foxp3⁺CD4⁺CD25⁺ T cells was observed in D-Pen treated BN rats, and it was considered possible to associate with skin rash. In addition, *Cd25/Il2ra* gene was also identified to be transiently up-regulated in toxicogenomic research of chemical sensitization (Adenuga et al., 2012). Although whether an increase in population of Foxp3⁺CD4⁺CD25⁺ T cells also occurred after administration of AMX and SMX remains unsolved, the evidence suggests that an increment of this population could be another predictive biomarker.

Any biomarker that is intended for use as a safety assessment tool must be accurate and reproducible. We demonstrated that germinal center hyperplasia only occurred after administration of test drugs with high potential of drug hypersensitivity in this study, and also it has been shown that HCB, which is an immune stimulant but devoid of allergic properties (Ezendam et al., 2005), did not induce germinal center formation in BN rats. Although variability exists in the incidence (as shown in Supplementary Table 1), reproducible positive responses were confirmed in BN rats when administered with D-Pen, PHT, and CBZ. Regarding sensitivity, dose level and duration seemed associated with the incidence of germinal center hyperplasia. We consider that the maximum tolerated dose level and 28 days of administration should be set to prevent producing a false-negative result in this model at present, but they should be optimized based on data to be collected in future. This has to be confirmed with more drugs, and a pharmacokinetic approach may be useful in this optimization process.

In summary, we demonstrated here that drugs that are associated with drug hypersensitivity in humans can be identified by using BN rat following repeated oral exposure in combination with histological examination of secondary lymphoid organ/tissue. The present research reveals promising data in the development of oral exposure models that are urgently needed for hazard identification of newly developed drugs with regard to their potential to induce drug hypersensitivity reactions in humans. The use of BN rat and standardized histological techniques enables observation of germinal center hyperplasia and makes this model a suitable tool for screening the immunosensitizing potential of new chemical entities during preclinical drug development. Future experiments that focus on what biomarkers best reflect the risk of various types of drug hypersensitivity reaction, and also on the development of high-throughput in vitro assays with these biomarkers will be needed.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2013.04.022>.

Conflict of interest

The authors declare no competing financial interests.

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