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Original Article

Strain differences in histopathological features of lymphoid tissues of SD and F344 rats in a T cell-dependent antibody response assay of cyclophosphamide

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Abstract: When conducting histopathological evaluation of lymphoid tissues, it is necessary to know the variability and strain differences in histological features of different sites of lymphoid tissues. To investigate in detail the variability of lymphoid tissues and strain differences of control rats as well as those of immune reactivity and sensitivity to immunosuppression, we performed a histopathological analysis of various lymphoid tissues in conjunction with the evaluation of immune function in a T cell-dependent antibody response (TDAR) assay with cyclophosphamide (CP) in Sprague Dawley (SD) and F344 rats. Six-week-old male SD and F344 rats were orally treated with CP at 0 (control) or 4 mg/kg/day for 28 days; keyhole limpet hemocyanin (KLH) was introduced intravenously on Days 14 and 23, and the serum concentrations of anti-KLH antibodies were measured. HE staining and immunohistochemistry for T-cell (CD3) and B-cell (CD45RA) markers were performed using tissues from the spleen, thymus, and various lymph nodes. In CP-treated rats of both strains, decreased concentrations of anti-KLH antibodies were observed. Histopathological analysis revealed decreased lymphocytes mainly in the B-cell area, and these changes induced by CP treatment were more prominent in the F344 rats than in the SD rats. The present study also demonstrated that some of the lymphoid tissues of the control F344 rats were less developed than those of the control SD rats, suggesting that F344 rats might be easily affected by CP-induced immunosuppression. This information concerning rat strain differences in lymphoid tissues will be useful in histopathological evaluation for drug-induced immunotoxicity. (DOI: 10.1293/tox.2018-0052; J Toxicol Pathol 2019; 32: 143–154)

Key words: rat, lymphoid tissues, strain difference, immunotoxicity, T cell-dependent antibody response assay, cyclophosphamide

Introduction

Evaluation of potential adverse effects on the immune system is very important in drug development. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) S8 guidelines[1] recommend a "weight of evidence" approach regarding the evaluation of immunotoxicity, and immune function evaluations are recommended if a test article is suspected to have immunotoxic potential based on standard toxicology parameters (e.g., immune organ weights, hematology, and histopathology). Histopathological examination in nonclinical toxicity studies plays an important role, especially in the initial screening for potential immunotoxicity. The guidelines also recommend that the spleen, thymus, draining lymph node (LN) and at least one additional LN, and bone marrow should be evaluated in histopathological examination in standard toxicity studies. For orally administered drugs, Peyer's patches that are exposed to a high concentration of a drug should also be evaluated histopathologically. As for the detailed method of histopathological examination, "semiquantitative" descriptions of changes in each compartment are recommended[1, 2]. Since the histological features of normal lymphoid tissues can be highly variable by species, strain, age, sex, and their location, matched controls and background information are important in toxicity studies[3, 4]. However, there are only a few reports that have investigated the histological features of various lymphoid tissues, and more information, including strain differences, is needed[5].

The T cell-dependent antibody response (TDAR) assay is an immunotoxicity study for evaluating immune function, and a TDAR assay is recommended when a test article is suspected to have immunotoxic potential but a specific target has not been identified[1]. As a T cell-dependent anti-

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gen, keyhole limpet hemocyanin (KLH) is now used more often than sheep red blood cells (SRBC) in the pharmaceutical industry because KLH is easier to handle and obtain and is more stable than SRBC[6, 7]. Measurement of the primary antibody response to KLH in rats using an ELISA is a standardized method for evaluating the immunotoxicity of a compound in the pharmaceutical industry[6]. The use of TDAR in various experimental animals (e.g., mice, rats, dogs, nonhuman primates) has been reported[3, 7, 8], and the immune response to a T cell-dependent antigen differs among not only species but also among strains[3, 9–11]. In rats, outbred rats have more obvious individual differences in antibody production than inbred ones[1, 9, 12]. Though the difference in reactivity to a T cell-dependent antigen seems to be related to genetic diversity, the detailed mechanism is not clear. At present, a detailed histopathological analysis performed in conjunction with the TDAR assay has not been reported, and only a few reports have interpreted limited histopathological examinations in TDAR studies[8].

In the present study, to investigate in detail the variability of lymphoid tissues and strain differences of control rats as well as those of immune reactivity and sensitivity to immunosuppression, we conducted a histopathological analysis of various lymphoid tissues in conjunction with evaluation of immune function in a TDAR study of cyclophosphamide (CP) in Sprague Dawley (SD) rats and F344 rats.

Materials and Methods

Chemicals and animals

As a T cell-dependent antigen, KLH was selected and purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). CP, which is an alkylating anticancer drug and has an immunosuppressive effect, was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

Sixteen 5-week-old male SD (Crl:CD [SD]) and F344 (F344/DuCrlCrlj) rats, as representative strains of outbred and inbred rats, respectively, were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan) and housed individually in polycarbonate cages with wood chip bedding in an air-conditioned animal room (temperature 23 ± 3°C, relative humidity 50 ± 20%) with a 12-h light/dark cycle. They were given a pellet basal diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and well water ad libitum.

Experimental design

Animals in each strain were divided into control and CP groups (n=8) and were orally treated with CP at a dose of 0 (control) or 4 mg/kg/day from the age of 6-weeks for 28 consecutive days (Day 1: day of first administration) (Fig. 1). Based on previous reports, a CP dose capable of producing minimal adverse effects, such as a decrease in body weight, was selected[13–15]. Physical examinations were conducted twice a day before and after dosing throughout the dosing period, and body weight was measured on Days 1, 3, 8, 15, 22, and 29. KLH dissolved in saline was administered intravenously at 300 μg per animal on Days 14 and 23, and blood samples for measuring the concentrations of KLH-specific immunoglobulins were collected on Days 20 and 29, based on the protocol described by Kawai *et al.*[16]. On Day 29, after collecting the blood samples for measuring the hematologic parameters and the flow cytometry analysis, animals were sacrificed by exsanguination from the abdominal aorta under deep isoflurane anesthesia and then necropsied. The following organs were weighed using an electric scale: spleen, thymus, mesenteric LN, mandibular LN, axillary LN, popliteal LN, and adrenal. Relative organ weight was calculated by dividing the organ weight by the final body weight. According to ICH S8 guidelines[1], the spleen, thymus, mesenteric LN as a draining LN, Peyer's patch in the ileum, mandibular LN, and bone marrow in the femur and sternum were collected. In addition to these routine lymphoid organs, some LNs, the axillary LN, popliteal LN, inguinal LN, pulmonary LN, and iliac LN, were also selected for detailed evaluation of immunotoxicity. The adrenal gland was also evaluated to confirm stress condition.

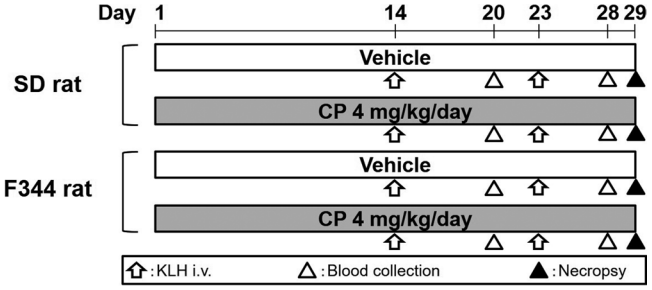

Fig. 1. Experimental design. Male SD and F344 rats were divided into control and cyclophosphamide (CP) groups (n=8 each) and were orally treated with CP at a dose of 0 (control) or 4 mg/kg/day beginning at the age of 6 weeks and continuing for 28 consecutive days. Keyhole limpet hemocyanin (KLH) was administered intravenously on Days 14 and 23, and blood samples were collected on Days 20 and 29. After blood collection on Day 29, the animals were sacrificed for necropsy.

This study was conducted in accordance with the Guidelines for Animal Experimentation specified by the Research Center of Taisho Pharmaceutical Co., Ltd.

Concentrations of serum anti-KLH IgM and IgG and hematology

The blood samples collected on Days 20 and 29 were centrifuged to obtain serum, and the concentrations of serum KLH-specific IgM (Days 20 and 29) and IgG (Day 29) were measured using an ELISA method with an Anti-KLH (TDAR) Rat ELISA KIT (AKRKM-010 for IgM, AKRKG-010 for IgG, Shibayagi, Gunma, Japan).

Hematological parameters including the white blood cell (WBC) count, differential WBC absolute counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells), red blood cell count, hemoglobin level, hematocrit level, reticulocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin

(MCH) level, mean corpuscular hemoglobin concentration (MCHC), and platelet count were measured using a hematology system (ADVIA 120, Siemens Healthcare Diagnostics K.K., Tokyo, Japan).

Immunophenotyping analysis using flow cytometry

To assess the cell subtypes, an immunophenotyping analysis was conducted using flow cytometry. The blood samples collected at necropsy were placed into EDTA-2K-treated tubes, centrifuged to isolate live cells, and treated with a mixture of labeled antibodies: FITC-labeled anti-CD3 (clone G4.18, BD Biosciences, Franklin Lakes, NJ, USA) with PE-labeled anti-CD45RA (clone OX-33, BD Biosciences), PE-labeled anti-CD8 (clone OX-8, BD Biosciences), or PE-labeled anti-CD4 (clone OX-35, BD Biosciences). A flow cytometry analysis was performed using a flow cytometer (EPICS-XL, Beckman Coulter, Inc., Brea, CA, USA). The percentages of CD3−/CD45RA+ (B cells), CD3+/CD45RA− (T cells), CD3+/CD4+ (helper T cells, Th) and CD3+/CD8+ (cytotoxic T cells, Tc) were determined, and the T-cell ratio (Th/Tc) was calculated.

HE staining

The organs collected at necropsy were fixed in 10% neutral buffered formalin. The bone marrow with femur and sternum were fixed in 10% neutral buffered formalin and were decalcified using a rapid decalcification method (Plank-Rychlo's method). After fixation, the tissues were embedded in paraffin, stained with hematoxylin and eosin (HE), and examined microscopically.

Immunohistochemistry and morphometry of immunopositive cells

The following lymphoid organs were trimmed before formalin fixation, frozen immediately, embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and stored in a deep freezer for immunohistochemistry: spleen, mesenteric LN, mandibular LN, axillary LN, and popliteal LN. Immunohistochemistry was performed on frozen sections with antibodies against CD3 (IR503, rabbit, 1:2, Dako, Glostrup, Denmark) and CD45RA (clone OX-33, mouse IgG1, 1:500, AbD Serotec, Bio-Rad Laboratories, Hercules, CA, USA). These primary antibodies were selected with reference to a previous report[17]. Immunodetection was performed using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3'-diaminobenzidine/H_2O_2 as the chromogen. Sections were then counterstained with hematoxylin and cover-slipped for microscopic examination. One immunohistochemistry section per organ per animal was prepared.

The immunohistochemistry sections were scanned using a Leica Aperio Scanscope XT (Leica Microsystems, Wetzlar, Germany), and the number of immunopositive cells (Np) in the whole tissue area of each staining section was measured by counting the number of immunopositive pixels with the Leica Aperio Positive Pixel Count Algorithm, version 9.1. The tissue area of each organ was measured using the Leica Aperio ImageScope digital slide viewing software, and the Np per tissue area (Np per area) was also calculated.

Statistical analysis

For each group, the mean and standard deviation were calculated for numerical data (body weights, concentrations of serum immunoglobulin, hematological parameters, immunophenotyping analysis, organ weights, and morphometric assessments of immunopositive cells). Statistical significance between the control and CP-treated rats was examined as follows. For each strain of rats, the homogeneity of variance was analyzed using an F-test; Student's *t*-test was then performed when the variance was proven to be homogenous. If a significant difference in variance was observed, Welch's *t*-test was performed.

Results

Clinical signs and body weights

No treatment-related changes in clinical signs were observed in any of the SD or F344 rats during the experimental period. In CP-treated rats of both strains, minimal body weight gain prevention was observed during the last week of the dosing period. Statistically significant decreases in mean body weight were observed on Day 22 (−6% vs. control) and Day 29 (−7% vs. control) in the CP-treated SD rats and on Day 29 (−4% vs. control) in the CP-treated F344 rats.

Concentrations of serum anti-KLH IgM and IgG and hematology

The mean concentrations of serum anti-KLH IgM and IgG are shown in Fig. 2. In CP-treated SD rats, a decrease in the concentration of serum anti-KLH IgM (−45% vs. control) was observed on Day 20, but the difference was not statistically significant. On Day 29, statistically significant decreases in the concentrations of serum anti-KLH IgM and IgG (−86% and −67% vs. control, respectively) were observed. On the other hand, in CP-treated F344 rats, a statistically significant decrease in the concentration of serum anti-KLH IgM (−97% vs. control) was observed from Day 20, and significant decreases in the concentrations of serum anti-KLH IgM and IgG (−98% and −100% vs. control, respectively) were observed on Day 29. Regarding inter-individual variability, the control SD rats exhibited a larger variation (standard deviation) than the control F344 rats. The mean values of hematologic parameters are shown in Table 1. Some changes in hematologic parameters related to the decrease in serum anti-KLH immunoglobulin were observed on Day 29. Concretely, in CP-treated SD and F344 rats, statistically significant decreases in mean white blood cells (−69% and −80% vs. control, respectively), especially in lymphocytes (−75% and −87% vs. control, respectively), were observed, and slight but statistically significant decreases in the numbers or percentages of other white blood cell parameters (monocytes, eosinophils, basophils, and large unstained cells) were also seen. In relation to the se-

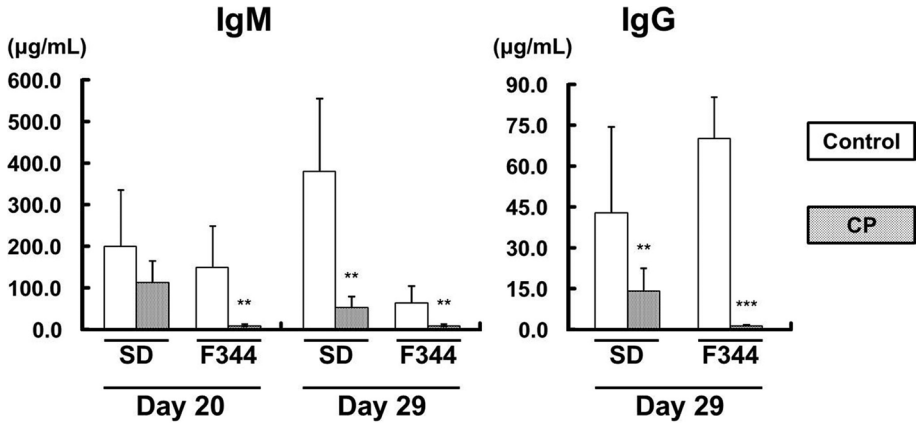

Fig. 2. Serum concentrations of anti-keyhole limpet hemocyanin (KLH) IgM and IgG. The concentrations of serum KLH-specific IgM (Days 20 and 29) and IgG (Day 29) are presented as the mean ± SD. **P*<0.05, ***P*<0.01, and ****P*<0.001 versus the control group of each strain (Welch's *t*-test).

Table 1. Hematologic Parameters of SD and F344 Rats Treated with Cyclophosphamide (CP) at 0 or 4 mg/kg/day for 28 Days

Strain		Crl:CD(SD)			F344/DuCrlCrlj		
Test article		Control	CP		Control	CP	
Dose (mg/kg)		0	4		0	4	
Number of animals		8	8		7	7	
White blood cells	×10³/μL	10.754 ± 2.819	3.321 ± 1.350	***	5.864 ± 1.630	1.151 ± 0.155	***
Red blood cells	×10⁶/μL	7.535 ± 0.189	7.519 ± 0.582		8.266 ± 0.230	7.771 ± 0.152	***
Hemoglobin	g/dL	14.83 ± 0.33	14.85 ± 0.57		14.94 ± 0.40	14.34 ± 0.29	**
Hematocrit	%	43.14 ± 0.82	42.69 ± 2.10		43.39 ± 0.97	41.27 ± 0.76	***
MCV	fL	57.30 ± 2.05	56.89 ± 2.15		52.49 ± 0.38	53.10 ± 0.29	**
MCH	pg	19.70 ± 0.79	19.81 ± 0.91		18.07 ± 0.24	18.47 ± 0.18	**
MCHC	g/dL	34.35 ± 0.49	34.83 ± 0.74		34.47 ± 0.51	34.79 ± 0.39	
Platelets	×10³/μL	1170.9 ± 127.7	1136.5 ± 142.3		798.4 ± 45.6	601.0 ± 181.1	*
Reticulocytes	%	2.73 ± 0.25	2.33 ± 0.40	*	2.76 ± 0.17	2.80 ± 0.51	
Neutrophils	%	13.14 ± 4.99	22.83 ± 12.59		14.71 ± 3.63	38.97 ± 6.62	***
	×10³/μL	1.365 ± 0.527	0.873 ± 0.849		0.859 ± 0.346	0.453 ± 0.126	*
Lymphocytes	%	82.04 ± 5.48	70.13 ± 14.08		80.90 ± 3.93	52.86 ± 7.28	***
	×10³/μL	8.870 ± 2.661	2.211 ± 0.647	***	4.741 ± 1.279	0.604 ± 0.080	***
Monocytes	%	3.14 ± 0.95	5.29 ± 1.96	*	3.19 ± 0.59	6.54 ± 1.25	***
	×10³/μL	0.333 ± 0.122	0.185 ± 0.126	*	0.191 ± 0.085	0.074 ± 0.020	*
Eosinophils	%	1.11 ± 0.50	1.31 ± 0.57		0.63 ± 0.10	1.24 ± 0.79	
	×10³/μL	0.119 ± 0.062	0.039 ± 0.011	**	0.036 ± 0.011	0.013 ± 0.008	***
Basophils	%	0.30 ± 0.09	0.15 ± 0.12	*	0.14 ± 0.08	0.23 ± 0.18	
	×10³/μL	0.035 ± 0.019	0.004 ± 0.005	**	0.010 ± 0.006	0.000 ± 0.000	**
Large unstained cells	%	0.31 ± 0.11	0.34 ± 0.15		0.41 ± 0.17	0.19 ± 0.11	*
	×10³/μL	0.039 ± 0.022	0.013 ± 0.009	*	0.027 ± 0.018	0.000 ± 0.000	**

Mean ± SD. Significantly different from the control (0 mg/kg) of the same strain: **P*<0.05; ***P*<0.01; ****P*<0.001. MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

vere decrease in lymphocytes, the percentages of neutrophils in F344 rats and of monocytes in SD and F344 rats were relatively increased. In addition, some of the red blood cell parameters were also changed. In CP-treated F344 rats, the number of red blood cells was slightly but significantly decreased, and other parameters related to red blood cells (hemoglobin, hematocrit, MCV, MCH, and platelets) were also changed. In CP-treated SD rats, a slight but statistically significant decrease in reticulocytes was observed.

Immunophenotyping analysis using flow cytometry

The mean percentages of immunopositive cells are shown in Table 2. In CP-treated SD rats, a severe decrease in CD3−/CD45RA+ cells (−78% vs. control) and a slight increase in CD3+/CD45RA- cells (+22% vs. control) were observed. In CP-treated F344 rats, a decrease in CD3−/CD45RA+ cells (−98% vs. control) was observed, and the change was much severer than that in CP-treated SD rats. In CP-treated F344 rats, decreases in CD3+/CD4+ cells and CD3+/CD8+ cells (−44% vs. control each) were also observed. In both strains, no remarkable changes in the T-cell ratio were seen.

Table 2. Immunophenotyping Analysis of Peripheral Blood of SD and F344 Rats Treated with Cyclophosphamide (CP) at 0 or 4 mg/kg/day for 28 Days

Strain		Crl:CD(SD)			F344/DuCrlCrlj		
Test article		Control	CP		Control	CP	
Dose (mg/kg)		0	4		0	4	
Number of animals		8	8		7	7	
$CD3^-$ $CD45RA^+$	%	36.4 ± 11.9	7.9 ± 2.4	***	15.6 ± 5.4	0.3 ± 0.1	***
$CD3^+$ $CD45RA^-$	%	42.9 ± 6.6	52.2 ± 9.1	*	57.8 ± 13.3	46.6 ± 9.2	
$CD3^+$ $CD4^+$	%	22.9 ± 4.2	25.1 ± 7.9		32.8 ± 3.8	18.3 ± 6.2	***
$CD3^+$ $CD8^+$	%	15.0 ± 2.8	19.3 ± 5.9	*	18.7 ± 2.2	10.4 ± 3.3	***
Th/Tc		1.6 ± 0.4	1.4 ± 0.5		1.8 ± 0.1	1.7 ± 0.1	

Mean ± SD. Significantly different from the control (0 mg/kg) of the same strain: *P<0.05; **P<0.01; ***P<0.001.

Table 3. Organ Weights of SD and F344 Rats Treated with Cyclophosphamide (CP) at 0 or 4 mg/kg/day for 28 Days

Strain		Crl:CD(SD)			F344/DuCrlCrlj		
Test article		Control	CP		Control	CP	
Dose (mg/kg)		0	4		0	4	
Number of animals		8	8		8[a]	8[a]	
Spleen	g	0.820 ± 0.039	0.499 ± 0.084	***	0.595 ± 0.032	0.324 ± 0.026	***
	g/100 g BW	0.195 ± 0.011	0.127 ± 0.019	***	0.261 ± 0.010	0.148 ± 0.010	***
Thymus	g	0.636 ± 0.125	0.481 ± 0.089	*	0.285 ± 0.023	0.133 ± 0.018	***
	g/100 g BW	0.152 ± 0.033	0.122 ± 0.019		0.125 ± 0.011	0.061 ± 0.008	***
Mesenteric LN	mg	155.0 ± 33.0	92.8 ± 24.2	***	116.0 ± 36.5	58.1 ± 21.8	**
	mg/100 g BW	37.0 ± 8.5	23.5 ± 5.5	**	51.1 ± 17.1	26.8 ± 10.4	**
Mandibular LN	mg	218.4 ± 34.8	120.6 ± 30.4	***	128.4 ± 28.4	56.8 ± 20.6	***
	mg/100 g BW	51.7 ± 6.1	30.7 ± 7.7	***	56.2 ± 11.9	26.0 ± 9.3	***
Axillary LN	mg	98.6 ± 35.3	68.1 ± 38.1		54.0 ± 18.3	22.4 ± 13.4	**
	mg/100 g BW	23.3 ± 7.7	17.4 ± 10.1		23.6 ± 7.7	10.2 ± 6.1	**
Popliteal LN	mg	20.1 ± 11.9	19.6 ± 8.0		7.7 ± 4.5	2.6 ± 0.8	*
	mg/100 g BW	4.8 ± 3.0	5.0 ± 2.0		3.4 ± 2.0	1.2 ± 0.4	*
Adrenal	mg	47.1 ± 7.8	45.6 ± 6.2		27.4 ± 1.1	28.9 ± 1.2	*
	mg/100 g BW	11.2 ± 1.7	11.6 ± 1.4		12.0 ± 0.4	13.2 ± 0.7	**

Mean ± SD. Significantly different from the control (0 mg/kg) of the same strain: *P<0.05; **P<0.01; ***P<0.001. LN, lymph node.
[a]For the axillary and popliteal LNs, the number of animals examined was seven.

Organ weights

The mean organ weights are shown in Table 3. In CP-treated rats of both strains, the absolute and relative weights of the spleen, mesenteric LN, and mandibular LN were significantly lower than those in each of the respective control groups. In addition, statistically significant decreases in the absolute and relative weights of the thymus, axillary LN, and popliteal LN were observed in CP-treated F344 rats. A slight but statistically significant increase in the adrenal weight was also observed in CP-treated F344 rats.

Histopathology (HE staining and immunohistochemistry)

The main histopathological findings that were obtained using HE staining and immunohistochemistry are summarized in Table 4. Various changes indicating immunosuppression were observed in the lymphoid tissues in CP-treated rats of both strains. In the spleen, decreases in lymphocytes in the white pulp (follicles, germinal centers, and periarteriolar lymphoid sheath [PALS]) and marginal zone (MZ) were observed (Fig. 3). In the mesenteric, mandibular, axillary, popliteal, inguinal, pulmonary, and iliac LN, decreases in lymphocytes in the cortex (follicles and germinal centers) were observed. In addition to the regions in the cortex, decreases in lymphocytes in the paracortex were observed in the axillary, popliteal, inguinal, pulmonary, or iliac LN (Fig. 4). Decreases in lymphocytes of follicles and germinal centers were also observed in Peyer's patch. In addition, a decrease in lymphocytes in the interfollicular region (IFR) was observed in CP-treated F344 rats (Fig. 4). In many of these lymphoid organs, the decreases in lymphocytes were more prominent in the F344 rats than in the SD rats. Regarding the thymus, decreases in lymphocytes in the cortex were observed in CP-treated F344 rats, but remarkable changes were not observed in the thymus of the CP-treated SD rats. Though differences in the sensitivity to CP immunosuppression were not observed clearly among these lymphoid organs, the changes in the mesenteric LN and Peyer's patch were milder than those in the other LNs or spleen. In other findings, some spontaneous changes were observed in the control and CP-treated groups in each strain. Increases in plasma cells in the sinus were observed in the mandibular LN, and this finding was more frequently observed in SD rats than in F344 rats. In the pulmonary LN, hemorrhage

Table 4. Histopathological Findings in SD and F344 Rats Treated with Cyclophosphamide (CP) at 0 or 4 mg/kg/day for 28 Days

Strain	Crl:CD(SD)								F344/DuCrlCrlj							
Test article / Dose	Control / 0 mg/kg				CP / 4 mg/kg				Control / 0 mg/kg				CP / 4 mg/kg			
Number of animals	8 [a]				8				8 [a]				8 [b]			
Grade	−	±	+	2+	−	±	+	2+	−	±	+	2+	−	±	+	2+
Spleen																
Follicles, decreased lymphocytes	8	0	0	0	0	0	8	0	8	0	0	0	0	0	0	8
Germinal centers, decreased	8	0	0	0	0	0	8	0	4	4	0	0	0	0	0	8
PALS, decreased lymphocytes	8	0	0	0	0	8	0	0	8	0	0	0	0	0	8	0
MZ decreased lymphocytes	8	0	0	0	0	0	8	0	8	0	0	0	0	0	0	8
Thymus																
Cortex, decreased lymphocytes	8	0	0	0	8	0	0	0	8	0	0	0	5	3	0	0
Mesenteric LN																
Follicles, decreased lymphocytes	8	0	0	0	3	5	0	0	8	0	0	0	0	0	8	0
Germinal centers, decreased	7	1	0	0	0	3	5	0	1	7	0	0	0	0	8	0
Mandibular LN																
Follicles, decreased lymphocytes	8	0	0	0	1	5	2	0	8	0	0	0	0	0	5	3
Germinal centers, decreased	8	0	0	0	0	6	2	0	8	0	0	0	0	0	5	3
Increased plasma cells	3	5	0	0	3	5	0	0	6	2	0	0	8	0	0	0
Axillary LN																
Follicles, decreased lymphocytes	8	0	0	0	4	3	1	0	8	0	0	0	0	0	4	3
Germinal centers, decreased	5	2	1	0	0	0	8	0	0	5	3	0	0	0	4	3
Paracortex, decreased lymphocytes	8	0	0	0	8	0	0	0	8	0	0	0	3	3	0	1
Popliteal LN																
Follicles, decreased lymphocytes	8	0	0	0	3	4	1	0	8	0	0	0	0	0	0	7
Germinal centers, decreased	6	2	0	0	0	1	7	0	0	2	6	0	0	0	0	7
Paracortex, decreased lymphocytes	8	0	0	0	8	0	0	0	8	0	0	0	0	1	2	4
Inguinal LN																
Follicles, decreased lymphocytes	7	0	0	0	2	2	3	1	7	0	0	0	0	1	2	3
Germinal centers, decreased	4	3	0	0	0	0	7	1	1	2	4	0	0	1	2	3
Paracortex, decreased lymphocytes	7	0	0	0	6	1	0	1	7	0	0	0	3	2	0	1
Pulmonary LN																
Follicles, decreased lymphocytes	8	0	0	0	1	2	4	1	8	0	0	0	0	1	3	4
Germinal centers, decreased	1	6	1	0	0	0	7	1	0	2	6	0	0	0	4	4
Paracortex, decreased lymphocytes	8	0	0	0	6	1	1	0	8	0	0	0	4	3	1	0
Hemorrhage	7	1	0	0	8	0	0	0	6	1	1	0	6	0	2	0
Iliac LN																
Follicles, decreased lymphocytes	8	0	0	0	2	1	3	2	4	2	2	0	0	0	2	4
Germinal centers, decreased	5	0	3	0	0	0	6	2	3	0	5	0	0	0	2	4
Paracortex, decreased lymphocytes	8	0	0	0	5	2	1	0	8	0	0	0	1	1	4	0
Peyer's patches																
Follicles, decreased lymphocytes	8	0	0	0	7	1	0	0	8	0	0	0	1	1	4	0
Germinal centers, decreased	8	0	0	0	3	5	0	0	8	0	0	0	0	2	4	0
IFR, decreased lymphocytes	8	0	0	0	8	0	0	0	8	0	0	0	2	4	0	0
Bone marrow (femur and sternum)																
Hypocellularity	8	0	0	0	8	0	0	0	0	8	0	0	0	8	0	0

Symbols: −, none/negligible; ±, minimal/slight; +, mild; 2+, moderate. LN, lymph node; PALS, periarteriolar lymphoid sheath; MZ,marginal zone; IFR, interfollicular region. [a]For the inguinal LNs, the number of animals examined was seven. [b]For the axillary and popliteal LNs, the number of animals examined was seven; it was six for the inguinal LNs, iliac LNs, and Peyer's patches.

with brown pigmented macrophages was observed in both strains, and this finding was observed to be more severe and more frequent in F344 rats than in SD rats. In the control groups for both strains, decreases in germinal centers were sometimes observed, and the incidence or degree of this change was higher in the axillary, popliteal, inguinal, pulmonary, or iliac LNs than in the mesenteric or mandibular LNs. In the control F344 rats, the incidences or degrees of decreased germinal centers in the spleen, mesenteric LN, axillary LN, popliteal LN, inguinal LN, and pulmonary LN were higher than those in the control SD rats. In addition, in F344 rats, decreases in lymphocytes in the cortex of the iliac LN were also observed in the control group (Fig. 4), and hypocellularity in the bone marrow was observed not only in the CP-treated rats but also in the control rats. There were no differences in the degree of hypocellularity in the bone marrow between the femur and sternum. No remarkable changes in the adrenal gland were observed in either strain.

Immunohistochemistry and morphometry of immunopositive cells

The mean numbers and mean numbers per area of immunopositive cells are shown in Table 5. The immuno-

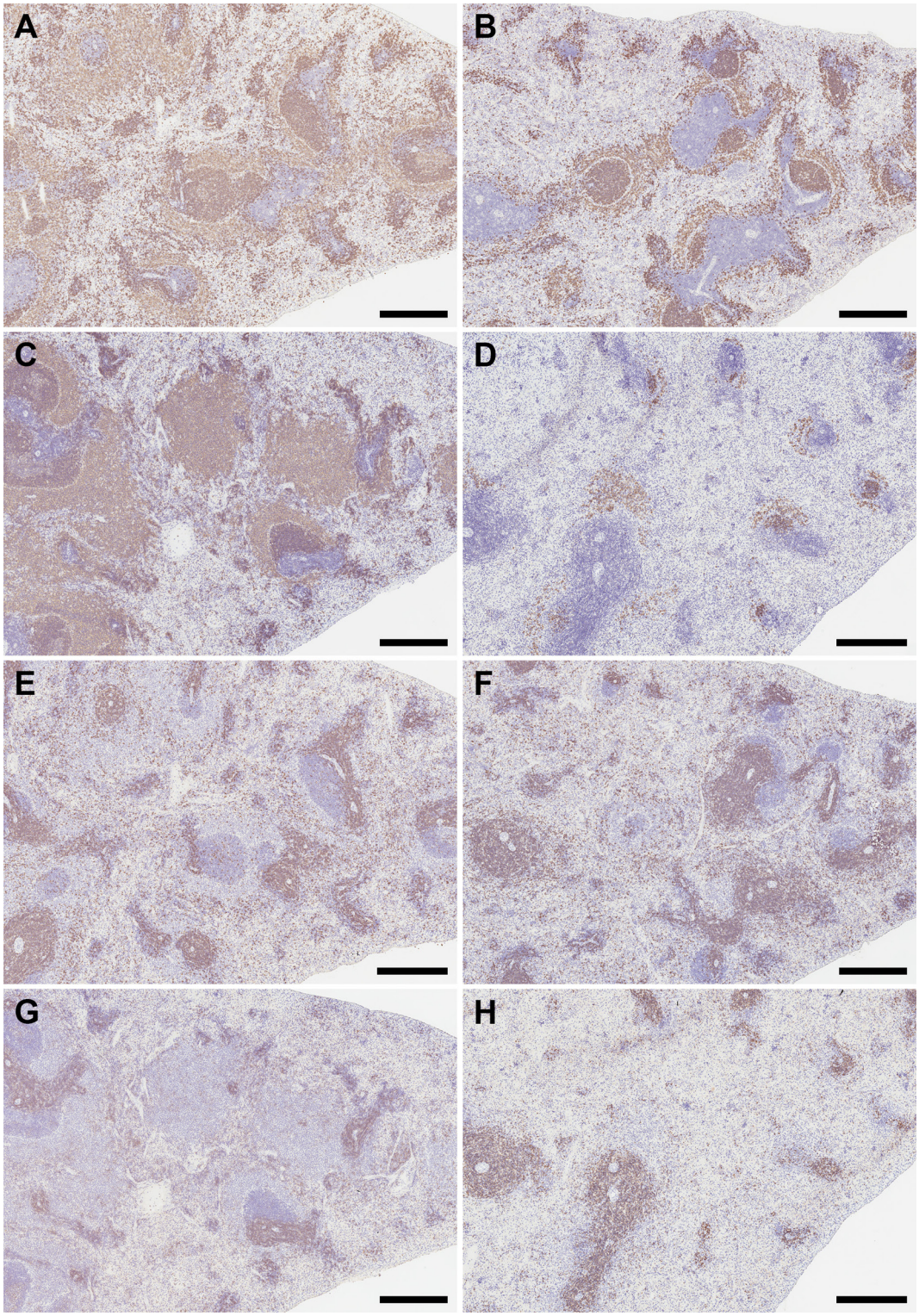

Fig. 3. Immunohistochemistry for CD45RA (A–D) and CD3 (E–H) in the spleen. (A, E) Control SD rats. (B, F) Cyclophosphamide (CP)-treated SD rats. (C, G) Control F344 rats. (D, H) CP-treated F344 rats. Compared with the control SD rats (A) and the control F344 rats (C), the numbers of CD45RA-positive cells in the CP-treated SD rats (B) and the CP-treated F344 rats (D) were lower, and the change was more noticeable in the F344 rats than in the SD rats. The numbers of CD3-positive cells in the CP-treated SD rats (F) and CP-treated F344 rats (H) were also lower, but the changes were smaller than those for the CD45RA-positive cells. Scale bar = 400 μm.

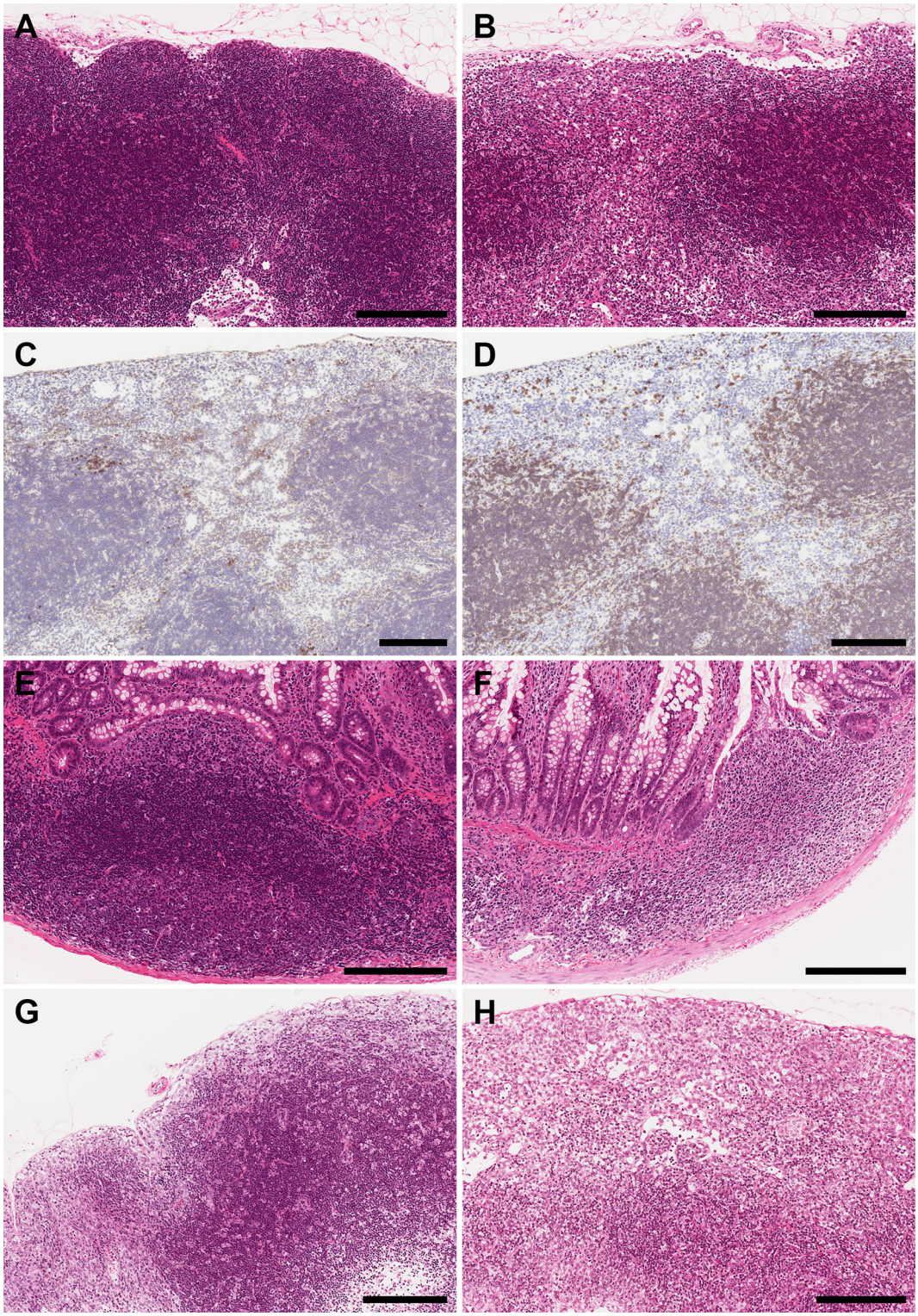

Fig. 4. HE staining and immunohistochemistry of the mesenteric lymph node (LN) (A–D), Peyer's patch (E, F), and iliac LN (G, H) in F344 rats. A, E, and G were tissues of the control rats. In the mesenteric LN, decreases in lymphocytes of follicles (B) and CD45RA-positive cells (C) were observed in cyclophosphamide (CP)-treated rats. Decreases in lymphocytes of paracortex and CD3-positive cells (D) were not so prominent. In the Peyer's patch in CP-treated rats (F), decreases in lymphocytes of follicles with germinal centers and in the interfollicular region (IFR) were observed. In the iliac LN in CP-treated rats (H), decreases in lymphocytes were observed more prominently than in the mesenteric LN. In F344 rats, decreases in germinal centers or follicles were sometimes observed in the LNs, even in the control animals (A, G). Scale bar = 200 μm.

Table 5. Immunohistochemistry and Morphometry of Immunopositive Cells of SD and F344 Rats Treated with Cyclophosphamide (CP) at 0 or 4 mg/kg/day for 28 Days

Strain		Crl:CD(SD)			F344/DuCrlCrlj		
Test article		Control	CP		Control	CP	
Dose (mg/kg)		0	4		0	4	
Number of animals		8	8		8[a]	8[b]	
CD45RA							
Spleen	$\times 10^6$	21.727 ± 7.913	8.204 ± 3.655	***	17.763 ± 5.143	1.269 ± 0.419	***
	/μm²	1.087 ± 0.287	0.542 ± 0.229	***	1.008 ± 0.242	0.107 ± 0.032	***
Mesenteric LN	$\times 10^6$	17.566 ± 6.267	6.853 ± 2.806	***	11.699 ± 5.594	1.847 ± 0.975	**
	/μm²	1.124 ± 0.193	0.662 ± 0.174	***	1.217 ± 0.231	0.357 ± 0.120	***
Mandibular LN	$\times 10^6$	18.193 ± 8.081	6.039 ± 1.552	**	10.468 ± 4.292	0.332 ± 0.135	***
	/μm²	0.732 ± 0.138	0.366 ± 0.089	***	0.632 ± 0.153	0.066 ± 0.022	***
Axillary LN	$\times 10^6$	7.657 ± 3.566	2.323 ± 1.357	**	4.408 ± 1.909	0.261 ± 0.278	**
	/μm²	0.746 ± 0.175	0.409 ± 0.163	**	0.573 ± 0.138	0.064 ± 0.050	***
Popliteal LN	$\times 10^6$	3.308 ± 1.168	1.481 ± 0.718	**	1.174 ± 0.602	0.079 ± 0.045	**
	/μm²	0.779 ± 0.226	0.380 ± 0.097	**	0.669 ± 0.150	0.111 ± 0.089	***
CD3							
Spleen	$\times 10^6$	11.987 ± 2.407	8.842 ± 2.132	*	6.218 ± 0.957	3.595 ± 1.380	***
	/μm²	0.593 ± 0.067	0.577 ± 0.090		0.339 ± 0.051	0.303 ± 0.122	
Mesenteric LN	$\times 10^6$	12.800 ± 4.780	10.475 ± 4.053		7.778 ± 4.358	4.359 ± 2.132	
	/μm²	0.856 ± 0.337	1.037 ± 0.127		0.920 ± 0.117	0.894 ± 0.119	
Mandibular LN	$\times 10^6$	14.486 ± 8.893	9.123 ± 4.223		9.481 ± 8.045	3.267 ± 1.241	
	/μm²	0.630 ± 0.410	0.552 ± 0.299		0.488 ± 0.231	0.670 ± 0.205	
Axillary LN	$\times 10^6$	12.508 ± 8.754	6.371 ± 5.107		6.698 ± 2.317	3.176 ± 2.200	*
	/μm²	1.048 ± 0.478	1.007 ± 0.486		0.994 ± 0.360	0.906 ± 0.227	
Popliteal LN	$\times 10^6$	4.704 ± 2.256	4.680 ± 3.073		1.717 ± 1.607	0.618 ± 0.472	
	/μm²	0.973 ± 0.191	1.028 ± 0.296		0.814 ± 0.295	0.678 ± 0.329	

Mean ± SD. Significantly different from the control (0 mg/kg) of the same strain: *P<0.05; **P<0.01; ***P<0.001. LN, lymph node. [a]For the axillary and popliteal LNs, the number of animals examined was seven. [b]For the axillary LNs, the number of animals examined was seven.

histochemistry analysis of the spleen revealed an apparent decrease in the number of CD45RA-positive cells in CP-treated rats, and this change was more noticeable in F344 rats than in SD rats. In both strains, the number of CD3-positive cells was also decreased, but the decreases in CD3-positive cells were mild compared with the decreases in CD45RA-positive cells. The number of CD3-positive cells per area was not remarkably different between the control and CP groups in each strain (Fig. 5). Various LNs showed changes similar to those observed in the spleen. In the mesenteric LN of CP-treated rats, a statistically significant decrease in the number of CD45RA-positive cells in the cortex was observed, and this change was more prominent in F344 rats (−84% vs. control) than in SD rats (−61% vs. control). The number of CD3-positive cells in the paracortex of the mesenteric LN was also reduced, but the difference was not statistically significant (Table 5). In other LNs, a statistically significant decrease in CD45RA-positive cells was also observed, and the changes were more noticeable in F344 rats than in SD rats (Table 5).

When the control groups for both strains were compared, the Np per area of CD3-positive cells in the spleen of F344 control rats was lower than that of SD control rats, but the Np per area of CD45RA-positive cells in the spleen of F344 controls was almost the same as that of the SD controls. In some LNs, the Nps per area of CD3- or CD45RA-positive cells of F344 control rats were also lower than those of SD control rats, though the changes were minimal and not significantly different (Table 5).

Discussion

In the present study, we conducted a histopathological analysis of various lymphoid tissues in conjunction with an evaluation of immune function in a TDAR study with CP in SD and F344 rats and investigated in detail the variability of lymphoid tissues and possible differences of control rats as well as those of immune reactivity and sensitivity to immunosuppression between the two strains.

In both the SD and F344 rats treated with CP, various changes related to CP-induced immunotoxicity were observed. Decreased concentrations of serum anti-KLH IgM and IgG were observed, as reported in other studies[9, 10]. In an immunophenotyping analysis of peripheral blood samples, severe decreases in the percentage of CD3−/CD45RA+ cells (B cells) were observed in both rat strains. In the CP-treated F344 rats, a decrease in the percentage of CD3+/CD45RA− cells (T cells) was also observed, and the change was much more severe than that in CP-treated SD rats. On the other hand, a slight increase in the percentage of T cells was observed in the CP-treated SD rats, but this change was considered a secondary effect of the severe decrease in the percentage of B cells with minimal biological significance. Similar to the results of the immunophenotyping analysis,

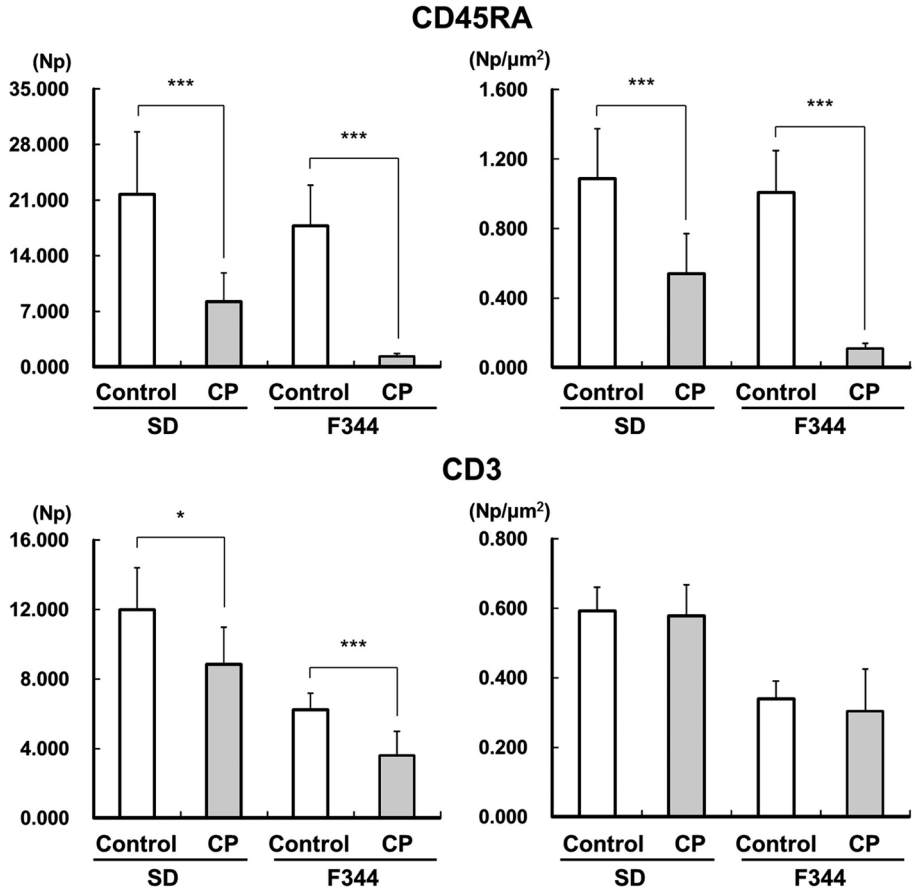

Fig. 5. Morphometry results for immunopositive cells in the spleen. The numbers of CD45RA- and CD3-positive cells (Np) and the Np per tissue area (Np/μm²) are presented as the mean ± SD. **P*<0.05; ****P*<0.001 (Whlch's *t*-test).

decreases in the weights of the spleen, thymus, and/or some of the lymph nodes were observed in the CP-treated rats of both strains. The histopathological examination revealed that decreases in the lymphocytes were noted mainly in the B-cell area (follicles, germinal centers, and MZ) in the HE staining sections of various lymphoid tissues and organs, consistent with the reduction in CD45RA-positive cells in the immunohistochemistry. CP is an alkylating anticancer drug that reportedly has an immunosuppressive effect predominantly on B cells, with a relatively minor effect on T cells; our results were consistent with these previous reports[18, 19].

Regarding the immunosuppressive effect of CP on T cells, some remarkable changes indicating a reduction in T cells were observed in the various lymphoid tissues in CP-treated F344 rats, though the effects of CP treatment on T cells were much milder than those on B cells in all the lymphoid tissues. In the spleen, decreases in lymphocytes of PALS were observed using HE staining and immunohistochemistry. In the thymus, decreases in the absolute weight, relative weight, and number of cortex lymphocytes were observed. In the axillary and popliteal LN, decreases in paracortex lymphocytes were observed using HE staining. In CP-treated F344 rats, the effect of the drug on T cells was slightly greater than that in CP-treated SD rats. A previous study reported that a higher dose of CP causes lymphoid reductions not only in B cells but also in T cells[19, 20], so the present results suggest that F344 rats are more sensitive to CP immunotoxicity than SD rats. On the other hand, the proliferation of T cells is well known to be suppressed by stress and following an increase in glucocorticoids, while an increase in adrenal weight arising from cellular hypertrophy of the zona fasciculata has been observed[21, 22]. In the present study, a slight decrease in body weight was observed in CP-treated rats of both strains, and a slight increase in adrenal weight was also observed in CP-treated F344 rats. However, no remarkable changes in the histopathological findings for the adrenal gland were observed, and no findings related to stress, such as a poor condition, were observed clinically. From these results, the immunosuppressive effects arising from stress were considered to be minimal in CP-treated rats in this study.

Regarding the differences among the lymphoid tissues in sensitivity to CP immunosuppression, clear differences were not observed in either strain. However, histopathological examination showed that the changes in the draining lymphoid organs (mesenteric LN and Peyer's patch) were milder than those in the other LNs or spleen. Since the dura-

tion of dosing in this study was relatively long and CP has strong immunosuppressive effects, almost all of the lymphoid organs seemed to be effected unanimously. Considering the results of HE staining and immunohistochemistry, some peripheral LNs and other small LNs that are resting under normal conditions might be fragile and sensitive to immunotoxicity. However, as described in previous review articles[2, 23], the peripheral LNs, except for the LNs that drain the site of xenobiotic application, should not be used for routine assessment of systemic immunotoxicity because of their histological variability and the difficulty in preparing specimens. In this study, some variability that overlapped the CP-induced changes, especially the variability in the number of germinal centers, was observed in these resting LNs of control rats. If needed to evaluate resting LNs as draining LNs of xenobiotic application, it would be very important to collect as many node from each site as possible or to utilize adequate numbers of animals.

The reasons for the strain differences in sensitivity to CP-induced immunosuppression were not clear in this study, but one of the most probable factors is considered to be the difference in genetic background. It was reported that the percentages of T cells in white blood cells were different between SD and F344 rats[24], and some articles reported a relationship between genetic differences and immune response or immunotoxicity in rats[14, 24, 25]; thus genetic difference is an important factor to consider when examining drug-induced effects on immune systems. On the other hand, it is also well known that the development of immune organs is affected by environmental pathogens or antigen exposure[3, 4]. As for the LNs, their histologic appearances are related to the level of immunologic stimulation, and the appearance of each LN is different[5]. For example, the axillary LN, one of the peripheral LNs, starts to develop later than the mesenteric LN and rarely has germinal centers in 6-week-old SD rats[5]. In this study, when the control groups of both strains were compared, the numbers of immunopositive cells in most of the lymphoid tissues of the control F344 rats, except for the spleen, were lower than those of the control SD rats. This is consistent with the results of the hematological analysis. In the hematologic parameters, the numbers of white blood cells and lymphocytes in the F344 rats were lower than those in the SD rats. In addition, in the control F344 rats, some of the histopathological findings that might have been related to the low numbers of white blood cells and lymphocytes were observed in the bone marrow (hypocellularity), spleen, and several LNs (decrease in lymphocytes in the follicles or germinal centers). These strain differences in immune organs, which might also have been derived from genetic differences, indicate that the baseline numbers of T and/or B cells in the lymphoid tissues were lower and that the lymphoid tissues were less developed in the control F344 rats than those of the control SD rats, suggesting that F344 rats might be more easily affected by CP-induced immunosuppression than SD rats. However, other factors, such as toxicokinetics or metabolism, should also be taken into account, since genetic differences in liver enzymes were reported and the effects of CP on cytochrome P450 expression were reportedly greater in F344 rats than in SD rats[25, 26]. Regarding other immunotoxicants, SD rats were reported to be more sensitive than F344 rats, although the basis for the differences in the sensitivities of rat immune systems was unknown[25]. Considering our study results and these previous studies, further studies and analyses using other immunosuppressive compounds are needed.

A previous TDAR study reported a low response in SD rats[9]. A similar response was also observed in this study, and the standard deviations for the concentrations of serum anti-KLH IgM and IgG levels were larger in the control SD rats than in the control F344 rats. Though we confirmed the histopathological features of the lymphoid organs in these low-response SD rats and compared them with those in normal-response SD rats, no remarkable histopathological differences were observed, and no clear relationship was seen between the concentrations of serum anti-KLH IgM and IgG and the numbers of CD45RA- or CD3-immunopositive cells in the histopathology findings.

A detailed histopathological analysis performed in conjunction with a TDAR assay, as done in the present study, has not been previously reported. In this study, the results of histopathological examinations in lymphoid organs were consistent with the results of the serum anti-KLH IgM and IgG concentrations as determined using an ELISA, and the histopathological analysis revealed that the immunosuppressive effects of CP were prominent in the spleen and LNs at various sites but less prominent in the thymus and bone marrow. Such information on the effects in specific organs might be useful when considering the mechanisms and specifying the target cells or organs of compound-induced immunotoxicity.

In conclusion, the results of our study showed that histopathological changes related to CP immunosuppression were more prominent in F344 rats than in SD rats, corresponding with the results for anti-KLH antibody concentrations. The histopathological changes in the mesenteric LN and Peyer's patch were milder than those in the spleen and other LNs. In addition, some lymphoid tissues of the control F344 rats were less developed than those of the age-matched control SD rats, suggesting that F344 rats might be easily affected by CP-induced immunosuppression. This background information concerning rat strain differences in lymphoid tissues will be useful in histopathological evaluation for drug-induced immunotoxicity.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

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