antagonist showed restoration of the FSHβ mRNA level in the pituitary, whereas no addition of T to the same animals decreased the FSHβ mRNA level [28]. To understand the 2-fold higher level of FSHβ subunit mRNA in the pituitary despite the absence of any change in serum FSH level in the present study, it should be remembered that the much higher levels of blood T in FM-treated animals increases the FSHβ mRNA content in the pituitary independently of the GnRH pulse.

The immediate early genes, c-fos and c-jun, encode two components forming activator protein-1 (AP1), a potential transcription factor, and their transcription is rapidly up-regulated by activation of cytoplasmic kinase [29, 30]. Expression of both genes is induced in the pituitary by GnRH stimulation, and they up-regulate LHβ and FSHβ gene transcription. In the present study, the mRNA levels of c-fos and c-jun on D8 were lower than in the control (although the difference in c-fos mRNA was not significant), suggesting that LHβ and FSHβ up-regulation may have already been reduced 2 days after the final FM injection. Egr1, another immediate early gene, has been reported to be rapidly induced in the pituitary by GnRH stimulation and has a responsive element in the LHβ gene promoter [31]. The somewhat lower Egr1 mRNA level (not significant) than in the control appears to be caused by the same reason as the c-jun reduction.

The increase in intratesticular T level is due to up-regulation of the 3β-hydroxysteroid dehydrogenase gene

Among the six steroidogenic enzyme genes assessed in this study (Fig. 3), the most obvious effect of FM was up-regulation (2.5-fold) of the 3β-hydroxysteroid dehydrogenase type I gene, an isoform showing the most dominant expression of all 3βHSD isoforms [32]. P450scc and P450c17 were also up-regulated, 2-fold and 1.8-fold, respectively, over the control, whereas 17β-hydroxysteroid dehydrogenase type III, the only 17βHSD isoform expressed in the testis [33], was reduced slightly, but with statistical significance. The 3βHSD-type I up-regulation appeared to cause the extraordinary increase in intratesticular T level. Consistent with the present study, early studies of FM reported that 3βHSD enzyme activity increased more than any of the other steroidogenic enzymes in male rats [34]. The up-regulation of the three steroidogenic enzyme genes must be due to an inhibitory action of FM on the negative feedback effect of T on the hypothalamus and pituitary, and subsequent increase in LH secretion.

An early endocrinological study reported an increase in P450scc enzyme activity in response to LH or hCG injection of intact male rats [35]. However, a single high dose of LH/hCG decreased testicular T synthesis, a phenomenon referred to as Leydig cell desensitization [36], which was revealed to be due to reduction of LHR expression and decreased levels of P450c17 enzyme activity [37]. In our study, there was no significant change in LHR mRNA expression, suggesting that an increased level of LH is insufficient to cause the desensitization. By contrast, repeated administration of a relatively low dose of LH/hCG for 6 days was reported to increase T synthesis and enzyme activities of P450c17 and 3βHSD with a slight decrease of 17βHSD [36]. The changes observed in mRNA expression levels of P450c17, 3βHSD, and 17βHSD in the present study strikingly resemble the changes in enzyme activity described in the earlier report, suggesting that the subacute FM administration caused medium amplification of pulsatile LH secretion.

It has been well-documented in a study that used a cultured Leydig cell line and promoter analysis that transcription of P450scc, P450c17, and 3βHSD genes, all of which were up-regulated by FM in our study, is mediated by cAMP, mostly due to LH signaling [38]. However, the nuclear factors modulating the steady-state or induced level of P450scc and P450c17 expression differ from each other [39]. Moreover, expression of the 3βHSD gene was found to be inhibited by T via AR in a trans-acting manner [40]. Based on these findings, a significant increase in 3βHSD, despite the 4-fold intratesticular T level in the present study, strongly suggests that the level of FM, probably hydroxyflutamide (OH-FM), was still high enough in the testis on D8 to antagonize T binding to AR and then blocked the inhibition of 3βHSD gene expression. Presumably, this inhibitory effect on the blockade of 3βHSD gene expression and enhancements of P450scc and P450c17 gene expression by increased levels of LH synergistically amplified T production in the FM-administered rats.

Marked decrease in AR and GR mRNA levels in the testis of rats subacutely administered FM

Although there was no change in ERα mRNA, both AR and GR mRNAs were down-regulated in the testis by FM (29% and 35%, respectively, of the control by β-actin standardization). The magnitude of AR mRNA down-regulation was the most dramatic change among the genes examined in the present study, and this is the first report of AR gene alteration by an anti-androgenic compound. In the adult rat testis, AR protein has been localized to Sertoli cells, peritubular myoid cells, and Leydig cells [41]. An *in situ* hybridization study showed that the signal for AR mRNA was most intense in the Sertoli cells at stage VII-VIII of the seminiferous tubule [42]. The AR gene itself is a target for androgen, and AR mRNA levels in the ventral prostate of adult rats have been shown to be down-regulated by androgens, suggesting the presence of an autoregulation system on the AR gene [43, 44]. There have been several studies on AR-autoregulation in the testis. In rats pretreated with ethane dimethane sulfonate (EDS), which specifically inhibits Leydig cell function subsequently inhibiting T production, the AR mRNA level in the testis was unchanged, suggesting that there is no autologous regulation by androgen in the testis [45]. However, in the GnRH-analogue-treated rat model, in which LH secretion and T production are inhibited, exogenous LH or synthetic androgen R1881 restored immunoexpression of AR protein in Sertoli cells [46]. On the other hand, a 4-fold increased in testicular AR mRNA level has been reported 15 days after hypophysectomy [41]. As the above findings suggest, autologous regulation of the AR gene in the testis is still a matter of controversy.

The AR mRNA down-regulation observed in the present study appears to be the result of the markedly higher level of intratesticular T, which seems to be due to the same mechanism as in the prostate. However, as described above, based on the trans-acting inhibitory effect of T on the 3βHSD gene, it is likely that the OH-FM level in the testis on D8 was still high enough to antagonize androgen-binding to AR [40]. Moreover, we could not detect any changes in the mRNA level of LNGFR, a subunit of NGF-receptor expressed on the Sertoli cell surface. The LNGFR gene has been well-documented to be directly down-regulated by T [47, 48]. A more than 10-fold increase in LNGFR mRNA level has been shown in EDS-treated (T free) or hypophysectomized rats [47], and the LNGFR gene has a promoter region that is responsive to T [48]. The absence of any significant change of LNGFR in the FM treated testis, in spite of the markedly higher level of intratesticular T, strongly suggests that T did not suppress the LNGFR gene. This appears to mean that an antagonistic activity of the higher level of OH-FM retained in the testis on D8 blocked the inhibitory effect of T on LNGFR expression. If the OH-FM level in the testis on D8 was still high enough to antagonize androgen-binding to AR, T probably maintains AR gene expression in the rat testis, rather than down-regulating it, as in the prostate.

In the testis, GR has been reported to be localized to Leydig cells [49]. Although there have been no reports on the regulation of GR gene expression by androgen in the testis, a glucocorticoid and GR complex has been reported to inhibit cAMP-dependent up-regulation of P450scc gene expression [50], suggesting that GR is one of the regulatory factors for T production in the testis. Therefore, down-regulation of GR mRNA by FM to the same level as AR mRNA seems to be implicated in the markedly higher level of T production after FM treatment.

Burnstein and coworkers [51, 52] are trying to identify the AR gene transcription machinery on a molecular basis. Using a prostate cancer cell line PC3, they identified two distinct AR responsive elements in exon D and exon E of the AR gene itself, that were found to be involved in the up-regulation of AR gene transcription by androgen [51]. They subsequently discovered that the elements contain specific DNA sequences bound to the proto-oncogene: immediate early gene products Myc and Max, suggesting that these proteins might be involved in the AR gene autoregulation system [52]. The significant up-regulation of c-myc mRNA by FM in the present study might also implicate Myc in the AR gene down-regulation.

Over production of T or down-regulation of AR gene expression may be involved in the reduced sperm production

It has recently been pointed out that abnormally high intratesticular T levels inhibit spermatogenesis [53–55]. Rats exposed to 6 Gy medium strength gamma irradiation showed

markedly increased levels of intratesticular T (3-fold) with complete loss of spermatogenesis. GnRH antagonist administration to these rats decreased the LH and intratesticular T levels and then stimulated spermatogonial reproliferation [54]. A mutant mouse, juvenile spermatogonial depletion (*jsd*), also showed a higher level of intratesticular T (3-fold) with complete loss of spermatogenesis. In this model, spermatogonial reproliferation was also stimulated by GnRH analogue [55]. The results of these series of studies by Meistrich and coworkers strongly suggest a greatly increased level of intratesticular T directly inhibits spermatogenesis. They hypothesized that the inhibition is due to apoptosis of spermatogonia by high T levels unrelated to gonadotropin [54]. More recently, they showed that this spermatogenic inhibition by high T levels is mediated by AR [56, 57]. In the present study, subacute FM administration produced a 4-fold intratesticular T level 2 days after the final administration (D8), but not one month later (D36). This temporary increase in the T level may affect the early stage of spermatogenesis through the same mechanism described in the studies by Meistrich and coworkers, and then reduce DSP on D36. Since we did not measure the concentration of OH-FM in the testis on D8 in the present study, we could not compare the testicular OH-FM with the T level. A study on FM metabolism in 6-week-old male rats showed that the plasma OH-FM level after a 10 mg/kg single dose remained above approximately 100 ng/ml for 24 hrs [58]. As described above, based on the report by Paul *et al.* [28] our finding that the pituitary FSHβ mRNA level had increased 2-fold despite the absence of any difference in serum FSH level suggested that the pituitary T level was higher than the OH-FM level. Based on the reports by Stalvey and Clavey [40] and Shan *et al.* [46], however, the results showing up-regulation of 3βHSD and down-regulation of AR suggest that the testicular T level is likely to be lower than the OH-FM level. Further investigations will be required to clarify this discrepancy. A possible explanation is that ABP secreted by Sertoli cells may neutralize intratesticular T, making the effective level of intratesticular T much lower than the measured value.

The gene expression profiles presented in this study should provide useful data for initial screening for anti-androgenic compounds *in vivo*. Future studies with other anti-androgenic drugs, such as Casodex, will be needed to compare the effects of FM and elucidate the differences between the mechanisms of action of different anti-androgenic compounds.

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Effects of Vinclozolin Administration on Sperm Production and Testosterone Biosynthetic Pathway in Adult Male Rat

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Abstract. The effect of vinclozolin (VCZ), used as a fungicide and known to have anti-androgenic effects on spermatogenesis and gene expression in the male rat testis was investigated. In Experiment 1, VCZ (100 mg/kg/day) or flutamide (FM, 25 mg/kg/day) was orally administered to male Holzman rats for six days. 8 days after the last administration (D8), a drastic increase in intratesticular testosterone was detected in FM (4.2-fold over control) but not in VCZ treated animals, whereas on D36 post-administration, both groups showed similar levels. Significant decreases in daily sperm production were seen in both VCZ and FM-treated rats on D36. Semiquantitative RT-PCR analysis with testicular and pituitary mRNAs on D8 revealed that LHβ and FSHβ mRNAs were increased in the pituitary by VCZ, as well as by FM. Among the four testicular steroidogenic enzyme genes, cytochrome P450 side chain cleavage (P450scc) and cytochrome P450 17α/$C_{17\text{-}20}$ lyase (P450c17) mRNAs were significantly increased, whereas 17β-hydroxysteroid dehydrogenase type III (17βHSD) mRNA was not changed. A significant increase in 3β-hydroxysteroid dehydrogenase type I (3βHSD) and a decrease in androgen receptor (AR) mRNA were observed only in FM treated rats. Immunohistochemistry demonstrated intense staining of P450scc in the interstitial cells of VCZ-treated testis on D8. In Experiment 2, hormone levels were measured at 1, 3, 6, 12 and 24 hours after VCZ (100 mg/kg) administration to Sprague-Dawley rats. Serum LH level remained constant for the first 3 hours and started to increase at 6 hrs. In contrast, serum and intratesticular testosterone levels increased 2-fold at 1 hr and maintained the level until 24 hrs. P450c17 mRNA level was 2-fold increased at all periods, whereas no obvious changes were detected in the other steroidogenic enzyme genes. Although not statistically significant, AR mRNA level increased 2-fold, 3 hrs after VCZ administration. These results indicate that VCZ affects the pituitary in a similar manner as FM, but functions differently on testicular gene expression.

Key words: Vinclozolin, Testis, Testosterone, Steroidogenic enzyme, Androgen receptor

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Vinclozolin (VCZ) is known as an endocrine disrupting chemical that has been shown to display an anti-androgenic activity [1]. In laboratory animals, an exposure to VCZ during sexual differentiation can produce malformations of the reproductive tract in male offsprings such as

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reduced anogenital distance, cleft phallus with hypospadias and suprainguinal ectopic scrota/testes [2-4]. These developmental defects are believed to result from antagonistic actions of VCZ and its metabolites, the two active forms M1 and M2, on mammalian androgen receptors (AR), where androgen-dependent gene expression is inhibited. *In vitro* studies revealed that VCZ and its metabolites bind to AR and inhibit AR-binding to DNA [1, 5, 6]. VCZ is used as a systemic dicarboximide fungicide in the United States and throughout Europe to control fungal disease in grapes, fruits, vegetables, hops, ornamental plants and turf. Although the production of VCZ is decreasing because of its adverse effect on fetal development of a wide variety of animals, the total amount of this compound in the environment seems to be still high [7]. Naturally, intake of VCZ via food and wine remains an important issue in terms of human health [8]. To date, reports on the endocrine disrupting effects of this compound in laboratory animals are limited to fetal exposure *in utero*, and there are no reports concerning adult exposure to VCZ. In the course of developing a screening system for anti-androgen like endocrine disrupting chemicals, more convenient and simple assays detailed in molecular aspects will be required in terms of so-called 'Toxicogenomics' [9, 10]. We previously reported a brief profiling of a change in gene expression in adult male rats given flutamide (FM), an anti-androgenic drug [11]. Consistent with earlier studies with FM [12–14], we confirmed a significant increase in intratesticular testosterone level shortly after administration and a decrease in sperm counts one month after administration. We also detected a decrease in AR mRNA and an increase in 3βHSD mRNA in testis. In our present study, in order to compare the anti-androgenic effects of FM to VCZ, we administered VCZ to adult male rats and searched for alterations in spermatogenesis, serum hormone and testicular testosterone levels, testicular steroidogenic enzyme and AR mRNA levels.

Materials and Methods

Materials

Vinclozolin (VCZ: [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine-2,4-dione]) and flutamide (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl]poropamide) were purchased from Wako Chemical Co., (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively. Corn oil used for dissolving flutamide (FM), vinclozolin (VCZ) and vehicle control was also purchased from Sigma. Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were determined by enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Serum and intratesticular testosterone levels were determined by enzyme immunoassay (EIA) system (Cayman Chemical Co., Ann Arbor, MI, USA). SuperScript™ II RNase H- Reverse Transcriptase and oligo(dT)$_{12-18}$ primer were obtained from Invitrogen (Carlsbad, CA, USA). Ex Taq™ with 10 x Ex Taq™ Buffer were purchased from TaKaRa Biomedicals (Otsu, Shiga, Japan). The pGEM-TEasy vector was obtained from Promega Corp. (Madison, WI). Rabbit anti-rat cytochrome P450scc polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from CHEMICON International, Inc. (Temecula, CA, USA) and Pierce Chemical Corp (Rockford, IL, USA), respectively.

Animals and treatment

Male Holtzman and Sprague-Dawley rats bred in our own facility were maintained in a controlled environment with the temperature at 24 ± 1 C, humidity at 45 ± 5%, and a 12/12 h light/dark cycle (08:00–20:00), and were given Rodent feed (CE-2, CLEA Japan) and distilled water *ad libitum*. In Experiment 1, 13-week-old Holtzman male rats weighing about 400 g were randomly selected and divided into six groups (n=8) so that the average body weight of each group was the same. The rats were orally administered vehicle (corn oil), VCZ (100 mg/kg/day in corn oil), or FM (25 mg/kg/day in corn oil) for 6 days between 12:00 and 15:00. 8 days (D8) and 36 days (D36) after the last administration (D0), the rats were sacrificed under ether anesthesia and abdominal aorta bleeding between 12:00 and 15:00. The serum was separated and stored at –20 C. Testes on both sides were excised and weighed after removal of surrounding adipose tissues. Right testis samples were frozen with liquid nitrogen immediately after dissection and stored at –80 C until measurement for daily sperm production, intratesticular testosterone concentration and RNA extraction. Left testis samples were fixed with Bouin's solution and

Table 1. Primers used for semiquantitative RT-PCR

Gene	Primer sequences		Product size (bp)	PCR cycle used	GenBank accession no.
	Forward primer (5' to 3')	Reverse primer (5' to 3')			
P450scc	CGCTCAGTGCTGGTCAAAA	TCTGGTAGACGGCGTCGAT	688	23	J05156
P450c17	GACCAAGGGAAAGGCGT	GCATCCACGATACCCTC	302	24	M22204
3βHSD	TTGGTGCAGGAGAAAGAAC	CCGCAAGTATCATGACAGA	547	24	M38178
17βHSD	TTCTGCAAGGCTTTACCAGG	ACAAACTCATCGGCGGTCTT	653	26	AF035156
AR	TGCTGCCTTGTTATCTAGTCTCA	ACCATATGGGACTTGATTAGCAG	570	26	M20133
LHβ	CTGGCTGCAGAGAATGAGT	GAAGGTCACAGGTCATTGG	292	20	J00749
FSHβ	TGTACGAGACCATAAGATTG	TTGAGTATCCTAACCTTGTG	757	22	D00577
CP	TCTGAGCACTGGGGAGAAAG	AGGGGAATGAGGAAAATATGG	524	18[a], 21[b]	M19533

Used for testicular mRNA (a) and pituitary mRNA (b).

embedded in paraffin for immunohistochemistry. Pituitary was also quickly removed and frozen with liquid nitrogen. In Experiment 2, 13-week-old Sprague-Dawley male rats were randomly selected and divided into six groups (n=5). The rats were orally administered vehicle (corn oil) or VCZ (100 mg/kg in corn oil) at between 12:00 and 15:00. At 0 (Non administrated group), 1, 3, 6, 12 and 24 hours after administration, the rats were sacrificed and serum and testis samples were collected following the same protocol as in Experiment 1.

Daily sperm production

Testes were homogenized in 10 mM phosphate buffered saline (pH 7.4) with a Polytron homogenizer. The number of homogenization-resistant spermatid nuclei per testis were determined with a hemocytometer, and daily testicular sperm production (DSP) was calculated by dividing this by 6.1 as normally described [15].

Hormone assay

Serum luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone levels and intratesticular testosterone level were determined with the corresponding EIA systems. Serum samples were directly applied to the kit, and processed following the manufacturer's descriptions. For the measurements of intratesticular testosterone levels, the frozen right testis was homogenized in 8 ml of 10 mM phosphate buffered saline (PBS, pH7.4) with a Polytron homogenizer. The homogenate (2 ml) was then extracted with diethylether and the ether fraction was dried in air. The intratesticular serum was also extracted with diethylether and the ether fraction was dried in air. The dried lipophilic substances were resuspended with an appropriate volume of the buffer in the kit, and processed for measurement following the manufacturer's descriptions.

Semiquantitative RT-PCR

Total RNA was extracted from testes and pituitaries following the basic protocol of Chomczynski and Sacchi [16]. RNA samples (testis, 8 μg; pituitary, 1.25 μg) were reverse-transcribed for 50 min at 42 C in a 40-μl reaction solution containing 400 units of SuperScript™ II reverse transcriptase and 1 μg of oligo(dT)$_{12\text{-}18}$ primer by the standard protocol of the supplier (Life Technologies, Rockville, MD, U.S.A.). PCR solution (50 μl) contained 0.2 mM of each dNTP mixture, 0.4 μM of each strand of primer, and 1 μl of reverse-transcribed cDNA described above. 1.25 unit of TaKaRa Ex Taq™ polymerase (TaKaRa Biochemicals, Otsu, Japan) diluted with 1 × Ex Taq™ Buffer (TaKaRa Biochemicals, Otsu, Japan) was added to the solution. PCR was performed by 94 C-30 sec denaturing, 60 C-30 sec annealing, 72 C-45 sec extention. Table 1 shows the primer sequences, PCR-product sizes, optimized cycles to semi-quantify the mRNA amount decided by preliminary experiments, and Taq polymerase in addition to GenBank accession numbers for all genes examined in this study: cytochrome P450 side chain cleavage (P450scc), cytochrome P450 17α/C$_{17\text{-}20}$ lyase (P450c17), 3β-hydroxysteroid dehydrogenase type I (3βHSD), 17β-hydroxysteroid dehydrogenase type III (17βHSD), androgen receptor (AR), β subunit of LH (LHβ), β subunit of FSH (FSHβ), and cyclophilin. The PCR products (5 μl) were then separated with 2% agarose gel containing ethidium bromide. The

Table 2. Effects of vinclozolin and flutamide administration on sperm count and hormone levels

	Body weight (g)	Paired testicular weight (g)	DSP ($\times 10^7$/testis)	Serum LH (ng/ml)	Serum FSH (ng/ml)	Serum testosterone (ng/ml)	Intratesticular testosteorne (ng/g testis)
D8							
Control	491 ± 11	3.71 ± 0.07	4.20 ± 0.01	19.21±0.34	129.0±13.5	1.40 ± 0.78	161± 38
Vinclozolin	480 ± 11	3.55 ± 0.11	4.15 ± 0.20	21.89±0.93*	149.6±12.0	2.43 ± 0.93	208± 24
Flutamide	470 ± 12	3.95 ± 0.09*	4.41 ± 0.12	23.62±0.83**	137.9± 7.3	4.73 ± 0.59**	716±146**
D36							
Control	518 ± 19	3.82 ± 0.08	4.10 ± 0.11	22.57±1.31	88.7±10.5	0.42 ± 0.20	90± 16
Vinclozolin	536 ± 16	3.64 ± 0.14	3.32 ± 0.11**	20.87±1.49	104.7± 5.5	0.53 ± 0.36	133± 32
Flutamide	528 ± 13	3.78 ± 0.27	3.26 ± 0.10**	22.25±2.40	109.4±10.4	0.68 ± 0.15	99± 22

Data are means ± SE. Statistically significant difference between means from control was analyzed by Student's *t*-test (*P<0.05; ** P<0.01).

bands on UV-transilluminated gel were transferred into digital images by a gel analyzer (ATTO Inc., Tokyo, Japan) and RT-PCR products were quantified with Scion Images software (Scion Corporation, Frederick, MD, USA). PCR products for cyclophilin were employed as an internal standard. To determine the sequences, the PCR product for each gene was subcloned into pGEM-T Easy vectors (Promega Corp., WI, USA) and sequenced by the dideoxynucleotide chain termination method with ABI Prism BigDye terminator cycle sequencing kit (PE-Biosystems, CA, USA).

Immunohistochemistry

Testes were fixed with Bouin's solution and embedded in paraffin. 4 μm thick sections were deparaffinized, and incubated with blocking solution containing 20% normal goat serum (Sigma, MO, USA), 1% bovine serum albumin (BSA), and 10 mM phosphate buffered saline (PBS, pH 7.4) for 1 h at room temperature (RT) to minimize non-specific staining. After rinsing with PBS, the sections were incubated for 1 h at RT with 40 μg/ml primary antibody for rat P450scc (Chemicon, CA, USA) suspended in PBS containing 0.1% BSA (BSA/PBS). They were then washed three times with PBS and incubated for 1 h at RT with 8 μg/ml HRP-conjugated goat anti-rabbit IgG(Pierce, IL, USA) suspended in BSA/PBS. After washing with PBS, 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in PBS were applied to the sections for 3 min to develop the peroxidase reaction. The sections were washed in PBS again, and counterstained with hematoxylin, dehydrated, and mounted with DPX(BDH Laboratory Supplies, Poole, UK).

Statistical analysis

For statistical analysis, StatView for Windows version 5.0 (SAS Institute, NC, USA) was used. All results represented are the means ± SE. In all experiments, data were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's PLSD test. P values less than 0.05 were considered as statistically significant.

Results

Effects of VCZ and FM on daily sperm production

Table 2 shows body weights, testicular weights, daily sperm production (DSP), serum-LH and FSH, and testosterone levels in each group. Body weight at D8 and D36 were not affected by 6-day administration of VCZ or FM. Although no changes were observed in DSP on D8, a significant decrease was observed on D36 in both groups (approximately 80% of control, P<0.01).

Effects of VCZ and FM on serum hormone and intratesticular testosterone levels

Serum LH level in control animals significantly increased with age from 19.2 ng/ml to 22.6 ng/ml, whereas LH level on D8 was slightly but significantly increased in the VCZ and FM treated groups (1.1-fold by VCZ (P<0.05), 1.2-fold by FM (P<0.01)), but no change was observed on D36 (Table 2). The FSH level in VCZ treated rats on D8 was slightly higher than in the control group, but this was not statistically significant. Serum and intratesticular testosterone levels in control animals

significantly decreased with age ($P<0.05$). Serum testosterone level of the FM treated animals on D8 was 3.4-fold increased compared to the control animals ($P<0.01$), whereas no significant difference was observed on D36. Similarly, intratesticular testosterone level on D8 showed a 4.2-fold increase (FM, 720 ng/g testis; control, 170 ng/g testis), but there were no statistically significant changes in serum and intratesticular testosterone levels caused by VCZ treatment either on D8 or D36.

Effects of VCZ and FM on testicular and pituitary mRNA levels

On D8 post-administration, testis and pituitary mRNA levels of several genes were measured by semiquantitative RT-PCR analysis. Significant increases in LHβ and FSHβ subunit mRNAs were detected both in VCZ and FM treatment groups in the pituitary(Fig. 1A). The magnitudes of increases were similar for VCZ and FM. Significant increases in P450scc and P450c17 mRNAs were detected in both VCZ and FM treated testes (Fig. 1B), although the degree of increase in VCZ-treated rats was smaller than those in FM-treated rats. 3βHSD mRNA level in the VCZ-treated group was similar to that in the control group, whereas mRNA level in the FM-treated group was 3-fold higher than in the control, which was the most drastic difference in the four steroidogenic enzyme genes. There were no significant changes in 17βHSD mRNA level in neither treatment group. AR mRNA level was not changed by VCZ treatment, but a significant decrease (30% of control) was observed in the FM-treated group (Fig. 1B).

Immunohistochemical changes in P450scc staining in the VCZ treated rats

To examine whether the increase in P450scc mRNA level corresponded with the protein expression level, immunohistochemistry was performed. Intense P450scc staining was detected in the interstitial cells, presumably Leydig cells, of VCZ-treated testis on D8, whereas no change was observed on D36 (Fig. 2).

Time course changes in LH and testosterone levels by VCZ administration

To examine the changes in hormone levels shortly after the VCZ treatment, the serum LH and testosterone and intratesticular testosterone levels were measured at 1, 3, 6, 12 and 24 hours after a single VCZ administration. The serum LH and testosterone levels of 13-week-old male SD rats were slightly lower than those of Holtzman rats, presumably due to strain difference. During the first 3 hrs, the serum LH level was not changed. However, a slight increase was detected 6 hrs after VCZ administration, and the increase was statistically significant at 24 hrs (Fig. 3A). Measurements of serum testosterone concentration indicated a transient sharp increase (1.9-fold) 1 hr

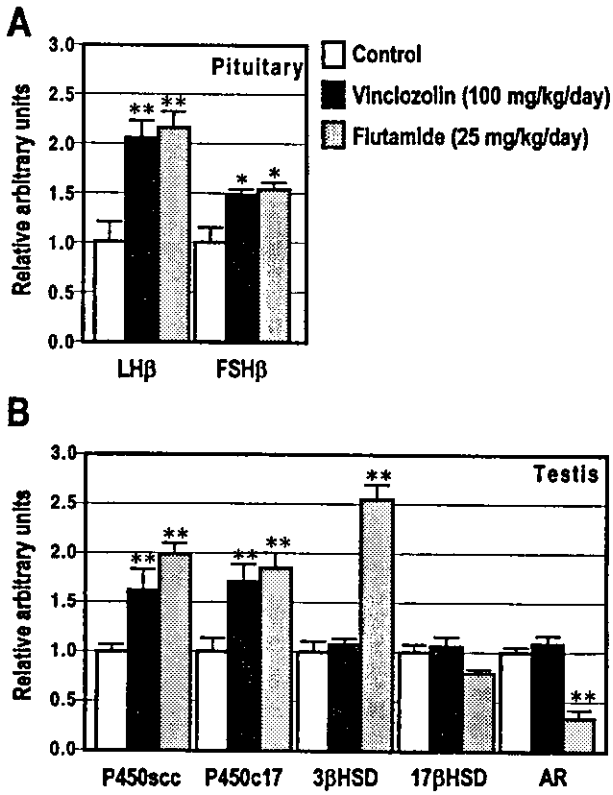

Fig. 1. Semiquantitative RT-PCR analysis of long term vinclozolin (VCZ) and flutamide (FM) administration on testicular and pituitary mRNA levels. Semiquantitative RT-PCR was performed to measure mRNA levels 48 hours after administration of VCZ (100 mg/kg/day) or FM (25 mg/kg/day) for 6 days. Relative amounts of RT-PCR products to the average of each control were evaluated by standardizing with internal control cyclophilin. A, Gonadotropin subunit (LHβ, FSHβ) in pituitary; B, Four steroidogenic enzymes (P450scc, P450c17, 3βHDS, 17βHSD) and AR in testis. The values expressed are the mean ± SE (n= 8). Note the significant increases in P450scc, and P450c17 mRNA levels in the VCZ treated testis. The values expressed are the mean ± SE (n=5). Statistically significant difference between means from control was analyzed by ANOVA followed by Fisher's PLSD test (*$P<0.05$, **$P<0.01$).

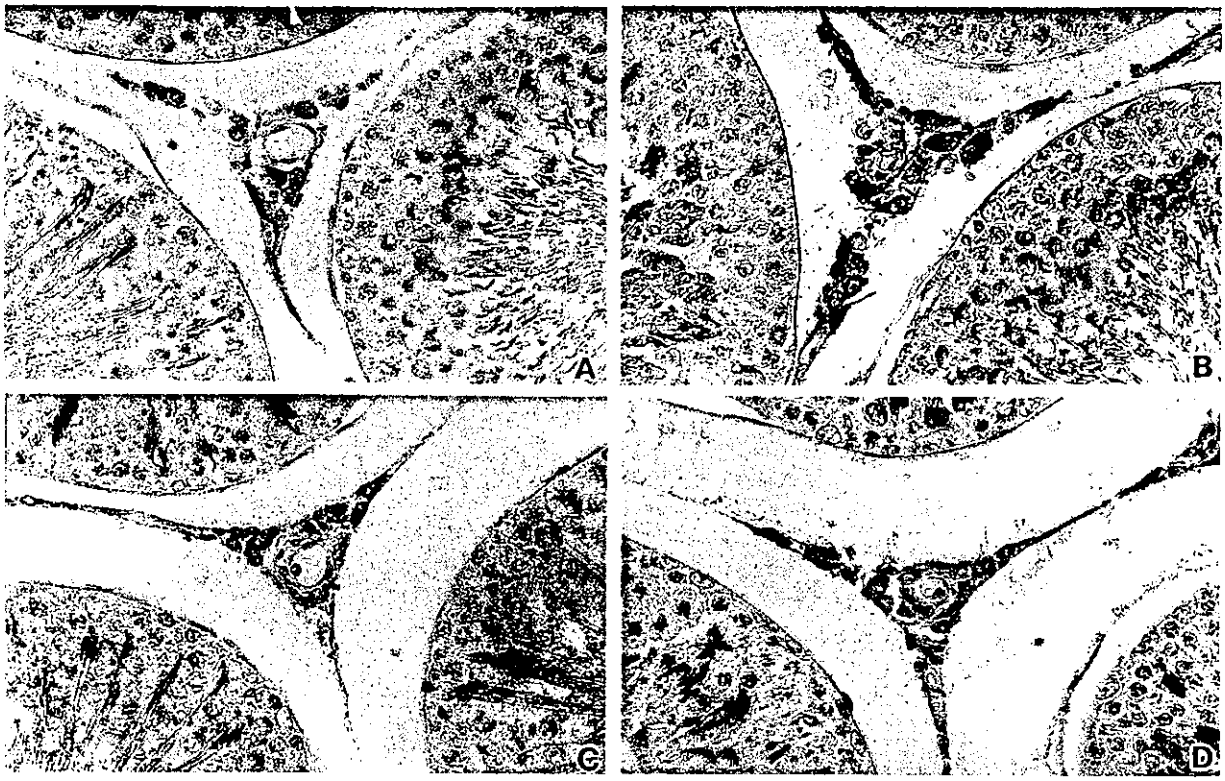

Fig. 2. Immunohistochemical comparison of P450scc protein expression in the testis of control and vinclozolin (VCZ) treated rats. The testis sections of control and VCZ treated rats were stained with anti-P450scc antibody as described in Materials and Methods. A, Control testis on D8; B, VCZ treated testis on D8; C, Control testis on D36; D, VCZ treated testis on D36. Note the intense and enlarged staining of interstitial cells in the VCZ treated testis on D8. Magnification ×200.

post administration of VCZ(Fig. 3B). The level was reduced at 3 hrs, but steadily increased at 6, 12 and 24 hrs. Intratesticular testosterone levels also showed a surgical increase 1 hr after VCZ administration, and decreased at 3 hrs, concurrent with the changes in the serum level (Fig. 3C). The highest increase compared to control was observed at 12 hrs (2.8-fold), though not statistically significant by ANOVA.

Time course changes in testicular mRNA levels by VCZ administration

Examination of steroidogenic enzyme mRNA levels shortly after VCZ treatment was conducted by subjecting the testis mRNAs to semiquantitative RT-PCR. No statistically significant difference was detected by ANOVA in any of the five genes. However, approximately 1.5-fold increase in P450scc mRNA level was observed at 24 hr after VCZ administration (Fig. 4A). In contrast, P450c17 mRNA level increased at 1 hr after VCZ administration, and the level was maintained until 24 hrs (Fig. 4B). Distinct changes were not detected in 3βHSD mRNA level throughout the time course (Fig. 4C). There was a slight increase in 17βHSD mRNA level at 6 hrs (Fig. 4D). AR mRNA level rose from 1 hr to 3 hrs after VCZ administration (approximately 2-fold), and then gradually lowered (Fig. 4E).

Discussion

Vinclozolin decreased testicular sperm production as well as flutamide

In the present study, we demonstrated that vinclozolin (VCZ), an endocrine disrupting chemical and a potent anti-androgen, affects spermatogenesis and reduces testicular sperm production in adult rats. In the rat seminiferous tubule, it has been well documented that the development of type A spermatogonia to step 19 spermatids, which are homogenization-resistant, requires 49 days [17]. Therefore, the reduction of

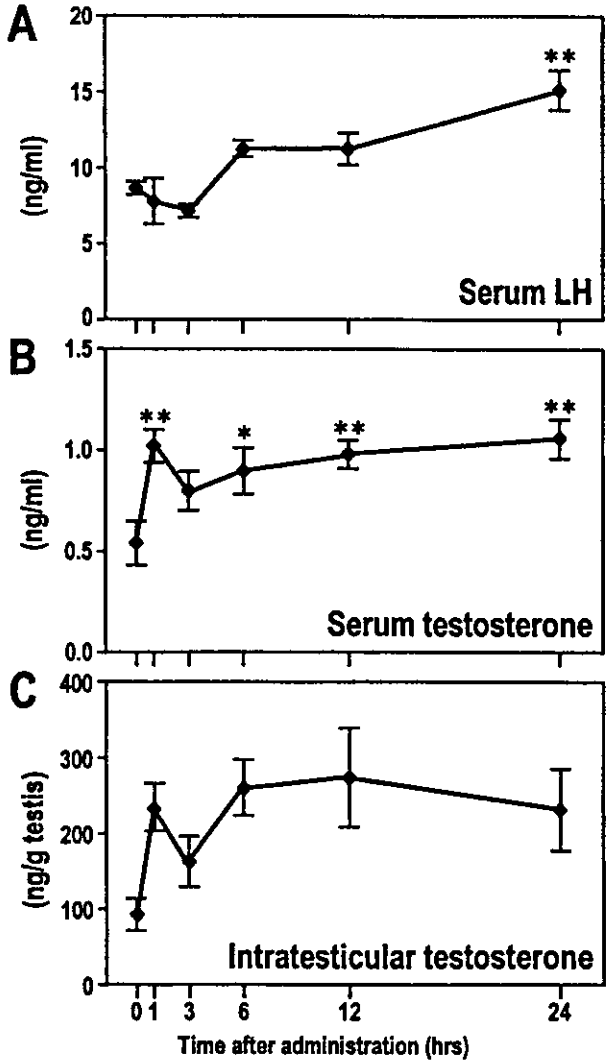

Fig. 3. Change in serum lutenizing hormone (LH) and testosterone and intratesticular testosterone levels in the time course shortly after a single vinclozolin (VCZ) administration between 12:00 and 15:00. Serum LH and testosterone and intratesticular testosterone levels 1, 3, 6, 12 and 24 hrs after VCZ administration were measured as described in Materials and Methods. A, Serum LH; B, Serum testosterone; C, Intratesticular testosterone. The values expressed are the mean ± SE (n=5). Statistically significant difference between means from control was analyzed by ANOVA followed by Fisher's PLSD test (*P<0.05, **P<0.01).

DSP on D36 but not on D8, indicates that VCZ administration suppressed the early stage of spermatogenesis, i.e. differentiation of spermatogonia or early phase spermatocytes. This is the first report to demonstrate an adverse effect of VCZ on the reproductive system of adult male

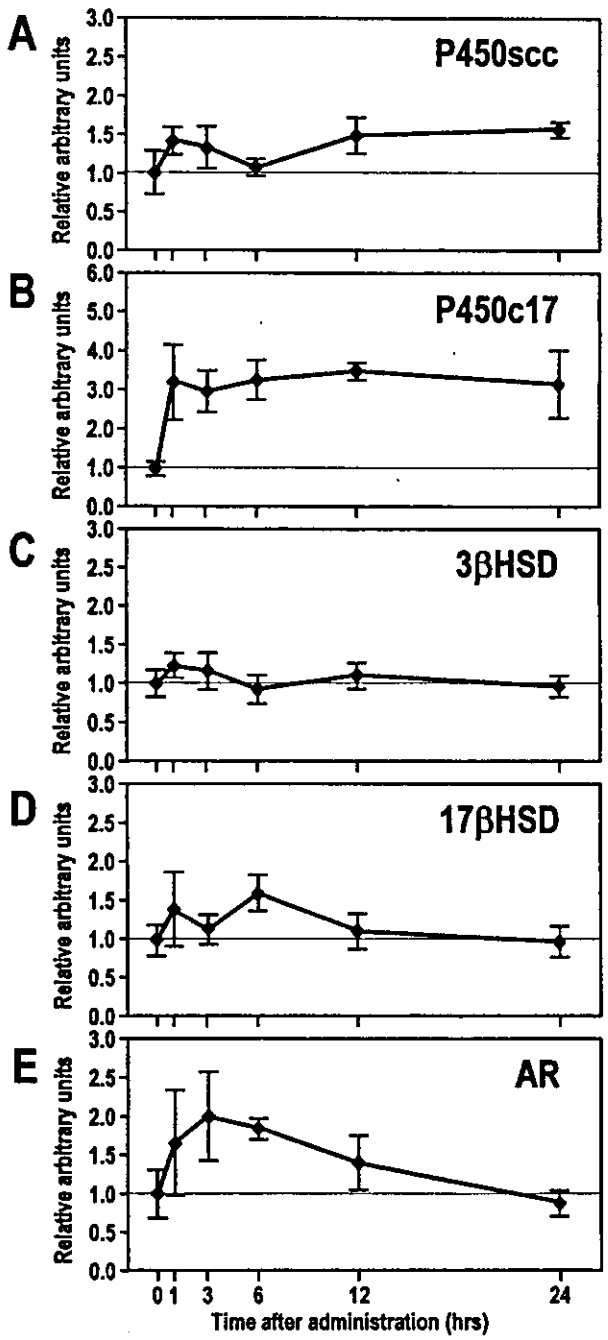

Fig. 4. Change in steroidogenic enzymes (P450scc, P450c17, 3βHDS, 17βHSD) and AR mRNA levels in the testis in the time course shortly after a single vinclozolin (VCZ) administration between 12:00 and 15:00. Semiquantitative RT-PCR was performed to measure mRNA levels after a single administration of VCZ. Amounts of RT-PCR products relative to the average of each control (0 hr) were evaluated by standardizing with internal control cyclophilin. A, P450scc; B, P450c17; C, 3βHSD; D, 17βHSD; E, AR. The values expressed are the mean ± SE (n=3). Statistically significant difference between means from control was analyzed by ANOVA followed by Fisher's PLSD test (*P<0.05, **P<0.01).

animals. In the present study, we employed flutamide (FM) as a positive control, which also causes a decrease of DSP at a similar level to VCZ. Previous reports showed that FM inhibited differentiation of spermatogonia [12, 13]. Our results indicated that VCZ exhibits similar effects as FM in the early stages of spermatogenesis in adult animals.

Vinclozolin increases testicular testosterone production immediately after administration

FM is considered to block the negative feed back of testosterone to the hypothalamus and pituitary as an anti-androgen. The increased level of GnRH pulse increases the transcription of gonadotropin subunit-genes [18] and increases LH secretion and consequently, enhances testosterone production in testis [19, 20]. Increase in serum and intratesticular testosterone levels caused by VCZ in the present study appeared to be mediated by LH, since serum LH and pituitary LHβ subunit mRNA levels were significantly increased by VCZ administration. Injection of LH or human chorionic gonadotropin (hCG) to intact male rats is reported to cause increased P450scc enzyme activity and testosterone overproduction [21]. In addition, repeated administration of a relatively low dose of LH/hCG for 6 days has been demonstrated to increase P450c17 enzyme activity [22]. It is well-documented, by using promoter analysis in a cultured Leydig cell line, that transcriptions of P450scc and P450c17 genes are mediated by cAMP through LH signaling [23]. Based on these reports, the significant increases in P450scc and P450c17 mRNAs, together with the increase of testosterone level induced by VCZ, appears to be mediated by an increase of LH secretion. Therefore, it is probable that VCZ acts on the hypothalamus/pituitary and induces an increase of LH secretion in a similar manner to FM. However, in Experiment 2, despite the unchanged serum LH level during the first 3 hours post administration, a significant increase in serum testosterone was observed 1 hour after VCZ treatment (Fig. 3). It is generally considered that LH secretion represents a pulsatile pattern in response to the GnRH pulse, and that increase of the frequency but not the amplitude of the LH pulse stimulates Leydig cell steroidogenesis [24]. Therefore, there is a possibility that LH-pulse frequency was increased during the first 3 hours even though the serum LH level was the same as that of the 0 hr control. At least, our finding that P450c17 mRNA levels were higher at all times from 1 hour after administration (Fig. 4B) suggested that the increase in testosterone production is due to an increase in P450c17.

The present study showed a 1.3-fold increase in the intratesticular testosterone level 48 hrs after a 6-day treatment with VCZ and a 2.8-fold increase 12 hours after a single VCZ treatment. These results of higher intratesticular testosterone level shortly after administration were very similar to the results observed with FM. Recently, it has been indicated that an abnormally high level of intratesticular testosterone inhibits spermatogenesis [25–28]. These consecutive studies conducted by Meistrich and coworkers, strongly suggest that a supra-higher level of intratesticular testosterone directly inhibits spermatogenesis. They report that this inhibition is due to apoptosis of spermatogonia caused by a high level of testosterone and is mediated by AR, irrelevant to gonadotropin [27, 28]. In the present study, we also detected a 3-fold increase in intratesticular testosterone level shortly after VCZ administration. This temporal increase of testosterone might have affected the early stage of spermatogenesis through the same mechanism as described by Meistrich, so that subsequently, DSP was reduced on D36. This speculation does not coincide with our previous discussion on DSP decrease, and in view of the data presented, both are possible. Further study on the direct effect of intratesticular testosterone on spermatogenesis is needed to decide which is the real mechanism in the decrease of DSP.

Possible differential mechanism of action of vinclozolin from flutamide

Although the amount of VCZ administered was 4-fold higher than that of FM, the increase in intratesticular testosterone level was smaller than that of FM, suggesting that the anti-androgenic effect of VCZ may be weaker than that of FM, consistent with a previous report showing a stronger effect of FM than that of VCZ [29]. Previous studies indicate that among the four testicular steroidogenic enzymes, testicular 3βHSD enzyme activity was predominantly affected by FM administration [30]. Consistent with these reports, we found 3βHSD gene expression to be most increased by FM (Fig. 1A). However, VCZ administration did not alter 3βHSD gene

expression at any time in this study. This was completely different from the case of FM administration. Moreover, as shown in Fig. 1A, AR mRNA level was dramatically decreased in the testis by FM administration. Changes in AR mRNA expression could be detected by VCZ administration, with a marked increase of approximately 2 fold, 3 hrs after VCZ administration (Fig. 4E). These results indicated that the action mechanism of VCZ in adult rats partially differs from that of FM. Since pituitary gonadotropin subunit (LHβ and FSHβ) and testicular P450c17 mRNAs levels of VCZ and FM treatment were almost the same, it may be suggested that VCZ acts similarly to FM in the hypothalamus/pituitary, but differently in testis.

Summary

In the present study, we demonstrated that anti-androgen VCZ, an environmental endocrine disrupting chemical, increased intratesticular testosterone level shortly after administration, as seen with FM. This is likely to be caused by VCZ blocking the negative feed back of testosterone to the hypothalamus and pituitary. Nevertheless, the effect of VCZ on testicular genes might be different from that of FM, since no increase in 3βHSD or a decrease in AR mRNA levels, clearly observed in FM-treated rats, was detected.

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Suppressive Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) on the High-Affinity Antibody Response in C57BL/6 Mice

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In the humoral immune response to an invasion of foreign antigens, B cells differentiate into low-affinity antibody-forming cells (AFCs) that mainly secrete IgM or, through germinal center (GC) formation, into high-affinity AFCs that secrete IgG-class antibodies with a higher affinity for the antigen. Previous studies have established the suppressive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on low-affinity antibody responses to antigens. However, whether and how TCDD affects the high-affinity antibody response to antigens has not yet been clarified. In this paper we investigate the effects of TCDD on GC formation, high-affinity AFC generation, and high-affinity antibody production in the primary humoral immune response. C57BL/6 mice were orally administered 0 or 20 μg/kg of TCDD and subsequently immunized with alum-precipitated ovalbumin (OVA) on day 0. Then the GC formation in the spleen and OVA-specific antibodies in the plasma, was evaluated until day 14 postimmunization. TCDD exposure reduced the production of OVA-specific IgG1 on days 10 and 14. GC formation in the spleen was also suppressed by TCDD exposure, and the suppression persisted from day 7 until day 14. In TCDD-administered mice, on day 7, cellular proliferation in the GCs was significantly suppressed, although apoptosis was not markedly affected. In order to measure high-affinity antibody and high-affinity AFCs, the mice were administered TCDD followed by immunization with alum-precipitated (4-hydroxy-3-nitrophenyl) acetyl linked to chicken γ-globulin (NP-CG). The frequency of high-affinity NP-specific AFCs that bind to low-haptenated antigen was clearly shown to be reduced in the spleen on days 10 and 14. Furthermore, the high-affinity anti-NP IgG1 levels on days 10 and 14 postimmunization were significantly reduced by TCDD exposure. Taken together, the results of this paper demonstrate that TCDD exposure inhibits the generation of high-affinity AFCs and high-affinity antibody production during the primary humoral immune response and suggest that these alterations were caused by the suppression of antigen-responding B-cell proliferation induced by TCDD during GC formation.

Key Words: TCDD; germinal center; high-affinity antibody-forming cell; high-affinity antibody; immune suppression; humoral immunity.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous and persistent environmental contaminant that has been shown to exert a wide spectrum of toxic effects (Tohyama, 2002; Van den Berg *et al.*, 1998). The immune system is recognized as being one of the most sensitive targets to the toxicity of TCDD (reviewed by Kerkvliet, 2002). In mice, TCDD has been demonstrated to induce thymus involution and the suppression of both humoral and cellular immunity (Holsapple *et al.*, 1991; Kerkvliet, 2002; Vos *et al.*, 1997/1998), mediated through high-affinity binding to the aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996; Staple *et al.*, 1998; Vorderstrasse *et al.*, 2001).

In the primary humoral immune response, B cells differentiate along two distinct pathways in response to protein-based antigens (reviewed by Kelsoe, 2000). After the activation of antigen-specific B cells by antigen-specific T cells in secondary lymphoid tissues such as the spleen, one pathway leads to the differentiation of antibody-forming cells (AFCs) that secrete IgM. Afterward, AFCs producing downstream immunoglobulin (Ig) classes such as IgG1 are generated. These AFCs secrete low-affinity antibodies encoded by variable gene segments that have not yet been mutated. The other differentiation pathway leads to the formation of germinal centers (GCs) and the subsequent generation of high-affinity AFCs. The activated B cells proliferate vigorously and form GCs. In these GCs, B cells expressing Igs with variable affinities for the antigen are generated by somatic hypermutations of the Ig genes. The cells with improved affinities are positively selected and continue on to become high-affinity AFCs in the early GC formation, while memory B cells are generated in the late GC formation. Within these reactions, cells with low-affinity Igs are eliminated by apoptosis. These processes, which increase the affinity of antibodies, are considered to be important for the effective and rapid elimination of an antigen in the primary humoral immune response.

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Previous studies have demonstrated the suppressive effects of TCDD on the IgM response to antigen exposure (Harper *et al.*, 1994; reviewed by Holsapple *et al.*, 1991; Smialowicz *et al.*, 1996). The fact that the antibody response against a T-cell–independent (TI) antigen is suppressed by TCDD (Harper *et al.*, 1994; Smialowicz *et al.*, 1996) suggests that B cells are the direct targets of TCDD toxicity. As regards the specific site of suppression, TCDD has been reported to inhibit IgM secretion by B cells, which are activated by anti-Ig antibody and growth factors, or by a superantigen *in vitro*; however, TCDD has been shown to exert only a slight effect on the proliferation of these activated B cells (Luster *et al.*, 1988; Wood and Holsapple, 1993). These previous studies suggest that TCDD directly inhibits the terminal stage of the B cell response during low-affinity antibody production. Furthermore, Sulentic *et al.* (2000) recently proposed, based on their results obtained using CH12.LX cells, that TCDD suppresses μ gene expression as well as IgM secretion by inducing the binding of the AhR to the xenobiotic-responsive element within the Ig heavy chain 3′ α-enhancer.

On the other hand, in prior studies, helper T-cell function was also suggested as a target of TCDD toxicity in the suppression of antibody production. The IgM response to a T-cell–dependent (TD) antigen has been reported to be more sensitively suppressed than that to a TI antigen (Kerkvliet *et al.*, 1990). The splenocyte antibody response in nu/nu athymic mice was more resistant than that observed in nu/+ littermates (Kerkvliet and Brauner, 1987). Since the interaction of helper T cells with antigen-stimulated B cells is indispensable for inducing not only the differentiation of low-affinity AFCs, but also for GC formation and subsequent B-cell differentiation into high-affinity AFCs (Garside *et al.*, 1998; Han *et al.*, 1995; Perez-Melgosa *et al.*, 1999; Takahashi *et al.*, 1998), the inhibition of T-cell function by TCDD may cause the suppression of high-affinity AFC production. Although previous studies have reported that TCDD suppresses IgG-class antibody production (Harper *et al.*, 1994; Ito *et al.*, 2002; Kerkvliet *et al.*, 1996; Lundberg *et al.*, 1991; Shepherd *et al.*, 2000; Warren *et al.*, 2000), it is not clear if only low-affinity IgG is sensitive to TCDD, or if high-affinity IgG production is also affected by TCDD. In the latter case, high-affinity AFCs may be affected in a mechanism that is different from low-affinity AFCs. To clarify whether and how TCDD affects the high-affinity antibody response to antigens, we carried out this study, focusing on the formation of GC, the generation of high-affinity AFCs, and the production of high-affinity antibodies in the primary humoral immune response of C57BL/6 mice.

MATERIALS AND METHODS

Animals. Female C57BL/6N mice (5 weeks old) were purchased from Clea Japan (Tokyo) and were acclimatized to the experimental environment for 1 week prior to their use. The mice were maintained in a controlled environment with the following conditions: The temperature was maintained at 24 ± 1°C, the humidity at 50 ± 10%, and the mice were kept on a 12/12-h light/dark cycle. The animals were given food and distilled water *ad libitum*. The mice were handled in a humane manner, according to NIES guidelines for animal experiments.

Mouse treatments. TCDD (purity, 98%) obtained from Cambridge Isotope Laboratory (Andover, MA) was a concentration of 50 μg/ml in nonane. The TCDD/nonane solution was further diluted with corn oil to render a dose volume of 10 μl/g body weight. Ovalbumin (OVA) (Grade VII; Sigma, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. An equal volume of 9% (w/v) $AlK(SO_4)_2$ solution was added to the OVA solution and the pH was adjusted to 6.5 with KOH. After repeated washings with PBS, the alum-precipitated OVA (OVA/alum) was resuspended in PBS at 0.5 mg OVA/ml. The mice were administered a single dose of TCDD (20 μg/kg) or vehicle by gavage and were subsequently immunized intraperitoneally with 100 μg of OVA/alum. On a specified day postimmunization, the animals were sacrificed, and their plasma samples and spleen were examined. In some experiments (4-hydroxy-3-nitrophenyl) acetyl linked to chicken γ-globulin (NP-CG) (NP:CG conjugation ratio 16:1) (Takahashi *et al.*, 2001) was used instead of OVA, and on a selected day the plasma samples, spleen, and bone marrow (BM) were examined.

Enzyme-linked immunosorbent assay. For the determination of OVA-specific IgM levels, 96-well plates were incubated overnight at 4°C after being coated with 1 mg/ml of OVA in PBS and were blocked with 3% BSA in PBS for 1 h at 37°C. The plates were incubated at room temperature for 2 h with serial dilutions (1:10 to 1:100) of each plasma sample. They were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 37°C and visualized with *o*-phenylenediamine dihydrochloride.

OVA-specific IgG1 was measured as described in Nohara *et al.* (2002) with some modifications. In brief, 96-well plates were coated with 10 μg/ml of OVA, washed, and blocked with BlockAce (Dainippon Pharmaceutical Co., Tokyo). A 100-μl volume of each plasma sample was added to the plates in serial dilutions from 1:500 to 1:2000. After being washed, the plates were incubated with HRP-conjugated anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) and visualized as described above. Vehicle control- and TCDD-treated groups were compared in the linear range of absorbance.

Total or high-affinity NP-specific IgG1 was measured by using high-NP–haptenated or low-NP–haptenated bovine serum albumin (NP:BSA conjugation ratio 25:1, NP25-BSA, or conjugation ratio 2:1, NP2-BSA, Biosearch Technologies, Inc., Novato, CA) as the coating antigen according to a previous report (Takahashi *et al.*, 1998) with some modifications. By using this method, the NP25-BSA ELISA detects the total antibodies, and the NP2-BSA ELISA detects the high-affinity antibodies. After 96-well plates were coated with 50 μg/ml of NP-BSA, they were washed and blocked with BlockAce. Then, a 100-μl volume of the diluted plasma samples (1:100 to 1:218,700) or standard (kindly provided by Dr. T Azuma, Tokyo University of Science) was added to the plates, and the bound antibodies were detected as described above. The concentration of plasma samples (diluted 1:2700) was determined from the standard curve.

Cell preparation. A single-cell suspension was prepared from the spleen as previously described (Nohara *et al.*, 2002). BM cells were isolated by flushing the femurs with 2% fetal calf serum (FCS) (Sigma) in PBS, using a 5-ml syringe with an attached 21-gauge needle. After centrifugation, the cells were hemolyzed by the method used for the splenocytes, and then the cells were washed twice and filtered through a stainless steel mesh for further manipulation.

Enzyme-linked immunospot. Total AFCs or high-affinity AFCs specific for NP hapten were detected by an enzyme-linked immunospot (ELISPOT), as described in Takahashi *et al.* (1998). Briefly, nitrocellulose filters (Osmonics, Minnetonka, MN) were coated with 50 μg/ml of NP25-BSA, NP2-BSA, or BSA in PBS and then blocked with 1% BSA in PBS. Splenocytes (6×10^5) or BM cells (1.2×10^6) were incubated on nitrocellulose filters in 96-well plates at 37°C in 5% CO_2 for 2 h. Nitrocellulose filters were washed and stained with alkaline phosphatase (AP)–conjugated anti-IgG1 antibody (Southern Biotech-

nology). The AP activity then was visualized as described in Takahashi *et al.* (1998). The frequency of total AFCs or high-affinity AFCs was determined from the NP25-BSA– or NP2-BSA–coated filters by subtracting the background level observed on the BSA-coated filters.

Flow cytometry. Splenocytes (1×10^6) were stained with fluorescein isothiocyanate (FITC)-conjugated anti-B220 mAb (Pharmingen, San Diego, CA; clone RA3-6B2) and phycoerythrin (PE)-conjugated anti-CD3 mAb (Pharmingen; clone 145–2C11) to detect B cells and T cells. GC B cells, which are known to be detectable with peanut agglutinin (PNA) (Shinall *et al.*, 2000), were stained with FITC-conjugated PNA (Honen Corporation, Tokyo) and PE-conjugated anti-B220 mAb (Pharmingen; clone RA3-6B2). After staining, the cells were treated with 7-aminoactionmycin D (Sigma) to label the dead cells and measured using a FACSCalibur (Becton Dickinson, Mountain View, CA) as described in Nohara *et al.* (2000). The cells were gated to exclude dead cells and cell debris and then were analyzed. Data were collected from 10,000 cells.

Histologic studies. Serial spleen cryosections (6 μm) and paraffin sections (4 μm) were prepared for histochemical staining. The GCs were visualized by incubating acetone-fixed cryosections with biotin-conjugated PNA (Vector, Burlingame, CA), followed by incubation with HRP-conjugated streptavidin (Vector) and 3,3′-diaminobenzidine (DAB) as the substrate. Apoptotic cell death in the GCs was estimated in cryosections by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method using an *in situ* apoptosis detection kit (TaKaRa Biomedicals, Tokyo), and DAB was used as the chromogen. To co-label TUNEL$^+$ cells with PNA, AP-conjugated streptavidin (Southern Biotechnology) was used instead of HRP-conjugated streptavidin, and the AP activity was visualized as described in Takahashi *et al.* (1998). Paraffin sections were examined to detect cellular proliferation in the spleen by an immunohistochemical method using anti-Ki-67 Ab (Santa Cruz Biotechnology, Santa Cruz, CA; clone M-19) as described in Weihua *et al.* (2000) with a slight modification. After the deparaffinization and subsequent rehydration of the sections, antigen retrieval was conducted by autoclave processing instead of boiling. To visualize Ki-67$^+$ cells, sections were incubated with anti-Ki-67 Ab followed by incubation with HRP-conjugated anti-goat IgG (Sigma) and DAB as the substrate. Then the sections were stained with hematoxylin to identify the white pulp. The stained sections were visualized under a Leitz DMRBE microscope (Leica, Wetzler), and the images were captured by a 3CCD color camera (Leica). Then the area of white pulp, Ki-67$^+$ cell clusters, and GCs were quantified by analyzing the digitized images using *Leica Qwin* Version 2.2A software. TUNEL$^+$ cells were counted using a Leitz DMRBE microscope. Three random fields per spleen were analyzed at a fivefold magnification for Ki-67$^+$ analysis, and 15 random fields per spleen were analyzed at a 40-fold magnification for TUNEL$^+$ analysis. The means of the values obtained per each spleen were used to calculate the ratio of Ki-67$^+$ area to the area of white pulp or the ratio of TUNEL$^+$ cell number to the GC area.

Statistical analysis. The results are expressed as the mean ± standard error of 5–8 mice per group. Significant differences between the vehicle-control group and the TCDD-treated group were determined by two-tailed Student's *t*-test, and a value of $p < 0.05$ was considered significant.

RESULTS

Time-Course of Antibody Production following TCDD Administration and Immunization

First, we examined the time-course of antigen-specific IgM and IgG1 production following TCDD administration and OVA/alum immunization. To this end, the mice were treated with 20 μg/kg of TCDD and subsequently immunized with OVA/alum. Then the levels of OVA-specific antibody in the plasma in the vehicle-control group and TCDD-treated group were determined until day 14 (Fig. 1). In the vehicle-control mice, a significant increase in the level of anti-OVA IgM was found on day 7, which was maintained up until day 10, whereupon the level again decreased on day 14. The level of anti-OVA IgG1 increased dramatically on day 10 and remained elevated on day 14. On the other hand, in the TCDD-treated mice, the level of anti-OVA IgM from days 7 through 14 was

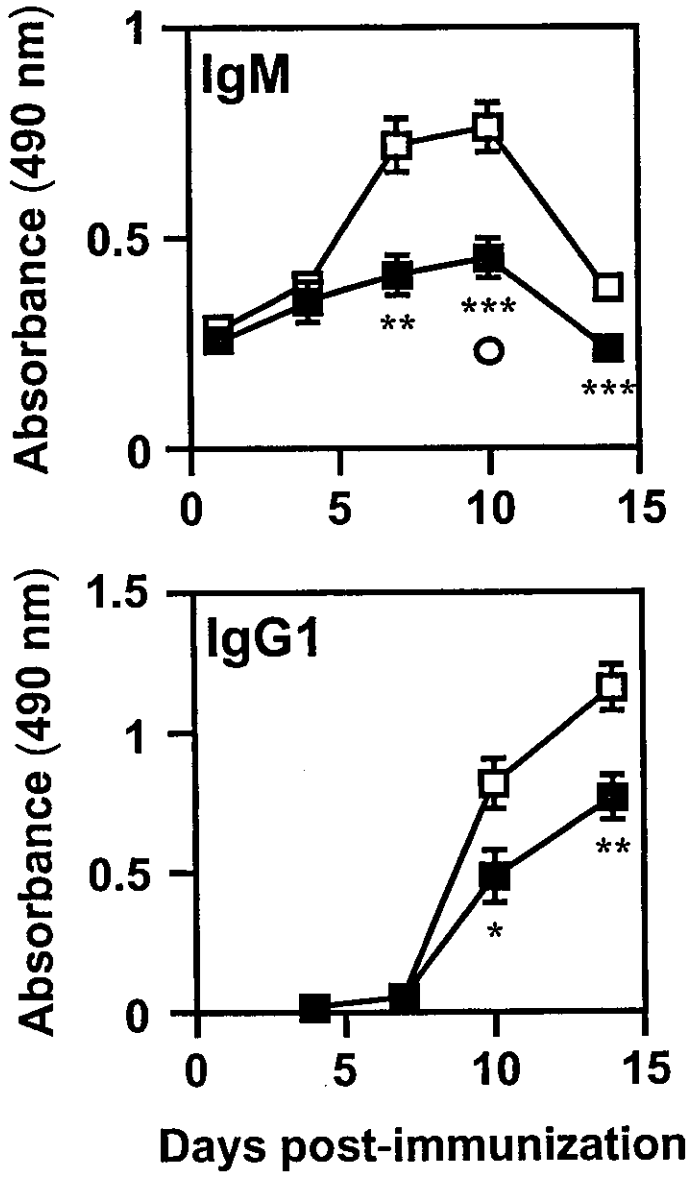

FIG. 1. Time-course of TCDD-induced suppression of OVA-specific antibody production. C57BL/6N mice were administered a single oral dose of 20-μg/kg TCDD (closed square) or corn oil as a vehicle (open square) and then were immunized with 100 μg of OVA/alum on day 0. Plasma samples were obtained from the vehicle-control and TCDD-treated groups on days 1, 4, 7, 10, and 14 or from a nonimmune (circle) group on day 10. OVA-specific IgM- and IgG1-class antibodies in the plasma were measured by ELISA as described in the Materials and Methods section. Data of IgM (diluted 1:30) or IgG1 (diluted 1:1000) are presented as means ± SE (n = 5–8). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

significantly reduced compared with that in the vehicle-control group. The level of anti-OVA IgG1 was also significantly reduced in the TCDD-treated mice (by 41% in comparison to the vehicle-control group on day 10 and by 35% on day 14).

Time-Course of TCDD-Induced Suppression of B Cell Expansion

Next we examined the cellularity of the spleens, where the AFCs are generated. Following the OVA/alum immunization, the total number of splenocytes increased after immunization in the vehicle-control mice, whereas such an increase was completely suppressed in the TCDD-administered group (Table 1). The number of B cells in the spleen increased following immunization in the vehicle-control group, as did the total number of splenocytes; such an increase was again completely suppressed by TCDD administration (Fig. 2A). TCDD exposure increased the percentage of B cells on day 4 (Fig. 2B), which may be attributed to a decrease in the number of $B220^-$ $CD3^-$ cells following TCDD exposure (data not shown). In contrast, the percentage of B cells was 5% less in the TCDD-treated group on day 10.

TCDD Exposure Suppresses GC Formation in the Spleen

To evaluate the effects of TCDD on GC formation in the spleen, we determined the frequency of PNA^+B220^+ GC B cells by flow cytometry (Fig. 3A). In the vehicle-control mice, GC B cells were detected from day 7 after immunization; this number reached a peak on day 10 and was followed by a subsequent decrease on day 14 (Fig. 3B). The time-course of changes in the percentage of GC B cells was similar to that of the changes in the number of GC B cells, with an increase on day 10 and a subsequent decrease noted on day 14 (Fig. 3C). TCDD exposure decreased the numbers of GC B cells by 60, 53, and 42% on days 7, 10, and 14, respectively. The percentages of GC B cells were also less in TCDD-treated mice by 20, 19, and 31% of the vehicle-control group on days 7, 10, and 14,

TABLE 1
Time-Course Changes of Spleen Cell Number in Vehicle-Control and TCDD-Treated Mice

	Cell number ($x10^7$)	
Days postimmunization	Vehicle	TCDD
1	5.2 ± 0.3	6.9 ± 0.7
4	6.6 ± 0.8	5.6 ± 0.4
7	9.6 ± 0.6	5.1 ± 0.4***
10	8.9 ± 0.9	5.2 ± 0.6**
14	6.8 ± 0.4	5.9 ± 0.8

Note. C57BL/6N mice were administered a single oral dose of TCDD or corn oil as a vehicle and then immunized with 100 μg of OVA/alum on day 0. The splenic cell number was measured on the indicated days. Data are presented as means ±S.E. (n = 5–8). $^{**}p < 0.01$, $^{***}p < 0.001$.

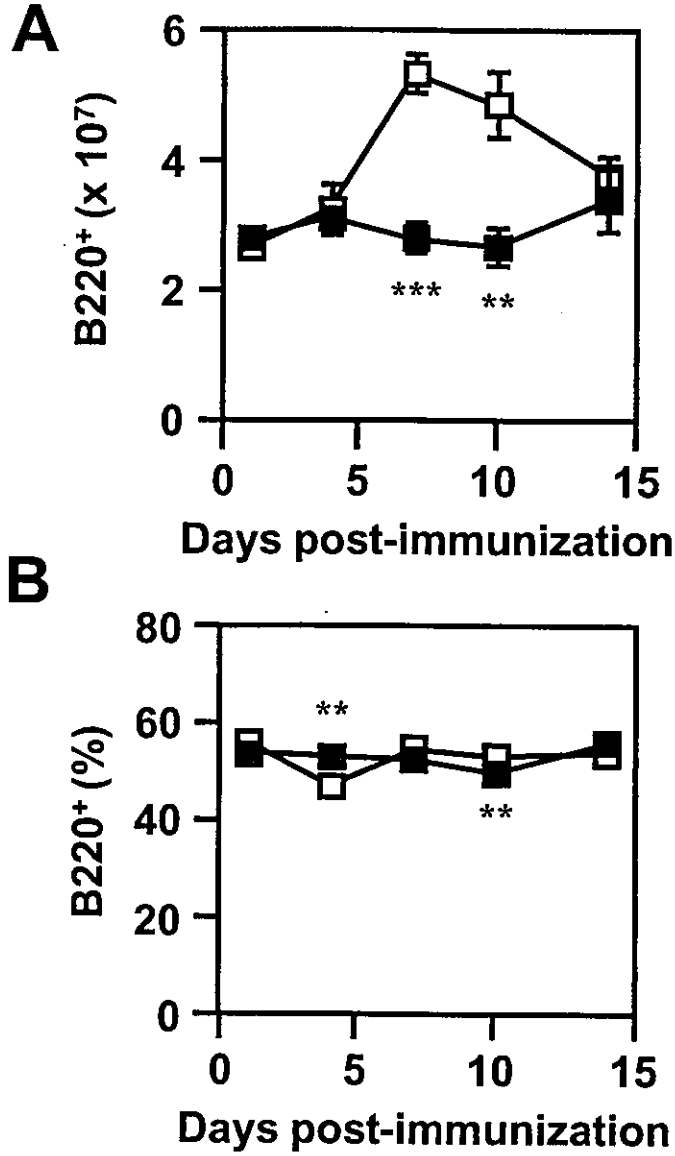

FIG. 2. Flow cytometric analysis of splenic B cells in vehicle-control and TCDD-treated mice. (A) Number and (B) percentage of splenic B cells. The mice were treated as described in Figure 1. Splenocytes obtained from the vehicle-control (open square) and TCDD-treated (closed square) groups on days 1, 4, 7, 10, and 14 were stained with anti-B220 mAb for B cells and analyzed by flow cytometry. The cell number was calculated as the percent of positives multiplied by the total spleen cell number. Data are presented as means ± SE. (n = 5–8). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

respectively. The TCDD-dependent decrease in the percentage of B cells on day 10, as described above (Fig. 2B), was shown to be due to a decrease in the percentage of PNA^+B220^+ GC B cells, since the percentage of PNA^-B220^+ cells was not found to be altered following the administration of TCDD (data not shown).

GC formation in the spleen was further examined by histological methods. In splenic sections prepared from vehicle-control mice on day 14, a large cluster of GCs was observed in

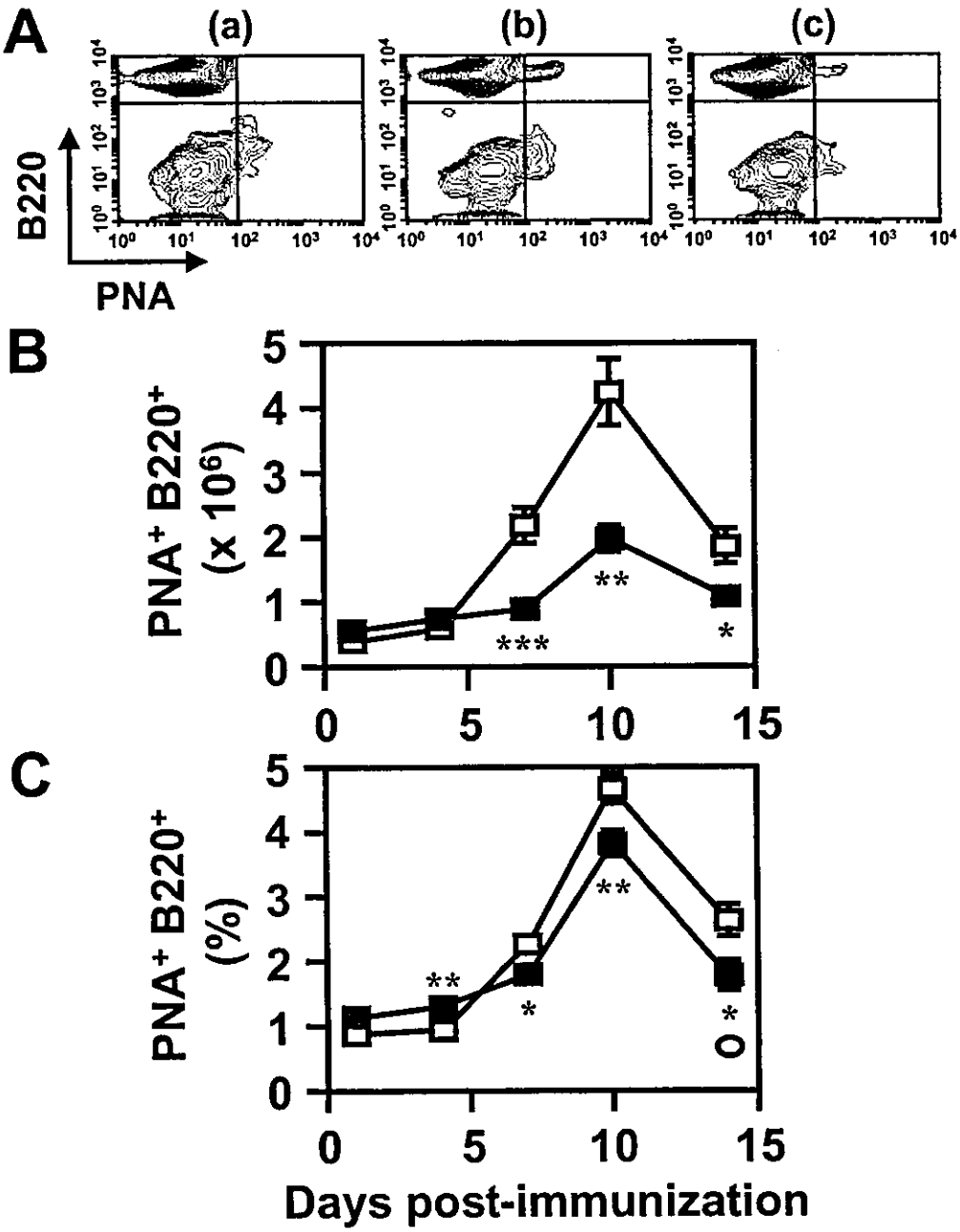

FIG. 3. Flow cytometric analysis of GC B cells in vehicle-control and TCDD-treated mice. Splenocytes obtained from the vehicle-control (open square) and TCDD-treated (closed square) groups on days 1, 4, 7, 10, and 14 or from nonimmune mice (circle) on day 14 were stained with PNA and anti-B220 mAb and analyzed by flow cytometry. (A) The contour plots show PNA$^+$B220$^+$ GC B cells on day 14 postimmunization: (a) nonimmune, (b) immunized vehicle-control, and (c) immunized TCDD-treated mouse. (B) Number and (C) percentage of PNA$^+$B220$^+$ GC B cells. The cell number was calculated as a percentage of the positives multiplied by the total spleen cell number. Data are presented as means ± SE. (n = 5–8). *p < 0.05, **p < 0.01, ***p < 0.001.

the white pulp (Fig. 4A), whereas, in the corresponding sections obtained from TCDD-treated mice, an apparent reduction in GC size was observed (Fig. 4B). These results revealed that TCDD significantly suppresses the formation of GCs following OVA/alum immunization.

TCDD Exposure Suppresses Cellular Proliferation but Does Not Alter Apoptosis in GCs

Since GC formation in the spleen was suppressed by TCDD exposure, we analyzed cellular proliferation and apoptosis in GCs to elucidate the mechanism of suppression of GC formation by TCDD. Cellular proliferation was estimated by an immunohistochemical method that detected the expression of the cell-cycle–associated nuclear antigen, Ki-67. As is shown in Figure 4C, large clusters of Ki-67$^+$ cells were observed in the white pulp of the vehicle-control mice on day 7. Serial sections indicated that the Ki-67$^+$ cell clusters were localized in the GCs (data not shown). In the TCDD-treated mice, an apparent decrease in the size of the Ki-67$^+$ cell clusters was detected in the white pulp (Fig. 4D). When the ratio of the area occupied by the Ki-67$^+$ cell clusters to that of the white pulp was analyzed, a 48% reduction was detected in the TCDD-