Effects of 6-Hydroxydopamine on the Development of the Immune System in Chickens

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ABSTRACT. It has been suggested that the sympathetic nervous system communicates with lymphocytes expressing cell surface receptors for neurotransmitters such as norepinephrine (NE), on the basis of the finding that neurotransmitters modify immune responses in mammalian species. We confirmed that chicken lymphocytes in the brusa of Fabricius, thymus and spleen expressed β-adrenergic receptor (β-AR) mRNA from embryonic day (E) 10 and that intracellular cAMP level was elevated by NE, suggesting that lymphocytes express functional β-AR on their surface at an early embryonal stage. To clarify whether the nervous system is involved in the development of the immune system, the effects of 6-hydroxydopamine (6-OHDA), one of sympathectomizing agents, on chicken lymphocytes was investigated. A single injection of 6-OHDA at a dose of 400 µg into a chicken embryo was carried out at E7 or 14 (as referred to E7 group and E14 group, respectively). NE level and the relative proportion of Bu-1a+, CD4+ and CD8+ cells in the spleen of 3-week-old chickens were not altered by 6-OHDA treatment. However, the proliferative responses and expression of IL-2 mRNA in spleen cells cultured with pokeweed mitogen were reduced in E7 group compared with those of control. Furthermore, in CD8+ spleen cells of E14 group of 3-week-old chickens, the expression of β-AR mRNA and the relative increase of intracellular cAMP stimulated with NE were significantly decreased. These results suggest that the sympathetic nervous system affects the development of the immune system.

KEY WORDS: β-adrenergic receptor, cytokine, 6-hydroxydopamine, lymphocyte proliferation, norepinephrine.

FULL PAPER

Immunology

It has been demonstrated that lymphoid organs, including the thymus, spleen and lymph nodes, are innervated by the sympathetic nervous system [9, 45]. Norepinephrine (NE), a signaling molecule of the nervous system, is released from sympathetic nerve terminals and binds to high affinity β-adrenergic receptors (β-AR) that are expressed on various immune cells. β-AR transduce signals from NE to G protein, which in turn activates the adenylate cyclase to produce the second messenger cAMP. Stimulation of β-AR by NE has been reported to be associated with the cytokine production [35, 36], cell proliferation [2], and the expression of surface molecules on immune cells [8, 11, 43].

In the chicken, it is also observed that catecholamine modulates immune responses such as antibody responses [6, 10], phytoshemaglutinin wattle response and lymphocyte migration [31]. Denno et al. [6] showed that in vivo NE administration suppressed IgM- and IgG-PFC and in vitro NE treatment of spleen lymphocytes reduced IgM-PFC. This inhibition of IgM-PFC was blocked by treatment with antagonists to α- and β-receptors, suggesting that there are α- and β-receptor sites on lymphocytes and that catecholamine has a regulatory role in immunoglobulin synthesis.

6-Hydroxydopamine (6-OHDA) is a neurotoxin that selectively destroys sympathetic nerve fibers with the catecholamine high affinity carrier found on nerve terminals in the peripheral nervous system [3]. In the rodent, 6-OHDA is often used to investigate the interaction between the nervous and immune systems, and it appears that 6-OHDA injection in vivo decreases NE levels and alters antibody responses [16, 18, 20], cytokine production [20, 34], and mitogen-induced lymphocyte proliferation [1, 29]. There is, however, little information concerning the effects of catecholamine on the development of the immune system at the embryonal stage. In the chicken, Mulder et al. [33] demonstrated that basal plasma NE level was significantly higher than epinephrine (EPI) at embryonic day (E) 10 and remained constant during the embryonal stage. On the other hand, plasma EPI level was undetectable at E10 but gradually increased from E10 to E19, indicating that high circulating levels of NE detected at the early developmental age of embryos may be derived from sympathetic nerve fibers. Therefore, it is considered that the sympathetic nervous system would be involved in the development of the immune system in the chicken. Regarding the avian lymphogenesis, the inflow of the lymphoid stem cells into the thymus rudiment starts on E6.5 [22] and the colonization of the bursal primordium by stem cells occurs from E8 to 14 [13].

The purpose of the present study was to investigate the effects of 6-OHDA treatment at E7 or E14 on lymphocyte development to elucidate the interactions between the nervous system and immune system at embryonal stage in the chicken.

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MATERIALS AND METHODS

Animals: Chickens and fertile eggs of White Leghorn inbred strain H.B2, maintained at the National Institute of Animal Health, Tsukuba, Japan, were used. Fertilized eggs were incubated at 38°C and 80% humidity and the embryonic age was determined by the length of the incubation period.

Treatment with 6-OHDA: Previous study showed that 400 µg of 6-OHDA treatment at E7 or E14 induced significantly reduced NE levels in the peripheral blood of 8-week-old [48], thus, same protocol was used in this study. 6-OHDA (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in sterile saline containing 0.01% ascorbate as an anti-oxidant. Embryos received a single injection of 400 µg of 6-OHDA into the chorioallantoic cavity at E7 or E14, as referred to E7 group and E14 group, respectively. Embryos injected with saline instead of 6-OHDA were used as a control group.

Cell suspensions: Cells were prepared from lymphoid organs by teasing the minced organs through a stainless steel mesh, and fractionated by centrifugation on a Percoll density gradient (Amersham Pharmacia Biotechn, Uppsala, Sweden) as described previously [30].

Immunofluorescence analysis and cell sorting: Indirect immunofluorescence staining was performed by incubation of cells with each supernatant of the relevant hybridoma cells (anti-Bu-1a, a B cell marker (clone L22) [39], anti-CD4 (clone 2–6) [27] or anti-CD8 (clone 11–39) [26]) and consecutively with FITC-conjugated goat anti-mouse Ig diluted 1:100 (Zymed, San Francisco, CA, U.S.A.). Relative immunofluorescence intensities were measured by flow cytometry using an Epics XL instrument (Beckman Coulter, Fullerton, U.S.A.). For RNA extraction, spleen cells from 3-week-old chickens were sorted into Bu-1a, CD4 and CD8 positive cell fractions with FITC- conjugated goat anti-mouse Ig. The purity was 70–80% for Bu-1a+ cells, 70–90% for CD4+ cells and 80–95% for CD8+ cells. For measurement of intracellular cAMP level, cells (10^6) were sorted by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) and the purity was >90%.

Measurement of NE and dopamine (DA) levels: Heparinized blood was centrifuged at 4,500 rpm at 4°C for 15 min, and plasma was stored at –80°C until analysis. NE and DA levels in plasma were determined as described previously [48].

Spleen was freshly frozen and stored at –80°C until analysis. The tissues were homogenized in 0.1 M HClO₄ containing 3,4-dihydroxybenzylamine (0.25 µM) that was used as an internal control, and spun at 15,000 rpm. To perform catecholamine extraction, 80 mg acid-wash aluminum was added to each supernatant, the pH was adjusted to 8.6 with Tris-HCl and then vortexed. The aluminum was then washed twice with distilled water, and catecholamine was extracted with 200 µl of 0.1 M HClO₄. Samples were frozen at –80°C until analysis by HPLC as described previously [48].

Cell proliferation responses to mitogen: Spleen cells were suspended in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 1% normal chicken serum (NCS), 5 × 10^-5 M 2-mercaptoethanol (Sigma), 100 µg/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan) and 100 U/ml penicillin (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan). The cells (2 × 10⁶ cells/ml) were cultured at 40°C in a humidified atmosphere of 5% CO₂ in the presence or absence of pokeweed mitogen (PWM, 50 µg/ml, Sigma) and concanavalin A (Con A, 5 µg/ml, Sigma). Mitogen-induced lymphocyte proliferation was determined by bromodeoxyuridine (BrdU) incorporation assay as described previously [32]. Cells were dispensed in duplicate (1 ml per well) in a flat-bottom 48 well plate (Nunc, U.S.A.). BrdU (18 µg/ml, Sigma) was added to cells for the final 20 hr. BrdU incorporation rate was measured by a flow cytometer (Epics XL, Beckman Coulter). Stimulation index was calculated by BrdU incorporation rates of cells cultured with mitogen divided by those of cells cultured without mitogen.

Reverse transcription polymerase chain reaction: The expression level of each of mRNA for β-AR and interleukin-2 (IL-2) was detected by the reverse transcription polymerase chain reaction (RT-PCR) method. The mRNA was prepared from cells with QuickPrep™ Micro mRNA purification kit (Amersham Pharmacia Biotech) by following the manufacturer’s instructions. The sense and antisense primers for β-AR used were designed on the basis of the published cDNA sequences [47]. The sense primer used was 5’-ATGATCTCTCGTGACTTCTGC-3’ (located from 736 to 756 in the cDNA sequence), and the antisense primer was 5’-CTCCATCTTCAGACTCGGAGCC-3’ (located from 1324 to 1344 in the cDNA sequence), and the predicted length of the amplified fragment is 611 bp. The primers for IL-2 [5] and β-actin [23] were used as published information. The cDNA was synthesized with random hexamer as primer by using SuperScript™ II reverse transcriptase (Gibco BRL, Gland Island, MD, U.S.A.). PCR amplification was conducted by GC-rich system (Roche Diagnostics, Mannhaim, Germany) containing 0.5 µM of sense primer, 0.5 µM of antisense. Cycling condition steps for the amplification reaction were a single 5-min heating step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 1 min, and extension at 72°C for 1 min. After the last cycle, the reaction mixture was incubated at 72°C for 7 min and cooled to 4°C. The PCR-amplified products were electrophoresed and visualized on 1% agarose gel containing 0.5 µg/ml of ethidium bromide. The intensities of amplified products were analyzed by image analysis system (AlphaImager, Alpha Innotech Corporation, San Leandro, U.S.A.).

Measurement of intracellular cAMP level: To evaluate the intracellular cAMP level, cells (1 × 10⁶ cells) suspended in 1 ml of 1% NCS-IMDM were incubated with NE (100 µM, Wako Pure Chemical Industries, Ltd., Osaka) in the presence or absence of propranolol (a β-adrenergic receptor antagonist, 1 µM, Sigma) for 15 min at 40°C. The concentration of NE and propranolol were referred to published
information [14, 42]. The intracellular cAMP level was measured by cAMP enzymeimmunoassay system (Amer-
sham Pharmacia Biotech). The relative increase of cAMP
upon β-AR stimulation with NE was calculated by subtrac-
tion of basal levels from stimulated ones divided by basal
levels.

Statistics: Results are expressed as mean ± standard error
(SE), and differences among the various parameters were
analyzed by ANOVA and post-hoc analysis. Significant
differences were defined at p<0.05.

RESULTS

Expression of β-AR in the bursa of Fabricius, thymus, and spleen: In order to clarify whether chicken lymphocytes
express β-AR and respond to NE, the expression of β-AR
mRNA and the intracellular cAMP levels stimulated with
NE were evaluated. It was found that lymphocytes
expressed β-AR mRNA in the bursa of Fabricius, thymus
and spleen at E10 at least, and the levels of its expression
increased with embryonal development (Fig. 1A). In
primary lymphoid organs, the relative increase of cAMP was
significantly high at E10 but decreased with development.
On the other hand, the relative increase of cAMP in the

Fig. 1. Expression of β-AR mRNA and NE-mediated intracellular cAMP elevation in the bursa of Fabri-
cius, thymus and spleen. (A) Expression of β-AR mRNA. E, embryonic day; H, hatch; w, weeks of age;
M, molecular marker (ΦX174 RF DNA/Hae III fragment). (B) The relative increase of intracellular
cAMP in cells stimulated with NE. ■, bursa of Fabricius; ○, thymus; △, spleen. (C) Effects of propra-
nolol on intracellular cAMP elevation by NE in E10 embryo. □, medium; ■, NE; ■, propranolol and
NE. Values are expressed as mean ± SE (n=3–7). *, p<0.01; **, p<0.05 when compared to medium.
spleen was almost constant from E10 to 3 weeks of age (Fig. 1B). NE-mediated intracellular cAMP elevation was blocked by propranolol, one of β-antagonists (Fig. 1C), indicating that lymphocytes express functional β-AR on their surface at E10 at least. These results suggested that the nervous system would influence lymphocyte development via β-AR by NE.

**Effects of 6-OHDA on NE and DA levels:** It has been demonstrated that chemical sympathectomy with 6-OHDA in mice significantly reduce NE levels in the spleen [1, 16, 18, 20, 29]. To evaluate effects of 6-OHDA treatment at E7 or E14, NE and DA levels in the spleen and peripheral blood of 3-week-old chickens were measured by HPLC. As shown in Fig. 2, there was no significant difference between control and 6-OHDA treatment groups in the spleen, however, NE levels in the peripheral blood of E7 and E14 groups were significantly decreased when compared with those of control. The result in the peripheral blood was in agreement with previous study [48]. These results indicated that effect of 6-OHDA treatment on NE and DA levels remained in the peripheral blood of 3-week-old chickens.

**Effects of 6-OHDA on lymphocyte proliferation responses to mitogens:** As shown in Table 1, lymphocyte proliferation responses to PWM in E7 group significantly decreased in both BrdU incorporation rate and stimulation index when compared with those of control. On the other hand, in E14 group, stimulation indexes in both PWM- and Con A-induced proliferation were slightly lower than those of control.

Table 1. Effects of 6-OHDA on mitogenic responses of spleen cells to pokeweed mitogen and concanavalin A

<table>
<thead>
<tr>
<th>Group</th>
<th>Mitogen</th>
<th>BrdU+ (%)</th>
<th>Stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Medium</td>
<td>4.8 ± 1.2</td>
<td>9.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>PWM</td>
<td>30.2 ± 3.2</td>
<td>15.4 ± 4.5</td>
</tr>
<tr>
<td>E7</td>
<td>Con A</td>
<td>51.0 ± 7.6</td>
<td>8.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>8.6 ± 2.4</td>
<td>6.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>PWM</td>
<td>14.0 ± 6.6</td>
<td>11.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Con A</td>
<td>46.3 ± 8.6</td>
<td>11.2 ± 2.2</td>
</tr>
<tr>
<td>E14</td>
<td>Con A</td>
<td>50.1 ± 6.4</td>
<td>11.2 ± 2.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE (n=7). *, p<0.01; **, p<0.05 when compared to control.

Alteration of T cell population in the spleen following 6-OHDA treatment was observed in adult mice [29]. To confirm whether altered T cell population contributed to the reduced proliferative responses, flow cytometric analysis of lymphocyte populations in the spleen was conducted. It was observed that the relative proportion of Bu-1α+, CD4+ and CD8+ cells in E7 group was similar to that of control (Table 2). In addition, there were no significant differences in the relative proportion of those surface marker antigens in the bursa of Fabricius and thymus of 3-week-old chickens (data not shown).

In chickens, PWM stimulates peripheral B cells as well as activating a T cell subset [38], and treatment of spleen cells with anti-CD4 antibody (clone CT-4) inhibits PWM-induced proliferation, indicating that CD4+ T cells produce growth factors such as IL-2 responses to PWM [4]. Consequently, it is suggested that lower lymphocyte proliferation responses to PWM in E7 group are caused by a decrease in IL-2 production. As shown in Fig. 3, the expression of IL-2
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mRNA of spleen cells cultivated with PWM for 24 hr in E7 groups was lower than that of control.

Taken together, these findings suggest that lower expression of IL-2 mRNA is associated with the reduced lymphocyte proliferation.

**Effects of 6-OHDA on the expression of β-AR mRNA and intracellular cAMP levels:** The inhibitory effects of elevated cAMP levels on T cell proliferation and effector functions are well recognized [15]. To elucidate whether an increase in the number of β-AR or intracellular cAMP level by 6-OHDA treatment causes the reduced lymphocyte proliferation, the expression of β-AR mRNA and intracellular cAMP levels were investigated. The expression of β-AR mRNA in Bu-1a⁺ and CD4⁺ cells did not alter by 6-OHDA treatment (Fig. 4A), and the basal intracellular cAMP level and NE-induced cAMP elevation were also within the range of control (data not shown). The expression of β-AR mRNA in CD8⁺ cells of E14 group was significantly decreased (Fig. 4A). Furthermore, its basal intracellular cAMP level tended to increase and the relative increase of cAMP was significantly lower than that of control (Fig. 4B). On the contrary, the expression of β-AR mRNA, basal intracellular cAMP level and NE-mediated cAMP elevation in CD8⁺ cells in E7 group were as same as control, indicating that reduced proliferation responses to PWM were not relevant to the elevated cAMP levels in T cells.

**DISCUSSION**

We used 6-OHDA to study the potential role of the sympathetic nervous system in ontogeny of the immune system in the chicken. To investigate the effects of 6-OHDA on lymphocyte development, the relative proportion of cell surface marker antigens, in vitro proliferation, expression of β-AR mRNA and intracellular cAMP levels were evaluated. The NE levels and relative proportion of cell surface marker antigens in the spleen did not change by 6-OHDA treatment. However, mitogen-induced lymphocyte proliferation and inducible intracellular cAMP levels upon stimulation of β-AR were significantly reduced by 6-OHDA treatment. The results of the present study demonstrated that 6-OHDA treatment at embryonal stage influenced lymphocyte functions.

In the bursa of Fabricius and thymus, the relative increase of cAMP was significantly high at E10 even though β-AR mRNA was not strongly expressed when compared to those of other stage. It is not confirmed whether the expression of β-AR mRNA correlates with the number of β-AR on cell surface. However, β-AR expression does not always correlate with generation of cAMP response to catecholamine on lymphocyte subpopulations [7]. At E10, more than 90% cells in the bursa of Fabricius and thymus is Bu-1a⁻ and CD4⁺ CD8⁻, respectively. It is considered that progenitor cells are more sensitive to NE.

Ho et al. [12] showed that 6-OHDA treatment in E3 chicken embryos caused the delay in the development of the sympathetic nervous system and significantly lower NE levels in the heart. In this study, the NE and DA levels in the spleen were not decreased by 6-OHDA treatment. Differences in strain, age at the time of 6-OHDA treatment and organ tissues analyzed may account for this discrepancy. It has been indicated that sympathetic nerve fibers reversibly reconstituted after 6-OHDA treatment in rats [25], on the basis of findings that the distribution of nerve fibers and NE levels in the spleen were recovered to control levels by 56 days postdenervation. Therefore, sympathetic nerve fibers in the chicken treated with 6-OHDA would also regenerate. 6-OHDA treatment at E7 or E14 may transiently decrease the NE levels and/or delay the development of sympathetic nervous system.

Gene targeting studies have shown that the lack of NE in Th (encoding tyrosine hydroxylase, Th) or Dbh (dopamine β-hydroxylase) germ line-mutant mice leads to death at mid-gestation [17, 37, 49]. In addition, loss of Gata3, essential for Th biosynthesis, causes NE depletion and impairs the development of heart and thymus [24]. Like NE deficient mice, cardiac malformation [12] and decrease of hatching rate (Co, 86.4 ± 6.03%; E7, 49.4 ± 8.5%, p<0.01; E14, 79.7
± 10.3% in six repeated experiments) by 6-OHDA treatment at the embryonal stage have also been observed in the chicken. Therefore, it is considered that reduced NE levels may affect the development of thymus. In this study, no alteration was observed in the development of thymus, indicating that a dose and/or age at the time of 6-OHDA treatment was not enough to induce thymus malformation. However, 6-OHDA treatment could affect microenvironment cells in the thymus.

Thymic epithelial cells (TEC) stimulate the proliferation of lymphoid stem cells and mediate their differentiation into thymocytes [21]. TEC have been shown to synthesize various cytokines such as IL-1 and IL-6 [46]. von Patay et al. [40] showed that NE stimulated IL-6 synthesis of TEC in vitro. Considering the finding that TEC express functional β-AR [21], various gene expressions including IL-6 in TEC would be regulated by stimulation of β-AR. In addition, CD4+ CD8+ and CD4+ CD8+ cell populations in the thymus were increased by administrating β-antagonist in old mice [28]. Hence, it is suggested that the sympathetic nervous system influences thymocytes directly or indirectly through modulation of the cytokine secretion by TEC.

Chronic β-adrenergic agonist treatment decreased β-AR density of lymphocyte [44]. However, in this study, NE level was not involved in decreased expression of β-AR mRNA, because no increase of NE levels both in the spleen and plasma was observed. The mechanisms underlying β-AR regulation on lymphocyte are still unclear, but it is considered that cytokines are involved in regulation of β-AR density [19]. IL-2 has an up-regulating effect of β-AR expression on CD8+ cells but not on CD4+ cells in vitro, indicating that cytokines exhibit a profound potential to modulate β-AR expression on lymphocytes [41]. Thus, the alteration of cytokine production by 6-OHDA treatment would affect β-AR expression on CD8+ cells.

Bartik et al. [2] have reported that there is correlation between the degree of inhibition of T cell proliferation and the level of cAMP elevation stimulated with β-agonist. The degree of β-AR expression may influence antibody production which is modulated by NE and intracellular cAMP. The reduced β-AR expression by 6-OHDA treatment might contribute to antibody responses in vivo.

In conclusion, our results showed that 6-OHDA treatment in chicken embryos reduced the lymphocyte proliferation response to mitogen, cytokine production and NE-induced intracellular cAMP elevation. These results suggest that the sympathetic nervous system has a key role in the development of the immune system. Further studies are required to define the mechanisms by which 6-OHDA treatment alters lymphocyte functions.

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