Structure-Function Relationship of T-2 Toxin and Its Metabolites in Inducing Thymic Apoptosis in Vivo in Mice

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Recently we found that a single administration of T-2 toxin (T-2), a trichothecene mycotoxin, into mice induced DNA fragmentation, a biochemical hallmark of apoptosis, in the thymus. In this study, we investigated the effective chemical structure(s) of T-2-derived metabolites capable of inducing thymic apoptosis in vivo in mice. Metabolic conversion of T-2 to 3'-hydroxy-T-2 toxin (3'-OH-T-2) (Fig. 1) did not diminish the apoptosis-inducing activity, since essentially the same level of fragmented DNA was detected in the thymus taken from mice injected with either T-2 or 3'-OH-T-2. In contrast, hydrolysis of T-2 and 3'-OH-T-2 at the carbon-4 (C-4) position to HT-2 toxin (HT-2) and 3'-hydroxy-HT-2 toxin (3'-OH-HT-2), respectively, greatly decreased the level of DNA fragmentation. Similarly, hydrolysis of T-2 at the carbon-8 (C-8) position to neosolaniol strongly diminished its ability to induce DNA fragmentation. T-2 tetraol, having no ester groups, was unable to induce apoptosis. Based on the data presented in this study, we concluded that both the acetyl group at the C-4 position and the isovaleryl or 3'-hydroxyisovaleryl group at the C-8 position of the T-2 molecule are important for inducing cell death through apoptosis in the thymus.

Key words: apoptosis; T-2 toxin; DNA fragmentation; trichothecene mycotoxin; mouse thymus

T-2 toxin (T-2), a trichothecene mycotoxin produced principally by Fusarium species, has been detected in a great number of field crops such as maize, wheat, and oats. T-2 has caused several outbreaks of mycotoxicosis in humans and animals after accidental ingestion via contaminated foods. Studies in animal models demonstrated that T-2 has strong immunosuppressive effects such as thymic atrophy, suppressed lymphocyte activation and clonal expansion, reduction of circulating numbers of both B and T lymphocytes, and significantly reduced circulating IgG and IgM antibody levels. Additional studies in the experimental models indicated that such immune effects led to clinically significant impairment of host immunity including decreased resistance to experimental infections and increased tumor incidence following challenge with tumor cell lines. At present, however, the mechanism of T-2-induced immunosuppression remains poorly understood.

Recently, we sought insight into the mechanism of T-2-induced immunosuppression, and found that T-2 exposure caused severe thymic atrophy in mice through apoptotic cell death. Trichothecene mycotoxins are separated into two groups on the basis of their structural characteristics. One is the non-macro cyclic group, composed of a tetracyclic 12,13-epoxytrichothecene (trichothecene nucleus), and the other is the macrocyclic group with a large ring accompanied by the trichothecene nucleus. Nine trichothecene mycotoxins, including six non-macro cyclic and three macro cyclic trichothecenes, have been compared with respect to their activities to induce thymic apoptosis. The results indicated that a substantial amount of DNA was cleaved into a ladder of discrete fragments in response to three non-macro cyclic trichothecenes including T-2, diacetoxyscirpenol, and nivalenol, and that T-2 appeared to be the most potent of these. Furthermore, it was of interest that macro cyclic groups (satratoxin G and baeccharinoids B-4 and B-5) were unable to induce thymocyte apoptosis, even though their toxicities in terms of LD50 values (satratoxin G, 1.23 mg/kg; baeccharinoids B-4 and B-5, 6 mg/kg) are more potent than (or as potent as) T-2 (LD50: 5.3 mg/kg). These results suggested that the potential to induce thymocyte apoptosis is not merely correlated with the LD50 values of trichothecenes but that a unique side chain structure(s) on the trichothecene nucleus seems to be of fundamental importance. Thus, the data presented in our previous study have allowed us to ask which structural component is responsible for the potent apoptotic effect of T-2. A number of metabolites of T-2 have been previously identified as occurring in vitro and in vivo (Fig. 1). In this study, we have chosen T-2 and five of its metabolites: 3'-hydroxy-T-2 toxin (3'-OH-T-2), HT-2 toxin (HT-2), 3'-hydroxy-HT-2 toxin (3'-OH-HT-2), neosolaniol (NEOS), and T-2 tetraol (TOL) (Fig. 1), and evaluated their abilities to induce apoptotic cell death in the thymus.

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Abbreviations: T-2, T-2 toxin; 3'-OH-T-2, 3'-hydroxy-T-2 toxin; HT-2, HT-2 toxin; 3'-OH-HT-2, 1'-hydroxy-HT-2 toxin; NEOS, neosolaniol; TOL, T-2 tetraol; iv, intravenous; PBS(−), Dulbeccco’s phosphate-buffered saline (without Mg2+ and Ca2+, pH 7.3); PI, propidium iodide.
Fig. 1. Metabolic Pathways of T-2 Toxin in Mammals.

The pathways shown by the solid-line arrows have been verified experimentally, and the dotted-line arrow indicates a hypothetical pathway.

Materials and Methods

Mycotoxins and reagents. Trichotheccene mycotoxins including T-2, 3'-OH-T-2, HT-2, 3'-OH-HT-2, NEOS, and TOL were prepared and purified in the laboratory of Dr. Yoshizawa, Kagawa University. The purity of these mycotoxins was 98% by gas-liquid chromatography. Propidium iodide (PI) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Animals and animal treatments. Four-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice, 34 ± 2 days old, were randomly divided into several groups with three or four mice in each group. They received a single intravenous injection of mycotoxin (1.56 mg/kg body weight) or vehicle alone (dimethyl sulfoxide: 156 μl/kg). PBS (−) was used as the diluent, and the volume administered was 0.25 ml.

Cell preparation. Following euthanization by cervical dislocation, the thymus was removed immediately. Single cells were prepared from the individual organs in ice-cold PBS (−) as described. Cells were collected by centrifugation (250 × g) at 4°C for 5 min. The cell pellet was resuspended in ice-cold PBS (−) and passed twice through a 40-μm nylon sieve.

Assay for DNA fragmentation. DNA from the thymus was extracted by the described method. Briefly, thymocytes (1 × 10⁷) were lysed with 100 μl of lysis buffer (10 mM Tris, 10 mM EDTA, pH 8.0) containing 0.5% Triton X-100. After centrifugation for 30 min at 13,000×g, the supernatant was collected and treated with RNase A and proteinase K (0.4 μg/μl each) separately for 1 h at 37°C. The fragmented DNA in the supernatant was precipitated in 50% isopropanol. Electrophoresis was done on 2% agarose gel in 90 mm of Tris-borate buffer containing 2 mM EDTA (pH 8.0), and the gel was stained with ethidium bromide (0.5 μg/ml).

DNA measurement. DNA fragmentation in the thymus was measured by a colorimetric reaction as described. Briefly, thymocytes (1 × 10⁶ cells) were lysed in 0.6 ml of lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0) at 4°C for 15 min and the lysates were centrifuged at 13,000×g for 30 min to separate intact from fragmented chromatin. Intact and fragmented DNA were treated with 5% perchloric acid, and this was followed by reaction with diphenylamine reagent to develop a dark blue color. Optical densities were measured at 600 nm and the percent of DNA fragmentation was calculated using the formula:

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\% \text{ fragmented DNA} = \frac{\text{OD supernatant}}{\text{OD supernatant} + \text{OD pellet}} \times 100.
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Flow cytometric measurement of the percent of apoptotic cells. The percentage of apoptotic cells in the thymuses of vehicle-, 3'-OH-T-2-, and T-2-treated mice was measured by flow cytometry using the PI-staining method.

Flow cytometry for T cell phenotypes. Thymocytes (2.5 × 10⁶) were incubated with 0.5 μl of fluorescein isothiocyanate-labeled anti-CD4 monoclonal antibody (Life Technology, Grand Island, NY, U.S.A.), or R-phycocerythrin-labeled anti-CD8 monoclonal antibody (Life Technology) at 4°C for 45 min. Stained cells were analyzed by a Coulter EPICS flow cytometer (Hialeah, FL, U.S.A.) as described.

Statistical analysis. In most cases, results were expressed as means ± SEM. Comparisons between the means of treatment groups were by two-tailed Student’s t-test.

Results

Time-dependent thymic apoptosis induced by 3'-OH-T-2

3'-OH-T-2 (1.56 mg/kg) was injected into different groups of mice. The DNA fragmentation pattern, a biochemical hallmark of apoptosis, was analyzed by agarose gel electrophoresis at different times. As shown in Fig. 2, definite DNA fragmentation appeared 1 h after injection and reached the maximum at 12 h. DNA fragmentation lasted for 24 h. Based on this experiment, the 12 h point was chosen for further studies.

Effects of T-2 and its metabolites on thymic apoptosis in vivo

To identify the essential structural moiety of T-2...
responsible for the induction of thymic apoptosis in vivo in mice, we injected T-2 and some of its metabolites the chemical structures of which are known (Fig. 1). Fragmented DNA induced by each metabolite was measured at 6 h (Fig. 3A) and 12 h (Fig. 3B) by colorimetric methods. The figures show that the percent of DNA fragmentation induced by T-2 and 3'-OH-T-2 was much higher both at 6 h and 12 h after treatment than that induced by the other T-2 metabolites tested or by the vehicle control. As is depicted in Fig. 3, the rank order of the potency to induce thymic apoptosis in this experimental system was found to be T-2 = 3'-OH-T-2 > HT-2 = 3'-OH-HT-2 > NEOS = TOL = vehicle control.

3'-OH-T-2 induction of thymic atrophy

Relative thymus weight expressed as milligrams of thymus per 100 g body weight (Fig. 4A) and cellularity expressed as total yield of thymic cells per thymus (Fig. 4B) were calculated to assess whether a single iv injection of 3'-OH-T-2 could elicit thymic atrophy. 3'-OH-T-2 (1.56 mg/kg) was injected into groups of female BALB/c mice and thymic atrophy was monitored at different times after administration of the mycotoxin. As is shown in Fig. 4, a maximal thymic atrophy was observed on day 4 and after that the atrophied thymus was found to recover gradually. It was found that thymic atrophy was no longer detectable 2 weeks after treatment with 3'-OH-T-2.

Measurements of percent apoptotic cells in the thymuses of 3'-OH-T-2-treated mice

To confirm that the thymocytes from T-2- and 3'-OH-T-2-treated mice were undergoing apoptosis, we measured the incidence of apoptotic cells by fluorescence emission of PI-stained cells at 6 h (Fig. 5A) and 12 h (Fig. 5B). From the flow cytometric data of the percentage of the hypodiploid DNA peak, it was found that the potencies of T-2 and 3'-OH-T-2 in inducing thymic apoptosis were essentially the same, and the hypodiploidy was 9% at 6 h and 21% at 12 h.

CD4^+CD8^+ double positive thymocytes as the main target of 3'-OH-T-2

To find whether 3'-OH-T-2 affected the predominant phenotypes of the thymocyte populations, a flow cytometric analysis for CD4/CD8 expression was done at different times after treatment. As shown in Fig. 6, when compared with untreated controls, the CD4^+CD8^+ double positive subsets were affected 6 h after treatment and the effect became maximal on day 2. It was also noticed that the affected subsets completely recovered by day 14. The other three kinds of subsets were not found to be altered significantly at any time.
Fig. 4. 3'-OH-T-2 Induces Time-Dependent Thymic Atrophy.

The thymus was removed from untreated mice or from mice treated with 3'-OH-T-2 (1.56 mg/kg, iv). Relative thymus weight as milligrams of thymus per 100 g body weight (A), and total cell yield per thymus (B) are shown. Results were from three individual mice per point and expressed as means ± SEM.

Fig. 5. Effects of T-2 and 3'-OH-T-2 on in vivo Thymic Apoptosis.

Mice, 4 mice per group, were injected iv with either vehicle, T-2 (1.56 mg/kg) or 3'-OH-T-2 (1.56 mg/kg), and they were killed 6 h (A) and 12 h (B) later. Flow cytometric data, as assessed by PI method, represent means ± SEM. *p<0.01 compared with the vehicle control.

Discussion

Apoptotic cell death is an important physiological process in most multicellular organisms. In contrast to cell necrosis, apoptosis is an active process of cellular self destruction that involves fragmentation of genomic DNA into integral multiples of 200-bp units by the activation of endonuclease. Exposure to an apopgen, a trigger of apoptosis, can increase apoptotic cell loss during homeostasis as can acute or chronic toxicities. We reported previously that a single injection of T-2 into mice caused apoptotic cell death in the thymus. At present, however, little is known about the mechanism by which trichothecene mycotoxins induce toxic effects through apoptosis. In this study, we investigated the structure-activity relationship within a series of metabolites of T-2.

Our study was designed to investigate which structural component of T-2 is responsible for induction of thymic apoptosis in vivo in mice. It was demonstrated that hydroxylation of T-2 at the 3'-carbon position did not diminish the magnitude of thymic apoptosis (Figs. 3 and 5). In contrast, both HT-2 and 3'-OH-HT-2 could induce apoptosis only marginally (Fig. 3). In addition, NEOS and TOL were incapable of inducing apoptosis (Fig. 3). In summary, the rank order of apoptosis-inducing activity was found to be T-2 = 3'-OH-T-2 > HT-2 = 3'-OH-HT-2 > NEOS = TOL = vehicle control, and it was concluded that both the acetyl group at the C-4 position and the isovaleryl or 3'-hydroxyisovaleryl group at the C-8 position of T-2 appear to be involved in the thymic apoptosis.

Little information concerning the relative toxicities of T-2 and 3'-OH-T-2 in vivo is available. The results presented here, which indicate that 3'-OH-T-2 is as potent as T-2, are consistent with previous findings. By using mitogen-induced blastogenesis in human lymphocytes, Forsell et al. reported that 3'-OH-T-2 was as potent as T-2, which is consistent with our present results. In the same experiment, however, they noticed that HT-2 was similar to 3'-OH-T-2 in terms of the inhibitory action to the blastogenesis, which is inconsistent with our data. Yoshizawa et al. reported the acute toxicity of trichothecenes including 3'-OH-T-2 and 3'-OH-HT-2 in vivo in mice. In their experiments, the rank order of median lethal dose was T-2 = 3'-OH-T-2 > HT-2 > 3'-OH-HT-2 = NEOS = TOL. This is essen-
Fig. 6. Changes in the Absolute Number of Thymocyte Subsets in 3'-OH-T-2-Treated Mice.

Mice, 4 mice per group, were injected iv with 3'-OH-T-2 (1.56 mg/kg) and they were killed at different times. Thymocytes from each mouse were analyzed after staining with fluorescent monolonal antibodies (for CD4 and CD8) as described under Materials and Methods. ▲, total thymocyte number; ◦, CD4+CD8+ double positive subset; ○, CD4+CD8- double negative subset; ●, CD4+ single positive subset; ■, CD8+ single positive subset. SEM<10%.

Initially consistent with our results except for HT-2, since this metabolite could induce thymic apoptosis only marginally in our system. The different results may imply that the acetyl ester at the C-4 position is not essential to a lethal effect, but that this side chain structure is important for inducing thymic apoptosis.

Perhaps our most significant finding is that 3'-OH-T-2 was capable of inducing thymic apoptosis in vivo as efficiently as T-2 (Figs. 3 and 5). Naseem et al. followed the fate of T-2 administered in monkeys.35 They made two findings. First, plasma HT-2 and 3'-OH-HT-2 levels detected were very low throughout the experiment. Secondly, the plasma levels of 3'-OH-T-2 were much higher than those of HT-2 and 3'-OH-HT-2 at all times (5 min, 2 h, 24 h). Based on the profile of the metabolites, the pathway through formation of 3'-OH-T-2 and 3'-OH-HT-2 (Fig. 1) could be the major one. Consequently, the other pathway which includes HT-2 is presumed to be a minor one (Fig. 1). Thus, the potent toxic effects of both T-2 and 3'-OH-T-2 in vivo may be explained as follows: 3'-OH-T-2 is produced rapidly from T-2, but further metabolic conversion of 3'-OH-T-2 is slow, and hence a potent 3'-OH-T-2 molecule is retained in the body for a long time. However, the question of whether in vivo hydroxylation of T-2 at the 3'-carbon of the isovaleryl group by the cytochrome P-450 dependent system is key to induce thymocyte apoptosis in our experimental system remains unresolved. A study using a cytochrome P-450 inhibitor will answer this question.

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References


