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# Inhibition of Mitogen-Induced Blastogenesis in Human Lymphocytes by T-2 Toxin and Its Metabolites†

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**Concentrations of T-2, HT-2, 3′-OH T-2, 3′-OH HT-2, T-2 triol, and T-2 tetraol toxins which inhibited [$^3$H]thymidine uptake in mitogen-stimulated human peripheral lymphocytes by 50% were 1.5, 3.5, 4.0, 50, 150, and 150 ng/ml, respectively. The results suggested that the initial hydrolysis of T-2 toxin to HT-2 toxin and the hydroxylation of T-2 toxin to 3′-OH T-2 toxin did not significantly decrease the immunotoxicity of the parent molecule, whereas further hydrolysis to T-2 triol and T-2 tetraol toxins or hydroxylation to 3′-OH HT-2 toxin decreased in vitro toxicity for human lymphocytes.**

Trichothecene mycotoxins are secondary metabolites produced in agricultural commodities by members of the fungal genera *Fusarium*, *Trichoderma*, *Myrothecium*, and *Stachybotrys* (16, 22). T-2 toxin, one of the most potent trichothecenes, has been implicated in episodes of human and animal toxicoses, the most significant human outbreaks being fatal alimentary aleukia in the Soviet Union (5). The biological mode of action of T-2 toxin and other trichothecenes is via protein synthesis inhibition (11), with actively dividing tissues such as bone marrow, lymph nodes, spleen, thymus, and intestinal mucosa being most susceptible to the toxin (5, 17). The immune system is particularly sensitive to trichothecene mycotoxins. Trichothecene-exposed mice are more susceptible to experimental infections and have decreased humoral responses to T-dependent antigens, increased skin graft rejection times, and decreased lymphocyte responses to B- and T-cell mitogens (4, 6, 14, 15). T-2 toxin inhibits DNA and protein syntheses and induces DNA single-strand breakage more effectively in lymphoid cells than in nonlymphoid cells both in vitro and in vivo (7, 13).

Because T-2 toxin metabolism and immunotoxicity cannot be evaluated experimentally in humans, it is necessary to test the toxin and its major metabolites, identified in animal studies, in human in vitro assay systems to better assess potential health risks. Mitogen-induced blastogenesis in human peripheral blood lymphocytes is inhibited by T-2 toxin (2). The inclusion of rat hepatocytes in this in vitro blastogenesis assay slightly reduces the lymphotoxicity of T-2 toxin, suggesting that the metabolism of T-2 toxin by liver microsomal enzymes decreases the immunotoxicity of this compound (2). A number of metabolites of T-2 toxin have been previously identified as occurring in vitro and in vivo, including HT-2, 3′-OH T-2, 3′-OH HT-2, and T-2 tetraol (10, 18–21). In this report, we evaluate the effects of T-2 toxin and its major metabolites on mitogen-induced blastogenesis in human peripheral lymphocytes.

T-2, HT-2, T-2 triol, and T-2 tetraol toxins were obtained from Sigma Chemical Co., St. Louis, Mo. The 3′-OH T-2 and 3′-OH HT-2 toxin metabolites were produced by the procedures of Yoshizawa et al. (19, 20). Sources of reagents and biological agents were as follows: Ficoll, concanavalin A (ConA), and leukoagglutinin (LA) were from Pharmacia Fine Chemicals, Piscataway, N.J.; $Ca^{2+}$- and $Mg^{2+}$-free Hanks balanced salt solution and RPMI-1640 containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from Microbiological Associates, Bethesda, Md.; penicillin-streptomycin solution and pokeweed mitogen (PWM) were from GIBCO Laboratories, Grand Island, N.Y.; fetal calf serum was from Sterile Systems, Inc., Logan, Utah; Hypaque was from Sterling Organics, New York, N.Y.; and [*methyl*-$^3$H]thymidine ([$^3$H]TdR) (6.7 Ci/mmol) was from New England Nuclear Corp., Boston, Mass.

Blood was collected from humans by venipuncture in sterile, evacuated tubes containing sodium heparin, and lymphocytes were separated from blood with Ficoll-Hypaque cushions (1). Lymphocytes recovered from the Ficoll-Hypaque cushions were washed two times with $Ca^{2+}$- and $Mg^{2+}$-free Hanks balanced salt solution by centrifugation at 200 × *g* for 15 min each time, followed by a third wash for 10 min. Platelets were removed with a fourth wash at 30 × *g* for 7 min, and the lymphocytes were then suspended in RPMI-1640 containing 25 mM HEPES, 5% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Lymphocytes were adjusted to $2 \times 10^6$/ml with a Coulter ZBI particle counter (Coulter Electronics, Inc., Hialeah, Fla.). Cell viability was greater than 98% as determined by trypan blue dye exclusion.

Mitogen-induced blastogenesis was carried out in triplicate by a microculture procedure (3). Lymphocytes ($2 \times 10^5$) were cultured in RPMI-1640 (supplemented as described above) in "U"-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). Three mitogens, LA, ConA, and PWM, were used to stimulate the lymphocytes. Blastogenic responses were studied at two levels for each mitogen as follows: LA, 5 and 0.25 μg/ml; ConA, 10 and 1.0 μg/ml; and PWM, 10 and 0.1 μg/ml. Toxins were dissolved in ethanol and added to cultures immediately after mitogens were added. The maximum amount of ethanol used to deliver a mycotoxin (2 μl) represented 1% of the final culture volume. Ethanol controls run at 1% had no effect on DNA synthesis. T-2 toxin analog concentrations ranged from 0.0005 ng/ml to 5 μg/ml. Lymphocyte cultures were incubated at 37°C in a 5% $CO_2$ humidified atmosphere for 72 h.

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TABLE 1. Inhibition of the incorporation of [$^{3}$H]TdR in mitogen-stimulated human lymphocytes by T-2 toxin

| T-2 toxin dose (ng/ml) | % Inhibition[a] | | | | | | Mean[b] |
|---|---|---|---|---|---|---|---|
| | LA | | ConA | | PWM | | |
| | 5.0 μg/ml | 0.25 μg/ml | 10.0 μg/ml | 1.0 μg/ml | 10.0 μg/ml | 0.1 μg/ml | |
| 0.0005 | 110 | 105 | 100 | 102 | 106 | 124 | 110 |
| 0.005 | 107 | 119 | 93 | 114 | 98 | 106 | 106 |
| 0.05 | 94 | 77 | 100 | 85 | 90 | 86 | 89 |
| 0.5 | 90 | 90 | 107 | 116 | 99 | 87 | 98 |
| 1.0 | 86 | 42 | 79 | 69 | 73 | 44 | 66 |
| 2.5 | 1 | 2 | 2 | 3 | 2 | 6 | 3 |
| 5.0 | 2 | 34 | 2 | 12 | 3 | 7 | 10 |
| 50 | 1 | 4 | 2 | 4 | 2 | 12 | 4 |
| 500 | 2 | 7 | 3 | 12 | 3 | 17 | 7 |

[a] Calculated as (counts per minute in test culture/counts per minute in control culture) × 100. All cultures were run in triplicate.
[b] No differences existed ($P > 0.05$) among mitogens or between mitogen levels.

At 48 h, cultures were pulsed with 25 μl of [$^{3}$H]TdR (0.25 μCi). Cultures were harvested on filter paper disks with a mini-multiple analysis sample harvester (mini-MASH; Microbiological Associates), and isotope incorporation was evaluated by scintillation counting. Results were expressed as the percentage of DNA synthesis in control lymphocytes which were stimulated by a mitogen in the absence of toxin.

The effective doses of T-2 toxin which caused a 50% reduction ($ED_{50}$) of [$^{3}$H]TdR incorporation were between 0.5 and 2.5 ng/ml for all mitogens and mitogen levels examined (Table 1). A 50% reduction of [$^{3}$H]TdR incorporation by T-2 toxin has been previously shown to occur in vitro in human lymphocytes at 1.5 ng/ml (2), in bovine lymphocytes at 1.4 ng/ml (9), and in mouse spleen cells and thymocytes at 1.0 to 2.5 ng/ml (8). Apparently, species differences do not affect the toxicity of T-2 toxin in lymphocyte culture systems. The observation that LA- and ConA-stimulated lymphocytes (primarily T-cells) and PWM-stimulated lymphocytes (B- and T-cells) were equally inhibited suggests that both peripheral T- and B-cells were affected by T-2 toxin. It has been previously demonstrated that phytohemagglutinin-stimulated lymphocytes (T-cells) and lipopolysaccharide-stimu-

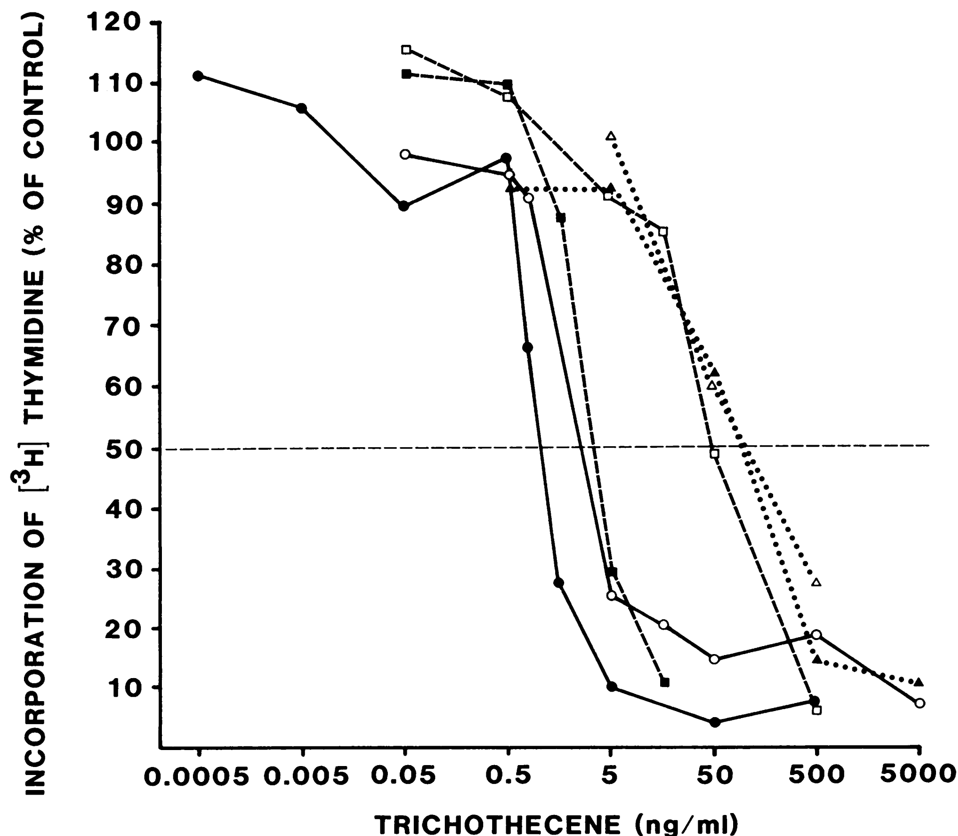


FIG. 1. Inhibition of [$^{3}$H]TdR incorporation in mitogen-stimulated human lymphocytes by T-2 toxin and its analogs. Each point represents the mean inhibition in lymphocytes stimulated by LA, PWM, and ConA at two concentrations each in triplicate (a total of 18 determinations). Symbols: ●, T-2 toxin; ○, HT-2 toxin; ■, 3′-OH T-2 toxin; □, 3′-OH HT-2 toxin; ▲, T-2 triol toxin; and △, T-2 tetraol toxin.

lated lymphocytes (B-cells) isolated from mice are inhibited by similar levels of T-2 toxin (8).

It is of interest to note that there was a slight stimulation of lymphoblastogenesis when low T-2 toxin concentrations ($5 \times 10^{-4}$ to $5 \times 10^{-3}$ ng/ml) were included in the assay (Table 1). These results are consistent with those of Lafarge-Frayssinet et al. (8), who found that mitogen-induced blastogenesis of murine spleen cells and thymocytes was stimulated by similar concentrations of T-2 toxin.

As found above for T-2 toxin, no differences in $ED_{50}$s occurred for any of the T-2 toxin derivatives when different mitogens or mitogen concentrations were used (data not shown). The lymphotoxicity of T-2 toxin and its metabolites is summarized in Fig. 1. In order of decreasing toxicity, the $ED_{50}$s (ng/ml) of the analogs were as follows: T-2, 1.5; HT-2, 3.5; 3′-OH T-2, 4.0; 3′-OH HT-2, 50; and T-2 triol and T-2 tetraol, 150. Apparently, the in vitro lymphotoxicity of the T-2 toxin analogs was associated with the degree of hydroxylation of the parent T-2 toxin molecule (20). Hydrolysis at the C-4 position of T-2 toxin to HT-2 toxin or hydroxylation at the C-3′ position of T-2 toxin to 3′-OH T-2 toxin had only a minimal effect on the toxicity relative to that of the parent compound. However, hydrolysis at position C-4 of 3′-OH T-2 toxin to 3′-OH HT-2 toxin or hydrolysis at position C-15 of HT-2 toxin to T-2 triol toxin greatly diminished the toxicity of the T-2 toxin molecule. Thus, although a single substitution of a hydroxyl at the C-4 or C-3′ position did not greatly affect lymphotoxicity, second substitutions of hydroxyl groups at these same positions decreased toxicity. However, hydrolysis at position C-4 of T-2 triol toxin to T-2 tetraol toxin did not further diminish the lymphotoxicity of the compound.

In liver homogenate and intestinal strips from rats, T-2 toxin is deacylated stepwise in a hydrolytic pathway at the C-4, C-8, and C-15 positions, thus initially forming HT-2 toxin and ultimately being converted into T-2 tetraol toxin (via 4-deacetylneosolaniol) (22). Although both HT-2 and T-2 tetraol toxins have also been identified as minor metabolites in bovine tissue and in cow and chicken excreta (18, 21), Yoshizawa et al. (19) determined that the major T-2 toxin metabolites identifiable in bovine plasma, milk, and excreta are 3′-OH T-2 and 3′-OH HT-2 toxins and further suggested that these may also correspond to unidentified major metabolites in excreta of chicks and rats fed T-2 toxin. In cows, T-2 toxin absorbed from the intestinal tract is rapidly metabolized to 3′-OH T-2, 3′-OH HT-2, and another newly identified major metabolite, 3′-OH-7-OH HT-2, which circulate in the blood and are distributed evenly in many tissues and organs (12, 18). Hydroxylation at the C-3′ position of T-2 and HT-2 toxins occurs in vitro in mouse and monkey liver homogenates only after the addition of an NADPH-generating system (20).

Our data indicate that the metabolism of T-2 toxin to 3′-OH T-2, HT-2, 3′-OH HT-2, and T-2 tetraol toxins would increase the $ED_{50}$s required to inhibit human lymphocyte blastogenesis by 2.3-, 2.7-, 33-, and 100-fold, respectively. Thus, although metabolic inactivation of T-2 toxin occurs after hydrolysis and hydroxylation, the resulting metabolites still retain toxicity. In humans, the entry of the abovementioned metabolites into blood and tissue could result in immunotoxicity. Lymphotoxicity by T-2 toxin metabolites may partially explain the extreme symptoms of leukopenia, bone marrow aplasia, and immunosuppression that are associated with fatal alimentary toxic aleukia in humans who have ingested grain heavily infected with toxigenic fusaria (5). Our results lend further support to the suggestion by other investigators (7, 8, 13, 14) that even low levels of T-2 toxin as a dietary contaminant in the food chain might function as a determinative factor in the resistance of humans to disease.

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