Immunochemical Studies on the Excretion of T-2 Toxin Metabolites in Rat and Cynomolgus Monkey Urine

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A generic antibody against group A trichothecence mycotoxins was used in a competitive enzyme-linked immunosorbent assay (ELISA) for the detection of T-2 toxin and its metabolites in the urine of rats and cynomolgus monkeys after a single dose of T-2 toxin (5 μg/kg). Of the total administered dose 49% was detected by ELISA in monkey urine with a peak at 24 h and persistent measurable toxin remaining until the fifth day. Tritiated T-2 toxin was used in the rat study. Whereas 50% of total tritium was found in the rat urine samples, only 33% of the administered dose was detected by the ELISA with a peak of excretion within the first 24 h. T-2 metabolites were detectable in the rat urine for the entire 9 days of the experiment. The results suggest that ELISA could be used to detect a sublethal dose of T-2 toxin intoxication in animals.

T-2 toxin is one of the most toxic naturally occurring trichothecene mycotoxins produced by a number of species of *Fusarium* (Anonymous, 1983; Bamburg and Strong, 1987; Ueno, 1983). After animals ingest the toxin (Matsumoto at al., 1978; Visconti and Mirocha, 1985; Yoshizawa et al., 1982), the toxin is rapidly metabolized to HT-2 toxin and T-2 tetraol by esterase and to several hydroxylated derivatives such as 3′-hydroxy-T-2 toxin (3′-OH-T-2) and 3′-hydroxy-HT-2 toxin (3′-OH-HT-2) by microsomal enzymes. Deepoxidation (Chatterjee et al., 1986) as well as glucuronidation of T-2 toxin and its metabolites have also been demonstrated (Corley et al., 1985; Pfeiffer et al., 1988). After metabolism, the toxin is rapidly excreted via feces or urine (Chi et al., 1978; Matsumoto et al., 1978; Pace et al., 1985; Pfeiffer et al., 1988; Robinson et al., 1979; Yoshizawa et al., 1981). Depending on the animal species and other factors such as dosage, route, and time of administration, the ratio of metabolites in feces and urine varied considerably (Pfeiffer et al., 1988). For example, almost all of the toxin and its metabolites was rapidly excreted into the urine and feces by 72 h (Yoshizawa et al., 1981) in a lactating cow intubated with tritiated T-2 toxin. Urinary excretion was fast, reached maximal level at 16 h after dosing, and was completed by 48 h. About 29% of administered toxin in the lactating cow was found in the urine (Yoshizawa, et al., 1981). Similar results were observed in mice after a single oral dose of tritiated T-2 toxin. A daily excretion curve of T-2 toxin in mice indicated a quick elimination of the toxin; 68% of the administered dose was eliminated into urine and feces during a 96-h study period (51% in the feces and 17% in the urine; Matsumoto et al., 1978). In guinea pigs, 75% of the radioactivity was excreted in the urine and feces at a ratio of 4:1 in a 5-day period after a single i.m. injection of tritiated T-2 toxin. Radioactivity in the urine peaked at 24 h after injection, rapidly decreased during the second day, and then gradually disappeared over the next 3 days (Pace et al., 1985).

With the accumulated data on the kinetics of excretion of T-2 toxin and metabolites in urine of different animal species (Beasley et al., 1986; Pfeiffer et al., 1988), it is apparent that monitoring T-2 toxin and its metabolites in urine and other body fluids could be used as a diagnostic index in T-2 toxin toxicosis. Nevertheless, chemical analysis of T-2 toxin and its metabolites is very complicated and insensitive and needs expensive instrumentation (Kamimura et al., 1981; Mirocha, 1986; Scott, 1982; Yagen et al., 1985). Although immunochemical methods were considered to be the most promising approaches (Chu, 1986, 1989), it was hindered by the complexity of the metabolism of T-2 toxin and by the specificity of the antibodies. Antibodies against T-2 toxin and its metabolites are very specific (Chu et al., 1979; Fan et al., 1987; Gendloff et al., 1987; Hack et al., 1987; Wei et al., 1986); thus, it is difficult to select an antibody that can detect all the metabolites. However, in a recent study, a new method for the production of an antibody with specificity toward the group A trichothecenes in common was reported (Wei and Chu, 1987). This antibody is most specific to T-2-4-ol-4-Ac in addition to being cross-reactive with most of the group A trichothecenes (Wei and Chu, 1987). A direct competitive enzyme-linked immunosorbent assay (ELISA), involving the use of this antibody, i.e., *anti*-3-acetylneosolaniol (*anti*-3-Ac-NEOS), and 3-Ac-NEOS-hemisuccinate–horseradish peroxidase (HS–HRP) was also developed (Lee et al., 1989). In the present study, the newly developed ELISA was tested for the analysis of the kinetics of excretion of T-2 toxin and its metabolites in the urine of rats and cynomolgus monkeys. The concentrations of the toxin and its metabolites in the urine samples were analyzed by both radioimmunoassay (RIA) and direct ELISA after conversion of all the toxin and metabolites including glucouronides to T-2-4-ol-4-Ac. Tritiated T-2 toxin was also used in the rat studies.

MATERIALS AND METHODS

Materials. T-2 toxin, T-2 tetraol tetraacetate (T-2-4-ol-4-Ac), tritiated T-2 toxin, and tritiated T-2-4-ol-4-Ac were prepared as described previously (Cullen et al., 1982; Wallace et al., 1977; Wei and Chu, 1987). Bovine serum albumin (BSA; RIA grade), β-glucuronidase (from limpets, Type L-II), Amberlite XAD-2, acetic anhydride, Tween 20, 30% hydrogen peroxide, and creatinine testing kit were purchased from Sigma Chemical Co. (St. Louis, MO). 4-(Dimethylamino)pyridine (DMP) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Microtiter plates and Minisorp RIA tubes were purchased from Nunc

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Co. (Roskilde, Denmark). The peroxidase substrate, *o*-phenylenediamine tablets (OPD; 4.0 mg/tablet), was supplied by Indexx (Portland, ME). The antibody against group A trichothecenes was produced as described previously (Wei and Chu, 1987). Female Sprague–Dawley rats were purchased from Harlan-Sprague-Dawley, Inc. (Madison, WI). All chemicals and organic solvents used were reagent grade or better.

Animal Treatment. *1. Tritiated T-2 Toxin in Rats.* Three female Sprague–Dawley rats weighing about 200 g each were housed separately in metabolic cages. Tritiated T-2 toxin together with nonradioactive T-2 toxin was dissolved in ethanol at a toxin concentration of 1 mg/mL. A single dose of toxin was injected i.p. into the rats at a level of 0.5 mg/kg body weight (3.2×10^7 dpm/kg), which is comparable with the monkey experiment. Feed and water were available ad libitum during the study period. The LD_{50} of T-2 toxin in this strain of rat is not known; assuming that it is in the same range as Fisher Dunnings, the level is around 0.5–1 mg/kg (Wannemacher et al., 1989).

2. T-2 Toxin in Cynomolgus Monkeys. Four cynomolgus monkeys weighing 4.2–6.1 kg were housed in individual metabolic cages and fed with monkey chow and water ad libitum during the experimental period. Three cynomolgus monkeys were each injected intramuscularly (i.m.) with 0.1 mL/kg body weight of a solution that contained 0.5 mg/0.1 mL of T-2 toxin dissolved in 50% ethanol. One cynomolgus monkey was injected i.m. with 0.1 mL/kg body weight of a 50% ethanol solution as a control. The LD_{50} of T-2 toxin in the monkey is approximately 0.8 mg/kg body weight (Wannemacher et al., 1989).

Sampling. *1. Rat Urine.* Pretreatment urine samples were collected immediately before toxin injection. After administration of the labeled toxin, urine samples were collected every 3 h for 12 h, and then every 12 h for the next 36 h and at 24-h intervals thereafter. The total study period was 9 days. Water (5–10 mL) was used to wash the collecting pan every time the urine was collected. The washing water and urine were pooled together, and the volume of each pooled urine sample was recorded. A 1-mL portion of each sample was subjected to enzyme hydrolysis and subsequent sample treatments. The radioactivity and the concentration of creatinine of each urine sample were also determined.

2. Cynomolgus Monkey Urine. Urine samples were collected every 24 h for 3 days before toxin administration. After the toxin injection, 8-h urine collections were made for the first 2 days, followed by 24-h urine collections until 10 days postexposure. The collecting pan was washed with 10 mL of water with each time collection, 1.7 g of sodium fluoride was placed in each sample collected. Volumes of urine samples were recorded, and 1 mL of each sample was hydrolyzed with β-glucuronidase.

Enzyme Hydrolysis. The protocol for enzyme hydrolysis was modified from Corley et al. (1985) and Gareis et al. (1986). An enzyme solution with an activity of 4500 units/0.5 mL was prepared by dissolving 0.03 g of β-glucuronidase in 10 mL of 0.1 M acetate buffer (12% 0.1 M sodium acetate solution in 0.1 M acetic acid, pH 3.8). To determine the amounts of glucuronide conjugates of T-2 toxin and its metabolites in the urine, 4 mL of 0.1 M acetate buffer was added to 1 mL of urine sample; the mixtures were heated at 90 °C for 30 min in a water bath to inactive enzyme inhibitors and enzymes/microorganisms that may further degrade T-2 toxin. After cooling, 0.5 mL of β-glucuronidase solution was added to each sample, followed by incubation at 37 °C for 24 h. After incubation, the enzymatic activity was stopped in an ice bath, and then the samples were subjected to an Amberlite XAD-2 column cleanup treatment.

For a control, equal amounts of the urine samples were treated in the same way except that 0.5 mL of 0.1 M acetate buffer was added instead of enzyme solution.

Sample Preparation, Acetylation, and Immunoassays. An XAD-2 column was used to recover T-2 toxin and its metabolites from urine samples with the same protocol as described before (Lee et al., 1989). In general, a 1-mL sample (with or without β-glucuronidase treatment) diluted with 10 mL of distilled water was added to a 1 × 5 cm XAD-2 column (Sigma A-7643) at a flow rate of 1.5 mL/min, followed by washing the column twice with 10 mL of distilled water. The toxin and its metabolites were eluted from the column with 10 mL of acetone (5 mL each, two times). The combined acetone eluates were air-dried.

To hydrolyze the sample extract, 0.5 mL of methanolic KOH (5% of 0.01 N KOH in methanol) was added to the dry sample and the mixture incubated at room temperature for 10–15 min (Wei et al., 1971). After reaction, the methanol and water were removed. For acetylation, 0.2 mL of acetic anhydride (5% solution in acetonitrile) and 0.2 mL of 4-(dimethylamino)pyridine (DMP) solution (10 mg/mL of acetonitrile) were added to the dried hydrolyzed sample and incubated at room temperature for 1 h. After reacting and air-drying, 0.1 mL of ethanol and 0.9 mL of 0.01 M PBS were added. This sample after appropriate dilutions in PBS was then processed with ELISA or RIA with the protocols we have previously described (Lee et al., 1989). The antibodies used in the assay had good cross-reactivity with acetyldiacetoxyscirpenol (Ac-DAS), T-2-4-ol-4-Ac, 3′-OH-Ac-T-2, 3-Ac-NEOS, and acetyl-T-2-8-one but less cross-reactivity with Ac-T-2 toxin and T-2 toxin. Cross-reaction of the antibodies used in the present study with the deepoxide T-2 toxin was also observed (Wei et al., 1988). The overall analytical recoveries for T-2 toxin, HT-2, T-2-4-ol, 3′-OH-HT-2, neosolaniol (NEOS), and a mixture of these five toxins added to the urine samples at a concentration of 0.05 and 0.2 μg/mL were 87 and 94%, respectively. The detection limits appeared to be around 10 ppb in the urine sample (Lee et al., 1989). No loss of radioactivity was observed when a tritiated T-2 toxin sample was subjected to the whole assay protocols (Lee et al., 1989).

In the rat study, we found that the recovery of T-2 toxin and its metabolites in the urine as determined by the total radioactivity was higher than the data obtained from ELISA. Thus, an additional experiment was carried out. The samples were passed through an Amberlite XAD-2 column as described above. After the column was washed with 20 mL of water, T-2 toxin and metabolites were then eluted from the column with acetone. The radioactivity as well as actual T-2 toxin metabolites in both acetone eluates and water portions were analyzed by scintillation counting and by ELISA.

Determination of Radioactivity. The total radioactivity in the rat urine samples was determined by counting the radioactivity of 0.1–1 mL of urine with use of 5 mL of Aquasol scintillation cocktail (New England Nuclear Corp., Boston, MA) in a Beckman Instruments, Inc. (Fullerton, CA), Model LS-5801 liquid scintillation spectrometer.

Determination of Creatinine Concentrations in Rat Urine. Because the urine samples collected from rats were diluted with water (used to wash the collecting pan), concentrations of creatinine in the urine samples were determined to correct the urine volume excreted. An assay kit for kinetic determination of creatinine was used in this study. Creatinine can react with picric acid under alkaline conditions to form a red complex, and the absorbance of the color produced, measured at 510 nm, is directly proportional to creatinine concentration in the samples (Fabiny and Ertingshausen, 1971).

RESULTS

Creatinine Concentrations of Rat Urine. Since the daily urinary excretion of creatinine is approximately constant, the amounts of creatinine in diluted urine samples were first determined to correct urine volume excreted from the rats and to calculate the actual concentrations of T-2 toxin and its metabolites in the rat urine. The average concentrations of creatinine in the urine of three rats were 85.4, 72.3, and 56.8 mg/mL, respectively. The amount of urine excreted by these rats varied from 12 to 16 mL/day.

Quantitation of T-2 Toxin Metabolites in Rat Urine by Direct ELISA. On the assumption that the molecular weights of T-2 toxin metabolites are comparable to that of T-2-4-ol-4-Ac, the concentrations of T-2 toxin and its metabolites in every urine sample collected were determined by direct ELISA. The results are summarized in Table I. The urinary excretion was rapid, reached maximal level (about 6 ppm) 6 h after toxin administration, and then gradually decreased. Although most of the excre-

Table I. Concentrations of T-2 Toxin Metabolites in the Urine of Rats after a Single Dose of T-2 Toxin

	concn of T-2 toxin metabolites,[a] μg/mL					
	ELISA anal.					
time, h	with enzyme hydrolysis	SD	no enzyme hydrolysis	SD	tritiated T-2 toxin equiv[b]	SD
0	0.01[c]	0.004			0.001	
3	3.82	1.46	3.48	0.94	7.95	0.81
6	5.99	1.20	5.98	0.62	10.83	1.34
9	3.60	1.21	4.00	1.63	4.33	1.69
12	2.09	0.84	2.15	0.97	2.57	0.80
24	1.15	0.19	1.10	0.26	1.89	0.72
36	0.52	0.14	0.57	0.18	0.94	0.26
48	0.44	0.21	0.47	0.23	0.59	0.31
72	0.19	0.10	0.19	0.11	0.26	0.19
96	0.08	0.03	0.08	0.02	0.09	0.05
120	0.05	0.01	0.05	0.005	0.05	0.02
168	0.05	0.02	0.05	0.02	0.05	0.01
192	0.04	0.007	0.03	0.006	0.05	0.006
216	0.04	0.005	0.03	0.003	0.03	0.02

[a] Average of three analyses. [b] Values converted from the radioactivity of urine samples using specific radioactivity of tritiated T-2 toxin. [c] Detection limit.

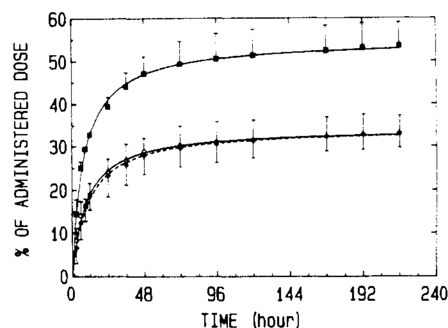

Figure 1. Cumulative excretion curves of T-2 toxin metabolites in rat urine. Urine samples were obtained from rats administered i.p. with tritiated T-2 toxin at a level of 0.5 mg/kg (3.2 × 10^7 dpm/kg). The total excretion of T-2 toxin and its metabolites in rat urine, in terms of the percent of the administered dose, was determined by converting the total radioactivity measured before XAD-2 column treatment to the T-2 equivalent (■) and by ELISA (♦). Of the total administered radioactivity, 53% was found in the urine, whereas 33.0% of the administered dose was detected by ELISA in rat urine. Tritiated T-2 toxin equivalents present in the acetone eluate (○) after XAD-2 column treatment were also converted as the percent of administered dose and compared with the data obtained from ELISA. A total of 33.1% of the administered dose was found in the acetone eluates. Each point is the average of the data obtained from three rats.

tion was completed by 48 h, there was still a detectable amount of T-2 toxin metabolites, about 40 ppb, present in the urine 9 days after dosing.

An incubation with β-glucuronidase was included in the urine sample treatment, but there was no significant difference ($p < 0.05$) between the results from samples treated with enzyme hydrolysis and those from control samples (see Table I).

Quantitation of T-2 Toxin Metabolites in Rat Urine by the Radioactivity. The concentrations of T-2 toxin metabolites present in the rat urine were estimated from the total radioactivity in the urine samples and the specific radioactivity of tritiated T-2 toxin (6.4 × 10^7 dpm/mg). The results, as they are expressed as T-2 equivalents (T-2 equiv), are also presented in Table I. The concentrations of T-2 toxin and its metabolites in the urine samples as estimated by the total radioactivity were 1.2–2 times higher than those detected by ELISA before any treatment of the urine samples.

Cumulative Excretion of T-2 Toxin Metabolites in Rat Urine. The total cumulative excretion curves of T-2 toxin metabolites in the urine of rats up to 216 h after a single administration of tritiated T-2 toxin at a level of 0.5 mg/kg (3.2 × 10^7 dpm/kg) are presented in Figure 1. The appearance of T-2 toxin metabolites in the urine was biphasic, with an initial slope of 2.04% of administered dose/h (2.04 μg/h) as analyzed by ELISA. Although the appearance of radioactivity in the urine was also biphasic, the initial slope of excretion as estimated from the specific radioactivity for the T-2 equivalents was 4.16% of administered dose/h, i.e. 4.16 μg of T-2 equiv/h. During the 9-day period, about 53% (53.3 μg of T-2 equiv) of the administered radioactivity was excreted into the urine; in contrast, only 33% (33.0 μg) of the administered T-2 toxin dose was detected by direct ELISA.

To determine the cause of this discrepancy, each urine sample was subjected to a cleanup treatment. After Amberlite XAD-2 column treatment, 15–40% and 53–84% of the total radioactivity in the urine sample were found in the aqueous fraction and acetone eluate, respectively. The results (Figure 1) showed that the amount of T-2 toxin and its metabolites in the acetone eluates, as estimated from the radioactivity counting, was comparable to the total amount of T-2 toxin in the urine samples determined by ELISA. The total cumulative amount of T-2 equivalents (radioactive materials) in the acetone eluates was 33.1% of administered radioactivity, indicating that nearly 100% of the T-2 equivalents in the acetone eluates was recovered by ELISA as T-2-4-ol-4-Ac. Direct ELISA analysis of the aqueous fraction revealed that no detectable T-2 toxin or its metabolites was present. Thin-layer chromatography of the aqueous fraction on a silica gel G plate in a solvent system of benzene–acetone (50:50, v/v) showed that 95% of the radioactivity stayed at the origin position. Under these TLC conditions, the major T-2 toxin metabolites have R_f values greater than 0.2 (Scott, 1982). In a controlled experiment, no radioactive material was found in the aqueous fraction when tritiated T-2 toxin was tested. Nearly 90% of the T-2 toxin was recovered in the acetone fraction as analyzed by TLC.

Quantitation of T-2 Toxin Metabolites in Cynomolgus Monkey Urine by Direct ELISA and RIA. Concentrations of T-2 toxin metabolites in the cynomolgus monkey urine samples treated with or without enzyme hydrolysis were determined by direct ELISA and RIA. The results for each individual cynomolgus monkey are presented in Figure 2. Each profile in this figure represented the urinary excretion of T-2 toxin and its metabolites of one cynomolgus monkey. There were no significant differences between the results obtained from direct ELISA and those obtained from RIA ($p < 0.05$). Urine samples from control cynomolgus monkeys, which did not receive any toxin treatment, tested negative by both direct ELISA and RIA (less than 10 ppb), as were the urine samples collected before toxin administration. The urinary excretion of toxin metabolites was rapid; 4.2–14.2 ppm was detected in urine samples collected only 8 h after dosing. The level of toxin metabolites in urine stayed high for about 1 day and then declined gradually. No toxin metabolites could be detected in the urine after 5 days. About 30% of T-2 toxin metabolites found in the urine was in the form of glucuronide conjugate in two of the three cynomolgus monkeys (Figure 2a,b) and 50% in the third one (Figure 2c).

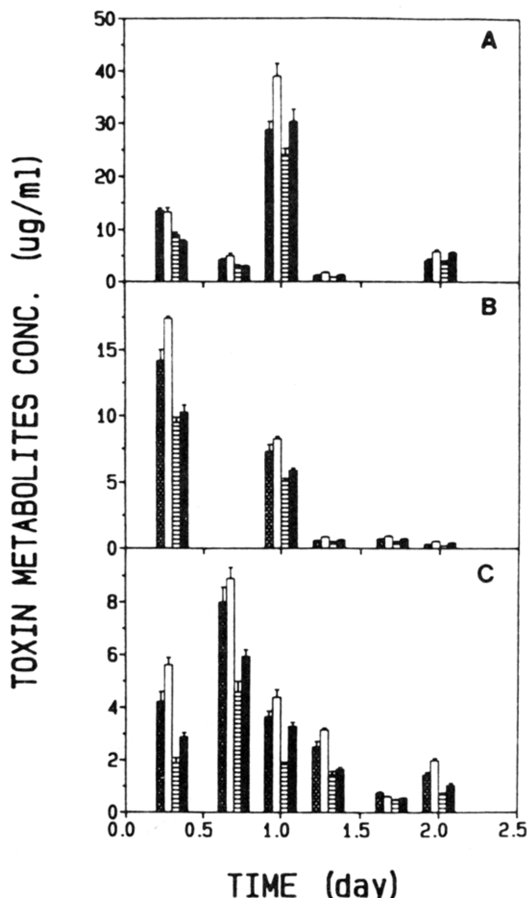

Figure 2. Excretion of T-2 toxin metabolites in the urine of cynomolgus monkeys (A, 5.72 kg; B, 4.34 kg; C, 4.91 kg) administered (i.m.) with T-2 toxin at a level of 0.5 mg/kg of body weight. The concentrations of T-2 toxin metabolites in the urine collected after toxin treatment were determined by ELISA with (▩) or without (▤) β-glucuronidase hydrolysis and by RIA with (□) or without (■) enzyme hydrolysis. The concentrations of toxin metabolites in the urine for cynomolgus monkeys A–C after 2 days were less than 0.02, 0.02, and 0.04 μg/mL, respectively.

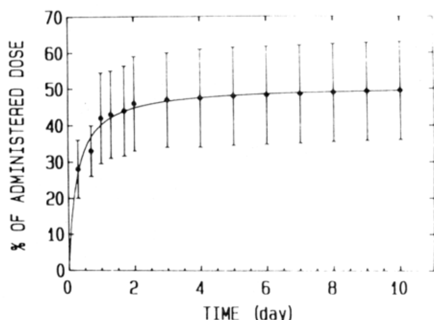

Figure 3. Cumulative excretion curves of T-2 toxin metabolites in cynomolgus monkey urine. Total amounts of T-2 toxin and its metabolites in the urine of cynomolgus monkeys treated with T-2 toxin at 0.5 mg/kg (●) were determined by ELISA and converted in terms of the percent of the administered dose. Each point is an average of data obtained from three cynomolgus monkeys.

Cumulative Excretion of T-2 Toxin Metabolites in Cynomolgus Monkey Urine. The cumulative excretion curves of T-2 toxin in the cynomolgus monkey urine are given in Figure 3. Each curve represents an average of data obtained from three cynomolgus monkeys. A total of 49.5% (0.25 mg/kg) of the administered dose (0.5 mg of T-2 toxin/kg of body weight) was eliminated into the urine during the total 10-day experimental period. The initial excretion was linear with a slope of 3.6% of administered dose/h (18 μg/kg per h).

DISCUSSION

Investigations in our laboratory have lead to a simple and sensitive ELISA test for the determination of total T-2 toxin metabolites in urine (Lee et al., 1989). The main objective of the present study was to determine whether this method could be applied in detecting or monitoring T-2 toxin exposure. In both rat and cynomolgus monkey studies, we found that the data are consistent with those reported by investigators using other techniques. Therefore, this newly developed immunochemical approach could be a very powerful diagnostic tool for T-2 toxin toxicosis.

Using rats as the animal model, approximately 30% of total T-2 toxin administered was detected as T-2 toxin metabolites in the urine by ELISA. Approximately 50% of the total radioactivity administered was recovered in the rat urine samples, 15–40% of which was found in the aqueous fraction after the sample was subjected to a XAD-2 column treatment. No toxin metabolites could be detected by ELISA in the aqueous washing fraction. After XAD-2 column treatment, the direct ELISA detected essentially 100% of the radioactivity in the acetone eluates as "T-2 equivalents". This accounted however for only 60–85% of the total radioactivity, and the balance remained in the more polar water-soluble fraction. This is likely a more polar conjugate (Sintov et al., 1988), although definitive chemistry has not been done. Our data agreed well with a recent study by Pfeiffer et al. (1988) who found that only 52% of the total radioactivity in rat urine was extractable and identified as T-2 toxin and different T-2 toxin metabolites such as HT-2, 3′-OH HT-2, 3′-OH T-2, and T-2 tetraol.

Garies et al. (1986) reported the excretion of T-2 toxin metabolites into the bile of rats primarily as glucuronide conjugates. However, conjugation with glucuronic acid did not seem to be a major metabolic pathway for T-2 toxin in rat urine. In cynomolgus monkeys, 13–50% of toxin metabolites was detected as the glucuronide adduct and the level of conjugation depended on individual animals.

The present study indicated that the urinary excretion of T-2 toxin metabolites in both animals was rapid and generally completed by 72 h after exposure, which is in close agreement with studies on other animals (Matsumoto et al., 1978; Pace et al., 1985; Robison et al., 1979; Yoshizawa et al., 1981). Although T-2 toxin is rapidly metabolized, the method developed in the present study still can detect the toxin metabolites (40 ppb) in the rat urine 9 days after dosing. In the cynomolgus monkey experiment, 21–28 ppb of T-2 toxin equivalents was detected 5 days after dosing with 0.5 mg of T-2 toxin/kg. This is within the detection limits (10 ppb; Lee et al., 1989). Since the toxin doses used in the monkey study were nonlethal, the immunoassays employed in this study could be used as an early detection of the exposure to T-2 toxin in animals and humans. No significant difference between the results obtained from RIA and those obtained from ELISA ($p < 0.05$) was found in the cynomolgus monkey study. Thus, both immunoassays are equally specific and sensitive methods. However, the RIA needs a radioactive ligand, which is a potential health hazard to humans. Disposal of radioactive compounds and expensive facilities are also problems associated with RIA. Furthermore, in the RIA, more time and more antibody are required for each assay than in direct ELISA.

Therefore, between these two immunoassays the direct ELISA is the preferred one.

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