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Production and Characterization of Antibodies against HT-2 Toxin and T-2 Tetraol Tetraacetate

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Three new immunogens which were prepared by conjugation of the carboxymethyl oxime (CMO) derivatives of HT-2 toxin, T-2 tetraol (T-2 4ol), and T-2 tetraol tetraacetate (T-2 4Ac) to bovine serum albumin (BSA) were tested for the production of antibodies against the major metabolites of T-2 toxin. Antibodies against HT-2 toxin and T-2 4Ac were obtained from rabbits 5 to 10 weeks after immunizing the animals with CMO-HT-2-BSA and CMO-T-2 4Ac-BSA conjugates. Immunization with CMO-T-2 4ol-BSA resulted in no antibody against T-2 4ol. The antibody produced against HT-2 toxin had great affinity for HT-2 toxin as well as good cross-reactivity with T-2 toxin. The relative cross-reactivities of anti-HT-2 toxin antibody with HT-2 toxin, T-2 toxin, iso-T-2 toxin, acetyl-T-2 toxin, 3′-OH HT-2, 3′-OH T-2, T-2 triol, and 3′-OH acetyl-T-2, were 100, 25, 10, 3.3, 0.25, 0.15, 0.12, and 0.08%, respectively. Antibody against CMO-T-2 4Ac was very specific for T-2 4Ac and had less than 0.1% cross-reactivity with T-2 toxin, HT-2 toxin, acetyl-T-2 toxin, diacetoxyscirpenol, deoxynivalenol, and deoxynivalenol triacetate as compared with T-2 4Ac. The detection limits for HT-2 toxin and T-2 4ol by radioimmunoassay were approximately 0.1 and 0.5 ng per assay, respectively.

T-2 toxin is one of the most toxic naturally occurring trichothecene mycotoxins produced by a number of fusaria (1, 18–20). After ingestion of T-2 toxin by animals, the toxin is metabolized rapidly to HT-2 toxin (8, 15, 21, 26) and then to T-2 tetraol (T-2 4ol) via 4-deacetylneosolaniol through a series of hydrolyses (28). Although most metabolites of low-molecular-weight drugs or toxins are detoxification products, metabolites of T-2 toxin are still toxic to animals (20). A new metabolite, 3′-OH T-2 toxin, which was formed as the result of hydroxylation by monooxygenases in microsomes, was found to be more toxic than the parent toxin (27). Since these metabolites are frequently found in the excreta and other biological fluids, monitoring such metabolites could provide important information regarding the level of exposure to T-2 toxin as well as being used as a diagnostic tool to monitor T-2 toxicosis. Consequently, there is a need for a rapid and specific method for the analysis of these metabolites in animal tissues, organs, and body fluids. Since immunoassays for T-2 toxin (3, 9–11, 13, 14, 16, 17) and several other trichothecenes (4, 5; Y. C. Xu, G. S. Zhang, and F. S. Chu, J. Assoc. Off. Anal. Chem., in press) have proved to be useful for the detection of the unmetabolized toxin in biological fluids (9, 14) as well as in foods and feeds (13, 16), efforts were made for the development of immunoassays for these metabolites in the present study. Unfortunately, the antibody used for the analysis of T-2 toxin is very specific to T-2 toxin. Although it has some cross-reactivity with HT-2 toxin (10 to 20% cross-reactivity [3]), it has less than 0.1% cross-reactivity with T-2 4ol. Investigations in our laboratory resulted in the development of a method for the production of specific antibody against 3′-OH T-2 toxin (23). As a first step in developing immunoassays for the T-2 toxin metabolites, methods for the production of specific antibodies against HT-2 toxin and T-2 4ol were investigated. Our approach included conversion of T-2 toxin to its carboxymethyl oxime derivative (CMO-T-2) and then to CMO-HT-2 or CMO-T-2 4-ol before conjugation to bovine serum albumin (BSA) for immunization. In the course of the present study, we found that CMO-T-2 4ol-BSA was not a good immunogen; therefore, CMO-T-2 tetraol tetraacetate (CMO-T-2 4Ac) was conjugated to BSA and used for immunization. Details of the preparation of immunogens and of the production and characterization of such antibodies are presented in this paper.

MATERIALS AND METHODS

Materials. T-2 toxin was produced by *Fusarium tricinctum* 696 (kindly provided by E. B. Smalley, University of Wisconsin) in cracked corn under the conditions described by Cullen et al. (6). T-2 toxin was extracted from the culture with methanol and purified on a silica gel column as previously described by Wei et al. (25). The T-2 toxin metabolites, including HT-2 toxin, T-2 triol, T-2 4ol, 3′-OH T-2, 3′-OH HT-2, acetyl-T-2 toxin, and 3′-OH acetyl-T-2, were either chemically converted or metabolized from T-2 toxin as previously described (24, 25). Deoxynivalenol (DON) and DON triacetate were prepared as described by Ehrlich and Lillejah (7). CMO-T-2 toxin was prepared by the method of Zhang et al. (29). Tritiated T-2 toxin with a specific radioactivity of 19 Ci/mmol was prepared by the procedure described previously (22). BSA (radioimmunoassay [RIA] grade) was purchased from Sigma Chemical Co. (St. Louis, Mo.). 1-(3-dimethylaminopropyl)-3,3-ethylcarbodiimide hydrochloride was purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Complete Freund adjuvant containing *Mycobacterium tuberculosis* H37Ra and incomplete Freund adjuvant were obtained from Difco Laboratories (Detroit, Mich.). Minisorp RIA tubes were purchased from Nunc (Roskilde, Denmark). Female albino rabbits were purchased from Smith's Rabbitry (Seymour, Wis.) and tested to be free of *Pasturella* species before use. All chemicals and organic solvents were reagent grade or better.

Preparation of CMO-HT-2 toxin-BSA conjugate. T-2 toxin was oxidized to 3-dehydro-T-2 toxin by pyridinium chlorochromate and purified by thin-layer chromatography (TLC) by the method of Wallace et al. (22) as modified by Chu et al.

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(4). Oximation of the 3-dehydro-T-2 toxin was carried out in a mixed solvent of methanol-water-pyridine (4:1:1, vol/vol/vol) in the presence of an equimolar concentration of carboxymethoxylamine hydrochloride at room temperature for 24 h. Under these conditions, the reaction was more favorable for the formation of CMO-HT-2 toxin than for CMO-T-2 toxin. A mixture of the two isomers of CMO-HT-2 toxin was then purified by TLC in solvent system A (ethyl acetate-methanol-acetate, 80:20:2) with a final yield of 78%. In TLC solvent system A, both isomers of CMO-HT-2 migrated as a single spot (R_f value of 0.4). Conjugation of CMO-HT-2 toxin (mixture of both isomers) to BSA was made by the water-soluble carbodiimide method under the conditions described before (30). The molar ratio of hapten/BSA, as determined by the method of Habeeb (12), was 17.

Preparation of CMO-T-2 4ol-BSA and CMO-T-2 4Ac-BSA conjugates. CMO-T-2 4ol was prepared from CMO-T-2 toxin after complete hydrolysis of this toxin in 0.1 N KOH (in 90% aqueous methanol) at room temperature for 32 h (25) and then purified by TLC (R_f value of the a isomer was 0.21 and that for the b isomer was 0.18 in solvent system A). Since the b isomer is the major product, it was conjugated to BSA by the water-soluble carbodiimide method.

CMO-T-2 4Ac was prepared from CMO-T-2 4ol by acetylation with acetic anhydride in the presence of pyridine at room temperature for 2 h and was purified by TLC (R_f value of the a isomer was 0.68 and that of the b isomer was 0.58 in solvent system A). Again, both derivatives were conjugated to BSA by the water-soluble carbodiimide method. The molar ratios of hapten to BSA were 9, 21, and 19 for the CMO-T-2 4ol and CMO-T-2 4Ac a isomers and the CMO-T-2 4Ac b isomer, respectively.

Preparation of radioactive tracers. TLC pure tritiated HT-2 toxin was prepared from tritiated T-2 toxin under limited hydrolysis conditions (25). In a typical experiment, 0.5 mCi of tritiated T-2 toxin in 0.5 ml of absolute ethanol was mixed with 3 ml of 0.01 N NaOH (in 90% aqueous ethanol). The reaction was allowed to proceed at room temperature for 50 min with gentle stirring. After reaction, the pH of mixture was adjusted to 6.0 with 0.1 N HCl, and the mixture was evaporated under vacuum to remove the ethanol. The reaction products were extracted with 5 ml of chloroform. TLC analysis revealed four major spots which were identified as T-2 toxin, HT-2 toxin, T-2 triol, and T-2 4ol. Further purification of tritiated HT-2 toxin was done by TLC with a resulting R_f value of 0.69 (R_f value of T-2 toxin was 0.82) in solvent system B (methanol-ethyl acetate-acetone; 1:8:4). About 12% of the tritiated T-2 toxin was hydrolyzed to HT-2 toxin. The purity of tritiated HT-2 toxin was also determined by autoradiography which showed one spot on TLC after being developed in solvent system B. The tritiated T-2 toxin and its derivatives were stored in ethanol at a concentration of 1 mCi/ml in a sealed ampule at 4°C. Tritiated T-2 toxin was stable for at least 2 years under these conditions.

TLC pure tritiated T-2 4ol was obtained from labeled T-2 toxin after complete hydrolysis of the toxin in a 0.1 N KOH solution under the same conditions used in the preparation of CMO-T-2 4ol. Tritiated T-2 4Ac toxin was prepared from tritiated T-2 4ol after acetylation of this derivative with acetic anhydride (0.5 ml) in the presence of pyridine (0.5 ml) at room temperature for 2 h. Labeled T-2 4Ac (R_f value of 0.5) was purified by TLC in solvent system C (ethyl acetate-toluene, 3:1).

Production of antibodies. The immunization method was essentially the same as that described previously (2). Three rabbits were used for each immunogen, and a multiple-site injection technique was used. The back of each rabbit was shaved and injected intradermally with 0.5 mg of immunogen which was prepared by dissolving the immunogen in 0.5 ml of PBS (0.01 M sodium phosphate buffer containing 0.85% NaCl, pH 7.4) and mixing with 1.5 ml of complete Freund adjuvant. Each rabbit received approximately 25 to 30 injections (0.05 to 0.1 ml of immunogen-adjuvant emulsion per injection) in the shaved area. For booster injections, each rabbit received intramuscularly 2 ml of emulsion that contained 0.5 mg of immunogen in 1.0 ml of PBS–1.0 ml of incomplete Freund adjuvant. Rabbits were bled weekly starting 3 weeks after the initial injection. The collected antisera were purified by an ammonium sulfate precipitation method (3), dialyzed against distilled water followed by PBS, and lyophilized.

RIA. RIA was carried out by the protocols previously used for other mycotoxins (3, 4, 28). Both tritiated HT-2 and tritiated T-2 toxins were used for monitoring antibody titers against HT-2 toxin. Tritiated T-2 4ol and T-2 4Ac were used to monitor antibody production against T-2 4ol and T-2 4Ac. An ammonium sulfate precipitation method was used to separate the free and bound labeled toxin. Briefly, 0.1 ml of labeled toxin (around 10,000 dpm) was incubated with 0.1 ml of purified antibody solution of various dilutions in PBS at room temperature for 30 min and then at 4°C overnight. After ammonium sulfate precipitation, the amount of radioactivity of the unbound labeled toxin (free toxin) was determined (3). The antibody titer was defined as the reciprocal of the amount of antibody required to give 50% binding of the tritiated toxin.

Analysis of antibody specificity. A competitive RIA was used to determine the specificity of the antibodies obtained from rabbits after immunization with different conjugates. In these assays, different T-2 toxin metabolites and other structurally related trichothecenes were used as competitors of the binding of the radioactive marker ligand (either tritiated HT-2 toxin or tritiated T-2 4Ac) with the respective antibody. These derivatives were dissolved in methanol and later diluted with PBS to a final methanol concentration of less than 5%. The toxin solution (0.1 ml) was mixed with labeled toxin (0.1 ml, 10,000 dpm) and an antibody solution (0.1 ml) which gave 50 to 60% binding of the tritiated toxin. The separation of free and bound toxin was essentially the same as in the RIA protocols described above.

Determination of radioactivity. Radioactivity was determined in a liquid scintillation spectrometer (model LS-5801; Beckman Instruments, Inc., Fullerton, Calif.) in 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) for aqueous samples and in OCS (Amersham Corp., Arlington Heights, Ill.) for organic samples.

RESULTS

Production of antibodies. The production of antibody against HT-2 toxin was monitored by a RIA with both labeled T-2 toxin and HT-2 toxin as the testing markers. The antibody titers for two rabbits tested over a period of 14 weeks are shown in Fig. 1. The titers increased significantly 6 weeks after immunization. Rabbit 1 gave a better response than the other rabbits tested; the antibody titers reached 1,600 and 1,200, respectively, when tritiated HT-2 toxin and tritiated T-2 toxin were used as marker ligands. After the first booster injection, a significant difference between T-2 toxin RIA titers and HT-2 toxin RIA titers was observed. The results indicated that the antibody had a higher affinity

toward HT-2 toxin than toward T-2 toxin after one booster injection. This observation was proved to be correct by subsequent analysis of the antibody specificity.

In two separate trials, five rabbits were immunized with a total of 1.5 mg of CMO-T-2 4ol-BSA conjugate per animal over a period of 13 weeks. Specific antibody against T-2 4ol was not detectable by a RIA with tritiated T-2 4ol as the testing ligand. Therefore, a CMO-T-2 4Ac-BSA conjugate was prepared and tested for its immunogenicity in eliciting antibody against T-2 4Ac.

Because two isomers of CMO-T-2 toxin were obtained in the preparation of this derivative from T-2 toxin (30), both isomers were used as the starting materials for the preparation of CMO-T-2 4ol and CMO-T-2 4Ac. For the preparation of the T-2 4ol immunogen, the major product b isomer CMO-T-2 4ol was conjugated to BSA. Both isomers of CMO-T-2 4Ac were conjugated to BSA for immunization. The two groups of rabbits that received the a and b isomers of CMO-T-2 4Ac-BSA immunogens separately showed a significant difference in antibody production. The results showed that the b isomer conjugate was a weaker immunogen; the titer was never greater than 200. Three rabbits that received four injections of the b isomer immunogen (total of 2 mg over 3 months) were rested for 3 months and then immunized with 0.5 mg of the a isomer-BSA conjugate. The same rabbit that showed a titer of 156 after the first booster injection of the b isomer antigen produced a titer of 277 at week 4 after immunization with the a isomer antigen (0.5 mg per rabbit). The response of the rabbits to the a isomer CMO-T-2 4Ac-BSA immunogen is presented in Fig. 2. A titer of 3,700 was obtained in one rabbit 14 weeks after initial immunization and two booster injections.

Specificity of antibody against HT-2 toxin. A competitive RIA with [^{3}H]HT-2 as the marker ligand was used to test the specificity of the anti-HT-2 toxin antibody. The results (Fig. 3) indicate that the antibody is specific for HT-2 toxin, but also cross-reacts with T-2 toxin. The concentrations causing 50% inhibition of the binding of this antibody to [^{3}H]HT-2 by

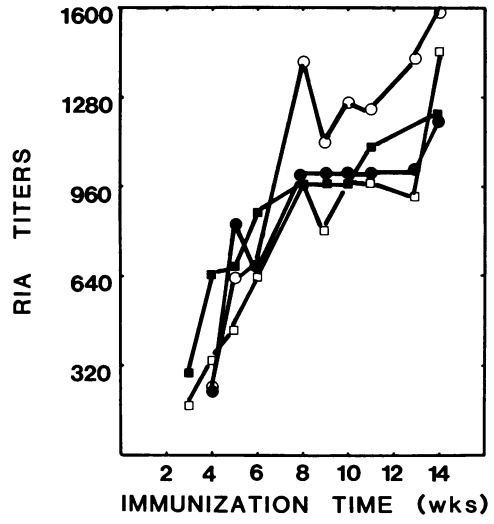

FIG. 1. Antibody titers of two representative rabbits (circles, rabbit 1; squares, rabbit 2) after immunization with CMO-HT-2-BSA conjugate. Both labeled T-2 toxin (●,■) and HT-2 toxin (○,□) were used to monitor the antibody titers. Antibody titer is defined as the reciprocal of the amount (milliliters) of antiserum required to give 50% binding of labeled ligand. The booster injection was given at week 8 postimmunization.

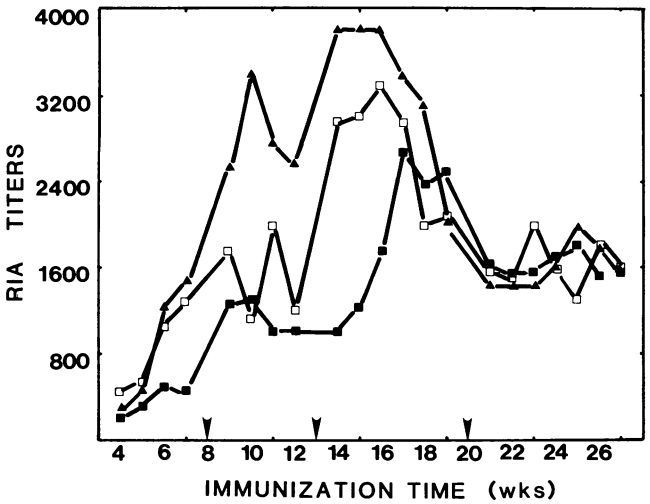

FIG. 2. Antibody titers of three rabbits after immunization with CMO-T-2 4Ac (a isomer)-BSA. The titer was determined by RIA with tritiated T-2 4Ac as the radioactive ligand, and an ammonium sulfate precipitation method was used to separate the free and bound ligands. Antibody titer is defined as the reciprocal of the amount (milliliters) of antiserum required to give 50% binding of tritiated T-2 4Ac. Arrows indicate the time of booster injections.

unlabeled HT-2 toxin, T-2 toxin, iso-T-2, acetyl-T-2, 3′-OH HT-2, 3′-OH T-2, T-2 triol, and 3′-OH acetyl-T-2 were 0.62, 2.5, 6.2, 18.6, 248, 420, 530, and 763 ng per assay (0.1 ml per assay), respectively. The relative cross-reactivity of this antiserum (or percent cross-reactivity) with HT-2 toxin, T-2 toxin, iso-T-2, acetyl-T-2, 3′-OH HT-2, 3′-OH T-2, T-2 triol, and 3′-OH acetyl-T-2 was 100, 25, 10, 3.3, 0.25, 0.12, and 0.08, respectively.

Specificity of antibody against T-2 4Ac. Results of a competitive RIA for T-2 4Ac are presented in Fig. 4. The T-2 4Ac antibody was highly specific for T-2 4Ac. The concentration causing 50% inhibition of binding of the antibody to radioactive T-2 4Ac by the unlabeled T-2 4Ac was 1 ng/ml (0.1 ml per assay), in comparison with more than 1 μg/ml for other trichothecenes including T-2 toxin, HT-2 toxin, diacetoxyscirpenol, acetyl-T-2 toxin, DON, and DON triacetate. Thus, the cross-reactivity of this antibody with other trichothecenes is at least 1,000 times weaker than with T-2 4Ac.

DISCUSSION

Conjugation of the CMO derivatives of metabolites of T-2 toxin to BSA proved to be an effective approach for the preparation of immunogens for antibody production. Although good antibody titers were obtained for HT-2 toxin after immunization, rabbits did not respond to CMO-T-2 4ol conjugate. However, once the hydroxyl groups were acetylated, the acetylated derivative was an effective immunogen. These results are not surprising because similar results were found in an earlier investigation (29). Zhang et al. (29) found that no antibody was obtained from rabbits when a CMO derivative of DON conjugated to BSA was used for eliciting antibody; nevertheless, once the DON was acetylated, high antibody titers against DON triacetate were found. The inability of the CMO-T-2 4ol conjugate to elicit antibody may be due to the haptenic structure containing several free hydroxyl groups. These polar groups may form hydrogen bonds with the side chains in the BSA molecule and thus alter the haptenic conformation.

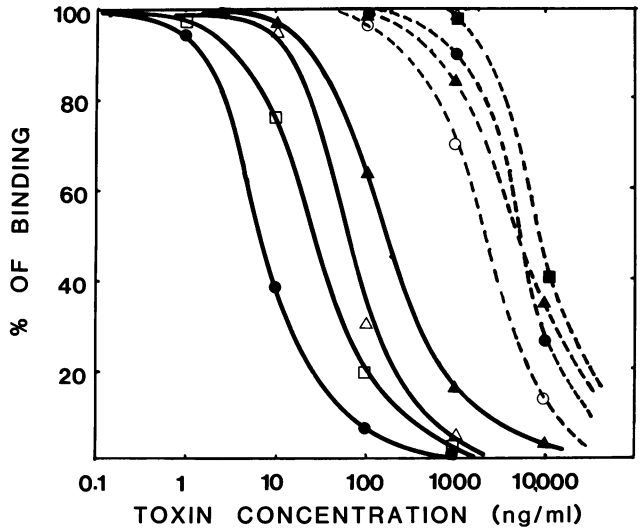

FIG. 3. Effects of different trichothecene mycotoxins on the binding of tritiated HT-2 toxin with antibody obtained from a rabbit after immunization with CMO-HT-2-BSA. A 1-to-100 dilution of antiserum (20-week bleeding) (100 μl) was incubated with 100 μl of tritiated HT-2 toxin (10,000 dpm) and 100 μl of different unlabeled trichothecene mycotoxins at various concentrations. The mixture was incubated at room temperature for 30 min and then at 4°C overnight. The binding of tritiated HT-2 toxin in the absence of unlabeled mycotoxin was 50 to 60%. The free and bound labeled HT-2 toxin was separated by an ammonium sulfate precipitation method. The relative cross-reactivity of the HT-2 toxin antiserum for the trichothecene mycotoxins, as defined by the amount of toxin (nanograms per assay) required for 50% inhibition of maximal binding of labeled HT-2 toxin, was as follows: HT-2 toxin (●——●), T-2 toxin (□——□), T-2 isomer (△——△), acetyl-T-2 toxin (▲——▲), 3′-OH HT-2 (○——○), 3′-OH T-2 (▲-----▲), T-2 triol (●-----●), and 3′-OH acetyl-T-2 (■-----■) were found to be 1, 4, 10, 30, 400, 677, 854 and 1,230 ng, respectively.

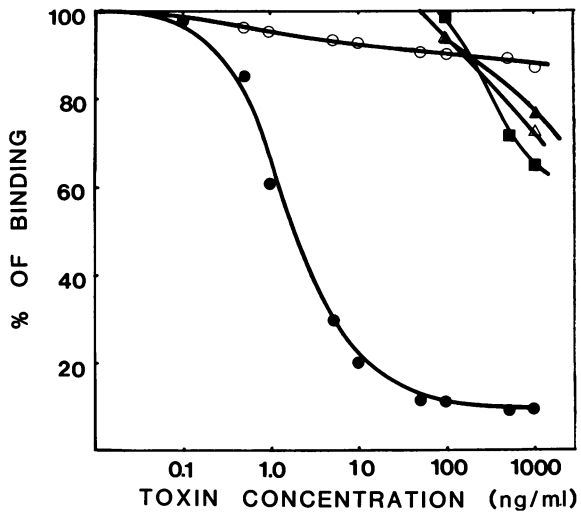

FIG. 4. Effects of different trichothecene mycotoxins on the binding of tritiated T-2 4Ac with antiserum obtained from a rabbit after immunization with CMO-T-2 4Ac (a isomer)-BSA. Experimental conditions for this study were the same as those described in the legend to Fig. 3. The antiserum dilution (16-week bleeding) was 1 to 200. Symbols: ▲, T-2 toxin; ○, HT-2 toxin; ■, diacetoxyscirpenol; △, acetyl-T-2 toxin; ●, T-2 4Ac. DON, acetyl-DON, and T-2 4ol showed no cross-reaction at a concentration of 1 μg/ml (data not shown).

It has been previously demonstrated that the side chains in the trichothecenes play an important role in eliciting specific antibodies within the trichothecene family (30). Present results further reiterate such importance. Whereas antibody against T-2 toxin is very specific for T-2 toxin, it does cross-react weakly (10 to 20%) with HT-2 toxin. The observation of good cross-reactivity of anti-HT-2 toxin antibody with T-2 toxin in the present study is consistent with the properties of anti-T-2 antibody. The presence of acetyl groups or other side chains provide the antibodies with high specificity; thus, anti-T-2 4Ac has little cross-reactivity with other metabolites.

The problem of the effectiveness of the two isomers of the CMO derivatives to be used as immunogens for conjugation was considered when the present study was designed. To avoid the use of too many different kinds of haptens, we used a mixture of the a and b isomers of CMO-HT-2 toxin. However, the present study indicates that rabbits responded to the a isomer CMO-T-2 4Ac conjugate more effectively than to the b isomer of CMO-T-2 4Ac. These results are consistent with the observations in a previous study in which both a and b isomers of CMO-T-2 toxin were used (30). Thus, a minor change in the conformation of the hapten could affect the antibody production as well as the antibody specificity.

From the inhibition curves (Fig. 3 and 4), it is apparent that the concentrations causing 10 to 20% inhibition of binding by the unlabeled HT-2 toxin and T-2 4Ac are approximately 0.1 and 0.5 ng per assay, respectively. Considering the standard deviation in the assay, it is reasonable to assume that the lower limits for detection of these two toxins by RIA are in these ranges. In view of the high cross-reactivity of anti-HT-2 with T-2 toxin, unless T-2 toxin is separated from HT-2 toxin by a chemical method (25), analysis of HT-2 toxin by any immunoassay should be run simultaneously with the anti-T-2 antibody so that the presence of both toxins can be calculated mathematically (T. S.-L. Fan, Ph.D. dissertation, University of Wisconsin–Madison, Madison, 1986). In comparison with the anti-HT-2 antibody, anti-T-2 4Ac is very specific for T-2 4Ac. Therefore, T-2 4ol could be analyzed immunochemically after converting this metabolite to T-2 4Ac by acetylation. Details for the use of the antibodies produced in the present study for the analysis of HT-2 and T-2 4ol in biological fluids by an indirect enzyme-linked immunosorbent assay are under study. Initial results indicate that the antibodies produced are very useful as immunoassay reagents for the detection of these metabolites in urine. It should also be noted that if a crude extract of a cereal-feed sample infected with *Fusarium* species was acetylated, a total T-2 toxin analog estimate could be made with the anti-T-2 4Ac antibody.

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