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Production and Characterization of Antibody Against Deoxyverrucarol

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Immunization of rabbits with deoxyverrucarol (DOVE) conjugated to bovine serum albumin resulted in antibodies bound with either tritiated DOVE or diacetoxyscirpenol (DAS), but not with T-2 toxin. The affinity of antibodies with DOVE was found to be much higher than with DAS. When [^{3}H]DOVE was used as a marking ligand in the competitive radioimmunoassay, the concentrations causing 50% inhibition of binding radioactivities by unlabeled DOVE, verrucarol, verrucarin A, and 4-monoacetoxyscirpenol were found to be 0.32, 1,070, 9,500, and 10,000 ng per assay, respectively. T-2 toxin, 15-monoacetoxyscirpenol, and deoxynivalenol gave less than 20% inhibition at 10 μg per assay. However, when [^{3}H]DAS was used as the marking ligand, the concentrations causing 50% inhibition by DOVE, DAS, and verrucarol were found to be in the 50 to 60 ng per assay range. The antibodies are thus highly specific to DOVE rather than a common trichothecene backbone. The possible use of this antiserum for assay of macrocyclic trichothecenes is discussed.

Recent studies in our laboratory and others have led to the production of specific antibodies against T-2 toxin (3) and diacetoxyscirpenol (DAS) (2), two of the most potent trichothecene mycotoxins produced by a number of fusarial species and other related fungi (1, 4, 15). With the availability of antisera, specific and sensitive immunoassays (10, 11, 13, 14) have been developed for the analysis of these mycotoxins in foods, feed, and biological fluids. Antiserum against T-2 toxin has also been used as a diagnostic tool to monitor T-2 toxicosis (12). Such developments have led to a great demand for large quantities of antisera. However, because of the toxicity and the immunosuppressive effect of these mycotoxins, the antibody titers in rabbits were relatively low as compared with other haptens, such as steroids. In addition, the antibodies obtained from rabbits after being immunized with the respective mycotoxin-protein conjugate were very specific (2, 3, 5, 14); thus, they cannot be used to determine other trichothecenes. To circumvent such problems, a recommendation was made by the Committee on Protection Against Mycotoxins of the National Research Council, urging use of deoxyverrucarol (DOVE)-protein conjugate as an immunogen for subsequent immunization (4). DOVE (Fig. 1) contains only the trichothecene backbone and is also of low toxicity to test animals (P. F. Schuda, S. J. Patlock, and R. W. Wannemacher, Jr., J. Nat. Prod., in press). The rationale for using this compound as a hapten was twofold: (i) as an attempt to obtain antibodies which may have diverse reactivities with a wide range of trichothecenes, and (ii) as an attempt to obtain higher titers in a reasonably short period of time. This task was undertaken in our laboratory. In this paper, details for the preparation of this conjugate and subsequent production of antibody as well as characteristics of the antisera obtained from rabbits after immunization with DOVE-bovine serum albumin (BSA) conjugate are presented.

MATERIALS AND METHODS

Materials. DOVE, verrucarol, and verrucarin A were prepared in the chemistry department of the University of Maryland according to procedures previously described (7–9). DAS and scirpentriol were purchased from Calbiochem-Behring, San Diego, Calif. 4-Monoacetoxyscirpenol (4-MAS) and 15-MAS were kindly provided by W. Roush of the Massachusetts Institute of Technology. Deoxynivalenol was kindly provided by R. Vesonder of the Northern Regional Research Center of the U.S. Department of Agriculture. Crystalline T-2 toxin was prepared in our laboratory by a previously described procedure (17). Tritiated sodium borohydride (77.9 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Tritiated DAS was prepared in our laboratory (19.4 Ci/mmol) (2). The purity of these mycotoxins was further analyzed by a thin-layer chromatography (TLC) method (14) and was shown as a single spot at concentrations of 5 to 10 μg per spot. *N*,*N*-Dimethylaminopyridine and succinic anhydride were obtained from Aldrich Chemical Co., Milwaukee, Wis., and recrystallized twice before using. 1-Ethyl-3,3-dimethylaminopropylcarbodiimide was purchased from Aldrich. BSA was a product of Sigma Chemical Co., St. Louis, Mo. (A-7511). All the other chemicals and organic solvents were reagent grade or better. TLC plates were precoated silica gel 60, F-254, with layer thicknesses of 0.25 and 0.5 mm (E. Merck, AG, Darmstadt, Germany). Complete Freund adjuvant containing *Mycobacterium tuberculosis* (H37 Ra) and incomplete Freund adjuvant were obtained from Difco Laboratories, Detroit, Mich. Female albino rabbits (weight, 5 lb [ca. 2.3 kg]) were purchased from Klubertanz Rabbit Farm, Edgerton, Wis., and tested to be negative for *Pasteurella* sp. before use.

Preparation of HS of DOVE. Hemisuccinate (HS) of DOVE was prepared according to the method previously described for the preparation of T-2 hemisuccinate (3). Briefly, 5.3 mg of DOVE in 8 ml of dry tetrahydrofuran was refluxed with 63.4 mg of succinic anhydride and 2.6 mg of *N*,*N*-dimethylaminopyridine overnight. After reaction, the mixture was diluted with 10 ml of distilled water and evaporated on a rotary evaporator to remove tetrahydrofuran. The aqueous solution was then acidified with 1 N HCl and extracted with 10 ml of chloroform. The chloroform solution was washed with 10 ml of distilled water twice, dried over anhydrous sodium sulfate, and concentrated.

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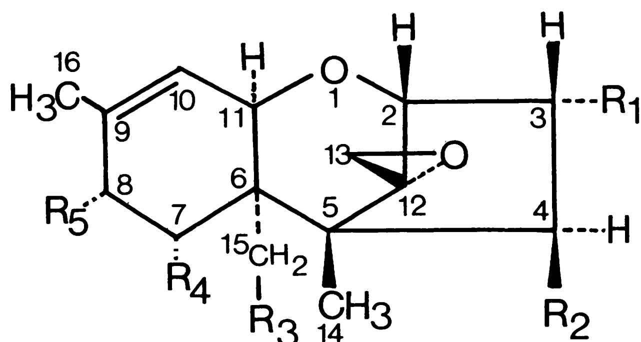

FIG. 1. Structure of DOVE and related trichothecenes. The side-chain residues for different trichothecenes used in the present study are given in Table 1.

Further purification was achieved by a preparative TLC method, using a silica gel plate. The plate was developed in a solvent containing 2.5% (volume) methanol in chloroform. The R_f values for DOVE and DOVE-HS under these conditions were 0.46 and 0.18, respectively. The silica gel bands containing either DOVE-HS or DOVE were removed from the TLC plate and extracted with 5% methanol in chloroform to yield 4.6 mg of DOVE-HS and 2 mg of unreacted DOVE. The isolated compound was further characterized by high-resolution mass spectral and nuclear magnetic resonance analyses and was shown to be DOVE-HS. Conjugation of DOVE to BSA was carried out in the presence of water-soluble carbodiimide, using the procedure previously described for T-2 toxin (3). The molar ratio for DOVE-BSA was estimated to be 16 from the free amino groups in the BSA before and after conjugation, using the procedure of Habeeb (6).

Preparation of tritiated DOVE. Tritiated DOVE was synthesized by a two-step reaction under conditions similar to those described for the preparation of tritiated T-2 toxin (3, 16). First, the hydroxyl group at the C-15 position of DOVE was oxidized to an aldehyde with $CrO_3 \cdot 2C_5H_5N$. The oxidized DOVE had an R_f of 0.83 in a solvent system containing 2.5% methanol in chloroform. The R_f of DOVE was 0.46 under the same conditions. Next, the aldehyde was reduced with tritiated $NaBH_4$. After reaction, the labeled DOVE was purified by preparative TLC under the same conditions described above. In a typical run, 9.4 mCi of tritiated DOVE was obtained from 5.2 mg of unlabeled DOVE after oxidation and subsequent reduction with 166 mCi of $NaBH_4$. The purified labeled DOVE was analyzed by TLC and exhibited as a single radioactive spot which had an R_f value the same as unlabeled DOVE.

TABLE 1. Side-chain residues for different trichothecenes shown in Fig. 1

Trichothecene	Side-chain residue[a]				
	R_1	R_2	R_3	R_4	R_5
DAS	OH	OAc	OAc	H	H
Deoxynivalenol	OH	H	OH	OH	=O
DOVE	H	H	OH	H	H
4-MAS	OH	OAc	OH	H	H
15-MAS	OH	OH	OAc	H	H
Scirpentriol	OH	OH	OH	H	H
T-2	OH	OAc	OAc	H	ISV
Verrucarin A[b]	H			H	H
Verrucarol	H	OH	OH	H	H

[a] OAc and ISV represent $OCOCH_3$ and $OCOCH_2CH(CH_3)_2$, respectively.
[b] Verrucarin A is a macrocyclic trichothecene and has a chain of atoms joining the C-4 and C-15 positions in which R_2 and R_3 become OCOCROCO (7).

Production of antibody. Immunization schedule and methods of immunization were essentially the same as those described for T-2 toxin (3). A multiple-site intradermal injection technique was used. Three rabbits were each injected on the back with 2.0 ml of immunogen emulsion at about 40 sites. The emulsion was prepared by emulsifying 500 μg of DOVE-HS-BSA (16 M DOVE per mol of BSA) in 0.5 ml of sterilized 0.9% NaCl with 1.5 ml of complete adjuvant. Bleedings and booster injections were made at appropriate times after the initial injection. For booster injection, 300 to 500 μg of immunogen in 0.5 ml of saline was emulsified with 1.5 ml of incomplete adjuvant and injected into the thigh. The antisera collected were precipitated with $(NH_4)_2SO_4$ to a final saturation of 33.3%, reconstituted to original volume with 0.1 M sodium phosphate buffer at pH 7.2, dialyzed against distilled water overnight, and then kept at −80°C or lyophilized.

Radioimmunoassay. Protocols for radioimmunoassay were essentially the same as those described for T-2 toxin in which an ammonium sulfate precipitation method was used to separate the free and bound toxins (3). In an initial experiment, the efficiencies of binding of tritiated DAS, DOVE, and T-2 toxin to the antiserum were tested. Because the binding efficiency of antiserum with tritiated T-2 toxin was low, only tritiated DAS and DOVE were used in the subsequent studies. In general, 0.1 ml of radioactive DAS or DOVE (1.5×10^4 to 2.0×10^4 dpm) was incubated with 0.1 ml of antiserum solution which was prepared by dilution of various amounts of antiserum (5 to 100 μl) in phosphate buffer (0.1 M; pH 7.2) at room temperature for 30 min and then in a cold room (6°C) overnight. Separation of the bound and free ligands was achieved by an ammonia precipitation method as described previously (3). Antibody titer was defined as the reciprocal of the amount (milliliters) of antisera which was required to give 50% binding of [^{3}H]DOVE or [^{3}H]DAS.

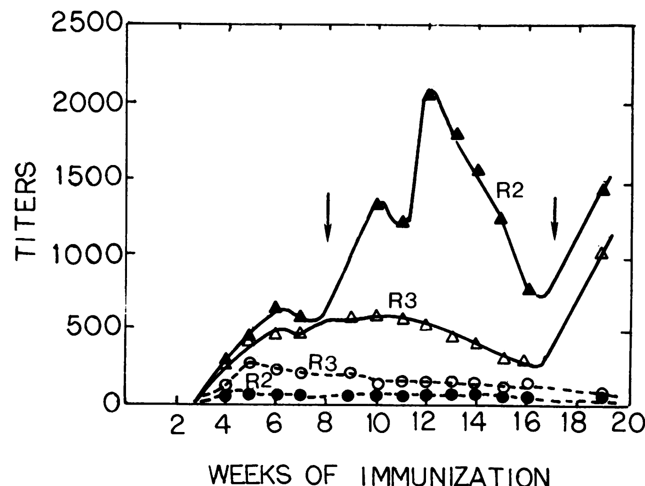

FIG. 2. Antibody titers of two representative rabbits (R2 and R3) after immunization with DOVE-HS-BSA. Both tritiated DAS (○, ●) and DOVE (△, ▲) were used as the marker ligands in the binding assay. An ammonia precipitation method was used to separate the free and bound ligands. Arrows indicate the times of booster injections.

Analysis of antibody specificity. Protocols for determination of antibody specificity were essentially the same as the titer determination except that unlabeled DOVE or various structurally related trichothecenes were present in the reaction mixture. Both tritiated DOVE and DAS were used in the test. Different derivatives were first dissolved in methanol and then diluted with 0.1 M sodium phosphate buffer (pH 7.2). The final volume of the reaction mixture was 0.2 ml.

Determination of radioactivity. For radioactivity determination, an appropriate amount of test solution, generally less than 1 ml, was mixed with 10 ml of Aquasol (New England Nuclear Corp), and the radioactivity was counted in a Beckman model LS-330 liquid scintillation spectrometer. All the counts were corrected to disintegrations per minute by a channel ratio method by using known standards of tritiated toluene. The counting efficiency was generally around 35 to 50%.

RESULTS AND DISCUSSION

Because one of our initial objectives was to produce antibodies which cross-react with most trichothecenes, the binding of different antiseral preparations with tritiated DAS, DOVE, and T-2 toxin was first examined. However, we found that the binding of antiserum with T-2 toxin was very poor. Therefore, only tritiated DAS and DOVE were subsequently used for monitoring the antibody titers. Results for the responses of two of the three rabbits during the first 20 weeks after immunization with DOVE-BSA are shown in Fig. 2. It is apparent that the antibody titers for DOVE increased rapidly after immunization (Fig. 2). The rabbits elicited antibodies as early as 4 weeks after immunization and kept them at a relatively constant level after

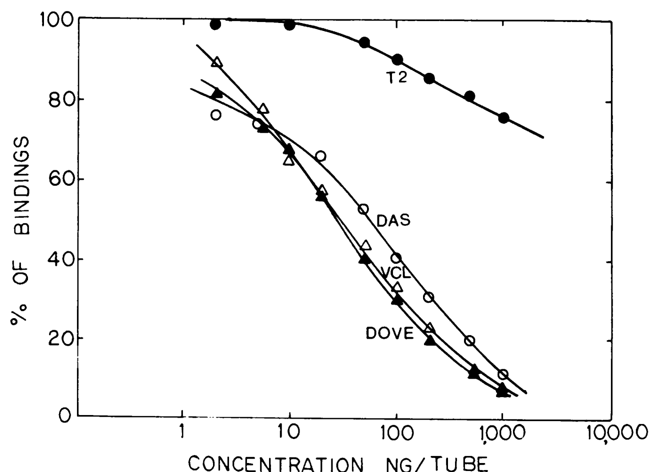

FIG. 4. Effect of different trichothecene mycotoxins on the binding of [^{3}H]DAS with immunoglobulin. The experimental conditions were the same as in Fig. 3, except that [^{3}H]DAS was used as the marking ligand in the assay. VCL, Verrucarol.

repeated booster injections. The antibody titers for DOVE of all the rabbits tested were substantially higher than titers for DAS (Fig. 2).

Variation of the response of antibody production by individual rabbits was also observed. For example, rabbit 2 produced a considerably higher amount of DOVE antibody. The ratios of DOVE titers to DAS titers for rabbit 2 at different bleedings were high and also were relatively constant, with an average of 35.4 ± 10.42 during the 27 weeks of immunization. In contrast to rabbit 2, rabbit 3 produced a slightly higher amount of DAS antibody in the earlier immunization period; thus, the ratios of DOVE titers to DAS titers at different bleedings were low during the first 16 weeks, with an average of 3.12 ± 0.83. The DOVE antibody titers of rabbit 2 increased considerably 16 weeks after immunization, whereas the DAS titer decreased, thus leading to a higher average DOVE titers/DAS titers ratio of 17.58 ± 3.92. Because of bacterial infection, rabbit 1 was killed at week 16.

The specificity of the antibody was determined by a competitive binding assay in which either radioactive DOVE or DAS was used as the marking ligand. Different structurally related unlabeled trichothecenes at various concentrations were used as the competitors for both assays. Results for the inhibition of binding of tritiated DOVE with antiserum by different trichothecenes are shown in Fig. 3. The concentrations to cause 50% inhibition of binding of [^{3}H]DOVE by unlabeled DOVE, verrucarol, verrucarin A, and 4-MAS were found to be 0.32, 10,670, 9,500, and 10,000 ng per assay, respectively. At a concentration of 10,000 ng per assay, T-2 toxin, 15-MAS, and deoxynivalenol gave less than 20% inhibition of binding. No inhibition on the binding of [^{3}H]DOVE with the antibody was observed when unlabeled DAS and scirpentriol were tested.

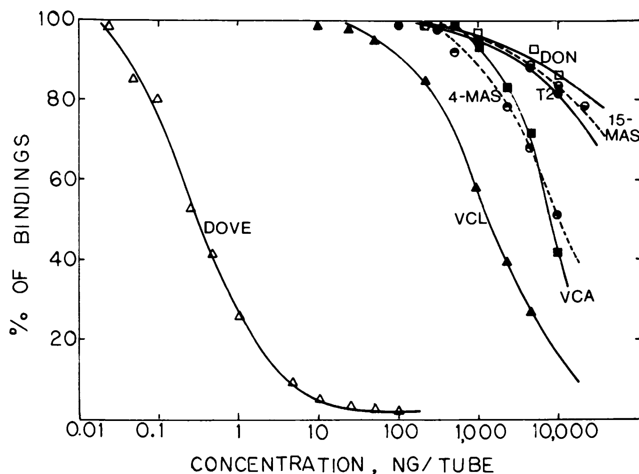

FIG. 3. Effect of different trichothecene mycotoxins on the binding of [^{3}H]DOVE with immunoglobulin G obtained from a rabbit after immunization with DOVE-HS-BSA (bleeding at 23 weeks). A 50-μl amount of 1 to 70 dilution antiserum was incubated with 50 μl of [^{3}H]DOVE (13,000 dpm) and 100 μl of different unlabeled trichothecene mycotoxins at different concentrations at room temperature for 30 min and then at 5°C overnight. The binding of [^{3}H]DOVE in the absence of unlabeled compounds was 55 to 60%. Separation of the free and bound ligands was achieved by an ammonia precipitation method. All the results were obtained from the average of three sets of experiments. The concentrations shown on the x axis of the figure are in log scale. DON, Deoxynivalenol; VCA, verrucarin A; VCL, verrucarol.

Results for the inhibition of binding tritiated DAS by different unlabeled trichothecenes are shown in Fig. 4. The concentrations to cause 50% inhibition of binding of DAS to the antiserum by unlabeled DOVE, DAS, and verrucarol were found to be in the range of 50 to 60 ng per assay. At a concentration of 1,000 ng per assay, T-2 toxin gave only 24% inhibition of binding of [^{3}H]DAS to the antibody. Because of the low affinity of the antiserum to DAS, a considerable amount of antiserum was used in this study as compared

with the last experiment in which DOVE was used as the marking ligand.

The present study demonstrated that high antibody titers for DOVE were obtained from rabbits after the animals were immunized with DOVE-HS-BSA. These results supported our hypothesis that a nontoxic trichothecene would be more favorable for antibody production. However, because the DOVE antibodies had high affinity to DOVE itself, they did not cross-react with most other types of trichothecenes. These antibodies have little use for the immunoassay of either T-2 toxin or DAS. Nonetheless, because of its high affinity to DOVE, the antiserum together with tritiated DOVE might be useful to monitor the unknown fungal metabolite which has a chemical structure similar to DOVE.

The possibility of using the antiseral preparation and tritiated DAS as immunological reagents also has been tested. The DAS antibody, however, shows good cross-reactivity with DOVE and verrucarol. Although the sensitivity for detecting these three mycotoxins still cannot be compared with other systems, it appears that at least 25 ng of DAS, DOVE, and verrucarol can be monitored (Fig. 4). Since no sensitive analytical method is currently available for the macrocyclic trichothecenes, it is possible to use this antibody to develop an immunoassay for verrucarol. The macrocyclic trichothecenes, such as verrucarin A, can be hydrolyzed to verrucarol for subsequent immunoassays.

Previous investigations in our laboratory and others (2, 3, 5, 14) have found that the side chains in the trichothecene played an important role in determining the specificity of antibody elicited. Thus, when either T-2 or DAS was conjugated to the protein for immunization, the antibodies obtained have affinity to either of the homologous compounds. Present results further reiterate the importance of side-chain epitopes in the haptens on the specificity of antibody. Such side chains may directly affect the conformation as well as the molecular size of this group of mycotoxins. Present results also emphasize that the selection of radioactive ligand is equally important for the monitoring of polyclonal antibody titers as well as for the immunoassays. Thus, when $[^3H]$DAS is used in the assay, the antibody may be useful in the analysis of verrucarol.

Since it has been demonstrated that the side-chain groups in the trichothecene molecule affect greatly the spectrum of the biological activity of different trichothecenes (1, 4), different highly specific antibodies may be used as biological probes for toxicity studies. With different antibodies having defined specificities available, studies on the interaction of trichothecenes with these antibodies may shed some light on the mode of action of trichothecenes.

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