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Production of Polyclonal Antibodies to the Trichothecene Mycotoxin 4,15-Diacetylnivalenol with the Carrier-Adjuvant Cholera Toxin

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The trichothecene mycotoxin 4,15-diacetylnivalenol (DNIV) was conjugated to cholera toxin (DNIV-CT) for use as an immunogen and as an adjuvant for specific antibody production. Repeated intravenous injection of 7.5 μg of the conjugate was effective at generating specific antibodies to DNIV in rabbits as determined by enzyme-linked immunosorbent assay (ELISA). When small amounts (1 to 10 μg per animal) of DNIV-CT were used to immunize mice, polyclonal antibodies were observed as early as 4 weeks of immunization. The relative affinity of the antibodies to DNIV increased with the immunogen dose in mice. Antibodies were not detectable in either rabbits or mice that were injected with DNIV conjugated to the carrier protein bovine serum albumin or when DNIV-CT was blocked with glutaraldehyde. Competitive ELISA of mouse and rabbit serum revealed that the antibodies were most specific for DNIV but reacted to a small extent with fusarenone-X, deoxynivalenol, and nivalenol. No reactivity was observed with 3- or 15-acetyldeoxynivalenol. The results suggest that specific polyclonal antibodies can be prepared against a trichothecene when CT is used as an adjuvant and carrier protein. DNIV antibodies will be useful for monitoring the compound in food in conjunction with other trichothecene antibodies, detection of DNIV-producing cultures, and investigation of 8-ketotrichothecene biosynthesis.

The trichothecenes mycotoxins are a group of naturally occurring sesquiterpenoids that include some of the most potent inhibitors of protein synthesis known (29). Manifestations of trichothecene toxicosis include emesis, hemorrhaging, feed refusal, and altered immune function (22, 30). The presence of the 8-ketotrichothecene (group B) mycotoxins in foods and feed is a worldwide problem (27). These mycotoxins include deoxynivalenol (DON), 3- and 15-acetyl-DON, fusarenone-X, and nivalenol. Although precursors of these compounds and metabolites of their biosynthetic pathways may be highly toxic (3), little is known about their occurrence in food. Notably, 4,15-diacetylnivalenol (DNIV) exhibits greater in vitro toxicity than DON or fusarenone-X does (3).

Trichothecene detection for food safety assurance is generally based on thin-layer chromatography (26, 28), gas chromatography-mass spectrometry (11), high-performance liquid chromatography (7), or mass spectrometry (5); however, it is now possible to use immunochemical assays (21). Work in our and other laboratories has focused on the generation of antibodies to detect the 8-ketotrichothecenes (6, 14, 20, 31).

Since mycotoxins are low-molecular-weight compounds, they do not independently induce a hyperimmune response. In most cases, they must be derivatized and then conjugated to a carrier protein such as bovine serum albumin to be rendered immunogenic. A major challenge for trichothecene antibody production has been to develop suitable conjugates which are immunogenic. In general, functional moieties such as carboxymethyloxime and hemisuccinate groups can be introduced and the derivative can be conjugated to proteins by carbodiimide, mixed-anhydride, or hydroxysuccinimide methods (8, 10). Derivatization of DON to a hemisuccinate involves chemical blocking or enzyme treatment because of the presence of multiple hydroxyl groups on the parent compound (6, 20).

Recently, we have attempted to develop antibodies against DNIV. However, several attempts with the standard protein carrier, bovine serum albumin (BSA) plus Freund's complete adjuvant, yielded negative results. Cholera toxin (CT), a protein enterotoxin produced by *Vibrio cholerae*, has been shown to have strong oral and systemic adjuvant properties when coadministered with unrelated antigens (17, 19); additionally, its large molecular size (≈85 kDa) makes it potentially useful as a carrier protein. CT has been successfully used as a carrier adjuvant to produce polyclonal and monoclonal antibodies for the fumonisin mycotoxins (1, 2). We report here the rapid production of rabbit and mouse polyclonal antibodies for the trichothecene mycotoxin DNIV by using an immunization procedure that takes advantage of the dual carrier and adjuvant properties of CT as well as application of the antibodies to enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Chemicals and reagents. All inorganic chemicals and organic solvents were reagent grade or better. BSA (fraction V), chicken egg albumin (ovalbumin [OA]; grade III and fraction VII), CT, Tween 20, 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS), hydrogen peroxide, sodium borohydride, glutaraldehyde, 1,3-dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide were obtained from Sigma

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Chemical Co., St. Louis, Mo. Complete and incomplete Freund adjuvants were purchased from Difco Laboratories, Detroit, Mich. Goat anti-mouse immunoglobulin G (IgG)–horseradish peroxidase conjugate and goat anti-rabbit IgG–horseradish peroxidase conjugate were obtained from Cappel Laboratories, West Chester, Pa. DNIV was synthesized from fusarenone-X (4-acetylnivalenol) by partial acetylation with acetic anhydride-pyridine. Its purity (98% as a trimethylsilyl ether derivative) was confirmed by gas chromatography and gas chromatography-mass spectrometry. DON, 3-acetyl-DON, nivalenol, and fusarenone-X were purchased from Romer Laboratories, Inc., Washington, Mo. 15-Acetyl-DON was prepared as described previously (24).

Preparation of immunogens. To produce a reactive group for attachment to the carrier protein CT, we converted DNIV to a hemisuccinate as follows. First, 20 mg of DNIV was dissolved in 0.5 ml of pyridine in a 2-ml reaction vial. Then 14 mg of succinic anhydride was added, and the vial was capped tightly and heated in a boiling-water bath for 2 h. Pyridine was evaporated under N_2, and the residue was dissolved in 10 ml of distilled water. The aqueous solution was washed with 10 ml of chloroform five times. Chloroform fractions were collected, passed through anhydrous sodium sulfate, and evaporated to dryness under N_2. DNIV-hemisuccinate was conjugated to CT (DNIV-CT) (Fig. 1) and to BSA (DNIV-BSA) for use as immunogens and to ovalbumin (DNIV-OA) for use as a solid-phase antigen for ELISA; the procedure of Kitagawa et al. (15), was used for the conjugation. DNIV-OA and DNIV-BSA conjugates were aliquoted in 1-mg fractions, lyophilized, and stored at −20°C. Because CT is sensitive to freezing, DNIV-CT was diluted in 0.01 M phosphate-buffered saline (PBS; pH 7.2), filter sterilized, dispensed into sterile screw-cap vials in 1-ml fractions, and stored at 4°C until required.

Some preparations of DNIV-CT were modified by adding an equal volume of glutaraldehyde (2%, vol/vol) dropwise with constant stirring to DNIV-CT (1 mg/ml) in 0.01 M phosphate buffer (pH 7.2) at 10°C. After 1 h, the reaction was stopped by the addition of sodium borohydride to a final concentration of 10 mg/ml. The final mixture was dialyzed for 72 h (three changes) against 4 liters of 0.01 M PBS (pH 7.2).

Immunization. New Zealand White female rabbits were obtained from the Baily Rabbitry, Alto, Mich. Six rabbits were divided into three groups (two rabbits each). Each rabbit in group I received 7.5 μg of DNIV-CT intravenously (i.v.) via the marginal ear vein, boosted weekly with 7.5 μg of DNIV-CT i.v. The group II rabbits were immunized and boosted the same way as the group I rabbits, but glutaraldehyde-blocked DNIV-CT was used. Each group III rabbit was immunized intradermally at 20 to 30 sites on a shaved back area with DNIV-BSA (500 μg) mixed with complete Freund adjuvant. A monthly intramuscular booster of 250 μg of the same conjugate mixed with incomplete Freund adjuvant was given to each group III rabbit. The rabbits were bled via the marginal ear vein at regular intervals, and sera were purified with 33% ammonium sulfate (13).

Female BALB/c mice, 6 to 8 weeks old, were purchased from Charles River Laboratories, Wilmington, Mass. Groups (three to five mice each) were immunized i.v. with different amounts of DNIV-CT (1, 2, 3, 5, or 10 μg per mouse) and boosted biweekly with an identical dose. A sixth group was injected with 10 μg of glutaraldehyde-treated DNIV-CT per mouse and boosted biweekly with 10 μg per mouse. For i.v. immunization, mice were injected in the lateral tail vein with multiple doses of DNIV-CT dissolved in

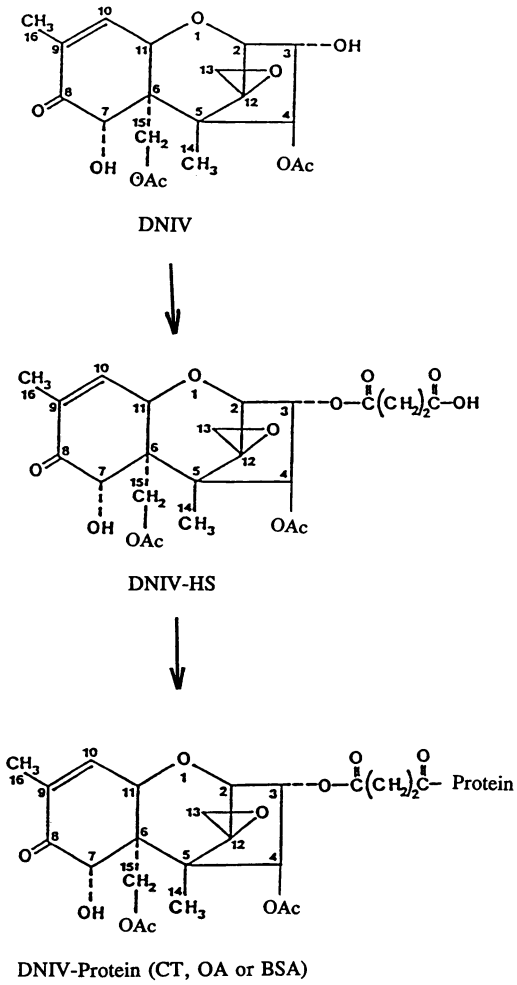


FIG. 1. Pathway for conjugation of DNIV to protein (CT, OA, or BSA). OAc, acetate.

0.2 ml of PBS. Ether-anesthetized mice were bled from the retrobulbar plexus, blood samples were kept overnight at 4°C, and sera were obtained after centrifugation at 1,000 × *g* for 15 min.

In a separate experiment, four groups of mice (five mice per group) were immunized subcutaneously (s.c.) or intraperitoneally (i.p.) with 25 or 100 μg of DNIV-BSA mixed with complete Freund adjuvant. Mice received biweekly injections of 25 or 100 μg of DNIV-BSA mixed with incomplete Freund adjuvant.

ELISA. For antiserum titer determination, wells of polystyrene microtiter plates (Immunolon 2-Removawells; Dynatech Laboratories, Alexandria, Va.) were coated overnight (4°C) with 100 μl of DNIV-OA (5 μg/ml) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). The plates were washed four times with 300 μl of 0.02% (vol/vol) Tween 20 in PBS (PBS-Tween). The wells were blocked for 30 min at 37°C with 300 μl of 1% (wt/vol) OA in PBS (OA-PBS) and then washed four times with PBS-Tween. Next, 50 μl of serially diluted serum was added to each well, and the wells were incubated for 1 h at 37°C. Unbound antibody was removed by washing four times with PBS-Tween, and 100 μl of goat anti-mouse IgG peroxidase conjugate or goat anti-rabbit IgG peroxidase conjugate (diluted 1:500 in OA-PBS)

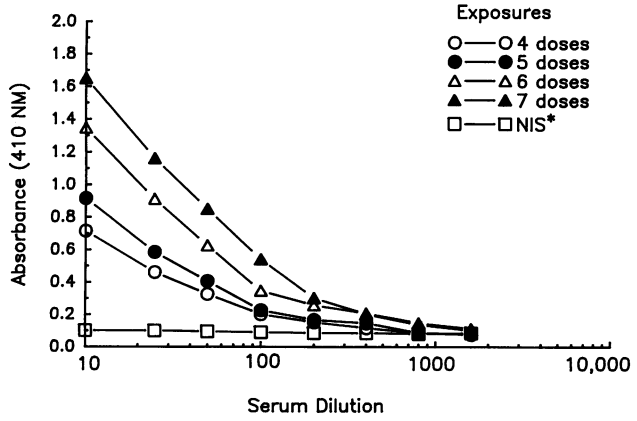


FIG. 2. Indirect ELISA titer determination of rabbit DNIV antibodies after multiple immunizations. NIS, nonimmune serum.

TABLE 1. Production of DNIV polyclonal antibodies from mice immunized with different conjugates

Conjugate	Route of administration	Dose (μg)	No. of mice:	
			Immunized	Producing antibodies
DNIV-CT	i.v.	1	3	1
	i.v.	2	3	1
	i.v.	3	3	2
	i.v.	5	3	3
	i.v.	10	5	5
DNIV-CT-GA[a]	i.v.	10	5	0
DNIV-BSA	i.p.	25	5	0
	i.p.	100	5	0
	s.c.	25	5	0
	s.c.	100	5	0

[a] GA, glutaraldehyde.

was added to each well. Plates were incubated for 30 min at 37°C and washed eight times with PBS-Tween, and bound peroxidase was determined with ABTS substrate as described by Pestka et al. (23). The A_{405} was read with a Minireader II (Dynatech), and the titer of each serum sample was arbitrarily designated as the maximum dilution that yielded at least twice the absorbance of the same dilution of nonimmune control serum.

A competitive indirect ELISA was used to assess the presence of specific DNIV antibodies in mouse and rabbit sera. Microtiter plates were coated and blocked as described for antiserum titer determination. Next, 50 μl of DNIV (or analogs) dissolved in PBS was simultaneously incubated with 50 μl of appropriate antiserum dilution in PBS over the DNIV-OA solid phase for 1 h at 37°C. Bound antibody was determined by the addition of anti-mouse or anti-rabbit IgG peroxidase conjugate as described above.

RESULTS AND DISCUSSION

Rabbits immunized with DNIV-CT produced specific antibodies against DNIV after four injections. Antibody titers increased with repeated immunization (Fig. 2). In contrast, sera from rabbits immunized with DNIV-BSA as the carrier protein and complete and incomplete Freund adjuvants were devoid of DNIV antibody reactivity. Antibody responses were similarly not observed with rabbits that were immunized with the glutaraldehyde-treated DNIV conjugate.

All groups of mice that were immunized with DNIV-CT exhibited antibody titers for DNIV, whereas DNIV-BSA was ineffective (Table 1). Each group of three mice immunized with 1 and 2 μg of DNIV-CT per mouse contained one positive mouse. As the dose increased to 3 μg per mouse, two of three mice produced specific antibodies for DNIV. All mice immunized with 5 or 10 μg of DNIV-CT readily produced DNIV antibodies; the sera of mice immunized with 10 μg had the highest relative affinity for DNIV (Fig. 3). Specific antibodies were not detectable in mice immunized with 10 μg of DNIV-CT treated with glutaraldehyde.

The reactivity of pooled serum antibodies of mice or rabbits toward analogs was also assessed by competitive indirect ELISA with DNIV-OA as the solid phase. When the ability of other 8-ketotrichothecenes (Fig. 4) to inhibit the binding of the antibody to the solid phase in the ELISA was compared with that found for free DNIV by using mouse (Fig. 5) and rabbit (Fig. 6) antisera, fusarenone-X was found to be cross-reactive. At 50 μg/ml, the highest concentration tested of the mycotoxin, a maximum of 30 and 33% binding inhibition was observed with fusarenone-X with rabbit and mouse antibodies, respectively. Slight cross-reactivity of DON and nivalenol was observed with both mouse and rabbit antibodies (8 to 22% inhibition when 50 μg of both mycotoxins per ml was tested). Cross-reactivity with 3-acetyl-DON and 15-acetyl-DON was not detected.

The presence of multiple hydroxyl groups in the trichothecenes has made the development of useful antibodies against these mycotoxins very difficult. For example, we found that after numerous failed attempts to produce a polyclonal antibody to a DON-hemisuccinate conjugate in rabbits, only 1 of 30 mice yielded a demonstrable immune response to the toxin (6). Zhang et al. (31) used a hemisuccinate derivative of 7,8-dihydroxycalonectrin coupled to BSA to produce antibodies specific for triacetyl-DON. Mills et al. (20) used an indirect approach to synthesize DON-hemiglutarate–BSA by using acetyl esterase to deacylate

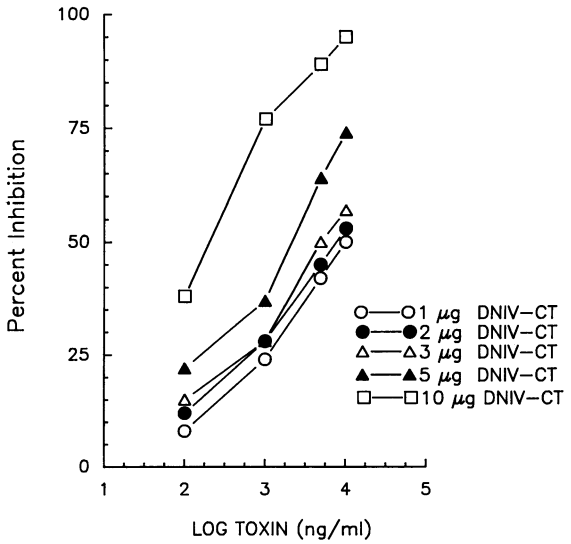
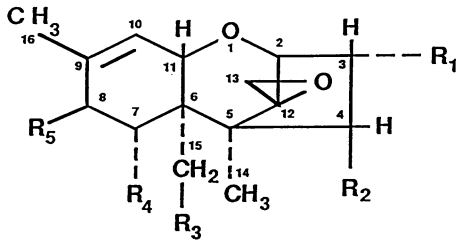


FIG. 3. Effect of immunization on competitive indirect ELISA for DNIV when using mouse antisera. Antisera were produced after four i.v. injections with DNIV-CT. A 1/400 dilution of pooled sera was used.



Compound	R_1	R_2	R_3	R_4	R_5
DNIV	OH	OAc[a]	OAc	OH	=O
Fusarenone X	OH	OAc	OH	OH	=O
Deoxynivalenol (DON)	OH	H	OH	OH	=O
3-Ac-DON	OAc	H	OH	OH	=O
15-Ac-DON	OH	H	OAc	OH	=O
Nivalenol	OH	OH	OH	OH	OH

[a] OAc = -OOCCH$_3$

FIG. 4. Structures of 8-ketotrichothecene mycotoxins.

3-acetyldeoxinivalenol hemiglutarate and coupling the product to BSA, which was used to generate polyclonal antibodies for DON. In an attempt to develop an ELISA for nivalenol, Ikebuchi et al. (14) used nivalenol tetraacetate conjugated to BSA; the resultant antibody was more specific for nivalenol tetraacetate than for nivalenol. In this study, we selected DNIV for conjugation to CT or BSA as the carrier protein and subsequent antibody development. Because DNIV contains fewer hydroxyl groups than other trichothecenes do, we had also hoped to generate antibodies

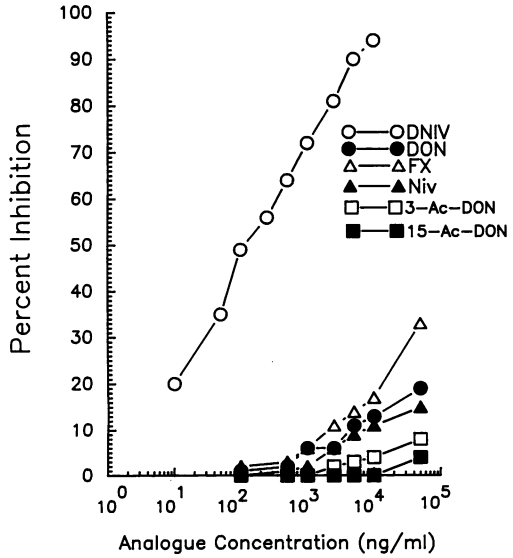


FIG. 5. Reactivity of mouse polyclonal antiserum toward DNIV analogs as determined by competitive indirect ELISA. Abbreviations: FX; fusarenon-X; Niv, nivalenol; 3-Ac-DON, 3-acetyl-deoxynivalenol; 15-Ac-DON, 15-acetyl-deoxynivalenol.

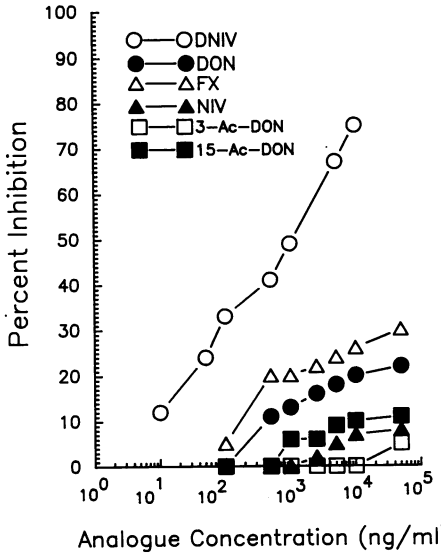


FIG. 6. Reactivity of rabbit polyclonal antiserum toward DNIV analogs as determined by competitive indirect ELISA. The abbreviations are the same as in Fig. 5.

that reacted with all 8-ketotrichothecenes. The C-7 hydroxyl remains underivatized since it is unreactive as a consequence of the hydrogen bonding to the C-8 ketone (12). However, the above data indicate that the resultant antibodies were highly specific for DNIV and are not applicable for general screening of the 8-ketotrichothecene group.

The results obtained from this investigation provide further evidence that use of CT as an adjuvant and carrier protein is of potential value for production of specific antibodies against haptenic compounds such as trichothecenes. CT has approximately the same number of lysine groups as BSA on a molar basis, thus facilitating its use as a carrier. CT was used as the carrier for 2-acetylaminofluorene in the production of rabbit mucosal antibodies (25). It has a strong systemic and mucosal adjuvant effect upon oral coadministration with unrelated antigens (9, 17, 18). Immunity to this antigen can last several years (19), and nominal concentrations of CT are required for immunization (16). Although the mechanism(s) by which CT exerts its potent adjuvant effect in the immune system is not fully understood, it has been shown that CT concomitantly stimulates antigen presentation and interleukin-1 production (4). Thus, the effectiveness of the DNIV-CT conjugate compared with the DNIV-BSA immunogen might be explained on the basis of the CT adjuvant effect.

The apparent dual properties shown for CT (carrier and adjuvant) in this and previous (1, 2) work suggest that CT may be useful in the preparation of antibodies to other haptens and peptides. This approach can be advantageous for several reasons. The procedure was rapid and yielded high-quality antibodies in comparison with the poor results achieved by standard protocols. Also, since animal health impairment is not observed at the low levels used in this work ($\leq$10 μg per animal), CT might be a humane alternative to Freund adjuvant, which typically gives rise to abscesses, ulcers, or granulomas at the injection site. Finally, the use of CT would also be valuable when hapten availability is limited, since, as has been shown here, relatively low doses of immunogen are required to induce a rapid and strong antibody response.

In conclusion, we report the rapid and efficient production of polyclonal antibodies to DNIV when a CT conjugate was used as the immunogen. Potential applications of DNIV antibodies and ELISA will include the assay of this metabolite in food and feed in conjunction with other trichothecene antibodies, detection of trichothecene-producing fungi, and study of 8-ketotrichothecene biosynthesis.

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