Production of a Monoclonal Antibody to T-2 Toxin with Strong Cross-Reactivity to T-2 Metabolites

E. H. Gendloff, J. J. Pestka, D. E. Dixon, and L. P. Hart

First and fourth authors, Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824; and the second and third authors, Department of Food Science and Human Nutrition, Michigan State University, East Lansing 48824.
Present address of first author: Agrigenetics Advanced Science Company, 5649 East Buckeye Road, Madison, WI 53716.
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ABSTRACT

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A monoclonal antibody against T-2 toxin was produced using a mouse immunized by subcutaneous injections into the shoulder with large immunogen doses. When this antibody was used in an indirect competitive enzyme immunoassay, sensitivity to T-2 toxin was 10 ng/ml (0.5 ng per assay). The antibody cross-reacted less to HT-2 toxin than T-2 antibodies previously described. Strong cross-reaction with the T-2 metabolites 3'OH T-2 and 3'OH HT-2 was noted.

Additional key words: competitive indirect enzyme immunoassay, ELISA, fungal metabolites, hapten, mycotoxicosis, trichothecene.

T-2 toxin (T-2) is a trichothecene mycotoxin produced by various plant pathogenic *Fusarium* spp., and is sometimes found in agricultural products (17). T-2 is a potent inhibitor of protein synthesis (1) and has been implicated in several cases of mycotoxicosis. Various metabolites of T-2 have been identified (21–23) that are also toxic (22) and inhibit lymphoblastogenesis (8). Because of difficulties inherent in chemical methods of detecting trichothecenes (17), immunological methods have been developed for the detection of T-2 (2,5,7,9,14,15,19) and other trichothecenes (3,4). Monoclonal antibodies against T-2 were recently produced (11). This was a significant development in trichothecene serology because, unlike serum antibodies, monoclonal antibodies are of invariate specificity and unlimited supply (16). A competitive indirect enzyme immunoassay (CIEIA) employing these monoclonal antibodies was less sensitive for T-2 than previously reported polyclonal antibody-based enzyme immunoassays (5,9,19), however. We describe herein the production of a new monoclonal antibody to T-2 employing alternative immunization and CIEIA methods. This antibody/CIEIA system allows 50-fold greater sensitivity to T-2 and cross-reacts with certain metabolites of T-2 that are diagnostic of T-2 toxicosis.

MATERIALS AND METHODS

Material. All inorganic chemicals and organic solvents were reagent grade or better. Bovine serum albumin (BSA) (fatty acid free and fraction V), polyoxyethylenesorbitan monolaurate (Tween 20), 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS), ovalbumin (crude and fraction VII), dicyclohexylcarbodiimide, N-hydroxysuccinimide, poly-1-lysine (MW 22000) (PLL), aminopterin, 8-azaguanine, polyethylene glycol MW 1450, sodium pyruvate, insulin, oxaloacetate, pristane (2,6,10,14-tetramethyl pentadecane), thymidine, and hypoxanthine were obtained from Sigma Chemical Co., St. Louis, MO; Freund's adjuvants from Difco, Detroit, MI; goat antimouse IgG conjugated to horseradish peroxidase (antimouse-peroxidase) from Cooper Biomedical, Malvern, PA.; immunoassay microtiter plates (immunoplates) from Nunc Intermed, Roskilde, Denmark; filter paper from Whatman, Inc., Clifton, NJ; and various cell culture reagents from GIBCO Laboratories, Grand Island, NY. Trichothecenes used in previous work (9) were obtained from the same sources, and the T-2 metabolites 3'OH T-2 and 3'OH HT-2 (22) were generously provided by T. Yoshizawa, Kagawa University, Kagawa, Japan.

Preparation of conjugates. T-2 was converted to T-2-hemisuccinate (T-2HS) and conjugated to fatty acid-free BSA by the method of Chu et al (2). T-2HS was also conjugated to ovalbumin (fraction VII) and PLL via an activated ester intermediate using the following modification of the method of Kitagawa et al (13). T-2HS was stirred with equimolar amounts of both dicyclohexylcarbodiimide and N-hydroxysuccinimide in 0.1 ml of dry tetrahydrofuran for 30–60 min at room temperature. Precipitates that formed were collected by filtration on Whatman No. 1 paper, and washed with 2–3 ml of tetrahydrofuran. The tetrahydrofuran was evaporated and the residue dissolved in 0.2 ml of dimethylformamide. This was added dropwise to 5 mg of ovalbumin dissolved in 0.5 ml of 0.13 M sodium bicarbonate. The mixture was stirred slowly for 30 min, then dialyzed for 3 days against frequent changes of 0.1 M sodium bicarbonate.

Immunization protocols. Two immunization protocols were compared. Both used the T-2HS-BSA conjugate described above and three female BALB/c mice. In the first group (Protocol A), 100 μg of conjugate in 0.1 ml of saline was emulsified with 0.1 ml of Freund's complete adjuvant and injected into the peritoneal cavity of each mouse. Booster injections were made at 1-mo intervals as above, except the Freund's adjuvant was incomplete. In the second group (Protocol B), 1 mg of conjugate in 0.5 ml of saline was injected subcutaneously into the shoulder. These were repeated at 2-wk intervals, except 0.5 mg of conjugate was used. All mice were bled through the tail vein 79 days after the initial injection and the serum was tested for T-2 antibody activity as described below.

CIEIA. A competitive indirect procedure was used to screen for antibody activity against T-2. T-2HS-PLL or T-2HS-ovalbumin conjugate (200 μl), diluted to 2.5 μg/ml in 50mM carbonate-bicarbonate buffer, pH 9.6, was placed in each well of 96-well immunoplates. The PLL conjugate was used for initial hybridoma screening and the ovalbumin conjugate was used later, with similar results. The plates were incubated overnight at 4 C, then washed

with sodium phosphate-buffered saline (PBS 0.1 M, pH 7.5) containing 0.05% Tween 20. After washing, 200 μl of PBS containing 1% (w/v) crude ovalbumin (PBS-ovalbumin) was added to each well (insoluble matter in the PBS-ovalbumin preparation was removed by low-speed centrifugation). After incubation for 30 min at 37 C, the plates were washed as above. When undiluted hybridoma supernatant fractions were being screened, 40 μl of either 10% methanol in PBS (PBS-MEOH) or T-2 toxin (10μg/ml) in PBS-MEOH was placed in each well, followed by 40 μl of hybridoma supernatant. Supernatant from each colony tested was put in four wells, two with free T-2 and two without. When preparations other than undiluted hybridoma supernatants were assayed, solutions added were (in order) 25 μl of PBS-ovalbumin, 50 μl of trichothecene in PBS-MEOH, and 50 μl of antibody preparation diluted in PBS. Washing followed a 1-hr incubation at 37 C. Next, antimouse-peroxidase (80 μl when screening hybridoma supernatants and 100 μl at other times), diluted 1/500 in PBS containing 1% fraction V BSA (w/v) and 0.1% Tween 20, was added, followed by a 30-min incubation at 37 C. After washing, bound peroxidase was determined by a 5–30 min incubation of 100 μl of ABTS-H$_2$O$_2$ substrate in each well. The reaction was terminated with 100 μl of stopping solution (18). Absorbance at 405 nm was determined on an EIA reader EL307 (Bio-Tech. Inc., Burlington, VT).

Hybridoma preparation. Dulbecco's modified eagle medium with 20% fetal bovine serum (FBS), 50 units of penicillin per milliliter, 50 μg of streptomycin per milliliter, 1% NCTC (Gibco), 5 μM oxaloacetate, 10 μM sodium pyruvate, and 75.5 mg of insulin per liter (20% FBS) was routinely used as cell culture media. After a 5-wk rest, the mouse with the highest serum titer was injected intraperitoneally with 300 μg of T-2HS-BSA in 300 μl of saline. The fusion protocol described by Oi and Herzenberg (16) and the spleen cell preparation method of Kennett (12) were followed. A total of 300 wells were seeded at 7×10^5 cells per well, with a B cell to myeloma ratio of 7:1. Supernatants were screened when the colonies were at least half-confluent. Positive colonies were expanded in 20% FBS, to which hypoxanthine and thymidine (HT) had been added. Half of this medium had been previously conditioned by myeloma growth in log phase for 2 days (half-strength conditioned medium). Cloning was by limiting dilution in either half-strength conditioned medium or 20% FBS + HT, which contained 30% myeloma conditioned and 20% macrophage-conditioned media (20). Ascites fluid was collected from pristane-primed mice injected intraperitoneally with 10^7 hybridoma cells in 0.5 ml of 20% FBS 12 days earlier.

Monoclonal antibody characterization. The supernatant from a colony cloned at one cell per well was precipitated three times in a 50% ammonium sulfate solution. The precipitate was dissolved to 16 times the original supernatant volume in saline and used in the

CIEIA described above. This fraction was also used with the subclass determination kit of Boehringer Mannheim Biochemicals (Indianapolis, IN) to determine the subclasses of the heavy and light chains of the antibody.

RESULTS

Immunization protocols. The results obtained when a dilution of the mouse sera was tested with CIEIA are summarized in Table 1. The mice injected by protocol A, a common immunization protocol, showed no activity against T-2 toxin, either in amount of antibody binding or in specific inhibition of antibody binding by free T-2. In contrast, strong specific binding was seen in the sera of mice 1 and 3 immunized by protocol B, because free T-2 at 1 μg/ml inhibited binding of antibody to levels occurring with uncoated wells. Serum from mouse 2 with protocol B showed nonspecific binding of antibody, because free T-2 could not inhibit antibody binding and wells coated with a conjugate that was not made with T-2 toxin had a similar amount of antibody binding (Table 1). At a dilution of 1/100, serum from mouse 3 of protocol B showed stronger T-2 antibody activity than mouse 1. The spleen from this mouse was therefore used to prepare hybridomas.

Hybridoma preparation. The fusion efficiency (number of wells with growing colonies per number of wells seeded) was greater than 95% (286/300). Supernatant from nine wells (3.1%) showed strong specific binding to the T-2HS-PLL solid phase. Other colonies showed an equivalent amount of binding, but this binding was not inhibited in the wells where free T-2 was present. The nine colonies showing specific binding were cloned at both one and five cells per well in half-strength conditioned media. None of these clonings yielded a colony with stable T-2 antibody activity and eight of these either lost activity before they could be frozen or were no longer active when they were thawed. One colony did retain activity on freezing and thawing, and this was successfully cloned (one positive clone) at one cell per well in 20% FBS + HT with 30% myeloma-conditioned and 20% macrophage-conditioned media. This positive clone was quite stable, and all three mice injected with this clone yielded ascites fluid that was active at a dilution of 1/5,000 after ammonium sulfate precipitation. When cloned a second time at one cell per well, more than 90% of the colonies that grew showed specific binding.

Antibody characterization. The anti T-2 monoclonal antibody was IgG$_1$ with kappa light chains. The cross-reactivity with other trichothecenes is summarized in Table 2. This antibody has less reactivity to HT-2 toxin relative to T-2 (2%) than other T-2 antibodies reported in the literature (2,9). A strong cross-reaction was found with the 3'OH metabolites of T-2 and HT-2 (about 100% and 20%, respectively) (Table 2). In contrast, rabbit polyclonal antibody prepared against T-2 had cross-reactivities with these

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TABLE 1. Antibody activity of diluted (1/400) mouse sera against T-2 toxin in a competitive indirect immunoassay (CIEIA)

| | | Absorbance (405nm) | | |
| | | T-2HS-PLL coat | | PLL coat[a] |
Protocol[b]	Mouse	No free T-2[c]	Free T-2[d]	...
A	1	0.17	0.17	0.17
	2	0.15	0.19	0.14
	3	0.12	0.12	0.14
B	1	0.47	0.21	0.23
	2	0.61	0.70	0.60
	3	0.44	0.23	0.19

[a] Wells coated with a similar conjugate to that prepared for the T-2HS-PLL conjugate, except no T-2HS used.
[b] Protocols: A = Intraperitoneal injections of 100 μg of T-2HS-BSA in 0.1 ml of saline and 0.1 ml of adjuvant at 1-mo intervals. B = Subcutaneous shoulder injections of 0.5–1.0 mg of T-2HS-BSA in 0.5 ml of saline at 2-wk intervals.
[c] 10% methanol in PBS (PBS-MEOH) added with equal volumes of diluted serum.
[d] 1 μg/ml of T-2 in PBS-MEOH with equal volumes of diluted serum.

TABLE 2. Sensitivity of T-2 reactive monoclonal antibody to various trichothecenes in competitive indirect enzyme immunoassay[a]

Trichothecene	Minimal inhibition[b]	50% inhibition[c]
T-2	0.01	0.023
T-2HS	0.01	0.072
Acetyl T-2	0.01	0.094
3'OH T-2	0.005	0.018
3'OH HT-2	0.05	0.100
HT-2	0.5	1.0
Neosolaniol	1.0	1.6
T-2 triol	5.0	14.9
T-2 tetraol	100.0	447.0
Verrucarol	500.0	>500.0
Deoxynivalanol	>1,000.0	>1,000.0
Roridin A	>5,000.0	>5,000.0

[a] All values in μg/ml of trichothecene in 10% methanol/PBS.
[b] μg/ml of trichothecene required for first significant inhibition of binding of antibody to the T-2HS-ovalbumin solid phase.
[c] μg/ml trichothecene required to inhibit binding of antibody by 50% to the T-2HS-ovalbumin solid phase. Calculated by regression analysis.

metabolites of about 2 and 1%, respectively (10). Other trichothecenes tested, with the exception of HT-2, cross-reacted comparably with rabbit polyclonal antibodies described (2,9). Sensitivity to T-2 was 10 ng/ml (0.5 ng per assay), which is more sensitive than the previously reported T-2 monoclonal antibody/CIEIA system described (11), but less sensitive than systems using rabbit antisera (2,5,7,9,19).

DISCUSSION

The immunization protocol used, in which large antigen doses were given subcutaneously without adjuvant, was clearly superior to the more traditional immunization protocol tested (Table 1). Sera from two of the three mice immunized with the former protocol showed titers at the dilution tested, whereas none of the mice immunized with the latter protocol did. The nonspecific binding shown by the serum of mouse B2 (Table 1) illustrates that antibody binding does not necessarily mean a specific reaction has taken place. As stated previously, when the hybridoma supernatant fractions were being tested, antibody binding would sometimes occur that could not be inhibited with free T-2 toxin. Therefore, we considered routine use of the competitive procedure crucial in avoiding false positives.

The major problem encountered was in maintaining a stable colony through cloning. Although the half-strength conditioned medium allowed very high cloning efficiencies when myeloma as well as these hybridoma lines were cloned (data not shown), a T-2 antibody-producing colony could not be obtained until macrophage-conditioned medium was used (20). Presumably, this hybridoma required growth factors present in the macrophage-conditioned medium but absent from the myeloma-conditioned medium.

Except for HT-2 and the 3'OH metabolites, this antibody had cross-reactivities similar to T-2 antibodies previously characterized (Table 2). These varying specificities could be useful in assaying for T-2 in biological systems. If samples were assayed with two antibodies of different specificities, the relative amounts of each trichothecene might be determined. This result also suggests that a series of monoclonals can be obtained, each with different specificities for many trichothecenes. This has been found with steroid haptens (6).

The strong cross-reactivities to the 3'OH metabolites of T-2 and HT-2 opens the possibility that this antibody may be used in assays for T-2 toxicosis, because these metabolites are considered diagnostic and are present in significant amounts when T-2 toxicosis occurs (21–23).

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