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Preparation and Characterization of Monoclonal Antibodies to the Trichothecene Mycotoxin T-2

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Two mouse immunoglobulin G1 monoclonal antibodies that bind to the trichothecene mycotoxin T-2 were prepared. These antibodies, designated 12C12 and 15H6, had affinities for T-2 of 3.5×10^6 and 5.8×10^7 liters/mol, respectively. A competitive inhibition enzyme immunoassay that employed these antibodies had a sensitivity for T-2 of 50 ng per assay. Both antibodies bound to the metabolite HT-2 but not to the related trichothecenes monoacetoxyscirpenol, diacetoxyscirpenol, deoxynivalenol, and deoxyverrucarol. Evidence is presented that T-2-protein conjugates inhibit protein synthesis in lymphoid cells and that this apparent immunotoxicity may be due to the release of T-2 from the protein carrier.

The trichothecene mycotoxin T-2 is a fungal metabolite known to contaminate stored grain and other agricultural products (22). This compound is a potent inhibitor of protein synthesis (1, 20) and has been implicated as the causative agent of several mycotoxicoses (12, 22). The lack of sensitivity and specificity of existing biological and physicochemical analysis methods for T-2 led Chu and co-workers to devise immunoassays for T-2 quantification (2, 17). Critical to the development of any chemical immunodetection system are antibodies to the target chemical. However, the titers of antisera to T-2 raised in rabbits have been uniformly low, although the antibodies in these antisera have been of sufficient affinity to allow adequate quantification (2, 7, 18). The low titers and inherent problems of standardization with antisera led us to prepare mouse monoclonal antibodies to T-2. In this paper, we offer a possible explanation for the low titers in anti-T-2 antisera and describe the preparation and characterization of two mouse monoclonal anti-T-2 antibodies.

MATERIALS AND METHODS

Chemicals. T-2, HT-2, diacetoxyscirpenol, monoacetoxyscirpenol, and deoxynivalenol were obtained from MycoLab Co., Chesterfield, Mo. Deoxyverrucarol was prepared and donated by Bruce Jarvis, University of Maryland.

Preparation of conjugates. T-2 was conjugated to bovine serum albumin (BSA), keyhole limpet hemocyanin, or goat immunoglobulin G (GIgG) by the method of Chu et al. (2). The epitope densities were between 10 and 20 mol of T-2 bound per mol of protein. All conjugates were dialyzed against at least 10 liters of phosphate buffer (pH 7.2) to remove unconjugated T-2.

EIA. Standard enzyme immunoassays (EIAs) and competitive inhibition EIAs (CIEIAs) were performed essentially as described previously (9, 10) except that microtiter plates were coated with T-2-BSA in 0.1 M Tris hydrochloride buffer (pH 8.2) and affinity-purified rabbit anti-mouse IgG1 was used (4). In the CIEIA, a final concentration of 5% methanol was present in every microwell, and all experiments were controlled for nonspecific binding of reagent antibodies. Logit-log plots of competitive inhibition data were generated with the computer program described by Rodbard and Lewald (19), and the molar concentration that inhibited 50% of antibody binding (IC_{50}) was calculated for each inhibitor compound. Minimal detectable concentrations of T-2 were calculated by the method of Miles (14).

Hybridoma preparation. A series of fusions were done over several months with various standard immunization regimens, but no hybridomas secreting antibodies to T-2 were found. Indeed, the fusion efficiency (number of wells with hybrids per number of wells seeded) was always less than 10% and often less than 1%. In an attempt to improve the fusion success rate, an immunological enhancement procedure that increases the frequency of antigen-reactive B lymphocytes was used. Details of the anti-IgD enhancement system have been published elsewhere (6). Briefly, T-2 is conjugated to GIgG by the method of Chu et al. (2), and female BALB/c mice are injected intravenously with 400 μg of exhaustively dialyzed T-2-GIgG concomitantly with 200 μg of goat anti-mouse IgD prepared by the method of Finkelman et al. (5). Sera from immunized mice were tested for anti-T-2 titers by EIA, and two mice with the highest titers were boosted on day 34 postimmunization with 100 μg of exhaustively dialyzed T-2-GIgG. Splenic lymphocytes were harvested 4 days later and fused as described previously (10). Hybridomas were screened by EIA and CIEIA; hybrids positive by both assays were cloned by limiting dilution, and the cloned hybridomas were adapted as ascites tumors in mice.

Affinity purification. Monoclonal anti-T-2 antibodies were precipitated from mouse ascites fluid by 50% $(NH_4)_2SO_4$, and the precipitate was dialyzed into 0.1 M phosphate buffer, pH 7.2. T-2-BSA was conjugated to CNBr-activated Sepharose 4b (Pharmacia Fine Chemicals, Piscataway, N.J.) by the instructions of the manufacturer. The washed immunoabsorbant was placed in a small column, and the immunoglobulin precipitate of the ascites fluid was passed slowly over the bed. Unbound antibody was removed by extensive washing, and the bound anti-T-2 antibody was

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TABLE 1. Inhibitory effect of T-2-BSA on protein synthesis by human B lymphoblastoid cells

Prepn added	Concn[a]	Protein synthesis (mean cpm ± SD)[b]
T-2	100	511 ± 49
	10	1,780 ± 366
	1	22,192 ± 1,230
	0.1	18,893 ± 3,515
T-2-BSA	50	178 ± 30
	5	439 ± 157
	0.5	2,689 ± 321
	0.05	9,372 ± 1,683
Dialyzed T-2-BSA[c]	50	294 ± 55
	5	1,295 ± 274
	0.5	11,818 ± 892
	0.05	20,224 ± 2,458
Medium only		29,184 ± 4,326

[a] T-2, Nanograms per milliliter; T-2-BSA and dialyzed T-2-BSA, micrograms per milliliter.

[b] Data represent the mean counts from incorporation of [^{3}H]leucine in triplicate cultures from one representative experiment.

[c] T-2-BSA (originally dialyzed against 10 liters of buffer after synthesis) was further dialyzed against 20 liters of phosphate-buffered saline, pH 7.4, immediately before testing.

eluted with glycine hydrochloride buffer, pH 2. The pH was quickly adjusted to pH 7.2 with NaOH, and then the affinity-purified anti-T-2 was dialyzed into phosphate buffer, pH 7.4. The protein content of the antibody was determined by the procedure of Lowry et al. (11). Although no attempt was made to optimize the purification procedure, 15 to 20 mg of affinity-purified antibody was routinely harvested from a column with the stated dimensions.

Immunochemical characterization. The affinity of anti-T-2 antibodies was measured by equilibrium dialysis as described by Eisen and Karush (3) with an equilibrium dialyzer (Spectrum Medical Industries Inc., New York, N.Y.) Tritiated T-2 (Amersham Corp., Arlington Heights, Ill.) was combined with unlabeled T-2 to obtain the desired specific activity (approximately 20 Ci/mol) and concentration. Binding data were plotted by the method of Sips (21) as modified by Nisonoff and Pressman (15). The isotype and subclass of the anti-T-2 monoclonal antibodies were determined by immunodiffusion against heavy-chain-specific rabbit anti-mouse antibodies purchased from Meloy Laboratories Inc., Springfield, Va., and Litton Bionetics, Kensington, Md.

Assays for protein synthesis. Protein synthesis was measured by the incorporation of [^{3}H]leucine (40 to 60 Ci/mmol, Amersham). The indicator cells were rapidly proliferating human B lymphoblastoid cells (USU-9-113-IIA7) cultured at densities of 10^5 cells per well in leucine-free RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μg of gentamicin per ml, and 10% fetal bovine serum (Hyclone; Sterile Systems Inc., Denver, Colo.). T-2 or T-2-BSA was added to freshly plated cultures of USU-9-113-IIA7 cells in flat-bottomed microtiter plates concomitantly with 0.5 μCi of [^{3}H]leucine per well. The cells were harvested on glass fiber filters after 18 h of culture at 37°C in 6% CO_2–94% air and washed thoroughly with distilled water; the filter plugs were then added to fluor and counted in a scintillation spectrometer. In some experiments, Marbrook chambers consisting of inner and outer chambers separated by a dialysis membrane (12 to 14 kilodalton exclusion) were used. The USU-9-113-IIA7 cells were cultured in the inner chambers on the dialysis membranes at densities of 3.0×10^5 cells per chamber under conditions identical to those described for microtiter plate cultures.

TABLE 2. Marbrook chamber analysis of protein synthesis inhibition by T-2-BSA

Prepn[a] added to:		Protein synthesis (cpm)[c]
Inner chamber[b]	Outer chamber	
T-2	T-2	6,151
T-2-BSA	T-2-BSA	6,297
None	T-2	7,936
None	T-2-BSA	7,033
None	None	179,066

[a] T-2, 5 ng/ml; T-2-BSA, 100 μg/ml; T-2-BSA was dialyzed against 20 liters of phosphate-buffered saline just before the experiment.

[b] The indicator cells (USU-9-113-IIA7) were cultured in the inner chamber on the surface of a dialysis membrane.

[c] Mean counts of [^{3}H]leucine incorporated over 18 h in one representative experiment.

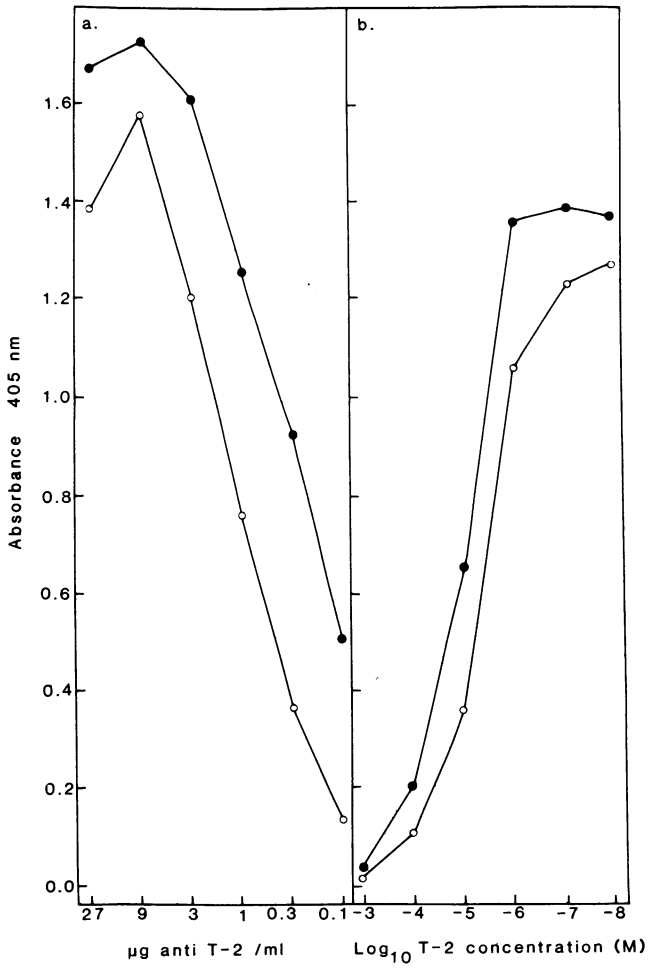
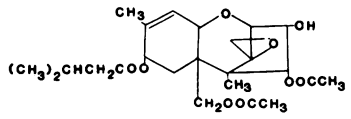
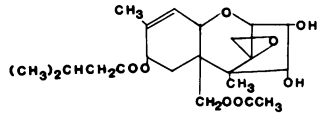
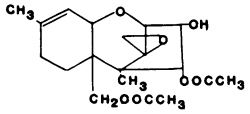
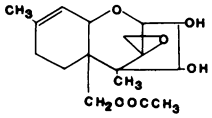
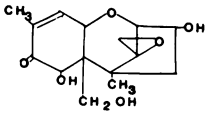
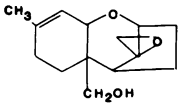


FIG. 1. (a) EIA titration curves for affinity-purified 12C12 (●) and 15H6 (○) on T-2-BSA-coated plates. Nonsaturation antibody concentrations chosen for use in the CIEIA were 2.5 and 5 μg/ml for 12C12 and 15H6, respectively. (b) CIEIA inhibition curves for 12C12 (●) and 15H6 (○) with various concentrations of T-2 as inhibitor.

TABLE 3. Specificity of monoclonal anti-T-2 antibodies

Compound name and structure	IC_{50} Inhibitor concentration X 10^{-6} molar	
	Antibody	
	12C12	15H6
T-2 Toxin 	3.59 ± .17	3.06 ± .56
HT-2 	11.59 ± .05	0.79 ± .20
Diacetoxyscirpenol 	0[a]	0
Monoacetoxyscirpenol 	0	0
Deoxynivalenol 	0	0
Deoxyverrucarol 	0	0

[a] $IC_{50} > 10^{-3}$ M.

RESULTS

Failure to produce viable hybridomas due to immunotoxicity of T-2-BSA. Since T-2 is a potent inhibitor of protein synthesis (1, 20), we examined whether the probable concentration of T-2-BSA present in the serum of the mice after a standard immunizing dose (50 to 100 μg/ml) was toxic to lymphoid cells. We chose to employ a human B lymphoblastoid cell line (USU-9-113-IIA7) to obviate the need for mitogen stimulation of mouse lymphoid cells. T-2 concentrations as low as 10 ng/ml caused profound inhibition of protein synthesis in USU-9-113-IIA7 cells (Table 1). Similarly, T-2-BSA demonstrated a dose-dependent inhibition of protein synthesis, and concentrations approximating those achieved in vivo nearly abrogated protein synthesis. When the T-2-BSA was tested immediately after dialysis against 24 liters of phosphate-buffered saline to remove residual free T-2, a substantial decrease in inhibition of protein synthesis was observed, but the dialyzed T-2-BSA still caused significant inhibition. These data indicated that residual T-2 was responsible for some inhibition of protein synthesis but suggested that the T-2-BSA conjugate might also be toxic.

We could not understand how T-2 conjugated to BSA could cross cell membranes and bind to peptidyl transferase on the ribosomes (20). An alternative explanation for the apparent toxic effect of T-2-BSA after exhaustive dialysis was release of free T-2 from the carrier BSA. To investigate this, we dialyzed a new batch of T-2-BSA against 24 liters of buffer and immediately tested its inhibitory potential in Marbrook chambers, a system for studying the effects of dialyzable materials on cells (13). The results (Table 2) demonstrate that some dialyzable (<12-kilodalton) substance associated with the T-2-BSA in the outer chamber crossed the membrane and inhibited protein synthesis by cells in the inner chamber. As the data also show that T-2 can readily cross the barrier, the most likely explanation is dissociation of toxic T-2 from the BSA either by nonspecific or enzyme-mediated hydrolysis of the hemisuccinate ester or amide linkage or by the release of noncovalently associated T-2.

Hybridoma preparation. In an attempt to overcome the apparent immunotoxicity of our immunogen, we employed a new immunomodulation method which has been shown to increase the clonal frequency of antigen-reactive B lymphocytes (6). This method requires that T-2 be conjugated to GIgG as the carrier protein. Hybridomas were prepared by the standard procedure from mice given this *immunization* regimen. Of 1,728 cultures seeded with fused cells, 57 (3.8%)

yielded viable hybridoma clones, but when the clones were screened by EIA and CIEIA, only 2 of the 57 cultures were positive for anti-T-2 antibody. These cultures, designated 12C12 and 15H6, proved to be stable and were cloned and adapted to ascites production in mice. Both clones produced in excess of 10 mg of specific anti-T-2 antibody per ml of ascites fluid.

Characterization of monoclonal anti-T-2 antibodies. Both 12C12 and 15H6 antibodies were purified from ascites fluid by affinity chromatography. Immunodiffusion in agar revealed that both antibodies were of the IgG1 heavy-chain subclass, and both had kappa-type light chains (data not shown). By standard equilibrium dialysis, 12C12 and 15H6 had affinities for T-2 of 3.5×10^6 and 5.8×10^7 liters/mol, respectively. Linear regression of Sips plot binding data (15) revealed correlation coefficients of 0.99 and 0.93 for 12C12 and 15H6, respectively, indicating significant homogeneity in binding sites (a presumptive indication of monoclonality).

Titration curves for 12C12 and 15H6 in EIAs against T-2-BSA are shown in Fig. 1a. Nonsaturating concentrations of antibodies for use in the quantitative CIEIA were 2.5 and 5 μg/ml for 12C12 and 15H6, respectively. Both antibodies yielded inhibition curves against T-2 that were linear over a 3-log range, and the sensitivity of the assay approached 10^{-6} M (Fig. 1b). It is interesting that the curve for 12C12 was shifted to the left, as would be predicted for a lower-affinity antibody, but this antibody appeared to bind better to T-2-BSA in the noncompetitive EIA (Fig. 1a). One possible explanation for this observation is that 12C12 has a higher affinity for T-2 conjugated to protein than 15H6, but lower affinity for free T-2.

To assess the specificity of the anti-T-2 monoclonal antibodies, several related trichothecene compounds were tested in the same CIEIA system (Table 3). Neither antibody reacted with diacetoxyscirpenol, monoacetoxyscirpenol, deoxynivalenol, or deoxyverrucarol at the highest concentration tested (10^{-3} M). Antibody 15H6 had a lower IC_{50} (indicating a higher affinity) for HT-2 than for T-2 and a lower IC_{50} for HT-2 than antibody 12C12. Both antibodies had similar IC_{50} values for T-2. Given the structural differences between these compounds, it is likely that the isovaleryoxy function common to T-2 and HT-2 confers the specificity to this antigen-antibody reaction.

DISCUSSION

In this paper, we have demonstrated that T-2-protein conjugates are immunotoxic and that this immunotoxicity is best explained by the release of T-2 from the protein carrier. The low titers of anti-T-2 antibodies in rabbits immunized with T-2-protein over many months (2, 7, 18) may have resulted from the continuous exposure of the animals to free T-2. As T-2 manifests a radiomimetic effect (23), it is understandable that the rapidly proliferating cells of the lymphoid system would be profoundly affected by this agent. It would seem probable that an alternative to the hemisuccinate linkage that would be more stable under physiological conditions would decrease or eliminate the immunotoxicity. Our own early failures to produce viable hybridomas were most likely due to the toxic effect of T-2. Our data also indicate that the toxic effect might be substantially reduced by dialyzing the T-2-BSA immunogen just before immunization.

We employed a novel immunomodulation scheme (6) to expand the frequency of occurrence of T-2-specific B lymphocytes, although the poor fusion efficiency and low yield of anti-T-2-secreting hybridomas suggest that the immunotoxicity problem was not eliminated with this method. Nevertheless, two hybridomas that secreted monoclonal anti-T-2 antibodies were identified, cloned, and adapted to ascites growth. Affinity purification of these antibodies yielded homogeneous populations of T-2-binding proteins that were incorporated into a CIEIA for T-2 detection. The specificity of the CIEIA with both 12C12 and 15H6 was somewhat different from that found with polyclonal antisera (2, 7, 18). Both monoclonal antibodies bound to T-2 and its primary metabolite, HT-2, with 15H6 actually binding better to HT-2. Neither 12C12 or 15H6 interacted with monoacetoxyscirpenol, diacetoxyscirpenol, deoxynivalenol, or deoxyverrucarol, which suggests that the isovaleryoxy function common to T-2 and HT-2 is the determinant of specificity for these antibodies. This is not unexpected, as the T-2 immunogen was conjugated through the C-3 position at the other end of the molecule, making the isovaleryoxy function the immunodominant structure. The affinities of the anti-T-2 antibodies were moderate, limiting the ultimate sensitivity of a quantitative immunoassay. In our hands, the CIEIA could reliably detect 50 ng per assay (12), somewhat more than the detection limit for anti-T-2 antisera (7, 8). Nevertheless, this level is sufficient for most analytical applications, and together with the unlimited production potential (>10 mg/ml in ascites), these monoclonal antibodies should be valuable analytical reagents.

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