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L. Patrick Hart

Major professor
Lynn Patrick Hart

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QUANTITATIVE CHARACTERISTICS OF RESISTANCE TO CORN EAR ROT CAUSED BY GIBBERELLA ZEAE (SCHW.) PETCH.

AND

IMMUNOCHEMISTRY OF T-2 TOXIN

By

Elie Hy Gendloff

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ABSTRACT

QUANTITATIVE CHARACTERISTICS OF RESISTANCE TO CORN EAR ROT
CAUSED BY GIBBERELLA ZEAE (SCHW.) PETCH.
AND
IMMUNOCHEMISTRY OF T-2 TOXIN

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Various generations of corn (Zea mays L.) derived from four inbred lines were inoculated over a three year period with Gibberella zeae (Schw.) Petch. and rated for disease severity. Analyses of the inbred and F1 generations revealed significant genetic and environmental effects. Generation means analysis indicated that additivity was the predominant genetic effect. Seven corn inbreds were also inoculated with one of eleven isolates of either G. zeae or Fusarium sporotrichioides. G. zeae was more virulent than F. sporotrichioides. Disease reaction of the inbreds followed similar rankings when inoculated with any of the isolates. These data suggest that stable resistance to Gibberella ear rot could be bred into hybrid corn lines.

Polylysine conjugates of three structurally unrelated mycotoxins were made via a mixed anhydride intermediate or an activated ester intermediate. Control conjugates, which included no mycotoxins, were also prepared. Two antisera elicited by mycotoxin-bovine serum albumin-mixed anhydride conjugates bound to all four polylysine-mixed anhydride conjugates but bound only to the polylysine-activated ester conjugates when homologous mycotoxin was used. Conjugation of an unwanted immune-reactive epitope onto polypeptides by the mixed anhydride procedure was hypothesised to account for these data.

A competitive enzyme-linked immunosorbent assay (direct ELISA) was used to screen for T-2 toxin (T-2) in Fusarium sporotrichioides-infected corn. The assay detected T-2 at concentrations of 0.05 ng/ml in extracts of corn samples. In infected corn samples, direct ELISA and gas-liquid chromatography estimations of T-2 concentrations were similar.

A polyclonal antibody was produced against T-2 by immunizing rabbits with a mixed anhydride conjugate of T-2HS and bovine serum albumin (T-2HS-BSA). The antibody was used to detect T-2 at 0.05 ng/ml by direct ELISA and 1 ng/ml by an indirect ELISA. Cross-reactivity of this antibody with trichothecenes other than T-2 was similar to previously described polyclonal antibodies. A monoclonal antibody against T-2 was produced using a T-2HS-BSA that was conjugated using a carbodiimide reagent. Mice were succesfully immunized using an unconventional immunization protocol involving large antigen doses without adjuvant. The monoclonal antibody was characterized by indirect ELISA. Sensitivity to T-2 was 10 ng/ml (0.5 pg/assay). The antibody cross-reacted less to HT-2 than previously described T-2 antibodies.

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TABLE OF CONTENTS

PART IV
PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES
AGAINST T-2 TOXIN

APPENDICES

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

The fungus Gibberella zeae (Schw.) Petch. (asexual state, Fusarium graminearum Schwabe) causes stalk rot, ear rot, and seedling blight in corn (Zea mays L.) in the United States (10,11) and other parts of the world (17,18). The most important aspect of the ear rot disease caused by this fungus is the production of mycotoxins, most commonly the trichothecene deoxynivalanol and the estrogenic lactone zearalanone (25). This dissertation reports research directed toward reducing the occurrence of these mycotoxins in the food and feed chain. Two approaches were taken. The first involved a genetic study of the components of resistance to ear mold in corn. The second involved immunochemical methods for detecting trichothecenes, using T-2 toxin as a model.

There are only scattered reports in the literature concerning resistance to corn ear rot caused by various Fusarium species. Genetic and environmental variation of resistance to G. zeae in segregating generations of corn inoculated over several years has not been reported previously. Data on the variation in virulence of various G. zeae isolates was also lacking. Studies in these areas are reported in Part I.

Many immunochemical methods have been developed for the detection of mycotoxins, particularly the aflatoxins (4,6,8,9,12,13,14,20,21,23), ochratoxin A (1,2,19,24), and T-2 toxin (3,5,7,15,16,22). Immunological

detection can be sensitive, specific, and rapid. Parts II, III, and IV of this dissertation describe work involving various aspects of the immunochemistry of mycotoxins, particularly T-2 toxin. Part II describes a cross-reaction that occurs among antisera produced by immunization with mycotoxin-protein conjugates produced by the same conjugation procedure. Part III describes immunological methods developed to detect T-2 toxin in corn infected by various isolates of Fusarium sporotrichioides. New methods for the production and characterization of polyclonal and monoclonal antibodies against T-2 toxin are described in Part IV. An appendix is included which describes factors influencing the effectiveness of an enzyme immunoassay for ochratoxin A and antibodies against ochratoxin A. The development of this assay was the result of studies described in Parts II and IV. A second appendix summarizes protocols used in the indirect and direct ELISA used in parts II, III, and IV. The third appendix gives data used to calculate the cross-reactivities of a monoclonal antibody with various trichothecenes.

A pertinent literature review is given at the beginning of each section.

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PART I

COMPONENTS OF RESISTANCE TO *GIBBERELLA ZEAE* IN FIELD CORN

ABSTRACT

Crosses involving various generations derived from two susceptible and two resistant inbred corn lines were inoculated with Gibberella zeae U5373 over a three year period. Analysis of variance of disease reaction in inbred and F1 generations revealed differences among lines and blocks, and a year x line interaction. Generation means analysis involving inbred, F1, F2, F3, backcross, and selfed backcross generations implicated additivity (lack of dominance) as the predominant genetic effect. A maternal influence was apparent in one set of reciprocal crosses. Seven inbred lines also were inoculated with seven G. zeae and four Fusarium sporotrichioides isolates in two blocks. G. zeae was generally more virulent than F. sporotrichioides. Inbred x isolate interactions were observed. Disease reaction of these inbred lines followed similar rankings regardless of the pathogen isolate tested.

INTRODUCTION

Gibberella ear rot of corn, caused by Gibberella zeae (Schwabe) Petch (anamorph=Fusarium graminearum) is sometimes epidemic in the midwestern United States (20). The disease is a cause for concern, even when no significant yield loss occurs, because the causal fungus often produces deoxynivalanol, a cytotoxic trichothecene (21), and zearalenone, an estrogenic lactone (18). Other Fusarium species, such as F. moniliforme, F. moniliforme var. subglutinans, F. culmorum (14), and F. sporotrichioides (=F. tricinctum) (8,14) cause similar ear rots, with or without the presence of mycotoxins.

Some work has been done in determining the nature of resistance to ear rots caused by various Fusarium species, but variation in experimental methods and pathogen species tested have limited the amount of useful information obtained. Early studies involved F. moniliforme isolates. Boling and Grogan (3) used generation means analysis of a susceptible x resistant cross and found differences between years in the significance of various genetic effects. There was also evidence of epistasis. Other workers found significant maternal effects using diallel analysis of resistance to F. moniliforme seedling blight (15). Variation in resistance to one or many Fusarium species among popular hybrids has also been found (2,6,16). Differences among isolates and isolate x hybrid interactions were apparent (2), but no large rank

reversals among these interactions were evident. Host morphological components have also been implicated as factors influencing resistance (6,13).

Recent work has focused on G. zeae, primarily because of concern about mycotoxins. Inbred and F1 analyses have been used to sort out significant genetic components. Cullen et al (5) found more resistance among F1's than among the inbreds from which they were derived.

Working along these lines, large differences in resistance were found among 58 inbreds (9). Diallel analysis among the 10 most resistant and susceptible inbreds revealed significant general combining ability but not specific combining ability effects. This work carries that analysis further, with a generation means analysis of some of those crosses. Environmental variation among inbred and F1 generations over three years also was analyzed. Lastly, isolates of G. zeae and F. sporotrichioides were compared for virulence on seven inbreds.

Generation means analysis. Generation means analysis is a method of determining significance of various genetic effects using the means of several generations. Hayman (8,9) developed this method to its current state by expanding the digenic epistatic theoretical models of Anderson and Kempthorne (1). Hayman (8), using data previously generated with wheat, tomato, and tobacco, also showed that digenic epistatic effects, or the interaction of two nonallelic genes, can be significant. Gamble (7) used this method with corn to determine that epistatic effects were important componants in corn yield. Boling and Grogan (3) also used this method with corn to show that resistance to Fusarium moniliforme ear rot involved additivity (a), dominance (d), and a x d epistasis.

Anderson and Kempthorne (1) and Hayman (8,9) showed that the expectations of the means of two inbred lines and their decendents can be listed (using the terminology of Gamble [7]) as follows:

P_1	$= m + a$	$- 1/2d$	$+ aa - ad$	$+ 1/4dd$
P_2	$= m - a$	$- 1/2d$	$+ aa + ad$	$+ 1/4dd$
F_1	$= m$	$+ 1/2d$		$+ 1/4dd$
F_2 $(=SF_1)$	$= m$			
P_1F_1 $(=BC_1)$	$= m + 1/2a$		$+ 1/4aa$	
P_2F_1 $(=BC_2)$	$= m - 1/2a$		$+ 1/4aa$	
F_3 $(=SF_2)$	$= m$	$- 1/4d$		$+ 1/16dd$
$S(P_1F_1)$	$= m + 1/2a$	$- 1/4d$	$+ 1/4aa - 1/4ad$	$+ 1/16dd$
$S(P_2F_1)$	$= m - 1/2a$	$- 1/4d$	$+ 1/4aa + 1/4ad$	$+ 1/16dd.$

Note that the mean (m) is a statistical midpoint, defined as the F_2 generation mean. The other generations are therefore defined in terms of the F_2 generation. Estimates of these genetic effects can be derived from the means of the generations tested by solving the equations listed above for each effect. For example, if means were obtained for the first six generations listed above, the various effects are estimated as (7):

Genetic effect	Means estimating the effect
m	F_2
a	$P_1F_1-P_2F_1$
d	$-1/2P_1-1/2P_2+F_1-4F_2+2P_1F_1+2P_2F_2$
aa	$-4F_2+2P_1F_1+2P_2F_1$
ad	$-1/2P_1+1/2P_2+P_1F_1-P_2F_1$
dd	$P_1+P_2+2F_1+4F_2-4P_1F_1-4P_2F_1$

The significance of each effect is tested by a 2-tailed t test,

where the variance for each effect is the variance of each generation summed as above. For example, the variance of aa would be $16(\text{var}.F_2) + 4(\text{var}.P_1F_1) + 4(\text{var}.P_2F_1)$.

MATERIALS AND METHODS

Inoculations were made as previously described (9), using the toothpick method modified from Young (22). Only the uppermost ear of each plant was inoculated. Disease ratings were taken after the first killing frost, as previously described (9). Husks were removed and disease was rated by amount of mycelium visible on the ear. The ratings were: 0 = no disease present, 0.1 = a few kernels around the inoculation point infected, 1 = 10% or less of the ear infected, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, and 5 = 75-100% of the ear infected.

For the genetic studies, various crosses of the inbreds B79, B73Ht, A509, and Pa347 were inoculated in each of three years (1982-1984). The first two inbreds (B79 and B73Ht) were consistently susceptible and the last two (A509 and Pa347) were consistently resistant in previous work (9). A randomized block design with three blocks was used. Each plot was a single row of one cross, and 30-70 plants in each row were inoculated with G. zeae, isolate W8 (Penn state # U5373,=F. graminearum R6576). Inbreds and all F1 combinations were inoculated in all three years. The only reciprocals included were those of A509 x B73Ht. In 1983 and 1984, F1 backcrosses (F1P1 and F1P2) and F2s were also included. In 1984, selfed F1 backcrosses (F1P1S1 and F1P2S1) and F3s were included when available. Significance of additive (a), dominance (d), and the three digenic epistatic (aa, ad, and dd)

effects were determined by the method of Hayman (8,9). This method is summarized in the Introduction of this section. Significance of interactions between each of the six genetic effects and the two years (ym, ya, yd, yaa, yad, ydd) also was determined (19).

Analysis of variance (19) of the inbred and F1 generations was used to determine environmental effects. The analysis included the reciprocal generation described above. Orthogonal comparisons (19) were sometimes made.

Seven inbreds were used to determine variability of the reaction to various isolates of G. zeae and F. sporotrichioides. Three (B79, B73Ht, and Mo17Ht) were susceptible and four (A509, Ms74, Pa347, and ND100) were resistant to isolate U5373 in previous work (9). Each inbred was inoculated by the toothpick method with each of eleven pathogen isolates. A split plot design (19) was used, with each plot containing one inbred inoculated with each isolate. Two blocks were used, and each block contained one row of 20-40 plants per inbred-isolate combination. Seven isolates (U5373, U5372, U5371, M3, S1, SA2, and VWA1) were G. zeae. The first four were from the culture collection of L. P. Hart; S1 was obtained from R. Stuckey, University of Kentucky; and SA2 and VWA1 were from D. Cullen, University of Wisconsin. The remaining four isolates (T-340, F27, NRRL3299, and F38) were F. sporotrichioides (= F. tricintum [8]) . T-340 was obtained from E. B. Smalley, University of Wisconsin; NRRL3299 and F38 were from C. J. Mirocha, University of Minnesota; and F27 was from the culture collection of L. P. Hart.

RESULTS

Variation in G. zeae resistance. Analyses of variance of disease ratings of the inbred and F1 generations for individual years and for the three years combined are shown in Table 1. There were significant differences among these lines, implying genetic differences. The significant block effects in 1983 and 1984 suggested that location within a field also affects disease severity. Differences in overall disease severity from year-to-year was not significant (Table 1d), although there were significant year x line interactions (Table 1d).

The mean of the disease ratings used in the previous analysis (Table 1) is shown in Table 2. Although some year-to-year differences occured within some lines, the rankings were similar from year to year. The disease severity of every F1 fell between its two parents. There were no differences between the reciprocals of the cross B73Ht x A509 (Table 2); therefore no maternal effect was evident among the F1 generation of this cross.

Generation means analysis. Table 3 shows the mean and variance of the disease rating for each generation and cross used in the generation means analysis. There was a large variance for some inbred and F1 generations, which are genetically homogeneous determinations. Table 4 summarizes the results of the generation means analysis. The major genetic effect in the susceptible x resistant crosses was the additive

Table 1. Analyses of variance for disease rating of Gibberella zeae ear rot on various corn lines. Only inbred and F1 generations are included. See text for lines used. NS = not significant at the 5% level of probability. ** = significant at the 1% level of probability.

a. 1982

Source	DF	MS	F	Significance
Blocks	2	.568	1.3	NS
Lines	10	5.133	11.9	**
Error	20	.432		
Within plots	1550	.035		

b. 1983

Source	DF	MS	F	Significance
Blocks	2	4.804	12.9	**
Lines	10	4.916	13.2	**
Error	19	.373		
Within plots	673	.104		

c. 1984

Source	DF	MS	F	Significance
Blocks	2	4.337	7.8	**
Lines	10	3.094	5.5	**
Error	20	.559		
Within plots	1961	.038		

d. All three years

Source	DF	MS	F	Significance
Years	2	6.839	2.3	NS
Blocks in Years	6	2.982	6.4	**
Lines	10	8.944	4.1	**
Year x Line	20	2.199	4.7	**
Error	59	.467		

Table 2. Mean disease ratings[1] for inbred and F1 generations of corn inoculated in the developing ear with Gibberella zeae U5373. The lines are ranked from most resistant to most susceptible over all three years. See text for rating system. Means followed by the same letter within a column are not significantly different at the 5% level by Duncan's multiple range test (17).

Line	All three years	1982	1983	1984
Pa347	.33a	.53ab	.23a	.33a
A509 x Pa347	.67ab	.13a	.40a	1.47ab
A509	1.47abc	.53ab	1.17ab	2.73 bc
Pa347 x B73Ht	1.57abc	.93ab	1.83 bcd	2.03 bc
B79 x Pa347	1.70abcd	.80ab	2.37 cd	1.94 bc
B73Ht(F) x A509	1.90 bcd	1.70 bc	1.80 bcd	2.17 bc
A509(F) x B73Ht	2.13 bcde	1.27abc	2.07 bcd	3.00 cd
A509 x B79	2.77 cde	1.57 bc	3.70 ef	3.03 cd
B73Ht	2.87 cde	2.17 c	2.77 de	3.23 cd
B79 x B73Ht	3.17 de	2.33 c	4.47 f	2.70 bc
B79	3.60 e	4.87 d	1.50 bc	4.17 d

[1]Disease ratings- 0 = no disease present; 0.1 = a few infected kernels around the inoculation point; 1 = 10% or less of the ear infected; 2 = 11-25%; 3 = 26-50%; 4 = 51-75%; and 5 = 75-100% of the ear infected.

Table 3. Means and variances of disease ratings[1] for various generations of corn crosses. In each year, three replications (rows) of 30-70 plants per row were inoculated in the ear with a toothpick infested with Gibberella zeae U5373, as described in the text. Plants were rated after the first frost.

		B79xPa347		A509xB79		B73(F)xA509[2]		B73(M)xA509		B73xPa347		B79xB73		A509xPa347	
Gen.[3]	Year	Mean	Var	Mean	Var	Mean	Var	Mean	Var	Mean	Var	Mean	Var	Mean	Var
P1	83	1.64	3.11	1.01	2.65	3.50	2.66	3.50	2.66	3.50	2.66	1.64	3.11	1.01	2.65
	84	4.07	2.73	2.75	3.19	3.17	4.00	-	-	3.17	4.00	4.07	2.73	2.79	3.19
P2	83	0.21	0.19	1.64	3.11	1.01	2.65	1.01	2.65	0.21	0.19	3.50	2.66	0.21	0.19
	84	0.31	0.35	4.07	2.73	2.79	3.19	-	-	0.31	0.35	3.17	4.00	0.31	0.35
F1	83	2.31	3.28	3.63	2.07	2.17	3.53	1.20	3.38	1.81	2.54	4.43	1.09	0.44	1.18
	84	1.93	3.46	3.05	2.71	2.57	2.42	-	-	2.14	4.02	2.74	3.89	1.47	1.95
F2	83	1.65	2.71	2.07	3.64	2.18	3.49	2.22	3.50	1.59	3.19	4.27	1.40	0.50	0.83
	84	1.48	2.44	1.76	3.35	1.25	2.80	-	-	1.21	2.38	3.24	3.90	1.83	2.76
P1F1	83	3.40	2.93	2.14	3.19	2.41	3.89	2.82	2.46	2.97	2.50	4.48	1.45	0.54	1.27
	84	3.21	2.94	2.18	3.70	2.85	1.80	-	-	-	-	3.66	3.54	1.66	3.23
P2F1	83	0.55	0.99	3.86	2.35	1.03	2.41	1.05	2.53	0.49	0.75	4.37	1.52	0.37	0.66
	84	1.63	3.18	3.25	2.55	2.77	3.54	-	-	1.38	2.51	2.97	3.31	1.53	2.59
F3	(84)	1.82	2.68	3.08	3.63	2.05	3.03	-	-	0.97	1.84	3.17	4.82	2.25	2.56
P1F1S1	(84)	2.98	3.21	2.68	3.32	2.53	2.89	-	-	2.22	4.86	3.57	3.65	1.46	2.46
P2F1S1	(84)	0.84	2.21	3.06	3.47	2.90	3.53	-	-	0.76	0.90	3.12	4.19	2.09	2.46

[1]Disease ratings- 0 = no disease present, 0.1 = a few kernels around the inoculation point infected, 1 = 10% or less of the ear infected, 2 = 11-25%, 3 = 26-50%, 4 = 51-75%, and 5 = 75-100% of the ear infected.

[2] In 1984, this included both reciprocals B73(F)xA509 and B73(M)xA509.

[3]P1= first parent listed. P2= second parent listed. P1F1= F1 back crossed to P1. P2F1= F1 backcrossed to P2. P1F1S1= P1F1 selfed. P2F1S1= P2F1 selfed.

Table 4. Significant genetic effects determined by generation means analysis. Means and variances listed on Table 3 were analyzed for six genetic effects by the method of Hayman (8,9). These effects were mean (m), additive (a), dominant (d), and the three epistatic effects (aa, ad, dd). Differences between the two years (y) and the interaction between years and genetic effects (ya, yd, etc.) were also analysed. The reciprocal crosses of B73Ht x A509 were analysed separately in 1983 but together in 1984; therefore no determination of year effects were made for these crosses.

Cross	Significant Effects[1]
B79 x Pa347	m a
A509 x B79	m a
B73Ht(M) x A509 (83)	m a d
B73Ht(F) x A509 (83)	m a aa ad
B73Ht x A509 (84)	m aa ad
B73Ht x Pa347	m a
B79 x B73Ht	m y ya
A509 x Pa347	m

[1]Significant at 5% level using Student's t test.

effect. No genetic effects were indicated in the two crosses of inbreds with the same disease reaction (B79 x B73Ht- both susceptible, and A509 x Pa347- both resistant). There were interactions between years and genetic effects (Table 4) in only one of the seven crosses: the cross involving the two susceptible inbreds. Genetic differences were found between the two reciprocal crosses of B73Ht x A509. This indicates a possible maternal effect in disease reaction to G. zeae in generations later than the F1, since there were no differences between the F1 reciprocals of this cross (Table 2).

An attempt was made to fit a relatively simple Mendelian gene model using the ratings obtained for the various crosses involving the most susceptible (B79) and most resistant (Pa347) inbreds. The ratings of the F1 generation of that cross, as a percentage of the total, were: 0 and 0.1 - 39.8%; 1 - 16.9%; 2 - 8.7%; 3 - 13.9%; 4 - 11.5%; 5 - 7.9%. Since all plants in the F1 generation are genetically identical, this variability must be due to environmental factors. I concluded, therefore, that this large environmental influence made it impossible to fit this data to a simple gene model.

Inbred x isolate interactions. Partitioning of the variation among inbred lines showed that the significant differences were between the resistant and susceptible groups and among the susceptible lines (Table 5). B79 was more susceptible than Mo17Ht and B73Ht when ratings were averaged over all isolates (Table 6), but there were no differences among the resistant inbreds (Table 5). Similarly, there were significant differences between isolates of G. zeae and F. sporotrichioides (Table 5), with the former the more virulent (Table 6). There were also

Table 5. Analysis of variance for corn inbred x isolate effects in 1984. Seven inbreds were inoculated with each of eleven pathogen isolates. Three inbreds were susceptible (=S) and four resistant (=R) to pathogen isolate U5373. Seven isolates were G. zeae (=Z) and four F. sporotrichioides (=F). Each inbred-isolate combination was represented as one row in each of two blocks. NS=not significant at the 5% level. *=significant at the 5% level. **=significant at the 1% level.

Source	DF	MS	F	Significance
Block	1	0.03	0.0	NS
Inbred	6	19.80	38.6	**
R vs S	1	82.77	162.3	**
Among R	3	1.11	2.2	NS
Among S	2	15.52	30.4	**
Error a	6	0.51		
Isolate	10	12.77	39.2	**
Z vs F	1	93.66	283.8	**
Among Z	6	6.05	18.3	**
Among F	3	0.03	0.1	NS
Inbred x Isolate	60	0.79	2.4	**
(R vs. S)x(Z vs. F)	1	6.67	20.2	**
(R vs. S)x(among Z)	6	3.25	9.8	**
(R vs. S)x(among F)	3	0.17	0.5	NS
(Z vs. F)x(among S)	2	0.95	2.9	NS
(among Z)x(among S)	12	0.55	1.7	NS
(among F)x(among S)	6	0.10	0.3	NS
(Z vs. F)x(among R)	3	1.22	3.7	*
(among Z)x(among R)	18	0.47	1.4	NS
(among F)x(among R)	9	0.02	0.1	NS
Error b	70	0.34		
Within plot	3804	0.16		

Table 6. Average disease ratings of corn inbred x isolate inoculations. a. Each combination ranked by disease rating. Means of inbreds or isolates followed by the same letter are not different by Duncan's multiple range test. b. Grouped by reaction on U5373 and fungal species.

a.

	Inbred							
	Susceptible				Resistant			
Isolate	B79	Mo17Ht	B73Ht	Ms74	ND100	A509	Pa347	Mean
G. zeae								
U5372	4.40	3.55	3.45	2.95	2.10	1.90	1.40	3.06a
S1	4.20	3.75	3.65	2.45	2.30	1.75	1.00	2.79a
U5373	4.75	4.05	4.55	1.25	0.60	0.55	0.25	2.42a
SA2	4.50	2.60	3.05	1.65	1.45	1.75	1.45	2.41a
VWA1	4.05	2.00	2.40	2.60	2.50	1.95	0.60	2.39a
U5371	3.65	3.55	2.70	2.45	0.75	2.40	1.70	2.18a
M3	3.00	0.30	0.80	0.40	0.45	0.40	0.55	1.05b
F. sporotrichioides								
F38	2.30	0.25	0.55	0.00	0.40	0.25	0.25	1.08b
T-340	2.85	0.80	0.45	0.00	0.20	0.30	0.30	0.79b
NRRL3299	2.50	0.85	0.35	0.10	0.35	0.20	0.30	0.78b
F27	2.10	0.45	0.50	0.20	0.50	0.20	0.25	0.62b
Mean	3.52a	2.07b	2.02b	1.33bc	1.07bc	1.04bc	0.71c	1.67

b.

	Reaction		
	Susceptible	Resistant	Mean
G. zeae	3.28	1.48	2.26a
F. sporotrichioides	1.16	0.24	0.63b
Mean	2.51a	1.03b	1.67

differences in virulence among G. zeae isolates (M3 was significantly less virulent), but not among F. sporotrichioides isolates. Although there were inbred x isolate interactions (Table 5), these did not involve major rank reversals; i.e. an inbred which was resistant to one isolate was never susceptible to another (Table 6). Therefore, the inbred rankings determined with isolate U5373 were similar to other isolates.

The inbred x isolate interaction involved differences among inbreds in the variation of their reactions to different isolates. For example, a large interaction was evident when comparing the difference in reaction of resistant lines to susceptible lines inoculated with G. zeae with the reaction of these lines inoculated with F. sporotrichioides (Table 5). An interaction was also evident when disease reaction of resistant lines were compared to susceptible lines when inoculated with the G. zeae isolates (Table 5). The only other significant inbred x isolate interaction occured when the virulence of the two species among the resistant inbreds was compared (Table 5). Among the resistant inbreds, therefore, the G. zeae isolates were more virulent than the F. sporotrichioides isolates (Table 6).

DISCUSSION

These data generally support the contention that environmental influences have an important effect on the reaction of corn to G. zeae ear rot (13). The differences between blocks in 1983 and again in 1984 as well as the year x line interaction among F1 and inbred generations (Table 1) clearly implicate an environmental effect. The large error variances in Table 1 also indicate a substantial variation within rows. Other indications of an environmental influence are the large variances among inbred and F1 generations (Table 3) and the inability to fit the cross B79xPa347 to a Mendelian model.

In spite of the environmental influence, significant genetic differences were consistent from year to year among the inbred and F1 generations (Table 2). As previously noted, all F1s analysed were rated intermediate to the inbred parents in disease reaction. This lack of overdominance (since the hybrid disease ratings were not outside the range defined by the inbreds) is contrary to the findings of Cullen et al (5), although they used different lines.

The generation means analysis indicated additivity was the predominant genetic effect, occuring in five out of the eight analyses conducted (Table 4). Other effects (aside from the mean effect) were sometimes significant, but less often then was additivity. This implies that most segregating genes for resistance exhibited little dominance or

digenic epistasis (interaction between two loci). One of the potential deficiencies or cautions of generation means analysis is an inability to identify when opposing effects cancel each other (10-12). Therefore, dominant genes for resistance and susceptibility could occur within a particular cross and would not be evident in the analysis due to their opposing effect. This possibility is minimized, however, when each inbred is analysed in more than one cross (12), as was done here. The mean, which is the statistical midpoint defined as the mean of the F2 generation, was significant in all cases. This means that the mean F1 rating is different from zero. There were no significant genetic effects between the two crosses of inbreds with the same disease reaction (B79 x B73Ht and A509 x Pa347), suggesting few genetic differences between these comparably rated inbred lines, since the environmental differences were greater than the segregating genetic differences. The predominance of additivity in resistance to G. zeae should make incorporation of resistance into agronomically useful corn lines relatively easy, since genes for a susceptible or resistant disease reaction would not be masked by other dominant or epistatic alleles (12).

Interesting in this analysis were the differences in significant genetic effects between the two reciprocals of the cross B73Ht x A509 (Table 4). As noted above, there were no differences between the two F1 reciprocals (Table 2). Therefore the genetic differences must have been evident in later generations, even though the differences between various crosses involving these reciprocals were generally not great (Tables 2 and 3).

The study of inbred x isolate effects (Table 5 and 6) clearly

showed that F. sporotrichioides (=F. tricinctum) isolates were less virulent than those of G. zeae. This has been reported previously (14). However, F. sporotrichioides was capable of causing significant disease on the highly susceptible inbred B79 (Table 6).

There were no major rank reversals in susceptibility of inbreds among different Fusarium isolates tested (Table 6). Since there were no major rank reversals among G. zeae or F. sporotrichioides isolates, it is probable that these two species have similar methods of causing disease, with G. zeae simply having more effective virulence genes. If these two species had different methods of causing disease, inbred rankings with G. zeae would likely be different than inbred rankings with F. sporotrichioides, which did not happen. Since each inbred was ranked similarly with each isolate, resistance incorporated into an inbred is likely to be stable (4).

In spite of the environmental influence, the large differences in resistance among inbreds and their crosses were readily evident. The large additivity component as well as the apparent "stable" reaction among the lines tested indicate that long lasting resistance to G. zeae could be easily incorporated into agronomically useful lines.

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PART II

MYCOTOXIN-PROTEIN CONJUGATES PREPARED BY THE MIXEDANHYDRIDE METHOD: CROSS-REACTIVE ANTIBODIES IN HETEROLOGOUS ANTISERA

ABSTRACT

Polylysine conjugates of three structurally unrelated mycotoxins were made by a mixed anhydride intermediate or an activated ester intermediate. Control conjugates, in which no mycotoxin was involved, were also prepared by each method. Two antisera, made by T-2-toxin- and aflatoxin B1-bovine serum albumin-mixed anhydride conjugates, bound to all mycotoxin- and the one control-polylysine-mixed anhydride conjugates, but bound only to the polylysine-activated ester conjugates produced using the same (homologous) mycotoxin that was used to produce the antibody. Binding of antisera to their homologous polylysine conjugates was always inhibited by free hapten when activated ester-mycotoxin conjugates where used to coat immunoplates, but not when mixed anhydride-mycotoxin conjugates were used. The origin of this cross-reactivity was hypothesised to be due to the conjugation of an unwanted immune-reactive epitope onto polypeptides by the mixed anhydride procedure, but this could not be proven.

INTRODUCTION

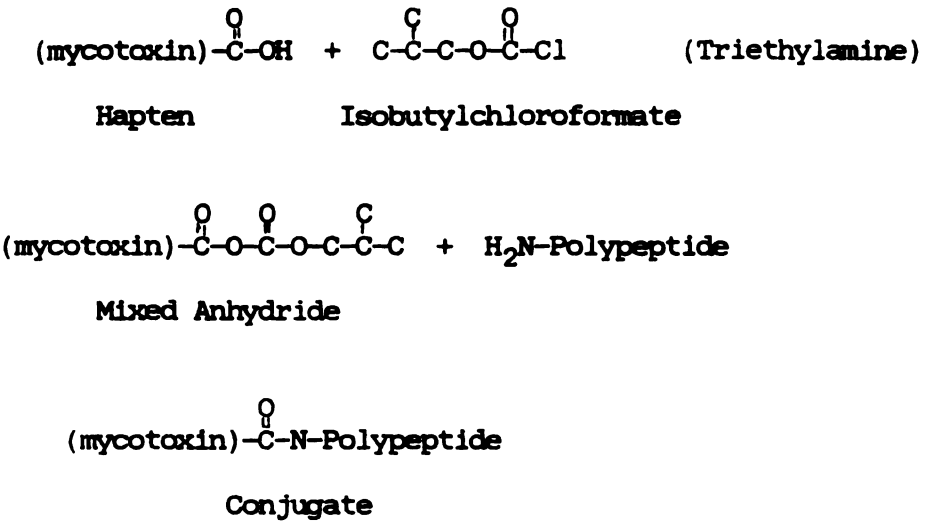
One of the goals of my immunochemical studies was to develop methods for the production of monoclonal antibodies to T-2 toxin (T-2) and other mycotoxins. Mycotoxins are low molecular weight compounds (haptens) which do not by themselves elicit an antibody response when injected into an animal. In order to elicit this response, they require conjugation to a larger immunogenic molecule, such as bovine serum albumin (BSA). Some of the antibodies elicited against this conjugate will be against the mycotoxin. To isolate hybridomas which produced antibodies to these toxins, a rapid, sensitive, and reliable assay for detection of these antibodies was required. Therefore, I investigated various published procedures to conjugate mycotoxins to poly-L-lysine (polylysine) for the development of an indirect enzyme immunoassay (ELISA) for mycotoxin-specific antibody detection. In the indirect ELISA the mycotoxin is immobilized on a solid phase, usually by conjugating the mycotoxin to a compound like polylysine, which readily sticks to plastic solid phases (immunoplates). As part of these investigations, I found that binding occured between polylysine-mycotoxin conjugates and antisera elicited by different (heterologous) protein-mycotoxin conjugates when both were prepared by a popular conjugation method involving a mixed anhydride intermediate. These cross reactions did not occur when conjugates were prepared via an activated ester intermediate.

The mixed anhydride procedure, originally developed for peptide preparation (19,20), has been extensively used in immunochemistry for conjugation of a variety of carboxyl-containing haptens to proteins, either for antigen preparation (5,10) or for enzyme labeling for enzyme immunoassay (3,11,15). This procedure has proved useful for preparing conjugates with a high ratio of hapten to protein (13).

The reaction proceeds in two steps (Figure 1A). First, the hapten containing a carboxyl group is conjugated to an alkylchlorocarbonate, usually isobutylchloroformate (=isobutylchlorocarbonate), in the presence of a tri-n-alkylamine, usually triethylamine or tributylamine, at low temperature and anhydrous conditions. This results in the formation of a mixed anhydride. The mixed anhydride is then added to a polypeptide solution where the hapten reacts with free amino groups, usually lysine side chains, linking the hapten to the polypeptide via an amide bond (4).

The activated ester method (12) is an alternative method for conjugating carboxyl-containing haptens. This method is illustrated in Figure 1B. The hapten is first conjugated ("activated") to N-hydroxysuccinimide with dicyclohexylcarbodiimide. This activated ester is then added to the polypeptide and a mycotoxin-polypeptide amide bond is formed which is equivalent to that formed by the mixed anhydride reaction (Figure 1A and 1B).

A



B

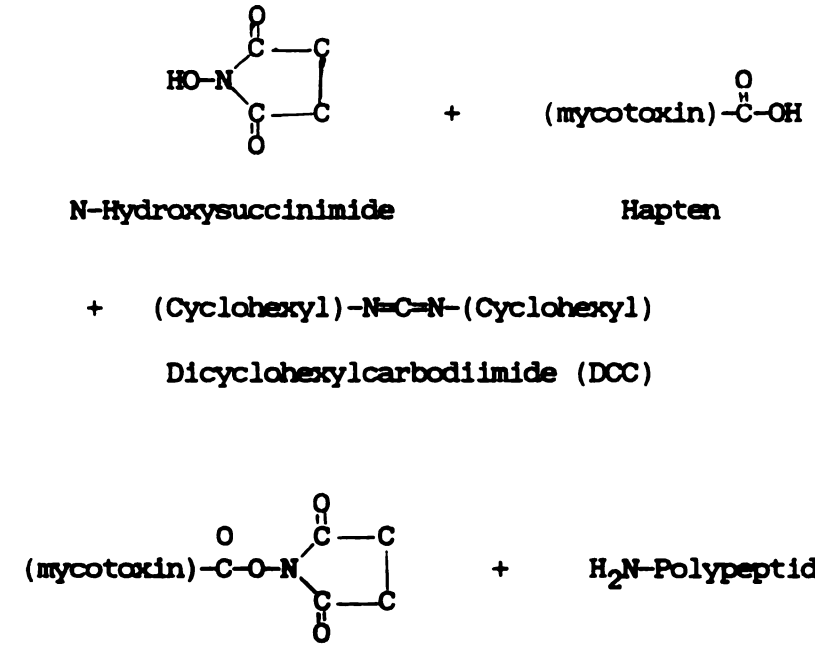


Figure 1. Mycotoxin-polypeptide conjugation procedures. A. Mixed anhydride. B. Activated ester.

MATERIALS AND METHODS

Materials. All inorganic chemicals and organic solvents were reagent grade or better. BSA (fatty acid free and fraction V), polyoxyethylenesorbitan monolaurate (Tween 20), 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) (ABTS), polylysine (M.W. 22,000), ochratoxin A (ochratoxin; crytallized from benzene), ovalbumin (crude), dicyclohexylcarbodiimide, and N-hydroxysuccinimide were obtained from Sigma Chemical Co., St. Louis, MO; Freund's adjuvants from Difco, Detroit, MI; isobutylchloroformate and triethylamine from Aldrich Chemical Co., Milwaukee, WI; goat antirabbit IgG conjugated to horseradish peroxidase (antirabbit-peroxidase) from Cooper Biomedical, Malvern, PA; immunoassay microtiter plates (immunoplates) from Nunc Intermed, Roskilde, Denmark; filter paper from Whatman, Inc., Clifton, NJ; T-2 toxin (T-2) from MycoLab Co., Chesterfield, MO; Adsorbosil (200/425 mesh) from the Anspec Co., Ann Arbor, MI; Silica gel-G thin layer chromatography (TLC) plates (Redi-plates) from Fisher Scientific Co., Pittsburgh, PA; antisera reactive against ochratoxin was provided by F. S. Chu, University of Wisconsin; polylysine conjugated to aflatoxin B1-oxime (polylysine-aflatoxin) by both the activated ester and mixed anhydride methods were provided by B. P. Ram, as was both mixed anhydride- and activated ester-produced antisera reactive to aflatoxin B1. Isobutylhemisuccinate was synthesized and conjugated to

polylysine by W. L. Casale.

Preparation of conjugates. A carboxyl group was introduced to T-2 by adding a hemisuccinate group to carbon C-3 (T2HS) (21). Conjugations of T-2HS to polylysine or BSA by the mixed anhydride method were carried out by the method of Lau, et al (13). Briefly, 10 mg of dried mycotoxin or carboxyl-containing derivative was dissolved in 5 ml tetrahydrofuran and cooled to -5 C. To this, 5 ul triethylamine and 5 ul isobutylchloroformate were added. After 20 min at -5 C, the mixture was slowly added to a stirring solution of 25 mg polypeptide (BSA or polylysine) in 15ml water plus 7.5 ml pyridine at 4 C. This mixture was stirred for 30 min at 4 C, then overnight at room temperature. Dialysis for 3 days against frequent changes (four liters per day) of distilled water followed.

Conjugations by the activated ester method were by a modification of the method of Kitagawa, et al. (12). Mycotoxin or carboxyl-containing derivative (1 mg) was mixed with an equimolar amount of both dicyclohexylcarbodiimide and N-hydroxysuccinimide in 0.1 ml dry tetrahydrofuran, then stirred 30- 60 min at room temperature. Precipitate was filtered using Whatman #1 paper, and then washed with 2-3 ml tetrahydrofuran. The tetrahydrofuran was evaporated and the residue dissolved in 0.2 ml dimethylformamide. Dropwise addition of the dissolved residue to 5 mg polypeptide (BSA or polylysine) dissolved in 0.5 ml of 0.13 M sodium bicarbonate followed. This mixture was slowly stirred 30 min then dialyzed for 3 days against four liters per day of 0.1 M sodium bicarbonate.

Control conjugates of polylysine prepared without mycotoxin were

made by both the mixed anhydride and activated ester methods to investigate the effects of these procedures on the polypeptide when used in the indirect ELISA described below.

Immunization protocols. T2HS-BSA conjugated by the mixed anhydride method was used as an immunogen. Initial immunization was by a modification of the multiple site method of Vaitukaitis et al (17). Here, 0.5 mg conjugate in 0.5 ml of 0.9% saline was emulsified with 1.5ml Freund's complete adjuvant. The preparation was injected intradermally into 30-40 sites on the shaved back of a New Zealand white doe rabbit. Subsequent injections were made at six week intervals using Freund's incomplete adjuvant emulsified in the same ratios and concentrations as described above, but at one-half the volume. Rabbits were bled through the marginal ear vein and sera purified by three ammonium sulfate (35% saturated) precipitations (8).

Indirect ELISA. This method is summarized as a flow chart in Appendix B, under indirect ELISA. Polylysine-mycotoxin conjugate (200ul), diluted to 5 ug/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6, was placed in each well of 96-well immunoplates. In some cases, polylysine or a polylysine control conjugate at the same concentration was substituted. The plates were incubated overnight at 4 C. They were then washed two times with sodium phosphate buffered saline (PBS- 0.1 M; pH 7.5) containing 0.05% Tween 20 as previously described (7), except a twelve-channel aspirator was used. After washing, 200 ul PBS containing 1% (wt/vol) ovalbumin (PBS-ovalbumin) was added to each well after insoluble matter in the preparation was removed by a low speed centrifugation. After incubation for 30 min at 37 C, the plates were

washed twice as above. Next, 25 ul PBS-ovalbumin was added to each well, followed by 50 ul purified antisera which had been adjusted to 100 ug/ml by the spectrophotometric method of Hurn and Chantler (9). Washing (four times) followed a 1 h incubation at 37 C. In some cases, a competitive procedure was used by adding 50 ul unconjugated mycotoxin at various dilutions prior to antibody addition. Next, 50 ul antirabbit-peroxidase (100 ul in the competitive procedure), diluted 1/2000 in PBS + 1% BSA (wt/vol) + 0.1% Tween 20 was added, followed by a 30 min incubation at 37C. After washing eight times, bound peroxidase was assayed by incubating 100 ul ABTS-H_2O_2 substrate in each well for 5-10 min; the reaction was terminated with 100ul stopping solution (14). Absorbance at 405 nm was determined on an EIA reader EL307 (Bio-Tech, Inc., Burlington, VT). There were three replications of each treatment.

RESULTS AND DISCUSSION

Characteristics of the indirect ELISA. Free T-2 inhibited binding of the anti-T-2 sera (produced using a mixed anhydride-conjugated immunogen) when an activated ester-T2HS-polylysine conjugate, but not when a mixed anhydride-T2HS-polylysine conjugate, was bound to the immunoplates (Figure 2), even though the T-2 antisera strongly binds to the mixed anhydride-T2HS-polylysine conjugate (Table 1). The regression analysis equation was $y = .52 - 0.0688 \log(x)$, where $y = A_{405}$ absorbance readings and x = concentration of free T-2, in ng/ml. The r^2 was 0.94. A similar phenomenon occured with aflatoxin, where free aflatoxin strongly inhibited binding of both mixed anhydride- and activated ester-produced aflatoxin antisera to the activated ester-polylysine-aflatoxin conjugate bound to the immunoplates. Free aflatoxin also strongly inhibited binding of the activated ester-aflatoxin antisera to the mixed anhydride-polylysine-aflatoxin bound to the immunoplates. However, free aflatoxin only weakly inhibited binding of the mixed anhydride-aflatoxin antisera to mixed anhydride-polylysine-aflatoxin bound to immunoplates (B. P. Ram, personal communication). This suggested the presence of another epitope on the mixed anhydride-polylysine conjugates that only mixed anhydride-produced antisera can bind.

Free ochratoxin inhibited binding of the ochratoxin antisera to the activated ester-ochratoxin-polylysine conjugate (Figure 2). The

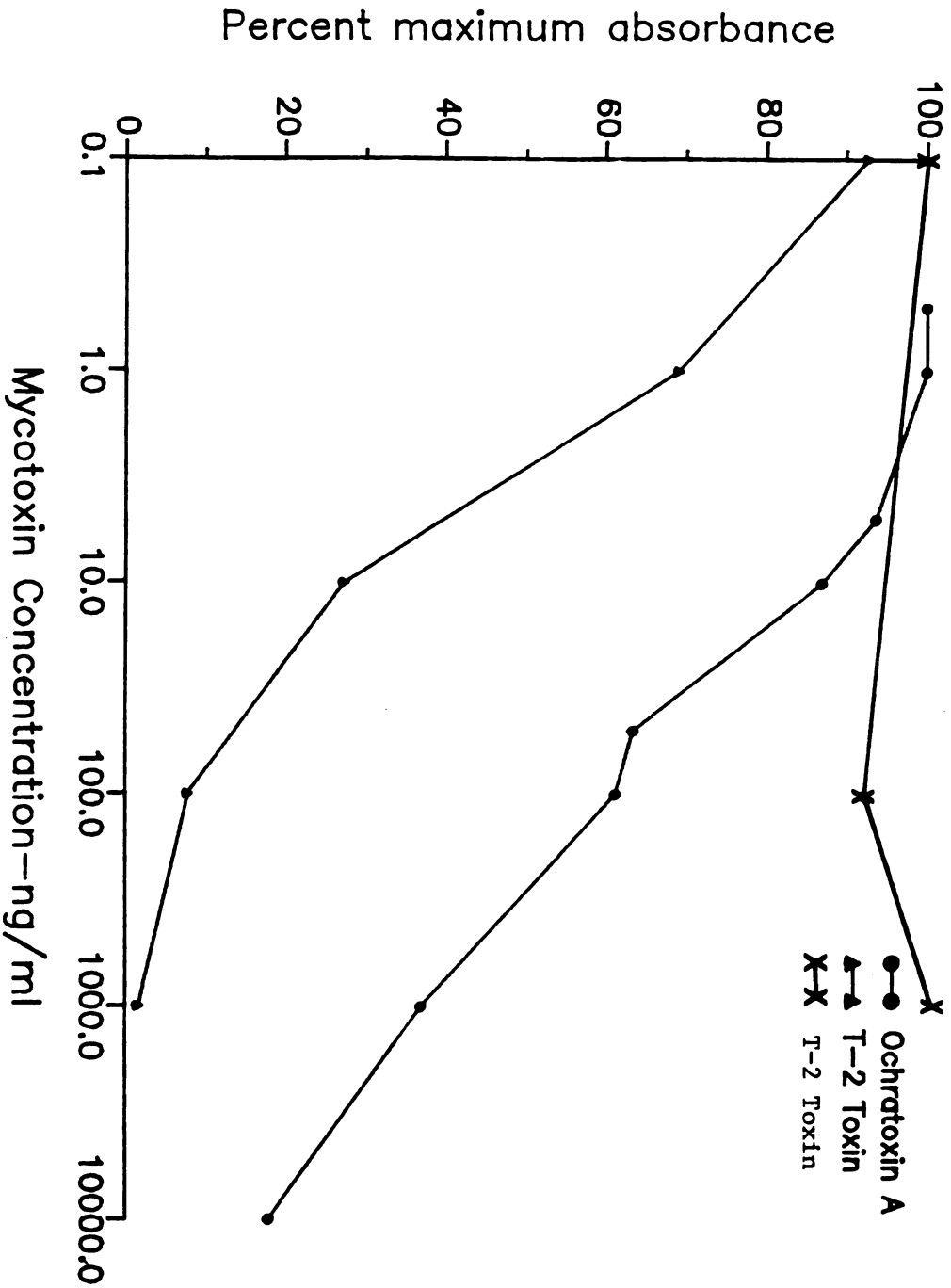
Table 1. Cross-reactivities of mixed anhydride (MA) and activated ester (AE) coating conjugates in indirect ELISA. The coating conjugate was bound to microtiter immunoplates, then treated with mycotoxin antibody. Bound antibody was determined with antirabbit-peroxidase followed by peroxidase substrate. All values are A405. Each value represents the mean of three replications. Standard deviations were always less than 0.1AU. The polylysine-aflatoxin assays were conducted separately from the others. N= preimmune rabbit serum.

Polylysine coating conjugate	Mycotoxin antibody	Method used to make coating conjugate: Mixed anhydride	Activated ester
T-2	T-2 (MA)	1.54	0.59
	Aflatoxin (MA)	1.36	0.09
	Aflatoxin (AE)	0.30	0.07
	N	0.03	0.02
Aflatoxin	T-2 (MA)	0.93	0.15
	Aflatoxin (MA)	0.93	0.56
	Aflatoxin (AE)	0.40	0.85
	N	0.01	0.01
Ochratoxin	T-2 (MA)	1.56	0.19
	Aflatoxin (MA)	1.40	0.07
	Aflatoxin (AE)	0.14	0.07
	N	0.02	0.02
Control conjugate (no mycotoxin)	T-2 (MA)	1.58	0.21
	Aflatoxin (MA)	1.37	0.21
	Aflatoxin (AE)	0.19	0.11
	N	0.04	0.03
Polylysine (no conjugation)	T-2 (MA)	0.14	
	Aflatoxin (MA)	0.08	
	Aflatoxin (HS)	0.10	
	N	0.05	

regression analysis equation was y = 0.278 - 0.021 log (x), where y = A_{405}, and x = concentration of free ochratoxin, in ng/ml. The r^2 was 0.73. Among all competitive indirect ELISAs described, structurally unrelated mycotoxins had no inhibitory effect on the binding of a specific antisera to its respective polylysine activated ester-conjugated mycotoxin. Since the activated ester conjugation method was useful in producing effective and specific polylysine coating conjugates with three unrelated mycotoxins (ochratoxin, aflatoxin, and T-2) (16) it appears to be an effective general method for conjugations of this type.

Cross reactivities of various conjugate-antisera combinations. All mixed anhydride-produced polylysine conjugates (including the control conjugates, which did not have mycotoxin attached) bound both the T-2- and aflatoxin-mixed anhydride-produced antisera (Table 1). In contrast, the activated ester-produced polylysine conjugates bound only antisera reactive to the homologous mycotoxin, regardless of the conjugation procedure used in producing the antisera. As noted above, the extensive antibody binding found in the mixed anhydride-polylysine/mixed anhydride antisera combinations was inhibited poorly or not at all when free homologous mycotoxin was added, in contrast to the strong competitive inhibition found with activated ester-polylysine/homologous antisera combinations (Figure 2). All these data were consistant with the hypothesis that mixed anhydride-specific cross-reactions resulted from an immunoreactive epitope being introduced onto polypeptides during the mixed anhydride procedure. Thus, antisera produced from a mixed anhydride-conjugated mycotoxin would contain antibodies that bound to this epitope on mixed anhydride-conjugated polylysine conjugates, and

Figure 2. Competitive indirect ELISA for Ochratoxin A (circles) and T-2 toxin (triangles and x's). Each point represents the mean of three replications. The coating conjugate was bound to microtiter immunoplates, then treated with free mycotoxin plus mycotoxin antibody. Bound antibody was determined with antirabbit peroxidase followed by peroxidase substrate. The assays employed polylysine-mycotoxin conjugates which were conjugated by the activated ester method (circles and triangles) or the mixed anhydride method (x's).

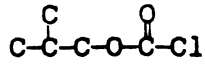

Percent maximum absorbance
0
20
40
60
80
100
0.1
1.0
10.0
100.0
1000.0
10000.0
Mycotoxin Concentration—ng/ml
Ochratoxin A
T-2 Toxin
T-2 Toxin

free homologous mycotoxin would not be expected to compete for antibodies specific to this epitope.

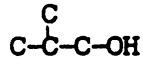
Attempts to determine the nature of mixed anhydride cross-reactivity. I hypothesized that the cross-reactivities described above were due to the conjugation of isobutylformate, the "other" side of the mixed anhydride (Figure 1A), to the polypeptide when the mixed anhydride was added to the polypeptide. This would give R'OCON-Polypeptide rather than RCON-Polypeptide, where R' is $(CH_3)_2CHCH_2$ and R is the desired hapten. If this R' epitope was immunoreactive, then antibodies raised against that would be available for reaction to the same epitope on the polylysine-mycotoxin coating conjugate when produced by the mixed anhydride method. This side reaction does occur when the mixed anhydride reaction is used in dipeptide synthesis (1). This hypothesis was tested by adding either free isobutylchloroformate (Figure 3A) or free isobutanol (Figure 3B) as a hapten in the competitive indirect ELISA as described earlier. Neither compound at 10 ug/ml inhibited binding of any mixed anhydride-produced antibodies to any mixed anhydride-produced polylysine conjugates. However, isobutylchloroformate may not have been an effective inhibitor because of possible nonspecific covalent binding with the coating conjugate, antibody, or ovalbumin blocking protein.

To further test the above hypothesis, W. L. Casale conjugated isobutylhemisuccinate to polylysine by the activated ester method. Isobutylhemisuccinate (Figure 3C) was selected because it has the same isobutylformate moiety as isobutylchloroformate as well as a carboxyl group for conjugation via the activated ester method. If isobutylformate was present in mixed anhydride-produced conjugates then antibodies made

A



B



C

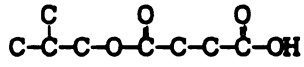


Figure 3. Isobutyl- compounds tested for reactivity to mixed anhydride-produced antibodies. A. Isobutylchloroformate. B. Isobutanol. C. Isobutylhemisuccinate.

by a mixed anhydride procedure should react to this isobutylhemisuccinate-polylysine. However, none of the antisera elicited against mixed anhydride-mycotoxin-BSA reacted with isobutylhemisuccinate-polylysine, nor did free isobutylhemisuccinate at 10ug/ml inhibit binding of either of the mixed anhydride-produced antibodies to the polylysine-mixed anhydride-control conjugate. There is, therefore, no evidence for an isobutylformate moiety present on mixed anhydride conjugates. Other explanations for the unusual cross-reactivities described here, such as a polypeptide alteration peculiar to the mixed anhydride procedure, should be examined.

To my knowledge, this is the first report of an apparent epitope associated with a hapten-polypeptide conjugation procedure. Due to the cross-reactivity described herein, I recommend that the mixed anhydride procedure not be used for both immunogen preparation and antibody detection. The mixed anhydride procedure has been succesfully used in each aspect of a system, however, both in indirect ELISA for aflatoxin, as indicated here, and in direct ELISA (18).

The problems encountered with the mixed anhydride method would not arise in immunoassays where this conjugation procedure is used only for immunogen preparation, such as radioimmunoassay. However, in light of the results reported here, I recommend that where the same conjugation procedure is used in immunogen preparation and antibody detection, proper controls ensuring detection of a similar phenomenon should be employed.

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PART III

DETECTION OF T-2 TOXIN IN *FUSARIUM SPOROTRICHIOIDES*-INFECTED CORN BY ENZYME-LINKED IMMUNOSORBENT ASSAY

ABSTRACT

A competitive enzyme-linked immunosorbent assay was used to screen for T-2 toxin in Fusarium sporotrichioides-infected corn. The assay detected T-2 toxin in diluted methanol extracts of corn samples at concentrations of 0.05 ng/ml. In infected corn samples, enzyme-linked immunosorbent assay and gas-liquid chromatography estimations of T-2 toxin concentrations were similar.

INTRODUCTION

Analytical methods developed to detect T-2 toxin (T-2) include biological assays, thin-layer chromatography, high-pressure liquid chromatography, gas-liquid chromatography (GLC), and gas chromatography-mass spectroscopy (6). These methods lack sensitivity and/or specificity, or are laborious and require expensive equipment, and are therefore inadequate as rapid screening assays. The development of a radioimmunoassay (2) and an enzyme linked immunosorbent assay (direct ELISA) (8) for T-2 has shown that immunological assays may be useful alternatives for detection of this toxin. In order to further establish the usefulness of immunological methods for detection of T-2 toxin, a comparison was made of an ELISA screening method for T-2 after a simple extraction of corn infected with T-2-producing strains of Fusarium sporotrichioides with a GLC analysis of the same corn after an extensive cleanup.

MATERIALS AND METHODS

Antisera reactive against T-2 was provided by J. J. Pestka, Michigan State University, and was purified by precipitation with ammonium sulfate (4) by means of a 35% saturated ammonium sulfate mixture. T-2 (MycoLab Co., Chesterfield, Mo.) was converted to T-2-hemisuccinate (T-2HS) by the method of Chu et al. (2). T-2HS was conjugated to horseradish peroxidase by means of a modification of the method of Pestka et al. (8). In this variation, 150 ug of T-2HS dissolved in 1.0 ml of ethanol-3.0 ml of water was mixed with 150 mg of 1-ethyl-3-(dimethylaminopropyl) carbodiimide (Sigma) and 3.0 mg of type VI horseradish peroxidase (Sigma) in 1.0 ml of 25% aqueous ethanol. The mixture was stirred for 30 min at room temperature, and then an additional 150 mg of carbodiimide was added. This mixture was stirred for 20 h at 4 C and then dialyzed against three changes of 0.01 M sodium phosphate buffer (pH 7.5) for 3 days. The T-2HS-horseradish peroxidase conjugate (0.3 mg/ml) was stored frozen in one ml aliquots. As needed, the aliquots were thawed and distributed into single-use aliquots (30 ul), and these were frozen. The 1:20 ratio of T-2HS:horseradish peroxidase used in preparation of the conjugate resulted in less nonspecific binding of the conjugate in the ELISA than did the 3:10 ratio used by Pestka et al (8).

Antisera titration and T-2 quantitation by direct ELISA were modified from the method of Pestka et al (8). Appendix B summarizes this

direct ELISA protocol in a flow diagram. Falcon 3070 polystyrene microtissue culture plates (Becton Dickinson and Co., Oxnard, Calif.) were prepared by air drying 50 ul of 0.2% (vol/vol) fraction V (Sigma) BSA solution (0.2 mg/ml of water) in each well. The wells were then reacted with 50 ul of 0.2% (vol/vol) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.5) for 30 min, washed extensively with distilled water, air dried, and stored under desiccation. Purified antisera were diluted fifty-fold in PBS, and 50 ul aliquots were air dried in each well under a warm air current (ca. 40 C) and then stored under desiccation. When used, the antisera coated plates were first washed three times by filling each well with 0.2 ml of 0.05% (vol/vol) Tween 20 in PBS (PBS-Tween 20) and aspirating the contents with a single-well aspirator under a vacuum of 580 mm Hg. Nonspecific binding was decreased by incubating each well for 1 h at 37 C with 0.2 ml of 1% (wt/vol) BSA in PBS, followed by two more washes with PBS-Tween 20.

Antisera were titrated by diluting the T-2HS-horseradish peroxidase conjugate 1:300 with 5% (wt/vol) BSA and 0.1% (vol/vol) Tween 20 in PBS (PBS-BSA-Tween 20) and adding 50 ul aliquots to wells previously treated with serial dilutions of antisera or preimmune sera. Incorporation of Tween 20 with the conjugate allowed less nonspecific conjugate binding than did the method of Pestka et al (8). In the competitive direct ELISA, this step was performed by simultaneously incubating 25 ul of T-2 standard or extracts of infected corn diluted in 10% methanol in PBS with 25 ul of the T-2HS-horseradish peroxidase conjugate diluted 1:150 in PBS-BSA-Tween 20. In both assays, incubation at 37 C for 1 h followed. The plates were then washed six times with PBS-Tween 20 as

described above. Bound horseradish peroxidase per well was assayed (7), and absorbance at 410 nm was determined with a Microelisa Mini Reader MR590 (Dynatech Laboratories, Inc., Alexandria, VA).

Since other trichothecenes might be present in samples of F. sporotrichioides-infected corn, various standards were tested by competitive direct ELISA to determine cross reactivity in the T-2 ELISA. The trichothecenes tested were HT-2 toxin, verrucarol, diacetoxyscirpenol, and roridin A, all from Sigma Chemical Co., deoxynivalanol from MycoLab, and acetyl T-2, neosolaniol, T-2 triol, and T-2 tetraol, prepared as previously described (1,11). The structures of these trichothecenes are shown in Figure 1. The effectiveness of direct ELISA in detecting T-2 in F. sporotrichioides-infected corn was tested. Ears of corn (inbred B79) were inoculated at the milk stage of development by inserting toothpicks infested with a single strain of F. sporotrichioides through the husks into the center of the ear (3). F. sporotrichioides strains used were NRRL 3299, F27, T-2, F38, and T-340. Strains T-2 and T-340 were obtained from E. B. Smalley and R. W. Caldwell (Department of Plant Pathology, University of Wisconsin); strains NRRL 3299 and F38 were obtained from C. J. Mirocha (Department of Plant Pathology, University of Minnesota); and strain F27 was from the culture collection of L. P. Hart.

For determinations by direct ELISA, 25 g of infected kernels were extracted by blending for 5 min with 250 ml of methanol-water (60:40). The solids were removed by filtration through no. 4 filter paper (Whatman, Inc., Clifton, N.J.), and the extracts were diluted to various degrees (up to 4 X 10^6 times) with 10% methanol in PBS.

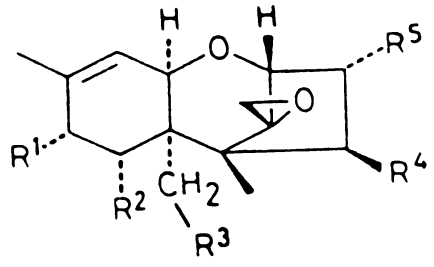
The detection of T-2 in corn by direct ELISA was compared with detection by GLC for nine different samples of corn inoculated in the field with various isolates of F. sporotrichioides. Visibly molded kernels were removed from infected ears and dried at 65°C for 2 days, followed by dry chopping for 45 s in a blender sat at low speed. For sample 1 (Table 2), the entire cob, which was extensively molded, was dry chopped for 90 s. Each sample was divided into two subsamples, and direct ELISA determination of T-2 concentration was made on one subsample after dilution of the methanol-water extract as described above. The other subsample was subjected to GLC with electron capture determination of toxin concentration by S. P. Swanson, University of Illinois, by the method of Scott et al (9). Thin-layer chromatography by the method of Takitani et al. (10) was used by S. P. Swanson to confirm the presence of the various trichothecenes tested.

RESULTS AND DISCUSSION

The reactivities of the trichothecenes in the direct ELISA relative to T-2, as indicated by the concentration required for 50% inhibition of conjugate binding, is summarized in Table 1. Both T-2HS and T-2 inhibited conjugate binding at 50 pg/ml. Only trichothecenes containing the isovaleroxy moity at the R^1 position (Figure 1) inhibited conjugate binding at a concentration comparable to that of T-2. As an illustration of this point, neosolaniol, similar in structure except for this moiety, inhibited conjugate binding at only 0.1% of the level of T-2. However, as indicated by the low affinity of T-2 triol for the T-2 antibody (Table 1), not all analogs with the isovaleroxy moiety are cross reactive. The minor reaction of trichothecenes without the C-8 isovaleroxy moiety would be insignificant in assays employing this direct ELISA method. The macrocyclic trichothecene roridin A was the only trichothecene tested that did not inhibit conjugate binding at 500 ug/ml. Similar cross-reactivities of various trichothecenes for T-2 antibody have been found in T-2 radioimmunoassay (2) (Table 1), suggesting that no significant differences in specificity occur when radiolabeled or horseradish peroxidase-labeled T-2 is used as an immunoassay ligand.

Concentration determinations of T-2 in 60% methanol-extracted, noninfected corn by direct ELISA revealed that the extract could be diluted minimally (sixfold) with PBS without causing significant

Figure 1. Structures of trichothecenes tested.



Name	R^1	R^2	R^3	R^4	R^5
T-2	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	OH
T-2HS	$OCOCH_2CH(CH_3)_2$	HO	$COCH_3$	$OCOCH_3$	$OCOCH_2CH_2COOH$
Acetyl T-2	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
HT-2	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	OH	OH
3'OH T-2*	$OCOCH_2COH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	OH
3'OH HT-2*	$OCOCH_2COH(CH_3)_2$	H	$OCOCH_3$	OH	OH
T-2 triol	$OCOCH_2CH(CH_3)_2$	H	OH	OH	OH
Neosolaniol	OH	H	$OCOCH_3$	$OCOCH_3$	OH
T-2 tetraol	OH	H	OH	OH	OH
Verrucarol	H	H	OH	OH	H
Diacetoxyscerpenol	H	H	$OCOCH_3$	$OCOCH_3$	OH
Deoxynivalanol	=O	OH	OH	H	OH
Roridin A	H	H	'a 14 C	'diester	H

*These compounds are studied in Section IV

Table 1. Specificity and sensitivity of T-2 antibody in ELISA for T-2 and other trichothecenes

Trichothecene	% Cross reactivity relative to T-2[a]	Minimum sensitivity (ng/ml)[b]
T-2	100 (100)[c]	0.05
T-2HS	100 (ND)	0.05
Acetyl T-2	100 (ND)	0.05
HT-2	3.4 (17.5)	0.1
Neosolaniol	0.1 (0.2)	100
T-2 triol	0.1 (2.1)	50
T-2 tetraol	<0.1 (0.07)	500
Deoxynivalanol	<0.001 (<0.01)	10,000
Verrucarol	0.003 (ND)	5,000
Diacetoxyscirpenol	0.001 (<0.01)	10,000
Roridin A	<0.001 (ND)	>100,000

[a] Nanograms of T-2 required for 50% inhibition/nanograms of trichothecene required for 50% inhibition X 100.

[b] Nanograms of trichothecene per milliliter required for first significant inhibition ($P = 0.05$ by Student's t test on four replications per ELISA) of peroxidase conjugate binding.

[c] Numbers in parentheses indicate percentage of cross-reactivity relative to T-2 determined for T-2 radioimmunoassay by Chu et al. (2). ND, Not determined.

interference of peroxidase conjugate bindir
the methanol was evaporated from the extra
extract to 10% (vol/vol), interference wit
occured. Therefore, preparations for dire
not include methanol evaporation. Prel
sporotrichioides F38-infected and T-2-spil
indicated that T-2 could be detected by di
by methanol extraction with recoveries of
spiked samples had equivalent concentra

The results obtained when direct ELI
the same samples are shown in Table 2. A
direct ELISA versus GLC for T-2 concentr
0.01 (df = 1,7;F = 13.95). The regression e
where X = T-2 concentration as determir
coefficient (r) was 0.816.

For sample 3 (Table 2), T-2 was not d
10.9 ppm was detected by GLC. This dispa
sampling problems. Further samples from the s
for reanalysis. It is also possible th
substances that interfere with the inter
during direct ELISA, or cause a peak at th
heptafluorobutyrate during GLC analysis.

This is the first report of T-2 dete
immunological methods. The results present
could be used as a semiquantitative tool f
sporotrichioides-infected corn. Acetyl

Table 2. Comparison of ELISA and GLC methods for determination of total trichothecenes in F. sporotrichioides-infected corn samples.[a]

Sample	F. sporotrichioides strain	ELISA-mean (3 determinations)[b]	GLC[c] T-2	GLC[c] HT-2	GLC[c] T-2 triol	GLC[c] Neosolaniol
1	NRRL 3299	20.0 (15, 20, 25)	9.5	10.0	tr[d]	
2	F27	23.3 (15, 25, 30)	11.4	1.6	tr	4.2
3	F27	<0.05 (all <0.05)	10.9	0.3		tr
4	T-2	0.08 (0.1, 0.1, 0.05)	1.4	1.3		
5	T-2	0.5 (0.3, 0.6, 0.6)	2.3	0.4		
6	T-2	25.0 (20, 25, 30)	19.9	21.2		
7	T-340	33.3 (15, 25, 60)	47.7	42.4	3.5	2.8
8	T-340	1.2 (1.0, 1.0, 1.5)	2.0	4.0		
9	T-340	0.3 (0.4, 0.4, 0.15)	1.0	0.9		

[a]All values are in parts per million. ELISA results are of total trichothecenes in the sample with affinity for the T-2 antibody.

[b]Each determination is the average of four replications of infected corn extract per ELISA plate compared with replications in the same plate of noninfected corn extracts diluted to the same degree and spiked with various amounts of T-2.

[c]Levels uncorrected for spike recoveries (85% for T-2, 76% for HT-2). GLC analysis was performed by S. P. Swanson by the method of Scott, et al. (9).

[d] tr, Trace (<0.5 ppm)

(5), also could be detected by direct ELISA (Table 1). The high sensitivity exhibited is much greater than is required to detect T-2 at toxic concentrations (6), and the extraction procedures are simpler than those used in other assays (6,8). This assay could therefore be very useful for routine screening of large numbers of corn samples for T-2 in the time required to analyze a much smaller number by conventional chemical means (6).

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PART IV

PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST T-2 TOXIN

ABSTRACT

A polyclonal antibody was produced against T-2 toxin by immunizing a rabbit with T-2HS conjugated to bovine serum albumin (T-2HS-BSA) by a mixed anhydride intermediate. The rabbit did not produce a titer detectable by direct ELISA until after one year of repeated immunization, and two other rabbits did not produce a titer after more than one year. A titer was detected by indirect ELISA in sera from the earliest bleedings, however. The antibody was used to detect T-2 toxin at 50 pg/ml by direct ELISA and 1 ng/ml by indirect ELISA. Cross-reactivity with other trichothecenes, determined by indirect ELISA, was similar to previously described polyclonal antibodies. Cross-reactivity against the two T-2 metabolites 3'OH T-2 and 3'OH HT-2 was 2% and 1%, respectively, that of T-2 toxin. A monoclonal antibody against T-2 toxin was produced using a T-2HS-BSA conjugated by a water-soluble carbodiimide method. Mice were succesfully immunized using a unconventional immunization protocol consisting of large antigen doses injected subcutaneously without adjuvant. This antibody was characterized by indirect ELISA. Sensitivity to T-2 toxin was 10ng/ml (0.5pg/assay). The antibody cross-reacted less to HT-2 toxin than previously described T-2 antibodies. There were strong cross-reaction to 3'OH T-2 and 3'OH HT-2.

INTRODUCTION

There are various difficulties in the most commonly used chemical methods for detecting trichothecenes (16). Therefore, immunological methods have been developed for the detection of T-2 (1,4,6,8,13,14,18) and other trichothecenes (2,3). My project, started before some of those reports appeared, had the goal of furthering the development of T-2 immunochemistry. Specifically, I wanted to produce polyclonal and monoclonal antibodies to T-2 and compare them to each other and to other T-2 antibodies described in the literature, with respect to both sensitivity and to specificity to T-2 and other trichothecenes. I hoped to ascertain the feasability of producing a number of different antibody preparations, each with different cross-reactivities to various trichothecenes. If this was possible, then samples contaminated with trichothecenes could be analyzed for the various trichothecenes by simply conducting a series of immunological tests with the sample and determining which antibodies reacted strongly and which weakly with the sample. By knowing the cross-reactivities of the antibodies with various trichothecenes, the trichothecene profile of the sample could be determined. I wanted to know if monoclonal antibodies had greater or less variability than polyclonal antibodies with regard to cross-reactivity to various trichothecenes. Large variation in cross-reactivity of monoclonal antibodies to haptens has been found in investigations with certain steroids (5), so this approach seemed

reasonable. Accomplishment of this goal involved investigations into conjugation procedures, ELISA optimization, and immunization protocols, as detailed in in other sections of this dissertation.

MATERIALS AND METHODS

Materials. All solvents and inorganic chemicals were reagent grade or better. Sources of materials used in mixed anhydride, water soluble carbodiimide, and activated ester conjugations of polypeptides to T-2HS are given in Part II of this dissertation. The trichothecenes 3'OH T-2 and 3'OH HT-2 were obtained from Steven Swanson, University of Illinois. Sources of other trichothecenes used are given in the Part III of this dissertation. Ovalbumin (fraction VII), aminopterin, 8-azaguanine, polyethylene glycol MW 1450, sodium pyruvate, insulin, B-mercaptoethanol, oxaloacetate, pristane, thymidine, and hypoxanthene were obtained from Sigma Chemical Co., St. Louis, MO; goat antimouse IgG conjugated to horseradish peroxidase (antimouse-peroxidase) from Cooper Biomedical, Malvern, PA; and various cell culture reagents from GIBCO Laboratories, Grand Island, NY.

Preparation of conjugates. Preparation of mixed anhydride and activated ester conjugates are described in Part II of this dissertation. T-2HS conjugation to ovalbumin (fraction VII) via the activated ester method was also described in Part II of this dissertation. Conjugation of T-2HS to bovine serum albumin via water soluble carbodiimide was by the method of Chu et al (1).

Rabbit immunization protocol. This protocol is described in Part II of this dissertation. Three rabbits were injected repeatedly for more

than one year. They were bled weekly and the titer was checked by the direct ELISA method described in Part III of this dissertation as well as in Appendix B. The titer was also checked by the competitive indirect ELISA described in Part II of this dissertation.

Mouse immunization protocols. Two immunization protocols were compared. Both used the T-2HS-BSA carbodiimide conjugate described above. Three BALB/c mice were injected by each protocol. In the first group, 100 ug T-2HS-BSA conjugate in 0.1 ml saline was emulsified with 0.1 ml Freund's complete adjuvant and injected into the peritoneal cavity of each mouse. Booster injections were made at one month intervals as above except that Freund's incomplete adjuvant was used. In the second group, 1 mg T-2HS-BSA conjugate in 0.5 ml saline was injected subcutaneously into the shoulder. These were repeated at two week intervals, except that 0.5 mg conjugate was used. Both groups were bled through the tail vein 79 days after the initial injection. The red cells were pelleted and the serum was diluted and tested for T-2 antibody activity as described below.

Competitive indirect ELISA. A competitive indirect ELISA procedure was always used in screening for mouse antibody activity against T-2. This procedure is detailed in a flow diagram in Appendix B under "Indirect ELISA". T-2HS-polylysine or T-2HS-ovalbumin conjugate (200 ul), diluted to 2.5 ug/ml in 50 mM carbonate-bicarbonate buffer, pH 9.6, was placed in each well of 96-well immunoplates. The polylysine conjugate was used in initial hybridoma screening and the ovalbumin conjugate was used later. The plates were incubated overnight at 4 C. They were then washed with sodium phosphate buffered saline (PBS- 0.1 M;

pH 7.5) containing 0.05% Tween 20 as previously described (8), except that a 12 hole aspirator was used. After washing, 200 ul PBS containing 1% (wt/vol) ovalbumin (PBS-ovalbumin) was added to each well after insoluble matter in the preparation was removed by a low speed centrifugation. After incubation for 30 min at 37C, the plates were washed as described above. Next, when undiluted hybridoma supernatant fractions were being screened, 40 ul of either 10% methanol in PBS (PBS-MEOH) or T-2 toxin (10 ug/ml) in PBS-MEOH were put in each well, followed by 40 ul of hybridoma supernatant. Supernatants from each colony tested were put in four wells, two with free T-2 and two without. Since often less than 200 ul of supernatant could be sampled from the hybridoma colonies, 40 ul was used here instead of 50 ul so that each colony could be tested in four wells. When preparations other than undiluted hybridoma supernatants were assayed, this step consisted of adding 25 ul PBS-ovalbumin to each well, followed by 50 ul trichothecene in PBS-MEOH, then 50 ul antibody preparation diluted in PBS, as described in Part II. The preparations were incubated for 1 h at 37 C, then were washed. Next, antimouse-peroxidase (80 ul when screening hybridoma supernatants and 100 ul at other times), diluted 1/500 in PBS + 1% fraction V BSA (wt/vol) + 0.1% Tween 20 was added; followed by a 30 min incubation at 37C. After washing, bound peroxidase was assayed by incubating 100ul ABTS-H_2O_2 substrate in each well for 5-30 min; the reaction was terminated with 100ul stopping solution (17). Absorbance at 405nm was determined on an EIA reader EL307 (Bio-Tech, Inc., Burlington, VT).

Hybridoma preparation. Dulbecco's modified eagle medium with 20%

fetal bovine serum, 50 units penicillin/ml, 50 ug streptomycin/ml, 10% NCTC (Gibco), 5 uM oxaloacetate, 5 uM sodium pyruvate, 75.5 mg insulin/L, and 50 uM B-mercaptoethanol (20% FBS) was routinely used as cell culture medium. The mouse with the highest titer was injected intraperitoneally after five weeks rest with 300 ug T-2HS-BSA in 300 ul saline. The fusion protocol described by Oi and Herzenberg (15) and the spleen cell preparation method of Kennett (11) were followed. The spleen cell to myeloma ratio in the fusion was 7:1. Three hundred wells were seeded at 7 x 10^5 total cells per well. Supernatants were screened when the colonies were at least half-confluent (ie. when at least one-half of the bottom of the microtiter plate well was covered with cells). Colonies positive for anti-T-2 antibody were expanded in 20% FBS, to which hypoxanthine and thymidine (HT) had been added. Half of this medium had previously been conditioned by myeloma growth in log phase for 2 days (half strength conditioned media). Cloning was performed by limiting dilution in either half strength conditioned medium or 20% FBS + HT which contained 30% myeloma conditioned and 20% macrophage conditioned (19) media. Ascites fluid was collected from pristane primed mice which were injected intraperitoneally with 10^7 hybridoma cells in 0.5ml 20% FBS twelve days earlier.

Monoclonal antibody characterization. The supernatant fraction from an anti-T-2 antibody producing colony that was cloned at 1 cell per well in the limiting dilution step was precipitated three times in a 50% ammonium sulfate solution. The precipitated fraction was diluted sixteen-fold over the original supernatant volume and this was used in the indirect ELISA described above. This fraction was also used with the

subclass determination kit of Boehringer Mannheim (Indianapolis, IN) to determine the subclasses of the heavy and light chains of the antibody.

RESULTS

Production and characterization of polyclonal antibody reactive against T-2. Only one of three rabbits repeatedly immunized for greater than one year showed a titer by direct ELISA. The earliest bleedings from all rabbits, made seven weeks after the initial injection, had a titer of at least 1/500 in the indirect ELISA, however. Sensitivity to T-2 toxin in direct ELISA (performed exactly as in Part III) was the same as for a previously characterized antibody (8), 0.05 ng/ml. The sensitivity of this antibody in the indirect ELISA to various trichothecenes is shown in Table 1. The structures of these trichothecenes are shown in Figure 1 of Part III. This antibody was used at a dilution of 1/1000. The sensitivity to T-2 in the indirect ELISA was 1 ng/ml, 1/20 that of the same antibody in the direct ELISA. The relative cross-reactivity to T-2 of the other trichothecenes tested was similar to previously characterized T-2 reactive rabbit antibodies (1,8).

Mouse immunization protocols. Table 2 summarizes the results obtained when a 1/400 dilution of the mouse sera was tested by indirect ELISA. The mice injected by protocol A, a common immunization protocol, showed no activity against T-2, either in amount of antibody binding or in specific inhibition of antibody binding by free T-2. In contrast, strong specific binding occured in mice 1 and 3, since free T-2 at 1

Table 1. Sensitivity of T-2 reactive rabbit antibody to various trichothecenes in competitive indirect enzyme immunoassay[a]

Trichothecene	minimal inhibition[b]	50% inhibition[c]
T-2	1.0	3.2
T-2HS	1.0	2.7
HT-2	1.0	18.5
3'OH T-2	50	300
3'OH HT-2	100	350
T-2 triol	1000	1150
Deoxynivalenol	10000	38000

[a]All values are in ng/ml of trichothecene in 10% methanol/PBS. See text for assay protocol.

[b]ng/ml trichothecene required for first significant inhibition of binding of antibody to the T-2HS-ovalbumin solid phase.

[c]ng/ml trichothecene required to inhibit binding of antibody by 50% to the T-2HS-ovalbumin solid phase. Calculated by regression analysis.

Table 2. Results of competitive indirect enzyme immunoassay testing of diluted (1/400) mouse sera for T-2 antibody activity.

		A_{405}		
Protocol[a]	Mouse	No free T-2[b]	free T-2[c]	No specific coat[d]
A	1	.17	.17	.17
	2	.15	.19	.14
	3	.12	.12	.14
B	1	.47	.21	.23
	2	.61	.70	.60
	3	.44	.23	.19

[a]Protocols: A = 100ug T-2HS-BSA in 0.1ml saline + 0.1ml adjuvant intraperitoneally at 1 month intervals. B = 0.5 to 1.0mg T-2HS-BSA in 0.5ml saline subcutaneously in the shoulder at two week intervals.

[b]10% methanol in PBS (PBS-MEOH) added with equal volumes of diluted serum.

[c]1ug/ml T-2 in PBS-MEOH added with equal volumes of diluted serum.

[d]These wells were coated with a conjugate prepared similar to the T-2HS-PLL conjugate, except no T-2HS was used.

ug/ml inhibited binding to levels occuring with uncoated wells. Mouse 2 with this protocol showed nonspecific binding of antibody, since free T-2 could not inhibit antibody binding and wells coated with a conjugate that was not made with T-2 had a similar amount of binding (Table 2). Mouse 3 under protocol B was used in the fusion; at a dilution of 1/100, serum from this mouse showed stronger T-2 antibody activity than did mouse 1.

Hybridoma preparation. The fusion efficiency (number of wells with clones/number of wells seeded) was greater than 95% (286/300). Of these, nine (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 upon 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 stable, and all three mice injected with this clone yielded ascites fluid active at a dilution of 1/5000 after ammonium sulfate precipitation. When cloned a second time at one cell per well, more than 90% of the colonies which grew showed specific binding.

Monoclonal antibody characterization. The antibody described above was an IgG_1 with a kappa light chain. Table 3 shows the results of tests

Table 3. 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	0.19
HT-2	0.5	1.0
Neosolaniol	1.0	1.6
T-2 triol	5.0	14.9
T-2 tetraol	100.	447.
Verrucarol	500.	>500.
Deoxynivalenol	>1000.	>1000.
Roridin A	>5000.	>5000.

[a]All values are in ug/ml of trichothecene in 10% methanol/PBS.See text for assay protocol.

[b]ug/ml trichothecene required for first significant inhibition of binding of antibody to the T-2HS-ovalbumin solid phase.

[c]ug/ml trichothecene required to inhibit binding of antibody by 50% to the T-2HS-ovalbumin solid phase. Calculated by regression analysis.

of various trichothecenes for antibody reactivity. The structures of the trichothecenes tested for cross-reactivity with this antibody are shown in Figure 1 of Part III. The data used to determine the cross-reactivity of the most cross-reactive trichothecenes listed in Table 5 is shown in Appendix C. This monoclonal antibody had less reactivity to HT-2 toxin relative to T-2 (2%) than did other monoclonal or polyclonal T-2 antibodies (1,8,10). A strong cross reaction was found with the 3'OH metabolites of T-2 and HT-2 as well as with acetyl T-2 (Table 3). These metabolites showed much greater cross-reactivity to this antibody than did the polyclonal antibody described above (Table 1). The other trichothecenes tested (other than HT-2) had similar percentage cross reactivities relative to T-2 as did other T-2 rabbit or monoclonal antibodies (1,8,10). Sensitivity to T-2, at 0.01ug/ml, was 0.5ng/assay, which is more sensitive than the previously described T-2 monoclonal antibody/ELISA system (10), but less sensitive than systems using rabbit antisera (1,4,6,8,18), including the one described above (Table 1).

DISCUSSION

The rabbit antibody described above produced a titer detectable by indirect ELISA quite easily. This does not agree with the assertion by Hunter et al (10) that T-2 is a relatively ineffective hapten. A titer detectable by direct ELISA could only be produced with great difficulty, however. This demonstrates the importance of the assay. The direct assay needed a much higher titered antisera, and this was only obtained after one year. Since the antibody could be used at 1/1000 in the indirect ELISA but only showed a minimal titer at 1/50 in the direct assay, this is also evidence that the indirect ELISA requires less antibody than does the direct ELISA, in agreement with Fan et al (4). However, the direct ELISA had a 20-fold greater sensitivity to T-2 than did the indirect ELISA.

The mouse immunization protocol used in monoclonal antibody production, in which large antigen doses were given without adjuvant, was clearly superior to the more traditional immunization protocol (Table 2). 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 were positive. The nonspecific binding shown by serum of mouse B2 (Table 2) 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, I considered routine employment of the competitive procedure crucial in identifying false positives.

I experienced none of the fusion problems found by Hunter et al (10). I had high fusion efficiency and a reasonable number of positive colonies. Perhaps the immunization protocol we used overcame the possible immunotoxicity experienced in their work. The major problem I had was in retaining a stable colony through cloning. Although cloning efficiencies approached 100% for both myeloma and hybridoma lines when half strength conditioned medium was used, no positive colony was obtained until macrophage conditioned medium (19) was used. Presumably, the hybridoma needed growth factors present in the macrophage conditioned medium and not present in the myeloma conditioned medium.

The monoclonal antibody has cross-reactivities similar to T-2 antibodies previously characterized (1,8,10), except for its relatively weak cross-reactivity to HT-2 and relatively strong cross-reactivities to the two 3'OH metabolites tested (Table 3). As explained in the introduction, these differing 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 may be determined. This also lends credence to the possibility that a series of monoclonals can be obtained, each with different specificities for many trichothecenes. The strong cross-reactivity of this monoclonal antibody to the 3'OH metabolites of T-2 and HT-2 opens the possibility that this antibody may be used in assays of T-2 toxicosis, since these metabolites are diagnostic and present in

significant amounts when T-2 toxicosis occurs (20-22).

The three monoclonal antibodies against T-2 described thus far (two by Hunter et al [10] and one here) all show more variability to HT-2 than do the polyclonal systems (1,8). This indicates a greater variability in cross-reactivities among monoclonal antibodies than among polyclonal antibodies. Therefore, a number of monoclonal antibodies against T-2 would probably give a greater range in cross-reactivities than the same number of different rabbit antisera.

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APPENDIX A

AN ELISA FOR OCHRATOXIN A

Ochratoxin A is a fungal metabolite first isolated from Aspergillus ochraceus (9) and subsequently found in various agricultural commodities (2). This nephrotoxin is the most toxic of the ochratoxins and is also produced by other Aspergillus as well as certain Penicillium species (2,5). As with the trichothecenes and aflatoxins, immunochemical methods have been developed to detect ochratoxin A more rapidly than by conventional chemical means (1,6,7,8).

As part of investigations detailed in Part II of this dissertation, an indirect ELISA was developed for ochratoxin A. Most of the details of this assay are discussed there, and the reader is referred there for materials, methods, and results of the assay when conducted as described.

I was interested in developing that assay further so that it might be used as a screen for hybridoma-produced antibodies against ochratoxin A, much as the equivalent assay was used with T-2 in Part III of this dissertation. As described, the assay could detect ochratoxin A at 50 ng/ml, since this was the toxin concentration giving the first significant inhibition of antibody binding (Figure 2, part II). I have found that various modifications to that assay affect this sensitivity. These modifications will now be described.

Since ochratoxin A is a rather polar molecule (5), I performed the assay by diluting the free ochratoxin A in PBS, rather than PBS with 10% methanol. This was effective, reducing the toxin concentration necessary to inhibit antibody binding to 10 ng/ml. I next eliminated the 25ul ovalbumin put in with the antibody and free toxin, since Chu (3,4) had found that ochratoxin A binds to bovine serum albumin (BSA). If the

toxin also binds to ovalbumin, this may prevent it from specifically binding to the antibody. When the ovalbumin was eliminated from this step, antibody binding was inhibited by a smaller concentration of ochratoxin A, 5 ng/ml. Since hybridoma supernatants normally contain 20% fetal bovine serum, there would be a considerable amount of BSA. Based on the above results, the BSA in the medium might interfere with an indirect screening proceedure for antibodies against ochratoxin if performed as described in Part III of this dissertation. The possibility of interference by media was tested by diluting the rabbit anti-ochratoxin A antibody in 20% FBS media. This increased the concentration of free toxin necessary to inhibit binding of the antibody to 500 ng/ml.

In conclusion, this assay is more effective if methanol and albumins are eliminated from the step where free toxin is added with antibody. If this is not possible, then greater concentrations of ochratoxin A must be used to get competitive inhibition. I recommend, therefore, that free ochratoxin A be diluted in PBS to a concentration of 10 ug/ml when using this assay to screen for anti-ochratoxin A antibodies in hybridoma supernatants.

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APPENDIX B

FLOW CHARTS FOR DIRECT AND INDIRECT ELISA

All quantities are per well of microtiter plate. Dilutions for reagents that vary from batch to batch (eg. antisera, enzyme conjugates) will not be given.

Direct ELISA-see Part III for reagent sources and conjugation protocols

50ul 2mg/ml BSA in H_2O

↓ air dry

50ul 0.2% (vol/vol) glutaraldehyde in PBS

↓ incubate 30min, room temperature;
wash in H_2O; air dry

air dry 50ul diluted, purified antisera

↓ wash 3x with PBS/0.05% Tween 20

200ul 1% BSA in PBS

↓ incubate 1h 37°C; wash 2x

25ul sample or T-2 standard in 10% methanol/PBS

+

25ul T-2HS-horseradish peroxidase in 5% BSA/PBS/0.1% Tween 20

↓ incubate 1h 37°C; wash 6x

100ul ABTS/H_2O_2 solution

↓ incubate 30min, room temperature

100ul HF/EDTA stopping solution

Read A_{405}

Indirect ELISA-see Parts II and III for reagent sources and conjugation protocols.

200ul T-2HS-polylysine or T-2HS-ovalbumin in 50mM carbonate-bicarbonate, pH 9.6

↓ incubate 4°C overnight; wash2x PBS/0.05% Tween 20

200ul 1% ovalbumin in PBS

↓ incubate 37°C 30min; wash 2x

25ul 1% ovalbumin in PBS

+

50ul purified antisera, diluted in PBS

+

50ul toxin in 10% methanol/PBS

↓ incubate 37°C 1h; wash 4x

100ul goat antirabbit (or mouse) diluted in 1% BSA/PBS/0.01% Tween 20

↓ incubate 37°C 30min; wash 8x

100ul ABTS/H_2O_2 solution

↓ incubate 30min, room temperature

100ul HF/EDTA stopping solution

Read A_{405}

APPENDIX C

DATA USED TO GENERATE TABLE 5 OF PART III

See text or Appendix B for assay protocol. Includes only trichothecenes with 50% inhibition at less than 10 ug/ml. All values are A_{405}

Trichothecene; high; low[a]	[ ][b]	Average (3 replications)
T-2 .815 .051	0.001	.828 (.748, .907, .830)
	0.005	.740 (.721, .756, .745)
	0.01	.631 (.656, .695, .543)
	0.05	.263 (.281, .275, .232)
	0.1	.159 (.155, .156, .165)
	1.0	.066 (.047, .099, .052)
T-2HS .451 .000	0.001	.416 (.304, .470, .474)
	0.005	.370 (.325, .442, .344)
	0.01	.365 (.323, .407, .365)
	0.05	.268 (.218, .234, .351)
	0.1	.131 (.119, .129, .145)
	0.5	.059 (.064, .054, .060)
	1.0	.052 (.044, .063, .048)
Acetyl T-2 .451 .000	0.005	.383 (.384, .347, .419)
	0.01	.341 (.314, .344, .366)
	0.05	.184 (.187, .187, .177)
	0.1	.126 (.112, .144, .121)
	0.5	.045 (.039, .050, .047)
	1.0	.040 (.037, .045, .039)
3'OH T-2 .708 .039	0.001	.635 (.632, .650, .622)
	0.005	.502 (.527, .455, .523)
	0.01	.436 (.418, .408, .484)
	0.05	.163 (.136, .166, .188)
	0.1	.082 (.079, .080, .086)
	0.5	.024 (.008, .032, .031)
	1.0	.012 (.011, .024, .002)
3'OH HT-2 .442 .000	0.001	.432 (.384, .433, .479)
	0.005	.459 (.431, .414, .531)
	0.01	.422 (.420, .444, .403)
	0.05	.363 (.356, .344, .389)
	0.1	.286 (.287, .264, .306)
	0.5	.132 (.124, .134, .137)
	1.0	.101 (.092, .101, .111)
	5.0	.078 (.054, .099, .081)

Trichothecene;	high;	low[a]	[ ][b]	Average (3 replications) A_{405}
HT-2	.710	.047	0.1	.774 (.721, .754, .846)
			0.5	.520 (.502, .524, .534)
			1.0	.454 (.440, .439, .484)
			5.0	.153 (.151, .148, .161)
			10.0	.087 (.093, .080, .089)
			50.0	.052 (.048, .057, .052)
Neosolaniol	.710	.047	0.5	.607 (.592, .642, .587)
			1.0	.480 (.499, .475, .465)
			5.0	.179 (.169, .193, .176)
			10.0	.105 (.094, .112, .109)
			50.0	.054 (.049, .051, .061)
			100.0	.046 (.042, .051, .046)

[a]high= average absorbance value when no free trichothecene is included (0 inhibition value); low= average absorbance value when plate is coated with bicarbonate coating buffer only (100% inhibition value).

[b]Concentration of free trichothecene, ug/ml.