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Production and Characterization of Monoclonal Antibodies to the Macrocyclic Trichothecene Roridin A

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Two murine monoclonal antibodies to the macrocyclic trichothecene roridin A are described. Screening for antibody production was performed on absorbed anti-mouse immunoglobulin serum as double-antibody solid phase, and further characterization was done on affinity-purified anti-mouse IgG serum. The antibodies, designated 5G11 and 4H10, had affinity constants for roridin A of 9.25×10^7 and 1.7×10^7 liters/mol, respectively. In monoclonal antibody-based direct enzyme immunoassays, these IgG1 antibodies had detection limits for roridin A of 0.4 ng/ml (0.02 ng per assay) and 1.8 ng/ml (0.09 ng per assay), respectively. Both antibodies were most specific for the tested macrocyclic trichothecenes. The relative cross-reactivities of antibody 5G11 with roridin A, roridin J, verrucarin A, satratoxin G, and satratoxin H were 100.0, 43.8, 16.7, 3.7, and 18.9%, respectively; for antibody 4H10 they were 100.0, 6.3, 64.0, 4.4, and 4.9%, respectively.

The trichothecene mycotoxins are known as sources of intoxication in humans and animals, and a lot of work has been done to develop methods for their rapid and efficient detection. The methods mainly involve physicochemical techniques, but immunological procedures have also been investigated. More recently, the production of monoclonal antibodies to T-2 toxin (8, 10, 12, 18) and diacetoxyscirpenol (19) has been reported.

The macrocyclic trichothecenes, however, have not been dealt with in this manner. This group, which includes more than 30 members (14), is characterized by a carbon-oxygen ring system at C-4 and C-15 of the basic trichothecene molecule (Fig. 1). They are produced by *Myrothecium* (1) and *Stachybotrys* (15) species but are also found in the higher plants *Baccharis megapotamica* (16) and *B. coridifolia* (2) in South America. In Brazil and Argentina these plants are known as causative agents of livestock poisoning (9), and in Hungary *Stachybotrys atra* was reported to cause intoxication in sheep (11). The latter was also proved to be responsible for unexplained illness in humans (3) in the United States.

Roridin A, one of these macrocyclic trichothecenes, appears to be one of the central members in the further biotransformation to most of the other macrocyclic trichothecenes (13). Therefore an enzyme immunoassay for the detection of roridin A based on a conventional rabbit antiserum was recently developed (17). The potential for improvement of immunochemical detection methods through the use of hybridoma technology led us to prepare murine monoclonal antibodies to roridin A. In this paper the production and characterization of monoclonal antibodies to roridin A are described, and these monoclonal antibodies are compared with the rabbit polyclonal anti-roridin A antibodies.

Female BALB/c mice were immunized with 50 μg of roridin A-human serum albumin conjugate (RA-HSA), prepared as recently described (17), dissolved in sodium phosphate-buffered saline (PBS [0.01 mol/liter, pH 7.2], NaCl [0.1 mol/liter]) and emulsified in complete Freund adjuvant. At days 21 and 50 they received a booster injection of the same amount of antigen in incomplete Freund adjuvant. At day 90, 3 days before removal of spleens, the animals got a final booster injection of 75 μg of antigen in PBS alone. All injections were given intraperitoneally, and 2 weeks after each injection test bleedings were performed through the orbital vein plexus. The splenocytes were fused with myeloma cells (X63-Ag8.653) in a ratio of 5:1 with a standard fusion protocol (4) and cultivated for 2 weeks in HAT medium containing 10 U of insulin per liter (5).

After this period, antibody-producing hybridomas were detected by a direct enzyme immunoassay with a double antibody technique as recently described (10) with the slight modification that anti-mouse immunoglobulin serum (Dakopatts, Hamburg, Federal Republic of Germany) was used instead of anti-mouse IgG serum for coating the plates. Briefly, after coating the plates with anti-mouse IgG serum (100 μl of a 10-μg/ml solution in carbonate-bicarbonate buffer; 0.01 mol/liter, pH 9.6) overnight, blocking with 150 μl of PBS containing 3% fetal calf serum (FCS) for 30 min, and a washing step, 100-μl portions of the tissue culture supernatant were incubated for 1 h. Following another washing step, 100 μl of roridin A-horseradish peroxidase conjugate (RA-HRP) (diluted 1:10,000 in PBS containing 1% FCS) prepared as recently described (17) was added and reacted for 1 h. After being washed, the plate was developed with substrate solution described elsewhere (7), the reaction was stopped after 10 to 20 min with 1 mol of sulfuric acid per liter (100 μl per well), and the A_{450} was measured. The antibody titers of serum, culture supernatant, and ascites were determined in the same manner by serial dilutions in PBS.

For confirmation of positive results a competitive direct enzyme immunoassay was used. In the test system described above, roridin A standard dilutions (50 μl per well in PBS containing 10% methanol) and RA-HRP (50 μl per well, 1:5,000 in PBS containing 1% FCS) were incubated simultaneously for 1 h. Colonies positive in both assays were considered to be specific for roridin A and were cloned at least twice by limiting dilution.

In an indirect enzyme immunoassay with plates coated with RA-HSA (100 μl of a solution of 10 μg/ml in carbonate-bicarbonate buffer per well) as the solid phase, the immunoglobulin class and subclass were determined. The assay was performed essentially as recently described (10). After identification of the immunoglobulin class, all further experiments were performed on plates coated with anti-mouse IgG

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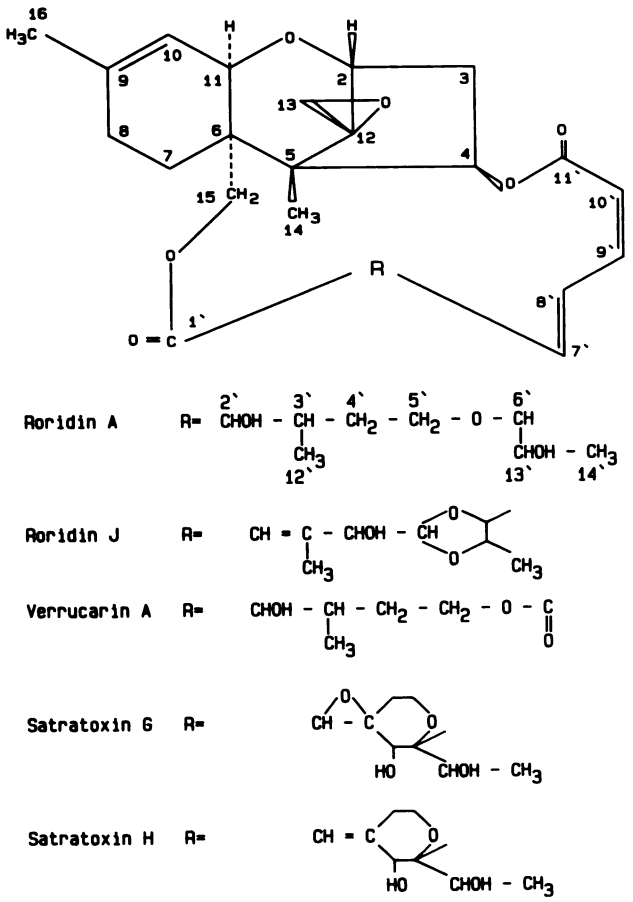

FIG. 1. Structures of the macrocyclic trichothecenes tested.

(developed in rabbits and purified by affinity chromatography by using mouse IgG coupled to cyanogen bromide-activated Sepharose 6B according to the instructions of the distributor [Sigma Chemical Co., Deisenhofen, Federal Republic of Germany]).

To check the specificity and sensitivity of the antibodies, five macrocyclic trichothecenes (roridin A and verrucarin A, purchased from Sigma, and roridin J, satratoxin G, and satratoxin H, from the Institute of Biochemistry, University of Mainz, Federal Republic of Germany) (Fig. 1) and nine nonmacrocyclic trichothecenes (T-2 toxin, T-2 tetraol, diacetoxyscirpenol, 15-acetoxyscirpenol, verrucarol, and diacetylverrucarol obtained from Sigma, and neosolaniol, nivalenol, and fusarenon X from Wako Chemical Co., Neuss, Federal Republic of Germany) were tested in the competitive enzyme immunoassay. All toxins were tested in a concentration range of 50,000 to 0.005 ng/ml in PBS containing 10% methanol and appropriate working dilutions of tissue culture supernatant (1:1,500 for antibody 5G11 and 1:100 for antibody 4H10) were used. The affinity constants were determined by the method described by Friguet et al. (6).

After several fusion experiments, three hybridomas showing antibody production against roridin A could be detected, and two of them, designated 5G11 and 4H10, were chosen for further characterization, whereas the third, 3E1, was neglected because of its low affinity to roridin A (complete inhibition in the competitive enzyme immunoassay at a concentration level of about 50,000 ng of roridin A per ml; data not shown). A screening procedure with anti-mouse immunoglobulin serum as the double antibody solid phase

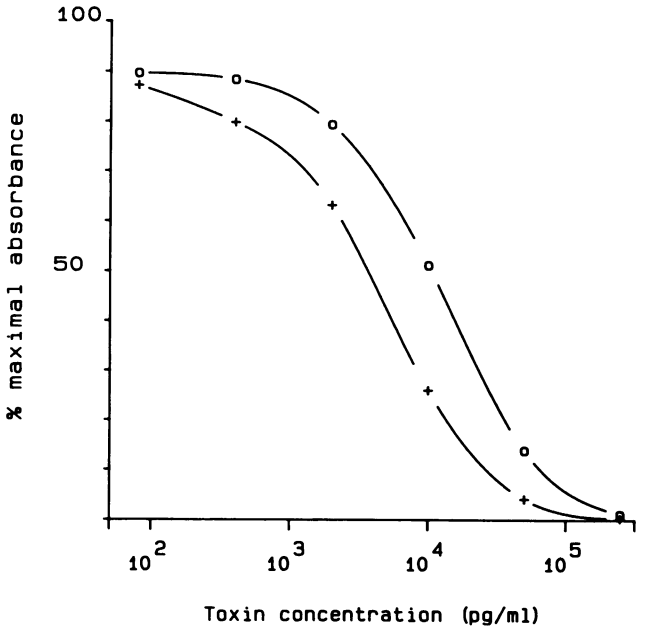

FIG. 2. Standard inhibition curves of the two monoclonal antibodies in the competitive enzyme immunoassay. Symbols: +, 5G11; O, 4H10.

was used to screen for all immunoglobulin classes. Since both antibodies belonged to the IgG1 subclass, further inhibition experiments were made on plates coated with anti-mouse IgG serum. From the standard inhibition curves (Fig. 2), the detection limits for antibodies 5G11 and 4H10 were found at 0.4 ng of roridin A per ml (0.02 ng per assay) and 1.8 ng of roridin A per ml (0.09 ng per assay), respectively. These data agree with the affinity measurements, in which the antibodies 5G11 and 4H10 had affinity constants for roridin A of 9.25×10^7 and 1.7×10^7 liters/mol, respectively. The detection limit was defined as the concentration at 80% of maximal absorbance in the competitive direct enzyme immunoassay. The intra-assay coefficients of variation ($n = 8$) for standard concentrations (50,000 to 16 pg/ml) were usually below 5%.

The specificity data for the antibodies, the concentrations causing 50% inhibition of binding of RA-HRP to the antibodies, and the relative cross-reactivities are shown in Table 1. Both antibodies were most specific for roridin A and for the other macrocyclic trichothecenes tested. This is not surprising since for the production of the antigen RA-HSA roridin A was first derivatized to a dihemisuccinate at positions C-2′ and C-13′ (Fig. 1) and then coupled to HSA, making the basic trichothecene skeleton together with the macrocyclic ring system the immunodominant structure. Differences in cross-reactivity, however, indicate that each antibody might prefer another part of the molecule. Antibody 5G11 reacted much more strongly with roridin J than with verrucarin A and satratoxin H, perhaps due to the different conformation of the toxins at position C-6′. The absence of the C-2 side chain led to a significant decrease in cross-reactivity from 43.8% to 16.7 and 18.9%, respectively. The additional epoxy group at C-2′ to C-3′ (satratoxin G) further reduced the cross-reactivity to 3.7%. The other trichothecenes tested did not show significant cross-reactivity. Therefore, this antibody seemed to recognize separately the right-hand side of the trichothecene molecule (Fig. 1).

The other antibody, 4H10, behaved completely differently. In contrast to the above findings, it reacted very

TABLE 1. Specificities of the two monoclonal antibodies determined in the competitive direct enzyme immunoassay and of the rabbit antiserum presented previously (17)

Toxin	50% inhibitory concn (μmol/liter)[a]		
	Monoclonal antibody		Rabbit antiserum
	5G11	4H10	
Roridin A	0.007 (100.0)	0.016 (100.0)	0.030 (100.0)
Roridin J	0.016 (43.6)	0.255 (6.3)	0.074 (40.7)
Verrucarin A	0.042 (16.7)	0.025 (64.0)	0.199 (15.1)
Satratoxin G	0.190 (3.7)	0.364 (4.4)	0.459 (6.5)
Satratoxin H	0.037 (18.7)	0.326 (4.9)	0.208 (14.8)
Diacetylverrucarol	13.208 (0.05)	8.377 (0.2)	19.046 (0.15)
Verrucarol	7.723 (0.09)	41.330 (0.04)	60.150 (0.05)
Diacetoxyscirpenol	>136.550 (<0.005)	2.003 (0.8)	>163.860 (<0.018)
15-Acetoxyscirpenol	>154.246 (<0.005)	65.660 (0.02)	>185.096 (<0.016)

[a] Numbers in parentheses indicate cross-reactivity relative to roridin A [(roridin A concentration for 50% inhibition/toxin concentration for 50% inhibition) × 100].

strongly with verrucarin A. Considering the weak cross-reactivity with roridin J and satratoxins G and H, for this antibody the most important part of the molecule seemed to be at the left-hand side (Fig. 1), including the position C-2′ to C-3′. The identical configuration of roridin A and verrucarin A at this site caused strong cross-reactivity (64.0%). By changing this part of the molecule to a carbon double bond (roridin J, satratoxin H) or an epoxy group (satratoxin G), the cross-reactivity decreased rapidly.

Comparing the monoclonal antibodies with the rabbit antiserum presented previously (17), it was remarkable that 5G11 reacted almost identically in terms of specificity (Table 1), whereas 4H10 behaved in the different manner described above. In regard to sensitivity of the enzyme immunoassays, both monoclonal antibodies showed lower 50% inhibition values than reported for the rabbit antiserum. Therefore, an enzyme immunoassay based on monoclonal antibodies could be developed which was at least as sensitive as an enzyme immunoassay based on the corresponding polyclonal antiserum. Thus, the hybridoma technology could be a versatile tool to improve the reliability and sensitivity of immunoassays for the detection of mycotoxins and may facilitate further investigations on the occurrence of these extremely toxic substances.

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