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Blockage of Cell Wall Receptors for Yeast Killer Toxin KT28 with Antimannoprotein Antibodies

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Binding of yeast killer toxin KT28 to its primary cell wall receptor was specifically blocked with polyclonal antimannoprotein antibodies which masked all toxin-binding sites on the surface of sensitive yeast cells. By indirect immunofluorescence, it was shown that KT28 binds to the cell wall mannoprotein and that the toxin resistance of mannoprotein mutants (*mnn*) of *Saccharomyces cerevisiae* was due to a lack of killer toxin-binding sites within the yeast cell wall. Structural analysis of acetylated mannoproteins from KT28-resistant mutant strains identified the outer mannotriose side chains as the actual killer toxin-binding domains.

Binding of *Saccharomyces cerevisiae* killer toxins to primary receptors within the cell wall of susceptible yeast strains represents the initial step in killer toxin action (14, 16). Depending on the type of killer toxin (K_1, K_2, K_3, or K_{28}), these primary toxin-binding sites are localized within the glucan (K_1 to K_3) or the mannan (K_{28}) fraction of the cell wall (7, 11) and play an important role in establishing killer toxin specificity (17). Mutations in either of the two chromosomal genes *KRE1* and *KRE2* lead to a toxin resistance at the level of the yeast cell wall, which is caused by a modification of β-1,6-linked glucan structures that are necessary for toxin binding (2). Since *kre* mutants are resistant to a wide range of different killer toxins, binding to cell wall glucans is thought to be a common initial step in killer toxin action.

Unlike killer toxins of the types K_1 and K_2, killer toxin KT28, secreted by *S. cerevisiae* strain 28, is a 16-kilodalton glycoprotein that does not bind to glucans but to a mannoprotein of the yeast cell wall (9, 11). Also, whereas the target of K_1 and K_2 toxins is the cytoplasmic membrane (5, 8), KT28 appears to inhibit DNA synthesis without having any detectable effect on membrane permeability (10). The energy-independent binding of KT28 to its primary cell wall receptor is a rapid process specific enough to be utilized in vitro for the purification of the killer protein, via a receptor-mediated affinity chromatographic technique (13). Mutations in the nuclear genes *MNN2* and *MNN5*, which are known to cause defined alterations in mannoprotein side chain structure (3), have been shown to prevent killer toxin binding to the yeast cell wall, thus conferring resistance to KT28 (12). Correspondingly, the appropriate mutants became fully sensitive after their cell walls had been enzymatically removed.

In this study, we investigated whether receptor-specific antibodies can be used to mask killer toxin-binding sites, thus giving immediate immunity to KT28. Antibodies against the purified 185-kilodalton cell wall mannoprotein (PMP) of the susceptible strain X2180-1Aa were raised in two New Zealand White rabbits, and the resulting immunoglobulin G fraction was purified by precipitation with ammonium sulfate and ion-exchange chromatography on Q-Sepharose (Pharmacia, Inc.). By using PMP as an antigen, receptor-specific antibodies (antimannoprotein antibodies [α-MP-ab]) were detected by an indirect enzyme-linked immunosorbent assay essentially as described previously (15). In this assay system, the titer of the α-MP-ab was $32{,}000^{-1}$ versus PMP, whereas titers versus purified cell wall glucans (β-1,3- or β-1,6-D-glucans) as well as the titer of the preimmune serum were zero. When cells of the susceptible strain *S. cerevisiae* 381 were treated simultaneously with a lethal concentration of KT28 toxin (1.5×10^3 U/ml) and the polyclonal α-MP-ab, the survival rate increased significantly, whereas the preimmune serum could not prevent the toxin-mediated cell death of the susceptible strain (Fig. 1). Further control experiments showed that the same susceptible strain remained fully susceptible to another killer toxin (K_1), no matter whether the cells had been treated with the α-MP-ab (data not shown). Since killer toxins of this type (K_1) are known to bind to β-1,6-D-glucans of the yeast cell wall (7) which have not been recognized or masked by our antibody, interference of antibodies directed against receptor-nonspecific antigens of the yeast cell surface with killer toxin cell wall binding does obviously not account for the significant effect observed with KT28-treated yeast cells (Fig. 1). Unlike the lectin concanavalin A, which prevents the lethal effect of killer toxin KT28 only when sensitive cells are preincubated with the lectin (12), the α-MP-ab caused an efficient, immediate, and specific blockage of all killer toxin-binding sites. A possible neutralization of KT28 by the polyclonal α-MP-ab could be ruled out because additional enzyme-linked immunosorbent assays revealed no detectable cross-reactivity with the killer toxin. Thus, it was possible to confer immunity to toxin-treated sensitive yeast cells just by adding both α-receptor antibodies and KT28 simultaneously.

To further characterize this KT28-mannoprotein interaction in vivo, we tried to localize the killer toxin-binding sites on the cell surface of toxin-treated and untreated yeast cells by indirect immunofluorescence. The susceptible strain *S. cerevisiae* X2180-1Aa was incubated with killer toxin KT28 for 10 min at room temperature and treated with α-MP-ab, followed by incubation with a fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G secondary antibody (Sigma Chemical Co.). Thereafter, the cells were incubated in the dark for 30 min, pelleted, washed, and photographed with an Olympus BH-5 microscope. Whereas killer toxin-treated yeast cells did not show any detectable

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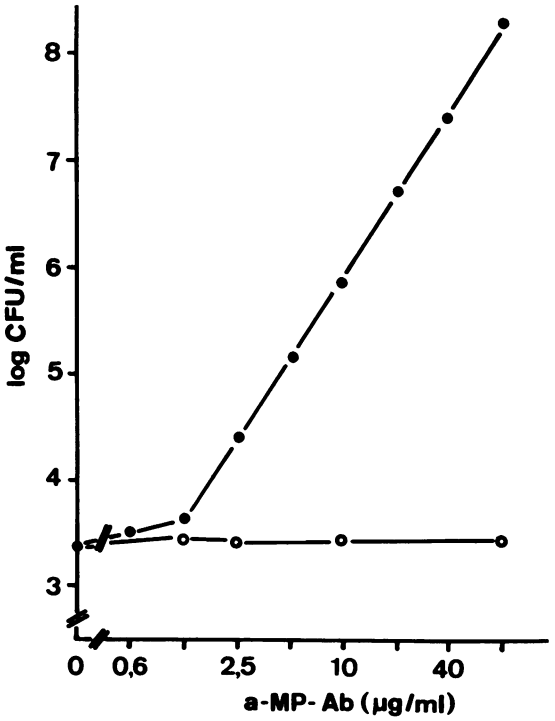

FIG. 1. Effects of receptor-specific α-MP-ab (●) versus normal rabbit serum (○) on the toxin-mediated cell death of *S. cerevisiae* 381. Receptor blockage was done by adding the polyclonal α-MP-ab to 10^6 exponentially growing yeast cells that were treated simultaneously with killer toxin KT28 (1.5×10^3 U/ml). Killer toxin activity is expressed in arbitrary units determined by the agar diffusion assay; 1 U corresponds to about 0.1 ng of purified killer toxin.

fluorescence when probed with the receptor-specific antibody, untreated cells gave a strong signal for the cell surface mannoprotein (Fig. 2). These data suggest that the specific binding of killer toxin KT28 to its primary cell wall receptor prevents the subsequent binding of the polyclonal α-MP-ab,

TABLE 1. Correlation of killer toxin-cell wall binding and indirect immunofluorescence of different KT28-treated wild-type and mutant (*mnn*) strains of *S. cerevisiae*

S. cerevisiae strain	Susceptibility to KT28 toxin[a]	Fluorescence		KT binding
		With KT	Without KT	
X2180-1Aa	S	−	+	+
mnn1	S	−	+	+
mnn2	R	+	+	−
mnn4	S	−	+	+
mnn5	R	+	+	−
512-WT	S	−	+	+
512-R7	R	+	+	−

[a] S, Susceptible; R, resistant.

thus causing the quench in fluorescence. Additional investigations of several receptor-defective mannoprotein mutants (*mnn*) of *S. cerevisiae* indicated that the resistance of these mutants is caused by a lack of killer toxin-binding sites on the cell surface (Table 1). Thus, this indirect immunofluorescence assay can be used as a fast and reliable test system for the identification and characterization of KT28-resistant *mnn* mutants. Furthermore, screening for toxin resistant-mutant strains should be a fruitful strategy to obtain and select new mannoprotein mutants in *S. cerevisiae*.

To identify the actual killer toxin-binding domain within the isolated KT28 cell wall receptor, we purified the 185-kilodalton mannoprotein PMP from different toxin-resistant mutants and analyzed their side chain patterns by gel filtration of the acetylated toxin receptors. Purification as well as controlled acetolysis of the isolated mannoprotein was done by methods described previously (4, 11, 12). For this study, we used mannoproteins from the mutants *mnn1* and *mnn5*, which are known to differ only in the number of mannose residues within the outer mannoprotein side chains (3, 6). Their side chain patterns as well as those of the KT28-resistant mutant 512-R7 (this work) and the corresponding sensitive wild type, 512-WT, are shown in Fig. 3. The acetylated mannoproteins of both mutants *mnn1* and *mnn5*

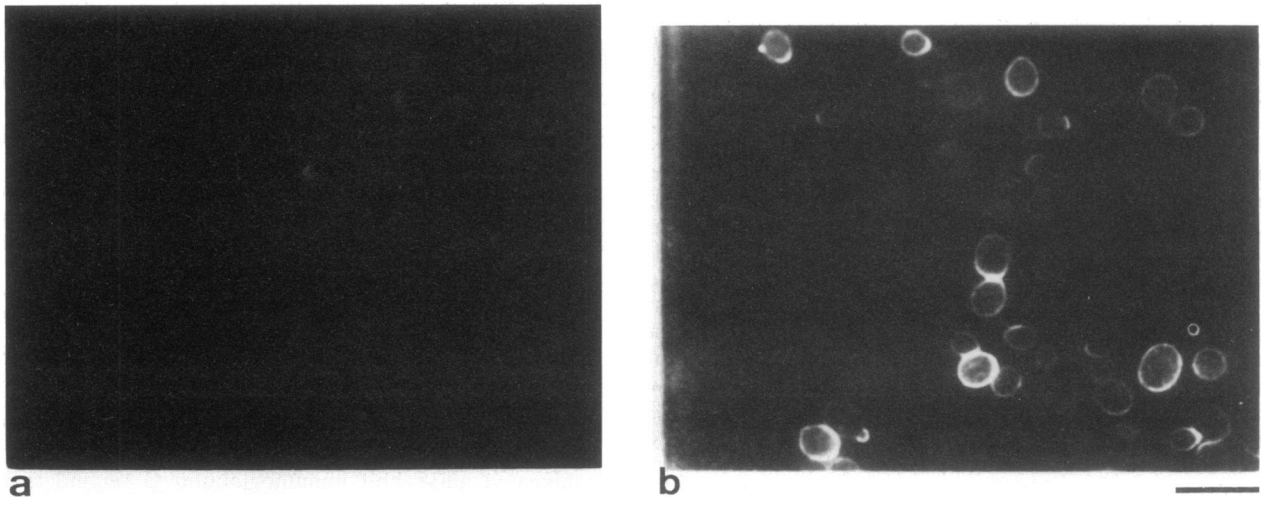

FIG. 2. Localization of killer toxin KT28 primary receptors on the surface of toxin-treated (panel a) and untreated (panel b) cells of the susceptible strain *S. cerevisiae* X2180-1Aa by indirect immunofluorescence. The indicated yeast strain was incubated with polyclonal α-MP-ab, followed by incubation with a fluorescein isothiocyanate-conjugated anti-rabbit-immunoglobulin G secondary antibody and analyzed by fluorescence microscopy. Bar, 10 μm.

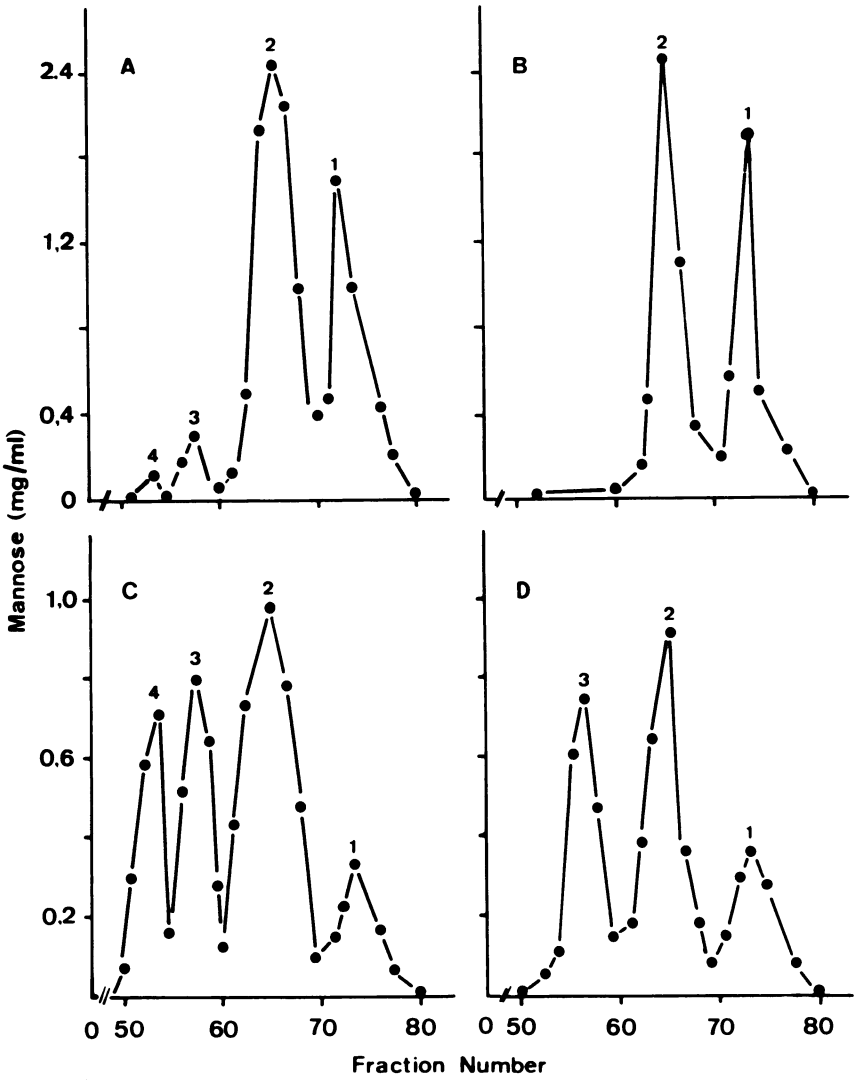

FIG. 3. Acetolysis fingerprints of purified mannoproteins from toxin-resistant mutants, *mnn1* (panel A), *mnn5* (panel B), and 512-R7 (panel D), and the susceptible wild type, 512-WT (panel C). Purified mannoproteins (100 mg of each) of the indicated strains were acetylated in a 1:1 mixture of pyridine and acetic anhydride for 8 h at 100°C before the acetylated products were recovered by evaporation of the solvents at 60°C on a rotary evaporator (11). The products were acetolyzed for 13 h at 40°C in a mixture (10:10:1) of acetic acid, acetic anhydride, and sulfuric acid, respectively. The reaction was terminated by adding pyridine (20 ml). The acetolyzed fragments were extracted twice with chloroform and deacetylated in dry methanol with sodium methoxide before fractionation on a column (2.5 by 100 cm) of BioGel P-2 (Bio-Rad Laboratories) by elution with 0.1 M Sorensen phosphate buffer (0.1 M KCl; pH 6.8). The carbohydrate peaks correspond to mannose (peak 1), mannobiose (peak 2), mannotriose (peak 3), and mannotetraose (peak 4).

(Fig. 3A and B) showed two major carbohydrate peaks, with retention volumes corresponding to the mannosides mannose (man_1) and mannobiose (man_2). The oligosaccharides mannotriose (man_3) and mannotetraose (man_4) which represent side chains consisting of two and three mannose residues, were only detectable in comparatively low quantities in mutant *mnn1* and not at all in mutant *mnn5*. This indicates that both mutants possess side chains mainly with one single mannose residue. The unexpected but low number of side chains containing three mannose units in mutant *mnn1* is likely to be the reason for its reduced killer toxin-binding capacity and the comparatively weak susceptibility of this strain (12). Analysis of side chain patterns from mannoproteins of the susceptible wild-type strain 512-WT and the corresponding resistant mutant 512-R7 (Fig. 3C and D) identified the latter as a prototype of a *mnn1* mutant, lacking all mannotetraose oligosaccharides in the acetolysis fingerprint. This omission, which represents a lack of mannotriose side chains in vivo, was shown to prevent successful binding of KT28 and to cause resistance to the toxin at the level of the cell wall mannoprotein. Binding capacities of crude and partially purified mannoproteins from the resistant mutant 512-R7 and the corresponding wild type were measured indirectly by determining their effects on the survival rate of toxin-treated sensitive yeast cells (Fig. 4). The mannoproteins from the mutant strain, lacking all mannotriose side chains in the outer part of the molecule, were unable to act as a primary KT28 receptor, whereas the unaltered mannoprotein of the susceptible wild type retained its killer toxin-binding capacity. This and additional adsorption experiments with intact cells of both strains (data not shown) led to the conclusion that the outer mannotriose side chains

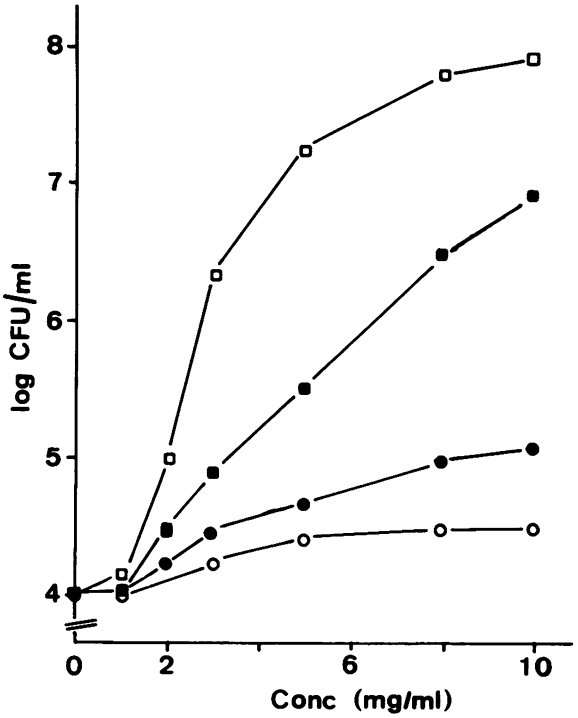

FIG. 4. Influence of crude and partially purified mannoproteins of the susceptible wild type 512-WT and the resistant mutant 512-R7 on the survival rate of killer toxin-treated cells of the susceptible strain *S. cerevisiae* 381. ■, Crude 512-WT; □, partially purified, 512-WT; ●, crude, 512-R7; ○, partially purified, 512-R7. The KT28-resistant mutant 512-R7 was isolated after mutagenesis of strain 512-WT with nitrosoguanidine at a concentration of 0.5 mg/ml (1). After an incubation at 30°C for 25 min, surviving cells (about 0.1%) were diluted into 10 volumes of yeast extract-peptone-dextrose medium (pH 5.0; 50 mM $CaCl_2$) and incubated at 30°C for 12 h to allow expression of the mutant phenotype. Thereafter, the cells were treated with killer toxin KT28 (2.8×10^3 U/ml) at 22°C for 48 h and toxin-resistant mutants were screened by replication onto methylene blue agar plates (pH 5.8) seeded with 10^5 cells of the susceptible strain, 381.

of the 185-kilodalton cell wall mannoprotein PMP represent the actual killer toxin-binding domains of the KT28 cell wall receptor.

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