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PRIMARY EFFECTS OF YEAST KILLER TOXIN

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SUMMARY

Killer toxin from Saccharomyces cerevisiae binds to sensitive cells immediately after addition to the cells. However, 50% mortality was obtained only after 40 minutes. Although it is thought that a lag phase is required for the killer to exert its action, we report experiments showing that the killer starts affecting the cell immediately after binding. Thus, shortly after addition the toxin was able to inhibit the transport of L-|^{3}H|leucine as well as that of protons which are cotransported with this amino-acid or with histidine. Moreover, killer toxin inhibited the pumping of protons to the medium by cells which were actively metabolizing glucose. These effects were a function of the concentration of toxin used. The results suggest that killer toxin acts by affecting the electrochemical proton gradient across the plasma membrane of yeast.

INTRODUCTION

Killer strains of yeasts have been known for several years(1). They produce an extracellular toxin that kills sensitive cells(2) and which only recently has been purified to homogeneity(3). Research on the mechanism of action of the toxin has been scant as compared to genetic studies(4) and evidence has been presented that treatment of sensitive cells with killer toxin induces a leakage of potassium ions(5) and ATP(6). Kotani et al.(7) have found that killer toxin from sake yeast is also able to induce ATP leakage from sensitive cells. However, all these effects were observed with a delay of 40 minutes to 1 hour after addition of the toxin to the cells. Since this is the time at which dead cells are observed, the above described effects might be a final consequence rather than a prima-

1) Abbreviations. DNP: 2,4 dinitrophenol; CCCP: carbonyl cyanide-m-chlorophenyl hydrazone; YEPD medium: medium containing 1% yeast extract, 2% peptone and 2% glucose; YEPAD: as YEPD plus 2% agar.

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ry effect. In this paper we report experiments showing that partially purified killer toxin from S. cerevisiae inhibits the transport of aminoacids which are cotransported with protons as well as the proton uptake and the efflux of protons from cells which are actively metabolizing glucose. These seem to be primary effects since they are observed immediately after binding. They are toxin dependent and toxin specific since identical preparations from an isogenic nonkiller strain do not affect sensitive cells.

MATERIALS AND METHODS

Strains and chemicals. All the strains of Saccharomyces cerevisiae used in this work were from the laboratory of Dr. G. Fink (Cornell University. Ithaca, N.Y.) and were a gift of Dr. J. Conde (Cervezas Cruzcampo, Sevilla). Strain X 17/17 (α,his 1) was used as sensitive strain. Strain A 8207 B was used as a killer producer and the nonkiller strain derived from it A 8207 B-NK1 (both α, his 4)(8) was used as control.

L-[^{3}H]Leucine was from The Radiochemical Centre, Amersham; CCCP[1] was from Calbiochem; DNP[1] and Glucose were from Merck and the aminoacids were obtained from Sigma.

Preparation of toxin and assay of activity. Toxin was obtained from cultures of strain A 8207 B grown at 22°C in ultrafiltrated YEPD[1] medium(3) supplemented with 400 mg/l of adenine, 30 mg/l of histidine and 30 mg/l of leucine. The medium was buffered with 0.25 M citrate phosphate pH 4.7. Culture supernatants were ultrafiltrated as described by Palfree and Bussey(3). After ultrafiltration a precipitation with 80% ammonium sulfate was carried out. Identical preparations from culture supernatants of the nonkiller isogenic strain A 8207 B-NK1 were used throughout the work. Killer toxin activity was assayed using the well-test method of Woods & Bevan(2). The central well was 5 mm in diameter and a linear relationship between the diameter zone from 8 to 18 mm and the logarithmic of toxin concentration was obtained. A unit of killer toxin was defined as the amount giving a clear zone of 13 mm.

Binding of killer toxin and counting of viable cells. Cells of strain X 17/17 were grown to logarithmic phase, collected and resuspended in the same medium containing killer activity. The final cell concentration was $3x10^7$ cells/ml. A control without toxin was run in parallel. At intervals, 100 µl aliquots were withdrawn in which the remaining killer activity in the supernatant was determined after separating the cells by centrifugation at 3000xg for 5 minutes. The number of viable cells was determined by colony forming activity on YEPAD[1] medium after the appropiate dilution of a different aliquot.

L-[^{3}H]Leucine transport assay. Cells from strain X 17/17 were collected at log phase and resuspended at a concentration of $2x10^8$ cells/ml. This cell suspension was incubated in 25 mM glucose,5 mM citrate phosphate buffer, pH 4.7 containing 100 mM NaCl. After 30 minutes the cells were washed with the same buffer without glucose and transport assayed as follows: 100 µl aliquots of cell suspen-

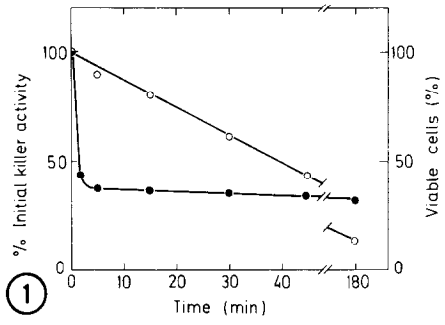
sion were preincubated at room temperature with killer toxin or uncouplers and subsequently L-|^{3}H|leucine (0.5 µCi, 100 µM final concentration) was added. At intervals, 2 ml of cold incubation buffer was added to one aliquot and immediately filtered through GF/C Whatman filters. After washing with another 2 ml of buffer and drying, the radioactivity was determined in the filter in a Beckman liquid scintillation counter.

Measurement of pH. pH was continously monitored with an electrode GK 240 (Radiometer) coupled to a pH meter PHM 64 (Radiometer). The determinations were made in a water-jacketed vessel in a total volume of 3 ml containing $4x10^8$ cells. Aminoacids were used at a final concentration of 0.8 mM and glucose concentration was 2.5 mM.

RESULTS

Binding of toxin and killing of sensitive cells. Figure 1 shows that killer toxin binds to sensitive cells quite rapidly and efficiently. Within 10 minutes most of the available toxin was already bound and the amount of toxin bound was a function of the cell number (not shown). However, when the number of viable cells was determined a totally different pattern was observed. Even though the toxin was bound, the number of viable cells decreased very slowly, with 50% mortality being obtained only after 40 minutes.

Effect of killer toxin on aminoacid and proton uptake. Since any effect observed after the onset of killing could be a general effect rather than a killer specific effect, we looked for killer effects which occurred immediately after the binding. Figure 2b shows that killer toxin inhibited the uptake of L-|^{3}H|leucine. Most importantly, the effect was comparable to that of proton conductors 2,4 dinitrophenol and CCCP. Since this aminoacid is cotransported with protons, it should be expected that the transport of the ion is also inhibited. Indeed, when leucine was added to a cell suspension and the pH monitored, an alkalinization of the medium was observed which was inhibited when killer toxin was present (Figure 2a). A similar effect was observed when another proton cotransported aminoacid (histidine) was used. It is important to note that a preparation from the isogenic nonkiller strain A 8207 B-NK1 did



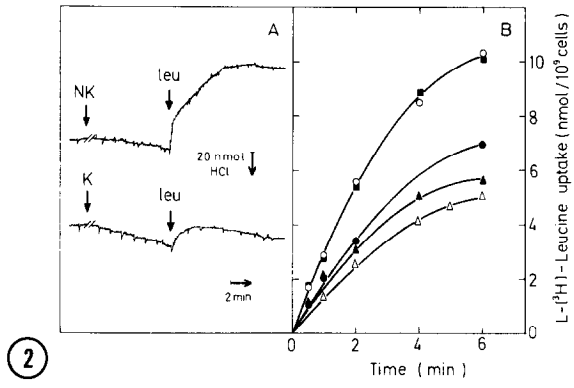


Figure 1.- Binding of killer toxin and killing of sensitive cells

Two ml of a cell suspension of strain X 17/17 was prepared and incubated with 60 U/ml of killer activity as described under Material and Methods. (●) Percentage of initial killer activity in the supernatant. (o) Percentage of viable cells as estimated after incubation for 48 hours at 28°C. Each point represents duplicated samples from two independent experiments.

Figure 2.- Effect of killer toxin on aminoacid and proton uptake

A) pH changes associated with leucine transport. At the times indicated by the arrows the following additions were made: (K) 100 µl of a killer preparation containing 30 units of activity. (NK) 100 µl of a preparation identical to that of killer but obtained from the nonkiller isogenic strain. (leu) Leucine to a final concentration of 0.8 mM.
B) L-[^{3}H]Leucine uptake by X 17/17 cells which have been previously treated for 10 min with: (●) 10 units of killer toxin. (▲) 1 mM DNP. (Δ) 100 µM CCCP. Controls without additions (o) and to which the nonkiller preparation was added (■) were run in parallel.

not have any effect, suggesting that the observed effects are killer specific.

Killer effect on proton pumping. When glucose was added to a yeast suspension an acidification of the medium was observed which is thought to be due to a proton pumping activity. The process, which can be followed by monitoring pH changes in the external medium with a sensitive pH meter, was inhibited almost immediately when killer toxin was added after glucose (Figure 3a). Again, when the preparation was from the nonkiller strain no effect was observed. Inhibition was also obtained by proton conductors; on the other hand, heat inactivated toxin was unable to inhibit the proton pumping. The decrease in the slope (i.e. the inhibition of pumping) was a function of the amount of toxin added (Fig. 3b).

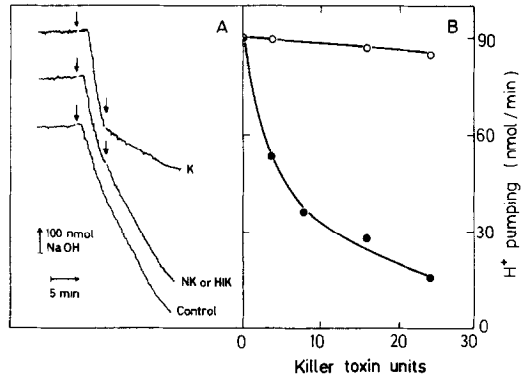


Figure 3.- Killer effect on proton pumping

Cell suspensions of strain X 17/17 containing $4x10^8$ cells in 3 ml of 5 mM citrate phosphate buffer, pH 4.7 containing 10 mM KCl were incubated as described in Material and Methods.
A) pH changes after addition of D-glucose (arrows on the left). At the times indicated (arrows on the right) 100 µl containing 16 units of killer toxin (K) or the same volume from the nonkiller preparation (NK) or a heat inactivated killer toxin (HIK) were added. No further addition was made to the control. Electrode disturbances during additions have not been incorporated into the figure.
B) Plot of proton pumping versus killer toxin concentration (●) or the corresponding volume of preparation from the nonkiller strain(o).

DISCUSSION

Consideration of the survival data together with that of the stability of the yeast potassium pool has led to the suggestion that two phases can be distinguished in killer action: the binding of killer toxin to the cell and the onset of membrane alteration(5). A similar conclusion could be drawn from the experiment shown in Figure 1. However, since a significant number of cells died after 40 minutes of the treatment with the toxin, any effect observed in this late phase might be a consequence of death rather than a primary effect of the toxin. Our results show alterations that are induced by the toxin immediately after binding. The reason why such a high number of cells remain alive for 30 to 40 minutes are not clear yet. It should be considered that the measurement of survival requires manipulation of the toxin treated cells, which could account for the difference ob-

served. It is also possible that there is a period of time during which the lesion initiated by the toxin can be repared, in agreement with the reversible and ireversible phases suggested by Kotani et al.(7) for sake yeast toxin. In any case, the concept of toxin being bound without exerting any action is now clearly unjustified.

The reported inhibition of aminoacid transport (the distinction between transport and incorporation should be emphasized) as well as that of proton transport could be interpreted as the toxin acting on the carrier. However, since the toxin was also able to inhibit proton pumping when glucose was added to a cell suspension (a process in which no carrier is implicated) that possibility is unlikely. The observed effects suggest that killer toxin is able to disrupt the electrochemical proton gradient. The creation of this electrochemical proton gradient has been attributed to an ATP-ase (9) and evidence for this has been presented(10). The results presented in this paper do not permit a distinction as to whether the toxin acts as a proton conductor or as a inhibitor of the proposed ATP-ase, but they could explain the late effects previously observed (5-7). Furthermore, although the suggestion has been made repeatedly(5-7) that killer toxin can be compared in its action to colicins (11), these results provide the first clear evidence: Killer toxin, as is the case for colicins, may act by disrupting an energized membrane state.

REFERENCES

1.- Somers, J.M. and Bevan, E.A. (1969) Genet. Res. 13, 71-83
2.- Woods, D.R. and Bevan, E.A. (1968) J. Gen. Microbiol. 51, 115-126
3.- Palfree, R.G.E. and Bussey, H. (1979) Eur. J. Biochem. 93, 487-493
4.- Wickner, R.B. (1979) Plasmid 2, 303-322
5.- Skipper, N. and Bussey, H. (1977) J. Bacteriol. 129, 668-677

6.- Bussey, H. and Sherman, D. (1973) Biochim. Biophys. Acta 298, 868-875
7.- Kotani, H.; Shinmyo, A. and Enatsu, T. (1977) J. Bacteriol. 129, 640-650
8.- Wodking, M.; Katterman, F. and Fink, G.R. (1974) J. Bacteriol. 117, 681-686
9.- Peña, A. (1975) Arch. Biochem. Biophys. 167, 397-409
10.- Serrano, R. (1980) Eur. J. Biochem. 105, 419-424
11.- Konisky, J. (1978) in The Bacteria; eds. Ornston, L.N. and Sokatch, J.R. (Academic, N.Y.) Vol 6 pp. 71-136