Killing of *Saccharomyces cerevisiae* by the Lysosomotropic Detergent *N*-Dodecylimidazole

MUSADDEQ HUSSAIN,¹ MICHAEL J. LEIBOWITZ,² AND JOHN LENARD^{1*}

Department of Physiology and Biophysics¹ and Department of Microbiology,² University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School,† Piscataway, New Jersey 08854-5635

Received 3 December 1986/Accepted 26 January 1987

The lysosomotropic detergent *N*-dodecylimidazole (C₁₂-Im) has previously been found to kill mammalian cells by concentrating in lysosomes, followed by lysosomal disruption and release of cytotoxic enzymes into the cytoplasm. The action of C₁₂-Im on *Saccharomyces cerevisiae* is described in this report. C₁₂-Im prevented growth of colonies when present in 1% yeast extract–2% Bacto-Peptone–2% glucose plates at concentrations of 5 µg/ml or above, or when present in a soft agar overlay at 20 µg/ml. Treatment of cells suspended in glucose-containing buffer (pH 8.0, 37°C) with C₁₂-Im (6 µg/ml) caused >95% cell death within 6 min. Dependence of killing on C₁₂-Im concentration was sigmoidal, suggesting a cooperative mode of action. Killing was pH dependent, being much more effective at pH 8.0 than at pH 5.0. Ammonium sulfate and imidazole protected against killing if added before, but not after, the addition of C₁₂-Im. Sensitivity to C₁₂-Im was strongly growth dependent: the cells were most sensitive at early to mid-logarithmic phase of growth and became progressively less sensitive during progression through late logarithmic and stationary phase. Vacuolar disruption by C₁₂-Im was demonstrated by using cells loaded with lucifer yellow CH or fluoresceinated dextran in their vacuoles; vacuoles of logarithmically growing cells were more sensitive than those of stationary-phase cells. These results suggest that vacuolar disruption by C₁₂-Im may underlie its cytotoxic effects.

DeDuve et al. applied the term “lysosomotropic” to various weakly basic amines that share the property of becoming concentrated, often several hundredfold, within the lysosomes of mammalian cells to which they are added (2). Lysosomotropic amines possess this property because they diffuse readily across membranes in their uncharged form, but become trapped in their protonated (nondiffusible) form in membrane-bound compartments of low pH. Following this concept, Firestone et al. synthesized several compounds designed to combine lysosomotropism with detergent activity (3–5). One effective lysosomotropic detergent is *N*-dodecylimidazole (C₁₂-Im), which combines imidazole, a base of intermediate pK, with a long hydrocarbon chain. It was reasoned that accumulation of the protonated form of this compound above a certain concentration (related to its critical micelle concentration) should cause it to acquire detergentlike properties, resulting in lysis of the lysosomal membrane and subsequent cell death. Consistent with this expectation, lysosomotropic detergents were found to be cytotoxic to several types of cultured mammalian cells at low concentrations. When added at the same concentrations to erythrocytes, which lack lysosomes, the lysosomotropic detergents had no hemolytic effect (3–5, 13).

Miller et al. have studied the mechanism whereby C₁₂-Im and related compounds kill BHK-21F cells, a line of cultured hamster fibroblasts (13). The results showed a strong association between lysosomal disruption and cytotoxicity. The compounds were shown to accumulate in lysosomes and rapidly to increase the permeability of the lysosomal membranes towards ions and small molecules. Some protection against cell killing was afforded by methylamine, nigericin, and monensin, agents which raise lysosomal pH without damaging lysosomal integrity. Further, effects on the

lysosomes preceded by at least 1 h any other indication of cytotoxicity, such as inhibition of protein or nucleic acid synthesis, cell rounding, or cell leakage (13). Finally, sensitivity of mammalian fibroblasts to C₁₂-Im can be controlled by altering lysosomal enzyme levels, suggesting that release of these enzymes from disrupted lysosomes into the cytoplasm is a cause of cell death (P. D. Wilson, R. A. Firestone, and J. Lenard, *J. Cell. Biol.*, in press).

The yeast *Saccharomyces cerevisiae*, in common with many other “lower” eucaryotes, possesses a vacuole which is considered functionally analogous to the mammalian lysosome: it is acidified by an ATP-driven proton pump, and it contains numerous hydrolytic enzymes (7, 8, 12, 18). These organisms should, therefore, be killed by lysosomotropic detergents in the same way as are mammalian cells. In addition, they offer the advantages over mammalian cells of rapid growth and the possibility of genetic analysis of function (7). This report shows that *S. cerevisiae* cells are killed by C₁₂-Im at substantially lower concentrations than are required to kill mammalian cells. The cytotoxic effects of C₁₂-Im are consistent with a vacuolar site of action.

MATERIALS AND METHODS

Reagents. C₁₂-Im was a gift from R. A. Firestone (Merck Sharp & Dohme Research Laboratories, Rahway, N.J.). Yeast extract, Bacto-Peptone, and Bacto-Agar were from Difco. ¹⁴C-labeled α-aminoisobutyric acid ([¹⁴C]AIB; specific activity, 60 mCi/mmol) was purchased from New England Nuclear Corp. All other chemicals were of reagent grade.

Organisms and growth conditions. *S. cerevisiae* X2180-1A (MATa mal gal2) was used. Some of the experiments were repeated with an isogenic strain of opposite mating type (X2180-1B). Both of these strains, the different *sec* mutants, and the *pep4-3* mutant were obtained from the Yeast Genetic

* Corresponding author.

† Formerly Rutgers Medical School.

Stock Center (Berkeley, Calif.). Cells were cultivated in 1% yeast extract–2% Bacto-Peptone–2% glucose (YPD) medium at 30°C with shaking. Growth was monitored by turbidity, measured as A_{650} .

Assays for C_{12} -Im action. In initial experiments cells were either plated on YPD plates containing different concentrations of C_{12} -Im, or diluted in soft agar (YPD with 0.5% Bacto-Agar) at 42°C, containing various concentrations of C_{12} -Im, and poured onto YPD plates. Colonies were counted after growth at suitable temperature for 3 days.

The following procedure was used to study the sensitivity of cells grown in liquid culture and to assess more precisely the effects of specific treatments. Cells were diluted in 20 ml of sterile buffer (0.2 M sodium phosphate, 2% glucose, pH 8.0, or as indicated) to a final concentration of 30,000 to 40,000 cells per ml and incubated at room temperature or 37°C for 5 to 60 min. To this suspension was added 20 μ l of an ethanolic solution of C_{12} -Im (6 mg/ml or as indicated). Immediately before addition of C_{12} -Im (0 time), and 2, 4, and 6 min after addition, portions of the cell suspension were withdrawn and diluted 10-fold with 0.2 M sodium phosphate–2% glucose (pH 6.5). One hundred microliters of the diluted cells was plated on YPD plates, and viable colonies were counted after growth. Reported counts represent the average of three identical plates.

Cells were loaded with fluorescein isothiocyanate-dextran or lucifer yellow CH by the method of Makarow (11) or Chvatchko et al. (1), respectively. Cells were suspended in a small volume of phosphate-buffered saline containing 50% glycerol and applied to microscope slides pretreated with 0.5 mg of concanavalin A (Pharmacia, Inc., Piscataway, N.J.) per ml.

Carboxypeptidase Y activity was assayed by the method of Herber (W. Herber, Ph.D. thesis, Rutgers University, Piscataway, N.J., 1983), which is a modified procedure of Jones (6).

Measurement of cell leakage. *S. cerevisiae* cells were loaded with [14 C]AIB, and the efflux was measured as described by Komor et al. (9) with some modifications. Cells were suspended in 3.5 ml of 0.2 M sodium phosphate–2% glucose (pH 6.0)–[14 C]AIB (43 μ Ci/ml) to a final A_{650} of 100 and incubated at 30°C for 2.5 h. Cells were then pelleted and washed with 20 ml of 0.2 M sodium phosphate–2% glucose (pH 8.0) and suspended in 2 ml of the same buffer. One hundred microliters of this cell suspension was then diluted to a final volume of 40 ml in the same buffer, with or without the detergent to be tested. Five milliliters of this suspension was filtered through a Millipore GS filter (0.22- μ m pore size) at stated time intervals, and the filter was then washed twice with the same buffer and counted (with Liquiscint) in a scintillation counter.

RESULTS

***S. cerevisiae* cell killing by C_{12} -Im.** *S. cerevisiae* cells failed to grow on YPD plates containing 5 μ g or more of C_{12} -Im per ml. In contrast, no inhibition of growth was seen on YPD plates containing 10 μ g of the denaturing detergent sodium dodecyl sulfate per ml. When cells were suspended in soft agar (3 ml) containing C_{12} -Im and poured on YPD plates (20 ml) containing no detergent, the presence of 20 μ g of C_{12} -Im per ml in the soft agar resulted in complete inhibition of growth. Cells of both mating types showed similar sensitivity.

Cell killing by C_{12} -Im was also tested in glucose-containing buffer at pH 8.0. Cell killing was detectable on concentra-

tions of C_{12} -Im as low as 3.56 μ g/ml and was nearly complete after 6 min of exposure to 7.12 μ g/ml (Fig. 1). If the reciprocal of the time needed for 50% killing is used as a measure of toxicity, a sigmoidal dose response is found (Fig. 1, inset). Sigmoidal dose dependence has been reported previously for mammalian cells and is expected if the cytotoxic action of C_{12} -Im is cooperative, i.e., if several molecules must act together to kill a cell (13). Note that the times shown in Fig. 1 are those at which the C_{12} -Im was effectively diluted out of the medium, and that survival was determined by subsequent growth. Killing, therefore, either is complete within these few minutes or, more likely, arises from an irreversible interaction between C_{12} -Im and the cells.

Effects of pH and amines. Killing by C_{12} -Im is strongly pH dependent (Fig. 2). Fifty percent killing occurred in less than 3 min at 5 μ g/ml at pH 8.0, but required ca. 20 min at 20 μ g/ml at pH 5.0; intermediate sensitivity was found at pH 6.5. Poole and Ohkuma (14–16) showed in mammalian cells that the final intralysosomal concentration of protonated amine depended upon the concentration of unprotonated amine present in the medium, and similar results have been reported in *S. cerevisiae* (10). Since the concentration of unprotonated C_{12} -Im increases dramatically over the pH range 5 to 8 (3–5), the observed pH dependence is consistent

FIG. 1. Dependence of cell survival upon time of exposure to different concentrations of C_{12} -Im. Cells were grown to early stationary phase ($A_{650} = 59$) and suspended in 2% glucose–0.2 M sodium phosphate buffer (pH 8.0). C_{12} -Im was added to achieve the following final concentrations (μ g/ml) at $t = 0$: $\bullet$, 0; $\circ$, 1.78; $\triangle$, 3.56; $\square$, 5.34; ∇ , 7.12. Inset: Dependence of $1/t_{50}$ (time required for 50% killing) upon C_{12} -Im concentration, taken from the data of Fig. 1.

FIG. 2. pH dependence of cell killing. Cells were grown to late logarithmic phase ($A_{650} = 25$) and suspended in 2% glucose–0.2 M sodium phosphate buffer adjusted to pH 5.0 (A), pH 6.5 (B), or pH 8.0 (C). The following concentrations of C_{12} -Im (in μ g/ml) were added at $t = 0$: $\circ$, 0; ∇ , 5; Δ , 10; $\square$, 20.

with mediation of cell killing by intravacuolar C_{12} -Im accumulation.

Accumulation of high levels of protonated C_{12} -Im in the vacuole should depend also upon low intravacuolar pH. Intravacuolar pH is expected to be raised by the addition of

FIG. 3. Protection by amines from C_{12} -Im-mediated cell killing. Cells in the logarithmic phase of growth ($A_{650} = 14$) were suspended in 2% glucose–0.2 M sodium phosphate (pH 8.0). Two minutes before the addition of C_{12} -Im (6 μ g/ml), amines were added to the indicated final concentrations: $\bullet$, no amine; $\square$, 30 mM imidazole; Δ , 30 mM ammonium sulfate; $\blacktriangle$, 100 mM ammonium sulfate; $\circ$, no C_{12} -Im, with or without addition of 30 mM imidazole or 100 mM ammonium sulfate.

nondetergent amines to the medium (14–16). Ammonium sulfate, and, less effectively, imidazole, provided protection against cell killing by C_{12} -Im (Fig. 3). Both agents protected the cells as shown when added 2 min before the addition of C_{12} -Im. When added 2 min after C_{12} -Im addition, however, they failed to protect the cells. Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone, a proton ionophore, also protected the cells from killing by C_{12} -Im (data not shown). These experiments are also consistent with the idea that cell killing results from the accumulation of lethal levels of C_{12} -Im in the acidic vacuolar lumen.

Vacuolar disruption induced by C_{12} -Im was directly visualized by fluorescence microscopy. Cells accumulated the fluorescent dye lucifer yellow CH in their vacuoles after 45 min of exposure to the dye (4 to 100 mg/ml) in the presence of high concentrations of dextran (200 mg/ml). In most cells, the dye was sequestered in discrete vacuoles after this treatment (Fig. 4B). Subsequent treatment with 6 μ g of C_{12} -Im per ml for 2 min disrupted most of the vacuoles (Fig. 4D) in logarithmically growing cells, leaving a diffuse fluorescence throughout the cell. Similar effects were seen when cells loaded with fluorescein isothiocyanate-dextran were used (not shown).

Growth dependence of C_{12} -Im killing. Cell killing depends strongly upon the growth state of the cells (Fig. 5). Cells were most sensitive during the early logarithmic phase of growth and became progressively less sensitive through late logarithmic and stationary phase. This is reminiscent of the behavior of mammalian monolayer cells, which became progressively more resistant to lysosomotropic detergents as they progressed from a sparse, rapidly growing condition through higher cell densities to confluency (13; Wilson et al., in press).

Komor et al. (9) have reported that several different organisms, including *S. cerevisiae*, became markedly resistant to leakage caused by Triton X-100 or deoxycholate when energy metabolism was inhibited by uncouplers or other agents. In view of the growth dependence of sensitivity in both *S. cerevisiae* (Fig. 5) and mammalian cells (13; Wilson et al., in press), it was of interest to determine whether the action of C_{12} -Im resembled that of Triton X-100. We therefore compared the effects of C_{12} -Im and Triton X-100 on AIB leakage and cell killing (Fig. 6). The rate of leakage of AIB from C_{12} -Im-treated cells actually lagged slightly behind the rate of cell killing (compare solid circles,

FIG. 4. Disruption of vacuoles by C_{12} -Im. Cells (3×10^7 /ml) in logarithmic phase were loaded with lucifer yellow CH, treated with C_{12} -Im (25 μ g/ml) for 2 min, and visualized by phase-contrast (A, C) or fluorescence (B, D) microscopy. (A, B) Cells before C_{12} -Im treatment, showing phase-dense material and lucifer yellow CH concentrated in vacuoles. (C, D) Cells after C_{12} -Im treatment, showing diffuse cytoplasmic staining by lucifer yellow CH due to vacuolar disruption.

Fig. 6A and B). Both killing and leakage were substantially prevented by the action of ammonium sulfate (open circles, Fig. 6A and B). The leakage of AIB induced by C_{12} -Im thus appears to follow cell killing and probably represents a consequence of cell death, as does release of the cytoplasmic enzyme lactate dehydrogenase from mammalian cells (13; Wilson et al., in press). In marked contrast, Triton X-100 induced substantial leakage of AIB, but only negligible cell death (solid circles, Fig. 6C and D). The addition of ammo-

nium sulfate substantially increased the rate of killing by Triton X-100 (for unknown reasons), but did not affect AIB leakage.

The most likely site of action of Triton X-100 in inducing leakage of AIB is the plasma membrane, since AIB accumulates in the cytoplasm. Partial disruption of the plasma membrane, sufficient to induce leakage of small molecules, is evidently not a lethal event (Fig. 6C). Comparison of C_{12} -Im and Triton X-100 therefore suggests that these compounds have quite different effects on *S. cerevisiae* cells.

Effects of defined mutations on sensitivity to C_{12} -Im killing. Schekman and co-workers have characterized 24 different temperature-sensitive mutants (*sec*) with defects in various aspects of protein secretion (17). We tested 23 of these (all except the *sec21* mutant) for resistance to C_{12} -Im at both restrictive and permissive temperatures; none differed significantly from wild type when compared with wild-type cells in the appropriate growth state.

Jones has described a mutant, bearing the *pep4-3* mutation, which is characterized by low levels of several vacuolar proteases (6, 7). This pleiotropic effect apparently arises because the *PEP4* gene product that is defective in this mutant is itself a protease, required for the proteolytic activation of some (but not all) of the vacuolar enzymes. Since specific lysosomal enzymes have been shown to be involved in the cytotoxicity of C_{12} -Im to mammalian cells (Wilson et al., in press), this mutant was examined. No differences in sensitivity were found between the *pep4-3* mutant and wild-type cells. Carboxypeptidase Y activity was ca. 10% as high in mutant as in wild-type cells and did not rise after C_{12} -Im treatment, ruling out the possibility that cytotoxic vacuolar enzymes were being activated after leak-

FIG. 5. Dependence of sensitivity to C_{12} -Im upon growth state. Cells were diluted and exposed to C_{12} -Im under standard conditions (see Materials and Methods) at points indicated in the growth curve (A). (B) Survival curves after dilution.

FIG. 6. Comparison of effects of C_{12} -Im and Triton X-100 on cell survival and AIB leakage. Cells were grown to late logarithmic phase ($A_{650} = 20$) and tested for cell killing (A, C) and AIB leakage (B, D) in suspensions of 3×10^7 to 4×10^7 cells per ml (ca. 1,000 times the number used in previous figures). (A, B) Effects of C_{12} -Im (6 μ g/ml) in the presence (Δ) or absence ($\bullet$) of 100 mM ammonium sulfate; $\circ$, no C_{12} -Im added. (C, D) Effect of Triton X-100 (0.3%, vol/vol) in the presence (Δ) or absence ($\bullet$) of 100 mM ammonium sulfate; $\square$, 100 mM ammonium sulfate alone; $\circ$, no additions.

age into the cytoplasm. It appears, therefore, that the vacuolar enzymes requiring *PEP4* for activation are not the cytotoxic agents.

DISCUSSION

In this paper, we report several experiments which, taken together, provide evidence that C_{12} -Im kills *S. cerevisiae* cells by interaction with the vacuoles, the only acidic organelles known in this organism; (i) killing occurred with a sigmoidal dose dependence on C_{12} -Im; (ii) killing was strongly pH dependent; (iii) ammonium sulfate and imidazole protected against killing by C_{12} -Im; (iv) vacuolar disruption by C_{12} -Im was directly observed by fluorescence microscopy; (v) the action of C_{12} -Im on cells was quite different from that of the nonionic detergent Triton X-100. This conclusion is similar to that reached previously for mammalian cells (13; Wilson et al., in press), although quite different experiments were done. *S. cerevisiae* cells offer a versatility and ease of handling that makes cell killing studies faster, easier, and more precise than the studies of mammalian cell monolayers previously described.

Growth dependence provides another point of similarity between mammalian cells and *S. cerevisiae* in their responses to C_{12} -Im. In this study, maximal sensitivity was found for *S. cerevisiae* in early to mid-logarithmic stage of growth, with increasing resistance as cells went through late logarithmic growth and into stationary phase. Mammalian cells are most sensitive in sparse, rapidly growing monolayers and become increasingly resistant as they approach confluency. The basis for this striking growth dependence is

not yet known, but may well be similar in *S. cerevisiae* and mammalian cells.

The studies reported here provide a basis for selection of lysosomotropic detergent-resistant mutants. These might be defective in important vacuolar proteins, thus providing valuable tools for the study of vacuolar function and proving the postulated vacuolar site of action of lysosomotropic detergents at the same time. Other mutants might provide an insight into the lethal events that are assumed to occur in the cytoplasm after vacuolar disruption and leakage.

It is interesting that *S. cerevisiae* cells are significantly more sensitive to killing by C_{12} -Im than the mammalian cells studied previously. Direct comparisons are difficult, owing to the sigmoidal dose dependence (Fig. 1), the pronounced dependence on growth state (Fig. 4), and the different assay techniques employed, but 5 to 10 times more C_{12} -Im is routinely used in studies of most mammalian cell lines. This difference cannot arise from differences in pH between lysosomes and vacuoles, since yeast vacuolar pH is considerably higher than mammalian lysosomal pH (N. Greenfield, M. Hussain, and J. Lenard, manuscript in preparation), resulting in a smaller pH gradient between extracellular medium and vacuoles than exists between medium and lysosomes in mammalian cells. Although the basis for the sensitivity difference is not known, it opens the possibility that antifungal applications might be found for these compounds.

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