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Submicellar Complexes May Initiate the Fungicidal Effects of Cationic Amphiphilic Compounds on *Candida albicans*

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The killing of *Candida albicans* by a series of amphiphilic quaternary ammonium compounds (QACs) with different hydrocarbon chain lengths was closely related to the binding of the compounds to the cells and damage of the cell membranes. The membrane damage was measured as the level of release of the UV-absorbing material into the medium in which the cells were suspended and as the level of uptake of propidium iodide in individual cells by flow cytometry. It was shown that of the compounds tested, hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide [CTAB]) bound most efficiently. Tetradecyl betainate chloride (B14), tetradecanoylcholine bromide (C14), tetradecyltrimethylammonium bromide (TTAB), and dodecyltrimethylammonium bromide (DTAB) followed and had declining degrees of binding efficiency. The proportion of CTAB bound was almost total at concentrations up to the critical micelle concentration (CMC) of the compound, whereas that of B14 was somewhat smaller. For the two remaining tetradecyl compounds (C14 and TTAB), still smaller proportions were bound at low concentrations, but the proportions rose disproportionally at increasing concentrations to a distinct maximum at concentrations of 0.2 to 0.5 times the CMC. We propose that interfacial micelle-like aggregates are formed at the cell surface as a step in the binding process. An analogous, but less conspicuous, maximum was seen for DTAB. Thus, great differences in the binding affinity of QACs with different hydrocarbon chains at different concentrations to *C. albicans* were observed. These differences were related to the CMC of the compound. In contrast, the binding of TTAB to *Salmonella typhimurium* 395 MS was almost total at low as well as high concentrations until saturation was attained, indicating fundamental differences between binding to the yeast and binding to gram-negative bacteria. The importance of lipid-type complexes or aggregates to the antifungal effect of membrane-active substances are discussed.

In recent years, invasive fungal infections have become a more frequent and serious threat, particularly in immunocompromised patients. Diagnosis is difficult, and only a few antifungal agents are available for treatment. To achieve a fungicidal effect, amphotericin B is often the only drug available. Due to its hydrophobicity, it is usually solubilized into deoxycholate or phospholipid complexes before injection. It causes, however, acute and chronic toxicities after intravenous injection. The type and severity of the toxic reactions are related to the mode of complexing.

Amphotericin B is an amphiphilic heptaene that is readily complexed with deoxycholate or phospholipids in vitro into aggregates. The shape and size of these aggregates are related to the compounds and concentrations used. The drug binds preferentially to ergosterol of the fungal cell membrane, forming pore-like structures and causing leakage of potassium ions and other cell constituents (3). The mechanism of transfer of amphotericin B from complex to cell membrane is, however, poorly understood. Such complexes may participate in the fungicidal process or may mainly serve as storage and transport vehicles. Since amphotericin B and related polyene compounds are difficult to synthesize and radiolabel, we have started some mechanistic and kinetic studies on the binding to and antimicrobial action of a series of amphiphilic quaternary ammonium compounds (QACs) on *Candida albicans*. These compounds, like amphotericin B, interact with membranes of mammalian, fungal, and bacterial cells, causing leakage of intracellular material and finally cell death. QACs as a compound group have an infinite number of structural variations. We have chosen to work with stable monoalkyltrimethyl ammonium salts, which have 12 to 16 carbon atoms in the alkyl chain, and also with similar substances in which a cleavable moiety has been incorporated into the QAC molecule, i.e., esters of betaine and choline. These esters are subject to base- and enzyme-catalyzed hydrolysis, respectively, into nontoxic components and may thus be used as soft antimicrobial agents (1, 7, 24) and for drug targeting. In the action on *C. albicans*, they may play both roles.

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MATERIALS AND METHODS

Chemicals and reagents. Dodecyltrimethylammonium bromide (DTAB) and tetradecyltrimethylammonium bromide (TTAB) were from Sigma Chemical Co. (St. Louis, Mo.). Hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide [CTAB]) was from Fluka AG (Buchs, Switzerland). The synthesis of ^{3}H-CTAB was described earlier (35), and ^{3}H-TTAB and ^{3}H-DTAB were synthesized by procedures (Fig. 1) analogous to those described previously (35).

Tetradecanoylcholine bromide (C14) and ^{3}H-C14 were synthesized as described earlier (5). Electrospray ionization mass spectrometry analysis of the compounds gave a spectrum containing only the M^{+} molecular ion (M = 314) (Fig. 1). The procedures used to synthesize tetradecyl betainate chloride (B14) and ^{3}H-B14 were slight modifications of versions described earlier (34). The radiochemical purities of the products were verified by high-pressure liquid chromatography with on-line β-radioactivity detection. All products were found to have >99% radiochemical purity (Fig. 1).

The nucleic acid-binding fluorochrome propidium iodide (PI) and the fluorescent reagent 1,6-diphenyl-1,3,5-hexatriene (DPH) were from Sigma Chemical Co.

Yeast strain and growth conditions. *C. albicans* CCUG 35309 (Culture Collection University of Göteborg) was a recent isolate from a clinical specimen and was previously found not to show any enzymatic activity toward alkanoylcholines (1). The strain was grown in Sabouraud broth on a rotary shaker at 37°C for 24 h.

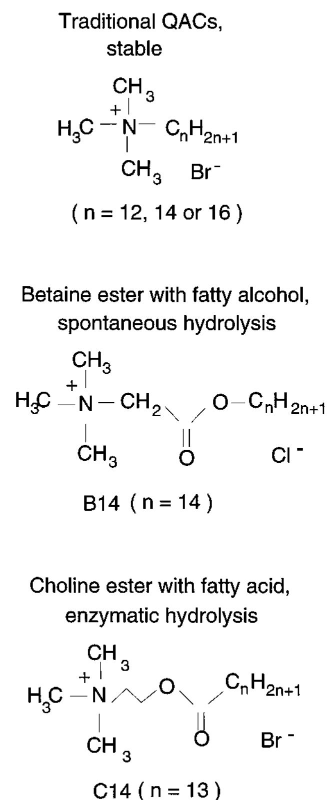

FIG. 1. Structures of the QACs studied.

The yeasts were harvested by centrifugation and washed once with phosphate-buffered saline (PBS) solution. The pelleted cells were diluted in 0.01 M phosphate buffer (pH 7.0) to a cell concentration of 2.0×10^8 to 2.2×10^8 CFU ml^{-1}. *C. albicans* appeared predominantly as blastoconidia.

The bacterial strain *Salmonella typhimurium* 395 MS, a smooth strain, has been described earlier (6). The bacteria were cultured in broth at 37°C for 16 h on a rotary shaker, washed once with PBS, and diluted in buffer to a cell concentration of 4.9×10^{10} CFU ml^{-1}.

Exposure of yeast cells to QACs. The susceptibility of *C. albicans* to the QACs was determined by exposing a 100-μl portion of the yeast suspension to serial dilutions of the compounds in 0.01 M phosphate buffer (900 μl) at pH 7.0 and 37°C for 10 min. Four different methods were used to study the effect.

(i) Measurement of the amount of substance bound to the cells. After incubation, the cells were sedimented by centrifugation at 12,500 rpm (Microfuge E; Beckman) for 5 min. The radioactivity in the supernatant was measured with a liquid scintillation counter (LKB 1217; LKB Wallac). The amount of bound substance was calculated by subtraction of the amount of radioactivity in the supernatant from the total amount added to the cells.

(ii) Release of intracellular material. After incubation and centrifugation the absorbance (A) of the supernatants was measured in a spectrophotometer (Shimadzu UV-240) at 260 and 280 nm. The highest value at 260 nm was taken as the maximum release (A_{Max}), and the percentage of liberated intracellular material was calculated by the following equation: $R = 100(A_{Obs} - A_{Contr})/(A_{Max} - A_{Contr})$, where R is the released fraction, A_{Obs} is the measured value for the sample, and A_{Contr} is the value for a sample not treated with a QAC.

(iii) PI uptake in individual cells. PI is a nucleic acid-binding fluorochrome largely excluded by intact cell membranes (10). After incubation of the yeast cells in a series of concentrations of QACs for 10 min as described above, the suspension was diluted 10-fold in PBS, PI was added to a concentration of 25 μg ml^{-1}, and the suspension was further incubated on a rotary shaker for 30 min at 37°C in the dark. Cells diluted in buffer alone and cells suspended in 70% ethanol before being exposed to PI were used as references for intact and permeated cells, respectively (10). The cell size and PI fluorescence intensity for 10,000 cells were determined by flow cytometry (FACScan; Becton Dickinson), and the percent PI-positive cells among 10,000 cells was calculated.

(iv) Viable count. After the 10-min incubation, the samples were diluted 100-fold in Letheen broth (Thiotone peptone [BBL Microbiology Systems, Cockeysville, Md.], 10.0 g; beef extract, 5.0 g; lecithin, 0.7 g; polysorbate 80, 5.0 g; sodium chloride, 5.0 g; distilled water, 1,000 ml). This medium inactivates QACs and is recommended as a neutralizing diluent for the evaluation of disinfectants containing cationic surfactants (2). The suspension and dilutions were then plated with a spiral plater (Spiral Plater model D; Spiral Systems Inc., Cincinnati, Ohio) onto Sabouraud agar plates. After incubation at 37°C for 2 days, the surviving fraction was determined.

Bactericidal activity toward *Salmonella* was measured by the same methods; however, cultivation was on nutrient agar. PI incorporation was not studied with bacteria.

CMC determination. The critical micelle concentrations (CMCs) of the QACs were determined with a fluorescent probe, DPH, in 0.01 M phosphate buffer (pH 7.0). At concentrations close to the CMC there is an increase in fluorescence intensity due to the entrapment of the fluorescent molecules in the hydrophobic environment of the micelles (4). Two milliliters of serial dilutions of the QACs was mixed with the probe (1 μl; stock solution of 10 mM in tetrahydrofuran). The test tubes were incubated for 30 min (with B14 for 5 min) in the dark at 37°C, and the fluorescence intensity was measured in a Shimadzu RF-535 spectrofluorimeter at an excitation wavelength of 358 nm and an emission wavelength of 430 nm.

Determination of aggregates on the cell surface. In order to determine if aggregate formation of the QAC molecules on the cell surface could be visualized microscopically, the fluorescence intensity of DPH in TTAB-treated cell suspensions was estimated. The temperatures of buffer solutions (450 μl) containing a series of concentrations of up to 0.4 mM TTAB were adjusted to 37°C. After 10 min, a 50-μl cell suspension was added and after an additional 1 min, 1 μl of DPH was added. After 2 min of incubation and a 5-s centrifugation step, the fluorescence intensity of the cells was observed in an epifluorescence microscope (Zeiss Standard 18) equipped with a UV filter set (Zeiss G365/FT395/LP420). The fluorescence intensity from similarly treated cell suspensions, without the centrifugation step, was measured in the spectrofluorimeter as described above for the CMC determination.

RESULTS

The binding isotherm for ^{3}H-CTAB showed that at the lowest concentration tested (0.003 mM) all ^{3}H-CTAB was bound to *Candida* cells (Fig. 2A and 3). When the concentration was increased in twofold increments to 0.1 mM, close to stoichiometric binding was seen, since nearly all CTAB bound to the yeast cells. Above that concentration, increasing proportions of the ^{3}H-CTAB were found in the soluble fraction: 50% at 1 mM and 80% at 2.6 mM (Fig. 3). A slight fungicidal effect was seen at 0.0125 mM CTAB, reaching 90% at 0.063 mM and close to complete killing at 0.1 mM. A similar concentration dependence was seen for the release of UV light-absorbing material. However, at concentrations of 0.8 mM CTAB and above, lower concentrations of solubilized UV-absorbing material was seen, probably due to precipitation of released material. Concentrations of CTAB approximately twofold higher than those needed for killing were required before the yeast cells were liable to uptake of PI, and slightly lower concentrations were required for cell leakage.

When the PI fluorescence of 10,000 individual *C. albicans* cells was measured by flow cytometry, two distinct populations of cells appeared when all the different QACs were tested. The nonfluorescent population (live cells), as represented by control cells (Fig. 4A), consisted of cells showing fluorescence intensities below 22 fluorescence units (FU), with a mean of ca. 8 FU. The fluorescent population (dead cells) was made up of cells showing fluorescence greater than 50 FU. In Fig. 4, the results for C14 are shown. The fraction of dead cells and the mean fluorescence intensity of the dead cells increased at increased concentrations of C14 (Fig. 4B to F). In the untreated *C. albicans* control cell suspension, >99% of the cells were nonfluorescent (Fig. 4A), whereas among cells treated with 70% ethanol, >99% were fluorescent (data not shown). Treatment with QACs increased the fraction of fluorescent cells, as shown in Fig. 2A to E and 4A to F.

The binding of ^{3}H-TTAB to *C. albicans* also increased with increasing concentrations, but in contrast to CTAB, the binding was nonstoichiometric (Fig. 2B). The proportion bound decreased from 55% at 0.0063 mM to a minimum of 22% at 0.05 mM, but it then increased again to a maximum of 52% at 0.2 mM (Fig. 3). The increased proportion bound can also be

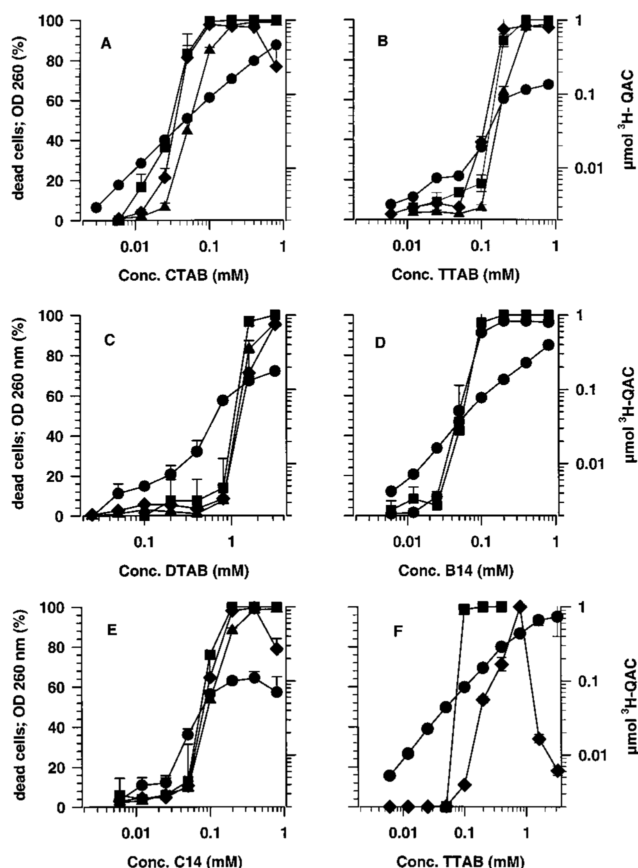

FIG. 2. Effects of different concentrations of QACs (CTAB [A], TTAB [B], DTAB [C], B14 [D], and C14 [E]) on the binding and damage to *C. albicans* and on *S. typhimurium* exposed to TTAB (F). ●, micromoles of ^{3}H-QAC bound to 2×10^7 *C. albicans* cells (A to E) and 4.9×10^9 *S. typhimurium* cells (F); ◆, release of intracellular material (percent) measured at 260 nm; the proportion of dead cells was calculated from plate counts (■) and by PI staining (▲). OD, optical density.

observed as a greater steepness of the binding isotherm in the part of the curve (0.05 to 0.2 mM) preceding the plateau level maximum (Fig. 2B). As with CTAB, the fungicidal effect and the release of UV-absorbing material by TTAB coincided (Fig. 2B). A 90% killing effect was seen with 0.20 mM TTAB. Slightly less than twice the killing concentration was required to cause uptake of PI into the same proportion of yeast cells.

The binding of ^{3}H-DTAB to *C. albicans* (Fig. 2C) was smaller than that of TTAB at corresponding concentrations. The proportion bound never exceeded 10% (Fig. 3). For clear-cut killing of *C. albicans*, 3.1 mM DTAB was required (the 90% killing effect was estimated to be 1.5 mM). In the concentration increment between 0.8 and 1.6 mM DTAB, drastic increases in the numbers of dead cells appeared, as indicated by viable counts, uptake of PI, and release of UV-absorbing substances, which accompanied one another rather closely.

The proportion of B14 bound to *C. albicans* showed a minimum of 60% at 0.0125 mM and a maximum of 80% at 0.1 mM (Fig. 3). The fungicidal effects studied by viable counts and release of UV-absorbing material were close. The 90% killing effect was seen at 0.09 mM (Fig. 2D).

The proportion of C14 bound to *C. albicans* showed the greatest variations among the different QACs within the concentration ranges tested. At 0.025 mM C14, 17% was bound, whereas at 0.1 mM, 72% was bound (Fig. 3). A conspicuous fungicidal effect, as tested by viable counts, a steep rise in the number of PI-stained cells and release of UV-absorbing material, also appeared at 0.1 mM. The 90% killing effect was seen at 0.15 mM (Fig. 2E).

The CMCs at 37°C were determined for the QACs by following the fluorescence of DPH (Table 1). For the stable compounds, lower CMCs were seen with longer hydrocarbon chains. The CMC of B14 (0.24 mM) was close to that of CTAB (0.21 mM), whereas that of C14 was higher (0.56 mM). When the log of the concentrations causing 10, 50, 90, and 99.9% killing of *C. albicans* were plotted against the log of the CMC, linear correlations were obtained ($r = 0.98$ for 50, 90, and 99.9% killing and $r = 0.91$ for 10% killing; Fig. 5). A similarly good linearity ($r = 0.99$) was seen between the hydrocarbon chain length and the 90% killing effect of the stable QACs. B14 and C14 were intermediate between TTAB and CTAB (Fig. 6).

Attempts to demonstrate aggregates on the surfaces of the yeast cells were performed by measuring the fluorescence intensity of DPH. Cells not exposed to TTAB showed nearly no fluorescence. At 0.1 mM TTAB, the cells were clearly fluorescent, and at 0.4 mM the fluorescence intensity of most cells was high (Table 2). The release of intracellular material was very low at 0.1 mM but increased almost to a maximum at 0.2 mM. The fluorescence from DPH faded extremely fast during illumination.

The binding of TTAB to the gram-negative bacterium *S. typhimurium* was almost total at 0.006 to 0.1 mM (Fig. 2F). From 0.2 to 0.8 mM, smaller portions were bound. Fairly high concentrations (0.05 mM) of TTAB bound to the bacteria without causing any release of intracellular material or cell death. Increasing the concentration to 0.1 mM reduced the live bacteria by ~2 log but released only 10% of the UV-absorbing material.

DISCUSSION

There were great differences between the different QACs with respect to both binding to *C. albicans* and fungicidal effect. As expected, the strongest binding and antifungal effect was shown by CTAB (16 carbon atoms in a straight hydrocar-

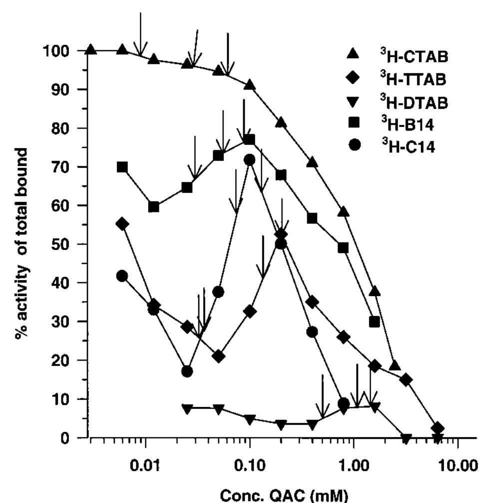

FIG. 3. Proportion of QAC bound when *C. albicans* (2×10^7 ml^{-1}) was exposed to different concentrations of CTAB, TTAB, DTAB, B14, and C14 in 10 mM phosphate buffer at 37°C for 10 min. After centrifugation at 12,500 rpm, the radioactivity in the supernatant was measured and the proportion bound was calculated by subtraction. The arrows show 10, 50, and 90% killing effects.

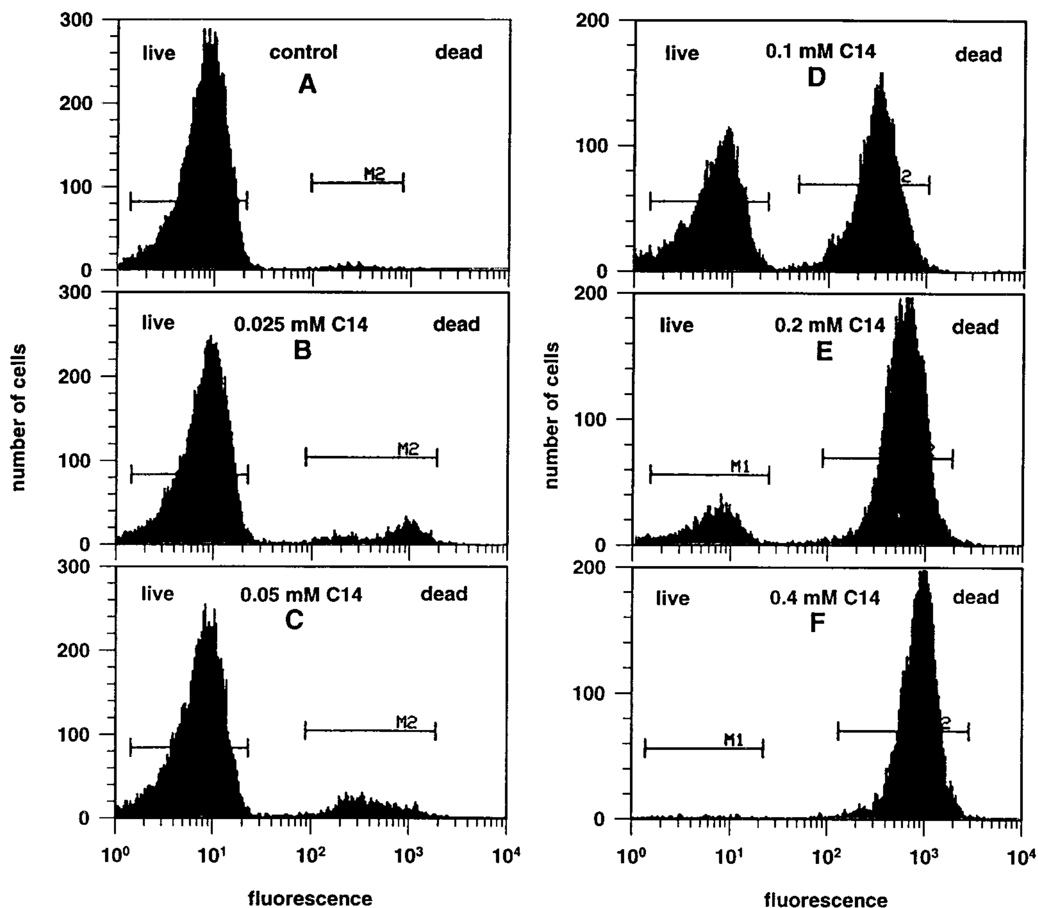

FIG. 4. Flow cytometry histograms of *C. albicans* cells treated with different concentrations of C14 after staining with 25 μg of PI ml^{-1}. The fluorescence intensities within the M1 regions were taken as live cells, and those within the M2 regions were taken as dead cells; >99% of control cells were within the M1 region, and >99% of cells treated with 70% ethanol were within the M2 region.

bon chain) and the weakest was shown by DTAB (12 carbon atoms). B14 (14-carbon fatty alcohol esterified with betaine), C14 (14-carbon fatty acid esterified with choline), and TTAB (14 straight carbon atoms) showed effects in between those of CTAB and DTAB. The concentrations required for the killing of *C. albicans* by DTAB amounted to 16- to 24-fold those for CTAB, irrespective of whether 90%, 99.9%, or complete killing was measured. The differences tended to be greater at the lower killing effects: at 50% killing it was 40-fold and at 10% killing it was ca. 55-fold. Since we consider the early events in the fungicidal effect to be of particular interest for elucidating the killing mechanism, we have focused our primary interest there.

The binding of CTAB to *C. albicans* was close to complete from the lowest concentration tested (3.1 μM). At 12.5 μM,

TABLE 1. CMC and concentration of QAC required for killing of *C. albicans*[a]

QAC	CMC concn (mM)	Concn of QAC (mM) required for the indicated proportion of killing				Complete killing[b]
		10%	50%	90%	99.9%	
CTAB	0.21	0.009	0.029	0.063	0.13	0.20
TTAB	1.79	0.031	0.130	0.20	0.32	0.50
DTAB	12.7	0.50	1.10	1.50	2.1	3.1
B14	0.24	0.028	0.053	0.090	0.12	0.20
C14	0.56	0.037	0.073	0.15	0.19	0.21

[a] The inoculum was ~2×10^7 CFU ml^{-1}. Incubation took place at 37°C for 10 min.

[b] Complete killing was <200 viable cells ml^{-1}.

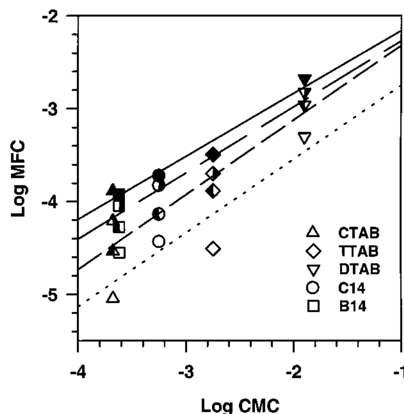

FIG. 5. Relationship between log CMC and log minimum fungicidal concentration (MFC) for *C. albicans*. The minimum fungicidal concentration was the concentration of QAC that killed 10% (symbols open; $r = 0.91$), 50% (symbols filled on the left; $r = 0.98$), 90% (symbols filled on the right; $r = 0.98$), and 99.9% (filled symbols; $r = 0.98$) of 2×10^7 cells ml^{-1} plotted against log CMC.

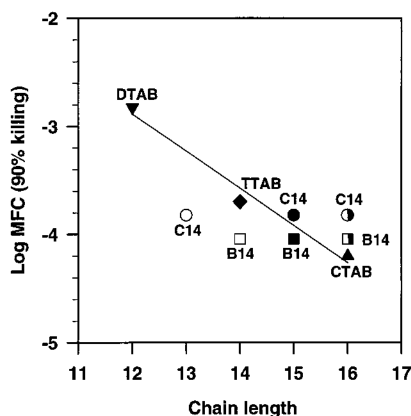

FIG. 6. Relationship between 90% killing concentration for *C. albicans* and chain length. Filled symbols, number of methylene groups in hydrocarbon chain bound to the quaternary nitrogen; open symbols, maximum number of consecutive methylene groups in the hydrocarbon chain; half-filled symbols, number of carbon atoms in the long chain bound to the quaternary nitrogen. The linear correlation for the stable QACs was $r = 0.99$.

the fungicidal effect was significant and reached 50% at 29 μM. At this quite low concentration, leakage of UV-absorbing material occurred at the same concentration as killing, indicating that damage to the cell membrane is closely related to the killing effect (26, 32). However, achievement of the PI signal in the same proportion of cells required twice the concentration of CTAB. The PI signal requires intercalation with DNA, and intact nuclear membranes may prevent access of PI, even though the cell is not viable, since at limiting fungicidal concentrations, most of the CTAB is bound at the yeast cell periphery. With the other QACs the concentrations causing PI uptake were closer to the fungicidal concentrations, being nearly identical for DTAB, the QAC with the shortest hydrocarbon chain tested. The proportion of this substance bound never exceeded 10%, which left large quantities free to react with the nuclear membranes after the cytoplasmic membrane had been disrupted. Thus, membrane damage and disruption of the barrier is an important mechanism in the killing of *C. albicans* by QACs, which is in agreement with the results of Green et al. (10). If each *C. albicans* cell in the suspension of 2×10^7 cells ml^{-1} is given a surface of 150 μm^{-2} (27) and 12 to 28 μM CTAB is bound, each cell will bind 3.6×10^8 to 8.4×10^8 CTAB molecules, meaning that there are two to six CTAB molecules nm^{-2}. Since the diameter of the cationic head group is ca. 0.31 nm (29) and the extension of the molecule is ca. 2.18 nm (8), there is a packing problem at the surface in case the full extension of the molecule is adsorbed, but there is enough space if the molecules are adsorbed head-on. Although higher concentrations of the other QACs were added to cause the same fungicidal effect (Table 1), approximately the same number of molecules was bound (Table 3); for 50% killing by DTAB, however, about three times as many molecules were required (Fig. 2A to E; Table 1).

TABLE 2. Fluorescence intensity from DPH-exposed *C. albicans* after treatment with TTAB[a]

TTAB concn (mM)	Fluorescence intensity		A_{260} (mean ± SD)
	Fluorimetry[b]	Microscopy	
0.000	49 ± 2	(−)	0.022 ± 0.006
0.025	146 ± 20	(+)	ND[c]
0.050	212 ± 55	+	0.033 ± 0.008
0.100	186 ± 19	++	0.047 ± 0.007
0.200	330 ± 20	+++	0.304 ± 0.004
0.400	382 ± 67	+++	ND

[a] DPH was used as a probe which fluoresces in a hydrophobic environment. Damage to the cell membranes was measured as the amount of UV-absorbing material released.
[b] Values are expressed in fluorescence units.
[c] ND, not done.

TABLE 3. Number of molecules of substance on a *C. albicans* cell in which the substance had a fungicidal effect on 90% for the whole population[a]

Compound	No. of molecules nm^{-2}	Concn (μM) for 90% fungicidal effect on 2×10^7 cells ml^{-1}
CTAB	12	63
TTAB	20	200
DTAB	26	1,500
B14	14	90
C14	19	150

[a] The whole population consisted of 2×10^7 cells ml^{-1}.

The concentration of a QAC required to accomplish a certain fungicidal effect showed a relationship to the CMC (Fig. 5) and the hydrocarbon chain length (Fig. 6). When the concentrations achieving 90% killing were plotted versus hydrocarbon chain length (Fig. 6), the data for the nonester compounds CTAB, TTAB, and DTAB fit in a straight line, with the data for the esters B14 and C14 appearing between those for CTAB (16 carbon atoms) and TTAB (14 carbon atoms). It has been shown that the CMCs of the stable QACs approximate those of betaine esters, which have two fewer methylene groups (30); those data fit ours, which showed that CTAB and B14 have similar CMCs. However, the same is not true for C14, which has a CMC that is markedly higher than that of CTAB (Table 1). One of the 14 carbon atoms in the choline derivative is a carbonyl carbon, thus giving only 13 true alkyl carbons. This is not the case with B14, in which the ester bound is inversed in the molecule, resulting in 14 alkyl carbons (Fig. 1 and 6). Even though B14 and CTAB have similar CMCs, their effects on *C. albicans* are different, as seen in Fig. 2 and 3 and Table 1. During micelle formation, the carbonyl carbon and the methylene group of betaine appear to function as two methylene groups (30). However, the dipole moment of the carbonyl group would be expected to enhance the hydrophilic properties of the molecule and affect its interaction with membranes (22). It would also be expected that the carbonyl group would give B14 better solubility than CTAB; thus, CTAB would be expected to interact with yeast cell walls at lower concentrations.

A linear relationship between the CMC and the fungicidal effect was seen at killing effects of 10, 50, 90, and 99.9% (Fig. 5), supporting the fact that the hydrophobic interaction is important in the fungicidal effect of QAC on *C. albicans*. At the lowest killing effect studied (10%), the fit was not as good ($r = 0.91$) with the ester compounds showing a poorer fungicidal effect in relation to the CMC. This might be due to greater interference of the ester group in the interaction with yeast cells than in that with the micelle. However, the method's precision at low killing effects might also have influenced the results.

Also, the binding kinetics of the QACs to *C. albicans* cells was very much influenced by the structure of the hydrocarbon chains (Fig. 2 and 3). The larger degree of binding of CTAB in comparison to that of the other QACs, particularly at low concentrations (Fig. 3), is probably due to its greater hydro-

phobicity and stronger tendency toward binding to hydrophobic structures at the cell surface, leading to aggregate formation before saturation of the negative sites (31). Almost all CTAB was bound up to 99.9% killing (Fig. 3). At higher concentrations, the proportion bound decreased rapidly, indicating saturation of binding sites. B14, C14, and TTAB showed quite different binding kinetics. At the lowest concentration tested, 0.0063 mM, all three bound in proportions amounting to 40 to 70% (Fig. 3). The proportion bound decreased at higher concentrations, reaching minima at 0.0125 mM (B14), 0.025 mM (C14), and 0.05 mM (TTAB), which are of the same order as the CMCs, namely, 0.24, 0.56, and 1.79 mM, respectively. No killing was seen at the binding minima. At higher concentrations, the proportion bound increased, reaching maxima of 0.1 mM (B14 and C14) and 0.2 mM (TTAB). These concentrations were the closest to those causing 90% killing (Fig. 3), implying that the enhanced binding is intimately related to the fungicidal effect. These concentrations are 10 to 40% of the respective CMCs. At such concentrations, submicellar aggregates at solid-liquid interfaces may be formed, as proposed for different nonbiological models by Fuerstenau and Herrera-Urbina (9), Harwell and colleagues (14, 15), and Gu and colleagues (11–13). Submicellar aggregates were also postulated when concentration-dependent base-catalyzed hydrolysis was observed for a series of amphiphilic betaine esters (33). For B14, such concentration dependence was seen from below 0.1 mM. Gu and Huang (12) proposed a model based on adsorption of cationic surfactants to negatively charged silica gel surfaces. In the very low concentration range, the adsorption results from an electrostatic interaction between the surface-active cation monomer and the negatively charged silica gel surface. When the surfactant concentration is increased to a certain concentration below the CMC, an aggregate is formed at the surface by the hydrophobic interaction between the hydrocarbon chains of the surfactant, which stabilizes the binding of the aggregate by the multiplicity of the interactions.

Tiberg and colleagues (36–38) investigated the adsorption kinetics of polyethylene glycol alkyl esters at hydrophilic silica surfaces by in situ ellipsometry, which measures the optical thickness. Those studies showed that the adsorbed layer was built up of discrete aggregates. Such surface micelle patches already had a well-defined thickness when a small proportion of the surface was covered as it was at concentrations in the solution below the CMC. Lewitz and Van Damme (23) made the conclusion that at below the CMC of the surfactant, silica acts as a solid surface precursor for the micellization process and that aggregates are created on the solid surface from isolated surfactants in the solution.

The cell surface of *C. albicans* is considered to be negatively charged due to terminal sialic acid moieties and shows only a certain degree of hydrophobicity (18, 20, 28). *C. albicans* cells grown at 37°C in vitro seem to be more hydrophilic than cells grown at 25°C (16, 17). In our system, the QACs may initially interact via electrostatic forces with the cell wall of *C. albicans* in a way similar to that in which it interacts with hydrophilic silica. With CTAB, additional hydrophobic interaction of the individual molecules with the cell wall might be enough for binding. For the less hydrophobic QACs, we suggest that when the concentration increases, aggregates of high charge density are formed at the cell surface due to the charge interaction between the QAC and the yeast cell and the hydrophobic interaction between the QAC molecules.

Concurrent with the increase in the proportion bound, the killing effect increased such that close to the maximum of the proportion of B14, C14, and TTAB bound (Fig. 3), a 90% killing effect was achieved. Thus, the enhanced binding engaged the cell membranes. It might be argued that the increase in the proportion bound was a consequence of new binding sites exposed by the cell barrier damage rather than the cause of it. However, the steep drop in the proportion of C14 bound at concentrations of 0.2 mM and above, when the 90% killing effect (0.12 mM) had barely been passed, indicates that *C. albicans* cells killed in this way did not soak up the QACs; rather, complexes were formed as initiating steps in the killing process. However, the topographical relationship between complex formation and the different components of the cell envelope is not known.

Intracellular UV-absorbing material from the yeast cells was released at the same QAC concentrations and at the same proportions as the cells killed (Fig. 2A to E), indicating that the killed cells released their contents. Also, the appearance of two distinct populations of cells with respect to PI staining (Fig. 4) indicates that for yeast the membrane disturbance by QACs causing the loss of viability is a distinct step rather than a gradual process (10). For bacteria, the release of UV-active substances became significant at higher QAC concentrations than those required to cause cell death (Fig. 2F). K^+ leakage may occur earlier (21). We did not perform the dye exclusion test with bacteria, but several investigators have used flow cytometry to distinguish between live and dead bacteria (19, 25). Thus, flow cytometry and the release of UV-active substances may, under certain conditions, be more rapid ways of assessing the proportion of dead cells than viable count cultivation tests.

Hydrophobic QACs such as CTAB interact strongly not only with *C. albicans* cells but also with proteins and other biological structures. With TTAB, B14, and C14, the uptake through the cell wall was favored from a submicellar state, thus supporting the conclusion that uptake of amphiphilic molecules by *Candida* cells does not need to take place from the monomer form but may be facilitated by groups of molecules. If such mechanisms are also operating in binding in the human body, it might have a great impact on the optimization of drug delivery systems. It might be more than a coincidence that the polyene structure of amphotericin B, which is considered to interact with the fungal cell membrane, also contains 14 carbon atoms and is antimicrobially efficient when it is presented in different complexes with lipid in vitro and in vivo.

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REFERENCES

1. **Ahlström, B., M. Chelminska-Bertilsson, R. A. Thompson, and L. Edebo.** 1995. Long-chain alkanoylcholines, a new category of soft antimicrobial agents that are enzymatically degradable. Antimicrob. Agents Chemother. **39:**50–55.
2. **Association of Official Analytical Chemists.** 1984. Disinfectants, p. 65–77. *In* Official methods of analysis, 14th ed. Association of Official Analytical Chemists, Arlington, Va.
3. **Bolard, J.** 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta **864:**257–304.
4. **Chattopadhyay, A., and E. London.** 1984. Fluorimetric determination of critical micelle concentration avoiding interference from detergent charge. Anal. Biochem. **139:**408–412.
5. **Chelminska-Bertilsson, M., and S. Allenmark.** 1992. Liquid chromatographic monitoring of pseudocholinesterase activity: comparison of methods. J. Chromatogr. **575:**237–242.
6. **Edebo, L., E. Kihlström, K.-E. Magnusson, and O. Stendahl.** 1980. The hydrophobic effect and charge effects in the adhesion of enterobacteria to animal cell surfaces and the influence of antibodies of different immunoglobulin classes, p. 65–101. *In* A. S. G. Curtis and J. D. Pitts (ed.), Cell adhesion and motility. Third Symposium of the British Society for Cell Biology. Cambridge University Press, Cambridge, England.

7. **Edebo, L., B. Ahlström, M. Chelminska-Bertilsson, S. Lange, M. Lindstedt, and R. A. Thompson.** 1992. Betaine esters: quaternary ammonium compounds with time-limited activity, p. 184–207. *In* D. R. Karsa (ed.), Industrial applications of surfactants III. The Royal Society of Chemistry, Cambridge, England.
8. **Evans, D. F., and H. Wennerström.** 1994. Solute and solvents, self-assembly of amphiphiles, p. 5–35. *In* D. F. Evans and H. Wennerström (ed.), The colloidal domain. VCH Publishers, Inc. New York, NY.
9. **Fuerstenau, D. W., and R. Herrera-Urbina.** 1991. Adsorption of cationic surfactants and the flotation of minerals, p. 407–447. *In* D. N. Rubingh and P. M. Holland (ed.), Cationic surfactants: physical chemistry. Marcel Dekker, Inc., New York, N.Y.
10. **Green, L., B. Petersen, L. Steimel, P. Haeber, and W. Current.** 1994. Rapid determination of antifungal activity by flow cytometry. J. Clin. Microbiol. **32:**1088–1091.
11. **Gu, T., Y. Gao, and L. He.** 1988. Hemimicelle formation of cationic surfactants at the silica gel-water interface. J. Chem. Soc. **84:**4471–4473.
12. **Gu, T., and Z. Huang.** 1989. Thermodynamics of hemimicellization of cetyltrimethylammonium bromide at the silica gel/water interface. Colloids Surfaces **40:**71–76.
13. **Gu, T., and H. Rupprecht.** 1990. Hemimicelle shape and size. Colloid Polymer Sci. **268:**1148–1150.
14. **Harwell, J. H., J. C. Hoskins, R. S. Schechter, and W. H. Wade.** 1985. Pseudophase separation model for surfactant adsorption: isomerically pure surfactants. Langmuir **1:**251–262.
15. **Harwell, J. H., and M. A. Yeskie.** 1989. Reply to comments on "On the structure of aggregates of adsorbed surfactants: the surface charge density at the hemimicelle/admicelle transition." J. Phys. Chem. **93:**3372–3373.
16. **Hazen, K. C., B. J. Plotkin, and D. M. Klimas.** 1986. Influence of growth conditions on cell surface hydrophobicity of *Candida albicans* and *Candida glabrata*. Infect. Immun. **54:**269–271.
17. **Hazen, K. C., J.-G. Lay, B. W. Hazen, R. C. Fu, and S. Murthy.** 1990. Partial biochemical characterization of cell surface hydrophobicity and hydrophilicity of *Candida albicans*. Infect. Immun. **58:**3469–3476.
18. **Hobden, C., C. Teevan, L. Jones, and P. O'Shea.** 1995. Hydrophobic properties of the cell surface of *Candida albicans*: a role in aggregation. Microbiology **141:**1875–1881.
19. **Jepras, R. I., J. Carter, S. C. Pearson, F. E. Paul, and M. J. Wilkinson.** 1995. Development of a robust flow cytometric assay for determining numbers of viable bacteria. Appl. Environ. Microbiol. **61:**2696–2701.
20. **Jones, L., and P. O'Shea.** 1994. The electrostatic nature of the cell surface of *Candida albicans*: a role in adhesion. Exp. Mycol. **18:**111–120.
21. **Kopecká-Leitmanová, A., F. Devínsky, D. Mlynarcik, and I. Lacko.** 1989. Interaction of amine oxides and quaternary ammonium salts with membrane and membrane-associated processes in *E. coli* cells: mode of action. Drug Metab. Drug Interact. **7:**29–51.
22. **Kuczera, J., T. Janas, S. Przestalski, S. Witek, and M. Oświecimska.** 1985. Influence of some amphiphilic derivatives of glycine ester on sulphate ion transport across lecithin liposome membrane. Studia Biophys. **105:**167–176.
23. **Lewitz, P., and H. Van Damme.** 1986. Fluorescence decay study of the adsorption nonionic surfactants at solid-liquid interface. 2. Influence of polar chain length. J. Phys. Chem. **90:**1302–1310.
24. **Lindstedt, M., S. Allenmark, R. A. Thompson, and L. Edebo.** 1990. Antimicrobial activity of betaine esters, quaternary ammonium amphiphiles which spontaneously hydrolyze into nontoxic components. Antimicrob. Agents Chemother. **34:**1949–1954.
25. **Mason, D. J., R. Lopéz-Amorós, R. Allman, J. M. Stark, and D. Lloyd.** 1995. The ability of membrane potential dyes and Calcafluor White to distinguish between viable and non-viable bacteria. J. Appl. Bacteriol. **78:**309–315.
26. **Merianos, J. J.** 1991. Quaternary ammonium antimicrobial compounds, p. 225–255. *In* S. Block (ed.), Disinfection, sterilization and preservation, 4th ed. Lea & Febiger, Philadelphia, Pa.
27. **Meyer, S. A., D. G. Ahearn, and D. Yarrow.** 1984. *Candida* Berkhout, p. 611. *In* N. J. W. Kreger-van Rij (ed.), The yeasts—a taxonomic study, 3rd ed. Elsevier Science Publisher BV, Amsterdam, The Netherlands.
28. **O'Shea, P.** 1991. The role of electrostatic and electrodynamic forces in fungal morphogenesis and host infection, p. 286–302. *In* J. P. Latagé and D. Boucias (ed.), Fungal cell wall and immune response. NATO AS Series, vol. H 53. Springer-Verlag, Berlin, Germany.
29. **Rennie, A. R., E. M. Lee, E. A. Simister, and R. K. Thomas.** 1990. Structure of a cationic surfactant layer at the silica-water interface. Langmuir **6:**1031–1034.
30. **Rózycka-Roszak, B., S. Przestalski, and S. Witek.** 1988. Calorimetric studies of micellization of some amphiphilic betaine ester derivatives. J. Colloid Interface Sci. **125:**80–85.
31. **Rupprecht, H., and T. Gu.** 1991. Structure of adsorption layers of ionic surfactants at solid/liquid interface. Colloid Polymer Sci. **269:**506–522.
32. **Russel, A. D.** 1982. Antifungal activity of biocides, p. 134–149. *In* A. D. Russel, W. B. Hugo, and G. A. J. Ayliffe (ed.), Principles and practise of disinfection, preservation and sterilisation, 2nd ed. Blackwell Scientific Publications, Ltd., Oxford, England.
33. **Thompson, R. A., and S. Allenmark.** 1989. Effects of molecular association on the rates of hydrolysis of long-chain alkyl betainates (alkoxycarbonyl-*N*,*N*,*N*-trialkylmethanaminium halides). Acta Chem. Scand. **43:**690–693.
34. **Thompson, R. A., M. Lindstedt, and S. Allenmark.** 1990. A liquid radiochromatographic method for following the synthesis and hydrolysis of a double (^{3}H, ^{14}C) radiolabeled alkyl betainate. Anal. Lett. **23:**787–798.
35. **Thompson, R. A., and S. Allenmark.** 1992. The effect of long-chain alkyl betainates on the arylesteras-like activity of bovine serum albumin. Bioorg. Chem. **20:**356–365.
36. **Tiberg, F., and M. Landgren.** 1993. Characterization of thin non-ionic surfactant films at the silica/water interface by means of ellipsometry. Langmuir **9:**927–932.
37. **Tiberg, F., B. Jönsson, J. Tang, and B. Lindman.** 1994. Ellipsometry studies of the self-assembly of non-ionic surfactants at silica-water interface: equilibrium aspects. Langmuir **10:**2294–2300.
38. **Tiberg, F., B. Jönsson, and B. Lindman.** 1994. Ellipsometry studies of the self-assembly of non-ionic surfactants at silica-water interface: kinetic aspects. Langmuir **10:**3714–3722.