ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1972, p. 114-121

Vol. 2, No. 3
Printed in U.S.A.

In Vitro Studies of 5-Fluorocytosine Resistance in *Candida albicans* and *Torulopsis glabrata*

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Received for publication 17 May 1972

Spontaneous mutants of *Candida albicans* resistant to 5-fluorocytosine (5-FC) were isolated from a strain susceptible to 5-FC. These mutants were compared with 5-FC-resistant strains of *C. albicans* and *Torulopsis glabrata* isolated from patients treated with the drug and from untreated patients. Resistance to 5-FC was in all cases followed by a decreased susceptibility to 5-fluorouracil. Most strains were also more resistant to 5-fluorouridine and had a lowered incorporation of uridine. In one spontaneous mutant totally resistant to all three 5-fluoropyrimidines, the uridine monophosphate pyrophosphorylase activity was greatly decreased. The 5-FC-resistant strains were of two main phenotypic classes. Class one was unaffected by 5-FC at the highest concentration tested. The growth rate of strains belonging to the other class was markedly decreased by low concentrations of 5-FC, but these strains were still able to form colonies after 7 days of incubation on plates containing very high concentrations of the drug (2,000 μg/ml). The frequency of spontaneous mutation of a susceptible strain to 5-FC resistance was fairly high. The number of mutants growing on media containing 5-FC was unchanged by concentrations ranging from 25 to 500 μg/ml.

5-Fluorocytosine (5-FC) was originally intended as a cytostatic agent (2), but in animal experiments it proved to lack both cytostatic properties and the capacity to produce leucopenia (6). However, in 1964 it was discovered (5) that the compound had a fungistatic effect in certain experimental fungus infections in mice. Experience has since shown that 5-FC should be regarded as one of the most important agents in the treatment of deep *Candida* infections (13), especially of the urinary tract (13; J. Schönebeck, Proc. 5th Congr. ISHAM, Paris) and that it is also effective against infections due to *Torulopsis glabrata* (17). It is relatively nontoxic (13, 14), but clinical experience has shown that the development of resistance is common (15). Emergence of microbial drug resistance was at first thought to be a problem mainly in the treatment of cryptococcosis, and only occasionally in the treatment of *Candida* infections (15). More recent studies, however, have shown that resistance frequently develops during the treatment of candidiasis. Schönebeck and Ånséhn (*in preparation*) found that 10 of 135 clinical isolates of *C. albicans* and 3 of 50 *T. glabrata* isolates showed a low degree of susceptibility to 5-FC. From 18 patients treated with the drug, four *C. albicans* and two *T. glabrata* strains were isolated which in vitro showed a greater degree of resistance to 5-FC. Resistant strains could be isolated more often from patients treated with low concentrations of the drug at the site of infection. This has been observed also by Shadomy (15).

5-FC is converted to 5-fluorouracil (5-FU) by a cytosine deaminase in *C. albicans* and *Saccharomyces cerevisiae* before it is incorporated into ribonucleic acid (RNA) as 5-fluorouracil riboside (7, 8). The 5-fluoropyrimidines, 5-FC, 5-FU, and 5-fluorouridine (5-FUI), have been shown to have antifungal properties against *S. cerevisiae* (7). Mutants of this fungus resistant to 5-fluoropyrimidines were recently described by Jund and Lacroute (7).

5-FC-resistant mutants of a susceptible strain of *C. albicans* were isolated by plating on different concentrations of 5-FC without the use of mutagens. The mechanism underlying the development of resistance has been studied, in part in these spontaneous mutants and in part in strains of *C. albicans* and *T. glabrata* isolated from treated and untreated patients.

MATERIALS AND METHODS

Microbial strains. The *C. albicans* strain H1200 was supplied by Hoffmann-La Roche, Inc., Basle, Switzerland. Strain H12R3 and strains S1 to S47 are 5-FC-resistant mutants isolated from stationary-phase cultures of strain H1200 by plating on different concen-

trations of 5-FC. To minimize the possibility of double mutations, no mutagens were used. The 5-FC-susceptible *C. albicans* strains, CA7, CA8, and CA9, and strains CA14, CA15, and CA16 with decreased susceptibility to 5-FC were isolated from untreated patients. Strains CA10 to CA13 were isolated from the urine of a patient with asymptomatic *Candida* infection of the urinary tract before (CA10), during (CA11), and after (CA12 and CA13) 5-FC treatment. Strains CA17 to CA19 were obtained from a patient with pulmonary candidiasis before therapy (CA17) and 1 week (CA18) and 1 month (CA19) after cessation of therapy. The *T. glabrata* strains were cultivated from the urine of untreated (TG1, TG2, and TG3) and treated (TG4 and TG5) patients.

Media and growth conditions. The following media were used: LB of Bertani (1) supplemented with medium E (16) and 0.2% glucose; Casamino Acids medium containing 0.2% casein hydrolysate, medium E, and 0.2% glucose; and Difco yeast-nitrogen base medium (YNB) with 0.1% glucose. The solid media used were yeast morphology agar (YMA) and Casamino Acids medium solidified with 1.5% agar.

The experiments were performed at 37 C. The yeast strains were cultivated on a rotary shaker (100 rev/min), and optical density was measured with a Klett-Summerson colorimeter with filter W66 or with a Zeiss spectrophotometer at a wavelength of 450 nm. The number of yeast cells per cellaggregate was determined with a Zeiss phase-contrast microscope.

Materials. The 5-fluoropyrimidines were kindly supplied by Hoffmann-La Roche, Inc., Basle, Switzerland. ^{3}H-labeled uracil and uridine (1.0 Ci/mmole) were purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England.

Determination of resistance. The 5-fluoropyrimidine resistance of mutants isolated in the laboratory and of clinical isolates was tested by replication from autoclavable microculture containers made of nylon (Bertani, *personal communication*). The containers consist of 25 square wells and fit into 9-cm petri dishes. A 0.5-ml amount of YNB medium was added to each well. The wells were then individually inoculated from yeast colonies by means of sterile toothpicks. Overnight microcultures were diluted by transfer to sterile microculture containers with 0.5 ml of YNB in each well. After dilution, a replicator with steel needles was used to replicate one drop from each culture, containing 5 to 50 viable cell units, onto YMA plates with different concentrations of the respective 5-fluoropyrimidine. The plates were wrapped in aluminium foil and incubated at 37 C. Results were read after 2, 3, 4, and 7 days of incubation. Resistance was determined as the highest concentration permitting development of colonies of the same number and approximate size as on a control plate without 5-fluoropyrimidine.

Measurement of uracil and uridine incorporation. To assay for acid-insoluble radioactivity of uracil or uridine in RNA, cells were grown overnight at 37 C in 5 ml of Casamino Acids or LB medium containing ^{3}H-labeled uracil or uridine. Growth was measured with a Klett-Summerson colorimeter. The cultures were mixed with 5 ml of ice-cold 10% trichloroacetic acid and were allowed to precipitate at 0 C for at least 1 hr. Each culture was then filtered on a Sartorius membrane filter (23 mm) and washed five times with 5 ml of 0.1 M HCl. The filters were dried and the number of cells was determined in a liquid scintillation counter (Nuclear-Chicago Mark 1).

Enzyme assays. Yeast cells were grown in 2 liters of LB medium overnight. After centrifugation, the cells were resuspended in 10 ml 0.1 M tris(hydroxymethyl)-aminomethane-hydrochloride, *p*H 7.2, and broken in a French press. Intact cells and cell walls were centrifuged at 20,000 × *g* for 20 min. Uridine monophosphate (UMP) pyrophosphorylase activity was tested according to Reichard and Sköld (11). UMP was separated from uracil on a Dowex-1-X2 column (3). Uridine kinase activity was tested as described by Reichard and Sköld (12). The protein content was measured by the method of Lowry et al. (9).

RESULTS

Characterization of the 5-FC-resistant strain H12R3. *C. albicans* H1200 was grown overnight in LB medium and washed; samples were plated on YMA containing 5-FC (100 μg/ml). After 2 days of incubation, the number of visible resistant colonies was 1 per 10^8 viable clones of strain H1200. In a phase-contrast microscope, the average number of individual cells observed per cell aggregate was 2.5. Resistant colonies therefore occurred at a frequency of 4 per 10^9 cell units. The nature of the 5-FC resistance in one of the resistant mutants, H12R3, was established by plating on YMA containing different concentrations of 5-FC, 5-FU, or 5-FUI. As can be seen in Table 1, strain H12R3 was highly resistant to all three 5-fluoropyrimidines. This finding suggested that the mutation had occurred after the conversion of 5-FC to 5-FU. Furthermore, when compared with the wild-type parent strain H1200, the mutant H12R3 had a lowered incorporation of both ^{3}H-uracil and ^{3}H-uridine into trichloroacetic

TABLE 1. *Resistance of the wild strain H1200 and the 5-fluorocytosine-resistant mutant H12R3 to 5-fluoropyrimidines*[a]

Strain	Resistance (μg/ml)		
	5-Fluorocytosine	5-Fluorouracil	5-Fluorouridine
H1200.....	<0.5	25	<0.5
H12R3....	>2,000	>2,000	>1,000

[a] Resistance was determined after 3 days of incubation on YMA containing increasing concentrations of one of the 5-fluoropyrimidines. Resistance was defined as the upper limit on which the strain was able to form colonies of the same number and of approximately the same size as on control plates without 5-fluoropyrimidines.

acid-insoluble material (Fig. 1). The resistance of H12R3 to 5-FU suggested the possibility of a block in the UMP pyrophosphorylase-catalyzed conversion of uracil to UMP. The ability to convert uracil and uridine to UMP was tested in cell-free extracts of the mutant H12R3 and the wild-type strain H1200. Table 2 shows that the mutant was considerably impaired in UMP pyrophosphorylase activity, whereas the uridine kinase activity was approximately the same in both strains.

The mass doubling time of mutant and parent strain was compared during steady-state growth in LB and YNB medium. No difference in growth rate was found. Moreover, the mutant and the wild-type strain reached the same density during the stationary phase.

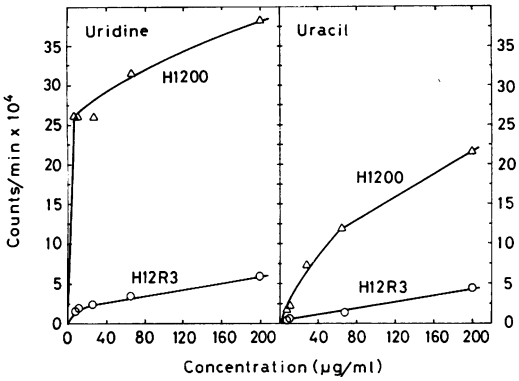

FIG. 1. *Incorporation of ^{3}H-uridine and ^{3}H-uracil in the wild-type strain H1200 and the 5-FC-resistant mutant H12R3. Cells were grown to the stationary phase in 5 ml of Casamino Acids medium with different concentrations of ^{3}H-labeled uracil and uridine. The specific activity was 0.013 µCi/mmole of uracil and 0.006 µCi/mmole of uridine. The cells were grown to the same cell mass. Ice-cold trichloroacetic acid (5 ml) was added to each culture and was allowed to precipitate for more than 1 hr. The precipitate was collected on a membrane filter (Millipore Corp.) and extensively washed, and the radioactivity was measured.*

TABLE 2. *Uridine kinase and UMP pyrophosphorylase activity[a] in the H12R3 (mutant) and H1200 (wild type) strains of C. albicans*

Strain	Specific activity[b]	
	Uridine kinase	UMP pyrophosphorylase
H1200.....	0.37	0.18
H12R3.....	0.30	0.04

[a] Measured as described in Materials and Methods.

[b] Expressed as nanomoles of UMP formed per minute and milligram of protein.

5-FC-resistant mutants of strain H1200 isolated on plates with different 5-FC concentrations. The number of 5-FC-resistant mutants in a batch culture of strain H1200 and the levels of their resistance were tested by plating samples of a stationary-phase culture on YMA containing different concentrations of 5-FC. The number of visible clones was counted after 2, 4, and 7 days (66, 90, and 170 hr) of incubation. As shown in Fig. 2, the number of mutant clones was virtually the same in the range of 25 to 500 µg/ml. Below 25 µg/ml, a larger number of resistant mutants were observed. In the plateau region (25 to 500 µg/ml), the number of mutant clones increased by a factor of 5 between 2 and 7 days of incubation. With a concentration of 1,000 µg/ml, a 20-fold increase was observed. Thus, it is apparent that about 80% of the mutants recovered after 7 days of incubation were affected by the drug, and were equally affected by concentrations ranging from 25 to 500 µg/ml. We assumed that after 7 days of incubation the total number of growing clones was very close to the total number of 5-FC-resistant mutants, since in separate experiments we

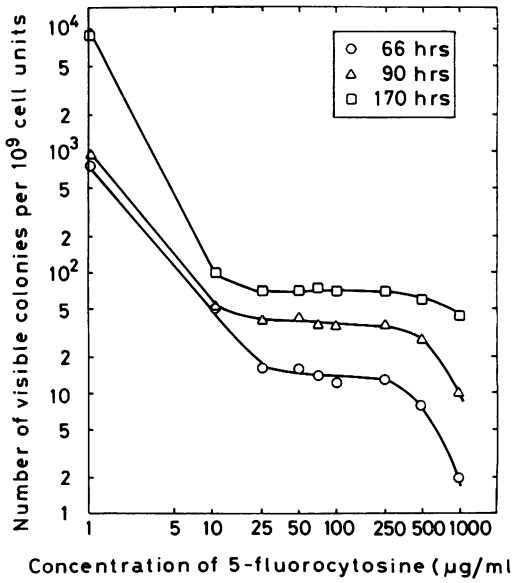

FIG. 2. *Number of 5-FC-resistant mutants of H1200 forming visible colonies on YMA plates containing 5-FC at different concentrations. C. albicans H1200 was grown overnight to the stationary phase in LB medium. The cells were centrifuged and washed twice in 0.15 M NaCl solution before plating. The plates were wrapped in aluminium foil. The number of visible colonies was read after 66, 90, and 170 hr of incubation. The total number of H1200 colonies was multiplied by 2.5 to get the number of yeast cells in the culture.*

did not find any appreciable increase in the number of mutants after more than 7 days of incubation. After 7 days, the number of 5-FC-resistant mutants was in the plateau region calculated to be approximately 7 per 10^8 cell units.

After 7 days of incubation, 47 clones taken from plates with different 5-FC concentrations were incubated and thereafter plated on YMA containing different amounts of 5-fluoropyrimidines (Table 3). After 2 days of incubation, all mutants selected at a 5-FC concentration of 1 μg/ml appeared to be susceptible to the agent. At a concentration of 10 μg/ml, 3 of 10 mutants appeared susceptible, 5 were resistant, and 2 showed an intermediate degree of resistance. In the selection range of 25 to 1,000 μg/ml, 12 of 27 mutants did not form colonies after 2 days of incubation on 1 μg of 5-FC/ml, whereas 11 were highly resistant. When the incubation time was prolonged to 7 days, 35 of 37 mutants selected in the concentration range of 10 to 1,000 μg/ml were able to grow on 2,000 μg of 5-FC/ml. The remaining two mutants tolerated 1,000 μg/ml. Only 2 of 10 mutants selected at 1 μg/ml were able to grow on 2,000 μg of 5-FC/ml. The other eight mutants were resistant to 500 or 1,000 μg/ml, but they produced very small colonies after 7 days of incubation on those plates. Moreover, from the data in Table 3 it could be concluded that all mutants with increased tolerance to 5-FC showed increased tolerance to 5-FU and that the great majority were also more resistant to 5-FUI. The common finding of 5-FUI resistance suggested that the major part of this compound is converted to 5-FU before being incorporated into RNA. Incorporation studies of the mutants done with ^{3}H-uridine showed that only five mutants incorporated ^{3}H-uridine to the same extent as the

TABLE 3. *Resistance to 5-fluoropyrimidines of spontaneous 5-fluorocytosine-resistant mutants of strain H1200*[a]

Strains	Selection concn (μg/ml)	Resistance (μg/ml)					
		5-Fluorocytosine		5-Fluorouracil		5-Fluorouridine	
		2 days	7 days	2 days	7 days	2 days	7 days
S5	1	<0.5	500	50	100	<0.5	0.5
S1, S4, S9	1	<0.5	1,000	50	500	<0.5	0.5
S2, S6, S7, S8	1	<0.5	1,000	50	500	0.5	>1,000
S3, S10	1	<0.5	>2,000	50	500	0.5	>1,000
S11	10	<0.5	1,000	100	500	<0.5	0.5
S14	10	<0.5	>2,000	100	500	0.5	10
S15	10	<0.5	>2,000	50	>2,000	0.5	>1,000
S17	10	25	>2,000	250	500	0.5	25
S18	10	500	>2,000	250	1,000	0.5	>1,000
S13, S20	10	>2,000	>2,000	250	>2,000	0.5	>1,000
S19	10	>2,000	>2,000	>2,000	>2,000	0.5	>1,000
S12, S16	10	>2,000	>2,000	>2,000	>2,000	>1,000	>1,000
S22	25–1,000	<0.5	1,000	50	500	<0.5	0.5
S41	25–1,000	<0.5	>2,000	100	>2,000	<0.5	1
S32	25–1,000	<0.5	>2,000	50	>2,000	<0.5	100
S26, S27, S31	25–1,000	0.5	>2,000	50	>2,000	<0.5	>1,000
S28, S30, S35, S39, S42, S43	25–1,000	0.5	>2,000	50	>2,000	1	>1,000
S23, S24	25–1,000	1	>2,000	250	1,000	0.5	>1,000
S21, S25	25–1,000	1	>2,000	250	>2,000	<0.5	>1,000
S37, S45, S46, S47	25–1,000	>2,000	>2,000	500	>2,000	1	>1,000
S29, S33, S34, S36, S38, S40, S44	25–1,000	>2,000	>2,000	>2,000	>2,000	1	>1,000
H1200	—	0.5	0.5	25	50	<0.5	0.5
H12R3	—	2,000	2,000	>2,000	>2,000	>1,000	>1,000

[a] Resistance to 5-fluoropyrimidines was determined on YMA plates after 2 and 7 days of incubation as described in Materials and Methods.

wild-type strain H1200; the rest showed a moderately to markedly lowered capacity to utilize uridine present in the LB medium (Table 4).

In no case could resistance to the 5-fluoropyrimidines be explained by an excretion of pyrimidines, since no mutant was able to feed the *pyrB Escherichia coli* strain La5.

Characterization of C. albicans and T. glabrata strains with decreased susceptibility to 5-FC isolated from treated and untreated patients. The *C. albicans* strains CA7 to CA19 and the *T. glabrata* strains TG1 to TG5 were characterized by testing their resistance to 5-fluoropyrimidines and their ability to incorporate ^{3}H-uridine (Table 5). CA7, CA8, and CA9 were susceptible strains taken from untreated patients, as were strains CA14 to CA16 and TG1 to TG3 which showed a decreased 5-FC susceptibility. TG4 and TG5 were isolated from the urine of a patient after 5-FC treatment. In two patients, the strains were isolated before (CA10 and CA17), during (CA11), and after (CA12, CA13, CA18, and CA19) treatment. All strains of *T. glabrata* were completely resistant to 5-FC and 5-FU and had an increased tolerance to 5-FUI (Table 5). Moreover, their incorporation of ^{3}H-uridine was considerably lower than that of *C. albicans* H1200. The 5-FC-susceptible *C. albicans* strains were as susceptible to 5-FU and 5-FUI as the wild-type strain H1200. They also had a high ^{3}H-uridine incorporation. Of eight *C. albicans* strains with decreased susceptibility to 5-FC, four were resistant to all three 5-fluoropyrimidines after 3 days of incubation. Two of them had a very low uridine incorporation (CA15 and CA16). The other two (CA18 and CA19) were isolated from the same 5-FC-treated patient after an interval of 3 weeks. The resistance patterns and incorporation values suggest that the two strains were identical. The incorporation of uridine was fairly high but much lower than in the strain isolated before treatment (CA17). The other four strains (CA11 to CA14) appeared susceptible to 5-FC after 3 days of incubation. After 7 days, however, they formed colonies on 5-FC concentrations of 250 to 1,000 μg/ml. Strain CA10 originated from the same patient as strains CA11

TABLE 4. *Relative incorporation of ^{3}H-uridine into spontaneous 5-FC-resistant mutants of the C. albicans strain H1200*[a]

Strains	Relative ^{3}H-uridine incorporation
S1–3, S5, S7–8, S10–12, S14, S16, S18, S19–20, S23–24, S26, S28, S31, S32, S34–43, S46–47, H12R3	<0.4
S6, S15, S21, S25, S27, S29, S30, S33, S44	0.4–0.8
S4, S9, S13, S17, S22	>0.8
H1200	1.0

[a] The respective mutant strain was grown over night in 5 ml of LB medium containing ^{3}H-uridine. Each culture was cooled, and 5 ml of 10% ice-cold trichloroacetic acid was added and allowed to precipitate at 0 C for at least 1 hr. The precipitates were collected on membrane filters (Millipore Corp.) and washed five times with 0.1 M HCl; radioactivity was then measured. Strain H1200 was chosen as unit.

TABLE 5. *Resistance to 5-fluoropyrimidines and incorporation of ^{3}H-uridine in Candida albicans and Torulopsis glabrata* strains isolated from patients[a]

Strains	Resistance (μg/ml)						^{3}H-uridine
	5-Fluorocytosine		5-Fluorouracil		5-Fluorouridine		
	3 days	7 days	3 days	7 days	3 days	7 days	
TG1, TG2, TG3, TG4	>2,000	>2,000	>1,000	>1,000	10–100	10–100	0.07–0.17
TG5	>2,000	>2,000	>1,000	>1,000	>1,000	>1,000	0.11
CA7, CA8, CA9	<0.5	0.5	25–50	25–50	<0.5	<0.5–1	
CA10	<0.5	0.5	50	50	<0.5	<0.5	1.2
CA11, CA12, CA13	<0.5	250	50	250	<0.5	5	0.74–1.1
CA14	1	1,000	100	500	>1,000	>1,000	0.21
CA15, CA16	>2,000	>2,000	>1,000	>1,000	>1,000	>1,000	0.17–0.30
CA17	<0.5	0.5	50	50	<0.5	<0.5	1.6
CA18, CA19	>2,000	>2,000	>1,000	>1,000	>1,000	>1,000	0.65–0.75
H1200	<0.5	0.5	50	50	<0.5	0.5	1.0
H12R3	>2,000	>2,000	>1,000	>1,000	>1,000	>1,000	0.13

[a] Resistance to 5-fluoropyrimidines was determined on YMA plates after 3 and 7 days of incubation as described in Materials and Methods. The relative incorporation of ^{3}H-uridine was determined as described in Table 3.

to CA13. Since the three strains (CA11 to CA13) showed a similar increase in 5-FU and 5-FUI resistance, they are probably identical. Strain CA14 was highly resistant to 5-FUI after 3 days and also had a very low capacity to incorporate uridine, despite the fact that it appeared quite susceptible to 5-FC and 5-FU after this incubation period.

None of the *Candida* strains isolated from patients was able to feed the pyrimidine-requiring *E. coli* strain La5.

Growth in the presence of 5-FC of a clinical C. albicans strain and of resistant H1200 mutants. A common finding with clinical isolates of *C. albicans*, as well as with the H1200 mutants, was strains which were affected by low concentrations of the drug though they were still able to form colonies on very high concentrations if the time of incubation was prolonged. The growth in the presence of 5-FC of two such strains, one isolated from a patient (CA12) and one H1200 mutant (S23), was compared with that of strains H1200 and H12R3. The four strains were incubated in flasks containing YNB and different concentrations of 5-FC. As shown in Fig. 3 the growth of strain H12R3 was unaffected by 5-FC at the highest concentration tested, 1,000 μg/ml. The growth rate of the mutant S23 and of the clinical strain CA12 was markedly affected even by 1 μg of 5-FC per ml. However, growth, although reduced, was not completely inhibited at any concentration tested. The 5-FC-susceptible strain H1200 was able to grow and to produce two mass doublings in the presence of 1 to 50 μg of 5-FC per ml. The cell cultures were tested afterwards for their resistance to 5-FC on plates. No mutants were detected except in strain S23 cultivated in the presence of 1,000 μg of 5-FC/ml. These 5-FC-resistant mutants were probably responsible for the increased growth rate observed after 90 hr in this flask (Fig. 3).

DISCUSSION

The metabolism of pyrimidines and their incorporation into RNA has been studied in *Salmonella* and *E. coli* (for review, see 10). Cytosine can be deaminated to uracil, which is then converted to UMP. Uridine is either transformed to uracil or directly phosphorylated to UMP. The mechanisms of 5-FC resistance in *S. cerevisiae* have been elegantly worked out by Grenson (4) and by Jund and Lacroute (7). They showed that resistance developed as a result of four types of mutation: (i) mutation in the cell membrane affecting a cytosine specific permease, (ii) mutation affecting cytosine deaminase production, (iii) mutation affecting UMP pyrophosphorylase activity, (iv) mutation giving rise to an increased de novo synthesis of pyrimidines (Fig. 4). The first two types of mutation specifically affect 5-FC resistance; the latter two also affect resistance to 5-FU and, to a varying extent, resistance to 5-FUI.

The *C. albicans* strains most thoroughly investigated in our study were the wild strain H1200

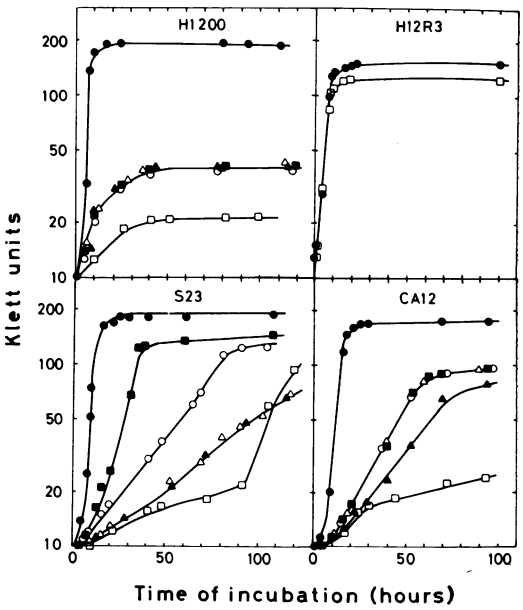

FIG. 3. *Growth of 5-FC-resistant C. albicans strains in the presence of different concentrations of 5-FC. Strains H1200, H12R3, S23, and CA12 were grown to the logarithmic phase in YNB medium and thereafter inoculated into prewarmed YNB medium containing 5-FC at the following concentrations (μg/ml): 0 (●), 1 (■), 5 (○), 10 (△), 50 (▲), and 1,000 (□). Growth was followed in a Klett-Summerson colorimeter with a W66 filter.*

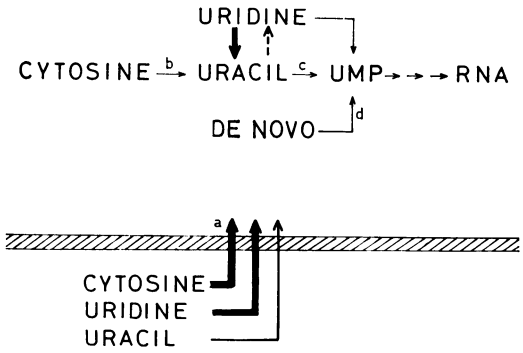

FIG. 4. *Presumptive scheme of uptake and utilization of pyrimidines in C. albicans. The scheme is partly based on the data for S. cerevisiae presented by Grenson (4) and Jund and Lacroute (7). The letters a–d indicate possible mutations increasing the tolerance to 5-FC.*

and the spontaneous in vitro mutant H12R3. For obvious reasons, this type of mutation is of the greatest clinical interest, since the mutation to 5-FC resistance in strain H12R3 did not affect growth rate. Moreover, growth was not affected by a 5-FC concentration of 1,000 μg/ml (Fig. 3). Strain H12R3 was completely resistant to all three 5-fluoropyrimidines. In this respect, it resembled the FUR1-1 mutant of Jund and Lacroute (7). Since no mutagens were used, we are fairly convinced that resistance to both 5-FU and 5-FUI in strain H12R3 was caused by the same mutational event, though it cannot be proven genetically. In growing cells, neither uridine nor uracil was incorporated into trichloroacetic acid-insoluble material (Fig. 1). In cell-free extracts, however, uridine could be converted to UMP to about the same extent as in the parent strain H1200. The ability to catalyze the conversion of uracil to UMP was, however, impaired. This strongly suggests that the 5-FC resistance of H12R3 was caused by a mutation in the gene coding for UMP pyrophosphorylase. The mechanism behind the absolute FUI resistance is not clear, but it appears that in *C. albicans* the major part of 5-FUI is transferred to 5-FU before being converted to 5-FUMP.

The analysis of 47 5-FC-resistant mutants of strain H1200 showed that they all also had increased resistance to 5-FU. After 7 days of incubation, most mutants were resistant to 5-FUI, and most of them also had a considerably lowered incorporation of uridine into RNA. This suggests that in no mutant was the specific cytosine permease or the cytosine deaminase affected. No mutant, including H12R3, was able to feed the pyrimidine-requiring *E. coli* strain La5, showing that pyrimidines were not excreted into the growth medium. The accompanying resistance to 5-FU could suggest that the conversion of 5-FU to 5-FUMP was affected in all mutants.

The strains isolated from patients showed resistance patterns similar to those of the H1200 mutants. Of five *T. glabrata* strains isolated, one obtained from a patient treated with 5-FC was totally resistant to all 5-fluoropyrimidines. The other four were completely resistant to 5-FC and 5-FU and had increased resistance to 5-FUI compared with *C. albicans* H1200. Three of these four strains were isolated from nontreated patients. All five strains had a low incorporation of ^{3}H-uridine.

The 5-FC-resistant *C. albicans* strains isolated from patients were of two main phenotypic classes. The size and number of phenotypic class one colonies was, after 3 days of incubation, not affected by any tested concentration of the 5-fluoropyrimidines (CA15, CA16, CA18, CA19). Strains CA15 and CA16 had a low incorporation of ^{3}H-uridine, but strains CA18 and CA19 were intermediate. This could be explained by one of the enzymes converting uridine to UMP being more specific in the latter strains, leading to utilization of uridine but not of its 5-fluoroanalogue. The strains in the other phenotypic class (CA11 to CA14) were significantly affected by moderately low concentrations of 5-FC and appeared to be as susceptible as strain H1200 to 5-FC and 5-FU after 3 days of incubation. After 7 days, however, these mutants formed colonies on 5-FC concentrations ranging between 250 and 1,000 μg/ml. The H1200 mutants could also be divided into these two main phenotypic classes. Since the number of visible mutants increased by a factor of 5 (Fig. 2) between the second and seventh days of incubation, at least 80% of the total number of mutants selected in the concentration range of 25 to 500 μg/ml were of the latter type. Selecting mutants on 1 μg of 5-FC/ml considerably increased the number of mutants appearing to be susceptible after 2 days of incubation but still able to form colonies on high concentrations of 5-FC after 7 days of incubation (Fig. 2 and Table 3). When two strains of this type, one clinical isolate (CA12) and one H1200 mutant (S23), were compared with strains H1200 and H12R3, it was shown that the growth of strains CA12 and S23 was considerably less on 1 μg of 5-FC/ml (Fig. 3). The increase in cell mass on this 5-FC concentration was, for the first 30 hr of treatment, not greater than that of the 5-FC-susceptible strain H1200.

The distinction between these two phenotypic classes of 5-FC-resistant *Candida* mutants should be of clinical importance. When resistant mutants which form colonies of normal or nearly normal appearance after 2 days of incubation on 5-FC-containing plates are isolated, it is our belief that the therapy should not be continued, for increased concentrations of the drug probably would be useless against infections with this class of resistant organisms. Infections caused by strains of the other class, although also forming normal or nearly normal colonies after 7 days of incubation on 5-FC-containing plates, may still be controlled by 5-FC at concentrations of 25 to 75 μg/ml. One such patient, a 70-year-old man with *Candida* in the urinary tract, was successfully treated with 5-FC. The infection was caused by a *Candida* strain which after 3 days of incubation formed visible but small colonies on 409 μg of 5-FC per ml and nearly normal-sized colonies after 7 days of incubation on 1,638 μg of 5-FC per ml (Schönebeck and Ånséhn, *unpublished data*).

The frequency of mutation to 5-FC resistance was found to be high. The possibility that 5-FC in

itself might act as a mutagen is unlikely, since no radioactivity appeared in deoxyribonucleic acid after the susceptible H1200 strain was grown in the presence of ^{14}C-labeled 5-FC (Normark and Schönebeck, *unpublished data*).

Since the agent possesses a low degree of toxicity, the occurrence of resistant strains is the main disadvantage of the drug. Our experiments clearly show that in cultures of H1200 the number of isolated mutants is constant over a wide range of 5-FC concentrations (25 to 500 μg/ml). The number of resistant mutants occurring can therefore not be controlled by increasing concentrations of 5-FC above 25 μg/ml. Below this concentration, the number of isolated mutants was considerably higher. Thus, one may assume that the concentrations used in vivo for therapeutic purposes should not be lower than this.

ACKNOWLEDGMENTS

We thank Hoffmann-La Roche, Inc., Basle, Switzerland, for the generous supply of 5-fluoropyrimidines.

This work was supported by grants from The Swedish Cancer Society (grant to H. G. Boman) and The Swedish Natural Science Research Council (grant to H. G. Boman).

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