INFECTION AND IMMUNITY, Apr. 1993, p. 1500–1508
0019-9567/93/041500-09$02.00/0

Vol. 61, No. 4

Filamentous Growth and Elevated Vaginopathic Potential of a Nongerminative Variant of *Candida albicans* Expressing Low Virulence in Systemic Infection

FLAVIA DE BERNARDIS,[1] DANIELA ADRIANI,[1] RODOLFO LORENZINI,[2] EUGENIO PONTIERI,[3] GIUSEPPE CARRUBA,[3] AND ANTONIO CASSONE[1]*

Laboratories of Bacteriology and Medical Mycology[1] and Veterinary Medicine,[2] Istituto Superiore di Sanità, Rome, and Department of Experimental Medicine, University of L'Aquila, Aquila,[3] Italy

Received 28 September 1992/Accepted 8 January 1993

The vaginopathic potential and the intravaginal morphology of a nongerminative variant of *Candida albicans*, strain CA-2, were studied in a rat vaginitis model. Although it expressed low virulence in systemic infections, strain CA-2 was capable of causing a vaginal infection of the same duration and extent as that obtained in rats challenged with the germ-tube-forming strain *C. albicans* 3153 from the stock collection or with a fresh clinical isolate of *C. albicans* from a case of human vaginitis. During the experimental infection, the CA-2 cells did not maintain their yeast morphology but gave rise to single enlarged-elongated elements (1 to 2 days) which grew predominantly as coarse, short, pseudomycelium-like filaments (2 to 3 days) and then as long threads (7 days). These latter filaments were ultimately indistinguishable from the hyphal filaments formed by the germ-tube-forming strains, which, however, initially developed in the vagina by typical germ tube formation. This peculiar morphological development of strain CA-2 was not observed in organs of systemically infected mice, where, in contrast to strain 3153 which formed typical hyphae, strain CA-2 maintained a typical pattern of yeast growth. Vaginal isolates of strain CA-2 taken at different days of infection were found to be identical to the challenging CA-2 cells, in terms of biochemical characteristics, inability to form germ tubes in any medium at 37°C in vitro, echinocandin resistance, DNA biotype, and low virulence in systemic infections in mice. Thus, experimental vaginitis by strain CA-2 is associated with a peculiar filamentous growth in the vagina, through an apparently novel morphological development bypassing classical germ tube formation but ultimately leading to ordinary hyphae. The elevated vaginopathic potential of strain CA-2, in contrast to its low virulence in systemic infection, also suggests that different *Candida* virulence factors (and host responses) come into play in local and disseminated candidal infections.

Vulvovaginitis is one of the most frequent diseases caused by *Candida albicans*. Approximately two-thirds of women experience at least one acute attack of this disease during their life span, yet the mechanisms by which the fungus infects and spreads into the vaginal cavity have not been elucidated (20, 25). Although some authors claim that candidal colonization is usually associated with vaginal symptoms (18), it is widely held that *C. albicans* may persist in the vagina without causing overt disease, and it is often unclear why some individuals develop acute symptomatic candidal vaginitis (24, 25). Thus, virulence factors of the fungus are likely to play a role in the inflammatory process, and it is possible that they are dynamically expressed in the vaginal environment as a consequence of antigenic variations and phenotypic switching (4, 21, 28).

Among the recognized or putative virulence factors of *C. albicans*, adherence to mucosal cells, secretion of the aspartyl proteinase enzyme(s), and germ tube formation, factors which are possibly interrelated (8, 12, 15, 16, 26, 27; reviewed in reference 25), are candidates to favor the vaginal implant of the fungus. Germ tube formation has been particularly associated with virulence expression, since a variant of *C. albicans* unable to form germ tubes at 37°C was less able than germ-tube-forming strains to give an experimental vaginal infection in pseudoestrus rats (26).

We have used here a germ-tube-negative strain of *C. albicans*, CA-2, selected primarily for resistance to echinocandin from a mutagenized culture of strain 3153 of *C. albicans* (9). Strain CA-2 has a low degree of virulence in terms of animal lethality after an intravenous challenge but is capable of sustaining a nonlethal chronic infection in mice (3, 22). During this infection, nonspecific immunity against several microbial pathogens is acquired by complex mechanisms that are still incompletely known (3, 10). The initial purpose of the present investigation was to assess the experimental vaginopathic potential of this interesting strain of *C. albicans* in comparison with germ-tube-forming, systemically lethal strains. We found that strain CA-2 was at least as vaginopathic as the germinative counterparts in an experimental rat vaginitis model. Unexpectedly, however, we also found that this strain did not maintain the yeast form of growth during vaginal infection but developed coarse filamentous forms resembling the hyphae of the germinative strains but which were not formed through the classical transitional stage of germ tube formation.

MATERIALS AND METHODS

Organisms and growth conditions. *C. albicans* CA-2, an echinocandin-resistant agerminative strain of *C. albicans* originally derived from a chemically mutagenized culture of *C. albicans* 3153 (9), *C. albicans* 3153 itself, and SA-512, a fresh *C. albicans* isolate from a typical case of human vaginitis, were used throughout this study. They were stored on malt extract agar (1.5% [wt/vol] malt extract broth, 2%

* Corresponding author.

[wt/vol] agar) at 4°C and subcultured. For the infectious challenges, cells of both strains were grown in Winge broth (2% [wt/vol] glucose, 0.3% [wt/vol] yeast extract) for 48 h at 28°C on a shaker at 200 rpm (New Brunswick Scientific Co., Edison, N.J.). Growth was measured by hemocytometer counts, and the yeast suspension was diluted appropriately in physiological saline before animal inoculation.

Morphological and biochemical characterization. All strains employed were subjected repeatedly to the established morphological and biochemical tests for *C. albicans* identification, in particular, formation of germ tube in serum, formation of chlamydospores in cornmeal agar with 1% (vol/vol) Tween 80, and assimilation with the API 20C gallery system (Ayerst Italiana, Milan, Italy). The identification was also confirmed by more classic assimilation and fermentation tests (19) and by slide agglutination tests with the polyclonal antibodies (*Candida* Check; Iatron, Tokyo, Japan). Apart from the lack of germ tube formation in any medium in vitro, strain CA-2 met all other criteria for *C. albicans* identification. To check the stability of resistance of the vaginal isolates of strain CA-2 to echinocandin, yeast nitrogen base (0.15% [wt/vol] L-asparagine, 1% dextrose [wt/vol]) plus 20 μg of echinocandin (kindly provided by Eli Lilly Italia, Florence, Italy) was used.

Molecular biotyping techniques. The methods for biotyping *Candida* isolates have been described in detail elsewhere (6). Briefly, *Candida* cells were grown in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] dextrose) at 30°C with good aeration to the stationary phase (optical density at 600 nm, 1 cm = 1.5). For spheroplast formation, the cells were suspended in SE medium (1.2 M sorbitol, 0.1 M EDTA [pH 7.5]) and treated with Lyticase (Sigma; 100 μl of stock solution at 1 mg/ml) for 1 h at 37°C.

For DNA extraction, the spheroplasts were centrifuged (1,800 × *g*, 5 min) and incubated in a lysis buffer (50 mM Tris HCl, 20 mM EDTA, 1% [wt/vol] sodium dodecyl sulfate [pH 7.5]), in a final volume of 4 ml, for 30 min at 65°C. Potassium acetate (1.5 ml, 3 M) was added, and the mixture was kept on ice for 1 h and then centrifuged at 12,000 × *g* for 15 min. The pellet was discarded, and the supernatant was treated for DNA purification as described elsewhere (6).

For restriction endonuclease analysis, the total DNA was incubated with restriction endonucleases (*Eco*RI, *Hin*dIII) obtained from Boehringer (Mannheim, Germany). Twenty units of each enzyme was used, as described in a previously established method (6).

The electrophoretic karyotype of each isolate was determined by transverse alternating field electrophoresis performed with a GenLine apparatus from Beckman. Briefly, four consecutive runs were done on each gel (10 by 7.5 cm, 0.5 cm thick, 1% agarose; Bio-Rad) containing the DNA samples in agarose inserts and immersed in running buffer (24.2 g of Tris, 2.9 g of EDTA, 5 ml of glacial acetic acid). The parameters of each run were: time, 24 h; voltage, 70 mA; temperature, 10°C; time intervals, 120, 240, 420, and 600 s for the first, second, third, and fourth runs, respectively. After electrophoresis, the gel was stained with ethidium bromide and photographed (6).

Experimental rat vaginitis. Ovariectomized female Wistar rats weighing 80 to 100 g (Charles River Breeding Laboratories, Calco, Va, Italy) were maintained in pseudoestrus by injection of estradiol benzoate (0.5 mg subcutaneously) (Benzatron; Samil, Rome, Italy) every 2 days. Six days after the first estradiol dose, the animals were inoculated intravaginally with 10^7 *C. albicans* cells in 0.1 ml of saline solution, which was administered to each animal through a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy). Vaginal fluid was taken from each animal every 2 days with a calibrated (1-μl) plastic loop (Dispoinoc; PBI) inserted and removed from the vagina. As reported elsewhere (12–14), this procedure offered a reproducible and consistent determination of fungal burden in the vagina. Some fluids were stained by the periodic acid-Schiff (PAS) method for microscopic examination, while other fluids (one vaginal sample per rat) were used for measurement of vaginal colonization. To this end, the contents of each loop were vigorously suspended in 0.1 ml of phosphate-buffered saline (PBS), and then aliquots were streaked on Sabouraud dextrose agar containing chloramphenicol (20 μg/ml) to calculate the CFU per milliliter that developed after incubation of the plates at 30°C for 48 h. At intervals during the experimental infection, the colonies developed on agar were subjected to species identification (see above). In some experiments, echinocandin (20 μg/ml) was added to the medium used for vaginal CFU enumeration. For the assay of the hypha-inducing ability of the vaginal fluid, the vaginal cavity of oophorectomized, estrogen-treated rats (6 days after the first estradiol administration [see above]) was flooded with 0.5 ml of PBS, and the fluid was harvested and centrifuged at low speed to remove any vaginal debris. To 0.9 ml of cell-free supernatant, with or without estradiol addition, was added 0.1 ml of a suspension of *C. albicans* yeast cells to reach a final density of 10^6 cells per ml of vaginal fluid, and the mixture was incubated for 48 h at 37°C.

Systemic mouse infections. Groups of eight inbred male CD_2F1 mice (18 to 21 g: Charles River Breeding Laboratories) were injected intravenously with 10^6 cells of each strain of *C. albicans* (from Winge broth cultures; see above) in a final volume of 0.2 ml. At intervals during the experimental infection, *C. albicans* growth in mouse organs was monitored by CFU enumeration as described elsewhere (3, 14). Some organs were also fixed in 10% (vol/vol) formalin, and sections of paraffin-embedded tissues were examined after treatment with PAS and Van Gieson stains. In other experiments, graded doses of *C. albicans* strains were injected intravenously into CD_2F1 mice, and mortality was assessed over a period of 60 days (3, 14).

RESULTS

Rat vaginal infection. The extent of vaginal infection by strains CA-2 and 3153 of *C. albicans* was evaluated in three independent experiments involving five, five, and seven rats, respectively, infected by either strain. The results of each experiment, expressed in terms of CFU per milliliter of vaginal fluid, are presented separately in Fig. 1a to c, while Table 1 shows the results expressed in terms of the number of rats infected relative to the total number challenged. In each experiment, all rats were consistently infected by the two strains for at least the first 2 weeks (Table 1). On the last day of observation (day 29), in two experiments, fewer rats were infected by strain CA-2 than by strain 3153 (four of five versus one of five, and three of four versus two of four, respectively), but the difference was not statistically significant ($P > 0.05$; Fisher's exact test) even after the data of the two experiments were combined. When the number of CFU was monitored as the mean ± standard error of the mean for all infected rats, vaginal colonization by the two strains was found to be similar in each experiment, since the difference in the mean number of CFU, on most of the days it was measured, was statistically insignificant. At the few time points at which a statistically significant difference was

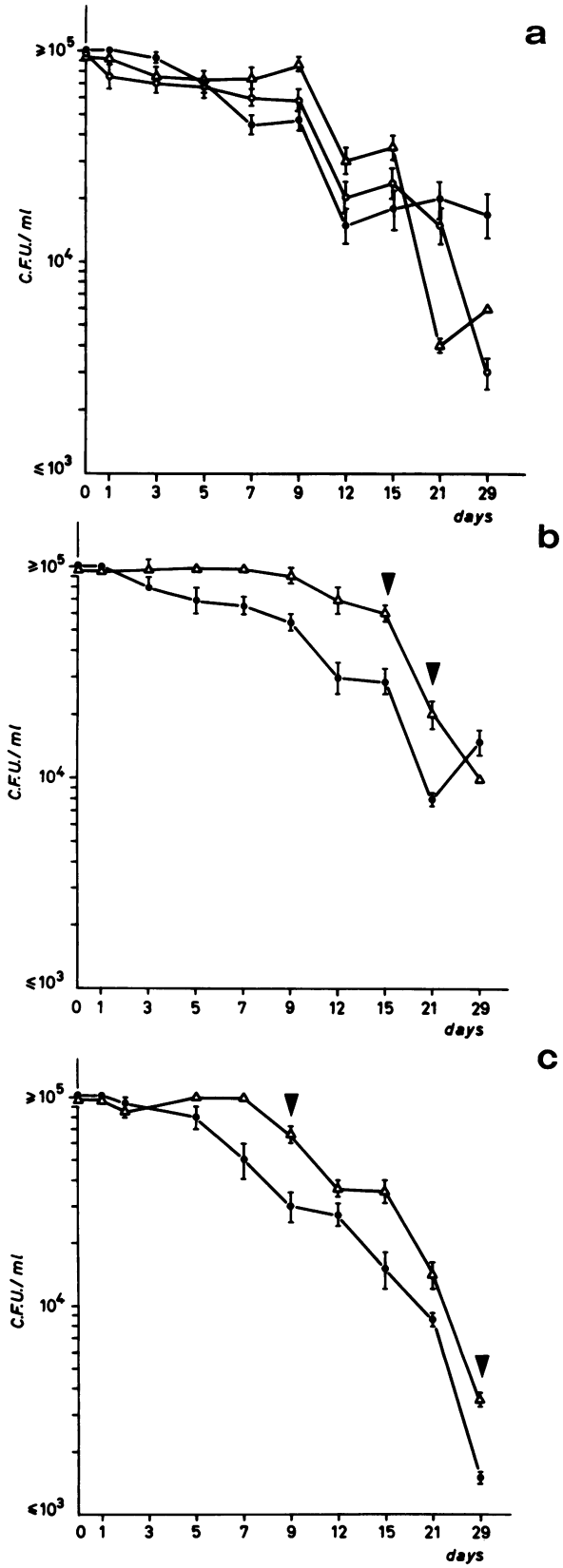

FIG. 1. Vaginal infections in oophorectomized pseudoestrus rats challenged with *C. albicans* CA-2 (△), 3153 (●), and SA-512 (○). Panels a, b, and c refer to three independent experiments. The bars indicate the standard errors of the means, and the arrowheads indicate statistically significant differences ($P < 0.05$, Student's t test; only for panels b and c).

TABLE 1. Vaginal infection, expressed as the number of animals infected relative to the total number, in pseudoestrus rats challenged with strains 3153 and CA-2 of *C. albicans*[a]

Day	No. of rats infected/total no.					
	Expt 1		Expt 2		Expt 3[b]	
	3153	CA-2	3153	CA-2	3153	CA-2
1	5/5	5/5	5/5	5/5	7/7	7/7
5	5/5	5/5	5/5	5/5	6/6	5/6
9	5/5	5/5	5/5	5/5	5/5	5/5
12	5/5	5/5	5/5	5/5	4/4	4/4
19	5/5	3/5	ND[c]	ND	4/4	3/4
22	4/5	3/5	5/5	5/5	4/4	3/4
29	4/5	1/5	4/5	4/5	3/4	2/4

[a] A rat was defined as infected when its vaginal fluid contained $>10^3$ CFU of *Candida* cells per ml. For other details, see the text.

[b] Some rats were, in a random fashion, sacrificed for histological examinations at different times during the whole experimental period. These rats were not counted, and this accounts for the reduction in denominator values during the experiment.

[c] ND, not determined.

noticed (Fig. 1b and c, arrowheads), the number of CFU indicated greater vaginal colonization by CA-2. The interexperiment reproducibility (calculated for the time period in which all rats were infected) was satisfactory for both strains. For instance, on day 7, the mean CFU (± standard errors of the mean) of the vaginal counts in the three experiments in which rats were infected by strain 3153 were $(45 \pm 9.8) \times 10^4$, $(75 \pm 16.9) \times 10^4$, and $(50 \pm 13.9) \times 10^4$. Conversely, the CFU counts in the vaginal fluids of rats infected by strain CA-2 were $63.4 \times 10^4 \pm 5.3 \times 10^4$, $>10^5$, and $>10^5$, respectively. In one experiment, the vaginal CFU of strains 3153 and CA-2 were compared with the CFU of a fresh clinical isolate (SA-512) of *C. albicans* which was obtained from a typical case of human vaginitis and was highly germinative. As shown in Fig. 1a, the CFU were markedly similar for all three strains and did not differ statistically for the whole period during which all rats remained infected (12 days).

Colonies (at least five) of both strains CA-2 and 3153, which developed in Sabouraud plates seeded with the infected vaginal fluids after 1, 2, and 3 weeks during the infection, were subjected to fungal reidentification. They were also tested for the ability to form germ tubes in serum. All isolates were reidentified as *C. albicans*, and none of the CA-2 isolates was able to form germ tubes, regardless of the isolation time (see also below), in contrast to the germ-tube-forming ability of all colonies tested of strains 3153 and SA-512.

Morphology of strains 3153 and CA-2 of *C. albicans* during vaginal infection. The results of the preceding section demonstrated that a nongerminative low-virulence strain of *C. albicans* was able to produce a vaginal infection in pseudoestrus rats that was totally comparable in extent and duration to that given by germinative virulent strains of this fungus. This prompted us to examine more closely the morphology of strain CA-2 during the vaginal infection. Vaginal scrapings were taken from each infected rat each day postinfection and separately examined after PAS-van Gieson staining. Figure 2 illustrates the morphology of CA-2 cells in rat vaginal scrapings taken on days 0, 1, and 7 after challenge, in comparison with the morphology of the parental strain on the same days. One hour after vaginal inoculation, both strains showed a typical yeast-like form, corresponding to

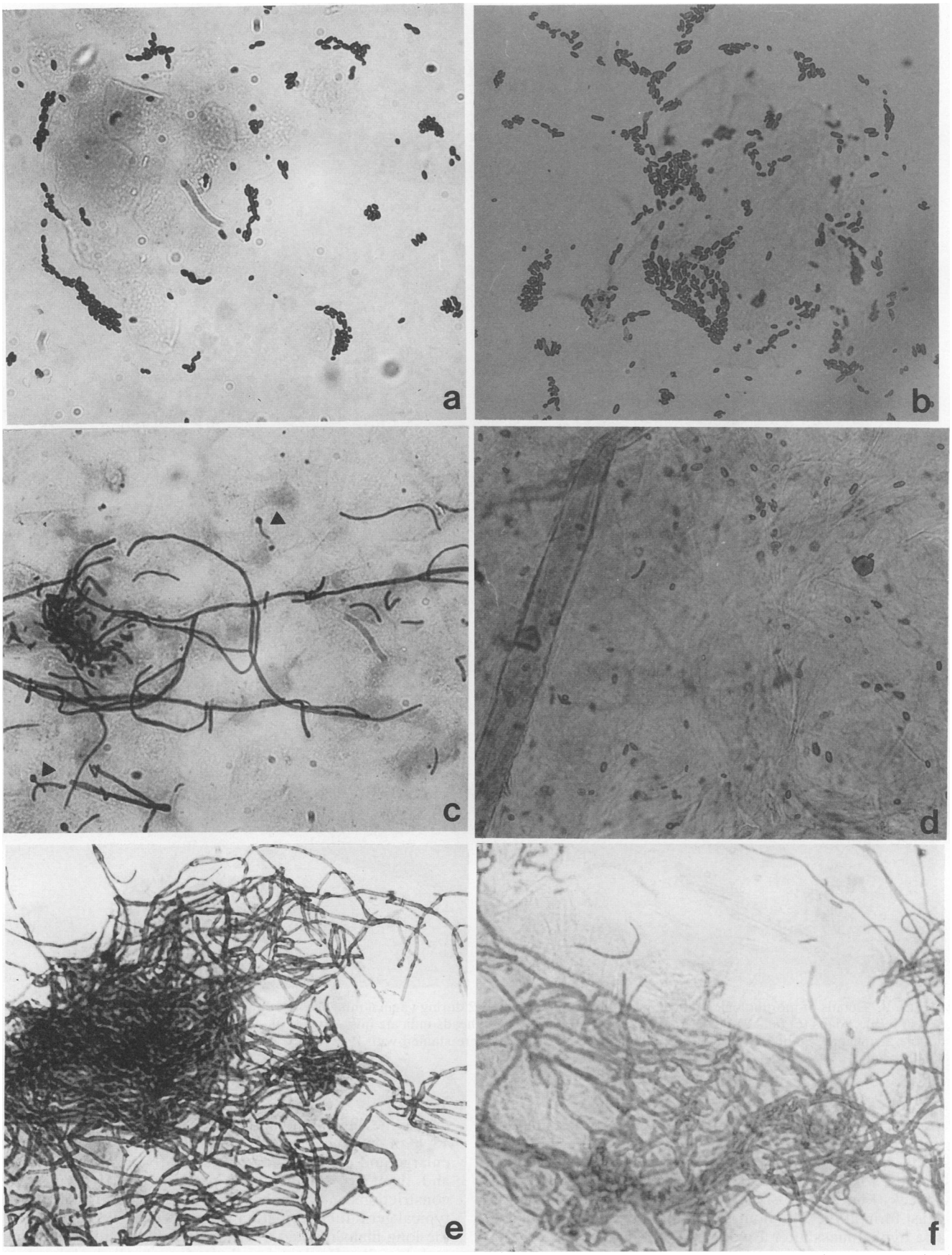

FIG. 2. Morphology patterns of *C. albicans* 3153 (a, c, and e) and CA-2 (b, d, and f) during vaginal infection of oophorectomized pseudoestrus rats after 1 h (a and b), 24 h (c and d), and 5 days (e and f). Arrowheads in panel c indicate typical germ tubes, never observed with CA-2 (see Fig. 3 and text). The vaginal smears were stained by the PAS-Van Gieson method. Magnification, ×240.

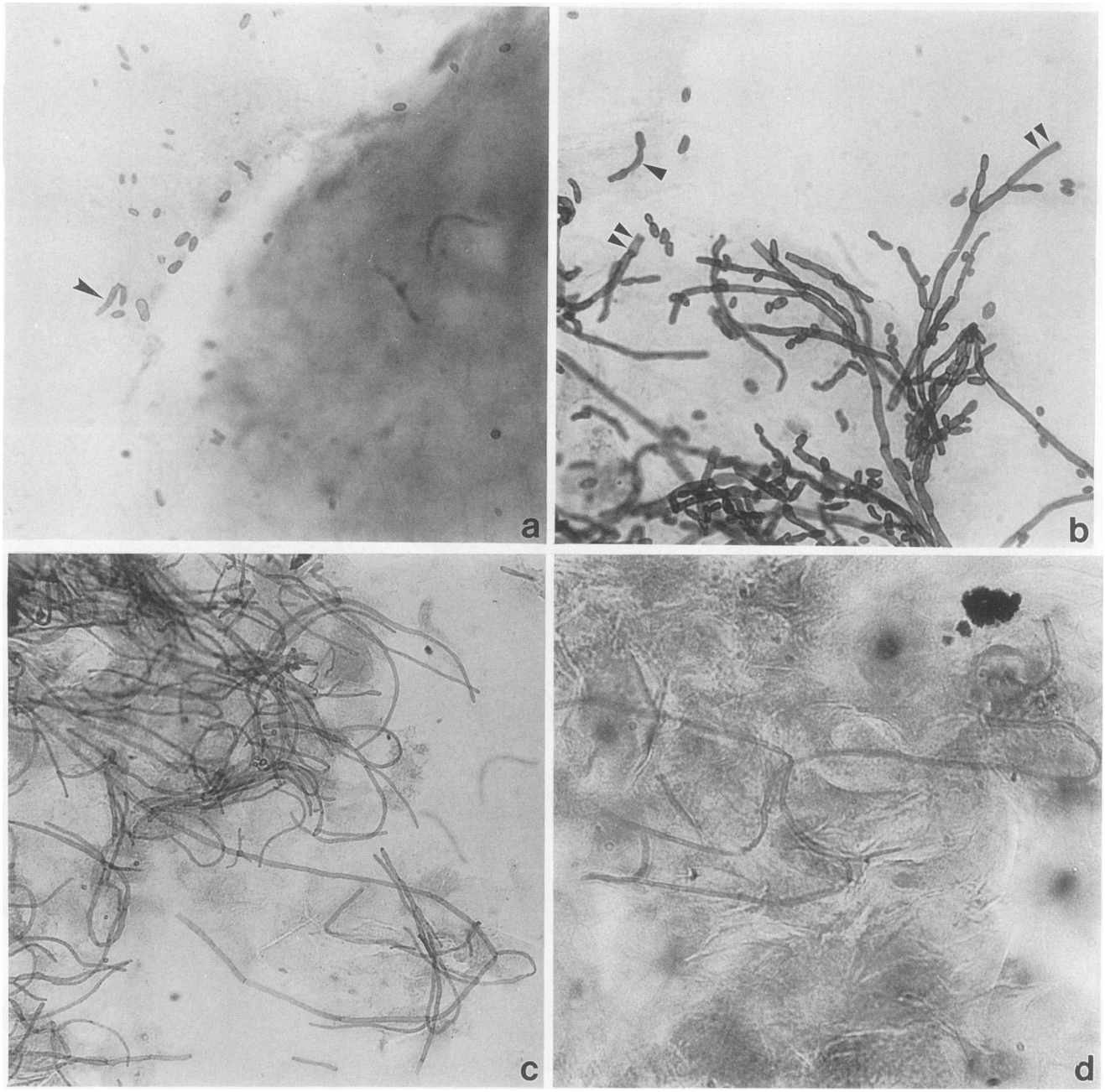

FIG. 3. Details of morphological development of *C. albicans* CA-2 during vaginal infection in oophorectomized pseudoestrus rats at 24 h (a), 48 h (b), 7 days (c), and 21 days (d) after challenge. Single arrowheads indicate initial stages of elongation and filament formation, while double arrowheads indicate typical sharp filament ends. Samples were stained with PAS-Van Gieson. Magnifications, ×340 (a and b) and ×240 (c and d).

the morphology in their respective in vitro cultures at 28°C (Fig. 2a and b). One day after challenge, the germinative strain had developed both germ tubes and long hyphal threads, which gave rise to a mat of interwoven hyphae after 1 week (Fig. 2c and e). Strain CA-2 grossly maintained its yeast form at the 24th hour (Fig. 2d); however, it developed as a hyphal mass after 1 week in a morphology pattern that was coarsely similar to that shown by strain 3153 (Fig. 2e and f).

Details of the morphological development of strain CA-2 during the vaginal infection are shown in Fig. 3. From 24 to 48 h of in vivo growth, the yeast-like cells appeared to enlarge and elongate, forming short (two to four elements) and then long filaments of pseudomycelial aspect, with constrictions and branches (Fig. 3a and b). No evidence of a typical germ tube formation was ever observed, and most of the long filaments had a terminal element with a flat, sharp end (Fig. 3b). By days 5 to 7, the fungal cells had developed into an intricate mass of elongated septate threads, in which few yeast cells were occasionally observed (Fig. 3c). This

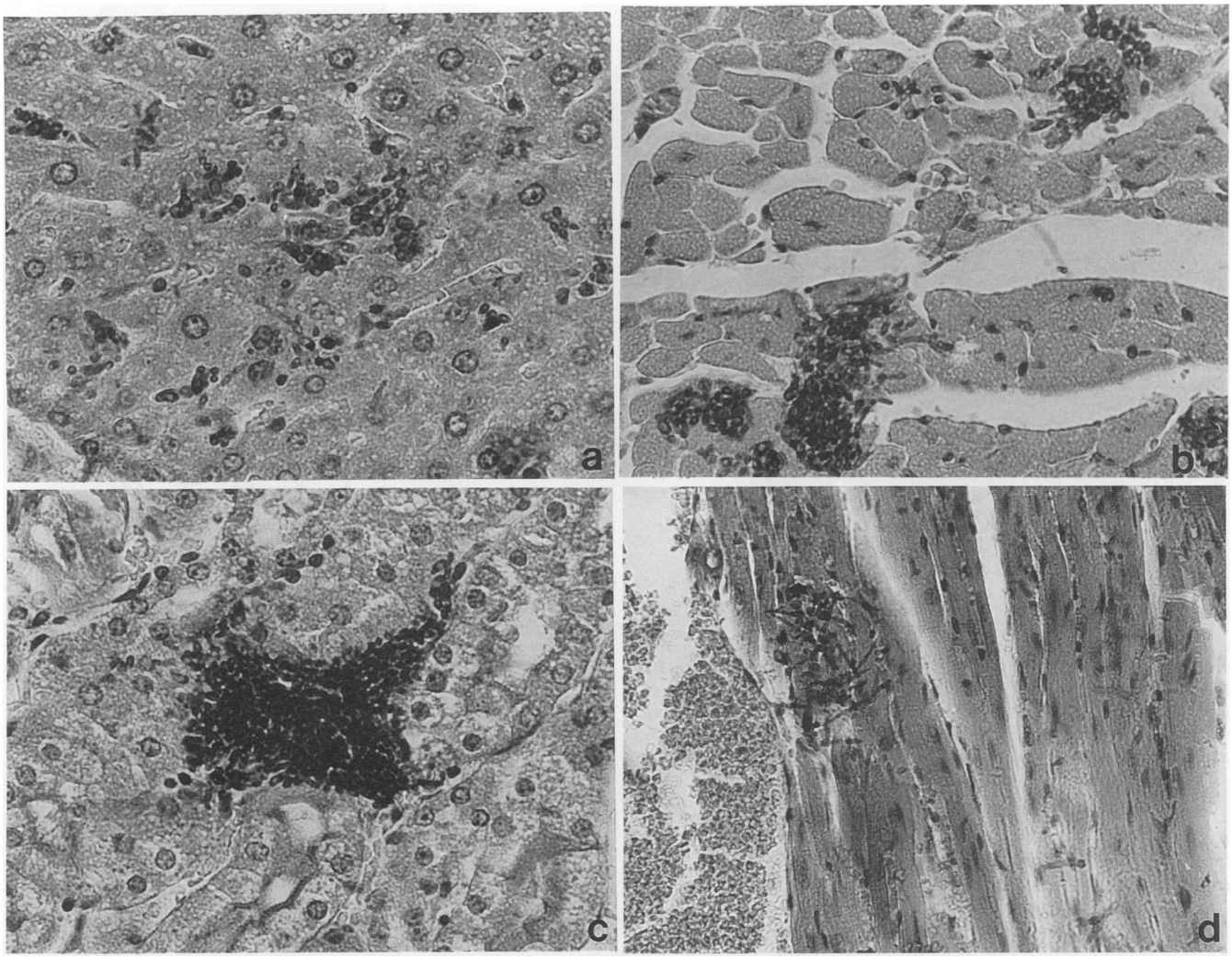

FIG. 4. PAS-Van Gieson-stained sections of kidneys and hearts of CD_2F1 mice challenged with *C. albicans* CA-2 (a, b, and c) and 3153 (d). Mice were sacrificed on day 7 (strain CA-2) or day 3 (strain 3153) to prepare the histological sections. Magnification, ×350.

condition persisted for the remaining period of infection until fungal elimination (Fig. 3d). The morphological development of strains 3153 and CA-2 described above was identical in all infected rats.

Systemic infection of mice with strain CA-2. To ascertain whether the pattern of morphological development of CA-2 described above was typical of the vaginal environment (or infection) or simply reflected the modalities of in vivo growth of this in vitro nongerminative strain, we reproduced a previously established chronic nonlethal infection model in mice (3) and looked at the morphological appearance of strain CA-2 in histological sections of various organs from mice sacrificed at intervals during the infection. *C. albicans* 3153 was used as a control. Strain CA-2 grew with yeast-like morphology in the organs of chronically infected mice (Fig. 4), in a pattern similar to that described for this mutant by others (23). Yeast colonies were particularly prominent in the cortical kidney area, sometimes occupying the whole glomerulus, with no evidence of inflammatory response. The liver and spleen were also abundantly parasitized by yeast fungal cells (data not shown). In contrast, the organs of mice infected with strain 3153 showed the usual germ tube and hyphal development (see, as example, a section of the heart in Fig. 4). Table 2 shows the mortality of mice inoculated intravenously with graded doses of both 3153 and CA-2 cells from exponentially growing cultures. In this systemic infection, CA-2 was substantially avirulent up to an inoculum size

TABLE 2. Mortality of mice challenged with *C. albicans* 3153 and CA-2 yeast cells[a]

Strain	Mortality after challenge with:					
	10^4 cells		10^5 cells		10^6 cells	
	MST	D/T	MST	D/T	MST	D/T
3153	>60	3/10	7	10/10	3	10/10
CA-2	ND	ND	>60	0/10	>60	1/10

[a] Groups of 10 CD_2F1 male mice (12 weeks old) were inoculated via the tail vein with the indicated number of 3153 or CA-2 yeast cells harvested from an exponentially growing culture in Winge broth (see Materials and Methods). Mice were observed daily for 60 days, and the mortality was evaluated as the median survival time (MST, in days) and the number of dead animals relative to the total number (D/T) at day 60.

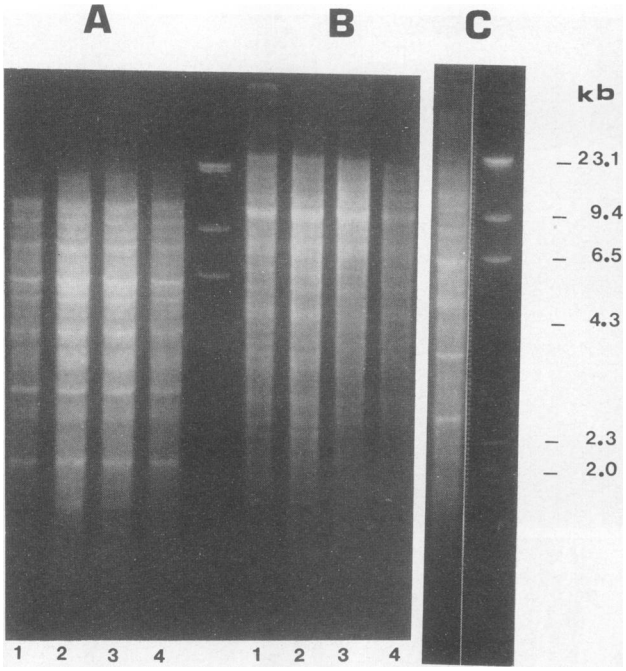

FIG. 5. DNA fingerprinting of rat vaginal isolates of CA-2 cells on days 1, 7, 14, and 21 (lanes 1 to 4, respectively) during the experimental infection. (A) *Eco*RI-digested DNA; (B) *Hin*dIII-digested DNA; (C) *Eco*RI digest of DNA from cells of CA-2 culture used for infection, in a separate electrophoretic run. Molecular weight standards (*Hin*dIII digest of lambda DNA) are shown in the lane between panels A and B and in the right-hand lane of panel C.

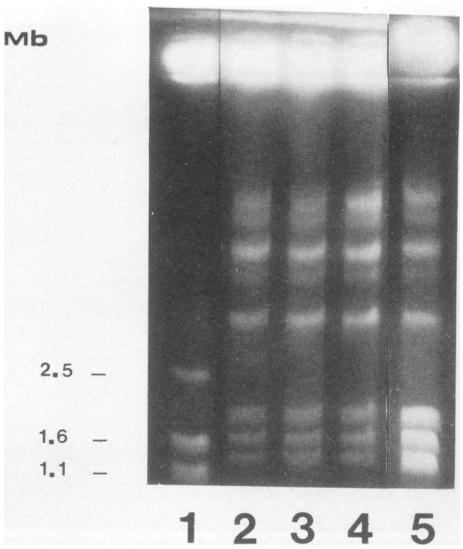

FIG. 6. Electrophoretic karyotype of vaginal isolates of CA-2 cells on days 1, 7, and 14 (lanes 2, 3, and 4, respectively) during the experimental infection as well as CA-2 cells taken from the culture used to infect the rats in a separate run (lane 5). Lane 1 shows the higher portion of the *Saccharomyces cerevisiae* karyotype used as the chromosomal standard. The karyotype was determined by transverse alternating field electrophoresis (see Materials and Methods).

of 10^6 cells per mouse. In contrast, *C. albicans* 3153 was capable of killing all animals inoculated with 10^5 cells and an appreciable proportion of those inoculated with 10^4 cells.

Strain identification during vaginal infection. The data described above could not totally rule out the possibility that the rats infected by strain CA-2 quickly eliminated most of the yeast cells, and this infection was superseded by another (endogenous or exogenous) fungal contaminant, ultimately giving rise to the filamentous growth typical of the germinative strains of *C. albicans*. This question was approached in several ways. In the three experiments reported in "Rat vaginal infection," 50 distinct isolates, at different time intervals, from the vaginas of infected rats were reidentified as *C. albicans*, and their API 20C profiles were exactly the same as those of the challenging fungal cells. In the experiment used to monitor the morphological development of strain CA-2 in the rat vagina (see above), multiple isolates of this fungus were taken at various times during the infection and subjected to DNA fingerprinting and karyotype analysis in pulse-field electrophoresis. All of these distinct isolates proved to have the same fingerprinting and electrophoretic karyotype, which were similar to those of distinct colonies of CA-2 cells taken from the culture used to challenge the rats. Figures 5 and 6 show examples of the identity of some of the isolates studied. We also took advantage of the antimycotic (echinocandin) resistance of strain CA-2. Thus, vaginal samples from rats infected with either strain 3153 or CA-2, at different times during the infection, were streaked in parallel onto Sabouraud dextrose agar plates, one of which contained 10 μg of echinocandin per ml, a dose sufficient to inhibit totally the growth of the drug-susceptible strain 3153 while not affecting the growth of strain CA-2 (2). The mean CFU of this latter strain did not significantly differ when the fungi were seeded in Sabouraud dextrose agar or Sabouraud dextrose agar plus echinocandin at any time interval considered. In contrast, the vaginal fluids from rats inoculated with strain 3153 gave no or only occasional colonies when streaked on the plates containing echinocandin (data not shown).

Growth of strain CA-2 in vitro. In view of the peculiar morphological behavior of CA-2 cells during vaginal infection, as opposed to their typical yeast growth in vitro or in the organs of systemically infected mice, we wondered whether cell-free vaginal fluids from ovariectomized, estrogen-treated rats could promote hypha formation by CA-2 cells in vitro. The effect of estradiol addition to the vaginal fluid was also examined. The results of these experiments showed that vaginal fluids, with or without exogenous estradiol addition (in a dose range of 5 to 100 μg/ml), did not induce CA-2 cells to form hyphae over a 48-h incubation period at 37°C. Interestingly, vaginal fluid induced some germ tube formation in the germinative strain 3153. In a typical experiment, the fluid pooled from vaginal washes of five ovariectomized, estrogen-treated rats (see Materials and Methods) induced 40 to 50% germination in cells of strain 3153 after 5 h of incubation at 37°C, as compared with 80 to 90% of germ-tube-bearing cells after incubation in fetal calf serum (10% [vol/vol] in PBS). The addition of estradiol (5 or 100 μg/ml) to the vaginal fluid did not appreciably increase the percentage of germ tubes, and the results were comparable whether the cells were from a subculture of strain 3153 in Winge medium or freshly isolated from rat vagina (data not shown).

DISCUSSION

As recently emphasized by Sobel (25), germ tube and hyphal development of *C. albicans* is likely to play a role in

the pathogenesis of acute candidal vaginitis. A widely accepted suggestion holds that the yeast form of this fungus colonizes the healthy vagina asymptomatically, whereas hyphal or pseudohyphal development is associated with the onset of disease (20, 24–26). Mechanisms by which hyphal growth can favor fungal infection, such as the increase of fungal adherence to vaginal epithelial cells, have also been postulated (15, 27). From in vitro and in vivo studies, it is clear that hyphal development is accompanied by remarkable changes in the antigens expressed on the cell surface (4, 21). These antigenic variations may have particular relevance in consideration of the recognized role of T-cell-mediated immune responses in the control of mucosal candidiasis (5, 7, 11, 30) and of the fact that the modulated antigens in vitro include some which are the main targets of T-cell responses in humans (9a, 29). Production of the enzyme aspartyl proteinase(s) is also advocated as a characteristic of the most vaginopathic *Candida* strains (7, 8, 12–14), but the relationship between proteinase production and germ tube formation is not clear (see also below).

We originally thought it might be relevant to the pathogenicity mechanisms in vaginitis that strain CA-2 was unable to form germ tubes in any medium at 37°C in vitro (17). Thus, we assessed the vaginopathic potential of this strain in the rat vaginitis model. The data reported in this article demonstrate that this strain of *C. albicans*, of manifestly low virulence potential in systemic infections, is as capable as the fully germinative strains to give a prolonged, extensive vaginal colonization in oophorectomized, pseudoestrus rats. Importantly, strain CA-2 gave rise to a peculiar filamentous growth in the rat vagina.

The modes by which CA-2 yeast cells elaborated this filamentous growth were different from those of typical germ tube formation. Initially, the growth pattern was rather polymorphic and coarsely resembled a pseudomycelium-like, cell elongation development. However, the final net result was a filamentous pattern not distinct from the hyphal mass formed by the parental, germ-tube-forming strain. This mode of growth of CA-2 cells in the vaginal environment highlights an unusual, alternative way of making hyphal filaments, bypassing the more classic germ tube formation. We speculate that this alternative pathway could be an extreme characteristic of the nongerminative strains of *C. albicans* under those in vivo situations, with strong selective pressure for hyphal filaments. The vaginal cavity may be such an environment, since this peculiar morphological development of CA-2 cells was not found in internal organs of chronically infected mice and was totally absent in any simple or complex medium including serum or organ-extract-enriched medium at 37°C (17). Nonetheless, attempts ~~at in~~ducing filamentous growth at 37°C in vitro in the presence of vaginal fluid from oophorectomized, estrogen-treated rats, with or without exogenous estradiol addition, were unsuccessful. Although the morphogenic factor(s) in the rat vagina remains to be identified, the peculiar hyphal development of CA-2 intravaginally is likely to bear relevance to the high vaginopathic potential of this strain since the non-germ-tube-forming avirulent strains used by Sobel et al. (26) were reported to maintain a typical yeast form in the vagina. The equal vaginopathic capacities of strains CA-2 and 3153 of *C. albicans*, as opposed to their markedly different potentials for systemic infections, also argue for the differential expression of virulence factors (and host responses) in the two different pathologies. In this context, it is of interest that *Candida parapsilosis* gives the same pattern of rat vaginitis as *C. albicans*, although it maintains a pure yeast form of growth in the vagina (13, 14). Another germ-tube-negative species, *Torulopsis glabrata*, was totally unable to produce an experimental vaginitis in the rat model used here (1, 13, 14). Therefore, hyphal development, whatever the type of transitional morphological stage (classical germ tube formation, the new one described here, or others), is not an absolute requirement for successful vaginal infection in rats. Its apparent relevance as a vaginopathic factor for *C. albicans* can be possibly replaced by the expression of other virulence factors (e.g., the secretion of aspartyl proteinase) in a species like *C. parapsilosis*, which is incapable of forming hyphal filaments (12–14).

Interestingly, strain CA-2 is a low proteinase producer and maintains this property during rat vaginitis (14a). We have previously suggested that proteinase secretion is likely to play a role in human vaginitis (8, 12). In experimental rat vaginitis, a proteinase-negative, but fully germinative, mutant of *C. albicans* was less pathogenic than the parental, highly proteolytic strain but still capable to moderately infect the rat vagina (12). The virulence of *C. albicans*, at least in vaginitis, seems to be pleiotropically determined, and it is possible that a single virulence determinant like germ tube formation or aspartyl proteinase secretion, because of its elevated penetrance, accounts for most of the virulence in a particular strain. The relationship between germ tube formation and expression of one or more aspartyl proteinase genes in candidal vaginitis is currently being investigated in our laboratory.

In our experiments, we drew particular attention to the demonstration that the nongerminative fungus growing into a filamentous form in vivo was indeed strain CA-2 and not another strain of *C. albicans* occasionally contaminating the cultures or originally colonizing the rat vagina. Thus, we resorted to both echinocandin resistance and highly discriminatory molecular biotyping in identifying the vaginal isolates of CA-2 during the experimental infection. All of the data coherently indicate that the filamentous fungus growing in the rat vagina was indeed the progeny of the challenging strain CA-2. In fact, it would seem quite unlikely that a contaminant *C. albicans* could combine the same phenotype, drug resistance, DNA fingerprinting (with two endonucleases), and electrophoretic karyotype of strain CA-2 used for infection. Moreover, the inability of all CA-2 isolates from the rat vagina to form germ tubes and hyphae in vitro demonstrates that CA-2 did not simply revert to the putative parental hypha-forming strain during the infection.

It is not clear whether the intravaginal filamentous growth is a peculiar property of strain CA-2 of *C. albicans* or reflects more general properties of all those strains of *C. albicans* which have been qualified as nongerminative in vitro. Strain CA-2 was originally isolated from cultures of chemically mutagenized strain 3153 of *C. albicans* and selected for echinocandin resistance (9). Strain CA-2 differs from strain 3153 in many aspects other than this characteristic, including the pathological and morphological ones documented here. Thus, strain CA-2 is likely to be a pleiotropic variant with multiple mutations or even genomic deletions. Further studies will require the genetic characterization of strain CA-2 as well as experiments to determine the in vivo growth and vaginopathic potential of other, genetically better characterized, nongerminative variants of *C. albicans*.

ACKNOWLEDGMENTS

We are greatly indebted to J. E. Cutler, Department of Microbiology, University of Montana, for helpful advice and critical reading

of the paper. F. Baschieri helped in the preparation of the manuscript.

This work was supported by grants obtained from I.S.S.-Ministero della Sanità (Rome, Italy) (Special Project A.I.D.S., contract 62.06-021) and C.N.R. Italy (Special Project F.A.T.M.A., contract 91.00276.41).

REFERENCES

1. **Agatensi, L., F. Franchi, F. Mondello, R. L. Bevilacqua, T. Ceddia, F. De Bernardis, and A. Cassone.** 1991. Vaginopathic and proteolytic *Candida* species in outpatients attending a gynaecology clinic. J. Clin. Pathol. **44:**826–830.
2. **Angiolella, L., C. Bromuro, N. Simonetti, and A. Cassone.** Glucan synthesis and its inhibition by cilofungin in susceptible and resistant strains of *Candida albicans*. J. Med. Vet. Mycol., in press.
3. **Bistoni, F., A. Vecchiarelli, E. Cenci, P. Puccetti, P. Marconi, and A. Cassone.** 1986. Evidence for macrophage-mediated protection against lethal *Candida albicans* infection. Infect. Immun. **51:**668–674.
4. **Brawner, D., and J. Cutler.** 1986. Ultrastructural and biochemical studies of two dynamically expressed cell surface determinants on *Candida albicans*. Infect. Immun. **51:**327–336.
5. **Cantorna, M. B., and E. Balish.** 1990. Mucosal and systemic candidiasis in congenitally immunodeficient mice. Infect. Immun. **58:**1093–1100.
6. **Carruba, G., E. Pontieri, F. De Bernardis, P. Martino, and A. Cassone.** 1991. DNA fingerprinting and electrophoretic karyotype of environmental and clinical isolates of *Candida parapsilosis*. J. Clin. Microbiol. **29:**916–922.
7. **Cassone, A.** 1989. Cell wall of *Candida albicans*, its functions and its impact on the host. Curr. Top. Med. Mycol. **3:**249–314.
8. **Cassone, A., F. De Bernardis, F. Mondello, T. Ceddia, and L. Agatensi.** 1987. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J. Infect. Dis. **156:**777–783.
9. **Cassone, A., R. E. Mason, and D. Kerridge.** 1981. Lysis of growing yeast-form cells of *Candida albicans* by echinocandin: a cytological study. Sabouraudia **19:**97–110.

9a. **Cassone, A., et al.** Unpublished data.

10. **Cenci, E., L. Romani, A. Vecchiarelli, P. Puccetti, and F. Bistoni.** 1989. Role of L3T4 lymphocytes in protective immunity to systemic *Candida albicans* infection in mice. Infect. Immun. **57:**3581–3587.
11. **Cutler, J. E.** 1976. Acute systemic candidiasis in normal and congenitally thymic-deficient (nude) mice. RES J. Reticuloendothel. Soc. **19:**121–124.
12. **De Bernardis, F., L. Agatensi, I. K. Ross, G. W. Emerson, R. Lorenzini, A. Sullivan, and A. Cassone.** 1990. Evidence for a role for secreted aspartate proteinase of *Candida albicans* in vulvovaginal candidosis. J. Infect. Dis. **161:**1276–1283.
13. **De Bernardis, F., R. Lorenzini, R. Verticchio, L. Agatensi, and A. Cassone.** 1989. Isolation, acid proteinase secretion and experimental pathogenicity of *Candida parapsilosis* from outpatients with vaginitis. J. Clin. Microbiol. **27:**2598–2603.
14. **De Bernardis, F., L. Morelli, T. Ceddia, R. Lorenzini, and A. Cassone.** 1990. Experimental pathogenicity and acid proteinase secretion of vulvovaginal isolates of *Candida parapsilosis*. J. Med. Vet. Mycol. **28:**125–137.

14a. **De Bernardis, F., et al.** Unpublished data.

15. **King, R. D., J. C. Lee, and A. L. Morris.** 1980. Adherence of *Candida albicans* and other *Candida* species to mucosal epithelial cells. Infect. Immun. **28:**667–674.
16. **Kinsman, O. S., and A. E. Collard.** 1986. Hormonal factors in vaginal candidiasis in rats. Infect. Immun. **53:**498–504.
17. **Mattia, E., G. Carruba, L. Angiolella, and A. Cassone.** 1982. Induction of germ-tube formation by *N*-acetyl D-glucosamine in *Candida albicans*: uptake of inducer and germinative response. J. Bacteriol. **152:**555–562.
18. **McCormak, W. M., K. M. Starko, and S. H. Zinner.** 1988. Symptoms associated with vaginal colonization with yeast. Am. J. Obstet. Gynecol. **158:**31–33.
19. **Meyer, S. A., G. Ahearn, and D. Yarrow.** 1984. Genus 4 *Candida*, p. 764–767. *In* I. N. Berkhout and N. J. W. Kreger (ed.), The yeasts, a taxonomic study. Elsevier Science Publishing, Inc., Amsterdam.
20. **Odds, F. C.** 1988. *Candida* and candidosis. Leicester University Press, Leicester, United Kingdom.
21. **Poulain, D., V. Hopwood, and A. Vernes.** 1985. Antigenic variability of *Candida albicans*. Crit. Rev. Microbiol. **12:**223–270.
22. **Ross, I. K., F. De Bernardis, G. W. Emerson, A. Cassone, and P. A. Sullivan.** 1990. The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. J. Gen. Microbiol. **136:**687–694.
23. **Ryley, J. F., and N. G. Ryley.** 1990. *Candida albicans*—do mycelial matter? J. Med. Vet. Mycol. **28:**225–239.
24. **Sobel, J. D.** 1985. Epidemiology and pathogenesis of recurrent vulvovaginal candidiasis. Am. J. Obstet. Gynecol. **152:**924–935.
25. **Sobel, J. D.** 1989. Pathogenesis of *Candida* vulvovaginitis, p. 86–108. *In* M. R. McGinnis, and M. Borges (ed.), Current topics in medical mycology. Springer-Verlag, Stuttgart, Germany.
26. **Sobel, J. D., G. Muller, and H. Buckley.** 1984. Critical role of germ tube formation in the pathogenesis of candidal vaginitis. Infect. Immun. **44:**576–580.
27. **Sobel, J. D., P. G. Myers, D. Kaye, and M. Levison.** 1981. Adherence of *Candida albicans* to human vaginal and buccal epithelial cells. Infect. Immun. **143:**76–82.
28. **Soll, D. R., C. J. Langtimm, J. McDowell, J. Hicks, and R. Galask.** 1987. High-frequency switching in *Candida* strains isolated from vaginitis patients. J. Clin. Microbiol. **25:**1611–1622.
29. **Torosantucci, A., C. Palma, M. Boccanera, C. M. Ausiello, G. C. Spagnoli, and A. Cassone.** 1990. Lymphoproliferative and cytotoxic responses of human peripheral blood mononuclear cells to mannoprotein constituents of *Candida albicans*. J. Gen. Microbiol. **136:**2155–2163.
30. **Witkin, S. S., J. Hirsh, and W. J. Ledger.** 1986. A macrophage defect in women with recurrent *Candida* vaginitis and its reversal *in vitro* by prostaglandin inhibitors. Am. J. Obst. Gynecol. **155:**790–795.