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Series Preface

Current Topics in Medical Mycology, is intended to summarize current research areas in medical mycology for medical mycologists and other scientists who are working in microbiology and immunology. Topics to be included in each volume will serve as contemporary reviews, summaries of current advancements and future directions, and mechanisms to enhance the interdisciplinary use of medically important fungi in understanding pathogenesis, epidemiology, mycotoxins, taxonomy, and other areas where basic, applied, and clinical sciences are used.

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1—Animal Models for Dermatomycotic Infections

JAN VAN CUTSEM

Under natural conditions the skin of man and animals is exposed to numerous agents and may be subject to infections by various pathogenic organisms, including viruses, bacteria, and fungi. In predisposed individuals or those exposed to large concentrations of pathogens, the infection is more pronounced, more invasive, more extended, and less susceptible to therapeutic agents. There are a number of uncommon organisms that are often considered to be saprophytic yet turn to be infectious agents. In this chapter the most common fungal pathogens used as test species in animal models for dermatomycoses are discussed, and the usefulness of the animal models for studying infection and screening antifungal compounds is evaluated.

Most dermatomycoses are caused by dermatophytes and yeasts, mainly *Candida* spp. and *Malassezia* sp.. The increasing incidence of acquired immunodeficiency syndrome (AIDS) and its secondary infections have led to other skin infections, e.g., cryptococcosis, becoming more common. These fungi are the ones most frequently used in experimental animal models of dermatomycosis. Infection by the dermatophytes *Candida* spp., *Malassezia* sp., *Cryptococcus* sp., and miscellaneous agents are discussed in detail in this chapter.

The objectives when using an animal infection model are to study the pathogenicity of the strain and the immunity problems, to evaluate therapeutic agents by screening methods, and to determine the in vivo potency of a compound under experimental conditions in order to obtain information about its antifungal value. The information obtained is used to direct the synthesis of new antifungal agents via substitution or modification of active structures, enabling new candidates to be selected for clinical trials. These models therefore must ensure that extrapolation of the infection and the results after prophylactic or therapeutic treatment to natural and spontaneous infection in man and animals is possible.

A screening model must be standardized to ensure reproducibility, that all animals used in the same experiment have identical lesions, and that the

infection shows the same evolution. No differences in intensity of the infection may occur over a period of years.

First, the animals must be housed in isolated rooms and in separate cages. These cages have smooth walls with the bottom consisting of wire meshes, and they must be easy to disinfect. The next requirement is small, inexpensive animals that are docile, easy to manipulate, and inexpensive to maintain. They must be highly sensitive to the infection, and their body weight must be low so that only a small amount of test compound is needed. Species that make their toilet by licking or that scratch or bite itching or irritated lesions are not used if possible. This requirement disqualifies the mouse, rat, hamster, and rabbit. Taking all these recommendations into consideration, the most valuable candidate is the guinea pig.

Spontaneous cure with persistence or reappearance of chronic lesions may occur, especially in models with high inflammatory reactions. Yet the need for chronic noninflammatory infection models persists. Unfortunately, most animals develop acute inflammatory reactions.

Dermatophytes

Dermatophytes are keratinophilic fungi that affect mainly the keratinized layers of the skin, i.e., the hair and nails of man and animals. Other parts of the body may be invaded, however, although mainly in predisposed and immunocompromised patients. The geographical distribution of the dermatophytes largely depends on the species. Some species are ubiquitous, whereas others are present in delimited areas. Yet, owing to migrations and traveling, the geographical delimitation of these dermatophytes is becoming increasingly blurred and cosmopolitan. It is especially true for anthropophilic dermatophytes, which are host-specific; only rarely are animal infections caused by true anthropophilic dermatophytes found. Natural occurrence of infections in animals by *Trichophyton rubrum*, *Epidermophyton floccosum*, and others have been described (7,38,41,74,77), but infection with anthropophilic agents in experimental animals is difficult (63) and almost not reproducible. It has been possible to infect rabbits with *T. rubrum* (61) after exposure to irradiation or after castration, but the infection was not homogeneous in all animals.

Fujita and Matsuyama (19) were able to obtain superficial invasion of the upper two-thirds of the horny layer of the plantar part of the hind foot of guinea pigs after inoculation under occlusion with anthropophilic strains of *Trichophyton mentagrophytes*. No inflammatory response was recorded, and hyperkeratosis and desquamation were absent; the infection remained silent. The same authors also used zoophilic isolates of *T. mentagrophytes* and concluded that these strains were consistently more invasive and

spread more intensively, producing a strong inflammatory response, erythema, and formation of thick scales.

It is generally accepted therefore that zoophilic dermatophytes are more pathogenic to laboratory animals than anthropophilic strains. Some geophilic agents are also pathogenic in experimental infections, especially *Microsporum gypseum* and *M. nanum*. *Microsporum canis* or *T. mentagrophytes* are most often selected for animal models of dermatophytosis, and most studies are performed on the abraded or nonabraded skin of guinea pigs. (28,63,76).

The experimental infection of cattle by *T. verrucosum* resulted in a clearing phase after the inflammatory phase (42,43). The evolution of *M. nanum* infection on the pig skin also led to a spontaneous recovery (22). *Trichophyton mentagrophytes* var. *quinckeanum*, the agent of mouse favus, is highly pathogenic for the mouse; but on the abraded and nonabraded skin of mice, resolution of the disease occurred within 2 to 3 weeks, producing scutulum and inflammation in the stratum corneum (30,65). *Trichophyton mentagrophytes* var. *quinckeanum* was also pathogenic in inbred strains of mice: The most sensitive were BALB/K mice (30).

The infection of *T. verrucosum* on guinea pig skin was successful and produced the typical evolution of inflammation, as observed with other dermatophytes (40,46). A guinea pig skin graft on athymic mice infected with *T. mentagrophytes* underwent the same evolution. Yet an acute and a chronic phase did not spread to the mouse skin (27). In guinea pigs, there was no difference between the occluded and the nonoccluded *T. mentagrophytes* infection skin (39).

If corticoids or methotrexate were used or if germ-free animals were infected, the infected was not prolonged (26,31). Delayed hypersensitivity, a specific type of cell-mediated immunity, correlated well with the onset of intense inflammation, limitation of surface spread at inoculated sites, and ultimate rejection of infection. Acquisition of specific delayed hypersensitivity was seen in conjunction with enhanced resistance to reinfection (30,31,35,36,43,46).

Guinea pigs infected with *M. canis* or *T. mentagrophytes* on scarified skin using homogenized infected hairs and scales taken from infected guinea pigs demonstrate irregular skin infections. Moreover, it is difficult to obtain adequate quantities for a large series of animals. The most regular infections are obtained with cultures growing at 25°C for 12–14 days on Sabouraud glucose agar in tubes with low glucose content (1% or 2%). For some slow-developing dermatophytes, 21 days of incubation are needed (64,80). After incubation, the aerial sporulated mycelium is removed and suspended in saline or in a mixture of bees' honey and saline (50:50). A 1-ml aliquot is used for each tube, a quantity sufficient for inoculation of two guinea pigs. Standardization of the inoculum is obtained by pooling the collected material of at least 20 tubes and homogenizing it in an ultra-

turrax (20,000 rpm) for 15–30 seconds. Diluted bees' honey is preferred over pure saline; the inoculum mats better on the scarified skin (although eventually on nonscarified skin), and germination of the conidia is faster. An inoculum of *T. verrucosum* grown at 37°C is moderately more pathogenic then an inoculum produced at 25°C in the guinea pig. It may be due to the more prolific development of the fungus and to a profuse production of conidia, especially chlamydoconidia and aleurioconidia.

Large series of nonpredisposed Swiss mice, Wistar rats, and Fisher rats were infected on clipped abraded and nonabraded skin. The infection on the abraded skin was more regular, but the course of the infection was not as extensive as in guinea pigs. Moreover, both mouse and rat often licked the inoculum, the infection site, and the topically applied preparations. They also scratched the itching lesions, producing satellite lesions.

The rabbit is more sensitive but presents the same disadvantages as the mouse and rat. The higher cost of the animal, its maintenance, and especially the larger amounts of test compound needed for treatment make its use in great numbers unpractical. It is, however, the most sensitive animal for *T. schoenleinii*.

Infection on the comb of cocks can be reasonably obtained with *M. canis*, *T. mentagrophytes*, and *M. gallinae* after scarification, but topical treatment is difficult. If a fluid excipient is used it does not adhere sufficiently, and if a viscous cream is applied the chicken feed forms crusts on the inoculated part.

Infection on scarified skin of dermatophyte-free dogs is uniform, but the dogs have to be muzzled in order to avoid licking. Overall, mongrels seem to be more sensitive than beagles. We found no difference in pathogenicity among *M. canis*, *M. gypseum*, and *T. mentagrophytes* for dogs. Various sites can be inoculated over a large skin surface area (Fig. 1-1), but dissemination and interference make the use of such a model questionable.

We have also inoculated calves with *T. verrucosum* on skin that was either scarified or unscarified. Natural infections were used as controls and persisted longer than the experimental ones.

The albino guinea pig is sensitive to dermatophyte infections with zoopathogenic strains and theoretically may be infected at various sites (19). In this case the animals rub against the walls of the cage; and therefore when different inocula or different medications are applied, they may become mixed. In addition, if various medications are applied, percutaneous absorption may also occur for some substances, and so interference cannot be excluded.

Scarification or abrasion of the clipped skin provides the most uniform and consistent infection (Figs. 1-2 and 1-3). Depilation with sodium sulfide (36 g/100 ml of water) applied for 30 seconds is also useful. A slight abrasion of the skin is obtained, and this method is interesting if occlusive dressing with polyethylene film and sealing with adhesive tape is used. We have compared occluded with nonoccluded skin of guinea pigs infected with *M.*

FIG. 1-1. *Microsporum gypseum* infection on the scarified skin of the dog, 14 days after infection.

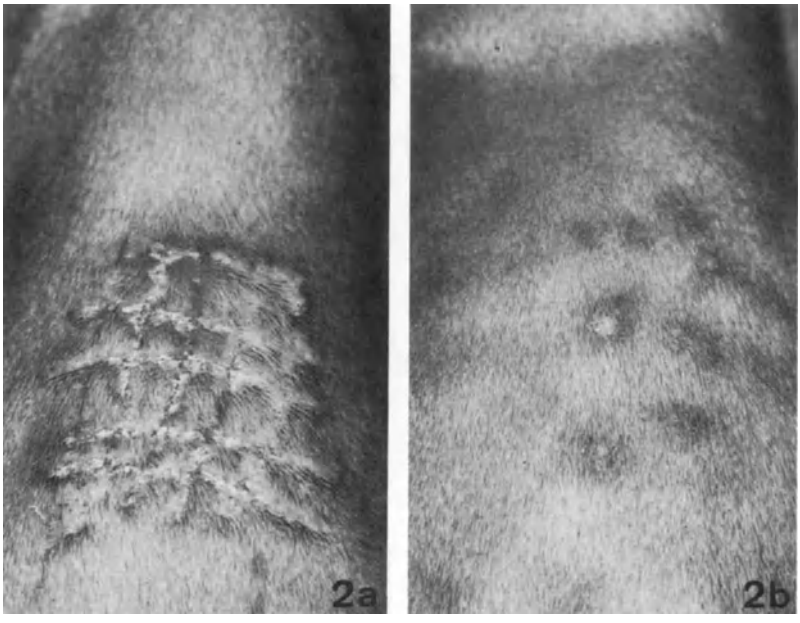
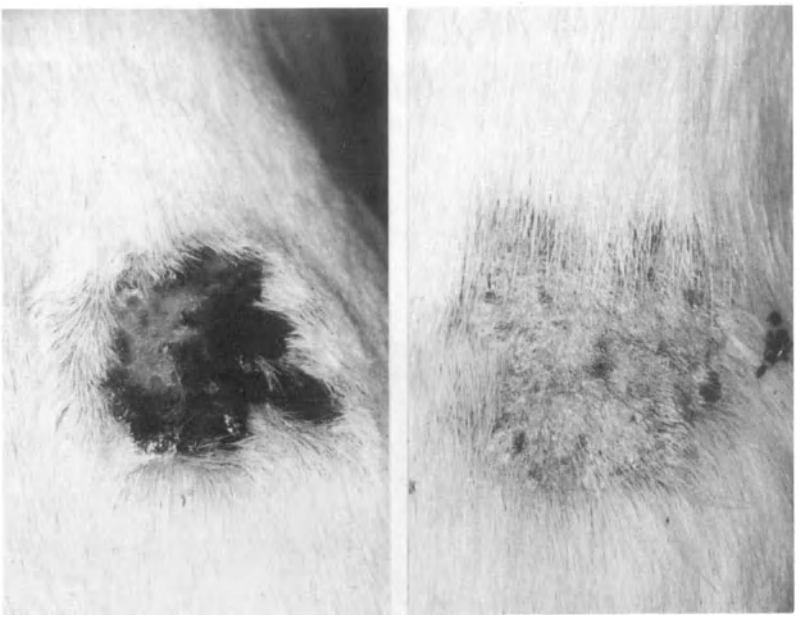


FIG. 1-2. *Trichophyton mentagrophytes* infection on the skin of the guinea pig, 7 days after infection. (a) Scarified skin. (b) Intact skin.



(a) (b)

FIG. 1-3. *Microsporum canis* infection on the scarified skin of the guinea pig, 21 days after infection. (a) Inflammatory lesion. (b) Noninflammatory lesion.

canis or *T. mentagrophytes*, and major differences in the evolution of the infection were noted.

In other experiments we administered either hydrocortisone acetate or prednisolone acetate daily by the intramuscular route at 10 $mg \cdot kg^{-1}$ from day −7 to day +7; metronidazole daily by the intraperitoneal route at 20 $mg \cdot kg^{-1}$ from day −7 to day +7; alloxan once intramuscularly at 200 $mg \cdot kg^{-1}$ 24 hours before infection; chloramphenicol at 50 $mg \cdot kg^{-1}$ and streptomycin at 40 $mg \cdot kg^{-1}$ orally on alternate days from day −7 to day +14, combined or not with prednisolone acetate intramuscularly at 10 $mg \cdot kg^{-1}$ from day −7; or four subcutaneous injections of estradiol undecylate in female guinea pigs at 2 $mg \cdot kg^{-1}$ weekly, starting 1 week before infection. None of these predisposing factors was able to modify the normal course of *M. canis* or *T. mentagrophytes* infections of the scarified skin of guinea pigs.

The normal evolution of dermatophytosis in the guinea pig may be clearly divided into four phases (35): incubation, spreading, inflammation, and healing or clearing extending from days 25 to 60. The length of each phase depends on the mode of infection, the fungus species, and the strain used. In our experiments, after a second inoculation the onset of the infection was more irregular (occurring 1–2 days earlier), the inflammation was mild-

er, and the course of the active disease was shorter. After a third inoculation the course of the infection was even shorter. Our results confirmed those obtained by many other investigators (36,40,43).

In all reported experiments, the infected animals were observed daily, and the lesions were scored weekly. The infection was checked microscopically, by cultures, and in some instances histologically. In order to study the pathogenicity on the scarified skin of nonpredisposed albino guinea pigs, 136 strains belonging to 17 dermatophyte species (Table 1-1) were inoculated. At least six animals were used per strain. Of these strains only the species *M. canis*, *M. gypseum*, zoophilic *T. mentagrophytes*, and *T. mentagrophytes* var. *quinckeanum* may be considered to be pathogenic for the guinea pig. Anthropophilic species were devoid of real pathogenicity under these experimental conditions. Some strains, however, produced superficial, transitory invasion. In other experiments, eight species of anthropophilic (41 strains), six species of zoophilic (34 strains), and two species of geophilic dermatophytes (6 strains) were infected on the scarified skin of guinea pigs and intravenously in male guinea pigs (Tables 1-2 and 1-3).

The standard inoculum used for skin infection was aerial sporulated mycelium, grown for 12–14 days at 25°C. For *T. concentricum*, *T. vio-*

TABLE 1-1. Pathogenicity* of dermatophytes on the scarified skin of guinea pigs.

Dermatophytes	No. of strains				
		With ringworm			
	Tested	0	+	++	+++
Microsporum canis	42	0	1	6	35
M. gypseum	4	0	1	0	3
M. equinum	2	1	1	0	0
M. audouinii	2	2	0	0	0
M. gallinae	1	0	1	0	0
Trichophyton ajelloi	6	6	0	0	0
T. concentricum	8	8	0	0	0
T. equinum	3	1	2	0	0
T. mentagrophytes anthropophilic	17	13	3	0	1
T. mentagrophytes zoophilic	5	0	0	1	4
T. mentagrophytes var. *quinckeanum*	1	0	0	0	1
T. rubrum	18	16	2	0	0
T. schoenleinii	2	1	1	0	0
T. tonsurans	4	3	1	0	0
T. verrucosum	9	6	3	0	0
T. violaceum	6	6	0	0	0
Epidermophyton floccosum	7	7	0	0	0

*Pathogenicity: 0 = no invasion of hair follicles and no skin reaction; + = limited invasion of the hair with sporadic invasion of the epidermis and slight hyperkeratosis; ++ = extended invasion of the hair with moderate inflammation and erythema and moderate crust formation; +++ = severe inflammatory response, erythema, crusts, and scaling followed by alopecia.

TABLE 1-2. Pathogenicity of anthropophilic dermatophytes in the guinea pig.

Dermatophyte	No. of strains		Cutaneous route (abraded skin)		Intravenous route					
					No. of animals		Positive cultures			
	Tested	Origin*	No. of strains	Pathogenicity†	Total	With cutaneous lesions	Skin	Lungs	Liver	Kidneys
Microsporum audouinii	1	1 Scalp	1	0	6	0	0	0	0	0
Trichophyton concentricum	6	6 Skin	6	0	36	0	0	0	0	0
T. rubrum	12	4 AF	4	0	32	0	0	0	0	0
		4 Nail	4	0	30	0	0	0	0	0
		2 EMH	2	0	16	0	0	1	0	0
		2 Skin	1	0	6	0	0	1	0	0
			1	+	8	0	0	0	0	0
T. mentagrophytes	12	5 AF	4	0	30	0	16	10	9	6
			1	+	12	0	11	9	5	3
		2 Nail	2	0	12	0	0	1	0	0
		4 EMH	4	0	24	0	4	4	1	2
		1 Skin	1	+++	12	2	6	5	0	0
T. schoenleinii	1	1 Scalp	1	+	6	0	0	3	1	0
T. tonsurans	2	1 AF	1	0	6	0	0	0	0	0
		1 Skin	1	+	6	0	0	1	0	0
T. violaceum	3	2 Scalp	2	0	20	0	0	0	0	0
		1 Skin	1	0	4	0	0	0	0	0
Epidermophyton floccosum	4	2 AF 2 EMH	4	0	20	0	0	0	0	0

*AF = athlete's foot; EMH = eczema marginatum of Hebra.

†Pathogenicity: 0 = no invasion of hair follicles and no skin reaction; + = limited invasion of hair (not for *T. schoenleinii*) with sporadic invasion of the epidermis and slight hyperkeratosis; ++ = extended invasion of the hair with moderate inflammation and erythema and moderate crust formation; +++ = severe inflammatory response, erythema, crusts, and scaling followed by alopecia.

TABLE 1-3. Pathogenicity of zoophilic and geophilic dermatophytes in the guinea pig.

Dermatophyte	No. of strains		Cutaneous route (abraded skin)		Intravenous route					
					No. of animals		Positive cultures			
	Tested	Origin	No. of strains	Pathogenicity*	Total	With cutaneous lesions	Skin	Lungs	Liver	Kidneys
Microsporum canis	19	8 Dog	7	++	6	0	1	2	1	1
				+++	42	9	26	17	2	4
		1 Cat	1	+++	8	0	6	7	1	2
		4 Rabbit	4	+++	28	2	3	19	7	5
		6 Man	5	+++	38	3	17	24	5	7
			1	++	6	0	1	1	0	0
M. gypseum	2	1 Raccoon	1	+++	18	12	14	3	1	1
		1 Soil	1	+	6	0	0	1	1	0
M. equinum	1	1 Horse	1	+	6	0	0	0	0	0
M. gallinae	1	1 Chicken	1	+	4	0	0	0	0	0
Trichophyton ajelloi	4	2 Horse	2	0	12	0	0	0	0	0
		2 Soil	2	0	12	0	0	0	0	0
T. equinum	1	1 Horse	1	+	6	0	0	0	0	0
T. mentagrophytes	2	1 Dog	1	+++	10	3	9	1	1	1
		2 Dog†	1	+++	12	12	12	11	6	4
T. verrucosum	6	2 Man	2	+	20	3	3	0	0	0
		4 Cattle	3	0	12	0	0	0	0	0
			1	+	4	0	0	0	0	0

†*T. mentagrophytes* B 32663.

*Pathogenicity: 0 = no invasion of hair follicles and no skin reaction; + = limited invasion of the hair with sporadic invasion of the epidermis and slight hyperkeratosis; ++ = extended invasion of the hair with moderate inflammation and erythema and moderate crust formation; +++ = severe inflammatory response, erythema, crusts, and scaling followed by alopecia.

laceum, *T. schoenleinii*, and *T. verrucosum* the cultures were grown for an extra week. Two guinea pigs were inoculated with the aerial mycelium, and conidia were harvested from one 16 × 160 mm tube. For intravenous infection a standard inoculum of 15,000 colony-forming units (CFU) was administered per gram of body weight (BW).

Of the human isolates evaluated, one *T. mentagrophytes* strain produced the typical invasion of the stratum corneum, hair follicles, and hair shaft after skin infection. This strain was the only one that was able to produce skin ringworm in two guinea pigs after intravenous infection. Lung granulomas with positive cultures were found in 6 of 12 animals. After intravenous infection with the other human *T. mentagrophytes* isolates, a significant number of positive cultures were obtained from skin samples of asymptomatic animals (40%) as well as from the lungs (31%), liver (19%), and kidneys (14%). No dissemination to the skin occurred with other strains, but the lungs were positive for individual animals: two with *T. rubrum*, one with *T. tonsurans*, and three with *T. schoenleinii*. *Trichophyton Schoenleinii* was isolated from the liver of one infected guinea pig. Bloch and Massini (5,6) were able to produce deep dermatophytosis with dissemination using anthropophilic dermatophytes, but these experiments could not be reproduced, and the infection cured spontaneously.

In our experiments, none of the guinea pigs was positive after inoculation with *T. ajelloi*. One strain of *M. gypseum* was poorly pathogenic, and another produced typical dermatophyte lesions after local infection and typical ringworm lesions in 67% of the animals after intravenous infection. Cultures of skin and hair samples were positive in 78% of the cases, but lungs, liver, and kidneys were poorly invaded.

Some strains of *M. equinum*, *T. equinum*, *M. gallinae*, and *T. verrucosum*, infected on scarified skin, produced marginal invasion of the skin without reaction in the hair follicle and with rejection of the infected scales within 2 weeks. Intravenous infection produced small ringworm lesions in 3 of 36 guinea pigs infected with *T. verrucosum*, which disappeared within 4 weeks. These animals had positive microscopic and culture examinations.

Skin inoculation of albino guinea pigs with *M. canis* always gives positive results with small differences between strains. Intravenous inoculation produced ringworm lesions in 11%, but hair and skin microscopy and cultures were positive in 42% of the animals during the experimental period of 4–8 weeks, corresponding to 55% positive lung, 13% positive liver, and 15% positive kidney cultures. Histopathological examination of macroscopic necrotic foci of lungs demonstrated fungi in the capillary bed of the pulmonary alveoli, with polymorphonuclear (PMN) and round cells, masses of short hyphae, and conidia in the zones of consolidation. Microabscesses with fungal elements were present in the hair root sheath and the epidermis: hyphae were later observed in the keratinized layers of the epidermis, and conidia and hyphae were seen in the hair shaft itself.

Cutaneous inoculation with two zoophilic *T. mentagrophytes* strains was

TABLE 1-4. Cutaneous and intravenous infection with *Trichophyton mentagrophytes* B 32663 in various animal species.*

Animal		Pathogenicity (after scarification)†	Intravenous infection‡						
					No. of animals				
						Positive cultures			
Species	Strain or race		No. of animals	Experimental period (days)	Ringworm lesions	Skin	Lungs	Liver	Kidneys
Chicken	Arbor acre	+++	24	28–42	0	1	15	1	0
Dog	*Canis vulgaris*	+++	2	28	1	2	2	0	0
Guinea pig	Pirbright	+++	1,174	7–17	1,174	1,169	1,069	378	357
Mouse	Swiss	+++	98	28–35	0	7	80	94	58
Rabbit	New Zealand	+++	16	28–49	15	15	7	4	3
Rat	Wistar	+++	38	28–35	0	3	14	21	6

*Modified from Van Cutsem and Janssen (88).
†Scarification of the comb for chickens and of the skin for other species.
‡Intravenous infection with 15,000 CFU/g.

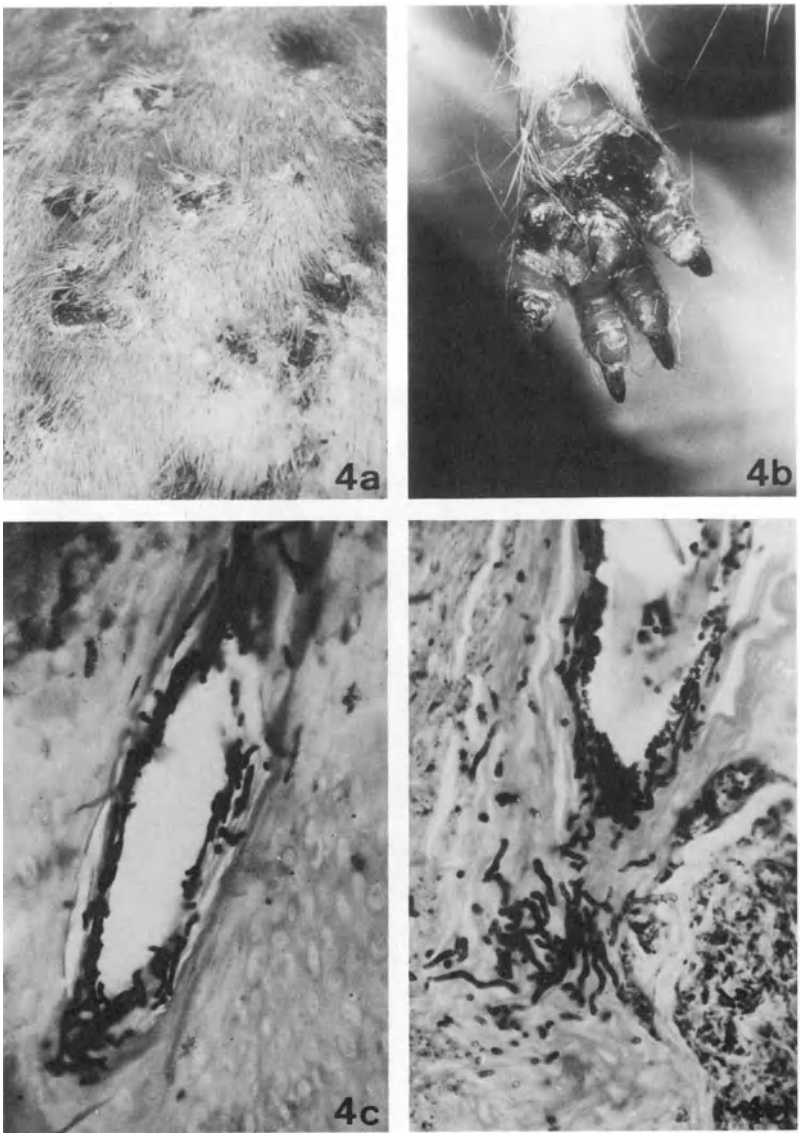


FIG. 1-4. *Trichophyton mentagrophytes* infected intravenously into the guinea pig. (a) Skin: ringworm lesions, 21 days after infection. (b) Foot sole: lesions, 28 days after infection. (c) Fungal elements in the hair root sheath. (d) Microabscess and fungal elements in the hair root sheath.

successful, one producing ringworm lesions after intravenous infection in 3 of 10 animals. However, the second strain, inoculated by the intravenous route, resulted in generalized skin dermatophytosis in all guinea pigs and production of fungal aggregates in various organs, especially the lungs. The latter strain, *T. mentagrophytes* var. *granulare* B 32663, was used for further research. The production of ringworm lesions and the culture results of intravenous infection in guinea pigs as a function of the inoculum size have been reported in previous studies (84,88). The sensitivity of various animal species to infection on the scarified skin was similar (Table 1-4). Yet spontaneous recovery started a few days earlier in mice and rats than in guinea pigs. Disseminated ringworm was constant in all guinea pigs intravenously infected with 15,000 CFU/g BW (Fig. 1-4), whereas discrete skin lesions were present in 15 of 16 rabbits and one of two mongrel dogs. Cultures of skin and hairs were positive in almost all guinea pigs, rabbits, and dogs but were sparsely positive in other animal species. The results of invasion of internal organs by *T. mentagrophytes* are give in Table 1-4. Dermatophyte granulomas in the lungs, containing large amounts of fungal elements, and pneumonia were observed in 91% of guinea pigs. The guinea pigs infected intravenously with *T. mentagrophytes* B 32663 showed ringworm and lung granulomas over a period of 7–77 days (Table 1-5). First, dermatophyte skin eruptions became visible 2–4 days after inoculation, depending on the inoculum size. Samples taken from these lesions were positive by microscopy and on culture. Development into specific ringworm took 7–14 days, and the infection persisted 6–7 weeks. Sections of skin revealed microabscesses with parakeratosis within the epidermis. Some hairs remained unaffected, but most were invaded by hyphae and spores within the hairshaft, accompanied by an inflammatory reaction. In-

TABLE 1-5. Disseminated trichophytosis in guinea pigs infected intravenously.*†

Experimental period (days)	No. of animals	No. of animals				
			Positive cultures			
		Ringworm lesions	Skin	Lungs	Liver	Kidneys
7	24	24	24	24	24	24
14	46	46	46	46	29	30
21	72	72	72	67	30	27
28	826	826	825	763	232	211
35	72	72	70	60	22	18
42	92	92	92	75	29	36
49	18	18	17	15	6	6
56	18	18	17	14	5	4
77	6	6	6	5	1	1

*Modified from Van Cutsem and Janssen (88).
†Intravenous infection with *Trichophyton mentagrophytes* B 32663 15,000 CFU/g.

flammatory reaction of the foot sole started 21–28 days after inoculation. The lesions became more hyperkeratotic, containing large amounts of fungal elements in the scales. Perionychomycosis was present, and onychomycosis started about 7–8 weeks after infection.

In intravenously infected guinea pigs in which cure of skin dermatophytosis had been confirmed after treatment but in which the fungus had not yet been eliminated from the lungs, relapses of ringworm were frequent, and they were considered to spread from the deeper-seated organ lesions.

Routes of infection other than the cutaneous and intravenous ones were not successful or only sporadically so. Comparing skin inoculation to intravenous infection, it may be concluded that zoophilic or geophilic dermatophytes are more pathogenic than anthropophilic agents. Also, cutaneous infection is often of the inflammatory type with an evolution to spontaneous recovery, whereas a stable infection in the guinea pig is obtained after a nonconventional intravenous infection with dermatophytes.

Candida and *Torulopsis*

Members of the genus *Candida* are widely spread yeasts, present as endosaprophytes in the gastrointestinal tract of most mammals and birds. They can also be isolated regularly from external sources. A limited number are considered to be important medical species causing infections in man or animals (32). Various *Candida* spp. as well as other yeasts, especially *Torulopsis glabrata*, may also be a cause of morbidity, mainly in immunocompromised individuals. Under such conditions any organ or part of the body may be involved, but superficial infections constitute by far the most common manifestations (49). In this study, only experimental skin infections in animals, after inoculation on the skin or invasion by other routes, are considered.

Various models have been established on intact or scarified skin, occluded or not (11,50,71,75), and have been reviewed by Ray (57) and Guentzel et al. (29). After epicutaneous infection with occlusion in newborn rats and mice, only *C. albicans* and *C. stellatoidea* were able to produce subcorneal abscesses (58,59). Morphogenetic transformation from yeast to pseudohyphae was observed in the lesions, and only these two yeast species could penetrate the intact stratum corneum. Other species, e.g., *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. guilliermondii*, remained uninvasive.

Hurley (33) inoculated mice by the subcutaneous route: an indurated area appeared at the site of injection during the first week followed by a shallow ulcer during the second week in 9 of 12 animals. Recovery was observed by the end of the third week.

Intracutaneous infection in mice (21) elicited an acute inflammatory response, large inocula (10^7 blastoconidia) inducing large ulcerations. Such inocula promote healing with wide variations in lesion size within the group. Smaller inocula (5×10^5 to 5×10^6 blastoconidia) resulted in reproducible lesions, resolving with 2–3 weeks.

In a model of cutaneous infection with *C. albicans* under occlusive dressing in mongrel dogs, the inoculation resulted after 1 day in minimal oozing and after 2 days in maceration, ulceration, and suppuration. *Candida albicans* was not isolated for more than 5–6 days during occlusion: but if the occlusive dressing was removed earlier, cultures were positive for only 2 days after its removal (68). Maestrone and Semar (45) administered triamcinolone acetonide subcutaneously prior to and after inoculation of New Zealand white rabbits. The hairs were clipped, and the inoculum was mixed with alumdum powder and rubbed on the inside of the ear lobe and on the clipped skin until erythema developed. The lesions were of the acute inflammatory type for about 1 week, becoming chronic with crust formation on the back and desquamation of the epidermis on the ear. Lesions remained clinically evident and culturally positive for 28–35 days.

Wildfeuer (97) inoculated rabbits with *C. albicans* on depilated and scarified skin. Edema appeared within 24 hours with scales and crusts. Pustules were produced within 3 days. Fourteen days after infection the lesions cleared. The topical application of a lotion containing 0.5% hydrocortisone and 0.25% neomycin exacerbated the disease and caused more profuse invasion by hyphae (96). Wildfeuer (98) also compared, in the cutaneous rabbit model, the pathogenicity of 14 yeast species belonging to the genera *Candida*, *Cryptococcus*, *Saccharomyces*, *Torulopsis*, *Rhodotorula*, and *Trichosporon*. He concluded that *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *T. glabrata* possessed a dermotropic affinity for the skin of the rabbit, but the epidermal affinity of *C. stellatoidea*, *C. kefyr*, and *C. guilliermondii* was poor.

Young and adult guinea pigs either received (or did not) alloxan 200 $mg \cdot kg^{-1}$ intramuscularly 24 hours before infection (81). The hairs were clipped before infection and weekly thereafter. One-half of the guinea pigs were scarified on the back. Infection with *C. albicans* on the scarified skin of non-alloxan-pretreated animals produced acute erythema within 3 days and pustules and hyperkeratosis by day 7, spontaneous healing started within 10–12 days. Lesions were more pronounced in adult than in young animals. The skin candidiasis was more severe and more regular in alloxan-pretreated than in nonpretreated guinea pigs. Infection with *C. albicans* on the intact skin of alloxan-pretreated adult guinea pigs resulted in minute papules within 3–4 days, which developed into small vesicles containing a clear exudate within 6–8 days. Erythema and edema were pronounced. Hyperkeratosis and acute desquamation followed after 21 days, and recovery was reached between 35 and 40 days. Scales of all animals contained large numbers of yeasts and pseudohyphae for about 2 weeks, but after 3

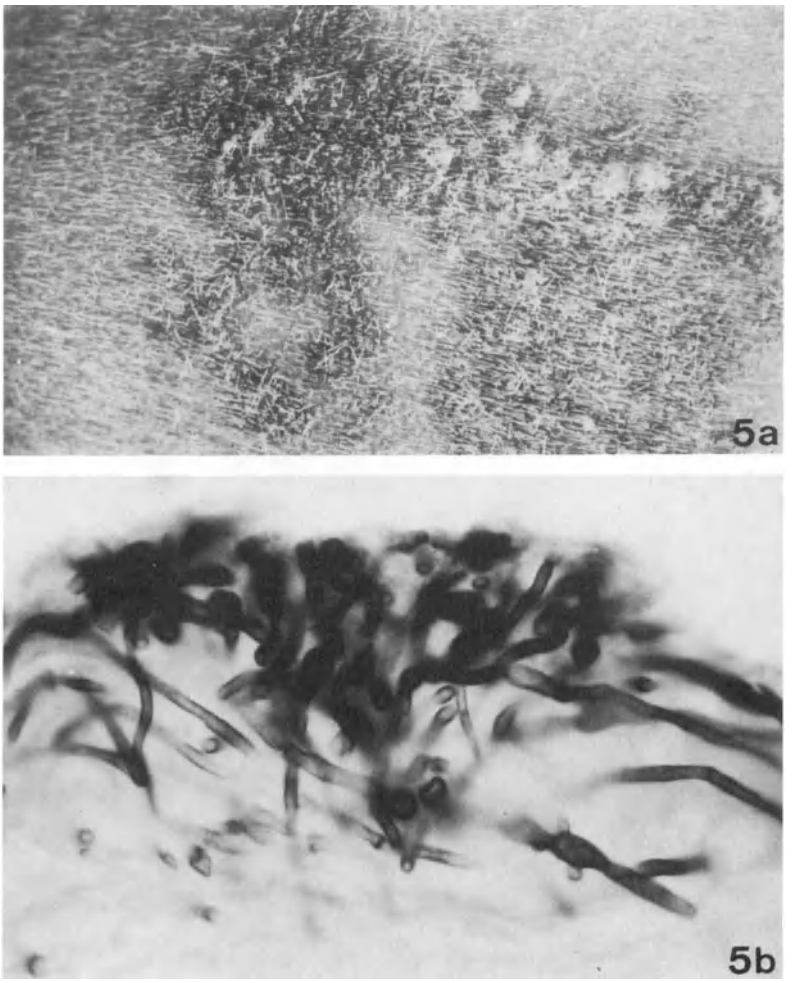


FIG. 1-5. *Candida albicans* infection in the intact skin of the alloxan-diabetic guinea pig, 14 days after infection. (a) Skin lesion. (b) Fungal elements in the keratin.

weeks only 50% of the samples were positive. In histological sections the fungal elements were observed in the stratum corneum and in necrotic foci of the epidermis (Fig. 1-5). The infection was not influenced by occlusive dressings. We also compared the alloxan predisposition to pretreatment with intramuscular hydrocortisone acetate, intramuscular prednisolone acetate, and metronidazole given intraperitoneally. None of these immunomodulating or predisposing agents proved to have a better *Candida* infection-promoting potency in guinea pigs than alloxan.

The pathogenicity of 212 strains (Table 1-6) belonging to seven *Candida*

TABLE 1-6. Pathogenicity of *Candida* spp., *Torulopsis glabrata*, and *Rhodotorula rubra* on the intact skin of guinea pigs with alloxan-induced diabetes.

Species	No. tested	No. with skin lesions*		
		0	Moderate	Pronounced
C. albicans	162	1	3	158
C. stellatoidea	4	0	0	4
C. tropicalis	8	6	2	0
C. kefyr	8	6	2	0
C. krusei	6	6	0	0
C. parapsilosis	6	5	1	0
C. guilliermondii	6	4	2	0
T. glabrata	9	5	4	0
R. rubra	3	2	1	0

*Moderate skin eruptions healed spontaneously in 7–10 days; pronounced skin eruptions: large erythematous and inflammatory lesions.

species, *T. glabrata*, and *R. rubra* was evaluated on the intact nonoccluded skin of alloxan-pretreated albino guinea pigs. In general, six or more guinea pigs were infected per strain, although for a few strains only four animals were used. Of 162 strains of *C. albicans* isolated from various human and animal sources, 158 produced the same pathologic course as the earlier described evolution. Three strains of *C. albicans* showed only a moderate transitory infection and one, isolated from dog feces, was not pathogenic. The skin candidiasis of the four strains of *C. stellatoidea* was comparable to that obtained with *C. albicans*. Inoculation with the other species of *Candida*, *T. glabrata*, and *R. rubra* provoked some slight transitory inflammation and pustules that resolved rapidly.

Candida folliculitis has often been described in man, especially in heroin addicts. We were able to reproduce skin folliculitis in animals after intravenous infection but not by any other infection routes. A total of 182 strains of the same yeast species as above were injected into a lateral vein of the penis of nonpredisposed guinea pigs using an inoculum form 8,000–32,000 CFU/g BW (Table 1-7). Several organs were infected, but dissemination to the skin occurred only with *C. albicans* and *C. stellatoidea*, with folliculitis being established (Fig. 1-6).

At 2–4 days after infection with *C. albicans*, small eruptions appeared on the skin that rapidly transformed to vesicles. Nodules appeared, coalescing between days 7 and 14 after infection, and crusts were formed. The vesicles and the crusts contained large numbers of yeasts, pseudohyphae, and hyphae. The climax of the folliculitis was reached within 12–15 days, and resolution began 3–5 weeks after infection, although all animals remained highly positive during this experimental period, clinically as well as mycologically. The folliculitis was directly correlated with invasion of various other organs.

TABLE 1-7. Skin folliculitis in guinea pigs after intravenous infection with *Candida* spp., *Torulopsis glabrata*, and *Rhodotorula rubra*.

Species	Inoculum (CFU × 10^3 g)	No. with skin folliculitis		
		No. tested	Absent	Present
C. albicans	8	155	1	154
C. stellatoidea	8	4	0	4
C. tropicalis	8	4	4	0
C. tropicalis	16	3	3	0
C. kefyr	16	1	1	0
C. krusei	16	3	3	0
C. parapsilosis	16	2	2	0
C. guilliermondii	16	2	2	0
T. glabrata	16	4	4	0
T. glabrata	32	2	2	0
R. rubra	32	2	2	0

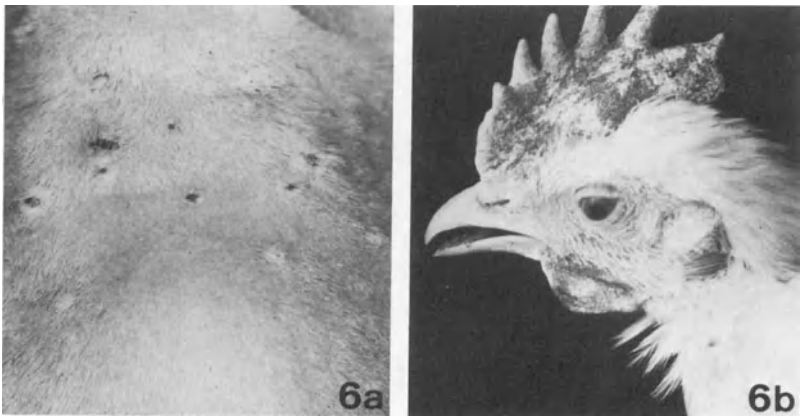


FIG. 1-6. *Candida albicans* intravenous infection, 14 days after infection. (a) *Candida* skin folliculitis in the guinea pig after 500 CFU/g BW. (b) *Candida* lesions in the chicken, on the comb and the wattles, after 200,000 CFU/g body weight.

Histopathologically, the early lesions observed after 1 day started as an embolus in the capillaries that projected into the dermal papillary layer. Accumulation of PMNs was observed. Budding of yeasts and hyphal development started. After 2 days, inflammatory infiltration and minute bleedings became more defined. Then the focal breaking up of either the basal lamina, epidermis, or glassy membrane delimiting the hair follicles started. Some filaments were already present inside the keratinized layers. On day 3 microvesicles and microabscesses with accumulation of PMNs were observed at the level of the stratum spinosum and within the external

hair root sheath. Concomitantly, filaments reached the deeper layers of the stratum corneum or the hair cuticle. The fast-developing filaments soon filled the entire cortex of the hair shaft and reached the antrum, growing upward in the hair itself. At about the time the filaments reached the upper epidermis, the clinical lesions appeared. Seven days after infection sporulation and filamentation was observed within the cornified layers. During the second and third weeks after infection, lesions were stabilized histologically, but an increased break-up and shedding of hairs with atrophy of the bulbus was shown. Hyphal elements were still present in the hyperkeratotic crusts. On day 35, patches with a slightly thickened epithelium, devoid of dermal papillae and with a fibrous underlying dermis, were observed (18,84,86).

The pathogenicity of *C. albicans* ATCC 44858 was also evaluated on the intact comb of cocks and the intact skin of mongrel dogs, guinea pigs, golden hamsters, monkeys, mice, rabbits, and rats (Table 1-8). Some of these animals were pretreated with alloxan, but, in addition, all species were infected without pretreatment. Guinea pigs and rabbits were equally susceptible for local inoculation on intact skin. Intravenous inoculation resulted in profuse invasion of internal organs in various animal species as well as in heavy folliculitis in the guinea pig. In the chicken, lesions of the comb and wattles and intertrigo between the toes were most often observed. In rabbits the folliculitis was limited and transitory. In other animal species occasionally minute eruptions healed rapidly and spontaneously. The inoculum size of *C. albicans* ATCC 44858 injected intravenously in the guinea pigs was evaluated as a function of the folliculitis (Table 1-9). When a 32,000 CFU/g BW dose was injected, the mortality reached 89% between days 2 and 5 after infection. Infection with 16,000, 8,000, 6,000, and 4,000 CFU/g BW gave mortality rates of 67%, 25%, 17%, and 13% respectively. With smaller inocula no mortality was observed, although folliculitis and positive cultures were still present in high numbers, which was directly related to the number of yeasts injected. After inoculation with small inocula the size and inflammatory reactions of the eruptions were reduced.

In the guinea pig model, using 8,000 CFU of *C. albicans* per gram BW intravenously, skin candidiasis was constant and thus may offer a new approach to studying the disease, especially in highly compromised patients.

Malassezia

The genus *Malassezia* (syn. *Pityrosporum*) includes the medically important yeasts *M. furfur* (syn. *P. ovale*, *P. orbiculare*) and *M. pachydermatis* (syn. *P. canis*), considered to be episaprophytes (1,56,69,99). The epi-

TABLE 1-8. Cutaneous and intravenous infection with *Candida albicans* ATCC 44858 in various animal species.

Species	Strain or race	Pathogenicity on intact skin*		Intravenous infection								
		Alloxan†		Inoculum	No. of infected animals		No. of survivors					
							Skin folliculitis lesions score§				Positive cultures at sacrifice	
		None	Plus	(CFU × 10^3/g)	Total	Survivors‡	0	+	++	+++	Skin	Kidneys
Chicken	Arbor acre	+	−‖	400	24	20	0	6	9	5	20	1
Dog	*Canis vulgaris*	+	−	8	6	5	0	2	0	0	2	4
Guinea pig	Pirbright	++	+++	8	920	688	0	1	4	683	687	685
Golden hamster	*Mesocricetus auratus*	+	−	8	8	4	4	0	0	0	1	4
Monkey	*Cercopithecus aethiops*	+	−	8	6	6	0	2	1	0	3	5
Mouse	Swiss	(+)	+	40	80	36	34	2	0	0	5	36
Rabbit	New Zealand	++	+++	2	16	13	3	3	7	0	11	13
Rat	Wistar	(+)	+	200	24	20	19	1	0	0	4	20

*Pathogenicity (comb for chickens and skin for other animals): 0 = absent; + = discrete eruptions and scaling; ++ = pronounced redness, vesicles, and scaling; +++ = erythema, vesiculation, inflammation, and scaling.

†Alloxan: 200 mg/kg IM once 24 hours before infection.

‡Survivors: duration of experiment 17 days for guinea pigs and 21 days for others; kidney cultures were performed for dead animals and all were positive except for two chickens.

§Lesions on the comb and wattles or intertrigo for chickens; skin folliculitis for others.

‖= no data.

Parentheses indicate presence of rare microscopical pinpoint eruptions.

TABLE 1-9. Intravenous infection with *Candida albicans* ATCC 44858 in guinea pigs: skin folliculitis and cultures.*

Inoculum (CFU/g)	No. of infected animals		No. of survivors					
			Skin folliculitis lesions score†				Positive cultures at sacrifice	
	Total	Survivors	0	+	++	+++	Skin	Kidneys
32,000	18	2	0	0	0	2	2	2
16,000	30	10	0	0	0	10	10	10
8,000	920	688	0	1	4	683	687	685
6,000	36	30	0	0	6	24	30	29
4,000	40	35	0	1	5	29	34	32
2,000	12	12	0	2	9	1	1	10
1,000	12	12	2	7	3	0	4	9
500	6	6	1	4	1	0	2	2
250	12	12	6	6	0	0	2	3
125	6	6	2	4	0	0	3	1
63	12	12	10	2	0	0	0	0

*Modified from Van Cutsem et al. (84).
†Skin folliculitis: lesions and eruptions on the back. 0 = absent; + = 1–5; ++ = 6–20; +++ = 21–innumerable.

saprophytism and pathological aspects of *M. furfur* are described in relation to other microorganisms in pityriasis versicolor, dandruff, seborrheic dermatitis, folliculitis, and other *Malassezia* infections in man (44,54,79). *Malassezia pachydermatis* is present as a saprophyte on the skin and in the cerumen of various animal species, but it is often isolated from patients with dermatitis and otitis (4,13).

Several investigators inoculated *M. furfur* scales or cultures in man with poor results in many cases. Burke (8) reproduced pityriasis versicolor with cutaneous application of a culture of *M. furfur* (as *P. orbiculare*) in patients with Cushing syndrome or undergoing corticotherapy. Randjandiche (56) was unsuccessful in inoculating Swiss and nude mice on nonoccluded skin.

Faergemann (16,17) inoculated 14 individuals with a history of pityriasis versicolor and six volunteers who had not had the disease before. None had any underlying disease. Two weeks after successful topical treatment of the patients, they were inoculated on their arms, on which no natural infection or medication had been previously present. The patients were infected with *M. furfur* either under occlusive dressing for 7 days or not, with or without the addition of olive oil. When occlusion was not used, all parameters were negative: lesions, Wood's light examination, microscopy, and cultures. After occlusion for 1 week, the lesions, Wood's light examination, and microscopy were positive in some patients, but samples from all of the subjects gave positive cultures. Additional application of olive oil produced positivity of all parameters, including more profuse de-

velopment of retrocultures from scales of all patients. Faergemann also inoculated rabbits on the unshaven inside of the ear with *M. furfur*. No occlusion was used in some, and in others occlusion was used with and without the addition of olive oil. Results after inoculation without occlusion were poor. The best results were obtained with occlusion and olive oil, under which conditions the animals remained positive for 3 weeks.

Our experiments used guinea pigs. We infected 12 albino guinea pigs with *M. furfur* (as *P. ovale*), 12 with *M. furfur* (as *P. orbiculare*), and 6 with *M. pachydermatis* (95). The animals were not pretreated; the hairs of the back were clipped, and an inoculum of 1.2×10^6 to 5.1×10^6 CFU was applied on the intact skin. The infected back was occluded for 5 days with an occlusive dressing (Oclufol: Lohman KG Fahr, Rhein Germany) and maintained with Dermiclear tape (Johnson & Johnson). Eruptions and inflammatory lesions were observed during the first week, followed by hyperkeratosis and scaling (Fig. 1-7a). The results 2 weeks after infection are represented in Table 1-10. At that point all animals had clinical lesions. The cultures on Sabouraud glucose ager plus antibacterial antimicrobics and cycloheximide were positive for five of six animals infected with *M. pachydermatis* and for 23 of 24 animals infected with *M. furfur* on Dixon medium supplemented with antibacterial antimicrobics and cycloheximide. Histologically, yeasts were found to be present in the stratum corneum (Fig. 1-7b).

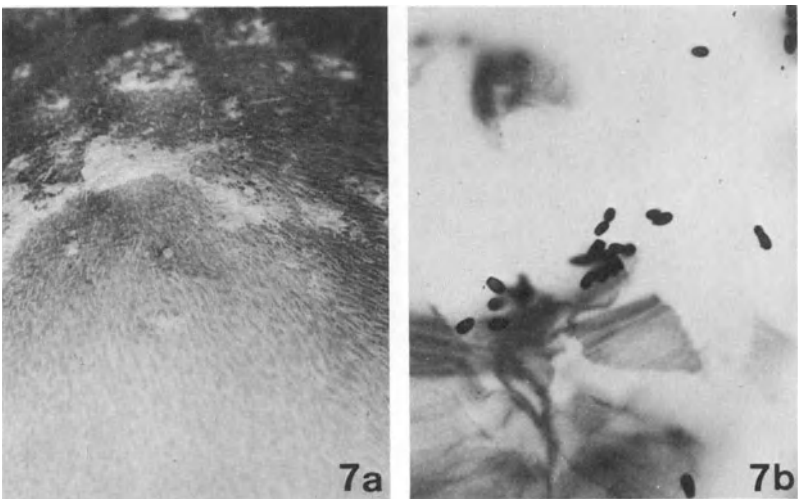


FIG. 1-7. *Malassezia furfur* infection on the intact occluded skin of guinea pig. (a) Skin lesions. (b) Yeasts in the keratinous layer.

TABLE 1-10. *Malassezia* on the occluded intact skin* of guinea pigs.

Malassezia species	No. of animals	No. of guinea pigs (2 weeks after infection)				
		Lesion score†			Culture	
		0	+	++	Positive	Negative
M. furfur	12	0	5	7	12	0
M. furfur	12	0	3	9	11	1
P. pachydermatis	6	0	4	2	5	1

*Occlusion for 5 days.
†Hyperkeratotic lesions and scaling. 0 = absent; + = moderate; ++ = pronounced.

Cryptococcus

The normal portal of entry for cryptococcal infection is the lung, but at least one-third of the patients are asymptomatic and many cases remain unrecognized (68). When Swiss mice reside on soil inoculated with a culture of *Cryptococcus neoformans*, they may contract cryptococcosis via inhalation of airborne conidia (70). According to Graybill (25), approximately 90% of the patients with cryptococcosis have dissemination to various organs including the meninges.

Cutaneous cryptococcosis is recognized as a sentinel of disseminated disease in about 10% of cases (10,66)—before AIDS was described—most often with underlying disease (67). In an increasing number of AIDS patients in Central Africa with meningeal and disseminated cryptococcosis, cutaneous cryptococcosis is diagnosed as well (37). Maculopapular pruritic skin eruptions were reported in 75% of 32 AIDS patients with cryptococcal meningitis (37). Primary cutaneous cryptococcosis is infrequently described, but the number of cases is increasing (3).

Most of the animal models of cryptococcosis were set up to study the disseminated and meningeal disease and to evaluate therapeutic agents. With these models, normal mice, athymic hairless nude mice, and irradiated or immunodepressed mice were inoculated intranasally, intracerebrally, intramuscularly, intraperitoneally, or intravenously (25,73).

After subcutaneous infection of immunocompetent mice with *C. neoformans*, the yeasts survived and multiplied in the lesions for 4 weeks, but elimination started during the fifth week, and it was a benign experience for most of the animals (14). Intracutaneous inoculation in normal mice produced a localized infection for 5–6 weeks with considerable variations (48).

Song (72) inoculated immunocompetent mice subcutaneously. Other groups were infected on the scarified skin. Half of the animals received corticoid treatment (four times before and twice after the infection). After

subcutaneous inoculation, a generalized infection appeared for at least 2 weeks, with some mortality, and then regressed up to 50 days after infection. At that moment, yeasts were found only in regressed skin lesions and eventually in the liver of the cortisone-treated mice. If *C. neoformans* was applied on the scarified skin of mice and guinea pigs the lesions were limited to the skin and regressed more rapidly in animals without cortisone treatment. Rabbits receiving high doses of corticoids were receptive to meningeal cryptococcosis by intravenous inoculation (51). Intraperitoneal infection of guinea pigs with *C. neoformans* (12,62) resulted in disseminated disease: no skin invasion was mentioned.

In various experiments we attempted to establish superficial cryptococcosis in small laboratory animals. Swiss mice, Wistar rats, New Zealand white rabbits, and Pirbright albino guinea pigs were infected on intact and scarified skin. Some of the animals were under corticoid treatment. The results were variable and self-limiting, and recovery was spontaneous in most cases. Exceptionally, an animal died as a result of dissemination. Because of the variability of skin infection with *C. neoformans*, such models cannot be used for the therapeutic evaluation of compounds. Other animals were infected intramuscularly and subcutaneously: In general, a localized induration subsisted for variabel periods.

The mouse has been preferred by many investigators for models of meningeal and disseminated cryptococcosis to study the disease and to evaluate the activity of compounds. In search of more realistic models, we infected nonpretreated albino guinea pigs with *C. neoformans* by various routes. After intracardial and intravenous infection with *C. neoformans* in male and female guinea pigs, skin eruptions appeared on days 12–14 after infection. The pathogenicity of the strain and the size of the inoculum were responsible for an early or delayed appearance of the eruptions. Intracardial infection, however, is a hazardous and time-consuming technique for the infection of large numbers of animals. Intraperitoneal infection with *C. neoformans* always produced dissemination, but involvement of the skin and the outgrowth of granulomas were more irregular. For these reasons the intravenous route was selected. Guinea pigs were infected with various strains of *C. neoformans* using 200–20,000 CFU/g BW (Table 1-11). Most of the organs, including the brain, were invaded by the organism 24–72 hours after infection. In the guinea pigs infected with *C. neoformans* strain B 42419 200 CFU/g BW, some fungal elements and inflammatory cells were observed in the dermis after 7 days. The small skin eruptions, present after 2 weeks, developed into ulcerative dermatitis and granulomas within 5 weeks (Fig. 1-8c, d). Ulcerative vesicles on the mucosae of the nose, mouth, eyelids, and preputium were present. Indian ink microscopic examination of exudates from cryptococcal vesicles and granulomas showed a massive presence of large encapsulated yeasts. Cultures also produced luxuriant cryptococcal growth. Numerous encapsulated yeasts were observed histologically on day 21, and their number increased during the experiment. The dermis was infiltrated by macrophages and PMNs. Col-

TABLE 1-11. Skin cryptococcomata in guinea pigs after intravenous inoculation.

Cryptococcus neoformans strain	CFU/g	No. of animals	No. of guinea pigs with skin cryptococcomata	
			Absent	Present
B 38334	2.10	6	0	6
	2.10^2	12	0	12
	2.10^3	6	0	6
B 39395	2.10^2	6	3	3
	2.10^3	6	0	6
	2.10^4	6	0	6
B 39919	2.10^2	6	2	4
	2.10^3	6	0	6
	2.10^4	6	0	6
B 40363	2.10^2	6	5	1
	2.10^3	6	3	3
	2.10^4	6	0	6
B 42419	2.10	12	5	9
	2.10^2	236	0	236
	2.10^3	26	0	26
	2.10^4	14	0	14

lagen bundles were degenerated or atrophied under the pressure of cellular reaction and fungi. Fungal development and the cellular reaction in the dermis resulted in an ulcus of the overlying epidermis. Within the necrotic epidermal layers, fungal elements and many neutrophils were present, and after 35 days the granulomas became apparent. The epidermis was focally erosive and ulcerated. At the periphery of the lesions, the epidermis was acanthotic and infiltrated with PMNs. In the epidermal lesions the dermis was invaded by numerous cryptococci, often with large capsules. A fibrogranulomatous reaction with many macrophages and giant cells became visible. Some hair root sheaths were infiltrated by PMNs, although they were not invaded by *Cryptococcus*. Up to 56 days after infection about 9% of guinea pigs infected intravenously with *C. neoformans* B 42419 200 CFU/g BW died from the infection. All others were in poor condition with progression of the infection. The sacrificed animals proved to be highly positive by microscopy, in cultures, and histologically. With more concentrated inoculum the infection was more intense and death appeared earlier.

No difference in sensitivity to *C. neoformans* was observed between sexes contrary to earlier reported observations (12). Male guinea pigs can be infected with no problems by way of a lateral vein of the penis. For this reason the male guinea pig was selected for further experiments.

To compare the dermotropism of *C. neoformans* after intravenous infection, guinea pigs, mice, rats, and rabbits were infected with *C. neoformans* strain B 42419 at various concentrations (Table 1-12). In the mice, dissemination to organs, including the meninges, occurred, but skin manifesta-

TABLE 1-12. Dermatomycosis after intravenous infection with *Cryptococcus neoformans* B 42419 in various nonpredisposed animal species.

Species	CFU/g	No. of animals	No. of animals with skin lesions			
			Absent	Rash	Acneiform eruptions	Granulomas
Mouse (Swiss)	2.10^2	6	6	0	0	0
	2.10^3	6	6	0	0	0
	2.10^4	6	6	0	0	0
	2.10^5	6	6	0	0	0
Rat (Wistar)	2.10^2	4	4	0	0	0
	2.10^3	4	3	1*	0	0
	2.10^4	4	3	0	1*	0
Rabbit	2.10^2	8	0	0	8*	0
(New Zealand)	2.10^3	8	0	0	8*	0
	2.10^4	8	0	0	8*	0
Guinea pig	2.10	12	5	0	0	7
(Pirbright)	2.10^2	236	0	0	0	236
	2.10^3	26	0	0	0	26
	2.10^4	14	0	0	0	14

*Rash and acneiform eruptions, *Cryptococcus*-positive, resolved spontaneously.

tions were absent. A rash developed in 2 of 12 rats (Fig. 1-8a). The exudate contained a large number of yeasts. The rats recovered spontaneously. Acneiform eruptions that were dispersed over the whole body appeared within 2 weeks in all rabbits after intravenous infection (Fig. 1-8b). The eruptions were sparse in rabbits inoculated with 200 CFU/g BW and confluent in the animals that received the highest inoculum. Two weeks after infection *C. neoformans* was isolated from the homogenized skin in 9 of 12 sacrificed rabbits. All skin samples of the rabbits had become negative by day 42. The evolution of cryptococcosis after intravenous infection in the guinea pigs and the development of skin cryptococcomas is clearly not a result of transmigrating fungal cells that provoked transepithelial elimination (24).

This guinea pig model of cryptococcosis appears to be a stable model for evaluating compounds that must penetrate the blood-brain barrier to eliminate the fungus from the central nervous system. It is also of value as a cryptococcal dermatomycosis model (85,88,90).

Miscellaneous Organisms

A broad range of other fungal organisms can be isolated from skin lesions in animals and man that vary greatly in shape, size, and appearance. They may appear autonomously or be disseminated from an internal organ.

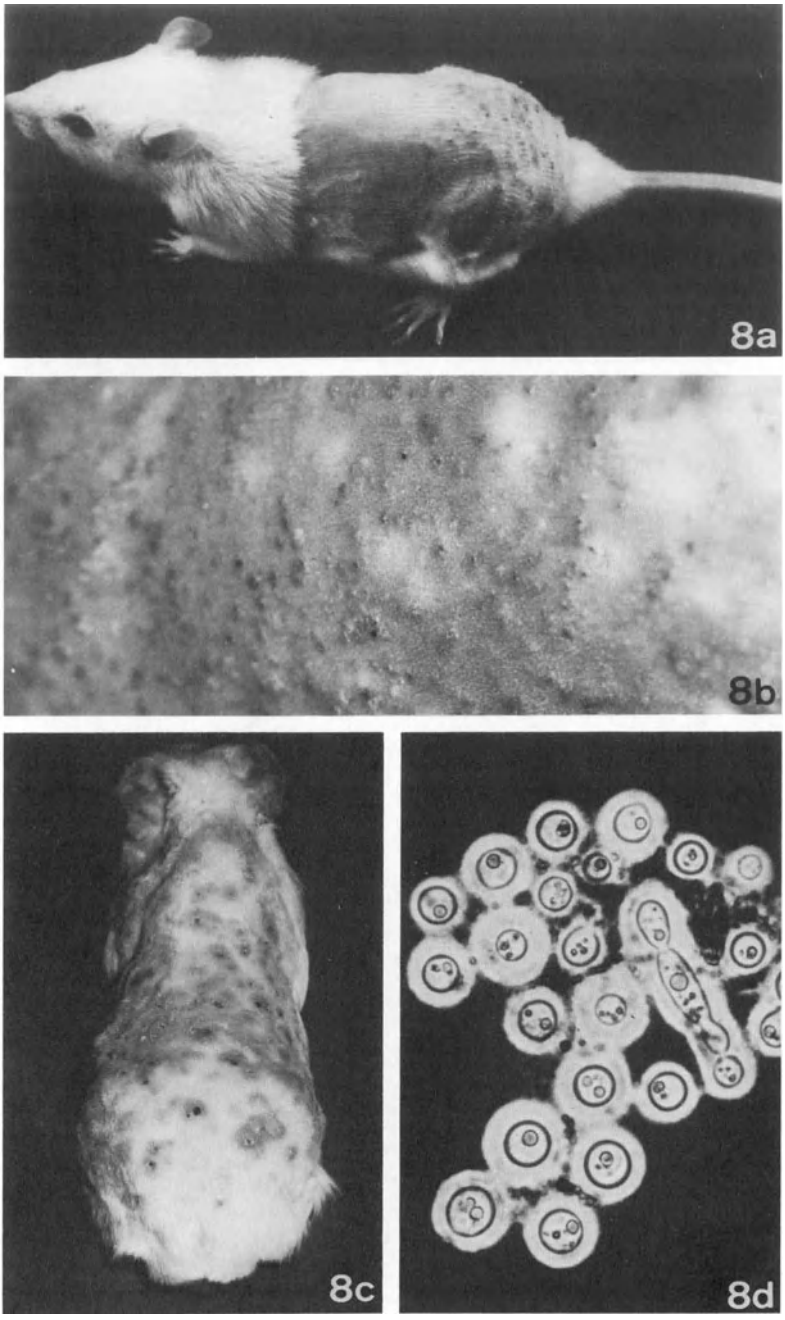


FIG. 1-8. *Cryptococcus neoformans* intravenous infection. (a) Rat with rash. (b) Rabbit with acneiform eruptions. (c) Guinea pig with skin granulomas. (d) Exudate of a skin granuloma: yeasts with polysaccharide capsules (Indian ink).

Polak (53) gave intravenous injections of *Fonsecaea pedrosoi* to Swiss mice that also received two injections of corticoids: an infection somewhat similar to human disease was produced. Granulomatous lesions and microabscesses of the lung, the brain, and kidneys were accompanied, after 3–4 weeks, by swollen legs and black spots on the tail. These spots increased in size, and after 7 weeks, in the final state, the tail and the legs became dark and full of nodules. Sclerotic bodies were present in the skin lesions and the subcutis.

Subcutaneous injection of zoospores of *Pythium insidiosum* (syn. *Hyphomyces destruens*) in immunocompetent rabbits and rabbits immunodepressed by administration of corticoids resulted in subcutaneous nodules and localized progressive abscessation. In the cortisone-treated rabbits, fungal hyphae were more abundant, but the infection was not enhanced. The authors concluded that this model mimicked somewhat the natural pythiosis in horses (47).

Infections by *Fusarium* spp. are reported regularly in the literature, mostly in leukemic or aplastic patients (2), but *Fusarium* spp. are also currently isolated from diseased nails, as are *Scopulariopsis brevicaulis*, *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., black fungi, and others. *Fusarium solani* or *F. oxysporum* is also reported frequently as an eye pathogen. Ishibashi et al. (34) infected corticoid-treated rabbits in the cornea with *F. solani* and where able to establish keratitis. This model may be considered to be an alternative for a dermatomycotic infection with *Fusarium*, which creates the possibility of evaluating chemotherapeutic agents topically, orally, or parenterally for cutaneous and subcutaneous fungal infections caused by dematiaceous and nondematiaceous fungi, which are being reported with increasing frequency (2). Wildfeuer (99) infected *Hendersonula toruloidea* on the depilated intact and scarified nonpretreated skin of albino guinea pigs and rabbits. The skin of the guinea pigs remained unaffected (99). In histological preparations of skin from infected rabbits the hyphae were seen in the stratum corneum and in superficial cell material. However, only 57% and 30% of the samples were positive for the scarified skin and 37% and 23% for the nonscarified skin on days 5 and 8 after infection, respectively.

Reddy et al. (60) infected nonpretreated guinea pigs and rabbits on the scarified skin or by intradermal inoculation with *Alternaria alternata.* In all scarified animals the inoculation sites were erythematous for 4 days. In some animals the skin became dry and scaly; in others it was moist and crusted. The infection was of the eczematoid type with peripheral spreading, and it was of short duration. The fungus was isolated in cultures of skin scrapings for 2 weeks. Rabbits developed more inflammatory lesions, and the infection lasted distinctly longer than in guinea pigs. After intradermal inoculation, a papule-like swelling persisted for 2 weeks, but the fungus was not reisolated from the inoculated area.

We infected *S. brevicaulis* on the scarified skin of nonpretreated albino

guinea pigs. The skin became hyperkeratotic without inflammation, and in 7–14 days the invaded epithelial scales were rejected.

A strain of *Rhizopus arrhizus*, injected intravenously and intraperitoneally into nonpretreated guinea pigs, produced largely dispersed skin eruptions within 2–3 days after injection. Most of the eruptions became dry and rapidly covered large parts of the body; some of them ulcerated: and all samples taken for microscopy, histology, and cultures were highly positive. The fungus was abundant in all layers of the skin and appeared as large, long hyphal forms as well as short, irregular, coenocytic hyphae; some elements were yeast-like. At the same time, almost all internal tissues and organs were invaded, producing multiple abscesses, necrotic foci, and plaques, especially in the liver, kidneys, and spleen. Lungs, brain, and eyes were also affected. This systemic mycosis associated with dermatomycosis presents a valuable model for evaluating orally and parenterally administered drugs (85).

It can be concluded that there is a real need to establish new and valuable animal models for investigation of the fungi that the often considered saprophytes but that are increasingly isolated from patients at risk or from patients with conditions that up to now have been insufficiently studied or perhaps neglected.

Analogous problems are present in veterinary medicine. The so-called pseudodermatophytosis that appears in various animals caused by *Alternaria*, *Trichothecium*, *Acremonium*, and other fungi may be used to illustrate this problem (15).

Extrapolation of the Animal Model to the Natural Disease

Natural fungal infections in man and animals have been intensely studied, but many problems remain unsolved and open to research. New approaches may help to better understand the life cycle of pathogenic and opportunistic fungi, the conditions under which they may invade tissues, and why and how some apparently saprophytic organisms cause disease. In vitro sensitivity studies are important for evaluating the fungistatic and fungicidal activities of compounds, determining the broadness of their spectrum of activity, and rapidly detecting the emergence of resistant strains. In vivo studies contribute to a better understanding of adherence and the fungus–host interaction, which in turn contribute to understanding their importance to the pathogenesis of the infection (55).

The introduction of griseofulvin to human therapy regimens was based on the experiments of Gentles (20). These experiments were performed with zoophilic dermatophytes. It was found that the drug was active not only against zoophilic dermatophytes but also against anthropophilic and geophilic dermatophytes.

Selection and development of topical azole broad-spectrum antifungal agents were guided by a comprehensive number of animal models (23,52,82). Using a wide range of animal models, it has been possible to select and introduce the first oral broad-spectrum antifungal drug, ketoconazole (78,81,83). Ketoconazole would not have been selected based on in vitro data derived from conventional tests. However, adapting the in vitro sensitivity tests to the in vivo potency of this compound demonstrated its efficacy. Progress in the development of antifungal therapy has been possible because of the continuous screening of new molecules. A strategy that consisted in a basic series and a detailed series of well established animal models, including animal models of immunodepression, allowed the selection of itraconazole—a step forward for antifungal therapy (90,92–94).

The animal model plays an important role in detecting and demonstrating antifungal activity of a drug as well as in demonstrating the absence of irritation, sensitization, photosensitivity, and other characteristics. The animal pharmacology contributes significantly to kinetic upgrading of the molecule in terms of tissue affinity and to the fine-tuning in terms of selectivity and toxicity (9).

It remains important to evaluate new compounds in animal models that have proved their value in large series of animals and have confirmed the results by repetition at various doses and concentrations of the molecule. Compounds that satisfy all requirements of safety and activity in the animal models may be considered to be candidates for clinical trials in animals and man.

A large number of animal models are of the acute type. The animal models of disseminated disease discussed in this chapter may contribute to the evaluation of chronic fungal infections. In this respect, the guinea pig, when possible, is the animal of choice; it has several various advantages, e.g., dissemination of parenterally injected fungi to the skin and the great susceptibility to both dermatophyte and yeast skin infections. It is inexpensive and is easy to manipulate, infect, and treat. The guinea pig also rarely licks or scratches the infected back or licks the topically applied compounds.

New drugs can be evaluated in animal models that allow extrapolation to most natural human and animal diseases. Over the years progress has been made in improving and adapting animal models for fungal diseases, especially dermatomycosis. There is still room for new models and for improvement of older ones, however, when dealing with opportunistic and saprophytic fungi in immunocompromised animals.

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2—Vaccines for the Management of Dermatophyte and Superficial Yeast Infections

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Superficial fungal infections involving hair, nail, skin, and mucosal surfaces are among the commonest infectious diseases known since antiquity to plague humanity. Infections caused by the dermatophytes were described by the Romans (2) and were among the first communicable diseases with a known etiology (5). Dermatophytoses are also a major medical problem in wild and domestic animals and, as such, can be a source for zoonoses transmitted to man, in addition to having economical implications. In addition to dermatophytes, yeasts, particularly species of the genus *Candida*, frequently, cause nail, skin, and mucosal infections. Thus these organisms have a leading role in the morbidity in humans and animals. In view of the proved efficacy of vaccines to combat, prevent, and even eradicate various bacterial and viral diseases, a similar approach to the management of superficial dermatophyte and yeast infections seems plausible.

Antidermatophyte Vaccines

Dermatophytes and Dermatophytoses

Dermatophytes comprise a group of keratinophilic molds that includes 40 species assigned to three genera: *Trichophyton, Microsporum*, and *Epidermophyton* (3,60). They also can be classified as anthropophilic, zoophilic, or geophilic depending on their origin (60). Dermatophytes thrive on human and animal keratinous structures and cause pathologic conditions in the skin, nails, and hair (28).

Although the exact prevalence of human dermatophytoses is unknown because these infections are not reportable, indirect evidence is available indicating that they have a high frequency of occurrence. For example, it is believed that in the United States (33) about 9% of the population may be

infected and that at least 40 million dollars are spent yearly for treatment of dermatophytoses (60).

Ringworm or dermatophytoses of wild and domestic animals are well recognized problems (36). Again, although the exact prevalence of such infections is unknown, it is generally believed that they are frequent. For example, it is estimated that the prevalence of ringworm in cattle is about 20% (52), and in some countries (Russia) it has been reported (62) that dermatophytosis (due to *T. verrucosum*) comprises more than 40% of all the infectious diseases of these animals. Morbidity of domestic animals such as cattle caused by dermatophytes has a marked economic impact on this industry, particularly in countries such as Norway, where infected animals are forbidden in to markets and public places (44). Furthermore, some zoophilic dermatophytes, e.g., *M. canis*, *T. mentagrophytes*, and *T. verrucosum*, are important sources of human infection (45,46,60).

The host response to a dermatophyte plays a major role in the pathogenesis of dermatophytoses. The clinical manifestation is mostly due to the reaction of the host to the invading fungal parasite (33). The clinical course and the outcome of the infection are highly associated with the immune response of the patient. In individuals with an acute inflammatory reaction that is correlated with mounting cell-mediated immunity (CMI) to the dermatophyte, the infection generally responds well to treatment or resolves spontaneously (34). This reaction is contrary to that seen frequently in individuals with chronic infections, who do not show a CMI response to the fungus. Moreover, apparently CMI, as expressed by the delayed-type hypersensitivity (DTH) response to dermatophyte antigens (trichophytin), accompanies acquired resistance to dermatophyte infections and is the major defense mechanism (11,12).

Some degree of acquired resistance in humans has been noted following dermatophyte infections (23). These observations were confirmed by data indicating that in animals infection may be followed by resistance to reinfection (20,26,43,58,75).

Because dermatophytoses pose a problem in humans and animals, the development of vaccines for management is important. Infection may result in partial resistance to reinfection, so attempts to induce protectivity by vaccination have been undertaken.

Experimental Animal and Human Dermatophyte Immunizations

Despite the abundance of dermatophyte infections in animals and humans, only sporadic studies describe attempts to induce protection against these infections. An early study by Huppert and Keeney (30) described vaccination attempts in humans. These investigators used a mycelial preparation from *T. mentagrophytes* incorporated into an inert base for topical vaccina-

tion of volunteers. When the vaccinated volunteers were challenged topically with live fungi, they developed tinea pedis at a much lower incidence on the "vaccinated" foot than on the "nonvaccinated," control foot (14% versus 57%). Another study in humans has been described by Beemer et al. (7) in which they treated patients suffering from chronic infections with intradermal vaccines administered twice weekly for 2 weeks, then once a week for a month, and subsequently once a fortnight for 2 months. The vaccines consisted of ground material prepared from an alcohol-treated mycelial mass of the fungus originally recovered from the patient. Three patients with prolonged chronic tinea pedis and tinea unguium caused by *T. mentagrophytes* and *T. rubrum* were treated and improved following the treatment.

Regarding experimental studies in animals, a study by Hussin and Smith (31) described attempts to vaccinate guinea pigs with three vaccines prepared from *T. mentagrophytes* var. *erinacei*: (a) a live vaccine; (b) hyphal cell walls; and (c) a soluble cytoplasmic extract. Of the three preparations administered subcutaneously, the live vaccine was most beneficial in protecting the animals prior to experimental infection. The course of infection following challenge of the vaccinated animals was similar to that observed in guinea pigs reinfected after recovery from a primary inoculation. The lesions were small, and fungal elements could not be detected microscopically in skin scrapings from day 6 to day 8 after inoculation, whereas in animals with primary infection hyphae were visible after 28 days. It appears that the live vaccine is most effective. However, if application in humans is considered, one should consider the reservations regarding the use of "live" vaccines with their potential risk.

These investigators also evaluated the immune response elicited by the various vaccinations and determined the antibody titer by an indirect immunofluorescence technique and by counterimmunoelectrophoresis. Antibodies were detected by both techniques in the sera from animals vaccinated with the cell walls or cytoplasmic extract but not in the sera from guinea pigs vaccinated with the live vaccine. The authors believed that their data supported the conclusion that CMI is a major defense system against dermatophytoses.

Earlier animal studies, e.g., that of Keeney and Huppert (37), showed that topical application of killed *T. mentagrophytes* induced in guinea pigs a short-lived resistance to reinfection. Resistance was also demonstrated by Arnold et al. (6) when they vaccinated guinea pigs with peptidopolysaccharide antigens of *T. mentagrophytes*.

However, none of the studies—neither animal studies nor human trials—were systematically continued. Thus there is not enough information to determine the potential merits of vaccination for management of dermatophytoses. Apparently successful results with a *T. verrucosum* vaccine for veterinary use are described in the following section.

Dermatophyte Vaccines for Veterinary Use

Bovine dermatophytoses caused by *T. verrucosum* have a worldwide distribution with an economical impact and concurrent public health problems. Infection in humans following contact with infected animals is well known (60).

Attempts for prophylaxis of such infections by vaccination with various preparations of *T. verrucosum* have been described by a number of investigators (13,19,24,40,49,50). They include vaccines consisting of organisms killed by heat (autoclave) or acid (13,49,50) and, later, by live attenuated fungi prepared by several Russian investigators (4,17,32,56,57,59,61,62,64).

Early studies with killed fungal preparations showed that intra- or subcutaneously immunized calves can be partialy protected prior to infection (13,19,49,50). Noskov (49), who used a hydrolyzed *T. verrucosum* preparation, showed the efficiency of this material in a small number of calves, which was confirmed later (13) when a large number of animals were vaccinated. In their field trial the vaccine was used in both healthy calves and those already infected. The authors reported that, among animals that were treated every 10 days with three or four subcutaneous injections of the vaccine, approximately 88% were resistant to infection, even though they were in close contact with infected animals. In addition to prophylaxis, management of already diseased animals was also attempted as a parallel study to the immunoprophylaxis experiment. The investigators concluded that the vaccinated animals showed clinical improvement.

Kielstein and Richter (38) used several immunogenic preparations of *T. verrucosum* and *T. mentagrophytes* in a field trial to vaccinate 229 calves, which were then compared with 193 unimmunized animals. The vaccines included killed and live preparations with and without adjuvants and were administered twice subcutaneously (10 ml dose) or intracutaneously (2 ml dose) at 2- to 3-week intervals. The animals were examined for development of infection and clinically evaluated for the degree of lesion severity. From this field trial it was concluded that although immunoprophylaxis is possible the protectivity achieved is only partial. Infection was either rarer or more moderate in degree than in unimmunized control animals. Among the control animals, 52.3% of calves developed infection and among the vaccinated animals 27% were infected. The authors stated that they could not reach definite conclusions regarding the most effective vaccine or optimal mode of application. However, analysis of the data led them to conclude that it is preferable to immunize subcutaneously with three doses of 10 ml of a hydrochloric acid-treated preparation of *T. verrucosum*, the chemical nature of which was not indicated.

A breakthough in the bovine vaccination research area occurred with the introduction by Sarkisov and colleagues (62–65) of a live vaccine prepared from *T. verrucosum*: LTF-130. They showed that after 2 weeks postvac-

cination the calves developed resistance to experimental infection, and the induced immunoprotection lasted up to 5 years. Following this discovery, the vaccine was evaluated on a large scale in various regions of the Soviet Union as well as other countries in Europe.

During the initial stages of Sarkisov et al.'s study, the investigations concentrated on selecting a highly immunogenic strain. A variant of *T. verrucosum*, was selected that, when cultured on malt agar, developed abundant microconidia, in contrast to the wild type. Whereas the wild type caused numerous typical persistent skin lesions, the variant (LTF-130) caused only superficial, relatively short-lived lesions.

Once the immunogen was selected, it was followed by a series of experimental immunizations in rabbits in which a live and a killed vaccine were compared for their capacity to protect the animals before challenge with virulent strains of *T. verrucosum*. Protection was evaluated clinically, histopathologically, and microbiologically, with the immune responses being assayed by measuring the titer of agglutinins. The experiments showed that the live vaccine was most effective. The best route of administration was intramuscular, which elicited high antibody titers (up to 1:1280).

After their initial work was completed, the researchers utilized calves. With this model, they evaluated dose, interval between injections, and animal response in comparison with a killed vaccine. These experiments showed that immunization with two doses of 5 ml of the live vaccine, administered intramuscularly with an interval of 10 days between doses, gave promising results. As with the rabbit model, the vaccine induced significant titers of dermatophyte agglutinins in the calves: ranging up to 1:640 versus 1:80 and 1:40 in calves immunized with a killed preparation or in unimmunized controls, respectively. Although antibody production was measured, the investigators did not clearly specify if they believed that a humoral response was the major mechanism responsible for the induced protectivity.

The conclusions from these experiments were next applied in large field trials. A group of 293 calves were vaccinated with two intramuscular injections, 5 ml each, administered 10 days apart; they were then evaluated over a period of 6 months. The researchers reported that during this period no case of dermatophytosis was observed among immunized animals who were infected experimentally or among those exposed to infected animals. Other long-term experiments established the duration of immunity, which was found to be up to 5 years. It was shown that the LTF-130 vaccine provided protection against infection with isolates of *T. verrucosum* that came from various regions of the Soviet Union.

The mass vaccination program began on farms located in various regions of Russia between 1968 and 1970. By the end of the 1970s more than 130 million vaccine doses were produced in that country. The vaccine was made available in a freeze-dried form that required storage at 2–10°C. In terms of the efficacy of the preparation, the vaccination program led to

eradication of large portion of the disease. Dermatophyte infections comprised 42% of all infectious diseases in cattle in 1969 but this figure decreased to 0.6% in 1979 as a result of the vaccination program.

The investigators believed that the vaccine can also be used for therapeutic purposes. Vaccine doses of 10–20 ml (depending on the age of the calves) could be administered, leading to recovery of animals presenting with dermatophytosis. The authors indicated that this treatment of ringworm lowers the costs of management of infected animals in comparison to the cost of antifungal drugs.

Since 1975 the vaccine has been widely used in other eastern European countries, e.g., Hungary (29), Bulgaria (71), Germany (76), and Czekoslovakia (53,54), and from the late 1970s to early 1980s in other European countries, e.g., The Netherlands (10,70), Sweden (72), and particularly Norway (1,44,47,48).

Pavlas et al. (54), in Czekoslovakia, reported in 1979 that vaccination of calves resulted in a reduction of the incidence of dermatophytosis to 0–13% within 3 to 5 months, compared to 36–100% in unimmunized animals under the same test conditions. Otcenacek and collaborators (53) confirmed in a 4-year study the efficacy of the vaccine as a means of controlling dermatophyte infections in cattle.

The Bulgarian scientists Stankushev et al. (71) reported that in a 3-year vaccination program (1976–1978), in which 653,666 calves were immunized for prophylactic purposes, no cases of dermatophytoses were observed. Of 62,121 animals immunized for therapeutic purposes, 95.4–98.8% of the animals recovered from the disease.

In 1982, Breuthouwer (10) described a well controlled trial with the LTF-130 vaccine in the Netherlands. Ten calves were immunized with a virulent *T. verrucosum* isolate, and the immunized animals did not develop infection. In a later study by Spanoghe et al. (70), it was observed that during the period of induction of immunity a number of vaccinated calves did develop disease when they came in contact with infected animals. Vaccination at an earlier age and in higher doses was therefore proved more effective.

A study conducted in Sweden (72), consisting of field trials on three farms used for beef production (about 400 animals), showed that vaccination by the recommended regimen (two 5-ml intramuscular injections at a 2-week interval) led to statistically significant protection in one group of animals. An increase in the vaccination dose (two 10-ml doses) led to significant protection—defined by the absence of infection in comparison to that in nonvaccinated cattle—on all the farms.

The most extensive studies with the LTF-130 vaccine outside Russia and other east European countries have been carried out in Norway (1,44,47,48). Immunizations were initiated in 1978–1979, and by 1985 approximately 350,000 animals had been vaccinated (44). The vaccination program significantly reduced the incidence of infection. The vaccine was

used for prophylactic and therapeutic purposes. Naess and Sandvik (48) in 1981 and Aamodt et al. (1) in 1982 concluded that the prophylactic effect is nearly 100%, although the therapeutic effect is less favorable. Aamodt et al. (1) did not recommend it as a therapeutic modality.

A report by Liven and Stenwig (44) indicated some drawbacks for the vaccine. Some cases of ringworm were found among vaccinated animals. The authors believed that these drawbacks may result from some factors in the vaccination procedure that can eventually be overcome.

Overall it appears that the published data confirm the efficacy of the LTF-130 live vaccine as a useful tool for managing zoophilic dermatophytoses caused by *T. verrucosum*, although the nature of immunity it induces and the basic biological aspects of the vaccine are unknown.

In summary, the literature may be evaluated by the following major points.

1. The studies concentrating on attempts to induce immunity in experimental animal models and the sporadic human trials were not systematic enough to yield sufficient information to evaluate the feasibility of using a vaccine against dermatophyte infections.
2. The LTF-130 vaccine seems to be an effective measure for controlling *T. verrucosum* infections in cattle.

Candida Vaccines

Cutaneous and Mucosal Infections

The genus *Candida* includes more than 80 species, with six major potential human pathogens: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, and *C. kefyr*. *Candida albicans*, the major pathogen of humans and a variety of animals, is an endogenous species. It inhabits the normal human buccal cavity, intestinal tract, female genital mucosa, and skin. Under certain conditions, however, it can cause a wide variety of clinical manifestations, ranging from superficial dermal or mucosal to deep-seated infections of internal organs to generalized disease. The superficial manifestations, which comprise most cases of candidiasis, involve nails, skin, and mucosal surfaces. These infections include the clinical entities oral thrush, denture stomatitis, vulvovaginitis, balanitis, paronychia, onychomycosis, diaper rash, cutaneous candidiasis, and chronic mucocutaneous candidiasis (CMCC) (17,51,59).

The transition from the commensal form to the pathogenic state occurs generally with the disruption of normal defense mechanisms, changes in the balance of the normal microbial flora, or damage to the integuments (17). Various external and environmental factors contribute to the increase in the frequency of mucocutaneous forms of candidiasis (51). Such factors

include the use of antimicrobics, hormones, and corticosteroids. Individuals with endocrinological changes or defects of the immune system are prone to these infections (9). Thus during pregnancy, with use of oral contraceptives, or in patients with diabetes, there is a significantly increased incidence of vaginal candidiasis (51). Patients with acquired immunodeficiency syndrome (AIDS) are especially prone to development of severe oral thrush or esophagitis (9). Chronic mucocutaneous candidiasis is also a syndrome associated with defects in the immune system, particularly in the area of the CMI response (17).

Although topical or systemic antifungal drugs, which have the ability to relieve symptoms, are available, a substantial proportion of patients either are resistant to treatment or are prone to recurrence, including many vaginitis patients or individuals with CMCC. Despite the available antimycotics for management of dermal and mucosal *Candida* infections, it is desirable to develop prophylactic measures for prevention of these infections. This goal seems achievable because superficial *Candida* infections are often not associated with deficiencies of the host's immune system.

Experimental Animal and Human *Candida* Immunization

The serious medical problem that the severe systemic *Candida* spp. infections pose in compromised individuals and the abundance of superficial mucocutaneous forms of candidiasis in uncompromised and compromised patients has stimulated numerous investigators (66) to attempt to induce protective immunity by vaccination. These attempts utilized various experimental animal models (e.g., mice, rats, rabbits), immunogenic preparations (whole live or dead yeasts killed by various methods; cell extracts; or subcellular fractions), and a variety of immunization procedures and regimens (intravenous, intraperitoneal, or subcutaneous single or multiple injections of the immunogen; various time intervals between the vaccine injections). The common denominator for these studies was the goal of inducing, by vaccination, protection against systemic *Candida* infections. Such infections, which appear infrequently, particularly plague patients with severe underlying disease (9). In contrast, attempts to vaccinate against superficial mucocutaneous forms of candidiasis, which constitute the bulk of the *Candida* infections, are relatively few and sporadic.

Among the experimental studies focusing on candidiasis of other than systemic infections were those of Giger et al. (21) and Pearsal et al. (55), who used murine cutaneous and thigh models, respectively, and that of Sohnle et al. (69), who used a cutaneous model in guinea pigs. Pearsal et al. (55) developed an experimental candidiasis model in the mouse using a thigh lesion, the size of the lesion being a parameter of infection. The investigators found that animals recovering from the lesions developed partial immunity based on the diminished size of the lesion when the ani-

mals were reinfected with live organisms. They also observed that passive transfer of serum from mice immunized with a *Candida* cell sonicate to naive recipients conferred on the latter a state of immunity, which again was demonstrated by the smaller size of their thigh lesions compared to unimmunized control animals (not receiving the immune serum).

Giger et al. (21) showed in their murine cutaneous model that inoculation with a sublethal dose of live *C. albicans* cells rendered the animals immune prior to attempts at reinfection. Inoculation with heat-killed yeast cells was less effective. Sohnle et al. (69), using a cutaneous guinea pig infection model, observed that resistance to infection of the skin involved T-cell-mediated immunity and activation of complement.

An interesting study was reported by Kuttin et al. (41), who vaccinated geese intradermally or intramuscularly with ethanol-killed *C. albicans* cells to prevent a venereal infection caused by the yeast. It was claimed that the vaccination resulted in significantly reduced morbidity among the immunized geese, compared to the unimmunized controls (0% versus 17.1–44.8% 3.5 months after vaccination and 6.3% versus 13.9% 4.5 months after vaccination).

Sporadic reports regarding immunization studies involving humans have appeared in the literature. *Candida* vaginitis comprises a significant proportion of candidiasis cases. It poses a medical problem, particularly because of the high percentage of recurrence among women suffering from the disease. In view of its clinical importance, the study of Waldman et al. (75) involving immunizing women against *C. albians* assumes importance. These investigators vaccinated ten female volunteers intravaginally with a lyophilized *C. albicans* culture-filtrate. The induced immunity was evaluated by measuring *Candida* antibody in serum and vaginal secretions. The vaccination resulted in the appearance of antibodies in vaginal secretions and an increase in the level of antibody in serum in six of the ten immunized women. Whether the antibodies had a protective role was not clearly established.

Gravina-Sanvitale et al. (25) used an autovaccine to treat a patient with onychomycosis, a treatment that was claimed by the authors to give favorable results. *Candida* skin infections have also been treated with a vaccine prepared by Harada (27) and Beemer et al. (7). The latter investigators claimed to have protected the patients from prolonged chronic infections caused by dermatophytes and *Candida* utilizing vaccination with a combined vaccine of ethanol-killed organisms.

Cancer patients undergoing therapy suffer frequently from severe oral thrush. Valdazo (73) attempted to protect such patients by administering a preparation of a yeast (referred to as *Saccharomyces boulardii*). The author believed that this yeast serves as an unspecific booster on the immune system that may act as an antagonist to *C. albicans* (15). Boostering the immune system was also attempted by Kirkpatrick and others (39,51) in CMCC patients using a leukocyte factor ("transfer factor"). The results,

although variable, led to partial immunological reconstitution in some patients.

In the context of preventing mucocutaneous *Candida* infections, Segal and colleagues (42,66), working with an experimental murine vaginitis model, showed that the infection could be prevented by blocking the adhesion of the fungus to the mucosa. Blocking was accomplished by competitive binging of *Candia* adhesive-like substances (substances involved in the adhesion) to the putative receptor sites on the mucosa. This approach, although not qualifying as vaccination per se, may partially answer the need for prophylaxis.

In conclusion, despite the need of prophylaxis for management of frequent *Candida* mucocutaneous infections, current data do not seem to indicate that a vaccine for prophylaxis of these infections is forthcoming.

Pityriasis Versicolor

Pityriasis versicolor, a skin infection that involves the superficial layers of the stratum corneum, is caused by the lipohilic yeast *Malassezia furfur* (29,61). The infection, which is frequently seen in young adults, generally affects the chest and abdomen and is manifested by skin patches of variable pigmentation. Although in many instances it is primarily a cosmetic problem, it causes frequent visits to dermatological clinics.

Malassezia furfur is a human commensal organism (22,28,61) that under certain conditions causes disease. The factors contributing to this situation are only partially known and possibly include disturbances in normal turnover of the dermal epithelium (8,35) or some defect in CMI (68).

Although the infection responds to treatment with various antifungal or keratinolytic agents, it is associated with a high rate of recurrence. Hence this infection also qualifies as a candidate for prophylaxis in individuals prone to infection. Unfortunately, no efforts in this direction have been described in the literature. Furthermore, there are only sporadic reports of experimental infections in animals (16,74) and few studies evaluating the immune responses evoked by this fungus (68).

Summary and Conclusions

Efforts have been made to induce protective immunity to the infections caused by dermatophytes, *Candida*, and *Malassezia*. The following conclusions can be drawn from the studies reviewed.

1. The research directed toward the management of dermatophyte infections by vaccination have yielded a promising vaccine for prevention of *T. verrucosum* infections in cattle.

2. A number of investigators have attempted, by various methods and with different immunogenic preparations, to prevent superficial mucocutaneous candidiasis. The results of those investigations do not indicate that a vaccine for prophylaxis of this disease is forthcoming.
3. No vaccination attempts for prevention of pityriasis versicolor have been described.

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3—Tissue-Bound Immunity: A Mechanism of Candidacidal Activity

HISASHI TAKAHASHI, KENICHIRO CHIKAKANE, MASARU OKUDA, MARIKO HATANO, MANABU HOSHINO, OSAMU KANEKO, AND YOKO KAWA

As reported in preliminary proceedings (34), we recognized that crude extracts of guinea pig epidermis displayed candidacidal activity in vitro. We have since fractionated and partially purified the activity substances, and here we introduce the methods of extraction and the characterization of the substances (33). The substances are basic proteins and polypeptides that show similarities to the microbicidal substances in lysosomal extracts of polymorphonuclear leukocytes (PMNs), as reported by Spitznagel (30) and Lehrer et al. (14). They also show similarities to the basic substances extracted from other organs by Bloom and Blake (1) and Hirsch and Dubos (9). The widespread occurrence of microbicidal basic polypeptides suggests that basic tissue-associated polypeptides and proteins may act together to defend the body against invasions by pathogenic organisms. In this chapter we deal with the candidacidal substances of the epidermis in relation to the superficial mycoses such as candidiasis and dermatophytoses, and we discuss the biological defense mechanisms provided by these basic substances.

Preparation of Guinea Pig Epidermal Extract Sample Solution

Guinea pigs (Hartley strain) weighing 300–500 g were clipped, and their skins were widely excised under ether anesthesia. The peeled skins were put on a 60°C hot plate, surface downward, for a few seconds until the epidermis became heat-separated. Then the epidermis was homogenized in 0.34 *M* sucrose with 0.1 *M* citric acid buffer and centrifuged at $400 \times g$ for 10 minutes. The process was repeated two times, and the collected supernatant was certrifuged at $8{,}700 \times g$ for 15 minutes. The resultant sediment was freeze-thawed five times in 0.25 *M* sucrose solution containing Triton-X and then examined by electron microscopy. The extraction method was

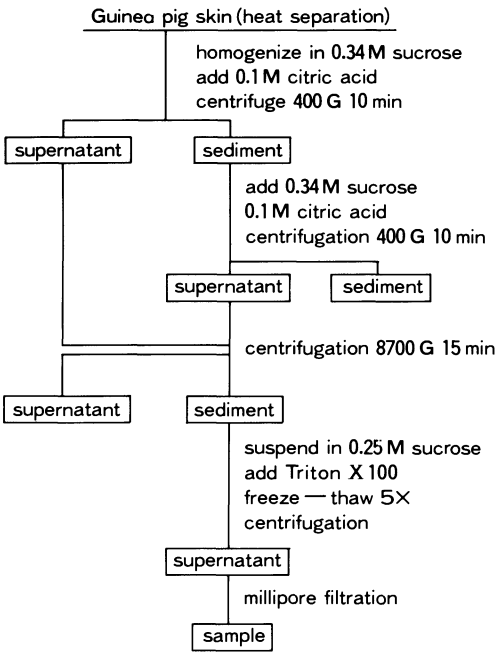


FIG. 3-1. Process of extraction of candidacidal sample solution from guinea pig epidermis.

that used by Matoltsy and Matoltsy (18) for extraction of keratohyalin granules. Our results coincided well with theirs, as we obtained a solution that was rich in keratohyalin granules. Because our procedure also resembled the technique used by Lehrer (12) for extracting lysosomes from PMNs for the study of nonoxidative candidacidal substances, this method was favorable for the subsequent candidacidal assay. The extracted solution, rich in keratohyalin substance, was Millipore-filtered and used as a sample solution (Fig. 3-1).

Candidacidal Assay by the Sample Solution

Candida albicans ATCC 16261 was shake-cultured in liquid medium [neopeptone (Difco), ad. aq. 1,000 ml] at 30°C. Organisms were harvested in the logarithmic stage 6 hours later and washed with sterile water by centrifugation. Small tapered test tubes containing 0.1 ml of *Candida* suspension, 0.8 ml of 0.1 *M* citric acid buffer solution, and 0.1 ml of sample solution of various dilutions were incubated by gentle shaking in a 37°C water bath for 1 hour. Viable organisms were then counted by the trypan blue dye exclusion method.

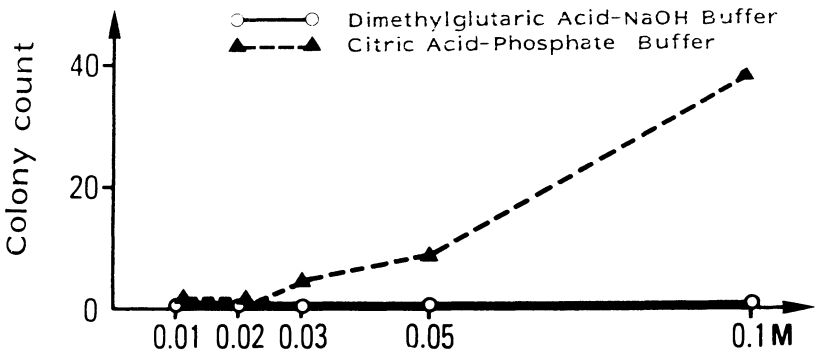


FIG. 3-2. Comparison of the influences of two buffers on the candidacidal activities of sample solutions. Sample solution 8 μg/ml, pH 4.5.

Fractionation of Sample Solution

The obtained sample solution was fractionated on a sephadex G50 column and eluted by 0.01% citrate-phosphate buffer at pH 4.7. During earlier experiments we used a 0.1 *M* citric acid buffer pH 4.5. Because we noted that high concentrations of citric acid buffer decreased candidacidal activity, we later changed the buffer to 0.03 *M* dimethylglutaric acid–NaOH buffer to provide a low pH and low ionic strength buffer action (Fig. 3-2).

Candidacidal Effects of the Test Sample

The fungicidal activities of a sample solution containing 10 μg/ml in citric acid buffer pH 4.5 were 72% for *C. albicans* and 98% for *C. tropicalis*, respectively. However, the organisms both revealed a 16% spontaneous death rate under these conditions, so no conclusion could be drawn about the relative resistance of the two *Candida* species. Because the small size of *Sporothrix schenckii* made its trypan blue intake less recognizable, we could not obtain clear results with this organism (Table 3-1).

The relation between the protein concentration and the candidacidal rate of the sample solution, summarized in Table 3-2, revealed a high candidacidal potency at pH 4.5 with citric acid buffer. Protein concentrations exceeding 10 μg/ml (Lowry) killed 100% of *Candida* within 1 hour whereas concentrations of less than 5 μg/ml killed only 50% of *Candida*. The candidacidal effect occurred rapidly. A 20 μg/ml sample solution killed 94% of *Candida* cells within 15 minutes (Table 3-3) and 100% with 30 minutes. The activity was heat-stable: 70% of the original activity remained after heating at 90°C for 10 minutes.

TABLE 3-1. Fungicidal rates of sample solutions on *Candida albicans*, *C. tropicalis*, and *Sporothrix schenckii*.

Fungus	Fungicidal rate (%)	Control (%)
C. albicans	72	1
C. tropicalis	98	16
S. schenckii	16	13

Sample solution 10 μg/ml, citric acid buffer pH 4.5, 37°C, 1 hour.

TABLE 3-2. Influence of concentrations of sample solution on the candidacidal rates.

Protein concentration (μg/ml)	Candidacidal rate (%)
40	100
20	100
10	100
5	87
2.5	40.5
1.25	6.5
0.625	1
0.3125	0

Citric acid buffer pH 4.5, 37°C, 1 hour.

TABLE 3-3. Time course of candidacidal process.

Time (min)	Candidacidal rate (%)
15	94
30	100
60	100
120	100

Citric acid buffer pH 4.5, sample solution 20 μg/ml.

The hitherto presented data resulted from experiments carried out using the eight fold diluted McIlvaine buffer (0.1 *M* citric acid with 0.2 *M* Na_2HPO_4) as used by Lehrer (12). With this buffer the highest rate of candidacidal activity was obtained at pH 4.5 or below (Table 3-4), and the sample solution suddenly lost its activity at higher pH. The activity of the sample solution was also dependent on the ionic strength of the buffer. By using 0.03 *M* dimethylglutaric acid–NaOH buffer instead of citric acid buffer, we could study the relation between the two buffers at higher pH and observed a candidacidal activity between 50 and 60% at pH 7.0 for this buffer (Fig. 3-3).

TABLE 3-4. Effect of pH on candidacidal activity of sample solution.

pH	Candidacidal rate (%)
3.7	100
4.5	100
6.0	0

Citric acid buffer 20 μg/ml, 37°C, 1 hour.

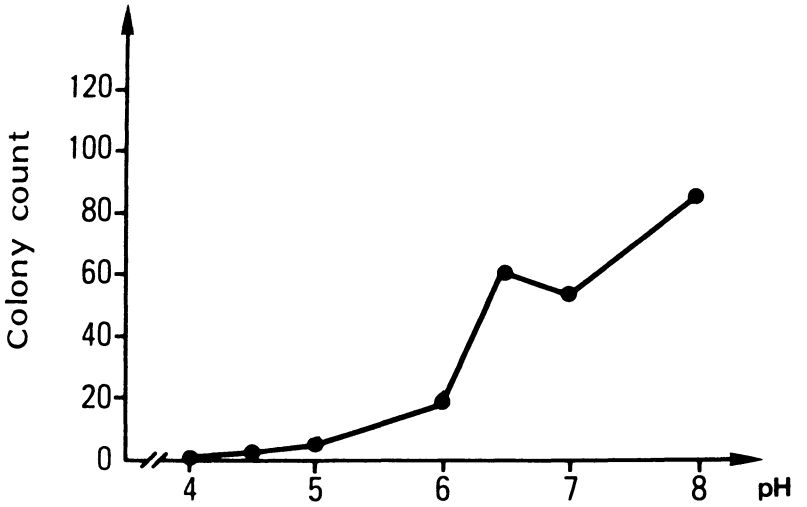


FIG. 3-3. Effect of pH on candidacidal activity of sample solution (10 μg/ml) 0.03 *M* dimethylglutaric acid–NaOH buffer.

Ultrastructural Correlates

Candida albicans specimens were first prepared for electron microscopy with Karnovsky's fixative, and then we applied two methods of processing the cells. With the first process postfixation was done with 1% OsO_4 and the samples underwent dual staining by the uranium–lead method before examination in a JEM 100-S electron microscope (Nippon Denshi Co.). With the second process the samples were postfixed in 1% $KMnO_4$ for 1 hour and then further stained with alkaline bismuth prior to electron microscopic examination.

With the first method, it was observed that electron-dense material surrounded fungus (Fig. 3-4), and the second method showed a breakdown of the nuclear membrance and swelling of mitochondria (Fig. 3-5).

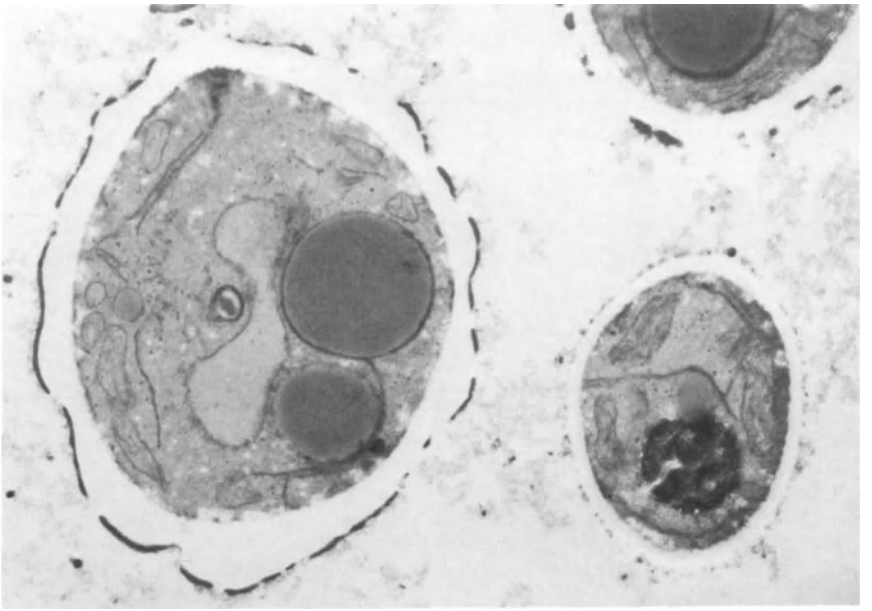


FIG. 3-4. Electron microscopic findings of treated *Candida* with epidermal extract. Method 1 of the processing revealed that *C. albicans* organisms are surrounded by electron-dense material.

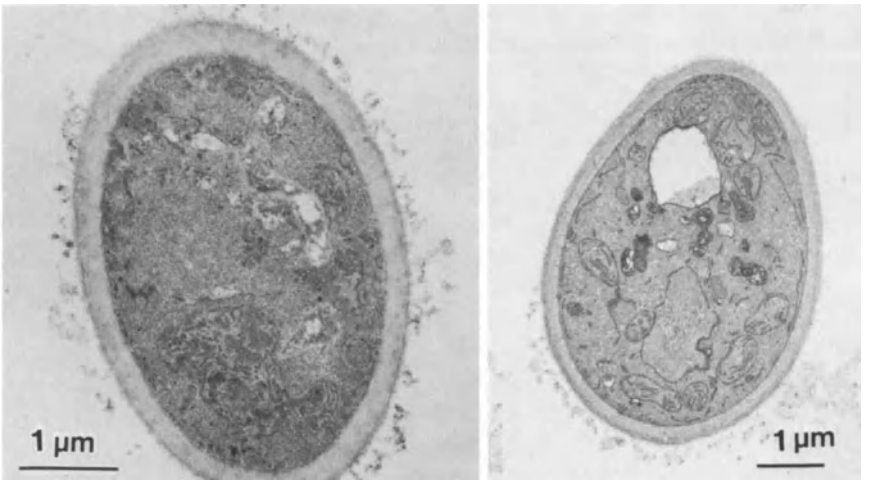


FIG. 3-5. Electron microscopic findings of treated *Candida* (left) and intact control (right), processed by method 2. Nuclear membrane breakdown and mitochondrial swelling were noted.

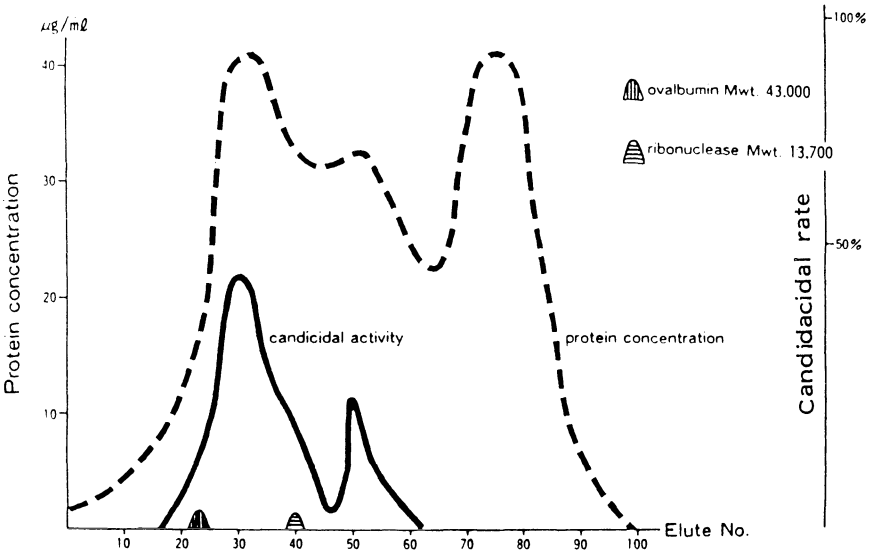


FIG. 3-6. Candidacidal activities of fractionated sample solution.

Fractionation of Sample Solution in Relation to Candidacidal Activity

The fractionated sample solution revealed three major peaks in protein concentration (Lowry), and two of them coincided with the peaks of candidacidal activity (Fig. 3-6). The molecular weight of these two peaks was about 20,000 and somewhat less than 10,000, respectively.

Mode of Action of Microbicidal Activity of Basic Proteins

A number of heterogeneous basic agents, e.g., cationic detergents, polymyxin B, protamine sulfate, histones, and lysozyme, have potent antimicrobial activities. Sahl (25) reported the mode of action of the basic substances. According to this report, a basic protein with a staphylococcin-like peptide, Pep5, attacks cytoplasmic memberanes as the primary target, and the treated bacteria become unable to transport amino acids actively. Subsequently, there is leakage of low-molecular-weight substances such as potassium ions, adenosine triphosphate (ATP), and amino acids. This leakage, in turn, causes cessation of biosynthesis and blockage of active transport through the membrane. Basic proteins are also highly active against gram-positive bacteria, probably because they have affinity sites on their surfaces, e.g., teichoic acid on staphylococci.

The cell wall of *Candida* is negatively charged except for the apex of its growing germ tube, so the mode of action of the candidacidal activity can be interpreted in a similar way. The basic substances are also highly microbiocidal against gram-negative bacilli, e.g., *Escherichia coli* (39). Sahl (26) and Ruhr and Sahl (24) attributed the toxic activity of the basic proteins to the electrostatic charge of the bacterial cell surface, but it seems likely that there may be another factor involved in killing *Candida*. In the disease called acrodermatitis enteropathica, proliferation of *Candida* on the skin occurs secondary to zinc deficiency, suggesting some relation with the activity of zinc compounds (rich in the epidermis) and candidacidal activity. More detailed explanations may remain elusive until the relevant biological actions of metalloproteins are established.

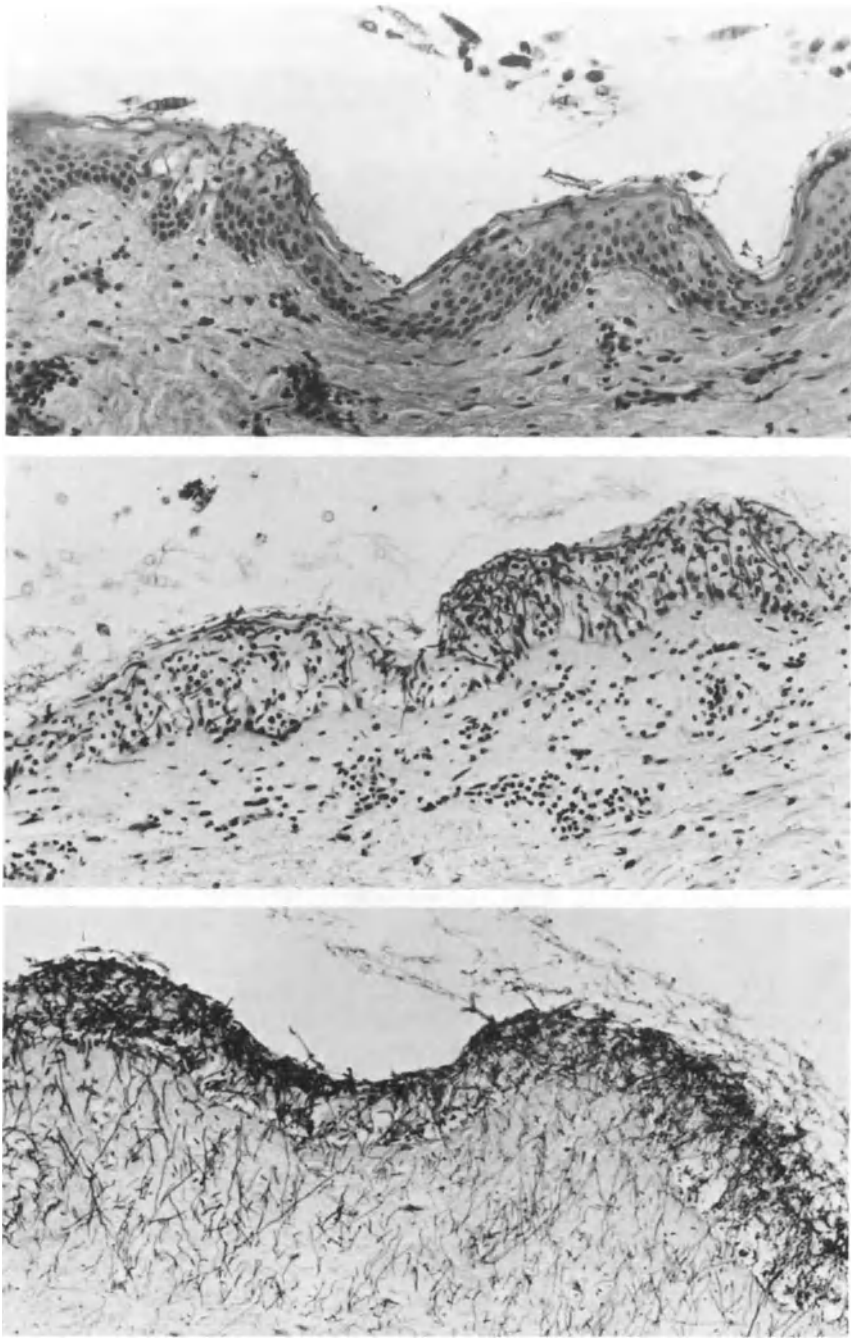
Epidermal Basic Protein and Superficial Fungus Infection

The reason fungi do not invade deeper into the skin in cases of superficial candidiasis, pityriasis versicolor, and dermatophytosis remains obscure, although it was previously attributed to serum factors. Roth et al. (23) evaluated the fungistatic activity of serum with respect to the incidence of the superficial mycoses. Louria et al. (16) and Lorincz et al. (15) found both anticandidal and antidermatophytic activities in serum.

During the 1970s Hendry and Bakerspeigel (7) and Carlisle et al. (5) also found such factors in serum. Because the addition of iron abolishes the antimicrobial activity of serum, the activity was attributed to transferrin by some researchers (17,29). Keratinophilic proteinase (keratinase) inhibitors such as α_2-macroglobulin (38) or some inhibitor existing in the epidermis (37) have been highlighted as the defending substances. Our substances, extracted from a keratohyalin-rich fraction of the epidermis, may also serve as a substance that prevents invasion of fungal elements into the granular layer of the skin. In our experience, the activity of the substance is lost within 1 day after the excision of the skin (Figs. 3-7, 3-8, and 3-9). From this fact it can be concluded that the active fungicidal substance may not be the keratohyalin itself but some substance continuously formed in association with keratohyalin.

Phylogenetic Aspects of Biological Defense Substances

What kinds of substance, present in higher plants and animals, protect them against invasion of lower microorganisms by inhibiting their host-specfic toxins? The term *pathogenicity* implies that the toxins or toxic prop-



FIGS. 3-7 to 3-9. Invasion of *Microsporum gypseum* into excised guinea pig skin (× 200). FIG. 3-7. After 2 days. FIG. 3-8. After 5 days. FIG. 3-9. After 8 days.

erties of the parasite overcome the resisting host substances that should inhibit parasite proliferation.

Fungus infection offers a good model of a host–parasite relationship. Nishimura, a plant pathologist (19), elucidated the chemical conformations of some "host-specific toxins" produced by pathogenic fungi, e.g., AF toxin (strawberry), AT toxin (tobacco), AM toxin (apple), AC toxin (citrus fruits), AK toxin (pear), and AL toxin (tomato). These toxin-producing fungi manifested specific pathogenicities against the respective plants. For example, among mammals *Trichophyton verrucosum* attacks cattle, *T. rubrum* and *Epidermophyton floccosum* humans, and *Microsporum nanum* swine, but their host-specific toxins have not yet been identified. In contrast to phytoalexins of plants, which have some microbicidal activities and of which the chemical composition has been defined as stated above (35), no exact chemical substance involved in defense mechanisms has yet been determined in mammals.

In lower animals, e.g., *Bombyx mori*, several antimicrobics are induced in the larvae (10), and one of them is reported to be a basic substance of molecular weight 5,000 and Ip 7.7. The substance has a minimal inhibitory concentration (MIC) of 1.0–1.6 μg/ml against gram-negative and gram-positive bacteria and has the property of disrupting the membrane of an invading organism. According to Wago (36), lower animals without immunological systems can distinguish self and not self, and can recognize and reject foreign bodies despite the lack of an immunological organ.

Defense mechanisms have been studied in mammals since the nineteenth century using cell-free blood. The detailed reports by Buchner (3,4) were especially excellent articles and are still valuable to date. At that time two substances were considered to engage in biological defense mechanisms. One was the heat-labile substance now called *complement* (Ehrlich) and formerly named alexin (Buchner) or *α-lysin* (Petterson). The other was a heat-stable substance called *β-lysin.* Studies on the former have been vast and constitute a major area of modern immunology. Studies on the latter are being continued by investigators such as Bloom and Blake (1,2), Hirsch and Dubos (8,9), and Donaldson and Ters (6). The substances were extracted mainly from PMN lysosomes (12–14,28,30–32,40,41) as well as from other organs, e.g., parotids (21), testis (11), and sperm (22). Spitznagel et al. (30–32,40,41) reported that the bactericidal substance of PMNs was a cationic protein residing in the azurophil granule. Using zone electrophoretic studies, they fractionated the substance into I–VII bands. The most basic bands, I–III, showed a high activity, in contrast to band IV (lysozyme), which had no microbicidal activity against *Escherichia coli.* The studies, using fungi as the targets, were the work of Lehrer et al. (12–14,28). They found that the PMN lysosomes from a myeloperoxidase-deficient patient possessed adequate candidacidal activities. That is, addition of potassium iodide and hydrogen peroxide to the normal lysosomal extract yielded one peak of candidacidal activity, even

though the patient failed to rerveal the myeloperoxidase-dependent candidacidal fraction. Yet the patient did not suffer from candidiasis because another fraction was identified in PMN extracts with candidacidal activity in vitro. They referred to the lysosomal substance of PMNs other than myeloperoxidose as the second mechaism of candidacidal activity (12) or nonoxidative fungicidal mechanism (14). This substance was basic and more active at pH 5.0 than at pH 7.0, and *C. parapsilosis* was more susceptible to the substance than *C. albicans*. More recently, they extracted similar basic substances from rabbit alveolar macrophages (20,27).

The substance we extracted from epidermis is similar to the basic substance extracted from the PMN lysosomes in that (a) both are active at acidic pH and (b) both tend to adhere to the surface of the target. The main difference is that our substance is histidine-rich, whereas the PMN substance is arginine-rich.

Significance of Basic Proteins as Defense Mechanism

The ubiquitous nature of the basic substances in animals means that there are enormous possibilities for such substances to compose a defense mechanism for the body against invading microorganisms. These substances are "tissue-bound" and have been extracted by many investigators from various tissues. Yet according to our usual concepts, the major defense mechanisms are dependent on immunological (humoral, cellular) substances such as interferons, lymphokines, and interleukins. Other substances, irrespective of present knowledge of immunology, have not been taken seriously. However, a defense mechanism in mammals has evolved from immunity systems of invertebrates, and these primordial defense mechanisms may persist even in the higher animals. Based on this fact, it seems necessary to study both kinds of immune mechanism as well as the relation between them to obtain a full picture on immunity (Fig. 3-10).

To protect against the invasion of harmful microorganisms, tissues are required to meet the following characteristics: (a) recognition of harmful microorganisms; (b) adherence to them by some antagonistic substances; and (c) breakdown via enzymatic activities or other toxic processes of the substances, e.g., ligand metals of metalloproteins.

Like the activated complement system, which finally perforates the target cell membrane, basic polypeptides also damage the target cell's wall conformation, and they have in common the tendency to "recognize" and adhere to or aggregate the target organisms. Whereas immunological recognition is more precise and sensitive, the primordial defense mechanism may also have some ability to recognize self and not self. From the viewpoint hitherto reviewed, the defense mechanisms show similarities and may cooperate for the defense of higher animals.

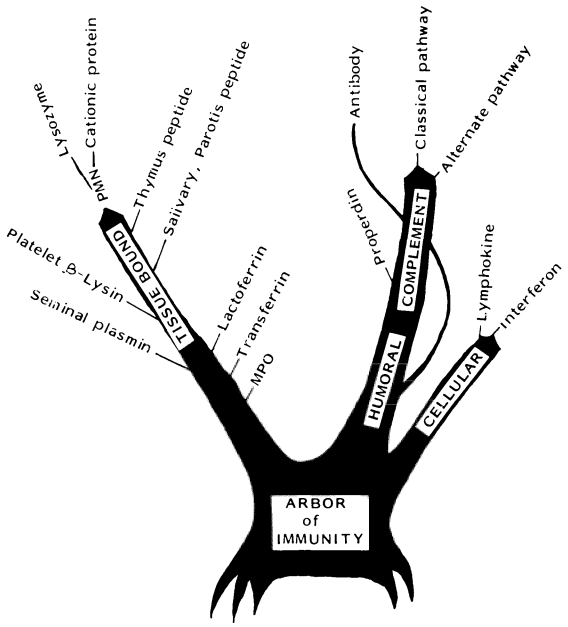


FIG. 3-10. Tree of defense mechanisms.

Acknowledgment

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4—Rhinosporidiosis

MERANI THIANPRASIT AND KLEOPHANT THAGERNGPOL

Rhinosporidiosis, an infection of the nasal mucocutaneous tissue and skin, was first described in 1900 by Guillermo Seeber (53). Seeber considered the organism to be related to the agent of Posadas's disease. In 1903 O'Kinealy reported a case from India under the name "localized psorospermosis" (49). In 1905 Minchin and Fantham (36) studied the detailed structure of this organism, which had its life cycle in the host only. They described the organism as a sporozoan that was characterized by the lack of locomotor organs in the adult stages. In Argentinian publications it was named *Rhinosporidium kinealyi* (49). Later, many reports were published with various opinions regarding the nature and nomenclature of this organism. In 1923 Ashworth (4) studied this disease in detail and concluded that the causative organism belonged to the fungi known as the Phycomycetes. He thought it should be named *Rhinosporidium seeberi* because it reproduced by endosporulation in the host tissue, giving rise to sporangia and spores. The organism often occurs on the mucosa of exposed body areas such as the eye and the urethra (30,49).

Conjunctival rhinosporidiosis was first described by Elliot and Ingram in 1912 in India, and this report was followed by descriptions by Kirkpatrick in 1916, Wright in 1922, Duggan in 1928, Kurup in 1931, Rao in 1931, and Allen and Dave in 1936 (11). Dhayagude reported the first case of urethral rhinosporidiosis in 1941 (12). Between then and 1983, only 11 cases of urethral infection have been recorded, according to Palaniswami and Bhandari (42). In 1987 Sasidharan et al. published a series of 27 cases of urethral rhinosporidiosis in both men and women (50). Rare cases of this disease have been reported affecting the laryngeal area, trachea, vagina, vulva, and anus; and it has been seen in disseminated form (22,30,34,49). Cultures have been attempted in various media without success (10,23, 45,48), and animal inoculation has failed to yield infection (10,14,28,50, 62).

Distribution

There have been reports on the prevalence of rhinopsporidiosis in large endemic studies, covering about 2,000 cases from India and Sri Lanka (2,19,30,42,50,51). However, sporadic cases have also been reported from nearly every part of the world (3,6,13,23,24,29,32,40,43,49,61). In Thailand the recorded number of rhinosporidiosis cases up until now is six; five cases were of the nasal form, and one case was a cutaneous granuloma on the face (56).

Age

The age of patients with rhinosporidiosis varies from 3 to 90 years. The average patient age when diagnosed is 20–40 years (49).

Sex

About 70–80% of the nasal cases occur in men. Eye infections seem to be more common in women (49), but nearly all of the cases of urethral infection are in men (42).

Route of Infection of *R. seeberi*

The exact mode of infection by *R. seeberi* is unknown. The organism cannot be cultured in artificial media, but it is known to commence its life cycle in the host tissue. Many authors have postulated that it is present in soil, dust, and natural water, and that nasal and eye infections occur by rubbing with fingers contaminated by soil or water (23,49). In other patients the habit of removing the last drops of urine following micturition by cleaning the penile meatus with a stone may have predisposed them to contamination causing infection (49). Dust storms may also induce rhinosporidiosis of the nasal cavity and eyes. The rural background of most patients supports the postulation that contaminated water and soil are the most likely sources of infection. This disease is not contagious, nor does it seem to be transmitted from animal to humans (33,50).

Immunology

Only a few reports can be found in the literature on the immune response of the host against *R. seeberi*. Chitravel et al. (10,11) studied the cell-mediated immune (CMI) response and humoral antibodies in human rhinosporidiosis. The antigen used was a filtered suspension of crude homogenate from infected tissues. The CMI response was studied by leukocyte migration inhibition (LMI) and lymphocyte count in 37 patients. The results showed that the LMI titer was higher than in control subjects without the disease; the titer was maximum in patients who had already had the illness for 3–9 years. It decreased considerably in patients whose chronicity of infection exceeded more than 10 years.

The humoral antibody test was undertaken to demonstrate the presence of antibodies and circulating antigen in the serum of 69 patients detected by counterimmunoelectrophoresis and immunodiffusion agar methods. The results showed that no antibodies are found in the serum of these patients. On the other hand, rhinosporidial circulating antigen was detected (10,11).

In conclusion, the immunological defense mechanisms of the host do not play an important role in the fight against this infection. The main defense mechanism is concentrated in phagocytic activity via an inflammatory process and granuloma.

Clinical Features

Of 2,000 cases of rhinosporidiosis reviewed by Karunaratne (30), about 70% were nasal infections, 15% eye infections, and 8% infections of some other mucosal area such as the urethra, vagina, or anus. There were some rare cases of cutaneous infection. Another study reported a 75% incidence of nasal infection (24). The most common sites of nasal infection are the mucosa of the septum, the interior turbinate part, and the nasal floor (49). The lesions appear as friable, sessile or pedunculated polyps on the surface of the mucosa. Fully mature polyps may hang down through the nasal meatus, or lesions may extend through the nasopharynx. The surface color of the polyps varies from pink to purplish red, with white spots suggesting colonies of organisms. In some cases, lesions may grow progressively toward the larynx, trachea, and bronchus (38,49).

Rhinosporidiosis of the eye has been reported in India by Kuriakose, who noted that 64% of cases affected the conjuctiva, 24% the lacrimal sac, 4% the canaliculi, 4% the lids, and 4% the sclera (15). Conjuctival lesions are characterized by polypoid or flattened growths of an indolent nature on the upper or lower palpebrae or on the bulbar conjunctiva. The infected

polyps are painless, pink, and soft. White spots may occur on the surface of the mass from which a creamy pus exudes.

The occurrence of urethral rhinosporidiosis appears to be rare. However, Palaniswami and Bhandari (42) reported 11 cases in 1983, and Sasidharan et al. (50) reported 27 cases in 1987. Urethral rhinosporidiosis is usually restricted to the fossa navicularis and the external urethral meatus. Few cases have shown ascending infection to the spongy urethra as far as the penoscrotal junction. The most common urethral lesions are solitary, pinkish, tongue-shaped, and pedunculated, and they arise from the floor or walls of the fossa navicularis (12,19,31,41,50).

Cutaneous rhinosporidiosis is rare. Skin lesions begin as tiny papules and become verrucous and granulomatous with age. Lesions can also be found on the external ear (49), and one report mentioned painless verruciform nodules on the face (56). The cutaneous lesions rarely form peduncles. Cases of fatal disseminated rhinosporidiosis due to hematogenous dissemination have been reported. The lesions are described as firm, hard, subcutaneous indolent nodules (1,9,22,34,44).

There is little information in the literature on the exact structure and components of *R. seeberi*. However, some studies with different viewpoints have been presented concerning the structure of this organism. (7,16,18, 20,26–28,37,52,56,60,64).

Grover (20) attempted to culture this organism in the liquid tissue culture medium 199 of Morgan et al. (39). It was observed that the immature spores developed to maturity in about 10 days, after which they degenerated. Because of the paucity of information on components and the lack of success in cultivation, we were prompted to study this disease with regard to its histopathogenesis, histochemistry, ultrastructure, and cultivation.

The subjects for the study were two patients with intranasal rhinosporidiosis and one patient with cutaneous granulomatous rhinosporidiosis of the right side of the nose (Figs. 4-1 and 4-2). Biopsy specimens were taken from the lesions of three patients and were prepared for histopathological and histochemical studies, electron microscopy, and cultivation.

Histopathology and Histochemistry

The specimens of the infected tissues were fixed in 10% formalin and embedded in paraffin. Staining techniques used for the histopathological and histochemical studies included hematoxylin–eosin; Gomori methenamine for hexosarmine; mucicarmine and alcian blue (pH 3.5) for acid mucopolysaccharides; toluidine blue and Giemsa for the metachromatic reaction; PAS and PAS-diastase for polysaccharides and cellulose, respectively; Feulgen for DNA; Lugol's solution for starch; Sudan 4 for lipids;

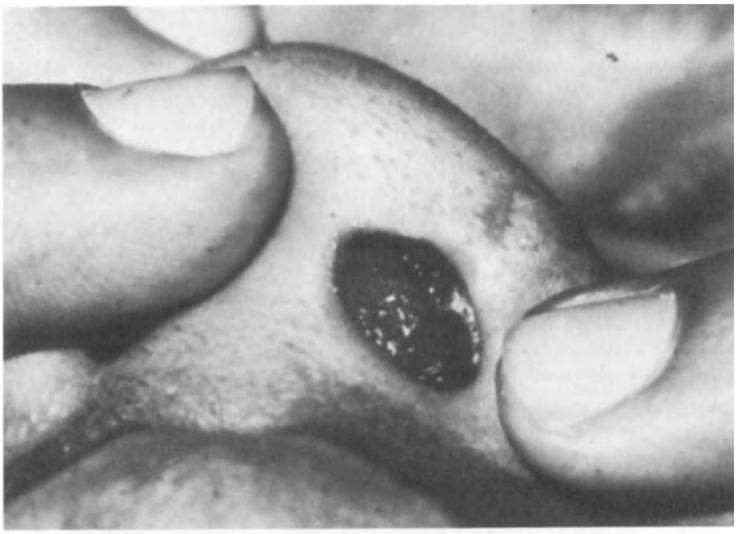

FIG. 4-1. Intranasal polyp of rhinosporidiosis.

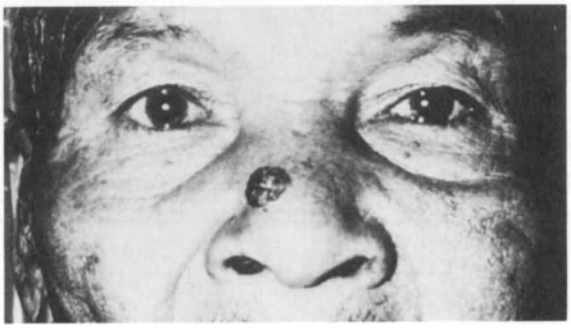

FIG. 4-2. Granulomatous nodule of cutaneous rhinosporidiosis.

and Baker for phospholipids, as for the enzymes NADHase (nicotinamide adenine dinucleotide, reduced form) and NADPHase (nicotinamide adenine dinucleotide phosphate, reduced form). Cobalt sulfide was used to detect alkali phosphatase, and fast garnet salts were used to detect acid phosphatase. The results are shown in Table 4-1.

Histopathological findings from nasal mucosa specimens stained with hematoxylin showed mucosal hyperplasis forming polyps and some areas of ulceration. The polyps consisted of fibrovascular and fibromyxomatoid tissues including various stages of round organisms without hyphae. By using polarized light, the cell walls of both sporangia and trophocytes were

TABLE 4-1. Histochemical reactions with *Rhinosporidium seeberi*.

Test	Trophocyte		Sporangium		Spore		Spheroid body
	CW	Cyt	CW	Cyt	CW	Cyt	
Hematoxylin–eosin	M	E	M	E	M	E	M
Gomori	S	W	S	W	S	W	S
Mucicarmine	W	W	W	W	S	W	–
Alcian blue	–	–	–	M	S	–	–
Toluidine blue*	–	S	–	–	S	M	–
Giemsa*	–	W	–	W	S	–	–
PAS	M	M	W–M	–	S	S	M
PAS-diastase	M	M	W–M	–	S	S	M
Sudan 4	–	S	–	M	–	W	–
Baker	–	M	–	M	–	W	–
Feulgen	–	M†	–	–	–	–	S
NADH	–	W	–	W	–	W	–
NADPH	–	W	–	W	–	W	–
Alkaline phosphatase	–	–	–	–	–	–	–
Acid phosphatase	–	–	–	–	–	–	–

*Metachromasia.
†Chromatin granules.
CW = cell wall; Cyt = cytoplasm; S = strong; M = moderate; W = weak; – = negative.

shown to be birefringent. In some areas, invagination of epithelial layers, fibrous tissue surrounding exudates, fibrins, mucoid materials, and organisms forming a pseudocystic abscess could be observed. The submucosal stroma was granulomatous and consisted of inflammatory cells, predominantly neutrophils, lymphocytes, histiocytes, plasma cells, and epithelioid cells (Fig. 4-3). Eosinophils were not as numerous as seen in zygomycosis caused by *Basidiobolus* and *Conidiobolus* spp. Foreign body giant cells were few in number.

The histopathological study of cutaneous specimens stained with hematoxylin–eosin revealed pseudoepitheliomatous hyperplasia with hyperkeratosis and papillomatosis. There was also a dermal reaction comprising a mixed-cell granuloma, as was the case in the specimens taken from the nasal mucosa, except that foreign body giant cells were abundant. One of the interesting findings was that the organisms did not penetrate deeper than the lower layer of the dermis but were pushed out to the surface of the mucosa or skin by transepidermal elimination and phagocytosis.

Distinct stages of *R. seeberi* (i.e., trophocytes or the trophic stage of Ashworth and sporangia, the mature trophocytes) were seen throughout the infected lesions (Fig. 4-4). Trophocytes were round and varied in size between 30 and 80 μm in diameter. In the earliest developmental stages, they contained a single nucleus and eosinophilic materials. Their walls were thin and became bilaminated in mature stages. Older trophocytes

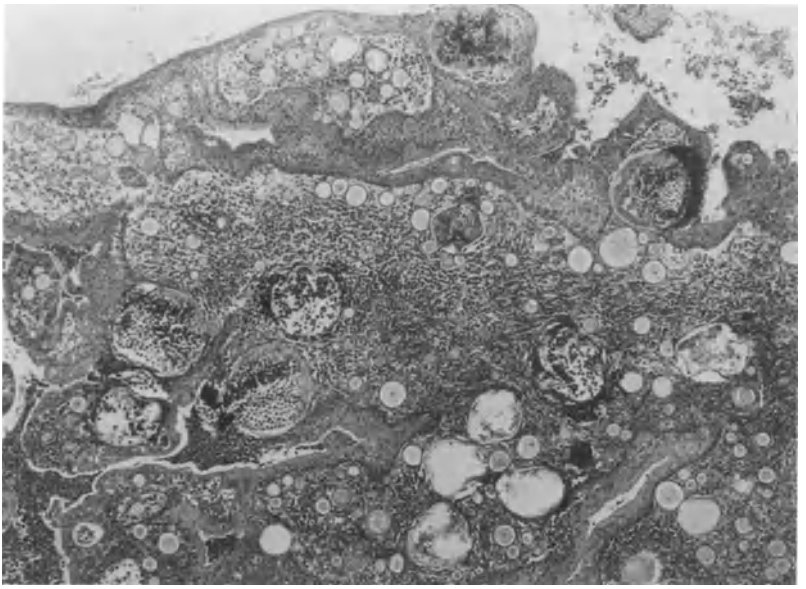

FIG. 4-3. Mixed cell granuloma with a pseudocystic abscess in the submucosa. Sporangia and trophocytes of *Rhinosporidium seeberi* appear in a polyp. (× 20, HE)

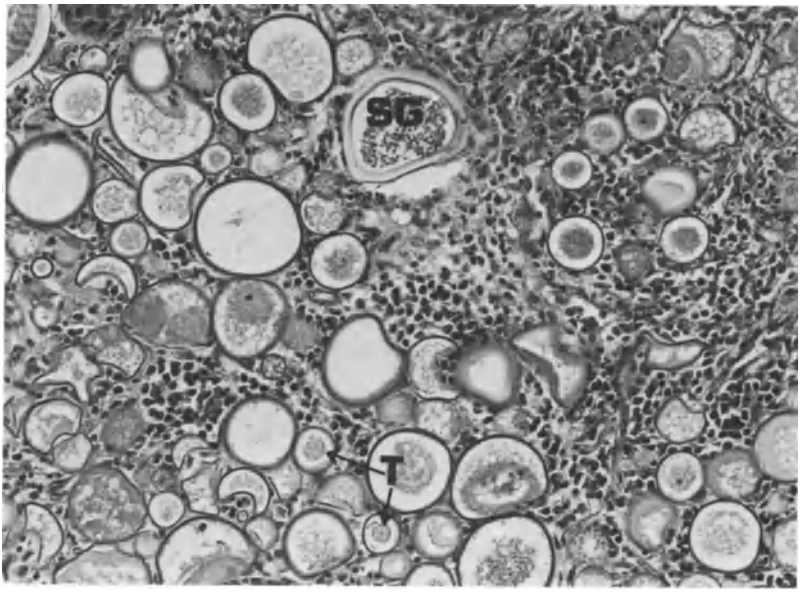

FIG. 4-4. Stages of trophocytes (T) and a sporangium (SG). (× 100, HE)

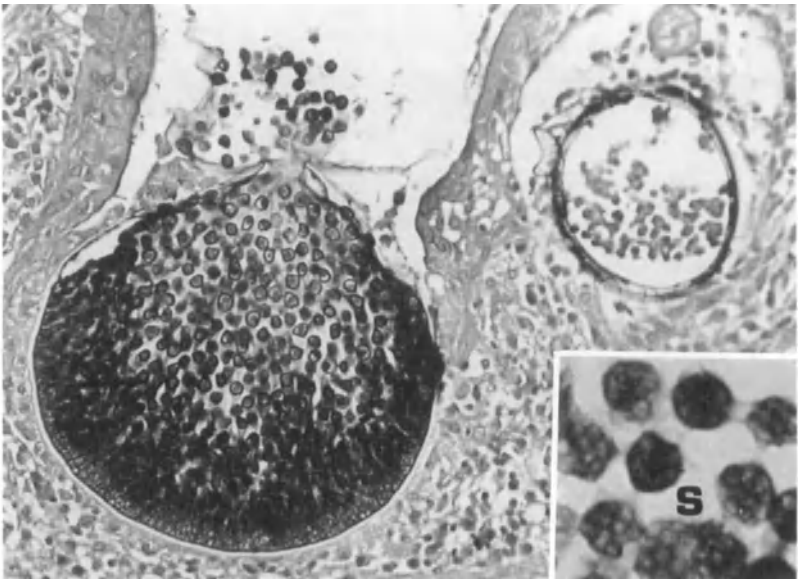

FIG. 4-5. Spores are released through the pore of a ruptured sporangium. Young spores appear at the bottom (× 100, PAS). (INSET) S = enlargement of spores containing clusters of spheroid bodies. (× 400, PAS)

consisted of young spores, lipid globules, and eosinophilic granules. Mature trophocytes or sporangia were 100–400 μm in diameter and were characterized by numerous encapsulated spores. Young spores migrated toward the other side (Fig. 4-5). At maturity the spores were 7–9 μm in diameter and contained clusters of multiple indistinct, round bodies (Fig. 4-5). As the sporangium matured, its wall became thin and then ruptured. Spores were released through the ruptured pore and became young trophocytes in the host tissue.

The cell wall of the sporangia, the spores, and the trophocytes of *R. seeberi* stained well with hematoxylin, especially in frozen sections or smears. The black staining of silver precipitation by Gomori's methenamine method demonstrated the presence of hexosamine in the wall of mature sporangia, trophocytes, and spores. However, in the young stages, staining was faint. Mature spores were joined by an intersporal mucoid substance, and there was no direct contact between the cell walls. This mucoid substance gave a positive reaction to mucicarmine and alcian blue staining, suggesting the presence of acid mucopolysaccharides (Fig. 4-6). The organism showed metachromasia after toluidine blue and Giemsa staining. The outer and inner walls of the sporangia, trophocytes, and spores exhibited strongly positive periodic acid-Schiff (PAS) and PAS-

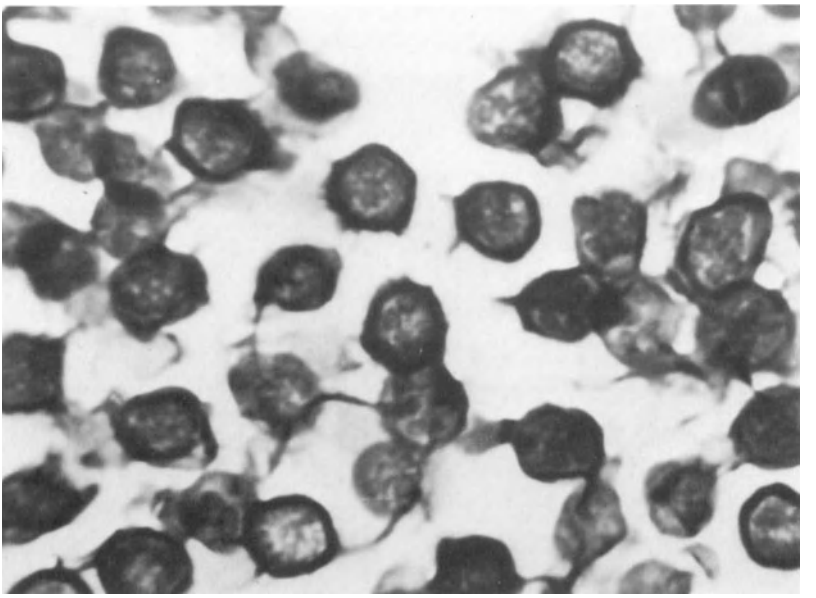

FIG. 4-6. Spores with mucoid substance showing an intersporal network. (× 1000, Alcian blue)

diastase reactions. The cytoplasm of the trophocytes stained well with PAS, revealing fine granules of glycogen and glycoprotein (Fig. 4-5). Small sporangia were formed in clusters and displayed a positive reaction to Feulgen, suggesting the presence of DNA components. Using Lugol's solution, starch granules could not be detected as in *Cryptococcus neoformans*.

In frozen sections stained with Sudan 4 and Baker's methods, abundant lipid and phospholipid granules were observed in the cytoplasm of sporangia, trophocytes, and spores (Fig. 4-7).

Only weak activity of oxidative enzymes (e.g., nicotinamide adenine dinucleotide, reduced form, and nicotinamide adenine dinucleotide phosphate, reduced form) could be demonstrated in the cytoplasm of trophocytes of *R. seeberi*, whereas the hydrolytic enzymes (alkaline phosphatase and acid phosphatase) were asbent.

Electron Microscopy

Biopsy specimens were immediately fixed in 4% glutaraldehyde, buffered with Milloning's phosphate at pH 7.4, and postfixed in 2% buffered osmium tetroxide; they were then dehydrated in graded concentrations of ethanol followed by propylene oxide treatment and embedding in Epon.

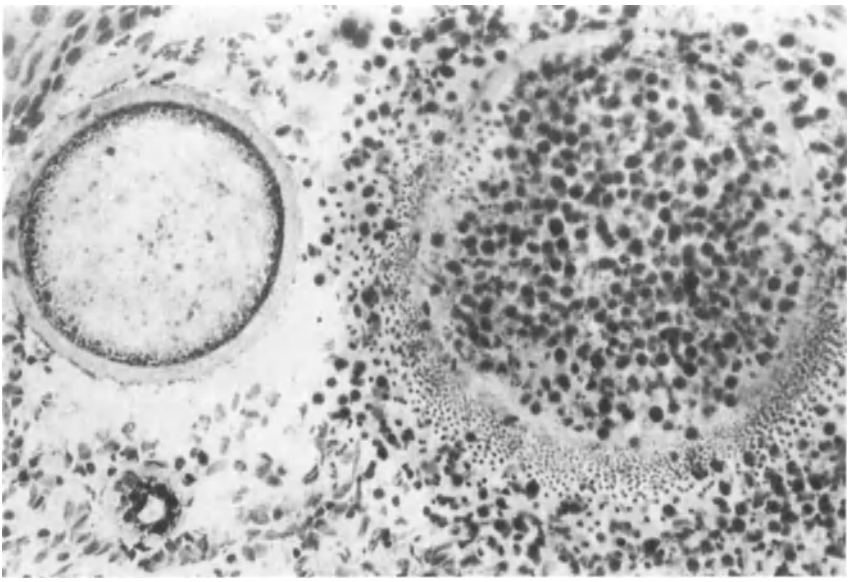

FIG. 4-7. Lipid droplets disperse in a trophocyte (left) and sporangium (right). (× 200, Sudan 4)

Sections 1 μm thick were cut on an LKB Ultratome V using a glass knife. The sections were then stained with toluidine blue at pH 11 and examined under a light microscope. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined at 80 kV in a Jeol 100 SX electron microscope.

For scanning electron microscopy, the paraffin-embedded sections were cut at 5 μm. Following deparaffinization, pieces of glass slides containing the tissues were mounted on stubs using conducting silver paste. They were then coated with a thin layer of gold and examined under a Jeol JSM-25S microscope.

Scanning electron microscopy of the infected tissues revealed infective sporangia and trophocytes. The organisms were surrounded by fibrous tissue of the host. Spores were encapsulated by multiple layers of fibrillary sheaths that adhered to intersporal fibrillary substances (Fig. 4-8).

Transmission electron microscopy revealed that the walls of the sporangia were trilaminated, whereas those of the trophocytes were bilaminated with a less dense electron appearance. In both sporangia and trophocytes, the inner walls were about one-third the width of the outer walls, and they appeared to be composed of homogeneous electron-dense material. The outer wall was composed of a myriad of curvilinear structures (Fig. 4-9). The walls of the trophocytes were bilaminated in the immature stages and trilaminated in mature stages.

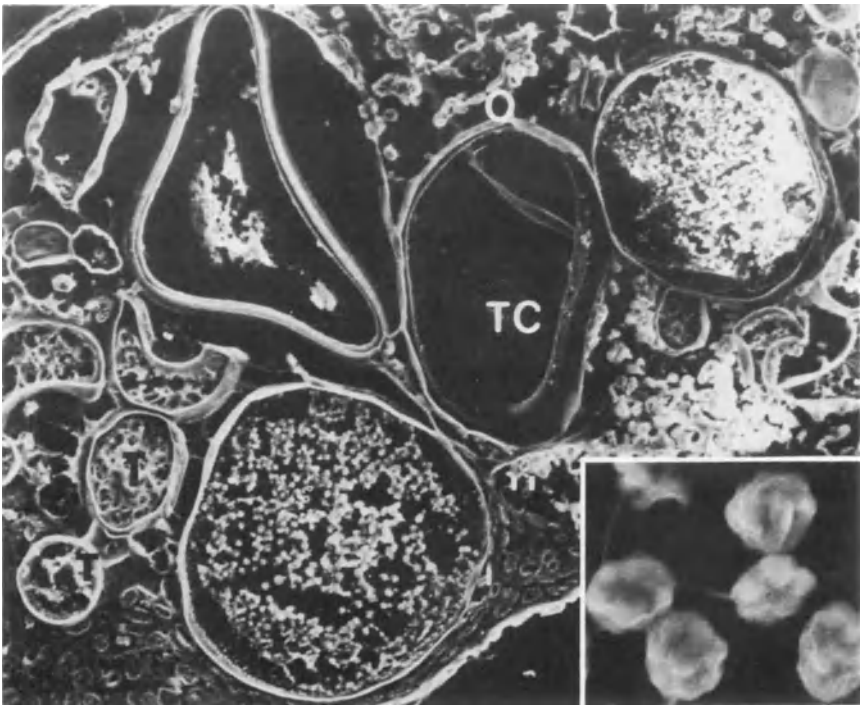

FIG. 4-8. Numerous spores with spheroid bodies in the sporangium connected by an intersporal fibrillary network (lower right) and various stages of trophocytes (T) as well as a trophic cyst (TC). (× 200, SEM)

The spores inside the sporangia varied in size and number owing to differences in maturity. Spore walls were composed of multiple layers of granular, fibrillary, and mucoid membranes that adhered to the intersporal network. The ultrastructure of each spore comprised an oval or round membrane-bound nucleus with a nucleolus, mitochondria with plate-like cristae, endoplasmic reticulum, vacuoles, lipid bodies, chromatin granules, and electron-dense spherules or round bodies (Fig. 4-10).

We observed that released spores in the infected tissue commenced a life cycle by forming young trophocytes. The surrounding fibrillary capsule was transformed into a true wall with a bilaminated homogeneous appearance (Fig. 4-9). At the same time, the sphere-like bodies inside the spore degenerated and disappeared.

There were abundant multilamellar bodies in various stages in the trophocytes. Some consisted of a single layer and some of multiple concentric layers. The outermost of the mature multilamellar bodies showed electron-dense rings suggesting cell walls. These bodies multiplied by

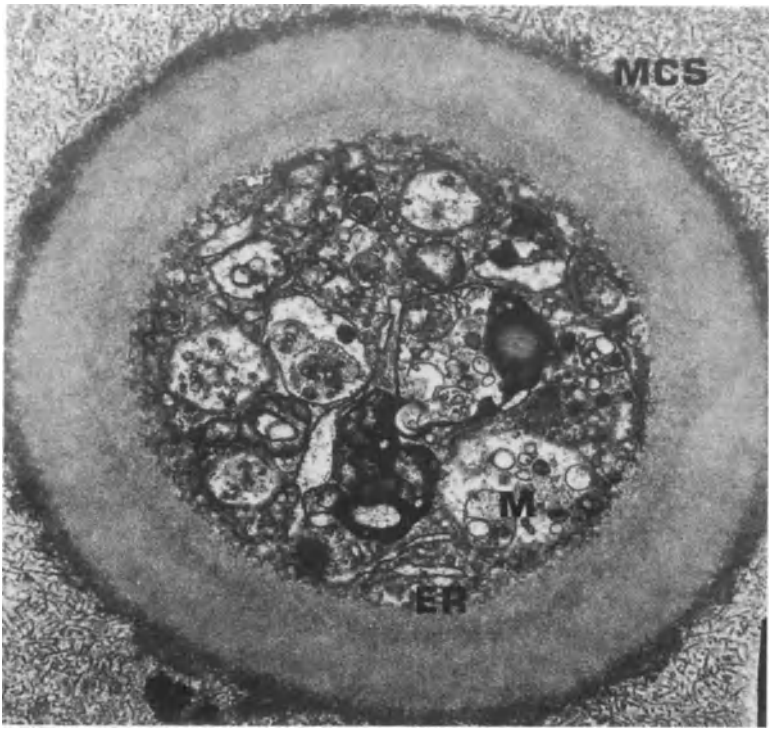

FIG. 4-9. Curvilinear structures (MCS) around the outer wall of a young trophocyte. Mitochondria (M) and endoplasmic reticulum (ER) appear in the cytoplasm. (× 15,400, TEM)

undergoing divisional development without nuclear division (Fig. 4-11). Furthermore, mitochondria with plate-like cristae, endoplasmic reticulum, lysosomes, and lipid bodies are also observed in the cytoplasm of trophocytes. Phospholipids showed a whorl, or onion-like, appearance (Fig. 4-12).

During the sporulation stage the multilamellar bodies (the earliest stage of sporoblasts) commenced their life cycle. The inner concentric layers began to disappear, and dark chromatin granules dispersed throughout the cytoplasm and gradually developed into young sporoblasts with an irregular outline (Fig. 4-13). The developed sporoblasts increased in size and formed a membrane-bound nucleus. In the cytoplasm were mitochondria, endoplasmic reticulum, chromatin granules, vacuoles, lipid droplets, and electron-dense organelles (Fig. 4-14). At this stage the trophocytes formed true cell walls with a bilaminated appearance. As sporoblasts reached maturity, walls with multiple fibrillary layers were formed. Finally, spheroid bodies developed in the sporoblasts (Fig. 4-15). Some trophocytes

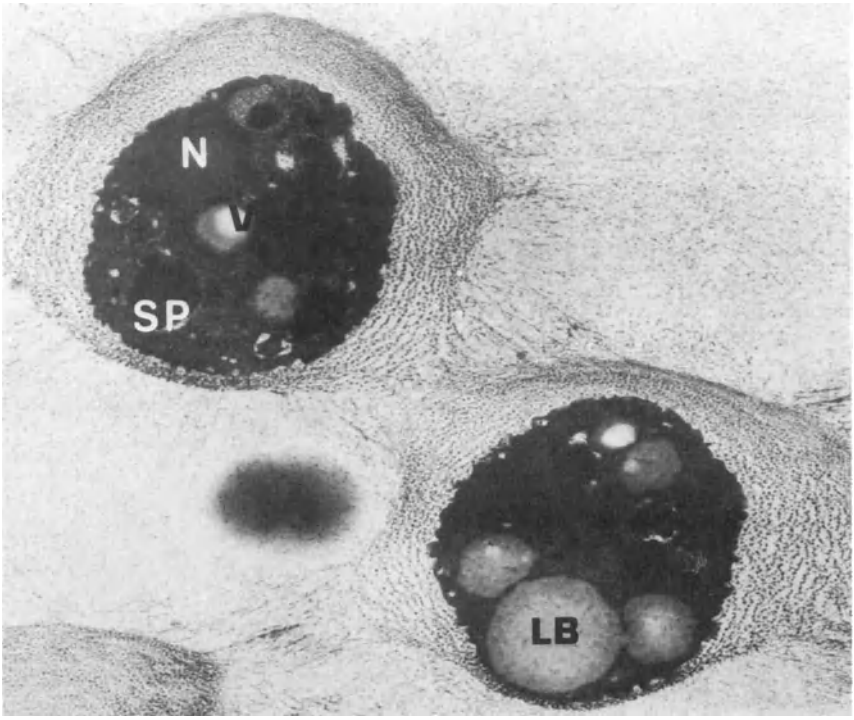

FIG. 4-10. Spores joined by granular intersporal fibrillary substances. They contain a nucleus (N) with a nucleolus, vacuoles (v), lipid bodies (LB), and spheroid bodies (SP). (× 10,560, TEM)

failed to produce living cytoplasmic organelles at the beginning of sporulation, and they increased in size to maturation as abortive forms or trophic cysts but finally collapsed (Fig. 4-8). Protoplasmids and plasmids could not be detected in the cytoplasm of this organism.

Cultivation

We have made attempts to culture biopsy specimens of *R. seeberi* on the following media.

1. Sabouraud glucose agar and broth
2. Czapex Dox's agar and broth
3. Corn meal agar
4. Human blood agar
5. 20% Soil agar and broth
6. Human serum
7. Morgan, Campbell, and Morton liquid media TC 199

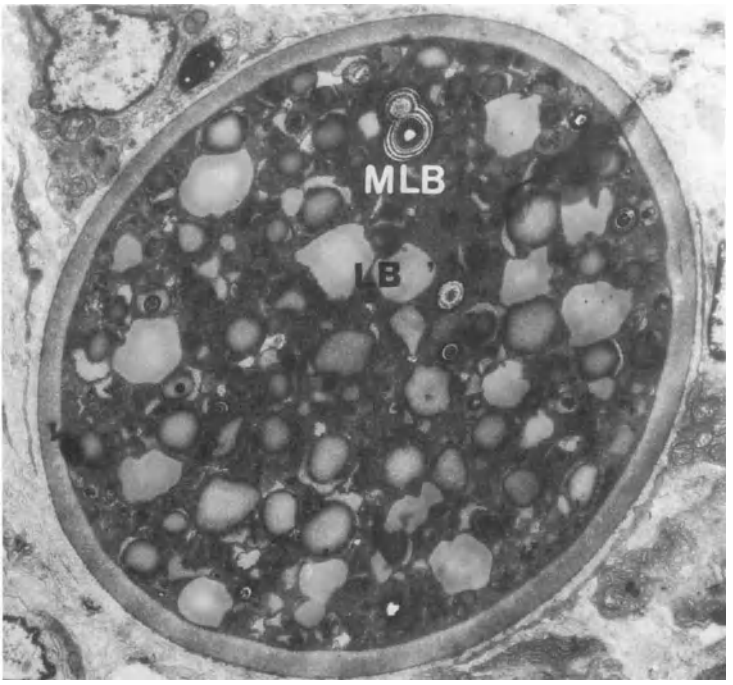

FIG. 4-11. Abundant multilamellar bodies (MLB) with electron-dense concentric rings and lipid bodies (LB) in a young trophocyte. One multilamellar body shows divisional development. (× 4,400, TEM)

Biopsy specimens were washed several times in sterile saline without antibiotics and then were cut into as small pieces as possible with a surgical blade and crushed with a glass crusher. The crushed tissues were then inoculated on the agar media and into the broth. Some were incubated at room temperature (28°C), and some were stored at 2°C in a refrigerator. The inoculated media were examined for growth of *R. seeberi* by light microscopy every day for 3 months.

The organisms inoculated on agar media and in broth (media 1–6, above) did not change morphology during the first 48 hours of incubation at 28°C. After 5 days of incubation, however, they began to degenerate, although in tissue culture medium 199 they maintained their morphology for 1–3 weeks, especially at 2°C. In conclusion, *R. seeberi* failed to grow in all the media tested (media 1–7, above).

Levy et al. (33) and Easly et al (16), on the other hand, have cultivated *R. seeberi* in tissue culture medium containing 20% fetal bovine serum plus 1% sodium bicarbonate (75%) and a confluent monolayer of human rectal tumor cells. This culture was incubated at 34°C under an atmosphere of 5% CO_2 in humidified air. The authors observed that within 3–35 days

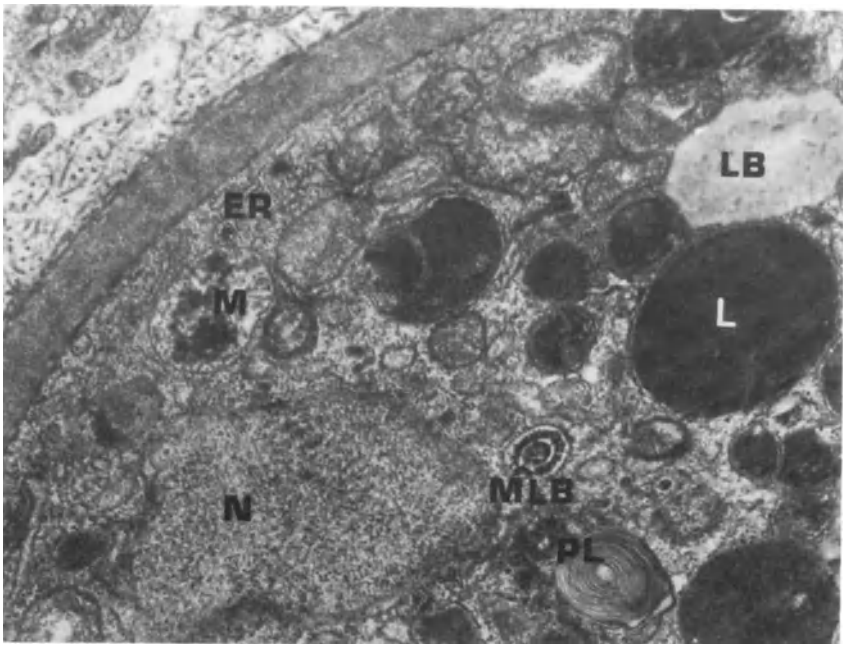

FIG. 4-12. Trophocyte demonstrates a large membrane-bound nucleus (N), multilamellar bodies (MLB), endoplasmic reticulum (ER), mitochondria (M), lysosome (L), lipid bodies (LB), and a whorl-like phospholipid body (PL). (× 10,560, TEM)

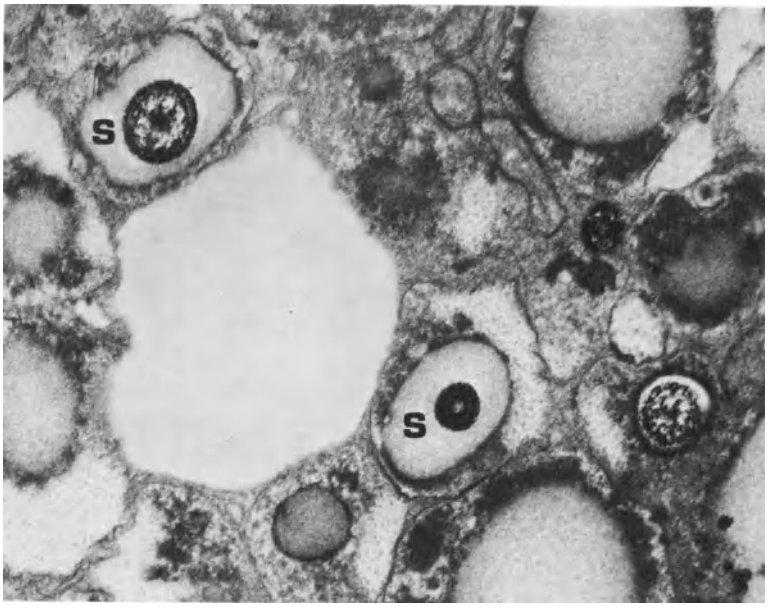

FIG. 4-13. Young sporoblasts (S) originate from multilamellar bodies. (× 22,000, TEM)

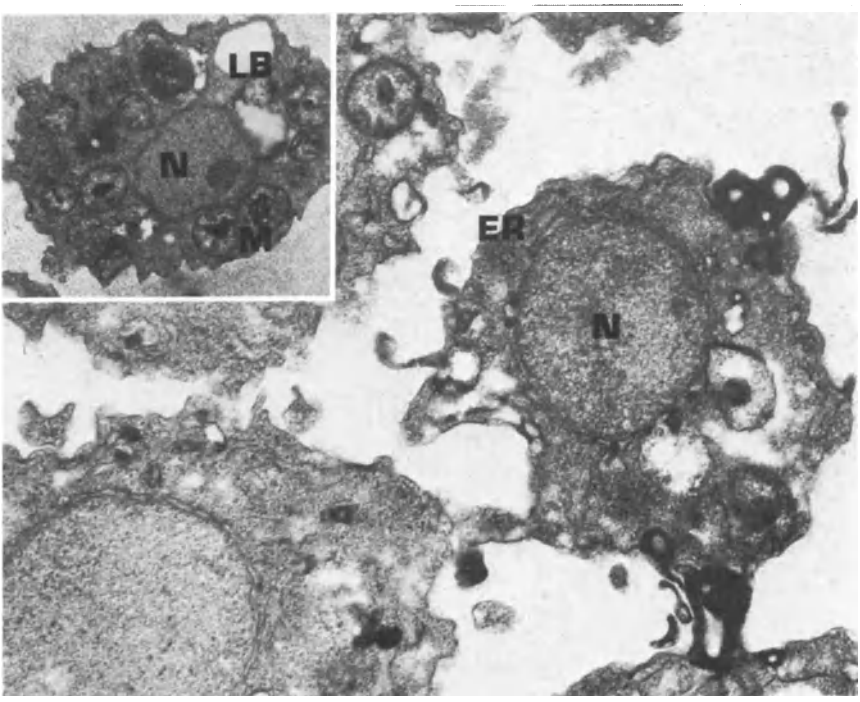

FIG. 4-14. Young sporoblasts contain a nucleus (N) with nucleolus, endoplasmic reticulum (ER), mitochondria (M), lipid bodies (LB), and irregular cytoplasmic membrane. (× 30,800, TEM; inset × 15,400)

growth of the orgainsm was similar to that in host tissue. The human rectal tumor cells responded by showing polyp formation. This experiment appeared to be the first successful cultivation of *R. seebseri* in vitro.

In general, the histopathological findings of rhinosporidiosis, in both the mucosal and the cutaneous forms, show a mixed-cell granulomatous reaction consisting predominantly of neutrophils, lymphocytes, histiocytes, plasma cells, and foreign body giant cells. Eosinophils are not as numerous as in infections caused by members of the Entomophthorales (55,58,63), indicating the presence of a host defense mechanism of cell-mediated immunity. There is also formation of fibrous tissue around *R. seeberi* that limits its spread. The host pushes the organisms away from the mucosa or skin by transepidermal elimination.

According to Ashworth, a sporangium contains numerous spores in which dark round bodies (spheroid bodies) are found (4). We and others have found that *R. seeberi* exhibits distinct morphological and histochemical characteristics (4,5,16,46,56). Even though it reproduces by endosporulation in tissues in a manner similar to that exhibited by *Coccidioides immitis*, it differs from the latter by the presence of laminating walls,

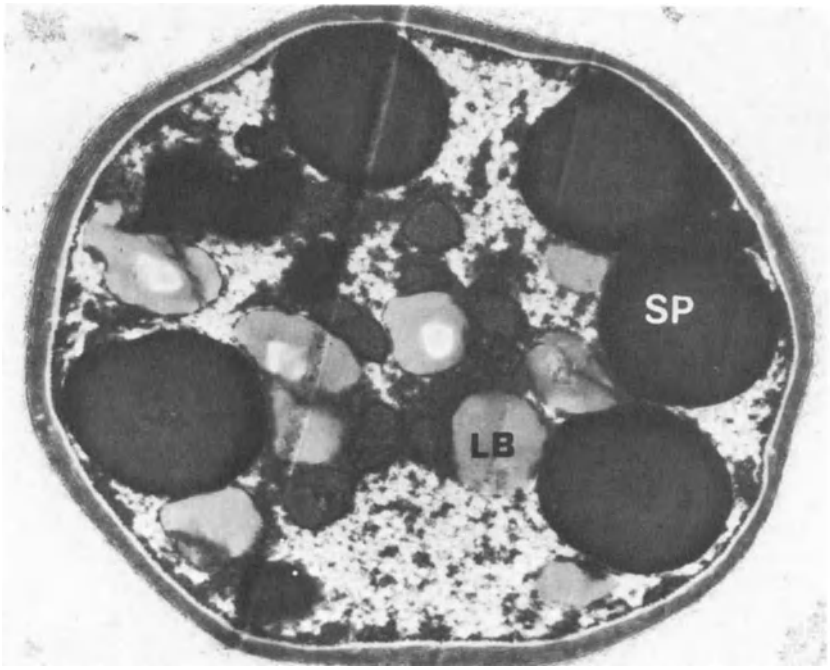

FIG. 4-15. Spheroid bodies (SP), lipid bodies (LB), and cytoplasmic electron-dense granules appear in a mature sporoblast (spore). (× 15,400, TEM)

mucoid materials, and encapsulated fibrillary spores. It is also much larger than *C. immitis*. Lastly, it does not grow in artificial media, whereas *C. immitis* does. *Rhinosporidium seeberi* can be differentiated from acholoric algae such as *Prototheca* spp. by its distinct morphology (57). The mature sporangium of *R. seeberi* is much larger than that of *Prototheca* spp. Endosporulation in *R. seeberi* produces hundreds to thousands of spores at maturity, whereas *C. immitis* and *Prototheca* spp. produce only up to approximately 64 spores. In addition, the spores of *Prototheca* spp. do not have encoated sporangial walls like *R. seeberi* (49).

The walls of sporangia and trophocytes stain well with both PAS and PAS-diastase, and they are birefringent, which suggests that they consist of hemicellulose or cellulose. By contrast, the spore walls are composed of acid mucopolysaccharides (5,46,64). Spheroid bodies contain DNA, as demonstrated by Feulgen's reaction (5,46). They disappear when the spores break from the sporangium and start a new life cycle. Thus the spheroid bodies are not the precursors of sporangia.

From our studies and those of others we can summarize the life cycle of *R. seeberi* as follows. In the early stages of sporulation, multilamellar bodies are precursors of trophocytes, which in turn develop into sporangia

during the late stages of sporulation. The walls of trophocytes are bilaminated with a myriad of curvilinear structures lying at the outer part (52). The central dense chromatin granules, seen at the center of the multilamellar bodies, are the origin of sporulation. At a later stage, these chromatin granules disperse and form young sporoblasts (26–28,52). At the same time, fibrillary walls are formed around these sporoblasts. During late sporulation, young spores develop into mature spores that contain cytoplasmic organelles including the spheroid bodies previously described. At this stage, the mature trophocytes or sporangia with numerous spores have trilaminated walls with the same myriad of curvilinear structures (52). Young, abortive trophocytes develop to mature stages by losing cytoplasmic organelles until they become empty and finally collapse. This summary is consistent with data reported by other investigators (16,26–28,52,56, 60,64).

The complex walls of the sporangium and trophocytes, which are composed of thick cellulose material, cannot be destroyed by the host defense cells. Spores released into infected tissues have thick fibrillary walls with mucoid substances that also resist phagocytosis by host cells. It probably also renders drug penetration improbable (27,64). In our hands, treatment with various systemic antimycotics (e.g., amphotericin B, ketoconazole, 5-fluorocytosine, and potassium iodide) or with the antibacterial clotrimoxazole has not been successful.

The cell walls of most pathogenic fungi contain polysaccharides that are digested by diastase, and they are not bifringent. The walls of *R. seeberi* are PAS-diastase-resistant and bifringent, suggesting the presence of hemicellulose or cellulose (46,60). Most pathogenic fungi, e.g., dermatophytes or Cryptococcaceae, reveal strong oxidative and hydrolytic enzymatic activities (35,54,59). In contrast, *R. seeberi* shows only weak oxidative enzymatic activity and no hydrolytic enzymatic activity.

To date, it has not been possible to cultivate *R. seeberi* on ordinary culture media. Thus knowledge concerning its life cycle is limited largely to that gained from studies in host tissues. This situation has resulted in it being classified as a sporozoan, an alga, or a fungus, according to the interpretation of the author (4,10,23,36,48–50,53,60).

Classification

After Seeber (53) first described this disease, Minchin and Fantham (36) concentrated their attention on the causative organism. They concluded that it was a sporozoan on the grounds that the organism was present in host tissues only and because of the lack of locomotor organs in the mature state. This opinion was accepted for a long time until Asworth suggested that *R. seeberi* was not a sporozoan at all but a fungus that multiplied by

endosporulation (4). This opinion was generally accepted in 1964 (30). Vanbreuseghem (60) has shown that even though *R. seeberi* does not synthesize chloroplasts, it does produce precursors of chlorophyll. Thus it cannot be excluded as a pathogenic alga without chloroplasts. Furthermore, the presence of hemicellulose of chitin in the sporangial wall of *R. seeberi* is an indication that it could very well be an alga. It is true that when we try to classify the organism as a fungus it is difficult to classify it in any of the currently recognized fungal groups.

Yet, studies on the ultrastructure of *R. seeberi* have not been able to shown the presence of protoplastids or plastids in the cytoplasm of the organism, although these structures have been found to be important components of plant or algal cells (7,8). Thus *R. seeberi* should be classified as a fungus after all (7,17,26–28,30,47,52).

Ashworth (4) placed *R. seeberi* in the old Class Phycomycetes, Order Chytridiales because of its endosporulation. Later, Dodge (49) and Rieth (48) placed *R. seeberi* in the Class Ascomycetes, Order Endomycetales because the spherules bodies in the spores resembled ascospores in an ascus.

We believe that there are many obvious reasons not to classify *R. seeberi* as an oomycete; indeed, aquatic oomycetes possess zoospores and oospores, and *Rhinosporidium* possesses neither. If it were in the Order Mucorales of the zygomycetes, there should be hyphae present in the host. It cannot be classified in the Family Endomycetales either on the basis that spheroid bodies and spores resemble ascospores and asci, as it has been proved by histochemical methods that the spheroid bodies are not ascospores but merely DNA in a *Morula*-like structure (5). In conclusion, we believe that *R. seeberi* should still be considered a fungus that as yet cannot be definitely placed in any existing taxonomic group (10,23,50,56,60).

Summary

Rhinosporidium seeberi, the causative organism of rhinosporidiosis of the nasal mucosa and skin was reviewed with regard to its pathogenesis and histopathology, histochemistry, ultrastructure, life cycle, and cultivation. The pathological findings from infected tissues reveal a granulomatous reaction comprising mixed cell granuloma, pseudocystic abscesses, fibrosis around the causative organism (*R. seeberi*), and transepidermal elimination.

The cell walls of trophocytes and sporangia exhibit the presence of cellulose. The spore wall is encapsulated with granular fibrillary substances consisting of acid mucopolysaccharides. Spheroid bodies have proved to be DNA surrounded by a thin membrane-bound layer. In the cytoplasm of the organism, various substances can be detected by histochemical

methods (e.g., glycogen, glycoprotein, acid mucopolysaccharides, neutral lipids, and phospholipids). The walls of the sporangia are found to be trilaminated, whereas those of trophocytes are bilaminated. There is a myriad of curvilinear structures around the outer wall of both forms. The ultrastructure of a trophocyte shows it to be comprised of sporoblasts containing oval or round membrane-bound nuclei with nucleoli, mitochondria, endoplasmic reticulum, chromatin granules, vacuoles, lipid bodies, and spherules. We suggest that the multilamellar bodies are precursors of trophocytes and sporangia. Abortive trophocytes without cytoplasmic organelles are seen, and they collapse at the end of the maturation process. *Rhinosporidium seeberi* fails to grow in any of the artificial media used but can be maintained through its life cycle in tissue cultures.

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5—Pathogenesis of *Candida* Vulvovaginitis

JACK D. SOBEL

There are unfortunately no reliable figures defining the incidence of *Candida* vulvovaginitis (CVV) in the United States mainly because this common clinical entity is not reportable and most public health authorities do not monitor the prevalence of CVV. Statistical data in Great Britain derived from patients diagnosed at genitourinary medicine centers reveal a sharp increase in the incidence in CVV during the period 1975–1984 (2). Incidence increased from 118 per 110,000 to 200 per 100,000 women over this 10-year period. CVV is found worldwide and is the commonest cause of acute vaginitis in tropical climates. In the United States, *Candida* spp. are now the second commonest cause of vaginal infection, with bacterial vaginosis (the most common diagnostic entity) occurring almost twice as often as CVV (11).

Hurley and De Louvois estimated that 75% of women experience at least one episode of CVV during their child-bearing period (36,37), and approximately 40–50% of these women experience a second attack (35). A small subpopulation of women of undetermined magnitude, probably less than 5% of the adult female population, suffers from repeated, recurrent, often intractable episodes of *Candida* vaginitis.

Microbiology

Between 85 and 90% of yeasts isolated from the vagina are isolates of *Candida albicans* (28,36,69,62). The remainder are due to *Candida* species other than *C. albicans*, the commonest of which is *Torulopsis glabrata* (67). Other species, including *C. tropicalis* (34), *C. guilliermondii*, *C. parapsilosis*, *C. krusei*, and *C. kefyr* (syn. *C. pseudotropicalis*), appear capable of inducing clinical symptomatic vaginitis including rare cases caused by *Saccharomyces cerevisiae*. Studies attempting to identify the existence of "vaginotropic" or "vaginopathic" strains of *Candida* were in the past hand-

icapped by the lack of a reliable typing system. The method of *C. albicans* strain identification or biotyping established by Warnock et al. (97) and Odds and Abbott (64) has provided a useful method of strain typing. Although more than 200 strains of *C. albicans* have been identified by this method, there is no evidence of strain tropism selecting for strains with a predilection to colonize the vagina or to cause vaginitis (63). Thus all isolates studied to date appear to be equally capable of colonization and causing vaginitis, and uniquely virulent strains have not been identified (65,66). It is likely that the biotyping method described above will be replaced by the more reliable molecular DNA hybridization techniques. The most important application of a reliable typing system is (a) to study the epidemiology of CVV, in particular for differentiating of relapse from reinfection in women with recurrent infection (61), and (b) to evaluate virulence factors of strains of organisms identified in symptomatic vaginitis in contrast to asymptomatic colonization of the vagina. As dimorphic organisms vivo, *Candida* may be found in different phenotypic phases in the vagina. As a generalization, blastoconidia represent the phenotypic form responsible for transmission or spread, including the bloodstream phase, as well as the form associated with asymptomatic colonization of the vagina. In contrast, germinated yeast cells with the subsequent production of mycelium most commonly constitute the tissue-invasive form and are usually identified in the presence of symptomatic disease.

Candida Virulence Factors

Candida albicans is a polymorphic fungus. Depending on the environmental and nutritional conditions, it may grow in a budding, yeast-like form, as filaments consisting of elongated attached blastoconidaia (pseudohyphae) or as true mycelium consisting of apical-growing filaments (62).

Adherence

In order for *Candida* spp. cells to colonize the vaginal mucosa they must first adhere to the vaginal epithelial cells; and for the yeast to survive on the mucosa of the vagina, a specific means of adhesion and anchorage is required to prevent its dislodgement and eradication by the normal fluid and secretion kinetic flow. *Candida albicans* adheres in significantly higher numbers to vaginal epithelial cells than do *C. tropicalis*, *C. krusei*, or *C. kefyr* (41), which may explain the relative infrequency of the latter strains in vaginitis. All clinical *C. albicans* strains appear to adhere equally well to both exfoliated vaginal and buccal epithelial cells. In contrast, there is considerable person-to-person variation in terms of vaginal cell receptivity to *Candida* in adherence assays (88). Nevertheless, vaginal cells from women with idiopathic recurrent VVC were not shown to have increased cell avid-

TABLE 5-1. *Candida* virulence factors in the pathogenesis of *Candida* vaginitis.

Adherence
Germination
Proteinases
Mycotoxins
"Switching" colonies

ity or affinity kinetics for *Candida* (94). Segal et al., however, described enhanced in vitro attachment of *Candida* to vaginal epithelial cells of diabetic women (82). Although adherence as a virulence characteristic (Table 5-1) may explain the paucity of non-*C. albicans* strains found, all *C. albicans* strains appear capable of colonizing mucosal surfaces. The significance of adherence in the pathogenesis of vaginitis is suggested by the failure of a cerulenin-resistant mutant of *C. albicans*, which adhered poorly, to induce vaginitis in the experimental murine model of candidiasis (47). Currently, no epithelial cell receptor for *Candida* ligands has been identified, and the yeast adhesin function appears to reside with the yeast surface mannoprotein (46,78,79,88). The role of mannan-containing moieties in adherence was first suggested by studies utilizing the lectin concanavalin A, which not only induced yeast agglutination but significantly reduced adherence of *C. albicans* to mucosal cells (79). This conclusion was supported by additional studies using yeast cells treated by mild alkali and acid extraction of mannan present in the cell wall (49, 78). In a study primarily devoted to the phospholipases of *C. albicans*, virulence of isolates correlated well with enhanced adherence as well as with the capacity of the organisms to produce more phospholipases (6).

Germ Tube and Mycelium Formation

A germ tube constitutes the intermediate stage between the blastoconidium and the filamentous (hyphal, mycelial) element. In vitro studies have shown that germ tube formation enhances the capacity of *Candida* to adhere to buccal and vaginal exfoliated epithelial cells (40,88). This fact suggests that, after introduction of *Candida* into the vaginal lumen, germ tube formation may facilitate the initial establishment of the organism and enhance colonization. Moreover, the experimental animal model of *Candida* vaginitis has been used to confirm that germ tube and mycelium formation facilitates vaginal mucosal tissue invasion (85). Electron and scanning microscopy have demonstrated the invasive capacity of both the yeast and mycelial phases of *Candida*. Using a mutant strain of *C. albicans* that failed to germinate at 37°C but retained this capacity at 28°C, Sobel et al. demonstrated in vivo in estrogenized rats that the nongerminating mutant was incapable of inducing experimental vaginitis (86). Similarly, pretreatment

of susceptible rats with subinhibitory concentrations of ketoconazole, which did, however, prevent germ tube formation, protected the rats from acquiring experimentally induced *Candida* vaginitis (85).

The critical role of germination in enhancing the capacity of commensal organisms to cause symptomatic vaginitis is the foundation of many studies investigating the pathogenesis of acute and especially recurrent *Candida* vaginitis. Factors, both exogenous and endogenous, that enhance or facilitate germination tend to precipitate symptomatic vaginitis, whereas measures that inhibit germination may prevent acute vaginitis in women who are asymptomatic carriers of yeast.

Proteinase Secretion

Several pH-dependent proteolytic enzymes have been isolated from *Candida* species. It is noteworthy that several acid proteinases have been identified that are optimally active at normal vaginal pH 4.0–4.5. Studies with mutant strains of *Candida* deficient in acid protease demonstrated reduced capacity to adhere. The role of these hydrolytic enzymes in vivo in the pathogenesis of vaginitis is obscure, but they may facilitate germ tube penetration of intact mucosal epithelial cells. Of interest was the report by Cassone et al. that fresh isolates of *C. albicans* obtained from patients with acute symptomatic vaginitis were more proteolytic in vitro than those from asymtomatic carriers (9). Whether increased proteinase secretion is a universal consequence of the transformation from the blastoconidium-colonization phase to the germinated invasive vaginitis stage (26) or is an independent virulence factor that determines which asymptomatic carriers go on to synptomatic vaginitis is unknown.

With regard to toxin production, Stanley and Hurley (91) noted no direct or indirect toxic effects of *C. albicans* on tissue culture. Cutler et al. (13), however, found that only the cell wall glycoproteins of *Candida* had toxic properties; and although Iwata (38) described a canditoxin, it appears to be confined to occasional strains only.

Switching Colonies

Since the original description of switching colonies by Soll et al. (83,90), these investigators have observed additional systems of in vitro switching of colonies. The original report described several morphological variations in the colony type of a single strain of *C. albicans* due to high-frequency heritable phenotypic changes that occurred when *Candida* was grown on amino-acid-rich agar in vitro at 24°C. Subsequently, the white/opaque switch system appears to occur at the highest frequency (10^{-3} to 10^{-2}). Once the white colony switches to an opaque colony, it contains individual cells that vary from the original white-colony-containing cells in terms of

possessing or acquiring new virulence characteristics. They include the capacity to adhere as well as the increased ability to germinate, elaborate proteases, and form mycelium (90). The opaque cell appears to represent a transient phenotype that has evolved specifically for tissue penetration. Soll et al. reported the presence of switching colonies in vitro from fresh isolates obtained from patients with candidal vaginitis (90). Most vaginitis isolates were found to be in a high frequency mode of switching.

Whether genetic switching to more virulent cells occurs in vivo at 37°C remains to be clarified; however, the fundamental concept that has emerged is that yeasts colonizing asymptomatic carriers represent but one phase of an organism which, given changes in the environment, transforms to a more virulent phase capable of inducing disease. The recognition by Soll et al. of hereditable spontaneous switching raises the possibility that this transformation may also occur spontaneously in vivo.

Asymptomatic Vaginal Colonization

Point prevalence studies of healthy asymptomatic women reveal a wide range of colonization rates, from 10 to 55% (22). Most investigators agree that the usual figure is between 15 and 25% of young nongpregnant premenopausal women. The natural history of asumptomatic colonization is unknown, as no longitudinal studies have been done and the data reported have included typing studies to differentiate chronic carriage of multiple consecutively acquired strains of *Candida*. Anecdotally, clinicians have observed women with asymptomatic chronic colonization with repeated positive cultures obtained consistently month after month.

Candida spp. gains access to the vaginal lumen and secretions predominantly from the adjacent perianal area. This entry has been borne out by several epidemiological (22,64) and typing (57,61,97) studies. Animal studies reveal that only a small inoculum, of 10^2 cells, is necessary to establish colonization (87). The prepubertal and postmenopausal woman appears more resistant to colonization given the marked differences in the vaginal environment, which provides the first hint of the hormonal dependence of this infection. Other mechanisms whereby *Candida* may reach the vaginal lumen are poorly understood and may include the fingers, male genitalia, and inert objects such as a contraceptive diaphragm.

After colonization has occurred, the organisms appear to remain in low numbers and predominantly in the yeast phase. Differences between *Candida* isolated from asymptomatic carriers and from women with overt vaginitis are summarized in Table 5-2. This summary represents an oversimplification, as one occasionally identifies asymptomatic women with high concentrations of yeasts that are predominantly in the hyphal phase as well as women with severe symptoms with few organisms on quantitative culture (56). The latter observation suggests that more than one pathogenic

TABLE 5-2. Comparison of asymptomatic vaginal colonization and symptomatic vaginitis.

Parameter	Asymptomatic colonization	Symptomatic vaginitis
Candida strain type	Identical	Identical
Predominant phenotype	Blastoconidia and budding	Germ tube and hyphae
Titer	$\leq 10^3$/ml	$\geq 10^4$/ml
Proteolytic activity	+ to ++	+++ to ++++
White/opaque colonies	Fewer opaque	More opaque

mechanism may be responsible for the development of inflammation in women with CVV.

The question of yeast numbers in the presence or absence of symptomatic infection remains controversial. Several investigators (67) found no association between numbers and symptoms of vaginitis, the latter presenting at either very low or high counts. In contrast, Odds and co-workers found a clear-cut correlation between high titers (usually exceeding 10^3/ml of secretion) and symptoms, in contrast to significantly lower titers in asymptomatic women (66). Part of the discrepancy among investigators relates to the fact that one occasionally sees asymptomatic women apparently tolerating large numbers of organisms often in the filamentous form (personal observation). More important, however, is the realization that candidal vaginitis represents a spectrum of disease. At one end of the spectrum is typical vulvovaginal thrush, which is almost invariably associated with high numbers of germinated yeasts (66). At the other end of the spectrum the women with severe vulvar symptoms, including prominent pruritus and vulvitis but no clinical evidence of typical thrush, and yeasts are found in low numbers (56).

Factors that facilitate vaginal colonization with *Candida* are outlined in Table 5-3. The listed factors are the same as those associated with the

TABLE 5-3. Factors associated with increased *Candida* asymptomatic vaginal colonization and *Candida* vaginitis.

Pregnancy
Uncontrolled diabetes mellitus
High-estrogen-containing oral contraceptives
Corticosteroid therapy
Tight-fitting synthetic underclothing
Antimicrobial therapy
    Oral/systemic
    Topical/local
Increase frequency of coitus (vaginitis only)
"Candy binge" (vaginitis only)
Idiopathic

occurrence or the precipitation of symptomatic vaginitis. The various factors act to facilitate both colonization and transformation to vaginitis by altering the vaginal microenvironment to facilitate yeast multiplication, germination, and the expression of virulence factors. It is achieved by (a) increasing the available nutrient substrates; (b) altering pH, trace metals (zinc), CO_2, and oxygen availability; or (c) removing natural restraining mechanisms, i.e., biological brakes provided by the resident bacterial flora and mucosal immune system. Although *Candida* species are considered to grow poorly in vitro under anaerobic conditions, the anaerobic acidic environment of the vagina indicates that *Candida* is more than capable of thriving under anaerobic conditions. This fact was confirmed by the in vitro studies of Webster and Odds, which showed growth of all species of *Candida* under elevated concentrations of CO_2 and in anaerobic gas jars (98). Little correlation has been found between pH conditions in the vagina and the presence or absence of *C. albicans*, which is able to grow over a wide pH range. Drake et al. found a mean vaginal pH of 4.3–4.6 in a group of 141 women irrespective of whether *Candida* was present (21). There was no change in vaginal pH when the *C. albicans* infections were eliminated by miconazole or nystatin therapy. The normal pH observed with *Candida* vaginitis is a useful diagnostic marker, in contrast to bacterial vaginosis and trichomoniasis, where pH is elevated.

Strain typing of organisms isolated from women with asymptomatic carriage has not shown any difference in distribution frequency when compared with strains obtained from symptomatic patients (63,65). Thus there is little evidence to incriminate "vaginopathic" strains that are more virulent and more likely to induce disease in women when all other factors are equal. In the animal model all strains of *C. albicans* tested, irrespective of their anatomical site, appeared equally capable of causing experimental vaginitis (87). The model, however, requires that large inocula of *Candida* be used, and hence isolate differences may not be recognized.

From a historical perspective, in contrast to the thesis that *C. albicans* and other *Candida* spp. may be a normal if not transient component of the vaginal flora, Hurley and De Louvois (35–37) fostered the view that *Candida* was never a commensal in the vagina and always a pathogen. Given more recent epidemiological studies as well as the observation that *Candida* is frequently isolated from totally asymptomatic women in whom physical examination is completely normal, Hurley's views are no longer acceptable, particularly when low numbers of organisms are found.

Transformation to Symptomatic Vaginitis

Although a high prevalence of CVV has been observed among women of low socio-economic status, CVV is widely represented in all socio-economic groups throughout the world. *Candida*-associated vaginitis is

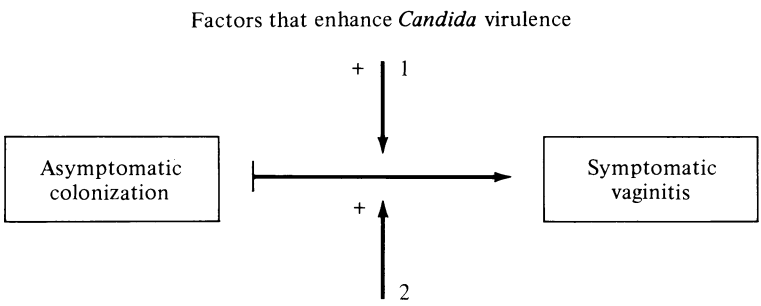

FIG. 5-1. Pathogenesis of acute vaginitis.

TABLE 5-4. Pathogenesis of acute vaginitis.

Factors that enhance *Candida* virulence	Factors associated with loss of local defense mechanisms
Pregnancy	Local/systemic antimicrobial agents
Uncontrolled diabetes	AIDS
Exgenous hormones (estrogens, corticosteroids)	Sexual intercourse (?)
Use of tight-fitting noncotton underclothing	Role of hypersensitivity reactions (?)
"Candy binge"	
Switching colonies	

seen predominantly in women of childbearing age; it rarely occurs in prepubertal girls and is less commonly encountered in postmenopausal women, emphasizing the hormonal dependence of *Candida* infection.

The issue critical to understanding the pathogenesis of *Candida* vaginitis is the mechanism whereby asymptomatic colonization of the vagina transforms to symptomatic vaginitis. During the phase of asymptomatic carriage, *Candida* exists predominantly in the nonfilamentous form and in relatively low numbers. Under these circumstances there exists a delicate equilibrium between *Candida* and the resident protective bacterial flora and other local restraining mechanisms that represent local vaginal defense mechanisms (Fig. 5-1). Symptomatic vaginitis develops in the presence of factors that enhance *Candida* virulence factors (Table 5-4) or as a result of loss of local defense mechanisms.

The exact mechanism whereby *Candida* induces inflammation is unclear. It is also conceivable that, based on the varying numbers of organisms seen and the variable clinical picture, more than one mechanism exists. *Candida albicans* may cause cell damage and resulting inflammation by direct

FIG. 5-2. Scanning electron micrographs of vaginal mucosa obtained by biopsy showing acute *Candida* vaginitis. The appearance is that of extensive hyphal formation and disruption of superficial vaginal squamous cells. INSET: *Candida* hyphae invading into deeper layers of mucosa.

hyphal invasion of epithelial tissue (Fig. 5-2). This fact has been documented in vivo (87) and in in vitro tissue culture systems (27). Light and electron microscopy of exfoliated vaginal cells and cells in tissue culture have shown the hyphae passing through cell membranes (30). Scanning electron micrographs reveal holes created by invading hyphal elements. It is possible that proteases and other hydrolytic enzymes facilitate cell penetration. Cell penetration and mucosal invasion are not confined to hyphae, however, and blastoconidia are clearly capable of achieving tissue invasion.

Tissue invasion with induction of mucosal inflammation results in mucosal swelling, erythema, and desquamation or exfoliation of vaginal epithelial cells. The nonhomogeneous vaginal discharge consists of a conglomerate of hyphal elements and exfoliated nonviable epithelial cells. Few polymorphonuclear leukocytes are visualized by Gram stain, saline preparations, or scanning electron microscopy (SEM) photomicrographs, although mucosal biopsy reveals the presence of leukocytes in the lamina propria (87).

Other than direct tissue invasion and resulting exudative inflammation, *Candida* species may induce symptoms by possible hypersensitivity or allergic reaction, particularly in women with idiopathic recurrent vulvovaginal candidiasis.

In most women with occasional, infrequent episodes of CVV, clinicians can usually identify a precipitating event. Not infrequently, however, no cause is found.

Host Predisposing Factors

Pregnancy

The vagina is more susceptible to infection during pregnancy, resulting in a higher incidence of colonization as well as symptomatic vaginitis (7). Vaginal colonization rates of *Candida* species during pregnancy is approximately 30%, almost twice the incidence in nonpregnant women (55). In particular, the clinical attack rate is maximally increased during the third trimester, and symptomatic recurrence is also more common during pregnancy (7,34). It is generally thought that the high levels of reproductive hormones, by providing a higher glycogen content in the vaginal environment, provide an excellent carbon source for *Candida* growth, germination, and adherence (7,53). A more complex mechanism is likely, however, in that estrogens enhance vaginal epithelial cell avidity for *Candida* adherence; and a cytosol receptor or binding system for female reproductive hormones has now been documented (71). These investigators and others demonstrated in vitro binding to *Candida* spp. of these female hormones as well as the capacity of certain hormones to enhance yeast myce-

lium formation and hence virulence (42,71). Accordingly, it is postulated that the high levels of reproductive hormones encountered during pregnancy directly enhance yeast virulence. Not surprisingly, therefore, clinical cure rates are significantly lower during pregnancy (62).

Oral Contraceptives

Several studies have shown increased vaginal colonization rates with *Candida* following high-estrogen-content oral contraceptive use (28,62). Almost certainly, the same mechanism operative during pregnancy applies to these subjects. Studies utilizing low-estrogen-content oral contraceptives have not found a increase in *Candida* vaginitis (3,18).

Diabetes Mellitus

Vaginal colonization with *Candida* is more frequent in diabetic women. Although uncontrolled diabetes predisposes to symptomatic vaginitis, most diabetics are not afflicted by repeated infections (29,62). It has become traditional to perform a glucose tolerance test in all women with recurrent CVV. The yield of these expensive studies is low, however, and testing therefore is not justified in premenopausal women. Occasionally women with recurrent CVV describe an association between "candy binges" and exacerbation of symptomatic vaginitis. For the most part, however, dietary restrictions have no place in the routine management of yeast vaginitis.

Antimicrobics

The onset of symptomatic CVV is frequently observed during or following a course of oral antimicrobics. Although no antimicrobial agent is immune from this complication, the broad-spectrum antimicrobics (e.g., tetracyclines, ampicillin, oral cephalosporins) are mainly responsible for exacerbation of symptoms. Not only is symptomatic vaginitis frequently precipitated, vaginal colonization rates increase from approximatelu 10% to 30% (8,68). Antimicrobics, both systemic and topical agents, are thought to act by eliminating the normal protective vaginal bacterial flora (48). Thus the natural flora are thought to provide a colonization-resistance mechanism, as well as to prevent *Candida* germination and hence superficial mucosal invasion. In particular, aerobic and anaerobic resident *Lactobacillus* spp. have been singled out as providing this protective function. It is of interest therefore that Auger and Joly found reduced titers of *Lactobacillus* species in vaginal cultures obtained from women with symptomatic vaginitis (5). Current concepts of the *Lactobacillus–Candida* interaction include competition for nutrients, as well as stearic interference by lactoba-

cilli of *Candida* adherence of vaginal epithelial cells (88). Other mechanisms include the elaboration of bacteriocins by lactobacilli, which inhibit yeast proliferation and germination as well as a direct antimicrobic-induced stimulatory effect on *Candida* growth kinetics (60).

Miscellaneous Factors

Among the factors that have contributed to the increased incidence of *Candida* vaginitis in western societies has been the use of tight, restricting, poorly ventilated clothing and nylon underclothing with increased local, perineal moisture and temperature (25,28,37). The use of well ventilated clothing and cotton underwear may be of value for preventing reinfection (35). There is no evidence confirming that iron deficiency predisposes to infection (16). There is anecdotal evidence, however, suggesting that the use of commercial douches, perfumed toilet paper, feminine hygiene sprays, and swimming in chlorinated pools contribute to symptomatic vaginitis. Chemical contact, local allergy, or hypersensitivity reactions may alter the vaginal milieu and permit the transformation from asymptomatic colonization to symptomatic vaginitis. The role of a contaminated cervical cap or diaphragm in recolonization of the vagina with *Candida* remains speculative and undocumented; however, one clinical study demonstrated that women using the contraceptive sponge have an increased likelihood of a vaginal infection with *Candida* (75).

Vaginal Defense Mechanisms

Humoral System

Agglutinating antibodies to *C. albicans* can be found in the serum of 20–64% of healthy adults (99). Patients with profound immunoglobulin deficiencies are not susceptible to vaginal yeast infections. Following acute vaginal candidiasis, a systemic and local [immunoglobulins M and G (IgM, IgG)] serological response is elicited, the latter consisting predominantly of IgA, particularly in cervical secretions (52,96). The protective role of local antibodies is unknown, as patients with recurrent infection do not lack antibodies (31). Somewhat lower local antibody titers have been described during active vaginal infections, but they may reflect an adsorption effect.

Phagocytic System

Although polymorphonuclear leukocytes and monocytes play an important role in limiting systemic *Candida* infection and deep tissue invasion (20), these phagocytic cells are characteristically absent from vaginal secretions

during *Candida* vaginitis. Accordingly, these cells are not thought to influence *Candida* mucosal colonization or even prevent superficial invasion of the vaginal mucosa. In the rat model of experimental vaginal candidiasis, as in humans, histology of the vagina fails to demonstrate leukocytes in the vaginal secretions or stratified squamous epithelium, which remains intact (87). Polymorphonuclear cells can be seen concentrating within the underlying lamina propria but appear not to be presented with a chemotactic signal that induces migration into more superficial layers and hence vaginal secretions.

Cell-Mediated Immunity

Oral thrush correlates well with depressed cell-mediated immunity (CMI) in debilitated or immunosuppressed patients (1). It is particularly evident in patients with chronic mucocutaneous candidiasis. In this context, *Candida* is typically an opportunistic pathogen. Accordingly, one might anticipate that lymphocytes similarly contribute to normal vaginal defense mechanisms preventing mucosal invasion by *Candida* species. In support of this concept is the increased frequency of *Candida* vaginitis in women receiving corticosteroids and those with acquired immunodeficiency syndrome (AIDS) (72).

How mononuclear cells and lymphocytes act to maintain normal mucosal immunity against *Candida* is unknown. However, Sobel and Opitz presented evidence of T-cell-associated in vitro inhibition of *Candida* proliferation and germ tube formation (89).

Vaginal Flora

Probably the most important defense against both *Candida* colonization and symptomatic inflammation is the normal natural bacterial flora. Newly arrived *Candida* cells, in order to survive and persist, must initially adhere to epithelial cells and then grow, proliferate, and germinate to successfully colonize the vaginal mucosa. Although microbial competition for nutrients has long been considered the most important source of competition, animal studies suggest that lactobacilli and *Candida* frequently survive side by side (80). The role of bacteriocins, possibly bacterial polyamines, in inhibiting yeast growth and germination requires further investigation (48,60). Lactobacilli may also prevent extensive colonization of the vagina mucosa by interfering with *Candida* adherence to epithelial cells (88).

Miscellaneous Factors

Although not studied in the vagina, various natural secretions have been shown to possess considerable antifungal activity. Pollack et al. (70) re-

ported fungistatic and fungicidal activity against *C. albicans* by human parotid salivary histidine-rich polypeptides.

Pathogenesis of Recurrent and Chronic *Candida* Vaginitis

Careful evaluation of women with recurrent vaginitis usually fails to reveal any precipitating or causal mechanism (84). These desperate women avoid antimicrobics, oral contraceptives, tight-fitting clothing, and hormone therapy, and they have normal glucose tolerance tests. In the past, clinicians attributed the frequent episodes to repeated fungal reinoculation of the vagina from a persistent intestinal source (58) or to sexual transmission (93).

Intestinal Reservoir

The intestinal reservoir theory is based on the report of recovery of *Candida* on rectal culture in almost 100% of women with acute *Candida* vaginitis (19,58). In support of this concept is the fact that biotyping of simultaneously obtained vaginal and rectal cultures almost invariably reveals identical strains (57,61,63). Nevertheless, this theory has been criticized. Several authors have much lower concordance between rectal and vaginal cultures in patients with recurrent *Candida* vaginitis (32,61). In particular, in a longitudinal study of women with recurrent vaginitis receiving long-term ketoconazole, recurrence of symptomatic and mycologically proved *Candida* vaginitis frequently occurred in the presence of negative rectal cultures for *Candida* (61,84). Two controlled studies using oral nystatin treatment, which reduces intestinal yeast carriage, failed to prevent symptomatic recurrence of vaginal candidiasis (17,95). Furthermore, some women had persistent intestinal yeast carriage and failed to develop vaginal colonization (17,61).

Sexual Transmission

Penile colonization with *Candida* is present in approximately 20% of male partners of women with recurrent vaginal candidiasis (15,61,73). Usually asymptomatic, the microorganisms are most commonly found in uncircumcised men in the vicinity of the coronal sulcus. Asymptomatic male genital colonization is four times more common in male sexual partners of infected women (94). Strain typing techniques indicate that infected partners usually carry identical strains (61).

Despite the aforementioned circumstantial evidence, confirmation that sexual transmission occurs is still lacking, and the contribution of sexual transmission to the pathogenesis of infection remains unknown. Based on the prevalence of positive penile cultures, the role of sexual spread appears

limited, and hence routine therapy of male partners, even those of women with recurrent candidal vaginitis, is unlikely to substantially reduce recurrence rates. Not one controlled study has shown that treatment of men prevents recurrence in women. Anecdotal evidence is available that anogenital and particularly orogenital contact may transmit infection, but adequate documentation is lacking.

Vaginal Relapse

Vaginal relapse infers that incomplete eradication or clearance of *Candida* from the vagina occurs following topical antimycotic therapy that may be sufficient to drastically reduce the numbers of *Candida* in the lumen and alleviate signs and symptoms of inflammation. Accordingly, organisms would persist in small numbers in the vagina and result in continued carriage of the organism. When host environmental conditions permitted, the colonizing organisms would increase in number and undergo mycelial transformation, and a new clinical episode would result (30).

In favor of this concept is the observation by Odds that women with acute vaginal candidiasis, after receiving conventional antifungal therapy, usually become culture-negative (>90%); moreover, within 4–6 weeks of completion of therapy 20–25% of the asymptomatic women become culture-positive, with typing revealing an identical strain (62). This rapid rate of reacquiring positive cultures appears inconsistent with vaginal reinfection from a gastrointestinal tract source. In a longitudinal study of women receiving maintenance ketoconazole, women were found to reacquire positive vaginal cultures without simultaneously positive rectal cultures (61).

Whatever the source of vaginal reinfection or relapse, it is apparent that women with recurrent candidal vaginitis differ from women with infrequent episodes by virtue of their inability to tolerate small numbers of *Candida* reintroduced or persisting in the vagina. On the basis of serotyping of organisms, women with recurrent, infrequent infection share the same distribution frequency of organisms as do women without symptoms (63,65,66). Likewise, *Candida* strains isolated from women with intractable disease express identical virulence factors and antifungal susceptibility results. The switching colony phenomenon, which may occur in vivo and may constitute an explanation for more virulent strains developing spontaneously, has yet to be addressed in women with unexplained recurrent CVV (83,90).

Host factors responsible for the frequent episodes are not clearly delineated, and more than one mechanism may be operative. There is no evidence of complement, phagocytic cells, or immunoglobulin deficiency in these patients. Recurrent CVV is not due to polyene or azole drug resistance; in fact, only three cases are as yet documented (77). Current

theories as to the pathogenesis of recurrent disease remain unproved but include the following.

1. Qualitative and quantitative deficiency in the normal protective vaginal bacterial flora that permits unsuppressed growth, proliferation, and germination of colonizing yeast cells.
2. An acquired, often transient, antigen-specific deficiency in T lymphocyte function that similarly permits unchecked yeast proliferation and germination (Fig. 5-3). According to Witkin et al., reduced T lymphocyte reactivity to *Candida* antigen is the result of elaboration by the patient's macrophages of prostaglandin E_2, which blocks lymphocyte proliferation possibly by inhibiting interleukin-2 production (101–103). They further postulated that abnormal macrophage function could be the result of local IgE *Candida* antibodies or a serum factor (100). This hypothesis requires confirmation; nevertheless, numerous investigators have observed decreased *Candida*-specific lymphocyte reactivity in vitro (33,92). With regard to the possibility of an acquired *Candida* antigen-specific defect in the CMI response, recurrent CVV in women is analogous to chronic mucocutaneous candidiasis (4). With both conditions, *Candida* infections are incal-

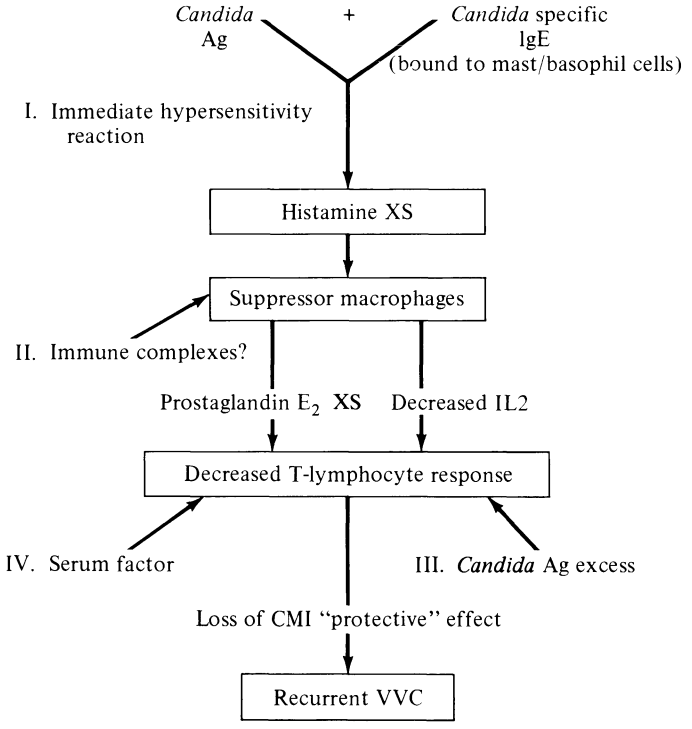

FIG. 5-3. Immunopathogenesis of recurrent VVC (101–103).

citrant, remain confined to mucosal surfaces, rarely become disseminated, and respond to ketoconazole maintenance therapy (44,84). Patients with chronic mucocutaneous candidiasis demonstrate several possible immunological defects in vitro, the commonest of which is failure of the lymphoblastic response only in response to *Candida* antigen (44). Investigations have documented the existence of a clone of suppressor T lymphocytes in many of these patients. The suppressive activity appears to be both mannan-specific and mannan-induced, unrelated to prostaglandin synthesis, and possibly due to mannan excess (23). According to this hypothesis, women with repeated antimicrobic-induced CVV would, as a result of chronic *Candida* colonization, carriage, and hence antigen excess, induce a suppressor population of lympthocytes that would block the normal lymphoblastic response to *Candida* antigen and acquire a specific form of nonreactivity to *Candida*. Hence *Candida* would proliferate unimpeded by local defense mechanisms.

Zinc deficiency is associated with depressed CMI and increased susceptibility to infections, particularly with *C. albicans* (12). Cornell et al., however, measured plasma and leukocyte zinc concentrations in a small series of women with recurrent CVV and found no evidence of zinc deficiency (12). Edman and Sobel, on the other hand, reported a mild zinc deficiency in women with recurrent CVV, although the severity of the deficiency was not such that it was likely to impair CMI (24).

3. The third concept is that there is an acquired acute hypersensitivity reaction to *Candida* antigen that develops and may be accompanied by elevated vaginal titers of *Candida*-antigen-specific IgE (45,50,51,69). This theory has a clinical basis in that patients with recurrent CVV often present with severe vulvar manifestations (rash, erythema, swelling, pruritus) with minimal exudative vaginal changes, little discharge, and low titers of organisms. So far, limited uncontrolled studies using *Candida*-antigen desensitization has been found to be helpful for reducing the frequency of recurrent episodes of vaginitis (74). As yet, only a small percentage of women with recurrent CVV have been shown to have elevated *Candida*-specific vaginal IgE (100). Allergic responses to *Candida* have been reported to involve the male genitalia following coitus with a *Candida*-infected woman; they are characterized by an acute onset of erythema, edema, severe pruritus, and irritation of the penis (10). Similarly, acute hypersensitivity reactions have been described in the oral cavity (14) and respiratory tract (39).

Experimental *Candida* Vaginitis

Mice, rats, and guinea pigs have been used in experimental models for studying aspects of the pathogenesis and treatment of *Candida* vaginitis (43,81). The hormone dependence of the infection in humans and animals

is emphasized by the role of estrogen in these models. Oophorectomized rats are resistant to infection, and estrogenization (pseudoestrus) is essential before infection can be induced (87). In the absence of estrogen, the vaginal mucosa is lined by a thin layer of cuboidal epithelium that is resistant to *Candida* colonization (87). Within 48 hours of estrogen administration, the mucosa is composed of stratified squamous epithelium, and infection with *Candida* can be established. A relatively small inoculum of 10^2 organisms is required to induce florid vaginitis (87). Once established, the infection may persist for several weeks or months, although lower titers are found with time. At any time following the withdrawal of exogenous estrogen in the oophorectomized animal, infection is rapidly controlled, with a prompt decrease in the number of *Candida* cells cultured from the vaginal lumen; and spontaneous cure can be anticipated within a few days (87). Low-grade uterine infections occur in about one-half of the experimental animals (76).

Criticism of the various animal models relates to differences in vaginal pH, natural bacterial vaginal flora, and the presence of keratin produced by stratified squamous epithelium lining the vaginal lumen (54). Nevertheless, the response to both topical and systemic antimycotic agents correlates well with human clinical studies (54). Accordingly, estradiol-treated mice and estradiol-treated ovariectomized rats are widely used in the course of screening is evaluating compounds for antifungal activity. Treatment efficacy is evaluated by several methods including homogenization of the vagina, vaginal lavage (85,87), and more recently utilization of a wire loop and immediate plating on agar (76).

This model has also been useful for studying fungal virulence factors, including the use of mutant strains and their revertants. Mutant strains with reduced capacity to adhere (47) or germinate have been found to express reduced capacity to induce persistent infections in the animal model (86). Sobel et al., utilizing multiple clinical isolates of *C. albicans* obtained from multiple sites, demonstrated that all the strains studied appear capable of inducing experimental vaginitis and achieving high in vivo number of *Candida* (87). However, these observations should be tempered by the fact that the experimental model usually utilizes an inoculum of 10^7 organisms, which is considerably higher than that required to induce an infection in vivo (87). Interestingly, no one has been successful in inducing experimental vaginitis with *Torulopsis glabrata*.

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6—Control of Extension of the Hyphal Apex

NEIL A.R. GOW

Fungal hyphae grow by extending their tips. Although we have known it is the case for half a century (142) we are only recently beginning to appreciate the complexity of the process of hyphal growth and how it is regulated. Superficially, the process appears to be straightforward. The hypha extends by depositing new membrane, enzymes, and wall precursors at the tapering zone at the extreme apex. These components are mostly organized in membranous vesicles, which encapsulate soluble proteins and polysaccharides and carry hydrophobic molecules in the membrane. Thus to obtain tip growth these vesicles must be brought to the tip and made to fuse just at this place. Cytoplasmic turgor pressure expands the tip. At the same time, the cytoplasm and its constituent organelles must be moved forward in order to maintain their position relative to the hyphal tip.

To understand how the hypha grows we must therefore endeavor to establish how cytoplasmic components are transported in a polarized fashion and how the vesicles are encouraged to fuse selectively with the membrane at the apex. There are many ramifications of these problems. For example, it is of interest to determine how tip growth is initiated in the germination of fungal conidia, the formation of hyphal branches, and the transition from budding to hyphal growth in dimorphic fungi. Studies of hyphal growth inevitably spill over into general issues regarding the control of cellular polarity, tropic growth, cell shape, morphogenesis, membrane biochemistry, wall biosynthesis, and antibiosis. Here the discussion is concerned with the control of vegetative hyphal growth in its steady state. Hyphal differentiation, including conidium production, the synthesis of multicellular tissues, and the integrated growth of the constituent hyphae in a mycelium, have been well dealt with elsewhere (2,81,153,155).

This review is designed to address hyphal growth of fungi in general, as there is no reason to suppose that hyphae of clinically important fungi differ greatly from those of saprophytic organisms and because most work in this area has centered on nonpathogenic fungi. No separate mention is made of germ tube and hyphal growth of dimorphic fungi, as the process of cell

extension is in essence the same as for the filamentous molds. There are, of course, significant differences in the ultrastructure, wall composition, and physiology of organisms that have the mycelial growth habit as a common denominator. Indeed, the hypha may be an example of evolutionary convergence rather than the exclusive invention of fungi. One study describing the ribosomal RNA sequences of selected mycelial organisms shows that the oomycetes are not close relatives of the true hyphal fungi but are, instead, akin to the golden algae (53). There are also striking parallels between the growth of fungal hyphae and that of other tip-growing structures such as the pollen tubes of higher plants, the protonema cells of mosses, and in some respects the hyphae of filamentous bacteria such as the streptomycetes. The hyphae may therefore be an example of a structure that has arisen a number of times during evolution, and the details of the underlying mechanisms may not always be the same. The insights that have been gained from studies of a specific aspect of hyphal growth in a particular fungus may not always be representative of all species of fungi. They do, however, define some of the possible mechanisms and the scope of this field.

Growth Kinetics and Hyphal Organization

Hyphae are tubular cells that are infinitely variable in length and variable in diameter over a limited range. In general, wide hyphae of a particular fungus extend more rapidly than thin ones. The tip is tapering and hemiellipsoidal and not hemispheric in shape, and the length of the tapering zone at the apex coincides with the zone of hyphal extension (132,156). A clear demonstration of this fact is evident from studies in which fungal hyphae are fed radiolabeled precursors of wall polysaccharides (14.35). The precursors uridine diphosphate (UDF)-glucose and UDP-*N*-acetylglucosamine are water-soluble, but they are rendered insoluble when they are incorporated into the crystaline polysaccharides of the cell wall. Autoradiographs of hyphae that were exposed to a short pulse of the radiolabel showed that the process of incorporation occurred for the most part in the extension zone. In *Neurospora crassa* more than 95% of the silver grains that were counted on autoradiographs were deposited within 10 μm of the apex, and most occurred at the extreme tip (35). Behind the apex the hypha gradually widens owing to thickening of the wall (22,154). Wall biosynthesis is not therefore wholely apical. The wall is thinnest at the hyphal tip, where the hypha is actively extending. Hyphae of *Allomyces macrogynus* can exhibit what amounts to reversed hyphal growth in which hyphae widen from the tip backward by an active biosynthetic process (169). The polarity of hyphal growth is not therefore fixed in every case. In

the ascomycetes, basidiomycetes, and most fungi imperfecti, hyphae are partitioned into compartments by septa that have a range of possible structures and may be perforate or complete (52). The zygogmycetes have few septa in the vegetative mycelium, and in the oomycetes septa are formed only during differentiation or sporulation when they separate the differentiating region from the mycelium. For hyphae with septa that have pores, the cytoplasm is continuous over long distances, spanning many hyphal compartments, and cytoplasm is free to flow between them. Some hyphae, e.g., those in certain dimorphic fungi, have pores that are only about 25 nm in diameter (47). These "micropores" may allow some cytoplasmic movement between adjacent compartments but are too small to permit organelle migration. In filamentous moulds such as *N. crassa* cytoplasm may flow through as many as 24 compartments en route to the hyphal apex (153), and organelles such as nuclei are able to squeeze through the central pore, which is nearly 500 μm wide (75). The question of cytoplasmic and organelle transport is considered later.

Branches are formed at intervals from hyphae. Branch formation can be regarded as the mycelial equivalent of cell division, and it is the regular formation of hyphal branches that accounts for the exponential growth of mycelial fungi on solid and in liquid cultures (151, 152). Branching is normally subapical, although apical branching occurs during the initial growth of germlings of ascomycetes such as *N. crassa* (145).

From the above discussion it is clear that extension, the making of a new hypha, is confined to a limited zone at the extreme apex. *Biosynthesis*—the producion of new proteins, polysaccharides, nucleic acids, and lipids—however, is not as polarized within the hypha. The endoplasmic reticula and Golgi apparatus (or Golgi equivalents in those fungi without true Golgi) are found throughout the cytoplasm, although not at the hyphal tips (51,68). Also there are biosynthetic events within the lateral wall behind the tip, as mentioned previously. However, the tip can be regarded as the principle sink for this biosynthetic potential that is generated in the subapical cytoplasm, as the fate of much of this potential is adsorbed at the apex, where enzymes, proteins, membrane lipids, and polysaccharides are put together to extend the hypha. The amount or volume of cytoplasm that can be sequestered for growth of each hyphal tip can be regarded as the functional unit cell of the hypha. This region has been given the name *peripheral growth zone*, meaning the length of hypha that supports the extending apex (151). The peripheral growth zone is a measurable parameter and one that helps in understanding the kinetics of hyphal extension. It has been measured in three ways.

1. Trinci showed that when hyphal tips were severed from the distal mycelium with a razor blade the tip continued at an uninterrupted extension rate provided the cut was not made close to the apex (151). The

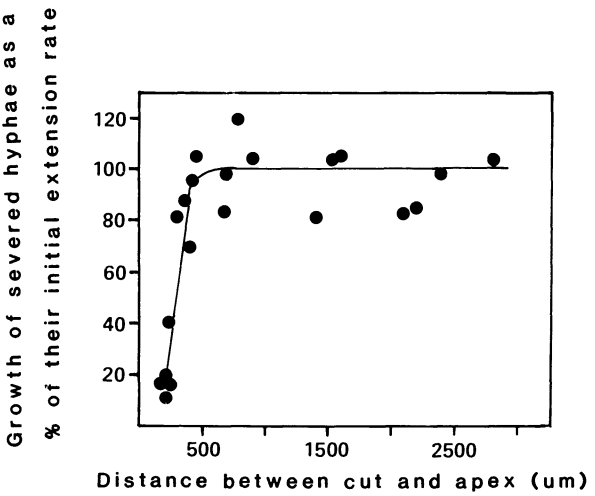


FIG. 6-1. Determination of the peripheral growth zone in *Achlya bisexualis* according be the method described in the text. Here the peripheral growth zone is estimated as 500–700 μm because cuts that are made any closer to the tip caused a reduction in the rate of hyphal extension.

minimum distance the cut could be made away from the tip without consequence to the extension rate defined the peripheral growth zone, as this portion of the hypha must have been contributing to the overall rate of extension. When the cut was close to the tip, it isolated some of the hypha that had been contributing to growth, and so the hypha slowed down or stopped (Fig. 6-1).

2. A direct consequence of the "apical extension, subapical biosynthesis" model of hyphal growth is evident in the exponential kinetics of germ tubes following germination of fungal conidia and spores (150). The kinetics of germ tube growth can also be used to determine the peripheral growth zone. As a newly formed germ tube extends, the biosynthetic capacity increases but the size of the sink does not. An increasing volume of biosynthetically active cytoplasm is generated, and this expanding resource is made available to a single germ tube tip. Germ tube growth is therefore said to be *autocatalytic* (118). It follows that the tip will be able to grow faster as it gets longer until the length of the germ tube approaches that of the peripheral growth zone. Once this critical length is achieved, the cytoplasm most distant from the apex is no longer able to contribute to tip extension, and the hypha then continues at a constant maximum extension rate. The length of the germ tube when extension changes from an exponential to a linear extension rate defines the peripheral growth zone (149).

3. The peripheral growth zone can also be measured indirectly by divid-

ing the measured specific growth rate in liquid cultured by the extension rate of the hypha under the same cultural conditions (151).

The extension of hyphae from germ tubes of filamentous moulds such as *Aspergillus fumigatus* (responsible for aspergillosis) and the dermatophytic fungi are likely to be initially exponential, then linear, for the above reasons. The observed linear growth of germ tubes of fungi such as *Candida albicans* (44) appear at first not to fit the model. Hyphae of *C. albicans* have been shown to incorporate wall precursors selectively at the tip (14), and work in which latex beads were used to map surface growth of germ-tube-forming yeasts showed that 90% of wall growth was confined to the apex (144). However, during germ tube formation in *C. albicans*, little new cytoplasm is synthesized and the effective biosynthetic capacity or peripheral growth zone is constant; hence growth is not autocatalytic (41,42,46,48). Instead of an increase in cytoplasmic volume there is a compensating increase in the vacuolar volume in the parent yeast cell and germ tube. The net result is that a fixed volume of cytoplasm flows from the parent yeast cell into the extending germ tube, leaving behind a vacuolated anterior—a literal example of the late Philip Gregory's contention that fungi were akin to "plasmodia moving around in a system of tubes" (50). A few other fungi, including *Uromyces* and *Basidiobolus* species, also have hyphae in which extension is supported by a terminal volume of cytoplasm that migrates forward with the extending apex. The peripheral growth zone in these types of fungi can be regarded as being equivalent to the terminal volume of cytoplasm. Cytoplasmic movement in these fungi is considered later.

The peripheral growth zone is the region in which hyphae turn the nutrients they absorb into new hyphae. The ultrastructure of this region reveals some of the key components that drive the process of hyphal extension.

Ultrastructure of the Hypha

Apical Vesicles

As seen by phase contrast microscopy the cytoplasm in the tip of an extending hyphae often has a high refractive index. This "structure" has been called the *Spitzenkörper* (33). This dark phase appearance is probably due to the accumulation of membranous vesicles at the apex, as there is a complementary relation between the arrangement of apical vesicles and the presence and shape of the Spitzenkörper (68). For example, where the packing of the apical vesicles is loose (as in the oomycetes) the Spitzenkörper is less evident, and in the zygomycetes both vesicle packing and Spitzenkörper are crescent-shaped (51). These vesicles were first revealed in

thin sections of fixed material by Girbardt (34) and Grove et al. (51). Vesicles do not always survive well in conventional electron microscopic preparations. The primary reason is the difficulty of obtaining good fixation of cells with thick cell walls, as it prevents rapid penetration of preferred fixatives such as osmium tetroxide and glutaraldehyde. More recently a technique involving rapid freezing of specimens in liquid propane, Freon, or nitrogen, followed by substitution of the cell water with an organic solvent of low freezing point (acetone, ethanol, methanol, tetrahydrofuran) in which chemical fixatives are dissolved has allowed rapid immobilization of the cell interior and improved preservation of labile cytoplasmic structures such as apical vesicles (Figs. 6-2 and 6-3). This technique, called freeze-substitution, has also had a revolutionary impact in other aspects of fungal ultrastructure, as in studies of the cytoskeleton, which are poorly preserved in conventionally fixed material (see later). For detailed reviews of the technique of freeze-substitution and fungal ultrastructure, see the reviews of Hoch (62) and Howard and O'Donnell (72).

Apical vesicles have been found at the hyphal tips of all filamentous and dimorphic fungi that have been examined in detail. They are thought to represent the major vehicles for the intracellular transport of membrane-bound and extracellular enzymes and for new cell membrane. Cytochemical techniques have shown the presence of polysaccharides in the vesicles in a variety of oomycetes, including *Achlya bisexualis*, *A. ambisexualis*, and *Saprolegnia monoica* (24,28,61). Vesicles have also been stained cytochemically for enzymes. Inosine diphosphatase and acid phosphatase and cellulase have been seen in some of the apical vesicles of *A. ambisexualis* (61,106). Alkaline phosphatase has been localized in vesicles of *A. bisexualis* (24). The vesicles are made at the endoplasmic reticulum and Golgi apparatus (or Golgi equivalents in those fungi without true Golgi) and are then transportd to and deposited at the tip where they fuse with the apical plasmalemma and exocytose their contents. Apical vesicles of chitin-containing fungi that have been examined by freeze-substitution are mostly spherical (63,68,71), although microvesicles that are hexagonal in cross section have also been observed (68). Oomycetes also have tubular vesicles (58). Because the vesicles supply the growing tip with new membrane their rate of absorption at the tip can be estimated by measuring the rate of surface extension and then calculating how many vesicles of a given surface area are required to account for the extension. In *N. crassa* around 38,000 vesicles must fuse with the tip every minute (22), whereas in *Aspergillus nidulans*, which is slower-growing and narrower, there must still be around 500 vesicle fusions per minute (118). Freeze-substitution studies suggest that the process of vesicle fusion is rapid. The rate of cooling is so quick that complete immobilization is achieved within milliseconds. Sections of freeze-substituted hyphal tips show few fusion profiles (68). Because there are many vesicles in the apex at any given instant the fusion of any one of these structures must occupy only a small fraction of a millisecond.

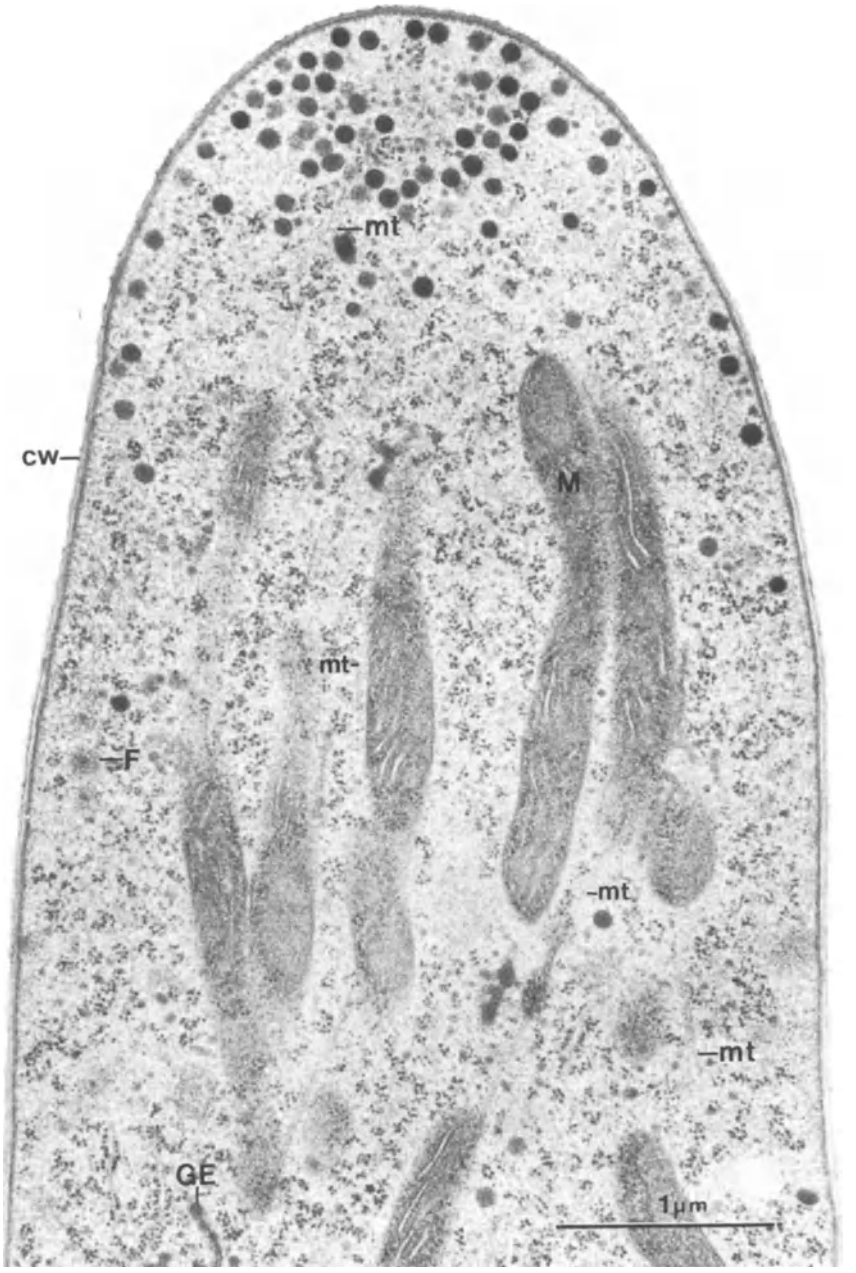


FIG. 6-2. Section of a hypha of *Fusarium acuminatum* prepared by freeze-substitution and showing accumulated apical macrovesicles and microvesicles. Microtubules (mt), mitochondrion (m), filasome (F), Golgi-like endomembrane (GE), and the cell wall (cw) are noted. (Reproduced from *The Journal of Cell Biology*, 1980, 87:55–64, by copyright permission of the Rockefeller University Press.)

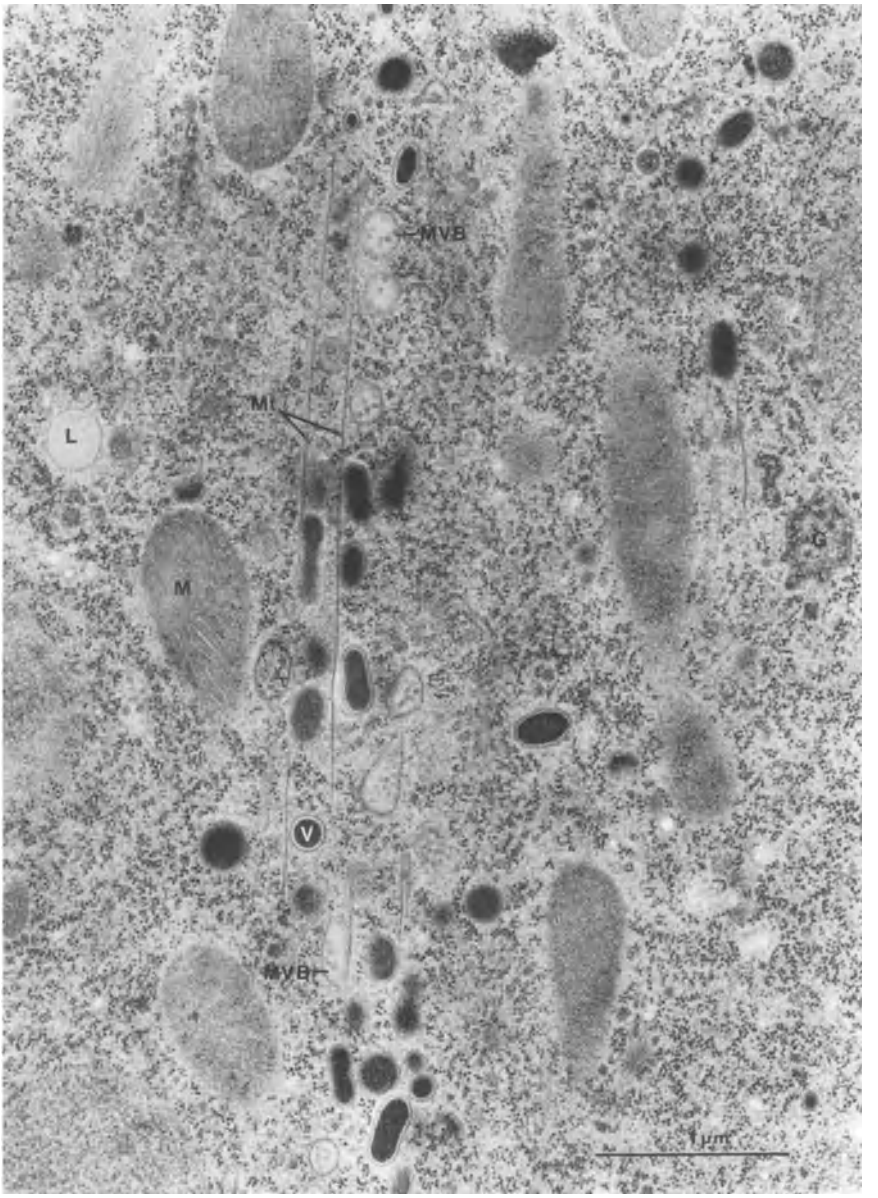


FIG. 6-3. Section of a freeze-substituted hypha of *Gilbertella persicaria* approximately 150 μm behind the tip showing a close association between microtubules (Mt), vesicles (v), and multivesicular bodies (MVB). The collapse of microtubules is an apparent artifact of specimen preparation. Mitochondrion (M), lipid body (L), and smooth endomembrane cisterna, possibly a Golgi-equivalent (G), are noted. (From R. Howard, unpublished.)

Macrovesicles

Although the sizes of apical vesicles vary according to whether they are measured in conventional or freeze-substituted preparations (they are generally smaller in the latter), it appears as though there are two size classes: microvesicles with a diameter of about 40–70 nm and macrovesicles with a diameter of more than 100 nm (7,38). The macrovesicles are more numerous and probably contribute most of the new membrane for tip expansion. They may also transport preformed nonfibrillar matrix polysaccharides and glycoproteins to the cell surface and be responsible for the exocytosis of extracellular enzymes. A great deal of information regarding the control of macrovesicle synthesis, membrane trafficking, and the control of exocytosis is being revealed in studies of *Saccharomyces cerevisiae*, and the reader is referred to the excellent work of Schekman (134). Much less is known about macrovesicle biochemistry and physiology in filamentous fungi.

Microvesicles and Chitosomes

The microvesicles of hyphae have been the subject of intensive investigations and have been given the name *chitosomes* (13). This name reflects the discovery that vesicles in this size range that are isolated from broken hyphae have only one measurable activity: the ability to synthesize chitin (10,12). Chitosomes have been described in representative fungi from all taxonomic groups that contain fungi with chitin in their walls (12). They are normally isolated by first breaking mycelium with glass beads and then separating the microvesicles from other organelles and vesicles using sucrose gradients (127). Density gradient sedimentation and isopycnic centrifugation yield preparations of microvesicles that also contain other particles, notably ribosomes; gradient fractions from such preparations contain many hundreds of proteins when analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Lending et al. (92) developed a protocol involving two isopycnic centrifugations that yielded chitosomes that were essentially free of other visible material (Fig. 6-4). Further subfractionations of ultrapure chitosomes produced a fraction of high chitin synthase specific activity that contained only five polypeptide bands (92). It is not yet known which band or bands correspond to chitin synthase.

These microvesicles can be reversibly dissociated with the detergent digitonin into subunits that have a sedimentation of 16S and apparent molecular weight of around 500,000 daltons. When the digitonin is removed by dialysis, these subunits self-assemble into normal vesicular structures (126). Chitosomes contain chitin synthase in a zymogenic form (128). When isolated chitosomes are incubated with a suitable protease activator and supplied with the substrate UDP-*N*-acetylglucosamine, they manufac-

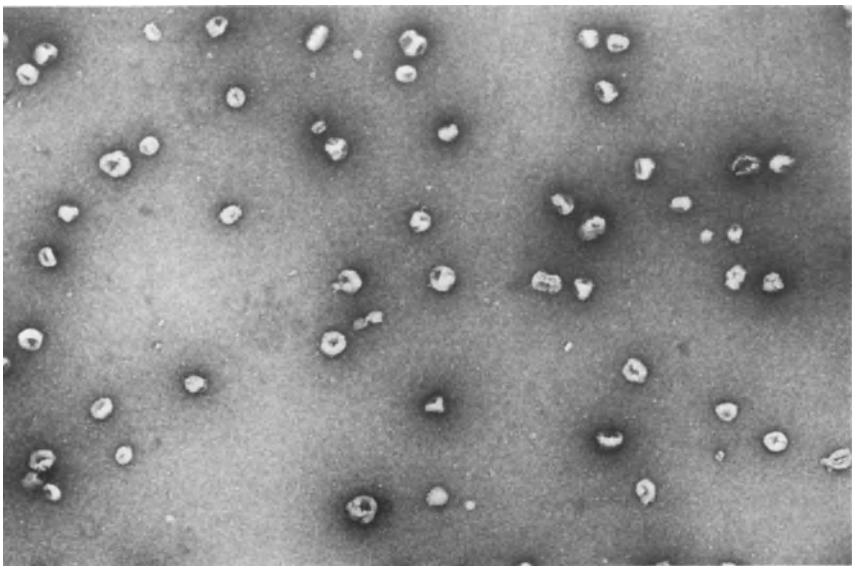


FIG. 6-4. Negative stain of purified chitosomes prepared by successive isopycnic centrifugations. The chitosomes are 40–70 nm in diameter. (From Flores-Martinez A, Lopez-Romero E, Martinez JP, Bracker CE, Ruiz-Herrera J, Bartnicki-Garcia, S. In preparation.)

ture long chitin microfibrils, one microfibril per chitosome (13). Because chitin is synthesized only at the cell surface it is proposed that activation of the chitin synthase in vivo occurs after the microvesicles fuse with the apical plasmalemma. It has been suggested that the activating protease is transported separately in the lumen of macrovesicles and that proteolysis of chitin synthase zymogen occurs in the periplasm where the enzyme and activator are brought together (7).

From the above discussion it is not surprising that chitin synthase zymogen can be found in both the cytoplasm (in microvesicles) and the cell membranes of fungi (18,127). The question of precisely how much enzyme is normally cytoplasmic and how much is associated with the membrane has been an issue of some debate. The debate is of importance because it calls into question the nature of the chitosome. In filamentous fungi the issue has not been contested, and the available information suggests that the microvesicular/cytoplasmic pool is the larger (7,12). For *S. cerevisiae* different methodologies have produced a different picture regarding the cellular location of chitin synthase. When the wall of this yeast was first removed with lytic enzymes and the protoplast membrane stabilized using the lectin concanavalin A, osmotic lysis produced sheets of plasma membrane that were so large that each protoplast was calculated to give rise to only one or two membrane fragments (26). When the chitin synthase activ-

ity in the membrane and soluble fractions were compared, almost all of the activity partitioned with the cell membrane. Experiments in which the same yeast was broken by glass bead homogenization produced conflicting data, with most of the activity in the soluble cytoplasmic fraction (12). On the basis of these experiments it has been suggested (a) that chitosome microvesicles may in fact be artifacts produced by physical disruption of the plasmalemma during ballistic cell breakage, and (b) that the main cellular reserve for chitin synthetase resides in the cell membrane, at least for *S. cerevisiae* (18). The suggestion that chitosomes may be artifacts is of course also of importance to the consideration of hyphal physiology.

At least four reasons can be given in support of the view that chitosomes are genuine microvesicular organelles of the type seen at the hyphal apex. (a) Chitosomes have a different buoyant density than the plasmalemma (127). (b) They lack enzyme activities associated with the plasmalemma or other membranous organelles (10,128). (c) They lack the concanavalin A binding sites that the plasmalemma and other cytoplasmic organelles share (9). (d) Chitosomes have been isolated from a wall-less "slime" mutant of *N. crassa* by both ballistic glass bead homogenization and osmotic lysis. The overall yield of chitosomal enzyme was not affected by the method of protoplast breakage, and so it seems unlikely that the chitosomes were derived from broken fragments of plasma membrane (11). The virtual absence of microvesicular chitin synthase in protoplasts of *S. cerevisiae* may be due to disruption of the microvesicles or their integration with the plasmalemma during the preparation of protoplasts with wall-degrading enzymes. The issue may be finally be resolved only when specific antibodies are available to search for the enzyme immunocytochemically.

Coated and Endocytotic Vesicles

Many higher eukaryotic cells have been found to contain a specialized form of vesicle that is surrounded by a basket-like lattice of a fibrous protein called clathrin (115). The clathrin forms a polyhedral network, or clathrin coat, over the vesicle that can be visualized using negative staining. These coated vesicles have been identified in *S. cerevisiae* (104) and more recently in hyphae of the ascomycete *N. crassa* and the basidiomycete *Uromyces phaseoli* (148). They may well turn out to be ubiquitous in the filamentous fungi. Two sizes of coated vesicles have been recognized in the fungi (148): large vesicles of the filamentous fungi (100–180 nm), similar in size to vertebrate vesicles, and small ones (50–80 nm), corresponding to the sizes reported for plants and for *S. cerevisiae* (102,104). The coated vesicles were not found at the extreme tips of the hyphae but, instead, were located subapically. Because the clathrin has been shown to inhibit fusion of these vesicles with other membranes (4) it may be necessary for them to be uncoated before they can deliver material to the apical plasma membrane (148). Their precise role in the process of hyphal growth is at present un-

known, but they may function in transporting specific molecules between organelles and cell surface or in recycling proteins in the lateral cell membrane to the endomembranes in the cytoplasm. With reference to the latter, it is of interest that endocytosis has been demonstrated in a fungus, albeit in a yeast (122). *Saccharomyces cerevisiae* took up a nontransportable water-soluble dye, lucifer yellow, which was ultimately accumulated in cytoplasmic vacuoles. Uptake was mostly confined to cells in the late-logarithmic phase of growth. Lucifer yellow was also shown to be taken up by hyphae of *N. crassa* (Harold and Harold, unpublished). These studies intimate that endocytosis and endocytotic vesicles might eventually be recognized in filamentous fungi and heralds the formulation of a dynamic model of hyphal growth in which vesicle fusion at the tip is counterbalanced by active membrane recycling in the subapical hypha.

Cytoskeleton

When a hypha grows, the cytoplasm must move forward in relation to the lateral cell wall at a rate approximately equal to the rate of tip extension. The organelles must also be correctly positioned so they can be properly partitioned by the laying down of septa and the formation of branches. It is also self-evident that cytoplasmic microvesicles that move to and exocytose at the tip must move at a rate that is faster than the rate of hyphal extension. When a growing hypha is observed by light microscopy, two types of cytoplasmic transport can be observed. First, there are rapid and erratic movements of particles of a size that is just resolvable under high magnification. This darting, highly variable movement has been called *saltatory movement* (120) or *organelle movement* (98). In addition, there are smooth, flowing, unidirectional movements that are characteristic of nuclei and mitochondria and that maintain the overall position of these organelles in the hypha. This alternative type of motion is called *organelle positioning.* These cytoplasmic movements are accomplished by the integrated activities of the cytoskeleton, consisting of microfilaments and microtubules. Intermediate filaments, which have been described in animal cells, have not so far been recognized in fungi. There has, however, been a burst of activity centered on the organization and function of the fungal cytoskeleton, and the discussion here summarizes the advances that have been made in this area.

Microfilaments

Microfilaments are produced from a 43-kilodalton (kD) protein called actin. The monomeric form of the protein (G-actin) polymerizes with itself to form F-actin filaments, which have a diameter of about 4–9 nm and

may be several microns in length. The two ends of the actin filaments appear different by electron microscopy, one end being pointed and the other end barbed. Although polymerization can occur at both ends, it is generally faster at the barbed end (23). Polymerization is promoted by ATP, and after hydrolysis the adenosine diphosphate (ADP) remains bound to the actin. In certain circumstances actin microfilaments can "treadmill"—they can lengthen at the barbed end by incorporating G-actin monomers that are released from the pointed ends [for a review see Wegner (165)]. Actin of animal cells is associated with a wide range of actin-binding proteins, yet none of these proteins have so far been found in fungi. Myosin, a protein that along with actin controls contraction in muscle and other animal cells, has been found in *S. cerevisiae* (164) and may therefore also be present in filamentous fungi. Actin microfilaments have both elastic and tensile properties and a range of possible roles in cell growth. They may serve in passively guiding and channeling the movements of vesicles or organelles or actively moving cytoplasmic components by means of contraction or translocation. The ways in which actin may be involved in hyphal extension are discussed below.

The distribution of microfilaments in fungal hyphae has been determined but only in a few fungi. Chemically fixed and even freeze-substituted fungal material seems to cause considerable fragmentation of microfilaments. Consequently, there are relatively few electron microscopic studies of microfilaments, although they have been seen at the hyphal apex (65,68), free in the cytoplasm (2,65,68), and in association with microvesicles (65,68), organelles (65,68), and septa (32). In hyphae of *Fusarium acuminatum* and *U. phaseoli* that have been examined by electron microscopy after freeze-substitution. Vesicles have been shown in association with aggregates of microfilaments (65,68). These structures have been called *filasomes*. Filasomes have not been seen in freeze-substituted hyphae of *Saprolegnia ferax* (58). Phallotoxins from certain agarics have been used to make fluorescent stains for actin. Phalloidin that has been conjugated to the fluorescent molecule rhodamine has been of particular use with fungi. The material is usually fixed in formaldehyde or paraformaldehyde, and the specimen is then stained with rhodamine–phalloidin. This stain is not able to permeate the walls of all fungal hyphae (Reilly and Gooday, unpublished); however, when it does work, it reveals much more filamentous actin than is seen in specimens examined in other ways.

Fluorescence microscopy shows that actin is concentrated in the hyphal apex (5,56,66,129) (Figs 6-5 and 6-6) but is also seen in the subapical hypha and in association with nuclei (64,66) (Fig. 6-7). Most of the actin is seen at the periphery of the hypha, not throughout the entire depth of the cytoplasm. Even in the hyphal tip the staining material is found predominantly around the inside surface of the apex (56,66). Taken at face value, it indicates that microfilaments are not associated with most of the vesicles in the Spitzenkörper. Three staining patterns are seen with rhodamine–

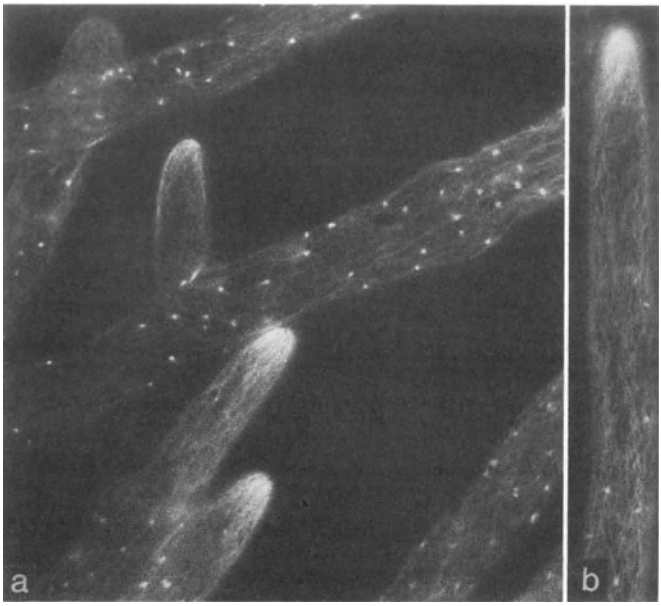


FIG. 6-5. Hyphae and branches of *Saprolegnia ferax* after fixation in 4% formaldehyde in PIPES buffer and staining of actin with rhodamine–phalloidin. Fibrous apical caps (a,b) and the apical area are free of actin spots, which occur along with actin fibrils in the older regions of the hyphae (b). (a × 1,605; b × 2,000) (From Heath [56].)

phalloidin: spots or plaques, filaments, and thick fibers or cables. In most fungi the spots predominate and are concentrated at the tips of hyphae of mycelial or dimorphic fungi, or in the buds or growth zones of budding or fission yeasts (1,5,64,66,83,94,129). Rhodamine–phalloidin staining of hyphal tips of *S. ferax* and *U. phaseoli* is shown in Figures 6-5 to 6-7. In the oomycete *S. ferax*, the hyphal tip contains a cap of fine filaments, without spots, and the filaments ramify into the distal cytoplasm where plaques are found (56) (Fig. 6-5). In *U. phaseoli* the hyphal tips contain spots, filaments, and more diffuse staining (64) (Fig. 6-7), similar to the pattern seen in the human pathogen *Candida albicans* (5) and most mycelial fungi (98,129). In all hyphae that have been examined, the filaments and cables run predominantly parallel to the long axis of the hypha. Actin cables may be artifactual aggregates of less organized actin, as they are not seen in freeze-substituted material (65). One study showed that some of these staining patterns are interconvertible (56) (Fig. 6-6). For example, pretreatment of hyphae of *S. ferax* with buffered saline for 10 minutes caused the filaments in the apical cap to be converted to spots, and exposure to the detergent sodium dodecyl sulfate (SDS) or potassium iodide caused the formation of actin cables (Fig. 6-6). PIPES buffer was also found to preserve the fine

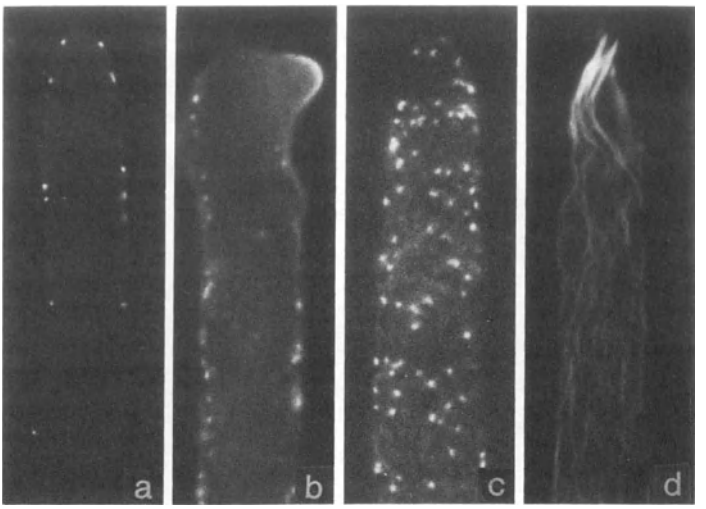


FIG. 6-6. Effects of various pretreatments on actin staining of *Saprolegnia ferax* with rhodamine–phalloidin. (a) Hyphal tip was treated with physiological buffered saline for 10 minutes and then fixed in phosphate-buffered formaldehyde. The tip retains a normal shape, but in place of an actin cap there are spots extending throughout the apical zone. (b) Regrowth of a tip after recovery of a hypha treated as in (a) in an organic medium. The old tip region contains spots, whereas the regrowing apex (top right) has an actin cap. (c) Hyphal tip fixed with PIPES-buffered formaldehyde after 10 minutes in saline. Actin spots are evident; but in contrast to the results with phosphate-buffered fixation, a disordered remnant of the fibrillar system is present. (d) Hyphal tip treated for 10 seconds with sodium dodecyl sulfate (SDS) prior to PIPES-buffered fixation. The fibrils are aggregated into coarse fibrils (compare with Fig. 6-5), with those at the extreme tip appearing as separate pointed bundles (cables). (a–d ×2000) (From Heath [56].)

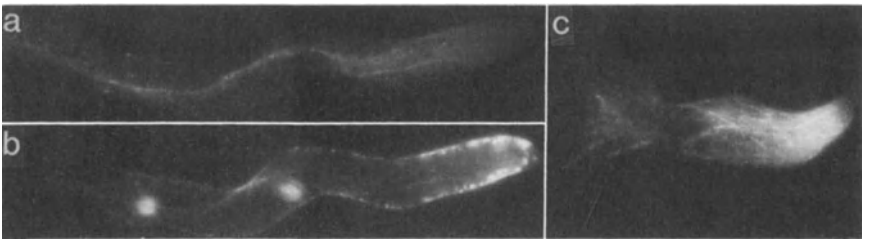


FIG. 6-7. Hyphal apices of *Uromyces phaseoli* stained with FITC-conjugated yeast antitubulin monoclonal antibody (a,c) or rhodamine–phalloidin (b). (a,b) Same cell is stained for microtubules (a) and F-actin (b). Many of the microtubule and F-actin profiles occupy similar sites. (b) Two F-actin nuclear inclusions are seen just out of the optical plane of focus. (c) Microtubules are readily seen at the hyphal apex by freeze-substitution in acetone. (a–c ×1,650) (From Hoch and Staples [64].)

filaments of actin better than phosphate buffer, although actin spots were present in the subapical cytoplasm even in PIPES-buffered preparations (Fig. 6-6). Clearly, the microfibrillar array in the cortex of the cytoplasm is highly labile, and great care must be taken to guard against the production of possible artifacts during the preparation of cells for rhodamine–phalloidin staining. It is worth noting that actin plaques are not generally found in rhodamine–phalloidin-stained cells that have no wall, and that all reports in which apical actin spots have been described used buffers based on phosphate. It is therefore possible that the actin spots at the hyphal apex of many fungi might have been induced during preparation. However, the filasomes that have been identified in some fungi by electron microscopy may give actin plaques and spots an ultrastructural basis. Alternatively, spots and plaques may represent foci of attachment of actin to the cell membrane (1). The question of how actin microfilaments might be involved in hyphal growth is considered later.

Microtubules

Microtubules are polymers of a family of homologous proteins called tubulins that have a molecular weight of around 50–55 kD. Assembly of the microtubule is nucleated from either microtubule organizing centers or nucleus-associated organelles and usually proceeds unidirectionaly. Microtubules have a diameter of 30–40 nm, and their maximum length varies from around 1 μm in some fungi including *S. ferax* (98) and *U. phaseoli* (57) to several micrometers in others (71). Fungal microtubules are in general considerably shorter than those found in animal cells. Polymerization is in many ways analogous to that for microfilaments, but guanosine triphosphate (GTP) is used in place of ATP. Like microfilaments, microtubules can treadmill (125) and occur in association with a large number of regulatory proteins. Few of these proteins have been demonstrated to be present in fungi. In animal cells microtubules are found frequently in association with vesicles and other organelles (65,68). In the much studied squid axoplasm system, organelles have been visualized moving unidirectionally or bidirectionally along isolated microtubules (3,103). The direction of axonal transport depends on the age of the extruded axoplasm, concentration of ATP, and presence of cytoplasmic translocation factors (3,159,160). In this system an ancillary protein called kinesin has been identified that joins the vesicles and organelles to the microtubule and imparts the ability for these structures to be transported unidirectionally (159,160). This protein can even be used to coat inert particles such as polystyrene beads, which are then translocated along microtubules (160). Kinesin also allows microtubules themselves to move along a surface and so may have a second role in microtubule movement. As well as having a role in cytoplasmic transport, microtubules may function in generating

tensile strength and structural organization to the cytoplasm (see later). Cytoplasmic transport in fungal hyphae is essentially unidirectional. It is not known if proteins such as kinesin are present in fungi.

Microtubules have been seen in conventional, chemically fixed thin-sectioned material, although freeze-substitution preserves more and longer microtubules (65,68,101) (Fig. 6-3). More recently (FITC)-conjugated monoclonal antibodies to fungal β-tubulin have been used to stain tubules immunocytochemically (68,129) (Fig. 6-7). Immunofluorescence staining is, however, limited by the necessity to permeabilize the cell with enzymes prior to staining with this large protein. In the studies of squid axoplasm mentioned above, computer-enhanced video recordings based on differential interference microscopy have been used to observe the movements of organelles on individual microtubules of extruded cytoplasm of axons (3,103). This exciting innovation has been used with living fungal hyphae. Preliminary results again show organelle movement along distinct tracks that were thought to be microtubule-based (R. Howard, personal communication).

Unlike microfilaments microtubules are found dispersed throughout the depth of the cytoplasm (Fig. 6-2) and not just at the periphery (65,64,68). They are normally orientated parallel to the surface of the hypha (Figs. 6-2 and 6-7). In germ tubes of the bean rust fungus *U. phaseoli*, more microtubules were found near the hypha–substrate interface than in the rest of the cytoplasm (65). As yet, no similar arrangement has been reported for surface-colonizing pathogenic fungi of animals. Microtubules are sometimes but not always seen to be concentrated at the apex (64) (Fig. 6-7). Studies in *U. phaseoli* of the repolymerization of microtubules after treatment with depolymerizing agents such as cold, demecolcine, griseofulvin, and nocodazole showed that repolymerization occurred first apically and then proceeded into the subapical cytoplasm. This finding indicates that the microtubule organizing centers may be located in the tip of this fungus (64). Microtubules have been reported in association with microvesicles (68) (Fig. 6-3), nuclei (65,68), and mitochondria (65), although some of these associations have been called into question. For example, it has been reported that there is no preferential association of microtubules with microvesicles (58) or with nuclear membranes (100) in *S. ferax* and *Basidiobolus ranarum* (as *B. haptosporus*), respectively.

Before considering the possible roles of microfilaments and microtubules in the control of hyphal extension it is worth considering the overall structure of the cytoskeleton. It is possible that the actin- and tubulin-based elements are reversibly cross-linked via ancillary proteins. In animal cells detergent-extracted cytoplasm, which is then freeze-etched or freeze-dried, leaves behind the insolube, fibrous elements of the cytoskeleton. Pictures of these whole cytoskeletons seem to show microfilaments, microtubules, and intermediate filaments cross-linked by fine filaments and associated proteins. This cytoskeletal complex has been called the micro-

trabecular lattice (117). Microtrabeculae are not seen in sectioned material, and the identity of the protein that forms the meshwork of connecting fine filaments has not yet been established. However, a similar detergent-resistant lattice has been prepared from the cytoplasm of hyphae of *B. ranarum* (as *B. magnus*) (97). The various components of the fungal cytoskeleton may therefore also have an interlinked microtrabecular-like organization. Although the various components may interconnect, they can also operate independently. For example, antimicrotubule drugs have been shown to destroy microtubules in *U. phaseoli* without affecting the functioning of the actin (59). Also, the experiments described below show that actin and microtubule-based cytoplasmic transport can function independently of each other.

Cytoskeleton and Hyphal Growth

Having considered how the cytoskeleton of fungi is organized, it is important to determine how this arrangement of microfilaments and microtubules might be orchestrated in the process of hyphal growth. The information summarized below suggests that the cytoskeleton is likely to be important in the transport of apical vesicles, cytoplasm, and constituent organelles to the hyphal apex and in providing the cytoplasm and hyphal tip with tensile strength and structural support.

Vesicle Transport

There is evidence implicating both microtubules and microfilaments in vesicle transport. In *U. phaseoli* and *F. acuminatum*, microtubules have been reported in intimate association with microvesicles (65,68) (Fig. 6-3), but in *S. ferax* no preferential association was found (58). The micrograph of a freeze-substituted hypha of *Gilbertella perisicaria* (Fig. 6-3) shows an array of vesicular bodies studded along cytoplasmic microtubules. Some of the microtubule-associated vesicles have an elongated shape as if they are flattened laterally by attachment sites on the microtubules. Video-enhanced light microscopic techniques also show intracytoplasmic movement of vesicles of this size on tracks presumed to represent tracks of cytoplasmic microtubules (R. Howard, personal communication). Microfilament–vesicle associations also have been reported, although not in all fungi (see above). In *F. acuminatum*, depolymerization of microtubules with methyl benzimidazole-2-ylcarbamate (MBC, benomyl) led to the dispersal of vesicles in the apex and concomitant inhibition of hyhal extension (69,70). Analogously, in *N. crassa*, *U. phaseoli*, and other fungi, cytochalasins cause disruption of microfilaments and inhibition of hyphal growth (2,70,157). Other studies in a range of fungi supported an actin-

based model of vesicle transport and site selection for vesicle exocytosis. In *C. albicans* the site of germ tube and bud formation is preceded by an accumulation of actin spots at that site, suggesting that actin may be necessary for tip formation and growth (5). A similar phenomenon has been described in bud formation in *Saccharomyces cerevisiae* (1). Mutations in the actin structural genes caused delocalized chitin deposition in the bud of *S. cerevisiae*, as if the mutants were unable to target vesicle transport (107). Finally, β-tubulin mutants of *A. nidulans* and germ tubes of *U. phaseoli* that were treated with antimicrotubule drugs could still undergo normal hyphal extension (110–112). In summary, actin- and tubulin-based systems have been implicated in the transport of apical vesicles, although we know little about the precise mechanisms. The ultrastructural evidence provides more convincing evidence for microtubule-based transport, as microtubules are present throughout the subapical cytoplasm and actin is for the most part confined to the periphery. However, physiological and genetic studies are more persuasive that actin plays the major role. It is possible that vesicle translocation may be achieved by different mechanisms in different fungi or by different mechanisms under different conditions in the same fungus.

Organelle Transport and Positioning

As mentioned previously, organelles such as nuclei and mitochondria have been observed in close association with microtubules (65,68) and microfilaments (64). In *A. nidulans*, nuclear movement was inhibited by mutations in the β-tubulin gene (111,112) and by the microtubule inhibitor benomyl (112), suggesting that microtubules may be involved in nuclear movement. Benomyl also caused displacement of mitochondria from the apices of *F. acuminatum* (70), thus providing tentative evidence for microtubule-based movement of mitochondria. However, Oakley and Rinehart showed in *A. nidulans* that mitochondrial movement was unaffected by benomyl or by β-tubulin mutations that eliminated nuclear motility (112). This finding suggested some alternative mechanism for mitochondrial transport in this fungus, perhaps one based on actin. The suggestion that nuclei might be moved by microtubules is, however, in contrast to the the findings of experiments described below where ultraviolet irradiation was used to depolymerize microtubules in the hyphae of *B. ranarum* (as *B. haptosporus*) (99,101). Here nuclear migration continued even after most of the microtubules surrounding the nucleus had been depolymerized. Also it must be borne in mind that the microtubules of fungi are short compared to the length of hyphae and the peripheral growth zone. Cytoplasmic transport on microtubule rails would necessarily involve frequent transitions between adjacent tubules (98). Therefore, as found in studies of vesicle movement, the picture that emerges suggests that different fungi may use

different mechanisms to transport organelles and that more than one mechanism may be used by individual organisms.

Cytoplasmic Movement and Structure

When fungal hyphae extend, the cytoplasm streams forward at a rate equal to or greater than that of the hyphal tip; and organelles such as the nucleus maintain a more or less constant position within the cytoplasm. Work using ultraviolet microbeam irradiation demonstrated that the cytoplasm has contractile and structural properties that are important in the control of these processes (99,101). This technique involves irradiating a small region of a hypha with ultraviolet light, which damages the cytoskeleton and causes depolymerization of microtubules (99). McKerracher and Heath demonstrated that irradiation of hyphae of *B. ranarum* caused the cytoplasm to contract as though an opposing tensile force had been weakened by the ultraviolet light. Concomitantly, cytoplasmic flow and saltatory movement were temporarily inhibited. Because the only ultrastuctural effect that could be seen after irradiation was a local decrease in the number of cytoplasmic microtubules it was assumed that the reduction in cytoplasmic tension was due to microtubule depolymerization (98). The subapical nucleus of this fungus was found to have a constant number of cytoplasmic microtubules along its length and half this number in front of and behind the nucleus (99). If hyphae were irradiated behind or at the site of the nucleus, the nucleus accelerated forward as the cytoplasm contracted (Fig. 6-8). Anterior irradiation caused the nucleus to stop or to move backward. After these initial events the nucleus continued to move forward at the same rate as before. The results therefore suggested that the microtubules in front of and behind the nucleus were involved in its positioning and imparted a state of structural tension that normally opposed the contractile properties of the cytoplasm (99,101). Because the nuclei and cytoplasm continued to migrate after irradiation, it appeared as though normal nuclear movement and cytoplasmic flow were not microtubule-dependent. By implication, actin may be responsible for these functions in this organism. In a following study they showed that these effects appeared to be mediated by calcium, as the removal of extracellular calcium prevented the effects induced by ultraviolet irradiation (101).

If cytoplasmic movement is indeed generated by contractile actin microfilaments, the actin must be arranged in such a way as to allow cytoplasm to be moved unidirectionally toward the apex. One possible model that would encompass what is known about the distribution of actin would be to have the apical actin anchored to the membrane or cell wall so that the microfilaments drag the cytoplasm forward with the tip by a process of controlled contraction (98). In fungi there are no reports of microfilaments causing organelle movement by a mechanism of lateral translocation, i.e.,

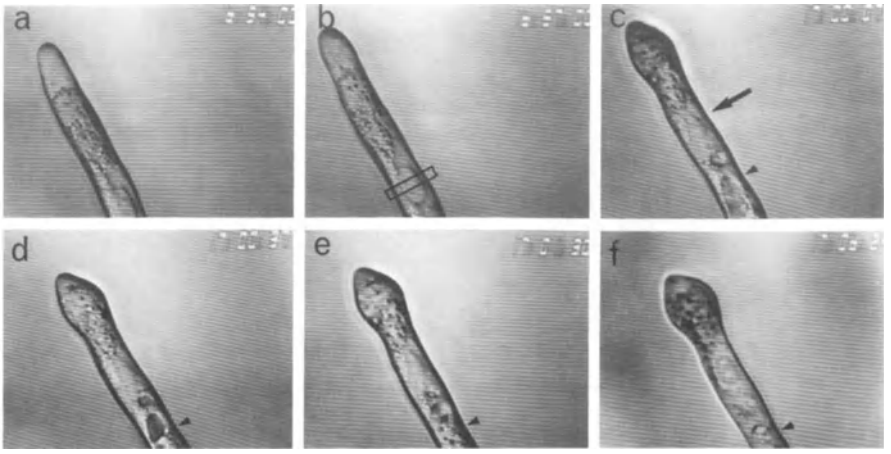


FIG. 6-8. Microbeam irradiation of a hypha of *Basidiobolus ranarum* with ultraviolet light. Irradiation was for 67 seconds starting just after the photograph (b) was taken at the position indicated by the strip corresponding to the midnuclear zone. Following irradiation, the nucleus responded in the same manner as for posterior irradiation: The nucleus (arrow) accelerates toward the tip, causing a decrease in the tip-to-nucleus distance (c–f). The arrowhead marks the site of former irradiation. The cytoplasm has contracted, as the posterior vacuolar cytoplasm has moved forward into the field of view (c). Subsequently, the cytoplasm, as shown by the vacuoles, stretches out again (f). (× 540). (From McKerracher and Heath [99].)

by causing the organelle to move along an actin track in the same way that has been suggested for microtubules. However, in the algae, genera *Chara* and *Nitella*, translocation of heavy-meromyosin-coated beads along actin cables has been observed (85,135). It is therefore not inconceivable that the role of actin in intracytoplasmic transport may involve contractile and translocatory mechanisms.

In addition to the mechanical functions of actin, microfilaments may have structural roles important to hyphal growth. Picton and Steer have proposed that the actin cap in pollen tubes may function in resisting the osmotic pressure of the cytoplasm (116), and the cap of actin described in hyphae of *S. ferax* may have a similar role (56). Finally, it is possible that the peripheral actin may control where exocytosis takes place, perhaps by regulating vesicle–membrane interactions. For example, the cortical microfilaments behind the tip may prevent vesicles from exocytosing with the subapical membrane. If so, however, there may be additional controls operating in the apex to permit exocytosis. The properties of actin gels are greatly affected by the calcium concentration and pH. Possible regulatory effects of these factors in the control of cytoskeletal function in the hyphal tip are considered in the next section.

Ionic Currents and Hyphal Growth

Hyphae are spatially differentiated along their length. The hyphal apex is, however, not only morphologically and ultrastructurally distinct from the region behind it, it is also physiologically different. It has been said that the key to the hypha lies in its tip (36). Thus physiological functions that differentiate the apices from the rest of the hyphae are of interest because they may reveal something of the mechanism that enables growth to be localized. One physiological property of the apex of fungi is that it drives an electrical current into itself (6,40,43,67,90). In 12 fungi that have been examined to date, electrical current flows into the growing tip in every case except one (170): current flows out of the nongrowing subapical hypha. In electrophysiological terms, an electric current is taken to mean the direction of flow of positive charge, so this finding indicates that in extending hyphae there is a net movement of cations into the hyphal tip or of anions out of it. The electrical current is small (0.05–2.50 $\mu A/cm^2$ for most fungi) and requires a specialized ultrasensitive microelectrode to measure it. This type of microelectrode is called a vibrating probe because the electrode tip vibrates during the course of measurement. The vibrating probe is held with a micromanipulator, which is used to move the electrode around the specimen. The technique is noninvasive, as all measurements are made extracellularly. The ultrasensitivity of the vibrating probe is related to the way in which the electrical signal of the specimen is processed. The technical details are complex, but the basis of the technique is not. The signal is measured only at the frequency at which the electrode oscillates, and extraneous noise is filtered out by an amplifier that locks into that frequency. For more details of the instrumentation and technical background to this area, see refs. 77,79,108,109. Before considering the relation between ionic currents and hyphal extension it is important to note that these electrical currents have been found in cells, tissues, and organs of organisms representing all the major groups of eukaryotes (78,109). For once, fungi are among the most extensively studied organisms. As yet, fungal pathogens of animals have not been studied in this way, as the hyphae of these organisms are often smaller and slower-growing than is desirable for electrophysiological work.

Five types of experimental observation suggest there may be a connection between the control of hyphal tip extension and the electrical activities of the cell. All of these observations are essentially correlative and consequently equivocal. Although the discussion here is concerned with fungal systems, it should be noted that electrical currents have been well characterized in other tip-growing cells, particularly in the developing egg cells of fucoid algae (77).

1. The profile of current flow around hyphae is symmetrical with respect to the axis of hyphal polarity for all fungi (6,40,43,67,90,170).

2. Most tip-growing organisms that have been investigated circulate currents in the same way; current enters the site of growth. Aside from fungal hyphae, this group includes the rhizoids of algae and chytridiomycetous fungi, pollen tubes, plant roots and root hairs, and nerve cells. (For a review see ref. 109.) There are three notable exceptions to this pattern. During the formation of lateral branches, the current of the parent hyphal tip of *A. bisexualis* has sometimes been found to turn outward (90). Outward currents have also been seen rarely at the tips of hyphae of *N. crassa* (96) and are always found at the hyphal apex of *Allomyces macrogynus* (170). In *B. ranarum* and *N. crassa* there was weak correlation between the the length of the zone of inward current and the peripheral growth zone (MacCulloch and Gow, unpublished).

3. In general, growing hyphae drive currents, and nongrowing hyphae do not. In *A. bisexualis* the mechanism of current generation has been well characterized, and the current can be turned off experimentally. When this is done, the hyphae stop extending (48,88). An exception is *N. crassa*, where nongrowing hyphae have been found with normal currents (96).

4. Currents precede hyphal branch formation. These currents are directed into the trunk of hyphae at sites where the branches will emerge. This phenomenon has been shown for vegetative branch formation in *A. bisexualis* (90) and sexual, antheridial branch formation in *A. ambisexualis* (43). Because the currents precede morphological events it is conceivable that they actually determine where they take place.

5. Hyphae generate endogenous electrical currents and electrical fields during polarized growth. Reciprocally applied, exogenous electrical fields actively polarize the growth of mycelial fungi (39,95). In *N. crassa* electrical fields affect a number of processes that relate to the polarity of cell growth. Germination of conidia is hastened, synchronized, and polarized; hyphal extension and branch formation are polarized; and branching is stimulated (95). In both *N. crassa* and *A. bisexualis* (Fig. 6-9) hyphae are oriented toward the anode of external electrical fields; however, *Mucor mucedo* and *A. nidulans* exhibit tropism toward the cathode (95). Because the endogenous electrical polarity of all four of these fungi is the same there is no simple correlation between the endogenous electrical field generated by a hypha and the response of the hypha to an exogenous one. Regenerating protoplasts of *Schizophyllum commune* have been shown to form germ tubes preferentially toward the anode (25), and in this system autoradiographic techniques have shown that chitin synthase activity is not affected by the electrical field. For a detailed discussion of the effects that electrical fields induce in fungi, see ref. 39.

Mechanisms of Current Generation in Hyphae

The fact that an ionic current flows through a hypha implies that ion transport systems in the cell membrane are spatially segregated. In *A. bisexualis*

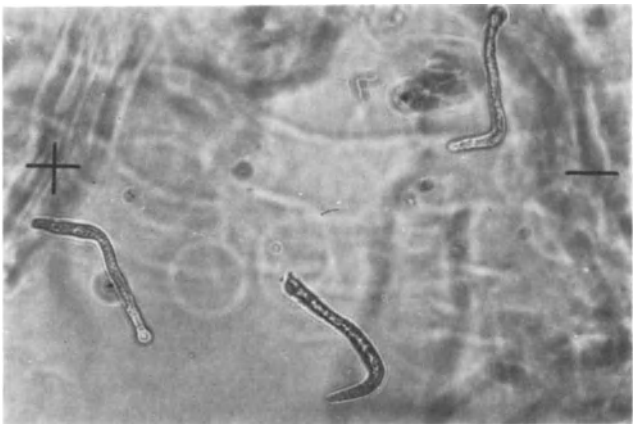


FIG. 6-9. Tropism of germ tubes of *Achlya bisexualis* toward the anode of an applied electrical field of 15 V/cm. The zoospores were first allowed to germinate in the absence of the electrical field and were initially randomly oriented. (From McGillivray and Gow [95].)

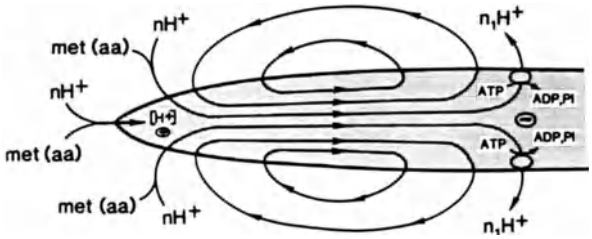


FIG. 6-10. Model for the mechanism of transhyphal electrical current generation in *Achlya bisexualis* in which the electrical current is carried by protons, which enter the apex in symport with methionine and other amino acids and exit at the rear through proton-ATPases. The membrane potential at the apex is relatively depolarized (more positive). (Reproduced from *The Journal of Cell Biology*, 1986, 102:1209–1216, by copyright permission of the Rockefeller University Press.)

the circulating current is carried by protons that enter the hyphal apex by amino acid–proton cotransport/symport and are expelled behind the tip by proton-pumping adenosine triphosphatases (ATPases) (Fig. 6-10) (49,54,87,88). The apex of *A. bisexualis* can therefore be said to be leaky to protons, as they flow into the hyphal tip down an electrochemical proton gradient. This current is therefore a spatially extended chemiosmotic system in which proton pumps are excluded from the apex and proton leaks are inserted preferentially into it. The evidence for this model in *A. bisexualis* is based on electrophysiological studies using three types of electrode: (a) The vibrating probe was used to show that inward electrical current was

abolished by the removal of amino acids from the growth medium or by increasing external pH but not by the removal of other inorganic ions (88). (b) A pH–microelectrode system showed that the growth medium was made alkaline around sites of inward electrical current and acidic at sites of outward current. The alkalinity around the apex was lost when amino acids were removed or the pH of the growth medium was increased (49). (c) Intracellular microelectrodes showed that the membrane potential was less negative at the tip, where positive charges in the form of protons were entering. When amino acids were removed from outside, the gradient of membrane potential was abolished and the membrane became hyperpolarized (87). This finding therefore supported the view that the process of amino acid/proton symport was localized at the tip. In those experiments where the circulating proton current was turned off. e.g., by removing amino acids, the hypha immediately stopped extending. Restoration of amino acids restored tip growth simultaneously (49,88). In these studies it is apparent that methionine carried more current than other amino acids, although other amino acids can support an electrical current (54). It is also likely that there is more than one mechanism of current generation in *A. bisexualis*. If this organism is placed in a medium containing no added amino acids,it forms long, thin branchless hyphae that still drive a proton current (Gow, unpublished experiments).

The current of *N. crassa* has also been examined. The presence of a transcellular current verified the finding of Slayman and Slayman (141), who measured a gradient of membrane potential in this organism using intracellular microelectrodes. Again, the transhyphal electrical current was carried by protons, but this time amino acid transport was not involved in generating inward current (96; Takeuchi et al., in press). Although the current of *N. crassa* requires glucose, glucose symport is probably not involved, as it is known that this transport process is completely repressed by the moderate or high glucose concentrations employed in the experiments (96). Phosphate may also comprise part of the current circulation in this fungus, and calcium ions are required for tip extension but not for the current (96). Experiments with pH-sensitive, permeable dyes showed that the cytoplasm at the hyphal tip of *N. crassa* was relatively acidic (96,158). These experiments and theoretical calculations based on the measured rate of proton influx and the diffusibility of protons in the cytoplasm suggest that the apex of *N. crassa* may be more than one pH unit more acidic than the cytoplasm a few hundred micrometers behind it. The establishment of cytoplasmic gradients of particular ions is therefore a consequence of the circulation of electrical currents.

A proton-based transhyphal ionic current has been proposed to flow through hyphae of the marine fungus *Dendryphiella salina* as a result of the spatial segregation of ATPases (29). It is suggested that this current also involves transport systems for potassium and sodium ions (81).

Significance of Ionic Currents in Hyphal Extension

There are many possible ways by which these electrical and ionic fluxes might bring about polarized cell growth, although at present there is little evidence for any of them. Indeed, there is a real possibility that the electrical current *sensu stricto* is not important. First, it has been suggested that the natural electrical field generated by segregating transport systems may cause the electrophoresis of charged components in the cytoplasm or membrane in a way that controls polarity (76,80). The cytoplasmic electrical fields in hyphae of *A. bisexualis* and *N. crassa* have been estimated at 0.4 and 0.5 V/cm, respectively (87,141); theoretically these voltages are sufficiently large to electrophorese proteins and organelles of a moderate negative charge to the hyphal tip at a rate that can keep up with hyphal extension. There is some evidence for the electrophoretic sorting of proteins in electrically active tissues of certain insects (105,168), but equivalent experiments have not been attempted in fungi. A preliminary study showed that the isoelectric point of chitosomes of *N. crassa* and *Mucor rouxii* were such that they would be negatively charged at physiological pH values and therefore capable of cytoplasmic electrophoresis to an electropositive apex (Gow, Bartnicki-Garcia, Lopez-Romero, Leal-Morales, and Bracker, unpublished). It is also possible that electrical currents cause electrophoretic water flow and movement of solute toward the apex (81). Again, there is no direct evidence that membrane components are moved in the electrical field of a fungus, although it is perhaps easiest to interpret the polarizing effects of applied fields as operating in this way. Elsewhere, electrical fields of physiological strength have been shown to influence the distribution of membrane proteins (39). Alternatively, the ionic consequences of the electrical activities of hyphae may be invoked. Gradients of pH and calcium have been shown to exist in the hyphae of fungi (96,121,158); they may arise as a consequence of the spatial localization of ion transport systems. Also, the formation of branches in *A. bisexualis* can be stimulated using proton or calcium ionophores (55). Calcium ions are not a component of the net flow of electrical current through the hyphae of *A. bisexualis* or *N. crassa*, but membrane-associated calcium has been shown to be locally high at the hyphal apex of *Achlya* using chlorotetracycline fluorescence (121). In *Chaetomium*, X-ray microanalysis has also demonstrated locally high calcium levels at the apex (30). The role of calcium in hyphal extension is not clear. For example, hyphae of *A. bisexualis* and *A. macrogynus* extend over the short term in media lacking calcium and containing 1 m*M* of the calcium chelator ethylene guanosine tetraacetate (EGTA) (88,170). Under the same conditions, extension of the hyphae of *N. crassa* is completely inhibited (96). Gradients of protons or calcium may affect the function and organization of the cytoskeleton or the activities of enzymes or secondary messengers. There is evidence suggesting that there may be an interplay between ionic currents and the cytoskeleton. For ex-

ample, during the process of polarization of the zygote of the alga *Pelvetia* it has been shown that cytochalasin inhibits the influx of calcium ions and that calcium influx appears to stimulate actin polymerization (15). As mentioned previously, calcium ions were also implicated in the control of ultraviolet-light-induced perturbation of the fungal cytoskeleton (101). Clearly, a great deal stands to be gained from bridging the electrophysiological and cytoskeletal perspectives of hyphal growth.

The evidence that has been gained so far from studies of fungi and in particular the water moulds allows some evaluation of the plausibility of the possible mechanisms mentioned above. For example, it seems increasingly unlikely that the flow of electrical current is essential for tip growth. In *A. bisexualis* the current of branching hyphae sometimes turns outward transiently, yet tip growth continues unabated (90). Also, media containing only thioglycolate and urea support normal rates of extension and normal-sized hyphae but greatly reduced currents (54). In *N. crassa*, growing hyphae with outward currents and nongrowing hyphae with normal-sized currents have been found (96). An illuminating counterexample to the normal inward currents of hyphae has been found in the chytridiomycete *A. macrogynus*, where growing and nongrowing hyphae and hyphae undergoing reversed growth were studied. In the latter, hyphae were not extending but, instead, widened their hyphae from the apex and backward toward the encysted spore from which the hypha was produced (170). All these hyphae had outward currents (170). In this fungus, inward current was found around the branching rhizoids and so followed the current pattern reported for the other chytrid, *Blastocladiella emersonii* (Fig. 6-11). Nutrient transport in *B. emersonii* has been shown to occur predominantly into the rhizoids and that in *A. bisexualis* into the hyphal apex (89). In *A. macrogynus* the rhizoids are more strongly chemotropic than the hyphae, and so nutrient transport might be restricted to the rhizoids. Comparison of the current patterns of these three organisms in the light of these findings suggests that the zones of inward current may indicate sites of nutrient uptake by some electrogenic mechanism such as proton symport (Fig. 6-11). On close examination, therefore, the correlation between inward current and hyphal growth seems to break down.

Although there are several reported instances where tip growth and inward electrical current have been found to be dissociated, there is a much better correlation between proton entry and hyphal extension. The alkalinity measured in the growth medium adjacent to tips of *A. bisexualis* has been interpreted as being a manifestation of the inwardly directed proton flux (49). This apical alkalinity was found to persist even in branching hyphae, which might have been expected to have transitory outward currents (49). Growing hyphae therefore continued to take up protons even when the net flow of electrical current was reversed. The available evidence supports the view that proton currents and pH gradients are more important to vegetative hyphal growth than are electrical currents and vol-

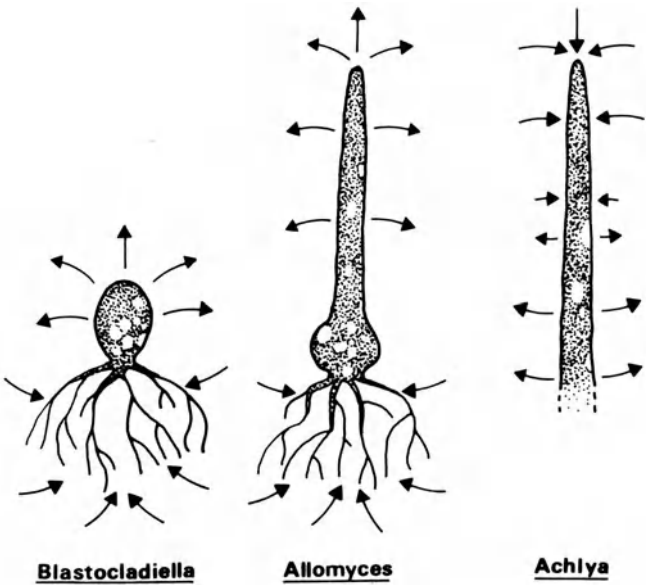


FIG. 6-11. Profiles of circulating currents in *Blastocladiella emersonii*, *Allomyces macrogynus*, and *Achlya bisexualis*. Positive current enters the branching rhizoids of *Blastocladiella* and *Allomyces* and the hyphal apex of *Achlya*. Current leaves the hyphal tip of *Allomyces*. Inward current correlates with the sites at which nutrients enter the organisms, i.e., the rhizoids of the chytridiomycetes and the hyphal tips of mycelial oomycetes. (From Youatt et al. [170].)

tage gradients. Standing gradients of free calcium may also be important in the regulation of hyphal extension; however, the relation between calcium and hyphal growth is not clear.

Wall Biosynthesis and Hyphal Extension

A significant proportion of research that has been concerned with the study of hyphal growth has devoted itself to the biochemistry of cell wall synthesis. A great deal is known about the composition of fungal cell walls, although the enzymology of hyphal growth is less well understood. These areas have been summarized elsewhere, and the reader is directed to the excellent reviews by Gooday (36), Gooday and Trinci (38), Farkas (27), Trinci (153), Bartnicki-Garcia (8), and most recently Wessels (166) and Cabib (18). Germ tube and hyphal wall biosynthesis in dimorphic fungi have been also been well reviewed (113,131,136,137,146) and are not dealt with here in any detail.

The walls of fungi consist mainly of polysaccharides; chitin, chitosan, α- and β-glucan; mannan; and in the oomycetes cellulose (27). These polysaccharides are arranged in overlapping layers that are embedded in an amorphous matrix of protein and glycoprotein. The mature hyphal wall is rigid and opposes the turgor pressure exerted on it by the cytoplasm. As described later, the wall at the hyphal tip is thought to be plastic so that it can be expanded by the turgor of the cell. To describe the process of wall synthesis during hyphal extension, it is necessary to understand something of the properties of the individual enzymes that are involved as well as how the activities of these enzymes are coordinated and controlled. To this end, there have been many advances that have improved and widened our understanding of the biochemistry and molecular biology of specific enzymes that are concerned with wall growth and the spatial organization and interplay between these enzymes. Particular attention has been paid to the synthesis of chitin and β(1-3)-glucan. Much less is known about the biosynthesis of β(1-6)-glucan, α-glucan, mannan, and cellulose.

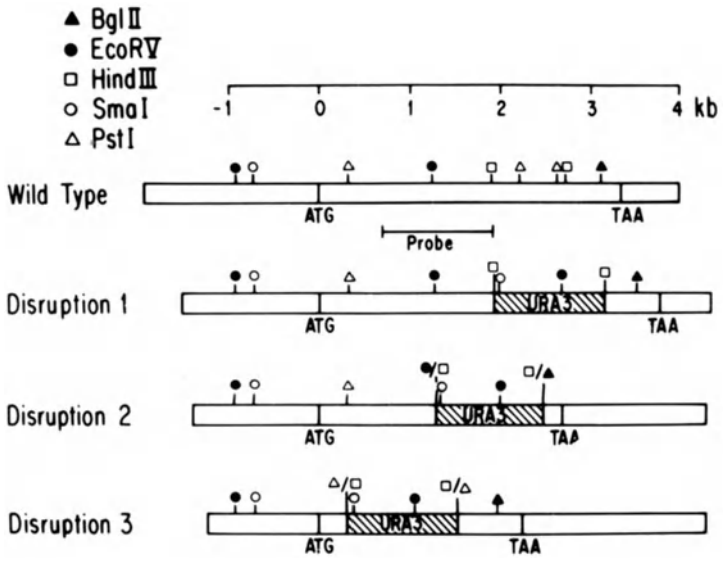
Chitin and β-Glucan Synthesis

Chitin and β(1-3)-glucan, along with cellulose, are the dominant structural polysaccharides in filamentous fungi and yeasts. For this reason these polysaccharides are of particular importance to hyphal structure and tip extension. At the hyphal tip the thickness of the wall is more or less constant in the hemiellipsoidal zone of extension (154) and then thickens progressively in the subapical region. In *N. crassa* and *F. acuminatum* the secondary thickening occurs mainly in the outer layers and not in the innermost chitin-containing layers (68,154). In *N. crassa* and most other filamentous fungi that have been examined the chitin at the apex was found to be similar to that in the lateral walls, in that it is organized as distinct microfibrils (17). These microfibrils are easily examined in shadow-cast preparations for electron microscopy, as other polysaccharides e.g., the various glucans and mannan, can be removed by solubilization in strong alkali and weak acids. In *S. commune*, however, the chitin is less fibrillar and more loosely arranged at the apex (162). This apical chitin is also susceptible to solubilization in chitinase and acids that had little effect on the mature chitin microfibrils in the subapical wall (161). In *N. crassa* the microfibrils are wider behind the apex (17). Chitin microfibrils in dimorphic fungi are shorter than those in obligately mycelial organisms (45). In all fungi the chitin synthase appears to be in a zymogenic form in the cytoplasm, and chitin is synthesized only after the enzyme is activated by proteolysis at the cell membrane. The substrate for chitin synthase is UDP-*N*-acetylglucosamine; activity requires magnesium ions and is stimulated by *N*-acetylglucosamine (36).

In two reports that are certain to become landmarks in the field, Bulawa

et al. (16) and Sburlati and Cabib (133) demonstrated that *Saccharomyces cerevisiae* has two structural genes for chitin synthase. These studies began with the selection of temperature-sensitive mutants with decreased chitin synthase activity. The mutant genotype was called Chs1. Restoration of chitin synthase activity by transformation of one of these strains with fragments of wild-type DNA from a genomic library provided a specific vector that carried the chitin synthase gene. This gene was cloned, amplified in *Escherichia coli*, and sequenced. The vector carrying the gene could be transformed into and expressed in *Schizosaccharomyces pombe*, which does not have any chitin or chitin synthase of its own. Perhaps most interesting of all, it was found that the Chs1 mutant strains were not morphologically abnormal. Moreover, the wild-type gene of the parent strain could be disrupted by inserting the URA3 gene into the chitin synthase structural gene. Gene-disrupted strains were selected on the basis of uracil prototrophy (Fig. 6-12). The chs1 : URA3-disrupted strains all lacked chitin synthase activity in vitro, yet were viable, had normal morphology (Fig. 6-12), had a normal level of total chitin, and were able to mate and sporulate efficiently. The only observable dysfunction was a tendency for buds to lyse when in the stationary phase of growth in minimal medium. Because the mutant strains still had a functional chitin synthase enzyme the wild type must have two chitin synthases. Later, a second biosynthetic enzyme (Chs2) was characterized in the gene-disrupted mutant. This enzyme had only 5% of the activity of Chs1 in wild-type cells and differed from Chs1 enzyme in its pH optimum and its divalent cation specificity (133). The chitin content in Chs1 cells was normal, suggesting that the Chs2 enzyme was responsible for its synthesis. It is possible that the two enzymes have different functions and that the Chs2 enzyme synthesizes the bud scars and Chs1 the thin lateral walls of *S. cerevisiae*. Sburlati and Cabib argued that this finding may be related to the way in which yeasts have evolved. In the filamentous fungi, the lateral walls and septa are chitin-rich. In *S. cerevisiae* much more chitin is in the bud scars than in the wall. If this yeast is descended from a filamentous fungus, it is possible that the ability to activate the zymogenic chitin synthase in the lateral wall was lost at some stage. It will be fascinating to see if filamentous fungi also turn out to have two chitin synthases and if the more active one is homologous to the Chs1 of *S. cerevisiae*, as predicted from these results. The implications with regard to the control of yeast-to-hypha transitions in the dimorphic fungi are no less exciting.

In filamentous fungi, with the exception of the zygomycetes, the wall β-glucans occur as a mixture of $\beta(1\text{-}3)$ and $\beta(1\text{-}6)$ glycosidic linkages. The $\beta(1\text{-}6)$ linkages form the branches of the $\beta(1\text{-}3)$-glucan polymer; reciprocally, $\beta(1\text{-}3)$ linkages form branch points in $\beta(1\text{-}6)$-glucan (27,136). Cell-free extracts tend to synthesize microfibrils of unbranched $\beta(1\text{-}3)$-glucan exclusively (91,119), and so the control of branching and $\beta(1\text{-}6)$-glucan production is not understood. The $\beta(1\text{-}3)/\beta(1\text{-}6)$-glucan in vivo is probably



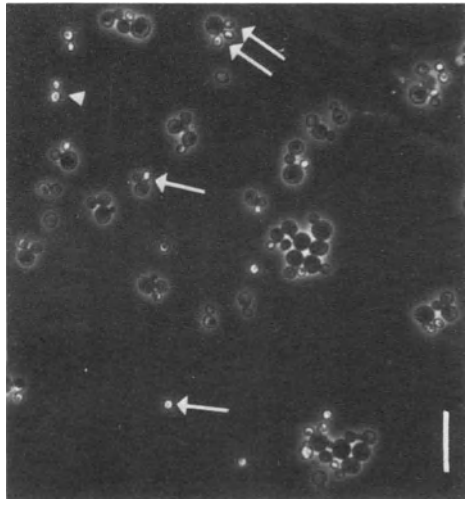


FIG. 6-12. Three chitin synthase gene-disrupted mutants of *Saccharomyces cerevisiae* in which the URA3 gene was inserted into the reading frame of the structural gene. The photograph on the right shows the morphology of cells with disrupted CHS1 genes. The cells grew and budded normally and had a normal chitin content. The only observable phenotypic abnormality was an increased tendency for buds to burst in minimal medium. The arrows indicate refractile lysed cells; the scale bar is 20 μm. (From Bulawa et al. [16].)

not microfibrillar but rather, a gel-like matrix, that in *S. commune* can also be covalently linked to chitin. β(1-3)-Glucan also appears to be synthesized at the apex (140), although preformed glucans have been seen in the vesicles of *Saprolegnia monoica* (28). There is now evidence in a range of filamentous and yeast-like fungi that the enzyme β(1-3)-glucan synthetase is probably another membrane-associated enzyme (31,82,147,151); however, unlike chitin synthase, it is not a zymogen. Regulation instead seems to be mediated by activation by nucleoside and nucleotide triphosphates (147) and inactivation by phosphatases (119). There are differences in this enzyme in different fungi, as GTP has been shown to be a powerful acivator of this enzyme in *N. crassa*, *A. ambisexualis*, *S. cerevisiae*, *S. commune*, *Cryptococcus laurentii*, *Hansenula anomala*, *Wangiella dermatitidis* (147), *C. albicans* (114), yet inhibitory in *A. ambisexualis* (147), *Aphanomyces astaci* (20), and *Paracoccidioides brasiliensis* (130). Perhaps this research heralds the recognition of multiple genes for β(1-3)-glucan synthetase with different functions as has been found for chitin synthase in *S. cerevisiae* (16,133). In *S. cerevisiae* there is evidence that the guanine-nucleotide binding function may be the property of a separate cytoplasmic protein, which may be instrumental in the regulation of cell wall morphogenesis (82). The substrate for β(1-3)-glucan synthetase is UDP-glucose; the enzyme does not require metal ion for activity (119).

Chitin and β-Glucan Lysis

The need for wall lysis in the initiation of hyphal branching is generally accepted, but there is some controversy regarding the putative functioning of autolysins in the process of hyphal extension. One view is that the expansion of the hyphal tip occurs by wall synthesis, which is in delicate balance with wall lysis (8). Accordingly, control over the assembly of chitin and β-glucan at the tip depends not only on biosynthesis but also on controlled autolysis, with the lytic enzymes allowing the insertion of new polymers within the polysaccharide chains and maintenance of a tip of sufficient plasticity to permit turgor-driven expansion. A membrane-bound, zymogenic chitinase that required phospholipid for activity and that was closely associated with chitin synthase has been reported in hyphae of *M. mucedo* (37,73,74). This chitinase has many of the attributes of a lytic enzyme that is involved in tip morphogenesis, although its location in the hypha has not been determined. β(1-3)-Glucanase has been found in a number of filamentous fungi (28) and yeasts (19). In hyphae of *Sclerotinium rolfsii* this enzyme was localized at the hyphal tip and at other sites of wall biosynthesis using immunocytochemical methods (86). In other cases the activity was not localized within the intact hypha but was known to be associated with the membrane, walls, or periplasm (28). Some of these examples may have roles unrelated to tip morphogenesis.

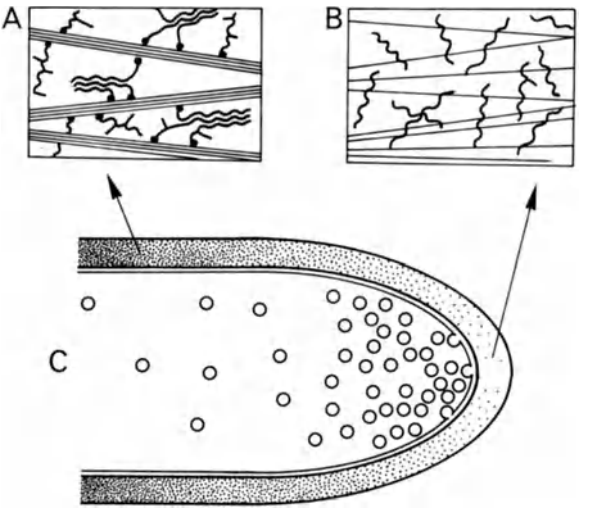


FIG. 6-13. Possible changes in wall structure in the growing hyphal apex of *Schizophyllum commune*. The increase in stippling in (C) indicates an increase in rigidity brought about by increased cross-linking and crystallinity of chitin and β-glucan chains. (A) Mature wall with chitin shown as distinct microfibrils that are cross-linked to partly crystallized β(1-3)-glucan chains (wavey lines), which have some β(1-6)-linked branches. (B) Extreme tip at which chitin chains (straight lines) are loosely organized and free from cross-linkages to β(1-3)-glucan, which does not have many β(1-6) branches. (From Wessels [166].)

Orchestration of Wall Synthesis and Lysis

Wessels and collaborators have produced an elegant model for control of the synthesis of chitin and β-glucan synthesis during hyphal tip extension of *S. commune* (138–140,143,161,162,166,167). This model does not require the invocation of lytic enzymes but, instead, suggests that the plasticity of the tip is related simply to differences in the arrangement of structural polysaccharides at the tip and in the subapical region. It was demonstrated using radiolabeled wall precursors and microautoradiographic techniques that the β-glucan was initially synthesized at the tip in the form of alkali-soluble β(1-3)-glucan (140,167). Behind the tip β(1-6) linkages were added, and the β(1-3)/β(1-3)-D-glucan became increasingly insoluble in alkali. The reason for the change in the β-glucan from an alkali-soluble form to an alkali-insoluble form could be attributed to the formation of covalent bonds between the glucan and the chitin microfibrils (167) (Fig. 6-13). The chitin at the apex, although alkali-insoluble, was susceptible to degradation by chitinase and solubilization in dilute mineral acid. Behind the tip chitin was little affected by these treatments suggesting that it may become more crystallized (161,162). The plasticity of the tip was therefore

reasoned to be due to the absence of chitin–glucan crosslinks and the presence of noncrystalline chitin. Further ultrastructural studies verified that the chitin was indeed loosely organized at the tip (162). The apices of nongrowing hyphae are thought to become rigid because they swell and must branch from the tip (167). The fact that they are unable to resume growth from the original tip is taken as evidence that it becomes irreversibly rigid. In nongrowing hyphal tips chitin–glucan covalent linkages extended right to the apex. This finding is also in accord with what is known about the events following the cessation of tip extension. These findings provide a precise biochemical explanation for the hypotheses developed during the 1960s by Robertson, who suggested on the basis of microscopic observations of the growth and inhibition of growth that hyphal extension occurred by apical synthesis and subapical rigidification (123). Analogous studies in hyphae of *C. albicans* have shown that chitin–glucan linkages probably form behind the tip, making the lateral wall more rigid (136).

The study above suggests that lytic enzymes are not required to plasticize the tip but in no way preclude them from being present. However, in the light of these findings it has been suggested that the inherent sensitivity of apical chitin and glucan to lytic enzymes makes their controlled lysis a precarious business (17,166). Elsewhere it has been shown that there is little wall turnover in growing hyphae (124) and that mutations in exo-β(1-3)-glucanase do not affect the morphology or growth of *S. cerevisiae* (19). These findings also cast doubt on the notion that hyphal extension involves controlled lysis. However, these arguments must be weighed against the finding of lytic enzymes, which apparently modulate biosynthesis (60,74, 93). The argument that lyticases endanger tip bursting must be evaluated in the light of suggestions that the elements opposing turgor pressure of the cytoplasm at the apex may be a function of the peripheral actin microfilaments. In other words, the tip might be supported by an endoskeleton and the lateral membrane by an external one. There is also some evidence to show that inhibition of autolysin activity affects hyphal extension. For example, in *S. monoica* the presence of the β-glucanase inhibitor gluconolactone inhibited hyphal extension and branching and caused an increase in hyphal diameter (28). The absence of equivalent inhibitory compounds for chitinase has so far prevented a complementary analysis for chitinase; however, the availability of the antichitinase antimicrobic allosamidin should make the requisite study possible. This compound has been shown to be a potent inhibitor of the chitinase of *N. crassa* (Gooday and McNab, unpublished).

Clearly, the hyphal tip is elastic in comparison with the lateral wall, and this elasticity allows the cytoplasmic turgor pressure to expand the tip. Mathematical models have been formulated in which the counteracting forces of turgor and surface stress and elasticity are examined (84,132). These models can accurately predict the shapes of hyphal tips and some of the effects of increasing or decreasing the wall plasticity or turgor pressure.

They do not, however, cast light on the nature of the mechanism, passive or active, that maintains the delicate biophysical condition of the hyphal apex.

Conclusions

Hyphal extension is achieved by mobilizing the biosynthetic capacity in the peripheral growth zone for localized growth at the extreme apex. Only wall biosynthesis is polarized, and exceptions to tip growth of hyphae do exist. The membrane and enzymes for tip growth arrive in vesicles of two size classes. The macrovesicles probably carry a diversity of enzymes and most of the membrane. The microvesicles are equivalent to chitosomes and carry only chitin synthase. Coated vesicles exist in filamentous fungi and, along with endocytotic vesicles, may be involved in active recycling of the cell membrane.

The organization and function of the microfilaments and microtubules in the cytoplasm is now being elucidated by fluorescence staining, immunocytochemistry freeze-substitution electron microscopy, and novel techniques such as ultraviolet microbeam analysis. Microfilaments are labile and prone to artifactual rearrangement during staining with rhodamine–phalloidin. Evidence so far suggests that most of the actin in hyphae is at the tip, where it occurs immediately under the cell membrane. Present evidence based on ultrastructural studies favors a microtubule-based mechanism for the intracytoplasmic transport of apical vesicles. Physiological and genetic studies implicate microfilaments in this capacity. Microtubules run longitudinally throughout the length and depth of the cytoplasm and are implicated in mitochondrial and nuclear movement in some fungi but not in others. Other evidence suggests that microtubules have a role in positioning the nucleus and providing structural support to the cytoplasm. Actin may be anchored to the apical cell membrane and move the bulk cytoplasm forward by a process of contraction that is regulated by local gradients of calcium and protons.

Ionic currents are probably ubiquitous in mycelial fungi. In most but not all cases, the current enters the growing apex and the presumptive sites of branches and is carried by protons in those fungi that have been examined. Hyphal growth is oriented in applied electrical fields, but it is not known how it relates to the process of polarized growth. Evidence to date suggests that the flow of electrical current through a hypha is inconsequential to its polarity but that ionic fluxes and ion gradients may be an important aspect of hyphal extension.

Molecular biological techniques have been used to investigate cell wall biosynthesis. Yeasts and possibly other fungi may have two chitin synthase genes, Chs1 and Chs2. These enzymes may synthesize the wall and septa,

respectively. The synthesis of the other major structural polysaccharide, β(1-3)-glucan, may be regulated via activation by nucleotide phosphates and inactivation by dephosphorylation. Dynamic models of apical wall growth propose that the plasticity of the tip is an inherent property of the apex, as chitin microfibrils are still relatively noncrystalline and the β(1-3)-glucan is not covalently bound to the chitin in this region. However, enzymological studies provide circumstantial evidence for hydrolytic enzymes such as chitinase, which may function in a way that controls the plasticity of the hyphal tip.

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7—Epidemiology and Ecology of Pityriasis Versicolor

JAN FAERGEMANN

Pityriasis versicolor is a chronic superficial fungal disease. It is usually localized on the upper part of the chest or on the back, neck, or upper arms. Lesions may be red, brown, or white. They are often slightly scaling, papular, or nummular, or they may coalesce to involve larger areas of the body. The conidia and short hyphae of the lipophilic yeast *Malassezia furfur* (syn. *Pityrosporum orbiculare*) can be detected by microscopy.

History

The fungus *Microsporum furfur* was described in skin scales from a patient with pityriasis versicolor by Robin in 1853 (54). Rivolta described the presence of round double-contoured budding cells in skin scales from a patient with psoriasis in 1873 (50), and in 1874 Malassez described round and oval budding cells in the stratum corneum of patients with various skin diseases (40). Bailon gave the fungus the name *Malassezia furfur* after Malassez. There were numerous attempts to culture the organisms, but Castellani and Chalmers were the first to be able to constantly culture the fungus from skin scales (9). They named the organism *Pityrosporum ovale*, and their description of the organism is based on both the description of the fungus in skin scales and on cultural characteristics (9). A nonlipophilic member of the genus, *Pityrosporum pachydermatis*, was cultured from animals and described by Weidman in 1925 (60). In 1951 Gordon isolated a round, double-contoured yeast producing spherical to oval cells from both pityriasis versicolor scales and normal skin (33,34). He named the yeast *P. orbiculare*. During recent years the identity of the oval and the round forms of the organism has been discussed, and there are now several reports in favor of them being synonymous (18,25,29,43,56). The oval and round forms may represent different stages in a cell cycle of the same organism.

Nomenclature

There has been considerable discussion regarding the proper generic nomenclature for this yeast. Some researchers believe that the name *Malassezia* should be used instead of *Pityrosporum*. It is my opinion that *Pityrosporum* should be used because Malassez was not the first to describe or culture the organism. *Pityrosporum ovale* was used in 1913 by Castellani and Chalmers who, for the first time, were able to describe the fungus in skin scales as well as add cultural characteristics to the description of the fungus (9). The term *P. pachydermatis* has been used for many years for the nonlipophilic member (60). However, for the rest of this chapter the name *Malassezia furfur* is used simply to maintain consistency in this review series.

Etiology

The etiological significance of *M. furfur* in pityriasis versicolor has been documented in several investigations (6,18,25,29,33,43).

Cultural Characteristics

M. furfur is lipophilic and requires the addition of lipids to the culture medium for optimal growth. The standard medium in our experiments for isolation and continuous growth is a glucose–neopeptone–yeast extract medium with the addition of olive oil (20 ml L^{-1}), Tween 80 (2 ml L^{-1}), and glycerol monostearate (2.5 gL^{-1}) (25). On this medium colonies are visible after 2 days' incubation at 37°C, and optimal growth is obtained after 3–4 days. The lipids are incorporated into the medium, and it is therefore easy to work with.

Although some workers observed no differences in lipid requirements and metabolism (45), others found the round variety of *M. furfur* to be more strictly lipid-dependent (60). It is able to grow in the temperature range 28–37°C (7,21,51,52,60), but most isolates grow better at 37°C (21,51,52). The optimal pH for growth is between 5.5 and 6.5. Optimal growth is obtained not only under aerobic conditions but also in an aerobic milieu with 7% CO_2 and under microaerophilic conditions (20).

Colonies are slightly raised with irregular edges, white to creamy in color, and 3–6 mm in diameter (21) (Fig. 7-1). Older colonies often turn yellow, especially if the humidity in the incubation chamber is low. Macroscopically, no difference is seen between the round and oval forms of the organism but their micromorphology is different. The round cells are 3–6 μm in diameter, usually producing single globose buds on a narrow base

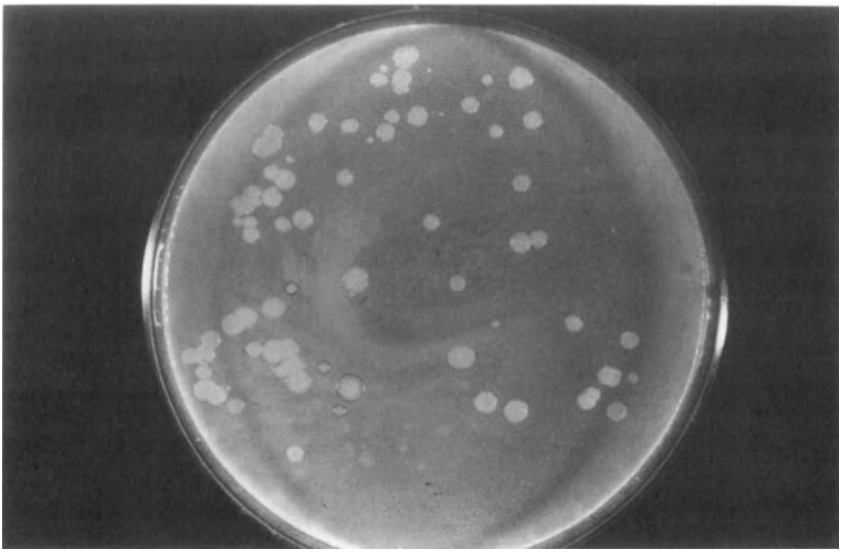

FIG. 7-1. Colonies of *Malassezia furfur* on glucose–neopeptone–yeast extract agar medium with lipid supplements.

(12) (Fig. 7-2). Sometimes several buds are produced in a row. The oval or cylindrical cells are 2–3 × 4–5 μm and produce single oval buds from a broad base (12) (Fig. 7-3). Although the micromorphology of the two forms is different, there have been reports that the forms are interchangable in culture (47,56).

Isolation and Distribution

M. furfur not only is the etiological agent of pityriasis versicolor, it is also a member of the normal human cutaneous flora in adults (21,34,52). It is uncommon on the skin of children (23): In one survey *M. furfur* could not be cultured on normal-looking skin taken from the backs of children under age 5 but was found in 93% of 15-year-old children (23). Colonization of normal skin with *M. furfur* begins with the increase in sebum excretion during prepuberty and puberty. *M. furfur* is most commonly isolated from the chest, upper back, and scalp (21,34,52). A method for quantitating a culture of *M. furfur*, uses a modification of the Williamson-Kligman scrub technique (14). Various skin locations were studied, and the highest number of organisms per square centimeter were found on the upper trunk (333) and the lowest on the hand (2) (19). This distribution parallels regional variations in sebum excretion and the distribution of pityriasis versicolor lesions. Using the quantitative culture technique, the number

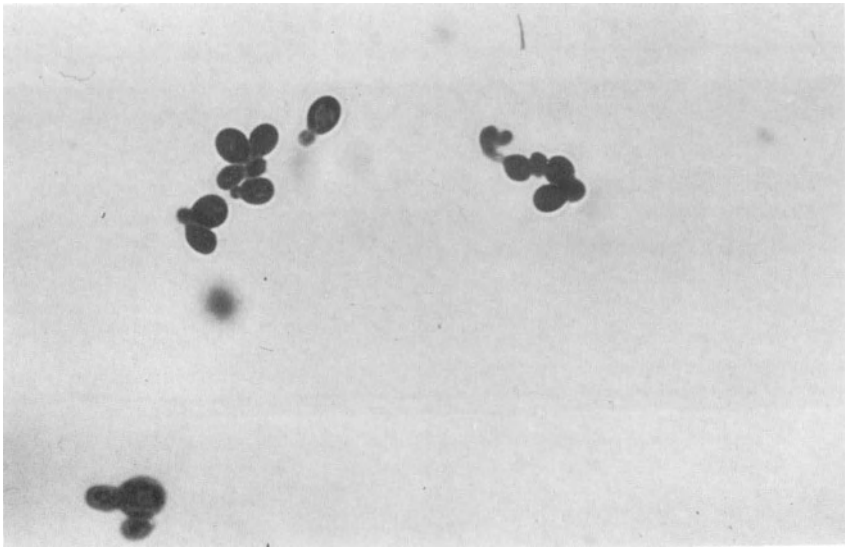

FIG. 7-2. Microscopic appearance of the round form of *Malassezia furfur*. (× 400)

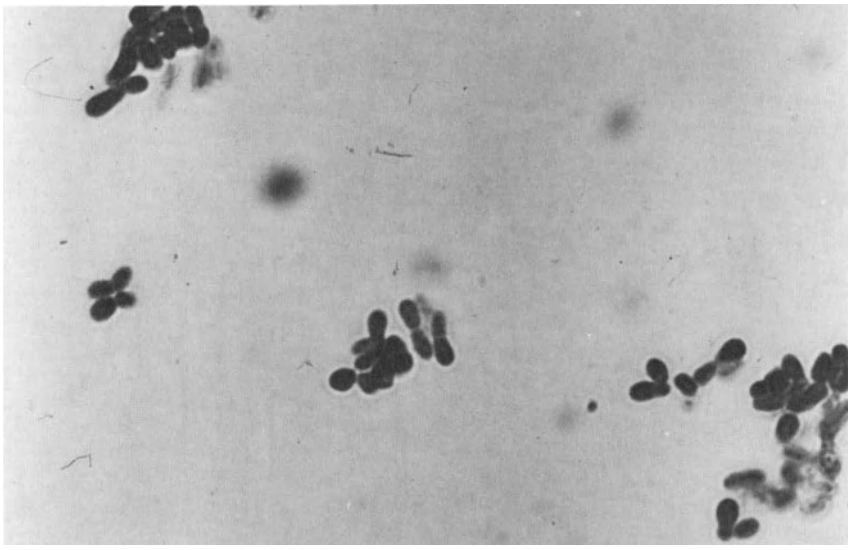

FIG. 7-3. Microscopic appearance of the oval form of *Malassezia furfur*. (× 400)

of organisms on skin (normal and diseased) was significantly higher in patients with pityriasis versicolor compared to normal skin in healthy individuals (14). In one investigation we quantitatively cultured normal-looking skin of the chest from healthy volunteers 30–80 years of age (unpublished data). There was a statistically significant reduction in the number of cultured organisms on the skin of the 80-year-old individuals compared to those on the 30-year-old subjects. These results, together with the results from the culture study in children (23), reflect the age distribution of pityriasis versicolor: The disease is most commonly seen in individuals 20–40 years of age (27,51).

Immunologic Characteristics

Serum antibodies (immunoglobulin G, IgG) against *M. furfur* are present in sera from healthy adults (11). Titers are very low in children (11) and are low in 80-year-old individuals compared to 30-year-old subjects (unpublished data). Antibodies against the yeast are produced when an individual becomes colonized with the yeast. Serum antibodies against *M. furfur* are found in the same titers in sera from both healthy adults and patients with pityriasis versicolor, indicating that determining titers is of no consequence (11). A cell-mediated immune (CMI) response to *M. furfur* has also been demonstrated in patients and healthy individuals. Both groups demonstrated positive lymphocyte transformation responses, but the response was significantly less in patients than in controls (61,62). Lymphocytes from patients with pityriasis versicolor produced significantly less leukocyte migration factor when stimulated with *M. furfur* extract (61).

M. furfur and Pityriasis Versicolor

There are now many reports indicating that the cultured *M. furfur* and the fungus seen in pityriasis versicolor lesions are identical. Antigenic similarities have been found between the round and the oval forms of the cultured yeast and the fungus seen in pityriasis versicolor lesions (29). Hyphae have been produced in vitro in both the round and oval forms (18,43). We were able to produce hyphae on human stratum corneum in vitro (18) with the longest hyphae being 50–60 μm that were produced from about 40% of the cells. We observed not only the production of hyphae from a large proportion of cells but also the conversion from one form to another (Fig. 7-4). Identical experimental infections that were clinically similar to pityriasis versicolor have been produced with both morphological varieties of the yeast on both humans and the inside of the ear of rabbits (25). Infections were produced with plastic occlusion, and the lesions healed spontaneously within 2 weeks after the occlusive bandages had been removed.

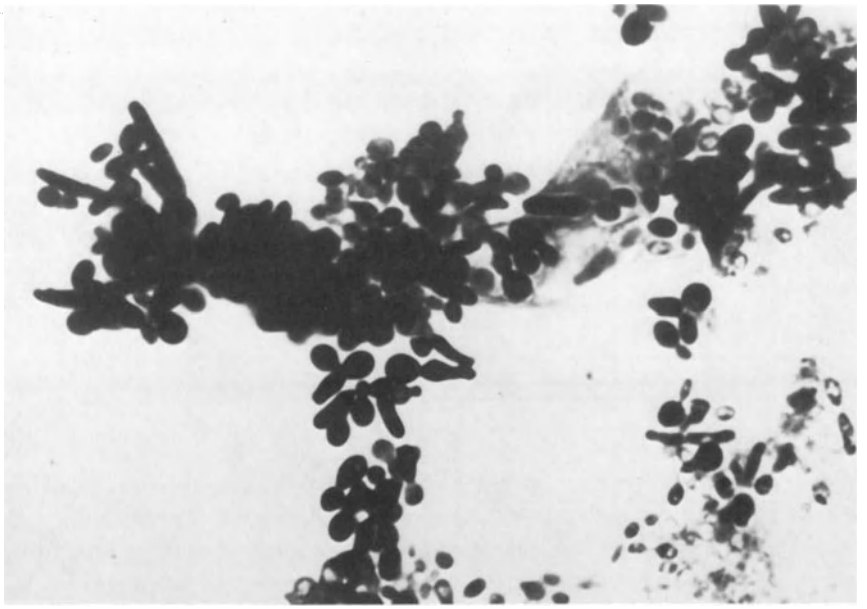

FIG. 7-4. Induction of hyphae and the conversion from the round to the oval form of *Malassezia furfur* on human stratum corneum in vitro.

Microscopically, the production of hyphae and variations between the different forms were observed (25).

M. furfur and Other Diseases

M. furfur is also an etiological agent in *Malassezia* folliculitis and seborrheic dermatitis. Its role in confluent and reticulate papillomatosis (Gougerot-Carteaud), some forms of atopic dermatitis, and psoriasis is also suspected. Even systemic infections with this yeast have been reported.

Malassezia folliculitis is a chronic disease consisting in pruritic follicular papules and pustules located primarily on the upper trunk, neck, and upper arms (3,30). In skin sections, abundant round budding yeast cells of *M. furfur* and sometimes even hyphae are seen in dilated follicles. The disease improves rapidly during antimycotic therapy.

Seborrheic dermatitis is characterized by yellow, greasy scales covering red, inflamed skin. It is located most commonly on the scalp, face, and chest. There are now several studies indicating an association between the lipophilic yeast *M. furfur* and seborrheic dermatitis (15,59). Most are treatment studies that report a good effect of antimycotics (15,59), but there have also been reports of experimental infections in animals (10). Others

have reported high serum IgG antibodies in patients compared to those in controls (2). Clearing of seborrheic dermatitis with antimycotics is accompanied by a reduction in the number of yeasts; recurrence of the disease is characterized by an increase of yeast cells (15).

Confluent and reticulate papillomatosis is a rare disease first described by Gougerot and Carteaud in 1927 (35). It consists in grayish-brown pigmented papules that later coalesce and are most often localized in the intermammary and interscapular regions, neck, or abdomen of young women. *M. furfur* conidia are often found in the scales, and several patients have been cured after topical treatment with antimycotics (28,53).

Psoriasis is a disease of multifocal etiology. Poststreptococcal psoriasis, especially the guttate variety, is well known. Rosenberg and co-workers believe that *M. furfur* may play a role in some forms of psoriasis (55) and reported that antimycotics had a good effect in some patients. This beneficial effect of antimycotics has also been reported for psoriasis localized to seborrheic areas (sebopsoriasis) (17).

Hjorth and Clemmensen have associated *M. furfur* with a special form of atopic dermatitis localized to the scalp, face, and neck and with an associated type I allergic reaction to *M. furfur* antigens (38). In a double-blind study these patients were cured with ketoconazole given orally (38).

M. furfur is not only found on the skin and in hair follicles but has been associated with systemic disease. It has been found and cultured from the peritoneal dialysis fluid of a patient on peritoneal dialysis having recurrent episodes of peritonitis (64). It has also been isolated from the nasopharynx of a patient who had received long-term antimicrobic treatment for maxillary sinus osteitis (44). A fatal case of pulmonary vasculitis caused by *M. furfur* in a premature girl baby has been reported (49) in which the patient was on intralipid therapy, and abundant yeast cells were found throughout the vessel wall. In another premature infant on intralipid therapy who developed sepsis, *M. furfur* was cultured not only from the blood but also from the central venous catheter (46).

Pathogenesis

Under the influence of predisposing factors (both exogenous and endogenous), *M. furfur* changes in pityriasis versicolor from the round blastoconidial form to the mycelial form. The most important exogenous factors are high temperatures and a high relative humidity, which probably explain the fact that pityriasis versicolor is more common in tropical areas (5,41). A contributing factor may also be that inhabitants in tropical countries often apply palm oil to their skin. Lesions are commonly seen on occluded skin areas, but in tropical areas they are also often located on the face, probably due to high temperature and high relative humidity (5,41).

In temperate climates endogenous factors such as greasy skin, hyperhidrosis, hereditary factors, corticosteroid treatment, and immunodeficiency are of major importance (21,27,33,51). Pityriasis versicolor is common in patients with seborrheic dermatitis, (27), *Malassezia* colliculitis (3), and hyperhidrosis, and it is difficult to eradicate in patients who sweat excessively (27,51). Pityriasis versicolor is seen more often than expected in family members of patients (6,27,36). A CMI defect may be of importance (61,62). The depigmentation seen in many patients may be explained by the presence of dicarboxylic acids (the main component of which is azalaic acid), which are produced by *M. furfur* (42). These acids have both a tyrosinase inhibitor effect and a cytotoxic effect on melanocytes (4).

Incidence

Pityriasis versicolor is commonly seen in patients living in tropical areas. In a survey from western Samoa, Marples found the incidence to be 49% in adults and 39% in schoolboys (41). It is also commonly seen in other tropical or subtropical areas (5,32). The incidence is much lower in temperate climates, i.e., 3.7% in Italy and 1.1% in Sweden (8,27). Pityriasis versicolor in young individuals is more frequent in surveys from the tropics than in studies from temperate countries (5,41). The sex ratio also varies among studies, one showing a predominance of female patients (27) and another of male patients (51).

Clinical Appearance

Pityriasis versicolor may be associated with all skin areas having active sebaceous glands. In temperate climates it is most often seen on seborrheic areas of the trunk naturally occluded by cloth. In the tropics, with high temperatures and high relative humidity, pityriasis versicolor is often seen on the face and scalp (5,13,41). Patients with immunodeficiency, e.g., acquired immunodeficiency syndrome (AIDS), are often affected, but pityriasis versicolor is also seen in generally healthy people. It is uncommon in infants and small children, being seen most often during puberty and adulthood, when the sebaceous glands are most active. Lesions are slightly scaling, papular, and nummular, sometimes coalescing to cover most of the trunk with only small areas of normally pigmented skin in between (Fig. 7-5). Early lesions are often red or brown (Fig. 7-6) and later become depigmented. In people with darker skin the lesions may turn dark and sometimes almost black. The name versicolor is due to this variation in color. Why lesions become depigmented in some and darken in others may be due to a difference in the inflammatory response. The main complaint is often cosmetic disfiguration, but about one-third of the patients also com-

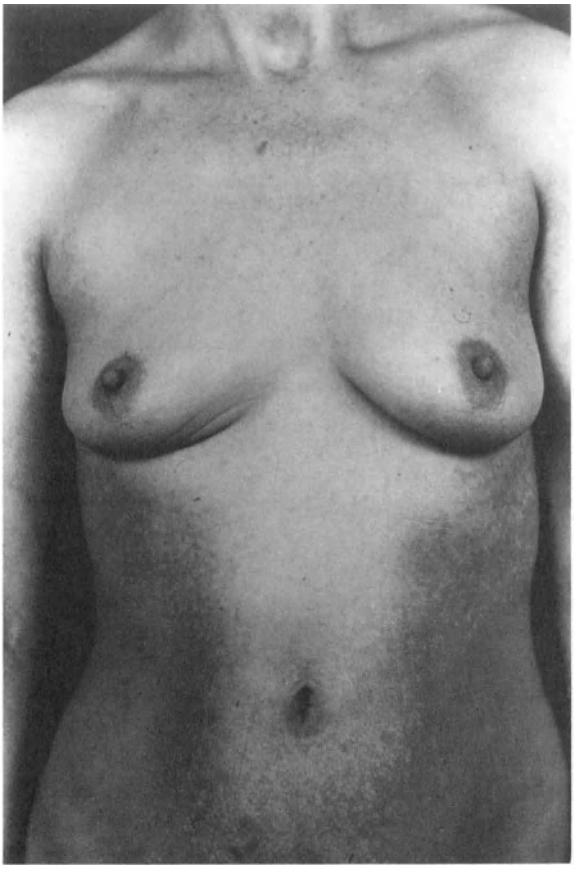

FIG. 7-5. Extensive depigmented lesions of pityriasis versicolor.

plain of moderate to severe pruritus (27). Itching is more pronounced when the patients are warm and sweating. Lesions are sometimes follicular, but most of these lesions are not pityriasis versicolor but *Malassezia* folliculitis. There is a correlation between these two diseases as well as between the diseases and seborrheic dermatitis. Pityriasis versicolor is found in about 15% and seborrheic dermatitis in about 18% of patients with *Malassezia* folliculitis (3).

Diagnosis

Diagnosis is based on the typical clinical picture, yellow fluorescence under Wood's light, and positive direct microscopy. Histopathology and culture are usually not necessary but may be helpful for difficult cases.

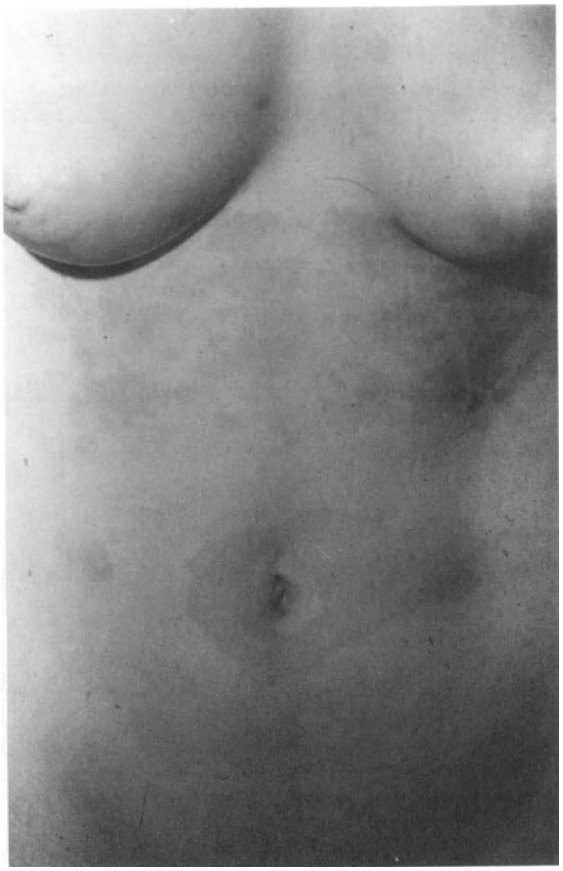

FIG. 7-6. Nummular red to brown lesions of pityriasis versicolor.

The typical clinical picture consists of a slightly scaling brown to depigmented nummular lesion on the upper trunk. The greasy scale often loosens as a sheet in a characteristic way, known as the coup d'angle, when scratched with a wooden stick or fingernail. Under Wood's light, lesions show a yellow to yellow-blue fluorescence, and lesions not visible to the naked eye are often found. If patients have taken a shower recently and especially if they have brushed their skin intensively, fluorescence may be weak or not visible. An easy and reliable method that facilitates direct microscopy is the use of Scotch Tape. The tape is pressed firmly against the skin, and a thin layer of epidermal cells containing the fungus is obtained. The tape is then stained with methylene blue 1% for 1 minute, mounted on a slide, and placed under the microscope. The round, budding cells and short hyphae—"spaghetti and meat balls"—can easily be identified (Fig. 7-7).

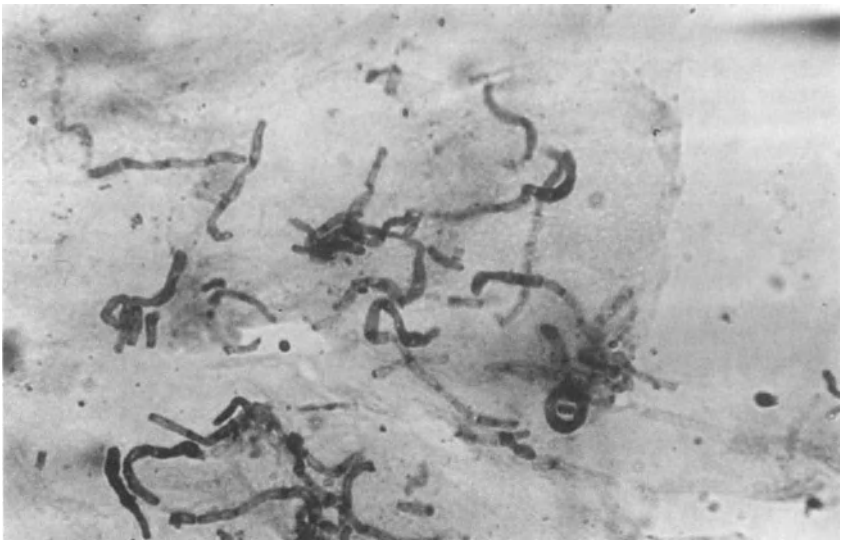

FIG. 7-7. Direct microscopy of a sample from a patient with pityriasis versicolor showing the round yeast cells and short hyphae of *Malassezia furfur*.

Culture is usually not necessary for routine diagnosis but may be included for the final diagnosis of the lipophilic yeast *M. furfur*. A quantitative culture provides the best information because *M. furfur* is, as previously mentioned, present on normal-looking skin. The scrub method is difficult to use for routine purposes (14). The use of contact plates has been described in detail elsewhere (16). Briefly, the plate is pressed firmly against the skin for 15 seconds and then incubated in a Bio-Bag Cfj or a normal plastic bag at 37°C for 6 days. The number of colonies is significantly higher in patients with pityriasis versicolor (14,16).

If the diagnosis is unclear, histopathologic examination may be included. With both periodic acid-Schiff (PAS) and methenamine silver nitrate stains, round budding cells and hyphae are seen throughout the stratum corneum and even inside the keratinocytic cells (4,21). In hematoxylin–eosin-stained sections, hyperkeratosis and slight acanthosis are seen in the epidermis, and there may be moderate and essentially perivascular infiltrates of lymphocytes, plasma cells, and histiocytes in the dermis (4).

Differential Diagnosis

The difficulty of diagnosis is due partly to the color of lesions and partly to the location. The pityriasis alba variety of atopic dermatitis and vitiligo

must sometimes be considered. Direct microscopy is often the best method for final confirmation. Seborrheic dermatitis and psoriasis may give differential diagnostic problems and may coexist with pityriasis versicolor (12). For lesions in the groin and axillae, erythrasma and dermatophytosis must be considered.

Treatment

There are numerous ways of treating pityriasis versicolor topically and systemically (1,22,24,26,31,48,58,63). Regardless of the active material used in the topical preparation, it is preferable to use solutions or lathering vehicles such as shampoos because they are easy to apply extensively to affected body areas. The patients should treat the entire trunk, neck, arms, and legs down to the knees even when only small areas are affected. The impression that pityriasis versicolor is more common in persons using poor hygiene is not true. Rigorous washing and scrubbing—which can make the diagnosis more difficult does not cure pityriasis versicolor.

My present standard treatment is application of propylene glycol 50% in water twice daily for 2 weeks (26). This treatment is effective, inexpensive, and cosmetically elegant, and it presents little risk of skin irritation (26). Another effective treatment is zinc pyrithione shampoo (24). The patient applies the shampoo to the above-mentioned areas and lets it work in for 5 minutes before showering. This procedure is repeated every evening for 2 weeks. Alternative topical treatments are selenium sulfide shampoo and the imidazoles, preferably used as shampoos or solutions (1,31,57,58,63).

Systemic therapy is indicated for extensive lesions, for lesions resistant to topical treatment, and in the case of frequent relapse. Ketoconazole, an orally active imidazole derivate, is effective (22,37,48). However, a potential risk with ketoconazole is its liver toxicity; approximately 1 per 15,000 patients develops hepatitis, probably due to an idiosyncrasy (39). Overall results have shown cure rates of 92% with a mean treatment of 4 weeks (39). Treatment for 5 days with 200-mg tablets once daily has also been effective (37), as has treatment with a single 400-mg does of ketoconazole (48). The risk of systemic side effects is minimized with short-term therapy. It is important to inform the patients that the depigmented spots may remain for several months after treatment, especially during the winter.

Prophylaxis and Prognosis

Pityriasis versicolor is not a contagious disease. The transformation of *M. furfur* from saprophyte to pathogen depends on predisposing factors, and

such factors may be difficult to eradicate. This problem thus explains the difficulty of permanently curing patients as well as the chronicity of the disorder. Prophylactic treatment is mandatory to avoid recurrence. Topical treatment schedules with application once or twice monthly would probably be effective, but patient compliance is often lacking. One effective prophylactic treatment schedule is one 200-mg ketoconazole tablet for three consecutive days every month (22). A 400-mg dose once monthly has also been used as prophylaxis (48).

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8—Fungal Cell Wall Synthesis and Assembly

José Ruiz-Herrera and Rafael Sentandreu

The importance of cell walls must not be underestimated. Once considered to be a nonliving excretion of the cell, its importance for maintaining cell viability is now fully established. Cell walls are essential structures of most eukaryotic organisms and nearly all prokaryotes. As clearly stated by Bartnicki-Garcia (19), "Three of [the] five kingdoms [proposed by Whittaker] (Monera, Fungi and Plantae) are made almost entirely of walled organisms. And in a fourth one (Protista) cell walls are essential in some stage in the life cycle of a majority of the species. Clearly, in members and variety, the walled kingdoms dominate the biological world." It is evident that during evolution the acquisition of a wall permitted the cells to survive in aqueous environments with dilute solute concentrations and to colonize media otherwise harmful for naked wall-less cells. Evolution of the cell wall to the impressive fabric of interwoven polysaccharide microfibrils that surround some eukaryotic organisms, including fungi, undoubtedly represented a breakthrough that permitted the appearance of large multicellular organisms, of which trees represent the epitome. The success of this cell wall is evidenced by its conservation and wide distribution in organisms of today.

As in any other living cell, the biological success of fungi can be measured by their ability to colonize new ecological niches. Starting from propagules growing on an appropriate substrate (which in some strict fungal parasites is the only nutritional source), fungi develop as long hyphae advancing through different environments, many harmful or aggressive, in search of new food sources. Along these tunnels composed of resistant cell walls, the living protoplasm can survive in various media and later colonize a new substratum.

Therefore to speak of the biology of fungi is to refer to the synthesis, structure, and organization of the cell wall. This structure protects the protoplasm and endows the organism with the ability to adopt its different forms, which are necessary for different functions: nutrition, mating, reproduction, dispersal, host invasion, and so on. The observation that

naked protoplasts do not carry out cytokinesis, thus linking wall growth with cell division, is illustrative.

From a practical point of view, the fungal cell wall is attractive to researchers interested in the control of fungal diseases. Of all the cell components, it is the wall that most comes into contact with the host. In phytopathogenic fungi the components derived from the cell wall act as elicitors of phytoalexins, provoking an active response toward the parasite. In animals, surface antigens on the fungi elicit the formation of antibodies, which induces host resistance, cause allergic reactions, or are useful for diagnosis. Moreover, of all the molecular components of host and parasite, some cell wall components are the only ones specifically absent in the invaded organisms. The use of compounds that selectively inhibit the synthesis of chitin, for example, would therefore block fungal growth without affecting the host. Such drugs would be the equivalent of the penicillins used for treatment of some bacterial infections.

Chemical Composition and Structure of the Cell Wall

Polysaccharides

Fungal cell walls predominantly consist of polysaccharides, which consitute nearly 80% of their dry weight. The rest is made up of proteins, lipids, inorganic salts, and pigments. Based on the two most abundant wall polysaccharides, Bartinicki-Garcia (15) divided fungal walls into eight groups. The fact that wall chemotypes closely correlated with other accepted taxonomic indicators for fungi suggested an evolutionary pathway for the cell wall coincident with other phylogenetic markers (120). By far the most abundant and most widely distributed sugar in the fungal cell walls is glucose (Table 8-1), which is present in all of the fungal taxonomic groups. Galactose, glucosamine (both free and acetylated), and mannose are the next most abundant sugars, and pentoses and deoxyhexoses are present in small amounts only. A more comprehensive synopsis of sugar abundance and distribution in the cell walls of fungi is shown in Table 8-2.

Chitin is the most characteristic polysaccharide of the fungal cell wall, but glucans are more abundant. Most glucans contain β(1-3) and β(1-6) linkages. Cellulose is present in only a limited number of taxonomic groups. Mannans (the carbohydrate moiety of glycoproteins), polyuronides, polygalactosamine, chitosan, and a number of mixed polymers complete the spectrum of fungal cell wall polysaccharides.

Proteins

Proteins are present in smaller amounts, ranging from 3 to 20% of the wall dry weight, but higher values (e.g., 30% and even 65%) have been re-

TABLE 8-1. Main sugars present in fungal cell walls.*

Sugar	Relative abundance	Presence in taxonomic groups
Glucose	++++	12 (all)
Galactose	+++	11
Glucosamine	+++	10
Mannose	++	11
Galactosamine	+	7
Glucuronic acid	+	5
Rhamnose	+	7
Xylose	+	9
Arabinose	Trace	7
Fucose	Trace	6
Ribose	Trace	4

*From Sentandreu et al. (211a).

ported (for a review, see ref. 18). Neither a special amino acid composition nor the presence of rare amino acids has been reported for wall proteins. Most of the fungal wall proteins are glycoproteins, and some have enzymatic activity. Of them, yeast invertase is perhaps the most thoroughly studied (85). Invertase from *Saccharomyces* is only weakly joined to the cell wall, whereas in *Neurospora crassa* it is firmly attached and survives the whole process of cell wall purification (48). α-Glucosidase is also tightly bound to the cell walls of *Mucor rouxii* (81). The role of these and other hydrolases present in the cell wall is to degrade compounds that are unable to permeate the cells, forming readily permeable products (121,185).

Lipids

Lipids exist in the cell walls in low amounts (1–10% of the dry weight). Some can be extracted by organic solvents, but others require acid hydrolysis first. It is unknown what kind of linkages maintain these "bound" lipids joined to the cell wall. Once thought to be cytoplasm or membrane contaminants, lipids are now considered bona fide components of the cell walls. The often cited observations of Dyke (67) on the lipid composition of cell walls from the yeast *Nadsonia elongata* support this idea. This author reported that cell walls contain 7.5% dry weight of lipids but lack palmitoleic acid, which is widely abundant in the protoplast, and that most fatty acids from the cell walls are saturated, exactly the opposite from the total cell lipids. Along the same lines, Domer and Harmon (63) observed that distribution of fatty acids in two species of *Sepedonium* obtained from different batches of cell walls were more or less constant, whereas those obtained from the cell sap were highly variable. The role of cell wall lipids remains unknown, but the hydrophobicity of several fungi is due to the presence of lipids in their outer coats.

TABLE 8-2. Sugar distribution in fungal cell walls.

Taxonomic group	Glc	Gal	GlcN	Man	GalN	Glucur	Rham	Fuc	Xyl	Ara	Rib
Acrasiales	++++	0	0	0	0	0	0	0	0	0	0
Myxomycetes	++	tr	0	tr	+++	0	tr	0	tr	0	0
Trichomycetes	++	+	++	+	+++	0	0	0	tr	0	0
Oomycetes	++++*	tr†	+‡	tr	tr	tr	tr	tr	tr†	tr	tr
Chitridiomycetes	++	+	+++	tr	0	0	tr	±	±	±	0
Zygomycetes	+§	+	+++	+	0	++	tr	+	0	0	tr
Hemiascomycetes	+++	+	+	+++	0	0	0	0	0	0	0
Euascomycetes	+++	++	++	+	+	+	±	0	tr	tr	0
Loculoascomycetes	+++	++	++	++	+	0	tr	tr	tr	tr	0
Homobasidiomycetes	++++	tr	++	+	tr	0	0	0	tr	tr	0
Heterobasidiomycetes	+++	tr	+	++	0	+	0	tr	tr	tr	±
Fungi Imperfecti	+++	+	++	++	tr	+	±	tr	tr	tr	tr

tr = <0.5%; ± = 0.6–1.0%; + = 1.1–5.0%; ++ = 5.1–20%; +++ = 20.1–50%; ++++ = >50%.

*With the exception of members of family Thraustochytriaceae, which have minimal values.

†Higher values for members of family Thraustochytriaceae.

‡Higher values for members of Leptomytales.

§High values only in spores.

Architecture of the Cell Wall

It has been repeatedly mentioned that, structurally, eukaryotic cell walls resemble reinforced concrete structures where fibrillar polysaccharides play the role of iron rods, providing resistance to tensions, whereas amorphous compounds play the role of mortar, giving resistance to pressure. Structural polysaccharides exist in the form of long, interwoven microfibrils whose structure becomes apparent by electron microscopic observation of tangential sections (34) or after enzymatic degradation of other wall components (224). As mentioned above, the most common microfibrillar structural polysaccharides are chitin followed by some β-glucans and cellulose. These substances are embedded in the amorphous components of the cell wall. In sections observed by electron microscopy most walls appear multilayered (Fig. 8-1). Treatment with specific hydrolytic enzymes, use of cytochemical methods, or staining with lectins tagged with colloidal gold has demonstrated that layers with a distinct degree of electron density are enriched with different chemical components. Characteristically, microfibrillar compounds constitute most of the inner layer, whereas outer layers contain mostly cementing or amorphous components. This observation suggests that these compounds associate into a coherent structure and are not merely being trapped in the microfibrillar fabric.

Chitin Biosynthesis

Structure of Chitin

Chitin is a linear homopolymer formed by *N*-acetylglucosamine (GlcNAc) residues. Considering that GlcNAc molecules are joined through β(1-4) bonds, the structural component of the molecule is in fact the disaccharide diacetylchitobiose (Fig. 8-2). The length of the chitin chains is uncertain considering that the polymer is insoluble, so that the usual methods for molecular size determination cannot be applied. For *Saccharomyces cerevisiae*, short chains of about 100 GlcNAc residues have been calculated (127), whereas chitin chains synthesized in vitro by chitosomes from *M. rouxii* contain about 2,000 GlcNAc units (46). Chains connecting with each other through hydrogen bonding constitute microfibrils. These structures are formed by 40–200 poly-GlcNAc chains. According to the orientation of the chains in the microfibrils, three crystalline forms of chitin have been described, among which the α-form is present in all fungi studied. In this crystalline variety the neighboring chains of the polysaccharide are antiparallel and organized in an orthorhombic unit cell with the following dimensions: a = 4.7 Å, b = 18.86 Å, and c = 10.32 Å.

That the crucial role of chitin is to keep the shape and rigidity of the walls is supported by several lines of evidence. Removal of the amorphous

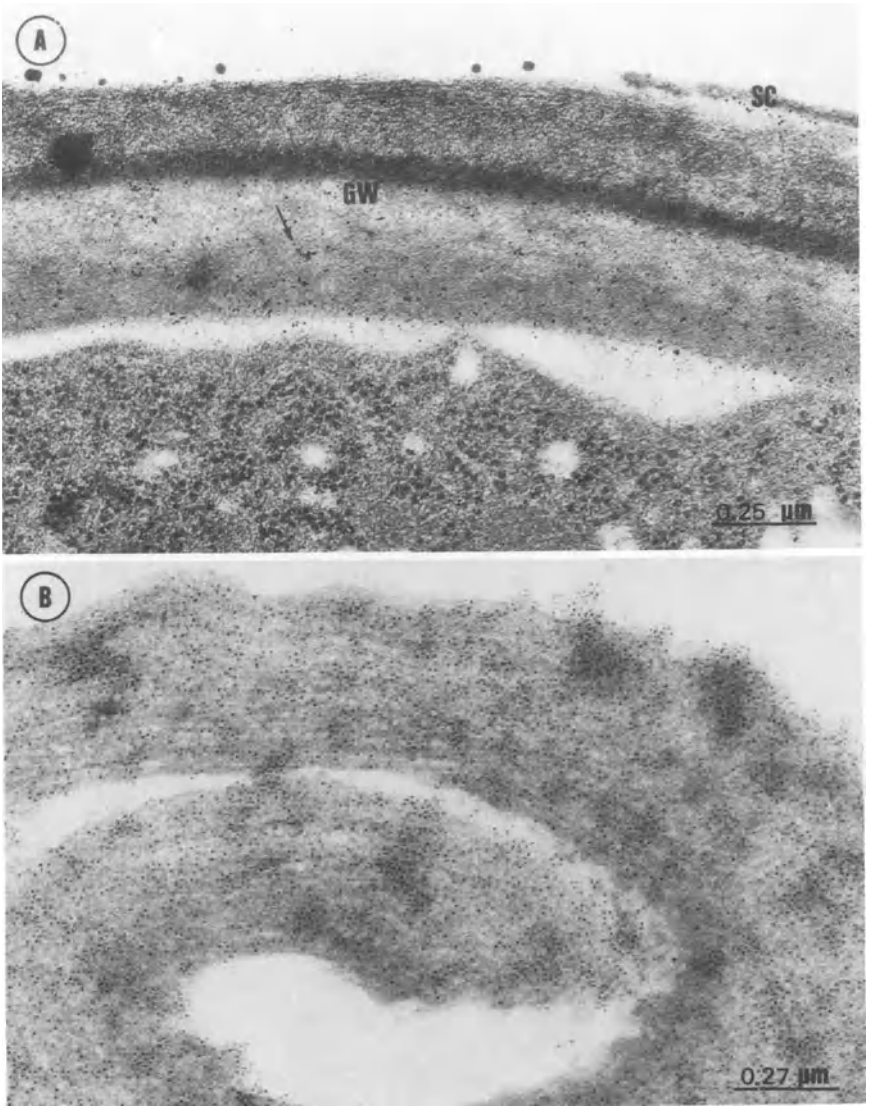

FIG. 8-1. (A) Section of a germinating sporangiospore from *Mucor rouxii* showing the fractured outer layer of the original spore coat (SC) and the newly formed cell wall (GW). Chitin was tagged with gold-labeled wheat germ lectin (Au-WGL) (dark points, arrow). (From A. Carabez-Trejo and J. Ruiz-Herrera.) (B) Section of a purified cell wall from *M. rouxii* tagged with Au-WGL. (From C.E. Bracker, S. Bartnicki-Garcia, and J. Ruiz-Herrera.)

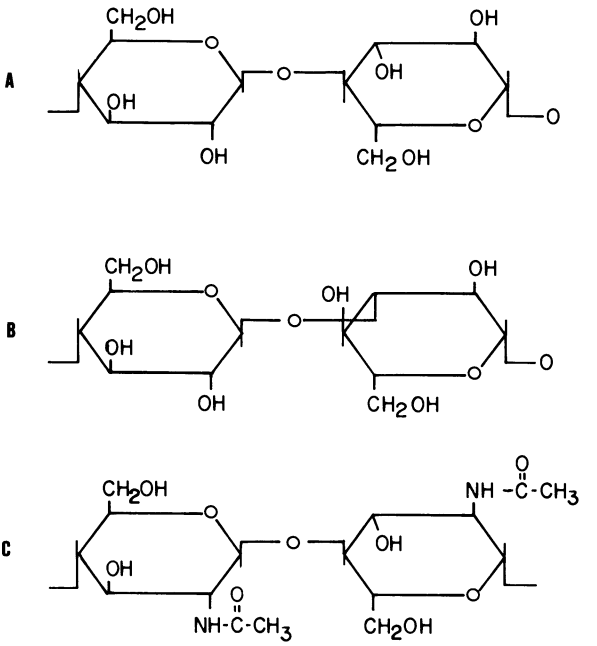

FIG. 8-2. Disaccharyl units of the main wall polysaccharides. (A) Cellobiosyl unit of cellulose. (B) Laminaribiosyl unit of β(1-3) of glucans. (C) Diacetylchitobiosyl unit of chitin.

components under conditions where only chitin remains unaffected does not alter the original shape of the cell wall in several fungi (190), nor does it affect the organization of the chitin microfibrillar mesh (41). On the other hand, fungal mutants unable to synthesize chitin in vivo are osmotically fragile (73,128). Polyoxins (74) or nikkomycins (54), which are specific inhibitors of chitin synthetase, affect growth of fungi and induce morphological alterations (25,31,165).

Biochemical Aspects

Chitin is synthesized, as are most polysaccharides, by a transglycosylation reaction using a nucleotide sugar as the glycosyl donor. For chitin biosynthesis, uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is the only effective substrate. The reaction is catalyzed by an enzyme called chitin synthetase. Chitin synthetases from different fungal sources share several properties, summarized in Table 8-3 (for a review, see ref. 191). Chitin synthetases require a divalent metal for their activity, Mg^{2+} being the most efficient one at concentrations ranging between 2.5 and 25.0 m*M*, but Mn^{2+} and Co^{2+} may be as effective as Mg^{2+} in some systems (196). Chitin

TABLE 8-3. General properties of chitin synthetases of fungal origin.

1. Kinetics show a cooperative effect.
2. Km values are in the millimolar range.
3. The enzyme requires a divalent metal for activity: Mg^{2+}, Mn^{2+}, or Co^{2+}.
4. GlcNAc and especially UDP-GlcNAc are allosteric activators.
5. There is no high-energy lipid intermediate in the reaction.
6. UDP and other nucleotides are competitive inhibitors with Ki in the range of Km.
7. Polyoxins and nikkomycins are competitive inhibitors with Ki values two to three orders of magnitude less than Km values.
8. The enzyme does not utilize exogenous acceptors.
9. Chitin synthetase is activated by unspecific proteases.
10. The enzyme is present in the cell in a particulate form.

synthetases show unconventional kinetics, and double reciprocal plots of 1/v versus 1/s are sigmoidal owing to a cooperative effect. Apparent Km values for UDP-GlcNAc are in the millimolar range. These values may appear high, but concentrations of UDP-GlcNAc in this order of magnitude have been reported to be present in the cytoplasm of fungi (68,137,206).

Stimulation of chitin synthetase by free GlcNAc was initially observed by Glaser and Brown (89), who reported chitin synthesis in vitro for the first time. This stimulation has been confirmed in most systems analyzed (191). GlcNAc is not incorporated into chitin and probably acts as an artificial allosteric activator, mimicking the effect of the natural activator, probably UDP-GlcNAc (151,196). Biosynthesis of chitin does not require an exogenous primer. Unconfirmed results suggested that chitin synthesis by particles from *Blastocladiella emersonii* utilized a diglycosyl diglyceride as primer (156), whereas a protein acceptor was reported in *M. rouxii* (27). Unlike the biosynthesis of other polysaccharides, synthesis of chitin does not involve a lipid intermediate (191).

Zymogenicity of Chitin Synthetase

An important observation, first made by Cabib and Farkas (45) in *Saccharomyces*, was that chitin synthetase mostly existed in an inactive form, requiring limited proteolysis to become fully active. Activation was achieved using endogenous or exogenous proteases. This observation has been extended to most fungal chitin synthetase preparations (43,191). Although chitin synthetase activation by proteases seems to be an unspecific phenomenon, the efficiency of several proteases as activators may be different (22). We have purified an acid protease from *M. rouxii* that only inactivates chitin synthetase from the fungus (62). Whether proteolytic activation of chitin synthetase plays a physiological role in vivo remains uncertain. We have observed that chitin synthetase present in crude

preparations of *Phycomyces blakesleeanus* was activated by calcium and calmodulin. This activation was not prevented by protease inhibitors, but the calmodulin antagonist trifluoperazine blocked activation brought about by calmodulin (150). It is interesting to note that different calmodulin-regulated enzymes were activated unphysiologically by trypsin (153). The similarity with chitin synthetase seems to be more than just a coincidence.

Chitin Synthetase Inhibitors

Several nucleotides inhibit chitin synthetase; of them, UDP, one of the products from the chitin synthetase reaction, is the most effective (191). Hori et al. (113) conducted a kinetic analysis of UDP inhibition of chitin synthetase from *Piricularia oryzae* and concluded that the enzyme had a pyrimidine-specific site for UDP-GlcNAc, where nucleotides competed.

The most efficient inhibitors of chitin synthetase are the nucleoside-peptide antimicrobics known as polyoxins (74) and nikkomycins (54), which are analogs of UDP-GlcNAc. Ki values for these antimicrobics are about 1,000 times smaller than the Km for UDP-GlcNAc. Hori et al. (113,114) concluded that polyoxins bind to the enzyme through their pyrimidine nucleoside moieties, and that an amino and a free carboxyl group are required for their inhibitory activity. It has been suggested that polyoxins and nikkomycins enter the cell through a specific dipeptide permease (91,165). By analyzing the antagonistic effect of dipeptides and tripeptides on the in vivo action of nikkomycin X, it was suggested that uptake of the nucleoside-peptide inhibitors involved the transpeptidase reaction of the γ-glutamyl cycle (83). It is likely that variations in the uptake of these antibiotics are responsible for their different effectiveness observed in vivo against several fungi.

Benzoylphenyl ureas are insecticidal compounds that inhibit chitin synthesis in vivo. The most thoroughly studied is diflubenzuron. Once considered to be inhibitors of chitin synthetase (see (52) for a review and discussion), it is now known that the effect of these ureas is due to inhibition of UDP-GlcNAc transport across the cell membranes (158).

Microfibril Biosynthesis

Before 1974, evidence for chitin biosynthesis by cell-free preparations rested on radiotracer studies, which measured the incorporation of minute amounts of GlcNAc into an insoluble product characterized as chitin because of its resistance to acids and alkalis and its susceptibility to chitinases. Whether it represented growth of preexisting chains or de novo formed chitin was uncertain. Physical characteristics of the product also remained

unknown. At that time, exposure of membrane fractions from *M. rouxii* to high concentrations of UDP-GlcNAc rendered part of the chitin synthetase free from the membranes. With this "solubilized" preparation, microfibrils indistinguishable from those isolated from the cell walls and identified as α-chitin by x-ray diffraction were synthesized in vitro for the first time by Ruiz-Herrera and Bartnicki-Garcia (193). Later, chitin microfibrils were synthesized in vitro by preparations obtained from *N. crassa* (22), *Agaricus brunnescens* (as *A. bisporus*) (22,100), *Allomyces macrogynus* (22), *S. cerevisiae* (22,66), *B. emersonii* (156), and *Schizophyllum commune* (229).

Enzyme Purification and Genetic Control

Chitin synthetase has been highly purified in the form of chitosomes (see below) from different fungi. Several detergents have been used to solubilize the enzyme, but only digitonin was found to effectively extract active enzyme from membrane fractions of *Coprinus cinereus* (92). This technique was applied successfully to extract chitin synthetase from membrane fractions of *S. cerevisiae* (66), *Candida albicans* (37), *S. commune* (231), and *A. brunnescens* (99) and from chitosomes from *M. rouxii* (194). Dissociated forms of chitin synthetase have a similar size—about 500 kilodaltons (kD)—and sedimentation coefficients of 16S–18S. They synthesize chitin microfibrils but with a short, needle-like appearance, different from the characteristic long ribbons synthesized by chitosomes. Starting from these preparations, chitin synthetase was purified from *C. cinereus* (161) and *S. cerevisiae* (127) using two completely different procedures. The enzyme from *C. cinereus* was purified in the last step by chromatography in a Cu^{2+} chelate column, whereas that from *S. cerevisiae* was purified by trapping it into its product, chitin. Both preparations showed a major polypeptide band with similar molecular weight: 67 kD for the enzyme from *C. cinereus* and 63 kD for the enzyme from *Saccharomyces*. Other minor polypeptide bands were observed in both preparations.

The gene for chitin synthetase (CHS1) from *S. cerevisiae* was cloned in a plasmid using chitin synthetase-less mutants as recipient (40). When a plasmid containing the chitin synthetase gene fused with lac Z was introduced into *Schyzosaccharomyces pombe*, which lacks chitin synthetase activity, both activities (those of chitin synthetase and β-galactosidase) were expressed in vitro in the same ratio as in the donor strain, suggesting that CHS1 is the structural gene for the enzyme. *S. pombe* transformants, however, do not contain chitin; accordingly, some other gene products are required for the in vivo expression of the structural gene of the enzyme. CHS1 gene has an open reading frame of 3,400 bases that encodes a protein of 130 kD. Because the major protein band observed in purified preparations of the active enzyme from *S. cerevisiae* has a molecular weight of 63 kD, and the gene contains no consensus splice sites, it is possible that pro-

teolytic activation is responsible for the split of the theoretical 130-kD polypeptide. Sequencing of the protein will throw some light onto this item. Disruption of the CHS1 gene with a URA3 insert did not affect chitin biosynthesis in vivo (40). This observation led the authors to suggest the presence of a second chitin synthetase activity normally not revealed in the in vitro assays. In cell-free extracts of strains containing the disrupted CHS1 gene, low levels of chitin synthetase activity were observed (172, 205). Kinetic behavior and response to proteolysis of this enzyme were different from those of chitin synthetase 1. The authors have suggested that this second enzyme is the one physiologically responsible for chitin synthesis in vivo. The role for the other enzymatic activity, which had been the subject of extensive research, therefore remains obscure.

Chitosomes

Evidence indicates that chitin synthetase that accumulates in the cytoplasm of fungi exists in the form of specialized microvesicles called chitosomes (20,22,29,35,100,149,192). Chitosomes comprise a population of microvesicles, homogeneous in both size (40–70 nm in diameter) and buoyant density (1.125–1.145 according to the species). They are delimited by a thin membrane (6.5–7.0 nm thick). In thin sections they have the same morphology displayed by apical microvesicles present in a number of fungi (Fig. 8-3). When incubated with substrate and activators, chitosomes synthesize chitin microfibrils in vitro (35) (Fig. 8-4). Isopycnic sedimentation of cell-free extracts from the yeast form of *M. rouxii* revealed that 80–85% of the total chitin synthetase of the cell sedimented as a sharp peak of chitosomes (195). The rest sedimented as two small, heavier peaks. Whether this enzyme was bound to plasmalemma, as has been claimed for *Saccharomyces* (65), remains unknown because of the tendency of chitosomes to aggregate with other structures, depending on the buffer used (195). Chitosomes were originally isolated from cells broken by ballistic disruption, but later they were extracted from the giant sporangiophores of *P. blakesleeanus* by pressure extrusion or with a microsyringe (104). Similar yields of identically appearing chitosomes were obtained from the wall-less "slime" variant of *N. crassa*, broken by osmotic lysis or ballistic disruption (21). More recently, chitosomes were purified from this strain lysed under isotonic conditions by treatment with triethanolamine buffer (197). Chitosomes from *M. rouxii* contain about two-thirds protein and one-third lipid. Roughly equal amounts of polar and neutral lipids were detected. From the latter, sterols and their esters are the most abundant ones (102). Qualitative differences in the sterol composition from chitosomes and whole cells have been noted (140). Phosphatidylcholine and phosphatidylethanolamine are the most abundant phospholipids in the chitosome. Ma-

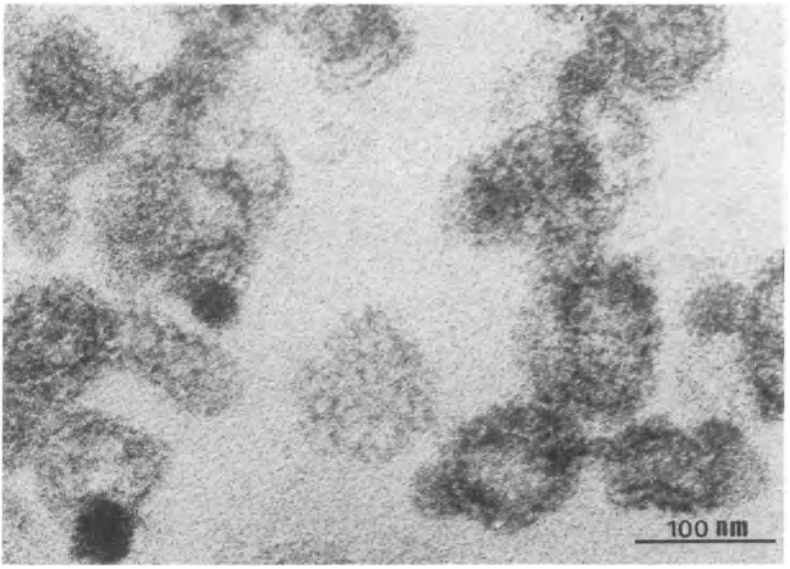

FIG. 8-3. Section of purified chitosomes from *M. rouxii*. (From C.E. Bracker, S. Bartnicki-Garcia, and J. Ruiz-Herrera.)

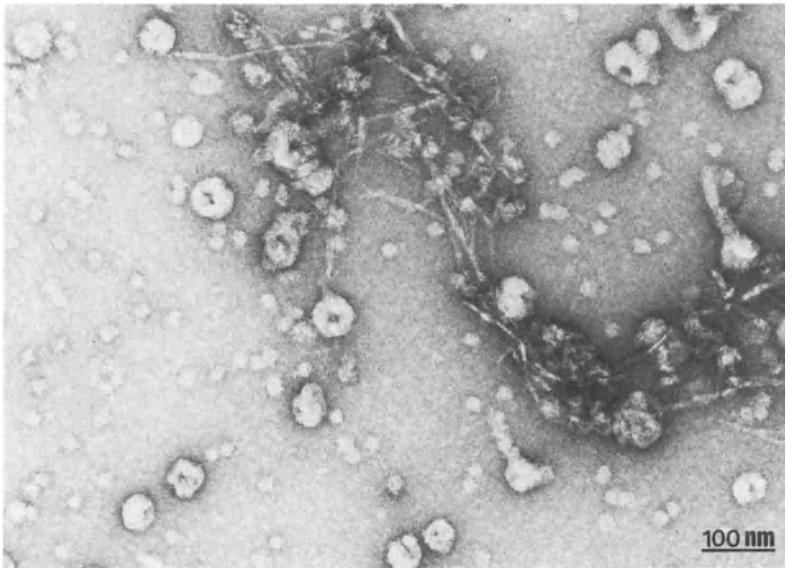

FIG. 8-4. Chitin microfibrils synthesized in vitro by purified chitosomes from *S. cerevisiae*. (From C.E. Bracker, S. Bartnicki-Garcia, and J. Ruiz-Herrera.)

cell structures of the same strain (239). As expected, the degree of crystallinity from β-glucans is variable; most glucans exhibit broad, diffuse reflections when subjected to x-ray diffraction analysis. "Hydroglucan," a microfibrillar form extracted from *Saccharomyces* cell walls subjected to HCl treatment, shows a higher order of crystallinity (78,132). Apparently, boiling in HCl strips the β(1-6) branches, permitting a better package of the β(1-3) chains. A triple-helical structure has been proposed for the arrangement of linear β(1-3) chains in a plant glucan (33).

The other β-glucan present in cell walls, specifically in Acrasiales, Oomycetes, and Chitridiomycetes, is cellulose, which by x-ray diffraction appears indistinguishable from plant cellulose (15).

α(1-3)-Glucan has been reported to be present in the cell walls of several fungi: *Cryptococcus* sp., *Schizosaccharomyces* sp., and *Polyporus* sp. (8); *S. commune* (239); and *Paracoccidioides brasiliensis* (125). In the latter organism, the presence of this polysaccharide in the outer layer of the wall of the yeast phase is relevant to the virulence of the fungus (202).

Enzymological Aspects of Glucan Synthesis

The biosynthesis of β(1-3)-glucans in vitro has been described in a number of fungal systems, including *Phytophtora cinnamomi* (233), *Cochliobolus miyabeanus* (110,111), *Saccharomyces* (10,135,141,142,208,213,214), *P. brasiliensis* (200,201), *Phytophthora palmivora* (235), *Saprolegnia monoica* (76,77,87), *Candida albicans* (173), *N. crassa* (137,157,180,181), and *Aphanomyces astaci* (50). One important observation is that only a limited number of properties are shared by the preparations analyzed. Thus all the studied systems utilize uridine diphosphate glucose (UDP-Glc) as substrate, with the exception of an unconfirmed report where guanosine diphosphate glucose (GDP-Glc) was found to be more efficient than UDP-Glc (10); no lipid intermediates have been found to intervene in the reaction. All preparations also have in common their instability. The *S. cerevisiae* enzyme, for example, had a half-life of less than 1 hour at 24°C (142), whereas the half-life of the *N. crassa* enzyme was 12 minutes at 14°C (181). Other properties of glucan synthetases are dissimilar. Km values for UDP-Glc reportedly vary from 0.12 m*M* in *S. cerevisiae* (142) to 4.5 m*M* in *A. astaci* (50). In contrast to chitin synthetase, glucan synthetase from *S. cerevisiae* showed classical michaelian kinetics. Glucan synthetases from plant origin are stimulated by cellobiose; similarly, cellobiose stimulation was noted for glucan synthetases from the oomycetes *Phytophthora* (234,235) and *A. astaci* (50), whereas no effect was observed in other fungi. Requirement for divalent metals is also variable: Glucan synthetase from *P. palmivora* was stimulated 6- to 14-fold by 20–40 m*M* Mg^{2+} (235) and a slight stimulation by Mg^{2+} was also noted in some preparations of *S. cerevisiae* (142) and *Saprolegnia* (76); in other systems, however, Mg^{2+} either had no

jor differences in the lipid composition from chitosomes and whole cells are the absence of phosphatidylserine and the abundance of galactolipids in chitosomes (102). Sodium dodecyl sulfate (SDS) electrophoresis of purified chitosomes from *M. rouxii* shows six to eight polypeptide bands, the most notorious having molecular weights of about 60, 55, and 18 kD (E. Lopez-Romero, A. Flores, S. Bartnicki-Garcia, C.E. Bracker, and J. Ruiz-Herrera, unpublished data). Chitosomes from the slime variant of *N. crassa* are enriched in four polypeptide bands with molecular weights of 22, 39, 38, and 53 kD (197). We have shown that chitosomes from *M. rouxii* do not contain adenosine triphosphatase (ATPase) activity (195). By differential and gradient centrifugation, chitosomes from *N. crassa* could be separated from secretory vesicles, appearing free of enzymatic markers characteristic for plasma membrane, endoplasmic reticulum, or vacuoles (197).

As described above, chitosomes can be dissociated by digitonin into subunits retaining chitin synthetase activity with a molecular weight of about 500 kD (194). The abundance of sterols in their composition provides the structural basis for their dissociation by the saponin. Chitosomal subunits show the remarkable capacity to reaggregate once digitonin is removed by centrifugation in saponin-free sucrose gradients (29,99).

Glucan Biosynthesis

Types of Glucan

β-Glucans comprise a heterogeneous population of polysaccharides that constitute an important fraction of fungal cell walls. Most β-glucans have been characterized as polymers of glucose moieties joined through β(1-3) linkages. Some of them contain branches joined through β(1-6) linkages. The degree of branching, the molecular weight, and their macromolecular structure are variable. Thus cell walls from *S. commune* are covered by a mucilage composed of a water-soluble, highly branched β-glucan (239), whereas β(1-3)-glucans present in the cell walls of *Saccharomyces* appear as a fine net of microfibrils. These nets are more apparent during protoplast regeneration (132). In this organism most of the glucan is a branched β(1-3) polymer of high molecular weight containing a small proportion of β(1-6) glycosidic interchain linkages (146). A small fraction is a highly branched glucan with a proportion of β(1-6) glycosidic linkages and a smaller amount of β(1-3) interchain and interresidue linkages (147). Part of this glucan is soluble in water, but most is water-insoluble; in *S. commune*, in addition to the water-soluble glucan described above, cell walls contain an insoluble, highly branched β-glucan with (1-3) and (1-6) linkages (R-glucan). The proportion of linkage types, chain lengths, and degree of cross-linking are highly variable depending on the strains and the various

effect or was inhibitory. Glucan synthetase from *P. brasiliensis* was stimulated two- to threefold by several divalent metals following the order Ca<Mg<Mn<Fe (200).

Activators

Glucan synthetase from various sources is activated by adenosine triphosphate (ATP) and guanosine triphosphate (GTP), but the effect is variable. For *S. cerevisiae*, GTP and ATP at very low concentrations are strong stimulators of β-glucan synthetase but apparently act by different mechanisms: ATP through phosphorylation and GTP by binding to the enzyme (214). Treatment with tergitol-NaCl caused dissociation, from membrane fractions of *Hansenula anomala* and *N. crassa*, of a soluble GTP-binding protein that activated the membrane-bound glucan synthetase (126). However, GTP has no stimulatory effect on the glucan synthetases from *N. crassa* (181) or *A. astaci* (50). In *Saprolegnia*, membrane-bound glucan synthetase was stimulated by GTP and ATP; however, when an enzyme preparation extracted with digitonin was used, it was found that concentrations below 0.1 m*M* had no effect on the enzyme, and higher concentrations were highly inhibitory (77). These results suggest that purine nucleotides do not act on the enzyme itself but on a regulatory system that operates when glucan synthetase is membrane-bound. Unlike chitin synthetase, proteolytic treatment of β-glucan synthetases either has no effect or at high concentrations is deleterious, with the exception of the enzyme from *Phytophthora*, which was stimulated by trypsin (234,235). Fluoride was found to be a strong stimulator of glucan synthetase from *S. cerevisiae* (135). This effect was later confirmed with the enzyme from *C. albicans* (174) and *N. crassa* (180). Apparently, fluoride acts in two ways: as an inhibitor of phosphatases, and directly on the enzyme potentiating the effect of adenine nucleotides (135,136). Addition of glycerol (213) or sucrose (135) stimulates and partially stabilizes the enzyme. The effect of sucrose was in part due to protection of the substrate (UDP-Glc) from the action of degrading enzymes present in osmotically fragile vacuoles (135). Leal et al. (136), via a detailed kinetic analysis, demonstrated that glucan synthetase behaves as a dissociating complex. Dissociation leads to the irreversible degradation of one of the components of the complex; sucrose prevents dissociation of the enzyme. Guillen et al. (98) have described the presence of endogenous, low-molecular-weight compounds that, by activation and inactivation, modulate yeast glucan synthetase in vitro.

Inhibitors

Uridine diphosphate (UDP) is a competitive inhibitor of glucan synthetase with a Ki of 0.22 m*M* for *N. crassa* (180), 0.45 m*M* for *S. cerevisiae* (142),

and 1 m*M* for *A. astaci* (50). Uridine monophosphate (UMP) and triphosphate (UTP) are also competitive inhibitors of the glucan synthetase from *N. crassa*, with Ki values of 0.21 and 0.24 m*M*, respectively (180). Gluconolactone, which is an inhibitor of glucanases, was also found to inhibit glucan synthetase from *S. cerevisiae* by an uncompetitive mechanism (142). Similar results were obtained with *N. crassa*, where a Ki of 1.2 m*M* was calculated (180). Papulacandin B was shown to be a noncompetitive inhibitor of glucan synthetase from *S. cerevisiae* with a Ki of 1.2 m*M* (9) [although there have been contradictory reports where no inhibition was detected (189,222)], and glucan synthetase from *S. pombe* (228). This compound and aculeacin also inhibited the enzyme from *Geotrichum lactis* (179). Echinocandin inhibited glucan synthetase from *C. albicans* by a mixed mechanism (204).

In *N. crassa* Quigley and Selitrennikoff (180) found that papulacandin B behaved as an uncompetitive inhibitor of glucan synthetase with a Ki of 120 μM, and echinocandin B behaved similarly with a Ki of 1.1 μM. After careful kinetic analysis, these authors suggested that sorbose and gluconolactone bound to the same nonsubstrate site of the enzyme, whereas papulacandin and echinocandin bound to a different site.

Cellular Location of Glucan Synthetase

Glucan synthetase activity has been found in association with membrane and wall fractions. In preparations obtained from *S. cerevisiae* protoplasts, it has been found associated with the plasmalemma (213), whereas in extracts from *Saprolegnia* the enzyme appeared associated with the endoplasmic reticulum; moreover, synthesized microfibrils appeared associated with cisternae and dictyosomes (77). Larriba et al. (135) described the biosynthesis of glucan microfibrils by a nonsedimentable fraction from *S. cerevisiae* cell-free extracts, but its instability precluded its characterization. A fraction of glucan synthetase with a lower specific gravity (1.1501–1.160) than plasmalemma has been obtained by centrifugation of cell-free extracts from *S. cerevisiae* and *N. crassa* in sucrose isopycnic gradients (C. Leal-Morales and S. Bartnicki-Garcia, personal communication).

Glucan synthetase has been extracted from mixed membrane fractions of *Saprolegnia* using digitonin, but the physical properties of this preparation have not been studied (77). Also, partial solubilization of membrane-bound β-glucan synthetase from yeast and mycelial membrane preparations of *P. brasiliensis* using several detergents has been described (201).

Nature of the Products Synthesized In Vitro

As described for chitin biosynthesis, early experiments on glucan synthesis in vitro measured only the incorporation of small amounts of radioactive

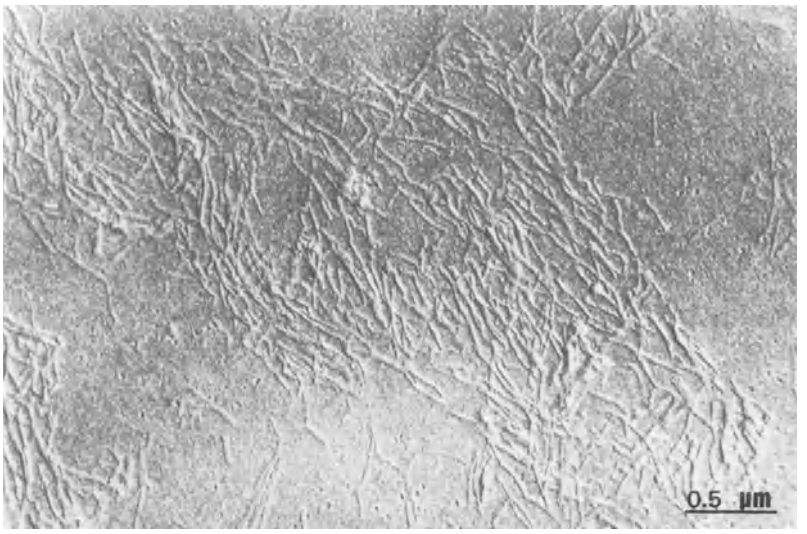

FIG. 8-5. Glucan microfibrils synthesized in vitro by a high-speed supernatant obtained from *S. cerevisiae*. (From B. Chavez, G. Larriba, M. Morales, and J. Ruiz-Herrera.)

precursors. Identification of the type of linkages relied on the use of specific hydrolytic enzymes. Most of these studies confirmed that the products were mainly β(1-3)-glucans; however, variable amounts of β(1-6) linkages in the products have been reported. Balint et al. (10) noted that most of the glucan synthesized by *S. cerevisiae* extracts from GDP-Glc contained β(1-6) bonds. A significant proportion of β(1-6) linkages was also obtained using wall fractions from *P. cinnamomi* (233) and *S. cerevisiae* (141). The fact that wall-containing preparations synthesized a polymer richer in β(1-6)-linked residues led us to suggest that 1,6-β-glucosyl transferase could be a cell-wall-bound enzyme acting perhaps as a branching enzyme (141).

β-Glucan microfibrils were synthesized in vitro for the first time by Wang and Bartnicki-Garcia, who used membrane fractions from *P. cinnamomi* (234). Glucan microfibrils have also been synthesized in vitro using cell-free extracts from *S. monoica* (77) and a high-speed supernatant from *S. cerevisiae* (135) (Fig. 8-5). In all cases, microfibrils were composed of glucan containing β(1-3) linkages only. Glucan microfibrils synthesized by *S. cerevisiae* were composed of chains containig around 700 glucose units, a value that closely agreed with those reported for the alkali-soluble glucan extracted from the cell walls of the organism (78). No glucose was detected at the reducing end, suggesting that glucan was bound to an acceptor of different chemical nature. On the other hand, preparations of *S. cerevisiae* obtained from prototoplasts synthesized a short-chain glucan, with glucose present at the reducing end (213). Reasons for this discrepancy have now

been clarified. It was found that glucan synthetase preparations obtained from protoplasts of *S. cerevisiae* or *C. albicans* contained an active β-glucanase (whether it is native or it comes from contamination of the glucanase employed to prepare the protoplasts remains unknown), which breaks down the synthesized glucan into shorter chains containing glucose at the reducing end (as expected). Fractions obtained by mechanical breakage of the cells, on the other hand, did not contain detectable glucanase activity, and they synthesized glucan with no glucose at the reducing end, which was bound to a molecule of a different nature (3). This association is established during biosynthesis of the polymer, as glucan synthetase utilizes a protein as acceptor for the growth of the glucan chains (4) by a mechanism similar to that described for starch and glycogen biosynthesis. The synthesis of a protein-bound β(1-6)-glucan by a particulate enzyme preparation of *S. cerevisiae* has been reported (10), this kind of bond has been detected in β(1-6)-glucans synthesized in vivo by the organism (222).

A different kind of polymer, a phosphorylated β(1-3)-glucan (phosphoglucan or mycolaminaran phosphate) was synthesized by extracts prepared from zoospores of *P. palmivora* (235). No reports exist on the in vitro synthesis of cellulose *sensu strictu*, i.e., identified by x-ray diffraction using cell-free systems from either fungi or other organisms, but synthesis of β(1-4)-glucans has been achieved in different systems (61), including cell-free extracts from *S. monoica* using low UDP-Glc and high Mg^{2+} concentrations (75,77). This activity was slightly stimulated by GTP and ATP and was susceptible to proteolytic activation.

Fungal Wall Glycoproteins

Chemical Composition and Structure of Glycoproteins

Glycoproteins represent, both quantitatively and qualitatively, an important fraction of fungal cell walls; in contrast to chitin and glucans, however, they do not play a structural role. They are cementing compounds that compact the wall into an organized structure essential for cell growth. As described above, some have enzymatic activity (e.g., invertase, acid phosphatase). In *Saccharomyces*, removal of the external portion of the carbohydrate moiety of wall glycoproteins by mutation makes the cells physiologically abnormal; they grow slowly, lyse, and have distorted walls (13); mutations that affect earlier steps in the synthesis of the carbohydrate moiety of glycoproteins may be lethal (119). Glycoproteins present on the cell surface of several yeasts also play important roles in sexual agglutination between cells of opposite mating type (242) and in their antigenic or pathogenic properties. Thus in *C. albicans* they define serological type (82,220) and are responsible for attachment to epithelial cells favoring

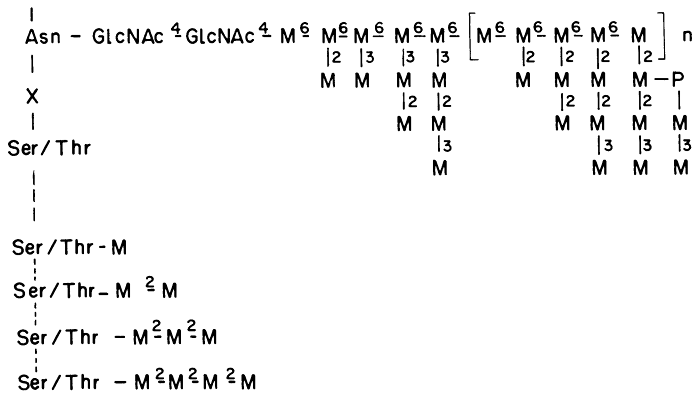

FIG. 8-6. General structure of the carbohydrate moiety of cell wall glycoproteins from *S. cerevisiae*. Asn = asparagine; Ser = serine; Thr = threonine; GlcNAc = *N*-acetylglucosamine; M = mannose; P = phosphate. [After C.E. Ballou (modified).]

invasion of the host (138) [although Lehrer et al. (139) suggested that chitin is more important than proteins to cell adherence]. The size of the carbohydrate moiety in glycoproteins is variable, and characteristically purified glycoproteins (i.e., invertase, acid phosphatase) are microheterogeneous owing to the different size of the carbohydrate portion. Considering the existence of several fungal wall chemotypes (see above), it would be expected that glycoproteins present in the various groups of fungi contained different sugars in variable proportions. However, there is general agreement that their initial biosynthetic steps are similar. The best studied fungal wall glycoproteins are the "mannans" from yeasts, mainly from *S. cerevisiae*. They are glycoproteins that comprise an important amount of the cell wall, with molecular weights ranging from 25 to 1,000 kD. As expected from their generic name, mannose is the main sugar present in these compounds, although minor amounts of glucose, galactose, xylose, arabinose, glucuronic acid, fucose, rhamnose, and glucosamine are also present (11). Early studies by Sentandreu and Northcote (210) established the similarity between yeast mannan and animal glycoproteins—mainly that the mannose chains were bound in two ways to the protein: Small oligosaccharides were linked through *o*-mannosyl bonds with serine or threonine residues, and larger moieties were linked to aspartic acid through glucosamine residues. This structure was refined thanks to the now-classic studies of Ballou and co-workers (11,12,14). According to their studies, short oligosaccharide chains ranging from one to four sugar units are linked to serine or threonine, whereas a large, branched structure is joined to asparagine through a diacetyl-chitobiosyl unit (Fig. 8-6). They recognized that this structure was made of a backbone of mannosyl units bound through α(1-6) linkages. A variable number of side chains joined to

this backbone through α(1-2) and α(1-3) bonds. The size and number of these chains are species- and strain-specific. Phosphodiester bonds have been identified in some branches as well. The abundance and size of branches were determined through gel filtration analyses of the products obtained by acetolysis of the mannoprotein ("fingerprinting"); acetolysis strips off the branches by the selective cleavage of α(1-6) bonds of the glycoprotein (131). Use of a "nearest neighbor" analysis suggested that the order of the side chains was not random (11). Using mutants it was disclosed the presence of two different regions in the carbohydrate moiety linked to asparagine residues: the core and the outer chain (Fig. 8-6). Chemically, the differences are that in the core some side chains are joined by α(1-3) linkages and it is devoid of phosphodiester bonds. This inner core is similar to the carbohydrate moiety present in several animal glycoproteins (55,134).

Mechanism of Glycoprotein Biosynthesis and Genetic Control

Advancement in the comprehension of glycoprotein biosynthesis came from four areas of investigation: (a) establishment of the role of dolichols in the process; (b) discovery of the basic mechanisms involved in protein secretion; (c) use of specific inhibitors; and (d) use of mutants affected at precise steps of the process. The first three aspects were developed mainly in animal systems, with the yeast and fungal systems lagging behind; with the development of studies with mutants, the yeast model has gained ground, as it has allowed demonstration of the suggested pathways and permitted more thorough analysis of the problem. An important contribution of the study with different models is the demonstration that the initial steps of glycoprotein biosynthesis are common among eukaryotic cells.

Demonstration of the role of polyprenols in the synthesis of bacterial cell walls prompted study of a family of isoprenoid compounds present in the nonsaponifiable fraction of the lipids from animals and plants. These compounds have received the generic name "dolichols" and differ from bactoprenols in that they have a larger number of isoprene residues and their α-isoprene unit is saturated. Sharma et al. (212) demonstrated synthesis of dolichyl-phosphate-mannose (Dol-P-Man) in *Saccharomyces* via incubation of GDP-Man and Dol-P, and its further transfer to a serine or threonine residue from an endogenous acceptor. Only the first mannose unit utilized Dol-P-Man as a donor; further mannose moieties came from GDP-Man.

Synthesis of the *N*-linked chains is more complicated. From a historical point of view comprehension of the mechanism of synthesis of the outer chain of yeast mannoproteins preceded analysis of the biosynthetic mechanism of the core. It was made possible by the studies performed by Ballou's group, which led to the isolation and the chemical and biochemi-

cal analyses of the first mutants with altered mannoproteins. However, for simplicity, we describe briefly the mechanism of the *N*-linked chains biosynthesis as it occurs in the cell. Four steps can be recognized in the process: (a) synthesis of the dolichol-oligosaccharide; (b) transfer to the acceptor protein; (c) processing or trimming; and (d) growth of the outer chain. The first three steps were initially disclosed in animal cells, but with the isolation of the *alg* (asparagine-linked glycosylation) group of mutants the mechanism was also confirmed in yeasts. The process has been comprehensively reviewed elsewhere (134).

Steps in the first part of the pathway (a) are represented in Figure 8-7. Mutants affected in the growth of the lipid-linked oligosaccharide have been classified in seven complementation groups: *alg*1, *alg*2, *alg*3, *alg*4, *alg*5, *alg*6, and *alg*8. It was concluded that only genes ALG1 and ALG2 are essential for growth (134). Once the oligosaccharide has grown to achieve the structure $Glc_3Man_9GlcNAc_2$, it is transferred to a protein acceptor with the sequence Asn-X-Ser-Thr, where X is any amino acid except proline (30), in agreement with the results obtained in animal cells (148).

Trimming reactions involve removal of the three glucose units and one mannose by glucosidases I and II (129) and a specific 1,2-mannosidase (42). The resulting oligosaccharide moiety $Man_8GlcNAc_2$ is further mannosylated to terminate the core and initiate synthesis of the outer chain. This mechanism has been studied by analysis of the oligosaccharides present in the invertase forms secreted by several *mnn* mutants (225).

The outer chains of the glycoproteins are the structures mainly responsible for the immunogenic characteristics of yeasts. The most important determinants characterized by Ballou (12) are shown in Figure 8-8. Using agglutination of wild-type cells with the corresponding antisera, mutants with altered outer chains were enriched, and the structure of their mannoproteins was studied (12). This approach has been useful for understanding the mechanisms involved in outer chain biosynthesis. Mutants completely lacking the outer chain were classified into four complementation groups *mnn*-0-*mnn*10 (13). As mentioned above, these mutants are affected in growth, wall structure, and sporulation. Mnn2 mutants conserve the α1-6-linked polymannose backbone but do not synthesize side chains because they are affected in the α(1-2)-mannosyl transferase, which joins the first mannose residue of the branches. Addition of the second α(1-2)-linked mannose is affected in *mnn*5 mutants, whereas in *mnn*1 mutants joining of the α(1-3)-linked mannose molecules to both the outer chain and the *O*-linked oligosaccharides is impaired. Finally, *mnn*4 and *mnn*6 mutants are affected in the binding of the phosphodiester branches.

Cytological Aspects of Glycoprotein Biosynthesis

There is evidence that glycoprotein biosynthesis occurs in the endoplasmic reticulum and in the Golgi apparatus or its equivalent. In *Saccharomyces*,

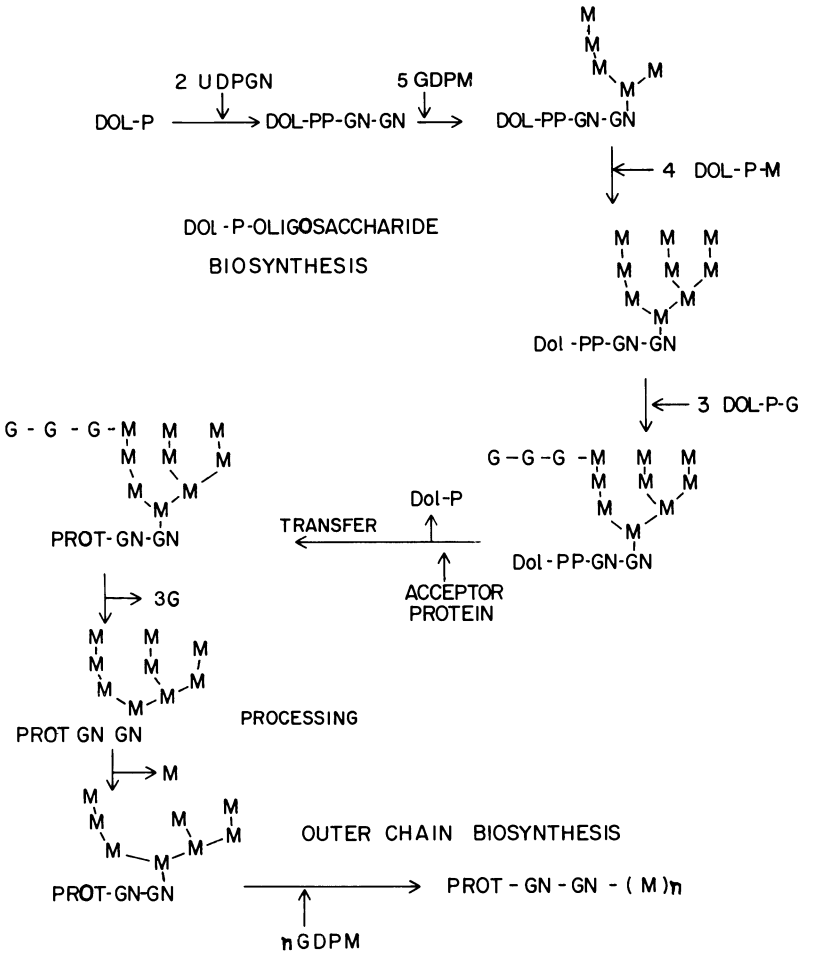

FIG. 8-7. Mechanism of glycoprotein biosynthesis in yeasts. DOL-P = dolichol-phosphate; UDPGN = uridine-diphosphate-*N*-acetylglucosamine; GDPM = guanosine-diphosphate-mannose; DOL-P-M = dolichyl-phosphate-mannose; DOL-P-G = dolichyl-phosphate-glucose; PROT = protein; M = mannose; GN = *N*-acetylglucosamine; G = glucose.

using *sec* mutants, it became evident that *O*-glycosylation occurs in the endoplasmic reticulum, and *N*-glycosylation must be terminated during transport of the glycoprotein to the cell surface (243). Synthesis of the lipid-linked oligosaccharide probably takes place in the endoplasmic reticulum, where transfer to the asparagine residues of the growing protein also occurs, i.e., co-translationally. This conclusion was evidenced in yeasts by the fact that mannose-labeled nascent polypeptides were released from polysomes by puromycin treatment (199).

One interesting question is whether synthesis of the oligosaccharide

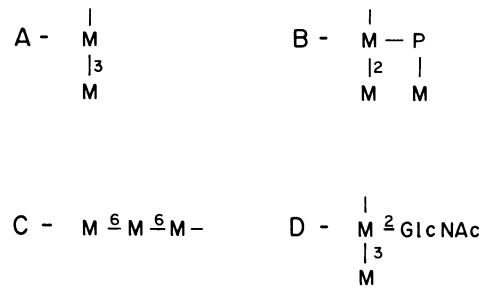

FIG. 8-8. Most important antigenic determinants from *S. cerevisiae* glycoproteins. [After C.E. Ballou (modified).]

moiety occurs in the cytoplasmic or the luminal sides of the endoplasmic reticulum. This question was reviewed by Hirschberg and Snider (112), who concluded that Dol-PP-$GlcNAc_2$ and the larger oligosaccharides are synthesized on the luminal side, whereas shorter oligosaccharides face the cytoplasmic side of the endoplamic reticulum. One problem that arises when explaining glycosylation in the lumen of the endomembrane system concerns the mechanism of access of the sugar donors. Haselbeck and Tanner (101) incorporated the mannosyl transferase involved in the synthesis of Dol-P-Man into liposomes containing Dol-P and demonstrated that when supplied with GDP-Man on one side the sugar could be transferred via the Dol-P to GDP located on the opposite side. These results demonstrated the role of Dol-P as a transport mechanism for sugars across the membranes and offered an explanation for the mechanism of sugar transfer from the cytoplasm to the interior of the endomembrane system. More intriguing is the demonstration that UDP-Glc and UDP-GlcNAc can be transported across the endoplasmic reticulum membranes by a mechanism dependent on temperature that is saturable and displays very low KmS (177,178). Translocation of a large number of nucleotide sugars into Golgi vesicles has also been demonstrated (112). The systems involved are specific, are temperature-dependent, have KmS values in the order of 1–10 μM, and are susceptible to inhibition by nucleotides.

Inhibitors of Glycoprotein Biosynthesis

Considering the complexity in the enzymatic array involved in glycoprotein biosynthesis, it should be expected that the process might be inhibited by a large number of compounds in the several biosynthetic steps. This expectation is fulfilled by the description of the numerous inhibitors that block the process. They include the classical inhibitors of protein biosynthesis and those that affect general membrane functions, which are not considered here. Specific inhibitors of the synthesis of the polysaccharide moiety in-

volve: (a) those blocking early steps in the synthesis of the precursors; (b) those interfering with the formation of the lipid-linked oligosaccharide; and (c) those blocking glycoprotein processing (55,69). Tetaine (or bacilysin or bacillin), an antimicrobic produced by *Bacillus subtilis* "theta" is a potent inhibitor of the growth of *Candida albicans* [minimal inhibitory concentration (MIC) 0.6–3.0 μg/ml], causing agglutination, deformation, and inhibition of septum formation (155). In the cell, tetaine is cleaved by peptidases producing a C-terminal epoxy amino acid called anticapsin, which binds to and inactivates glucosamine-6-phosphate synthetase. Anticapsin itself is produced by *Streptomyces griseoplanus* (168). The result of the inhibition of glucosamine-6-phosphate synthetase is a shortage of UDP-GlcNAc in the fungus and inhibition of chitin and glycoprotein biosynthesis (154).

Possibly the best studied inhibitor of glycoprotein biosynthesis is tunicamycin, an antibiotic produced by *S. lysosuperficus* (221) which selectively inhibits the transfer of GlcNAc-1P from UDP-GlcNAc to dolichol, the first step in the synthesis of the lipid-linked oligosaccharide (223). Four chemically related antibiotics—mycospocidin, streptovirudin, antibiotic 24010, and antibiotic MM 19290—have the same effect as tunicamycin. The antibiotic has been employed in a large number of studies to determine the role of the carbohydrate moiety in the secretion or function of selected proteins. These studies have not yielded a coherent image of the problem, as tunicamycin may inhibit synthesis of some proteins, affect their proteolytic cleavage, or not alter their secretion or stability at all. This different effect is apparently due to the specific proteins involved, their extent of glycosylation, their time of residence in the endomembrane system, and the cell studied (69).

There are several antimicrobics that form complexes with Dol-P and thus inhibit glycosylation reactions; among them are amphomycin, tsushimycin, and bacitracin (69). Also, sugar analogs inhibit glycoprotein biosynthesis, mainly after they are metabolized to form the corresponding nucleotide or dolichyl analogs. For example 2-deoxy-D-glucose (dGlc) is transformed into GDP-dGlc, UDP-dGlc, and Dol-P-dGlc; 2-deoxy-2-fluoroglucose (2-FGlc) is transformed into GDP-2-FGlc and UDP-2-FGlc; and 2-deoxy-2-fluoro-D-mannose (2-FMan) is transformed into GDP-2-FMan, UDP-2-FMan, and Dol-P-2-FMan, which are incorporated into abnormal dolichyl-oligosaccharides, and stop further elongation (57). Fluoroglucose, on the other hand, is not incorporated into lipid-linked oligosaccharides and inhibits synthesis of Dol-P-Glc and Dol-P-Man (58).

Inhibition of the oligosaccharide processing is brought about by blocking the trimming reactions. Nojirimycin (an antimicrobic produced by several *Streptomyces* strains), its reduced form deoxynojirimycin (produced by *Bacillus*), and its derivative *N*-methyldeoxynojirimycin all inhibit glucosidase I from several sources including *Saccharomyces* (188,203). However, further studies have revealed that deoxynojirimycin may also block the

synthesis of the $Glc_3Mann_9GlcNAc_2$-PP-Dol (187). The same effect was exerted by manno-1-deoxynojirimycin and 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (186). Finally, we must cite bromoconduritol, which inhibits trimming of the most internal glucose molecule (56).

Synthesis of Other Cell Wall Polysaccharides

Polyuronides

Glucuronic acid is present in variable amounts in the cell walls of fungi (15). Zygomycetes characteristically contain a large proportion of polyuronides; two glucuronic acid-containing polysaccharides have been reported in the cell wall of *Mucor*: a homopolymer composed of glucuronic acid, and a heteropolymer containing, in addition, mannose, galactose, fucose, and glucose (23,28). Incorporation of glucuronic acid (GlcUA) from UDP-GlcUA into an acid-insoluble product was successful using particulate fractions from *M. rouxii* (64,80). Endogenous acceptors present in the membrane fractions were extracted using neutral detergents, and the enzyme became dependent on the addition of exogenous polyuronides for activity (79). Addition of UDP-GlcNAc, UDP-Glc, and UDP-Gal gave rise to the synthesis of a heteropolysaccharide (mucoran). Glucuronosyl transferase required a divalent metal, Mn^{2+} being the most efficient; kinetics were of the cooperative type with a Km of 0.73 m*M* for UDP-GlcUA; optimum temperature and pH were 26°C and 7.0, respectively.

Chitosan

Chitosan, a β(1-4)-linked homopolymer of glucosamine, is an important constituent of the cell wall from zygomycetes, and its abundance may even exceed that of chitin (15). In contrast to studies on chitin, there are only a few reports regarding the mechanism of chitosan biosynthesis. Araki and Ito (5,6) described the presence of a deacetylase in *M. rouxii* that catalyzed the deacetylation of *N*-acetyl oligomers and glycol chitin. These authors suggested that the enzyme was involved in chitosan biosynthesis; however, because no deacetylation of chitin was observed, this possibility was not confirmed. We found that membrane fractions from *M. rouxii* incubated with UDP-GlcNAc synthesized a deacetylated polymer (chitosan) and suggested that deacetylation occurred in the early stages of the synthesis (47). The situation was clarified by Davis and Bartnicki-Garcia (59,60), who ruled out the possibility that a special substrate (UDP-GlcN) was the precursor for chitosan; they found that, whereas crystalline chitin was resistant to deacetylation, nascent chitin was susceptible to being deacetylated; thus a mixture of chitin synthetase and deacetylase synthesized chitosan with

high efficiency. These results have been confirmed using a particulate deacetylase which, it has been hypothesized, regulates the relative amounts of chitin and chitosan synthesis, binding to the nascent chains of chitin (46). These results also suggested that deacetylation occurs while the polyGlcNAc chains are being synthesized, and clarified that cell-free extracts synthesize two polymers, one fully acetylated (chitin) and one partially deacetylated (chitosan) of about equal size. It may be interesting to mention that chitosomes do not synthesize chitosan, only chitin.

Cytological Aspects of Cell Wall Biosynthesis

Vesicular Apparatus

Synthesis of the cell wall poses interesting topological problems, as precursors must be synthesized intracellularly, but deposition of the components and all macromolecular organization occur outside the permeability barrier of the cell. Therefore there must be special mechanisms to convey the precursors and the synthesizing machinery to the cell surface. Characteristically, filamentous fungi grow at the apical zone (see below). A unique feature of the hyphal apices is the presence of a large number of vesicles (88,95; for reviews, 17,93). Based on size and electron density, two populations of vesicles may be distinguished: (a) small (30–100 nm) and electron-dense; and (b) large (100–400 nm) and electron-lucid. By Thiery's staining, the larger ones are seen to contain polysaccharide material in the interior. This vesicular apparatus has been equated with the "Spitzenkorper," a densely stained apical corpuscle described by Brunswick (39) that is considered responsible for transfer to the cell surface of enzymes and precursors necessary to sustain fungal growth (93,192). Two characteristics of the vesicular apparatus are (a) its extreme lability (88,95) (even washing the cells leads to their disappearence) and (b) the rapid rate at which it is formed and fuses with the plasmalemma. Collinge and Trinci (53) calculated that about 40,000 vesicles are needed to sustain the growth of *N. crassa* for 1 minute. We have presented evidence that chitosomes constitute the population of vesicles responsible for the transport of chitin synthetase to the cell surface (192). According to their size (40–70 nm), chitosomes might correspond to the small apical vesicles.

With regard to the origin of these vesicles, it has been suggested that they are formed in the subapical region and are continuously transported to the apex (53). Girbardt (88) suggested that they are derived from the smooth endoplasmic reticulum, although dictyosomes have also been proposed as the site of vesicle formation in oomycetes (96).

Further knowledge obtained through a genetic approach is in agreement with the general idea about the role of the endomembrane system in the

secretory pathway, already established in animal cells. By analyzing a large number of nonsecretory mutants of *S. cerevisiae*, Novick et al. (170) distinguished 23 complementation groups. These authors found that a group of mutants are unable to incorporate the nascent glycoprotein chains into the endoplasmic reticulum. Another set of mutants accumulate glycoproteins in the endoplasmic reticulum, others accumulate abnormal structures ("Berkeley bodies," probably derived from the equivalent of the dictyosomes), and still another group of mutants stop the glycoprotein flow in vesicles, which then accumulate in the cytoplasm (169). The large number of complementation groups involved in the secretory pathway indicates the complexity of the process. According to the results of Novick et al., at least nine gene products (called *sec*) plus energy are involved in the transfer of glycoproteins from the endoplasmic reticulum to the Golgi equivalent. Two gene products and energy are required for their further transfer to vesicles, after which at least ten more gene products and energy are required for the final secretion process (169).

Mechanisms of Vesicle Transport

With regard to the mechanism involved in vesicle migration, several possibilities have been entertained. Howard and Aist (116,117) implied a role for microtubules in vesicle migration based on the observation that benzimidazole-2-yl-carbamate (MBC) severely affected the microtubular organization and vesicle distribution in *Fusarium acuminatum*. The role of microtubules in mitosis and nuclear migration has been clearly established, but an absence of discernible microtubular structures in the apex of fungi had prevented their implication in vesicle migration. However, these negative results are now considered to be due to methodological problems. Apical microtubules have been detected in hyphae by freeze substitution (118) and by immunocytochemistry using monoclonal antibodies directed against yeast or mammalian tubulins (130). It has been demonstrated that microtubules have associated ATP-driven "motors" capable of moving vesicles along (207). Microfilaments have also been involved in vesicle migration, as cytochalasins inhibit hyphal tip growth and alter the pattern of wall synthesis in several fungi (2,97,103).

Electrical currents and ion gradients have attracted the attention of many investigators as the driving force for vesicle migration. Slayman and Slayman (216) detected a potential difference along the hyphae of *N. crassa*. Further studies have revealed that transhyphal currents exist in growing mycelia of a large number of fungal species (7,94,115,133). It has been suggested that these positive currents, entering at the apex and leaving at the subapical zone, are carried by protons (94), although in other systems the inward current is carried in part by calcium and potassium ions (171,236). The origin of these currents remains unknown, but the possibil-

ity of an asymmetrical distribution of proton ATPases or H^+ symporters involved in nutrient uptake has been entertained. Several possible mechanisms by which ion currents drive vesicle migration have been suggested. Most appealing is the establishment of intracellular electrical fields charging the cytoplasm or the plasma membrane, displacing the vesicle population through electrophoresis (122). Jennings (124) suggested that a possible operative mechanism was an electroosmotic flow. The possibility that electrical currents influence the cytoskeleton and thus affect vesicle migration is also feasible (144). Gow (94) discussed the possibility that the ionic species rather than the electrical current was the important factor; in this sense, it has been found that calcium plays an important role. Reiss and Herth (183) demonstrated the existence of a calcium gradient, with accumulation at the tip, in a number of apical-growing cells. By x-ray microanalysis, Galpin et al. (84) showed that calcium accumulated in the apical zone of *Chaetomium*. Calcium accumulation in the apical region of stage I sporangiophores of *P. blakesleeanus* was observed by electron microscopy of specimens treated with pyroanthimonate (M. Morales and J. Ruiz-Herrera, in press). Addition of the calcium ionophore A23187 collapses the calcium gradient in pollen tubes of *Lilium longiflorum* (184), stops apical growth (108), and causes disorganization and a reduction in the number of apical vesicles (184). We have observed (198) that the calcium ionophore A23187 stops growth of *Phycomyces* germlings, and within a matter of seconds it also stops chitin, but not protein, biosynthesis. Effect on chitin bioynthesis was not due to permeability problems or to alterations in sugar metabolism or chitin synthetase formation of activity. Secretion of invertase, a wall-bound glycoprotein was also stopped immediately by the ionophore. Addition of exogenous calcium did not revert the phenomenon. These effects were traced back to a sudden decrease in the number of apical microvesicles induced by the ionophore (Fig. 8-9). These results indicate the important role played by calcium gradients and electrical currents in vesicle formation and migration. They are also important for the comprehension of fungal apical growth (see below).

Organization and Assembly of Cell Wall Constituents

Assembly of Microfibrils

Accumulated evidence indicates that assembly of microfibrillar polysaccharides occurs in two discernible steps. Polysaccharide chains are synthesized during the first step, a reaction that precedes further organization of the chains into microfibrils. This phenomenon has been deduced from studies on the effect of Congo red and calcofluor white on the process of

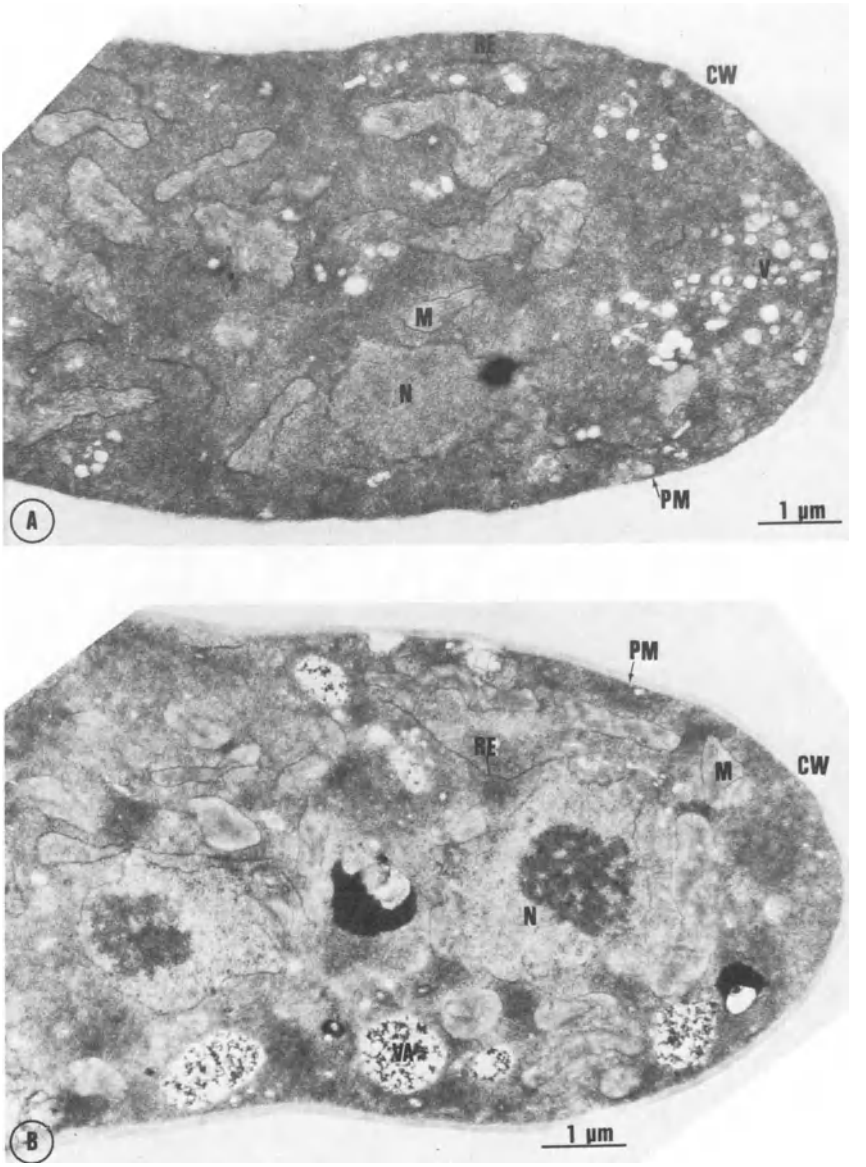

FIG. 8-9. Sections of the apical region of *Phycomyces* germlings. (A) Control cell. (B) Germling treated for 2 minutes with 10 μM calcium ionophore A23187. CW = cell wall; PM = plasma membrane; M = mitochondrion; N = nucleus; RE = endoplasmic reticulum; V = apical vesicle, VA = vacuole.

microfibril formation in vivo in the alga *Poterioochromonas stipitata* (107) and on the cell wall assembly of *S. cerevisiae* and *Candida* spp. (72,227). These dipole molecules introduce themselves among the sugar chains, interfering with the correct hydrogen bonding and crystallization of the polysaccharides. Vermeulen and Wessels (230) have demonstrated that chitin synthesized in the presence of cellufluor® is sensitive to chitinase and does not show the characteristic reflections of α-chitin when analyzed by x-ray diffraction, unless it is dried. Nascent chitin is susceptible to degradation by chitinases (143,160,230) and deacetylases (46,60). Once crystallized, chitin becomes resistant to enzymatic attack. This behavior has been taken as further evidence for the gap that undoubtedly allows the antiparallel organization of poly-GlcNAc chains, which are characteristic for α-chitin. It also may give rise to the formation of covalent bonding of chitin with other compounds of the cell wall (see below).

It is generally assumed that the synthesis of β-glucans and chitin occur at the cell surface through the operation of vectorial mechanisms catalyzed by membrane-bound enzymes. Evidence obtained for cellulose biosynthesis comes from the observation of microfibril impressions associated with intramembrane particles in freeze-etched algal and plant cells (86,162,163). Unfortunately, these cytological observations have not been accompanied by biochemical evidence that sustains the hypothesis that observed particles are indeed cellulose-synthesizing enzymes or that impressions come from cellulose microfibrils [for a discussion see (145)]. Chitin synthetase activity has been detected in significant amounts in the plasmalemma of yeasts and mycelial fungi (36,65,229), and using such preparations a vectorial synthesis of chitin oriented toward the outer face of the membrane was observed in *Saccharomyces* (44). However, the possibility of artifactual association of the enzyme in such preparations has not been indisputably demonstrated, as significant amounts of chitin synthetase have been found artifactually associated with walls, mitochondria, and microsomes by various authors (192). Although usually overlooked, intracellular synthesis of cellulose and chitin is not a rare phenomenon. In the alga *Pleurochrysis scherffelii*, cellulose synthesis and microfibril assembly occur in Golgi cisternae, which release the polysaccharide (bound to different components) to the cell surface by an exocytic process (38). Choi and O'Day (51) observed the accumulation of cellulose in vesicles distributed throughout the cytoplasm during the encystment of *Polysphondylium pallidum*. These vesicles moved toward the plasmalemma and discharged their content over the cell surface. A similar phenomenon occurs in the ciliate *Eufolliculina ohligi*, where chitin synthesized intracellularly is conveyed in an amorphous stage to the surface by large vesicles. After discharge, chitin crystallizes in the β-form to give rise to typical microfibrils (164). Which factors maintain these polysaccharides without crystallizing remains unknown. The existence of supersaturated "solutions" of cellulose and chitin that crystallize by a "flash" mechanism upon subtle alterations in the medium is

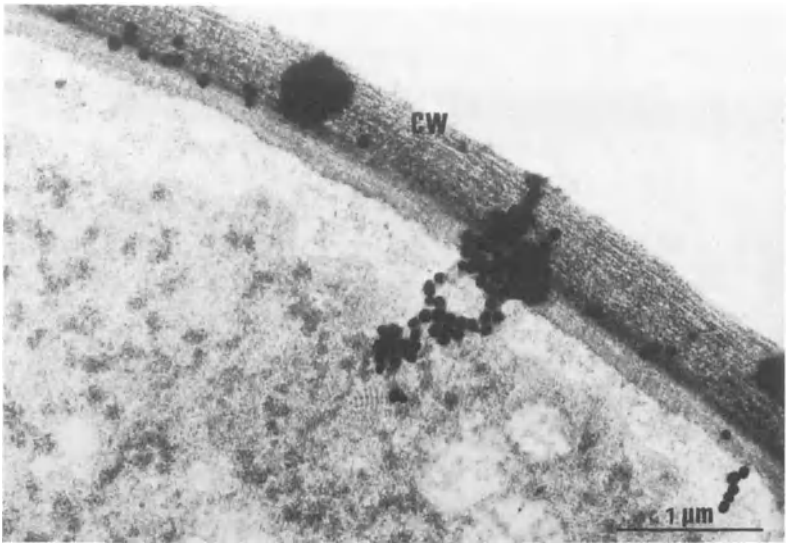

FIG. 8-10. Labeling of cell sections of *M. rouxii* germlings with Au-WGL. Note the intracellular gold grains. CW = cell wall.

known; moreover, the presence of other compounds accumulated in the vesicles may impair microfibril formation. Whether a similar mechanism operates normally for the synthesis of cellulose and chitin in fungi remains unknown. A common criticism of this mechanism is the inability to demonstrate accumulation of chitin within the cells by normal cytochemical staining of hyphal sections or by autoradiographic studies; but considering the astonishing velocity of vesicle synthesis and migration [40,000/minute, calculated as an underestimation, by Collinge and Trinci (53), for *N. crassa*], such an expectancy is probably overly optimistic. In sections of *Mucor* cells we have observed cytoplasm labeling with gold-tagged wheat germ lectin, which recognizes chitin (A. Carabez-Trejo, A. Arroyo Begovich, and J. Ruiz-Herrera, unpublished data) (Fig. 8-10), and we have also detected an intracellular insoluble radioactive label (possibly chitin) in the giant sporangiophore from *P. blakesleeanus* injected with the chitin precursor UDP-[^{3}H]-GlcNAc (B. Chavez, L. Herrera Estrella, and J. Ruiz-Herrera, unpublished) (Fig. 8-11).

In vitro studies using purified preparations of chitosomes have provided valuable information on the process of fibrillogenesis, as it is one of the few examples where both the enzymatic complex and the product are amenable to simultaneous biochemical and ultrastructural analysis (35). Incubations as short as 30 seconds in the presence of substrate and activators give rise to clusters of microfibrils associated with the chitosome shell. This observation reveals that microfibril formation is a rapid process. The observation

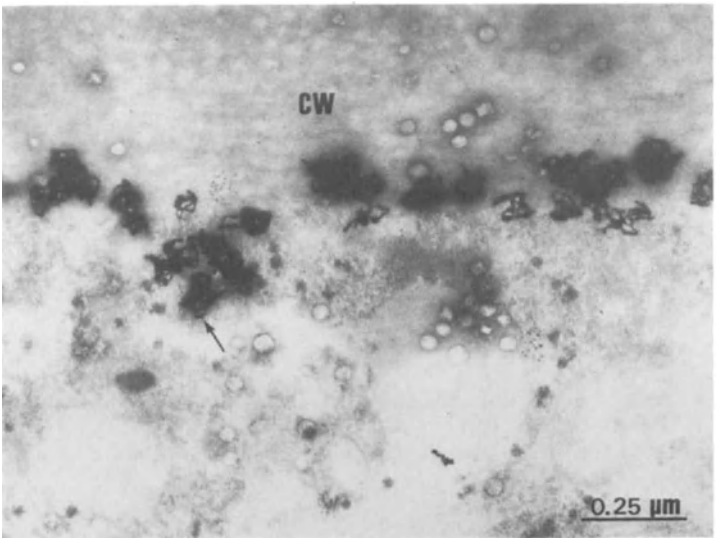

FIG. 8-11. Radioactive label in sections from *P. blakesleeanus* sporangiophores injected with the chitin precursor UDP-[^{3}H]-GlcNAc. Note the label accumulation in the cytoplasm wall interphase and in the cytoplasm (arrow). CW = cell wall.

of thin microfibrils in early stages suggests that microfibril formation involves the sequential lateral association of poly-GlcNAc chains to conform to the more robust structures, which have the same dimensions as microfibrils observed in native walls (93).

Assembly of Cell Wall Glycoproteins

Within the cell wall, microfibrils are embedded in a matrix of "cementing" compounds, which differ in nature among the several taxonomic fungal groups. However, glycoproteins are components universally distributed in fungal walls. It has been generally assumed that these glycoproteins are amorphous components physically trapped by the microfibrillar mesh of the cell wall. Evidence, however, does not support such a simplistic model. Analysis of the cell walls from several fungal species including *Zygossaccharomyces rouxii, Hansenula wingeii, Schizosaccharomyces pombe, Pichia scolyti, C. albicans,* and *S. cerevisiae* revealed the existence of a selective population of glycoproteins that are released only after enzymatic digestion of the glucans from the cell walls (71,106,176). These results suggest that the proteins are bound to glucans through covalent bonds. In all of these species, two populations of glycoproteins can be recognized: One comprises high-molecular-weight polydisperse glycoproteins containing low amounts of protein and a large carbohydrate portion, and the other

is made up of low-molecular-weight proteins (31–34 kD) with small amounts of carbohydrate bound through *N*-glycosidic linkages. These low-molecular-weight proteins seem to be highly conserved, as antibodies prepared against the *Saccharomyces* protein cross-react with the similar proteins from *Candida* and *H. wingeii* (106).

Interestingly, differences in the high-molecular-weight glycoproteins were observed between the walls from the yeast and mycelial phases of *C. albicans*. Yeast walls contained four glycoproteins species of 160,205 kD, and larger molecular mass, whereas mycelial walls showed only the two glycoproteins with smaller masses (71).

When glucan synthesis was inhibited by papulacandin B in *C. albicans*, incorporation of the covalently linked glycoproteins into the cell wall was prevented; on the other hand, incorporation of other glycoproteins, apparently joined to glucan through hydrogen bonding, was not affected. Cellufluor, which, as described above, interferes with the correct crystallization of structural polysaccharides, had exactly the opposite effect; it did not affect the incorporation of covalently bound glycoproteins but inhibited association of the other glycoproteins (166). Inhibition of the synthesis of the carbohydrate moiety of glycoproteins by tunicamycin also blocked the incorporation of covalently bound glycoproteins into the nascent cell wall of *C. albicans* regenerating protoplasts. Under these conditions, proteins of lower molecular weight, probably precursors, were released into the medium (167). These results suggest that covalently bound glycoproteins play an important role in wall assembly.

We (P. Martinez, M. Casanova, M.L. Gil, R. Sentandreu, and J. Ruiz-Herrera, in press) have observed that the wall-less slime variant of *N. crassa* secretes a proteinaceous material into the medium. This material, which contains no lipids, aggregates in the form of large sheets that acquire different configurations: straight, convoluted, and almost vesiculoid. The most important property of this material is that after being dissolved with urea or guanidine it can reaggregate once the solubilizing agents are dialyzed out, giving rise to lamellar structures indistinguishable from the original material. This behavior is similar to the self-assembly capacity of the proteinaceous cell wall from *Chlamydomonas* (109) and oblige us to revise our current concepts of cell wall assembly, which consider that microfibrillar polysaccharides are the components responsible for organization of a coherent cell wall.

Covalent Bonding Between Wall Components

As mentioned above, glucans (and possibly chitin) are bound to proteinaceous material. The establishment of covalent bonding between the several wall polymers may then be an important process in wall organization. In *S. commune* and *Saccharomyces* it has been demonstrated that

glucans are covalently bound to chitin (159,218), and in *Trichoderma viride* incorporation of β-glucans into the cell wall was partially prevented by polyoxin D (31), suggesting an association between the polymers.

The importance of this molecular association for cell wall assembly was suggested by the experiments of Elorza et al. (70) with regenerating *Candida* protoplasts. Using differential labeling of the several macromolecules, an initial organization of a chitin layer was observed followed by alkali-soluble and alkali-insoluble glucans and glycoprotein deposition. In the presence of nikkomycin, chitin was not synthesized, glucans remained alkali-soluble, and high-molecular-weight mannoproteins were not incorporated, whereas papulacandin B affected glucan biosynthesis and glycoprotein incorporation into the nascent wall. The nature of the linkages between the several macromolecules remains unknown, but a presumptive coupling polypeptide with 50% of the residues represented by lysine has been described (238). The mechanism for establishment of these covalent links is only speculative, although it is accepted it must occur in the wall itself. Wessels (238) has suggested that coupling via radicals produced by oxidases is a possibility and cited as examples the cross-linking between lysine residues in animal collagen and between tryptophane residues in extensin of plant cell walls (241).

Cell Wall Growth and Cellular Morphogenesis

Apical Growth

The shape of fungal cells depends on the shape of the cell wall itself. The latter, in turn, is not due to its chemical composition alone but also to the dynamics of its biosynthesis. Spherical cells grow isodiametrically; that is, biosynthetic units are uniformly distributed all over the cell surface; cylindrical cells, on the other hand, grow apically (24). The apical mechanism of growth, suggested some time ago by Reinhardt (182) using surface markers, was later confirmed in fungi by radioactive chitin precursors (24,90). Several aspects may be considered when trying to understand the apical mechanism of wall expansion, which in turn is fundamental for the comprehension of fungal growth. Among these factors we may refer to topological and kinetic aspects, the difference in physicochemical properties of the newly synthesized and the mature walls, and the chemical and biochemical factors in play during cell wall maturation.

Apices from fungal hyphae resemble half-ellipsoids of revolution, with extension zones ranging from 2 to 30 μm in length (219). In order to generate a cylindrical lateral wall, the specific rate of area expansion of the tip wall should be maximal at the apical pole and decrease gradually to become minimal at the base of the dome. This rate is proportional to the

cotangent of the angle formed between the longitudinal axis of the hyphae and a line traced from any point on the apex to the center of the dome base (226). This phenomenon was demonstrated by means of autoradiographic studies (24,90). Apical growth, which, as described above, depends on the continuous supply of apical vesicles, must be a rapid process. Turnover of the apical region occurs within a matter of 2–3 minutes for fast-growing fungi such as species of *Neurospora* and *Mucor*. Such speed is not surprising when one realizes that a whole microfibrillar alkali-resistant cell wall is synthesized by the zoospores from *P. palmivora* within a matter of 2 minutes (16) and that a whole chitin microfibrillar mesh is synthesized by chitosomes from *M. rouxii* in only 30 seconds (35). Therefore in order to sustain apical growth the cell must contain a reservoir of substrate and short-lived polysaccharide-synthesizing enzymes that should remain briefly active in the apical dome and be rapidly deactivated. We mentioned above that cellular concentrations of UDP-GlcNAc and UDP-Glc are in the millimolar range (137). The existence of a pool of chitin synthetase is demonstrated by the fact that inhibition of protein biosynthesis does not stop synthesis of chitin in vivo until 2–3 hours later (211). We have demonstrated that exhaustion of chitin synthesis in cycloheximide-treated germlings is accompanied by a drop in the number of apical vesicles (A. Obregon and J. Ruiz-Herrera, unpublished data). Chitin synthetase is in the form of a cytoplasmic reservoir, as demonstrated by autoradiographic visualization of the enzyme (209). Apical localization of chitin synthetase has been demonstrated by autoradiography of chitin accumulated in cell wall fragments (123,152). Demonstration of the localization of the enzyme itself represented a difficult task in view of the small size of fungal hyphae, but enzyme assays performed in sections obtained from the giant sporangiophore of *P. blakesleeanus* revealed the preferential localization of chitin synthetase in the apical region (105). As predicted, chitin synthetase in vivo once activated is a short-lived enzyme, evidenced by the data described above on the effect of the calcium ionophore A23187 on chitin synthesis in vivo by *Phycomyces* germlings (198).

Physicochemical and Chemical Properties of Nascent and Mature Cell Walls

Growing and nongrowing regions of the cell wall have different viscoelastic properties. Using a specially designed Instron instrument to perform uniaxial extension experiments with the sporangiophore from *Phycomyces*, Ahlquist and Gamow (1) demonstrated that the growing zone was elastic, whereas the nongrowing region was stiff. Cell walls from dead sporangiophores were also rigid. Using external markers, the authors measured the degree of extensibility along the growing region more accurately. Their data (1) followed exactly the pattern observed in other systems for chitin

biosynthesis (see above), i.e., maximal at the tip and minimal at the basal region of the growing zone; these data agree with the general observations that hyphal apexes are more sensitive to osmotic shock than the rest of the cell. Accordingly, we may conclude that cell wall present in the apical zone is plastic and becomes rigidified during its transport to the lateral cylindrical portion of the hyphae.

Cell Wall Expansion

Two general hypotheses have been forwarded to explain the difference in physicochemical properties of growing and nongrowing cell wall regions and to explain cell wall elongation. Based on earlier ideas, Bartnicki-Garcia (17) proposed a model including a delicate balance between wall synthesis and wall lysis. According to his ideas, intussusception of new wall material in the apical growing region requires previous softening of the preformed microfibrillar structure by lytic enzymes. The evidence obtained for this model comes from studies of bursting of hyphal apices induced by different types of solutions, and the fact that the temperature coefficient of bursting in water was 1.3–2.0, which suggests a biochemical reaction, rather than a purely physical phenomenon (26); moreover, the fact that polyoxin induced the same bursting of the hyphal apical region (25) suggested that a weakened structure was more susceptible to the action of lytic enzymes than the normal cell wall.

Based on the different structural properties of the hyphal apexes, Wessels and his colleagues have proposed a steady-state model of apical wall growth (237,238). As mentioned above, physicochemical properties are different between growing and non-growing zones, and structural and chemical differences have also been noticed. For example, wheat germ lectin binds only to the apical region of stage I sporangiophores from *Phycomyces*; and it is only after chitosan is extracted by HNO_2 that the agglutinin binds to the whole sporangiophore wall (105). It has been observed that the apical region of *S. commune* does not contain chitin microfibrils and that the polysaccharide is extremely sensitive to chitinase degradation and dissolution with dilute HCl, whereas in the non-growing zone or in dead hyphae, microfibrils are readily observed and chitin becomes resistant to the above mentioned treatments (232). In a series of papers, Wessels and co-workers (159,215,217,218,240), have also clearly established that newly-synthesized β(1-3) glucans present in the apical region are water-soluble and that after some time they become insoluble in water and then insoluble in alkali. The latter process depends on their covalent bonding to chitin, since it is inhibited by polyoxin D. Moreover, after digestion with chitinase, all the glucan becomes alkali-soluble. Appearence of β(1-6) linkages occurs not in the apical, but in the subapical region, in agreement with our earlier proposal on the synthesis of β(1-6) glucans (see above).

According to the steady-state model, lytic enzymes play no role; it is suggested instead that chitin and β-glucans extruded into the wall are highly hydrated and organize in the form of a visco-elastic structure which yields to the turgor pressure of the cell, stretching and displacing continuously to subapical regions during wall expansion. During this process, the cell wall becomes rigidified by establishment of covalent bonding between the different wall components and by crystallization of the wall polymers (238). In line with this model we may add the possibility that self-assembly of the glycoproteic matrix described above may be an organizer for the correct establishment of the microfibrillar mesh into the newly-synthesized cell wall. Which of the two hypotheses is closer to the mechanism operating in vivo is difficult to discern at the present time. As it usually happens, perhaps both mechanisms are substantial, and the real process involves elements from both hypotheses. It is a fact that lytic enzymes are involved in wall metabolism at certain times; fusion of hyphae during mating, heterokaryon formation, and clamp connecting undoubtedly involve lytic enzymes present in the growing region of hyphae. Moreover, branching demands the operation of such systems. The consideration that wall maturation involves physical and chemical modifications only excludes the operation of regulatory mechanisms of wall growth at this level. In this sense it is known that symmetrical stimuli applied to the giant sporangiophore from *Phycomyces* induce transient changes in growth velocity, whereas asymmetrical stimuli produce changes in growth direction (32,49). These responses are due to changes in the mechanoelastic properties of the cell wall. Ortega et al. (175) demonstrated a weakening in the growing zone of the sporangiophore following its illumination; and we observed that it was due to breakage of chitin microfibrils (105). Chitin biosynthesis was stimulated after this initial weakening, resulting in an increase in wall growth rate. Accordingly, it is possible that under normal conditions wall growth occurs, as suggested by the steady-state model, and that lytic enzymes play a regulatory role to increase or reduce wall growth rate and to induce changes in the direction of hyphal expansion.

Concluding Remarks

We hope that in this brief review of the mechanisms involved in the biosynthesis and expansion of the fungal cell wall we have left the reader with an image of an active, ever-changing composite, rather than the concept of a rigid and metabolically inert structure.

It is clear that great gaps in our knowledge still exist. As such, we have been unable to obtain bona fide purified preparations of the enzymes involved in the synthesis of microfibrillar polysaccharides. We also ignore the intimate mechanisms involved in microfibril biosynthesis and their deposi-

tion outside the permeability barrier of the cell; and our knowledge of the reactions involved in the organization of the various polymers to give rise to such a complex architectural structure as the cell wall remains fragmentary. On the other hand, we must realize that we have gained much knowledge on the structure, synthesis, and organization of the cell walls of fungi owing to the development of new techniques and the introduction of fresh ideas coming from several fields outside strict mycology. Certainly research on the fungal cell wall will remain an active field in the future, and we will obtain a comprehensive understanding on this structure, which is vital for the existence of fungi.

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9—Lipids and Lipoidal Mycotoxins of Fungi

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The presence of lipids in fungi is necessary for maintenance of proper membrane structure and function, storage of energy in the form of triglycerides, and the production of specialized molecules, e.g., hormones and toxins. Species of fungi are able to establish themselves in virtually all types of environments, including on and in both animals and plants. Under most conditions the immune system of animals is capable of controlling systemic fungal infections, which is fortunate because the similarity in physiology that exists between the fungus and its host makes treatment and management of the disease state most difficult. Immunosuppressed states due to disease, e.g, acquired immunodeficiency syndrome (AIDS), or immunosuppressive therapy, e.g., that administered in conjunction with organ transplants, have led to a dramatic increase in the frequency of certain types of fungal infection. Chief among these diseases are those caused by *Candida* and *Aspergillus* species, both of which are found abundantly in nature.

In addition to causing infection in man, fungi are also deleterious to human health because of their formation of mycotoxins. Some of these compounds, e.g., aflatoxin, are among the most toxic and carcinogenic substances known. The toxins are found on or in the foodstuffs on which the fungi have grown, being produced as secondary metabolites of the established fungal growth.

Lipids and Fungal Pathogenicity

The possibile association of lipids and fungal pathogenicity was first proposed by Peck (134), who found that virulent but not avirulent strains of *Blastomyces dermatitidis* and *Candida albicans* contained an unusual carbohydrate-containing phospholipid. Complete characterization of this compound was never reported. Subsequent investigations found that the

total lipid (54) and phospholipid contents (43) of a virulent strain of *B. dermatitidis* were higher than those in an avirulent strain. In contrast, analyses of *Histoplasma capsulatum* (107) and *Paracoccidioides brasiliensis* (125) revealed that lipid content was not associated with virulence.

More recent evidence suggests that steroidal hormones may play a role in regulating the conversion of the dimorphic fungus *P. brasiliensis* from its nonpathogenic mycelial form to its pathogenic yeast form. This organism is the causative agent of paracoccidioidomycosis, a disease that has a 75-fold higher level of occurrence in men than in women despite equivalent exposure to the fungus. Loose et al. (100) have found that *P. brasiliensis* contains estrogen receptors but not androgen receptors. Further studies revealed that conversion of the mycelial form (i.e., the inhaled form) to the yeast form is inhibited by estrogen, suggesting that *P. brasiliensis* may utilize an endogenously produced equivalent to estrogen to regulate conversion from mycelium to yeast. It will be interesting to learn if these findings can be extended to other dimorphic pathogens.

Fatty Acid Composition

The lipid content of fungi, including their fatty acid, phospholipid, and sterol composition, has been thoroughly reviewed by Weete (197). Therefore the fatty acids and phospholipids of only those fungi that are human pathogens are covered here. The lipids of pathogenic fungi have also been reviewed by others (37).

The fatty acid composition of some pathogenic fungi is given in Table 9-1. The fatty acids found in these organisms are identical to those found in nonpathogens. The predominant saturated fatty acids are palmitate (16:0) and stearate (18:0), and oleate (18:1) and linoleate (18:2) are the principal unsaturated fatty acids. On average, palmitate comprises about 18% (range 3–35%) of the total cellular fatty acid. When present, stearate averages 7% (range 1–28%); it was not detected in several species of *Candida* and *Cryptococcus*. Oleate is the most abundant fatty acid, averaging 39% of total fatty acids, and linoleate (when present) averages 28%. Palmitoleate (16:1) is consistently observed in *Candida* species and is a major component in several species. Linolenate (18:3) is a major component in several *Mucor* and *Rhizopus* species. Minor amounts of short-chain (<14 carbons) and long-chain (>18 carbons) fatty acids have been reported for many species. In some species small amounts of odd-chain-length fatty acids, especially 15:0 and 17:0, have also been reported.

In general, there is considerable variation in the amount of a particular fatty acid within a specific genus as well as significant variation among genera. The lipid content of fungi and especially the fatty acid composition can be influenced by culture age and a variety of environmental factors,

TABLE 9-1. Fatty acids of selected pathogenic fungi.

Organism	Short chain	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	Long chain	Ref.
Aspergillus													
A. flavus	—	—	—	tr	11.2	—	tr	2.1	25.6	60.7	—	—	146
A. flavus	0.5	0.2	0.2	—	12.6	—	—	17.6	37	31.9	—	—	165
A. fumigatus	—	—	—	—	17.1	—	—	28.4	25.1	29.2	—	—	14
A. fumigatus	—	—	—	—	19	0.8	—	3.1	35.2	41.9	—	—	197
A. nidulans	0.1	tr	—	0.6	18	0.3	0.4	6.4	17.9	53.8	—	2.5	61
A. niger	—	0.3	—	0.2	15.8	0.7	—	7.2	21.3	37.8	15.6	—	197
A. niger	1.8	0.8	0.7	—	14.2	2	—	8.2	27.8	35.6	8.9	—	165
A. parasiticus	—	—	—	—	11.4	—	tr	5.2	45.9	37.3	—	—	146
Blastomyces													
B. dermatitidis (mycelium)	—	—	—	—	13.7	0.9	—	5	34.1	46.2	—	—	55
B. dermatitidis (yeast)	—	—	—	—	13.3	1.7	—	4.1	52.6	28.3	—	—	55
Candida													
C. albicans (yeast)	—	6.6	5.1	—	28.5	10.4	—	14.5	14.7	5.5	5.7	—	71
C. albicans	—	0.6	—	—	11.6	8	—	6.8	35.9	25	9.6	—	41
C. albicans	0.5	0.7	—	—	14	8.5	2.3	6.3	43.5	17.3	1.5	1.5	116
C. albicans	5	10.1	—	—	28.3	9.3	2.6	6.1	30.2	—	—	3.7	68
C. curvata	—	3	—	—	17	1.5	—	3.5	36.5	27.5	6.5	1	116
C. curvata	3.8	—	—	—	13	39.2	—	—	43.2	—	—	—	68
C. guilliermondii	—	—	—	—	17.3	7.8	1.5	3.8	45	14	5	2.3	116
C. guilliermondii	11.1	12	—	—	19.6	4.6	—	2.2	48.9	—	—	—	68
C. intermedia	10.2	—	—	—	20.3	7.4	—	—	62.1	—	—	—	68
C. krusei	—	—	—	—	10.5	26.7	—	—	22.1	35.4	5.3	—	83
C. krusei	—	—	—	—	15	7	1	2	40	17	15	1	116
C. krusei	tr	tr	—	tr	15	6.2	tr	1.1	48.2	14.9	13.7	tr	84
C. krusei	10	—	—	—	19.6	5.2	—	—	65.2	—	—	—	68

C. lipolytica	—	—	—	—	13.7	10	0.3	2.7	42.7	22.3	—	2	116
C. lipolytica	7.6	—	—	—	12.5	4.7	—	—	75.2	—	—	—	68
C. parapsilosis	—	—	—	—	22	4	5	8	40	10	5	1	116
C. parapsilosis	5.8	—	—	—	22.6	10.2	—	—	61.6	—	—	—	68
C. pseudotropicalis	—	0.3	—	—	15.3	10.7	—	4	35.7	20.7	7.7	—	116
C. pseudotropicalis	12.4	—	—	—	29.2	2.2	—	—	55.8	—	—	—	68
C. rugosa	—	1	—	—	17	16	—	4	42	11	4	—	116
C. rugosa	12.1	—	—	—	18.6	3.4	—	—	65.9	—	—	—	68
C. tropicalis	—	—	—	—	28.1	13.8	1.9	—	55.8	—	—	—	68
C. tropicalis	—	0.4	—	—	12.2	5.8	—	4.3	44.1	22.7	—	—	175
C. tropicalis	tr	tr	—	1.2	21.8	5.4	2.6	9.4	28.6	26.2	4.4	tr	84
C. tropicalis	0.3	1	—	—	19	10	0.3	8.7	43	13	2	1	116
C. utilis	—	—	—	—	19	6	—	2.5	35	24	12	—	48
Coccidioides													
C. immitis (avirulent mutant)	0.19	0.18	—	0.24	32.1	—	1.7	8.7	13.6	1.2	—	42.1	3
C. immitis (wild type)	0.11	0.83	—	0.35	15.7	1	2.9	17.6	40.3	6	—	15.26	3
Cryptococcus													
C. gastricus	—	—	—	—	11	—	—	3	28	53	—	1	116
C. gastricus	3.6	—	—	—	16.2	—	—	—	81.1	—	—	—	68
C. laurentii	—	tr	—	tr	17.2	tr	tr	8.5	37.5	36.5	—	tr	84
C. neoformans	—	—	—	—	18.5	—	—	7.5	42.5	27	—	1.5	116
C. neoformans	—	tr	—	tr	13.5	tr	tr	1.9	38.1	46.6	—	—	197
C. neoformans	2	—	—	—	16.8	—	—	—	81.1	—	—	—	68
C. terreus	—	—	—	—	18	1	1	11	37	27	—	2	116
C. terreus	2.8	—	—	—	14.6	—	—	—	82.5	—	—	—	68
Epidermophyton floccosum	tr	tr	—	—	36.6	3.8	tr	9.2	15.2	33.2	—	—	88
Histoplasma													
H. capsulatum (mycelium)	—	—	—	—	18.7	1.6	—	6.5	33.9	39	—	—	55

TABLE 9-1. *Continued*

Organism	Short chain	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	Long chain	Ref.
H. capsulatum (yeast)	—	—	—	—	15.3	2	—	2.3	56.1	24.2	—	—	55
Microsporum													
M. cookei	—	1	—	—	28.9	1.4	—	7.1	11.9	50	—	—	89
M. gypseum	—	—	—	—	17.2	—	—	7.7	10.4	64.7	—	—	200
Mucor													
M. hiemalis	—	1.8	—	—	15.5	2	—	17.2	28.6	18.5	18.2	—	173
M. javanicus	—	3.2	0.4	—	20.3	4.7	—	8.3	26.8	12.6	13.7	—	165
M. mucedo	—	1.1	—	—	16.8	1.4	—	11.3	30.5	32.9	6.4	—	173
M. racemosus	—	3.1	—	—	17.8	3.3	—	5.9	38.2	12.7	18.8	—	173
M. ramannianus	—	1.6	—	—	17.6	3.1	—	5.9	28.7	13.1	29.1	—	173
Rhizopus													
R. arrhizus	—	1.2	—	—	18.4	3.7	—	11	29.4	16.3	0.2	—	197
R. arrhizus	1	0.7	1.3	—	19.9	3.5	—	7.4	40.6	15.8	9.8	—	165
R. stolonifer	—	tr	—	—	15.7	3.4	—	3.9	30.4	19.1	27.5	—	197
R. stolonifer	4.9	3.6	1.9	—	20.8	3.9	—	5.2	34.3	7.8	15.6	—	165
Torulopsis													
T. glabrata	—	—	—	—	2.8	33.6	—	7.2	55	—	—	—	68
T. glabrata	—	0.25	—	—	6	32.5	0.25	6	53.3	—	—	—	116
Trichophyton													
T. mentagrophytes	tr	tr	—	—	32.8	6.8	—	8.4	3.9	51.8	—	—	89
T. rubrum	tr	0.8	—	2.1	23.8	—	—	7.4	13.1	52.4	—	tr	199
T. rubrum	3.6	0.8	0.1	0.5	17.6	2.6	0.8	16.1	19.2	35.6	—	2.4	37
Trichosporon beigelii	—	tr	—	tr	24	tr	tr	6.1	35.0	36.3	3.4	—	84

— = none detected or not reported; tr = trace amounts.

including temperature, oxygen, nutrient availability, and pH. This point deserves emphasis, as many clinical laboratories utilize fatty acid composition as a criterion to characterize and identify parasitic organisms. In *Aspergillus nidulans* an amazing amount of variation was observed in the proportion of linolenate, which increased 15-fold during the first 24 hours of growth from conidia to reach a level of 5.6% of the total fatty acid content (61). This same fatty acid totally disappeared by the fourth day of growth. Other species in which fatty acid composition has been observed to change as a function of culture age include *Penicillium atrovenetum* (193), *Tricholoma nudum* (93), *Phycomyces blakesleeanus* (20a), *Candida utilis* (48), *Mucor genevensis* (72), and several species of *Entomophthora* (142, 143). Alterations in total amounts of lipid as a function of culture age have been noted in *Paecilomyces persicinus* (130a). The inter- and intraspecies variability in fatty acid composition could be attributed to one or more of the factors cited above.

Phospholipid Composition

In the pathogenic species analyzed to date, the predominant phospholipids are phosphatidylcholine and phosphatidylethanolamine, typically accounting for 50–80% of the total phospholipid (Table 9-2). Phosphatidylserine and phosphatidylinositol generally comprise 5–15% of the total phospholipid. *Histoplasma capsulatum* is a notable exception to this pattern in that it possesses a high level of phosphatidylinositol (49.3%) and a low level of phosphatidylcholine (7.8%). Other exceptions include *Aspergillus niger*, in which phosphatidylinositol was not detected, and *Trichophyton rubrum*, which contains a high level of phosphatidylserine (27.7%). In many species, the phospholipid fraction also contains minor amounts (<10%) of phosphatidic acid, cardiolipin, and a variable percentage of unidentified material. The amounts of individual phospholipids can also change as a function of culture age (130a), again suggesting the need for caution in the use of lipid composition as a determinative factor in species identification.

The fatty acids and phospholipids of pathogenic fungi appear to be both quantitatively and qualitatively similar to those of nonpathogens. There is no direct evidence indicating that the presence or absence of a particular fatty acid or phospholipid contributes to the pathogenicity of an organism. However, it is possible that some as yet unidentified lipid component(s) may be associated with fungal virulence.

Sterols

Sterols are structurally related compounds derived from isoprene (5-C) units. They are essential constituents of, and play critical roles in, the func-

TABLE 9-2. Phospholipid composition of pathogenic fungi.

Organism	Percent of total phospholipid							Ref.
	PC	PE	PI	PS	PA	CL	Other	
Aspergillus								
A. nidulans	47.0	37.5	10.5	4.0	NR	NR	1.0*	44
A. niger	51.0	28.5	ND	5.0	ND	2.3	10.5†	97
Candida								
C. albicans	40.0	25.3	NR	12.0	ND	5.3	13.3*	169
C. albicans (mycelium)	28.8	12.7	11.4	9.9	7.3	ND	NR	71
C. albicans (yeast)	25.6	11.4	10.9	8.8	9.0	ND	NR	71
C. krusei	33.0	17.0	20.0	9.0	<3.5	6.0	11.5*	197
C. parapsilosis	39.8	21.1	11.6	4.2	10.8	8.7	3.7‡	130
C. tropicalis	31.0	19.0	15.0	14.0	<3.5	8.0	9.5*	197
C. utilis	32.0	27.0	13.0	12.0	<3.5	8.0	4.5*	197
Cryptococcus neoformans	49.0	28.0	7.0	8.0	<3.5	4.0	4.0*	197
Epidermophyton floccosum	38.3	28.5	2.6	10.7	ND	3.3	16.9§	88
Histoplasma capsulatum	7.8	24.3	49.3	10.1	ND	ND	8.1‖	107
Microsporum gypseum	23.1	29.8	3.7	19.4	ND	ND	24.0¶	87
Paracoccidioides brasiliensis	25.0	20.9	14.7	12.1	2.6	7.0	17.8**	106
Torulopsis glabrata	29.0	16.0	16.0	14.0	<3.5	8.0	13.5*	197
Trichonopsis variabilis	41.0	18.0	12.0	12.0	<3.5	7.0	10.0*	197
Trichophyton rubrum	23.5	18.8	13.3	27.7	8.7	ND	8.1††	46
Trichosporon beigelii	29.0	15.0	11.0	12.0	<3.5	6.0	23.5*	197

PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PA = phosphatidic acid; CL = cardiolipin; NR = not reported; ND = not detected.
* Unidentified.
† Polyprenyl phosphate.
‡ Lyophospholipid.
§ Lyophosphatidylcholine (7.2%), unidentified (9.7%).
‖ Sphingomyelin.
¶ Lyophosphatidylcholine (11%), unidentified (13%).
** Cerebrosides (15.7%), unidentified (2.1%).
†† Polyphosphatidylinositol.

tional complexity of eukaryotic biological membranes (50,131). Their major functions include the maintenance of adequate levels of membrane fluidity (50,91,94,153), permeability (13,90), and modulation of the activities of certain membrane-bound enzymes (40,50,91,168,197). Moreover, the biochemical pathway of sterol synthesis is also implicated in providing isopentenyl groups to adenosine for tRNA structures and the side chain of dolichol and ubiquinone (28,145).

The knowledge of sterol biosynthetic process has been used to help ameliorate the problem of excessive cholesterol in humans as well as to achieve effective methods of controlling medically and agriculturally important fungi. Weete (197), Parks (131), and Nes and McKean (121) have reviewed in depth the sterols in fungi; Kato (85), Leroux and Benveniste (95), and Van den Bossche (187) have reviewed the inhibitors of sterol synthesis and their mode of action.

Ergosterol is the predominant sterol in most fungi (197), including *A. fumigatus* (74), *C. albicans* (190), *Monilina fructigena* (86), *Neurospora crassa* (147), *Penicillium expansum* (96), *A. nidulans* (59,164), and *A. niger*, and *Spicaria elegans* (198). Lognay et al. (99) extracted six sterols from lipid-accumulating *Candida curvata* and found that about 69% of the total sterol consisted of ergosterol. Other sterols are found in related species, e.g., episterol and brassicasterol in *Aspergillus oryzae* (66). The vegetative cells of pythiaceous (oomycetes) fungi (*Pythium* and *Phytopthora* spp.) do not produce sterols despite the fact that they require exogenously supplied sterol for sexual or zoosporangial reproduction (73,79,196). Among the oomycetes, species of Saprolegniales and Leptoniales contain cholesterol, desmosterol, 24-methylenecholesterol, and fucosterol. A strain of *Haliphthoros milfordensis* (marine oomycete) not requiring any lipid growth factor contains 24-methylenecholesterol and fucosterol, and *Atkinsiella dubia*, a related marine oomycete (not requiring any lipid growth factor), contains desmosterol, 24-methylenecholesterol, and fucosterol (102). The near universality of ergosterol as the fungal sterol has made disruption of its biosynthetic pathway a major means of arresting fungal growth.

Ergosterol is synthesized in four major phases. The first stage involves the successive condensation of three acetyl coenzyme A (CoA) molecules using the cytosolic enzymes acetoacetyl CoA thiolase and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase. The second phase of sterol synthesis is the conversion of mevalonic acid to squalene. Phase 3 is the cyclization of squalene to lanosterol, which requires the oxidation of squalene to 2,3-oxidosqualene (squalene-2,3-epoxide). The last phase is the modification of lanosterol to yield the final sterol product, usually ergosterol.

The condensation of acetyl CoA molecules is the most important pathway to HMG-CoA, although alternative routes have been reported for a few fungi (197). Servouse et al. (163) isolated a *Saccharomyces cerevisiae*, ergosterol-requiring mutants defective in HMG-CoA synthase and acetoacetyl CoA thiolase. The ergosterol auxotrophy suggests an insufficiency of other biochemical pathways, e.g., the degradation of amino acids, to produce HMG-CoA.

HMG-CoA is reduced to mevalonic acid by the action of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and NADPH; it is the rate-limiting step in the sterol synthetic pathway. *Aspergillus nidulans* appears to possess a pattern of HMGR-specific activity consistent with the pattern of total sterol production (59,60). The pattern, however, is different from that observed in *S. cerevisiae* (131) in which the amount of ergosterol continues to increase even in the stationary phase, although much of this ergosterol produced at this late growth stage is packaged into steryl esters. The HMGR of *A. nidulans* appears to be feedback-regulated, in that the activity decreases with addition of exogenous ergosterol (60). Exogenous ergosterol was shown to inhibit HMGR activity in *S. cerevisiae* strain *ole*3

by approximately 50% (12), but no inhibition was observed in the wild-type strain (180). The decrease in activity of HMGR in *A. nidulans* by ergosterol is more pronounced in the presence of miconazole (60).

Ergosterol does inhibit the activity of the acetoacetyl CoA thiolase and HMG-CoA synthase (180). Specific activities of HMG-CoA synthase and acetoacetyl CoA thiolase are increased in *S. cerevisiae* deprived of ergosterol (162) and are decreased strongly in anaerobically cells grown with excess ergosterol. HMGR activity is only slightly affected.

Although in mammals the HMGR has been demonstrated to be the product of a single gene, two functional genes have been reported in *S. cerevisiae* encoding two HMGR isozymes, designated HMG 1 and HMG 2, which are differentially expressed depending on growth conditions (17). These proteins may be localized to different organelles or have different kinetic properties. In *A. nidulans*, two pH optima for HMGR activity (pH 5.0 and 7.0) have been reported (60) in the $100{,}000 \times g$ supernatant and the pellet, suggesting that both forms of the enzyme exist. Multiple forms of HMGR (soluble and particulate) have also been reported in *Fusarium oxysporum* (104). In *N. crassa* (82) the reductase activity is associated with the microsomal pellet, whereas in *S. cerevisiae* the enzyme has been reported to be located in the mitochondrial matrix (167,180).

The thiocarbamate antimycotics tolnaftate and tolciclate, which have been used clinically as topical treatments against dermatophytic infections, block ergosterol biosynthesis in *C. albicans* cells and in cell extracts (159). The accumulation of squalene suggests that the site of action is the microsomal squalene epoxidase. Barrett-Bee et al. (15) also reported that tolnaftate is active against squalene epoxidation in broken cells of *C. albicans*. This compound and the allylamines also inhibit squalene epoxidation in the fungus *Trichophyton mentagrophytes*. The allylamine drugs terbinafine and naftifine and naphthiomate also inhibit the microsomal epoxidase enzyme in fungi, including *T. mentagrophytes*, *C. albicans*, and *C. parapsilosis* (115, 130,137,154–157,160).

The 2,3-oxidosqualene is cyclized to form lanosterol. Oxidosqualene cyclase, which catalyzes this reaction, has been isolated from a fungal system (49,113,166). Cattel el al. (33) reported the existence of two fundamental types of the cyclase enzyme: (a) the cyclase responsible for the cyclization of chair-boat-chair-boat (cbcb) folded squalene providing lanosterol, cycloartenol, parkeol, and cucurbitadienol in various organisms; and (b) the cyclase, which includes α- and β-amyrin synthetase, which is involved in the cyclization of cccb folded 2,3-oxidosqualene. β-Amyrin, a pentacyclic triterpene, has been found as an alternative cyclization product to lanosterol in *A. nidulans* (70).

An attempt has been made to produce antifungal drugs that control via the 2,3-oxidosqualene cyclase. 2-Aza-2,3-dihydrosqualene (and derivatives) inhibits the cyclase in *S. cerevisiae* (33) and *C. albicans* (158). These inhibitors are also active against liver microsomal oxidosqualene cyclase.

This lack of specificity has ruled out their use as potential antifungal agents.

In fungi ergosterol may be synthesized from lanosterol via several alternative pathways (85,119,197). Because of its clinical and agricultural importance, the mechanism of the sterol biosynthetic pathway has been studied as a means of controlling mycoses. Kato (85) has classified the inhibitors of ergosterol biosynthesis into six groups based on their mode of action: 14α demethylation, $\Delta^{8,7}$ isomerization, Δ^{14} reduction, Δ^{22} unsaturation, C-24 transmethylation, and $\Delta^{24(28)}$ reduction inhibitors.

Demethylation at C-4 and C-14

Ergosterol biosynthesis in fungi involves the oxidative removal of the 14α-methyl group (C-32) of lanosterol. In *C. utilis* and *C. albicans*, the methylation at the lanosterol level is minor (85). In *S. cerevisiae* and *C. albicans* C-14 demethylation is the first step in the pathway of lanosterol to ergosterol (140). It is accompanied by loss of C-15 hydrogen, resulting in the formation of 14 (15) double bond. The 14α-methyl group is removed as formic acid, and the resulting metabolite is 4,4-dimethyl-5-cholesta-8,14,24-trien-3β-ol (6).

Morita and Nozawa (115) reported that miconazole, which is known to inhibit C-14 demethylation, caused C-24 methylene-dihydrolanosterol accumulation in *T. mentagrophytes*, and in *C. albicans* the drug caused accumulation of lanosterol. Apparently, in miconazole-treated *T. mentagrophytes*, C-14 demethylation is preceded by the introduction of the C-24 methylene group in the biosynthesis of ergosterol (115,190). The question is unclear, however, because Polak-Wyss et al. (140) reported that metabolism of lanosterol in *C. albicans* takes place, stopping at 24-methylene dihydrosterols despite the inhibition of C-14 demethylation by oxiconazole.

The sterol biosynthetic inhibitors that primarily affect C-14 demethylation form a complex between their heterocyclic moiety and the protoheme iron of the microsomal cytochrome P-450 involved in the process of lanosterol demethylation (5,52,67). It has been hypothesized that the inhibitory activity of such antimycotic drugs as itraconazole, imidazole, and triazole is derived from their interaction with this enzyme system (25,108,188,189,191,192). A form of the enzyme that catalyzes 14α demethylation of lanosterol in the presence of NADPH, molecular oxygen, and NADPH cytochrome P-450 reductase was purified from microsomes of *S. cerevisiae* and was named "P-450$_{14DM}$" (6,203). Itraconazole, unlike ketoconazole, does not significantly affect the cytochrome P-450 isozyme involved in testicular and adrenal steroidogenesis of mammalian cells (188).

Miconazole and ketoconazole modulate HMGR activity in cultured cells

with the formation of oxylanosterol, an intermediate that is necessary for the inhibition. An elevated concentration of ketoconazole, but not miconazole, caused accumulation of 24, 25-epoxylanosterol in cultured Chinese hamster ovary cells, demonstrating a second site of inhibition for ketoconazole. Miconazole treatment of cells regulate HMGR without the obligate formation of the epoxidized sterol (63,181). Results indicated the lanosterol 14α-methyl demethylation, but not 24,25-epoxy lanosterol formation, is required to suppress HMGR. Nicholas et al. (124) have isolated a mutant of *Torulopsis glabrata*, designated G4/M1, resistant to miconazole. Though the results have pointed to an apparently general effect, the mechanism of resistance is not yet clear. There appears to be a higher phospholipid/sterol ratio in *C. albicans* strains that are azole-resistant than in those that are azole-sensitive (80).

$\Delta^{8,7}$-Isomerase and 14-α-Reductase

Tridemorph is shown to be a strong inhibitor of the $\Delta^{8,7}$-isomerase, and fenpropimorph and fenpropidin are found to be potent inhibitors of both $\Delta^{8,7}$-isomerase and 14α-reductase (10,11). The treatment with the morpholine fungicides lead to the accumulation of two main classes of sterols: those retaining a single nuclear Δ^{8} double bond and those retaining the $\Delta^{8,14}$ dienoid system (11). Inhibition of the $\Delta^{8,7}$-isomerase by these fungicides results in the accumulation of fecosterol in the yeast *S. cerevisiae*. In *Ustilago maydis* inhibition of the isomerase results in the accumulation of (a) fecosterol, ergost-8-enol, and ergosta-8,22-dienol (tridemorph treatment); (b) ergost-8-enol and ergosta-8,22-dienol (fenpropimorph); and (c) ergost-8-enol (fenpropidin). Inhibition of the reductase enzyme by only fenpropimorph- and fenpropidin-treated cultures of *U. maydis* is indicated by the accumulation of ignosterol. In *S. cerevisiae* the reductase inhibition by the three fungicides results in the accumulation of 4,4-dimethylcholesta-8,14,24-trienol, 4,4-dimethylcholesta-8,14 dienol, and ignosterol.

The structure–activity relation of these fungicides has been discussed by Baloch and Mercer (10). They found that the *cis*-methylated form of fenpropimorph displays effective inhibitory activities, but the demethylated or *trans*-methylated form of this compound and derivatives exhibit a lesser extent of inhibition. The dual site of action, i.e, isomerase and reductase, reduces the risk of resistance development.

Introduction of Double Bonds

Pimaricin-resistant mutants of *A. nidulans* have been isolated that lack ergosterol owing to a defect in sterol C-22 desaturation (205). The sterols 5,7-ergostadienol, 5,7,24(28)-ergostatrienol, and 5,8-ergostadienol are

accumulated in these mutants. C-22 desaturation reaction is mediated by a cytochrome P-450 (78). Ziogas et al. (205) pointed out that this reaction may be the last event in sterol synthesis in *A. nidulans*, or at least all transformations between lanosterol and ergosterol can be made on precursors saturated at C-22.

Introduction of double bonds at C-5 and at C-22 positions may precede reduction of the $\Delta^{24(28)}$ double bond. A nystatin-resistant mutant strain of *S. cerevisiae* defective in Δ^5 desaturation and $\Delta^{24(28)}$ reduction has been analyzed (117). Ergosta-7,22,24(28)-trienol accumulates in this strain.

C-24 Alkylation

24-Epiiminolanosterol inhibits side-chain methylation (C-24) in cultures of *Gibberella fujikuroi* with mycelial accumulation of lanosterol and 24-desalkyl sterols (118). The growth inhibition was caused by aberrant mycelial membranes resulting from the altered sterol composition. Similar results have been obtained by Oehlschlager and co-researchers (128) in yeasts in which accumulation of 24-desalkysterols is noted when growth is reduced by 25-azalanosterol.

Gibberella fujikuroi synthesizes multiple 24β-methylsterol endproducts, whereas in *S. cerevisiae* a single 24β-methylsterol endproduct, ergosterol, is synthesized (118). Three endproducts, ergosterol (24β-methylcholesta-5,7,22E-trien-3β-ol), brassicasterol (24β-methylcholesta-5,22E-dien-3β-ol), and 22(23)-dihydrobrassicasterol (24β-methylcholesterol), are found to be noninterconvertible during the exponential phase in *G. fujikuroi* (119). Based on this finding, Nes and Heupel ruled out the metabolic route $\Delta^{5,7,22}$-24β-CH_3 $\Rightarrow$ $\Delta^{5,22}$-24β-CH_3 $\Rightarrow$ Δ^5-24β-CH_3.

Epiiminolanosterol, which is structurally related to azasteroids, is believed to act on *S*-adenosyl-methyltransferase. *S*-Adenosyl-methionine (SAM) serves as the methyl donor for C-24 methylation in ergosterol biosynthesis (132). Methionine may also serve as a methyl donor in yeast *S. cerevisiae* (110,129,172) and *C. albicans* (154).

Sterol Synthesis and Fungal Growth

Although ergosterol content is frequently used as an indication of fungal mass (123), experimental findings suggest different synthetic patterns appear in at least some species. Ergosterol is continuously synthesized and stored in the yeast *S. cerevisiae* (131), and steryl esters are accumulated in *S. cerevisiae* (9,176,178) and *P. blakesleeanus* (16). Steryl esters are formed between a long-chain fatty acid, e.g., oleate or linoleate, and sterol (C-3 position) via an ester linkage. They may function in maintenance of the intracellular pool of free sterol within the physiological limits required for proper membrane function (22). Distribution of accumulated sterols by

*erg*6 mutants of *S. cerevisiae* (defective in ergosterol synthesis) varies with growth phase and between free and esterified fraction (111). The steryl ester concentrations of the mutant in exponential growth is higher than those of the wild type.

Sterol production in *A. nidulans* is highest during rapid growth and decreases during the stationary phase (60). Evans and Gealt (60) found that HMGR specific activity was decreased in the stationary phase and suggested that it may represent a regulatory behavior due to a reduced cellular demand for ergosterol. In both stationary and exponential phases of *A. nidulans* cultures, the sterol is found primarily in the form of free sterol and not the steryl ester. Virtually no steryl esters were observed by Buchanan et al. (29) in *Penicillium urticae* (up to 10 days). The low levels of steryl esters in these fungi are due to either the lack of efficient synthesis or the existence of an efficient system for hydrolyzing the ester bond.

Role of Sterols

An exogenous source of ergosterol is required by the *S. cerevisiae* when it is grown anaerobically (4,98,101). The requirement for membrane sterol is not specific for ergosterol, however, and other sterols may be substituted for ergosterol (122,138).

Because the biosynthesis of sterols is an aerobic process, it is possible to cause sterol concentration to be depleted by anaerobic growth. The oxygen-dependent reactions in the sterol biosynthesis include squalene epoxidation and desaturation and demethylation of lanosterol (8,78). Using an anaerobic growth experimental system, Nes et al. (120,122) found that a selectivity exists in the sterols that can be accommodated. The anaerobically grown cells take up the normal E-17(20) but not the Z-17(20) conformation of dehydrocholesterol. Van den Bossche et al. (190) have postulated that integration of 14-methylsterols such as lanosterol, 14-methylfecosterol, and 4,14-dimethylzymosterol may not be as efficient as the planar demethylated sterols such as ergosterol and cholesterol. The requirement for ergosterol and the obstructive effect of some ergosterol precursors in *C. albicans* have also been reported by Polak-Wyss et al. (140). Shimokawa et al. (168) have isolated polyene-resistant mutants of *C. albicans* that accumulate 14-methyl sterols in place of ergosterol but are defective in hyphal growth. They concluded that an intrinsic relation between sterol composition and morphogenesis exists.

Marishal et al. (108) have found that itraconazole is more inhibitory than ketoconazole in *A. fumigatus* and *A. niger*. They postulated that the accumulation of 4,14-dimethyl and 4,4′,14-trimethyl sterols in the treated cultures may result in an uncoordinated activity of membrane-bound enzyme systems such as chitin synthase. Pesti (135) isolated ergosterol-negative mutants of *C. albicans* with increased membrane-bound chitin synthase

activity compared to that in the ergosterol-producing parental strain. Chiew et al. (36) reported that high concentrations of ergosterol are inhibitory to the enzyme chitin synthase. Van den Bossche et al. suggested that the toxic activity of azoles may originate from the reduced ergosterol biosynthesis that leads to deteriorated membranes, disturbed membrane-bound enzymes, and uncoordinated synthesis of chitin (191).

Hancock and Weete (77) suggested that reductions in the ergosterol content mediate localized alterations in fatty acid composition, which reduces the functional capacity of the membranes, resulting in reduction of the growth potential of the treated fungus. Dexter and Cooke (53) compared mesophilic and psychrophilic *Mucor* spp. for ergosterol and fatty acid content. They found that the mesophiles' ergosterol level is higher and fatty acids are more saturated than those of the psychrophiles. A similar relation has been reported by Hammonds and Smith (76) in *Mucor* with a decrease in membrane phospholipid unsaturation from psychrophilic to mesophilic species. Total lipid, however, differed little when these species (*M. psychrophiles, M. hiemalis*, and *Rhizomucor pusillus*) were compared. Pesti et al. (136) investigated ergosterol biosynthesis in several mutants of *C. albicans* in which the sterol biosynthesis was blocked beyond zymosterol. The alterations in sterol composition caused a slight increase in saturation and a decrease in the chain length of fatty acids, decreases in phosphatidylcholine and phosphatidylserine, and increases in phosphatidylinositol and phosphatidic acid. These mutants exhibited higher plasma membrane order parameters than their ergosterol-producing parental strain during exponential growth.

A change in sterol profile may be the deleterious feature for the growth inhibition in fungi (119). It is evidenced in *G. fujikuroi* treated with epiiminolanosterol, which blocks the introduction of a methyl group into sterol side chains at C-24. Sufficient bulk sterol (e.g., lanosterol) is synthesized, but the appropriate sparking 24-methylsterol is not biosynthesized. The altered sterol composition leads to growth inhibition due to changes in the mycelial membranes (119). In *G. fujikuroi*, unlike yeast, ergosterol cannot play a dual role (bulk and regulatory), and apparently the two other sterol endproducts (brassicasterol and 22(23)-dihydrobrassicasterol) exert a regulatory function on the fungus (119).

In *S. cerevisiae* sterol functions in two roles: (a) the nonspecific bulk membrane; and (b) the high specificity "sparking" role (26,139,149,150). The latter role is satisfied by minute amounts of sterol possessing a nuclear unsaturation at the C5,6 position (Δ^5-sterol), and the former role requires large quantities of either sterol or stanol. Bottema et al. (26) have indicated that sparking sterol is not required for modulating overall bulk lipid properties of the plasma membrane. Rodriguez and Parks (149) reported that in *S. cerevisiae* the sterols possessing only the C-28 methyl group and a C5,6 unsaturation or that are capable of being desaturated at C5 fulfill the high-specificity sparking requirement, whereas the requirement for bulk

membrane of the sterols is broad. The results by McCammon et al. (111), however, have shown that in the mutant strain (*erg6*) of *S. cerevisiae* C-28 methylated sterols are not required for growth. Yeast sterol auxotrophs do not require C-28 methyl sterols for either bulk membrane or the "sparking" functions (149).

The multifunction utilization of sterol molecules by fungi suggests that more sophisticated and more specific antifungal agents may eventually be produced that interrupt the "sparking" function specifically. These agents presumably will have less cross-reactivity with the enzymes of the fungal host's biosynthetic processes and hence be less toxic.

Mycotoxins

Mycotoxins are metabolites of toxigenic fungi associated with the illness in humans and animals exposed to them via ingestion (contaminated food or feed), inhalation (toxins or spores), or dermal contact (32,81,171,182). Most of these compounds are referred to as "secondary metabolites" because their biosynthetic pathways appear to be a diversion from primary metabolism, or their role(s) in the physiology of the mold is not yet clear.

These compounds have become an important health issue since the discovery of aflatoxins, the causative agent of "turkey-X disease" in 1960 (31a). Because aflatoxins are potent hepatocarcinogens, the goal of many interdisciplinary studies is understanding the biosynthesis, metabolism, and mechanism of carcinogenicity of this group of mycotoxins (51).

Mycotoxins are synthesized from fungi of many taxonomic genera and species and may exert different biological effects. Some of the mycotoxins are placed in one group because their chemical structures are similar or their biosynthetic pathways are related. For example, aflatoxins B_1, B_2, G_1, G_2, and so on possess similar basic skeletons, i.e., a condensed bisfuran/coumarin ring system (27). Trichothecenes, another group of mycotoxins that are produced by various genera and species of fungi, contain the tricyclic trichothene skeleton with an epoxide ring at C-12 and C-13 (185).

Biosynthesis of Mycotoxins

Polyketides are one of the most common fungal end-products of acetate metabolism (7). Aflatoxins are polyketides synthesized via the acetate-malonate pathway, analogus to fatty acid synthesis but lacking the systematic dehydration and reduction steps (23,24). The chain is initiated with acetyl CoA, which is condensed to two molecules of malonyl CoA in the presence of NADPH to form an enzyme-bound hexanoyl starter (19). The chain is then elongated with the addition of malonyl CoA. Cyclization and

aromatization of the C20 polyketide chain results in formation of an anthrone (194), which then undergoes oxidation at C10 to norsolorinic acid, a polyhydroxyanthraquinone.

Using mutants of *A. parasiticus*, Bennett et al. (20) demonstrated that norsolorinic acid is reduced to the pigment averantin ($C_{20}H_{20}O_7$). Averufin is then formed from averantin by successive oxidations at C5′ and spontaneous intramolecular ketal formation (179). McCormick et al. (112) placed averufanin in the pathway between averantin and averufin. Hydroxylation of averufin produces nidurufin. Versiconal hemiacetal acetate is formed from the rearrangement of nidurufin (19) and is then converted to versicolorin A by losing its acetyl group. Versicolorin synthesis is absolutely dependent on Zn^{2+} (42,62).

Versicolorin A is then converted to the xanthone sterigmatocystin (ST) (65). Dutton et al. (56,57) reported that aflatoxin B_1 (AFB_1) and AFB_2 originated independently from versicolorin A and versicolorin C, respectively. Bhatnager et al. (21) reported that *O*-methylsterigmatocystin (OMST) is an intermediate between ST and AFB_1. The experimental results obtained by Cleveland et al. (39) indicated that B_1, B_2, and G_1 arise from separate branches in the aflatoxin biosynthetic pathway.

Regulation of mycotoxin biosynthesis is somewhat unclear. Abdollahi and Buchanan (1,2) developed a nutritional shift protocol and observed that carbohydrate metabolism is an essential factor for aflatoxin induction. These findings were confirmed in subsequent investigations. However, a relation between overall energy status and the induction or initiation of aflatoxin could not be identified (30). However, electron microscopic evaluations indicated that the toxin production occurred in association with a glucose-mediated inactivation of mitochondria, suggesting that the synthesis may be controlled by the energy status of specific subcellular compartments (30). It has been determined by Buchanan and Lewis (31) that a nutritional shift from a non-aflatoxin-supporting medium to an aflatoxin-supporting medium alters the activities of various glycolytic and tricarboxylic acid cycle enzymes.

Biological Effects of Mycotoxins

The biological effects of some important mycotoxins are summarized in Table 9-3. AFB_1 is a procarcinogen and promutagen and requires metabolic activation to exert its effect (161). The metabolic pathways of aflatoxin include production of less toxic hydroxylated compounds, AFM_1 (microsomal cytochrome P448-mediated), AFQ (P450-mediated), and AFP_1 (hydroxylated and demethylated). Another pathway involves a P-450-mediated synthesis of AFB_1 epoxide, which may undergo a microsomal epoxide-hydrolase-mediated or nonenzymatic reaction into less toxic AFB_1 dihydrodiol, or it may undergo a cytosolic glutathione *S*-transferase-

TABLE 9-3. Mycotoxins and their biological effects.

Mycotoxin (with refs.)	Biological effect	Organism
Aflatoxins (19)	Hepatocarcinogen	*A. parasiticus, A. flavus*
Trichothecenes (T-2, nivalenol, deoxynivalenol, satratoxins, DAS*) (127,152,182, 109a)	Dermal toxicity; impairment of immune system; inhibit protein synthesis, teratogen*	*Fusarium, Acremonium, Stachyobotris, Trichoderma, Trichothecium, Verticimonosporium*
Anthraquinones (luteoskyrin, rugulosin, iridoskyrin, emodin)	Hepatotoxic, hepatocarcinogen, or mutagen	*Penicillium, Aspergillus*
Citreoviridin (18,47,182)	Neurotoxin; inhibits mitochondrial ATPase activity; reduces glycogen synthase activity	*Penicillium*
Naphtoquinones (xanthomeganin, viomellein) (148,182,201)	Hepatotoxic	*Trichophyton, Penicillium viridicatum*
Ochratoxins (151,182)	Inhibit phe-tRNA synthase	*Aspergillus, Penicillium*
Zearalenone (F-2) (38,184)	Estrogenic, teratogenic	*Fusarium graminearum*
Chloropeptide (islanditoxin) (182)	Hepatotoxin, decreases hepatic glycogen synthase activity	*Penicillium islandicum*

*DAS = diacetoxy-12, 13-epoxytrichotec-9-ene.

mediated reaction into a nontoxic AFB_1-glutathione. AFB_1 epoxide may eventually react with DNA and exert its carcinogenicity effect (105). Sulfhydryl (SH) compounds such as glutathione (GSH) suppress the AFB_1 mutagenicity and carcinogenicity by either reacting with AFB_1-8,9-oxide and forming inactive conjugates (126) or reducing the amount of AFB_1 bound to DNA (186). Ethoxyquin increases the glutathione *S*-transferase (GSTase) activity of the liver cytosol fractions (105). Mice are resistant to aflatoxins (19), probably owing to increased epoxide-GSTase activity (182). R-goitrin and butylated hydroxyanisole also increase GSTase activity and reduce AFB binding to DNA (35). Phenolic compounds inhibited AFB_1-induced mutagenesis in *Salmonella typhimurium* only when they were concurrently administered with the mutagen (AFB_1 plus the rat liver microsomes [S9]) (161). San and Chan (161) concluded that the phenolic compounds do not react covalently with AFB_1, and the inhibitory effect may be due to the inhibition of activation enzymes. The effect of GSH on penicillic acid, a carcinogenic mycotoxin, has similarly been discussed (34). Chan and Hayes pointed out that an active metabolite that can be detoxified by GSH may be involved in the toxicity of penicillic acid (34).

Cullen et al. (45) have reported that AFM_1 is a weak hepatocarcinogen compared to AFB and possesses intestinal carcinogenicity because it is more polar and is therefore poorly absorbed from the digestive tract. However, the acute toxicity of AFM was found to be equivalent to that of AFB in rats and ducklings (141,144).

As mentioned above, AFB_1 is metabolized into a reactive form, AFB_1-8,9 epoxide by the action of the microsomal and nuclear cytochrome P-450 system (204). Essigmann et al. (58) incubated AFB_1 in vitro with DNA, microsomes, and NADPH and identified the principal aflatoxin–DNA adduct as *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1. The extent of covalent binding of AFB_1 to hepatic DNA closely parallels the susceptibility of the various animal strains and species to hepatocarcinogenesis by this mycotoxin (103,183,202). Mandel et al. (105) concluded that the reduced hepatocarcinogenesis resulting from feeding ethoxyquin plus AFB_1 is due to the reduction in DNA adduct formation, which in turn is due to increased detoxifying metabolism in the microsomal, cytosolic, and plasma membrane compartments of the liver cells. AFB_1-N^7G residue is chemically unstable (75,92,195), and in DNA a large portion of the N^7 adducts are readily removed spontaneously or enzymatically to yield apurinic sites (81). In *S. typhimurium*, however, aflatoxin B can induce base substitutions and exhibit some ability to revert frameshift mutations (69).

T-2 toxin, which possesses dermal toxicity and inhibits protein synthesis, is biotransformed in animal tissues using the hepatic microsomal esterase(s) that hydrolyzes the acyl group (182). This toxin has also been found to damage liver (174). Metabolism of T-2 toxin has been studied in several experimental animals. Sintov et al. (170) summarized the literature on T-2 toxin metabolism and suggested that T-2 toxin may be transformed via different pathways: (a) deacylation of the toxin into HT-2 toxin, which is then deacylated to T-2 tetraol via 4-deacetyl neosolaniol; (b) hydroxylation of the C-8 isovaleroxy residue of T-2 toxin and HT-2 toxin, which gives rise to 3′-hydroxy HT-2 toxins, respectively; (c) acetylation of T-2 toxin to acetyl T-2 toxin, which is then deacetylated via iso-T-2 toxin to HT-2 toxin; (d) glucuronide conjugation of T-2 toxin to very polar T-2 metabolites glucuronide conjugates; and (e) deepoxidation.

Emodin, a monoanthraquinone, is hydroxylated by the rat liver microsomal P-450 system into metabolites, including 2-, 4-, and 5-hydroxyemodin, among which 2-hydroxyemodin is a direct mutagen (109). Ochratoxins are a group of closely related fungal metabolites that are composed of a dihydroisocoumarin moiety whose 7′-carboxyl group is linked by an α-amide bond to one molecule of phenylalanine (151). The phenylalanine moiety is important for the toxicity of ochratoxin A.

Zearalenone (F-2) mycotoxin, although not steroidal, exhibits estrogenic activity via vertebrate cytoplasmic estrogen receptors (114). Similarly, an endogenous ligand, 17β-estradiol, which has been extracted from *S.*

cerevisiae, exhibits the ability to displace [^{3}H]estradiol from both mammalian estrogen receptor preparations and the estrogen binding protein in *S. cerevisiae* (64). Zearalenone is biotransformed to α- and β-zearalenone in rats and pigs by multiple forms of NADH- and NADPH-dependent zearalenone reductases localized in both the cytosol and microsomes (177,186). The α-form of zearalenone is more active than zearalenone itself.

Conclusion

Interactions of moulds with man may be deleterious to the life of the latter. Man is an adequate food source for the pathogenic fungi, whereas for man the fungus has a physiology that is so similar to his own that it is difficult to develop methods for combat that do not kill both combatants. In addition to direct attacks by the fungi, man also suffers from fungal by-products—the mycotoxins—which may cause deleterious alterations of metabolism or modifications of the DNA, which in turn may lead to cancer.

One of the areas that has been exploited in an attempt to control fungal growth has been the differences that exist between man and fungus in the synthesis of lipids, especially sterols. In part, this method of control has been successful because fungi contain sterols that are different from those of man. In addition, for the most part, fungi have only a single sterol, ergosterol, which means that the number of targets we need to hit is small. The fatty acid and phospholipid content of fungi has proved to be a less useful site of control for several reasons. First, there is great variation in the proportions of individual fatty acid or phospholipid components among species as well as within the life cycle of a single species. Second, the types of compound found within the fungi are, in most cases, the same as those synthesized by man, which limits the ability to use drugs to inhibit the metabolic pathways. Additional analysis of the regulation of fungal lipid synthesis, especially at the genetic level, may one day yield inhibitors that are more specific for the fungi than for their hosts.

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10—Cell Wall of *Candida albicans*: Its Functions and Its Impact on the Host

ANTONIO CASSONE

The purpose of this chapter is to summarize and critically compare current ideas about the cell wall of *Candida albicans*, its functions in the microorganism, and its impact on the host. *Candida albicans* is an important human commensal that has received much attention as a model for studies of cell biology and microbial opportunism (164). This review deals mostly with the second aspect, as it is of great relevance for medical mycologists. Thus the role of cell wall components in morphogenesis, pathogencity, and immunity receives the focus here, although at times more basic questions concerning cell wall chemistry and organization of wall constituents are addressed, especially when the answers to these questions are pertinent to understanding the pathogenicity of the microorganism and the host response. Those who wish to know more about the basic aspects of cell biology and wall biosynthesis may consult a number of excellent reviews elsewhere (13,33,55,84,86,100,165,168,183,192,196,201,202,230). The growing interest in *C. albicans* and related pathogens has led to a dramatic increase in publications on *Candida* biopathology, and when writing this review selection was inevitably based on personal assessments and ideas.

Cell Wall Functions and Definitions

The presence of a cell wall is essential to almost all aspects of biology and pathology of *C. albicans*. Table 10-1 outlines the main general functions of the cell wall and anticipates the attribution of these functions to specific cell wall constituents. The definition of *cell wall* is simple from a cytological point of view. Electron microscopy shows that *C. albicans*, as other fungal cells, possesses a layered structure lying outside the plasma membrane, visible in both chemically fixed and frozen-etched specimens (Fig. 10-1a,b). If the microorganism is mechanically disintegrated through vortex treatment with glass beads, by passage through presses, or, less efficiently,

TABLE 10-1. Basic functions of the cell wall with its components.

Function	Putative involved component
Morphogenesis and growth	Chitin, glucan
Protection against nonspecific and specific injuries	
Osmotic lysis	Glucan, glucan–chitin network
Immunological factors	
Antimycotics	glucan, mannoprotein
Nonselective permeability barrier	Mannoprotein, glucan
Nutritional	
Housing and adhesion to inanimate surfaces	Mannoprotein
Exoenzymes	
Host–parasite relationship	
Adherence and adhesins	Mannoprotein, chitin?
Toxins	
Germ-tube formation	Chitin, glucan
Shedding of structural components and virulence enzymes	Mannoprotein
Receptors for host's factors	Mannoprotein
Antigens, inducers, and modulators of humoral and cellular immune responses	Mannoprotein, glucan, chitin?

by sonication, a particulate body that retains the morphology of the whole intact cell can be separated from the cytoplasm (Fig. 10-1c). This organelle can be removed from the cell by means of enzymic degradation of its constituents, giving rise to *spheroplasts* or *protoplasts*. Although remnants of wall components may still be present (spheroplasts), these wall-devoid cells have an invariably spherical form, are osmotically sensitive, and are nonpathogenic, properties that emphasize the prominent functions of the cell wall.

Cell Wall and Cellular Morphology

With its cell wall in place, *C. albicans* has a multivariate morphology and occurs in different forms (39,165,202). Table 10-2 defines the forms in which the microorganism can present. There has been a good deal of confusion about terms such as mycelial and pseudomycelial. As emphasized by Odds (165), pure cultures of any single form are difficult to obtain. There are no satisfactory reports that a pure pseudomycelial culture has ever been obtained, but it is often seen to coexist with the yeast form. The word itself, "pseudomycelial," is equivocal because no relation exists between pseudomycelium and true hyphal expansion, as is shown by the fact that variants of *C. albicans* that are unable to convert yeast to the mycelial form

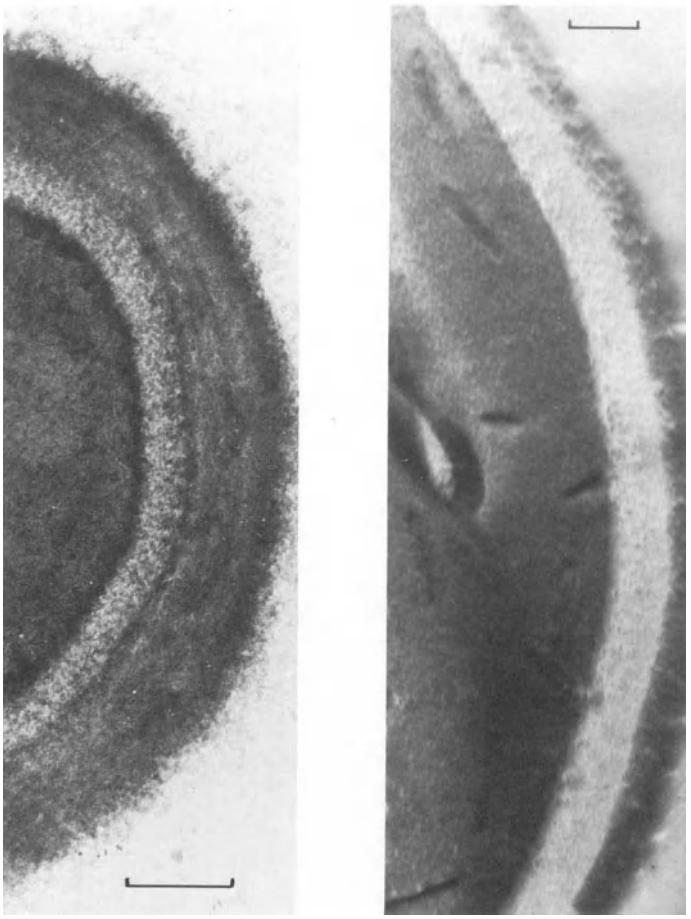
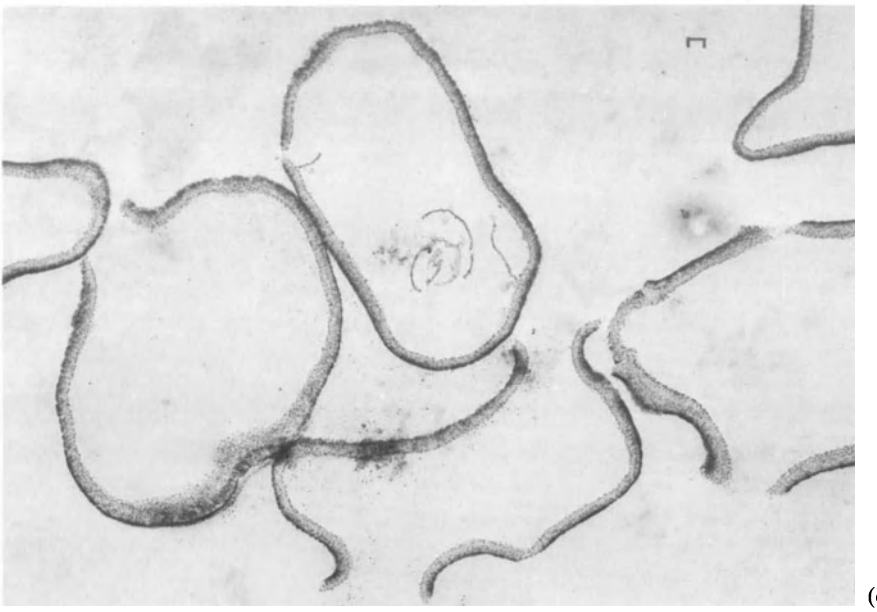


FIG. 10-1. Ultrastructure of the cell wall of *C. albicans* in situ. (a) Chemically fixed specimen. (b) Frozen-etched specimen. (c) Isolated from glass-beads-broken cells. Bar = 0.2 μm.

TABLE 10-2. Definition of the various forms of *C. albicans*.

Form	Definition
Yeast	Ovoid, unicellular, budding
Pseudomycelium	Elongated yeasts, attracted end-to-end and branching
Mycelium	Septate hyphal element, apically growing
Germ tube	Intermediary stage of the mycelial or hyphal form originating from the yeast
Chlamydospore	Round, asexual, refractile spore with thick cell wall



(c)

FIG. 10-1. *Continued*

are nonetheless able to develop into a pseudomycelial form (143,239). For the purpose of this review, yeast, hyphal forms, and chlamydospores are most relevant, as the cell wall changes most significantly during the transitions among these forms. Chlamydosporogenesis is of particular interest because it shows that the fungus is able to alter completely the organization of the cell wall concomitantly with a dramatically different function assigned to this organelle (165).

Chemical Constituents of the Cell Wall

The many diverse cell wall functions point to the presence in this organelle of more than one chemical constituent. Because protoplasts lyse in hypotonic media, whereas intact cells do not, at least one of the cell wall constituents must be endowed with such strong tensile and resistant properties as to withstand internal osmotic pressure. This role is invariably played in all cell-wall-possessing microorganisms by a polysaccharide or polysaccharide-derived macromolecules in β-configuration (33,100). Although some information about the chemical nature of the cell wall constituents can be obtained by in situ cytochemistry (see later), precise qualitative measurements are performed on isolated walls from mechanically disintegrated cells. Table 10-3 compares some of the analytical determinations on cell

walls performed by various investigators. Because different strains, grown in different media under different physicochemical conditions and for different lengths of time, have been employed, it may sound naive to anticipate that the chemistry of the cell wall is different in each case. The difference, however, concerns quantitative aspects, as uniform qualitative results are evident. Indeed the cell wall is, in all cases, constituted of polysaccharides and proteins (15,39,201) in a proportion of around 9:1.

Three basic constituents have been found in the polysaccharides: polymers of mannose (*mannan*), polymers of glucose (*glucan*), and the classical α-chitin (19,52,80,134,143,183,219). Although minor constituents (e.g., lipids, ions, and some other low-molecular-weight substances) may also play a significant role in cell wall organization and functions (8,90,94,95), we may at the present state of knowledge only imagine cell wall organization and functions in terms of position, amount, synthesis, assembly, and interrelations of the polysaccharide and protein complexes mentioned above. In other words, to truly understand cell wall biology, we should be able to separate and identify each major component, define its position and hierarchy, if any, in the overall structure, and assign to it, alone or in combination with other constitutents, a precise function. The following discussion shows that we are far from reaching any one of the above targets. However, we know enough of the cell wall constituents, for them to be used as a foundation for future research.

Cell Wall Chemistry and Taxonomy

In his classical review, Bartnicki-Garcia (15) assigned fungi to chemical categories on the basis of the type of polysaccharide present in the cell wall. In *C. albicans*, around two-thirds of polysaccharides belong to the β-glucan class and the residual one-third (or slightly more) is protein-associated mannan (Table 10-3). Overall, the proportions of the single constituents in the cell wall coincide well with those of Bartnicki-Garcia's class VI (glucan-mannan), which also includes one of the most thoroughly investigated yeasts, i.e., *Saccharomyces cerevisiae*. There is a wide consensus about the fundamental similarities in terms of cell wall chemistry and functions between *C. albicans* and *S. cerevisiae*, although this similarity should not be pushed to an extreme. We shall see later that there are structurally visible layers in the cell wall of *C. albicans* that appear to be absent in the *S. cerevisiae* cell wall and other significant differences in the content of chitin in the lateral wall, in mannoproteins (78,108,205,206,247), and in protoplast formation and regeneration (124,156). After all, *S. cerevisiae* is unable to form pseudomycelial or true mycelial cells and is nonpathogenic—except certain cases of compromised hosts (82). These events are strongly dependent on cell wall structure and composition.

TABLE 10-3. Chemical composition of the cell wall of *C. albicans*.

Form of growth	Mannan	Glucan			Chitin	Protein
		Alkali	Acid	Insoluble		
Yeast*	31.8; 32.4	6.1; 4.3		45.0; 47.0	2.7; 2.9	36.8; 22.5
Mycelium*	12.9; 24.0	17.1; 17.6	—	36.0; 45.3	9.9; 10.6	9.0; 8.8
Yeast†	22.0	5.0	52.0	20.0	—	—
Yeast‡	25.0		(73.3)		1.6	8.7
Mycelium‡	18.7		(72.8)		8.4	6.4
Yeast§	26.6	6.3	34.2	32.9		3.8
Germ tubes§	26.6	10.7	33.3	29.3		5.2
Yeast‖	19.0		(71)		9	
Mycelium‖	4		(74)		21	

Values are given as percent composition.
*Data from ref. 52; the two values refer to two culture conditions and have been extrapolated from the monosaccharide content of the cell wall fractions. A significant amount of mannan was in the alkali-insoluble cell wall fraction and has not been considered here as mannan. The protein value appears exceedingly high and was calculated by the authors from the total nitrogen of the fraction.
†Data from ref. 44; cells in the exponential phase of growth.
‡Data from ref. 143; the glucan value (in parentheses) refers to the sum of alkali-soluble plus insoluble material. The protein value was not reported in the original publication.
§Data from ref. 219; the chitin value is expressed as the percent of cell wall dry weight.
‖Data from ref. 78; the values were derived from incorporation of radioactive precursors. The glucan value (in parentheses) refers to total glucose polymer.

Apart from providing a clear differentiation between ascomycetous and basidiomycetous yeasts (15,128,129), cell wall structure is also of value for discriminating among *Candida* spp., some of which appear to be close to *Cryptococcus* species. Such discrimination is based mostly on their susceptibility to lysis by hydrolytic enzymes and reducing agents (17). Furthermore, at least some ascomycetous yeasts and related anamorphic forms contain in their wall a characteristic 31.5- to 34.0-kilodalton (kD) mannoprotein with antigenic cross-reactivity between *S. cerevisiae* and *C. albicans* (108) (see next section).

Mannoproteins

As in *S. cerevisiae*, the mannan constituent of the cell wall of *C. albicans* does not exist as such but in covalent association with proteins. Ballou (12,13) differentiated the mannoproteins into inducible and primarily secreted enzymes such as invertase, acid phosphatase, and acid proteinase (in *C. albicans*), also called *functional* mannoproteins, and similar constituents that are believed to be intrinsic structural components of the cell wall

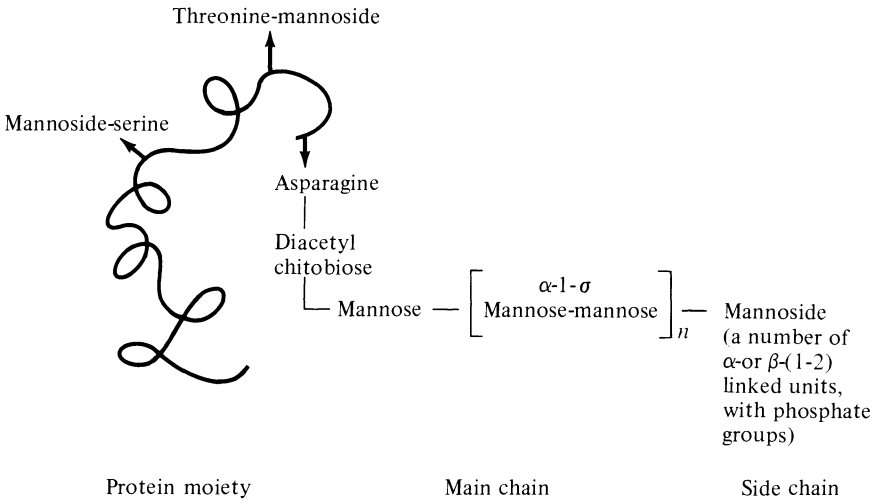


FIG. 10-2. Simplified structure of mannoprotein of *C. albicans*. (Based on data in refs. 12,13,203.)

(*structural* mannoproteins). Regardless of their specific role, all mannoproteins examined so far are molecules with a *main chain* composed of *D*-mannose residues linked by an α(1-6) bond, from which protrudes a varied number of *side chains* of *D*-mannose residues linked to each other by α(1-2), α(1-3), α(1-4) (and, more rarely, in β-configuration). The main chain(s) is covalently linked to the protein moiety through the characteristic, tunicamycin-sensitive bridge involving asparagine and two *N*-acetyl-*D*-glucosamine residues (di-*N*-acetylchitobiose) (13,232,233). At places along the protein backbone, other short, *secondary mannosyl chains* are attached by an *O*-glycosidic linkage with the hydroxy amino acids threonine or serine, forming the alkali-labile, β-eliminable ether bond, glycosyl serine, or glycosyl threonine (Fig. 10-2).

The mannan of *C. albicans* is more phosphorylated than that of *S. cerevisiae* (12,166,205,206), the phosphate group linking mannosyl residues in phosphodiester bonds of a variable number of side chains with serological specificity (246). From investigations performed with *Saccharomyces* and other yeasts (12), it is well known that many variations exist on the general theme of mannoprotein structure outlined above, and the lack of knowledge of precise details of mannoprotein structure and polymorphism in *C. albicans* and other pathogenic *Candida* species has been emphasized repeatedly. As shown below, investigation is focused on filling this gap for both the polysaccharide and the protein moiety.

Mannan

It has long been recognized that acetolysis patterns of mannan from *Candida* are more complex than those from most species of *Saccharomyces*, giving up to eight peaks of chromatographically separated oligosaccharides (227–229,261). Suzuki and collaborators (227–229) showed that side-chain mannosides separated by gel filtration contained both α(1-3) and α(1-2) linkages and that the acetolysis fragments of mannan from strains of *C. albicans* belonging to serotype A were longer (on average) than those of serotype B mannan. In both serotypes, as in *S. cerevisiae*, the main chain consisted of α1-6-linked mannosyl residues (13,227). Kogan et al. (121a), confirming most of the above data, also reported the existence of a substantial amount of branching hexamannosides in the side chains, mostly prevalent in B serotype. A severe limitation of all these studies is that the mannoprotein component was isolated from cell wall by rather strong, potentially degradative extraction procedures including hot alkali or strong acid solutions. The polymannose constituent of both serotypes of *C. albicans* has been characterized after mannoprotein separation from a hot aqueous extract of whole cells by a mild, conservative fractional precipitation with Cetavlon (204) or after extraction from viable cells through the action of zymolyase 100-T, a β(1-3)-glucanase (205). The predominant mannan constituent purified by the former procedure contained mannose in excess of 90%, 5% protein, and phosphate ranging between 0.72 and 1.60%. Both *N*- and *O*-glycosidic linkages were present in each serotype, as expected, but an acid- and alkali-labile oligomannosyl residue with mannose linked via β(1-2) bonds and connected to the main chain through a phosphate group was also detected.

The acid-labile β(1-2)-oligomannosides were the major determinants to react with specific antibodies, whereas the *O*-linked glycosyl chains did not appear to participate in the antigen. This observation is intriguing, on the one hand, in view of the well-established inhibition of serological reactivity of homologous antisera by α(1-3)- and α(1-2)-linked mannosyl haptens (183,221,229). It suggests that either this serological reactivity was not discriminative of polymers in α- or β-configurations or that different antibodies, specific for α- or β-linked mannose, were present in the anti-cell-wall sera. That this second possibility may be more realistic has been demonstrated by Tojo et al. (234a), who produced monoclonal antibodies able to discriminate between β(1-2)-oligomannosides and α-linked mannoside units. On the other hand, the existence of mannan chains in β-configurations may help explain the inability of the exo-α-mannosidases to hydrolyze mannan from *C. albicans* while being most effective in the hydrolysis of *S. cerevisiae* mannan (118). In zymolyase-extracted and Cetavlon-purified mannoproteins (205). 1-*O*-phosphorylated, β(1-2)-linked oligomannosyl residues were around 10% of total mannan of *C.*

albicans in the yeast form but, interestingly, significantly less in the mannoproteins solubilized by the mycelial forms. Also, the overall mannan extracted by the above procedure from mycelium of *C. albicans* was about one-half that from yeast cells (205). The findings are in accord with growing observations by others that suggest significant variations in the mannoprotein composition and structure between the two forms of growth of *C. albicans*. (For a critical discussion of this point, see below.)

Mutants with abnormal synthesis or structural defects in manoproteins would be of great help in elucidating the structure and function of these molecules, as has occurred with *S. cerevisiae* mannoproteins (13,86,88, 137,163). A few mutants of this kind have been reported for *C. albicans* serotype A, most of them characterized by a rough-colony morphology. Those isolated by Shimokawa and Nakayama (207,208) had a reduced mannan content associated with either a lower (about 10%) degree of phosphorylation of alterations in the α(1-6) main chain.

Protein

The analysis of the protein moiety, which is manifestly essential for understanding the biological significance of mannoproteins in the cell wall of *C. albicans*, has been hampered by the unavailability of nondegradative extraction methods and fine resolving analytical techniques. Recently, the application of glucanolytic enzymes and reducing agents as extractants coupled with high-resolution and high-sensitivity protein detection methods (Western blotting) has made approaches to protein structure of mannoprotein more feasible.

Sentandreu and his collaborators (78,79,108,155,156,247) examined the mannoproteins from *S. cerevisiae* and *C. albicans* (and other fungi) after solubilization with chemical reagents or glucanases acting on isolated walls. In both microorganism, numerous proteins were extracted by agents such as sodium dodecyl sulfate (SDS) and urea, most of which have a molecular weight in the range of 20–50 kD. Relatively low-molecular-weight proteins together with one polydispersed or several defined proteins of high molecular weight (>120 kD) were also solubilized by glucanase in the presence of the proteinase inhibitor PMSF (79). In *S. cerevisiae* and in *C. albicans*, a major, serologically cross-reactive mannoprotein, liberated by glucanase digestion but also by boiling in SDS buffer, banded in the 33- to 34-kD region (79,108). Interestingly, a protein of similar molecular weight is one SDS-polyacrylamide gel electrophoresis-(PAGE)-detectable component of a strong mannoprotein immunomodulator extracted from *C. albicans* (9,191; Cassone et al., manuscript in preparation) (see also below). These data should be considered together with those of Zlotnick et al. (262), who showed that most of the proteins solubilized from clean walls of *S. cerevisiae* by a protease-free β(1-3)-glucanase were more than 67 kD.

The latter authors produced some evidence indicating that a large portion of mannoproteins were covalently linked to β-glucan, and therefore reagents such as dithiothreitol (DTT) and SDS could not bring them into solution. On the other hand, it has been shown that SDS-extracted proteins from washed cell walls of *C. albicans* bind to concanavalin A (Con A), indicating their nature of mannoproteins (79). Apart from some contrasting data obtained in *S. cerevisiae*, it seems sensible to conclude that two categories of mannoproteins are present in the cell wall of *C. albicans*, one weakly bonded and therefore detergent-releasable and the other with prevalent high-molecular-weight constituents and covalently linked to β-glucan. In addition, much the same protein can be extracted by both glucanase digestion and hot SDS buffer (79,88,108,247), suggesting that the type of intramural bonding of a mannoprotein might undergo a dynamic expression. The exact type of bond(s) linking the polymannose or the protein moiety to glucan is presently unknown. The conclusion about the two categories of mannoproteins should also be tempered by the possibility that part of the hot SDS-solubilized proteins are not intrinsic components of the cell wall but originate from contaminating cytoplasm or membrane material. Some of the proteins solubilized by hot SDS from apparently clean cell walls of *C. albicans* and running in SDS-PAGE in a range of 80 kD to lower bands were also efficiently solubilized by lithium chloride in the cold, had too little mannan to be stained by periodic acid-Schiff (PAS), and showed an electrophoretic behavior of low- or nonglycosylated proteins (Fig. 10-3) (Cassone et al., unpublished data).

Murgui et al. (155,156) and Elorza et al. (78) have also evaluated the effect of inhibitors of synthesis and assembly of cell wall components on the incorporation of newly synthesized mannoproteins in the cell wall proper or in the wall regenerating net of protoplasts. More or less as expected, the glucanase-extractable mannoproteins were not incorporated into the cell wall when the synthesis of the glucan component was inhibited by papulocandin B, whereas the proteins that are held in place by noncovalent linkages were not affected by the inhibitor. In regenerating protoplasts, the SDS-extractable mannoproteins were synthesized and localized in the primary chitin skeleton of the net well before the glucan-linked mannoproteins. In the absence of a functional *N*-glycosylation (due to the inhibitory effect of added tunicamycin or in conditions of reduced glucan synthesis, the latter (but not the former) mannoproteins were not assembled in the regenerating fibrillar network but were released into the medium (155). In protoplasts of *S. cerevisiae* which, in contrast to those of *C. albicans*, are able to regenerate their walls in the presence of inhibitors of β(1-3)-glucan synthase (124,247), some mannoproteins were regularly incorporated in the wall, whereas others required formation of a network of β-glucan for their incorporation. These results further suggest that two categories of mannoproteins are present in the cell wall of *C. albicans*, but the possibility of a metabolic relation between them is still open to investigation.

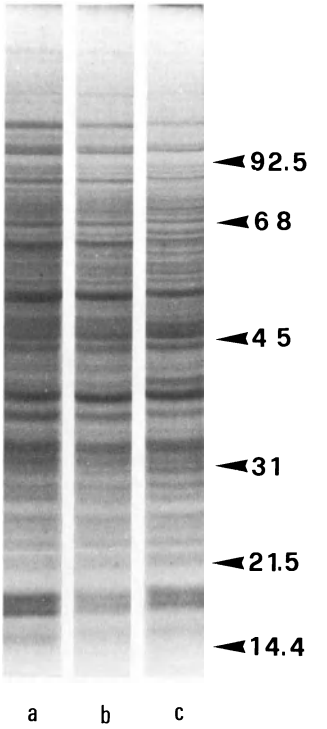


FIG. 10-3. SDS-PAGE of proteins extracted by hot (100°C) SDS from H_2O-washed, clean cell wall preparations of *C. albicans*. The purity of the wall preparation may be assessed from Figure 10-1c. The arrow heads point to molecular weight standards. Lanes: a, yeasts; b, 90-minute germ tubes; c, 240-minute hyphal cells. Induction of germ tubes and hyphal cells was as reported in ref. 143.

In most of the aforementioned studies concerning isolated cell walls, it was reported that S–S disrupting agents such as mercaptoethanol or DTT are unable to release a significant amount of mannoproteins. However, a number of investigations have furnished evidence that mannoproteins can easily be liberated by the above agents from intact yeasts as well as from nets of regenerating protoplasts (45,53,119,174,247). Both polysaccharides and proteins were released from cell walls of *C. albicans* by treatment with DTT, with the proteins being not (or hardly) mannosylated (174,175). These materials were supposed to come from the outer proteinaceous layers of the cell wall; but because cell viability after DTT treatments was not measured, it is not unreasonable to suspect that part of the DTT-extracted material may also have originated from periplasm or even the cytoplasm of dead or moribund cells following the however gentle extraction procedure. It was certainly the case in delicate-tip hyphal cells from which more proteins were extracted (174). At any rate, it seems clear that S–S disrupting reagents are able to extract cell wall constituents from the

intact cells, and we are faced with at least two distinct possibilities. Either most of the DTT-releasable proteins are not present in the isolated walls because they are lost during the preparation (41) or there is a DTT-activated process in intact cells that detaches the mannoproteins from other cell wall constituents. The releasing agent may also act through activation of cell-wall-associated β-glucanases. Work by Notario et al. (160,161) showed that thiol reagents activated cell-wall-associated β-glucanases in *C. albicans*, whereas SH-blocking agents such as *N*-ethylmaleimide inhibited the activation. Because the activity of free, purified β-glucanase was not markedly enhanced by reducing agents, SH-reagent-mediated enzyme activation must be indirect (through an action on mannoproteins or other wall targets). It has been suggested that glucanases are generated from inactive precursors and that their activation occurs in the cell wall during the secretion process. (150).

Proteins of Cell Wall and Their Mannosylation: Some Critical Considerations

Data in Table 3-3 show that cell wall preparations of *C. albicans* have a protein content that varies from 3 to 6%, depending on the phase and form of growth (219). Their content has generally been established by the Folin phenol method, which does not reveal if they are glycosylated. Some secretory proteins of *C. albicans* are mannosylated, but others are not or have a low degree of mannosylation (165,201,203).

When studying cell wall protein, attention should be paid to the preparation of the material for analysis, as even the cleanest of cell wall preparations (judged by electron microscopy or chemical methods) may be contaminated with cytoplasmic material, with its high relative protein content. On the other hand, extensive washings of cell wall with water, ionic solvents, and, as by Zlotnick et al. (262), SDS (a detergent that has been used by others just to extract cell wall proteins) may remove proteins that are intrinsic but weakly associated with the other mural constituents. Some proteins could be sheared from their anchorage to the cell wall during the process of cell breaking with glass beads followed by extensive washing but not after ultrasonic treatment (41).

Several authors have reported the existence of numerous proteins in the cell wall following extraction with hot SDS (Fig. 10-3). Although some maintain that these proteins are not extensively mannosylated [as demonstrated by the absence of staining with PAS (51,66) or by immunological techniques (35,174)], others have shown that most of the proteins bind Con A, suggesting significant glycosylation (79). Interestingly, both Chaffin and Stocco (51) and Elorza et al. (79) have quantified in more than 40 polypeptide bands extracted by SDS from clean cell walls and visualized by SDS-PAGE. Although a direct comparison is not possible, most of

these excess 40 bands were found in relatively low-molecular-weight protein regions. Nonmannosylated and mannosylated proteins were also present in the wall extracts from yeast and mycelial cells obtained by DTT extraction of whole cells (174,175,223).

It has been suggested that fungal cell walls are not necessarily inert structures (90): If only most of the SDS-extractable proteins are truly intrinsic cell wall constituents, the cell wall must be regarded as a metabolically active organelle. Some of these SDS-extractable cell wall proteins may act as β-glucanases, which have the potential for thriving autolysis of the cell wall structure, thus releasing mannoproteins (79,87,108,109,247). Along this line, SDS has been shown to be the strongest of several detergents capable of accelerating wall autolysis and releasing glucanases in *C. albicans* (160).

Glucans

Cell wall molecules, collectively called *glucan*, are made up of extended fibers of *D*-glucose in β-configuration (yeast cellulose). They are fundamental in cell wall construction and function, providing, together with chitin, the rigid framework that is ultimately responsible for most of the cell wall resistance and morphological properties (Table 10-1). In *C. albicans*, glucans account for about 60% of cell wall dry weight and can be grouped into at least three molecular species (74,86,201). Two of them are extracted, with different ease and yield, by alkaline and acidic solvents, leaving an insoluble constituent that grossly retains the shape of the original cell (19,45,74,260). It has been suggested (210,211) that the soluble species of glucan is the precursor of the insoluble one, and the insolubility is acquired concomitantly with glucan linkage to chitin. In general, the attention of scientists to glucans seems to be in inverse relation with the presumed importance of these molecules, probably as a consequence of the difficulty encountered when treating with these polysaccharides, as has been emphasized by Duffus et al. (74). There is no doubt, however, that comprehension of the organization and structure of the cell wall will be reached only when detailed knowledge of glucan constituents becomes available.

Gopal et al. (102) addressed the problem of glucan composition in the various forms of *C. albicans*. An acid-soluble fraction and an insoluble fraction extracted from whole cells or isolated walls according to Duffus et al. (74) received most of the focus. In each form (yeast, mycelial, and transitional germ tube) the ratio between the alkali/acid-insoluble and the acid-soluble glucans was about 2.7:1.0, and glucose was the only sugar identified. The insoluble glucan was a highly branched chain of β(1-3)-glucan with β(1-6) extra-chain residues, at variance with glucan from *S. cerevisiae*, which has fewer β(1-6) bonds and a minor degree of branching

(74,102). Most of the germ-tube-insoluble glucan was of the β(1-3)-linked type, pointing to an important modulation of this highly fibrous, highly "morphogenic," rigid constituent in the transition from yeast to mycelium. However, because the ratios of the β(1-3) and β(1-6) bonds were similar in the yeast and mycelial walls (102), the increased content of β(1-3)-glucan of the germ-tube form has only a transitional morphogenic value, in analogy with the aberrant wall of regenerating protoplasts (78,101,124,156), probably fulfilling the increased requirement for cell wall rigidity at critical stages of cell wall construction and remodeling. The acid-soluble glucan fraction was composed predominantly of β(1-6)-glucan, with a relatively small amount of β(1-3) bond residues; and its content did not differ substantially between the two forms of growth. Less acid-soluble glucan was reported to be present in the aging cell of *C. albicans* (44). Less than 10% of total cell wall glucan is represented by the alkali-soluble species, in a β(1-6) configuration (102,219). Molina et al. noted a decrease in the activity of exo β(1-3)-glucanase during mycelial development (150), but similar diminutions occurred under cultural conditions unrelated to hyphal morphogenesis.

Hydrolysis of the β(1-3)-glucan constituent by the action of β(1-3)-glucanases in isolated walls or in intact cells after removal of the outermost mannoprotein shield (78,101,240,262) is required for cell wall dissolution and protoplast formation. A potentially fundamental aspect of cell wall glucans resides in their well known association with β-glucanases, which are widely distributed in all glucan-based fungi (87,250). Two β-glucanases, one exo- and one endo-β(1-3)-hydrolase, of 150 and 4g kD, respectively, were found to be associated with the cell wall in *C. albicans* (160), whereas six different cell-wall-associated exo- or endo-β(1-3)-glucanase enzymes have been reported in *S. cerevisiae* (109). They hydrolyzed glucan into fragments of different lengths, probably due to fine differences in the specific hydrolyzed substrate. The activity of β-glucanase can be controlled by the type of association with cell wall polymers, including lipids and extent of reduction (90,160).

A variety of other enzymes may be found more or less firmly associated to glucan fibers, and, as for β-glucanase, this linkage may protect them from inactivation (65). Protein–glucan association have been reported in yeasts with a linear β(1-3)-glucan (65), but such a highly branched β(1-3)-glucan as the one found in *C. albicans* (102) might prove still more suitable for this molecular association. Binding of glucanases, other proteins, or mannoproteins is likely to have profound implications for wall construction and the processes of remodeling of the fibrillar skeleton during cell wall morphogenesis, but at present it is only a matter of speculation. To my knowledge, well characterized mutants in β-glucans of *C. albicans* have not yet been reported, but one of these mutants has been described in *S. cerevisiae* (209). It was sensitive to mild alkali treatment and underwent lysis on treatment with a β(1-3)-glucanase without pretreatment with thiols or

proteases. Analysis of the alkali/acid-insoluble glucan fraction showed that the mutant lacked the highly branched glucan with predominant β(1-6) linkage. The linear β(1-3)-glucan was much the same in the parent and mutant strains. Unfortunately, the mannoprotein component of the cell wall was not investigated. The high sensitivity of the mutant cells to mild alkali treatment and direct hydrolysis by glucanase (209) suggests that some modification in the organization of mannoproteins and their relation with glucan might have occurred.

Chitin and Its Relation with Glucans

The remarkable interest in chitin, a minor constituent of the cell wall of *C. albicans* (Table 10-3), by those active in the field of *Candida* biology and pathogenicity stems from essentially three observations.

The first one is that this polymer is about three times more abundant in the mycelial wall than the yeast cell wall (52). Second, a major ultrastructural change during wall morphogenesis in germ-tube formation is enrichment of an inner electron-transparent constituent that is supposed to be a chitin-rich layer (47) necessary to support the increased demand for rigidity of the emerging wall. This ultrastructural observation led to the use of *N*-acetylglucosamine, the monomer and allosteric activator of chitin synthase (31,33,100), as an effective inducer of germ-tube formation (although the two facts have never been shown to be causally related) (49,143). Third, significant experimental evidence has been reported by Cabib and his collaborators (31–33,75) concerning the control of chitin synthesis as a key morphogenetic element in the construction of a primary septum in *S. cerevisiae*.

That chitin may have a fundamental morphogenic role in *C. albicans* has been confirmed by a variety of reports, although the level of biochemical investigation attained in *C. albicans* so far as chitin synthesis and regulation is concerned has not yet reached the degree of biochemical and genetic sophistication achieved for *S. cerevisiae* (30,31,167,190).

Chitin is a linear polymer of β(1-4)-linked *N*-acetylglucosamine residues. The average chain length of this polymer is not known in *C. albicans*, but in *S. cerevisiae* it was calculated to be about 100 acetylglucosamynil residues (31). Chains are held together in fibers by a high number of H bonds and slide in antiparallel directions to form α-chitin (Fig. 10-4).

Various authors have studied the regulation of chitin synthesis and the chitin synthase activity of *C. albicans*. Almost all agree that chitin synthase is bound to the cytoplasmic membrance (27,54,75,201) where it exists in an inactive (zymogen) form, activatable by partial proteolysis through either natural or artificial activators (31), although none of the putative endogenous activators has been isolated in *C. albicans*. However, Gozalbo et al. (104) endorsed the idea that zymogenic synthase of *C. albicans* is located in

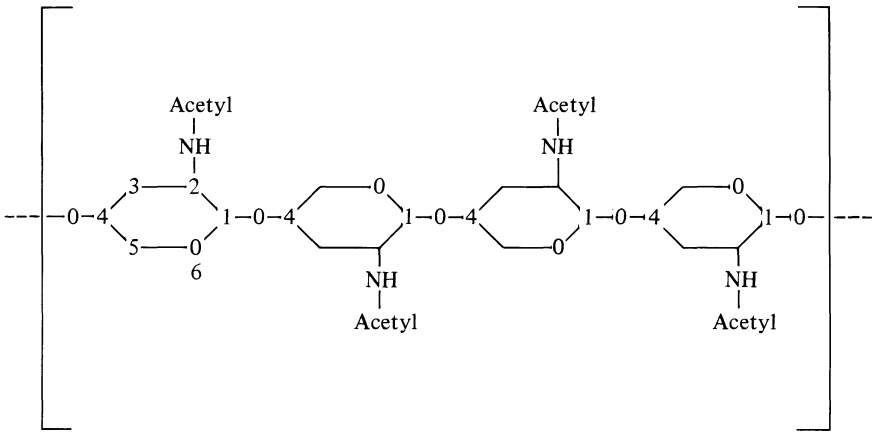


FIG. 10-4. Structure of the chitin unit (33).

microvesicular structures called *chitosomes*, as are suggested to occur in other fungi (16), which can be secondarily incorporated in the plasma membrance. An early, puzzling observation by Chiew et al. (54) that chitin synthase from germ-tube-forming cells was more stable to heat inactivation than the enzyme from the yeast-form cells of *C. albicans* merits special consideration and some speculation in view of the discovery of the presence of two chitin synthase in *S. cerevisiae* (30,167,190); only one of these enzymes is normally active in vivo (190), although its levels are far less than those of the other. If more than one chitin synthase exists in *C. albicans* (10,37), it would not be surprising that the two enzymes were differentially activated in the different morphogenetic forms of this fungus. One preliminary indication that it may indeed be the case has already been reported (37). The study of the cytological distribution of the two types of chitin synthase might also help resolve the controversy of the subcellular localization of these enzymes in yeasts.

In *C. albicans*, as in other fungi, chitin is covalently linked to glucan. In experiments reviewed by Shepherd (201), evidence has been provided that the linkage between the two insoluble components of the cell wall does not involve short peptides (210) but takes place directly between acetylglucosamine and glucose via a β(1-6) bond.

Lipids

Apart from the initial contribution by Bianchi (18), insight into the presence and composition of cell wall lipids in *C. albicans* can be derived from the work of Gale and collaborators (90) aimed at unraveling the mechanisms of the phenotypic resistance of the fungus to polyene antifungals dur-

ing cell aging in stationary phase (see also below). It has been disputed (8) that wall lipids may come entirely from the membrane; however, although a certain degree of contamination is likely, most authors agree on the cell wall containing true lipids. These lipids belong to the same categories found in the cells (18,259) and have been examined by Ghannoum et al. (95): neutral lipids composed of sterol, sterol esters, triglyceride, and free fatty acids, as well as phospholipids, with significant changes in the amount of each of these fractions being detected between young and old cell walls. It was not possible to correlate the lipid content of the cell wall to the emergence of phenotypic resistance to antimycotics (90).

Ultrastructure of the Cell Wall

The knowledge of the main macromolecular constituents of the cell wall of *C. albicans* and some of their mutual chemical relations, as outlined in the preceding section, may be useful for tracing possible ultrastructural patterns of cell wall organization. The relevant feature of macromolecular wall chemistry in *C. albicans* is a predominance of polysaccharides that are stable but poorly reactive to the ordinary fixatives and stains used for transmission electron microscopy. There are also a few mannoprotein or protein components, of which some are phosphorylated; they are usually denatured but largely preserved, and they stain by conventional techniques. Therefore unless special cytochemical stains or contrasting agents are used, the ultrastructural observation of the cell wall in thin sections of cells in routine preparation for electron microscopy invariably gives a paucity of well defined ultrastructural details. Most early and sometimes current reports on the ultrastructure of the cell wall of ascomycetous yeasts (including both *C. albicans* and *S. cerevisiae*) substantially conform to the above expectations (2,4,114,129,197).

On the other hand, the organization of the cell wall can be envisioned either as a homogenous intermixing of the various chemicals throughout the thickness of the cell wall or, at the other extreme, as a laminated structure with a topographical separation of mural constituents in concentric zones, the so-called *layers*.

Layering in Morphological Terms

It should be recognized that some degree of layering in the cell wall of *C. albicans* and related species was discernible, although not consistently, even in the early electron microscopic preparations, which were poor in ultrastructural detail (11,91,198,236,237). Taking advantage of the use of highly reactive cross-linking reagents included in the fixation schedule, Djaczenko and Cassone (67) and then Cassone et al. (47) gave ultra-

structural evidence for a multilayered wall structure of *C. albicans*, with morphologically definable, amorphous, granular and fibrous elements characterizing each layer (Fig. 10-5). This multilayered wall organization, with substantially uniform descriptions of the constitutive elements of each layer, has since been confirmed or extended using a variety of generic fixatives, stains, and mordants for electron microscopy (24,26,53,64,73,110, 113,152,170,174,177,193,240).

There is no consensus about the exact number of layers in the cell wall, which is hardly surprising as no standard definition of "layer" exists and the overall matter is liable to subjectivity. More important is that *C. albicans* is a morphologically dynamic organism with a dynamic cell wall. Cell wall layering, by its very nature of reflecting a metabolically active organelle, may undergo dramatic changes with the age of the culture (41) and the form of growth or terminal differentiation (46,47,115,200). It also depends on many other culture conditions or the distinctive properties of the strain used. Five (47,67), six (170), or even eight (177,178) layers have been described in the cell wall of *C. albicans*, although some of the layers were morphologically discernible only in particularly old cells or under peculiar situations (177). Some degree of artifactual layering is also evident when cells are treated with metabolic inhibitors, e.g., antimycotics (21, 24,26,258).

In order to verify the presence of cell wall stratification, the application of electron microscopic preparative techniques, which do not rely exclusively or predominantly on chemical fixation, is more important than academic dispute about the number of layers seen in chemically fixed specimens. Freeze-etching procedures, although confirming some layering and the presence of an outermost fibrillar constituent (172) (Fig. 10-1), have not furnished a wealth of details on the cell wall of *C. albicans*. Much more promising is the application of rapid freezing and freeze-substitution methods for scanning and transmission electron microscopy (238). This technique has allowed high-contrast, well resolved electron microscopic observations of chemically unfixed or osmium-fixed cells (Fig. 10-6). At least three layers of the cell wall have been clearly observed, and the above techniques appear distinctively advantageous for the ultrastructural preservation of the outermost capsular layer with its delicate fibrillar structures (238).

A comparison of cell wall morphology in *C. albicans*, *S. cerevisiae*, and *Cryptococcus neoformans*, a basidiomycetous yeast—each prepared by an identical fixation and staining schedule for transmission electron microscopy of thin sections—may be of some interest (Fig. 10-7). Roughly, the only significant difference in cell wall appearance between the two isolates of the ascomycetous yeasts employed is, in *S. cerevisiae*, the absence or a much reduced content of the outermost fibrillar layer that is so prominent in *C. albicans*, the overall wall stratification in the former showing an almost exact correspondence to the inner four layers of *C. albicans* cell wall

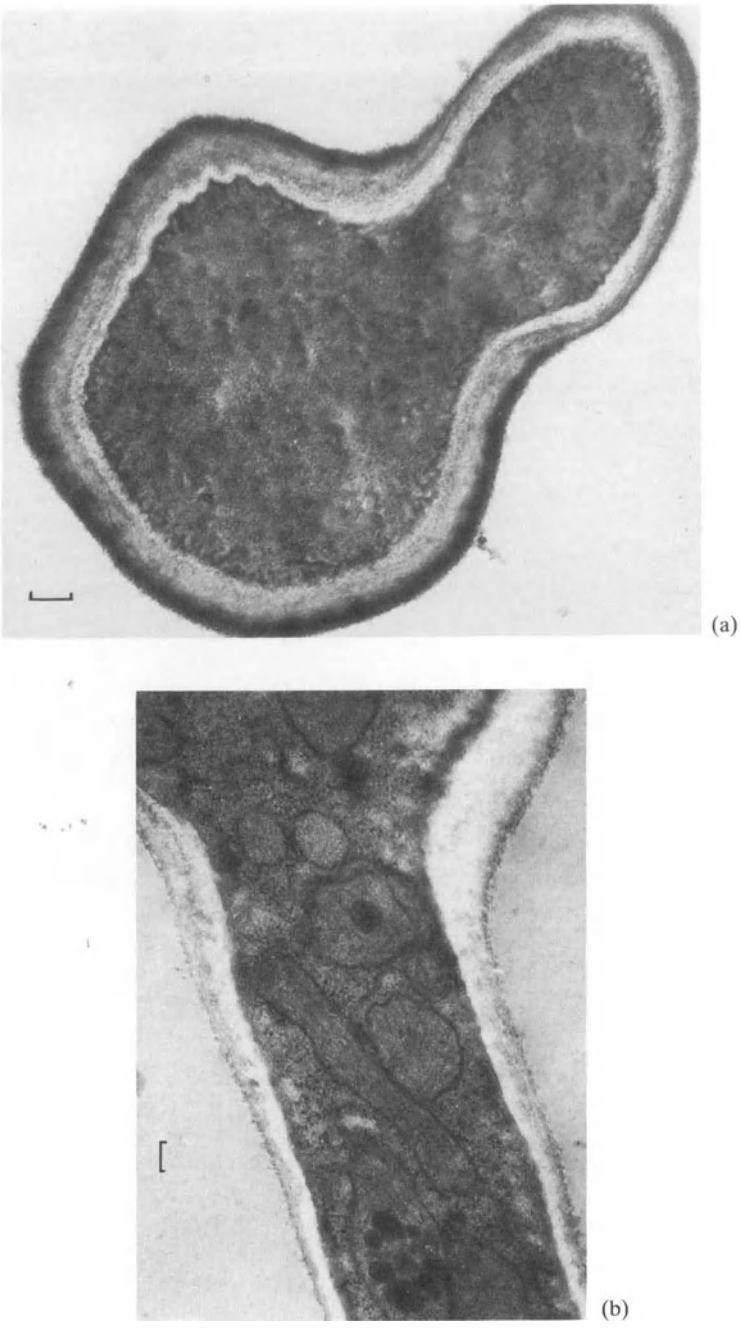


FIG. 10-5. Multilayered organization of the cell wall of yeast (a) and mycelial (b) forms of *C. albicans*. Bar = 0.2 μm.

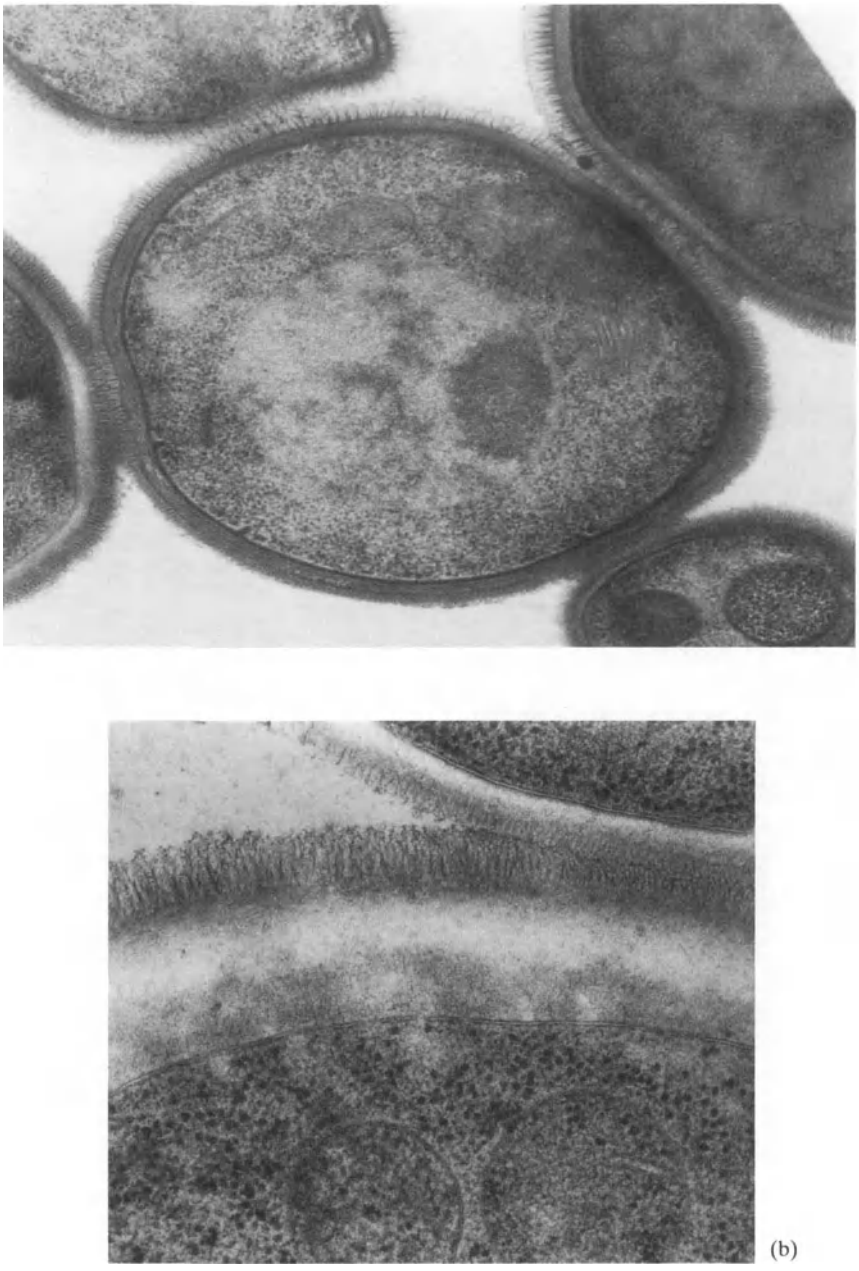


FIG. 10-6. Ultrastructural profile of the cell wall of *C. albicans* as seen by freeze-substitution fixation methods. Note in the enlarged section of (b) the details of a perfectly preserved capsular layer. (From Tokunaga et al. [238].)

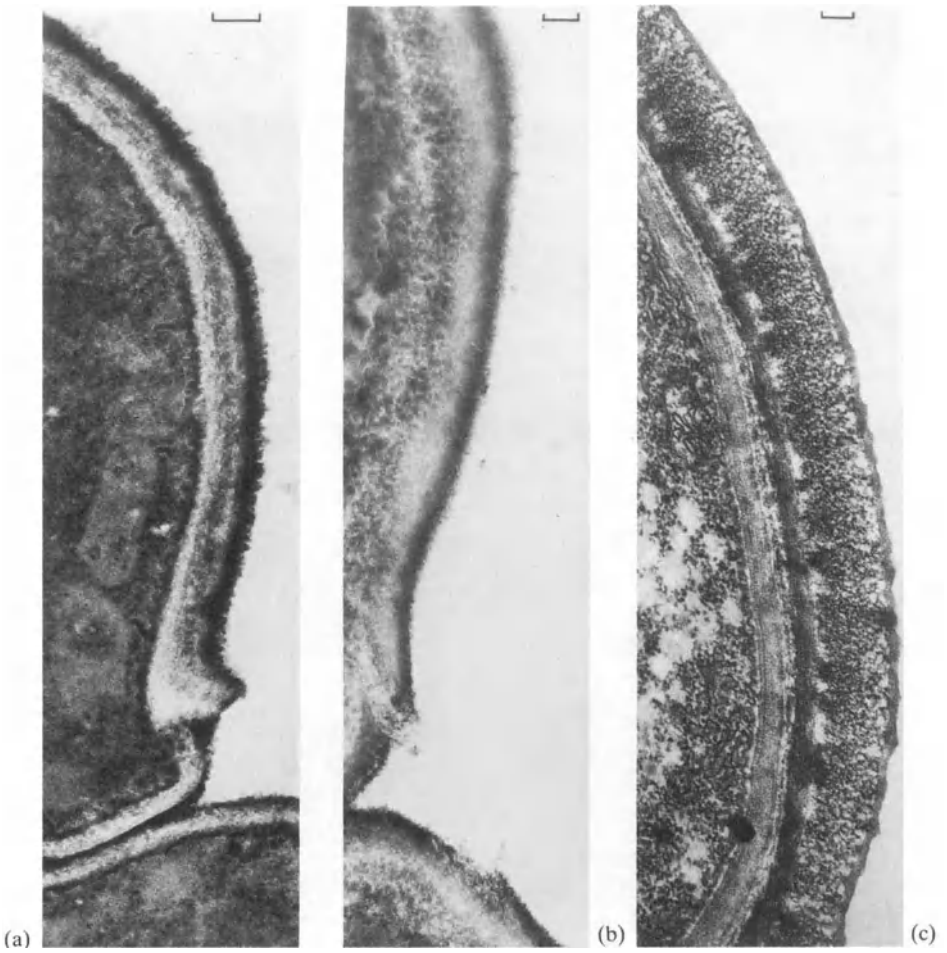


FIG. 10-7. Comparison of the ultrastructural appearance of the cell wall of *C. albicans*, yeast form (a), *S. cerevisiae* (b), and *Cr. neoformans* (c) fixed and processed for electron microscopy by similar methods (40,41). Bar = 0.1 μm.

(40,47,67). On the other hand, the cell wall ultrastructure of *Cr. neoformans* appears markedly different from that of both *S. cerevisiae* and *C. albicans*, in keeping with the marked differences in wall chemistry noted previously (15,48,183). Regardless of the outstanding presence of a thick capsule in *Cr. neoformans* (which, however, it may be taken to correspond to the outermost fibrillar component of *C. albicans*), the cell wall proper of *Cr. neoformans* shows electron-transparent regions external to fibrous laminations, a situation that appears *inverted* with respect to ascomycetous yeasts. This differential organization of wall constituents probably has some implications for the distinct mode of budding in the various types of

TABLE 10-4. Techniques employed for studies of localization of the main components in morphologically distinct layers of the cell wall of *C. albicans*.

Technical approach	Visualized component	Ref.
Cytochemical stains		
Thiery's method (vicglycol oxidation)	Mannan and β (1–6)-glucan	41,83,85,132,177,242
Phosphatase	Mannoprotein	
Lectin-stains		
Con A-ferritin, Con A-enzyme, or Con A-gold	Mannan	78,110,112,149,153,154, 178,231,241–243
Wheat germ agglutinin (WGA)-enzyme or WGA-gold	Chitin	
Immuno-stains		
Polyclonal-antibody-ferritin	Mannan	28,50,249,176,235,238
Polyclonal or monoclonal-antibody-gold		
Antibody-protein A-ferritin or gold		
Extraction of cell wall components with thiol reagents, acid and alkali solvents, and enzymes	All	45,53,77,83,149,178,149, 261

yeast (128,129). When critically assessing cell wall layering in morphological terms, it is of some importance that a given electron microscopic procedure, applied identically to the three fungi, revealed different ultrastructural layering. These findings conformed to the differences in cell wall chemistry and growth that are minor between *Candida* and *Saccharomyces* but consistently greater between these yeasts and *Cryptococcus*.

Layering and Localization of Wall Constituents

Because of the nonspecificity of the fixatives, mordants, and stains used in conventional electron microscopy, no immediate correlation can be made between the ultrastructural appearance of each layer of the cell wall and its chemical composition. It would be highly misleading to imagine a priori that each layer *must* have a fully distinctive chemical composition. Several experimental approaches were therefore used in an attempt to correlate ultrastructure with chemical composition, i.e., to find the localization of each major chemical constituent in the cell wall. The principal techniques employed to date are summarized in Table 10-4.

Some investigators have extracted intact organisms as well as isolated walls with solvents and hydrolytic enzymes known for their action on one or more of the wall constituents (45,53,73,77,83,123,126,149,178,179,262). A relatively mild alkaline extraction deranged wall layering completely, with the cell wall still exposing mannan to a sufficient density to bind Con A (45,188). After removal of the totality of mannoproteins by strong alkali (45) or hot neutral buffers (169,179), the cell wall appeared to be significantly thinner with loss of any appreciable layering (Fig. 10-8a,b). This loss was accompanied by the absence of electron-dense components to ordinary electron microscopy stains, Con A binding sites, and Thiery's positive staining for mannoprotein and β(1-6)-glucan (45,179). Because these cell walls maintained a full complement of alkali-insoluble β-glucan and chitin in the form of clearly visible fibrous elements (Fig. 10-8c), it seems inevitable to conclude that the wall layering, as seen by ordinary chemical fixation and staining, is essentially due to the distribution of plastic, alkali-soluble components at various levels of, and mostly externally to, the fibrous glucan–chitin skeleton, which remained practically unstainable. In unextracted cells, techniques the allow direct cytochemical staining of α-mannan and β(1-6)-glucan but not of β(1-3)-glucan or chitin (41,83, 177,178) visualized a prominent layering in the cell wall of intact cells, although the reactivity was concentrated in the outer regions (83,178).

Treatment with enzymes, coupled with specific cytochemical stains, have suggested at least a preferential localization of the major wall constituents in different layers (45,177,178,241–243). Immunolabeling techniques employing Con A or anti-mannan antibodies coupled with ferritin, horseradish peroxidase, or protein A–colloidal gold (112,153,154,178,231, 235,238,241,242,249) have consistently shown that mannoprotein constituents are located at the cell surface, where they probably represent the fundamental constituent of the capsular layer (see next section). The cell surface location of mannoprotein is made more likely by the immunolabeling with monoclonal antibodies, which are directed against distinctive mannan epitopes associated with the outermost fibrillar or floccular surface (28,50,176) (Fig. 10-9). In *S. cerevisiae*, treatment with a well defined purified proteinase (z-protease) that specifically digests yeast mannoproteins removed the outer region of the cell wall, apparently leaving intact the inner one (262).

In order to find their exact location in the outer region of the cell wall (mostly at the outermost layer, where they are probably in equilibrium with secretory constituents), mannoproteins must "travel" through the cell wall from the membrane and periplasmic space. Because secretory mannoproteins may be a main part of total cell wall mannoproteins (12,163,192,196), it is likely that some of them are found in inner areas of the cell wall. An elegant electron microscopic observation of this mannoprotein traffic has been reported by Poulain et al. (176), who used gold-monoclonal antibody labeling (Fig. 10-10). What is interesting is to know whether some mannoproteins form distinctive "layers" in the inner

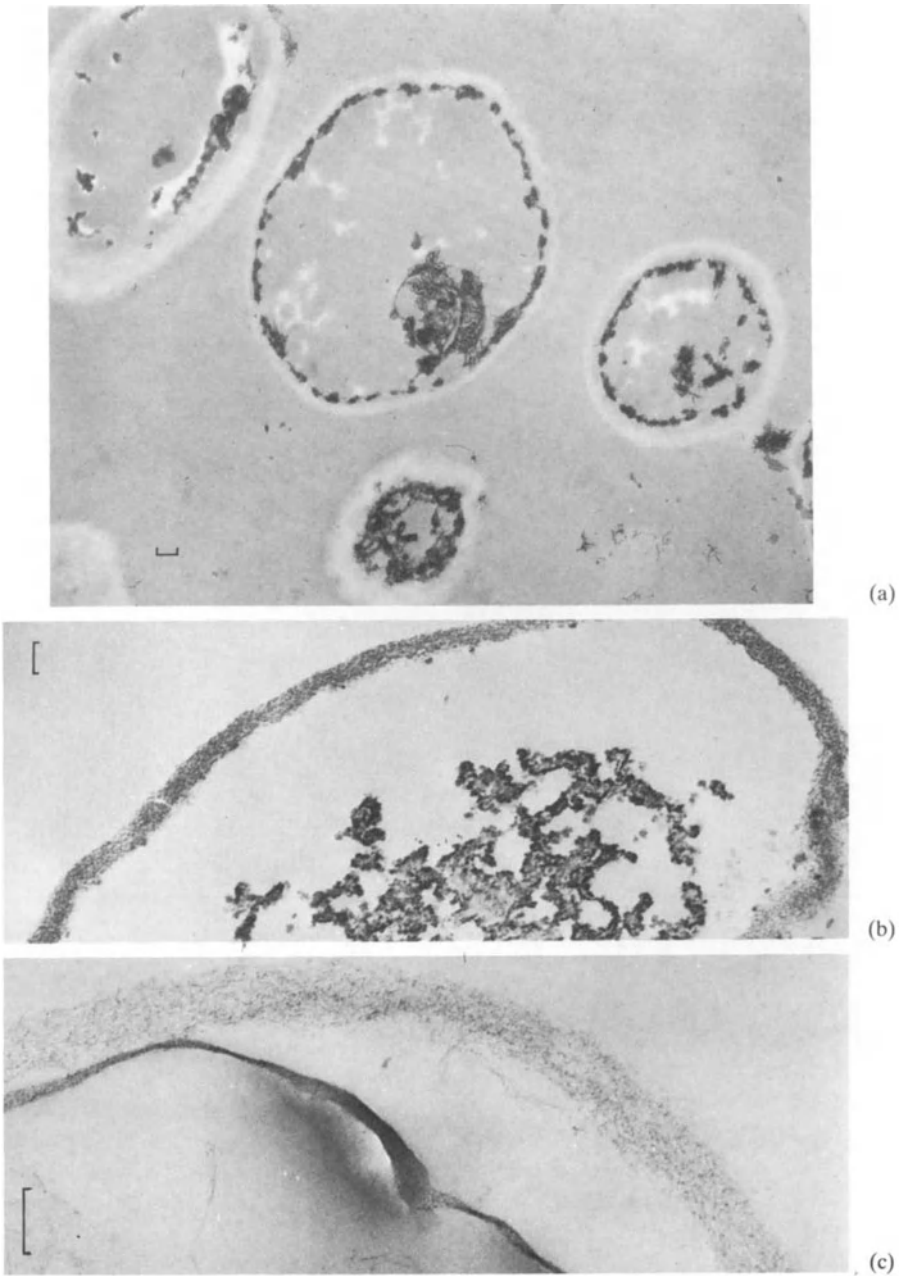
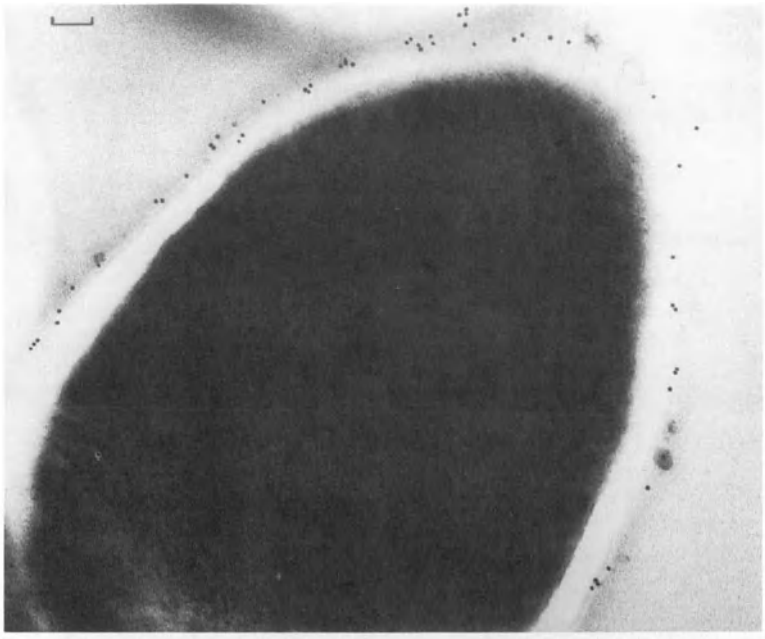
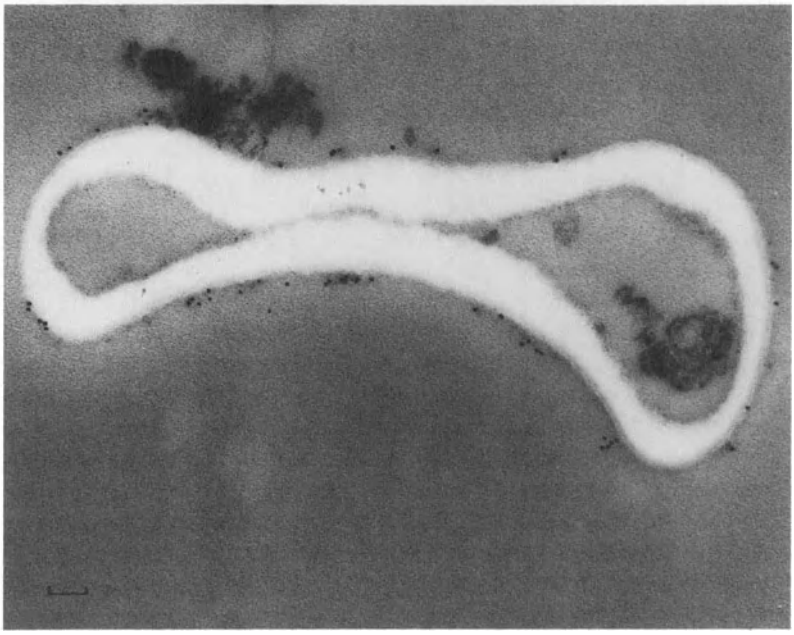


FIG. 10-8. Ultrastructural aspects of the cell wall of *C. albicans* extracted with cycles of hot alkali and stained lead citrate/uranyl acetate (a) or 0.01% phosphotungstic acid (b) or stained with lead citrate/uranyl acetate after partial digestion with helicase, enzyme preparation (c) (for details, see refs. 43 and 44). Note the fibrous appearance of the glucan-chitin skeleton in such particular preparations. Bar = 0.1 μm.



(a)



(b)

FIG. 10-9. Immunogold detection of a surface mannoprotein epitope recognized by a monoclonal anitbody (mAb AF-1) raised by immunization with a soluble mannoprotein extract of *C. albicans* (28,50,179). (a) Whole cells. (b) Isolated cell wall. Note in (b) the absence of gold labels on the inner side of the cell wall. Bar = 0.1 μm.

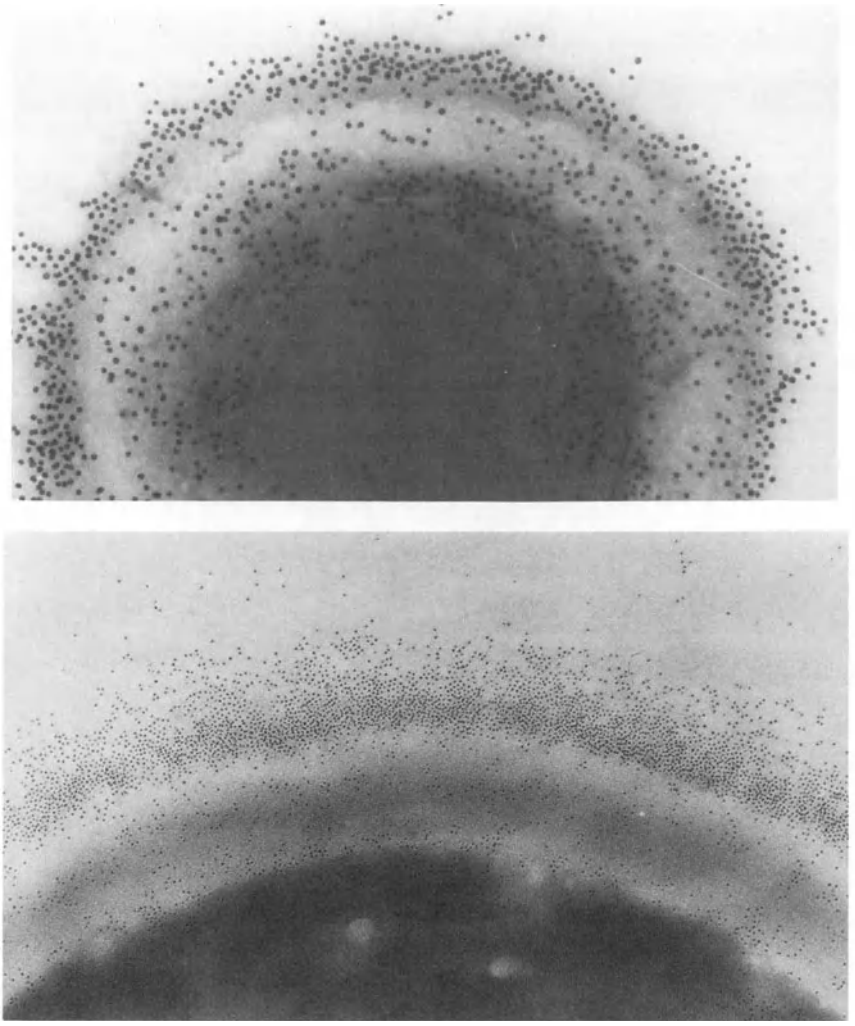


FIG. 10-10. Distribution of a mannoprotein antigen and its secretion through the cell wall of *C. albicans* as revealed by the immunogold technique employing a monoclonal antibody directly coupled with gold particles and applied to thin sections. The material stained is mostly present in the outer layer but is also scattered in intermediate regions. (Courtesy of D. Poulain, INSERM, France.) (b) Distribution of putative mannoproteins identified by polyclonal reagents coupled with ferritin particles. (Courtesy of M. Tokunaga, Japan.)

cell wall or are randomly distributed and if they are associated with β-glucan inside the rigid framework of the wall. When solvent extractions, enzyme hydrolysis, and cytochemical stains are applied to thin sections of intact cells, mannoprotein constituents are also usually detected, though in various consistencies, in inner zones of the cell wall but not as definite layers.

It was shown that cell wall layers can be progressively peeled away by sequential treatments with sulfhydryl (SH) reagents, proteases, and glucanases, but the cells still had unimpaired Con A agglutination (45), supporting the idea that the mannan Con A receptor may span the entire wall. In *S. cerevisiae* a specific mannoprotein, the acid phosphatase, was found to be located in the outermost layer and in an inner one (85,132). There are various reports on cell wall location of acid phosphatase in *C. albicans* (53,64) but the general mannoprotein location indicated by a Con A-reactive stain (242) corresponds fairly well to the acid phosphatase layer in *S. cerevisiae* and the pattern of cell growth (85,132). In this yeast, as well as in *Candida utilis*, other circumstantial evidence has been gathered about the presence of mannoproteins in covalent association with β(1-3)-glucan in the inner regions of the cell wall (112).

Some of the aforementioned technical approaches for mannoprotein topography have also been addressed to isolated, clean cell walls, but in this case the results are by no means clear. In contrast to whole cells, Thiery's stain of isolated walls did not neatly distinguish layers, although the stain accumulated at the outermost rim (41). Gold immunolabeling with a monoclonal antibody identified a widely distributed, major iterative mannan epitope of *C. albicans* (50) that marked the outermost layer but not the inner region of broken walls (Fig. 10-9b). This finding suggests that either mannoproteins were absent from that area or, less likely, that the innermost zone of the isolated wall contains mannoproteins with distinctive epitopes. Asymmetry in mannoprotein distribution is also evident in *S. cerevisiae*. Ready accessibility of a glucanase preparation to its substrate without prior elimination of a mannoprotein shield was seen in isolated walls but no in intact cells (262). As noted previously (41) in studies with isolated walls, the possible loss of noncovalently linked cell wall proteins or mannoproteins should be considered; as judged from the extractive effects of reagents unable to disrupt covalent linkages (45,174), these proteins form a considerable portion of the cell wall constituents. Moreover, isolated walls have lost their periplasmic constituent, which contributes to cell wall layering and contains mannoproteins (119,142). In the "aberrant" cell walls isolated from regenerating protoplasts of *C. albicans*, however, mannoproteins were detected by Con A–ferritin labels on both external and internal sides (78).

Studies on chitin location in the lateral wall and in the septum of *S. cerevisiae* and *C. albicans* have mostly been performed using immunolabeling detection based on wheat germ agglutinin (WGA) as the specific chitin marker (98). In all cases, labeling was largely if not exclusively confined to

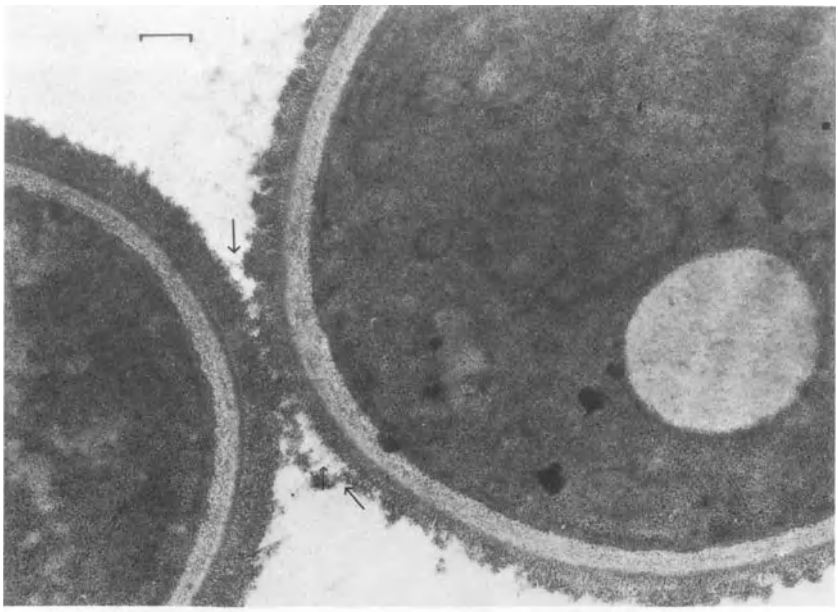
inner regions of the cell wall with an abundance of marker over septa and in bud scars (110,112,149,243). The WGA–gold-labeled areas roughly correspond to those layers that were mostly electron-transparent and unreactive to periodate-silver stains (Thiery's stain) (47,117). Dual, simultaneous label for mannan and chitin showed a differential location of these constituents in the cell wall of *C. utilis* (112). Some WGA-positive sites have been detected at the cell periphery of *C. albicans* (243), but the authors are inclined to interpret this finding as due to labeling of the diacetylchitobiose bridge of mannoproteins rather than to chitin. Extraction of the outer layers with solvents or removal of mannoprotein consituent from the cell wall by proteolytic digestion did not greatly affect the ultrastructural detection of chitin, confirming its inner location in the cell wall and its separation from the mannoprotein sites (149,243).

No antibody, lectin, or enzyme marker suitable for a specific electron microscopic cytochemical detection of glucans have so far been discovered. The inability of glucanolytic enzymes to hydrolyze this constituent in intact cells without prior removal of mannoprotein has been confirmed repeatedly. It seems logical to infer from these observations that glucan, or at least much of it, is internally localized in the cell wall, in the company of its covalently attached chitinous component.

Cell Wall Coat or Capsule

Some emphasis has been placed on the difference in the outermost layer of the cell walls of *C. albicans* and *S. cerevisiae*. When optimally preserved, the surface material of *C. albicans* has a fibrillar or flocculent aspect with thin, delicate filaments to an overall picture that is highly suggestive of a true capsule (67) (Figs. 10-1, 10-5, 10-6, and 10-11). This material, likely including the "fimbriae" (72,92), is mostly present in virulent isolates (146) and is consistently developed during in vivo colonization and tissue invasion (113,151,244), similar to the behavior of truly encapsulated organisms. It appears to mediate adhesion to surfaces, as it is more abundant in more adhesive strains (131,145,146). Like capsular or slime consituents, it is easily detached from the supporting wall and is shed to the external medium in high quantity during in vitro as well as in vivo growth of *C. albicans*, figuring as a prominent circulating antigen of the fungus in systemic disease (66,136). In the heavily encapsulated *Cr. neoformans*, the capsular material is a heteropolymeric peptidomannan with glucuronic acid, xylose, and mannose as sugar constituents; the polymer is *O*-acetylated to various degrees, depending on serotype (183). In *C. albicans* this capsular or slime material would mostly consists of peptidomannan (mannoprotein) with mannose as the predominant sugar; the molecule would be phosphorylated rather than acetylated. Similar fibrillar material has been noted in other pathogenic *Candida* species (238). Tronchin et al. (242) used the

(a)



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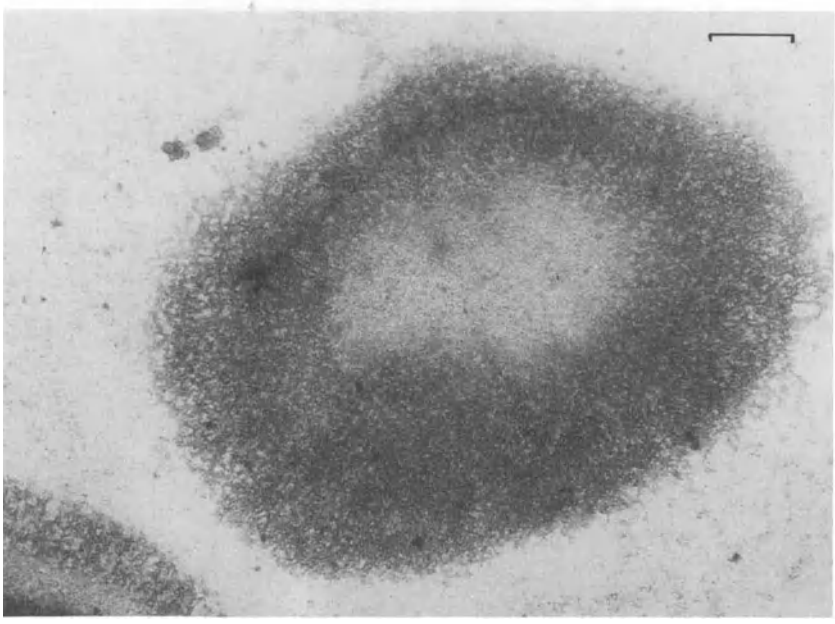


FIG. 10-11. Outermost capsular layer and fibrous appendages (arrows) of the cell wall of a fresh clinical isolate of *C. albicans* from a vaginitis patient (prepared for electron microscopy as in ref. 67), as seen in an orthogonal (a) and a tangential (b) section. Note the perfectly visible mucilaginous mat.

term "cell wall coat" to indicate a layer external to the cell wall proper and visible only in thick cryostatic sections. Acid phosphatase was detected therein (53,242), and an acid phosphomonoesterase is also present in the capsule of *Cr. neoformans* (138). Other authors have used "coat" or "fuzzy coat" to indicate the mucilaginous layer, but this terminology seems more appropriate for the animal cells, which are devoid of cell wall, to indicate the thin ectocellular component covering the plasma membrane (133). Cassone et al. (45,47) have called this outermost component of *C. albicans* "capsule-like," but in view of the evidence summarized above I would rather call it simply "capsule." Accordingly, *C. albicans* (and possibly other pathogenic *Candida* species) should be regarded as an "encapsulated" yeast, although the dimensions and consistency of this capsule may be less than those of the classically recognized capsulated fungi.

Ultrastructural Organization of the Cell Wall: Critical Comments and Exercise Models

By far the most informative technique for morphological observation of the cell wall of *C. albicans* has been electron microscopy of ultrathin sections of chemically fixed cells. Using this approach, the microorganism (harvested from an almost endless variety of cultural conditions) is processed through a number of chemical steps intended to preserve as much as possible the chemical constituents of each cell in the sample and to allow cutting the cell in sections of a few nanometers. Because the goal is not only to preserve structure but also to have it contrasted enough to be observed under the electron microscopy (EM) beam, suitable stains for EM study are introduced at one or several stages of the preparation. Even assuming that the EM observation does not itself introduce significant artifacts, it is nonetheless obvious that the process of sample preparation for EM has somewhat altered the *native* state of the cell and that what we observe is simply the result of the interaction between the original structure and the chemicophysical "aggression" to which it has been subjected. All other methods of visualizing the cell wall by EM have limitations and distorting effects. If we add here that *literally* each investigator or group of investigators has used a preparation schedule for EM that bears few or no similarities with those of other investigators, there appear to be many strong arguments for those who regard with skepticism the conclusions reached by electron microscopists about the complex ultrastructural organization of the cell wall of *C. albicans*. Thus the layering has been suspected to be, at least in part, illusory (165), as it would not reflect qualitative differences in the topology of wall polymers. The reasons qualitative differences are not a prerequisite for a layered cell wall organization and how the stratification can be supported by a differential distribution of even a single class of stainable polymers (mannoproteins) have already been discussed.

TABLE 10-5. Reasons for assigning a degree or "reality" to cell wall layering of *C. albicans* as revealed by electron microscopy of chemically fixed material.

1. There has been a widely heterogeneous use of largely different schedules of fixation and staining: The *basic* note of layered organization (outer EM-dense, inner EM-transparent) has been *always* detected.
2. The layered, ultrastructural pattern of the cell wall has always shown a basic correlate in chemical composition and taxonomic position of the fungus.
3. A range of ultrastructural alterations, from subtle (germ tube) to rather dramatic (chlamydospore) ones, are detected during morphogenesis. The pattern is coherent with chemical changes. Items 2 and 3 would not be expected were the layering largely artifactual.
4. When truly specific enzymes, lectins, cytochemical stains, or immunolabels have been applied, the localization of at least two fundamental constituents as mannan and chitin has been found to be in discrete zones (layers) rather than uniformly scattered throughout the cell wall.

Most investigators have expressed written or unwritten doubts on cell wall ultrastructure because of the variability due to the different staining, fixation, and cytochemical and microbiological factors (73,152,180). However, if the extraordinary variations on both the microbiological side (strain, form, and medium of growth; age and physical state of the culture; temperature of growth; and many others) and the technical side (all EM preparation steps) are properly considered, these investigators would more likely be surprised, as I am, of the substantial *constancy* of the basic, layered ultrastructure of the cell wall. Regardless of the exact, perhaps ephemeral number of layers, there is a rather firm body of evidence that the layered organization of the cell wall as seen in EM studies is basically correct. Table 10-5 summarizes the main reasons for this statement.

Layering should be taken to reflect a dynamic feature of the organelle rather than an unchangeable organization. There is some relevant amount of mannoprotein that traffics through the cell wall before being assembled at the outermost region or shedded from it: These secretory constituents can dynamically contribute to the layering, perhaps more than structural elements. There is a degree of covalent association among the main polymers of the cell walls (mannan with β-glucan and the latter with chitin) coupled with a certain amount of wall material, mostly mannoproteins, which are more weakly bound to wall rigid structures. The ratio between these two entities may also reflect dynamic cell wall morphology, probably according to the age of the cell (41) and its form of growth (47,79,174,205,222). The modulatory activities of cell-wall-associated β-glucanases (see above) and the (interrelated?) mannoprotein traffic may be crucial in the cell wall organization. The distribution of mannoproteins and chitin at various levels of the cell wall has been discussed. However, the location of one (and the most abundant) of the cell wall constituents, i.e., glucan in its various molecular forms (74,102), has not yet been addressed. There is no specific lectin for this molecule to use as a probe; and because

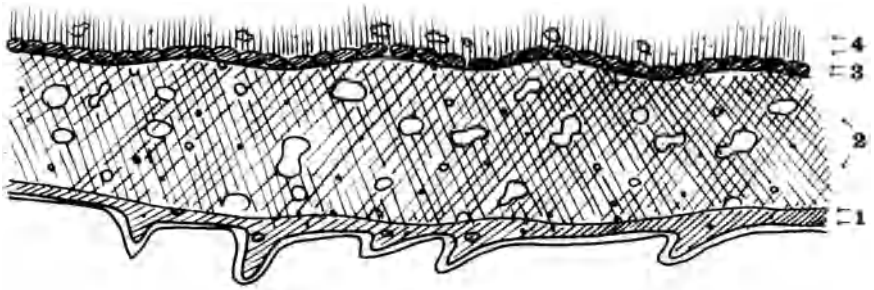


FIG. 10-12. Hypothetical model for the cell wall of *C. albicans*. The model proposes three basic layers. The largest (middle) layer (2) is a network of glucan–chitin fibers with some intermixed soluble mannoprotein constituents that have both secretory and structural (covalently linked to glucan) functions. The innermost layer (1) is the boundary between the plasmalemma and the fibrous cell wall proper. The outer layer (3) is composed mainly of mannoprotein constituents. The outermost component of this layour—of variable composition and extent, and markedly influenced by the age and form of the cell as well as by the nature of the strain—is the capsular layer (4), with fibers that are presumably mannan in nature.

pure glucans have low or no antigenicity, the use of targeted polyclonal or monoclonal antibodies to localize these constituents seems to be precluded or difficult. Targeting of glucanases, including the endogenous ones, has not yet been exploited. We know, however, that chitin is covalently linked to glucan in the cell wall, and that there are large areas in the wall of intact cells that are not stained by reagents that recognize *vic*-glycol groups: These areas, mostly comprising inner layers of the cell wall (47,67,177), are likely to contain β(1-3)-glucan with associated chitin, both of which polymers are unreactive to those reagents. Several authors have proposed cell wall models (84,119,177,196,201,262). Figure 10-12 shows my working model of cell wall organization in *C. albicans*. Although it accommodates all the major findings about location of wall polymers and ultrastructure, it is presented with the implicit assumption that a more realistic model must await the results of in-depth investigations of the localization of wall constituents and, in particular, of the various species of glucans.

Cell Wall Growth and Morphogenesis

Because of the fundamental importance of the cell wall as ultimate determinant of cellular morphology, a great deal of attention has focused on cell well modifications during the diverse processes of growth and morphological differentiation in *C. albicans*. The results of these investigations have revealed that changes in cell wall structure and chemical content are indeed expressed in morphogenesis. These changes may range from subtle, quan-

titative modifications confined to some chemical constituent to dramatic alterations leading to diversified structure of the cell wall, as in the case of chlamydosporulation. Those alterations that are detectable during growth in the yeast form (exponential or stationary) are examined separately from those connected with hyphal transformation (dimorphism). The construction of the chlamydospore cell wall and the regeneration of the cell wall from protoplasts are also summarized.

Cell Wall Morphogenesis in Yeast and Mycelial Forms

Considering the accentuated polymorphism of *C. albicans*, this fungus is supposed to have several physiological machanisms operative in the choice of cell wall structure and metabolism, fitting the possible mode of growth to a determined environment. Most investigations have dealt with changes of the cell wall in the initial stages of the emerging germ tube or early bud: The observations of Odds (165) about the limitations of this approach are sensible. It should be noted that morphogenic processes (except perhaps chlamydospore formation) are transient ones. For example, the cell wall of a fully developed hyphal cell tends to regain the general outline of the yeast cell wall, with only quantitative differences in thickness, probably depending on the specific segment of the hyphae examined (53). There is a continuum of variations in cell wall structure and metabolism that accommodate the flux of events related to the cell cycle. For some aspects of the cell wall, the differences between young, rapidly growing yeast and the old, aged cell in the stationary phase are ultrastructurally more clear-cut than the differences between a yeast cell wall and that of the mature hyphal cell (41,47).

Before analyzing changes in the cell wall during morphogenesis, it is useful to summarize current ideas about how the cell wall is built up during the two fundamental modes of fungal growth, e.g., yeast and mycelium. Soll and co-workers (7,215,218) integrated a multiplicity of approaches in a model where spatial and temporal controls of wall biogenesis cooperate to produce the final shape. In particular, growth of the cell wall was monitored in single cells growing as buds or germlings in perfusion chambers by determining the position of a marker (polystyrene bead) attached to the wall.

The main conclusion from this study was that the formation of new wall in the bud occurs during two temporal steps. During the initial stages, about two-thirds of the cell wall were deposited in an apical zone, and the residual one-third of the new wall material was equally distributed all around the bud. During the second stage, corresponding to the bud maturation, apical growth stops and the bud is enlarged by a general expansion process. As expected, practically the entire cell wall of the emerging germ

tube was localized apically, the apical growth process retaining full activity so long as the hyphal mode of growth continued (218). The mechanism of de-activation of the apical growth process must be somehow related to the size of the bud because when the latter reaches a circumference of about 64 μm apical growth is shut down and it becomes impossible for the fungus to initiate mycelial development without prior completion of the budding cycle. In contrast, smaller buds, still having an active apical zone of growth, may shift to mycelial development without traversing the budding cycle if the cell is moved to germ-tube-permissive conditions.

Commitment stages in morphology have been noted by others (165), but one interesting aspect of the above morphogenetic model is that the decision to become yeast or mycelium is not taken early (immediately after or soon after the cell experiences the *new* environmental condition) but, rather, late in development and without stopping it. Moreover, the critical event for mycelium development is the decision of *continuing* or *discontinuing* the apical mode of growth, a fact that also suits the proposal by Odds (165) about temporal differences in secondary wall synthesis between yeast and mycelial forms.

Many previous findings had indicated that apical growth is not less typical of young buds than hyphae. Both chitin (27,81) and mannan (13,84,234) synthesis in the young bud of *C. albicans* and *S. cerevisiae* take place by apical processes. Cell growth inhibitors that interfere with synthesis of β-glucan fully (echinocandin, papulocandin) or partly (deoxyglucose) cause cell wall lysis at the bud apex that is not distinct from that caused at the hyphal tip (44,78,117,258). A consequence of the studies by Soll and collaborators is that the mode of the early bud or germ-tube emergence should not substantially differ, provided that when constructing a bud or a germling the apical growth machinery may be operating at a different extent or rate or even using different combinations of the ordinary building blocks of wall components. The above authors changed the pH of the culture medium to induce bud or germ-tube growth. This pH-dependent dimorphism is only one of the many possible ways by which new yeast or mycelial growth is obtained in vitro. This subject has been reviewed by Szaniszlo (230), Odds (165), Cassone et al. (49), and Shepherd (201,202). In particular, early germ-tube formation may be accompanied by vigorous outgrowth and effective macromolecular synthesis as in the studies by Soll et al. (215), but it also occurs in the absence of net growth and adenosine triphosphate (ATP) synthesis (199) and diminished rate of protein synthesis (143,239), a situation more suggestive of cytoplasmic *movement* from the mother cell to the germ tube than of real growth (103). It would be interesting to see if and to what degree the machinery that allows selection and temporal regulation of the apical mode of growth versus the generalized one is influenced by these nongrowing but highly morphogenic conditions.

Using a fluorochrome-labeled phalloidin, it was possible to follow the distribution of actin, a major cytoskeletal protein of eukaryotes (1), during

yeast or mycelial growth of *C. albicans* (7). Actin filaments were seen to closely follow cell wall growth, coordinating well with apical synthesis or general expansion. Thus the actin localized diffusely in an outer lamellar zone (cortex) of the cytoplasm in budding cells, whereas it aggregated in bundles oriented subapically in growing germlings (7). The behavior of actin, and possibly of other cytoskeletal proteins, is critical for wall biogenesis in fungi. Disruption of the gene encoding for actin synthesis impedes a normal pattern of deposition of cell wall materials by affecting the ordered sequence of events that lead to initiation of cell wall growth (162).

Ultrastructure and Biochemistry of Budding and Germ-Tube Formation

In the light of previous discussion, ultrastructural and biochemical differences in the generation of new cell wall between the two fundamental forms of growth of *C. albicans* are expected to, and do indeed, occur. The studies published on these aspects, however, have rarely compared cells of the same strain, grown to equivalent stages of development in either form, or under conditions minimizing environmental influences on the morphology. If much of new cell wall is synthesized by a single mechanism (apical) in the early bud or germ tube emergence, it cannot be expected to pick up coarse qualitative differences early in ultrastructural development.

Differences in cell wall organization are observed when buds or germ tubes acquire their distinctive morphology as seen by light microscopy. The inner cell wall of the bud follows in regular continuity that of the mother cell wall, a continuity that is apparently broken or altered in the cell wall of the germ tube (40,47,67,193,197). In addition, extensive rearrangements of, and material shedding from, the outermost wall layers are clearly visible in the germ tube (Fig. 10-13), suggesting more accentuated metabolism and deeper alterations of cell wall components than are seen with the bud. Most authors (3,128,129,140,165,193) claimed that, in *C. albicans* as well as in *S. cerevisiae*, a direct, homogeneous extension of the whole mother cell wall into the bud cell wall takes place; but this conclusion was reached when the complicated layering of the cell wall was not yet unraveled. Gay and Martin (93) described two modes of cell wall behavior during bud formation in *S. cerevisiae*, depending on the age of the mother cell. In "young" cells, the bud was seen to be a direct continuation of the mother cell wall, but in older cells the cell wall of the bud seemed to originate from the innermost, electron-transparent layer, an observation corresponding to the description of the emergence of the germ tube wall in *C. albicans* and other organisms (47,128,129,140). This second, more disruptive mode of growth was characteristic for the cells that showed a number of bud scars adjacent to the area of bud evagination and therefore a less plastic, less extensible cell wall.

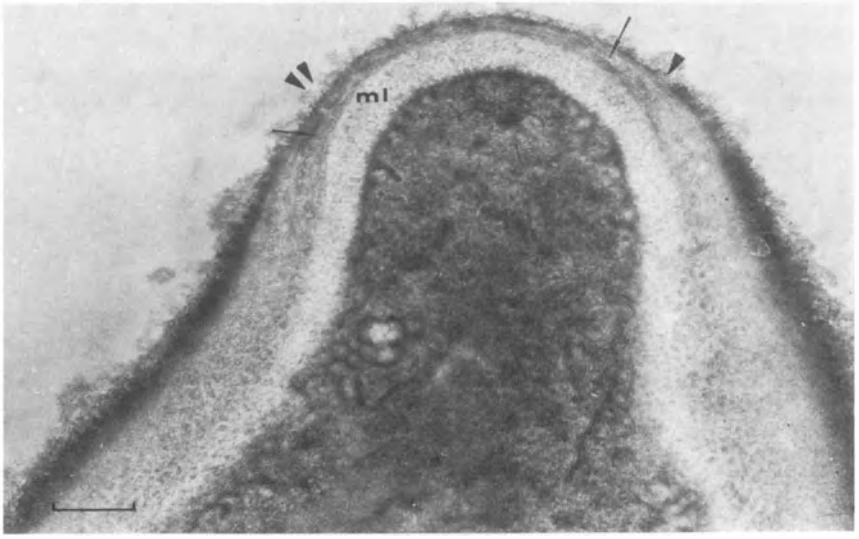


FIG. 10-13. Stage of germ-tube development from the yeast cell of *C. albicans*. Note the extensive shedding of the outermost surface constituent (arrow heads), with rearrangement of underlaying fibrous components (arrows) and the formation of a regular, prominent electron-transparent zone with fine granular constituents (ml). Bar = 0.2 μm. For other details, see ref. 40.

Based on electron-density properties after ordinary fixation and staining as well as on the absence of Thiery's stain for polysaccharides, it was hypothesized that the major morphogenic inner cell wall layer of the emerging germ tube (47) was rich in chitin and β(1-3)-glucan. Chemical determinations are in keeping with the ultrastructure in showing that both these constituents are indeed present in significantly greater amounts in the cell wall of hyphal cells than in yeasts, at least in the early stages of mycelium development (germ-tube formation) (52,54,80,102,143,219).

By using WGA–gold labeling, it was shown (110,112,243) that chitin was mostly present in the inner zone of the lateral wall of the germ tube. The technique was not suitable for a quantitative measurement, but it was observed (52,54,143,219) that the germ-tube wall had three to six times more chitin than the yeast cell wall, and that chitin synthesis was activated during germ-tube formation (27,54). Most of the chitin augmentation was measured early (30–60 min) during initiation of germ-tube formation (143). Pertinent data are also expected from studies aimed at detecting any differential expression of distinct chitin synthase enzymes in morphogenesis (10,37).

A slight decrease in mannan constituent relative to the total polysaccharide of the cell wall during germ-tube formation has occasionally been reported (143). The rate of mannan synthesis was higher in the yeast than in

the mycelial form even though yeast cells were grown at a lower temperature (80). Using a sensitive engyme-linked immunosorbent assay (ELISA) with both polyclonal and monoclonal antimannan sera, markedly increased release of total mannan from the cell wall early in germ-tube formation was measured (Cassone et al., manuscript submitted). Until recently, however, the analysis was too coarse to detect putative small yet significant differences in the mannoprotein structure and organization of yeast and mycelial forms. Solubilization of mannoproteins from the cell wall by chemical or enzymic methods coupled to potent cytochemical immune detection employing polyclonal or monoclonal antibodies (99,174,205,223–225) has suggested that some qualitative alterations in these fundamental constituents of the cell wall may occur during morphogenesis.

Of four high-molecular-weight mannoproteins solubilized from the yeast cell wall by glucanolytic enzymes, only two (those with the lower molecular weight) were detected in the mycelial wall (79). Following extraction with dithiothreitol (DTT), a polysaccharide–protein complex of 235–250 kD and a protein of 19 kD were detected in the mycelial but not in the yeast cell wall digest (174). These results are in accord with those of Sundstrom and co-workers (223–225), who suggested the presence of germ-tube-specific antigenic determinants associated with surface mannoproteins from zymolyase-digested cells; these determinants were found in the protein moiety (224). Human sera preadsorbed with yeast-form cells and enriched in antibodies against germ-tube-specific antigens have been claimed to be predictive for the diagnosis of systemic candidiasis (181). A number of authors have demonstrated that the fine structure of mannan antigens may change profoundly during germ-tube formation. Shibata et al. (205) showed that the structure of mannan of the hyphal cell is *incomplete* compared to that of yeast cell wall, with a reduced amount of a β-linked side-chain oligomannoside, probably due to some suppression of those mannosyltransferases involved in the synthesis of terminal chain residues of β(1-2)- and α(1-3)-bonded mannosides. In addition to possible changes in the protein moiety (224), it may well be another structural reason for antigenicity differences between yeast and mycelial forms. The use of gold immunolabeling and blotting techniques with monoclonal antibodies has made it possible to detect modulations in the distribution of specific epitopes [some of which were shown to be saccharide (mannan?) in nature (28,50,50a,111a)] over the surface of the yeast and hyphal cell. Epitope modulations are also detectable during growth in the yeast form (111a,225), suggesting that what happens at the cell surface during dimorphic transition is only an expansion or amplification of an ordinary, highly dynamic antigenic expression of the cell surface. However, no monoclonal antibody, exclusively directed against the hyphal surface, has been reported thus far (225). Extensive rearrangements and shedding of wall surface material were noted in the first ultrastructural studies of cell wall changes in germ-tube formation (Figs. 10-13 and 10-14). Immunoenzymatic

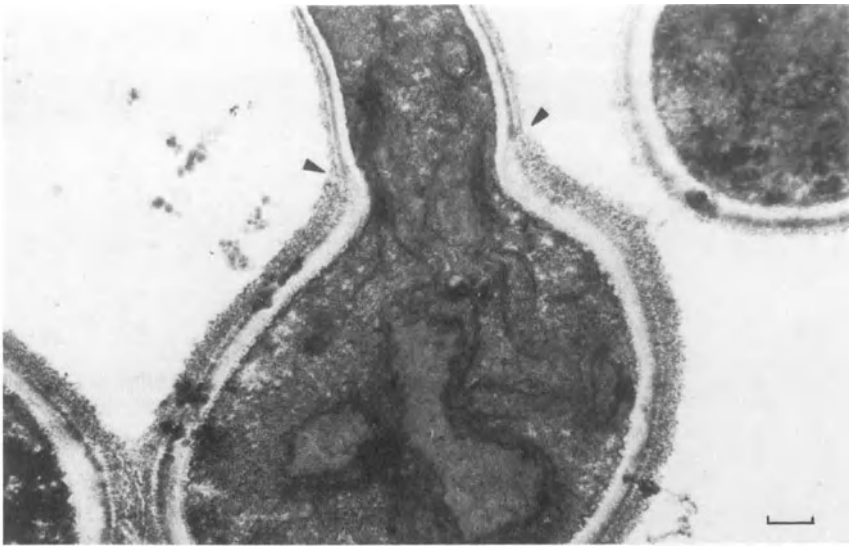


FIG. 10-14. Germ-tube section of a fresh clinical isolate of *C. albicans*. Note the consistency of the outermost capsular layer, with apparent breaking and alterations at the conjunction of the germling with the mother cell (arrow heads). Bar = 0.2 μm. For technical details, see refs. 40 and 67.

(Cassone et al., manuscript submitted) and immunoelectron microscopic approaches (28,176) with monoclonal reagents have greatly expanded perevious suggestions, showing that *C. albicans*, not unlike other symbionts or parasites (130), has a highly versatile antigenic behavior. This characteristic might be linked to the switching phenotype (6,213,216), and both may be important to explain the success of *C. albicans* as an animal commensal and opportunistic pathogen (216). Strain- and culture-dependent epitope modulations have also been reported (35,225). It remains to be investigated if and to what extent the above modulations play a role in the acquisition of specific cell wall morphology, the synthesis of new receptors for adherence to the host's cell surface and host proteins (see below), and, in general, the host-parasite relationship.

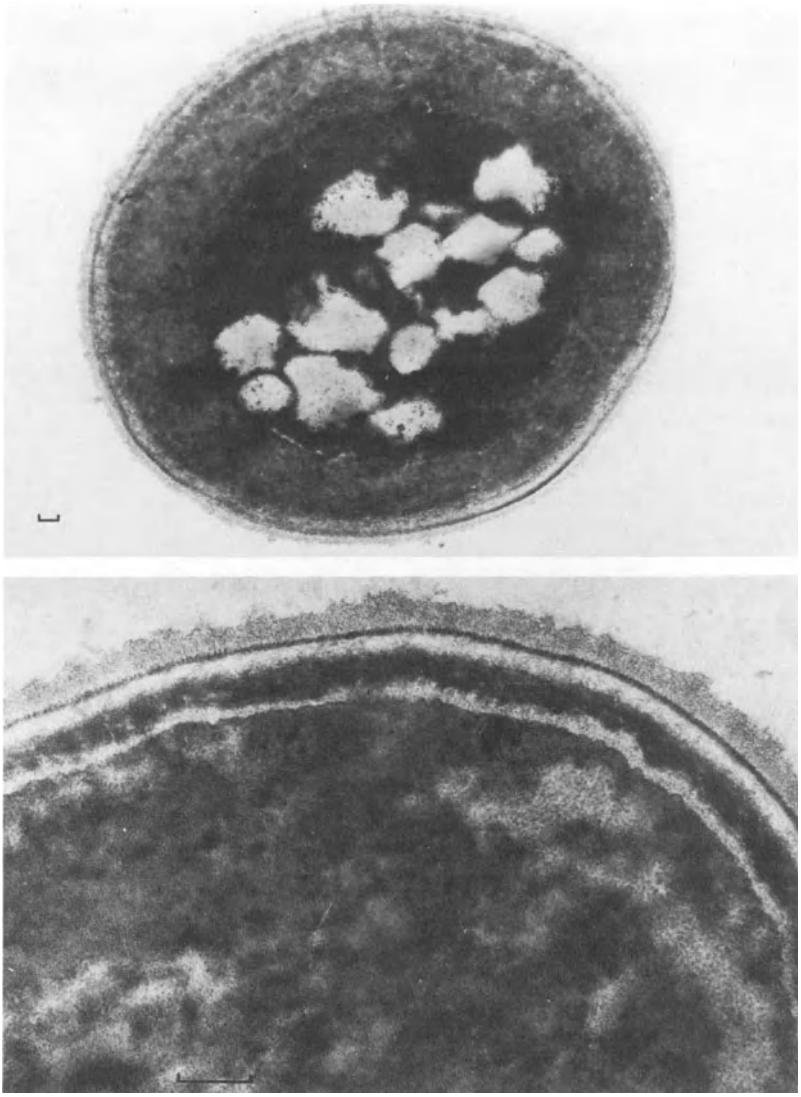
Cell Wall Morphogenesis During Protoplast Regeneration

As previously noted, efficient protoplast formation in *C. albicans* requires the use of S–S reducing agents, followed by proteases and β-glucanases (78,101,240). Cell wall regeneration in *C. albicans*, in partial contrast to *S. cerevisiae*, occurs in simple liquid media. One of the most interesting aspects of wall regeneration in the above media is that the protoplast gives rise to an intermediate, "*aberrant*" wall before acquiring the final one.

The composition of the aberrant cell wall is unique for its high chitin content, relatively low amount of glucan, and SDS- and DTT-extractable mannoproteins with symmetrical distribution on both sides of the wall (79,80,156). The results of these studies evidenced a sort of hierarchy of cell wall components in this peculiar model of wall growth. Thus chitin appears to be synthesized prior than glucan. Blockade of its synthesis by a specific inhibitor such as nikkomycin (144) resulted in the arrest (of the formation) of the other rigid cell wall (79,80,156). In turn, the formation of a glucan skeleton was necessary for deposition of mannoprotein and reversion to a normal cell wall (156), which is indeed expected because of the existence of covalent linkages between glucan and mannoproteins, as discussed above. Moreover, in the absence of chitin synthesis, some form of soluble glucan is synthesized, suggesting that chitin is necessary for the insolubilization of glucan, as suggested by Sietsma and Wessels (210,211). They worked with *Schizophyllum commune*, a fungus taxonomically and biochemically distinct from *C. albicans*. There is a relative abundance of structural information concerning the fibrous net of regenerating protoplasts of *S. cerevisiae* (124,127), in particular concerning β(1-3)-glucan microfibrils (124,126), which can also be regenerated by anucleated yeast protoplasts (125). The long, DNA-independent metabolic half-life of the glucan synthesizing machinery of the yeast cells has been reported. It seems to be the rule that the synthesis of rigid cell wall components precedes that of plastic ones, without apparent exception, even in fungi that have different cell wall chemistries and that are devoid of chitin. It would be unwise to draw direct parallels between cell wall regeneration from protoplasts of *C. albicans* and any of the morphogenetic processes that do not start from naked protoplasts. Nonetheless, early chitinogenesis appears as a constant event in many modes of wall growth in yeasts, and the increased secretion of mannan constituents to external media is impressive in both germ-tube construction and protoplast regeneration.

Cell Wall and Chlamydosporogenesis

Under rather particular culture conditions, *C. albicans* is able to form asexual propagules called chlamydospores, which are characterized by their refractility, perfect roundness, replenishment of reserve materials, and thick walling (Fig. 10-15). The significance of these elements in the life cycle of the fungus is unknown. Some believe that chlamydospores are dead units of a terminal differentiation process, whereas others say they are capable of budding or germinating upon stimulation with selected nutrients at a suitable temperature (25,46,116,182,237). Difficulty of interpretation may have originated from the basically different properties of chlamydospores in an early (148) or late (147) stage of maturation, with only the relatively young elements being capable of reproduction.



(a)

(b)

FIG. 10-15. Chlamydospore of *C. albicans* (a) and an enlarged section of the cell wall during chlamydosporogenesis (b). For other details, see ref 42. Bar = 0.2 μm.

It is usually reported that chlamydospores arise on hyphal segments, but Torosantucci and Cassone (239) showed that they may also originate from pseudomycelium-like differentiation clusters without the need of a "suspensor" (116), an observation that finds its confirmation in the report of "contiguously arranged" chlamydospores from clinical isolates of *C. albicans* (5).

In the context of this review chlamydosporogenesis is important, as it exemplifies a fungal cell wall organization that differs markedly from the one we have shown above for yeast and mycelial cells. Because chlamydospores are fragile and are not formed quantitatively in liquid media, direct chemical analysis is difficult. What we know, therefore, derives mostly from cytochemical and ultrastructural studies.

The ultrastructural appearance of the cell wall of a mature chlamydospore is shown in Figure 10-15. Surface appendages in the form of tiny fibrillar elements surround the spore, but most characteristic is a highly electron-opaque, fibrous, multilaminated constituent that develops as a precipitous structure within the inner electron-transparent area of the cell wall (46,200). The older the chlamydospore, the thicker is this layer, which is probably composed of a proteinaceous material (115). Its extraction with alkali produces the loss of wall refractility but not of the spore form, suggesting that the protein is embedded in a glucan–chitin skeleton (46,115). During germination of the young chlamydospore, the outermost wall layers and the proteinaceous middle component are extensively degraded (46,182,237). Chlamydospores were claimed to contain no mannan but glucan in the outermost layer (115), which would be an unusual situation for the cell wall of *C. albicans*. As a matter of fact, the outermost constituents of the chlamydospore wall were seen as a continuation of the corresponding elements of the mother cell wall of which they retained the ultrastructural aspects (46,237). By an indirect immunofluorescence technique, a monoclonal antibody directed against a major mannoprotein component of the yeast cell wall (50) was seen to effectively stain the chlamydospore surface, demonstrating that some mannan is indeed present (Cassone et al., manuscript submitted).

Cell Wall and Cell Aging

Significant changes in the ultrastructural organization of the cell wall of *C. albicans* are seen during aging of yeast cells in the stationary phase (41). Yeasts from batch cultures 144 hours old appeared to have lost all traces of a layered distribution of cell wall components, and their walls were markedly thicker than those of exponentially growing or early stationary-phase cells. If the layering of the cell wall has been correctly interpreted (see above), the absence of a layered cell wall organization in aged cells means either a decrease in, or a redistribution of, plastic soluble wall constituents in a more tightly interwoven fibrous matrix of insoluble glucan–chitin materials. Several studies (41,90,161) have presented evidence that the above events may indeed occur during cell wall aging. There was a decrease of soluble glucan early during starvation, and isolated walls from "old" cells had weaker and unlayered staining of mannan and β(1-6)-glucan constituents (41). Most of the wall-associated exo- and endo-β(1-3)-

glucanase activity was found in an inactive state as the cells progressed to the stationary phase (160,161). If glucanases play a role in breakdown and resynthesis of the cell wall during growth (250), glucanase inactivation in the nongrowing (aged) cells would inevitably lead to, or be a consequence of, more intense cross-linking of glucan fibers and a lesser amount of opened chains for insertion points (201,202). In addition, the outermost mannoprotein constituents may not be synthesized and efficiently secreted in aged cells, and glucan itself may be subjected to extensive turnover because of decreased efficiency of the cellular biosynthetic machinery (90).

When studying the phenotypic resistance of aged cells in the stationary phase to amphotericin B, it was hypothesized (90) that a highly cross-linked β(1-3)-glucan network performed as a barrier to antimicrobic penetration. Studies have pointed to the outermost mannoprotein shield as the structure impeding permeation of relatively large molecules (29,77,201,262) (see above). The two ideas are not mutually exclusive. The acitivty of cell-wall-associated glucanase is certainly a key factor for siting mannoproteins in covalent linkages to glucan, as demonstrated by the release of several mannoprotein constituents through the activity of endogenous glucanases in clean cell wall preparations (79,247). There is also some initial evidence that the release of outermost mannoprotein, which is easily obtained by treating cells with SH− reagents may be in part due to activation of cell-wall-associated glucanases (160; Cassone et al., unpublished results). Thus the activity of these enzymes in cells of young cultures of *C. albicans* not only may furnish a less tightly, less cross-linked, and more plastic cell wall but may also contribute to a significant shedding of mannoproteins to the cell exterior. Both these factors may play a role in allowing access of the antimycotic agent into the cell.

Cell Wall and Its Role in Pathogenicity

As outlined in Table 10-1, cell wall constituents are important to the virulence of *C. albicans*. This role may be played in several ways. First, some cell wall components may display primary toxicity for the host. This possibility was shown in experiments by Cutler et al. (61), who demonstrated that cell wall glycoproteins exert toxic effects on intact or drug-pretreated animals. Reports of nonspecific toxicity of glucomannoprotein-rich extracts from *C. albicans* are plentiful in the literature (164), but the distinction between the toxicity proper of the above components and that of any potential contaminating bacterial endotoxin has not always been rigorous. Mannan from *C. albicans* (and *S. cerevisiae*) has been advocated as a toxic compound with an LD_{50} for the normal outbred mouse in the order of a few milligrams per kilogram (157). The mechanism attributed to this toxicity was that of an anaphylaxis-like reaction. Nonphosphorylated, low-

protein mannan has been shown to be the most toxic, and its toxicity was prevented by corticosteroids but not by antiinflammatory, nonsteroidal drugs (157). Cell wall toxicity appears to be shared by the other main cell wall component, i.e., glucan; but in this case an endotoxin-like mechanism of action has been suggested (111). By employing a sensitive chromogenic assay with a Japanese amebocyte lysate reagent, glucan from *C. albicans* (and from other fungi) gave a positive endotoxin reaction seemingly not due to contaminating bacterial lipopolysaccharide (111). The exact composition of the glucan extract was not noted, and it is possible that nonglucan constituents participated in the endotoxin-like reaction.

Other distinct possibilities to explain how the cell wall of *C. albicans* contributes to the virulence of this fungus are indirect ones and concern the role played by mannoprotein enzymes and hyphal morphogenesis. Enzymes such as acid proteinase, phosphomonoesterase, and phospholipase (14,135,158,187) are not dealt with here. Apart from the direct activity of these enzymes, the mere secretion of mannoprotein antigens may play an intriguing role in pathogenicity. These cell wall constituents, by combining with antibodies and receptors on hosts, phagocytes, or other immunoeffectors, are likely to perturb the immune system and give the fungus more chances to evade the immune response of the host.

There is ultrastructural (28,47) and immunochemical (28,176) evidence for a remarkable shedding of mannoprotein antigens from the capsular surface of the fungus. The extent of secretion, quantified by sensitive ELISA-competition experiments using poly- or monoclonal anti-mannoprotein antibodies (Cassone et al., manuscript submitted) may reach several micrograms per milligram of cellular mass, an amount that is compatible with the doses of these constituents that modulate the activity of immunoeffectors in vivo and in vitro (see below).

The cell wall has a direct, specific impact on yet another fundamental aspect of microbial virulence, i.e., its capacity to adhere to host cells. Adherence is a property that, for all non-exotoxin-producing pathogens, is thought to be mandatory for colonization and invasion.

Cell Wall Constituents as Adhesins

Anyone working with *C. albicans* has surely experienced how strongly this microorganism "sticks" to a variety of surfaces. It adheres easily to epithelial and endothelial cells, fibrin–platelet matrices, and plastics. There are therefore some cell wall constituents that mediate the attachment; as in bacteria, they are called *adhesins* (57,72,186). It may seem naive to anticipate that components of the outermost cell surface play the role of adhesins, although they do not need to be the same for each adhesive event or surface. We previously discussed the surface localization of mannoproteins in *C. albicans*, and a consistent body of evidence points to these molecules

as the most likely mediators of the adhesion of fungal cells (72). Ultrastructurally, adhesins are expressed as spiky or fimbria-like appendages in, or finely protruding from, the capsular layer (Figs. 10-6 and 10-11). Thus yeast cells treated with Con A are much less adhesive to mucosal cells than their untreated counterparts, and secreted mannoproteins inhibit adhesion, at least when their protein moiety is not altered (145,189). Removal of putative mannoprotein molecules from the yeast surface by a mild alkali extraction method (45) abolished the adhesive properties of the fungus (180). Interestingly, sufficient mannan receptors remained on the cell surface for cell agglutinability by Con A (189), suggesting that some specific, alkali-labile mannoprotein molecules were involved in adhesion. Finally, treatment of yeast cells with proteolytic enzymes or reducing compounds known for their ability to extract the outermost mannoprotein constituents caused derangement of their ordered organization on the wall surface (45) and severely reduced their adhesion to vaginal cells (131,214).

As argued by Douglas (72), some mechanism is likely to operate in vivo for selection of the most adhesive cells among the yeast population, in view of the "sweeping away" effect of most organic fluids that bathe the cells and the shedding of the mucosal cells themselves during the physiological cycle. If this selective pressure does indeed operate, and surface-located mannoproteins mediate the adherence, it is expected that the outermost capsular layer of the yeast cell wall would be much more conspicuous in vivo than in vitro. This theory has indeed been verified (34,113,151,244), and in some instances the outermost layer is so developed in vivo as to suggest the ability by *C. albicans* to form a true, prominent capsule (see above). Growth of the fungus in saccharide-rich media promoted both adherence and formation of a thich capsular layer (146), with associated increased resistance to spheroplasting enzymes, in keeping with the role of external mannoproteins for accessibility of glucanolytic enzymes to the inner layers of the cell wall (262). Lee and King (131) and Critchley and Douglas (57) isolated mannoprotein molecules that inhibit the attachment of *C. albicans* to epithelial cells. On the basis of treatments that affect either the saccharide or the protein moiety of these putative adhesions, it has been suggested that the latter constituent is essential for adhesin (57,131). This conclusion is in accord with the high specificity of the inhibitory activity of the mannoprotein, in contrast to the rather uniform structure of the mannan moiety, as evidenced by the serological specificity. It is also a common experience of those who use mannoprotein antigens for ELISA that low-protein mannan preparations or deproteinated antigens do not attach to the plastic wells (50), a fact confirmed by experiments involving peptide elimination from mannan antigens by $NaBH_4$ treatment (234b). Others have shown that adherence to vaginal epithelial cells is diminished in mannosidase-treated fragments of the cell wall of *C. albicans* (131). Moreover, in models of adhesion to fibrin and endothelial cells (186,212) several findings supported the idea that the

carbohydrate moiety of the mannoprotein plays an essential role. These data are in no way contradictory, however, as different adhesins may be involved with different surfaces or, at the extreme, the *same* adhesin may bring into play various of its molecular parts in response to the type of animal cell surface receptor. In addition, the net result of any enzyme treatment is not only elimination of the receptor-specific mannan or protein moiety but also a configurational effect on the whole molecule, and this effect may well be the critical one in the loss of adhesion capacity.

A problem encountered when it is assumed that mannoproteins are the mediators of adherence of yeast cells in vivo (i.e., in an infective focus) is that, as previously mentioned, there is a remarkable shedding of this cell wall constituent by growing cells. Theoretically, this material could compete for adhesion of *Candida* cells (72). It can be envisioned that shedding, however large, is insufficient to block the receptors, or even that most of the shedded mannoprotein does not contain the true adhesins. Alternatively, the released mannoproteins may favor adherence by acting as an efficient bridge between *Candida* and the infected cell. The suggested "branched-tree" structure of mannoprotein, in contrast to the more classic "comb-like" one (227,228), may facilitate multiple bridging and adhesion.

Segal and collaboators (194) have shown that chitin, chitin hydrolysates, and amino sugars inhibit adhesion of *C. albicans* to vaginal cells. The inhibitory activity of *N*-acetyl-D-glucosamine, together with that of mannose and inositol, against adhesion to buccal epithelial cells has been confirmed by Ghannoum et al. (94). On the basis of these and other results, chitin was proposed as an adhesin, a role that is not compatible with the localization of chitin in the inner layers of the cell wall. Other *Candida* components, for which there is no evidence concerning their surface location (e.g., lipids), have been claimed to play a role in adhesion (94). In a report by Sandin (188), adherence to oral epithelial cells was completely inhibited after a mild alkali extraction that could hardly remove chitin or any chitinous constituent from the cell wall. It has also been shown (154) that *C. albicans* adheres efficiently to Con A–Sepharose, which does not bind chitin. However, some cytological observations have indicated that the fungus may also come into close contact with the host cell through its inner wall layers (131,151). Most of the evidence for a role of any cell wall constituents in adherence is based on experiments of inhibition by analogs or competitors of the postulated cell wall adhesin. These studies are sometimes misleading with respect to adhesin identification because (a) the tested inhibitor may be more analog of the animal cell receptor than of the adhesin (57,58) and (b) the compound added in the adherence assay may not specifically alter some of the environmental parameters for adhesion (72,171). These facts should be carefully considered when the added inhibitor provides relatively little (although statistically significant) reduction of adherence.

Cell Wall Constituents as Receptors for Soluble Factors

If it is true that *C. albicans* is likely to adhere to various substrates, the opposite is also true, i.e., many macromolecular constituents of host cells or fluids may strongly "adhere" to *Candida*. Two of these soluble factors have been investigated in some detail. Heidenreich and Diedrich (107) were the first to accidentally observe that fractions C3d and iC3b of complement bind to *C. albicans* but not to other less pathogenic species of *Candida*; the yeast forms rosettes with C3d- and iC3b-coated red blood cells. The receptors for C3d were identified by Calderone et al. (36) as 62- to 70-kD mannoproteins, with saccharide moiety probably not involved in recognition of the complement fraction.

Bonaly et al. (22,23) and Tronchin et al. (245) investigated binding of fibrinogen to *C. albicans*. The fungal receptor was suggested to be a DTT-releasable surface mannoprotein (23,245). Of particular interest is the fact that in all cases the receptors for the above plasma proteins were predominantly or even exclusively expressed in pseudomycelial cells or germ tubes (hyphal forms) (36,96a,245). This finding suggests that modulations in mannoprotein expression may mediate, or contribute to, the observed increase in the adherence of filamentous forms of *C. albicans* compared to those of the yeast forms (121,188,214). The implications of the possession of receptors for binding plasma proteins involved in fundamental processes of blood physiology or immune homeostasis are immediately understandable. Studies on the origin, nature, release, and functions of these receptors may prove useful for understanding colonization and pathogenicity. Gilmore and Hostetter suggested that the receptor for iC3d complement fraction may be able to inhibit phagocytosis of *C. albicans* (96a,97), a finding that should be considered together with the experimental evidence on cell wall mannan-mediated inhibition of phagocytosis (see below). These data also offer some clue to the mechanisms by which the hyphal forms of *C. albicans* predominate in infected tissues.

Cell Wall and Immunomodulation

As the cell wall represents the prime contact between the fungus and the host, it is obvious that the host will try to stage an efficient immune response against this cellular organelle. The outermost cell wall constituents form a mosaic of antigenic determinants (89,105,106,220,229), as demonstrated by the almost universal occurrence of antimannan antibodies in all normal individuals, their induction in vitro (76,254), and the lymphocyte proliferation in vitro and skin test reactivity in vivo in response to mannoprotein stimulation; these reactions are specific signs of the involvement of cell-mediated immunity to *C. albicans* (9,56,173). Although immuno-

modulation (e.g., the capacity of influencing the immune response) is by its nature intrinsic to all mitogen- or antigen-induced responses, *C. albicans* contains in its cell well constitutents that may not be recognized as antigenic *strictu sensu*. Nonetheless, they are able to nonspecifically activate or depress a variety of immune responses. The first (and strongest) immunomodulator is the *Candida* cell itself, which has been shown to modify the outcome of immune responses after being administered to the experimental animal in suitable forms, doses, and routes. The main effects reported are summarized in Table 10-6.

In order to understand the possible potential of the cell wall components of *C. albicans* for wide-rangeing immunomodulation, it is important to recognize the human commensal nature of this microorganism (164), with its associated intrinsic potential for immunosurveillance (42). The various arrays of responses that can be expected of intestinal colonization or even systemic spread (when compatible with normal functioning of the immune system) of *C. albicans* have been reported in the mouse. It has been demonstrated (70) that artificially induced gastrointestinal colonization with *C. albicans* primes the mice to the development of specific immunity and protection against a challenge with virulent microorganisms. This specific immune response was interpreted as being due to intestinal colonization rather than systemic spread. Using an agerminative variant of *C. albicans* inoculated intravenously and producing a systemic, persistent, but nonlethal infection. Bistoni et al. (20) have shown that an antiinfectious capacity develops throughout, so that lethal challenge by a number of highly virulent microorganisms is successfully opposed. This sort of classical "vaccination" with a live, low-virulence variant recognized activated macrophages and perhaps polymorphonuclear leukocytes (PMNs) as nonspecific mediators; the same results were obtained in congenitally athymic, nude mice (20a). The role, if any, of antibodies was not investigated, but Cutler and Lloyd (62) have demonstrated enhanced antibody response to *Candida*-unrelated antigens on injection of viable cells or isolated wall or even cell wall extracts from *C. albicans*. Although these studies emphasize the induction, by *Candida* colonization or infection, of protective, specific or nonspecific immunity, other investigators found at least transient immunosuppression of cell-mediated immunity, which is reminiscent, for several aspects, of the immunodepression seen in chronically affected candidiasis patients: The range of *Candida*-depressed responses included T cell mitogenesis (185) and antibody formation in response to T-dependent but not to T-independent immunogens (248).

The use of heat-killed or otherwise-inactivated cells of *C. albicans* in the intact mouse has produced two sets of results (activation of depression), depending on the type of response study and the conditions of the study. Some authors reported that induction or enhancement of immune responses led to increased resistance to aggressive tumors (46,251). It was shown in particular that for an antilymphoma response to occur, prior sen-

TABLE 10-6. *C. albicans* as biological response modifier and immunomodulator.

Effect	Mediator	Immunoeffector	Ref.
Antigenic or mitogenic stimulation or suppression of cell-mediated immunity	Mannoprotein	B or T lymphocytes	9,59,60,156,159,173,184,185,217
Activation of antitumor responses in vitro and in vivo	Mannoprotein, glucan	NK or lymphokine-activated killer lymphocytes; activated macrophages	42,43,46,139,141,191,251
Activation of anti-infection specific and nonspecific potential	Glucan, chitin	Activated macrophages; PMNs (through NK regulation?)	20,122,195,226,252
Enhancement or suppression of antibody response to *Candida* related or unrelated T-dependent or T-independent antigens	Mannoproteins	B cells	38,60,62,68,69,71,248,254
Inhibition or enhancement of PMN function	Mannan, glucan	PMN	63,68,253,256,257

sitization of mouse to *Candida* was an absolute requirement (43). Activation or site-recruitment of non-MHC-restricted antitumor immunoeffectors such as natural killer (NK) cells (141) or macrophages (251) may be one mechanism of protection.

In apparent contrast to the findings above, mice given chemically inactivated *Candida* cells also showed several markedly reduced immune responses. Roger and his group in Philadelphia (184) documented a *Candida*-induced suppression, in vivo and in vitro, of a proliferative response to T-cell lectins or antibody formation to T-dependent antigens but not of B cell mitogenesis due to lipopolysaccharide. The suppressor cells elicited by *Candida* had the characteristics of a cell in B lymphocyte lineage (184) and could also be induced in vitro (60).

Any attempt to reconcile these apparently diverse results may prove difficult because they are probably *not reconcilable* but simply denote the coexistence in the *Candida*-induced immunomodulation of enhancing and suppressive effects that may well be simultaneously expressed but are picked up separately by the various investigators with their different objectives and experimental approaches. It is known, for instance, that NK and macrophage effectors, which are so effectively induced by inactivated *Candida* (141,251,255), have the potential of enhancing or suppressing a diversified set of immune responses, including B and T cell responses (96,120) and anti-*Candida* activity of PMNs (68). A fascinating question on (and more pertinent to) the topic of this review (among those arising from these studies with intact *Candida* cells) is the following: Which *Candida* component(s) is responsible for the immunomodulatory effects exhibited by the intact microorganism? The answer to this question is not only relevant in the context of the host-parasite relationship in candidiasis but may also be essential for the preparation of purified material, which may add to the armamentarium of adjuvants, immunomodulators, or biological response modifiers of microbial origin potentially useful in tumor immunology and infectious diseases. Interestigly, the answers obtained so far, although preliminary, tend to indicate that mannan (or mannoproteins) and glucan (and perhaps chitin), which are the three fundamental cell wall constituents, do potentially participate in this role.

Mannan Constituents as Immunomodulators

As emphasized by Domer et al. (71), mannan-enriched fractions from the cell wall of *C. albicans* have mainly been associated with immunosuppressive effects that were detected in vivo and, more frequently, in vitro. In one study (38) the specific immunosuppressive effects of a gluco-mannoprotein fraction on the development of sensitization (with concomitant immunity) to *C. albicans* in vivo and lymphoproliferation induced by ethylenediamine-extracted mannoprotein in vitro was noted. The mannoprotein immuno-

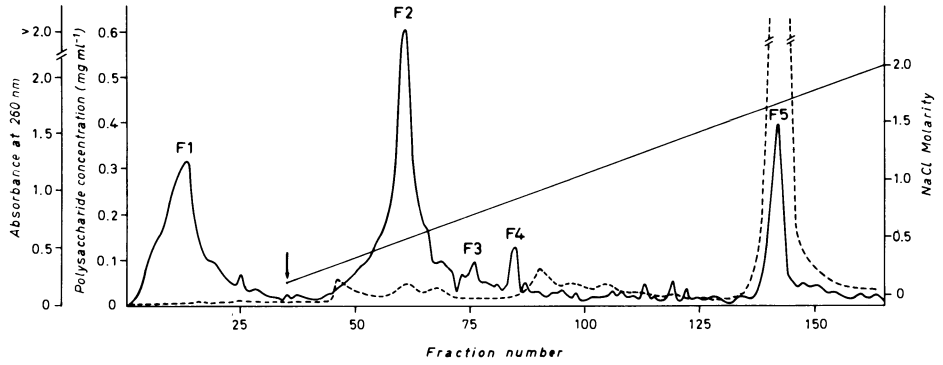


FIG. 10-16. Separation of mannoprotein extract (GMP) from the cell wall of *C. albicans* by ion-exchange chromatography DEAE-Sephadex A50 (50). Solid line = polysaccharide measured by the Dubois method; dotted line = absorbance at 280 nm. Each of the distinct five peaks bound polyclonal human anti-*Candida* serum, but only the first four peaks were recognized by a monoclonal antibody to GMP extract. For other details, see ref. 50.

suppression was attributed to the protein moiety of the molecule. A classical mannan (low protein) preparation (169) was devoid of effects, similar to what occurs with "antigenic" lymphoproliferation of human peripheral blood mononuclear cells (9; Ausiello et al., manuscript submitted). Interestingly, the same preparation used by Ausiello et al. as the antigenic activator of lymphoproliferation used by Ausiello et al. as the antigenic activator of lymphoproliferation and γ-interferon production (9,217) has been shown by Cuff et al. (59) to have strong immunosuppressive effects on mitogen-induced proliferation and antibody responses in vitro. The latter authors have also confirmed that, among their various cell wall preparations only the mannoprotein-rich fractions were immunosuppressive (59). All mannoprotein fractions cited above were rather crude mixtures of different constituents (including some containing glucan) than can be easily separated by ion-exchange or affinity chromatography with Con A—Sepharose or by gel size filtration. An example of separation of a whole-cell-wall mannoprotein by charge is shown in Figure 10-16, where four mannoprotein constituents are clearly observable. In these separations, only the first two mannoprotein fractions were capable of inducing human lymphocyte proliferation; and for each of the two, proteolytic degradation of the protein moiety abolished the effect (Ausiello et al., manuscript submitted). Using an approach of this type, Domer et al. (69,71) were able to separate distinct mannoproteins, some of which enhanced and others suppressed the in vivo antibody response to T-dependent or T-independent antigens. The unseparated mannan had both stimulatory and suppressive effects depending on the dose (71).

Clearly, crude mannan preparations may be contaminated by other cell wall or even cytoplasmic components of *C. albicans*: one of the contaminants may be glucan, although it cannot be inferred by the presence of a minimal amount of glucose in the preparation if the fact that some mannan is covalently linked to glucan in the cell wall is considered. Glucan contamination may modify the immunomodulation by mannan, as well as "modulate" immunosuppression, although glucan itself has never been shown to act as an immunosuppressive agent. Fractions of mannan are different in their biological effects; thus one fraction may induce enhancement (e.g., fraction II) and another may markedly suppress (e.g., fraction IV) the antibody to sheep red blood cells (SRBCs) (71). It should be noted, however, that these fractions do not differ in the basic chemical composition; thus their configuration or a distinct protein constituent is the factor responsible for the different effects. Two other more complex glucomannan-based fractions obtained by different extraction procedures were also effective stimulants of the antibody response (69), demonstrating that the immunostimulatory portion of the cell wall polysaccharides largely exceeds the suppression. The finding does not fit in with all the evidence for the suppressive effects of mannan in other systems. Domer et al. (71) reported that the immunosuppressive fraction was only 14% of the total mannan recovered, whereas more than 50% was immuno-enhancing.

Mannan extracted from *S. cerevisiae* or *C. albicans* by Peat et al.'s procedure (169) has been studied extensively for its ability to suppress lymphoproliferation of peripheral blood mononuclear cells and neutrophil functions (63,159,253,256,257). In the latter case, inhibition is thought to be mediated by the antagonism between mannan and *Candida* cells in binding to surface receptors or a myeloperoxidase enzyme. An apparently analogous competitive mechanism may be postulated for the observation that mannan inhibits the lymphoproliferative response to *Candida* antigens (9,159). In addition, Nelson et al. (159) have shown that many nonspecific effects of *Candida* antigens may be due to the copper contaminating the preparation of mannan (precipitated with copper-containing Fehling reagent). The presence of copper, however, could not explain the modulatory effects of different mannan fractions on the anti-SRBC antibody response (71). The inhibitory effects of mannan on neutrophil functions do not concern the ingestion phase, which may indeed be stimulated by this polysaccharide as well as by glucan (253). This stimulation of unopsonized particles of zymosan (particulate cell wall fragment of *S. cerevisiae*) was not due to complement activation. Human PMNs have been shown to possess a trypsin-sensitive receptor for ingestion of zymosan and yeast glucan, a receptor with a remarkable degree of specificity for a given glucan configuration (253). The respiratory burst, however, was sensitive to the mannan component of zymosan (256).

Glucan and Chitin as Immunomodulators

Glucan is another cell wall immunoactive component of *C. albicans* (and *S. cerevisiae*). Most of the early studies on yeast glucan as immunomodulator and biological response modifier were performed by Di Luzio and coworkers (122,139,252), who showed the antitumor and antiinfectious potential (including the anti-*Candida* one) of the alkali- and acid-insoluble β(1-3)-glucan preparation from *S. cerevisiae*. The glucan ghosts (45) were the only cell constitutents that could substitute the intact cells of *C. albicans* in the antitumor, chemotherapy-synergistic effect in LSTRA-lymphoma-bearing mice (43). Both mannoprotein and glucan of *C. albicans* are powerful inducers of peritoneal NK and macrophage effectors in mice: The elicitation of these effectors by glucan was more intense and of longer duration than that obtained with the mannoprotein extract (191). Sensitive ELISA-inhibition tests excluded the possibility that the induction of these cytotoxic immunoeffectors by glucan was due to any contaminating mannoprotein. Glucan is a strong macrophage activator in vitro and in vivo (195,251,252), and it is generally believed that the antitumor and antiinfectious properties of this cell wall constituent are due to activated macrophages (139,252). The experiments reported by Scaringi et al. (191), while confirming this glucan activity, also suggested that NK effectors may be involved additionally in the protective effect of glucan. The immunomodulatory properties of *Candida* glucan are shared by other β(1-3)-linked glucose polymers present in other microorganisms and higher fungi. Glucan is heterogeneous in the *Candida* cell wall (see above), but so far there have been no investigations on the possible differences in the immunomodulatory properties of the different glucans and on their association with the other cell wall constituents. All or most of the insoluble β-glucan preparations from *C. albicans* contain some chitin. This constituent has been shown to activate macrophages to candidacidal activity and recruit peritoneal effectors after injection into the peritoneal cavity of mice (226). The amount of chitin (50 mg/kg) used to elicit these effects, however, was much larger than the doses of glucan that give optimal activation of NK cells or macrophages (1 mg/kg), suggesting that the effects reported by Scaringi et al. (191) with *Candida* glucan were not due to chitin. Chitosan, chitin derivatives, and synthetic chitin oligomers are under investigation for their immunomodulatory properties.

Conclusions

The cell wall is of fundamental importance for most aspects of the biological actions and pathogenicity of *C. albicans* as well as the immune response to it. Therefore cell wall constituents are essential to survival and growth in

an aqueous environment with low osmotic pressure as well as for colonization and invasion of host tissues. This fungus may use mannoproteins for anchorage to inanimate surfaces or for creating a barrier to the penetration of potentially offensive external proteins or drugs. At the same time, it may use the molecules for adhering to the host cell, thereby initiating colonization or a frank infective process; and it may also exploit mannoproteins as subtle but strong modifiers of the biological response of the host, or even shed them to evade the host's defense. The glucan constituent of the cell wall of *C. albicans* is mostly involved, together with chitin, in osmotic protection and acquisition of the specific habit of growth: The same component, or part of it, can confer on the host an increased microbicidal potential and modulate essential antitumor responses such as those mediated by NK cells or other immunoeffectors.

Chitin plays a "hierarchic" role in morphogenesis and may also be involved in immunomodulation, with or without glucan. Study of the cell wall of *C. albicans* is apt to satisfy the intellectual curiosity and interests of biologists and medical researchers, and it can be anticipated that a new generation of anti-*Candida* chemotherapeutic drugs will be based on the selective inhibition of synthesis or assembly of one or more cell wall constituents. *C. albicans* has long been one of the most studied microorganisms, and its cell wall attracts more and more investigators, who have come to recognize the essential functions it plays in the activity of this microorganism.

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11—Regulation of *Candida albicans* Populations in the Gastrointestinal Tract: Mechanisms and Significance in GI and Systemic Candidiasis

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Colonization of the gastrointestinal (GI) tract by *Candida* species, and *C. albicans* in particular, may play an important role in human health and disease, as the GI tract has been implicated as an important source of infection in cases of recurrent vaginitis (104) and systemic candidiasis (94,112,149–151,164). The overgrowth of *C. albicans* in the intestinal tract and its subsequent passage through the gut mucosa into the host bloodstream, for instance, is believed to be the proximate mechanism leading to systemic candidiasis (151), particularly in patients with acute leukemia (103,112,129). Similarly, colonization of the GI tract by *C. albicans* and other *Candida* spp. may lead to involvement in a number of other disease syndromes that include esophageal, gastric and intestinal thrush, gastric and intestinal ulceration, GI bleeding, diarrhea, peritonitis, perianal itch, napkin dermatitis, chronic "irritable bowel" syndrome, and auto-brewery syndrome (12,59,60,80,81,94,102,106,112,117,118,130,131,149–151,156, 164,165,171). The importance of GI colonization by *C. albicans* therefore should not be underestimated, especially in view of the suggestion that "the most important source of *Candida* species in human disease is endogenous" (118). On the other hand, GI colonization by *C. albicans* may also have important beneficial effects on the host in that it apparently results in stimulation of the immune system, leading to a protective response to systemic *Candida* infection in the noncompromised host (31,32). Furthermore, preliminary evidence from our laboratory suggests that colonization of the GI tract by *C. albicans* may lead to resistance to subsequent colonization by the other opportunistic fungal pathogens.

Because of the importance of *Candida* gut colonization to human health, then, there can be no reasonable doubt that it would be of great benefit to clinical medicine if colonization of the intestinal tract by *C. albicans* could be regulated in such a way that the beneficial effects are retained and the detrimental effects eliminated. This situation will be possible, however, only when the ecological mechanisms that control *C. albicans* in the gut are more thoroughly understood. To that end, much remains to be done be-

fore the determinants of GI colonization by *C. albicans* are completely defined. For example, it is well known that broad-spectrum antimicrobic treatment predisposes patients to GI mucosal infections by *C. albicans* (118), but it is not clear as to the specific members of the indigenous microflora that are responsible for the suppression of *C. albicans* in the gut (90). Moreover studies suggest that the suppression of *C. albicans* within the gut is likely to be the result of several interactions regulated by a complex indigenous microflora (85,86,89) and not to a single interaction by one or a few bacterial species, as has previously been suggested (73,74,76,114). The major reason the intensive research by a number of competent investigators has not brought us closer to understanding what controls *Candida* in the gut is probably the lack of coordination of individual aspects of the larger subject (133). Generally, isolated components of intestinal colonization, e.g., mucosal association, are enthusiastically and effectively studied; but too often the kinship to other "colonization" or "virulence" factors (146), which in many instances may be of equal or greater importance, is ignored.

This chapter presents an overview of the factors influencing intestinal colonization by *C. albicans* and emphasizes interactions between *Candida* and the indigenous intestinal microflora. It also endeavors to correlate these and other observations into a coherent hypothesis that is intended to serve as an initial general framework to explain the regulation of *Candida* populations in the GI tract. First, however, is a brief overview of the microbial ecology of the GI tract.

Microbial Ecology of Gastrointestinal Ecosystems

Because the succession, development, and composition of the microbial flora of the GI tract has been the subject of several excellent reviews (34,43,48–50,108,109,134,137), there is not a complete overview of these topics here. However, before discussing the factors or bacteria, that regulate *C. albicans* populations in the gut, it is necessary to list the major features of the GI microflora, which should allow the reader to become familiar with the complexity of the GI microflora, the diversity of the microbial species found in various niches of the GI tract, and factors that might influence the composition of the microflora. By beginning with this subject, it is hoped that the medical mycologist will gain an appreciation for some of the stringent details that are involved with and can influence the study of the survival, dissemination, and pathogenesis of *C. albicans* and other fungi in the GI tract. The following comments and several related papers (33,34,43,48–51,54,77,95,96,108–110,124,134,137,142,143, 145,147,154,172,173) should be helpful to those investigators interested in this area of study. The following paragraphs were modeled after an

excellent review by Freter (50) and were supplemented to include certain generalizations.

1. It is generally believed that the intraluminal contents of the normal human stomach is relatively sterile, with only low numbers ($\leq 10^3$ colonies/ml gastric contents) of a variety of organisms being present (43,45,58). Studies indicate that these low counts may well represent oral and ingested microorganisms, as counts tend to decrease to undetectable levels several hours after eating (35,45), which is most likely due to changes in gastric pH (58). Despite the apparent harshness of the stomach to colonization by microorganisms, some investigators have noted that certain microorganisms can be seen attached to the epithelium and deep in foveae in histological preparations viewed by light or electron microscopy (135). A similar description of the stomach flora of laboratory rodents has also been detailed, but the microbial species associated with the gastric mucosa are different (134,136). In fact, it should be noted that the indigenous gut microflora, for any portion of the GI tract, probably differs from host to host, even within the same species (110). Studies have shown, however, that α-hemolytic streptococci, anaerobic cocci, lactobacilli, *Staphylococcus epidermidis*, and *C. albicans* comprise most of the microorganisms that colonize the stomach (45,58). The microflora of the small intestine in the normal individual or laboratory animal, in contrast, contains numerous bacteria, but the populations are highly dependent on the location of sampling. Generally, the microbial counts range from being sterile (or very low) to very high as samples are taken beginning in the duodenum and descending to the ileum (43). Several studies have revealed that in the upper small intestine of normal healthy human subjects, low counts (0–$10^{4.5}$ colonies/ml) of both aerobic (streptococci, staphylococci, lactobacilli, yeasts) and anaerobic (streptococci, lactobacilli) microorganisms and almost a complete absence of coliforms and *Bacteroides* spp. (43). As samples are taken farther along the small intestine, at the distal ileum, mean bacterial counts increase significantly ($10^{3.5}$–$10^{6.5}$ colonies/ml), and the microflora more closely resemble colonic flora with higher counts of coliforms and *Bacteroides* spp. (43). The large intestinal flora of the healthy host consists of an average of at least 10^{11} colonies/g, containing some 400–500 (mostly strictly oxygen-sensitive anaerobes) bacterial species from more than 45 genera (34,72,108,137). Finegold et al. (43), in their review of the "normal" indigenous microflora, have analyzed and summarized more than 10 years of in-depth studies characterizing the colonic microflora from several hundred individuals with various disease states and who were on various diets. These authors noted that there was a remarkable similarity among the major bacterial groups in general despite major differences in diet, disease state, and nationality. Table 11-1 summarizes the mean bacterial counts and percentages of specimens positive for the major bacterial groups present in the fecal flora as described in these studies. Several

TABLE 11-1. Major bacterial groups present in human fecal flora.*

Bacterial group	Percent of total specimens positive	Log_{10} No. of organisms/g (dry wt)	
		Mean	Range
Actinomyces	8	9.2	3.8–11.1
Anaerobic cocci	94	10.5	4.1–13.4
Arachnia-Propionobacterium	9	9.0	4.3–12.0
Bacteroides	99	11.3	9.5–13.8
Bifidobacterium	74	10.2	5.0–13.6
Clostridium	100	9.8	3.8–13.1
Eubacterium	94	10.7	5.1–13.5
Fusobacterium	18	8.5	5.1–11.0
Gram-negative facultative anaerobes	98	8.5	4.0–11.0
Other facultative anaerobes	93	6.5	1.0–11.2
Lactobacillus	78	9.5	3.6–12.3
Streptococcus	99	8.9	4.0–12.8

* Adapted from Finegold et al. (42).

factors that may alter the composition or metabolic activity of the microbial flora of the gut include stress, diet, prolonged antibiotic and immunosuppressive therapy, surgery, the individual's genetic background, and a number of infections and debilitating diseases. All of these factors have been commented on elsewhere (22,35,48,57,60,65,108–110,137), but several may also predispose to colonization and infection with *Candida* (118).

2. The indigenous microflora of a given location within the GI tract, including various microsites within that location, has a stable composition because the GI tract is an ecosystem in the climax stage (172,173). Thus "invading" microorganisms are usually prevented from colonizing the gut unless of course they enter this site before the indigenous microflora has become established or after the microflora has somehow been disrupted (86). This phenomenon has been described using several terms, e.g., "bacterial antagonism" (47), "bacterial interference" (36), and "colonization resistance" (161), and constitutes a potent host defense mechanism (50). However, as noted by Freter (50), although the gut microflora is stable in its function, the stability of individual bacterial species is not absolute. Consistent with this statement is the finding that the composition of the colonic microflora varies considerably in different hosts (108–110). Furthermore, it has been noted that, owing to unknown factors, the population size of some indigenous bacterial species apparently may change from day to day within the same individual (108–110). One relevant example, as noted by Freter (50), is that some individuals harbor no recognizable "resident" strains of *Escherichia coli* but only a frequent succession of different "invader" or "transient" *E. coli* strains

(3,22,57,142,143,161), and there are occasional individuals who allow "invader" strains to transiently coexist with "resident" strains (3,22,57,142,143,161). A similar situation may exist for *C. albicans* (1), but no definitive studies have been conducted to follow up this isolated observation. Nevertheless, one major feature of the gut microflora is its ability to resist implantation by allochtonous microbial species.

3. There are two general habitats of colonization in GI ecosystems, i.e., the luminal contents and the mucosa (50,137). This pattern has been observed in several animal species and in man, and it has been found that both of these sites may contain common or unique microbial species (46,50,134,135). Furthermore, within the mucosa there may be two micro-sites, which include those cells directly attached to the epithelium and cells entrapped in what is usually described as mucus (46,50,91). Studies indicate further that these micro-sites are closely interrelated in that a primary layer of bacteria "attach" to the epithelium, and that several subsequent "layers" adhere to the primary one and to each other (50). As is noted below, for an area of the GI tract to function properly, at least in terms of resisting colonization to *Candida* and other invaders, the mucosa-associated flora must remain intact.

Survival and Implantation of *Candida* in the GI Tract

The clinical recognition of candidiasis of the GI tract has been traced by Odds (118) to the late 1700s, which was long before *C. albicans* was discovered (118,131). Nearly a century elapsed from this date until *C. albicans* was first isolated from a patient with candidiasis of the stomach and colon (118,131). The modern revival of this subject, however, had to wait nearly another century and can probably be credited, at least in part, to Krause and co-workers (94), who demonstrated the rapid "persorption" of *C. albicans* through the GI mucosa into the bloodstream when Krause drank a saline suspension containing about 10^{10} viable *C. albicans*. This report was followed by a number of studies that examined the role of the "normal" intestinal flora in inhibition of *C. albicans* in experimental animals (8,9,24, 67,75,76,121,122) and to several clinical studies of the occurrence of *Candida* gut colonization in various patient populations (29,149–151). Widespread acceptance of this idea, however, had to wait a few more years until Myerowitz et al. (112) demonstrated a correlation between GI colonization by *C. albicans* and systemic infection in leukemic patients and to Miles et al. (104) who showed a similar relation to recurrent vaginitis. The latter findings, especially when considered with more recent studies (7,40,41,68–70,88–90,98,127,158–160,169,170), has brought prominence to the idea that the GI tract, colonized by *C. albicans*, may serve as an important reservoir for both superficial and systemic candidiasis (118). Other data obtained from experimental animals reinforces this view and

TABLE 11-2. Fungal species found to spread systemically from the GI tract of animals and man.

Fungi	Ref.
Basidiobolus ranarum	23
Candida albicans	7,26,40,41,68,71,88–92,94,111,112,125–127,149–151,158–160,167,169,170
Candida lusitaniae	102
Candida parapsilosis	88
Candida kefyr	88
Candida tropicalis	88,164,169,170
Coccidioides immitis	
Cryptococcus neoformans	61,144
Histoplasma capsulatum	144
Metarrhizium anisopliae	30
Saccharomyces cerevisiae	44
Sporothrix schenckii	84,144
Torulopsis glabrata	88,167
Trichosporon spp.	66

has shown that a number of fungi can spread systemically from the GI tract (Table 11-2).

Odds (118) in his scholarly and promethean review of *Candida* and candidiasis, examined in detail clinical and pathological features of GI candidiasis and painstakingly summarized the incidence of GI carriage in "normal" and "patient" populations. Therefore these topics are not considered here. Nevertheless, two significant facts that are evident from this work should be noted before beginning a discussion on the survival and implantation of *Candida* in the GI tract. (a) Not all healthy individuals, regardless of their age, sex, or the number of attempts (or methods) used to isolate "yeasts" from the GI tract, harbor a "resident" yeast flora. According to Odds (118), about 40–50% of a given sample population can be shown to carry *Candida* throughout most of the length of the GI tract. The incidence of *Candida* carriage, furthermore, has been shown to be highest in the oral cavity and lowest in the colon (118). Because *C. albicans* can pass through the GI tract apparently without being "killed" (85,86), the percentage of healthy individuals harboring *C. albicans* in their gut is probably lower, as some positive fecal cultures may represent *Candida* removed from the oral cavity. Thus common statements such as "All of us acquire *Candida* at birth" or "All of us carry *Candida* as a part of our normal flora" are unfounded. (b) The incidence and population size of *C. albicans* in the gut nonetheless usually increases in individuals who are predisposed by illness, debility, or a local reduction in host defense mechanisms (118,131). Table 11-3 summarizes factors thought to remove, or at least lower, the "barrier" that inhibits the overgrowth of *C. albicans* in the GI tract. Table 11-4 summarizes some data on the carriage of *Candida* spp. in the gut as related to diet.

TABLE 11-3. General classification of factors thought to predispose to colonization and dissemination from the GI tract by *Candida* spp.*

Classification of predisposing factors	Explanation	Examples
Natural factors	Infectious, idiopathic, congenital, or other debilitating diseases and disorders	Microbial infections, endocrine dysfunctions, defects in immunity
	Digressions form normal hormonal or physiological status	Pregnancy, infancy
Dietary factors	Excess or deficiency of foodstuffs that may alter the composition of the indigenous microbial flora or the metabolic activity of the flora	Carbohydrate-rich diets, vitamin deficiencies
	Intake of foodstuffs that may alter local immunity	
Mechanical factors	Trauma	Stabbing
	Prolonged stay in a closed environment	Space flight
Iatrogenic medical factors	Treatment with drugs that alter the composition of the indigenous microbial flora or suppress host defenses	Antimicrobics, corticosteroids
	Surgical procedures	Bowel resections

* Adapted and modified from Odds (118).

It is apparent from these brief comments that an overgrowth of *C. albicans* in the GI tract and its subsequent pathological consequences could arise in a compromised individual either because of already harboring a resident *C. albicans* population or by acquiring a "hospital strain" because the barrier to implantation was reduced or eliminated altogether. There is good evidence for both conditions (1,21,25,86,127,146), but it is not the intent of this review to suggest that one event is more relevant than the other. Nevertheless, both positions underscore that *C. albicans* can become implanted in the gut either before the normal microflora has become established (e.g., during infancy) or after the ecological balance of the microflora has been disrupted (e.g., by antimicrobic treatment). The survival and implantation of *Candida* in the gut is therefore considered from both views. Unfortunately, there is no study of which this writer is aware that has followed a large group of human subjects from birth to adulthood for the specific purpose of monitering yeast flora population dynamics or the acquisition of new yeast strains. Therefore the comments below were derived almost entirely from data obtained from experimental animals.

A number of animal models of GI colonization and dissemination by

TABLE 11-4. Isolation of *C. albicans* and other yeasts in stool samples from healthy individuals on different diets.*

Diet	No. of subjects	Organism	Percent of total samples positive	Log_{10} No. of organisms/g	
				Mean	Range
Japanese	15	*C. albicans*	47	5.6	3.5–8.9
		Candida spp.	13	6.3	3.7–8.8
		Other yeasts	53	5.8	3.4–8.7
Strict vegetarian	13	*C. albicans*	15	4.9	3.5–6.3
		Candida spp.	0	—	—
		Other yeasts	23	5.6	4.3–7.8
Vegetarian, some meat	14	*C. albicans*	0	—	—
		Candida spp.	0	—	—
		Other yeasts	50	6.1	4.2–8.7
Western	62	*C. albicans*	18	5.4	3.6–9.4
		Candida spp.	8	4.4	3.7–5.1
		Other yeasts	31	5.2	3.6–8.1

*Data from Finegold et al. (43).

C. albicans have been developed and used for studies of antifungal chemotherapy, colonization, mucosal association and invasion, and pathogenesis (Table 11-5). These models have included several animal strains and species, and have included the use of both adult and neonatal conventional, specific pathogen-free, germfree, gnotobiotic, and athymic germfree or gnotobiotic animals (2,4,6–9,11,19,24,26,28,31,32,39–41,62, 64, 67–71, 73–76, 78, 88–92, 98, 99, 111, 113–116, 119, 122, 125–128, 155, 157–160,163,167,169–171). For obvious reasons, laboratory rodents, particularly mice, have been the most extensively used models described to date (63). Because Guentzel et al. (63) have described in some detail most of the currently available animal models for studying GI colonization and dissemination by *C. albicans*, no attempt is made here to describe any of these models in any particular detail. Certain salient features unique to a particular model that may aid in the identification and elucidation of the determinants of gut colonization or invasion of the intestinal mucosa by *C. albicans*, however, are noted where appropriate. The following discussion focuses on the ability of *C. albicans* to survive and implant in the gut and examines the effects various pre- or post-treatments have on colonization and systemic spread of *Candida* from the GI tract.

Several studies have been conducted that make it impossible in the space given here to completely describe and compare the numerous data that have been generated on gut colonization by *Candida*. Therefore it became apparent at this writing that the only way to make such a comparison, especially when considering the wide variation in experimental parameters

TABLE 11-5. Animal models for studying the survival, implantation, and dissemination of *C. albicans* from the GI tract.

Animal	Type of pretreatment or state of animal	Strain	Age*	Ref.
Chicken	None	K-137 Kimber strain	A,I	8,9
	Germfree state	K-137 Kimber strain	A,I	8,9
Dog, primates	None	Beagle, other?	A	149,151
	Antimicrobic treatment	Beagle, other?	A	149,151
Guinea pig	None	"Albino"	A	155
	Antimicrobic treatment	"Albino"	A	155
Hamster	None	Syrian	A	89
	Antimicrobic treatment	Syrian	A	89
Mouse	None	Balb/c, CBA/J, CD-1, CF-1,	A,I	2,4–7,10,11,24,26,31,32,39–41,
	Antimicrobic treatment	CF1 W74, CFW, C3H, C57BL/6N,	A,I	64,67–71,73–75,78,88–92,
	Antimicrobic + corticosteroids	DBA/2J, DDI, HAM/ICR,	A,I	98,99,113,114,116,119,122
	Antimicrobic + Athymic state	ICR-germfree, ICR-SPF, Lobound ND1 & ND4, MF-1, RAP,	A,I	125–128,133,157–160,167, 169,170
	Germfree state	"Swiss," Swiss Flow DUB/kr	A,I	
Rabbit		?	A	115
Rat		4CD, Sprague-Dawley, Wistar	A	19,28,62,111

*A = adult; I = infant.

that has been used between laboratories, was to construct a table and list nearly all the available published data on the survival, implantation, and dissemination of *Candida* spp. from the gut. Table 11-6 is the result of this effort, with only the essential and pertinent information being listed. It was prepared so that studies could be compared directly. For a more detailed discussion of any particular model or facet of GI colonization by *C. albicans*, the interested reader is referred to the papers cited.

These data do permit several generalizations to be made regarding colonization and dissemination of *C. albicans* and other *Candida* spp. from the GI tract. The foremost generalization evident from the data is that the indigenous microflora represents one of the most important (if not the most important) host defense mechanisms suppressing the colonization of *Candida* spp. in the GI tract. It is clearly demonstrated from several studies that showed conventional animals to be significantly more resistant to GI colonization than were germfree and antimicrobic-treated animals (2,7, 40,64,70,75,88–92,158–160,169,170). Another observation consistent with this view is that neonatal animals, which lack a complete indigenous microflora, are also readily colonized by *C. albicans* (26,31,32,41,64,68,69,71, 92,125–127). The few apparent contradictory results reported in the literature with regard to the effects of the normal intestinal microflora on GI colonization by *Candida* that are listed in Table 11-6 may be due to the animals selected for study or to other methodological pitfalls that accompany the study of interactions between microorganisms in the GI tract. For instance, the early findings of Balish and Phillips that *C. albicans* was established in "large numbers" in the gut of both germfree and conventional mice (8,122) may be explained by the finding that many strains of mice are unsuitable for research on intestinal floral interactions (53,56,90). Such mice are characterized, as are most commercially available strains of mice, by a lack of strictly oxygen-sensitive anaerobes and therefore lack a true indigenous microflora (53,56,123). Using a different strain of mice, Balish and co-workers (70,98,99) later reported that conventional animals were significantly more resistant to GI colonization by *C. albicans* than were antimicrobic-treated and germfree mice.

The data presented in Table 11-6 suggest further that the indigenous microflora is also an important defense mechanism, inhibiting the systemic spread of *Candida* from the lumen of the gut to other organs. Animals treated with certain antimicrobics, for instance, showed that large populations of *C. albicans* colonized their GI tracts, with a corresponding high incidence of dissemination to visceral organs (88–90). Animals that posed a complex indigenous microflora, in contrast, had low numbes of *Candida* organisms residing in their GI tracts, with a low percentage or none of the animals showing signs of *Candida* dissemination. The finding that not all antibiotics predisposed mice to GI colonization or dissemination may suggest that only certain components of the microflora are primarily responsible for the suppression of *Candida* in the gut. This subject is discussed in more detail below.

TABLE 11-6. Comparison GI colonization data by *Candida* spp. in various animal models (listed in order of publication).

Animal			Animal treatment			Presence of *Candida*					Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.			
C. albicans strain 412: Grown in casamino acids medium at 37°C for 48 hr													
Mouse: CF-1 (Cartworth Farms)	12–14 g	M + F	None	0.2 mg cell nitrogen/ ml (× 1)	Intragastric (IG)	Feces	5	7/87					75
			Chlortetracycline					25/89					
			Chlortetracycline + parabens					13/90					
			Chloromycetin					24/30					
			Oxytetracycline					25/30					
			Dihydrostreptomycin					21/29					
			Magnamycin					12/30					
Mouse: RAP (Rockland Farms)	12–14 g	M + F	None					26/93					
			Chlortetracycline					22/33					
			Chlortetracycline + parabens					49/76					
			Chloromycetin					17/19					
			Oxytetracycline					16/16					
			Dihydrostreptomycin					19/33					
			Magnamycine					24/52					
			Neomycin					10/17					
			Oxytetracycline-AMP					11/20					
			Erythromycin					9/17					
			Penicillin					17/20					
			Tetracycline					23/38					

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*					Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.			
C. albicans strain ATCC 10231: Grown in SDB at 37°C for 24 hr													
Chicken: white leghorn K-137 Marshall Brothers Hatchery	7 days	M + F	None	10^5 (× 1)	Oral, in food	Crop	38		6.4	Y			8
						Duodenum			6.0				
						Jejunum			6.3				
						Ileum			6.3				
						Cecum			7.0				
			Germfree	10^5 (× 1)		Crop			8.3	M			
						Duodenum			6.4				
						Jejunum			6.7				
						Ileum			6.8				
						Cecum			7.9				
Chicken: white leghorn K-137			Gnotobiotic (*E. coli*)	10^6 (× 1)	Oral, in food	Crop	30		6.5	M + Y			8
						Duodenum			5.2				
						Jejunum			5.1				
						Ileum			5.0				
						Cecum			5.3				
			Gnotobioticon (*S. faecalis*)			Crop			6.5	Y + M			
						Duodenum			6.0				
						Jejunum			5.2				
						Ileum			5.5				
						Cecum			5.3				
Mouse: "Swiss" (Blue Spruce Farm)	20–25 g	M	Fasting 18 hr and opium alkaloids (germfree)		PO	Feces		70/70				2/70	128
			Fasting 18 hr and opium alkaloids (germfree + *S. typhimurium*)					?				47/66	

C. albicans strain ATCC 10231: Grown in SDB at 37°C for 18 hr											
Mouse: ND 1 (Lobund, Lab.)	40 g	M + F	None (conventional)	10^6 (× 1)	Oral	Stomach	21			Y, H	122
						Feces	7			Y, H	
			None (germfree)			Stomach	21			Y	
						Feces	7			Y	
C. albicans strain No. 4: Grown on SDA at 37°C for 24 hr											
Mouse: ICR-SPF (Japan CLEA Co) ICR-germfree	6 wk	M + F ?	None	10 (× 1)	IG	Feces	5	2/3	<2.0	Y	114
				<10 CFU (× 1)		Feces	10–20	3/3	7.7–8.1	Y	
						Stomach				Y, PH	
						Cecum				Y, PH	
			Inoc. *E. coli* prior to *Candida*	2×10^7 (× 1)		Feces	5	4/4	4.2		
							15	3/4	2.3		
Mouse ICR-germfree	6 wk	M + F ?	None	2×10^7 (× 1)	IG	Stomach	11	3/3	6.8		114
						Duodenum		3/3	6.5		
						Ileocecum		3/3	6.2		
						Rectum		3/3	6.7		
			Inoc. *E. coli* prior to *Candida*	2×10^7 (× 2)		Stomach	19	0/4	<2		
						Duodenum		0/4	<2		
						Ileocecum		1/4	2.8		
						Rectum		1/4	2.5		
			Inoc. *Candida* prior to *E. coli*	1×10^3 (× 1)		Stomach	26	4/4	4.9		
						Duodenum		4/4	4.2		
						Ileocecum		4/4	4.2		
						Rectum		4/4	4.4		
C. albicans strain No. 374: Grown in 1% casamino acids 1% yeast extract 2% glucose broth at 37°C for 24 hr											
Mouse: Ham/ICR Swiss germfree (Charles River)	20–25 g	M	None	1×10^6 (× 1)	IG	Feces	Up to 156	100%			24
			Oxytetracycline				Up to 156	100%			
Ham/ICR Swiss conventional			None				4	5/6			
							12	2/6			
							32	0/6			
			Streptomycin				4	10/10			
							12	10/10			
							32	2/10			

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*							
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.	Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
C. albicans strain 11952: Grown in BHI broth at 37°C for 18 hr													
Mouse: ICR-germfree (A.R. Schmidt Co.)			None	10^6/ml	Oral, in drinking water	Feces			8.2				74
			Inoc. *E. coli* 11922 (10^8/ml) prior to *Candida*						6.5				
			Inoc. *E. coli* 11922 (10^8/ml) after *Candida*						6.2				
			Inoc. *E. coli* 11 (10^8/ml) prior to *Candida*						7.9				
			Inoc. *E. coli* 11 (10^8/ml) after *Candida*						8.0				
			Fed *E. coli* (11922) dialystate						6.5				
			Given gentamicin (20 mg/kg)						8.2 10.0				
			Burned						8.3				
			Burn + gentamicin						6.5				
			Fed *E. coli* 11922 (10^8/ml) and gentamicin						6.8				
			Fed *E. coli* 11922 and burned						6.9				
			Fed *E. coli* 11922, burned, and gentamicin						7.6				
			Fed *E. coli* 11 (10^8/ml) and gentamicin						6.5				
			Fed *E. coli* 11 and burned										
			Fed *E. coli* 11, burned, and given gentamicin						7.9				

				C. albicans strain 6713						
Mouse: CF-1/H conventional (Takeda Chem. Ind. Ltd)	18 g	M + F	None	10^8 (× 1)	IG	Feces	24 hr	3.7–6.7		78
				10^3 (× 1)				7–8		
				10^5 (× 1)				7–8		
				10^6 (× 1)				7–8		
				10^6 (× 1)		Stomach	24 hr?	6.8	Y & gt	0/1
						Sm. int.		6.8	Y	
						Cecum		7.3	Y	
						Colon		6.8	Y	
						Feces		7.3		
			Cortisone			Stomach	24 hr?	7.6	Y, gt & hy	1/1
						Sm. int.		7.6		
						Cecum		7.5		
						Colon		7.5		
						Feces		7.7		
			Amphotericin B			Stomach	24 hr?	2.2	Y	0/1
						Sm. int.		0	—	
						Cecum		0	—	
						Colon		0	Y	
						Feces		0		
				C. albicans, "patient isolate": Grown in SDB + NP at 37°C for 18 hr						
Mouse: CD-1 (Charles River)	25–30 g	M	None	~2 × 10^5 (× 1)	IG	Stomach		5.7		6
						Sm. int.		5.1		
						Cecum		5.9		
						Colon		6.0		
			Ampicillin			Stomach		5.7		
						Sm. int.		5.2		
						Cecum		5.5		
						Colon		5.8		
			Cortisone			Stomach		5.8		
						Sm. int.		5.6		
						Cecum		6.1		
						Colon		6.3		
			Estrogen		Oral-IG	Stomach		4.1		
						Sm. int.		4.1		
						Cecum		4.8		
						Colon		5.4		
		F	None			Stomach		4.6		
						Sm. int.		4.4		

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*					Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.			
						Cecum			5.1				
						Colon			5.5				
			Ampicillin			Stomach			5.4				
						Sm. int.			5.1				
						Cecum			6.0				
						Colon			6.2				
			Cortisone			Stomach			5.0				
						Sm. int.			4.7				
						Cecum			5.3				
						Colon			5.8				
			Estrogen			Stomach			3.8				
						Sm. int.			4.4				
						Cecum			4.9				
						Colon			5.6				
		PF	None			Stomach			5.3				
						Sm. int.			4.9				
						Cecum			5.7				
						Colon			5.8				
			Ampicillin			Stomach			5.6				
						Sm. int.			5.2				
						Cecum			5.8				
						Colon			6.1				
			Cortisone			Stomach			5.6				
						Sm. int.			5.3				
						Cecum			5.8				
						Colon			6.1				
			Estradiol			Stomach			4.0				
						Sm. int.			4.3				
						Cecum			4.9				
						Colon			5.5				
		F	None			Stomach			4.4				
						Sm. int.			4.6				
						Cecum			5.1				
						Colon			5.3				

C. albicans strain B311: Grown in LBC at 25°C for 18 hr

Rat: Holtzman (Charles River)	60 days	F	Cortisone + chloramphemicol + gentamicin	3×10^3 (× 1)	IG	Feces	4		6.3	28
							10		6.2	
							20		5.5	
							30		5.4	
			Cortisone only				4		5.4	
							10		3.8	
							20		4.3	
							26		4.8	
			Antibiotics only				4		4.9	
							10		4.3	
							20		4.2	
							26		5.1	

C. albicans strain A26: Grown in SDB at 37°C for 24 hr

Mouse: NG	Adult?	NG	None	~10^7 (× 1)	Oral gavage		3		4.7	157
							12		4.5	
			X-irradiation (400 R)				3		4.9	
							12		5.9	
			None			Feces	3		5.2	
							10		5.0	
			X-irradiation (400 R)				3		5.2	
							10		5.0	
			X-irradiation (400 R) + vancomycin				3		4.7	
							10		6.5	
			X-irradiation (400 R) + kanamycin				3		5.0	
							10		5.3	
			X-irradiation (400 R) + vancomycin + kanamycin				3		7.5	
							10		6.9	
			X-irradiation (400 R) + gentamicin				3		6.9	
							10		5.9	
			X-irradiation (400 R) + tobramycin				3		5.8	
							10		5.9	
			X-irradiation			Colon	3	10/10	5.6	
							5	9/10	5.1	
							7	8/10	4.9	
							9	9/10	4.7	
							12	7/10	5.5	

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*							
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.	Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
Mouse			X-irradiation (400 R) + tobramycin	~10^7 (× 1)	Oral gavage		12	4/4?	4.6	Y & M			157
						Stomach			1.0				
						Duodenum			3.6				
						Jejunum			3.7				
						Ileum			4.6				
						Cecum			4.1				
						Colon			4.4				
						Rectum							
				C. albicans: Grown on SDA?									
Mouse: CF1/W74	20–24 g		None	1×10^8 (× 1)	IG	Feces	3						157
							7						
							14						
							21						
							28						
Mouse: CF1/W74	20–24 g		None	1×10^8 (× 1)	IG	Feces	3		5.4				167
							7		4.9				
							14		3.9				
							21		3.6				
							28		2.0				
			Neomycin				3		6.0				
							7		5.6				
							14		4.6				
							21		4.7				
							28		4.5				
			Neomycin	1×10^6 (× 1)			3		4.7				
							7		4.5				
							14		3.8				
							21		3.5				
							28		3.7				

			Neomycin + estradiol	1×10^8 (× 1)			3	6.3				
							7	4.8				
							14	4.5				
							21	4.5				
							28	4.7				
C. albicans strain B311: Grown in SDB at 25°C for 4 days													
Mouse: BALB/c (Charles River)	60–90 days	M + F	None	10^6/ml (18 hr)	Oral	Cecum	2	~3.9	Y			70
							4	~4.3				
							16	<1.0				
			Tetracycline (1 mg/ml in drinking water				2	~5.7				
							4	~5.1				
Mouse: BALB/c germfree							16	~4.1				
							2	~7.0				
							4	~7.1				
							16	~7.0				
			Tetracycline (1 mg/ml in drinking water)				2	~7.7				
							4	~7.4				
Mouse: BALB/c athymic flora defined							16	~7.5				
							5	~4.9				
							10	~5.0				
							16	~4.9				
							5	~5.9				
							10	~6.0				
							16	~5.8				
C. albicans strain NS-33: Grown on SDA at 37°C for 24 hr													
Mouse: CFW	5–6 days	M + F	Fasted 2–4 hr	5×10^8 (× 1)	IG	Stomach	6 hr	8.1				127
							24 hr	~5.8		8/9	15/60	
							72 hr	~6.1		9/13	25/60	
							20 days	~5.1				
						Ileum	6 hr	~9.5	Y			
							24 hr	~6.2				
							72 hr	~6.0				
							20 days	~5.1				
						Cecum	6 hr	~9.3				
							24 hr	~5.9				
							72 hr	~5.1				
							20 days	~5.0				
						Colon	6 hr	~9.8				
							24 hr	~8.2				
							72 hr	~6.1				
							20 days	~4.3				

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*							
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.	Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
C. albicans: inoculum not specified													
Mouse: DDI	6 wk	F	Aminobenzylpenicillin (30 mg/mouse) & x-irradiation (600 rad)	5×10^7 (× 1)	Oral	Upper int.	2		5.8				160
							4		5.2				
							6		5.6				
						Lower int.	2		6.2				
							4		6.3				
							6		6.4				
			Aminobenzylpenicillin (30 mg/mouse) & dexamethasone (200 μg/mouse)			Upper int.	2		5.9				
							4		5.5				
							6		5.9				
						Lower int.	2		6.4				
							4		6.4				
							6		6.6				
			Aminobenzylpenicillin (30 mg/mouse), x-irradiation (600 rad) & dexamethasone (200 μg/mouse)			Upper int.	2		6.3				
							4		6.5				
							6		7.1				
						Lower int.	2		6.5				
							4		6.7				
							6		6.8				
C. albicans strains JHH-1995 and JH-U990: Grown in SDB at 37°C for 48 hr													
Strain JHH-1995													
Mouse: DBA/25 (Jackson, Lab)	6 weeks	F	None	1.3×10^7 (× 1)	PO						0/5		171
			Poly B	1.3×10^7 (× 1)	PO						0/10		
Stain JHH-U990													
Mouse: DBA/25 (Jackson, Lab)			Polymyxon B	3.1×10^6 (× 1)	PO						0/15		
			Gentamicin	4.8×10^7 (× 1)							1/8		
			Cytarabine	1.2×10^7 (× 1)							6/11		

C. tropicalis strain JHH-A034: Grown in SDB at 37°C for 48 hr										
Mouse: DBA/25 (Jackson, Lab)	6 wk	F	None	1.2×10^7 (× 1)	PO				0/5	171
			Polymyxon B + Gentamicin	1.2×10^7 (× 1)					0/9	
C. albicans (various strains): Grown on SDA at 37°C for 24 hr										
Strain B311A										
Mouse: CFW (Charles River)	5–6 days	M + F	None	9.4×10^7 (× 1)	IG		30 min		8/15	41
							180 min		6/9	
Strain CA 30										
Mouse: CFW				8.2×10^7 (× 1)			30 min		11/15	
							180 min		1/5	
Strain NS33										
Mouse: CFW				8.5×10^7 (× 1)			30 min		20/25	
							180 min		7/10	
Strain UT1015										
Mouse: CFW				1.8×10^8 (× 1)			30 min		4/10	
							180 min		5/10	
Strain NS33										
Mouse: CFW				8.8×10^6 (× 1)		Stomach	1	~4.5		
							3	~4.5		
							7	~4.3		
							14	~4.4		
							21	~4.8		
							28	<1.0		
						Sm. int.	1	~2.4		
							3	~2.0		
							7	~2.1		
							14	~3.3		
							21	~3.4		
							28	<1.0		
						Cecum	1	~2.5		
							3	~2.3		
							7	~2.5		
							14	~3.7		
							21	~3.5		
							28	<1.0		
						Colon	1	~6.0		
							3	~3.4		
							7	~3.5		
							14	~4.3		
							21	~3.3		
							28	<1.0		

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*							
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.	Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
C. albicans "patient isolate": Grown in TSB overnight													
Rat: SPF (Sprague Dawley)	4–6 wk/ 175–200 g	F	None	2×10^5/ml in drinking water (5 days)	Oral	Stomach	24 hr		2.3				111
						Cecum			2.7				
			Carbenicillin (400 mg/kg/day) & gentamicin (2 mg/kg/day) for 3 days			Stomach			4.6				
						Cecum			4.4				
			Cyclophosphamide (100 mg/kg, then 75 mg/kg 4 days later)			Stomach			3.6				
						Cecum			3.3				
			Antibiotics & cyclophosphamide (as above)			Stomach			4.7		3/30		
						Cecum			5.5				
			Antibiotics & cyclophosphamide (as above) & cortisone acetate (25 mg/kg day) for 3 days)								9/34		
C. albicans strains CA30 and NS33: Grown on SDA at 37°C for 24 hr													
Strain CA30													
Mouse: CFW (Charles River)	5–8 days	M + F	Fasted 2–4 hr	$1.2–3.8 \times 10^6$ (× 1)	IG	Stomach	1 wk		4.3				126
							2 wk		4.6				
							3 wk		5.0				
						Sm. int.	1 wk		3.4				
							2 wk		3.6				
							3 wk		3.8				
						Cecum	1 wk		4.9				
							2 wk		4.8				
							3 wk		4.5				
						Colon	1 wk		4.6				
							2 wk		4.4				
							3 wk		4.2				

Strain NS33 Mouse: CFW	↓	↓	↓	9×10^6 (× 1)	IG	Stomach	1 wk		5.0		
							2 wk		4.9		
							3 wk		5.4		
						Sm. int.	1 wk		3.6		
							2 wk		2.7		
							3 wk		4.2		
						Cecum	1 wk		4.2		
							2 wk		4.1		
							3 wk		5.0		
						Colon	1 wk		4.8		
							2 wk		4.3		
							3 wk		4.8		
C. albicans strain B311: Grown on SDA at 37°C for 24 hr											
Mouse: BALB/c germfree (Charles River)	40–60 days	M + F	None	1×10^5/ml in drinking water	Oral	Cecum	1		5.7	–	98
							3		7.6	–	
							7		7.3	+	
							12		7.6	+	
							20		6.3	+	
Mouse: BALB/c germfree athymic							1		5.8	–	
							3		7.6	–	
							7		7.3	+	
							12		7.1	–	
							20		6.9	+	
C. albicans strain CA30: Grown on SDA at 37°C for 24 hr											
Mouse: CFW (Charles River)	6 days	M + F	Fasted 6–8 hr	1×10^8 (× 1)	IG	Stomach	20	6/6	5.2	2/6	71
							30	17/17	4.5	1/17	
							60	7/14	2.0	2/14	
						Int.	20	6/6	4.8		
							30	17/17	4.0		
							60	8/14	2.2		
C. albicans strain 20A: Grown in TSBD at 37°C for 18 hr											
Mouse: CD-1 (Charles River)	1–5 days	M + F	None	2×10^8/ml (5 days)	Oral	Stomach	1 wk	3/3	3.8 ± 0.2	2/3	None 69
							3 wk	3/3	4.7 ± 0.1	3/3	
							4 wk	15/15	4.9 ± 0.1	4/15	
							6 wk	25/25	3.7 ± 0.3	1/25	

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*					Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.			
C. albicans strain CA30: Grown on SDA at 37°C for 24 hr													
Mouse: CFW (Charles River)	6 days	M + F	Fasted 6–8 hr	1×10^8 (× 1)	IG	Stomach	45	2/6	~3.8		1/6	~50%	64
						Int.	45	2/6	~5.1				
			Fasted 6–8 hr, then chloramphenicol (0.75 mg/day) for 30 days starting 15 days after inoc.			Stomach	45	6/6	~6.8		1/6		
						Int.	45	6/6	~6.8				
			Fasted 6–8 hr			Stomach	31	11/11	~5.2		0/11		
						Int.	31	11/11	~4.7				
			Fasted 6–8 hr then cortisone acetate (1.25 mg twice weekly, for 2 wk starting 10 days after inoc.			Stomach	31	10/10	~5.9		1/10		
						Int.	31	10/10	~5.5				
			Fasted 6–8 hr			Stomach	30	4/6	~5.2		0/4		
						Int.	30	4/6	~4.4				
			Fasted 6–8 hr, then given one dose of cyclophosphamide (0.2 mg/g IP) at 15 days after inoc.			Stomach	30	5/6	~5.4		0/5		
						Int.	30	5/6	~5.2				
			Fasted 6–8 hr			Stomach	24	9/11	~5.1		0/9		
						Int.	24	9/11	~4.8				
			Fasted 6–8 hr, then given cyclophosphamide (0.1 mg/g IP) twice weekly for 2 wk 9 days after inoc.			Stomach	24	12/12	~6.4		3/12		
						Int.	24	12/12	~5.6				
			Fasted 6–8 hr			Stomach	21	5/6	~5.0		0/6		
						Int.	21	5/6	~4.0				
			Fasted 6–8 hr, then given methotrexate (5 μg/g) 3 times per wk for 1 wk at 13 days after inoc.			Stomach	21	5/5	~6.6		5/5		
						Int.	21	5/5	~6.6				
			Fasted 6–8 hr			Stomach		6/6	~5.1		0/6		
						Int.		6/6	~4.7				
			Fasted 6–8 hr, then x-irradiation (400 rad) at 9 days after inoc.			Stomach		6/6	~4.9		0/6		
						Int.		6/6	~4.3				

			Fasted 6–8 hr, then x-irradiation (700 rad) at 9 days after inoc.			Stomach		6/6	~6.2		0/6		
						Int.		6/6	~6.1				
			Fasted 6–8 hr			Stomach	18	6/6	~5.0		0/6		
						Int.	18	6/6	~4.8				
			Fasted 6–8 hr, then given cyclohexamide (0.1 mg/g) and cortisone acetate (1.25 mg) 2 times for 1 wk 9 days after inoc.			Stomach	18	6/6	~6.7		5/6		
						Int.	18	6/6	~5.8				

C. albicans various strains and *C. parapsilosis*, *C. pseudotropicalis*, *C. tropicalis* "patient isolate": Grown in SDB at 37°C for 48 hr

Strain CA34 Mouse: C57BL/6N	3 mo	M + F	None	1.2×10^7 (× 1)	IG	Cecum	3 hr	3/3	5.6	Y	0/3	None	88
							12 hr	3/3	4.4		0/3		
							24 hr	3/3	3.1		0/3		
Strain CA2 Mouse: C57BL/6N			VAG: vancomycin (500 μg/ml) + ampicillin (1 mg/ml) + gentamicin (100 μg/ml) for 3 days in drinking water	3.2×10^6 (× 1)		Gl tract (stomach to rectum)	48 hr		7.4		9/10		
Strain CA34 Mouse: C57BL/6N				1.2×10^7 (× 1)					7.4		17/20		
C. parapsilosis pseudotropicalis tropicalis "patient isolate" Mouse: C57BL/6N				1.2×10^7 (× 1)					6.5		9/12		
				2×10^7 (× 1)					7.3		8/10		
				1.2×10^7 (× 1)					7.6		5/10		

C. albicans strain B311: Grown on SDA at 37°C for 24 hr

Mouse: BALB/c germfree (NIH)	0–5 days	M + F	None	1×10^5/ml in drinking water (for 2–4 hr)	Oral	Stomach	3		3.5	Hy	–	7
							10		4.7		+	
						Sm. int.	3		4.2	Y		
							10		6.5			
	6–10 days					Stomach	3		0	Hy	–	
							10		5.8		+	
						Sm. int.	3		3.7	Y		
							10		6.2			
	11–15 days					Stomach	3		5.5	Hy	+	
							10		7.3		+	
							21		4.5		–	
						Sm. int.	3		6.8	Y		
							10		7.5			
							21		5.4			

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*					Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.			
↓	16–21 days	↓		↓	↓	Stomach	3		5.5	Hy	–		
							10		7.9		+		
							21		6.1		+		
						Sm. int.	3		7.0	Y			
							10		7.4				
							21		7.3				
C. albicans strain CA 34: Grown in SDB at 37°C for 48 hr													
Mouse: Swiss Flow DUB/ kr (Eastern Mich. U.)	2 mo	M + F	None	1×10^7 ($\times$ 1)	IG	Cecal contents	24 hr		3.3	Y	0/10		90
						Cecal wall			2.3				
			Erythromycin (200 μg/ml) in drinking water for 3 days			Cecal contents			3.4		0/10		
						Cecal wall							
			Gentamicin (100 μg/ml) in drinking water for 3 days			Cecal contents			3.5		0/10		
						Cecal wall			2.4				
			Vancomycin (500 μg/ml) in drinking water for 3 days			Cecal contents			6.9		5/10		
						Cecal wall			5.1				
			Penicillin G (500 μg/ml) in drinking water for 3 days			Cecal contents			7.4		6/10		
						Cecal wall			5.5				
			Clindamycin (500 μg/ml) in drinking water for 3 days			Cecal contents			7.4		8/10		
						Cecal wall			5.2				

C. albicans strain CA34: Grown in SDB at 37°C for 24 hr												
Hamster: Syrian (Charles River)	100–200 g	M + F	None	1×10^7 (× 1)	IG	Cecum	24 hr		3.4	Y	0/20	None	90
			Vancomycin ampicillin & gentamicin (VAG) in drinking water for 3 days						7.7		13/15		
			VAG, then cecal filtrates						7.6		17/20		
			VAG, then cecal homogenates						3.8		2/20		
			None						6.2		8/15		
			Penicillin G (500 U/ml) in drinking water for 3 days						7.4		6/10		
C. albicans strain 20A: Grown in TSB-D at 37°C for 18 hr												
Mouse (Charles River): CD-1	8–12 wk	F	None	1×10^7 (× 1)	IG	Stomach	0	3/3	5.9		1/3		32
							1	3/3	2.8		1/3		
							6	3/3	3.2		1/3		
							14	0/3	0		0/3		
							21	1/3	0.8		0/3		
							28	2/3	1.7		0/3		
			Tetracycline (1 mg/ml) + penicillin (0.3 mg/ml) in drinking water				0	3/3	6.3		2/3		
							1	3/3	4.3		1/3		
							6	2/3	1.6		1/3		
							14	3/3	4.0		2/3		
							21	3/3	4.8		1/3		
							28	3/3	3.2		1/3		
Mouse (Charles River): CBA/J	8–12 wk	M	None				0	3/3	3.5		0/3		
							2	3/3	2.6		1/3		
							8	3/3	3.5		2/3		
							16	1/3	0.1		0/3		
							0	3/3	4.0		2/3		
							2	1/3	0/8		0/3		
							8	2/3	1.6		1/3		
							16	2/3	1.5		0/3		

TABLE 11-6. *Continued*

Animal			Animal treatment			Presence of *Candida*							
Species & strain (source)	Age/wt.	Sex	Pretreatment	Inoc. dose (frequency)	Route	GI section	Time after inoc. (days)	No. +/ No. tested	Quant. (mn. $\log_{10}$ CFU/g)	Morph.	Incidence of dissemination (No. positive/ No. tested)	Mortality	Ref.
C. albicans "blood culture isolate": Grown in TSB at 37°C for 24 hr													
Mouse: ICR (Charles River)	2–3 mo	M	None	~2 × 10^6 (× 1)	Oral (in drinking water)	Cecum			2.3				40
			Methotrexate + cortisone						2.4				
			Trimethoprin-sulfamethoxazole						2.5				
			Vancomycin						2.6				
			Cortisone						2.7				
			Methotrexate						3.0				
			Gentamicin						3.0				
			Chloramphenicol						3.0				
			Clindamicin						3.0				
			Tetracycline						3.0				
			Cyclophosphamide + cortisone						4.1				
			Cyclophosphamide						4.3				
			Clindamycin + gentamicin						4.7		1/5	1/24	
			Clindamycin + gentamicin + methotrexate						5.3		3/7	2/17	
			Clindamycin + gentamicin + prednisolone						5.5		5/5	0/13	
			Clindamycin + gentamicin + cyclophosphamide						6.5		3/9	8/26	
C. albicans strain CA34: Grown in SDB at 37°C for 24 hr													
Mouse: Swiss-Flow DUB/kr	1–2 mo	M + F	None	~10^7 (× 1)	IG	Cecal wall	72 hr		2.1	Yeast		None	91
						Cecal contents			3.2				
			Penicillin G (500 μg/ml) in drinking water for 3 days			Cecal wall			5.5				
						Cecal contents			7.4				
			Vancomycin (500 μg/ml) in drinking water for 3 days			Cecal wall			5.1				
						Cecal contents			6.9				

C. albicans strain B311: Grown in SDB at 37°C, overnight

Rat: Sprague-Dawley (Sasco)	~200 g	F	Chloramphenicol (50 mg) once then alternating gentamicin (20 mg) and chloramphenicol (25 mg) daily	5×10^6 (× 1)	IG	Feces	0	0	62
							1 wk	3.6	
							2 wk	3.3	
							3 wk	3.8	
							4 wk	3.8	
			As above + cyclophosphamide (100 mg), with injections (75 mg) 4 & 7 days later				0	0	
							2 wk	3.8	
							3 wk	6.2	
							4 wk	6.1	

C. albicans various strain: Grown on SMA overnight

Strain CA37 Mouse: CD-1	5 days	M + F	None	5×10^7–1×10^8 (× 1)	IG	Entire GI tract	8/8	4.2	92
Strain Allen Mouse: CD-1							8/8	4.5	
Strain C316P3 Mouse: CD-1							3/5	3.1	
Strain C316 Mouse: CD-1							3/5	3.3	
Strain CA37 Mouse: CD-1							4/8	~3.5	
Mouse: CD-1			Cortisone (1.25 mg/mouse) 3 times				7/8	~5.1	
Mouse: CD-1			Cyclophosphamide (3 mg/mouse) 3 times				8/8	~5.0	
Mouse: CD-1			Cortisone + cyclophosphamide				8/8	~5.4	

It also appears that an intact immune system may also be involved in the suppression of both colonization and dissemination of *Candida* from the GI tract. It can be seen from the studies of Ekenna and Sherertz (40), who showed that pretreatment with cyclophosphamide or cyclophosphamide plus cortisone acetate predisposed mice to cecal colonization by *C. albicans*. It should be noted, however, that it has also been shown that mice possessing a competitive bacterial flora are more effective than mice with an intact immune system in suppressing colonization with this fungus (70). Nonetheless, it may be that local antifungal immunity may act synergistically with bacterial antagonism in controlling *C. albicans* populations in the intestine. This view is consistent with studies by Shedlofsky and Freter (145), who demonstrated synergism between ecological and immunological control mechanisms regulating bacterial populations in the intestine. Pretreatment of mice with a combination of antibiotics and immunosuppressive agents was found to be more effective than either antibiotics or immunosuppressive agents alone at predisposing the animals to cecal colonization by *C. albicans* (28,40,111). The possibility that the immunosuppressive agents used in these studies somehow modified the indigenous microflora has not been demonstrated.

Interactions with Indigenous Microflora

Colonization of the GI tract by *C. albicans* is a multifactoral process that involves a complex series of separate but interdependent interactions that are influenced by the immunological, physiological, and nutritional status of the host (89). As one example, there are a large number of factors that may modify adhesion and association of *C. albicans* with intestinal mucosal surfaces in vivo (89,90), and there appear to be several distinct mechanisms by which *C. albicans* can associate with the mucosa (91). Nevertheless, although the determinants of GI tract colonization by *C. albicans* are not completely defined, it is known that certain members of the indigenous microflora suppress the growth of *C. albicans* within the gut. Several studies, in fact, have shown that certain intestinal bacterial species are inhibitory to the growth of *C. albicans* in vitro and in gnotobiotic experimental animals, but suppression of *C. albicans* in the normal host is likely to be the result of several interactions regulated by a complex indigenous microflora (85,86). In this section the mechanisms by which the indigenous intestinal microflora regulates *Candida* populations is detailed and evaluated. In addition, with evidence drawn largely from studies with animals, the members of the indigenous microflora that may control *C. albicans* in the gut are discussed.

Mechanisms by which Intestinal Flora Controls *Candida*

As was described above, *C. albicans* and other fungi are able to readily colonize the GI tract of germfree and antimicrobic-treated animals, whereas similar colonization of conventional animals or man is difficult to achieve. This finding, in addition to numerous reports in the literature showing a high correlation between *Candida* infections and antimicrobic therapy (118), has led to the now commonplace assumption that the incidence and severity of alimentary tract colonization by *C. albicans*, as well as pathological consequences that can be associated with this colonization, is somehow controlled by antagonistic interactions exerted by the numerous microbial species that normally inhabit the gut. As mentioned already, several studies support this hypothesis and have shown that certain intestinal bacteria are inhibitory to both in vitro growth and GI colonization by *Candida* and other fungi (5,8,9,24,73,76,114,120,121). Unfortunately, however, most of the studies to date, although supporting this hypothesis, cannot be relied on to reflect interactions as they normally occur in the GI tract. Nevertheless, although there may be little similarity between the inhibitory mechanisms observed in simple in vitro studies and those found in the intestine, these and more recent data do suggest that the suppression of *Candida* in vivo is likely to be the result of the interdependence of a complex set of interactions. Moreover, other data further suggest that these interactions are probably regulated, collectively, by a complex indigenous microflora and not by one or even a few bacterial species, as has been suggested previously (85,86,88,89).

In practice, two general approaches have been used to study interactions between *Candida* and the indigenous microflora of the GI tract. They have included, with rare exceptions, estimating *C. albicans* population levels in the presence of intestinal bacteria (both in vitro and in vivo) and determining how well *Candida* "grows" in a mileu after intestinal bacteria have been grown in it and then were removed or after certain chemicals have been added. Although both methods have been used with some success to elucidate particular aspects of GI colonization by *Candida*, each is limited and can be misleading at times because of a number of accompanying technical difficulties. It suggests that studies combining both methods may be required to accurately understand and describe colonization of the GI tract by *Candida* and other fungi. In this section, the interactions between *C. albicans* and the various bacterial populations that comprise the GI microflora are considered in detail. However, because an anaerobic continuous-flow (CF) culture model of the ecology of the large intestinal microflora (85) was used to generate a large portion of the data to be described, it is also discussed here to show the relevance of the model to the study of microbial factors that may regulate *C. albicans* population dynamics in the gut.

The study of interactions between the indigenous microflora and *C.*

albicans or other fungi in the intestinal tract historically has been hampered by numerous problems (85). Foremost among them is that virtually all in vitro studies to date, with the exception of some studies using CF cultures (85,86), have relied on simple static cultures of a diflora of, for example, *C. albicans* and a single bacterial species (5,8,73,74,76,114,120,121,133). It is important to again emphasize that these studies do not reflect interactions as they occur in the intestinal tract (48,50,89). For one thing, it is unlilkely that the suppression of *C. albicans* by a single bacterial species in vitro could duplicate to any degree the complexity of the physiochemical interactions exerted by the 400–500 distinct bacterial species that are indigenous to the large intestine (34,72,108,137). It is evident from the findings that suppression of *C. albicans* using simple in vitro cultures is dependent on culture conditions or the population size of the antagonizing bacterium (120,133). In other words, the interactions between *C. albicans* and bacteria depend to a large extent on the environment in which they take place. The best proof comes from a comparison of studies in which a monoflora of *E. coli* (or any other single bacterium species) antagonized *C. albicans* growth in the gut of gnotobiotic animals (8,73,74,114), but the presence of *E. coli* did little or nothing to inhibit *Candida* growth in the gut of animals that contained other bacteria (24). Bacteria under the former condition reach abnormally high numbers in the gut (13,15). *Escherichia coli* itself is suppressed by the strict anaerobes that dominate the intestinal microflora (51,153). Consequently, it is still not known if *E. coli* can exert an inhibitory mechanism(s) over *C. albicans* under normal in vivo conditions as has so often been suggested (8,73,74,76,114). In fact, Clark (24) showed that *C. albicans* grew unchecked for several weeks in the GI tracts of gnotobiotic mice containing an intestinal flora of *Bacteroides* spp., *Lactobacillus* spp., *Streptococcus faecalis, S. lactis,* and *E. coli.* Studies also indicate that enteric bacilli (including *E. coli*) do not inhibit the growth of *C. albicans* in the GI tracts of conventional mice (2,90). One may conclude with some certainty, therefore, that there is little similarity between the inhibitory mechanisms for *C. albicans* in simple in vitro systems and those found in the intestine, and that suppression of *C. albicans* by a single bacterial species cannot be expected to reflect interactions of a complex indigenous microflora (51).

Although other examples could be given, including numerous studies that indicate a lack of correlation between bacterial interactions in vitro and the interactions of these same bacteria in the intestines of conventional animals or man (48–50), it is clear from the above list that in vitro model systems must closely simulate the process occurring under "normal" in vivo conditions if meaningful results are to be gained from studies of bacterial–candidal interactions. One such model (CF culture) of the ecology of the large intestinal flora has been developed that meets these stringent requirements in that it reproduces a number of bacterial interactions that occur in the large intestine of mice (56). For instance, it was

TABLE 11-7. Similarity of some environmental parameters and the bacterial flora obtained from CF culture models of the large intestinal flora ecology and large intestine.

Factor	CF cultures	Large intestine or feces	Ref.
Environmental parameters			
pH (mouse)	~6.8–7.2	~6.8–7.2	Kennedy, unpublished data; 52
H_2S (mouse)	2.2×10^{-4}	3.1×10^{-4}	52
SCFA (m*M*)	Mouse/human	Mouse/human	
Acetic	79.3/70.5	116.1/77.7	52,85
Propionic	19.1/21.3	20.4/29.9	52,85
Isobutyric	3.9/5.4	4.5/4.1	52,85
Butyric	21.8/29.5	20.8/30.4	52,85
Isovaleric	7.4/8.0	3.8/9.2	52,85
Valeric	0.6/1.7	1.3/1.5	52,85
Floral parameters			
Total $\log_{10}$ No. of anaerobes cultured			56
Mouse	>9.9	>9.9	85
Human	>8.9	>8.9	85
Total $\log_{10}$ No. of facultative anaerobes			56
Mouse	8.3	8.4	85
Human	8.1	8.5	85
Proportion of culturable flora that are Enterobacteriaceae			
Mouse	0.02%	0.001%	56,85
Human	1.2%	0.4%	56,85

found that mixed populations of mouse cecal bacteria in CF cultures were able to suppress "invader" bacterial populations (e.g., *Clostridium difficile* or *E. coli*) to levels similar to those found after being fed to conventional mice (52,56,168), and that contents of CF cultures fed to germfree mice redressed several germfree abnormalities (e.g., cecal size and mucosal histology) (56). These and other types of evidence for similarities between bacterial interactions in CF cultures and the mouse intestine are summarized in Table 11-7 and Figure 11-1. The finding that this model system can reproduce a variety of microbial interactions that occur in the murine large intestine nevertheless makes it likely that the underlying mechanisms that control the bacterial populations in anaerobic CF cultures are similar to those operating in vivo (52,56). Because of this fact and due to the shortcomings of the simple in vitro systems mentioned above, experiments were designed to use an anaerobic CF culture model to study interactions between *C. albicans* and large intestinal bacteria (85).

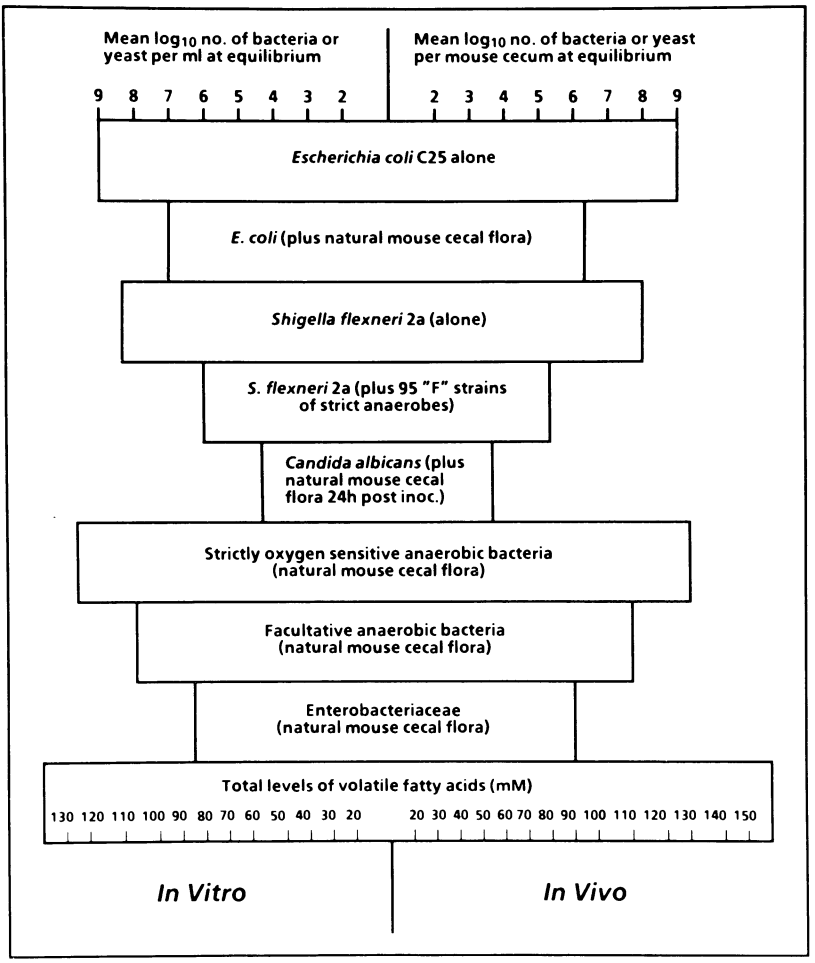

FIG. 11-1. Similarities between CF cultures of intestinal bacteria and the large intestine. (Data from refs. 52,56,85.)

In these studies, the similarity of the bacterial flora from CF cultures and mouse cecal contents or human feces was determined by making a number of comparisons. As summarized in Table 11-8, facultative and strictly anaerobic bacteria were found in almost identical numbers in CF cultures and in the ceca of mice or human feces. Likewise, the total numbers of Enterobacteriaceae found in CF cultures of human fecal flora and in human feces were also similar, although the numbers of Enterobacteriaceae were found to be slightly higher in CF cultures of mouse cecal flora than in those found in the mouse cecum. Therefore both the total number of bacteria and the populations of various types of intestinal bacteria in CF cultures were similar to their in vivo counterparts. This result is similar to the

TABLE 11-8. Population levels of intestinal bacteria in the ceca of test animals, human feces, and anaerobic CF cultures.*

Experimental group (*n*)	Log_{10} mean No. (± SD)/g (wet wt) or ml		
	Facultative bacteria	Enterobacteriaceae	Strictly anaerobic bacteria
Mouse cecal flora (10)	8.4 ± 0.4	4.9 ± 0.2	9.9 ± 0.1
CF culture of mouse cecal flora (4)	8.3 ± 0.3	6.3 ± 0.3	9.9 ± 0.2
Human fecal flora (2)	8.5 ± 0.9	6.5 ± 0.8	8.9 ± 0.5
CF culture of human fecal flora (2)	8.1 ± 0.8	6.8 ± 0.7	8.7 ± 0.4

*From Kennedy et al. (85).

findings of Freter et al. (56), who showed that the population levels of 37 strictly anaerobic bacteria, which had been chosen to form a representative sample of the genera (and unknown taxa) of bacteria from the large intestine of conventional mice, were maintained in CF cultures in the same proportion as found in vivo. Furthermore, Gram stains of cecal contents from CF cultures, mouse ceca, and human feces were also remarkably similar (85). In all instances the flora was characterized by a predominance of gram-negative rods, and in no instance did only one or a few strains "outgrow" and subsequently suppress all others (52,56,85). Another similarity between this model system and the large intestine was that dense layers of bacterial growth formed on the glass walls of the CF culture vessels, analogous to bacterial populations that colonize intestinal mucosa (56,85).

Another critical test to determine the similarity of interactions between *C. albicans* and the intestinal microflora in these systems, and if CF cultures would serve as good models of such interactions, came from monitoring *C. albicans* populations after inoculation into mice or CF cultures of mouse cecal flora. As may be seen in Figure 11-2, the elimination of *C. albicans* from the mouse cecum occurred at a rate similar to that observed when *C. albicans* was inoculated into a CF culture of mouse cecal flora, thus indicating that the CF cultures reproduced the populations of *C. albicans* as they occurred in mice under normal in vivo conditions.

As a final means of testing the abililty of CF cultures to maintain the indigenous bacterial flora, and therefore a number of interactions as they occur in vivo, the material from CF cultures of mouse cecal flora was fed to antibiotic-decontaminated mice to determine if they could redress several abnormalities that occur when the bacterial flora is eliminated (51,138,139), including the ability to resist colonization by *C. albicans*. This test was deemed important, as previous studies had shown that inoculation of whole cecal contents to antimicrobic-treated animals restored

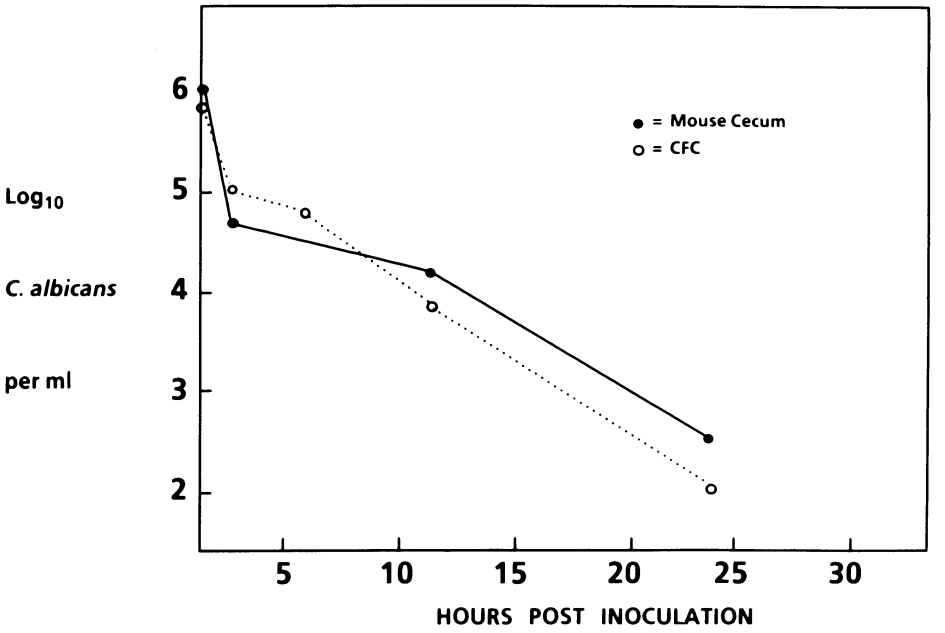

FIG. 11-2. Elimination of *Candida albicans* from CF culture of mouse cecal flora and mouse cecum. Note that the rate of removal is nearly identical in the two systems. (From Kennedy et al. [86].)

their ability to resist intestinal colonization to *C. albicans*, whereas the feeding of sterile cecal contents or a few bacteria did not (24,86,89). For these studies, mice were given a combination of antibiotics—vancomycin, ampicillin, and gentamicin (VAG)—an antibiotic regimen designed to eliminate most of their intestinal flora (84); subsequently, they were given oral and rectal injections of either CR culture material, cecal homogenates, or filtrates of cecal homogenates. These animals, as well as appropriate control animals, were then inoculated with *C. albicans* and cultured 24 hours postinoculation to determine the levels of *Candida* in their ceca. Antimicrobic-treated animals challenged with *C. albicans* had high levels of *C. albicans* colonizing their ceca, as did animals given antibiotics followed by cecal filtrates. Control animals not given antimicrobics; and antimicrobic-treated mice recolonized with the indigenous cecal microflora, in contrast, had significantly lower cecal populations of *C. albicans* ($p < 0.001$). Likewise, animals that had been given CF culture material also had levels of *Candida* colonizing their ceca that were significantly lower than those in mice given antimicrobics only ($p < 0.001$). These data are summarized in Table 11-9.

The effects of antimicrobics and recolonization on the indigenous cecal microflora and certain cecal characteristics was also studied. Animals

TABLE 11-9. Cecal population levels of *C. albicans* (CA34) in reconventionalized and control animals.*

Antimicrobic treatment	Log_{10} No. of *Candida*/g†
Antimicrobics only (VAG)‡	5.7 ± 1.2
Antimicrobics followed by cecal filtrates	5.8 ± 0.9
Antimicrobics followed by cecal homogenates	1.8 ± 0.9
Antimicrobics followed by CF culture material	2.3 ± 1.5
None	2.6 ± 1.6

*From Kennedy et al. (85).
†The values represent the means ± standard deviations of 5–10 mice per group cultured for *C. albicans* at 24 hours postinoculation.
‡Animals received vancomycin, ampicillin, and gentamicin (VAG) in the drinking water as described in the text.

treated with antimicrobics and those treated with antimicrobics followed by cecal filtrates had several cecal abnormalities. Gram stains, for instance, showed that these animals contained few bacteria (mostly gram-positive cocci), and there was complete disapperance of the predominant gram-negative and fusiform-shaped rods. In contrast, antimicrobic-treated mice, which had been recolonized with cecal bacteria isolated from the ceca of conventional mice or with CF cultures of conventional mouse cecal flora by oral and rectal injections, had bacterial flora that were similar to those found in conventional animals. Furthermore, dissection of test and control animals revealed other differences in cecal characteristics according to the experimental group examined. Animals that possessed a complex bacterial flora (i.e., normal mice or mice recolonized with mouse cecal contents or CF cultures of mouse cecal bacteria) had small ceca (approximately 1–2% of their total body weight) that contained thick, pasty contents. Mice given antimicrobics to eliminate the indigenous microflora, in contrast, had enlarged ceca (approximately 5–8% of their total body weight) with watery contents, a trait attributable to mice lacking a complex ("normal") intestinal microflora (38,51,138–140,153). These findings are summarized in Table 11-10.

It seems likely, then, that the above results, together with the exhaustive studies by Freter's group (51,52,54,56), strongly support the hypothesis that the major ecological mechanisms that control and maintain a balance among microbial populations of the large intestine are reproduced in CF cultures (85,86). The ability of CF cultures to maintain a bacterial flora that closely resembled that found in the mouse large intestine, for instance, is probably the most convincing of these data and constitutes significant evidence that CF cultures of mouse or human intestinal flora reproduce a number of bacterial interactions that occur in the large intestine (52,56). This finding is in contrast to the general finding that one or a few bacterial species usually overgrow all others when a clinical specimen is removed from the body and allowed to multiply in artificial nonselective culture

TABLE 11-10. Cecal characteristics of antimicrobic-treated, control, and reconventionalized animals.*

Animal treatment†	Gram stain‡	Predominant bacterial organisms§	Consistency of cecal contents	Cecal size‖ (%)
Antimicrobics only (VAG)	−	Gm− cocci and small Gm− rods	Soft	5–8
Antimicrobics followed by cecal filtrates	−	Gm− cocci and small Gm− rods	Soft	5–8
Antimicrobics followed by cecal homogenates	+	Large, fusiform-shaped Gm− rods	Thick & pasty	1–2
Antimicrobics followed by CF culture material	+	Large, fusiform-shaped Gm− rods	Thick & pasty	1–2
None	+	Large, fusiform-shaped Gm− rods	Thick & pasty	1–2

*From Kennedy et al. (85).
†Animals received vancomycin, ampicillin, and gentamicin (VAG) in the drinking water as described in the text.
‡Gram (Gm) stains: − = abnormal; + = normal compared to normal, untreated animals.
§Predominant organisms seen on gram-stained smears.
‖Percentage of total body weight.

(56). Because the major bacterial groups were present in similar numbers in CF cultures and in the ecosystems from which they were obtained, it is likely that the ecological control mechanisms operating in vitro were the same, or at least similar to, those controlling the microflora in vivo.

This interpretation was further supported by the finding that the qualitative and quantitative composition of various volatile fatty acids (VFA) present in CF cultures closely resembled that in the mouse cecum and human feces (Table 11-11). Because these acids are characteristic metabolic endproducts of the predominant anaerobic bacteria that colonize the large intestine (72), it may be concluded that the CF cultures were able to support the major metabolic activities of the predominant intestinal anaerobes. Therefore because it is unlikely that two mechanisms would bring about similar equilibria among a complex microflora (8), e.g., the intestinal microflora, one may conclude with some certainty that the predominant microbial interactions were reproduce in CF cultures as they occur in the large intestine. Experiments showing that the survival and passage of *C. albicans* through CF cultures of mouse cecal flora and the mouse cecum proper were remarkably similiar certainly support this view.

A final stringent test of the ability of CF cultures to propagate the numerous bacterial species of the cecal flora that are responsible for these

TABLE 11-11. Concentrations of volatile fatty acids in test animals, human feces, and anaerobic CF cultures.*

Experimental group	Volatile fatty acid concentration (m*M*) of contents (mean ± SD)					
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
Mouse cecal contents (n = 5)	116.1 ± 19.2	20.4 ± 4.5	4.5 ± 0.8	20.8 ± 5.7	3.8 ± 0.8	1.3 ± 0.6
CF culture of mouse cecal flora (n = 5)	79.3 ± 2.9	19.1 ± 6.0	3.9 ± 1.2	21.8 ± 3.4	7.4 ± 3.1	0.6 ± 0.8
Human feces (n = 2)	77.7 ± 12.6	29.9 ± 4.6	4.1 ± 0.8	30.4 ± 6.3	9.2 ± 0.7	1.5 ± 0.1
CF culture of human fecal flora (n = 2)	70.5 ± 15.1	21.3 ± 4.9	5.4 ± 1.1	29.5 ± 1.1	8.0 ± 1.7	1.7 ± 0.6

*From Kennedy et al. (85).

functions came from experiments showing that CF culture material could redress several cecal abnormalities of antibiotic-treated mice (51,138,139,153). For example, when CF culture material was fed to antimicrobic-treated mice, a complete reduction of the enlarged cecum to the normal size took place. Likewise, a bacterial flora resembling that of conventional mice not given antibiotics was confirmed by observing gram-stained smears of the cecal contents from normal and antibiotic-treated mice reconventionalized with CF culture suspension. Moreover, such reconventionalized mice were able to resist colonization by *C. albicans*, again suggesting that at least those organisms responsible for the suppression of *Candida* in the gut were maintained in this culture system.

It is significant to note that a complete reduction of the enlarged cecum to normal size and a reduciton of the intestinal population of *Candida* to levels found in conventional animals have never been fully achieved in germfree or antimicrobic-treated animals following implantation of one or a few bacterial species (8,24,73,74,114). Clark (24), for instance, showed that *C. albicans* grew unchecked for several weeks in the GI tracts of gnotobiotic mice containing an intestinal flora of *Bacteroides* spp., *Lactobacillus* spp., *S. faecalis*, *S. lactis*, and *E. coli*. Nevertheless, studies by Freter and co-workers (51,153) indicated that a complex intestinal flora, comprised predominantly of strict oxygen-sensitive anaerobes, was required to redress several ceca abnormalities of germfree mice. It was also found that a complex intestinal flora was required to maintain a balance among several bacterial populations similar to that found in conventional animals (56). In fact, close approximation of these parameters to normal values required the implantation of whole cecal contents or no less than 95 metabolically distinct strict anaerobes (51). The findings that whole cecal contents or CF cultures of the cecal microflora were able to convert antimicrobic-treated mice to the normal state and were able to reduce *C. albicans* populations to levels similar to those found in conventional animals, then, are also consistent with the view that a complex and diverse series of interactions is responsible for the control of *Candida* in the gut.

As stated above, *C. albicans* is eliminated from the CF culture of mouse cecal flora at a rate indistinguishable from that in the mouse cecum. To determine whether *Candida* organisms were being killed, were multiplying, or were simply being "washed out" with the flow of material through the CF culture system, the elimination of *C. albicans* from CF cultures was plotted against the dilution rate of the system. Figure 11-3 shows the passage of *C. albicans* through CF cultures of mouse cecal flora. As can be seen, *Candida* cells were removed from CF cultures at a rate faster than the rate of dilution (open squares). When inoculated into CF cultures of human fecal flora, in contrast, this same strains were eliminated more or less with the flow of broth through the system (Figure 11-4). Thus in both instances *C. albicans* failed to implant and was eliminated, but only in CF cultures of mouse cecal flora did "killing" of *C. albicans* appear to be

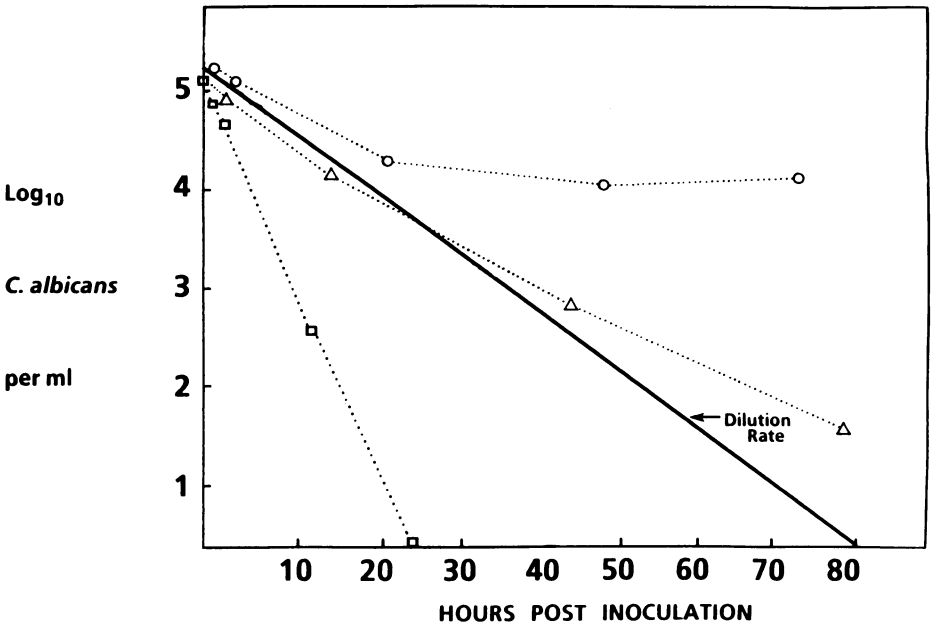

FIG. 11-3. Elimination of *Candida albicans* from CF culture of mouse cecal flora versus the dilution rate. *Candida* cells for two isolates were found to be removed faster than the dilution rate of the system, indicating that *Candida* cells were being "killed." Inoculation of *C. albicans* into a culture without cecal bacteria showed that viable bacteria (and not anaerobic condition alone) were responsible for controlling the *Candida* population. (From Kennedy et al. [86].)

taking place. The ability of *C. albicans* to colonize a sterile anaerobic CF culture (open circles) is also shown in Figure 11-3. In this instance, *C. albicans* was initially removed (for about 20 hours) from the system with the flow rate, but thereafter *Candida* cells began to multiply and maintained a steady population of about 10^4 cells ml^{-1}. This steady-state condition was maintained for more than 2 weeks.

As indicated in the discussion on the survival of *C. albicans* in the gut, *C. albicans* cannot easily be implanted into an established intestinal microflora, but *Candida* populations can be maintained if given prior to or during the succession and development of the intestinal flora (e.g., in infant mice). This phenomenon was also studied in CF cultures by inoculating *C. albicans* into a sterile CF culture simultaneously with 1.0 ml of an established CF culture of mouse cecal flora that is known to rapidly eliminate *C. albicans* (86). In this experiment, *C. albicans* was removed from the system with the flow rate for about the first 24 hours but thereafter was removed at a much slower rate. This finding indicated that *C. albicans* had become transiently implanted after an initial prolonged lag phase and may

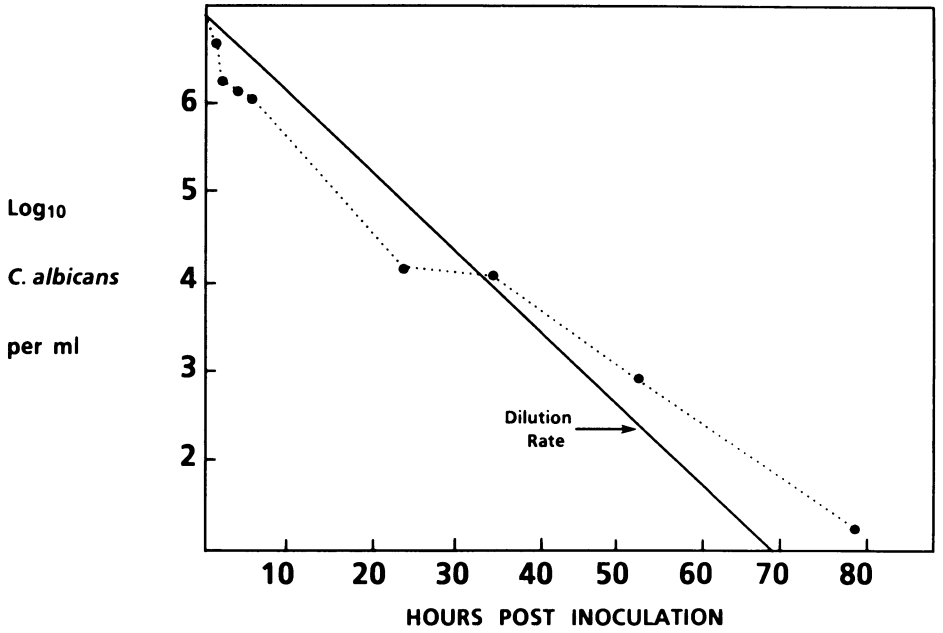

FIG. 11-4. Elimination of *Candida albicans* from CF cultures of human fecal flora. Note that *Candida* cells were removed more or less with the flow rate. (From Kennedy et al. [86].)

have been replicating very slowly in the presence of a functional indigenous microflora. The *C. albicans* population was eventually eliminated as the microflora reached equilibrium, probably because of a lack of adhesion sites available for *C. albicans* binding or the inability of *Candida* to bind to limited adhesion sites in the presence of intestinal bacteria, their metabolic product, or both (discussed below). The flora was found to be intact and functioning properly because the characteristic volatile fatty acids (VFAs) were being produced, although both the total and individual levels of VFAs were present in lower quantities than were found in the donor CF culture. It was not surprising, as the microflora had not yet reached equilibrium. Phase-contrast microscopy revealed that the predominant bacterial flora was indeed maintained.

To study the mechanism(s) that was responsible for causing the inhibition of *C. albicans* in CF cultures of conventional cecal microflora or human fecal microflora, the effluent from these cultures were used for growth studies (86). The effluent from each culture was sterilized by filtration through 0.22 μm Millipore filters inside the anaerobic chamber. The resulting filtrates were then inoculated with *C. albicans* (final concentration 10^5 cells ml^{-1}), incubated as static cultures aerobically and anaerobically 37°C, and the growth monitered for up to 5 days and compare to that in sterile

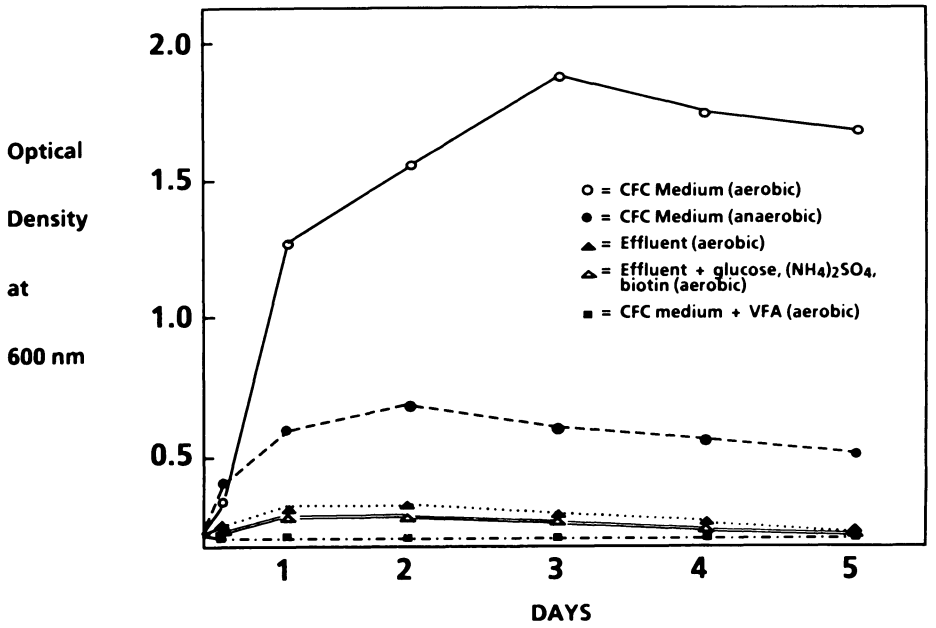

FIG. 11-5. Growth of *Candida albicans* in sterile effluent from CF cultures (CFC) of mouse cecal flora, sterile CFC effluent supplemented with vitamins, nitrogen and carbon sources, CFC medium, CFC medium under anaerobic conditions, and CFC medium supplemented with volatile fatty acids. (From Kennedy et al. [86].)

CF culture medium. It was found that *C. albicans* was unable to multiply in the culture filtrates from CF cultures of mouse or human intestinal microflora, regardless of whether the cultures were incubated aerobically or anaerobically (Figs. 11-5 and 11-6). To determine if the inability of *C. albicans* to multiply was due simply to the depletion of nutrients or micronutrients, carbon and nitrogen sources, vitamins, and trace elements were added to the effluent. It was found tha the inability of *Candida* to multiply in CF culture filtrates could not be reversed by adding any of these nutrients, either individually or collectively, to the cultures. Furthermore, this trend was not altered by changing the culture pH (to anywhere in the range of 6.0–8.0), incubating the cultures aerobically or anaerobically, or both. However, it was noted that the growth of *C. albicans* in sterile CF culture medium was much reduced when the cultures were incubated anaerobically. Nevertheless, the reduction in growth was not as severe as that in CF culture filtrates, thus indicating that something other than the anaerobic condition or substrate depletion, i.e., production of an inhibitory substance(s), was primarily responsible for controlling the growth of *C. albicans* in CF cultures.

In studies from our laboratory (85) it was found that both the total and individual levels of VFAs present in CF cultures coincided with the quanti-

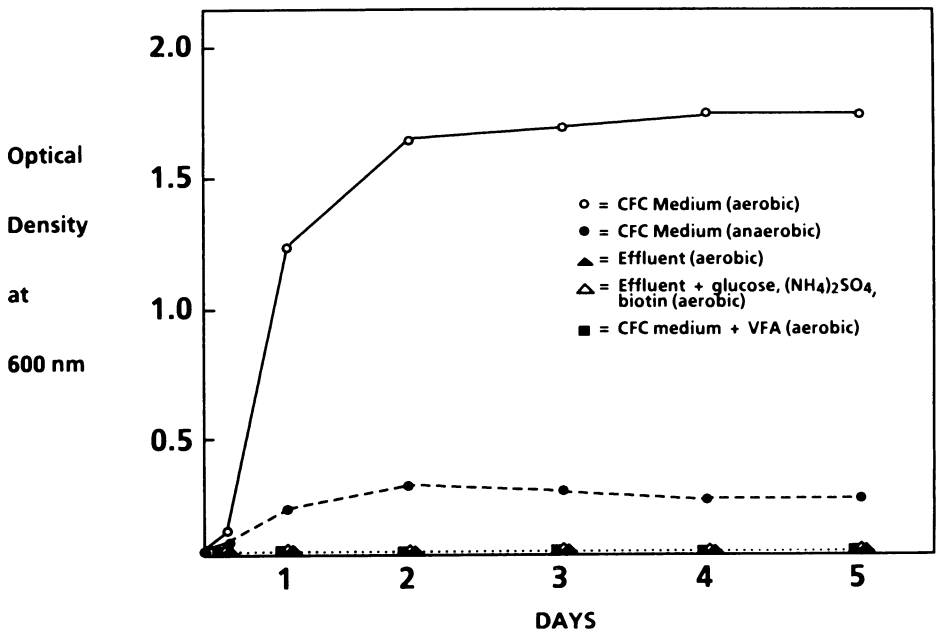

FIG. 11-6. Growth of *Candida albicans* in sterile effluent from CF cultures (CFC) of mouse cecal flora; sterile CFC effluent supplemented with vitamins, trace elements, and nitrogen and carbon sources; CFC medium aerobically; CFC medium anaerobically; and CFC medium supplemented with volatile fatty acids. (From Kennedy et al. [86].)

ty of VFAs found in the ceca of conventional mice or human feces used to establish the CF cultures. Therefore, because much has been speculated about the role of VFAs in the control of the intestinal microflora (17,20,95,100), and because short-chain fatty acids had previously been shown to be inhibitory to *Saccharomyces cerevisiae* (17,18,107), the possible role of VFAs in *C. albicans* suppression was assessed (86). Note that preliminary studies showed that the levels of VFAs were not reduced by filtration or subsequent incubation of the cultures. Figures 11-5 and 11-6 illustrate the results of experiments in which all six VFAs (at concentrations resembling those in CF cultures) were added to static cultures *C. albicans*. These data indicate that the VFAs added to fresh medium were able to inhibit the growth of *C. albicans* or at least cause a pronounced lag phase. By plating samples of these cultures at the end of the experiment, it was also determined that *C. albicans* remained viable. It should be noted that the inhibition of *C. albicans* was more pronounced when the pH of the medium was lowered to ≤ 6.0 (Kennedy, unpublished data). Furthermore, the inhibitory effect of VFAs for *Candida* was found to decrease if the cultures were not stirred. Nevertheless, from the above data it could not be concluded if only one short-chain acid was responsible for the observed

TABLE 11-12. Minimum inhibitory concentration of various short-chain fatty acids for *C. albicans*.*

	Minimal inhibitory concentration (m*M*) of SCFAs					
Strain identification	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
CA30	40	40	10	10	20	10
CA34	40	20	10	10	5	2.5

*From Kennedy et al. (86).

suppression or the mixture acted collectively to inhibit the growth of *C. albicans*. The minimum inhibitory concentration (MIC) for each VFA was therefore determined using the VFA mixture as a positive control. As is shown in Table 11-12, all of the short-chain fatty acids inhibited the growth of *C. albicans*. However, some of them did so only at levels that exceeded the levels found in the mouse cecum, human feces, or CF cultures. The possible significance of this finding is discussed in more detail below.

Up to this point, the discussion has considered interactions between *C. albicans* and bacteria in the intestinal lumen. The discussion now focuses on the mechanisms by which the intestinal bacteria inhibit *Candida*-mucosa association. These data were drawn largely from results obtained from studies using experimental animals or in an in vitro adhesion assay (89), but clinical correlations are noted where appropriate. The mechanisms by which *Candida* can associate with intestinal mucosa are considered in some detail below, but a discussion on inhibitory mechanisms would not be complete without considering this subject here.

The ability of *C. albicans* to associate with intestinal mucosal surfaces has been tested in vitro and in vivo (89–91). The adhesion assay used in the studies discussed below has been described in detail elsewhere (89), but it should be noted that the animals were sacrificed by cervical dislocation and placed in an anaerobic chamber, and the small intestine and ceca were aseptically removed (from either antimicrobic-treated or untreated hamsters) to avoid exposure to oxygen. This method is unique in that all other in vitro studies have been performed under aerobic conditions, which do not represent the anaerobic environment of the gut. Intestinal slices from either the small intestine or cecum, and *Candida* cells (at a final concentration of 10^6 CFU/ml) were incubated (with occasional agitation) in the anaerobic chamber at 37°C. Counts of the numbers of viable *C. albicans* associating with intestinal slices were performed, after inoculation and washing using standard procedures to determine the number of *Candida* organisms per slice (89). Assay solutions were also quantitatively cultured, and counts of the rinsed intestinal slices were related to the count of 1 ml of test solution and defined as the association index: $(t/t + k) \times 100$, where t is the number of *Candida* associating with intestinal slices after rinsing, and k is the number of viable yeasts per milliliter of assay solution after 2 hours of

incubation (89). To gain a better understanding of the inhibitory mechanisms, *Candida* adhesion was also examined using the adhesion assay in various test solutions, with intestinal slices from either untreated or antimicrobic-treated (VAG) conventional animals (89). To determine the number of *C. albicans* that associated with intestinal slices after incubation in the various test solutions, the slices and their surrounding solutions were quantitatively cultured and the association index calculated. The results of these experiments (Table 11-13) suggested that several inhibitory mechanisms were operating to inhibit the association of *Candida* with intestinal mucosa. When *Candida* organisms were incubated in PBS with intestinal slices from antimicrobic-treated animals (assay 3), large numbers of *Candida* were found to associate with intestinal slices, as indicated by association indexes for small intestine and cecal slices of 13.41 and 13.04, respectively. Conversely, when *Candida* cells were exposed to intestinal tissues from untreated hamsters (i.e., slices that contained an indigenous mucosa-associated microflora) low numbers of *C. albicans* were found to associate with the intestinal slices (assay 6). Low association indexes (0.70 and 0.73, respectively) were determined for small bowel and cecal tissues from this assay. Similarly, low numbers of *C. albicans* were observed to associate with intestinal slices when exposed to small bowel and cecal slices in the presence of intestinal contents, which contained the indigenous microflora (assays 1 and 4). Interestingly, the ability of *C. albicans* to associate with intestinal tissues from antimicrobic-treated hamsters was also reduced when the association assay was performed in intestinal contents from untreated animals that were filtered to remove the indigenous microflora (assays 2 and 5). It suggested that a certain unknown chemical substance(s) produced in the GI tract of untreated animals, probably by metabolic activities of those organisms that predominate in the gut, appeared to inhibit the ability of *Candida* organisms to associate with intestinal mucosal surfaces. In an attempt to identify such factors, VFAs and deconjugated bile acids (DCBAs) were tested int he mucosal association assay. It was found that both chemical substances reduced the ability of *C. albicans* to associate with intestinal mucosal tissues (assays 7 and 8).

The finding that certain chemical factors present in the "normal" gut environment inhibit the association of *C. albicans* with intestinal mucosal surfaces is not surprising, and it is an important observation. Because environmental parametes are known to influence *Candida* adhesion to vaginal and buccal epithelial cells (87,97), it follows that bacterial metabolic endproducts—which result from the activity of those organisms that control the indigenous microflora (52)—also reduced the ability of *Candida* cells to "attach" to certain mucosal structures or the mucus gel. DCBAs and VFAs, for example, both inhibited *Candida*–mucosa association, but the mechanism of inhibition is not known. These substances may have reduced the mucosal association of *C. albicans* by modifying *Candida* adhesin(s) or mucosal receptor(s) (55). When animals are given certain anti-

TABLE 11-13. Association of *C. albicans* with intestinal slices.*

Assay			Small intestine		Cecum	
System	Solution†	Source of intestinal slices‡	Log_{10} mean No. of *C. albicans*/slice	Association index§	Log_{10} mean No. of *C. albicans*/slice	Association index§
1	IC	Antimicrobic-treated hamsters	2.4	0.13	2.5	0.08
2	IF	Antimicrobic-treated hamsters	4.2	2.62	4.0	1.82
3	PBS	Antimicrobic-treated hamsters	5.0	13.41	4.8	13.04
4	IC	Untreated hamsters	2.5	0.09	2.4	0.06
5	IF	Untreated hamsters	3.8	0.60	3.4	0.25
6	PBS	Untreated hamsters	3.9	0.70	3.7	0.73
7	PBS + BA	Antimicrobic-treated hamsters	NT	NT	4.3	4.19
8	PBS + VFA	Antimicrobic-treated hamsters	4.1	1.17	4.1	1.86

*From Kennedy and Volz (89).
†PBS + BA = contains bile acids (lithocholic acid 3.0 m*M*; deoxcholic acid 2.6 m*M*); PBS + VFA = PBS contains VFA (valeric acid 1.2 m*M*; isovaleric acid 2.2 m*M*; butyric acid 12.4 m*M*; isobutyric acid 1.4 m*M*; propionic acid 20.1 m*M*; acetic acid 49.3 m*M*).
‡Antimicrobic-treated hamsters given vancomycin, ampicillin, and gentamicin for 3 days in the drinking water before experimentation.
§Association index = $(t/t = k) \times 100$, where t is the number of *C. albicans* associating with intestinal slices after rinsing, and k is the number of viable *C. albicans* per milliliter after 2 hours of incubation.
NT = not tested.

microbics that allow *C. albicans* to colonize the large intestine (90), there is a concomitant drop in intestinal levels of VFAs (Table 11-14). Likewise, antimicrobic treatment that predisposes to *C. albicans* colonization and dissemination has previously been shown to cause an increase in intestinal levels of conjugated bile acids and a decrease in the levels of DCBAs (40a). Further studies are necessary, however, to determine the exact nature of inhibition by these substances. Nevertheless, these data, together with the reported inhibitory activity of certain VFAs and DCBAs for *C. albicans* growth (86,101) represent an important host defense mechanism suppressing intestinal colonization by *C. albicans* and other fungi (86).

To test the ability of *C. albicans* to associate with intestinal mucosal surfaces in vivo and to obtain a feeling for the population dynamics of mucosal colonization, untreated and antimicrobic-treated (given penicillin G, 500 U/ml, for 3 days as described above) animals were injected with *C. albicans*, and the numbers of viable *Candida* were determined for cecal contents, cecal walls, and visceral organs. Several animals were sacrificed and their intestinal tissues examined by scanning electron microscopy. The ability of *C. albicans* to associate with intestinal mucosal surfaces in penicillin-treated and untreated animals was found to be significantly different (89,90). In penicillin-treated animals, large numbers of *C. albicans* were found in the intestinal contents and associated with intestinal mucosal surfaces (Table 11-15). Significantly lower numbers of *C. albicans*, in contrast, were found in the intestinal contents or associated with the mucosal surfaces of untreated animals ($p < 0.001$) (Table 11-15). Viable *C. albicans* was recovered only from the visceral organs of penicillin-treated animals (Table 11-15). Interestingly, the number of facultative enteric bacilli was likewise found to increase significantly in the intestinal contents and on host mucosal surfaces after antimicrobic treatment, concomitant with a reduction in the number of strictly anaerobic bacteria colonizing these same habitats (Table 11-15).

Scanning electron microscopy (SEM) studies revealed similarly that large numbers of yeast cells were present on the surface of the villi and mucous material in antibiotic-treated animals, whereas only small numbers of yeast cells were observed associating with host mucosal surfaces of untreated animals. However, yeast cells were often observed to penetrate deep into intestinal tissues of antimicrobic-treated animals but not untreated animals. Furthermore, yeasts were often seen attached to and embedded in mucus adjacent to intestinal villi only in antimicrobic-treated animals. In animals not given antimicrobics, yeast cells were probably associated with the loose top layer of mucus not preserved by the fixation procedure used for SEM studies. That is, most of the yeast cells found in untreated animals were probably associated with the thick layer of mucus gel covering the epithelium, whereas yeast cells were found throughout the cecal tissue of antimicrobic-treated animals. This finding was confirmed in additional experiments that studied the rate of *C. albicans* disassociation from intestinal tissues.

TABLE 11-14. Concentration of VFAs in the ceca of antimicrobic-treated and control hamsters.*

Treatment†	VFA conc. (m*M*) of cecal content‡						
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	Total
None (control)	118.6 ± 19.9	20.4 ± 4.5	4.4 ± 0.8	20.7 ± 6.3	3.6 ± 0.4	1.3 ± 0.1	168.2 ± 31.3
Penicillin	29.1 ± 3.9 (24.5)	6.9 ± 1.0 (33.8)	2.7 ± 1.0 (61.4)	2.7 ± 0.7 (13.0)	3.9 ± 0.7 (108.3)	0.5 ± 0.1 (38.4)	45.6 ± 4.9 (27.1)
VAG	25.8 ± 2.9 (21.8)	3.3 ± 0.4 (16.2)	16.3 ± 2.7 (370.4)	3.3 ± 2.1 (15.9)	4.0 ± 0.5 (111.1)	0.7 ± 0.6 (53.8)	53.4 ± 5.0 (31.7)

*From Kennedy and Volz (89).
†Animals were given nothing, penicillin, or vancomycin/ampicillin/gentamicin (VAG) ad libitum in the drinking water 3 days before *Candida* challenge.
‡Values are mean ± SD of five animals per group. Values in parentheses are percents of control group.

TABLE 11-15. Population of indigenous bacteria and *C. albicans* in cecal contents, cecal walls, and visceral organs of untreated and penicillin-treated animals challenged with 10^7 *C. albicans*.*

Animal treatment	Cecal contents/g (wet wt)			Cecal wall/per g (wet wt)			Visceral organs
	Enteric bacilli	Anaerobes	*C. albicans*	Enteric bacilli	Anaerobes	*C. albicans*	*C. albicans*
None	5.2 ± 0.5	9.3 ± 0.5	3.3 ± 0.3	3.2 ± 0.2	9.8 ± 0.3	2.1 ± 0.4	ND
Penicillin	9.6 ± 0.3	7.4 ± 0.7	7.4 ± 0.2	8.2 ± 0.2	8.1 ± 0.3	5.5 ± 0.3	2.06 ± 1.71 (6/10)†

*From Kennedy and Volz (89).
†Number of animals with *C. albicans* cultured from visceral organs/number of animals tested; this ratio was determined 24 hours after intragastric *Candida* challenge.
ND = not detectable.

In that study the ceca of antimicrobic-treated and untreated animals challenged with *C. albicans* were removed aseptically, opened, and the non-adherent fungi and digesta removed by washing with sterile PBS. Cecal tissues were then placed in 10 ml of sterile PBS and incubated on a gyrorotary shaker (at 10 rpm and 37°C). The number of viable *Candida* cells in the surrounding PBS solution was determined, and it was found that *C. albicans* cells were shed from intestinal mucosal surfaces at a faster rate in untreated animals than in antimicrobic-treated animals. Nearly 74% (range 58.7–76.2%) of the *Candida* cells that had associated with intestinal mucosal surfaces of untreated animals were removed by a 1-hour incubation, whereas only 31.3% (range 1.8–60.9%) of the *Candida* cells were removed from animals treated with antimicrobics. It should be noted, however, that it is not known if the in vitro results adequately reflect the disassociation rates of *C. albicans* in vivo, although it is likely that *Candida* disassociation from intestinal tissues would be higher from animals that possess an indigenous microflora, as *Candida* cells would not be able to penetrate the mucus gel to the same degree.

The findings thus described in this chapter strongly support the hypothesis that the indigenous intestinal microflora suppresses *C. albicans* and other fungi in the GI tract and reduces the incidence of dissemination from the intestinal lumen to visceral organs (88–90,112,149–151). The establishment of the normal flora before *Candida* challenge (discussed above) for instance, usually leads to a significant decrease in the intestinal population of *C. albicans* with a concomitant reduction in dissemination. Administration of intestinal contents containing indigenous bacteria following antimicrobic treatment significantly reduced *Candida* adhesion, colonization, and dissemination, whereas filtration of similar cecal contents through 0.20-μm Millipore filters eliminated their protective effect. This finding clearly indicates that viable bacteria are important in *Candida* suppression. Biochemical substances present in filtered intestinal contents that might suppress *Candida* growth probably would have been "washed out" of the gut without much effect, which can be deduced from the finding that intestinal tracers are rapidly removed from the GI tract by peristalsis (53,54). Viable bacteria injected with cecal contents, on the other hand, continue to multiply and colonize the intestinal lumen and mucosal surfaces (51,153) and would have remained until the time of *Candida* challenge.

Although it seems obvious that the normal flora is important in the suppression of gut colonization and dissemination by *C. albicans*, the data presented here also help to clear up previous contradictory results reported in the literature regarding the effects of the "normal" intestinal microflora on GI colonization by this fungus. In their early studies, for instance, Balish and Philips had reported that *C. albicans* was established in "large numbers" in the gut of both germfree and conventional animals (9,122), whereas De Maria et al. (28) and Helstrom and Balish (70) in later experiments found that conventional animals were significantly more resis-

tant to intestinal colonization than antibiotic-treated and germfree animals, respectively. Such discrepancies, however, may be due to the animals selected for study as many strains of mice are unsuitable for research on intestinal floral interactions because they lack a true indigenous microflora (53,56,90,123) or to the fact that different experimental conditions (e.g., *C. albicans* strain or phenotypic state of the organisms at the time of inoculation) were used, or both.

Moreover, from the above discussion it appears that the indigenous microflora inhibits *Candida* colonization and dissemination from the intestinal tract by at least two general mechanisms: (a) decreasing the size of the *Candida* population in the gut, and (b) inhibiting the mucosal association of *Candida* by forming thick layers of bacteria in the mucus gel (37) covering the epithelium. Both mechanisms are probably multifactoral in nature and depend on a complex and functional microflora to exert this control. Animals treated with antimicrobics, for instance, had high numbers of *C. albicans* opportunistically colonizing their GI tracts, with a high incidence of dissemination to visceral organs (88–91). In contrast, experimental animals that possessed an indigenous intestinal microflora had low numbers of *C. albicans* residing in their GI tracts, with a low percentage of animals showing signs of *Candida* dissemination. This finding has been confirmed in untreated animals that underwent multiple *Candida* challenges to maintain high gut populations, and in a human volunteer (89,94). In the former instance, viable *C. albicans* was recovered from the visceral organs of a significant number of animals. In the latter instance, the individual developed candidemia within a matter of a few hours. Thus suppression of the *Candida* population size appears to be an important factor controlling fungal dissemination from the GI tract (41,88–91). Again, it should be emphasized that it is probably the result of several interactions regulated by a complex intestinal microflora. Similarly, it has been shown that high intestinal populations of certain facultative and strictly anaerobic bacteria were required to promote their dissemination ("translocation") from the GI tract (13–16). When intestinal population levels dropped below the so-called threshold level due to antagonism by the indigenous intestinal microflora, these bacteria could no longer disseminate from the gut (13,16). Likewise, we have previously shown that fungi unable to maintain high population levels in the intestinal tracts of antimicrobic-treated mice could not disseminate to visceral organs, although viable fungal cells remained in the gut for several days (84,88). Other investigators have also suggested a direct relation between intestinal population levels of other microbes and systemic dissemination (148,161).

Nevertheless, it appears that the dense bacterial layers lining the mucosal epithelium provide an important defense mechanism that inhibits both *Candida* colonization and dissemination from the GI tract. For instance, it is apparent that the first step in mucosal association must be penetration of the mucus gel (46). In infant mice, which lack a complete bacterial flora

TABLE 11-16. Two general mechanisms that regulate *C. albicans* populations in the GI tract and inhibit the systemic spread to visceral organs.

Mechanism	Ref.
Suppression of *Candida* growth by	
Anaerobiosis	76,86,121,166
Prolonging the lag phase and doubling time	86
Production of inhibitor substances	86,89,121
Competition for limiting nutrients	86,120
Production of inhibitors that limit substrate availability	86
Inhibition of mucosal association by	
Blocking mucosal penetration and adhesion sites	89,91
Competing for adhesion sites	89,91
Prevention of adhesion synthesis or the production of digestive enzymes	89
Production of substances that modify *Candida* adhesions or host receptors	89

including the dense microbial populations in the mucus gel (27,141), *C. albicans* can readily associate with and pass through the gut wall to initiate systemic infection (41,127). In contrast, it was shown in other studies that intestinal tissues possessing an indigenous wall-associated microflora strongly inhibited mucosal association and dissemination of *Candida* from the intestinal tract. This inhibition of mucosal association by *Candida* appears to be regulated, as does the suppression of luminal *Candida* populations, by several independent but interrelated interactions. These interactions tend to fall into two general categories, as is summarized in Table 11-16. First, as already discussed, the intestinal microflora has been shown to prevent mucosal association of *Candida* by competing for adhesion sites and physically blocking the larger yeast cells from penetrating the mucous gel. For example, when intestinal slices from antimicrobic-treated animals, yeast cells, and intestinal bacteria were mixed at the same time (assay 1, Table 11-13), mucosal association by *C. albicans* was as strongly inhibited as when the mucosal association assay was performed with intestinal contents and slices from untreated animals (assay 4). Although it may seem surprising at first, it should be noted that most indigenous mucosal bacteria are motile and may have been chemotactically attracted to the mucosal surface rapidly (46,89); that is, indigenous bacteria may have reached attachment sites first by guided motility before yeast cells randomly "bumped" into intestinal tissues. In addition, SEM and disassociation studies suggest that in conventional animals *C. albicans* is associated with only the top layer of mucus gel lining the lumen. A large percentage of the yeast cells associated with the intestinal mucosa of such animals were found to be shed, whereas a much smaller percentage of associated *Candida* cells were shed from the intestinal walls of antimicrobic-treated animals. SEM analysis of intestinal mucosal surfaces also showed large numbers of

yeast cells attached to intestinal villi only in antimicrobic-treated animals. Presumably, then, the dense layer of bacteria colonizing the mucus gel in conventional animals acts as a resistance barrier to mucosal association and dissemination by *C. albicans*. Second, the production of certain metabolic end-products (e.g., secondary bile acids and short-chain fatty acids) may inhibit *Candida* adhesion by modifying *Candida* adhesins or mucosal receptors, inhibiting the production of mucinases, or all three.

Intestinal Microflora that Normally Controls *Candida* in the Gut

The finding by earlier workers that *E. coli* suppresses growth of *C. albicans* in vitro or in gnotobiotic mice has led to numerous erroneous conclusions regarding the identity of the organisms responsible for the suppression of *Candida* in the gut. It is due, as mentioned above, to the fact that nearly all studies to date have not reflected interactions as they occur in the intestinal tract. Therefore in the following paragraphs evidence has been drawn from several studies in an attempt to describe the components of the intestinal microflora that control *Candida* in the gut. They include (a) the effect of various antimicrobics on GI colonization and dissemination by *Candida*; (b) the survival of *Candida* in mice as the intestinal microflora goes through its normal succession until it reaches the climax stage; (c) the microecology of the GI microflora and the species of bacteria that predominate in the gut ecosystem; and (d) the mechanisms that control *Candida* populations in the gut. By taking a multifaceted approach to this topic, it is hoped that the discussion more clearly describes the complexity of the organisms that are probably involved with the regulation of *Candida* (and other fungi) in the gut as well as eliminates any bias this reviewer may have toward this subject.

It is well known that oral and parenteral administration of antibacterial agents disrupts the ecology of the indigenous intestinal microflora, allowing *C. albicans* and other "yeasts" to either increase in population size or colonize the gut (118). All antimicrobics, however, do not predispose the host to intestinal colonization or dissemination by *C. albicans* (90). Several investigators have described the effects of various antimicrobics on the ability of " *Candida*" or "yeasts" to colonize the GI tract. These results are difficult to interpret, primarily because in most studies there were no identifications or quantitative analyses of the "yeast" species present. In addition, the age of the subjects, their general state of health (if there was an underlying disease, or a GI disorder), the type of diet, if they were taking laxatives or other drugs, and so on were frequently not reported. Nonetheless, Table 11-17 summarizes the effects of antibacterial administration on "yeast carriage" in patients, and Table 11-18 summarizes the same type of data obtained from animal studies. It is evident from the data in these

TABLE 11-17. Summary of the effect of antimicrobial agents on human fecal flora.*

Drug	Aerobes and fecultative anaerobes	Gram-negative anaerobes (*Bacteroides*, *Fusobacterium*)	Gram-positive anaerobes	*"Candida"*	"Yeasts"
Sulfonamides					
Sulfasuxidine					NC
Sulfathalidine					NC
Sulfasalazine	NC	NC or dc	NC to IC		NC
Cotrimoxazone	Elim.				NC
Trimethoprim	Elim.				
Penicillins					
Phenoxymethyl penicillin (Pen V)	ic or NC	dc	NC to Elim.		IC
Phenoxyethyl penicillin	ic or NC	dc	NC to Elim.		ic
Ampicillin	NC to dc	NC to DC	dc to Elim.	NC to IC	NC to IC
Pivampicillin	ic	NC	NC?	ic?	
Hetacillin	NC to dc	NC to DC	dc to Elim.		ic?
Cyclacillin	NC to dc	NC to DC	dc to Elim.		NC?
Pivmecillinam	ic	dc	dc	NC	ic?
Carbenicillin	dc	NC	Lactobacilli ic		ic?
Dicloxacillin	ic	NC	NC, lactobacilli elim., anaerobic cocci ic		NC
Bacampicillin	NC	NC	NC		NC
Ampicillin + sulbactum	NC	dc	dc		
Cephalosporins and related drugs					
Cephaloglycine	dc	?	dc	NC	NC
Cephalexin	dc	NC	dc	NC to ic	NC
Cefoxitin	dc (Gm−)	dc	dc	NC to IC	NC
Moxalactam	dc to Elim.	dc to Elim.	dc?	ic to IC	NC
Cefoperazone	dc to Elim.	Elim.	Elim.	IC	NC

Ceftriaxone	dc to DC			NC?	IC
Thienamycin + MK 791	dc	NC?	NC	IC	NC
Tetracyclines					
Chlortetracycline	DC to Elim.	dc to Elim.	dc to Elim.	NC to IC	NC
Oxytetracycline	DC to Elim.	dc to Elim.	dc to Elim.	NC to ic	Nc to ic
Tetracycline	NC to ic	NC to Elim.	NC to Elim.	NC	NC
Pyrrolidinomethyltetracycline	NC to ic	NC to Elim.	NC to Elim.	NC	NC
Zicronyl tetracycline	NC to ic	NC to Elim.	NC to Elim.	NC	NC
Doxycycline	NC to dc	NC	NC?	NC to ic	NC
Chloramphenicol	NC to dc	NC	NC?	NC	NC to ic
Erythromycin	NC to DC	NC to DC	NC	NC	NC to ic
Colistin	DC to Elim.	NC	NC?	ic	NC to ic
Ristocetin	NC to ic (Gm−) DC to Elim (Gm+)	dc to Elim.	Elim.	NC	NC
Novobiocin	NC to Elim.	NC to ic	DC to Elim?	NC?	NC?
Aminoglycosides					
Streptomycin	Elim. then reappear	NC	?	NC to ic	NC
Dihydrostreptomycin	NC to DC	?	?	NC	NC to ic
Aminosidin	dc to DC	?	?	Usually NC	Usually NC
Paromomycin	dc to DC	?	dc to Elim.	?	NC to IC
Neomycin	dc to Elim.	NC to Elim.	?	ic?	ic
Kanamycin	Elim.	NC to Elim.	NC (Elim. *Clostridium*)	NC?	NC to IC
Lincosamides					
Lincomycin	DC to IC	Elim.	Elim.	IC	IC
Clindamycin	ic to dc	DC to Elim.	DC	NC to IC	NC to ic
Metronidazole	NC to IC	Usually NC	NC to dc	NC	NC
Fosfomycin	dc	?	?	NC	NC
Furazolidone	NC	NC	NC	NC	NC
Thiostrepton	NC	NC	NC (DC *Clostridium*)	NC	NC to ic
Hexetidine	NC	NC	NC (Elim. *Clostridium*)	NC	NC

TABLE 11-17. *Continued*

Drug	Aerobes and fecultative anaerobes	Gram-negative anaerobes (*Bacteroides*, *Fusobacterium*)	Gram-positive anaerobes	*"Candida"*	"Yeasts"
Chlorquinaldol	NC	NC	NC (DC *Clostridium*)	NC	NC
Bacitracin	NC to IC	?	?	NC	NC
Combinations					
Neomycin + oxytetracycline	Elim.	Elim.	EC to Elim.	IC	ic to IC
Neomycin + sulfathalidine + chloratetracycline	Elim.	Elim.	Elim.	IC	IC
Neomycin + tetracycline + nystatin	DC to Elim.	Elim.	Elim.	IC	?
Neomycin + erythromycin	Elim.	Elim.	Elim.	IC	ic to IC
Neomycin + bacitracin	Usually Elim.	NC?	?	ic?	ic?

*Adapted from Finegold et al. (42). The dosing regiment and references for the above findings are given in the paper of Finegold et al. (42).
dc = minor decrease; DC = major decrease; Elim. = eliminated; ic = minor increase; IC = major increase; NC = no change.

TABLE 11-18. Summary of the effect of antimicrobial and immunosuppressive agents on the implantation and dissemination of *C. albicans* in experimental animal models.

Drug or treatment	Dosage and duration	Effect			Ref.
		Colonization		Dissemination to visceral organs	
		Stomach	Intestine		
Persistent colonization initiated at infancy, then given drug					
Cortisone acetate	1.25 mg IP twice weekly for 2 weeks	Sign. Inc.	Sign. Inc.	NC	64
Cyclophosphamide	0.2 mg/g	Slt. Inc.	Sign. Inc.	NC	
Cyclophosphamide	0.3 mg/g	Sign. Inc.	Sign. Inc.	NC	
Cyclophosphamide	0.4 mg/g	Sign. Inc.	Sign. Inc.	NC	
Cortisone acetate + cyclophosphamide	1.25 mg and 0.10 mg, respectively, twice weekly for 1 week	Sign. Inc.	Sign. Inc.	Sign. Inc.	
Methotrexate	5 μg/g IP thrice weekly for 1 week	Sign. Inc.	Sign. Inc.	Sign. Inc.	
X-irradiation	100 rad	NC	NC	NC	
X-irradiation	400 rad	NC	NC	NC	
X-irradiation	700 rad	Sign. Inc.	Sign. Inc.	NC	
Chloramphenicol	0.75 mg/day PO for 30 days	Sign. Inc.	Sign. Inc.	NC	
Cortisone acetate	1.25 mg/mouse IP 3 times a week for 1–2 weeks		Sign. Inc.		92
Cyclophosphamide	3 mg/mouse IP 3 times a week for 1–2 weeks		Sign. Inc		
Cortisone acetate + cyclophosphamide	Same as above		Sign. Inc.	NC	
	0.01% solution in drinking water or SC at 50 mg/kg twice a day				
Augmentin	Drinking water		Sign. Inc.		
	Subcutaneously		Sign. Inc.		
Cefoperazone	Drinking water		Sign. Inc.		
	Subcutaneously		Sign. Inc.		

TABLE 11-18. *Continued*

Drug or treatment	Dosage and duration	Effect: Colonization: Stomach	Effect: Colonization: Intestine	Effect: Dissemination to visceral organs	Ref.
Ceftazidime pentahydrate	Drinking water		Sign. Inc.		
	Subcutaneously		Sign. Inc.		
Ceftizoxime	Drinking water		Sign. Inc.		
	Subcutaneously		Sign. Inc.		
Ceftriaxone	Drinking water		Sign. Inc.	Sign. Inc.	
	Subcutaneously		Sign. Inc.		
Gentamicin	Drinking water		NC		
	Subcutaneously		NC		
Metronidazole	Drinking water		NC		
	Subcutaneously		NC		
Moxalactam	Drinking water		Sign. Inc.		
	Subcutaneously		Slt. Inc.		
Vancomycin	Drinking water		Slt. Inc.		
	Subcutaneously		NC		
Ceftriakone + cortisone acetate + cyclophosphamide	Same as above			Sign. Inc.	
Adult animal given antibiotic, then *Candida*					
Chlorotetracycline HCl	Dose proportional to the dose a 70-kg man would normally receive		Sign. Inc.		75
Chloromycetin			Sign. Inc.		
Dihydrostreptomycin			Sign. Inc.		
Erythromycin			Slt. Inc.		

Magnamycin		Slt. Inc.		
Neomycin		Slt. Inc.		
Oxytetracycline HCl		Sign. Inc.		
Penicillin		Sign. Inc.		
Tetracycline		Sign. Inc.		
X-irradiation	400 rad for 24 hr	Slt. Inc.		157
X-irradiation + kanamycin	50 mg/kg/day	Slt. Inc.		
Vancomycin	50 mg/kg/day	Sign. Inc.		
Kanamycin + vancomycin	50 mg/kg/day for both drugs	Sign. Inc.		
Gentamicin	25 mg/kg/day	NC		
Toloramycin	25 mg/kg/day	NC		
Tetracycline	1 mg/ml in drinking water	Sign. Inc.		70
Aminobenzylpenicillin + X-irradiation	30 mg/mouse + 600 rad	Sign. Inc.		160
Aminobenzylpenicillin + dexamethasone	30 mg/mouse + 200 μg/mouse	Sign. Inc.		
Aminobenzylpenicillin + X-irradiation + dexamethasone	30 mg/mouse + 600 rad + 200 μg/mouse	Sign. Inc.		
Gentamicin	20 mg/kg/day SC for 24 hr		NC	170
Polymyxin B	50 mg/liter in drinking water for 48 hr		NC	
Cytarabine	25 mg/kg per dose IP at 3-hr intervals for 8 doses		NC	
Gentamicin + polymyxin B + cytarabine	As above		Sign. Inc.	
Vancomycin + ampicillin + gentamicin	500 μg/ml, 1 mg/ml, 100 μg/ml, respectively, in drinking water for 3 days	Sign. Inc.	Sign. Inc.	88

TABLE 11-18. *Continued*

Drug or treatment	Dosage and duration	Effect			Ref.
		Colonization		Dissemination to visceral organs	
		Stomach	Intestine		
Carbenicillin + gentamicin	400 mg/kg/day + 2 mg/kg/day, respectively, IM for 3 days	Sign. Inc.	Sign. Inc.	NC	111
Cyclophosphamide	100 mg/kg IP followed by a second IP inoculation of 75 μg/kg	Slt. Inc.	Slt. Inc.	NC	
Carbenicillin + gentamicin + cyclophosphamide	As above	Sign. Inc.	Sign. Inc.	Slt. Inc.	
Carbenicillin + gentamicin + cyclophosphamide + cortisone acetate	As above			Slt. Inc.	
Erythromycin	1% in food pellets, given ad libitum		NC		2
Clindamycin	500 μ/ml ad libitum in drinking water for		Sign. Inc.	Sign. Inc.	90
Erythromycin	200 μ/ml 3 days		NC	NC	
Gentamicin	100 μg/ml		NC	NC	
Penicillin G	500 μg/ml		Sign. Inc.	Sign. Inc.	
Vancomycin	500 μg/ml		Sign. Inc.	Sign. Inc.	
Vancomycin + ampicillin + gentamicin	500 μg/ml, 1 mg/ml, & 100 μg/ml, respectively, in drinking water for 3 days		Sign. Inc.	Sign. Inc.	89
Penicillin G	500 units/ml in drinking water for 3 days		Sign. Inc.	Sign. Inc.	
Penicillin + tetracycline	0.3 mg/ml + 1 mg/ml, respectively, in drinking water for 3 days	Slt. to Sign. Inc.		NC to Slt. Inc.	32

Trimethoprim + sulfamethoxazole	~0.1 mg/mouse + ~0.46 mg/mouse, respectively, in drinking water for 2 days	NC to Slt. Inc.		NC to Slt. Inc.	
Clindamycin	0.24 mg/ml in drinking water for 3 days		Sign. Inc.		40
Chloramphenicol	0.5 mg/ml in drinking water		Slt. Inc.		
Gentamicin	0.1 mg/ml in drinking water		Slt. Inc.		
Tetracycline	1 mg/ml in drinking water		Sign. Inc.		
Trimethoprim-sulfamethoxazole	0.06 mg/ml in drinking water		NC		
Vancomycin	0.2 mg/ml in drinking water		NC		
Clindamycin + gentamicin	As above		Sign. Inc.	Slt. Inc.	
Cortisone acetate	1 mg/mouse/day 2–3 times per week		NC		
Cyclophosphamide	3 mg/mouse/day 2–3 times per week		Sign. Inc.		
Methotrexate	0.15 mg/mouse/day 2–3 times per week		NC		
Clindamycin + gentamicin + cyclophosphamide	As above		Sign. Inc.	Sign. Inc.	
Clindamycin + gentamicin + methotrexate	As above		Sign. Inc.	Sign. Inc.	
Clindamycin + gentamicin + prednisolone	As above		Sign. Inc.	Sign. Inc.	
Cyclophosphamide + cortisone	As above		Sign. Inc.		
Methotrexate	As above		NC		

NC = no change; Sign. Inc. = significant increase; Slt. Inc. = slight increase. If left blank, data were not given in the reference cited. Note that some strains of *C. albicans* are unable to disseminate from the gut to visceral organs, even when high GI populations of the organism colonize the gut (Kennedy, unpublished data).

tables that several, but not all, antibacterial agents can predispose individuals to an increase in *Candida* colonization and population size. It is also clear that, of the drugs that do so, some render the host more susceptible than others.

Although it has generally been assumed that all antibacterial agents possess the ability to eliminate the microbial resistance barrier to *Candida* colonization, Tables 11-17 and 11-18 indicate that it is clearly not the case. Odds (118) has further noted that although it is often considered that "broad-spectrum" antibacterial agents are more likely to lead to *Candida* overgrowth than drugs with a more limited range of bacterial targets, it appears that drugs active against gram-negative anaerobes (e.g., clindamycin, metronidazole) are particularly effective in promoting *Candida* overgrowth. Therefore although many antimicrobics may enhance *C. albicans* carriage, those drugs that are active against specific components of the microflora (e.g., *Bacteroides* spp.) or a broad range of the anaerobic bacterial population significantly increase *Candida* colonization in the intestines (118), followed by antimicrobics that affect broad spectrum of both anaerobic and facultatively anaerobic bacteria in general (42). According to the literature, antimicrobics active against only facultative anaerobes or aerobes appear to have little or no effect on *Candida* carriage (Tables 11-17 and 11-18).

Studies with animal models also incriminate intestinal anaerobes as the main components of the intestinal microflora that suppress colonization and systemic spread by *C. albicans* (40,90). One study detailed the effects of various antibiotics on the indigenous microflora, the GI environment, the ability of *C. albicans* to associate with intestinal mucosal surfaces, as well as colonize and disseminate from the GI tract to visceral organs in mice (90). The effects of various antibiotics on the indigenous cecal microflora from that study are summarized in Table 11-19. Aerobic and anaerobic cultures of cecal contents and walls from mice treated with penicillin G or clindamycin, for example, revealed that the total anaerobic populations in the ceca were reduced by an average of 100-fold, whereas the enteric bacilli increased by an average of 10,000-fold ($p<0.001$). Animals treated with vancomycin also had lower total anaerobic population levels than conventional mice, with a concomitant increase in the facultative bacilli (e.g., *E. coli*) populations. For these test animals, counts of strictly anaerobic bacterial population levels were reduced by an average of ten fold, and the facultative bacilli populations were increased by an average of 10,000-fold ($p<0.001$). Dissection of all three groups of mice showed "enlarged" ceca (Table 11-20), a trait attributable to mice lacking a complex anaerobic microfiora (51,77,139,153). Aerobic and anaerobic cultures of intestinal contents from mice treated with gentamicin or erythromycin, in contrast, revealed that the total numbers of facultative and aerobic bacteria were significantly reduced, with no change in the total anaerobic population. Dissection of these mice showed ceca that grossly had a normal appearance (Table 11-20).

TABLE 11-19. Population levels of indigenous bacteria and *C. albicans* in ceca and visceral organs of untreated and antimicrobic-treated animals.*

Antimicrobic treatment†	Contents			Wall			Visceral organs
	Enteric bacilli	Anaerobes	*Candida*	Enteric bacilli	Anaerobes	*Candida*	*Candida*
None	4.92 ± 0.24‡	9.90 ± 0.10	3.26 ± 0.08	4.4 ± 0.22	10.3 ± 0.87	2.33 ± 0.37	0
Erythromycin	2.15 ± 0.26	9.83 ± 0.52	3.44 ± 0.79	ND	ND	ND	0
Gentamicin	5.20 ± 0.24	9.91 ± 0.16	3.52 ± 0.18	4.90 ± 0.49	9.90 ± 0.22	2.41 ± 0.42	0
Vancomycin	9.31 ± 0.90	8.90 ± 0.11	6.90 ± 0.17	7.60 ± 0.56	9.01 ± 0.15	5.07 ± 0.41	2.60 ± 0.10 (5/10)§
Penicillin G	9.70 ± 0.31	7.60 ± 0.12	7.38 ± 0.18	8.50 ± 0.67	8.2 ± 0.43	5.50 ± 0.31	2.06 ± 1.17 (6/10)
Clindamycin	9.50 ± 0.26	8.10 ± 0.27	7.44 ± 0.40	8.8 ± 0.53	8.3 ± 0.23	5.20 ± 0.25	9.95 ± 1.55 (8/10)

* From Kennedy and Volz (90).
† Mice received 3 days of antibiotic treatment before yeast inoculation.
‡ Log_{10} mean CFU/g organ ± 1 SD.
§ Number of mice with yeast cultured from visceral organs/number of animals tested.
0 = undetectable; ND = not determined.

TABLE 11-20. Cecal characteristics from antimicrobic-treated and control animals.*

Antimicrobic treatment†	Gram Stain‡	Predominant bacterial organisms	Consistency of cecal contents	Cecal size (% of body wt.)
None	+	Gm− rods§	Thick & pasty	1–2
Erythromycin	+	Gm− rods	Thick & pasty	1–2
Gentamicin	+	Gm− rods	Thick & pasty	1–2
Vancomycin	−	Large, Gm+ rods Small, Gm− rods	Soft	4–8
Penicillin G	−	Gm+ cocci and small Gm− rods	Soft	5–10
Clindamycin	−	Gm+ cocci and small Gm− rods	Soft	5–10

*From Kennedy and Volz (90).
†Mice received 3 days of antibiotic treatment.
‡Gram stains: − = abnormal; + = normal appearing.
§Predominant organisms seen on gram-stained smears.

From the same study it was also found that Gram stains of cecal contents from mice given clindamycin or penicillin G showed a drastic reduction in the numbers of "typical" fusiform and gram-negative rods (Table 11-20). The predominant bacteria from clindamycin-treated and penicillin-treated animals were gram-positive cocci and some small gram-negative rods, whereas vancomycin-treated animals had a cecal flora with some gram-negative and large gram-positive rods (Table 11-20). Gram stains of cecal contents from erythromycin-treated and gentamicin-treated animals showed a bacterial flora resembling that of untreated controls (Table 11-20).

The ability of *C. albicans* to opportunistically colonize and disseminate from the GI tracts of mice treated with various antibiotics is also summarized in Table 11-19. Mice treated with clindamycin, penicillin G, or vancomycin had high luminal and wall populations of *C. albicans*, and viable *Candida* were recovered from the visceral organs of 80%, 60%, and 50% of the animals, respectively. Animals treated with gentamicin or erythromycin and challenged with *C. albicans*, on the other hand, had significantly lower gut populations of yeast, and *Candida* cells could not be cultured from systemic organs. Likewise, visceral organs from control animals not given antibiotics were also found to be culture-negative for *C. albicans*, although low numbers of viable *C. albicans* were present in the GI tract (Table 11-19).

Each of the antimicrobics that predisposed animals to *Candida* colonization and dissemination from the GI tract produced characteristic disruptions of the ecology of the indigenous intestinal microflora. These changes can be summarized as follows: (a) total anaerobe population levels in cecal contents and on mucosal surfaces were significantly reduced; (b) enteric

bacilli population levels were significantly increased on mucosal surfaces and in cecal contents; and (c) high numbers of viable *C. albicans* were maintained in the intestinal contents and on host mucosal surfaces. Thus clindamycin, penicillin G, and vancomycin, but not gentamicin or erythromycin, disrupted the ecology of the indigenous microflora and decreased the numbers of strictly anaerobic bacteria, which allowed *C. albicans* to proliferate in the gut—reaching the hypothesized "threshold" yeast population levels required for fungal dissemination from the GI tract (118)—and spread systemically to visceral organs.

Treatment of mice with antimicrobics active against specific members of the indigenous intestinal microflora therefore suggests that it is the strict anaerobic bacteria (90) and not the facultative bacilli or aerobic bacteria that are responsible for the resistance of gut colonization and dissemination by *C. albicans*, as has been suggested previously (88–90). Treatment of mice with clindamycin, penicillin G, or vancomycin decreased the total populations of strictly anaerobic bacteria in the cecum, allowed an increased in the total facultative anaerobic and aerobic bacterial population levels, and allowed *C. albicans* to colonize and disseminate from the GI tract. Oral treatment with gentamicin or erythromycin, on the other hand, decreased populations of aerobic bacteria and facultative anaerobes, respectively, but did not affect the number of strictly anaerobic bacteria indigenous to the mouse cecum. Gentamicin and erythormycin treatment did not allow *C. albicans* to colonize or disseminate from the GI tract. These results are consistent with those reported previously by other laboratories, which showed that treatment of mice with gentamicin or erythromycin did not predispose mice to gut colonization or dissemination by *C. albicans* (2,28,170). Furthermore, several authors have observed an increase in the number of facultative anaerobes in the intestinal or fecal flora of animals and man treated with penicillin or clindamycin (14,65,123). Berg (14), for instance, found that treatment of mice with oral penicillin or clindamycin decreased the number of certain anaerobic bacteria in the GI tract and allowed *E. coli* and other enteric bacteria to overpopulate the ceca and disseminate to the mesenteric lymph nodes and other organs. Thus antagonism of the cecal population levels of *E. coli* by the strictly anaerobic bacteria that predominate in the gut ecosystem (51,153) appears also to be an important defense mechanism confining *E. coli* to the GI tract (13,14). Several studies suggest that the anaerobic bacteria of the indigenous intestinal microflora control *E. coli* population levles in the gut (51,95,153). Apparently, then, the strains of anaerobic bacteria (possibly using analogous mechanisms) that antagonize *E. coli* and other facultative anaerobes also antagonize *Candida* organisms in the GI tract.

Other evidence supports this hypothesis as well. For instance, it has been shown (41,69,127) that *C. albicans* disseminated from the GI tracts of 5- to 6-day-old infant mice and that stable *Candida* populations of 10^4 were maintained in the cecum for about the first 2 weeks of life (41). Thereafter

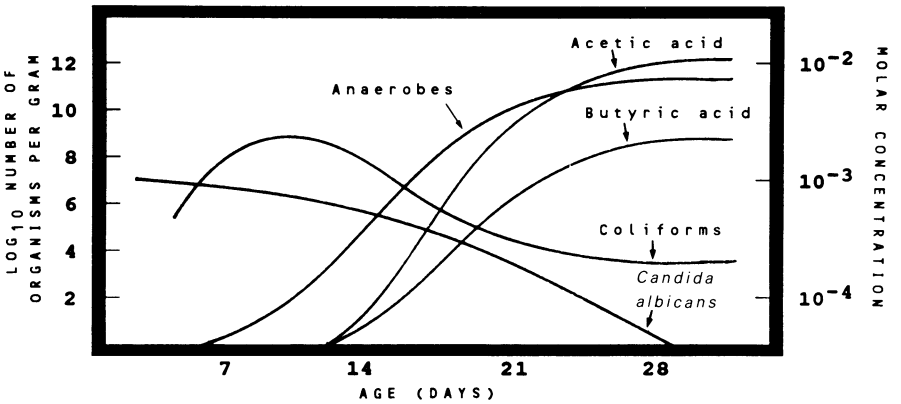

FIG. 11-7. Population dynamics of *Candida albicans* in the mouse cecum during ecological succession from infant to adult showing the population sizes of indigenous bacteria. (Data from refs. 37,41,140,141.)

Candida populations declined to undetectable levels by 4 weeks (41). The timing of elimination of *Candida* organisms in mice inoculated at infancy, then, coincides with the acquisition of intestinal anaerobes (27,141) (Figure 11-7). Lee and Gemmelle (95) found a similar relation between the decline in the number of facultative bacilli and the appearance of the strictly anaerobic bacteria that occurs in the ceca of mice at 2–3 weeks after birth. It is not surprising, as strictly anaerobic bacteria have been shown to be numerically dominant in the gut (34,51,108,141) and are the organisms that control the composition of the intestinal microflora itself (52,153). There apparently is also a correlation between the counts of *C. albicans* and strict anaerobes in the GI tract. It can be inferred by the statements above that *C. albicans* counts decrease from the stomach to the colon and that the counts of strict anaerobes increase from the stomach to the colon. Counts of *C. albicans* again peak at the rectum, whereas the number of oxygen-sensitive anaerobes decreases. Moreover, resistance to colonization by *C. albicans* in one patient appeared to depend largely on antagonism by the anaerobic portion of the intestinal microflora (162). In this patient, fecal counts of *C. albicans* exceeded 10^8 cells per gram for as long as the patient was on antimicrobic therapy. Only after stopping all antimicrobics, except for amphotericin B, and implanting an anaerobic microflora in the gut was *C. albicans* suppressed and seemed to "disappear" (fecal cultures and oral swabs became negative) (162).

Finally, the mechanisms by which the intestinal microflora antagonizes *C. albicans*, as described above, also implicate the strict anaerobic portion of the intestinal microbiota as the main agents responsible for resistance to colonization and systemic spread of *Candida* from the GI tract (85,86). For instance, hydrogen sulfide and short-chain fatty acids (which inhibit growth

and block adhesion) are produced by the metabolic activity of the strict anaerobes. Likewise, the numerically dominant mucosa-associated bacteria, which inhibit mucosal association by *Candida*, are probably strict anaerobes (89–91). Several genera and species of bacteria that may be involved with the suppression of *Candida* in the gut are listed in Table 11-21. It should be noted that these data do not rule out the possibility that certain facultative anaerobes (e.g., Enterobacteriaceae) that are a common component of the GI microflora, albeit in significantly lower numbers, may still exert some resistance to colonization by *C. albicans*. For example, such bacteria could compete with *Candida* for limiting adhesion sites when the microflora is intact and functioning properly (89). Therefore although several inhibitory mechanisms produced by the indigenous anaerobic microflora probably act collectively to suppress gut colonization and dissemination by *Candida*, studies to date cannot rule out the possibility that other components of the microflora may superimpose other secondary inhibitory mechanisms. Consequently, the importance of a single inhibitory mechanism should not be underestimated, as it may well supply that extra increment of resistance that is responsible for protection of the host to colonization and dissemination from the GI tract by *C. albicans* and other fungi. To that end, further studies conducted in gnotobiotic mice containing hundreds of well characterized species of bacteria, similar to the in-depth studies by Freter's group (51,52,54,56,153), are necessary to confirm or reject these hypotheses.

Interactions at Gastrointestinal Mucosal Surfaces

The association of *C. albicans* with GI mucosal surfaces is likely to play an important role in colonization and pathogenesis of GI and systemic *Candida* infections. For example, although the dynamics of luminal and wall-associated *Candida* populations have not been completely defined, studies have shown that in the large intestine only a small percentage of the total *Candida* population was associated with the gut wall early after challenge (91). Thereafter, however, as the intestinal microflora reestablished itself, mucosal *Candida* populations predominated (86). It seems likely, therefore, that mucosal association probably prevents *Candida* cells from being "washed out" of the GI tract due to antagonism by intestinal bacteria and the passage of material due to peristalsis. Considering further that the metabolic activity of the indigenous microflora causes *C. albicans* and other fungi to have a doubling time that can be significantly longer than this dilution rate (86) and that a significant lag phase is imparted to fungi upon entering the GI tract (86) suggests that mucosal association would not just simply facilitate survival in this harsh ecosystem but that it may actually be necessary for long-term implantation to occur (83). Likewise, passage of

TABLE 11-21. Indigenous intestinal bacteria of man that may play a role in the regulation of *Candida* populations in the gut.*

Gram-positive	Gram-negative
Cocci	Cocci
Peptococcus magnus	*Acidaminococcus fermentans*
Peptococcus prevotii	*Megasphaera elsdenii*
Peptostreptococcus productus	*Veillonella parvula*
Streptococcus constellatus	Rods
Streptococcus intermedius	*Bacteroides asaccharolyticus*
Streptococcus morbillorum	*Bacteroides capillous*
Rods	*Bacteroides distasonis*
Bifidobacterium adolescentis	*Bacteroides fragilis*
Bifidobacterium angulatum	*Bacteroides hypermegas*
Bifidobacterium bifidum	*Bacteroides melaninogenicus*
Bifidobacterium breve	*Bacteroides multiacidus*
Bifidobacterium catenulatum	*Bacteroides oralis*
Bifidobacterium cornutum	*Bacteroides ovatus*
Bifidobacterium dentium	*Bacteroides praeacutus*
Bifidobacterium infantis	*Bacteroides putredinis*
Bifidobacterium longum	*Bacteroides ruminicola*
Bifidobacterium pseudolongum	*Bacteroides splanchnicus*
Clostridium beijerinckii	*Bacteroides thetaiotaomicron*
Clostridium butyricum	*Bacteroides vulgatus*
Clostridium cadaveris	*Butyrivibrio crossotus*
Clostridium celatum	*Butyrivibrio fibrisolvens*
Clostridium clostridiiforme	*Desulfomonas pigra*
Clostridium innocuum	*Fusobacterium mortiferum*
Clostridium leptum	*Fusobacterium mortiferum*
Clostridium malenominatum	*Fusobacterium naviforme*
Clostridium nexile	*Fusobacterium necrogenes*
Clostridium paraputrificum	*Fusobacterium nucleatum*
Clostridium perfringens	*Fusobacterium planti*
Clostridium ramosum	*Fusobacterium prausnitzii*
Clostridium tertium	*Fusobacterium russii*
Eubacterium aerofaciens	*Fusobacterium symbiosum*
Eubacterium contortum	*Fusobacterium varium*
Eubacterium cylindroides	
Eubacterium limosum	
Lachnospira multiparus	
Propionibacterium acnes	
Propionibacterium granulosum	
Propionibacterium jensenii	

*Species of bacteria (72,152) found to produce one or more known inhibitors for *C. albicans*. It is significant to note that more than 99% of these bacteria, are strict anaerobes.

viable *Candida* organisms through the small intestinal mucosa to initiate systemic infection by the hematogenous route (111,112) could not take place in the face of the rapid passage of material through the small intestine (53,54) from which dissemination is thought to occur (83,86,91), unless these organisms could associate with the mucosa. Several other arguments could also be given to support the role of mucosal association in colonization and dissemination from the GI tract. Nevertheless, the above list does implicate the ecological and pathological importance of mucosal association and colonization. A previous review (82) included a detailed discussion on the molecular bases and physiochemical factors involved in the adhesion and association of *C. albicans* with mucosal surfaces. Therefore the discussion to follow makes no attempt to present an exhaustive biochemical review of *Candida* adhesion mechanisms but, instead, briefly describe adhesion and association mechanisms as they relate to mucosal colonization. In addition, the mechanisms that may allow *C. albicans* to pass through or penetrate intestinal mucosa are considered, as are certain interactions between *Candida* and mucosal bacteria.

Mechanisms of Adhesion and Association with GI Mucosa

The adhesion and association of *C. albicans* with GI mucosa has been examined in a number of in vitro and in vivo models (82). Such studies have included adhesion to tissue slices, isolated epithelial cells or mucus, and the ultrastructural characterization of attachment to and penetration of GI mucosal surfaces as it occurs in experimental animals. Similar studies have been performed on clinical specimens obtained from infected patients, although such studies have been limited and have focused primarily on diagnosis of infection and on upper portions of the alimentary tract (59, 79,105,106). Most studies, regardless of whether in experimental animals or patients, have involved examination of infected tissues by scanning (SEM) and transmission (TEM) electron microscopy, stained histological section, and quantitative methods to determine *C. albicans* population levels (63,82).

Animal models used to study colonization of the GI tract have yielded the most information on the adhesion and association of *C. albicans* with GI mucosa and have confirmed clinical findings that *Candida* can associte with the mucosa of all regions of the GI tract (7,26,41,89,91,125–127). A comparison of studies using experimental animals indicates that different preferential sites of colonization, as well as differences in *Candida* population dynamics, exist between various models of GI colonization by *C. albicans* (e.g., 7,91,125–127). Factors that can affect *Candida* gut colonization and mucosal association include age, species and strain of the animals, diet, microbiological and immunological status of the animal, if the animal is given compromising agents (e.g., antibiotics or immunosuppressive

TABLE 11-22. Factors affecting study of the survival, implantation, and dissemination of *Candida* species from the GI tract.

Factors	Ref.
Yeast factors	
Species and strain	41,88,125,169,170
Growth phase	Kennedy, unpublished data
Culture medium and growth conditions	Kennedy, unpublished data
"Cell type" and "phenotype"	Kennedy, unpublished data
Host factors	
Animal age	9,41,64
Animal species	89,90
Animal supplier	75
Animal strain	32,75,91
Diet	19
Immunological or physiological status	5,7,11,28,40,64,70,92,98,99,111,113, 116,170
Indigenous yeast present	4,39
Microbiological status	2,4–11,24,28,40,64,67–71,73–75, 78,88–92,99,111,113,114,116,119, 122,133,169
Experimental factors	
Diet and water preparation and availability to animal	19
Housing conditions	40

drugs), and the *Candida* strain, phenotype, and challenge dose (7,41,89–91,125–127). A complete list of factors that can influence the study of GI colonization and mucosal association by *Candida* is given in Table 11-22. Nevertheless, such studies have revealed that *Candida* organisms can associate with and attach to GI mucosal surfaces by several distinct mechanisms (91). As is summarized in Table 11-23, they include both adhesive and noadhesive, as well as direct and indirect, mechanisms.

Examination of stomach mucosa obtained from infant mice early after inoculation with *C. albicans* revealed that large numbers of *Candida* cells were attached to both keratinized and suamous epithelia (7,125,126). At later times, *Candida* cells were observed to be adherent to these surfaces but were also found to be embedded in and surrounded by mucus. Depending on the strain of *C. albicans* and the method used to prepare the inoculum for animal challenge, hyphal invasion of the keratinized region of the stomach can also occur in both infant and adult animals (7,41,125–127). Balish and co-workers (7), for instance, noted both yeast and hyphae in the cardial-atrium section of the stomach of adult gnotobiotic mice that had been monoassociated with *C. albicans*. This site appeared to be the preferential site of colonization and occasionally was observed to be the sole colonization and invasion site by *C. albicans* in the stomach (7). This

TABLE 11-23. Adhesion and association mechanisms by which *C. albicans* can "attach" to GI mucosal surfaces.*

Mechanism	Nature of mechanism			Ref.
	Active or positive	Specific or nonspecific	Direct or indirect	
Adhesin–receptor interaction	A	S	D,I	26,89,91
Nonspecific adhesion	A,P	N	D,I	91
Coadhesion to adherent organisms	A	S,N	I	89,91,125,126
Entrapment in mucus and tissue	P	N	D	89,91,126,127
Germ-tube penetration	A	N,S	D,I	7,9,70,79,98,99,122
Enzymatic digestion	A	S,N	D,I	26,91

*Modified from Kennedy (82).

finding is in agreement with studies using other animal models (41,125–127) and suggests that the cardial-atrium ridge may contain an abundant number of receptors, which causes *C. albicans* to colonize this region of the stomach preferentially (7).

Similar examination of small intestinal, cecal, and colonica mucosa by SEM and TEM has revealed that *Candida* cells also attach to and associate with these surfaces by several distinct mechanisms (91). Pope, Cole, and co-worker (125,126), for example, used an infant mouse model of GI candidiasis and reported that large numbers of *C. albicans* were clearly visible on the surface of villi. A closer examination revealed that *Candida* cells were adherent to the epithelium. In other studies using antimicrobic-treated adult mice, *Candida* cells were observed to be attached directly to the epithelium, probably by adhesion to the epithelial glycocalyx (91). Both studies, however, showed that *C. albicans* may attach to the epithelium via the epithelial glycocalyx, and that many yeast cells were seen frequently in association with mucus and appeared to be attached to, embedded in, and covered by a layer of mucus (Fig. 11-8).

It was also found that *C. albicans* associated with intestinal mucosa indirectly by attachment to other adherent organisms (91). Histological sections taken at early times after orointragastric inoculation of infant mice, for instance, showed that some yeast cells were associating with mucosa by attaching to yeast cells adherent to the epithelium (125,126). It was also observed in antimicrobic-treated adult animals challenged with *C. albicans* (91), but in these studies *Candida* cells were also observed associating with

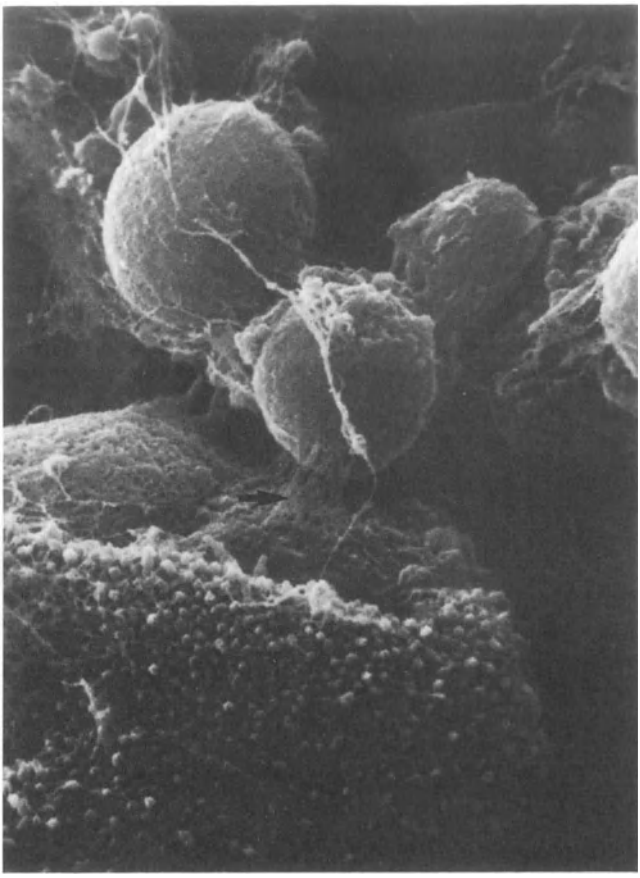

FIG. 11-8. SEM micrograph of cecal mucosa from an adult penicillin-treated mouse 72 hours after oral-intragastric inoculation with *Candida albicans* showing yeast associated with the epithelium, possibly by adhesion to the epithelial glycocalyx (arrow). (×10,000) (From Kennedy et al. [91].)

the mucosa by coadhesion to adherent bacteria. The possible importance of such interactions is apparent in Figure 11-9, which shows the establishment of microcolonies in the mucosa at 72 hours after oral challenge. If such microcolonies are formed in man, it may help to explain why isolated plaques are often seen on endoscopic or radiological examination of intestinal mucosa infected with *C. albicans* (79). Another mechanism by which *C. albicans* can associate with intestinal mucosa may also be important in plaque formation and may facilitate the penetration and invasion of *Candida* cells through the mucosa; it is the enzymatic lysis or digestion of mucus and the epithelium that has been observed in both infant and adult mice (26,91). Not only could this mechanism represent a unique association

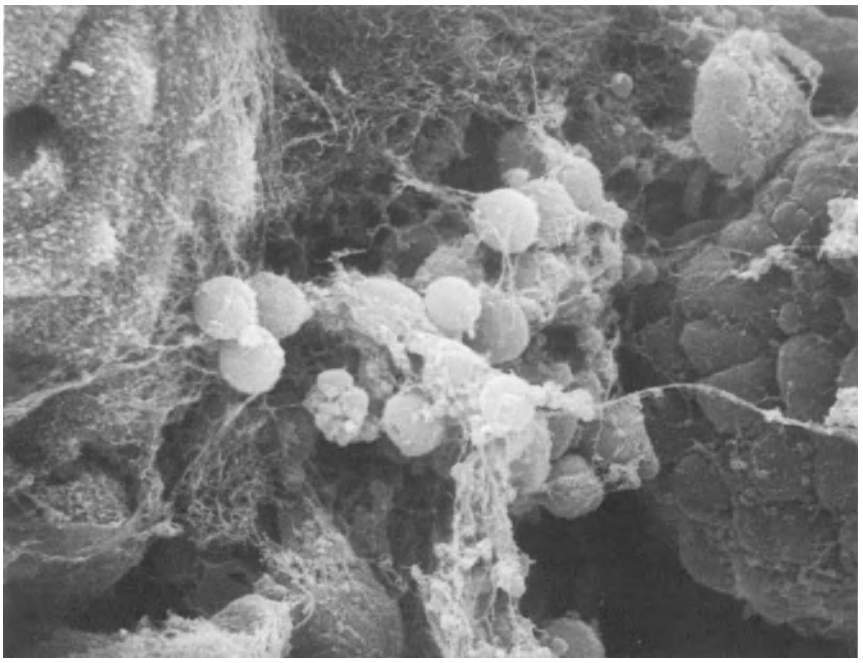

FIG. 11-9. SEM micrograph of cecal mucosa from an adult conventional mouse treated with penicillin 72 hours after oral-intragastric challenge with *Candida albicans* showing a microcolony on the mucosal surface. Note that some yeast cells are attached to the surface, whereas others are attached to adherent yeast. (×3,000) (From Kennedy et al. [91].)

mechanism in that it could retard the removal of *Candida* from the GI tract due to peristalsis, but enzymatic penetration of the epithelium may represent a distinct mechanism by which *Candida* can disseminate from the GI tract to other organs.

It is apparent from the data discussed above that in the GI tract it is possible to define three ecological sites for *C. albicans*, including the intestinal contents, the mucus gel, and the epithelium proper, which itself may contain three microsites (cells indirectly attached to the epithelium, cells directly attached to the epithelium, and cells penetrating the epithelium) (91). As noted already, the ecology of *C. albicans* gut colonization and the dynamics of luminal and mucosa-associated *Candida* populations have not been completely defined. Nevertheless, the data presented above, together with other reports on mucosal association by bacteria (46), emphasize the importance of three distinct steps in the association and passage of *C. albicans* and other fungi through intestinal mucosa: (a) contact with the surface of the mucus gel; (b) adhesion to and penetration or trapping in the mucus gel; and (c) adhesion to and penetration of the epithelial surface.

Mechanisms of Mucosal Invasion

The first step in mucosal association (and passage through the mucosa) by *C. albicans* is probably entirely random, whereas the latter steps are not in that they may involve the interaction of specific adhesin–receptor binding, enzymatic modification of the mucus gel and epithelial surface, or both for attachment to and penetration of intestinal mucosal surfaces by *C. albicans* (91). Ultrastructural examination of mucosal invasion in the GI tract of infant mice confirmed this view and showed that *C. albicans* yeast cells were apparently capable of progressive extracellular digestion of the intestinal mucus gel and microvillous layer (26). It was followed by invasion of columnar epithelial cells and the appearance of intraepithelial yeast cells in "vacuoles." Yeast cells were evident within the cytoplasm of the host cells within 3 hours, and no continuous membrane was noted at the interface between this vacuole-like space and host cytoplasm (26). Figure 11-10 shows this process in the jejunum of an infant mouse 3 hours after intragastric inoculation with *C. albicans*. It is interesting to note that germ tubes of hyphae were not observed in association with the mucosa of the small or large intestine in these or any other studies to date. Thus the mechanism operating in Figure 11-10 may well represent the primary method of invasion in the GI tract at regions below the stomach and may be similar to that observed for penetration of *C. albicans* into endothelial tissue (82).

Nonetheless, penetration of stomach mucosa by germ tubes may also play an important role in the systemic spread from the GI tract. Hyphal invasion of the keratinized region of the stomach, and the cardial-atrium ridge in particular, has been observed in both infant and adult animals (7,70,125,126). Although penetration of stomach mucosa by *Candida* has not been examined in detail, ultrastructural characterization indicates that the porcess may be similar to that for penetration of oral mucosa (82). Contact between *C. albicans* germ tubes and the epithelium in the latter case have shown that no alteration of the epithelial cell surface was noted either at the point of entry or as hyphae grew along the surface (82). These findings indicate that if enzymatic lysis is associated with the invasive process of hyphae, it is probably localized to the hyphae tips. Other reports support the role of hydrolytic enzyme activity in cell invasion of *Candida* blastoconidia and germ tubes or hyphae, and a number of proteinases and phospholipases have been implicated (82).

Neither of the aforementioned invasive mechanisms, however, can account for the rapid "persorption" of *Candida* from the GI tract that has been observed in man, dogs, monkeys, and infant mice (41,88,94,125–127). In all of these cases, *Candida* cells were recovered from the blood or systemic organs in large numbers within less than 2 hours. Therefore it seems unlikely that large numbers of *Candida* cells could be cleared from the stomach, associate with the jejunal mucosa, and degrade the mucus gel and microvillous layer to invade and pass through epithelial cells in

(a)

(b)

(c)

FIG. 11-10. TEM micrographs of thin sections of jejunal mucosa showing morphological aspects of mucosal invasion by *Candida albicans* in the infant mouse. (a) Apparent digestion of the microvillous layer. (b,c) Invasion of cytoplasm of epithelial cells and yeast associated with "pockets" in the microvillous layer are intraepithelial. The micrographs represent invasion in the jejunum of infant mice 1 hour (a), and 2 hours (b,c) after oral-intragastric challenge. (From Cole et al. [26].)

this length of time. More unlikely is the possibility that *Candida* cells could germinate, penetrate the epithelium, and generate large numbers of blastoconidia that would reach extraintestinal sites. It suggests that an additional mechanism may be responsible for the rapid dissemination of *Candida* from the gut as was noted in the studies mentioned above. One possibility is the paracellular passage of yeast cells that is apparently more frequent in the desquamation zones of the intestinal epithelium (63). This process has been observed by early workers for both starch granules that had been ingested by humans or rats (63) or in dogs that had been injected intraduodenally with large numbers of Fleischmann's yeasts (44). Appropriate controls and precise inoculation methods ruled out the possibility of aspiration as the means of *Candida* dissemination in these studies (63).

Apparently, this type of dissemination is regulated by the population level of yeast cells in the gut (88). For instance, significantly lower numbers of viable yeast cells were present in the GI tracts of animals that did not show signs of systemic spread to visceral organs compared to animals that did (88–90). Thus a "threshold" yeast population level appears to be a determinant for "passive" fungal dissemination (88,89). With long-term colonization of the GI tract, then, either germ-tube penetration or invasion by blastoconidia as the result of the activity of extracellular, hydrolytic enzymes may be the predominant forms of systemic spread. Odds (118) has noted antibodies to *Candida* proteinase(s) in both healthy individuals harboring *Candida* in their GI tract and patients with systemic candidiasis. It may be that *C. albicans* regularly and continually invades the bloodstream by one or both of these mechanisms from one or more sites along the alimentary tract (including the mouth and esophagus), similar to that of indigenous bacteria (50). This possibility lends support to the importance of bacterial antagonism in controlling *Candida* populations in the gut, which would keep yeasts that spread systemically at numbers that are manageable by the individual's immune system (132). This finding is consistent with those of Wingard et al. (170), who reported that treatment of mice with a combination of antimicrobics and cytarabine significantly increased the incidence of systemic spread by *C. albicans* and *C. tropicalis*.

Toward a General Theory to Explain the Regulation of *Candida* Populations in the GI Tract

Survival and implantation of *C. albicans* in the GI tract may be influenced by a number of factors, but the presence of an intact indigenous microflora is probably the most important determinent as to whether *Candida* can colonize the gut (86). The results described and discussed in this review strongly support this hypothesis and suggest that regulation of *Candida* populations in the GI tract is the result of a complex series of interactions

controlled by a complex indigenous microflora. This explanation stresses the importance of using experimental models that closely simulate the ecosystem (e.g., stomach, small or large intestine) and process (e.g., colonization or mucosal invasion) under study. The most convincing argument comes from the finding that more than one mechanism was found to suppress *C. albicans* in the large intestine. Studies in which a monoflora of *E. coli* (or any other single bacterial species) antagonized *C. albicans* in the gut of gnotobiotic animals (8,73,74,113,114,128) or similar studies performed in vitro (74,76,93,120,121) should be interpreted cautiously. Nevertheless, data from such studies together with more recent findings (85,86,89) indicate, as was alluded to above, that the indigenous microflora inhibits *Candida* colonization by two general mechanisms: (a) antagonizing and suppressing the growth of *C. albicans* in the gut, and (b) inhibiting the association of *C. albicans* with the GI mucosa.

The data described here concerning the passage of *C. albicans* through CF cultures and the growth of *Candida* in CF culture effluent, for instance, showed that the intestinal microflora caused *C. albicans* to remain in a prolonged lag phase (apparently up to several days) as well as to have a long doubling time. Furthermore, when *C. albicans* was inoculated into CF cultures of human fecal flora, the yeast cells were removed with the flow rate, whereas in sterile controls *Candida* cells maintained a steady population of about 10^4 cells ml^{-1}. Discussions in the literature of the possible inhibitory mechanisms involved with the suppression of *Candida* in the gut have suggested that anaerobiosis may be important (76,121). However, the anaerobic condition was only partially inhibitory to growth of *C. albicans* and did not prolong the lag phase as did CF culture filtrates, indicating that other inhibitory mechanisms also suppress the growth of *C. albicans*. The inhibitory effect on *Candida* growth in CF culture filtrates, moreover, could not be reversed by adding carbon or nitrogen sources, vitamins, or trace nutrients (individually or collectively) to CF culture filtrates and incubating the cultures (under varying pH levels) aerobically. Thus suppression of *C. albicans* in CF cultures was apparently not due solely to depletion of nutrients and anaerobiosis but appeared to be due to a combination of these factors in addition to the production of inhibitory substances by intestinal bacteria.

To begin to examine other factors that might be involved in the suppression of *Candida* growth, certain metabolic endproducts from the predominant anaerobic microflora were introduced into sterile CF culture medium, and the growth of *C. albicans* was monitored. It was found that both the total and individual levels of short-chain fatty acids present in CF cultures coincided with the quantity of acids found in the large intestine of conventional mice or human feces used to establish the CF cultures. Furthermore, short-chain fatty acids were also shown to inhibit the adhesion of *Candida* to intestinal mucosa (89) and were inhibitory to *Candida* growth (86). MICs for individual acids and "gut levels" or CF culture levels that were compared to determine if the entire mixture collectively was necessary for

suppression or if one or more acids individually could antagonize *Candida* growth showed that acetic and butyric acids antagonized *Candida* growth at levels that are present in these systems (86). Although it is not known if the in vitro results of that study adequately reflect the sensitivity of *C. albicans* to these acids in the GI tract or CF cultures, it is not likely that inhibitory concentrations of these short-chain fatty acids would be higher. Therefore these acids are likely to play a role in the suppression of *Candida* in the gut. A similar argument could be made to support the hypothesis that deconjugated bile acids play an important role in the suppression of *C. albicans* in the GI tract. For instance, deconjugated bile acids have been shown to inhibit both the in vitro growth and the adhesion of C. albicans to intestinal mucosal surfaces (89,101).

According to Freter (50), however, it is theoretically impossible to account for the total suppression of a sensitive microorganism, such as *C. albicans*, in the GI tract solely on the basis of the production of growth inhibitors. If it were the case, constant populations of the sensitive species could be maintained only at precise inhibitor concentration (50). Thus if too much inhibitor were present, *C. albicans* would be eliminated; or if too little were present, the *Candida* population would increase until it became limited by some other mechanism. Because *Candida* population levels in the GI tract have been shown to vary from day to day (40), it may be argued that other inhibitory mechanisms are also involved with the suppression of *C. albicans* in the gut. Considering that there must be fluctuations in the production of growth inhibitors and that short-chain fatty acids appear to be only fungistatic, it is likely to be the case. It should also be noted that there may be an enormous supply of growth inhibitors that can be produced by the indigenous intestinal microflora. For instance, several breakdown products from the large number of primary dietary or host-derived substrates in the gut may antagonize *C. albicans*, as might a number of substances synthesized by the microflora itself. Although *C. albicans* was apparently being "killed" when inoculated into established CF cultures (only mouse cecal flora), it was not "killed" when it was inoculated before or simultaneously with the microflora (86). Thus it may be that *Candida* can adapt itself to these "toxic" substances, which nevertheless are probably still inhibitory to *Candida* growth. This view is consistent with the finding that *C. albicans* was able to implant itself (albeit only for a short time) in CF cultures when inoculated simultaneously with mouse cecal flora (86).

The reason *C. albicans* could not permanently colonize CF cultures in large numbers was probably due to a lack of adhesion sites or the inability to attach to the glass vessel in the presence of, for example, short-chain fatty acids (86,89). It suggests that adhesion to the gut wall may be an important determinant to colonization for *C. albicans*, as association with the intestinal mucosa would probably allow *Candida* to remain in the gut even at severely depressed growth rates. Moreover, it may also be that

colonization of the mucosa would provide more sheltered colonization sites from growth inhibitors. The finding that mucosa-associated populations of *Candida* predominated after the intestinal microflora reestablished (86) is certainly consistent with this view. Therefore, as discussed above, the ability of the intestinal microflora to inhibit the association of *C. albicans* with the intestinal mucosa also represents an important defense mechanism that inhibits *Candida* colonization of (and systemic spread from) the GI tract.

In summary, then, the data described in this chapter suggest that *Candida* populations are controlled collectively by a number of mechanisms. First, *C. albicans* is apparently bombarded by several growth inhibitors (e.g., short-chain fatty acids, secondary bile acids) that cause *Candida* cells to remain in a prolonged lag phase and have a long doubling time upon entering the GI tract. This fact alone may cause most *Candida* organisms to be removed from the GI tract due to peristalsis and the flow of mucous (unless, of course, they are able to associate with the mucosa). Second, there are several mechanisms operating in concert to inhibit and suppress *Candida* cells from attaching to the gut wall, e.g., competition for adhesion sites, production of inhibitor substances, prevention of penetration into the mucus gel, and an increased disassociation rate (89). Finally, superimposed on and modifying this type of regulation is the metabolic competition for limiting nutrients, which probably becomes the predominant control mechanism if *Candida* cells become implanted in the gut. There is some evidence to suggest that the presence of certain metabolic endproducts produced by the predominant anaerobes restricts the range of substrates a given organism can efficiently utilize for anaerobic growth (Kennedy, unpublished data). Thus *C. albicans* is probably collectively controlled by growth inhibitors and anaerobiosis (which severely depresses its growth rate), competition for mucosal sites, and substrate competition. It is not known to what extent individual control mechanisms participate in the regulation of *C. albicans* in the gut, but the range of factors that can cause "overgrowth" of *Candida* in the GI tract confirms the idea that no single mechanism is responsible for the regulation of *C. albicans* populations. The present hypothesis therefore may help explain discrepancies in the literature regarding the components of the intestinal microflora as well as the "different" mechanisms that have been suggested to control *Candida* populations in the gut.

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12—Antifungal Drug Susceptibility Testing

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The continuing introduction of new antifungal drugs has increased the demand for methods of in vitro testing that can predict the effects of compounds in vivo. As with antibacterial drugs, tests designed to ascertain the minimum amount of drug needed to inhibit the growth of organisms in vitro (minimum inhibitory concentration, or MIC) have often been used in attempts to predict the clinical results of treatment. If growth of an organism is inhibited in vitro at drug concentrations lower than levels attained in vivo, it is usual to regard the organism as "sensitive" to that drug. If, however, growth is not inhibited at drug concentrations attained in vivo, the organism is regarded as "resistant." The results of treatment are often difficult to assess in infected patients. However, in situations where it can be assessed, there is often a good correlation between this interpretation and the clinical results of treatment with antibacterial drugs (19,46). In contrast, with most antifungal drugs, the correlation is either poor or absent.

Numerous factors have been identified that can affect the results of in vitro tests with antifungal drugs. In particular, the conditions under which the tests are performed often have a marked effect. The mechanisms of action of the drugs also have major implications for the design of test methods. In this chapter the methods of in vitro testing that have been developed for the various groups of antifungal drugs used to treat deep fungal infection are discussed. Attempts to develop methods that might give more meaningful results are also described.

Antifungal Drugs

Before 1950 no reliable or safe treatment existed for deep fungal infections, and treatment of superficial infections depended on empirical topical preparations. During the 1950s nystatin was introduced for the topical

treatment of candidiasis, griseofulvin was first used for oral treatment of dermatophytosis, and use of amphotericin B transformed the treatment of most forms of deep fungal infection. With the introduction of 5-fluorocytosine for treatment of candidiasis and cryptococcosis, drug resistance became an important mycological problem for the first time. The 1970s and 1980s have seen the introduction of a large number of broad-spectrum antifungal agents that are *N*-substituted imidazole or triazole compounds. The earliest members of this group, clotrimazole and miconazole, are useful for topical treatment of dermatophytosis and superficial forms of candidiasis, and it has remained the case for most of the similar compounds that have since appeared. However, miconazole is also available for parenteral administration, and some of the more recent azole compounds, e.g., ketoconazole, itraconazole, and fluconazole, are effective after oral administration.

Mechanisms of Action

Amphotericin B is a polyene macrolide antimicrobic. Members of this group of compounds consist of a large macrolide ring with well demarcated hydrophilic and hydrophobic regions. Amphotericin B interacts with sterols in fungal cell membranes, causing impairment of barrier function and leakage of cellular constituents. Metabolic disruption and subsequent death of the cell are consequent upon membrane alterations (43). At low concentrations of amphotericin B, leakage of cell constituents is restricted to small molecules or ions such as sodium and potassium and is still reversible. At higher concentrations, larger molecules are transported through the membrane, producing irreversible loss of cell constituents. At such concentrations, amphotericin B is fungicidal (44).

Investigation of the lipid composition of mutant strains of *Candida albicans* resistant to amphotericin B has shown that resistance is associated with alterations in the sterol composition of the cell membrane (58). The few strains of *C. tropicalis* and *C. parapsilosis* that have become resistant to amphotericin B during treatment have all contained reduced amounts of ergosterol (48,70,88).

Members of the azole group of antifungals have in common an imidazole or triazole ring with *N*-carbon substitution. This ring is responsible for the interaction of these drugs with certain target sites within the fungal cell. At low concentrations, these drugs interact with fungal cytochrome P-450, which results in inhibition of the 14-demethylation step in the biosynthesis of ergosterol. The depletion of ergosterol and concomitant accumulation of lanosterol or other 14α-methylsterols leads to alterations in a number of membrane-associated functions (43,85). At high concentrations, some azole antifungals such as miconazole, but not ketoconazole, interact with membrane lipids, causing direct membrane damage, which results in leak-

age of cellular constituents (43,85). It is believed that fungistatic effects result from the inhibition of membrane sterol synthesis and fungicidal effects from impairment of the barrier function of the membrane.

Two azole-resistant strains of *C. albicans* (37) have been shown to be impermeable to a triazole antifungal agent (68). Comparison of the lipid composition of these strains with that of several sensitive strains demonstrated a low phospholipid/nonesterified sterol ratio in the resistant strains (27). However, a third azole-resistant strain of *C. albicans* (86) had a high ratio and took up azole at about the same rate as sensitive strains (27). Hitchcock et al. (26) have reported that the sterol demethylase in this strain is less sensitive to azole, but other target sites might also be altered or absent. Initial findings on a fourth resistant *C. albicans* strain suggested that binding of azoles to cytochrome P-450 from this strain was reduced compared with that of sensitive strains (77).

5-Fluorocytosine is a fluorinated pyrimidine that is transported across the fungal cell membrane through the action of cytosine permease and is then deaminated to 5-fluorouracil by means of cytosine deaminase. Two independent mechanisms appear to account for the action of 5-fluorocytosine (43,73). The drug is metabolized to 5-fluorouridine triphosphate, which is incorporated into fungal RNA, leading to disruption of protein synthesis. The drug is also converted to 5-fluorodeoxyuredylic acid, which is a potent inhibitor of thymidylate synthetase. This conversion results in inhibition of DNA synthesis.

The most common cause of resistance to 5-fluorocytosine in *C. albicans* appears to be the loss of uridine monophosphate pyrophosphorylase (64,87). Loss of cytosine permease or deaminase can occur but appears to be infrequent (64).

Incidence of Clinical Resistance

Most isolates of most of the principal fungal pathogens of humans have MICs for amphotericin B ranging from 0.02 to 1.0 $mg \cdot L^{-1}$, which are similar to blood levels of the drug during treatment. For instance, Athar and Winner (2) tested 900 isolates of *C. albicans*, all of which had MICs of 0.25–1.0 $mg \cdot L^{-1}$, as did almost 300 isolates of *C. tropicalis* or *C. parapsilosis*. Amphotericin B MICs of less than 3 $mg \cdot L^{-1}$ for all isolates tested have been reported for *Crytococcus neoformans* (32). On the other hand, Artis and Baum (1) tested 24 isolates of *Histoplasma capsulatum* and found four that had MICs for amphotericin B of 10 $mg \cdot L^{-1}$ and one with an MIC of 100 $mg \cdot L^{-1}$. There are a few pathogens with MICs for amphotericin B of more than 10 $mg \cdot L^{-1}$, and these few must be considered resistant to the drug.

Treatment failure attributable to the development of amphotericin B resistance remains rare; it has been documented in two patients with *C.*

tropicalis infection (17,48), one with *C. parapsilosis* infection (15), one with *C. lusitaniae* infection (57), and one with *Torulopsis glabrata* infection (13). In most cases the strains had MICs of the order of 100–500 $mg \cdot L^{-1}$.

It is not surprising that reports of treatment failure attributable to the development of resistance to imidazole or triazole antifungals have been disputed, given the extent to which published MICs for particular organisms with individual compounds can differ. However, there have been a few convincing reports of clinical resistance arising in patients with *C. albicans* infection. In 1978 Holt and Azmi (35,36) described a strain of *C. albicans* resistant to miconazole (and other azoles) recovered from an infant who had received oral treatment with this drug for 2 months. The MIC prior to treatment had been 100-fold lower. Four instances have been described where patients who had undergone protracted oral treatment with ketoconazole for chronic mucocutaneous candidiasis relapsed despite achieving good blood levels of the drug (37,77,86). Extensive testing in vitro and in vivo has since demonstrated that the isolates of *C. albicans* recovered from these four patients are resistant to azole antifungals (26, 27,42,56,68,77).

Other reports of apparent azole resistance have been far less convincing. Ho et al. (28) described three "clotrimazole-resistant" isolates of *C. albicans*. Their criterion for resistance was an MIC of more than 8 $mg \cdot L^{-1}$, but no good reason was given for the selection of this concentration. Church et al. (12) attributed cutaneous ulcers in a child with chronic granulomatous disease to isolates of *C. tropicalis* and *C. parapsilosis* recovered from the lesions. The ulcers worsened during a short course of oral ketoconazole, and posttreatment strains showed MICs of 12.5 and 6.3 $mg \cdot L^{-1}$, respectively. Church et al. (12) judged it a treatment failure due to ketoconazole resistance, disregarding the fact that much higher MICs have sometimes been obtained with sensitive strains of the organism in question.

5-Fluorocytosine is the one antifungal drug that suffers from serious problems of resistance. It occurs with all organisms responsive to the drug, and resistant strains—defined as having MICs of 15–25 $mg \cdot L^{-1}$ after incubation at 30°C for 48 hours (74)—have been encountered both prior to and during treatment. For this reason it is prudent to perform MIC determinations on all isolates recovered from patients destined to receive the drug and all isolates recovered during treatment.

About 10% of *C. albicans* isolates obtained prior to treatment are resistant to 5-fluorocytosine (73,74). However, a higher rate of resistance has been found among serogroup B isolates than serogroup A (3,16,80). It has been attributed to a partial loss of uridine monophosphate pyrophosphorylase in serogroup B isolates (64). Fewer than 2% of *Cr. neoformans* isolates are resistant to 5-fluorocytosine prior to treatment (73,74). Emergence of resistance to 5-fluorocytosine during treatment is a common problem. However, marked differences in incidence have been noted for different organisms (73,74).

Methods for Testing Antifungal Drugs

The methods used for testing antifungal drugs in vitro are similar in design to those employed with antibacterial compounds. The most popular methods have been the traditional ones of broth dilution, broth microdilution, agar incorporation, and agar diffusion. The broth and agar dilution methods of MIC determination provide more numerical information than agar diffusion tests, but these methods are also more tedious and time-consuming. When selecting the methods to use, an important factor is the nature of the investigation being undertaken. If an individual isolate is being tested against a range of drugs, broth dilution is often most convenient. On the other hand, if numerous isolates are being tested against a single compound, some form of incorporation of diluted drug into solid medium is often preferred.

Correlation Between MIC Tests and Clinical Response

It seems logical that methods for determination of MICs would have been selected on the basis of good correlation with clinical results of treatment. In practice, there have been few attempts to test the extent of the correlation between MIC tests with antifungal drugs and the results of their administration to infected animals or patients with fungal infection.

Stiller et al. (79) tested the effects of 5-fluorocytosine against 40 isolates of *C. albicans* in vitro and in a murine model of candidiasis. Their results demonstrated a significant correlation between clinical response and both dilution MIC tests and agar diffusion methods. However, this correlation was far from perfect: The clinical responses of several of the most sensitive isolates overlapped with those of several of the least susceptible isolates.

Plempel (60) tested the effects of the triazole vibunazole against 59 isolates of *C. albicans* in vitro and in a murine model of candidiasis. MICs read after 48 hours of incubation showed no correlation with the results in the animal model. Polak et al. (63) tested the effects of ketoconazole against 58 isolates of *C. albicans* in vitro and in a murine candidiasis model. No significant correlations were detected among a number of methods of in vitro testing and the results in vivo.

Shadomy et al. (76) found no significant correlations between the results of MIC tests and the results of oral treatment with ketoconazole in patients with blastomycosis, coccidioidomycosis, cryptococcosis, histoplasmosis, or sporotrichosis. The MICs for 21 of 25 pretreatment isolates from patients who were later described as cured or improved were ≤ 3.1 mg·L^{-1}. The MICs of ketoconazole for 22 of 24 pretreatment isolates from patients who failed to respond to treatment or relapsed were also ≤ 3.1 mg·L^{-1}. Indeed,

the four highest MICs were recorded for isolates from patients who responded to treatment, and 12 strains with MICs of ≤ 0.1 mg·L^{-1} were from patients who failed to respond.

The problem of noncorrelation of MICs with clinical results of treatment has been most pronounced with dioxolane imidazole and triazole drugs such as ketoconazole, itraconazole, and fluconazole. This finding has led a number of manufacturers to screen for novel antifungal compounds in animal models of infection rather than depend on the results of tests in vitro (25,66,84).

Causes of Variation in MIC Tests

The poor correlation between MICs of antifungal drugs in vitro and effects of the compounds in vivo is due, at least in part, to the tremendous variation in MICs obtained under different test conditions. For instance, tests with *C. albicans* isolates have produced MIC ranges for miconazole with midpoints of 0.5–35.0 mg·L^{-1} depending on the conditions under which the tests were performed (52). With ketoconazole, even greater variations in MICs have been recorded, with midpoints in tests with *C. albicans* ranging from 1 to 80 mg·L^{-1} (52).

Various factors can affect the results of broth and agar dilution MIC methods with antibacterial drugs, including the size of the inoculum, pH and composition of the medium, incubation temperature, length of incubation, incubation atmospheric composition, and method of endpoint interpretation (18). Most if not all of these factors also affect tests with antifungal drugs.

Numerous studies attest to the fact that inoculum size affects MIC test results with 5-fluorocytosine (6,8,22) and the azoles (8,22,32,42,51, 59,61,62,67,90). In general, the MIC increases with the size of the fungal inoculum. In contrast, inoculum size appears to have minimal effects on the MICs of amphotericin B or nystatin (8,23).

The composition of the test medium has a pronounced effect on MIC results with 5-fluorocytosine (14,65). However, identical results have been obtained in tests in which MICs of *C. albicans* and *C. tropicalis* isolates were determined with liquid and solid media of similar composition (45,65). Inclusion of buffers in the medium can have a major effect on MICs: Calhoun and Galgiani (9) showed that MOPS (2-morpholinepropane sulfonic acid)/Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] buffer antagonizes the effect of 5-fluorocytosine. McKerrow et al. (47) have since reported that it is the Tris component of the buffer that produced this effect. Test format appears to have no effect on the MICs of amphotericin B (65), but medium composition can affect the results obtained (14,65).

Numerous reports have shown that medium composition has a marked effect on MIC results with imidazole and triazole drugs (14,29,30,34,39, 51,62,75,82,90). However, in several investigations in which MICs were determined with liquid and solid media of similar composition under standardized conditions, identical results were obtained in most instances (51,65). Inclusion of serum in the medium leads to higher MICs (51,72,83).

Medium pH, when lowered, has been associated with lower MICs of nystatin and amphotericin B (40). Effects of pH shifts are minimal in broth-dilution MIC tests with 5-fluorocytosine (9). In contrast, the pH of the medium has a marked effect on azole MICs. Most reports indicate that a neutral pH gives the lowest MICs (4,5,49,67).

Incubation temperature has not been shown to have a marked effect on MICs of ketoconazole (except with low inoculum sizes) or nystatin (8). With 5-fluorocytosine, elevated incubation temperatures can lead to lower MICs for *Cr. neoformans* (6). These variations appear to be due to differences in growth rates of organisms at different temperatures.

Length of incubation has a marked effect on MIC determinations with most antifungal drugs. MICs of 5-fluorocytosine tend to increase with prolonged incubation (6,8,14). Azole MICs, particularly in tests with *C. albicans*, show marked increases between 24 and 48 hours of incubation (8,14,42,51), and stable MICs seem to require as much as 96 hours of incubation (42,51). Except for situations where prolonged incubation leads to degradation of the drug (11,31), MICs of amphotericin B and nystatin tend not to alter with time of incubation (8,14,29).

Another problem encountered with imidazole and triazole compounds results from the fact that these drugs often cause partial inhibition of growth over a wide range of concentrations, making visual determination of endpoints in MIC tests difficult (20,24,33,39,50,53). This effect is most pronounced with *C. albicans* (20,50) and appears to account for the similar MICs obtained with azole-sensitive and azole-resistant strains of the fungus in some agar and broth dilution tests (38,42,68). One potential solution appears to be the inclusion in the test medium of antibacterial compounds that bind to 80S fungal ribosomes, thereby inhibiting protein synthesis (55).

The explanation for the inoculum effect seen in MIC tests with imidazole and triazole drugs lies in their mechanism of action and the method used to read the test results. Unlike tests with some antibacterial drugs, the rise in MICs with increasing inoculum is not due to the selection of resistant variants (78). Azole compounds inhibit ergosterol synthesis in proliferating fungal cells, causing gradual depletion of membrane sterols, which then leads to inhibition of further growth. It means that some growth occurs even with high drug concentrations, but the denser the initial inoculum in MIC tests, the higher is the drug concentration in which growth of cells reaches visible proportions before further growth is inhibited (59,78).

Alternative Methods of In Vitro Testing

The poor correlation of MICs for antifungal drugs in vitro with effects of the compounds in vivo has stimulated attempts to develop alternative methods of in vitro testing that avoid the problems of inoculum dependence and endpoint determination described in the previous section.

Agar disk diffusion tests have been described for a number of antifungal drugs (45,71,80,81,89). Tests with 5-fluorocytosine using *C. albicans* have shown that there is a good correlation between the diameter of the zone of inhibition and the MIC determined with an agar dilution method (45,80,81). The same cannot be said for other antifungal agents. With imidazoles the results of disk diffusion tests often fail to correlate with broth or agar dilution MICs (71), and substantial variations in results occur depending on the composition of the test medium (30).

One novel approach to in vitro testing that several manufacturers of azole antifungal agents have adopted is assessment of the effects of compounds on the hyphal form of *C. albicans* (7,59,69). Assessment of drug effects on hyphal elongation of *C. albicans* (41) appears to show a greater correlation with the effects of these compounds in vivo than MIC tests involving the blastoconidial form, and this method has permitted detection of resistance to ketoconazole (42,68,77,86).

Azole antifungal agents often cause marked reductions in fungal growth at concentrations far below the actual MIC (20,24,33,39,53), which could help to account for the poor correlation between classical MICs and clinical results with drugs such as ketoconazole. If reduction rather than cessation of growth is sufficient to bring about therapeutic effects in vivo, it would be worthwhile to investigate the range of concentrations over which fungal growth is reduced, rather than determine a straight MIC when evaluating the potential of novel antifungal compounds.

Turbidimetric methods that measure the antifungal drug concentration that causes 50% or 70% reduction of growth compared with controls (the IC_{50} and IC_{30} tests) take into account the partial inhibition of growth and avoid the problems of endpoint interpretation that impair classical MIC tests (22,23,42). In tests with *C. albicans*, turbidimetric measurements have shown a good correlation with cell counts (38). Because these methods are based on a comparison of growth rates rather than on levels of growth attained, the results are much less dependent on inoculum concentration, provided measurements are made during exponential growth (22,23,42). More work is needed to establish the extent of the correlation between turbidimetric endpoints and the clinical effects of antifungal compounds. However, this approach has proved useful for demonstrating resistance to ketoconazole in *C. albicans* (38,42,68,77).

Odds and Abbott (54) have devised a novel test method for antifungal agents that involves measuring the area under the dose-response curve between fixed upper and lower drug concentration limits: the "relative inhibition factor" (RIF) test. Again, this method takes into account the partical inhibition phenomenon seen with imidazole and triazole compounds. The tests are performed in Eagle's minimal essential medium with added serum under 5% CO_2 in air, and growth is measured with adenosine triphosphate (ATP) bioluminescence, so the method can be applied to filamentous organisms. In initial tests, RIF results correlated well with MICs for amphotericin B, nystatin, and 5-fluorocytosine but showed no correlation with MICs for five imidazoles (54). In a subsequent investigation (56) in which nine new azole antifungal agents were compared with five established azole compounds, the RIF results for the two groups were similar, but MIC tests showed most of the new antifungal agents in a poor light. Although the extent of the correlation between RIFs and clinical effects of azole antifungal agents remains unclear (63), this approach has proved useful for distinguishing azole-resistant isolates of *C. albicans* (56,77).

Standardization of Methods

By now it should be evident that the conditions under which tests with antifungal drugs are performed have a profound effect on the results obtained. If tests are performed under standardized conditions, however, can consistent results be achieved? It is an important point, but one that has seldom been studied. In 1986 Calhoun et al. (10) published a detailed evaluation of the precision of a broth dilution MIC method with several antifungal agents. The tests were performed with identical isolates under standardized conditions in seven laboratories. Although the results of repeated testing in the individual laboratories showed good precision, there were marked variations in MICs among the participants. This work suggests that laboratories wishing to perform tests with antifungal drugs should first establish their own normal ranges of MICs for sensitive isolates with each compound under standardized conditions before attempting to interpret their results to clinicians.

If different methods of testing a particular drug rank isolates in a consistent order, each method should show a similar correlation with the clinical results of treatment, making interpretation of MICs simpler. Published work suggests that different methods often give identical orders of ranking with *C. albicans* and 5-fluorocytosine (10,21) but not with ketoconazole (21). If the dissimilar patterns seen with the latter drug could be resolved if the conditions of testing and methods of endpoint interpretation were better standardized remains to be seen.

Conclusions

Fewer drugs are available for the treatment of fungal than bacterial infections, and yet their testing in vitro is a much more confusing and controversial subject, reflecting the problems with standardization and interpretation that have been encountered with all of them. Until there is some standardization of test methods, the uncommonness of resistance problems with amphotericin B and the azoles, and the lack of correlation between most methods of MIC testing and clinical outcome, indicate that laboratories dealing with few fungal infections would be best advised to establish good methods with 5-fluorocytosine and to follow clinical experience when administering other antifungal agents. Organisms should be preserved; and referral to specialist laboratories can be done in cases where unusual organisms or sites of infection or failure or recurrence makes other testing desirable.

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