Comparison of Immunosuppressive Effects of Cyclosporine A in a Murine Model of Systemic Candidiasis and of Localized Thrushlike Lesions

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*Candida albicans is an opportunistic human pathogen preferentially causing invasive and disseminated infection in patients with defective phagocytic defenses and serious mucocutaneous infection in patients with deficient T-cell function. Phagocytes appear to protect the host from fungal invasion even in the absence of adaptive immune mechanisms, while as-yet-undefined T-cell-dependent factors seem necessary for control of C. albicans on body surfaces. To study host defense mechanisms on body surfaces, we developed a new model of thrush in artificial pneumatized cysts in mice. Cyclosporine A, a relative selective suppressor of T-cell-mediated immunity and natural killer cell activity, promoted the formation of thrushlike lesions on cyst surfaces and impeded elimination of C. albicans from such lesions. As expected from the absence of an impairment of antifungal phagocytic activity, cyclosporine A had no effect on systemic candidiasis induced by intravenous inoculation. Surprisingly, athymic nude mice were not more susceptible to superficial candidiasis than control mice and were comparably affected by cyclosporine A. In contrast, beige mice, which in addition to phagocytic dysfunction have reduced natural killer cell activity, were more susceptible to thrushlike lesions, and cyclosporine A was correspondingly less active in this mouse strain. Immunosuppression with cyclosporine A affects host defense mechanisms which are operative against superficial candidiasis but appear superfluous in resistance to the invasive form of this mycosis, an indication for the divergent nature of host defense against the two forms of candidiasis.

Candida albicans is an opportunistic human pathogen causing serious infection, particularly in the immunocompromised host (6, 10, 16, 20, 36). Clinical observations indicate that the opportunistic proclivities of this fungus vary considerably with the nature of the immunologic defect of the victim. Patients with qualitative or quantitative defects of phagocytes are mainly prone to the invasive form of this mycosis (6, 10, 36). In contrast, defective T-cell-mediated immunity has been specifically associated with thrush and other forms of candidiasis limited to mucocutaneous surfaces (10, 11, 16, 20). It is particularly intriguing that in patients with congenital T-cell defects or with the acquired immune deficiency syndrome and suffering from severe mucocutaneous candidiasis, invasion and dissemination of C. albicans is notably absent (11, 16, 20), supposedly because of the divergent nature of host defense systems operative against C. albicans on mucocutaneous surfaces and in tissues. Phagocytes appear to protect the host from fungal invasion even in the absence of adaptive immune mechanisms (11, 31), and clinical observations indicate that T-cell-dependent, but hitherto undefined, host defense factors are necessary to control candidal growth on mucocutaneous surfaces (11, 16, 20). That host defense against invasion by, and dissemination of, C. albicans is independent from specific adaptive (i.e., acquired humoral or cell-mediated) immunity has been confirmed in various models of disseminated candidiasis in athymic mice (7, 29) or mice with combined severe immunodeficiency (22) or by analysis of adaptive immunity after infection with live C. albicans (17). Studies of host defense mechanisms operative against C. albicans on mucocutaneous surfaces have made less progress, probably because of a lack of convenient models of thrush. To study host defense mechanisms against C. albicans on body surfaces, we therefore developed a new model in which thrushlike candidal lesions are created in subcutaneous pneumatized cysts. This report summarizes our observations on the disparate nature of immune mechanisms operative against C. albicans in this model, compared to mechanisms operative against the disseminated form of this mycosis.

MATERIALS AND METHODS

Mice. If not stated otherwise, 8- to 9-week-old female ICR mice (Institut für Zuchtfehigkeits der Universität, Zurich, Switzerland) were used throughout the studies. Other mouse strains were 8- to 10-week-old female C57BL/6 bg/bg and C57BL/6 +/- mice (Harlan Olac Ltd., Bicester, England), 11- to 12-week-old female NMRI nulnu and NMRI +/- mice (Biologisches Zentralabotatorium, Universitätsspital Zurich, Zurich, Switzerland), and 5- to 6-week-old ICR nulnu mice (Tierzuchttinstitut, Züllinsdorf, Switzerland). Mice were housed in cages of 8 to 10 animals, and pellets (sterile for experiments with athymic mice) and acidified water were offered ad libitum.

Organism. C. albicans SD-#1, originally isolated from a patient with fungemia, was used throughout the studies. For inoculation of mice, C. albicans was grown in 100 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.) for 24 h at 35°C on a gyratory shaker, and yeast cells were harvested by centrifugation (200 × g), washed two times in 0.85% NaCl, and quantitated in a hemocytometer. The inoculum size was verified by quantitative culture of serial dilutions on tryptic soy agar (Difco). Listeria monocytogenes EGD was propagated as described previously (33).

Models of infection. Disseminated infection was provoked by inoculation of yeast cells, suspended in 0.5 ml of 0.85%
NaCl, into the lateral tail vein. For studies of candidiasis on a body surface, pneumatized subcutaneous cysts were formed. Cysts were prepared by injecting 3 to 3.5 ml of air through a hypodermic needle into the subcutis of the back. Initial studies indicated that neither anaesthesia nor shaving was necessary for this procedure. To maintain cysts pneumatized, air (usually 0.5 to 1 ml) was reinjected during the study every 3 or 4 days. Within 3 to 7 days, cysts were lined by an epithelioid cell layer of mesenchymal origin as shown previously by others (9). To provoke thrushlike lesions, 7-to 10-day-old cysts with an airfilled volume of about 3 ml were challenged with $2.8 \times 10^6$ to $2.5 \times 10^8$ yeast cells suspended in 0.2 ml of NaCl. Yeast infection was quantitated by culturing serial dilutions of homogenates, prepared from cysts and organs by individual homogenization with Teflon pestles, on tryptic soy agar as described previously (32). For the removal of intact cysts for culture, it was found advantageous to inject 0.05 ml of 1% trypan blue in 0.85% NaCl into the cyst prior to preparation. Listeriosis developing after intravenous (i.v.) injection was studied as described previously (33). Tissues were fixed in 4% buffered Formalin and stained with periodic acid-Schiff or hematoxylin-eosin stain for histologic evaluation.

Immunosuppressive regimens. Cyclosporine A was dissolved in olive oil and administered by gavage in a volume of 0.2 ml in a dose of usually 40 mg/kg per day from 2 days prior to challenge until sacrifice. This regimen has previously been shown to give maximal immunosuppression in murine listeriosis without toxicity (33). Cortisone acetate (Merck Sharp & Dohme, West Point, Pa.) was given in a dose of 2.5 mg/day beginning 2 days prior to challenge until sacrifice or death (32). Nitrogen mustard (Merck Sharp & Dohme) was diluted in 0.85% NaCl and injected i.v. in a dose of 40 $\mu$g per animal per day for 3 consecutive days, starting 2 days prior to challenge. This regimen has previously been found to induce profound neutropenia and complete suppression of an inflammatory cellular response for 5 to 6 days (32).

Statistical analysis. Results are given as mean values ± standard deviation (SD); mean values were compared by Student’s $t$ test.

RESULTS

Development of thrushlike lesions in pneumatized cysts. Upon challenge of pneumatized cysts with C. albicans, mice immunosuppressed with either nitrogen mustard, cortisone acetate, or cyclosporine A developed within 4 to 6 days distinct, macroscopic, thrushlike, white lesions on their cyst walls (Fig. 1 and 2). No such lesions were found in control animals receiving solvents without immunosuppressive drugs, unless high-challenge doses above $2 \times 10^6$ CFU were used, after which white membranes transiently formed even in normal animals.

FIG. 2. Thrushlike lesion 11 days after challenge with $5 \times 10^6$ CFU of C. albicans in a pneumatized cyst from a cyclosporine A-treated mouse (Hemalaun-eosin stain; original magnification, ×100). Note the minor inflammatory response to the yeast cells, which is limited to the loose granulation tissue beneath the membranelike fungal lesion. Insert: Densely packed yeast cells and pseudohyphae within the membrane (periodic acid-Schiff stain; original magnification, ×1,000).
To obtain objective quantitative observations on the effects of various immunodeficient states, complete cysts with their contents were homogenized and C. albicans was quantified by culture.

Effects of cyclosporine A on superficial candidal lesions and systemic candidiasis. Because we speculated that T-cell-dependent immune mechanisms were required for the control of thrushlike lesions, we first studied the effects of cyclosporine A on superficial candidiasis. Cyclosporine A was chosen because this immunosuppressive drug selectively impairs T-cell-mediated immunity and natural killer (NK) cell activity (2, 5, 15, 18, 34) without impairing nonspecific phagocytic resistance (2, 5, 33, 34). In several experiments, cyclosporine A decreased resistance against C. albicans growing on cyst surfaces (Fig. 3), documenting cyclosporine A-susceptible host defense operative against thrushlike lesions, an observation pointing to T-cell-dependent factors involved in defense against C. albicans on cyst surfaces.

Because T-cell-mediated immunity appears superfluous in resistance against experimental systemic candidiasis (7, 17, 22, 29, 31), we next examined whether cyclosporine A would lower resistance of mice against i.v.-injected yeast cells. In keeping with clinical observations on the distinct nature of host defense mechanisms operative against mucocutaneous and invasive candidiasis, cyclosporine A did not affect resistance of mice against the disseminated form of the mycosis. Thus, cyclosporine A-treated mice eliminated yeast cells from the spleen and liver comparably to control mice, and infection in the kidneys took an identical course in cyclosporine A-treated and control animals (Fig. 4). These differing effects on surface candidiasis and on the systemic mycosis appeared not to depend on the challenge dose and occurred either with a lethal i.v. challenge which matched the inoculum used for cyst infection (Fig. 5) or with much lower doses, resulting in indolent low-grade infection of the kidneys (Fig. 4 and 6). Thus, immunosuppression with cyclosporine A appeared to discriminate between host defense mechanisms operative against surface candidiasis, supposedly depending on T lymphocytes, and host defense mechanisms operative against systemic disease, supposedly of nonspecific, phagocytic nature, which were affected by cortisone and the cytotoxic agent nitrogen mustard but not by cyclosporine (Fig. 6). Next we wanted to exclude the possibility that the immunosuppressive effect of cyclosporine in the cyst model reflected mere nonspecific toxicity.

Mice receiving 80 mg of cyclosporine per kg per day gained weight in parallel to control mice and showed no signs of toxicity. At one quarter of this dose we found a near-maximal immunosuppressive effect of cyclosporine, indicating specific immunosuppression rather than nonspecific toxicity (Fig. 7).

Failure of cyclosporine to promote dissemination of candidiasis from thrushlike lesions. Cultures of homogenates from kidneys taken from cyclosporine-treated (or control) mice during the cyst experiments always remained sterile, indicating that cyclosporine A treatment did not lead to dissemination of C. albicans. This conclusion was confirmed in comparative studies of cyclosporine A, cortisone acetate, and immunosuppression with the cytotoxic agent nitrogen mustard (Fig. 8). Despite a challenge of cysts with high numbers of fungal cells, resulting in delayed elimination of fungi even from cysts in normal animals, dissemination from progressive local lesions to parenchymatous organs did not occur in cyclosporine A-treated mice. In contrast, in mice immunosuppressed with a cytotoxic agent or with cortisone, dissemination to the kidneys was frequently observed, even when localized lesions on the cyst surface were, compared with cyclosporine-treated mice, less well developed (Fig. 8).

![FIG. 3. Effect of an immunosuppression with cyclosporine A on the elimination of C. albicans from cysts. Each point represents the mean ± SD from four animals. The challenge dose was 1.1 × 10^6 CFU per cyst. Symbols: ●, cyclosporine A, 40 mg/kg per day; ■, solvent control.](./image)

![FIG. 4. Failure of cyclosporine A to affect systemic candidiasis following i.v. inoculation of yeast cells. (A) Control mice (solvent). (B) Cyclosporine A (40 mg/kg per day). The inoculum was 10^6 CFU per mouse. Each point represents the mean ± SD from four animals. Symbols: ●,●, kidneys; ▲,▲, liver; ●,○, spleen. All differences between cyclosporine and control animals were not significant.](./image)

![FIG. 5. Direct comparison of the immunosuppressive effect of cyclosporine A in the model of systemic candidiasis and thrush. (A) After an inoculum of 9.6 × 10^6 CFU, lethal renal candidiasis evolved rapidly in the i.v. model which was not affected by cyclosporine A. (B) In contrast, control of infection was affected by cyclosporine in the cyst model. Symbols: ●,●, cyclosporine A (40 mg/kg per day); ■,■, solvent control. After day 4, mice challenged i.v. started to succumb to the infection, and by day 7 all mice had died regardless of whether they were immunosuppressed or not.](./image)
FIG. 6. Comparison of the immunosuppressive effects of cortisone, the cytotoxic drug nitrogen mustard, and cyclosporine A on disseminated candidiasis after i.v. inoculation of 8.5 × 10⁴ CFU of yeast cells. (A) Control animals. While the low-grade infection in the kidneys (panel A) and elimination of yeast cells from the liver (panel B) were not affected by cyclosporine A (●), nitrogen mustard impaired control of the infection in the kidney but not early elimination of yeast cells from the reticuloendothelial system of the liver (▲). Cortisone promoted infection in both organs (○), resulting in death of mice between days 10 and 12. Each point represents the mean ± SD from four animals.

The capacity of an immunosuppressive drug to provoke dissemination from the cyst corresponded to its ability to promote disseminated disease in the i.v. model (Fig. 6 and 8).

Effects of cyclosporine on candidiasis in nude and beige mice. Next we investigated resistance of congenitally athymic nude mice against thrushlike lesions in the cyst model. Several authors had previously shown that nude mice are no more susceptible to disseminated candidiasis than normal littermates (7, 22, 29), as expected from the independence of host resistance against an i.v. inoculum of Candida sp. from adaptive specific immune mechanisms (17). Simultaneously, we intended to document in nude mice that cyclosporine A indeed affected T-cell-mediated host resistance as shown previously with listeriosis, an intracellular infection not affected by cyclosporine A in genetically athymic mice (33).

To our surprise, we found that nude mice were neither prone to develop candidal surface lesions in cysts nor exempt from the immunosuppressive effect of cyclosporine A (Fig. 9). Because we suspected that our nu/nu mice either had atypically high residual T-cell activity or displayed extrathymic T-cell maturation, we confirmed the experiment with 5- to 6-week-old athymic nude mice from another breeding colony and monitored their incompetence to mount an efficient T-cell-mediated immune response against L. monocytogenes. (Fourteen days after challenge with 1.1 × 10⁶ CFU of L. monocytogenes, we cultured 10⁶ to 10⁷ CFU of L. monocytogenes per liver or spleen in ICR nu/nu mice as compared with <10 CFU in ICR +/+ mice; P < 0.001.) Also in this batch of mice, we found that cyclosporine (50 mg/kg per day) prevented elimination of C. albicans from the cyst of ICR nu/nu mice challenged with 2.6 × 10⁵ CFU (log₁₀ CFU at day 14 after cyclosporine challenge, 5.606 ± 0.73, versus 2.4 ± 0.48 for control; P < 0.01), an immunosuppressive effect that was comparable to that seen in parallel in normal ICR mice.

Because cyclosporine A is known to affect NK cell activity (2, 18), and because NK cells might be important in host resistance against another yeast, Cryptococcus neoformans (23, 26), the question occurred whether cyclosporine A might interfere with host defense against C. albicans in the cyst model by affecting NK cells, a defense which is well developed in nude mice (1, 13). We therefore turned to beige mice, a mutant mouse strain with a lysosomal defect resulting in deficient phagocytic (14, 30) as well as deficient NK (1, 13, 23, 28) cell activity. Indeed, beige mice handled thrushlike lesions less well than their littermates (Fig. 10 and 11). Also, during the first days of infection, treatment with cyclosporine A failed to enhance thrushlike lesions (Fig. 10 and 11). Beige mice were also slightly more susceptible to systemic candidiasis, as shown previously by others (3, 12) and as expected from a genetic lysosomal defect which impairs phagocytes (Fig. 12). Taken together, these data suggested that in beige mice the phagocytic lysosomal defect affected resistance against the systemic form of the disease, but that in addition another defect was relevant to host defense of beige mice in the cyst model. This second resistance factor appeared to be impaired by cyclosporine A in normal littermates, but because of its absence in beige mice this factor could not be documented with cyclosporine in this mutant mouse strain.

DISCUSSION

These comparative studies on host defense mechanisms against C. albicans on an artificial body surface or in parenchymatous organs discriminated in murine models between host defense systems operative against the superficial and against the systemic form of the mycosis. Cyclosporine A, a substance shown to suppress T-cell function and NK cell activity (2, 5, 15, 18, 34), impeded the elimination of C. albicans from the surface of pneumatized artificial cysts and encouraged the development of superficial thrushlike lesions (Fig. 3, 5, and 7), but had no effect on the elimination of the fungus from parenchymatous organs such as lungs, liver, or spleen or the development of mycotic lesions in the kidneys (Fig. 4, 5, 6, and 8). These observations are in keeping with the clinical perception that host defenses against mucocutaneous candidiasis and against the invasive form of the mycosis are distinct: natural phagocytic resistance prevents invasion and dissemination of C. albicans even in the absence of adaptive specific immune mechanisms (7, 10, 22, 29, 31). In contrast, mucocutaneous candidiasis appears to require T-cell-dependent mechanisms (2, 5, 15, 18, 34). Accordingly, cyclosporine A, in contrast to immunosuppression with a cytotoxic drug or with cortisone, which both also promoted thrushlike lesions, did not result...
in dissemination of candidiasis from flourishing superficial lesions (Fig. 8).

Unexpectedly, nude mice were not more susceptible to superficial lesions caused by C. albicans in our cyst model. In addition, cyclosporine A affected resistance against thrushlike lesions in athymic mice comparably to its effect in normal thymus-bearing mice (Fig. 9). This observation raises the question whether T cells are the true target of the immunosuppressive effect of cyclosporine A in our model of thrush. Nude mice, however, display some thymus-independent T-cell functions (13, 35, 37), including secretion of lymphokines such as interleukin-2 (21, 35, 37) which could serve as possible cyclosporine targets.

A second candidate target for the observed cyclosporine effect might be nonspecific phagocytic resistance provided by mononuclear or polymorphonuclear phagocytes. However, cyclosporine A is not known to affect antimicrobial phagocyte function (5, 15, 33). Furthermore, it appears

FIG. 8. Effect of immunosuppression on dissemination of candidiasis from thrushlike lesions in pneumatized cysts. After challenge of cysts with $2.5 \times 10^6$ CFU, infection was monitored by culture in the cysts, kidneys, spleen, liver, and lungs. Dissemination was only seen in the kidneys of cortisone-treated (panel C) and nitrogen mustard-treated (panel D) mice. (A) Control mice; (B) 40 mg of cyclosporine A per kg per day.

FIG. 9. Comparison of the effect of cyclosporine A on thrushlike lesions in NMRI +/- (A) and athymic NMRI nu/nu (B) mice. Cyclosporine A at 40 mg/kg per day (dashed lines) had comparable effects in athymic and control mice. Mean ± SD from four animals per time point. The challenge dose was $8.8 \times 10^8$ CFU.

FIG. 10. Evaluation of the immunosuppressive effect of cyclosporine A on thrushlike lesions in beige (C57BL/6 bg/bg) mice (B) compared with the effect in C57BL control mice (A). Beige mice were less competent in eliminating C. albicans from the cyst surface (solid line in panel B compared with solid line in panel A). The effects of this congenital incompetence and immunosuppression by cyclosporine A (40 mg/kg per day) were not additive (dashed lines). Note the lack of a significant effect of cyclosporine A over the first 5 days of the experiment in bg/bg mice ($P > 0.5$). The differences between CFU values from C57BL (■) and beige (●) mice receiving only olive oil were statistically significantly different (day 5, $P < 0.003$; day 10, $P < 0.02$; day 13, $P = 0.05$). Mean ± SD from four animals per group and time point. The challenge dose was $2.8 \times 10^9$ CFU.
difficult to conceive an effect of cyclosporine A which would only affect phagocyte function against a superficial lesion, leaving resistance against *C. albicans* growing in tissues (known to depend on phagocytes) unaffected. Finally, histologic comparison of superficial lesions with abscesses formed after i.v. inoculation suggests that phagocytes are rather scarce in superficial lesions as compared with deep lesions.

Alternatively, a third possibility for a cyclosporine target could be NK cells. Cyclosporine affects NK activity (2, 18), which is well developed in nude mice (1, 28, 35). NK cells might play an important role in resistance to experimental infection with the yeast *C. neoformans* (23, 26), and human NK cells display secretory activities upon exposure to candidal yeast cells (8). Beige mice, which in addition to relevant phagocytic dysfunction (14, 30) display defective NK activity (13, 23, 26, 28), were indeed more susceptible to superficial candidiasis than their normal littermates, and cyclosporine A, particularly early on, had little effect on the development of surface lesions (Fig. 10 and 11). Taken together, these observations would be compatible with the hypothesis that NK cells play an important role in our model and that NK cells are the target for the immunosuppressive effect of cyclosporine A in candidal cyst infection. Under these assumptions it could even be speculated that the defective NK cell activity developing in progressing acquired immune deficiency syndrome (4, 24) contributes to the high vulnerability of these patients to mucocutaneous candidiasis. It is of note, however, that a direct activity of NK cells against *C. albicans* has not been documented.

The antifungal activity of cyclosporine requires also brief consideration. Cyclosporine has an impressive activity against *Coccidioides immitis* in vitro and in vivo (19), but is in vitro at least 10 times less active against *C. albicans* or *C. neoformans* (19, 27), which would appear to require unrealistically high blood or tissue levels to be affected by cyclosporine in vivo. Nevertheless, antifungal activity against *C. neoformans* has been reported in murine models of systemic or pulmonary cryptococcosis (25), cautioning against overinterpretation of in vitro data. Our finding that cyclosporine provokes candidiasis in the pouch model over a broad dose range, without affecting systemic candidiasis, directly speaks against the possibility that the difference between the immunosuppressive effect in the pouch model and the systemic model depends on antifungal activity of cyclosporine in the latter model only. Furthermore, studies are in progress which show that lipophilic, amphiphilic, and hydrophilic antifungal drugs are comparably active in the pouch and the systemic model (unpublished observations).

In conclusion, these studies disclose cyclosporine-susceptible host defense mechanisms against thrushlike candidal lesions which are disparate from the phagocytic defense mechanisms operative against the systemic disease. Further characterization of these mechanisms promises to give us more insight into the nature of host defense against superficial forms of candidiasis.

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LITERATURE CITED


