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Immunization with the *Candida albicans* Membrane Fraction and in Combination with Fluconazole Protects against Systemic Fungal Infections

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We studied the immunogenicity of a membrane fraction prepared from *Candida albicans* cells called *C. albicans* membrane antigen (CMA). The present study revealed that CMA immunization has antifungal activity in mouse models of systemic fungal infection. Immunization of mice by subcutaneous injections of CMA with incomplete Freund adjuvant induced resistance to infections caused not only by *C. albicans* but also by *Aspergillus fumigatus*. The level of resistance to candidiasis was as high as that induced by whole-cell immunization. The acquired resistance to candidiasis in the mice immunized with CMA was not diminished by immunosuppressive treatment with cyclophosphamide. The level of resistance to fungal infections was superior to that given by fluconazole (FLC) treatment alone and highly enhanced by the combination with FLC. When $CD4^+$ cells in CMA-immunized mice were depleted by a monoclonal antibody, the antifungal activity induced by the combination of CMA and FLC was significantly reduced. These results indicate that immunization with CMA is useful for preventing systemic fungal infections and in combination with FLC for increasing resistance after infection.

Immunologically compromised patients can suffer from mucosal, cutaneous, or systemic mycoses caused by opportunistic fungi such as *Candida* sp. and *Aspergillus fumigatus*. The frequency of life-threatening systemic fungal infections has increased substantially due to increasing numbers of patients with immunological disorders and due to the nature of the immunosuppressive therapies applied in transplantation and in treating malignancies (6, 14). The systemic antifungal chemotherapeutics used for treating such infections are not yet satisfactory in terms of efficacy, toxicity, antifungal spectrum, or the possibility of drug resistance. The frequent use of antifungal chemotherapeutics, including fluconazole, in humans has led to the development of resistant strains of *Candida* and has raised concerns regarding cross-resistance to azoles and other chemotherapeutics (22, 30).

Safe and reliable vaccines have generally failed to confer protective immunity against fungal infections. Studies of mouse models have revealed immunogenic molecules that confer systemic anticandida resistance. These include cell surface components such as the cell wall polysaccharide, mannan or mannoprotein (11, 18), intracellular components such as ribosomes (12, 25), and heat shock protein hsp90 (16), as well as antibodies to hsp90 or mannan (10, 16).

We have shown that immunizing mice with a membrane fraction prepared from *C. albicans* protoplast cells together with adjuvant confers protective immunity against systemic candidiasis, in which $CD4^+$ T cells are important (S. Mizutani, M. Endo, T. Ino-ue, M. Kurasawa, Y. Uno, H. Saito, K. Onogi, I. Kato, and K. Takesako, submitted for publication). The present study shows that immunization with the membrane fraction, *C. albicans* membrane antigen (CMA), prevents systemic candidiasis induced by treatment with an immunosuppressive agent and that the efficacy of CMA against fungal infections, including systemic aspergillosis, in combination with fluconazole is additive.

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MATERIALS AND METHODS

Preparation of CMA. We lysed protoplasts of *C. albicans* cells and isolated the membrane fraction, CMA, as described elsewhere (Mizutani et al., submitted). Briefly, *C. albicans* TIMM 1768 was cultured in YPD medium (yeast extract, 1%; polypeptone, 2%; glucose, 2%) at 30°C overnight. Cells harvested by centrifugation were washed and suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 M NaCl as a stabilizer. Cell walls were removed by digestion with Zymolyase-20T (Seikagaku Kogyo, Tokyo, Japan), followed by *Trichoderma* lysing enzymes (Sigma, St. Louis, Mo.). Protoplasts were washed by centrifugation with the same buffer containing 1 M NaCl and then osmotically lysed in saline; the lysate was then homogenized and separated by centrifugation at $10{,}000 \times g$. The precipitate containing the membrane fraction was suspended in saline, boiled in a water bath for 15 min, and then sonicated to yield CMA. All procedures were done under sterile conditions. Four-liter cultures containing 1.9×10^{12} cells generally yielded CMA containing 1.5 g of protein. After lyophilization, we determined the contents of protein, carbohydrate, and lipid, excluding NaCl, the content of which was 51%, which was determined as a residue upon ignition by heating with sulfuric acid.

Protein content was determined by the BCA assay kit (Pierce, Rockford, Ill.) by using bovine serum albumin as the standard. Carbohydrate content was determined as total sugar by phenol-sulfuric acid method (7) using mannose as the standard. Mannan content was determined by the Pastorex *Candida* kit (Fuji Rebio K. K., Tokyo, Japan) that uses a monoclonal antibody (MAb) against *Candida* mannan (α-1,2-tetramannose. Glucan content was determined by the Fungitec G test (Seikagaku Corp., Tokyo, Japan). Lipid content was determined as described by Bligh and Dyer (4). Endotoxin content, determined by using the Quantitative *Limulus* Amebocyte Lysate kit (BioWhittaker, Inc., Walkersville, Md.), was 0.1 pg/10 μg of protein.

Immunization. Specific-pathogen-free female C57BL/6 or BALB/c mice, 6 to 8 weeks old (Japan SLC, Inc., Shizuoka, Japan), were subcutaneously (s.c.) immunized by an initial injection of a mixture (0.1 ml) of CMA with an equal volume of incomplete Freund adjuvant (IFA; Difco, Detroit, Mich.) or of complete Freund adjuvant (CFA; Difco) at a dose of 20 μg of protein/mouse. The mice then received one or two booster injections of the same amount of CMA emulsified in IFA either at 7 days after or at both 7 and 14 days after the first immunization, respectively. As for live-cell immunization, mice were injected s.c. on days 0 and 7 with 0.1 ml (total volume) of a mixture of live *C. albicans* cells (5×10^6 cells/mouse) and IFA.

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Preparation of fungal cells for infection. *C. albicans* TIMM 1768 was cultured in Sabouraud dextrose broth in L tubes. After an overnight incubation at 35°C, cells were harvested by centrifugation, washed with saline, and adjusted to a cell density appropriate for injection by dilution with saline. *A. fumigatus* TIMM 1776 was cultured at 30°C for 3 days in tubes containing slants of potato dextrose agar (Nissui Seiyaku, Tokyo, Japan). Spores in one tube were suspended in saline (10 ml) containing 0.1% Tween 80, counted with a hemocytometer, and adjusted to a spore density appropriate for injection by dilution with saline.

Antifungal activity against systemic fungal infections. Seven to ten days after the last immunization, mice were infected intravenously (i.v.) with *C. albicans* cells or *A. fumigatus* spores in a volume of 0.5 ml via the tail vein. Protection was assessed as follows. Survival was monitored for 30 days after an injection of 1×10^4 to 2.5×10^5 *C. albicans* cells or 2×10^6 *A. fumigatus* spores per mouse, and the number of live mice and the mean survival days (MSD) of the 5 to 10 mice per group were recorded. We also determined the CFU of *C. albicans* cells in the kidneys 7 days after the injection of 10^5 *C. albicans* cells as described elsewhere (Mizutani et al., submitted). The number of viable *C. albicans* cells is expressed as the mean ± the standard deviation (SD) of $\log_{10}$ CFUs per homogenate of two kidneys of 5 or 10 mice per group.

Immunosuppression caused by CY. Cyclophosphamide (CY) was given to mice according to the following schedules at a dose of 200 mg/kg intraperitoneally (i.p.). (i) Mice immunized s.c. with CMA or saline received one injection of CY 4 days before i.v. infection with 1×10^4, 5×10^4, or 2.5×10^5 *C. albicans* cells 7 days after the last immunization. (ii) Mice received three CY injections 4 days before each of two immunizations and 4 days before infection with 10^5 *C. albicans* cells.

DTH assay. To measure delayed-type hypersensitivity (DTH) response, CMA (10 μg of protein/50 μl) was injected into the left footpads of mice. Footpad swelling 24 h later was measured by using calipers, and the difference in thickness compared with the right footpad was expressed as the mean ± the SD of the five to seven mice per group.

Treatment with FLC and its combination with CMA immunization. Fluconazole (FLC; Diflucan; Pfizer, Tokyo, Japan) was diluted with saline, and then 0.4 ml was given orally 4 h after infection and once daily for a further 3 days at doses of 0 (saline), 3.1, 12.5, or 50 mg/kg. We examined the combined therapeutic effect of FLC and CMA immunization as follows. Mice immunized with CMA or saline by s.c. injection were infected with fungal cells 1 or 3 weeks after the last immunization and then given FLC 4 h after infection and once daily for a further 3 days at the doses described above.

Depletion of $CD4^+$ or $CD8^+$ cells or IFN-γ with MAbs. Hybridomas GK1.5 (anti-CD4 [-L3T4]), 53-6.72 (anti-CD8 [-Lyt-2.2]), and R4-6A2 (anti-IFN-γ) (American Type Culture Collection, Rockville, Md.) were used to prepare the respective MAbs as described elsewhere (Mizutani et al., submitted). Immunized and control C57BL/6 mice received three injections of each purified MAb i.p. at a dose of 300 μg/mouse at 1 and 4 days before or 2 days after the *C. albicans* infection with cells injected 3 weeks after the last immunization. Depletion of $CD4^+$ or $CD8^+$ cells or interferon gamma (IFN-γ) was monitored on the day of and 7 days after infection by using mice similarly treated with the respective MAb. $CD4^+$ or $CD8^+$ cells were analyzed by using a FACScan flow cytometer (Ortho Diagnostic Systems K. K., Tokyo, Japan) in splenocytes that had been passed through a nylon wool column to enrich T cells. IFN-γ in serum was determined by enzyme-linked immunosorbent assay according to the instructions supplied with the kit (R&D Systems, Minneapolis, Minn.). Selective T cells were markedly depleted in the mice given anti-CD4 or anti-CD8 MAb. Serum IFN-γ levels were below 20% of those of control mice on both days.

Statistical analysis. Groups were compared by Student's *t* test with correction for unequal variance. Survival days were compared by using the Kaplan-Meier method, and the results were statistically evaluated by the generalized Wilcoxon test or Cox-Mantel test. The significance was set at a P value of <0.05 (two-tailed test).

RESULTS

Resistance to systemic fungal infections induced by CMA immunization. The membrane fraction consisted of 60% protein, 8% carbohydrate (2 and 0.05% of which were mannan and glucan, respectively) and 30% lipid. These chemical properties were quite different from the cell wall mannoprotein extract (24, 27), indicating sufficient separation from cell wall components. The protocol of two or three weekly s.c. injections of CMA emulsified in IFA or CFA conferred high resistance to systemic *C. albicans* infection in C57BL/6 mice (Table 1). The level of resistance was as high as that induced by immunization with whole cells (Table 1). Subcutaneous immunization with CMA also prolonged the survival of mice infected with *A. fumigatus*, and three injections appeared to induce higher antifungal activity against *A. fumigatus* infection than two (Table 1).

TABLE 1. Antifungal activity of immunization with CMA against systemic infections by *C. albicans* or *A. fumigatus*[a]

Antigen	*C. albicans*		*A. fumigatus*	
	MSD	No. of survivors/ no. treated (P)[b]	MSD	No. of survivors/ no. treated (P)[b]
Saline	8.6	0/5	5.3	0/6
CMA × 2	>30.0	5/5 (0.0052)	>18.8	2/6 (0.0048)
Live cells × 2	>27.1	7/9 (0.0014)	NT	
Saline	14.0	0/8	7.2	0/5
CMA × 3	>30.0	7/7 (<0.001)	>20.6	2/5 (0.0068)

[a] Mice received two (× 2) or three (× 3) weekly immunizations with CFA or IFA as described in Materials and Methods. Mice were infected with *C. albicans* (2.5×10^5 cells/mouse) 7 days after the last immunization. Survival was observed for 30 days after infection. NT, not tested.
[b] Generalized Wilcoxon test versus control group injected with saline.

Effect of immunosuppression by CY on the resistance induced by CMA immunization. Most humans, including patients with some diseases, harbor *C. albicans* as normal microbial flora or as a nonprominent, underlying infection. Cancer patients treated with immunosuppressive anticancer drugs such as CY develop neutropenia and often suffer systemic fungal infections. Injections of CY 4 or 3 days before CMA immunization and injection of the antigen induced neither a DTH reaction in mice (footpad swelling, $\times 10^{-2}$ mm: 3 ± 1 in CY-immunosuppressed mice versus 129 ± 16 in nonimmunosuppressed mice) nor resistance (CFU in kidneys: 2.1×10^5 in CY-immunosuppressed mice versus 3.9×10^2 in nonimmunosuppressed mice). To investigate the influence of this immunosuppression on the resistance acquired after CMA immunization, we immunized mice s.c. and then treated them with CY immediately before infection with *C. albicans*. Infection with a low number (5×10^4 to 1×10^5) of *C. albicans* TIMM 1768 cells caused little mortality in mice within 7 days. However, when infected with the same number of cells after administering CY, all nonimmunized mice soon died, probably as a result of systemic infection due to neutropenia. In contrast, mice immunized with CMA prior to CY resisted rapid infection (Table 2). These results suggest that CMA immunization will be useful for preventing systemic fungal infection associated with immunocompromised conditions.

Antifungal activity of CMA immunization in combination with FLC. FLC is frequently used to treat and prevent fungal infections. To compare the antifungal activity of CMA immunization with that of FLC and to investigate their combined effect, CMA-immunized mice were infected with *C. albicans* or *A. fumigatus* cells and then given FLC. Immunization with

TABLE 2. Effect of immunosuppression by CY on resistance to systemic candidiasis induced by CMA immunization[a]

Infection (no. of cells/mouse)	Immunization	MSD	No. of survivors/ no. treated (P^b)
1×10^4	Saline	>20.6	2/5
	CMA	>30.0	5/5 (<0.01)
5×10^4	Saline	1.9	0/10
	CMA	>24.8	7/10 (<0.01)
2.5×10^5	Saline	0	0/5
	CMA	6.8	1/5 (0.05)

[a] Mice immunized with CMA emulsified in IFA were infected i.v. with *C. albicans* cells 7 days after the last immunization. CY was given 4 days before infection. Survival was observed for 30 days after infection.
[b] Cox-Mantel test versus the respective control group injected with saline.

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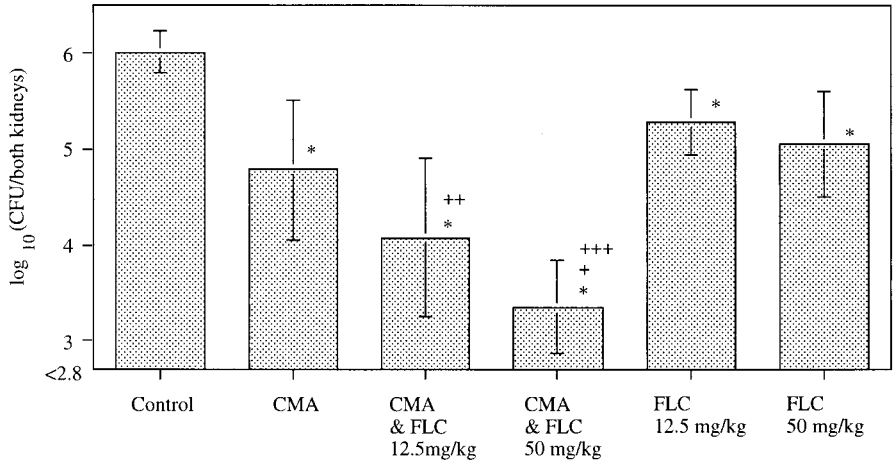

FIG. 1. Anticandida activity of CMA immunization combined with FLC. Mice (n = 10) immunized with CMA or saline emulsified in IFA were infected with *C. albicans* 7 days after the last immunization and given 4 oral administrations of FLC or saline as a control. Mice were sacrificed 7 days after the infection to determine CFU in kidneys. Student's t test results: *, $P < 0.001$ versus control group injected with saline; +, $P < 0.001$ versus CMA; ++, $P < 0.001$ versus FLC at 12.5 mg/kg; +++, $P < 0.001$ versus FLC at 50 mg/kg.

CMA alone conferred higher resistance not only to systemic candidiasis in terms of reduced CFU in the kidneys (Fig. 1) but also to systemic aspergillosis in terms of prolonged survival (Table 3) than did treatment with FLC alone. Furthermore, their combination was additive and therefore more effective in reducing CFU in the kidneys than either FLC or CMA immunization alone (Fig. 1). In addition, the combined antifungal activity against systemic *A. fumigatus* infection was more than additive (Table 3) and all mice given 50 mg/kg of FLC after CMA immunization survived for at least 30 days after infection. The DTH reaction to CMA was not affected by FLC plus CMA immunization (data not shown).

Effect of depletion of $CD4^+$ or $CD8^+$ cells or IFN-γ by MAb. We investigated the involvement of cell-mediated immunity in the additive effects of CMA immunization plus FLC. Mice immunized with CMA were depleted of $CD4^+$ or $CD8^+$ cells or of IFN-γ by three injections of the appropriate MAb starting 4 days before infection and then were given FLC. Depletion of $CD4^+$ cells caused a significant loss of DTH reaction to CMA (data not shown) and reduced resistance determined by CFU in the kidneys (Fig. 2). In contrast, depletion of $CD8^+$ cells or neutralization of IFN-γ after immunization immediately before infection did not reduce resistance.

TABLE 3. Prolonged survival of mice systemically infected with *A. fumigatus* after treatment with CMA, FLC, or a combination of both[a]

Group	CMA	FLC (mg/kg)	MSD	No. of survivors/ no. of treated mice	P^b versus:	
					Group 1	Groups 3, 4, and 5
1	−	0	7.0	0/5		
2	+	0	>20.6	2/5	0.0082	
3	−	3.1	8.4	0/5	0.4354	
4	−	12.5	11.6	0/5	0.0434	
5	−	50	>20.2	1/5	0.0086	
6	+	3.1	>25.6	3/5	0.0078	0.0098
7	+	12.5	>28.0	4/5	0.0068	0.0094
8	+	50	>30.0	5/5	0.0052	0.0188

[a] Mice that received three weekly immunizations with CMA (+) or saline (−) emulsified in CFA first and IFA thereafter were infected by *A. fumigatus* 7 days after the last immunization. Thereafter, they received FLC (3.1, 12.5, or 50 mg/kg once daily) for 4 consecutive days or saline (0 mg/kg) as a control. Survival was observed for 30 days after infection. Data are representative of two individual experiments.

[b] Generalized Wilcoxon test versus control (group 1) or the respective group treated with FLC (groups 3, 4, or 5). There were no significant differences between the CMA immunization only group (group 2) and the groups given CMA combined with FLC (groups 6, 7, or 8).

DISCUSSION

This study showed that a membrane fraction (CMA) prepared from protoplasts of *C. albicans* cells, conferred upon mice resistance to systemic aspergillosis as well as candidiasis after s.c. immunization with adjuvant. The results also showed that this immunization prevented mice from developing systemic candidiasis caused by immunosuppression with CY and indicated that it is useful during treatment with the chemotherapeutic agent FLC after infection. Although caution is needed in discussing these preliminary results, we stress the importance of studying CMA as an antigen preparation that may be useful for active immunization. We have elsewhere revealed the importance of the $CD4^+$ cells and the DTH reaction conferred by CMA immunization in resistance to systemic candidiasis (Mizutani et al., submitted). Stimulating spleen cells from CMA-immunized mice caused the release not only of IFN-γ but of nitric oxide (data not shown), which is produced by macrophages activated by IFN-γ. Activated nonspecific macrophages will generate resistance to aspergillosis and candidiasis similarly to that induced by i.v. injection of live attenuated *C. albicans* cells (3, 29).

Mice treated with several injections of CY prior to s.c. immunization with CMA neither resisted systemic candidiasis nor produced a DTH reaction. In contrast, mice that acquired resistance due to immunization were also resistant to systemic candidiasis induced by one injection of CY. Polymorphonuclear leukocytes are important in eradicating *C. albicans* cells, and these are decreased by CY (9). The noninhibitory effect of CY on the resistance of the immunized mice indicates that sufficient activated macrophages or neutrophils remain after CY treatment to eradicate *C. albicans* cells. CY injections prior to immunization and antigen administration inhibited induction of DTH response. This may be caused by a reduction in the number of lymphocytes at 3 days after the injection of CY

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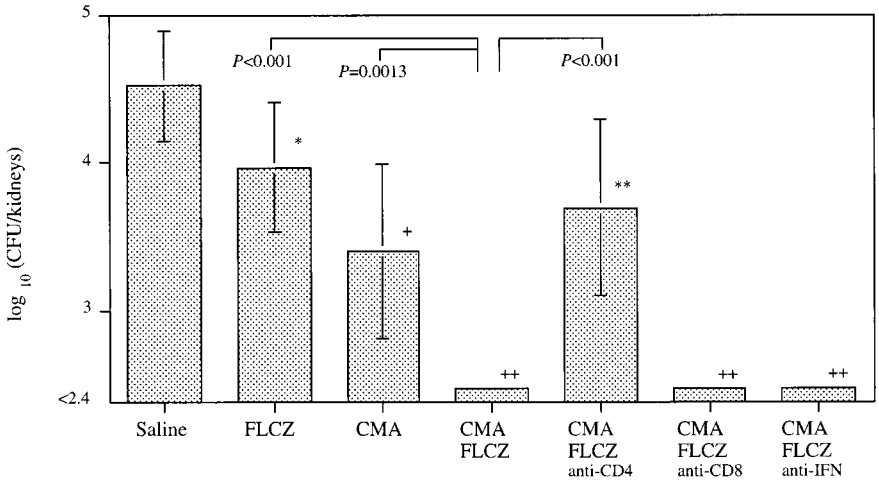

FIG. 2. Effect of depletion of $CD4^+$ or $CD8^+$ cells or IFN-γ by MAbs on the activity of combined CMA immunization and FLC. Mice ($n = 5$) that received two weekly immunizations with CMA or saline emulsified in IFA were infected with *C. albicans* 3 weeks after the last immunization and given four oral administrations of 50 mg of FLC or saline per kg as a control once daily. Mice were sacrificed 7 days after infection to determine the CFU in the kidneys. Mice were given each MAb three times as described in Materials and Methods. Data are representative of two individual experiments. Student's *t* test results: *, $P = 0.0264$; **, $P = 0.0088$; ***, $P = 0.0066$; +, $P = 0.0012$; ++, $P < 0.001$ versus control group injected with saline.

(20). However, a late increase in the number of neutrophils and lymphocytes caused by CY (1) may induce and activate resistance, leading to the long survival times as shown in Table 2.

The antifungal activity induced by CMA immunization was superior to the activity of FLC and was highly enhanced by combination with FLC. Depletion of $CD4^+$ cells reduced the level of combined antifungal activity. Polymorphonuclear neutrophils and macrophages activated by $CD4^+$ cells responding to CMA or cytokines may collaborate with the fungistatic activity of FLC to significantly increase the killing of fungi by these phagocytes. Antifungal substances such as nitric oxide produced by activated macrophages may enhance the antifungal activity of FLC (17). FLC inhibits the synthesis of sterols that are important for membrane formation (20, 26). Thus, fungal cells having an imperfect membrane formed in the presence of FLC may be more sensitive to neutrophils or macrophages activated by CMA immunization than are normal fungal cells. These effects would cause the high antifungal activity of FLC combined with CMA immunization. On the other hand, this increased activity may arise from FLC enhancing Th1-type cells induced by CMA, which activates production of IFN-γ and nitric oxide by spleen cells (4; Mizutani et al., submitted). This action will further enhance neutrophils and macrophages and finally enhance antifungal activity (3, 23, 29). Although we found that the DTH response was not enhanced, some stimulation of a protective Th1 response by FLC may be involved in the combined effect. Neutralization of IFN-γ after immunization and immediately before infection did not reduce resistance. This may result from incomplete neutralization of IFN-γ at local infection areas, including the kidneys, despite systemic reduction of this cytokine or from difficulties in inhibiting resistance in mice acquired by means of activated macrophages (21, 29). Administering anti-IFN-γ MAb during immunization will inhibit the acquisition of the resistance (29).

C. albicans is a constituent of the normal microbial flora that colonizes the mucocutaneous surfaces of the oral cavity, gastrointestinal tract, and vagina of many mammals and other animals (13). Almost all humans exhibit immune responses, including antibody production and the DTH reaction to *C. albicans* cells and their components. Passive or active immunotherapy with cell surface mannoproteins is effective against systemic candidiasis (10, 18). However, mannoproteins have nonspecific immunomodulatory and immunosuppressive functions (8) and modulate immune responses as inducers of human lymphocyte proliferation and neutrophil activation with cytokine production in vitro (18, 24). Cell wall glucans also have a variety of nonspecific immunomodulatory effects (24). Therefore, cell wall components, including mannoproteins and glucans, may cause adverse effects in humans. Membrane fractions such as CMA contain few cell wall components and have little nonspecific immunomodulatory functions (Mizutani et al., submitted), thus reducing the adverse effects. Recently, we isolated mitochondrial superoxide dismutase from the membrane fraction, characterized its antigenicity, and revealed its activity in inducing resistance to systemic candidiasis by s.c. immunization (K. Takesako et al., Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2162, 1999).

The recent frequent use of antifungal chemotherapeutics, including FLC, in humans has caused the emergence of resistant *Candida* strains and has led to the fear of developing cross-resistance to azoles and other chemotherapeutics (22, 30). Antifungals like amphotericin B that have immunomodulatory effect (2, 4) and azoles such as itraconazole may show synergism with CMA immunization. Immunotherapy with intracellular *Candida* constituents such as CMA will help to inhibit the emergence of resistant *Candida* strains and to treat fungal infections caused by chemotherapeutics after infection. Furthermore, the features of CMA immunization, including no loss of the acquired resistance after CY immunosuppression, indicate the potential of a vaccine with CMA as the antigen for the prophylaxis of fungal infections in patients with cancer and AIDS.

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