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Increase of Mouse Resistance to *Candida albicans* Infection by Thymosin α_1

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Studies were carried out to assess the ability of thymosin α_1 to prolong the survival of mice challenged with *Candida albicans*. Two- to four-month-old mice were treated with graded doses of thymosin α_1 before, after, or before and after intravenous challenge with *C. albicans*. Significant resistance to lethal infection was afforded by 100 μg of thymosin α_1 per kg given before or before and after challenge, whereas no protection was found in mice treated with thymosin α_1 administered at any dose level after inoculation. Pretreatment with thymosin α_1 also prevented the increased susceptibility to *C. albicans* infection of mice pretreated with cyclophosphamide on day −6. The results showed that thymosin α_1 was capable of protecting untreated or cyclophosphamide-pretreated mice from *C. albicans* infection at an optimal dose and schedule of administration.

Infections with opportunistic pathogens represent one of the major problems in the management of immunosuppressed hosts, especially in the case of cancer patients heavily treated with radiotherapy or chemotherapy. *Candida albicans* infections are rather frequent and can produce a fatal generalized disease (1, 9, 23, 24). Host resistance against disseminated *C. albicans* infections principally involves T-independent immune systems (3, 11, 12, 19, 21, 26), including natural resistance mediated by polymorphonucleates (4, 8, 12). Therefore, pharmacological manipulation of the host affecting cell populations that mediate innate resistance could influence profoundly anti-*C. albicans* resistance (4, 20).

The present report describes studies conducted in an animal model showing the influence of two immunoactive agents on resistance to intravenous challenge with *C. albicans*. In particular, a thymic factor such as thymosin α_1 (referred to hereafter as α_1), first isolated by A. Goldstein (6, 15), and an antitumor drug of large clinical application such as cyclophosphamide (Cy) have been tested alone or in combination on the survival times of infected mice. The results showed that the dose and treatment schedules of thymosin α_1 were of primary importance; α_1 was found to be capable of protecting untreated or Cy-pretreated recipient mice from *C. albicans* only at an optimal dose and schedule of administration.

MATERIALS AND METHODS

Mice. Inbred C3H Cr, BALB/c Cr, C57Bl/6 Cr, or hybrid (BALB/c Cr × DBA/2 Cr)F_1 (CD2F1) mice of both sexes, 2 to 4 months old, were obtained from the National Cancer Institute, National Institutes of Health, Bethesda, Md.

Drug. Cy was kindly supplied by V. L. Narayanan, National Cancer Institute. The drug was dissolved in 0.85% NaCl sterile solution immediately before use and injected intraperitoneally in a volume of 0.1 ml per 10 g of body weight.

Yeast cultures. *C. albicans* used throughout this study was isolated from a clinical specimen and identified by established taxonomic criteria (14, 16). The yeast cells were grown at 28°C under slight agitation in low-glucose Winge medium composed of 0.2% (wt/vol) glucose and 0.3 (wt/vol) yeast extract (BBL Microbiology Systems, Cockeysville, Md.) until stationary-phase growth was reached (about 24 h). Under these conditions the culture gave a yield of approximately 2.8×10^8 cells/ml, and the organisms grew as an essentially pure yeast-phase population (17). After growth, cells were harvested by low-speed centrifugation, washed twice in sterile distilled water, and diluted at the desired concentrations.

α_1. The polypeptide α_1 (RO 21-9199/002) was a kind gift from A. Ramael (Hoffman-LaRoche Inc., Nutley, N.J.). The lyophilized drug (2 mg/vial) was dissolved in 1.4% sterile solution of sodium bicarbonate, diluted to the desired concentration, and inoculated subcutaneously within 20 min.

Blood and organ culture for titration of *C. albicans* organisms. Blood samples of mice inoculated intravenously with *C. albicans* were collected from the retroorbital plexus and hemolyzed in distilled water.

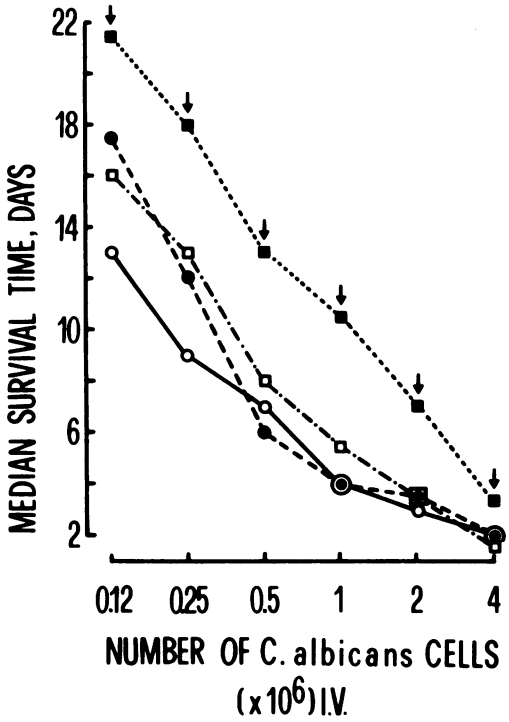

FIG. 1. Mortality of C57Bl/6 (■), BALB/c (□), C3H/HeJ (○), CD2F1 (●) male mice challenged intravenously with graded inocula of *C. albicans* cells. ↓, $P < 0.01$ comparing the survival times of C57Bl/6 mice with those of CD2F1 recipients. Similar results were obtained in two different experiments with female mice (data not shown).

The organs of mice (spleen, lungs and kidneys) were removed aseptically and placed in a tissue homogenizer with 3 ml of 0.85 M sterile NaCl solution. Each organ was homogenized and diluted with saline. The number of colony-forming units of *C. albicans* in the specimens was determined by a plate dilution method, using Sabouraud dextrose agar. The colonies were counted after 48 h of incubation at 37°C and expressed as number of colony-forming units per organ.

Statistical analysis. Differences in survival times were analyzed according to the Mann-Whitney *U*-test. Differences in colony-forming units in the various organs were determined according to Student's *t*-test.

RESULTS

Susceptibility of various mouse strains to *C. albicans* infection. Preliminary studies were performed to test the susceptibility of different mouse strains to infection with graded numbers of *C. albicans* injected intravenously. Inbred C3H and BALB/c and hybrid CD2F1 mice showed approximately similar degrees of susceptibility to the infection, whereas C57Bl/6 were slightly more resistant, as evidenced by their significantly longer survival times (Fig. 1). These results were highly reproducible and unaffected by the sex of animals used.

Death of the mice was always caused by generalized *C. albicans* infection as revealed by gross autopsy showing diffuse renal abscesses, positive for the presence of high titers of *C. albicans* as detected by histological examination (data not shown). Moreover, *C. albicans* bioassay showed elevated concentrations of the fungus in all of the organs tested (Fig. 2). Significant differences in the number of live yeast cells were observed between untreated and Cy-treated groups of mice 24 h after *C. albicans* challenge.

Effect of α_1 treatment on the survival of CD2F1 mice inoculated with graded doses of *C. albicans*. Two-month-old CD2F1 mice were treated with different doses of α_1 before (day −10, −8, −6, −4, or −2), after (day 1, 2, 3, 4, or 5), or before and after (day −10, −8, −6, −4, −2, 1, 2, 3, 4, or 5) intravenous challenge with graded doses of live *C. albicans*. The results obtained in four different experiments were highly reproducible and showed that α_1 was able to increase the survival times of treated mice with respect to those of untreated controls. This protective effect was dependent on the concentration and schedule of administration of α_1, but independent of the sex of the animals used (data not shown). Figure 3 shows the data obtained in one experiment in which 10 animals per group were used: the administration of α_1 before or before and after *C. albicans* administration showed a significant increase of the survival times of the mice challenged with 2×10^6 *C. albicans*. At lower doses, (10^6 and 5×10^5), the same treatment showed a protective effect not only in terms of increased survival time but also in terms of percentage of "cured" mice.

Administration of α_1 was effective only at a dose of 100 μg/kg per day, whereas no effect was found at a dose of 1 or 10 μg/kg per day, or when the substance, at a dose of 100 μg/kg per day, was given after *C. albicans* infection. Furthermore, no protection against *C. albicans* challenge was afforded if 2 instead of 10 injections of α_1 were delivered to CD2F1 mice (Table 1). Moreover, inactivated α_1 (110°C for 15 min) afforded no protective effect irrespective of the schedule of administration used (Table 1).

Effect of optimal treatment schedule of α_1 administration on survival of different strains of mice. The optimal treatment schedule found for α_1 administration was also tested in the strains of mice previously used for the experiments illustrated in Fig. 1. The results (Table 2) showed that α_1 was able to increase significantly the survival times with respect to those of untreated controls.

Effect of various treatment schedules of Cy on the survival of CD2F1 mice challenged with dif-

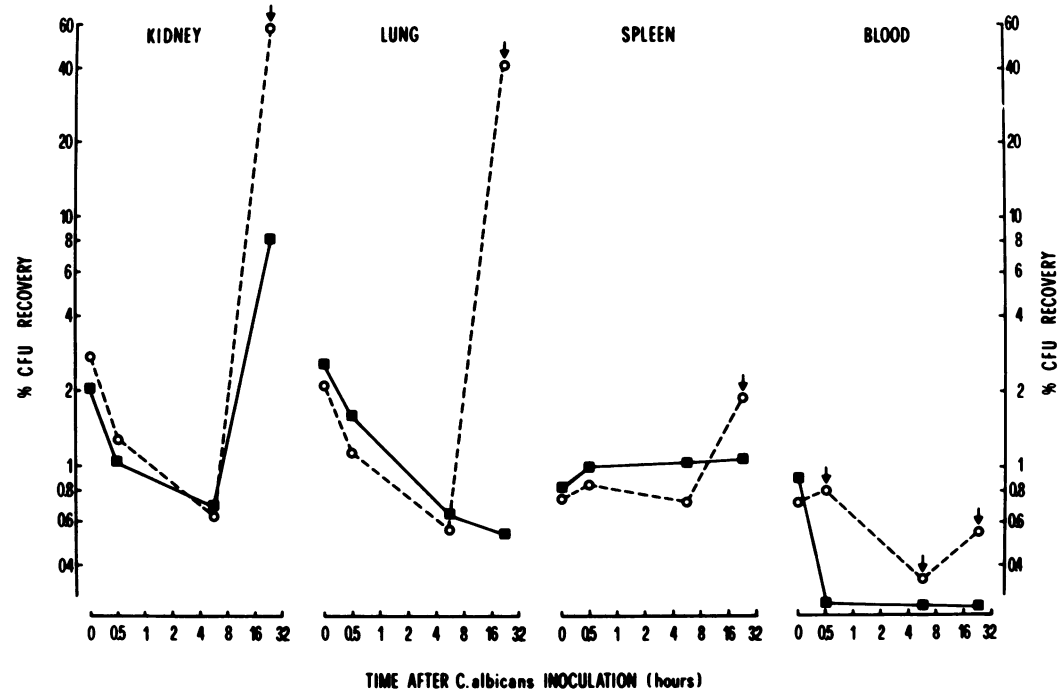

FIG. 2. Percentage of colony-forming units recovered from kidneys, lungs, spleen, and blood of Cy-pretreated CD2F1 male mice at different times after inoculation of 5×10^5 *C. albicans* cells. Results are expressed as percentage of recovery (geometric mean) of the *C. albicans* cells injected intravenously at time 0 (five mice for each determination). Symbols: ■, Untreated controls; ○, Cy (150 mg/kg) given on day −3 before *C. albicans* challenge; ↓, $P < 0.01$ comparing the number of colony-forming units of untreated controls with those of Cy-pretreated recipients according to Student's *t*-test analysis. Determined 5 min after *C. albicans* injection.

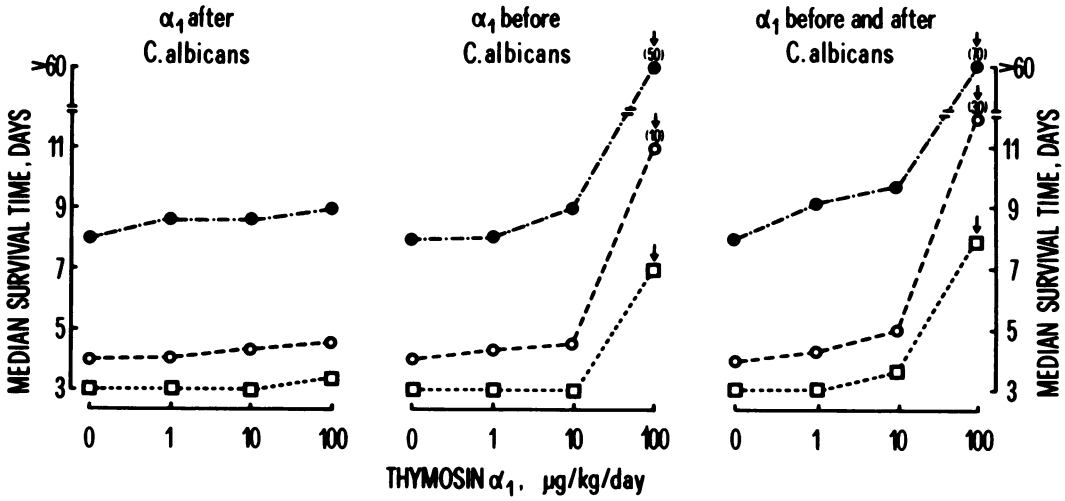

FIG. 3. Increased resistance induced by α_1 administered subcutaneously to CD2F1 male mice challenged intravenously with graded inocula of *C. albicans* cells. Symbols: ●, 5×10^5 organisms/mouse; ○, 10^6 organisms/mouse; □, 2×10^6 organisms/mouse. (Left) α_1 was administered subcutaneously on day 1, 2, 3, 4, or 5 with respect to the challenge day (day 0). (Middle) α_1 was administered subcutaneously on day −10, −8, −6, −4, or −2 with respect to challenge. (Right) α_1 was administered subcutaneously on day −10, −8, −6, −4, −2, 1, 2, 3, 4, or 5 with respect to challenge. ↓, $P < 0.01$ comparing the survival times of α_1-treated mice with those of untreated controls. In parentheses is given the percentage of surviving mice over a 60-day observation period (10 mice/group).

TABLE 1. Effect of heat inactivation on the antiinfectious activity of α_1 inoculated into CD2F1 male mice

α_1 treatment[a]	Median survival time (days)	No. dead[b]	P[c]
None	9	10	
−10, −8, −6, −4, −2, 1, 2, 3, 4, 5	60	4	<0.01
−10, −8, −6, −4, −2, 1, 2, 3, 4, 5 (inactivated)[d]	10	10	NS
−10, −8	9.5	10	NS
1, 2	8.5	10	NS

[a] α_1 (100 μg/kg) was administered subcutaneously on the days shown. The day of *C. albicans* challenge (5 × 10^5 organisms/mouse intravenously) was considered day 0.

[b] No. of dead mice at day 60 (10 animals tested).

[c] Probability was calculated by comparing the survival times of α_1-treated mice with those of untreated controls. NS, Not significant.

[d] α_1 was inactivated for 15 min at 110°C.

ferent doses of *C. albicans*. CD2F1 mice were treated with Cy (150 mg/kg) 6 h or 3, 6, or 9 days before *C. albicans* challenge. Cy pretreatment reduced significantly the survival time of the mice when given on day −3 or −6, but not 6 h or on day −9 before *C. albicans* challenge (Fig. 4). In addition, pretreatment with Cy was more effective in abrogating the hosts' resistance when given on day −3 rather than on day −6 before infection (Fig. 4). Bioassay studies performed on blood, spleen, kidneys, and lungs showed that the yeast cells rapidly disappeared from the blood, kidneys, and lungs of the control mice up to 6 h after challenge (Fig. 2). *C. albicans* titers declined less sharply in the blood of Cy-treated mice but showed similar patterns in the organs (Fig. 2). At 24 h after infection, low levels of the organism were detected in the blood and lungs of the control mice, whereas increased amounts were found in the kidneys. On the contrary, marked increases in *C. albicans* titers were found at 24 h in the same organs of Cy-treated recipients (Fig. 2). No change in the titers was detected in the spleens of the control mice at any time after challenge. On the other hand, a significant rise in colony-forming units was found in the spleens of Cy-pretreated mice 24 h after *C. albicans* challenge.

Effect of α_1 treatment schedules on CD2F1 mice immunosuppressed by Cy treatment and challenged intravenously with *C. albicans*. CD2F1 mice were treated with Cy (150 mg/kg intraperitoneally) on day −3 or −6 before *C. albicans* challenge (5 × 10^5 live cells). The survival times of all Cy-pretreated mice were significantly shorter than those of control groups (Table 3). Additional groups of Cy-pretreated mice were inoculated with α_1 before and after *C. albicans* challenge. In all cases, α_1 administration had no effect on the survival times of mice immunodepressed with Cy. Similar findings were obtained with a *C. albicans* inoculum of 5 × 10^4 instead of 5 × 10^5 (data not shown). However, when mice were pretreated with Cy on day −6 before the challenge of 5 × 10^5 organisms, administration

TABLE 2. Increased resistance induce by α_1 administered subcutaneously to different inbred or F_1 hybrid mice challenged intravenously with 10^6 *C. albicans*

Host[a]	Untreated		Treated with α_1[b]	
	MST[c] (days)	No. dead[d]	MST	No. dead
CD2F1	6	8	14	8
BALB/c	4.5	8	12	8
C57B1/6	9	8	17	8
C3H/HEJ	5	8	11.5	8

[a] Eight- to 12-week-old male mice.

[b] α_1 (100 μg/kg) was administered subcutaneously on days −10, −8, −6, −4, −2, 1, 2, 3, 4, and 5 with respect to *C. albicans* challenge (day 0). $P < 0.01$ for all groups tested.

[c] Median survival time.

[d] No. of dead mice at day 60 (eight animals tested).

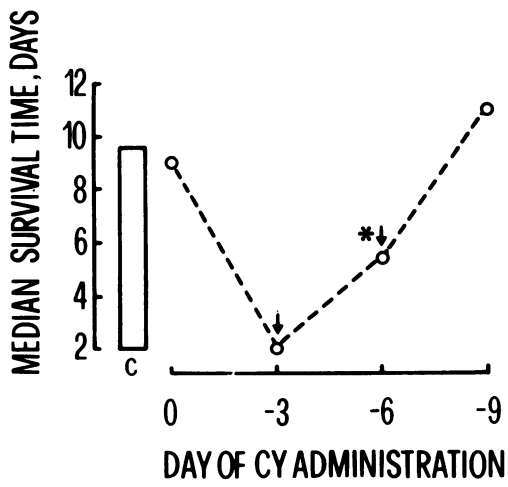

FIG. 4. Effect of the time of Cy administration on the resistance of CD2F1 male mice to intravenous challenge with 5 × 10^5 *C. albicans* cells. Symbols: □, Untreated control; ○, Cy (150 mg/kg) given at various intervals before *C. albicans* challenge. On day 0, *C. albicans* was given 6 h after drug administration. ↓, $P < 0.01$ comparing the survival times of Cy-pretreated mice with those of untreated controls. *, $P < 0.01$ comparing the survival times of mice treated with Cy on day −3 with those of animals treated with Cy on day −6 (10 mice/group).

TABLE 3. Effect of α_1 treatment on Cy-mediated depression of anti-infectious resistance in CD2F1 male mice challenged intravenously with 5×10^5 *C. albicans*

Treatment schedule[a]		MST[b]	No. dead[c]	P_1[d]	P_2[e]
α_1	Cy				
		10	8		
−10, −8, −6, −4, −2, 1, 2, 3, 4, 5		60	4	<0.01	
	−3	2	8	NS	
−10, −8, −6, −4, −2, 1, 2, 3, 4, 5	−3	2	8	NS	NS
	−6	4	8	NS	
−10, −8, −6, −4, −2, 1, 2, 3, 4, 5	−6	12	8	NS	<0.01

[a] α_1 (100 μg/kg) was administered subcutaneously on the days shown. (Time of *C. albicans* challenge was considered day 0.) Cy (150 mg/kg) was administered intraperitoneally as a single injection on the day shown.

[b,c] See footnotes *c* and *d*, Table 2.

[d] Probability was calculated by comparing the survival times of nontreated mice with those of α_1- and/or Cy-treated animals. NS, Not significant.

[e] Probability calculated as for footnote[d] comparing the survival times of Cy-treated mice with those of mice treated with α_1 and Cy.

of α_1 delayed mortality to values not significantly different from those found in non-immunodepressed controls.

DISCUSSION

This report shows that in vivo resistance to *C. albicans* infection is increased by treatment with α_1. In particular, significant resistance to lethal challenge was obtained by treatment with 100 μg of α_1 per kg given before or before and after *C. albicans* infection, whereas no protection was found in mice treated with α_1 administered at different doses after challenge. Treatment with α_1 increased resistance to the infection in all of the strains used, both in the more susceptible hosts (e.g., CD2F1, C3H, and BALB/c) and in the relatively resistant C57Bl/6 mice (see Table 2). The observation that treatment with α_1 was not capable of influencing the course of *C. albicans* infection when given after the challenge (Fig. 3) can be tentatively explained on a kinetic basis. It is conceivable that the rapid and invasive growth of *C. albicans* injected intravenously can overcome any increased resistance mediated by α_1 administration. Alternatively, it can be suggested that rapid antigen expansion occurring during *C. albicans* growth conditions a quite different response of the host to α_1 treatment. The finding that α_1 increases the resistance against *C. albicans* seems to suggest that mature T cells may be involved in resistance to infection. It is conceivable that increased levels of T-cell subpopulations induced by α_1 treatment (15, 27, 30) can potentiate a cell-mediated immunity against *C. albicans* organisms (25). Moreover, since macrophages appear to play a significant role in resistance to *C. albicans* (5, 13, 22, 29), a possible activation of macrophage function cannot be ruled out. In addition, natural resistance mediated by a variety of effector immunocytes, such as natural killer cells (7) or polymorphonuclear cells (10), should be considered. Our data also show the effect of Cy treatment on *C. albicans* infection, confirming previous observations (4, 18, 20). In particular, we found a marked decrease of resistance when Cy was administered 3 days before the *C. albicans* challenge, whereas a less marked effect was observed when the animals were pretreated 6 days before challenge (full recovery of resistance was observed 9 days after Cy treatment). Administration of α_1 was unable to affect the survival times of mice injected with Cy 3 days before the challenge, but was effective in restoring resistance in the group of animals pretreated with Cy 6 days before *C. albicans* challenge, when the level of immunodepression was less marked.

In conclusion, our data clearly indicate that a polypeptide of thymic origin such as α_1 can afford protection against lethal *C. albicans* septicemia when used according to appropriate dose and schedule of administration in both normal and immunodepressed mice. Whether this effect is mediated by increased efficiency of T-dependent immune function or through direct or indirect activation of natural resistance mechanisms (e.g., macrophages, polymorphonuclear cells, or natural killer cells) is not known at present. Although the mechanism underlying the anti-infectious properties of α_1 has not been elucidated, it appears that the factor may also have therapeutic applications in immunodepressed hosts treated with high doses of antineoplastic agents.

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