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Evidence for Macrophage-Mediated Protection against Lethal *Candida albicans* Infection

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Systemic infection of mice with a *Candida albicans* strain (PCA-2) incapable of yeast-mycelial conversion conferred protection against a subsequent intravenous challenge with the pathogenic strain of the parent organism, strain CA-6. Protection was nonspecific since it was also detected upon challenge of mice with *Staphylococcus aureus*. Moreover, the PCA-2 organisms had to be viable, their effects being most evident when they were given intravenously at a dose of 10^6 cells 7 to 14 days prior to microbial challenge. Thus, all mice pretreated with PCA-2 and challenged 14 days later with viable CA-6 cells lived through a 60-day observation period, whereas all control mice not treated with PCA-2 died within 3 days. In an attempt to correlate the immunostimulatory effects observed in vivo with possible modifications in in vitro functions, it was found that administration of PCA-2 was accompanied by an increase in the number of peripheral blood polymorphonuclear cells and by the activation in the spleen of cells with highly candidacidal activity in vitro. Moreover, the adoptive transfer of plastic-adherent cells from PCA-2-infected mice into histocompatible recipients conferred considerable protection against subsequent CA-6 challenge.

Candida albicans is a member of the normal flora of humans which lives commensally with its host until some precipitating event creates an environment favorable for invasion. The mechanisms by which normal individuals resist disease are poorly understood, but both the humoral (24, 27) and cellular (7, 10, 15, 17, 18, 32) arms of the immune system are believed to play a role. More recently, our laboratory has called attention to the possible relevance of innate resistance factors operating both in normal (2) and immunomodulated (1, 3, 5, 6) hosts challenged experimentally with *C. albicans*.

As a dimorphic fungus, *C. albicans* undergoes yeast-mycelial conversion through the intermediary formation of a germ tube, and such a conversion is suspected to play a crucial role in the pathogenicity of the fungus, the mycelial form being regarded as endowed with greater invasiveness and resistance to host defense mechanisms (30).

In the present study, we investigated the effect of host infection with a poorly virulent agerminative (23) strain of *C. albicans* (strain PCA-2) on subsequent challenge with a highly virulent germ tube-positive strain (strain CA-6). An increase in resistance was observed which was largely nonspecific and appeared to be mediated by cells in the granulocyte and macrophage lineages.

MATERIALS AND METHODS

Mice. Hybrid (BALB/cCr × DBA/2 Cr)F_1 (CD2F1:*H-2*^{d}/*H-2*^{d}) mice were obtained from Charles River Breeding Laboratories, Inc., Calco, Milan, Italy.

Drugs. Amphotericin B (Fungizone), kindly supplied by E. R. Squibb & Sons, Princeton, N.J., was provided in vials containing 50 mg of amphotericin B and 41 mg of sodium deoxycholate with 25.2 mg of sodium phosphate as a buffer. The drug was dissolved in sterile, nonpyrogenic 5% glucose in water and injected intraperitoneally in a volume of 0.1 ml/10 g of body weight.

Microorganisms. (i) Yeasts. Three strains of *C. albicans* (laboratory identification names, CA-6, 3153A, and PCA-2), all with identical sugar assimilation and fermentation patterns (33), were used throughout this study. Strain CA-6 was isolated from a clinical specimen (21), and strains 3153A and PCA-2 were kindly supplied by D. Kerridge, Department of Biochemistry, University of Cambridge, Cambridge, England. The agerminative strain PCA-2 (23) is an echinocandin-resistant mutant of the parental strain 3153A. The 50% lethal doses for strains CA-6, 3153A, and PCA-2 were 0.2×10^5, 1.0×10^5, and 2.5×10^6, respectively. In selected experiments, a *Candida tropicalis* strain, isolated from a clinical specimen and identified as described above, was also used. All yeasts were grown at 28°C under slight agitation in low-glucose Winge medium (23) composed of 0.2% (wt/vol) glucose and 0.3% (wt/vol) yeast extract (BBL Microbiology Systems, Cockeysville, Md.) until a stationary phase of growth was reached (about 24 h). Under these conditions, cultures gave a yield of approximately 3×10^8 cells per ml, and the organisms grew as an essentially pure yeast-phase population. After the 24 h culture, cells were harvested by low-speed centrifugation (1,000 × *g*), washed twice in saline, and diluted to the desired density.

(ii) *Staphylococcus aureus*. A coagulase-positive *S. aureus* strain (Cowan, NCTC, Colindale) was grown at 37°C on Mannitol Salt Agar (BBL). After the 24-h culture, microorganisms were harvested by low-speed centrifugation, washed twice in saline, and diluted to the desired number of CFU per milliliter. In all in vivo infection experiments, yeast and bacterial cell suspensions were injected intravenously (i.v.) via the tail vein at a volume of 0.5 ml per mouse. Each experimental group consisted of at least 10 mice.

Leukocyte counts. Mice were bled from the retroorbital sinus. Cell counts were determined by diluting anticoagu-

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lated blood 1:20 in Türk solution and counting the cells in a corpuscle-counting chamber. Differential cell counts were made on blood smears after May-Grünwald-Giesma staining by counting 200 leukocytes per slide.

Cell fractionation procedures. (i) Plastic adherence. Effector cells (4×10^7) suspended in a volume of 10 ml of RPMI 1640 medium were incubated for 3 h at 37°C in a 5% CO_2 atmosphere in 93-mm petri dishes (Nunc Inter Med, Roskilde, Denmark). At the end of the incubation, the dishes were extensively washed with RPMI 1640 medium to remove the nonadherent cells. The adherent cells were recovered by scraping with a rubber policeman, and then they were washed and suspended (viability, 80 to 90%) in RPMI 1640 medium (Eurobio Laboratories, Paris, France) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Eurobio), and 0.1% gentamicin sulfate (hereafter referred to as complete RPMI 1640 medium). More than 98% of the recovered cells had the morphology of macrophages on Giemsa stain.

(ii) Nylon column. Effector cells were passed over a nylon fiber column as previously described (14). Briefly, the sterile nylon columns were rinsed with 20 ml of RPMI 1640 medium supplemented with 5% fetal calf serum. The columns were drained of excess medium and then placed in sterile syringe covers and put in a CO_2 incubator at 37°C at least 1 h before loading of the cells. Then 10^8 cells in a volume of 2 ml were added to the column and washed in the nylon wool with 0.5 to 1 ml of warm (37°C) medium. The columns were replaced in the sterile syringe covers and were left for 45 min at 37°C. The columns were then washed slowly with medium (37°C), the first 25 ml of effluent was collected in 50-ml conical tubes, and then the cells were pelleted at $290 \times g$ for 10 min at 4°C. Cell recovery was about 30%, of which only 1 to 2% showed the morphology of macrophages on Giemsa stains.

(iii) Carbonyl-iron powder and magnet. Removal of phagocytic cells from the effector cell population was performed as previously described (19). Briefly, 25 ml of spleen cell suspensions (10^7 cells per ml) was incubated with 25 mg of carbonyl-iron powder (G. A. F. Corp., New York, N.Y.) in a 50-ml conical tube (Becton Dickinson Labware, Oxnard, Calif.) for 60 min at 37°C. To remove the cells that ingested iron particles, the tube was placed on the top of a magnet, and the supernatant was removed. This last step was repeated six to eight times. The cells were then washed and used as effectors in the microcytotoxicity assays. Cell recovery was about 60%, of which <1% appeared on morphological analysis to be macrophages.

(iv) Treatment with anti-Thy 1.2 antiserum plus complement. Monoclonal antibodies to the Thy 1.2 antigen were purchased from New England Nuclear Corp., Boston, Mass. (lot LK 114). Spleen cells (3×10^7) were preincubated with anti-Thy 1.2 antiserum diluted 1:100 in complete RPMI 1640 medium for 30 min at room temperature, washed once in complete RPMI 1640 medium, suspended in a 1:4 dilution of low-tox-m rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada), and incubated for 45 min at 37°C. The surviving cells were then washed twice and counted. In all experiments a complement control was performed in which the first incubation was in medium alone and the second incubation was with complement. Cell recovery was between 50 and 60%. The effectiveness of treatment in abrogating T-cell-mediated responses in vitro was assessed as inhibition of the cytotoxic activity of in vivo-primed alloreactive splenocytes in a ^{51}Cr release assay.

(v) Treatment with anti-Asialo GM_1 antiserum plus complement. Anti-Asialo GM_1 antiserum, previously shown to react selectively with mouse NK cells (16), was purchased from Wako Chemicals Gmbh, Dusseldorf, Federal Republic of Germany. Spleen cells were treated with a 1:200 dilution of antiserum in RPMI 1640 medium for 30 min at room temperature, washed twice, suspended in a 1:4 dilution of low-tox-m rabbit complement, and incubated for 1 h at 37°C. The surviving cells were then washed, counted, and tested for residual activity in the microcytotoxicity assays. The effectiveness of treatment in abrogating natural killer cell-mediated responses in vitro was assessed as inhibition of the cytotoxic activity of spleen cells from mice that were highly reactive to natural killer cells against YAC-1 tumor targets (1).

In vitro cytotoxicity assay: ^{51}Cr release assay against *C. albicans* or other *Candida* species. Candidacidal activity of various effector cell populations was assessed by means of a previously described technique (4). Briefly, single-cell suspensions were prepared from spleens, and various numbers of effector cells in 0.1 ml of suspension were mixed in U-shaped 96-well microtiter plates with 0.1 ml of a suspension of 5×10^4 ^{51}Cr-labeled yeast cells (2×10^8 cells with 300 μCi of $Na_2{}^{51}CrO_4$ for 2 h). After a 4-h incubation at 37°C in 5% CO_2, the plates were centrifuged at $800 \times g$ for 10 min, and the radioactivity in 0.1 ml of the supernatant was measured on a γ-scintillation counter (Auto Gamma 500C; Packard Instrument Co., Inc., Downers Grove, Ill.). The base-line ^{51}Cr release was that of yeast cells incubated alone in complete RPMI 1640 medium, and in no case did the release exceed 20% of total counts per minute (cpm) incorporated by target cells. The experimental results are expressed as the percentage of killing in the experimental group (quadruplicate samples) above the base-line control according to the following formula: % specific ^{51}Cr release = [(cpm experimental group − cpm spontaneous group)/(total cpm/2)] × 100, in which total cpm is the radioactivity incorporated by 5×10^4 *Candida* cells.

Plate counts. Phagocytic cells (5×10^5 in 0.1 ml of suspension per well) were infected with unlabeled *C. albicans* cells (5×10^4 cells in 0.1 ml of suspension per well). After 1 h of incubation at 37°C under 5% CO_2, the plates were vigorously shaken, and serial dilutions were made in distilled water from each well. Plates (duplicate samples) were made by spreading each sample on Sabouraud glucose

TABLE 1. Effect of dose and viability of strain PCA-2 cell administration on survival of CD2F1 mice undergoing systemic challenge with *C. albicans*

Kind of PCA-2 cells used for in vivo treatment[a]	Dose	Results after challenge with 10^6 *C. albicans*[b] cells	
		MST[c]	D/T[d]
Untreated control		3.5	10/10
Live	1×10^4	3	10/10
Live	1×10^5	3	10/10
Live	5×10^5	14[e]	6/10
Live	1×10^6	>60[e]	0/10
Heat inactivated	1×10^6	3	10/10
Merthiolate inactivated	1×10^6	3	10/10

[a] PCA-2 yeast cells were given as a single i.v. injection 14 days before challenge (day 0).
[b] *C. albicans* CA-6 was injected by the i.v. route.
[c] MST, Median survival time (days).
[d] D/T, No. of dead mice at 60 days/total no. of animals tested.
[e] $P < 0.01$ (PCA-2-treated mice versus controls).

TABLE 2. Effects of time and route of strain PCA-2 live-cell administration on survival of CD2F1 mice undergoing systemic challenge with *C. albicans*

Day of in vivo treatment with PCA-2[a]	Route[b]	Results after challenge with 10^6 *C. albicans*[c] cells	
		MST[d]	D/T[e]
Untreated control		3	10/10
3	i.v.	5	10/10
7	i.v.	10[f]	10/10
10	i.v.	>60[f]	3/10
14	i.v.	>60[f]	0/10
14	i.p.	4	10/10
14	s.c.	3.5	10/10

[a] PCA-2 (10^6 cells per mouse) was given as a single injection a number of days before infection (day 0).
[b] i.p., Intraperitoneal; s.c., subcutaneous.
[c] *C. albicans* CA-6 was injected by the i.v. route.
[d] MST, Median survival time (days).
[e] D/T, No. of dead mice at 60 days/total no. of animals tested.
[f] $P < 0.01$ (PCA-2-treated mice versus controls).

agar. The number of CFU was determined after 18 h of incubation at 37°C. Control cultures consisted of *C. albicans* cells incubated without effector cells.

Phagocytic activity. Microscopic examination of phagocytic cell function was performed as follows. Equal volumes of effector cells, the indicator dye methylene blue, and yeast cells were mixed in sterile polypropylene tubes (Becton Dickinson); an effector/yeast cell ratio of 1:1 was maintained. The tubes (duplicate samples) were incubated for 4 h at 37°C and suspended in 20 μl of RPMI 1640 medium, and a wet mount was prepared on slides with cover slips. The phagocytic activity of effector cells was expressed according to the following formula: % phagocytic activity = number of cells containing 1 or more yeast cells/100 cells.

Statistical analysis. Differences in survival times were analyzed by the Mann-Whitney U test. Differences in the numbers of CFU in the kidneys or the amounts of specific radiolabel release in the in vitro microcytotoxicity assays were determined according to the Student *t* test. Each experiment was repeated three to five times.

RESULTS

Effect of strain PCA-2 live-cell administration on resistance of mice to microbial challenge. CD2F1 mice were inoculated with different numbers of PCA-2 cells 14 days before i.v. challenge with 10^6 CA-6 cells. Mortality data are shown in Table 1. It is apparent that the injection of PCA-2 yeast cells conferred considerable protection against the microbial challenge. However, only an inoculum as large as 10^6 could result in protection against CA-6 cells. In addition, inactivation of PCA-2 cells by heat or Merthiolate abolished their ability to confer protection (Table 1).

Experiments were performed to ascertain the optimal conditions under which the use of PCA-2 cells could confer protection against CA-6 cells. To this end, the influences of time and route of PCA-2 administration as well as number of cells administered were evaluated.

In a first series of experiments, mice were treated i.v. with 10^6 PCA-2 cells on different days (day 3, 7, 10, or 14) before challenge with 10^6 CA-6 cells. In addition, groups of mice were treated according to the same schedule with PCA-2 by either the intraperitoneal or subcutaneous route. Protection was afforded only when PCA-2 was administered i.v., and the degree of protection was maximal when PCA-2 was given on days 10 to 14 before infection with CA-6 cells (Table 2).

In other experiments, the effect of PCA-2 administration on resistance of mice to challenge with different microorganisms was studied. Mice were given a single i.v. injection of 10^6 PCA-2 cells 14 days before i.v. challenge with 10^6 CA-6 or 3153A, 10^7 *C. tropicalis*, or 6.2×10^7 *S. aureus* cells. Mortality data are shown in Table 3. It was found that the injection of PCA-2 yeast cells conferred protection against systemic challenge with all of the pathogens. In particular, the PCA-2-induced protection was as effective against infection with parental strain 3153A as against the more virulent, unrelated CA-6 strain. All subsequent experiments were therefore conducted with the PCA-2 and CA-6 strains.

Cellular parameters of response induced by PCA-2 in the host. Resistance of intact and immunomodulated mice to *C. albicans* is known to be largely mediated by phagocytic cells of the granulocyte and monocyte lineages. We therefore decided to monitor hematological as well as immunological parameters of mice undergoing infection with strain PCA-2. At 7 to 14 days after injection of 10^6 PCA-2 cells, there was a significant increase in the number of peripheral blood polymorphonuclear cells (PMN), whereas only minor fluctuations could be found in the counts of lymphocytes and monocytes (Table 4). Splenic macrophages are regarded as major anti-*Candida* effectors in immunomodulated mice (1, 6). Table 4 also reports the effect of PCA-2 administration on the in vitro candidacidal activity of spleen cells. Mice given 10^6 PCA-2 cells at different times before testing were used as a source of splenic effectors to be reacted in vitro in a release assay employing radiolabeled *Candida* cells and in a CFU test. It is apparent that host sensitization with PCA-2 7 to 14 days before infection resulted in considerable activation of splenic candidacidal effectors.

TABLE 3. Effect of PCA-2 live-cell administration on survival of CD2F1 mice undergoing systemic challenge with *C. albicans* CA-6 or 3153A, *C. tropicalis*, or *S. aureus* microorganisms[a]

In vivo treatment with PCA-2[b]	Results after challenge with:							
	10^6 CA-6		10^6 3153A		10^7 *C. tropicalis*		6.2×10^7 *S. aureus*	
	MST[c]	D/T[d]	MST	D/T	MST	D/T	MST	D/T
−	3	10/10	5	10/10	8	10/10	8.5	10/10
+	>60[e]	0/10	>60[e]	0/10	>60[e]	0/10	>60[e]	3/10

[a] *C. albicans* CA-6 and 3153A, *C. tropicalis*, and *S. aureus* were injected by the i.v. route.
[b] PCA-2 (10^6 cells per mouse) was given as a single i.v. injection 14 days before challenge.
[c] MST, Median survival time (days).
[d] D/T, No. of dead mice at 60 days/total no. of animals tested.
[e] $P < 0.01$ (PCA-2-treated mice versus controls).

TABLE 4. Effect of time of strain PCA-2 yeast live-cell administration both on the number of peripheral leukocytes and on candidacidal activity of spleen cells

Day of in vivo treatment with PCA-2[a]	No. of peripheral leukocyte cells[b]			Spleen cellularity (10^6)[c]	% ^{51}Cr specific release at ratio[d] of:		% CFU inhibition at ratio[d] of:	
	Lymphocytes	PMN	Monocytes		10:1	2.5:1	10:1	2.5:1
None	4,301	1,596	217	86	6.2	2.8	57.8	28.8
3	2,718[e]	2,278	276	112	5.8	2.0	59.8	30.4
7	3,086[e]	5,572[e]	402[e]	119	18.5[e]	9.3[e]	78.8[e]	48.2[e]
14	3,184[e]	4,438[e]	387[e]	108	20.7[e]	10.6[e]	80.3[e]	51.7[e]
20	2,117[e]	3,139[e]	262	89	11.4	5.4	64.1	31.0

[a] PCA-2 live cells (10^6) were given as a single i.v. injection a number of days before the in vitro assay (day 0).
[b] Three to 20 days after PCA-2 administration, groups of 10 mice were killed for assessment of peripheral leukocyte counts. At no time did standard errors exceed 10% of the mean, and they have therefore been omitted.
[c] Splenocytes from normal or PCA-2-treated mice were collected and counted before the in vitro assay.
[d] Ratios of effector to target (CA-6) cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.
[e] $P < 0.01$ (PCA-2-treated mice versus untreated controls), according to Student's t test.

Characterization of the splenic candidacidal effectors induced by PCA-2 administration. To characterize the effector cell responsible for cytotoxic activity against *C. albicans* in the spleen 7 to 14 days after exposure of mice to strain PCA-2, we studied the effects of removal of nylon-adherent or phagocytic cells from the splenic population. Cells from mice injected with 10^6 PCA-2 cells 14 days before infection were thus fractionated according to their ability to adhere to plastic, nylon wool, or phagocyte carbonyl-iron powder, as described in Materials and Methods. The resulting populations were then reacted against ^{51}Cr-labeled *Candida* cells (Table 5). It is apparent that the removal of adherent or phagocytic cells considerably decreased reactivity. Moreover, various antisera directed against specific cell membrane determinants were used to characterize the cell responsible for candidacidal activity in vitro. Treatment with anti-Thy 1.2 serum plus complement or anti-Asialo GM_1 plus complement had no major effect on the cytotoxic activity of the PCA-2-induced effectors (Table 5).

Effects of amphotericin B treatment on induction of protection by PCA-2. Attempts were made to establish whether interference with the development of PCA-2 infection would adversely affect the onset of protection against CA-6 cells. Mice injected with 10^6 PCA-2 cells (day 14) were treated with amphotericin B (2.5 mg/kg) at different times before challenge with CA-6 (day 0) or testing of the in vitro splenic candidacidal activity in a ^{51}Cr release assay (Table 6). Early treatment (days -14 to -11) with amphotericin B greatly impaired the development of anti-*Candida* cytotoxic activity both in vitro and in vivo. Later exposures to amphotericin B (days -9 to -4), on the other hand, had only minor effects on the induction of resistance.

Adoptive transfer of adherent spleen cells from PCA-2-treated mice into recipient hosts challenged with CA-6. Finally, experiments were designed to investigate the effect of the adoptive transfer of plastic-adherent spleen cells from mice injected with PCA-2 into recipient hosts challenged with CA-6 (5×10^5 or 1×10^5 cells). Mice given an i.v. injection of 10^6 PCA-2 cells 14 days before infection were used as donors of plastic-adherent spleen cells which were infused (i.v., 1.5×10^7 cells per mouse) into recipient hosts challenged with CA-6 3 h after the adoptive transfer (Table 7). It is apparent that the infusion of adherent cells conferred considerable protection against CA-6 challenge. The degree of protection was dependent on the size of the CA-6 inoculum, however.

DISCUSSION

In recent years, a large body of evidence has accumulated showing that cell-mediated immunity plays a major role in protection against candidiasis in intact (9, 20, 25) as well as

TABLE 5. Effect of different treatments on the candidacidal activity of splenocytes from normal or PCA-2-treated CD2F1 mice

Effector cell population	In vivo treatment with PCA-2[a]	% Phagocytic activity[b]	% ^{51}Cr specific release at ratio[c] of:	
			10:1	2.5:1
Untreated cells	−	15.1 ± 1.8	18.4	6.5
Plastic-adherent cells	−	95.4 ± 2.2	28.4[d]	10.6[d]
Nylon wool-nonadherent cells	−	2.3 ± 2.6	4.6[d]	3.8[d]
Carbonyl-iron plus magnet-treated cells	−	1.5 ± 2.3	1.4[d]	0.8[d]
Anti-Thy 1.2 plus complement-treated[e] cells	−	ND	19.1	5.4
Anti-Asialo GM_1 plus complement-treated cells	−	ND	20.0	5.9
Untreated cells	+	18.2 ± 2.3	44.3	24.9
Plastic-adherent cells	+	96.3 ± 1.9	46.2	30.5
Nylon wool-nonadherent cells	+	3.1 ± 2.5	5.8[d]	3.7[d]
Carbonyl-iron plus magnet-treated cells	+	1.8 ± 2.0	2.6[d]	1.0[d]
Anti-Thy 1.2 plus complement-treated cells	+	ND	48.0	24.5
Anti-Asialo GM_1 plus complement-treated cells	+	ND	41.2	25.7

[a] PCA-2 live cells (10^6) were given by the i.v. route as a single injection 14 days before the in vitro assay (day 0).
[b] Mean of duplicate samples ± standard error. ND, Not done.
[c] Ratios of effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.
[d] $P < 0.01$ (fractionated or treated versus unfractionated or untreated cells, respectively).
[e] Values for cells treated with complement alone were not significantly different from those for controls.

immunomodulated (1, 3, 6) hosts. However, the relative contributions of thymus-dependent and -independent responses to overall protection have not been fully elucidated. Some reports emphasize that direct evidence is lacking to support the concept of a major role of T-cell-mediated immunity (8, 13, 28), and there are also suggestions that specific humoral rather than cellular immunity contributes to anti-*Candida* protection (25, 27). Our laboratory has been focusing for some time on the possible relevance of natural cell-mediated immunity in the in vivo resistance of mice against candidiasis. In this regard, evidence was presented that the candidacidal activity of cells in the granulocyte and monocyte lineages may be a primary defense mechanism of intact (2) and immunomodulated (1, 6) mice against challenge with the yeast. Along this line of research, in this study we investigated the effect of host infection with a poorly pathogenic strain of *C. albicans* (PCA-2) on subsequent challenge with highly pathogenic CA-6 yeast cells.

Our results clearly show that systemic infection of mice with PCA-2, a strain with a particularly low virulence, can confer protection against subsequent challenge with a highly pathogenic *Candida* strain, and this protective activity is possibly related to the chronic nature of the infection sustained by the PCA-2 strain. Our findings also support the theory that a nonspecific immune mechanism is a major factor contributing to the increase in resistance. Firstly, the protection was evident upon challenge of mice with another species of *Candida* (*C. tropicalis*) and with a bacterium (*S. aureus*). Secondly, a series of in vitro assays suggested a crucial involvement of cellular components of the immune system usually regarded as innate resistance factors against microbial infections. Granulocytosis developed at 7 to 14 days after PCA-2 infection; an increase in the number of peripheral blood PMN has often been associated with augmented anti-*Candida* resistance (12, 13, 29). Although in our study the activity of individual PMN was not increased in the ^{51}Cr release assay employing labeled yeast cells (data not shown), it is possible that the overall killing potential of PMN in vivo was greatly enhanced after infection with PCA-2. Nevertheless, in animals treated with cyclophosphamide and challenged with CA-6 after PCA-2 sensitization, lack of a strict correlation was found between PMN counts and degree of protection conferred by PCA-2 (data not shown). Thus, other factors in addition to PMN might play a role in our model. Interestingly enough, we also found that PCA-2 infection resulted in the activation in the spleen of cells with highly candidacidal activity in vitro. These cells were characterized as macrophages, a finding in line with previous results on the anti-*Candida* resistance of immunomodulated mice (1). Thirdly, adoptively transferred macrophages from PCA-2-infected donors induced considerable protection against systemic challenge of recipient hosts with CA-6 cells.

Thus it appeared that the exposure of mice to PCA-2 induced a state of macrophage activation and increased antimicrobial activity, possibly as a result of the peculiar interaction of PCA-2 with its host. Further experiments helped clarify this point. Live PCA-2 cells could be recovered from the kidneys of infected mice for up to 40 days after yeast injection (data not shown). Moreover, PCA-2 cells had to persist in the organs of the infected mice to confer protection, as shown by the antagonistic effect of an early treatment with amphotericin B on the induction of resistance. These data seem to suggest that the continued presence of a large number of PCA-2 cells in the host leads to nonspecific activation of immune mechanisms capable of inhibitory effects on the growth of pathogens such as CA-6 and *S. aureus*. These findings are perhaps not surprising if one considers that *C. albicans* displays considerable immunoadjuvant activity in murine lymphoma models (22). In this regard, the PCA-2 infection model might provide optimal conditions for studying the immune mechanisms triggered by *Candida* infection, since the sustained presence of a large number of PCA-2 cells in the organs of infected hosts is compatible with survival of the latter and allows for an immunological follow-up.

There are several reports in the literature of acquired resistance to *C. albicans* induced by viable inocula (9, 11, 26) or ribosomes (31). In most of these studies, however, no data were presented on the immune status and in particular on natural cell-mediated reactions of the animals at the time of challenge. In other investigations, in which the presence of specific responses was evaluated in immunized mice, lack of

TABLE 6. Effect of time of amphotericin B administration on increased candidacidal activity induced by PCA-2 treatment

Day of in vivo treatment with:		% ^{51}Cr specific release at ratio[a] of:		Results with mice challenged with 10^5 *C. albicans* cells[b]	
PCA-2[c]	AmB[d]	10:1	2.5:1	MST[e]	D/T[f]
None	None	13.2	5.1	9	10/10
−14	None	42.4	13.6	>60	0/10
−14	−14[b]	29.5[g]	7.6[g]	8[g]	10/10
−14	−13	28.3[g]	7.9[g]	12[g]	7/10
−14	−11	31.8[g]	15.9[g]	>60	0/10
−14	−9	36.3	12.2	>60	0/10
−14	−7	35.1	14.5	>60	0/10
−14	−4	45.8	16.5	>60	0/10

[a] Ratios of effector to target cells. Data are the means of quadruplicate samples. Standard errors, usually <1.5%, have been omitted.
[b] Means at 3 h after PCA-2 injection.
[c] PCA-2 cells (10^6) were given by the i.v. route as a single injection 14 days before the in vitro assay or the in vivo challenge (day 0).
[d] AmB, Amphotericin B. A dose of 2.5 mg/kg was given as a single i.v. injection on different days before day 0.
[e] MST, Median survival time (days).
[f] D/T, No. of dead mice at 60 days/total no. of animals tested.
[g] $P < 0.01$ (PCA-2-plus amphotericin B-treated mice versus PCA-2-treated mice).

TABLE 7. Effect of transfer of adherent spleen cells from untreated or PCA-2-treated mice to normal recipients systemically infected with *C. albicans*

Treatment of donors before transfer[a]	Results after challenge with strain CA-6 at inoculum density of:			
	5×10^5		1×10^5	
	MST[b]	D/T[c]	MST	D/T
Control (no transfer)	4	10/10	7	10/10
None	4.5	10/10	7	10/10
PCA-2	10.5[d]	10/10	>60[d]	4/10

[a] CD2F1 mice were injected i.v. with 10^6 PCA-2 yeast live cells 14 days before the assay. On day 0, spleens from normal or PCA-2-treated donor animals were collected, and, after separation, adherent cells were injected (1.5×10^7 per mouse) by the i.v. route into recipient mice. Three hours later, recipient and untreated control mice were injected i.v. with *C. albicans* CA-6 cells.
[b] MST, Median survival time (days).
[c] D/T, No. of dead mice at 60 days/total no. of animals tested.
[d] $P < 0.01$ (adoptively transferred mice versus untreated controls).

correlation was consistently found between delayed-type hypersensitivity and resistance against systemic *Candida* challenge (13). All of these studies emphasized the necessity for a viable *Candida* inoculum to induce protection (9). Our present results are largely in agreement with those previous reports and suggest that nonspecifically activated candidacidal cells, i.e., granulocytes and monocytes, are crucial to a successful defense against systemic candidiasis in our murine model.

Finally, it is of some interest that the protective effect has been obtained with a strain of *C. albicans* unable to form germ tubes, i.e., morphological elements which may play a role in the pathogenicity of *C. albicans*. It should be noted here that the agerminative variant, although significantly less virulent than germ tube-forming strains as judged from 50% lethal dose values on systemic challenge, was capable of infecting a normal mouse persistently and that this chronic infection was not cleared by the activation of the nonspecific immune mechanisms. The relationship between morphogenesis and infection by *C. albicans* remains controversial, and our infection model may be useful for gaining further insight into this interesting problem.

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