Immunomodulation by *Candida albicans*: Crucial Role of Organ Colonization and Chronic Infection with an Attenuated Agerminative Strain of *C. albicans* for Establishment of Anti-infectious Protection

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Intravenous inoculation of an attenuated agerminative strain of *Candida albicans* (PCA-2) of low virulence, but not of two other species of *Candida* of low virulence (*C. parapsilosis* and *C. viswanathii*) into CD2F1 mice conferred protection against the highly virulent microbes *C. albicans* CA-6, *Staphylococcus aureus* and *Aspergillus fumigatus*. To provide protection, a definite inoculum size (10^6 cells per mouse) resulting in organ colonization and establishment of a long-lasting chronic infection with PCA-2 was needed. An inoculum of 10^5 cells gave rise to transient kidney colonization whereas inocula greater than 10^6 cells led to acute septicaemia and eventual death. Chronic infection of mice following inoculation of 10^6 PCA-2 cells was accompanied by detectable mannoprotein antigen levels in the serum ($30\text{--}70\text{ ng ml}^{-1}$) while specific antibodies did not appear until 14 d after inoculation, at which time low antimannan antibody was present (ELISA titre 1:40–1:80). Chronic infection was characterized by the presence in the kidneys of $2\text{--}3 \times 10^6$ c.f.u. of PCA-2 for at least 40 d after inoculation. Pharmacological modulation of the host through administration of either an anti-*Candida* drug, amphotericin B, or an immunosuppressive agent, cyclophosphamide, strongly supported the premise that the anti-infectious state conferred by PCA-2 ‘immunization’ correlated with the maintenance of a sufficient number of PCA-2 *in vivo*. Protection was ‘switched on’ when $2\text{--}3 \times 10^5$ cells were present in the kidneys. It was maximal at a kidney count of $2\text{--}3 \times 10^6$ c.f.u. of PCA-2, and promptly declined when the number of PCA-2 cells in the kidneys fell below 2×10^5 . Mice chronically infected with PCA-2 had splenic macrophages with pronounced candidacidal activity *in vitro*. Modulation of the growth of PCA-2 *in vivo*, which determined activation or deactivation of the protective state, was paralleled by a similar modulation in macrophage activation, showing that in all cases resistance to virulent organisms persisted as long as macrophage activation was present. The results demonstrate that a critical *in vivo* antigenic load is crucial for the occurrence of resistance to infection and suggests that macrophages could be involved in this protection.

INTRODUCTION

Candida albicans is a micro-organism indigenous to humans, which can give rise to serious, often life-threatening disease in the immunocompromised or otherwise modified host (Odds, 1979). For a better understanding of the host–parasite relationship in candidosis, it seems crucial to investigate the mechanisms by which normal individuals resist *Candida* infection and,

Abbreviations: AmB, amphotericin B; Cy, cyclophosphamide; i.p., intraperitoneal(ly); i.v., intravenous(ly).

in particular, its transition from saprophytic status to that of systemic spread and invasion of target organs. One approach to this issue is manipulation of host responsiveness to experimental infection by either selective depletion (Bistoni *et al.*, 1984; Moser & Domer, 1980) or enhancement (Baccarini *et al.*, 1983*a*; Bistoni *et al.*, 1986) of immune functions with the intent of highlighting the relative contributions of different anti-*Candida* immune components. We have recently described an experimental model of mouse 'immunization' where a poorly virulent agerminative strain of *C. albicans* (PCA-2) was compared against a highly virulent, germ-tube-positive strain (CA-6). We observed a substantial increase in antimicrobial resistance which was largely non-specific, detectable in both intact (Bistoni *et al.*, 1986) and immunodepressed mice (unpublished results) and appeared to be mediated by cells in the monocyte-macrophage lineage (Bistoni *et al.*, 1986; Sasada & Johnston, 1980). Activated macrophages from PCA-2-treated mice could confer anti-*Candida* protection upon intact recipients following challenge of the latter with CA-6 cells. In an attempt to gain further insight into the nature of this protection, we examined selected microbiological and immunological parameters of infection sustained by PCA-2 cells. We attempted to correlate the kinetics of PCA-2 growth *in vivo* with macrophage activation and resistance to both bacterial and fungal challenge.

METHODS

Mice. Hybrid (BALB/cCr × DBA/2 Cr) F1 (CD2F1 : H-2^d/H-2^d) mice of both sexes, 18–22 g, were obtained from Charles River Breeding Laboratories.

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

Cyclophosphamide was dissolved in sterile 0.85% NaCl solution immediately before use and injected i.p. in a volume of 0.1 ml per 10 g body weight.

Micro-organisms. *C. parapsilosis* and *C. viswanathii* were obtained from the established type collection of the Istituto Superiore di Sanità, Rome, Italy. Two strains of *C. albicans* (CA-6 and PCA-2), with identical sugar assimilation and fermentation patterns, were used. Strain CA-6 was isolated from a clinical specimen and strain PCA-2 was kindly supplied by D. Kerridge, Department of Biochemistry, University of Cambridge, Cambridge, UK. The agerminative strain PCA-2 is an echinocandin-resistant mutant of the parental *C. albicans* reference strain 3153, serotype A. It grows as a pure yeast form *in vitro* at 28 °C or 37 °C in conventional mycological media. Under conditions which promote germ-tube formation *in vitro* (37 °C), or *in vivo*, this strain grows as a yeast and short pseudomycelium. The 50% lethal doses of strains CA-6 and PCA-2 are 0.2×10^5 and 2.5×10^6 , respectively. All yeasts were grown at 28 °C with slight agitation in low-glucose Winge medium composed of 0.2% (w/v) glucose and 0.3% (w/v) yeast extract (BBL) until the stationary phase was reached (approximately 24 h). Under these conditions, cultures gave a yield of approximately 3×10^8 yeast cells ml⁻¹ and the cells had the morphology described by Mattia *et al.* (1982). Cells were harvested by centrifugation at 1000 g, washed twice in saline, and diluted to the desired density.

A coagulase-positive *Staphylococcus aureus* strain (Cowan, NCTC, Colindale, UK) was grown at 37 °C on Mannitol Salt Agar (MSA, BBL) for 24 h. The bacteria were harvested by centrifugation at 1000 g, washed twice in saline, and diluted to the desired number of c.f.u. ml⁻¹.

The strain of *Aspergillus fumigatus* used was isolated from a clinical specimen. Conidia were harvested with a wire loop from 4 d cultures grown at 37 °C on Sabouraud dextrose agar (SDA) plates supplemented with 100 µg streptomycin ml⁻¹ and 100 U penicillin G ml⁻¹. The cells were washed three times in 0.85% NaCl solution plus 1% (v/v) Tween 80 and the suspension was filtered through cotton gauze. This procedure gave suspensions of 100% conidia with >95% as single cells.

In all *in vivo* infection experiments, yeast and bacterial cell suspensions were injected intravenously (i.v.) via the tail vein in a volume of 0.5 ml per mouse. Each experimental group consisted of at least 10 male mice.

Determination of c.f.u. in kidneys. Both kidneys of each mouse were removed aseptically and placed in tissue homogenizers with 3 ml 0.85% sterile NaCl solution. The number of c.f.u. of *C. albicans*, *A. fumigatus* or *S. aureus* was determined by a plate dilution method, using SDA for *C. albicans* and *A. fumigatus* and MSA for *S. aureus*. Alternative techniques for determining the biomass of filamentous organisms have been proposed (Cockayne & Odds, 1984; Gooday, 1986) but were not used in the present study. The differential counts between *C. albicans* CA-6 and PCA-2 recovered from the kidneys were based on morphological examination of the colonies grown on chlamydospore-agar (Biolife Italiana). The colonies were counted after incubation at 37 °C for 48 h and results were expressed as number of c.f.u. per pair of kidneys.

In vitro cytotoxicity assay: ^{51}Cr release assay against *C. albicans*. Candidacidal activity of various effector cell populations was assessed by means of a previously described technique (Bistoni *et al.*, 1982). Briefly, single-cell suspensions were prepared from spleens, and 5×10^5 to 10^5 effector cells in 0.1 ml of suspension were mixed in U-shaped 96-well microtitre plates with 0.1 ml of a suspension of 5×10^4 ^{51}Cr -labelled yeast cells (2×10^8 cells with $300 \mu\text{Ci}$ of $\text{Na}_2^{51}\text{CrO}_4$ for 2 h). After 4 h incubation at 37°C in 5% CO_2 -in-air, the plates were centrifuged at 800 g for 10 min, and the radioactivity in 0.1 ml of the supernatant was measured in a γ -scintillation counter (Auto Gamma 500C; Packard Instrument Co.). The base-line ^{51}Cr release was that of yeast cells incubated alone in RPMI 1640 medium (Eurobio Laboratories) supplemented with 10% (v/v) foetal calf serum (GIBCO Laboratories), 25 mM-HEPES buffer (Eurobio), and 0.1% gentamicin sulphate (hereafter referred to as complete RPMI 1640 medium). Spontaneous release never exceeded 20% of total c.p.m. incorporated by target cells. Experimental results are expressed as reported elsewhere (Bistoni *et al.*, 1982, 1986).

Measurement of antibody and PCA-2 antigen by indirect ELISA. Anti-PCA-2 serum antibodies in mice were evaluated by indirect ELISA using methods and reagents as described elsewhere (Ausiello *et al.*, 1986). Briefly, 200 μl of PCA-2 glucomannan protein antigen prepared as previously reported (Ausiello *et al.*, 1986), at a concentration of $5 \mu\text{g ml}^{-1}$, was added to wells of a polystyrene microtitration plate (Dynatech), held overnight at 4°C and subsequently rinsed with 0.1 M-PBS, pH 7.4, containing Tween 20 (0.05%). Then 200 μl of the test serum was added at the desired dilution and the plates were incubated for 1 h at room temperature. After careful washings with Tween 20/PBS buffer, 200 μl of a 1:350 dilution of goat anti-mouse Ig-alkaline phosphatase conjugate (Sigma) was added for 1 h and the enzyme activity determined by adding nitrophenylphosphate reagent (Sigma). The reaction was terminated by adding 50 μl of a 3 M-NaOH solution after 20 min. The absorbance was read on a Titertek Multiscan set at 405 nm and blanked against air. Tests were performed in triplicate and the A_{405} of the well without coating antigen (ranging from 0.16 to 0.18) was taken as the background reading. A positive test at any serum dilution was one with at least twice the absorbance of the background reading and serum titre was taken as the reciprocal of the highest dilution giving a positive ELISA reading. Pooled sera from groups of five mice randomly selected before PCA-2 inoculation were used as negative controls. These sera gave A_{405} readings in the ELISA assay ranging from 0.18 to 0.22. An ELISA technique was also used to assess the presence of serum circulating antigen during PCA-2 infection. Briefly, serum collected on different days after PCA-2 injection (10^6 cells i.v.) was treated to dissociate PCA-2 mannan protein according to a slightly modified protease-digestion schedule (Ausiello *et al.*, 1986). Serum (500 μl) was added with 50 μl of a protease solution (pronase, 1 mg ml^{-1} ; Sigma) and incubated overnight at 37°C , with slight agitation, then the mixture was boiled (15 min) and centrifuged to collect the supernatant. The amount of antigen was calculated by an ELISA-inhibition technique (Jones, 1980) using a standard curve of glucomannan protein antigen. The sensitivity of the assay was about 1–2 ng antigen ml^{-1} , and the assay itself did not depend on the source of coating antigen used (from CA-6 or PCA-2) as the anti-mannan antibodies were fully cross-reactive with these antigens.

Adherence of spleen cells to plastic. Unfractionated spleen cells (4×10^7) suspended in 10 ml complete RPMI 1640 medium were incubated for 3 h at 37°C in 5% CO_2 -in-air in 93 mm Petri dishes (Nunc Inter Med). The dishes were then washed extensively with complete RPMI 1640 medium to remove the nonadherent cells while the adherent cells were recovered by scraping with a rubber policeman, then washed and suspended (viability, 80–90%) in fresh medium. More than 98% of the recovered cells were recognized as macrophages in Giemsa-stained preparations.

C.f.u. inhibition assay. Plastic-adherent spleen cells from normal or previously infected mice (5×10^5 in 0.1 ml of suspension per well) were infected with *C. albicans* CA-6, *A. fumigatus* or *S. aureus*: 5×10^4 in 0.1 ml of suspension per well. After 4 h incubation at 37°C in 5% CO_2 -in-air, distilled water was added to each well and the microtitration plates were vigorously shaken. After lysis of adherent splenocytes (microscopically checked), serial dilutions were made in distilled water from each well. Viable counts were made by spreading each sample on SDA for *C. albicans* and *A. fumigatus* or MSA for *S. aureus*, in triplicate. The number of c.f.u. was determined after 24 h incubation at 37°C . Control cultures consisted of *C. albicans*, *A. fumigatus* and *S. aureus* cells incubated without effector cells.

Statistical analysis. Differences in survival times were analysed by the Mann-Whitney U test. Differences in the numbers of c.f.u., in the numbers of c.f.u. from the kidneys and in the amounts of specific radiolabel release in the *in vitro* microcytotoxicity assays were determined by Student's *t*-test. Each experiment was repeated three to five times.

RESULTS

Effect of PCA-2 inoculum size and different species of Candida on survival of CD2F1 mice following systemic microbial challenge

To test the effect of PCA-2 inoculum size on the level of protection against challenge with the virulent CA-6 strain of *C. albicans*, and against experimental infection with other fungi (*A. fumigatus*) or bacteria (*S. aureus*), mice were given a single i.v. injection of two different

Table 1. *Effect of administration of different species of Candida on survival of CD2F1 mice after systemic challenge with C. albicans (CA-6), S. aureus or A. fumigatus*

The results are shown as median survival time in days (MST), and as dead mice at 60 d over total animals tested (D/T).

Treatment (day -14)*		Challenge i.v. (day 0):					
		CA-6 1×10^6		<i>S. aureus</i> 1.3×10^8		<i>A. fumigatus</i> 5×10^6	
		MST	D/T	MST	D/T	MST	D/T
None		3	10/10	3	10/10	3	10/10
<i>C. parapsilosis</i>	1×10^6	3	10/10	3	10/10	3	10/10
<i>C. viswanathii</i>	1×10^6	5	10/10	3	10/10	3	10/10
PCA-2	1×10^6	>60†	0/10	>60†	2/10	>60†	1/10
PCA-2	1×10^5	4	10/10	3	10/10	4	10/10

* Live yeast cells were given as a single i.v. injection 14 d before challenge.

† $P < 0.01$ (yeast-treated versus controls).

Table 2. *Viable counts of CA-6, A. fumigatus and S. aureus from kidneys of mice treated or not treated with PCA-2*

Treatment with PCA-2*	Systemic challenge with	C.f.u. from kidneys†		
		Day +1	Day +4	Day +8
—	CA-6	12×10^3 (± 920)	2.4×10^6 ($\pm 1.9 \times 10^5$)	Dead
+	CA-6	100 (± 12)‡	14×10^3 ($\pm 1.5 \times 10^3$)‡	0
—	<i>S. aureus</i>	6×10^5 ($\pm 71 \times 10^3$)	35×10^6 ($\pm 2.8 \times 10^6$)	Dead
+	<i>S. aureus</i>	1×10^3 (± 130)‡	3×10^5 ($\pm 41 \times 10^3$)‡	300 (± 26)
—	<i>A. fumigatus</i>	9.5×10^3 (± 802)	4.8×10^5 ($\pm 35 \times 10^3$)	Dead
+	<i>A. fumigatus</i>	800 (± 65)‡	36×10^3 ($\pm 4.1 \times 10^3$)‡	0

* Live cells of PCA-2 were given i.v. as a single injection on day -14.

† C.f.u. values are the means of three separate experiments. Standard errors are given in parentheses.

‡ $P < 0.01$ (PCA-2-treated versus PCA-2 untreated).

doses (10^6 or 10^5) of PCA-2. Other mice alternatively received 10^6 cells of *C. viswanathii* or *C. parapsilosis*. All the mice were challenged 14 d later with CA-6 (1×10^6 cells), *S. aureus* (1.3×10^8) or *A. fumigatus* (5×10^6). The mortality data in Table 1 clearly demonstrate that only the higher PCA-2 inoculum conferred the capacity to resist challenge with any of the micro-organisms used. The administration of *C. parapsilosis* and *C. viswanathii* at the same dose failed to confer protection. The data in Table 2 show that non-specific resistance of PCA-2-immunized mice to challenge, in terms of mortality, was associated with the striking decrease in the number of viable challenge organisms in the kidneys. In these animals the virulent strain of *C. albicans* was not capable of sustained growth and was cleared from the kidneys by one week after challenge, in contrast to unimmunized mice where the challenge organism grew progressively in the kidneys until death of the infected animal.

Relationship between inoculum size and in vivo growth pattern of PCA-2

Since there appeared to be a critical PCA-2 inoculum size for development of protection, we decided to assess the kinetics of PCA-2 growth *in vivo* and determine the relationship between inoculum size, growth rate and infectious load in the organs. Yeast proliferation in the kidneys was an indication of general PCA-2 growth *in vivo*, as colonization of this organ has been shown to correlate with the outcome of systemic infection (Hurtrel *et al.*, 1980).

Groups of mice were given 10^5 or 10^6 PCA-2 or 10^6 *C. viswanathii* cells i.v. and the numbers of c.f.u. in the kidneys were determined at different times (Fig. 1). There was a close correlation between inoculum size and establishment of chronic infection. With either size of inoculum,

Fig. 1

Fig. 1. Effect of i.v. injection of *C. viswanathii* or different doses of PCA-2 on numbers of c.f.u. recovered from kidneys of CD2F1 mice. *C. viswanathii* (Δ , 10^6) or PCA-2 live cells ($\bullet$, 10^5 ; $\square$, 10^6) were given as a single i.v. injection on day 0. $\downarrow$, $P < 0.01$ [PCA-2 (10^6) treated versus PCA-2 (10^5) treated mice]; *, $P < 0.01$ [PCA-2 (10^6) treated versus *C. viswanathii* (10^6) treated mice]. Standard errors were usually less than 15% of the mean.

Fig. 2

Fig. 2. Effect of PCA-2 treatment (10^6 cells) on the *in vitro* microbicidal activity, 14 d later, of plastic-adherent spleen cells against CA-6, *S. aureus* and *A. fumigatus*. The vertical bars indicate standard errors; *, $P < 0.01$ (PCA-2 treated versus controls).

PCA-2 cells grew in the kidneys at approximately the same rate during the first three days after inoculation. Thereafter, the smaller dose was cleared progressively from the organ. However, the higher inoculum induced a long-lasting infection which plateaued at $2-3 \times 10^6$ c.f.u. per pair of kidneys for at least two weeks after infection (Fig. 1), with persistent colonization for at least 40–45 d (data not shown). *C. viswanathii* was rapidly cleared from the kidneys, even though it was inoculated at the higher dose (10^6).

To be certain that PCA-2 cells, under our experimental conditions, were establishing a true disseminated (although chronic) infection, we measured c.f.u. in the heart and brain. PCA-2 cells were also present in both these organs (data not shown). We also used a sensitive ELISA-inhibition test to detect PCA-2 mannoprotein antigen in the blood. PCA-2 antigenaemia was indeed detected during the infectious period. Antibodies reactive with the PCA-2 mannoprotein antigen were also found 9–14 d after the antigen became detectable. The antibody titre was 1:40 (on day 14) in three separate experiments.

Activation of splenic macrophages in PCA-2-infected mice: effect of time and inoculum size

We showed previously that splenic macrophages from PCA-2-infected mice were highly candidacidal as compared to those of uninfected controls, and protection could be adoptively transferred to intact recipients by injection of plastic-adherent spleen cells from PCA-2-infected animals (Bistoni *et al.*, 1986). We therefore investigated the dependence of macrophage activation on PCA-2 inoculum size and possible persistence of a chronic infection. Mice were given 10^5 or 10^6 PCA-2 cells and, at different times over a period of 14 d, spleens were removed

Table 3. *Effect of two different doses of PCA-2 on mouse survival to challenge with CA-6 and on in vitro candidacidal activity of plastic-adherent spleen cells on different days after PCA-2 immunization*

Live PCA-2 cells were given i.v. as a single injection on day 0. The *in vitro* assays for candidacidal activity (specific chromium release) and growth inhibition were performed on the days indicated, as described in Methods, both with a ratio of effector to target (CA-6) cells of 10:1. The results for ^{51}Cr release and c.f.u. inhibition are means of quadruplicate and triplicate samples, respectively; standard errors were usually <1.5% and <5%, respectively, of the mean.

PCA-2 dose on day 0	Day +1		Day +3		Day +7		Day +14		Response to CA-6 challenge*	
	^{51}Cr release (%)	C.f.u. inhib. (%)	^{51}Cr release (%)	C.f.u. inhib. (%)	^{51}Cr release (%)	C.f.u. inhib. (%)	^{51}Cr release (%)	C.f.u. inhib. (%)	MST	D/T
None	12.5	38.5	9.7	28.7	9.5	30.5	14.6	31.3	3	8/8
10^5	12.0	30.8	12.2	32.8	12.5	39.7	14.1	36.1	3	8/8
10^6	14.5	36.5	18.4†	49.0†	20.7†	58.6†	29.0†	60.0†	>60†	1/8

* CA-6 cells (10^6) were injected i.v. 14 d after PCA-2 treatment. MST, median survival time (d); D/T, dead mice at 60 d over total animals tested.

† $P < 0.01$ (PCA-2-treated mice versus untreated controls).

and used as a source of plastic-adherent splenic effectors in an *in vitro* killing assay based on ^{51}Cr -release from *Candida* cells, and in a c.f.u. inhibition assay. Splenic effectors were activated in mice given the higher PCA-2 inoculum (Table 3), and this activation was maintained for almost 30 d in chronically infected mice (data not shown). Fig. 2 shows data of a killing assay based on c.f.u. inhibition. Splenic macrophages of PCA-2-immunized mice had significantly higher lytic potential against all three micro-organisms tested as compared to the same effectors from non-immunized mice.

Pharmacological manipulation of the anti-infectious state induced by PCA-2 treatment

The experiments presented above suggested that organ colonization by PCA-2, beyond a certain value, could be one critical factor for establishing a chronic, persistent PCA-2 infection and, as a consequence, for activation of the antimicrobial state by splenic effectors. If so, interference with growth of PCA-2 *in vivo* would be expected to affect adversely the protection against other micro-organisms and activation of candidacidal effectors. We have previously shown (Bistoni *et al.*, 1986) that the antifungal drug amphotericin B (AmB) could abrogate non-specific resistance to infections caused by PCA-2 vaccination. We now investigated the effect of AmB on modulation of the yeast burden in the kidneys. A dose of AmB was chosen (4 mg kg^{-1}) which caused a reduction of about one order of magnitude in the number of PCA-2 c.f.u. in the kidneys, irrespective of the total renal load of the yeast. Mice were injected with 10^6 PCA-2 cells (day -14), then treated with AmB on different days before challenge (day 0) with CA-6 cells; kidney c.f.u. counts of PCA-2 were also assessed (day 0). Early treatments with the drug (2 h before or 6 h after PCA-2 injection), which completely abrogated or markedly limited (below 10^5 c.f.u.) PCA-2 colonization of the kidneys, resulted in no subsequent protection against challenge. Later treatments with AmB (starting 3 d after PCA-2 injection) did not affect the establishment of protection induced by PCA-2 treatment; concurrently, higher counts of PCA-2 cells were recorded in the kidneys (from about 3×10^5 to more than 10^6 c.f.u. when AmB was given 3 to 7 d after PCA-2 inoculation, respectively). In order to gain more insight into this problem, AmB was also given 6 h or 3 d after PCA-2 treatment and fungal growth was assessed for 14 d (Fig. 3). In both cases AmB induced a similar reduction of c.f.u. (about tenfold), but with the later treatment (3 d), yeast in the kidneys had grown to the usual load (10^6 cells), as observed with the AmB-untreated control, on day 14, whereas in the case of early AmB treatment (6 h), PCA-2 was cleared from the kidneys. As expected, only those mice that received AmB 3 d after PCA-2 injection resisted challenge with CA-6 cells (see inset table, Fig. 3). In other experiments, AmB treatments were given to mice 4, 8 and 10 d after PCA-2 injection. The results clearly

Fig. 3. Effect of different schedules of AmB administration on growth kinetics of PCA-2 in the kidneys. Live PCA-2 cells (10^6) were given as a single i.v. injection. AmB (4 mg kg^{-1}) was given 6 h or 3 d later. $\square$, PCA-2 alone; $\bullet$, PCA-2 plus AmB after 3 d; $\circ$, PCA-2 plus AmB after 6 h. *, $P < 0.01$ (PCA-2-plus-AmB-treated mice versus PCA-2-treated mice). Standard errors were usually less than 15% of the mean. The inset table shows mortality data for mice receiving the various treatments and challenged with *C. albicans* CA-6 14 d after PCA-2 inoculation. MST, median survival time (d); D/T, dead mice at 60 d over total.

Table 4. Effect of Cy on mouse survival and on c.f.u. recovered from the kidneys after PCA-2 treatment

In vivo treatment with:			Results after i.v. challenge with		10 ⁻³ PCA-2 c.f.u. recovered from kidneys‡
Cy*	PCA-2 yeast cells		5 × 10 ⁵ CA-6 cells†		
Day	Day	Dose	MST	D/T	
-	-	-	8	10/10	-
-24	-	-	7	10/10	-
-	-21	10 ⁶	> 60	0/10	1.674 (± 111)
-	-21	10 ⁵	9	10/10	200 (± 18)
-	-21	10 ⁴	8	10/10	0
-24	-21	10 ⁵	> 60§	1/10	3.273 (± 308)
-24	-21	10 ⁴	> 60	0/10	1.368 (± 148)

* Cy was administered (150 mg kg^{-1}) as a single injection on the indicated day before the challenge or the *in vitro* assay, on day 0.

† MST, median survival time (d); D/T, dead mice at 60 d over total animals tested.

‡ The standard errors of the mean are given in parentheses.

§ $P < 0.01$ (line 6 versus line 4).

|| $P < 0.01$ (line 7 versus line 5).

indicated that only those treatments which reduced renal PCA-2 values to $< 10^5$ c.f.u. abrogated the protection and activation of splenic plastic-adherent effectors against CA-6. Data from the experiments with AmB strongly suggest that a critical PCA-2 load confers protection and macrophage activation at the same time.

Another way to influence growth of PCA-2 in mouse kidney is by pretreating animals with cyclophosphamide (Cy) (Bistoni *et al.*, 1983, 1984). In previous studies we demonstrated that Cy

treatment adversely affects the ability of mice to mount a defensive immune reaction against *C. albicans*, with subsequent increased susceptibility to infection (Bistoni *et al.*, 1983). On this basis we reasoned that a lower inoculum size, unable to give protection in the untreated animals, might permit organ colonization and development of the protective state in Cy-treated mice. Table 4 shows the results of a typical experiment in which mice were treated with Cy (150 mg kg^{-1}) on day -24 with respect to the challenge on day 0, and 3 d later (day -21) inoculated with various inocula of PCA-2 cells (10^4 to 10^6). Growth of PCA-2 in the kidneys and mortality of CA-6-challenged mice were assessed on day 0. In Cy-pretreated mice an inoculum of only 10^4 PCA-2 cells conferred protection against the CA-6 challenge; concurrently, growth in the kidneys reached 3×10^6 c.f.u., as observed in animals not pretreated with Cy but inoculated with 10^6 PCA-2. Cy-treated mice inoculated with 10^6 PCA-2 died before challenge because of elevated kidney growth ($> 10^7$ c.f.u.) owing to the immunodepression caused by Cy (data not shown).

DISCUSSION

Previously (Bistoni *et al.*, 1986), we showed that inoculation of mice with an attenuated agerminative variant (PCA-2) of *C. albicans* established a persistent infection and induced resistance to virulent pathogens. It was hypothesized that the continued presence of yeast cells in the host, associated with shedding of immunoactive microbial components, might lead to non-specific activation of macrophages capable of strong microbicidal activity *in vivo*. Our present data further substantiate this hypothesis. They also show that the chronic nature of the infection sustained by PCA-2 cells, which are not cleared from mouse organs for a prolonged period, cannot be accounted for by the reduced virulence *per se*, because other *Candida* species of low virulence are rapidly eliminated by the host when injected in similar amounts and fail to confer increased antimicrobial resistance.

One critical factor for development of the anti-infective state by PCA-2 is the size of its inoculum, with an apparent threshold value of approximately 10^6 cells per mouse. This may indicate the need for achievement of a critical yeast load in mouse organs, which, in the case of the kidneys, is exemplified by the occurrence of a steady number of about $2-3 \times 10^6$ cells per pair of kidneys. Lower PCA-2 inocula failed to give rise to persistent colonization of the kidneys and were incapable of 'switching on' the protection. The mechanisms controlling fungal growth in the host after experimental challenge apparently become fully operative only 3 d after injection, as suggested by the fact that with widely differing inoculum sizes, PCA-2 grows in the kidneys during the first 72 h after infection at a virtually identical rate. It is interesting that the anti-infectious mechanisms elicited by PCA-2 immunization, which are so effective in eradicating other pathogens, are unable to eliminate the immunizing agent itself from the mouse (although they might be 'controlling' its multiplication). This inability must reside in some *in vivo* factor since PCA-2 from immunized mice was highly susceptible to killing *in vitro*. Artificial manipulation of PCA-2 growth *in vivo* by treatments with drugs affecting the yeast (AmB) or the host (Cy), strongly confirmed that yeast renal load is critical for induction of the protective state.

The renal load of PCA-2 could first be allowed to increase and then be lowered below a certain value by selecting particular regimens of AmB treatment. The results obtained by this approach showed that significant anti-infective protection begins with a renal load of $2-3 \times 10^5$ cells and is maximal when $2-3 \times 10^6$ cells are present in the kidneys. Moreover, once reached, the protective state is maintained only if the critical load is also preserved, since complete loss of protection follows as a result of AmB treatment bringing the yeast renal burden below the critical value. Under the experimental conditions employed here AmB does not manifest immuno-modulatory properties (Bistoni *et al.*, 1985), although we can not completely rule out direct effects of the drug on host reactivity to PCA-2. However, experiments with Cy further support the contention that antimicrobial protection in our model is crucially dependent on the achievement of a critical yeast load *in vivo*. Thus, relatively low inocula, incapable of protective effects and macrophage activation in intact hosts, become fully protective when administered to Cy-treated mice in which the critical load of PCA-2 is reached with a smaller inoculum.

Previously (Bistoni *et al.*, 1986), we showed that infection of mice with PCA-2 is associated with a transient increase in the number of peripheral blood polymorphonuclear cells and activation in the spleen of candidacidal effectors. We hypothesized that the two events may underlie the *in vivo* induction of anti-*Candida* resistance. The experiments reported here confirm the occurrence of macrophage activation in PCA-2-treated animals and add further evidence for a possible role of these effectors in the decreased susceptibility to microbial challenge. In the experiments on activation and deactivation of the anti-infective state, there was always a close correlation between macrophage functional status as assessed *in vitro* and outcome of resistance after the *in vivo* microbial challenge. Both protection and macrophage activation obeyed the same rule of a 'critical' *in vivo* load of PCA-2, and a close temporal relationship was observed between the two phenomena. Together with the previous finding that the protective state elicited by PCA-2 immunization can be transferred to the unimmunized animal by splenic activated macrophages (Bistoni *et al.*, 1986), the overall evidence points to these immunoeffectors as likely mediators of, or contributors to, the anti-infective potential achievable by PCA-2 immunization in mice.

Apart from macrophages and PMN, other natural effectors could play a role in the immunopotentiality induced by PCA-2. Precursors in the monocyte-macrophage lineage (promonocytes), almost indistinguishable from large granular lymphocytes exerting natural cytotoxic activity *in vitro* against tumours, have been suggested as candidacidal effectors both in normal (Baccarini *et al.*, 1983*b*) and immunomodulated (Baccarini *et al.*, 1983*a*) mice. Herberman & Ortaldo (1981) also showed that natural killer lymphocytes may be endowed with fungicidal or bactericidal properties *in vitro*. Further studies are needed to establish the possible involvement of immune effectors other than activated macrophages in the immunopotentiality by PCA-2.

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