Anti-*Candida* resistance in the mouse brain and effect of intracerebral administration of interleukin 1

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The effects of intracerebral and intravenous *Candida albicans* infection on experimental meningo-encephalitis in mice were compared. Naive mice inoculated with two *C. albicans* strains of different pathogenicity (highly virulent CA-6 and poorly virulent PCA-2) were more resistant to infection when the yeasts were inoculated by the intracerebral rather than the intravenous route. In immunized mice, in which systemic immunity had been induced by long-term colonization with low-virulence PCA-2 cells, increased intracerebral resistance to challenge with virulent *Candida* was observed at about two weeks post-infection. In contrast, the inoculation of PCA-2 cells directly into the brain resulted in early, long-lasting activation of local microbicidal mechanisms against intracerebral challenge with CA-6, *Staphylococcus aureus* or *Aspergillus fumigatus*. Increased local anti-*Candida* resistance was also observed upon intracerebral injection of human recombinant interleukin 1. These data suggest that, in addition to the intracerebral expression of systemic antifungal immunity, microbial mechanisms may be locally activated in the brain, possibly through release of endogenous interleukin 1.

Introduction

Candida albicans is an important fungal pathogen, causing life-threatening infections in immunocompromised or severely debilitated patients. Conditions leading to systemic or mucocutaneous candidosis include a variety of underlying diseases and/or immunosuppressive regimens (Odds, 1988). In particular, severe mucocutaneous candidosis is often seen in association with defects in T-cell-dependent immunity (Klein *et al.*, 1984; Khardori, 1989; Murphy, 1990).

Several animal models of systemic *C. albicans* infection have been established to assess the relative contributions of specific and non-specific mechanisms in anti-*Candida* resistance. Much evidence has accumulated over the years for a major role of polymorphonuclear neutrophils and macrophages as potent candidacidal effectors (Rogers & Balish, 1980; Murphy, 1990). In addition, recent evidence suggests that specific T cells may also be important, perhaps due to T-cell-dependent, cytokine-mediated regulation of effector cell function (Domer, 1988; Cenci *et al.*, 1989, 1990; Cantorna & Balish, 1999).

Although *C. albicans* is the causative agent in about 40% of cerebral mycoses (Lipton *et al.*, 1984; Pendlebury *et al.*, 1989), little is known of the mechanisms of intracerebral resistance to *Candida* infections. In the present study, we first investigated the effect of *C. albicans* inoculated directly into the brains of mice with no underlying immunity to the yeast. The test was then extended to mice with systemic anti-*Candida* immunity. The poorly pathogenic aegermative strain of *C. albicans*, PCA-2, is able to induce long-term colonization which results in the development of specific T-dependent immunity to challenge with cells of the highly virulent *C. albicans* strain CA-6 (Bistoni *et al.*, 1986; Cenci *et al.*, 1989, 1990). In order for systemic protection to occur, a critical number of PCA-2 cells must be injected intravenously (i.v.) (Vecchiarelli *et al.*, 1988). In addition, due to the nature of the final effector cells involved in this protection (cytokine-activated macrophages and granulocytes), the response lacks efferent specificity, such that PCA-2 vaccination will also result in increased non-specific anti-infectious protection at about two weeks post-infection (Bistoni *et al.*, 1988). For this reason, we examined the effect of systemic PCA-2 infection on host susceptibility to intracerebral (i.c.) *Candida* challenge. In addition we studied the effects of i.c. injection of the low-virulence cells on the expression

Abbreviations: AmB, amphotericin B; i.c., intracerebral(ly); i.v., intravenous(ly); IL-1, interleukin 1.

of peripheral anti-*Candida* immunity. The effect of i.c. injection of interleukin 1 (IL-1) on resistance to intracerebral *Candida* challenge was also investigated.

Methods

Mice. Female CD-1 mice, weighing 18–22 g, were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy). The mice were used at 6 to 8 weeks of age.

Organisms. The origin and characteristics of the two strains of *C. albicans* (CA-6 and PCA-2) used in this study have already been described in detail (Bistoni *et al.*, 1986, 1988; Vecchiarelli *et al.*, 1988; Cenci *et al.*, 1989). Briefly, the highly pathogenic strain CA-6 was isolated from a clinical specimen; the agerminative low-virulence strain PCA-2 is an echinocandin-resistant mutant of the parent strain 3153A, and was kindly supplied by D. Kerridge, Department of Biochemistry, University of Cambridge, UK. All yeasts were grown to stationary phase 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 Microbiology Systems). The harvested cells were washed twice in saline (0.85% NaCl) and then diluted in saline to the desired density and injected i.v. into mice in 0.5 ml volumes. Specific protection against i.v. challenge with CA-6 cells (up to 10^6) was induced by the i.v. injection of 10^6 PCA-2 cells 14 d earlier (Bistoni *et al.*, 1986, 1988; Vecchiarelli *et al.*, 1988; Cenci *et al.*, 1989).

A coagulase-positive *Staphylococcus aureus* strain (Cowan; NCTC, Colindale, UK) and a strain of *Aspergillus fumigatus* (isolated from a clinical specimen) were also used in experiments aimed at assaying the specificity of the response induced by PCA-2. The characteristic growth conditions of the two micro-organisms have been described in detail elsewhere (Vecchiarelli *et al.*, 1988).

For i.c. challenge, micro-organisms (*S. aureus*, *A. fumigatus*) or *C. albicans* conidia were suspended in 0.03 ml pyrogen-free saline and injected under sterile conditions through the central area of the frontal bone to a depth of 2 mm with a 0.1 ml glass microsyringe and a 27-gauge disposable needle (Romani *et al.*, 1982). All the animals developed meningo-encephalitis as proven by clinical signs and histopathological examination. Control mice receiving saline alone fully recovered from trauma within 30–60 min. Surgical mortality was less than 3% and always occurred 1–5 min after injection.

Reagents. Amphotericin B (Fungizone), kindly supplied by E. R. Squibb & Sons, Princeton, NY, 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 deionized water, diluted in non-pyrogenic 5% (w/v) glucose in water and injected intraperitoneally at 10 mg kg⁻¹ in a volume of 0.1 ml per 10 g body weight. Recombinant human IL-1 α was provided by Janssen Biochimica (Beerse, Belgium). The IL-1 preparation used contained <0.14 mg endotoxin ml⁻¹ and had a specific activity of 13 Mio units mg⁻¹. The material, supplied at a concentration of 10000 U ml⁻¹, was dissolved in sterile pyrogen-free saline and injected i.c. at 30 U per mouse in a volume of 0.03 ml.

Histological analysis. Mice were killed 7 d after challenge and brain specimens were fixed in 10% (v/v) neutral formalin, dehydrated and embedded in paraffin. Five to seven sections of each brain were stained with hematoxylin/eosin or periodic acid/Schiff.

Quantification of *C. albicans* in organs. This procedure has been detailed elsewhere (Vecchiarelli *et al.*, 1988). Briefly, brain and kidneys from individual mice were removed aseptically and placed in a tissue homogenizer with 3–6 ml sterile saline. The number of colony-forming

Table 1. Survival of mice challenged i.c. or i.v. with different numbers of *C. albicans* cells

Challenge*			Median survival time (d)†	D/T‡
Strain	Dose (cells per mouse)	Route of inoculation		
CA-6	10^6	i.v.	5.5 (2–8)	10/10
	10^5	i.v.	9 (7–17)	10/10
	10^6	i.c.	8§ (4–10)	10/10
	10^5	i.c.	>60§ (–)	0/10
PCA-2	10^7	i.v.	2 (1–4)	10/10
	10^6	i.v.	>60 (–)	1/10
	10^5	i.v.	>60 (–)	0/10
	10^7	i.c.	4§ (3–6)	10/10
	10^6	i.c.	>60 (–)	0/10
	10^5	i.c.	>60 (–)	0/10

* High- (CA-6) or low- (PCA-2) virulence *Candida* cells were given as a single i.v. or i.c. injection on day 0.

† The range of survival times is given in parentheses.

‡ D/T, dead mice over total animals tested.

§ $P < 0.01$ (CA-6 or PCA-2 i.c. versus i.v. treated mice).

units (c.f.u.) in the specimens (three to five mice per group) was determined by a plate dilution method, using Sabouraud dextrose agar. After 24–48 h of incubation at 37 °C, the colonies were counted; results (means $\pm$ SEM) were expressed as the number of c.f.u. per organ.

Statistical analysis. Differences in survival times were analysed by the Mann–Whitney U test. Differences in the numbers of c.f.u. in organs were analysed by Student's *t*-test. Each experiment was repeated three to five times.

Results

Survival of mice challenged by the i.v. or i.c. route using two different *C. albicans* strains

To investigate the resistance of mice to infection induced directly into the brain, we performed experiments in which naive CD-1 mice were inoculated with high- (CA-6) or low- (PCA-2) virulence *C. albicans* cells by the i.c. or i.v. route. The animals were then examined for survival time and for number of dead mice at 60 d over total animals injected. Table 1 shows that, for equal number of yeast cells, the resistance of mice to challenge with either strain was considerably greater when the cells were injected by the i.c. rather than i.v. route. In particular, after i.c. injection of 10^5 highly virulent CA-6 cells, the mice succeeded in resolving their infections, whereas all the mice that received 10^5 CA-6 cells i.v. died. In mice surviving i.c. challenge with either strain, histological examination of brain sections showed the occurrence of granulomas with prevalent participation of lymphomononuclear cells (data not shown). Transient candidaemia could be detected 12–48 h after challenge by either

Fig. 1. C.f.u. recovery from brain (▲) and kidneys (△) at different times after i.c. inoculation of 10^6 live cells of CA-6. The values are the means of three separate experiments $\pm$ SEM.

route, but the fungus was not recoverable from the blood after that time (data not shown).

To better evaluate the course of i.c. infection and the spread of yeast cells from the brain after i.c. challenge, yeast cells in the kidneys and brain were titrated at different times after injection of 10^6 CA-6 cells (Fig. 1). A marked decrease in the number of c.f.u. in the brain was evident from day 3 post-infection, suggesting the occurrence of an effective clearance mechanism. In contrast, the kidneys were rapidly colonized, with a progressive increase in c.f.u. over the 7 d observation period. Interestingly, the number of c.f.u. in the kidneys was only 10-fold less than that in the brain at 24 h post-inoculation. This may have been due to the fact that a significant portion of the i.c. inoculum was probably delivered systemically. A similar course of *C. albicans* infection was observed when the low-virulence PCA-2 cells, which give rise to chronic infection when injected systemically, were given i.c. (10^6 cells per mouse) and the animals were observed for up to 14 d, as shown in Fig. 2. This figure also shows that upon i.v. injection of an equal number of PCA-2 cells, there was a rapid dissemination of the yeast in all animals to both brain and kidneys. However, as early as 3 d after infection, the brain began to actively clear the pathogen; no such effect was observed in the kidneys. In this experiment, on day 14, c.f.u. counts in the brains of the mice infected i.c. were significantly lower than those in mice infected i.v. ($P < 0.01$).

Fig. 2. C.f.u. recovery at different times from the brain or kidneys after i.c. or i.v. inoculation with 10^6 live cells of PCA-2. ●, Brain of mice challenged i.c.; ■, kidneys of mice challenged i.c.; ○, brain of mice challenged i.v.; □, kidneys of mice challenged i.v. The values are the means of three separate experiments $\pm$ SEM.

Table 2. Effect of different numbers of live PCA-2 cells administered at different times on mouse resistance to i.c. challenge with 10^7 CA-6 cells

PCA-2 i.c. inoculum (cells per mouse)	Day of PCA-2 administration	Challenge with CA-6*	
		Median survival time (d)†	D/T‡
None	—	3 (1-4)	10/10
10^6	-14	4 (2-5)	10/10
10^4	-14	2 (1-3)	10/10
10^2	-14	3 (1-4)	10/10
10^6	-7	> 60§ (-)	0/10
10^4	-7	5 (1-6)	10/10
10^2	-7	3 (1-4)	10/10
10^6	-3	15§ (5-21)	10/10
10^4	-3	12§ (4-15)	10/10
10^2	-3	6§ (1-7)	10/10

* Live CA-6 cells were given i.c. (10^7 cells per mouse) on day 0.

† The range of survival times is given in parentheses.

‡ D/T, dead mice over total animals tested.

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

Effect of PCA-2 treatment on CA-6 challenge

Next, we tested whether the inoculation of PCA-2 cells directly into the brain would lead to increased local resistance against virulent *C. albicans*. Mice were inoculated i.c. with different numbers of PCA-2 cells and then challenged at different times with 10^7 CA-6 cells. Increased resistance to i.c. *C. albicans* challenge could be detected at 3-7 d post-infection, and the effect did not appear to be strictly dependent on the PCA-2 inoculum size (Table 2). When the size of the CA-6 challenge

Table 3. Effect of i.c. injection of PCA-2 on survival of mice challenged i.c. with CA-6, *S. aureus* or *A. fumigatus*

Live PCA-2 cells were given i.c. (10^6 cells per mouse) 10 days before i.c. challenge with live cells of CA-6 (10^6 cells per mouse), *S. aureus* (10^7 cells per mouse) or *A. fumigatus* (3×10^6 cells per mouse).

Treatment	Challenge with					
	CA-6		<i>S. aureus</i>		<i>A. fumigatus</i>	
	Median survival time (d)	D/T*	Median survival time (d)	D/T*	Median survival time (d)	D/T*
None	8	10/10	3.5	10/10	5	10/10
PCA-2	>60†	0/10	>60†	2/10	>60†	3/10

* D/T, dead mice over total animals tested.

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

Table 4. Effect of i.c. inoculation of live PCA-2 cells, heat-inactivated PCA-2 cells or sterile saline solution on resistance to i.c. challenge with 10^6 CA-6 cells

Treatment* before challenge	Day of treatment	Challenge with CA-6†	
		Median survival time (d)‡	D/T§
None	–	8 (4–17)	10/10
PCA-2	–14	>60 (–)	0/10
PCA-2	–7	>60 (–)	0/10
PCA-2	–3	>60 (–)	0/10
HCA	–14	9.5 (10–16)	10/10
HCA	–7	10.5 (7–15)	10/10
HCA	–3	7 (4–19)	10/10
SS	–14	10 (5–15)	10/10
SS	–7	8.5 (3–10)	10/10
SS	–3	7 (5–16)	10/10

* PCA-2, live PCA-2 (10^6 cells per mouse); HCA, heat-inactivated PCA-2 (10^6 cells per mouse); SS, sterile, pyrogen-free saline (30 μ l per mouse).

† Live CA-6 cells were given i.c. (10^6 cells per mouse) on day 0.

‡ The range of survival times is given in parentheses.

§ D/T, dead mice over total animals tested.

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

inoculum was lowered to 10^6 cells, complete protection was induced by 10^2 , 10^4 or 10^6 PCA-2 cells, injected 3–14 d before challenge (data not shown).

To gain some insight into the specificity of the response induced by i.c. inoculation of PCA-2 cells, we inoculated 10^6 PCA-2 cells into the brains of mice and after 10 d the animals were challenged i.c. with virulent *C. albicans* CA-6 conidia, *S. aureus* or *A. fumigatus*. The results in Table 3 indicate that PCA-2 injection conferred a high degree of local protection against the virulent CA-6 strain as well as the unrelated micro-

Table 5. Resistance of PCA-2-treated mice to i.v. and i.c. challenge with CA-6

PCA-2 treatment*		10^6 CA-6 cells i.c.		10^6 CA-6 cells i.v.	
Route of inoculation	Day†	Median survival time (d)‡	D/T§	Median survival time (d) ‡	D/T§
None		8 (4–17)	10/10	5 (2–9)	10/10
i.c.	–14	60 (–)	0/10	6.5 (4–10)	10/10
i.c.	–7	60 (–)	0/10	11.5 (9–15)	10/10
i.c.	–3	60 (–)	0/10	8 (6–10)	10/10
i.v.	–14	60 (–)	0/10	60 (–)	0/10
i.v.	–7	7 (3–10)	10/10	12 (10–16)	8/10
i.v.	–3	7 (4–11)	10/10	6 (4–10)	10/10

* Live PCA-2 cells were given i.c. or i.v. (10^6 cells per mouse)

† Day of PCA-2 administration before challenge.

‡ The range of survival times is given in parentheses.

§ D/T, dead mice over total animals tested.

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

organisms. Since it is known that different stimuli in the brain can non-specifically activate cerebral macrophages (Giulian, 1987; Perry & Gordon, 1988), we also performed experiments in which sterile saline solution of heat-inactivated PCA-2 cells were injected into the brains of naive mice. Neither inflammatory stimulus was able to increase the resistance of mice to CA-6 challenge (Table 4).

In a subsequent series of experiments, we investigated the possibility of inducing systemic anti-*Candida* protection by i.c. injection of PCA-2 or, conversely, to obtain i.c. protection by i.v. injection of PCA-2. The results in Table 5 indicate that the i.c. treatment could confer local but not systemic protection. In contrast, systemic infection with PCA-2 (10^6 cells per mouse) conferred both systemic and i.c. resistance to CA-6 challenge. While a local anti-CA-6 protective response in the brain was induced by PCA-2 as early as 3 d post-infection, increased resistance to i.c. challenge with CA-6 following peripheral PCA-2 sensitization could be detected no earlier than two weeks post-infection.

Effect of amphotericin B administration on PCA-2-induced protection

We have previously demonstrated that amphotericin B (AmB), administered according to selected treatment schedules, can completely eliminate *C. albicans* from the kidneys of infected mice (Bistoni 1985). For this reason, mice treated with PCA-2 plus AmB are unable to resist subsequent CA-6 challenge. To investigate the effect of AmB in the i.c. infection model, we administered the

Table 6. *Effect of AmB administration on PCA-2-induced protection*

Treatment		Median survival time (d) after CA-6 challenge†		10 ⁻³ × No. of c.f.u.‡ in:	
PCA-2	AmB	i.c.	i.v.	Brain	Kidneys
—	—	9	6	—	—
—	+	8.5	7	—	—
+	—	>60§	>60§	82	1520
+	+	>60§	8	0.83	0

* PCA-2 was given i.v. (10⁶ cells per mouse) 14 d before CA-6 challenge; AmB was given 6 h after PCA-2 injection.

† The challenge dose was 10⁶ cells per mouse (day 0).

‡ Yeast cell (PCA-2) counts per organ on day 0. Standard errors which were <5% of the means, have been omitted.

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

Table 7. *Effect of i.c. administration of IL-1 on survival of mice challenged i.c. with CA-6*

Treatment	CA-6 challenge		10 ³ × No. of c.f.u. recovered from brain‡		
	Median survival time (d)*	D/T†	Day +1	Day +3	Day +5
None	10 (4–17)	10/10	500 ± 39	380 ± 29	25 ± 1.8
Saline	10 (3–19)	10/10	ND	ND	ND
IL-1§	16 (12–25)	6/10	230 ± 18	180 ± 12	8 ± 0.2

* The range of survival times is given in parentheses.

† D/T, dead mice over total animals tested.

‡ C.f.u. values are the means ± SEM of three separate experiments. ND, Not done.

§ IL-1 was given as a single i.c. injection 48 h prior to challenge.

|| $P < 0.01$ (IL-1-treated versus untreated mice).

drug shortly after injection of PCA-2 (10⁶ cells i.v., day –14), prior to CA-6 challenge on day 0 by the i.c. or i.v. route. Table 6 shows that AmB treatment abrogated the systemic effect of PCA-2-induced protection although it did not affect the expression of local resistance in the brain.

Effect of i.c. administration of IL-1 on survival of mice challenged i.c.

Taken together, the above results suggested the occurrence of local immune and/or inflammatory responses in the brain of the i.c.-challenged mice. This type of response could be distinct from, and perhaps combine with, peripherally generated anti-*Candida* responses. IL-1 is a major intermediary in immune and inflammatory

responses involving the activity of phagocytes, and can also be locally produced by the brain microglia (Giulian, 1987; Giulian *et al.*, 1988; Hetier *et al.*, 1988). For this reason, we studied the effect of i.c. administration of IL-1 on the response to i.c. yeast challenge. Mice were inoculated i.c. with IL-1 (30 U per mouse) and, 2 d later, challenged i.c. with 10⁶ CA-6 cells. A significant increase in survival occurred in the IL-1-treated mice with respect to the controls (Table 7). The number of c.f.u. recovered from the brain correlated with the degree of resistance observed in the survival experiments.

Discussion

Although the brain represents an important target organ for *C. albicans* infections (Lipton *et al.*, 1984; Khardori, 1989), little is known of natural and immune mechanisms active in antifungal resistance in the brain. Using two strains of *C. albicans* with different pathogenicity, we found that the hosts were more resistant to i.c. than to systemic infection with *C. albicans*. This suggested that inoculation of yeast cells into the brain might have triggered local clearance mechanisms inadequately activated, under these experimental conditions, by the haematogenous dissemination of yeast cells to the brain from peripheral sites.

In a previously described model of systemic infection of mice with CA-6 cells, we have shown that protective immunity to lethal challenge is induced by vaccinating the animals with aegerminative PCA-2 cells (Bistoni *et al.*, 1986, 1988). In this model, the establishment of effective protection requires a period of 7–14 d after vaccination, is accompanied by a state of chronic infection involving a critical antigenic load (Vecchiarelli *et al.*, 1988), and is apparently mediated via macrophages non-specifically activated by cytokines (e.g. IL-1, IFN- γ , TNF- α) (Bistoni *et al.*, 1986; Cenci *et al.*, 1989, 1990; Vecchiarelli *et al.*, 1989). IL-1 seems to play a major role in the afferent induction of this response (Vecchiarelli *et al.*, 1989). For this reason, we performed experiments to ascertain whether i.c. infection with PCA-2 would enhance local resistance to challenge with CA-6. Our results showed that PCA-2 infection induced non-specific antimicrobial protection when performed 3–14 d before challenge. The protective effect was detectable with a wide range of inocula of PCA-2, indicating that relatively low numbers of *C. albicans* cells were capable of triggering local fungicidal mechanisms. Both the early emergence of the i.c. reactivity and its activation by limited numbers of PCA-2 cells suggest that this response is at least in part different from the local expression of systemic anti-*Candida* immunity. Increased i.c. resistance was demonstrable under condi-

tions not necessarily associated with the development of systemic T-cell dependent immunity (for example i.c. PCA-2 infection, or i.v. vaccination concurrent with AmB treatment). Nevertheless, it is possible that the local and systemic responses cooperated in increasing i.c. resistance following PCA-2 vaccination by the i.v. route. It has been reported that microglia is the major cell type associated with immunological responses in the central nervous system. Several functions have been ascribed to microglia, including antigen presentation, production of IL-1, Fc receptor and class II major histocompatibility complex antigen expression (Giulian, 1987; Perry & Gordon, 1988). Because stimulated microglia produces IL-1 (Giulian *et al.*, 1988; Hetier *et al.*, 1988) and this cytokine is a well-known macrophage-activating factor, active in anti-*Candida* resistance both *in vitro* (Vecchiarelli *et al.*, 1989) and *in vivo* (Van't Wout *et al.*, 1988; Kullberg *et al.*, 1990), we performed experiments to evaluate whether i.c. inoculation of IL-1 would increase anti-*Candida* resistance. Our results showed that injection of IL-1 into the brain produced a significant enhancement in resistance, in terms of both increased survival and reduced c.f.u. recovery from the brains of the IL-1-treated mice. Although our present observation that i.c. injected IL-1 non-specifically increases local anti-*Candida* resistance may be of therapeutic value, much study will be needed to clarify the mechanisms underlying this effect.

In conclusion, the data of the present paper suggest that both innate and acquired immunity may play a role in resistance to *Candida* meningo-encephalitis in mice and that the innate mechanisms may involve local production of IL-1

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