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Protective Effect of Picolinic Acid on Mice Intracerebrally Infected with Lethal Doses of *Candida albicans*

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We have studied the effects of picolinic acid (PLA), a product of tryptophan degradation, on mouse susceptibility to intracerebral infection with *Candida albicans*. We show that intraperitoneal administration of PLA significantly enhances the median survival time of mice inoculated with the lethal challenge. Furthermore, intracerebral administration of this agent induces a protective state against the local lethal infection, the phenomenon depending upon the administration schedule and doses of PLA employed. According to survival data, yeast growth in the brain as well as yeast colonization of the kidneys are drastically reduced in PLA-treated mice compared with those for untreated controls. Northern (RNA) blot analysis of brain tissues demonstrates that mRNA levels specific for tumor necrosis factor and interleukin 1 are augmented and induced, respectively, after inoculation of PLA. These results indicate that PLA has a protective effect likely involving elicitation of a cytokine response in vivo against fungal infections.

Candida albicans is an important fungal pathogen causing life-threatening diseases in immunocompromised or severely debilitated hosts (22). In particular, this pathogen is the causative agent in about 40% of cerebral mycoses (17, 23). While there is much evidence for a major role of polymorphonuclear neutrophils and macrophages as potent candidacidal effectors against systemic infections (21, 26), little is known of the mechanisms involved in intracerebral (i.c.) resistance to local *Candida* infections. Nevertheless, a major role may be ascribed to microglia as a local antimicrobial effector system. Besides being professional phagocytes (1, 4), microglial cells exert potent antimicrobial activity, as documented by in vitro and in vivo studies (1, 3). In particular, i.c. transfer of an in vitro-established microglial cell line into syngeneic mice confers local protection against subsequent lethal challenge with *C. albicans* (3). Furthermore, increasing evidence exists on the role of cytokines in the immune and inflammatory responses during brain infections or injuries (24). In particular, tumor necrosis factor (TNF), interleukin 1 (IL-1), and IL-6 are produced locally and likely have an immunomodulatory function(s) during meningococcal infections (30). Direct proof for IL-1 as an immunopotentiating cytokine at the cerebral level has been provided (19), since i.c. administration of exogenous IL-1 produces a significant enhancement of local anti-*Candida* resistance, in terms of both increased survival to the lethal challenge and reduced CFU recovery from the brain. Overall, these findings imply that macrophage-mediated effector as well as secretory functions are precious innate mechanisms involved in local antimicrobial defense(s) in the brain compartment.

Picolinic acid (PLA), a naturally occurring product of tryptophan catabolism, is endowed with a variety of effects on ion traffic (8, 9), cell cycle (10, 15), bacterial growth (7, 11), and host immune responses (27, 29). In particular, the macrophage compartment promptly responds to PLA as shown by in vitro and in vivo experimental models (27, 29). Furthermore, PLA exerts antitumor effects in vivo in that tumor-bearing mice show reduced tumor size and augmented median survival time (MST) upon treatment with this agent (16).

The aim of our study was to evaluate the effects of PLA on host susceptibility to *C. albicans* infection, thus showing that i.c. administration of PLA induces a protective state against local infection with *C. albicans*. By survival criteria, yeast growth in the brain is drastically reduced in PLA-treated mice compared with growth in untreated mice. Moreover, PLA administration is associated with a local response in terms of enhanced levels of mRNA specific for IL-1 and TNF in brain tissues.

MATERIALS AND METHODS

Mice. Female C57BL/6 (*H-2*^{b}) mice, 6 to 8 weeks old, were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy.

Drugs. Alpha-PLA (approximately 99% pure) and pyridine-4-carboxylic acid (PCA) were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions, prepared with sterile pyrogen-free saline, were tested for endotoxin contamination by *Limulus* amebocyte lysate assay. Only preparations with undetectable endotoxin levels (less than 0.5 ng/ml) were stored at −80°C and used in the experiments described.

C. albicans. The strain of *C. albicans* serotype A used throughout this study was a kind gift of D. Kerridge, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom. *C. albicans* was maintained by daily passages on Sabouraud dextrose agar plates.

Inoculation. i.c. inoculations were performed on anesthetized mice, as previously described (19). Briefly, mice received inocula (30 μl per mouse) in the brain, 1 mm laterally and posteriorly to the bregma at a depth of 2 mm with a 0.1-ml glass microsyringe and a 27-gauge disposable needle. PLA-treated mice received PLA, and untreated mice received a placebo (saline) according to schedules and doses described in the text prior to the i.c. microbial challenge (30

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μl per mouse). Mice recovered from trauma within 30 to 60 min. Surgical mortality was less than 3% and always occurred within 1 to 5 min after infection.

Quantitation of *C. albicans* in organs. Brains and kidneys from individual mice (five to seven per group) were removed aseptically and placed in a tissue homogenizer with 3 to 6 ml of sterile distilled water. The number of CFU in the specimens was then determined by a plate dilution method with Sabouraud dextrose agar. After 24 h of incubation at 37°C, the colonies were counted and results were expressed as the number of CFU per organ.

RNA extraction and Northern (RNA) blot analysis. Total RNA was isolated from brain tissues by solubilization with guanidine isothiocyanate as previously described (6). Poly(A)$^+$ RNA was then selected by chromatography on oligo(dT) cellulose (Boehringer). RNA species were then separated by 1% agarose-formaldehyde gel electrophoresis, blotted onto nylon membranes (Amersham International, Amersham, United Kingdom), cross-linked by UV irradiation, and heated for 1 h at 60°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate. Filters were prehybridized for 6 h at 37°C in prehybridization buffer containing formamide and denatured salmon sperm DNA (100 μg/ml). Then, 10 ng of the specific ^{32}P-labeled probe was added for 18 h in hybridization buffer containing dextran sulfate. Filters were washed four times at room temperature for 5 min and four times at 60°C for 30 min in 1× SSC–0.5% sodium dodecyl sulfate and then autoradiographed with Kodak X-AR5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens at −80°C. Probes were labeled by nick translation with a commercial kit (Amersham) as suggested by the manufacturer. The specific activity was always higher than 8 cpm/μg. For TNF detection, the 1.2-kb *Pst*I-*Eco*RI fragment (cloned from the pUC9 plasmid) was used (5). For IL-1β, the 0.870-kb *Eco*RI fragment (cloned from the pBR322 plasmid) was used (18). For β-actin detection, the 2.1-kb *Bam*HI fragment (from the Okayama-Berg pcD-X plasmid) was used (12).

Statistical analysis. Differences in survival times were analyzed by the Mann-Whitney U test. Differences in the numbers of CFU in the organs were evaluated according to Student's *t* test. Each experiment was repeated several times. The results given in the figures and tables are combined data from three experiments (except for Fig. 3, where representative data are shown).

RESULTS

With the purpose of establishing the effects of PLA on mouse susceptibility to fungal infections, we performed experiments in which mice were treated intraperitoneally (i.p.) with PLA prior to i.c. injection with *C. albicans*. As shown in Fig. 1A, naive mice challenged with the lethal dose of 10^6 yeast cells showed an MST of 10 days with survival ranging between 5 and 13 days. PLA treatment resulted in a significant enhancement of the MST (29 days), while 40% of the animals survived challenge. Conversely, when a dose of 10^7 cells was administered, PLA treatment was ineffective (Fig. 1B).

Because of the results observed following i.p. treatment, it became of interest to establish whether major effects could be reached by inoculation of PLA directly into the brain prior to the local challenge. In order to address this issue, experiments in which PLA was inoculated i.c. prior to the lethal challenge were designed. The results depicted in Table 1 indicate that following i.c. PLA administration (72, 24, or 3 h before challenge; 10^6 yeast cells per mouse), most if not all mice survived. The protective effect was evident at all time points tested, with 100% survival when PLA was given 3 h before infection. When the challenging dose was increased to 10^7 yeast cells per mouse, no protection occurred; yet, a significant enhancement of MST was observed. Again, the treatment at −3 h was the most effective (47% survival).

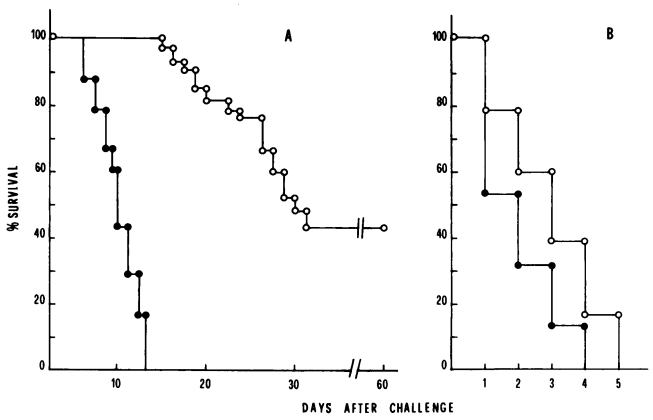

FIG. 1. Effect of PLA i.p. treatment on survival of mice challenged i.c. with *C. albicans*. Mice received a single i.p. injection of PLA (100 mg per kg of body weight) 3 days before i.c. challenge with *C. albicans*. PLA-treated (○) and untreated (●) animals were injected i.c. with 10^6 (A) or 10^7 (B) yeast cells per mouse on day 0.

In an attempt to protect mice against 10^7-cell *C. albicans* challenge, experiments in which the animals received double PLA treatment were carried out. As depicted in Table 2, the double PLA treatment, given i.c. 72 and 3 h or 24 and 3 h before 10^7 yeast-cell challenge, conferred local protection against fungal infection (MST, >60 days). In particular, the latter schedule was the most effective, since 100% of the animals survived challenge. The schedule of treatment at −72 and −24 h only increased MST, with 47% survival. In parallel groups, when the PLA analog PCA was employed, we failed to observe any significant enhancement in the MST and all mice succumbed to the challenge (Table 2). Neither PLA nor PCA alone had toxic effects, since all mice survived

TABLE 1. Effect of PLA i.c. treatment on survival of mice challenged with *C. albicans*

PLA treatment (h)[a]	Challenge dose[b]	Effect of treatment			
		MST (days)	Survival range (days)	No. of mice D/T[c]	% Survival
None	10^6	10	5–13	30/30	
−72	10^6	>60[d]	7–>60	4/30	87
−24	10^6	>60[d]	19–>60	4/30	87
−3	10^6	>60[d]		0/30	100
None	10^7	2	1–4	30/30	
−72	10^7	8[d]	1–>60	23/30	24
−24	10^7	6[d]	3–>60	26/30	14
−3	10^7	11[d]	4–>60	16/30	47

[a] Mice received a single i.c. injection of PLA (10 mg/kg) at the indicated times before the lethal local challenge with *C. albicans*.
[b] *C. albicans* (10^6 or 10^7 cells per mouse) was given i.c. on day zero.
[c] Number of dead mice at 60 days over total number of animals tested.
[d] $P < 0.01$ (PLA-treated versus untreated mice).

TABLE 2. Effect of double treatment with PLA or PCA on survival of mice challenged i.c. with *C. albicans*

Treatment (h)[a]	Challenge dose[b]	Effect of treatment			
		MST (days)	Survival range (days)	No. of mice D/T[c]	% Survival
None	10^7	2	1–4	30/30	
PLA					
−72, −24	10^7	6[d]	4–>60	16/30	47
−72, −3	10^7	>60[e]	15–>60	10/30	67
−24, −3	10^7	>60[e]		0/30	100
−72, −24	None	>60		0/30	100
−72, −3	None	>60		0/30	100
−24, −3	None	>60		0/30	100
PCA					
−72, −24	10^7	2	1–3	30/30	
−72, −3	10^7	2.5	1–4	30/30	
−24, −3	10^7	3	1–6	30/30	
−72, −24	None	>60		0/30	100
−72, −3	None	>60		0/30	100
−24, −3	None	>60		0/30	100

[a] Mice received double PLA or PCA (10 mg/kg, twice) treatment at the indicated times before i.c. challenge with *C. albicans*.
[b] *C. albicans* (10^7 cells per mouse) was given i.c. on day zero.
[c] Number of dead mice at 60 days over total number of animals tested.
[d] $P < 0.05$ (treated versus untreated mice).
[e] $P < 0.01$ (treated versus untreated mice).

treatment (Table 2) and showed no appreciable changes in ponderal body weight or behavior (data not shown).

Dose-response experiments in which mice received different doses of PLA i.c. 24 and 3 h before challenge were performed. We found that treatment with the PLA doses of 10 or 1 mg per kg of body weight resulted in 100 and 75% survival, respectively (Fig. 2). By lowering the dose of PLA to 0.1 mg/kg, we still observed 27% survival, whereas none of the mice survived when treated with a dose of 0.01 mg/kg, although significant MST enhancement was observed.

Using a CFU assay, we evaluated the effect of PLA on brain clearance and kidney colonization by the fungus. Table 3 shows the results after challenge with the lethal dose of 10^6

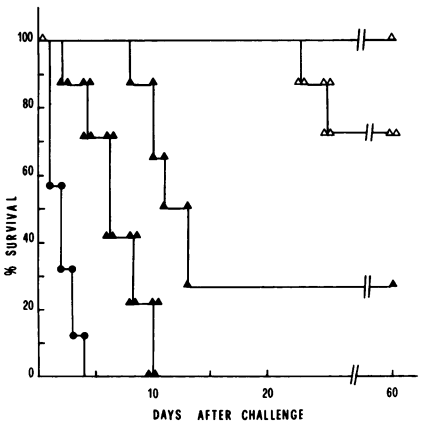

FIG. 2. Effect of various double doses of PLA on survival of mice challenged i.c. with *C. albicans*. Mice received double PLA i.c. injections 24 and 3 h before challenge. The doses used were 10 (△), 1 (△△), 0.1 (▲), and 0.01 (▲▲) mg per kg of body weight. PLA-treated and untreated (●) mice were injected i.c. with 10^7 yeast cells per mouse on day 0.

TABLE 3. *C. albicans* recovered from brain and kidneys of PLA-treated and untreated mice

Treatment[a]	*C. albicans* recovered (10^3 CFU)[b]							
	Brain				Kidneys			
	Day 1	Day 3	Day 7	Day 14	Day 1	Day 3	Day 7	Day 14
None	532.1	348.3	9.0	Dead	56.5	102.4	123.6	Dead
PLA	128.2	35.4	5.5	1.4	2.3	11.8	82.5	100.1

[a] Mice were treated i.c. with PLA (10 mg/kg) at 24 and 3 h before the i.c. challenge with *C. albicans* (10^6 cells per mouse).
[b] CFU recovery from organs was assessed for the indicated days as detailed in Materials and Methods. Dead, no cells were recovered because mice died before day 14.

C. albicans cells per mouse. Double PLA-treated mice exhibited a marked decrease in the number of CFU recovered from the brain at all the time points tested. When CFU in the kidneys were evaluated, we observed a progressive colonization of the organs both in double PLA-treated and untreated mice but to a different extent.

To establish whether PLA could have any direct antifungal effect on *C. albicans*, microorganisms were exposed in vitro to PLA for 3 h at a dose of 1 mg/ml, corresponding to the in vivo dose of 10 mg/kg. The yeast cells were then extensively washed and injected into naive mice. Table 4 shows that animals had comparable MSTs whether PLA-treated or untreated *C. albicans* was used. The survival ranges were also superimposable. The phenomenon was evident with injections of either 10^7 or 10^6 microorganisms per mouse.

In an attempt to analyze the events involved in the PLA-induced resistance against i.c. infection with *C. albicans*, experiments were performed to investigate whether the double PLA injection would affect the local response in terms of mRNA levels specific for TNF and IL-1β. Thus, Northern blot analysis was performed with poly(A)$^+$ RNA from brain tissues of PLA-treated and untreated mice. As shown in Fig. 3, brains from untreated mice had undetect-

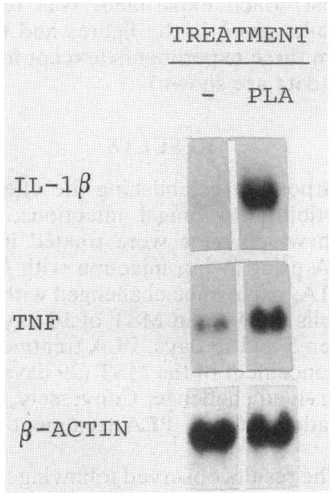

FIG. 3. Northern blot analysis of brain RNA from double PLA-treated or untreated mice. Total RNAs were purified from the brains of CD1 mice injected i.c. with a placebo (−) or PLA (10 mg/kg, twice) 24 and 3 h before Northern blot analysis. Blotted poly(A)$^+$ RNAs (10 μg per lane) were hybridized with ^{32}P-labeled probes specific for IL-1, TNF, and β-actin.

TABLE 4. Survival of mice challenged i.c. with *C. albicans* exposed to PLA in vitro

In vitro treatment[a]	Challenge dose[b]	Effect of treatment		
		MST (days)	Survival range (days)	No. of mice D/T[c]
None	10^7	3	1–4	30/30
PLA	10^7	2	1–3	30/30
None	10^6	8	4–15	30/30
PLA	10^6	9	5–14	30/30

[a] *C. albicans* was resuspended in RPMI medium supplemented with fetal calf serum (10%) at 3.3×10^8 or 3.3×10^7 cells per ml and incubated without or with PLA (1 mg/ml) for 3 h. The microorganisms were washed three times and resuspended in saline at the initial volume.

[b] *C. albicans* (30 μl per mouse) was given i.c. at a final concentration of 10^7 or 10^6 microorganisms per mouse.

[c] Number of dead mice at 60 days over total number of animals tested.

able levels of IL-1β-specific mRNA, and PLA treatment resulted in the induction of the message. When hybridization with the probe for TNF was performed, we observed detectable mRNA levels in control tissues and a consistent augmentation of TNF mRNA levels in tissues from PLA-treated mice. Under these conditions, β-actin-specific mRNA levels remained unchanged.

DISCUSSION

We have previously described an experimental model of i.c. infection by *C. albicans*, whose severity is closely related to the degree of pathogenicity of the *Candida* strain employed (19). In particular, we noted that 100% of the mice develop lethal disease following inoculation of the highly virulent strain. Interestingly, upon local administration of IL-1 or transfer of syngeneic microglial cells (3, 19), fungal clearance from the brain is rapidly achieved, no granuloma formation occurs, and most of the mice survive challenge. Here, we show that mouse susceptibility to lethal i.c. infection with *C. albicans* is affected by PLA administration.

Initial evidence for a PLA role as an immunomodulator in vivo has been provided by Leuthauser et al. (16), who show that PLA administration is effective in retarding tumor growth as well as in enhancing the MST of tumor-bearing mice. Our data ascribe a novel immunomodulatory property to PLA, that i.p. administration of this agent results in a significant enhancement of the MST of mice subsequently exposed to a lethal i.c. challenge with *C. albicans*. The phenomenon is accompanied by a rapid clearance of the fungus from the brain compartment (data not shown). Nevertheless, i.p. PLA treatment fails to protect animals from the lethal challenge. It is known that, under these conditions, PLA is rapidly excreted into the urine and has a half-life of 6 h (20). Thus, poor delivery of this agent to the brain compartment may explain the results obtained in our experimental model. This possibility is strongly supported by the demonstration that the efficacy of PLA is highly enhanced when the agent is given i.c. Mice receiving the lethal dose of 10^6 *C. albicans* cells per mouse survive challenge upon i.c. treatment with PLA (>80% survival). All time points of PLA treatment tested were comparable in terms of enhanced survival, with the one closest to the challenge being the most effective (100% survival). When the massive dose of 10^7 *C. albicans* cells per mouse (MST, 2 days) is employed, PLA treatment still produces some beneficial effects, since the MST is significantly enhanced (MST, 11 days) and 47% of the mice survive challenge after treatment on the optimal schedule (PLA treatment at −3 h). A direct antifungal effect of PLA in vivo seems unlikely, since (i) *C. albicans* exposed in vitro to PLA retains full pathogenicity when injected i.c. into naive mice and (ii) the dose employed in the present study is approximately threefold lower than the MIC (data not shown). Moreover, the fact that PLA, but not its analog PCA, affects mouse susceptibility to *Candida* infection allows us to exclude the possibility that aspecific mechanisms mediate the phenomenon described in the present paper. On these bases, we propose that the protective effect of PLA on *C. albicans*-challenged mice may depend upon activation of local host effector cells, particularly macrophages. This hypothesis is supported by the following considerations. First, brain macrophages exert a major defense role locally against *C. albicans* in that i.c. transfer of microglial cells confers protection (100% survival) to lethally challenged mice (3), the phenomenon being ascribed to a potent, local antifungal effect of these cells. Second, in vivo evidence ascribes to PLA a discrete immunopotentiating role in that peritoneal macrophages, but not other effectors such as natural killer cells, become activated in i.p. PLA-treated mice (27). Unlike what was previously shown in the experimental model of Ruffmann (27), we demonstrate that double PLA treatment is more effective than single treatment in enhancing the antifungal defenses. In particular, administration of PLA 24 and 3 h before challenge enhances the MST to >60 days with 100% survival. In our opinion, it is likely that multiple antimicrobial systems become activated, thus benefiting by multiple stimulation.

Brain clearance of the fungus is more rapid in PLA-treated mice than in untreated controls, strengthening the possibility that local effector mechanisms have become activated upon PLA treatment. No matter whether naive or PLA-treated mice are considered, some microorganisms escape local defenses and disseminate, preferentially colonizing the kidneys, as expected (19). This phenomenon is less pronounced in PLA-treated mice, which, although harboring yeast cells in the kidneys, will survive (100%) the challenge.

Northern blot analysis of RNA from the brains of control and PLA-treated mice indicates that local administration of this agent affects the levels of cytokine-specific transcripts in these tissues. In particular, IL-1β-specific mRNA levels are induced and TNF-specific mRNA levels are enhanced in PLA-treated mice compared with levels in placebo-treated mice. Such a phenomenon is detectable at time zero (i.e., when the lethal challenge is performed) and persists for at least 6 h (data not shown). The pattern of transcripts observed in the brain following PLA administration strengthens the hypothesis that the protective effects of PLA are indeed achieved through activation of local macrophages. If this is the case, we can suggest a model in which brain macrophages rapidly respond to PLA administration with enhanced cytokine production. This response would have profound consequences as the initial step triggering a cascade of autocrine and paracrine activation processes, since TNF and IL-1 are known to potentiate macrophage-mediated functions, including antimicrobial activity (2).

Although the mechanism of PLA action is still poorly understood, it has been shown that this compound enters into the cells and interferes with iron uptake (8, 9), cell cycle (10, 15), and RNA metabolism (29). Moreover, PLA is a potent costimulator, capable of synergizing with gamma interferon in activating macrophages in vitro (29). In this respect, if the in vitro phenomenon has a direct in vivo counterpart, we can speculate that in the brain compart-

ment, PLA could act as a costimulus with the endogenously produced cytokines to further enhance local defenses, namely, macrophage-mediated antifungal effects.

Finally, it is worth noting that alterations of L-tryptophan catabolism have been associated with brain injury and infections (25, 28, 31). In particular, while no direct evidence yet exists for PLA production during infection(s), increased concentrations of tryptophan and kynurenine pathway metabolites have been described in the cerebral cortex of endotoxin-treated mice (14) as well as in the cerebrospinal fluid of human immunodeficiency virus-infected patients (13). The neurological and immunological implications of these changes, including the potential presence of PLA as an endogenously produced metabolite, are still far from being elucidated. Our experimental model may provide a useful tool for studies aimed at establishing the pathophysiological significance of PLA as well as other tryptophan catabolites within the cerebral compartment.

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