Immunosuppressive effect of cyclosporin A on resistance to systemic infection with *Candida albicans*

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Summary. We studied the influence of cyclosporin-A (Cy-A) on resistance of mice to systemic infection with *Candida albicans*. Cy-A clearly inhibited resistance to *C. albicans*. The effect was dose-dependent and time and route of administration of the drug were important. This immunodepressive effect was due, at least in part, to an impairment of polymorphonuclear leucocyte (PMNL) candidacidal activity, as demonstrated *in vitro* by a reduction of phagocytic and cytotoxic activity and *in vivo* by protection when PMNL from untreated mice were transferred into cyclophosphamide-treated hosts challenged with *C. albicans*. The decreased activity of PMNL could be partly restored by adoptive transfer of normal T-lymphocytes into Cy-A-treated mice, as well as by exposure of PMNL to gamma-interferon (IFN- γ) *in vitro*.

Introduction

Cell-mediated immunity is a crucial factor in host defence against systemic infection with many fungi, including *Candida albicans* (Baccarini *et al.*, 1983a). The results of several experimental studies indicate that phagocytic cells, acting through non-specific mechanisms, are important in resistance to systemic *C. albicans* infection and are capable of phagocytosing and killing *C. albicans in vitro* (Lehrer, 1972; Sasada and Johnston, 1980; Bistoni *et al.*, 1982, 1986; Baccarini *et al.*, 1983b). In particular, previous observations suggest an important role for polymorphonuclear leucocytes (PMNL) and macrophages in resistance to *C. albicans* infections *in vivo* (Lehrer, 1969; Ruthe *et al.*, 1978; Hurtrel *et al.*, 1980; Baccarini *et al.*, 1985). The killing activity *in vitro* is not affected by treatment with anti-Thy 1 serum or anti-asialo GM-1 serum plus complement (Baccarini *et al.*, 1983a) and phagocytic cells isolated from nude mice are also actively candidacidal (Baccarini *et al.*, 1983b).

Immunomodulating agents have been employed previously to evaluate the possible role of different components of the immune system in experimental infections. In the case of experimental *C. albicans*

infection, both immunosuppressive and immunostimulating drugs have been studied (Moser and Domer, 1980; Baccarini *et al.*, 1983a; Bistoni *et al.*, 1983, 1984, 1985). We have examined the possible effect of cyclosporin A (Cy-A) on resistance to *C. albicans* challenge in mice. This drug has been reported to be a powerful immunosuppressant in all species tested; in man, it acts principally on T cells, and sometimes also on B cells, and has remarkably low myelotoxicity (Borel *et al.*, 1976; Larsson, 1980; Wiesinger and Borel, 1980; Britton and Palacios, 1982; Kaufman *et al.*, 1984; Hess and Colombani, 1986).

Materials and methods

Mice

Inbred BALB/c Cr (H^d) and hybrid (BALB/c Cr $\times$ DBA/2 Cr) F1 (CD2F1: H2^d/H2^d) mice, 2–4 months old, of both sexes, were obtained from Charles River Breeding Laboratories Inc., Calco, Milan, Italy.

Drugs

Cyclosporin A (Cy-A) was kindly provided as a gift by Sandoz Ltd, Basel, Switzerland. For administration *in vivo*, the drug was dissolved in Miglyol 812 (Dynamit Nobel, Witter, FRG), as described by Bout *et al.* (1986), to the desired concentration and injected by the intraperitoneal (i.p.), subcutaneous (s.c.) or intramuscular (i.m.)

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route in a volume of 0.1 ml/10 g of body weight. Control mice received Miglyol 812 alone. Cyclophosphamide (CY, Endoxan; Asta Werke, FRG) was dissolved in sterile NaCl 0.85% immediately before use and injected i.p. at a dose of 150 mg/kg in a volume of 0.1 ml/10 g of body weight.

Candida albicans

C. albicans, laboratory strain CA-6, was isolated from a clinical specimen (Marconi *et al.*, 1976). It was grown at 28°C with mild agitation in low-glucose Winge medium (Mattia *et al.*, 1982) containing glucose 0.2% w/v and yeast extract (BBL Microbiology Systems, Cockeysville, MD, USA) 0.3% w/v until the stationary phase of growth was reached at about 24 h. Under these conditions, cultures yielded $c. 3 \times 10^8$ cells/ml, and the organism grew as an essentially pure yeast-phase population. Cells were harvested from the 24-h culture by low-speed centrifugation (600 g), washed twice in saline and diluted to the desired density.

Leucocyte counts

Mice were bled from the retro-orbital sinus (10 mice were used for each determination). Cell counts were made by diluting anticoagulated blood 1 in 20 in Türk solution and counting the cells in a counting chamber. Differential cell counts were made on blood smears stained by the May-Grünwald-Giemsa method by counting 200 leucocytes on each slide. Standard errors never exceeded 10% of the mean and, therefore, have been omitted from the results.

Preparation of phagocytic cells

Peritoneal PMNL were obtained from the peritoneal cavities of mice 18 h after i.p. injection of 1 ml of thioglycolate broth 10%. Cells were harvested by peritoneal washing and approximately 85% of them were identified as PMNL on morphological criteria. When specified, experiments were performed with circulating PMNL separated from peripheral blood by Ficoll-Hypaque solution as described by Klock and Bainton (1976).

Spleen cells were harvested by adherence to plastic. Resident or thioglycolate-induced (i.p. injection of broth 4 days earlier) peritoneal cells were harvested as above and suspended (1×10^7 cells) in 10 ml of RPMI 1640 medium (Eurobio Laboratories, Paris) in 93-mm plastic petri dishes (Nunc InterMed, Roskilde, Denmark). After incubation for 2 h at 37°C in an atmosphere of CO₂ 5% in air, the dishes were washed thoroughly with RPMI 1640 medium to remove the non-adherent cells. The adherent cells were recovered by scraping the dish with a "rubber policeman", and were washed and suspended in RPMI 1640 medium supplemented with fetal calf serum (GIBCO Laboratories, Grand Island, NY, USA) 10%, 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-etha-

nesulphonic acid) buffer (Eurobio), and gentamicin sulphate 0.1% (hereafter referred to as complete RPMI 1640 medium). More than 99% of the recovered cells had the morphology of macrophages in Giemsa-stained smears and their viability was 85–90%.

Adoptive transfer of lymphocytes

The non-adherent cells separated by the plastic adherence fractionation method (see above) were passed over a nylon fibre column as described by Julius *et al.* (1973). The fraction that was recovered comprised 98–99% T-lymphocytes, as determined by their morphology in May-Grünwald-Giemsa stained smears and confirmed by immunofluorescence with fluorescein-labelled anti-Thy 1, 2 antibodies (New England Nuclear). These lymphocytes were injected intravenously (i.v.) into recipient mice (25×10^6 cells/mouse).

Interferon

Cloned murine gamma-interferon (IFN- γ) was kindly provided by Genentech, Inc. (South San Francisco, CA, USA).

Transfer of PMNL and C. albicans mixtures

C. albicans cells (1×10^4) were mixed with 20×10^4 PMNL from normal or Cy-A-treated mice and incubated at 37°C in air + CO₂ 5%. After contact for 1 h, the 0.5-ml volumes of the mixtures were injected i.v. into CY-immunodepressed mice.

Measurement of phagocytic activity in vitro

Microscopic examination of phagocytic cell function was performed by mixing equal volumes (0.1 ml) of effector cells, the indicator dye methylene blue, and a suspension of heat-inactivated yeast cells in sterile polypropylene tubes (Becton Dickinson) at an effector cell: yeast cell ratio of 1:10. Triplicate samples of the mixtures were incubated for different times at 37°C. Phagocytic cells were separated from non-phagocytosed *C. albicans* by centrifugation on a fetal bovine serum gradient (Shalaby *et al.*, 1985) and 0.1-ml samples of the harvested phagocytic cells were used to prepare wet mounts for microscopy. The phagocytic activity of effector cells was expressed according to the following formula: percentage phagocytic activity = number of cells containing one or more yeast cells/100 cells counted.

In vitro cytotoxicity assays

⁵¹Cr release assay. The candidacidal activity of various effector cell populations was assessed by the technique described by Bistoni *et al.* (1982). Briefly, PMNL or single-cell suspensions from the spleen were prepared and different numbers of effector cells in 0.1 ml of the suspension were mixed in U-shaped 96-well microtitra-

tion plates with 0.1 ml of a suspension of 5×10^4 ^{51}Cr -labelled yeast cells (2×10^8 cells were incubated with 300 μCi of $\text{Na}_2^{51}\text{CrO}_4$ for 2 h). After incubation for 4 h at 37°C in air + CO_2 5%, the plates were centrifuged at 800 g for 10 min, and the radioactivity in 0.1 ml of the supernate was measured in a γ -scintillation counter (Auto Gamma 500 C; Packard Instrument Co., Inc., Downers Grove, IL, USA). The base-line ^{51}Cr release was that of yeast cells incubated alone in complete RPMI 1640 medium; in no case did the release exceed 20% of the total counts/min (cpm) incorporated by target yeast cells. The experimental results are expressed as the percentage of killing of yeast cells in the experimental group (quadruplicate samples) represented by the ^{51}Cr release (cpm) above the base-line control according to the following formula: specific ^{51}Cr release (%) =

$$\left[\frac{(\text{cpm experimental group} - \text{cpm spontaneous group}) \times 2}{\text{total cpm}} \right] \times 100$$

in which total cpm is the radioactivity incorporated by 5×10^4 candida cells.

Inhibition assay by plate counts. Phagocytic cells (5×10^5 in 0.1 ml of suspension per well) were mixed in U-shaped 96-well microtitration plates (Greiner Labor-technik, Nürtingen) with *C. albicans* cells (5×10^4 cells in 0.1 ml of suspension/well). After incubation for 4 h at 37°C in air + CO_2 5%, the plates were shaken vigorously, and serial dilutions in distilled water were made from each well. Cell suspensions were incubated with distilled water until microscopic examination revealed the complete disruption of phagocytes. Triplicate samples from each dilution were spread on Sabouraud glucose agar. The numbers of cfu were counted after incubation for 18 h at 37°C . Control cultures were *C. albicans* cells incubated without phagocytes.

Culture of *C. albicans* from kidneys

Both kidneys of normal or Cy-A treated mice, challenged i.v. with 5×10^5 *C. albicans* cells, were removed aseptically and placed together in a tissue homogeniser with 3 ml of sterile NaCl 0.85%. The number of *C. albicans* cfu in a specimen was determined by a plate dilution method with culture on Sabouraud glucose agar. The colonies were counted after incubation at 37°C for 48 h, and the results were expressed as the number of cfu/organ.

Statistical analysis

Differences in survival times were assessed by the Mann-Whitney U test. Differences in the numbers of cfu or the amounts of the specific radiolabel release in the in-vitro microcytotoxicity assays were assessed by Student's *t* test. Each experiment was repeated three to five times.

Results

Effect of Cy-A treatment on the resistance of mice to systemic challenge with C. albicans

We performed experiments to test the influence of Cy-A treatment on the resistance of mice to i.v. injection of a lethal dose (5×10^5 cells) of *C. albicans* (fig. 1). Treatment, at different times before challenge and with different doses of Cy-A, caused a significant reduction of median survival time (MST) in some of the groups compared with untreated controls. In particular, doses of 50, 25, or 12.5 mg/kg, given 1 day before *C. albicans* challenge, or 50 or 25 mg/kg, given 3 days before, reduced the MST. By contrast, none of the four doses of Cy-A given 7 days before challenge significantly reduced MST when compared with untreated controls. These data indicate that Cy-A induces a rapid and transient depression of the mechanisms responsible for resistance to *C. albicans* challenge *in vivo*.

We further examined the effect of Cy-A treatment before challenging with different doses of *C. albicans* (table I). The administration of Cy-A 25 mg/kg, 1 or 3 days before challenge, significantly impaired host resistance to systemic infection following challenge with different doses of *C. albicans*. This suppressive effect was most evident when mice challenged with 5×10^4 *C. albicans* cells had been pretreated with Cy-A 1 or 3 days before challenge. In this case the MST was 17 days, while the control mice survived indefinitely (> 60 days).

Effect of Cy-A treatment on recovery of C. albicans from the kidneys of mice after i.v. challenge

We have shown previously that the number of *C. albicans* cfu recovered from the kidneys correlates well with the level of resistance of mice to challenge with *C. albicans* (Bistoni *et al.*, 1984). To examine further the nature of Cy-A-induced depression of resistance to *C. albicans* infection *in vivo*, we also compared the recovery of *C. albicans* cfu from the kidneys of untreated animals with those from Cy-A-treated animals (table II). The counts of cfu from the kidneys of animals treated with Cy-A 1 or 3 days before *C. albicans* challenge were compared with those of untreated animals. Cy-A pretreatment caused a significant increase in the number of cfu that was evident after 24 h and was more marked 48 h after challenge. As expected, Cy-A treatment caused a significant reduction in host resistance as shown by the comparison of MST of the different groups of treated and untreated animals.

Fig. 1. Effect of dose and time of administration of Cy-A on survival of CD2F1 mice challenged i.v. with 5×10^5 *C. albicans* cells: ■, untreated controls; □, mice (10 mice/group) treated with Cy-A 1(A), 3(B) or 7(C) days before challenge. Vertical bars represent the survival ranges (days); * $p < 0.01$ (Cy-A treated mice versus untreated).

Effect of Cy-A treatment on PMNL-mediated cytotoxic activity against C. albicans

We performed experiments to determine the number and candidacidal activity of PMNL in mice treated with Cy-A (table III). Treatment with Cy-A (25 mg/kg, i.p.), by any administration schedule, did not significantly alter the number of peripheral lymphocytes nor the number of mono-

cytes and PMNL. Therefore, we examined peripheral and thioglycolate-induced PMNL for their cytotoxicity against ^{51}Cr -labelled *C. albicans* *in vitro* as well as for their ability to inhibit *C. albicans* growth in a cfu assay, as previously described (Bistoni *et al.*, 1982). A significant reduction of cytotoxic activity and reduction of inhibition of *C. albicans* cfu recovery by peripheral and thioglycolate-induced PMNL was evident in the groups of

Table I. Survival of normal or Cy-A-treated CD2F1 mice after systemic challenge with *C. albicans*

Treatment with Cy-A* before challenge (days)	Number of <i>C. albicans</i>	MST†	Number of mice dead (10 per group)	Survival range (days) of fatal cases
No treatment	5×10^4	60	0	...
7	5×10^4	60	3	11-19
3	5×10^4	17‡	7	9-18
1	5×10^4	17‡	9	10-18
No treatment	10^5	12	10	9-14
7	10^5	10	10	8-14
3	10^5	8‡	10	7-9
1	10^5	7‡	10	7-9
No treatment	5×10^5	8	10	6-8
7	5×10^5	8	10	6-9
3	5×10^5	3‡	10	3-5
1	5×10^5	4‡	10	4-5

*, Cy-A 25 mg/kg as a single i.p. injection.

†, MST, median survival time (days).

‡ $p < 0.01$ (Cy-A-treated mice versus controls).

Table II. Survival, and recovery of *C. albicans* from kidneys, of normal or Cy-A-treated CD2F1 mice after systemic challenge

Treatment with Cy-A* before challenge (days)	MST	Number of mice dead (10 per group)	Survival range (days) of fatal cases	<i>C. albicans</i> (cfu $\times 10^3$) recovered from kidneys		
				8 h†	24 h†	48 h†
No treatment	8	10	7-8	0.9	5.7	167.3
3	4‡	10	3-5	1.2	85.5‡	306.0‡
1	3‡	10	3-5	1.0	268.1‡	2394.9‡

* Cy-A 25 mg/kg as a single i.p. injection before challenge with 5×10^5 *C. albicans* cells/mouse.

† Time after challenge; data are means of triplicate samples; standard errors, usually <15% of the value, have been omitted.

‡ $p < 0.01$ (Cy-A-treated mice *versus* controls).

Table III. Effect of Cy-A administration on the number of peripheral leucocytes and the candidacidal activity of peripheral and thioglycolate-induced PMNL of CD2F1 mice *in vitro*

Treatment with Cy-A* before challenge (days)	Candidacidal activity of								
	Peripheral leucocyte counts				peripheral PMNL		thioglycolate-induced PMNL		
					Percentage ^{51}Cr specific release at ratio†		Percentage ^{51}Cr specific release at ratio†		Percentage cfu inhibition
	Total WBC	lymphocytes	PMNL	monocytes	10:1	2.5:1	10:1	2.5:1	(SE) at ratio† 10:1
No treatment	3629	2150	1261	218	30.2	21.4	80.0 (5.0)	35.2	29.0
7	3613	1889	1473	251	28.5	23.6	73.2 (7.3)	29.5	23.1
3	3310	2002	1104	204	16.9‡	12.6‡	36.4‡ (4.0)	19.3‡	14.8‡
1	3059	1660	1214	185	17.3‡	10.1‡	33.5‡ (6.3)	15.6‡	12.3‡

* Cy-A 25 mg/kg as a single i.p. injection before the *in-vitro* assay and peripheral leucocyte counts.

† Ratios of effector to target cells; data are the means of quadruplicate samples; standard errors of ^{51}Cr -release assay, usually <1.5%, have been omitted.

‡ $p < 0.01$ (Cy-A-treated mice *versus* controls).

animals treated 1 or 3 days before with Cy-A 25 mg/kg. We also evaluated the phagocytic activity of PMNL from Cy-A treated mice. As shown in fig. 2, PMNL from animals treated with Cy-A 3 days before the PMNL were harvested showed significantly reduced phagocytic activity. Our data suggest that Cy-A treatment did not alter the number of circulating PMNL but significantly reduced their ability to phagocytose and inhibit the growth of *C. albicans*.

On the basis of these observations, we investigated the possibility that the candidacidal activity of resident, thioglycolate-induced macrophages and splenic macrophages could be affected also. The results are shown in table IV. The candidacidal activity of these different macrophage populations

was not affected by Cy-A treatment, suggesting that the reduction of resistance to *C. albicans* challenge *in vivo* after Cy-A treatment was due to the effects on candidacidal activity of PMNL but not of macrophages.

Protective ability of PMNL from Cy-A-treated mice in transfer experiments

We reported previously that it is possible to increase significantly the MST of immunodepressed mice infected with *C. albicans* by adoptive transfer of PMNL (Baccarini *et al.*, 1985). To document further the effect of treatment with Cy-A on PMNL activity, we performed transfer experiments with PMNL from untreated or Cy-A-treated animals

Fig. 2. Effect of Cy-A administration on in-vitro phagocytic efficiency of PMNL incubated with inactivated *C. albicans*. Cy-A (25 mg/kg) was given as a single i.p. injection 3 days before collection of PMNL for the assay. PMNL from normal (○-○) or Cy-A-treated (●-●) CD2F1 mice were incubated *in vitro* with inactivated *C. albicans* at a ratio of 1:10.

The results were obtained by counting 100 PMNL for each incubation period; $p < 0.01$ for phagocytosis by PMNL from Cy-A treated mice versus controls.

(table V). The i.v. injection of PMNL from untreated donors into CY-treated recipients significantly increased the MST of animals challenged with *C. albicans* (group 2) compared with control animals (group 1). In contrast, the adoptive transfer of PMNL from Cy-A-treated (25 mg/kg 3 and 1 day before the assay) donors did not result in any significant protection against *C. albicans* challenge (groups 3 and 4). These results demonstrate that PMNL of Cy-A-treated animals, unlike PMNL from normal donors, are not able to protect immunodepressed mice.

Effect of different routes of Cy-A administration on PMNL activity

We also tested the possible effect of Cy-A administration by different routes. Cy-A doses were injected i.p., s.c. or i.m. and we evaluated both resistance to *C. albicans* challenge *in vivo* and cytotoxic activity of PMNL *in vitro*. As shown in

Table IV. Effect of Cy-A administration on in-vitro candidacidal activity of peritoneal resident or thioglycolate-induced macrophages and plastic-adherent spleen cells of CD2F1 mice

Treatment with Cy-A* before challenge (days)	Percentage cfu inhibition (SE) with†		
	peritoneal resident macrophages	thioglycolate-induced macrophages	adherent spleen cells
None	25.2 (5.3)	74.3 (8.2)	51.2 (4.0)
7	30.2 (6.5)	76.8 (6.3)	56.0 (2.5)
3	27.5 (3.8)	65.2 (7.8)	56.0 (3.7)
1	28.9 (4.0)	60.8 (5.6)	48.2 (4.2)

* Cy-A 25 mg/kg as a single i.p. injection a number of days before the *in vitro* assay (day 0).

† Ratio of effector: target cells was 10:1; data are the means of quadruplicate samples.

table VI, the i.p. or i.m. injection of Cy-A significantly decreased the resistance to *C. albicans* challenge *in vivo* as well as the candidacidal activity of PMNL *in vitro*. However, s.c. administration failed to cause any significant effect. These results further indicate the relevance of Cy-A inhibition of PMNL activity as a possible mechanism responsible for the depression of resistance *in vivo*.

Partial reconstitution of PMNL activity in Cy-A-treated animals by transfer of T lymphocytes

Cy-A selectively inhibits T-cell functions (Borel *et al.*, 1976; Larsson, 1980; Wiesinger and Borel, 1980; Bunjes *et al.*, 1981; Britton and Palacios, 1982; Kauffman *et al.*, 1984). To study further the effect of Cy-A on PMNL activity, we examined the possible effect of T-cell transfer on PMNL activity in Cy-A-treated mice. Animals were treated with Cy-A 50 mg/kg 3 days before the assay; 2 days before the assay, one group of mice was given 2.5×10^7 cells of a T-lymphocyte enriched population from normal donors (see *Material and methods*) by i.v. injection. Both the ^{51}Cr -release assay and the cfu-inhibition assay indicated that lymphocyte transfer partly reconstituted the PMNL activity (table VII). These data suggest that the Cy-A-induced decline of PMNL activity could be due, at least in part, to the effect of Cy-A on T lymphocytes.

Partial reconstitution of PMNL activity in Cy-A-treated mice by IFN- γ

It was reported recently that IFN- γ increased PMNL-mediated candidacidal activity (Djeu *et al.*,

Table V. Correlation between in-vitro candidacidal activity of PMNL from normal or Cy-A-treated CD2F1 mice and protective activity against *C. albicans* infection in immunodepressed mice*

Group	Challenge†	Percentage ⁵¹ Cr specific release by PMNL <i>in vitro</i> at ratio		Results of <i>C. albicans</i> challenge		
		10:1‡	2.5:1‡	MST	Number of mice dead (10 per group)	Survival range (days)
1	1 × 10 ⁴ <i>C. albicans</i> cells	...	...	2.5§	10	2-3
2	1 × 10 ⁴ <i>C. albicans</i> cells after incubation for 1 h with PMNL from untreated mice	35.2	30.5	7.0	10	6-8
3	1 × 10 ⁴ <i>C. albicans</i> cells after incubation for 1 h with PMNL from mice treated with Cy-A 25 mg/kg 3 days before the assay	18.3	14.5	2.0§	10	2-3
4	1 × 10 ⁴ <i>C. albicans</i> cells after incubation for 1 h with PMNL from mice treated with Cy-A 25 mg/kg 1 day before the assay	17.5	12.6	2.5§	10	2-3

*The same PMNL population was used as an effector in the in-vitro assay and in the in-vivo transfer experiment

†Mice immunodepressed with cyclophosphamide (150 mg/kg 3 days before challenge) were given by i.v. injection 1 × 10⁴ *C. albicans* cells previously incubated for 1 h with RPMI medium (group 1), or with PMNL from untreated (group 2) or Cy-A-treated (groups 3 and 4) mice.

‡Ratios of effector: target cells; data are the means of quadruplicate samples; standard errors, usually <1.5%, have been omitted.

||p < 0.01 (groups 3 and 4 versus group 2).

§p < 0.01 (groups 1, 3 and 4 versus group 2).

Table VI. Effect of different routes of Cy-A administration on PMNL candidacidal activity and resistance of CD2F1 mice to systemic challenge with *C. albicans*

Treatment with Cy-A* before challenge		<i>C. albicans</i> challenge			Percentage ⁵¹ Cr specific release at ratio		
Days	Route	MST	Number of mice dead (10 per group)	Survival range (days)	10:1†	5:1†	2.5:1†
	None	8	10	6-9	33.1	29.9	20.3
3	i.p.	4‡	10	2-5	17.5‡	15.3‡	12.2‡
1	i.p.	2‡	10	1-4	10.8‡	9.8‡	7.7‡
3	s.c.	9	10	7-9	32.1	23.7	20.7
1	s.c.	7	10	6-10	28.0	25.6	20.5
3	i.m.	5‡	10	2-5	12.1‡	9.9‡	8.6‡
1	i.m.	4‡	10	1-5	12.5‡	11.7‡	8.9‡

*Cy-A 25 mg/kg was given as a single i.p. injection days before challenge with 5 × 10⁵ *C. albicans* cells/mouse.

†Ratios of effector: target cells; data are the means of quadruplicate samples; standard errors, usually <1.5%, have been omitted.

‡p < 0.01 (Cy-A treated mice versus controls).

1986). To examine further the possible mechanism of Cy-A-induced inhibition of PMNL activity, we measured the effect of IFN-γ on the activity, *in vitro*, of PMNL from Cy-A-treated and normal control mice (fig. 3). The incubation of PMNL with different doses of IFN-γ resulted in a significant

augmentation of their activity as evaluated by both ⁵¹Cr-release and cfu-inhibition assay. This potentiating effect was more marked for PMNL from Cy-A-treated animals than for those from normal animals. These data could indicate that lack of IFN-γ production in Cy-A-treated mice may ac-

Table VII. Effect of lymphocyte transfer on candidacidal activity of PMNL from normal or Cy-A-treated mice

Group	Origin of PMNL	Candidacidal activity of PMNL			
		Percentage ⁵¹ Cr specific release at ratio†			Percentage CFU inhibition (SE) at ratio† 10:1
		10:1	5:1	2.5:1	
1	normal mice	42.9‡	32.5‡	20.3‡	80.8‡ (8.2)
2	mice treated with Cy-A 50 mg/kg 3 days before the assay	18.3	15.8	8.9	38.0 (2.7)
3*	normal mice that received lymphocytes (2.5 × 10 ⁷ cells/mouse) 2 days before the assay	40.2‡	39.0‡	29.5‡	77.8‡ (4.3)
4*	mice treated with Cy-A 50 mg/kg 3 days before and given lymphocytes (2.5 × 10 ⁷ cells/mouse) 2 days before the assay	30.5‡	23.0‡	15.2‡	63.4‡ (2.2)

*The transferred lymphocyte population from normal mice was 98% Thyl+ T lymphocytes (see *Materials and methods*).

†Ratios of effector: target cells; data are the means of quadruplicate samples. Standard errors of the ⁵¹Cr-release assay, <15%, have been omitted.

‡p < 0.01 (groups 1, 3 and 4 versus group 2).

count, at least in part, for the decrease of PMNL candidacidal activity.

Discussion

We have shown that phagocytic cells play an important role in resistance to experimental *C. albicans* infection *in vivo* (Baccarini *et al.*, 1983a; Bistoni *et al.*, 1986). We also found that modulation of numbers of macrophages and PMNL or their activity, or both, may influence this resistance, suggesting that the phagocytic killing mediated by macrophages and PMNL could play a role. Previous observations indicate that macrophages and PMNL are able to kill *C. albicans in vitro* as well as inhibit colony formation (Lehrer, 1972). Our observations tend to exclude a direct role for T cells and natural killer (NK) cells in host resistance against infection as well as in killing or growth inhibition, or both, of *C. albicans in vitro*. Furthermore, anti-candida effector cells lack typical markers of either T or NK cells and their *in vivo* reactivity shows characteristics which differ from those of NK activity, including age and genetic influences (Baccarini *et al.*, 1983b).

To analyse further the mechanisms that may be involved in resistance to *C. albicans* infection, we manipulated host reactivity by treating animals with Cy-A. Unlike other agents, Cy-A does not attack all lymphocyte populations indiscriminately, but acts only against selected ones. It has been reported that Cy-A selectively depletes T cells, and,

in particular, T-helper and T-cytotoxic cells. More specifically, it acts at an early stage in the life cycle of T lymphocytes, inhibiting their activation by blocking the intercellular message carried by interleukin-2 (IL-2) (Bunjes *et al.*, 1981; Kaufmann *et al.*, 1984; Hess and Colombani, 1986). It has been proposed that Cy-A spares both myeloid tissue and the non-specific arm of the defence system and that it has no effect on neutrophils or macrophages (Weinbaum *et al.*, 1984; Lun *et al.*, 1985). Although additional actions may yet be defined, the major effect of Cy-A seems to be its ability to inhibit T-cell functions by acting at three distinct but related stages in the process of T-cell activation: (1) inhibiting T cell help of accessory cells for the synthesis of IL-1; (2) preventing IL-2-producing T cells from expressing receptors for IL-1 and suppressing the synthesis of IL-2; (3) rendering T cells unresponsive to IL-2 (Borel *et al.*, 1976; Bunjes *et al.*, 1981; Hess and Colombani, 1986).

The data reported here indicate that Cy-A decreases the spontaneous reactivity of PMNL against *C. albicans* as evaluated by *in-vivo* and *in-vitro* experiments. Evidence for this depressive effect on PMNL was a significantly decreased cytotoxic activity *in vitro*, as shown by the cfu assay and by a 4-h ⁵¹Cr-release assay against radiolabelled *C. albicans*. Furthermore, PMNL from Cy-A treated mice also showed significantly decreased phagocytic activity *in vitro* (fig. 2) which could account for the decreased cytotoxic activity observed (table III). This depressed activity was also

Fig. 3. Candidacidal activity of PMNL from normal or Cy-A-treated CD2F1 mice after exposure *in vitro* to IFN- γ . PMNL from normal mice ($\square$ - $\square$, $\blacksquare$ - $\blacksquare$) or Cy-A-treated mice ($\circ$ - $\circ$, $\bullet$ - $\bullet$) were exposed to mouse recombinant IFN- γ for 2 h before the assay and during the 4-h incubation period. The ^{51}Cr release assay and cfu-inhibition assay were performed at effector: target ratios of 10:1. Standard errors of the ^{51}Cr release assay, usually <1.5%, have been omitted. Vertical bars represent the standard errors of the cfu-inhibition assay; *, $p < 0.01$; #, $p < 0.05$ for IFN- γ -treated PMNL *versus* controls.

evident *in vivo*. Cy-A decreased resistance to *C. albicans* challenge (fig. 1; tables I and VI) and also reduced significantly the protective capability of PMNL transferred from Cy-A-treated mice into immunodepressed mice to a subsequent challenge with *C. albicans* (table V). The decreased resistance to *C. albicans* challenge *in vivo* also indicates that Cy-A impairs the activity of PMNL *in vivo*. However, Cy-A did not affect the viability and candidacidal activity of PMNL when incubated *in vitro* for different times and with different drug concentrations (data not shown).

Because of previous observations that T lympho-

cytes are the cell population mainly affected by Cy-A treatment (Borel *et al.*, 1976; Larsson, 1980; Wiesinger and Borel, 1980; Bunjes *et al.*, 1981; Britton and Palacios, 1982; Kauffman *et al.*, 1984), we tested the possibility that the depressive effect on PMNL activity was a consequence of the drug-mediated effect on T lymphocytes. We evaluated the effect of treatment with Cy-A (25 mg/kg 1 or 3 days before assay) on T-cell mediated alloreactivity *in vivo* or cytotoxic T-lymphocyte generation *in vitro*, as well as the proliferative response to PHA. Our results showed that the treatments that reduced PMNL candidacidal activity also inhibited these T-cell functions (data not shown).

These observations suggested a possible relationship between the effects of Cy-A on T-cell activity and the effects on PMNL candidacidal activity. Therefore, we performed transfer experiments in an attempt to reconstitute PMNL activity in Cy-A-treated mice. Our results (table VII) show clearly that transfer of nylon non-adherent, T-cell enriched, spleen cells partly reconstituted the PMNL candidacidal activity *in vivo*.

We also examined the effect of treatment of PMNL from Cy-A-treated mice with IFN- γ *in vitro*. Our data (fig. 3) show that IFN- γ treatment partly reconstituted the PMNL candidacidal activity. On the basis of our data and previously reported observations that Cy-A strongly affects IL-2 production by T lymphocytes and that IL-2 may, in turn, stimulate the production of IFN- γ , we suggest that Cy-A-induced depression of PMNL activity could be due, at least in part, to inhibition of T-cell dependent IL-2 and IFN- γ production (Farrar *et al.*, 1986). Moreover, recently IFN- γ has been reported to stimulate PMNL-mediated candidacidal activity (Djeu *et al.*, 1986), which suggests a possible physiological role of IFN- γ in maintaining levels of PMNL activity necessary for host defence against infections. These proposed mechanisms may account, in part, for the Cy-A-induced decrease of resistance to *C. albicans* infection, although other cells and factors may also be involved in this phenomenon.

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