***In vitro* survival and germination of *Candida albicans* in the presence of nitrogen compounds**

Fernando Abaitua, Aitor Rementería, Rosario San Millan, Ainhoa Eguzkiza, Jose Antonio Rodriguez, José Pontón and María Jesús Sevilla

Author for correspondence: María Jesús Sevilla. Tel: +34 946012688. Fax: +34 944648500.
e-mail: ippsegam@lg.chu.es

Departamento de
Inmunología,
Microbiología y
Parasitología, Universidad
del País Vasco, Apartado
644, 48080-Bilbao, Spain

The *in vitro* effect of nitric oxide (NO) and nitrite on blastoconidia and hyphae of *Candida albicans* was studied. Sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) were used as NO donors. Both minimal and complex media at two pH values, 7.0 and 4.5, were used for the assays. Blastoconidia were more susceptible than hyphae to NO. The NO effect on blastoconidia was greater at acidic pH. Nitrite affected the viability of blastoconidia in complex medium. The percentage germination and the relative rate of elongation of hyphae were both enhanced when NO was present in acidic conditions.

Keywords: Nitric oxide, sodium nitroprusside, candidacidal activity, dimorphism, *Candida albicans*

INTRODUCTION

Phagocytosis is probably the most important mechanism in protecting the non-compromised host against invasive candidiasis (Domer & Carrow, 1989). Polymorphonuclear leukocytes and macrophages, the main phagocytic cells involved in the clearance of fungal pathogens (Levitz, 1992), have different candidacidal mechanisms and different abilities to kill *Candida albicans*.

Nitric oxide (NO) is generated by the NO synthase of activated macrophages, but its real contribution to the candidacidal activity of murine macrophages is not clear, although it is certainly involved (Blasi *et al.*, 1995; Cenci *et al.*, 1993; Rementería *et al.*, 1995; Vazquez-Torres *et al.*, 1994). Thus some authors have correlated candidacidal activity of murine macrophages with peroxynitrite resulting from the interaction between NO and the superoxide anion (Vazquez-Torres *et al.*, 1996). Others argue that the two compounds do not occur simultaneously in murine macrophages (Assreuy *et al.*, 1994). In fact, there are few data about the susceptibility of *C. albicans* to nitric oxide *in vitro* in the absence of macrophages (Fierro *et al.*, 1996).

C. albicans is a dimorphic fungus which can cause severe infections in immunocompromised patients. The ability of the fungus to modify its morphology from blastoconidia to hyphae is considered a virulence factor. Blastoconidia and hyphae show different susceptibility to phagocytic cells (Baccarini *et al.*, 1985; Blasi *et al.*, 1995; Cutler & Poor, 1981), so they are probably diversely affected by macrophage products.

Our purpose was to study the *in vitro* susceptibility of blastoconidia and hyphae of *C. albicans* to NO in nutrient and pH conditions similar to those found in the phagolysosome or outside the macrophage, and the effect of this compound on the germination process of the fungus.

METHODS

Assay media. Two different assay media were used: (1) a minimal medium (MM) containing 1 g glucose l⁻¹ and 0.5 g biotin l⁻¹ in 0.08 M citrate/phosphate buffer; and (2) a complex medium, consisting of Dulbecco modified Eagle's medium in 0.08 M citrate/phosphate buffer (DMEB). The pH of both media was adjusted to 4.5 or 7.0. Sodium nitroprusside (SNP), sodium nitrite or S-nitroso-N-acetylpenicillamine (SNAP), at the concentrations indicated in the results, were added when necessary.

Micro-organism, maintenance and inoculum. The following

Abbreviations: SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside.

C. albicans strains were used: CA-2 (agerminative mutant, kindly provided by A. Cassone, Istituto Superiore di Sanità, Rome, Italy); CA-6 (isolated from a clinical specimen, kindly provided by E. Blasi, University of Modena, Italy); 3153 (National Collection of Pathogenic Fungi, Public Health Laboratory, Bristol, UK); C2T, C9S and 2282 (Universidad del País Vasco culture collection). The strains were maintained on GYE agar (20 g glucose l⁻¹; 10 g yeast extract l⁻¹; 20 g agar l⁻¹) by weekly transfer. Blastospores were obtained from 24 h cultures on GYE agar, washed with saline and adjusted to the desired concentration by direct counting under the microscope. Hyphae were obtained from CA-6 blastospores (4 × 10⁵ cells ml⁻¹) germinated in 96-well microassay plates in DMEB pH 7.0 (100 µl per well) at 37 °C for 3 h.

Anti-*C. albicans* blastospores (anti-Y) assay. *C. albicans* CA-2 (5 × 10⁵ blastospores ml⁻¹) were incubated in the assay media at 37 °C for 90 min; the viability of the blastospores was determined as colony forming units (c.f.u.) on Sabouraud agar plates after incubation at 37 °C for 24 h. The anti-Y activity was calculated as follows:

$$\text{Anti-Y activity} = [1 - (\text{c.f.u. in assay} / \text{c.f.u. in control})] \times 100$$

Anti-*C. albicans* hyphae (anti-H) assay. CA-6 hyphae were incubated in the assay media at 37 °C for 90 min; the viability of the hyphae was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction method (Mosmann, 1983). Briefly, 100 µl DMEB and 10 µl MTT (5 mg ml⁻¹ in 10 mM sodium phosphate buffer pH 7.2 with 0.85 % sodium chloride) were added to each well and the plate was incubated at 37 °C for 3 h. The medium was then removed and 100 µl DMSO was added to each well. The absorbance at 540 nm was determined. The anti-H activity was calculated as follows:

$$\text{Anti-H activity} = [1 - (A_{540} \text{ in assay} / A_{540} \text{ in control})] \times 100$$

Morphology studies. *C. albicans* blastospores (1 × 10⁷ cells ml⁻¹) were incubated in 200 µl assay media in siliconized tubes at 37 °C for 3 h. Fixing solution (10 % sodium lauryl sulfate, 10 % formaldehyde; 20 µl) was added, and the samples kept at room temperature until necessary. Morphology was examined under an image analysis system (VIDS-IV) connected to a phase-contrast microscope. The percentage germination and the relative rate of hyphal elongation were determined as follows:

$$\% \text{ Germination} = (\text{number of hyphae} / \text{total number of cells}) \times 100$$

$$\text{Relative rate of hyphal elongation} = (\text{length of hyphae in assay} / \text{length of hyphae in control}) \times 100$$

The length of at least 100 hyphae across the slide was determined.

NO determination. NO was indirectly determined as nitrite using the Griess reagent (Granger *et al.*, 1990). Briefly, 0.4 ml 1 % (w/v) sulfanilamide in 2.5 % (v/v) phosphoric acid and 0.4 ml 0.5 % (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5 % (v/v) phosphoric acid were added to 0.2 ml of sample. After 10 min at room temperature the absorbance at 540 nm was determined. Sodium nitrite (0–25 µM) was used for the calibration curve.

Statistics. The Student's *t*-test was applied to the data. The differences were considered significant when *P* < 0.05.

RESULTS

NO generation by SNP

First, the production of NO by SNP in the assay media without cells was quantified (Tables 1 and 2). In both media the NO generated was time-dependent and there was a good linear correlation between SNP concentration and NO produced. There were no significant differences in NO production between the two pHs or between the two media used. In all cases, the pH of the medium remained constant during the assay (data not shown).

Effect of SNP and nitrite on *C. albicans* viability

Once the linear relationship between SNP concentration and NO had been determined, we studied the effect of SNP and nitrite on the viability of blastospores and hyphae of *C. albicans*. SNP had a clear candidacidal effect against blastospores of *C. albicans* in MM at both pHs (Fig. 1). The effect was much greater at the acidic pH. When the assay was run in DMEB in acidic conditions, an anti-Y effect of SNP was also observed, while at neutral pH only 4 mM SNP showed a significant, though small, effect.

Table 1. NO (as nitrite, µM) generated by different concentrations of SNP in MM

Data represent the mean ± SD of at least three independent determinations.

SNP (mM)	pH 4.5		pH 7.0	
	1.5 h	3 h	1.5 h	3 h
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	4.77 ± 1.32	7.02 ± 0.62	4.94 ± 0.63	6.41 ± 0.22
2	7.24 ± 2.16	13.61 ± 2.32	7.16 ± 1.06	12.06 ± 1.76
4	14.53 ± 1.12	27.5 ± 2.41	15.32 ± 3.39	24.92 ± 1.66
8	38.80 ± 6.74	65.58 ± 10.60	41.04 ± 14.98	53.85 ± 2.74

Table 2. NO (as nitrite, µM) generated by different concentrations of SNP in DMEB

Data represent the mean ± SD of at least three independent determinations.

SNP (mM)	pH 4.5		pH 7.0	
	1.5 h	3 h	1.5 h	3 h
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	5.65 ± 0.25	9.50 ± 1.95	5.32 ± 0.25	7.79 ± 1.62
2	10.19 ± 2.40	21.25 ± 3.26	10.66 ± 1.29	16.83 ± 2.11
4	21.29 ± 6.89	32.83 ± 13.72	21.48 ± 5.79	27.90 ± 5.57
8	55.35 ± 8.18	59.30 ± 2.22	50.19 ± 11.10	57.77 ± 4.17

Fig. 1. Effect of SNP on the viability of *C. albicans* CA-2 yeast cells incubated at pH 4.5 (▨) or 7.0 (□) in MM or DMEB. Data represent the mean $\pm$ SD of at least three independent determinations. Statistically significant differences with respect to controls are marked with an asterisk.

Fig. 2. Effect of sodium nitrite on the viability of *C. albicans* CA-2 yeast cells incubated at pH 4.5 (▨) or 7.0 (□) in MM or DMEB. Data represent the mean $\pm$ SD of at least three independent determinations. Statistically significant differences with respect to controls are marked with an asterisk.

Conversely, nitrite had no anti-Y effect when the incubation medium was MM (Fig. 2) but it was candidacidal against blastoconidia when the incubation medium was DMEB, and in that case, the effect was

Fig. 3. Effect of SNP on the viability of *C. albicans* CA-6 hyphae at pH 4.5 (▨) or 7.0 (□) in MM or DMEB. Data represent the mean $\pm$ SD of at least three independent determinations. Statistically significant differences with respect to controls are marked with an asterisk.

Fig. 4. Effect of SNP on the percentage germination of *C. albicans* CA-6 at pH 4.5 (▨) or 7.0 (□) in MM or DMEB. Data represent the mean $\pm$ SD of at least three independent determinations. Statistically significant differences with respect to controls are marked with an asterisk.

greater at neutral pH. We cannot explain the lack of significance of the activity of 5 mM sodium nitrite in DMEB pH 7.0, apart from the wide dispersion of the data.

The effect of SNP on *C. albicans* hyphae was much lower than the effect on blastoconidia, both in MM and in DMEB, and the differences between pH values were not significant (Fig. 3). Nitrite, on the other hand, had practically no anti-H effect in the different conditions

Fig. 5. Effect of sodium nitrite on the percentage germination of *C. albicans* CA-6 at pH 4.5 (▨) or 7.0 (□) in MM or DMEB. Data represent the mean $\pm$ SD of at least three independent determinations. Statistically significant differences with respect to controls are marked with an asterisk.

studied, except for 10 μ M nitrite in MM pH 7.0, where a small anti-H effect (less than 10%) was observed (data not shown).

Effects of SNP and nitrite on *C. albicans* morphology

The germination of *C. albicans* CA-6 was low in MM and in DMEB in acidic conditions, but it was 100% in DMEB pH 7.0 (Fig. 4). In DMEB pH 4.5, but not in MM, pseudohyphae were frequently observed, although they were not quantified nor included in the percentage of true germination. The addition of SNP enhanced the germination of CA-6 in MM and DMEB at pH 4.5 in a dose-dependent pattern, although 100% germination was not achieved (Fig. 4); the increase in the percentage germination, attributed to NO, was simultaneous with the decrease in the proportion of pseudohyphae. No effect was detected in MM pH 7.0, nor, obviously, in DMEB pH 7.0 where the starting point was already 100%.

Nitrite added to MM had a minor effect on the germination of CA-6 (Fig. 5), but the differences in comparison to the control, though statistically significant, were very small. When this compound was added to DMEB, no effect on the morphology was observed at any of the pHs studied.

The effect of SNP on the germination of strain CA-6 was so conspicuous that we tested five more strains, in-

Table 3. Effect of SNP on the percentage germination of different *C. albicans* strains in DMEB pH 4.5

Data represent the mean $\pm$ SD of at least three independent determinations.

Strain no.	Control	SNP (4 mM)*
C9s	21.5 $\pm$ 2.1	85.0 $\pm$ 2.8
3153	51.0 $\pm$ 11.3	74.0 $\pm$ 5.7
C2T	5.0 $\pm$ 4.2	52.5 $\pm$ 6.4
2282	14.0 $\pm$ 0.0	31.5 $\pm$ 7.8
CA-2	0.0 $\pm$ 0.0	6.5 $\pm$ 3.5

* All values showed statistically significant differences with respect to controls.

Fig. 6. Effect of SNP on the relative rate of elongation of *C. albicans* CA-6 germ tubes at pH 4.5 (▨) or 7.0 (□) in MM or DMEB. Data represent the mean $\pm$ SD of at least three independent determinations. Statistically significant differences with respect to controls are marked with an asterisk.

cluding the agerminative mutant CA-2, in DMEB pH 4.5, to see if the effect was strain-specific. All strains tested showed a significant increase in the percentage germination when the NO donor was present (Table 3). In the case of CA-2, the increase in percentage germination was very low, but significant.

Finally we studied the effect of SNP and sodium nitrite on the elongation rate of *C. albicans* CA-6 hyphae. SNP significantly enhanced the elongation rate in MM, particularly in acidic conditions (Fig. 6). No significant

effect of SNP was observed in DMEB. Nitrite had no significant effect on the elongation rate of *C. albicans* hyphae in any of the conditions used (data not shown).

Effect of SNAP on the viability and morphology of *C. albicans*

To prove more directly the involvement of NO in the observed effects of SNP on the viability and morphology of *C. albicans*, we studied the effect of another NO donor, SNAP. First, we observed that NO production by 5 mM SNAP was quite variable, but it was at least as high as the amount produced by 8 mM SNP in similar conditions, and frequently much greater (nitrite = $230.24 \pm 136.07 \mu\text{M}$). The pH of the media remained the same at the end of each experiment (data not shown).

The anti-Y activity of SNAP 5 mM was qualitatively similar to the effect of SNP, with a greater candidacidal effect in acidic conditions (between 55% in DMEB and 75% in MM) than in the neutral ones (less than 20% in DMEB and 65% in MM).

SNAP had no effect against *C. albicans* hyphae in any of the conditions tested (data not shown). On the contrary, it enhanced germination in DMEB pH 4.5, increasing the percentage germination from 54% in the absence of SNAP to 91% when the compound was present.

DISCUSSION

NO production has been proposed as one of the major antimicrobial mechanisms of murine macrophages (MacMicking *et al.*, 1997), which are active against different kinds of pathogens, such as viruses, bacteria, fungi, protozoa and helminths. The role of NO (or related compounds) in macrophage anti-*Candida* activities seems to be well-established (Rementería *et al.*, 1995; Vazquez-Torres & Balish, 1997). However, NO is a very labile molecule and there is no agreement on the anti-*Candida* effect of the different intermediates and derivatives.

Our purpose was to study the susceptibility of blastoconidia and hyphae of *C. albicans* to NO and the effect of this compound on the germination process. We designed a cell-free system with SNP or SNAP as NO donors, in different pH conditions and in different media to mimic, to some degree, the phagolysosomal (MM, pH 4.5) and the extracellular (DMEB, pH 7.0) environments with respect to pH and nutrient availability. We studied the effect of sodium nitrite in similar conditions, because nitrite is always present in NO aqueous solutions (Granger *et al.*, 1990) and is also reputed to be a microbicidal compound. In fact, we detected NO as nitrite since we could not distinguish clearly between the two compounds. A second NO donor was used to be sure that the effects attributed to NO were really due to this compound. In order to study the differential susceptibility of yeast and hyphal morphologies, we used two different *C. albicans* strains, CA-2, an

agerminative mutant, and CA-6, a fully germinative strain. By using an agerminative mutant we were able to maintain the yeast phase in the assay conditions. We cannot exclude the possibility that differences between CA-6 and CA-2 may influence their interactions with the compounds tested in this study.

First, we demonstrated that NO production by SNP was time- and dose-dependent, whilst other assay conditions, such as pH or medium composition, did not significantly affect NO generation. We therefore used SNP concentration as an indicator of NO concentration in the assays.

C. albicans blastoconidia were susceptible to SNP and SNAP, especially in acidic conditions similar to those found in the phagolysosomal environment. *C. albicans* hyphae were more resistant to the nitrogen compounds in all the conditions tested. Little correlation was observed between the effect of the NO donors, SNP and SNAP, and the effect of nitrite on the viability of *C. albicans*. Consequently, we attribute the effect of SNP and SNAP mainly to NO, rather than nitrite. The smaller effects of nitrite could be partially due to NO when the pH was acid (Stuehr & Nathan, 1989), and may be due to the nitrite itself when the pH was neutral.

The effect of pH on the susceptibility of different microorganisms to NO has been previously reported by other authors (Buchmüller-Rouiller *et al.*, 1994; Kono *et al.*, 1994). A decrease in *C. albicans* viability in the presence of different NO donors was shown by Fierro *et al.* (1996), but no mention was made of a different susceptibility of yeast and hyphae. Blasi *et al.* (1995) reported that *C. albicans* hyphae are sensitive to the extracellular killing mechanism of murine macrophages mediated by nitrogen compounds but blastoconidia are not. This apparent contradiction with our results could be explained by the fact that macrophages produce not just NO, but a collection of toxic compounds whose synergistic effect could be totally different from the effect of each one separately. So the susceptibility of hyphae reported by Blasi *et al.* (1995) could be attributable to this synergistic effect. The resistance of *C. albicans* blastoconidia to extracellular NO could be explained in the same way or, perhaps, could be attributable to an insufficiently low pH in the assay. By contrast, evidence for the susceptibility of *C. albicans* blastoconidia to killing by NO-producing murine macrophages has already been published (Cenci *et al.*, 1993; Vazquez-Torres *et al.*, 1994, 1995) and it is generally accepted that *C. albicans* hyphae are more resistant to macrophage killing than blastoconidia (Cutler & Poor, 1981).

The ability to produce hyphae by *C. albicans* blastoconidia is important as far as virulence is concerned (Richardson & Smith, 1981; Saltarelli *et al.*, 1975). The infectious unit is usually in the yeast form, and germination occurs in animal tissues after infection. Accordingly, we were interested in determining the effect of nitrogen intermediates on the germination process in addition to their effect on the viability of both morpho-

logies. For that purpose, we used strain CA-6 as well as other *C. albicans* strains obtained from human patients. Although the germinative ability of these strains was diverse, in general they produced a maximum percentage of germ tubes in complex medium pH 7.0, whilst at pH 4.5 the percentage germination was significantly reduced. This is in agreement with data reported by Kaur & Mishra (1994).

Whilst nitrite did not affect the germination process, NO derived from SNP increased the percentage germination of all the *C. albicans* strains tested in acidic conditions. SNAP also enhanced the germination of CA-6 strain. Not only the percentage germination but even the elongation of hyphae of CA-6 was significantly enhanced by SNP in conditions equivalent to the intraphagolysosomal environment (pH 4.5, MM). These observations suggest that *C. albicans* may be able to avoid the microbiocidal mechanisms of macrophages by changing its growth to the most resistant morphology, not to mention the fact that the intracellular growing hyphae destroy the macrophage (Evron, 1980). This would mean that NO-generating macrophages would help in the extension of the infection, as has been proposed by Brummer & Stevens (1994). In fact, macrophages from mice infected with *C. albicans* produce significant amounts of NO (Rementería *et al.*, 1995).

However, the *in vivo* data are not easy to reconcile with our *in vitro* studies. It has been reported that activated macrophages are able to retard the germination of yeast forms of *C. albicans* for several hours (Maiti *et al.*, 1980). Additional experimental evidence is needed in order to extrapolate our data, obtained *in vitro*, to what happens *in vivo*.

In summary, we conclude that (1) *C. albicans* blastoconidia are more sensitive than hyphae to NO; (2) *C. albicans* blastoconidia are susceptible to nitrite; (3) NO enhances germination; and (4) NO is more efficient in acidic conditions.

ACKNOWLEDGEMENTS

We thank Professor David Hallet for his revision of the English version of the manuscript.

This research was supported by grant UPV 093.327EB131/96 from the Universidad del País Vasco and grant EX97/4 from the Departamento de Educación, Universidades e Investigación del Gobierno Vasco.

REFERENCES

- Assreuy, J., Cunha, F. Q., Epperlein, M., Noronha-Dutra, A., O'Donnell, C. A., Liew, F. Y. & Moncada, S. (1994). Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. *Eur J Immunol* **24**, 672–676.
- Baccarini, M., Vecchiarelli, A., Cassone, A. & Bistoni, F. (1985). Killing of yeast, germ-tube and mycelial forms of *Candida albicans* by murine effectors as measured by radiolabel release microassay. *J Gen Microbiol* **131**, 505–513.
- Blasi, E., Pitzurra, L., Puliti, M., Chimienti, A. R., Mazzola, R., Barluzzi, R. & Bistoni, F. (1995). Differential susceptibility of yeast and hyphal forms of *Candida albicans* to macrophage-derived nitrogen-containing compounds. *Infect Immun* **63**, 1806–1809.
- Brummer, E. & Stevens, D. A. (1994). Anticryptococcal activity of macrophages: role of mouse strain, C5, contact, phagocytosis, and L-arginine. *Cell Immunol* **157**, 1–10.
- Buchmüller-Rouiller, Y., Corradin, S. B., Smith, J. & Maüel, J. (1994). Effect of increasing intravesicular pH on nitrite production and leishmanicidal activity of activated macrophages. *Biochem J* **301**, 243–247.
- Cenci, E., Romani, L., Mencacci, A., Spaccapelo, R., Schiaffella, E., Puccetti, P. & Bistoni, F. (1993). Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of *Candida albicans*. *Eur J Immunol* **23**, 1034–1038.
- Cutler, J. E. & Poor, A. H. (1981). Effect of mouse phagocytes on *Candida albicans* in *in vitro* chambers. *Infect Immun* **31**, 1110–1116.
- Domer, J. E. & Carrow, E. W. (1989). Candidiasis. In *Immunology of the Fungal Diseases*, pp. 57–92. Edited by R. A. Cox. Boca Raton, FL: CRC Press.
- Evron, R. (1980). *In vitro* phagocytosis of *Candida albicans* by peritoneal mouse macrophages. *Infect Immun* **28**, 963–971.
- Fierro, I. M., Barja-Fidalgo, C., Cunha, F. Q. & Ferreira, S. H. (1996). The involvement of nitric oxide in the anti-*Candida albicans* activity of rat neutrophils. *Immunology* **89**, 295–300.
- Granger, D. L., Hibbs, J. B. J., Perfect, J. R. & Durack, D. T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J Clin Invest* **85**, 264–273.
- Kaur, S. & Mishra, P. (1994). Differential increase in cytoplasmic pH at bud and germ tube formation in *Candida albicans*: studies of a nongerminative variant. *Can J Microbiol* **40**, 720–723.
- Kono, Y., Shibata, H., Adachi, K. & Tanaka, K. (1994). Lactate-dependent killing of *Escherichia coli* by nitrite plus hydrogen peroxide: a possible role of nitrogen dioxide. *Arch Biochem Biophys* **311**, 153–159.
- Levitz, S. M. (1992). Overview of host defenses in fungal infections. *Clin Infect Dis* **14** (suppl.1), S37–S42.
- MacMicking, J., Xie, Q. & Nathan, C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol* **15**, 323–350.
- Maiti, P. K., Kumar, R. & Mohapatra, L. N. (1980). Candidacidal activity of mouse macrophages *in vitro*. *Infect Immun* **29**, 477–482.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55–63.
- Rementería, A., García-Tobalina, R. & Sevilla, M. J. (1995). Nitric oxide-dependent killing of *Candida albicans* by murine peritoneal cells during an experimental infection. *FEMS Immunol Med Microbiol* **11**, 157–162.
- Richardson, M. D. & Smith, H. (1981). Production of germ tubes by virulent and attenuated strains of *Candida albicans*. *J Infect Dis* **144**, 565–569.
- Saltarelli, C. G., Gentile, K. A. & Mancuso, S. C. (1975). Lethality of *Candida* strains as influenced by the host. *Can J Microbiol* **21**, 648–654.
- Stuehr, D. J. & Nathan, C. F. (1989). Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J Exp Med* **169**, 1543–1555.
- Vazquez-Torres, A. & Balish, E. (1997). Macrophages in resistance to candidiasis. *Microbiol Mol Biol Rev* **61**, 170–192.
- Vazquez-Torres, A., Jones-Carson, J. & Balish, E. (1994).

Candidacidal activity of macrophages from immunocompetent and congenitally immunodeficient mice. *J Infect Dis* **170**, 180–188.

Vazquez-Torres, A., Jones-Carson, J., Warner, T. & Balish, E. (1995). Nitric oxide enhances resistance of SCID mice to mucosal candidiasis. *J Infect Dis* **172**, 192–198.

Vazquez-Torres, A., Jones-Carson, J. & Balish, E. (1996). Peroxy-

nitrite contributes to the candidacidal activity of nitric oxide-producing macrophages. *Infect Immun* **64**, 3127–3133.

.....
Received 23 October 1998; revised 8 March 1999; accepted 22 March 1999.