Increased resistance in BALB/c mice to reinfection with *Candida albicans* is due to immunoneutralization of a virulence-associated immunomodulatory protein

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Here, it is shown that immunoneutralization of p43, a virulence-associated immunomodulatory protein secreted by *Candida albicans*, is responsible for immunoprotection against candidiasis after spontaneous healing of mice inoculated with 10⁶ *C. albicans* blastoconidia. p43 is produced by the pathogenic *Candida* blastoconidia, and neither immunoprotection nor immunoneutralization can be elicited by priming the mice with attenuated or heat-killed *C. albicans* blastoconidia. The immunoprotection against systemic candidiasis was positively correlated with (i) serum levels of antibodies against p43 and (ii) a high ratio between antibodies against p43 and antibodies against *C. albicans* structural antigens. Immunoprotection against candidiasis can be induced in mice primed with heat-killed *C. albicans*, after treatment of the animals with anti-p43 antibodies. The data described here provide a biological explanation for active immunoprotection achieved after spontaneous healing of infectious diseases, namely in candidiasis.

INTRODUCTION

Human vaccination based on the stimulation of the immune system with attenuated microbes or with constitutive microbial epitopes has been rather disappointing for most infectious diseases. There is no single licensed human vaccine against fungi, protozoa, helminthes and rickettsias, and protection against the majority of pathogenic viruses or bacteria can not be achieved using this rationale. Furthermore, the most effective human vaccines, which prevent tetanus and diphtheria, are directed against the bacterial toxins rather than against the structural epitopes of the microbes. Therefore, these vaccines do not follow the classical strategy of induction of immunoprotection against infectious agents.

Lifelong immunoprotection against the aetiologic microorganisms of infectious disorders may occur after natural clearance of infectious agents for which there are no available treatments, as in the case of measles, German measles, mumps or hepatitis A and B. Subclinical infections caused by very small numbers of pathogenic microbes may also explain the higher levels of resistance to infections that are found among some populations of health professionals. Similarly, naturally acquired high protection can be achieved in cattle or in guinea pigs after clearance of sublethal inocula of bacteria (Blander & Horwitz, 1989; Pal & Horwitz, 1992; Stevens et al., 1995), fungi (Timoney et al., 1988) and protozoa (Urquhart et al., 1998). It is also pertinent to recall that the exceptionally efficient smallpox vaccine was made of virus particles that were not fully attenuated, since skin infectious vesicles and subsequent scars usually occurred after the vaccination.

In experimental candidiasis, the best protective effects have been achieved by immunization with viable cells from virulent (Cassone et al., 1995; Giger et al., 1978; Mourad & Friedman, 1961) strains of *Candida albicans*, while immunization with killed cells or subcellular components of the organism has been moderately successful (Banerjee et al., 1985; Eckstein et al., 1997; Levy et al., 1985; Mencacci et al., 1994).

We have previously observed that bacteria (Ferreira et al., 1997; Lima et al., 1992; Santarem et al., 1987), viruses (Arala-Chaves et al., 1988) and fungi (Tavares et al., 1993) secrete virulence-associated immunomodulatory proteins (VIPs). These VIPs are only produced by micro-organisms in their pathogenic form. They are immunomodulatory because they are simultaneously mitogens for T- and B-lymphocytes and immunosuppressive for foreign antigens (Arala-Chaves
et al., 1988; Ferreira et al., 1988; Lima et al., 1992; Pal & Horwitz, 1992; Ribeiro et al., 1991; Soares et al., 1990; Tavares et al., 1993). We have shown the importance of VIPs for the survival of pathogenic micro-organisms to be due to the fact that they potentiate microbial growth (Lima et al., 1992; Santarem et al., 1987; Tavares et al., 1993) and because mice immunized with VIPs become resistant to infection (Soares et al., 1990; Tavares et al., 1995).

In the last decade, other authors have also isolated microbial VIPs from different bacteria and viruses. Thus, virulent (Balaban et al., 1998; Pal & Horwitz, 1992), immunosuppressive (Kierszenbaum et al., 1990; Klapproth et al., 1995; Sun et al., 1998), T-lymphocyte (Bruno et al., 1998; Itoh et al., 1992; Miyoshi-Akiyama et al., 1993; Mody et al., 1995) and B-lymphocyte (Dugas et al., 1991; Feng & Lo, 1994; Ying & Weis, 1993) mitogenic proteins have been described. Moreover, immunoprotection achieved with proteins secreted by Mycobacterium tuberculosis (Horwitz et al., 1995) and Staphylococcus aureus (Balaban et al., 1998) has also been reported.

All of the aforementioned observations have led us to investigate whether naturally acquired immunoprotection against systemic candidiasis, developed in mice that recovered from a fungal inoculation with $10^6$ Candida blastoconidia, was due to immunoneutralization of p43, a VIP produced by C. albicans.

**Methods**

**Mice.** Male BALB/c mice were purchased from the Gulbenkian Institute of Science, Oeiras, Portugal. All mice were 6–8 weeks old at the time of the experiments.

**C. albicans infection and/or priming protocols.** Pathogenic C. albicans was isolated and maintained as described previously (Tavares et al., 1993). Some of the pathogenic C. albicans blastoconidia were heat-killed at 100˚C for 30 min. The attenuated form of the same fungal strain was obtained after 60 passages in Sabouraud glucose agar (Difco) over 8 months, as previously described in detail (Tavares et al., 1993). Naïve BALB/c mice were primed with $10^6$ Candida blastoconidia by the intraperitoneal (i.p.) route with pathogenic C. albicans, the attenuated fungus or the heat-killed fungus. Control mice received the same volume of vehicle (PBS minus C. albicans) as the test mice via the i.p. route. One month later, all groups of mice were infected i.p. with an inoculum of $10^7$ pathogenic C. albicans blastoconidia. Kidneys were removed aseptically at selected times after fungal priming or after infection with $10^7$ pathogenic C. albicans blastoconidia. The organs were homogenized in 3 ml of PBS and the homogenates were serially diluted 10-fold. Plating of the homogenates was started at an initial dilution of 1:100. Colony-forming units (c.f.u.) of C. albicans were counted after 48 h incubation at 37˚C on mycobiotic agar (Difco) in duplicate cultures for each serial dilution. Only the number of c.f.u. found in the kidneys is shown because kidney is the organ of maximum fungal growth after systemic C. albicans infection (Louria, 1985). Furthermore, as shown in Table 1, there was a good correlation between fungal load in the kidneys and in the liver, the spleen and several other organs scored (data not shown).

**Preparation of p43 and Candida structural antigens (Cs).** Isolation and purification of p43 have been previously described in detail (Tavares et al., 1993). C. albicans was cultured on Sabouraud glucose agar medium for 24 h at 28˚C and then transferred to a medium containing 0-3 % (w/v) yeast extract (Difco) and 0-2 % (w/v) glucose for 60 h at 37˚C with agitation. The cultures were then centrifuged at 2500 g for 30 min. The supernatants were filtered through a 0.45 μm Millipore filter, concentrated by vacuum dialysis and fractionated by ion-exchange chromatography. Mannoside constituents were removed by passage through a concanavalin A-Sepharose column (Sigma Chemical). Finally, after PAGE, p43 was eluted by electrophoresis. All preparations of p43 were LPS-free as assessed by the Limulus amebocyte lysate (LAL) kit (E-Toxate; Sigma). Briefly, the evidence of gelator indicating the presence of a minimum of 0-05 to 0-1 endotoxin units (EU) (ml endotoxin) $^{-1}$ was tested in samples of 10 μg of p43, using as positive and negative controls serial dilutions of endotoxin standard stock solutions and endotoxin-free water, respectively. Cs were prepared as described previously (Tavares et al., 1995). Briefly, Candida blastoconidia were disrupted by sonication (10 cycles of 30 s at 100 W) on ice with a Branson cell disruptor (model W 185 D).

**Table 1.** Demonstrative example of four independent experiments of the number of c.f.u. found in the kidney, liver and spleen of BALB/c mice after i.p. infection with $10^7$ C. albicans blastoconidia.

Results are means of six mice per group $\pm$ 1 SD.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean response (log c.f.u. $\pm$ log SD)</th>
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<tr>
<td></td>
<td>Kidney</td>
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<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>I</td>
<td>$3.98$ $\pm$ $3.28$</td>
</tr>
<tr>
<td>II</td>
<td>$4.40$ $\pm$ $3.48$</td>
</tr>
<tr>
<td>III</td>
<td>$4.41$ $\pm$ $3.48$</td>
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<tr>
<td>IV</td>
<td>$4.08$ $\pm$ $3.30$</td>
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ND, Not detected.

*Control mice.

†Mice primed with $10^6$ heat-killed C. albicans blastoconidia.

‡Mice primed with $10^6$ attenuated C. albicans blastoconidia.

§Mice primed with $10^6$ pathogenic C. albicans blastoconidia.
centrifuged at 29000 g for 30 min and filtered through a 0.22 µm Millipore filter. Full disruption of blastoconidia was confirmed by light microscopy. The protein content was determined by the method of Lowry et al. (1951).

**Antibody detection.** A standard ELISA was done to quantify specific antibodies in mice sera. Briefly, polystyrene microtitre plates (Nunc) were coated with p43 (10 µg ml\(^{-1}\)) or Cs overnight at 4°C. The wells were saturated for 30 min with 1% (w/v) BSA in PBS at room temperature. Appropriate serial dilutions of the serum samples were then incubated in the plates for 2 h at room temperature. After washing, the bound antibodies were revealed by the addition of peroxidase-labelled goat-anti-mouse IgG (Southern Biotechnology Associates) for 1 h at room temperature. O-Phenylenediamine (Sigma) and H₂O₂ were used to develop the reaction; it was stopped by the addition of SDS. The colorimetric change was measured with a Biotek Chromoscan at 450 nm. The ELISA antibody titre was expressed as the reciprocal of the highest dilution giving an \(A_{450}\) value of 0-1 above that of the control (no serum added).

**Immunizations with p43 or Cs and passive serum administration.** BALB/c mice were immunized twice with intradermal injections of 10 µg of p43 or 10 µg of Cs in 0-2 ml PBS with a 3-week interval between injections. p43-immunized mice were treated i.p. with 0-5 ml of sterile Ig enriched fraction (IgF) (ammonium sulphate precipitation) obtained from the same strain of mice immunized with p43, or unimmunized, at the same time (day 0) and one day after (day +1) p43 immunization. One month later, both groups of mice were infected i.p. with 10⁷ pathogenic *Candida* blastoconidia. Mice primed with heat-killed *Candida* blastoconidia were treated i.p. with 0-5 ml of IgF obtained from the same strain of mice immunized with p43 or Cs, or unimmunized one day before (day −1), at the same time (day 0) and one day after (day +1) infection with 10⁷ pathogenic *Candida* blastoconidia. Each injection of IgF was obtained from 0:25 to 0:45 ml of mice sera. The IgF obtained from immunized mice was adjusted with balanced salt solution to 2500 ELISA titres against the respective antigen. IgF from unimmunized mice was adjusted to the same protein concentration as the anti-p43 IgF (27 mg ml\(^{-1}\)), whereas the concentration of IgF with anti-Cs was 18 mg protein ml\(^{-1}\).

**Statistical analysis.** In all cases, each experiment was repeated at least three times. Data were statistically analysed by Student’s t-test. *P* values were considered statistically significant if they were less than 0.05.

**RESULTS**

**Mice infected with a small inoculum of pathogenic *C. albicans* develop a mild systemic candidiasis**

To evaluate the level of infectiousness of the small inoculum of pathogenic *Candida* blastoconidia, we compared the fungal growth in BALB/c mice infected with 10⁶ pathogenic or attenuated *Candida* blastoconidia. As expected, the number of c.f.u. found in the kidneys was much higher 5 days after infection with the pathogenic (3.79 ± 2.68) than with the attenuated form of the fungus (2.65 ± 1.82). Moreover, no fungal c.f.u. were observed in the kidneys of mice infected 8 days before with the attenuated form of the fungus, whereas c.f.u. were still detected in the kidneys of mice infected with the pathogenic *C. albicans* (2.93 ± 1.68). No fungal c.f.u. were observed in the kidneys of the latter group of mice on day 15 after infection.

**Resistance to systemic candidiasis is induced in BALB/c mice primed with a pathogenic small fungal inoculum**

To evaluate the importance of fungal pathogenicity on priming of mice with *Candida* blastoconidia, we infected BALB/c mice with 10⁷ pathogenic *Candida* blastoconidia 1 month after the animals were primed with 10⁶ pathogenic, attenuated or heat-killed blastoconidia.

As shown in Fig. 1(a), mice primed i.p. 1 month before with 10⁶ heat-killed or attenuated *C. albicans* blastoconidia were as heavily infected as control (non-primed) mice, 5 days after i.p. infection with the large inoculum of pathogenic *Candida* blastoconidia. In contrast, the numbers of *C. albicans* c.f.u. recovered from the kidneys were markedly reduced in all mice primed with the small inoculum of pathogenic *Candida* blastoconidia. As depicted in Fig. 1(b), all control mice or mice primed with heat-killed or attenuated *Candida* blastoconidia were colonized to a similar extent or even more colonized at 15 days after infection than 5 days after the infection. In contrast, all mice primed with the pathogenic inoculum had cleared the fungus 15 days after infection (Fig. 1b).

**Immunoprotection against *C. albicans* infection is associated with a high ratio between serum anti-p43 and anti-Cs antibodies**

To investigate the role of antibodies in the immunoprotection against systemic candidiasis, the titres of serum anti-p43 and anti-Cs antibodies were quantified in mice sera by ELISA. The results are shown in Fig. 1(a). The titres of antibodies differed between groups I and IV. In group I, mice immunized with pathogenic *C. albicans* blastoconidia, the titres of antibodies were significantly higher than in group IV mice primed with 10⁷ heat-killed *C. albicans* blastoconidia.

**Fig. 1.** Comparison of *C. albicans* c.f.u. recovered from both kidneys of BALB/c mice (a) 5 and (b) 15 days after fungal infection with 10⁷ pathogenic *Candida* blastoconidia of control (I) and of mice primed with 10⁶ *C. albicans* heat-killed (II), attenuated (III) and pathogenic (IV) blastoconidia 30 days before fungal infection. Results are arithmetic means and the SD of six mice per group analysed individually. The differences between group I and group IV mice were statistically significant (*P*<0.05) but the differences between group I, II and III mice were not statistically significant (*P*>0.1). This figure and Fig. 2 show results of one of three representative independent experiments.
antibodies against p43 or against Cs were determined in the control and the different groups of primed mice before or after infection. The ELISA titres of serum IgG antibodies specific for p43 in control mice or mice primed with heat-killed or attenuated Candida blastoconidia were markedly lower than those observed in mice primed with the small inoculum of pathogenic C. albicans before being challenged with a large fungal infection (Fig. 2a). The titres of anti-Cs were also higher in mice primed with pathogenic Candida blastoconidia than in mice primed with heat-killed or attenuated C. albicans that presumably contained as many fungal structural antigens as the pathogenic fungus. Nevertheless, the ratio between the anti-p43 and anti-Cs antibody titres observed in the mice primed with pathogenic C. albicans was roughly 10 times higher than in the remaining groups of mice (3 as compared to 0-3) (Fig. 2a).

As depicted in Fig. 2(b), the ELISA titres of serum antibodies of both anti-p43 and anti-Cs still increased 15 days after mice primed with pathogenic C. albicans were infected with the larger fungal inoculum. This enhancement in the titres of anti-p43 and anti-Cs antibodies was at the same rate of proportionality before and after 15 days of infection. The absolute titres of anti-Cs antibodies increased considerably in the remaining groups of mice (controls and animals primed with heat-killed or attenuated C. albicans) 15 days after infection with the larger fungal inoculum (Fig. 2b). In contrast, the titres of anti-p43 antibodies were only slightly changed after infection in the same groups of mice, the ratio between anti-p43 and anti-Cs being reduced from 0·3 to 0·1 (Fig. 2b). Therefore, the ratio between the titres of anti-p43 and anti-Cs antibodies observed in the immunoprotected mice was roughly 30 times larger than the one observed in the non-immunoprotected mice after C. albicans infection (Fig. 2b).

**Passive administration of anti-p43 but not of anti-Cs antibodies induces resistance to systemic fungal infection**

To further analyse the role of antibodies against p43 in the immunoprotection against systemic candidiasis, the fungal load was evaluated in BALB/c mice that passively received anti-p43, anti-Cs or unrelated antibodies 30 days after priming with heat-killed Candida blastoconidia. The fungal load was markedly decreased in the group of mice that received IgF containing anti-p43 antibodies (1·2 × 10^5 c.f.u. in both kidneys) as compared to mice that received IgF containing anti-Cs (1·0 × 10^5 c.f.u. in both kidneys) or unrelated antibodies (between 1×10^5 and 1×10^6 c.f.u. in both kidneys). Candida cells were no longer detectable in the kidneys of half of the mice treated with IgF anti-p43 antibodies 5 days after infection with the larger fungal inoculum, whereas the fungus was only barely detectable in the remaining half of the mice of this group. In contrast, the majority of mice in each of the two groups that were treated with IgF, containing either anti-Cs or unrelated antibodies, showed a considerably higher fungal load in the kidney on the same day of observation. Even the mice of these two groups that presented a milder fungal infection showed a higher number of Candida cells in the kidneys than that found in mice treated with IgF containing anti-p43 antibodies. The differences between the mice that received the IgF containing unrelated antibodies or anti-p43 were statistically significant (P < 0·05; n = 6), whereas those between the mice that received IgF containing unrelated antibodies or anti-Cs were not (P > 0·1; n = 6).

**Immunoprotection against systemic candidiasis induced by p43 immunization is neutralized by the passive administration of anti-p43 antibodies at the same time of immunization**

To show the crucial role of anti-p43 antibodies against systemic candidiasis in mice, BALB/c mice were immunized with p43 or immunized with p43 and treated with anti-p43 antibodies in order to neutralize the production of anti-p43 antibodies. All mice immunized with p43 and treated with IgF from serum containing specific anti-p43 antibodies became susceptible to the fungal infection, whereas all mice immunized with p43 and treated with IgF from sera of unimmunized mice were immunoprotected against systemic candidiasis. Thus, the c.f.u. count from the kidneys of mice immunized with p43 and treated with IgF from serum containing specific anti-p43 antibodies was comparable to that of unimmunized mice [1×10^5 c.f.u. in both kidneys 5 days after infection with 10^7 pathogenic Candida blastoconidia; results between these two groups were not significant (P > 0·5; n = 6)]. Differences between unimmunized mice and those immunized with p43
and passively transferred with IgF with normal serum were statistically significant ($P<0.05; n=6$).

**DISCUSSION**

The present study reveals that fully efficient vaccination against systemic candidiasis is observed in mice that recovered from a systemic infection with a sublethal dose of *C. albicans* blastoconidia. This immunoprotection is correlated with high titres of anti-p43 antibodies, suggesting that the vaccination is associated with immunization against p43, the VIP secreted by the fungus during the infection. This is supported by the findings that mice primed with heat-killed *Candida* or with attenuated cells, which are non- or poor p43 producers, were not protected against infection with a lethal dose. This observation can be explained because p43 is produced by the pathogenic *C. albicans* whereas very little or no production of this VIP is produced by heat-killed or attenuated fungi (Tavares *et al.*, 1993). This inability to produce p43 is indirectly confirmed by the extremely low titres of anti-p43 antibodies found in mice primed with heat-killed or attenuated *C. albicans*.

Most likely, the higher increase in the serum antibodies against Cs observed in mice primed with a small inoculum of pathogenic *C. albicans* was due to the polyclonal B-cell non-specific effect induced by p43 (Tavares *et al.*, 1993). However, the values of the ratio between the anti-p43 and the anti-Cs antibodies seem to be more important than the absolute titres of anti-p43 antibodies to identify immunoprotection against the fungal infection. This is evident when comparing mice primed with heat-killed or attenuated *C. albicans* with control mice, since the first groups developed higher titres against p43 than the second and were equally susceptible to *C. albicans* infection. In agreement with these results, we have previously reported that specific immunization against VIPs (but not against other antigenic components of the fungus) results in impaired protection and adoptive transfer of anti-p43 antibodies whereas antibodies against Cs facilitated systemic *C. albicans* infection (Tavares *et al.*, 1995). Our results provide further evidence in support of the crucial role of anti-p43 antibodies in the protection against *C. albicans* infection. Therefore, immunoprotection against candidiasis can be induced in mice primed with an inoculum of heat-killed fungus by treating the mice with anti-p43 antibodies, whereas treatments with anti-Cs or irrelevant antibodies are not protective against the infection. Also, the resistance to systemic candidiasis induced by the active immunization with p43 was abrogated by the neutralization of anti-p43 antibody production.

The role of the humoral immune response in resistance to systemic candidiasis is controversial. In clinical systemic candidiasis, the titres of serum antibodies detected are mainly against Cs, and specific antibodies against *C. albicans* antigens have been described as non-protective (Elin *et al.*, 1974; Hector *et al.*, 1982; Mahanty *et al.*, 1988; Reitnauer *et al.*, 1986). Moreover, we have previously observed (Tavares *et al.*, 1995) in experimental systemic candidiasis that passive administration of high titres of anti-Cs antibodies markedly increased the susceptibility to *C. albicans* infection. Also, as it is well known, high levels of antibody anti-micro-organism epitopes facilitating micro-bial growth may reflect, in some cases, easier opsonization of the micro-organism in the target phagocytic cells. However, several studies on experimental candidiasis have shown that certain antibodies might be protective (Casadevall, 1995; Casadavell *et al.*, 1998; Mathews & Burnie, 1996). It has also been described that immunization of mice with *C. albicans* surface mannan complexes or with mannan–protein conjugates induced a protective antibody response that could be passively transferred to naïve animals (Han & Cutler, 1995, 1997; Han *et al.*, 1998, 2000). It is well known that mannan adheres responsible for the attachment of *C. albicans* to the host cells are an important factor in virulence; thus, antibodies specific for the adhesin fraction could be responsible for the protection. We demonstrated, some years ago, that p43 also was an important virulence factor for *C. albicans* by immunomodulation of the host immune system (Tavares *et al.*, 1993). Furthermore, a close relationship between the biological effects of p43 and the pathogenicity of *C. albicans* was reported (Tavares *et al.*, 2000). In this study, we have demonstrated that the clearance of systemic candidiasis seems to be associated with the development of an immune response against p43, which elicits an effector instead of a suppressive response to the fungus. Immunization against microbial VIPs may also explain the immunoprotection that occurs after the spontaneous healing of an infection with the pathogenic fungus.

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