Neutrophil depletion increases susceptibility to systemic and vaginal candidiasis in mice, and reveals differences between brain and kidney in mechanisms of host resistance

Alma Fulurija, Robert B. Ashman and John M. Papadimitriou

Infections caused by the yeast Candida albicans represent an increasing threat to debilitated and immunosuppressed patients, and neutropenia is an important risk factor. Monoclonal antibody depletion of neutrophils in mice was used to study the role of these cells in host resistance. Ablation of neutrophils increased susceptibility to both systemic and vaginal challenge. The fungal burden in the kidney increased threefold on day 1, and 100-fold on day 4, and infection was associated with extensive tissue destruction. However, a striking feature of the disseminated disease in neutrophil-depleted animals was the altered pattern of organ involvement. The brain, which is one of the primary target organs in normal mice, was little affected. There was a threefold increase in the number of organisms recovered from the brains of neutrophil-depleted mice on day 4 after infection, but detectable abscesses were rare. In contrast, the heart, which in normal mice shows only minor lesions, developed severe tissue damage following neutrophil depletion. Mice deficient in \( \text{C5} \) demonstrated both qualitative and quantitative increases in the severity of infection after neutrophil depletion when compared with \( \text{C5} \)-sufficient strains. The results are interpreted as reflecting organ-specific differences in the mechanisms of host resistance.

Keywords: Candida albicans, inflammation, neutrophils, complement

INTRODUCTION

Systemic infections with the yeast Candida albicans have increased by more than 500% during the past decade (Pfaller, 1995), and represent an increasingly serious clinical problem. Disseminated candidiasis occurs most commonly in patients whose bodily defences have been compromised by cancer, by major surgery, or by treatment with cytotoxic or immunosuppressive drugs, but important predisposing factors are dysfunctional neutrophils (Kim et al., 1969) or neutropenia (Bodey, 1966; Lehrer & Cline, 1971). Systemic infection usually occurs by introduction of the yeast directly into the circulation, from whence it can lodge in all major tissues and organs of the body (Odds, 1988). However, the brain and the kidney are the sites in which infection is most frequently established.

Host resistance to infection with C. albicans involves a number of effector mechanisms, acting at different levels (Ashman & Papadimitriou, 1995). The relative contributions of specific and non-specific factors have been explored most extensively in mouse models of the infection, which reproduce closely in nature and severity the lesions seen in the human disease (Papadimitriou & Ashman, 1986). Neutrophils are an important component of the host response to systemic C. albicans infections in mice (Hurtrel et al., 1980b; Jensen et al., 1994; van ‘t Wout et al., 1988; Vecchiarelli et al., 1989), and their recruitment to the site of infection is mediated by chemotactic factors produced by the activation of complement. A/J mice, which are genetically deficient in C5, are highly susceptible to lethal challenge with the yeast (Hector et al., 1982); however, this susceptibility is associated with a significant increase in the fungal burden.
in the kidney in C5-deficient strains (Ashman et al., 1996), resulting in death from acute pyelonephritis. Tissue damage in other organs, such as the brain, is not markedly affected by the presence or absence of C5 (Ashman et al., 1993), although careful examination of brain lesions reveals a significant reduction in the recruitment of inflammatory cells to the site.

Vaginal infections are not readily established in mice, and the mechanisms of recovery are poorly understood (Ashman & Papadimitriou, 1995). In humans, chronic mucocutaneous infections are associated with deficiencies in cell-mediated immune responses (Kirkpatrick et al., 1971), but an analysis of host resistance to vaginal candidiasis in oestrogen-prepared mice failed to provide evidence of a role for either CD4+ or CD8+ lymphocytes in recovery from primary infection (Fidel et al., 1995). Nevertheless, the predominance of neutrophils and T lymphocytes in the vaginal epithelium (Nandi & Allison, 1993) suggests that both of these cell types play a part in protection of the mucosal epithelium from infection with the yeast.

The observation that there were differences in the susceptibility of various organs within a single mouse strain, as well as between strains, prompted an examination of the contribution of neutrophils and complement C5 to the susceptibility and resistance of various anatomical regions to infection by C. albicans. This paper describes quantitative and qualitative changes in patterns of fungal growth and tissue damage after neutrophil depletion in C5-sufficient and C5-deficient mice.

**METHODS**

**Mice.** Mice of four inbred strains, A/J, AKR, BALB/c and CBA/CaH, were purchased from the Animal Resources Centre, Perth, Australia. The animals are bred under specific-pathogen-free conditions and do not carry C. albicans in the gut. Only female mice, 6–8 weeks old, were used in experiments. All animals were housed and used in accordance with the NH & MRC/CSIRO/Australian Agricultural Council's Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia, 1985. The experimental protocols were approved by the University of Western Australia Animal Ethics Committee, and in accordance with its guidelines, the minimum number of mice necessary to obtain statistically valid results have been used.

**Yeast.** C. albicans 3630 is a clinical isolate that was obtained from the Mycology Reference Laboratory at the Royal North Shore Hospital, Sydney. It has been used for more than 10 years in this laboratory, and the responses of inbred mouse strains to infection with this isolate have been well characterized (Ashman & Papadimitriou, 1989). The stock is kept in small aliquots at −70°C in 15% (v/v) glycerol in Sabouraud broth. Prior to use, a loop of the frozen stock was inoculated into Sabouraud broth, and grown for 2 d at room temperature, with constant agitation. The yeast cells were washed in PBS (0.145 M NaCl; 0.007 M Na2HPO4, 0.003 M NaH2PO4, H2O) and adjusted to the appropriate concentration for use.

**Infection.** Mice were infected with viable yeasts prepared as above. The inocula were administered either intravenously (i.v.) via the lateral tail vein, or intravaginally. For the latter procedure, 5 × 10³ yeast cells in 25 μl PBS were placed directly into the vagina using a sterile disposable plastic pipette tip. The concentrations of the yeast suspensions were routinely checked by plating samples on Sabouraud agar.

**Immunization.** BALB/c and CBA/CaH mice were injected i.v. with 1 × 10⁶ C. albicans yeast cells in PBS, and rested for 6–8 weeks before use.

**Histology.** Tissue samples were taken at various times after infection, fixed in formalin, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin, or by the periodic acid/Schiff (PAS) reaction.

**Candida clearance.** Mice were killed at various times after infection, and tissues samples were homogenized in 1 ml PBS using an Ultra Turrax T-25 homogenizer (IKB Labotechnik) running at 15 300 r.p.m. at room temperature. The samples were diluted appropriately, and 100 μl samples were plated in duplicate on Sabouraud agar containing chloramphenicol. The plates were incubated at 37°C for 2 d, and the colonies were counted. The data represent the mean counts g⁻¹ from duplicate assays using a minimum of five mice.

**Preparation of neutrophil-specific antibody.** A cell line (RB6-8C5) that secretes a monoclonal antibody specific for mouse neutrophils (obtained courtesy of Dr. R. Coffman, DNA Institute, Palo Alto, CA, USA) was grown in RPMI-1640 tissue culture medium supplemented with 10% (v/v) foetal calf serum. The antibody was prepared as described previously (Fulurija et al., 1996). Nude rats were primed with 1 ml pristane intraperitoneally (i.p.), and injected with 1 × 10⁷ tumour cells i.p. 14 d later. Ascitic fluid was collected from the peritoneal cavity, pooled, centrifuged at 3000 g for 30 min, and concentrated by precipitation with saturated ammonium sulphate, followed by dialysis against PBS. Complete depletion of polymorphonuclear leucocytes, as determined by blood smears taken 1 and 4 d after treatment, was obtained by i.p. administration of two equal doses of 500 μg of the antibody preparation at 48 h intervals.

**Neutrophil depletion.** Mice were depleted of neutrophils as described above. Control mice received a similar course of injections of isotype-matched immunoglobulin (Sigma; no. 54131) or PBS. Mice were challenged with 1 × 10⁶ yeast cells 6 h
after the second injection of antibody. The histological appearance of the tissue, and the number of fungal units present, were assessed at 1 and 4 d after infection. There were no differences between immunoglobulin-treated and saline-treated control mice in the histological appearance of the lesions, or the number of colonies recovered from infected organs.

Statistics. The data were analysed using one-way analysis of variance, or Student's t-test. Differences were considered significant at a level of $P < 0.05$.

**RESULTS**

Tissue damage after neutrophil depletion was evaluated in four inbred strains: A/J, AKR, BALB/c and CBA/CaH. Some characteristics of these strains, and their responses to *C. albicans*, are summarized in Table 1. Preliminary experiments had shown that neutrophil-depleted mice infected with $1 \times 10^5$ *C. albicans* i.v. died within 48 h, so the challenge was reduced to $1 \times 10^4$ yeast cells, adminis-
tered 6 h after the final injection of the RB6-8C5 antibody. At this dose, no mice of any strain died during the course of the experiment. Control mice showed evidence of some mild damage in the hearts and kidneys (Fig. 1), but only minor differences were detected between the C5-sufficient and C5-deficient groups.

**Histology of systemic infection**

Depletion of neutrophils prior to systemic challenge with $1 \times 10^4$ *C. albicans* yeasts resulted in the development of severe lesions, with numerous large areas of necrosis, in both the kidney and heart of all four mouse strains. By the
fourth day of infection, tissue damage was pronounced, and histological evaluation was easily performed at that time point. A comparison of the four strains showed that tissue destruction was most extensive in the C5-deficient AKR and A/J mice strains. The latter displayed severe pyelonephritis and myocarditis (Fig. 1), and developed foci of fungal invasion in the liver (Fig. 2a), spleen (Fig. 2b) and lung (Fig. 3). Lesions in these three sites were not seen in the C5-sufficient strains. The inflammatory infiltrate in the lesions of the neutrophil-depleted mice...
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**Day 4 - Brain**

- A/J
- AKR
- BALB/c
- CBA

**Day 4 - Kidney**

- A/J
- AKR
- BALB/c
- CBA

**Day 1 - Brain**

- A/J
- AKR
- BALB/c
- CBA

**Day 1 - Kidney**

- A/J
- AKR
- BALB/c
- CBA

Mouse strain

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Consisted predominantly of mononuclear phagocytes and lymphocytes. As expected, very few polymorphonuclear leucocytes were identified.

In contrast to its effect in kidney and heart, neutrophil depletion did not alter the severity of brain involvement (Fig. 4). After infection with $3 \times 10^9$ C. albicans yeasts, CBA/CaH and AKR mice developed severe lesions in the brain (Table 1; Fig. 4a), but few lesions were detected at the very low challenge dose ($1 \times 10^4$ yeasts) used in these experiments (Fig. 4b), and there were no obvious differences between any of the four strains in the severity of cerebral damage (data not shown). Ablation of neutrophils did not cause any substantial change in the severity of the cerebral lesions. Only a few scattered areas of necrosis, with varying numbers of inflammatory cells and small aggregates of yeasts and mycelia, were found (Fig. 4c).

**Quantification of infection**

Significantly more yeasts were recovered from the kidney of the C5-deficient control mice when compared with C5-sufficient animals (Fig. 5), but the presence or absence of C5 did not influence the severity of infection in the brain. Neutrophil depletion resulted in a substantial and highly significant ($P < 0.01$) increase in the fungal burden in the kidney of all four mouse strains, but this effect was less evident, although still statistically significant ($P < 0.05$), in the brain. A deficiency of C5 in neutrophil-depleted mice was also associated with a significant ($P < 0.05$) increase in the number of fungal units present in the kidney, but not in the brain.

**Vaginal infection**

Severe infections became established after vaginal inoculation of C. albicans in neutrophil-depleted mice (Fig. 6). Many mycelia invaded the cornified epithelium, and occasionally penetrated the keratinocytic layer. There were no differences between C5-deficient and C5-sufficient mice in the severity of vaginal infections.

**Effect of immunization**

Mice that have recovered from an initial infection with C. albicans develop protective antibody responses. These are detected more readily in strains such as CBA/CaH, which develop severe lesions, than in strains such as BALB/c, which show milder tissue damage (Ashman & Papadimitriou, 1987). Immunized BALB/c and CBA/CaH mice were depleted of neutrophils, challenged with $1 \times 10^4$ yeasts i.v., and the course of infection was monitored in brain and kidney. No lesions were seen in the tissues of either the neutrophil-depleted or control mice (data not shown). Quantitation confirmed that the levels of organ infection in immune animals were much reduced in comparison to normal mice (Table 2), and that neutrophil depletion had no effect on the fungal burden in the tissues.
DISCUSSION

Neutrophils are an important component of the inflammatory response, and are the first cells recruited to the site of injury or infection. However, these present data show that their contributions to host resistance against *C. albicans* are related to the anatomical region in which infection has occurred. Thus, the kidney and the heart show a dramatic increase in the severity of tissue damage in neutrophil-depleted mice, whereas tissue damage in the brain is not significantly different from that in the control animals.

The failure of neutrophil depletion to exacerbate tissue damage in the brain is not related to a more efficient clearance of the smaller inoculum, as the fungal burden increases substantially between days 1 and 4 in both neutrophil-depleted mice and in controls. This similarity in the growth curves suggests that clearance of *Candida* yeasts from the brain is mediated predominantly by
neutrophil-independent effector mechanisms. The brain possesses an extensive population of mononuclear phagocytes (microglialcytes), and cell lines of microglial origin have been shown to kill \textit{C. albicans} more efficiently than cells from other anatomical regions (Blasi \textit{et al.}, 1994). However, unlike macrophages derived from other tissues, microglial cells do not produce tumour necrosis factor (TNF) after activation with either yeast or hyphal growth forms. TNF is important in the activation of neutrophils for \textit{Candida} killing (Ferrante, 1989), but also causes a range of deleterious effects (Remick & Kunkel, 1989). It has been suggested (Blasi \textit{et al.}, 1994) that the production of TNF is under much tighter regulation in the brain than in other tissues, so that activation of neutrophils may be limited, leading to a reduction in their candidacidal activity and functional significance at this site.

Inbred strains show a clear dichotomy in the number and severity of lesions that develop in the brain after systemic infection with \textit{C. albicans}. For example, CBA/CaH mice develop numerous large abscesses, whereas in BALB/c, the lesions are small and infrequent (Ashman & Papadimitriou, 1987). The factors that contribute to the difference in tissue damage between the strains have not yet been identified, but they are not linked to the major histocompatibility complex (Ashman & Papadimitriou, 1989), are independent of the function of T lymphocytes (A. Fulurija and others, unpublished data), and are most probably related to genetically determined differences in the effector functions of mononuclear phagocytes or granulocytes.

The candidacidal activity of mononuclear phagocytes \textit{in vitro} is influenced by the \textit{Bcg} locus (Puliti \textit{et al.}, 1995), which regulates susceptibility and resistance to \textit{Leishmania} and some other bacterial infections (Vidal \textit{et al.}, 1995). However, the strain distribution of mild and severe tissue damage in \textit{C. albicans} infection (Ashman \textit{et al.}, 1993) is not consistent with the involvement of this locus. The role of neutrophils in determining strain differences in the severity of tissue damage could not be definitively established, as neutrophil-depleted mice given challenge doses that discriminated between control mice of the different strains died before the brain lesions developed to a point at which comparative assessment was possible. Nevertheless, it is clear that neutrophil depletion does not exacerbate cerebral lesions, and this leaves open the possibility that the severity of tissue damage in the brains of inbred mice after systemic challenge may be associated with differences in the numbers or functions of neutrophils recruited to the site of infection.

In the kidney, phagocytic effector mechanisms are capable of eliminating a large proportion of \textit{C. albicans} yeasts within 5 h after systemic infection (Baghian & Lee, 1991). However, lesions continue to develop, and the fungal burden increases, reaching a peak at about day 4 after infection (Ashman \textit{et al.}, 1996). There is evidence that the ability of the kidney to limit growth of the yeast is the major determinant of mortality after infection (Hurtrel \textit{et al.}, 1980a), and that this is linked to the presence or absence of C5 in any particular mouse strain (Ashman \textit{et al.}, 1996). However, the role of C5 in \textit{C. albicans} infection has not been definitively established. It does not act as an opsonin, but the degradation product C5a is a potent chemotactic factor for neutrophils and circulating monocytes. The increased severity of infection after neutrophil depletion in the kidney of C5-deficient, as compared to C5-sufficient strains of mice, suggests that C5-mediated recruitment of mononuclear phagocytes (Okamoto \textit{et al.}, 1992) may represent a significant component of the kidney’s defence mechanisms.

Although the depletion experiments provide convincing evidence that neutrophils and other phagocytic cells are essential for control of fungal proliferation in the kidney,
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A reduced recruitment of inflammatory cells to the kidney may not account completely for the pattern of early mortality after intravenous challenge in C5-deficient mice. A possible explanation is suggested by the observation that TNF mRNA expression and bioactivity is enhanced in macrophages incubated in C5-sufficient mouse serum before activation (Barton & Warren, 1993). These workers also found that C5-deficient mice challenged with lipopolysaccharide developed lower serum levels of TNF than controls. As high levels of TNF are associated with increased vascular permeability, the lower levels present in C5-deficient mice may be associated with a reduction in the passage of yeasts into the renal parenchyma. The resulting increase in growth within the tubules would quickly impede kidney function, leading to the gross oedema, severe pyelonephritis and early death documented in these strains.

The contribution of neutrophils to the resistance of different tissues and organs is not related simply to the anatomical region in which infection is established. Neutrophil depletion markedly alters organ susceptibility to listeriosis (Conlan & North, 1994), but the patterns of organ susceptibility are very different, in that infection is exacerbated in the liver, but not in the spleen or peritoneal cavity. In *C. albicans* infection, neutrophil depletion dramatically decreased the resistance of the heart, which contains few resident phagocytes, and in C5-deficient mice, caused the development in the liver, lungs and spleen, of lesions that were not seen in C5-sufficient strains. It is interesting that lesions in the lung and liver were also observed after intragastric inoculation of the yeast into mice immunocompromised by administration of both cyclophosphamide and cortisone acetate (Cole *et al.*, 1989). The latter drug was found to exert a profound inhibitory effect on the candidicidal activities of pulmonary alveolar macrophages *in vitro* (Sawyer & Harmsen, 1989), and further experiments showed that mice treated with cortisone acetate were able to clear some, but not all, of a pulmonary challenge. The implication is that these organs, despite possessing large populations of resident phagocytic cells, are dependent on an influx of circulating monocytes to achieve rapid, early control of *C. albicans* infection, and that this recruitment involves the chemotactic activities of C5 and its degradation products.

There was a substantial increase in *C. albicans*, predominantly of the mycelial growth form, in the vagina of neutrophil-depleted mice. Depletion of neutrophils increases susceptibility to orogastic candidiasis and disseminated candidiasis of endogenous origin (Jensen *et al.*, 1993), but although some hyphae had penetrated through the cornified epithelium of the vagina in the present experiments, there was no evidence of extensive invasion of the deeper tissues, nor were there detectable differences between strains in the severity of the mucosal lesions. Neutrophils represent a major component of the cells resident in the vagina (Nandi & Allison, 1993), and the presence of some neutrophils in the inflammatory infiltrate suggests that depletion was not absolute in this anatomical region. It is unclear at present whether the limited dissemination of the infection was associated with the activity of residual neutrophils, or whether there is a greater emphasis on the actions of other cell populations, such as the γ/δ subset of T lymphocytes (Jones-Carbon *et al.*, 1995), in protecting the vaginal mucosa from invasion by the yeast.

Recovery from systemic infection with *C. albicans* in mice induces an antibody response that protects the animals against further challenge (Ashman & Papadimitriou, 1988). Protection is independent of the isolate used for immunization (R. B. Ashman, unpublished data), but the protective effect is more readily detected in mice, such as CBA/CaH, which develop severe lesions (Ashman & Papadimitriou, 1993). Depletion of neutrophils in these immune mice had no effect either on the numbers of viable yeast cells in the tissues, or on the severity of tissue damage. It appears that opsonization by *Candida*-specific antibody and phagocytosis by macrophages in the liver and lungs provided efficient protection against the relatively small challenge dose used in these experiments, and masked or prevented the expression of organ-specific differences in susceptibility after neutrophil depletion.

In conclusion, the results demonstrate substantial differences between organs in the contribution of neutrophils to protection against *C. albicans*, and suggest that these cells may also be involved in the expression of genetically controlled differences in the severity of tissue damage in inbred strains of mice.

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