Acute susceptibility of aged mice to infection with Candida albicans

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The effect of aging on host resistance to systemic candidosis was assessed by monitoring the course of infection in 16-month-old CBA/CaH mice (aged non-immune) and in a comparable group that had been infected with a sublethal dose of Candida albicans at 6 weeks of age (aged immune). Aged non-immune mice showed rapid progression of the disease, with a marked increase in the number of mycelia in the brain and kidney, and early morbidity. Foci of myocardial necrosis were evident, but inflammatory cells were sparse. The histological picture in the aged immune mice was similar to that in the aged non-immune group, although fewer mycelial aggregates were seen. Both groups of aged mice showed a significantly lower fungal burden in the brain on day 1 of infection, but on day 4, colony counts increased significantly in the aged non-immune mice. Comparison of cytokine gene expression in the infected brains showed that the relative amount of interferon-γ and tumour necrosis factor-α cDNA were similar in all three groups. Interleukin-6 was elevated in both infected non-immune and uninfected aged mice. Aged immune mice showed no morbidity after challenge, and both colonisation and tissue damage were reduced in comparison with the aged non-immune animals.

Introduction

Immunocompetence declines with advancing age [1] and this is associated with a general increase in susceptibility to infection. Epidemiological investigations have shown that the elderly are at higher risk for pneumococcal and other respiratory infections [2], and experimental studies have demonstrated an increased susceptibility of aging mice to organisms as diverse as Leishmania major [3], Trypanosoma musculi [4], Legionella pneumophila [5] and Sendai virus [6].

Candida albicans is an opportunistic yeast that is carried as a commensal by the majority of the human population. Mucosal candidosis (oral and vaginal thrush) is common in the general population, although chronic infections usually occur in conjunction with deficiencies or abnormalities in the cell-mediated immune response [7, 8]. Systemic, or disseminated candidosis is rare outside the hospital environment and is typically associated with dysfunctional neutrophils [9] or neutropenia [10, 11]. Oral infections with C. albicans are common in the elderly [12], but although advanced age increases susceptibility of mice to intravenous challenge with Cryptococcus neoformans [13], it is not a recognised factor for disseminated candidosis.

This paper reports the effect of aging in CBA/CaH mice on susceptibility to systemic challenge with C. albicans and the effect of immunity induced as a consequence of infection with the yeast early in adult life on the severity of infection induced by re-challenge in aged animals.

Materials and methods

Mice

Specific-pathogen-free female CBA/CaH mice were purchased from the Animal Resources Centre, Perth, Australia. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and performed in accordance with the NH&MRC/CSIRO/Australian Agricultural Council’s Code of Practice for the Care

Yeast preparation

*C. albicans* isolate 3630 was obtained from the Mycology Reference Laboratory at the Royal North Shore Hospital, Sydney, Australia, and stored at −70°C in Sabouraud’s broth with glycerol 15%. Yeasts were grown in Sabouraud’s broth for 2 days at room temperature, with constant agitation. Blastospores were washed in phosphate-buffered saline (PBS) and adjusted to the appropriate concentration for inoculation.

Infection and immunisation

Mice were immunised with 3 × 10⁵ blastoconidia administered in 0.2 ml of PBS via the lateral tail vein at 6 weeks of age. The same yeast isolate, dose and route of administration were used both for infection of the aged non-immune mice and re-challenge of the aged immune animals.

Histology

Tissue samples were fixed in formalin, sectioned and stained with haematoxylin and eosin (H&E), or periodic acid-Schiff (PAS). The sections were coded, examined blind and re-evaluated when the code had been broken.

Clearance of *C. albicans*

Mice were killed at 1, 4, 8 and 14 days after infection. A transverse slice 2–3 mm thick was cut from the central portion of the brain and placed in 1 ml of PBS. Additional brain tissue was stored in liquid nitrogen for mRNA extraction and analysis of cytokine gene expression. Each tissue sample was weighed and disrupted with an Ultra Turrax T-25 homogeniser (IKA Labortechnik, Staufen, Germany) running at 13 500 rpm at room temperature. The samples were diluted appropriately and 100-µl volumes were plated on Sabouraud’s agar containing chloramphenicol. The plates were incubated at 37°C for 2 days and the colonies were counted. Each determination was performed in duplicate in a minimum of five mice. The results were calculated as log₁₀ cfu/g of tissue.

RNA preparation and reverse transcription

Total cellular RNA was prepared from infected brain tissue with ‘Ultraspec’ RNA Isolation Reagent (Biotex Laboratories, Houston, TX, USA) according to the manufacturer’s instructions. The concentration and purity of the RNA samples were determined by spectrophotometry at 260 and 280 nm. cDNA was prepared by reverse transcription of 2 µg of each RNA, with an oligo d(T)$_{15}$ primer and AMV reverse transcriptase, according to the manufacturer’s instructions (Promega Corporation, Madison, WI, USA). Briefly, 5 mM MgCl$_2$, 1 × reverse transcription buffer, 1 mM each dNTP, 0.5 U RNAasin, 15 UAMV reverse transcriptase and 0.5 µg oligo (dT)$_{15}$ primer were incubated in a 20-µl reaction mix at 42°C for 1 h, heated to 99°C for 5 min, then cooled on ice. The cDNA was stored at −20°C until used.

PCR

Primer sequences for interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) were obtained from published data [14], and the PCR was performed as described previously [15]. The amplification mix consisted of 50 ng DNA, 200 mM dNTPs, 0.5 U Taq polymerase (Biotech International, Perth, Western Australia), 1× reaction buffer, either 1 mM or 2 mM MgCl$_2$ and either 1 ml or 2 ml of primers in a total volume of 25 µl. The mixture was overlaid with paraffin oil, then amplified with a PTC-100 thermal cycler (MJ Research, Waltham, MA, USA). The amplification protocol was 94°C, 1 min; 60°C, 2 min; 72°C, 2 min; for 35–40 cycles. Following amplification, 10 µl of product were analysed by electrophoresis through agarose 3% gels. The gels were stained with ethidium bromide and the bands were visualised with a UV transilluminator.

Competitive PCR for comparison of cytokine cDNA

Plasmid MCQ (pMCQ) was generously donated by Dr Cornelia Platzer, Humboldt University, Berlin, Germany. This plasmid (control fragment) contains the primer sequences for interleukins 1–6, IFN-γ, TNF, lymphotoxin and β-actin, arranged so that the products amplified by cytokine-specific primers differ in size from those amplified from the target DNA, and thus can be distinguished by electrophoresis through an agarose gel [16]. Samples showing reactivity with cytokine primers were subjected to semi-quantitative analysis as described previously [15]. Briefly, the cDNA was adjusted to equal concentrations by co-amplification of a fixed amount of the cDNA with 10-fold, and then two-fold, dilutions of the control fragment (pMCQ), with primers for β-actin. For quantification and comparison of cytokine mRNA expression, equal amounts of cDNA were amplified in the presence of 10-fold and then three-fold dilutions of the control fragment. The linearised plasmid was used at dilutions ranging from 10⁵ to 10⁷ for the standardisation of the cDNA and from 10⁵ to 10⁶ for comparison of cytokine concentrations. The experiments were repeated three times. The end-points for the aged mice, expressed in logarithms, were subtracted from those for the young controls, and the resultant value gave a factor by which the cDNA concentrations in the aged mice were greater or less than those in the controls. A positive value indicated that the concentra-
tions in the aged mice were greater than in the young animals and vice versa.

Statistical analysis

The statistical significance of differences between groups in the number of yeast cells recovered from infected brains was determined by Scheffe’s test on the one-way analysis of variance. Cytokines were compared as follows. The mean and standard deviation of the factors by which the aged mice differed from the controls were calculated for each data set, and the hypothesis that the cDNA concentrations in young and aged mice were not significantly different was tested by determining whether the 95% confidence intervals included zero.

Results

Long-term effects of systemic infection

Female CBA/CaH mice infected with \(3 \times 10^5\) C. albicans yeast cells at 6–8 weeks of age and a group of uninfected, age-matched control mice were maintained in conventional animal facilities for 16 months. The mean weight (30.2 SD 6.1 g) of the aged immune mice was significantly less (\(p < 0.01\)) than that of the uninfected controls (35.2 SD 3.9 g), but they were otherwise active and healthy. Within 3 days after intravenous challenge with \(3 \times 10^5\) C. albicans yeast cells, the aged non-immune mice displayed visible signs of distress and this entire group was killed on day 4 for ethical reasons. In contrast, the old immune mice and a group of 6-week-old control mice given a similar challenge remained outwardly healthy and showed no unusual symptomatology at any time during the 14-day period of observation.

Histology of the lesions

The histological features of disseminated C. albicans infection in 6–8-week-old CBA/CaH mice were as described previously [17]. Briefly, mycelia were readily detected in the brains of the young control mice on the first day after infection. They were present to a lesser extent in the kidney, but were rarely found in the heart, although a focal myocarditis had already developed. By day 4, the encephalitis, pyelonephritis and myocarditis had increased in severity. The brain exhibited abscesses (Fig. 1A) containing predominantly yeasts, with some mycelial growth forms, the latter being present in greater numbers than on day 1. At day 14, mycelia were difficult to find. The myocarditis had abated, but the encephalitis and pyelonephritis had increased in severity.

On day 1 after infection of the aged non-immune mice, mycelia were seen in brain, kidney and heart, in numbers similar to those in the young controls. In the heart, scattered foci of myocardial necrosis were surrounded by a few inflammatory cells. By the fourth day of infection, the number of mycelia in the brain and kidney had increased markedly in comparison with those in the young mice, the brain being the most severely affected organ (Fig. 1B). Necrotic debris was seen in the vicinity of the mycelia, but only a few inflammatory cells were detected. Mycelia were not seen in the heart, but foci of myocardial necrosis, including calcified cardiac myocytes, were readily found (Fig. 2). Again, inflammatory cells were sparse.

The aged immune mice were similar to the other two groups in terms of the numbers of mycelia in the brain and kidney at 24 h after infection. By the fourth day, the mycelial population in the brain (Fig. 1C) and kidney had increased, but to a lesser extent than in the aged non-immune mice. Polymorphonuclear leucocytes (PMNLs) were easily detected at the periphery of the mycelial aggregates in the brain (Fig. 3), while in the heart they were present as focal infiltrates in the interstitium. Abscesses had formed in both brain and kidney at this time point, but the myocarditis remained unchanged. Calcified myocytes were detected, but at a much lower frequency than in the non-immune aged mice. By day 14, mycelia were only detectable in the kidney. The focal encephalitis, pyelonephritis and myocarditis were reduced in intensity compared with the aged non-immune mice, but were more severe than lesions in the young controls at the same time point.

Kinetics of fungal clearance

The fungal burden in the brain of both the aged non-immune and the aged immune mice was significantly less than in the young mice on the first day after infection (\(p < 0.01\)), but by day 4 the number of colonies recovered from the brains of aged non-immune mice was significantly greater (\(p < 0.01\)) than that in the other two groups (Fig. 4). After this time, the number of colonies recovered from the brains of the aged immune mice declined to levels similar to those in the young controls.

Comparison of cytokine cDNA profiles

It was of interest to determine whether the increased susceptibility of the aged mice was associated with a change in the relative gene expression in the infected brain of cytokines that are known to play a part in host defence against C. albicans infection. The cytokine profiles were assessed by comparison of the end-points of competitive PCR assays. The relative amounts of IFN-\(\gamma\) and TNF-\(\alpha\) cDNA in the aged non-immune mice were not different from those in the young controls (Fig. 5), but IL-6 cDNA levels were significantly higher in the aged infected mice. However, comparison of IL-6 gene expression in young and aged uninfected mice showed a 3–5-fold increase in aged animals (data not shown). There were no significant differences between aged immune mice and the young controls in the
Fig. 1. (A) Brain of a 6–8-week-old CBA/CaH mouse 4 days after i.v. challenge with $3 \times 10^5$ C. albicans yeast cells. A large abscess contains fungal elements and inflammatory infiltrates. (B) Brain of aged non-immune CBA/CaH mouse 4 days after challenge as above. There is extensive fungal proliferation, with prominent mycelial elements. Few inflammatory cells are present in the lesion. (C) Brain of aged immune CBA/CaH mouse, 4 days after challenge as above. An abscess shows yeast and mycelial forms, and a dense inflammatory infiltrate. All sections stained with periodic acid-Schiff ($\times 380$).
relative expression of IL-6, IFN-γ or TNF-α (data not shown). IL-4 was detected in the brains of all three control mice on day 4 after infection, but was not found in either the aged non-immune (Fig. 6) or aged immune mice (data not shown) at any time point.

Discussion

Systemic infection with *C. albicans* in aged mice causes acute disease and early death. The rapid onset of morbidity, within 2–3 days after challenge, suggested that the animals may have suffered some kind of septic shock, a condition that has also been associated with candidaemia in elderly patients [18]. Although there was a quantitative increase in the fungal burden in the brains of the aged non-immune mice at 4 days after challenge, the striking feature of the infection in these animals was the widespread appearance of mycelial growth forms, which are potent stimulators of TNF-α production by both macrophage cell lines [19] and macrophages obtained from different anatomical regions throughout the body [20].
Fig. 4. Number of *C. albicans* in the brains of normal (young; ○), aged non-immune ( ●), and aged immune ( ●) CBA/CaH mice, at various times after intravenous challenge with $3 \times 10^5$ yeast cells. Each bar shows the mean and SEM of $\log_{10}$ cfu/gram of tissue.

Fig. 5. Relative amounts of cytokine cDNA in the brain of aged non-immune CBA/CaH mice: IFN-γ (day 1, ○; day 4, ●), IL-6 (day 1, ○; day 4, ●), TNF-α (day 1, △; day 4, ▲). Each point represents the mean and 95% confidence intervals of the difference in cytokine cDNA concentrations between old and young animals, as estimated by competitive PCR with equivalent amounts of cDNA. mRNA was prepared separately from each of three mice per group and each determination was carried out at least twice.

TNF-α is a known mediator of septic shock syndromes [21] and accumulates to high levels in the serum of infected mice [22, 23]. Although aged mice are more susceptible than younger mice to the lethal toxicity of TNF-α [24], the relative levels of TNF-α cDNA in the brains of the aging non-immune mice were not significantly different from those in the young control group. This suggested that TNF-α was not implicated in the early morbidity and mortality of these animals. A recent study of *C. albicans* septic
Nevertheless, cytokine production and function in C. albicans are not limited to the young. Evidence for the induction of protective immunity consequent upon recovery from an initial sublethal infection is most readily demonstrated in mice of the CBA/CaH strain [38, 39]. The present data confirm that protective immunity elicited by systemic infection of young mice persists for at least 12 months, and probably for the life of the mouse. Colony counts in the brains of the aged immune mice were reduced by about 0.5 log_{10} relative to the aged non-immune group, which was similar to the effect achieved by immunisation of young animals [38]. Nevertheless, the increased susceptibility demonstrated by the aged non-immune mice was also evident in the aged immune mice when compared with the responses of younger animals. It is unclear whether this reflects an overall decline in the candidacidal potential of phagocytic effector cells, or a reduction in function of some of the antibody-dependent mechanisms that accelerate clearance of the yeast in immune mice.

In summary, these studies in the mouse provide evidence that the changes in host defence mechanisms associated with advanced age markedly increase the severity of disseminated candidiasis. It follows that the increasing prevalence of infections with Candida spp. in the hospital environment [40] may represent a threat not only to the debilitated and immunosuppressed, but also to the elderly patient.

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1102 R.B. ASHMAN, J.M. PAPADIMITRIOU AND A. FULURIJA.


