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Virulence Comparisons of *Aspergillus nidulans* Mutants Are Confounded by the Inflammatory Response of p47$^{phox-/-}$ Mice

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While investigating the requirement for phagosomal alkalinization in the host defense against pulmonary aspergillosis, we observed high morbidity of p47$^{phox-/-}$ mice infected with pH-insensitive *Aspergillus nidulans* mutants despite a paucity of fungal growth. Fatal infection also resulted from a normally avirulent *p*-aminobenzoate auxotroph. This demonstrates that p47$^{phox-/-}$ murine immunity contributes significantly to *A. nidulans* lethality. These data have wider implications for microbial virulence studies with p47$^{phox-/-}$ mice.

The elimination of invading microbes by professional phagocytes is crucial to mammalian immunity. An essential role for the phagocyte NADPH oxidase enzyme complex in this process has been well established from in vitro studies (2, 12, 13, 22) and the human condition chronic granulomatous disorder (CGD) (14, 16, 18). CGD polymorphonuclear leukocytes (PMNs) fail to alkalinize following phagocytosis (5, 15). Targeted deletion of murine p47^{phox} (6) or gp91^{phox} (11) NADPH oxidase subunits mimics CGD and results in susceptibility to *Staphylococcus aureus* (6, 11) and *Aspergillus fumigatus* (11) infection characterized by persistent granulomatous inflammation (11, 6).

We recently characterized the role of *Aspergillus nidulans* pH signal transduction in virulence, finding that mutants lacking the processed form of the pH-responsive transcription factor PacC (1, 19) have alkali-sensitive phenotypes and are dramatically attenuated in neutropenic mice (3). A plausible role for PacC during pathogenesis is the mediation of adaptation to phagolysosomal alkalinization. Such adaptation should be dispensable in CGD, as NADPH deficient PMNs have abnormally acidic phagolysosomes (5, 15).

We tested this hypothesis using p47$^{phox-/-}$ mice and *A. nidulans* pH response mutants C209 and C14 (3), which, as a consequence of *pacC* mutations, have non-alkaline-responsive and alkaline-adapted phenotypes, respectively. Murine infection regimens and histopathological analyses were as previously described (3) except that neither p47$^{phox-/-}$ nor parental 129sv mice were chemotherapeutically immunosuppressed. A 20% reduction in body weight measured from the day of infection was used as a surrogate marker of survival, at which point mice were sacrificed. This coincided with emergence of other indicators of severe infection, such as hunched posture, labored breathing, or a moribund state. Log rank analysis was performed to test the significance of virulence data, using Prism3 software. In survival comparisons ($n = 10$) following infection with 10^6 *A. nidulans* wild-type (EBPN17) (3) C14, or C209 conidiospores, neither C209 ($P = 0.53$) nor C14 ($P = 0.53$) differed significantly from the wild type (data not shown), demonstrating that alkaline adaptation is dispensable for *A. nidulans* virulence in p47$^{phox-/-}$ mice. There was 100% survival in parental 129sv mice receiving 10^6 *A. nidulans* EBPN17 conidiospores (3) and in p47$^{phox-/-}$ mice receiving saline.

We were unable to detect any differences between *A. nidulans* wild-type-, C209-, or C14-induced pulmonary histopathology at any infectious dose tested. In a time course experiment we examined two mice each with EBPN17, C209, and C14 at each of three time points: 24, 48, and 72 h. Representative samples from the 48-h time point are shown in Fig. 1. In a separate experiment using 10^4 conidiospores (in which most mice survived), we examined four mice for each of the three strains at 14 days postinfection (data not shown). In addition, we examined multiple samples from unmatched time points between 17 h and 14 days postinfection for all three strains (data not shown). For every lung studied histopathologically, we examined 32 slides, derived by quadruple sampling (for each of two staining treatments) at each of four lateral sectioning levels. At 48 h postinfection, consolidated inflammation was evident across large regions of lung tissue and mass migration of PMNs into the lung parenchyma prevented the visualization of pulmonary architecture (Fig. 1). Grocott's methanamine silver (GMS) staining revealed the presence of numerous, evenly distributed hyphal elements, some of which had branched. Throughout the study hyphal growth in vivo was independent of the genotype of the infecting strain. These findings indicate that, unlike in neutropenic murine infection (3), PacC is not required for *A. nidulans* virulence in p47$^{phox-/-}$ mice.

A. fumigatus infection of gp91$^{phox-/-}$ mice causes abnormal inflammatory provocation, even following inoculation with sterile hyphae (9). To determine the contribution of the aberrant inflammatory response (Fig. 1) to experimental outcome in our model, we examined wild-type *A. nidulans* disease progression and infection characteristics (Fig. 2). p47$^{phox-/-}$ mu-

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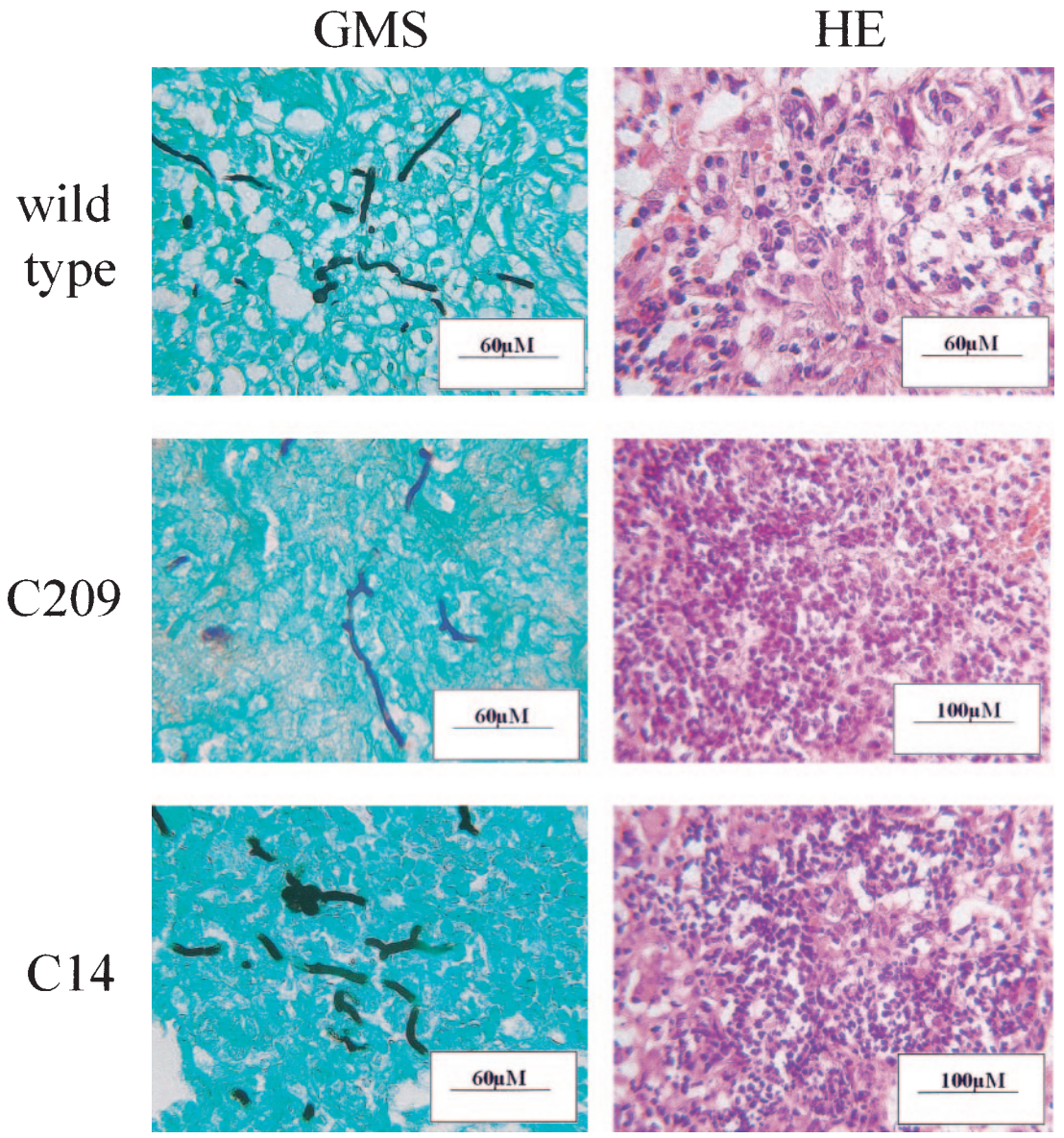

FIG. 1. *A. nidulans* pH mutants cause disease similar to that caused by the wild type in p47$^{phox-/-}$ mice. Wild-type, alkaline-nonresponsive, and alkaline-adapted *A. nidulans* strains show similar histopathological features of disease progression in p47$^{phox-/-}$ mice. Lungs were removed 48 h after infection with 10^6 *A. nidulans* conidiospores of the wild-type, alkaline-nonresponsive (C209), and alkaline-adapted (C14) strains. GMS-stained sections reveal equivalent fungal burdens, while hematoxylin-and-eosin (HE) staining shows a massive influx of PMNs.

rine weight loss and body temperature reductions ($n = 18$) were dramatic following infection with 10^5 EBPN17 (3) spores (Fig. 2A and B). Extensive bronchopneumonia characterized by large areas of neutrophil-saturated tissue was evident by 48 h postinfection, and the majority of animals in this group had been culled by day 4 (Fig. 2C). Infection with 10^4 conidiospores was less severe, with 50% of mice surviving to day 21 despite the formation of numerous granulomatous lesions consisting of neutrophils surrounded by epithelioid mononuclear cells (Fig. 2A to C). Although infection with 10^3 spores was nonfatal, pulmonary tissue granulomas formed throughout the lung parenchyma (Fig. 2A to C).

To characterize further the role of the p47$^{phox-/-}$ immune response to the experimental outcome of *A. nidulans* infection, we studied the pathogenicity of an attenuated *A. nidulans p*-aminobenzoate auxotroph, strain 302, which is unable to germinate and grow in vivo (17). To avoid possible ambiguity with a sublethal inoculum, we used an infectious dose expected to result in 100% mortality, as found for the wild-type isolate EBPN17 (3). By day 6 all mice ($n = 5$) receiving 10^6 strain 302 conidia had been culled. Histological examination of infected lungs revealed a degree of pulmonary inflammation similar to that induced by an inoculum of 10^6 wild-type, C209, or C14 conidia (Fig. 1 and 2C). However, GMS staining revealed the absence, in mice culled at 4 and 5 days postinfection, of *A. nidulans* germination (Fig. 2C), demonstrating that fatal infection in p47$^{phox-/-}$ mice is possible in the absence of fungal growth and sup-

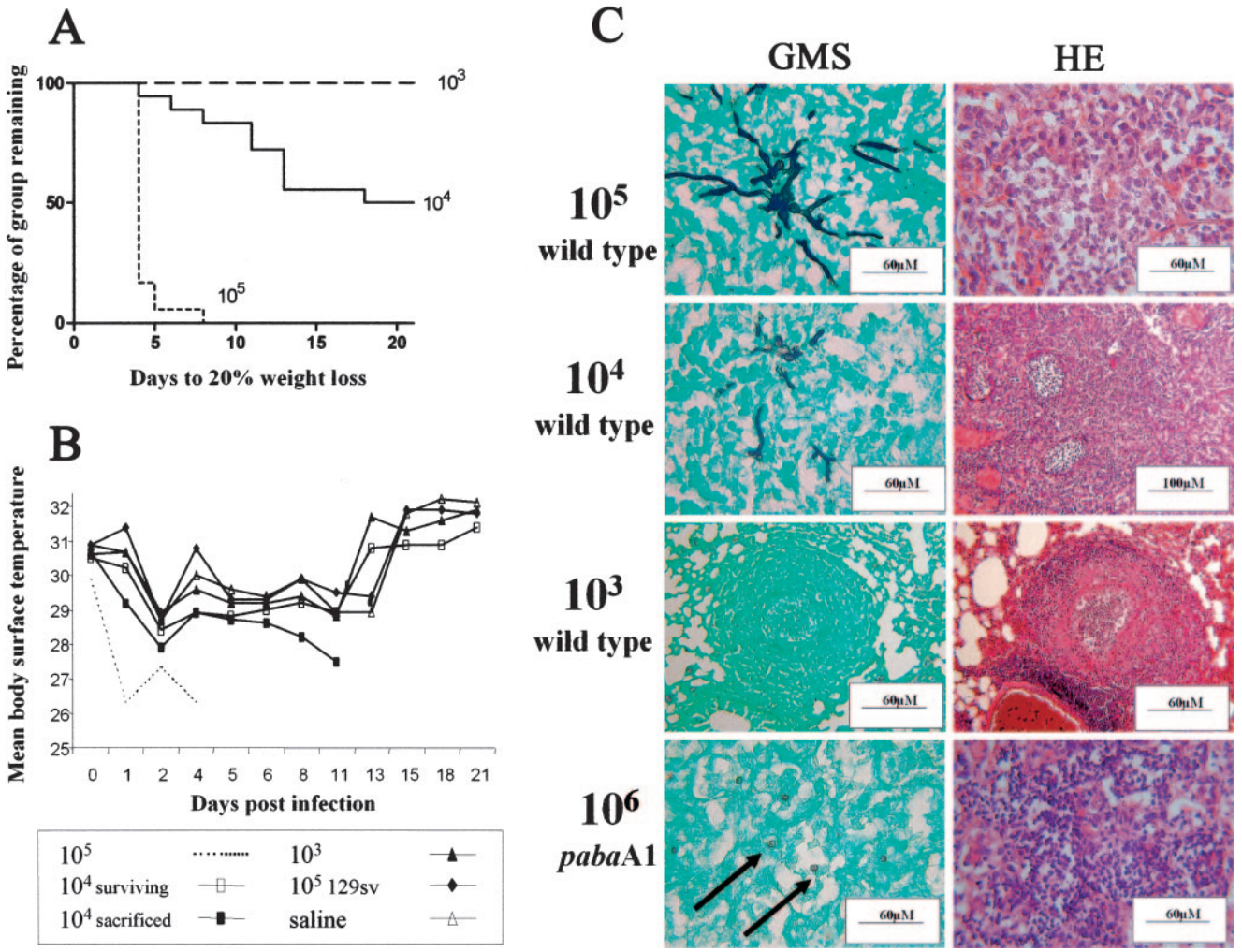

FIG. 2. Characterization of pulmonary aspergillosis in p47$^{phox-/-}$ mice. (A) Time to 20% weight loss of p47$^{phox-/-}$ mice following intranasal infection with 10^5, 10^4, or 10^3 *A. nidulans* wild-type conidiospores. (B) Mean body temperature (°C) of p47$^{phox-/-}$ mice following *A. nidulans* wild-type infection with 10^5, 10^4 (fatal infection and nonfatal infection), and 10^3 conidiospores. 129sv parental wild-type mice infected with 10^5 *A. nidulans* wild-type conidiospores and saline-inoculated p47$^{phox-/-}$ mice served as controls. (C) Histopathological analysis of pulmonary aspergillosis in p47$^{phox-/-}$ mice receiving 10^5 or 10^4 *A. nidulans* wild-type conidiospores at day 4 postinfection, 10^3 *A. nidulans* wild-type conidiospores at day 21 postinfection, or 10^6 *A. nidulans p*-aminobenzoate auxotroph 302 (*paba*A1) conidiospores at day 4 postinfection. Arrows indicate positioning of ungerminated conidiospores.

porting a role for aberrant inflammation in murine lethality in this model.

Virulence of normally attenuated mutants in NADPH oxidase-deficient mice has a precedent. Several *Salmonella enterica* serovar Typhimurium comparative virulence studies (4, 8, 10, 20, 21) have reported that NADPH oxidase-deficient mice have acute sensitivity to *S. enterica* serovar Typhimurium challenge compared to mice with fully competent respiratory burst activity. Some interpretations of such data attribute resistance to the phagocyte respiratory burst to the gene under investigation. However, our data demonstrate that, in whole-animal virulence studies, the complex inflammatory consequences of NADPH oxidase dysfunction seriously confound comparative virulence studies, which therefore require careful histopathological examination, cautious interpretation, and appropriate controls, including, where possible, an appropriately attenuated mutant. gp91$^{phox-/-}$ and p47$^{phox-/-}$ survival studies previously conducted in the absence of such controls or supporting data (such as those derived from cellular assays) may therefore require reevaluation. Infection using sublethal inocula in p47$^{phox-/-}$ mice may be one means of identifying differences in virulence or host response between strains. We were unable, at sublethal doses, to distinguish any differences between *A. nidulans* wild-type, C209, or C14 infection in p47$^{phox-/-}$ mice (data not shown).

The majority of the research into the diagnosis, pathology, and treatment of *Aspergillus* infection has used neutropenic mice (7). The extrapolation of data obtained in this way to the human condition of CGD may be of limited value in terms of improving prospects for this clinical cohort.

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